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Matrix metalloproteinases and proteinase inhibitors in antigen-induced arthritis of the juvenile temporomandibular joint

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MATRIX METALLOPROTEINASES AND PROTEINASE INHIBITORS IN ANTIGEN-INDUCED ARTHRITIS OF THE JUVENILE TEMPOROMANDIBULAR JOINT

by

SUNIL KAPILA

DISSERTATION

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of the

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DEDICATION

To my wife, Yvonne Kapila, and my parents, Dharam Inder and Vedi Kapila

PREFACE

Parts of these studies have been presented at meetings of the American and International Associations for Dental Research and the American College of Rheumatology. Several abstracts from these investigations have also been published in the Journal of Dental Research. A compilation of the studies described in Chapters II, IV and V received the Hatton Award from the American Association for Dental Research in 1993 for a paper titled "Mechanisms of matrix degradation of the temporomandibular joint disc" presented at the International Association for Dental Research and the American Association for Dental Research meeting in Chicago, Illinois.

I express my sincerest thanks to a number of faculty and colleagues whose tireless efforts and assistance have helped bring this work to its culmination. I am extremely grateful to my mentors and thesis committee members, Dr. David Richards and Dr. Arthur Miller, each of whom provided me with friendly advice, support and encouragement, and taught me to be analytical and perceptive through different stages of my doctoral studies. I also express my gratitude to the other members of my dissertation committee, Dr. Arnold Kahn and Dr. Zena Werb, who gave critical and thoughtful insights during the planning, conduct and writing of these studies, and to Dr. Caroline Damsky, my graduate adviser, for her gentle and patient guidance through much of my tenure as a doctoral student. Additionally, I thank my friends and peers, Dr. Calvin Lee and Dr. Majid Tavakkoli Jou, for their assistance in the surgical retrieval of tissues used in these studies, and to Dr. Robert Boyd and Dr. Ib Leth Nielsen, who provided the much needed moral support during my educational endeavors. My special thanks go to Dr. John Greenspan for recruiting me to the doctoral program in Oral Biology and for being readily accessible to give advice, and also to Dr. John Greene, Dean University of California San Francisco School of Dentistry, for providing me with the Dean's fellowship during a major portion of my doctoral studies. I am also very grateful to the American Association of Orthodontists who provided a substantial portion of the funding to support these studies. Last but not least, I express my heartfelt thanks to my wife, Yvonne for her loving support, dedication, encouragement, and patience, and to my parents, Dharam Inder and Vedi Kapila, who have disciplined me to inquire and to learn in the face of all odds. I dedicate this dissertation to my wife and parents.

MATRIX METALLOPROTEINASES AND PROTEINASE INHIBITORS IN ANTIGEN-INDUCED ARTHRITIS OF THE JUVENILE TEMPOROMANDIBULAR JOINT

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ABSTRACT

The loss of bone and cartilage matrices in rheumatoid arthritis (RA) and juvenile rheumatoid arthritis (JRA) impacts significantly on normal joint function. In these studies we examined the role of matrix metalloproteinases (MMPs) and proteinase inhibitors (PIs) in the loss of bony and cartilaginous matrices in experimental arthritis of the temporomandibular joint (TMJ). The unifying hypothesis of our studies was that antigen-induced arthritis of the juvenile TMJ is characterized by an increase in the absolute and relative levels of MMPs to their inhibitors expressed by specific tissues of the joint.

Arthritis was induced in TMJs of 10-week-old New Zealand white rabbits by systemic sensitization and intra-articular challenge with ovalbumin. Both sham-treated and untreated animals were used as controls. Five to 55 days following the induction of arthritis, right TMJs were biopsied *en bloc* for histopathology and immunohistochemistry, while the synovia and disc from the left joint were cultured. Synovium- and disc-conditioned media were assayed for MMPs and PIs.

The antigen-challenged joints demonstrated chronic arthritis characterized by mononuclear cell infiltration, synovial lining and villous hyperplasia, and pannus formation. Additionally, an early and sustained generalized loss of glycosaminoglycans was noted in the TMJ disc, while loss of collagen was localized to sites where the pannus invaded into bone and fibrocartilage. These changes were accompanied by marked immunohistochemical staining for collagenase and stromelysin in cells of the synovial lining, synovial stroma and disc, as well as in articular chondrocytes in antigen-challenged joints.

We also demonstrate, for the first time, that TMJ disc explants and cultured disc cells from normal animals constitutively synthesize 92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin, as well as two PIs of 30- and 20-kDas. The expression of these four MMPs is increased in explanted arthritic synovia and discs when compared to tissues from control joints. In contrast, the levels of PIs synthesized by the arthritic synovia and disc are similar to those from their respective control tissues. In related studies we have found that the expression of 92-kDa gelatinase and 30-kDa inhibitor

by disc cells is regulated via a protein kinase C-mediated pathway. These observations are consistent with the hypothesis that tissue loss associated with antigen-induced arthritis of the TMJ results, at least in part, from an increase in MMPs and a net increase in MMP to PI ratios.

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BACKGROUND, SIGNIFICANCE AND SPECIFIC AIMS

CHAPTER I

BACKGROUND, SIGNIFICANCE AND SPECIFIC AIMS

A. INTRODUCTION

Rheumatoid arthritis (RA) and juvenile rheumatoid arthritis (JRA) are autoimmune-mediated arthropathies of unknown etiology whose onset is often acute followed by a chronic and progressive course (Hollister, 1979; Bywaters, 1982). These pathologies are characterized by proliferation of synoviocytes, formation of pannus and presence of inflammatory infiltrate (Barriga et al., 1974). The eventual consequence of these arthropathies may be the complete destruction and ankylosis of the affected joint(s). Susceptibility to RA is to a large extent genetically determined but may be initiated by environmental factors (Stastny 1978; Winchester, 1981; Pope et al., 1982; Vaughan et al., 1983; Smith and Arnett, 1991). As suggested by the name, JRA is a condition similar in many respects to RA but afflicts children and young adolescents below the age of 16 years (Bywaters, 1982).

It is estimated that juvenile rheumatoid arthritis (JRA) or juvenile chronic arthritis afflicts approximately 113 of every 100,000 children in U.S.A. (Lawrence et al., 1989). Although the incidence of TMJ involvement in JRA has yet to be determined, studies on RA patients indicate that approximately 43% to 70% of these patients demonstrate TMJ involvement (Ericson and Lundberg, 1968; Franks, 1969; Crum and Loiselle, 1970; Ogus, 1975). JRA of the TMJ frequently results in pain and dysfunction, and is often accompanied by severe mandibulofacial developmental aberrations, including mandibular retrognathia, anterior open-bite and mandibular dental crowding (Barriga et al., 1974; Larheim et al., 1981a and b; Larheim and Hannaes, 1981; Wenneberg, 1987; Borchorst et al., 1988). The resulting compromised function and esthetics make JRA of the temporomandibular joint both physically and psychologically debilitating.

Since much more is known about the pathogenesis of RA than JRA, a considerable part of the ensuing discussion refers to findings from RA rather than JRA. Although there is probably much in common in the pathogenesis of these two types of arthritides, important differences may also exist. Differences between adults and growing individuals, such as those arising from differences in matrix composition, the higher rates of tissue synthesis and less degradation, and the greater reparative capacities of growing versus mature tissues (Holmes et al., 1988; Fornieri, et al., 1989; Front et al., 1989; Millie et al., 1989; Martin et al., 1990; Termine, 1990; Mays et al., 1991), as well as the

differences in immunologic mechanisms and responses (Dorai et al., 1987; Gahring and Weigle, 1990; Ho et al., 1990; McElhancy et al., 1990; Pross, 1990; Sironi et al., 1990) between the two age groups could contribute to variations in the pathogenesis of RA and JRA. These factors should be borne in mind when evaluating the findings discussed in this chapter.

A clinically and functionally significant manifestation of RA and JRA is the destruction of tissues of the joint, primarily cartilage and bone, resulting from a net loss of their extracellular matrix (ECM) macromolecules. Since both intact cartilage and bone are essential for the normal functioning of the joint, the loss of these tissues is often accompanied by perturbations in joint movements, and in the ability of the joint to withstand normal loading (reviewed in Christensen and Ziebert, 1986; Knets, 1987; Tammi et al., 1987). Since normal cellular function, including adhesion, migration, proliferation, differentiation and metabolism (Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1988a and b; McDonald, 1988; Werb et al., 1990; also reviewed in Toole, 1991), is modulated by the ECM, its loss may adversely affect these cellular activities. Several mechanisms have been proposed and investigated for the loss of matrix macromolecules in arthritis, resulting primarily from perturbed cell function which lead to altered matrix synthesis (Gillard and Lowther, 1976; Lowther and Gillard, 1978; Lowther et al., 1978; van den Berg et al., 1981; Kruijsen et al., 1985; Rubin and Roberts, 1987; Nuver-Zwart et al., 1988; Reddy and Dhar, 1992), increased matrix degradation (Wooley et al., 1977a and b; Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans, 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren et al., 1990; Firestein, 1992; Firestein and Paine, 1992 ; Hirose et al., 1992; Walakovits et al., 1992), or both. In recent years, substantial emphasis has been placed on elucidating the mechanisms for increased matrix degradation in arthritis (see preceding references). In this context, a family of matrix degrading proteinases, the matrix metalloproteinases (MMPs), has been specifically implicated. This family of MMPs includes enzymes such as interstitial collagenase (MMP-1), stromelysin (MMP-3), 72-kDa gelatinase (MMP-2) and 92-kDa gelatinase (MMP-9), whose activities towards matrix macromolecules range from specific to broadly reactive (reviewed in Matrisian, 1990; Alexander & Werb, 1989 and 1991; Murphy et al., 1991; Woessner, 1991; Birkedal-Hansen et al., 1993). Because of their substantial capabilities for ECM degradation, the activity of these proteinases is regulated both intracellularly, at the transcriptional level, and extracellularly, by their synthesis as inactive proenzymes and their inhibition by specific proteinase inhibitors (PIs).

Several clinical studies have demonstrated increased expression of the MMPs, collagenase and stromelysin, in arthritic joint tissues and synovial fluid (Wooley et al., 1977a and b; Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990; McCachren, 1991; Garvallese et al., 1991; Firestein et al., 1991; Firestein and Paine, 1992). However, investigations on human tissues often lack adequate or healthy controls, represent advanced stages of the pathology, have a large number of known and unknown variables, such as previous therapy, are not amenable to invasive procedures, and may not be adequately followed for longitudinal information (Wooley et al., 1977a and b; Cawston et al., 1984; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990 and 1991; Firestein et al., 1991; Garvallese et al., 1991; Firestein and Paine, 1992; Henderson et al., 1993). For these reasons, several animal models of arthritis have been developed over the years and utilized for studying various aspects of this arthropathy including clinical and histologic characterization of the models, the effects of anti-arthritic therapeutic agents, as well as the molecular immune mechanisms of arthritis (summarized in Table II). Limited information, however, has been gathered from these animal models on the role of MMPs and PIs in the loss of joint matrix molecules. Such information will enhance our more thorough understanding of the progressive mechanisms for matrix loss in arthritis, augment information currently available from human studies, examine the association between severity of arthritis and matrix degradative activities in tissues of the joint, and provide insights into the *in vivo* role of the extracellular balance between MMPs and PIs associated with matrix degradation of arthritis.

The overall purpose of these studies was to determine the matrix degradative activity in specific tissues of the joint during experimental arthritis of the juvenile rabbit temporomandibular joint (TMJ). In these series of investigations, the development and histopathologic characterization of a new model of antigen-induced arthritis of the juvenile rabbit TMJ is described. This model was then used to evaluate the progressive changes in matrix degradative activities with respect to synthesis of MMPs and their PIs in the arthritic synovium retrieved from these animals. We then demonstrate, for the first time, that a tissue relatively unique to the TMJ, the joint disc, expresses several proteinases and inhibitors, which were characterized and identified as MMPs and their PIs. The regulation of these MMPs and PIs by disc cells in response to a protein kinase-C agonist was also partially characterized. The final study evaluates the alterations in disc-cell derived MMPs and PIs in experimental arthritis of the TMJ. Reasons for changes in synthesis of these proteinases by disc cells were also examined by exposing cultured cells to a selected spectrum of recombinant cytokines. The findings of these studies provide an insight into

the mechanisms for matrix loss in antigen-induced arthritis of the TMJ, and will be useful in defining rational therapeutic approaches in preventing or alleviating matrix degradation which is a prominent feature of both RA and JRA. This experimental model of arthritis will also be useful for testing the efficacy of potential anti-arthritic therapies.

This chapter discusses the present day understanding on the anatomy, physiology and pathology of joints with a specific focus on the temporomandibular joint, the modalities for studying arthritis using various animal models, the sequelae of matrix loss, and the mechanisms by which matrices of joints may be lost during arthritis with a particular emphasis on the potential role of MMPs and PIs in this process. A review on the biochemistry and regulation of MMPs and PIs is also summarized to better acquaint the reader to this topic. Finally, an outline on the rational, hypothesis and specific aims of these studies is provided .

B. ANATOMY, FUNCTION AND BIOLOGY OF THE TEMPOROMANDIBULAR JOINT

In order to better understand the pathogenesis of RA, a thorough knowledge on the complex nature of the anatomy, organization and function of joints is necessary. Two basic types of articulations are found in the mammalian body: (1) synovial or diarthrodial joints which allow free movements and have a synovial membrane lining the joint cavity, and (2) synarthroses which demonstrate very little movement, and as such are of little consequence in the understanding of rheumatology. Typically, diarthrodial joints are mechanical systems which allow the relative movements of two body parts. These joints are composed of bones which are capped by articular cartilage and encapsulated by a fibrous tissue, the synovium. The inner surface of the joint is lined by a synovial lining formed by a layer of synoviocytes, 1 to 2 cells deep, while the outer layer is made up of loose connective tissue comprising the synovial stroma. The synovial fluid that lubricates these joints is viscous liquid composed of a vascular transudate admixed with hyaluronic acid. The articular cartilage does not contain blood vessels, lymphatics or nerves, but is metabolically active. The chondrocytes in this tissue are actively involved in turnover of cartilaginous matrix. Although, the TMJ in general conforms to this structural organization, specific differences exist between this joint and others found in the body. These differences as well as the function, development and microscopic anatomy of the TMJ are discussed in greater detail below.

Temporomandibular Joint Function

The TMJ is the only diarthrodial joint of the craniofacial skeleton and is necessary for mandibular movements. It is comprised of two articulating surfaces interposed by a fibrocartilaginous disc (Fig. 1). The superior articulating surface is formed by the temporal bone, while the condyle of the mandible constitutes the inferior articulation. These two structures are lined by articular fibrocartilage, and together with the intervening disc are encased in a synovial capsule which is reinforced by ligaments (Oberg and Carlsson., 1979; Christensen and Ziebert., 1986). This joint is considered to be a stress bearing joint (Hylander, 1979; Hylander and Bays, 1979; Brehnan et al., 1981; also reviewed in Hylander, 1985).

The TMJ is an anatomically and functionally complex joint because of the discordant or incongruent articular surfaces (Kubein et al., 1993) of the bilateral TMJs and the complete division of each joint into two cavities separated by a disc. Due to its complex organization, the TMJ provides for several types of joint movements which include antero-posterior gliding between the disc and temporal bone in the superior joint cavity producing antero-posterior condylar translation, and hinge and rotational movements between the disc and the condyle in the inferior joint space. The main function of this joint is in mandibular movements during mastication, deglutition and speech.

Development of the Temporomandibular Joint

Evolutionarily, the TMJ appeared relatively late, replacing the early jaw joint which became incorporated into the middle ear as part of the ossicular apparatus (Symons, 1952, Moffett, 1957). Embryologically, most components of this joint arise from mesenchyme of the second branchial arch. In the human, the mesenchyme lateral to the Meckel's cartilage condenses into the condylar and temporal blastemas by 6 weeks intrauterine (I.U.) which develop at different rates and move towards each other (Baume, 1962; Youdelis, 1966a and b; Baume & Holz, 1970; Keith, 1982; Perry et al., 1985). The formation and ossification of the condylar blastema precedes that of the temporal blastema. By 8 weeks, the condylar blastema is penetrated by an extension of membranous bone from the body of the mandible. Initially, the condylar and temporal mesenchymal condensations are widely separated but subsequent growth of the condylar primordia leads to a closer approximation of the two surfaces, which reach their closest approximation at 12 weeks. A narrow fibrous extension, first observed at 8 weeks, forms between the temporal and condylar primordia and subsequently develops into the lateral aspect of the joint capsule. At the dorsal end of the condylar process a cartilaginous core capped by highly condensed fibrous layer is observed at about 10 weeks. This tissue and its cells of mesenchymal origin develop into the secondary cartilage of the condyle, and postnatally provide a protective

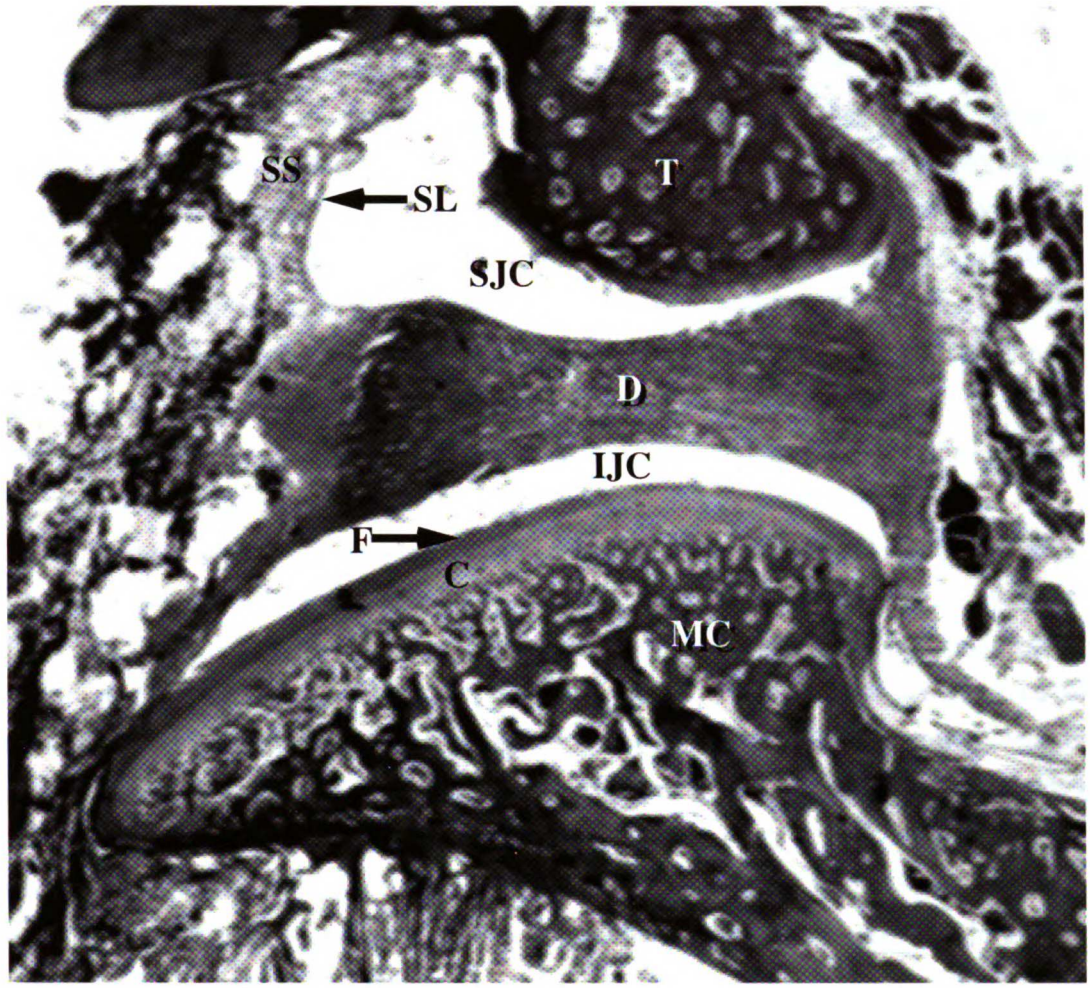


Figure 1: Organization and histology of the mammalian TMJ. (T=temporal articular surface; D=articular disc; MC=mandibular condyle; F=fibrous layer and C=cartilaginous layer of mandibular condyle; SS=synovial stroma; SL=synovial lining; SJC=superior joint cavity; IJC=inferior joint cavity).

articular surface and serve as a growth site for the mandible.

A band of highly condensed mesenchyme lying between the temporal and condylar primordia and joined to the aponeurosis of the lateral pterygoid muscle is also evident by the eighth week. There is some controversy as to the precise origins of these cells which have been presumed to arise either from the developing lateral pterygoid muscle, or partly from the condylar and partly from the temporal blastema, or forms from a separate mesenchymal condensation (Keith, 1982). This mesenchyme will form the future articular disc of the joint. At 10 weeks, a horizontal cleft develops between this mesenchymal condensation and the condylar blastema as the first sign of the inferior joint space. Two weeks later a similar cleft is observed between the temporal and disc primordia and is the first sign of the developing superior joint cavity. Recent immunohistochemical studies on

the developing rabbit TMJ have shown the presence of collagenase, stromelysin and gelatinase in the region of the presumptive joint space, suggesting that these proteinases may contribute to joint space cavitation (Breckon et al., 1990 and 1991).

A rapid increase in inter-condylar distance is observed after the 12th week as the neurocranium expands transversely (Youdelis, 1966a). Much growth of the condyle is observed from 14 weeks onwards due to expansion of the growth cartilage in all directions (Youdelis, 1966a and b). The articular disc and joint cavities become better defined by 14 weeks (Youdelis, 1966a and b; Baume and Holz, 1970; Keith et al., 1982). By this time most of the structures of the TMJ have been established and the joint resembles that of a full-term fetus. Subsequent development in the TMJ involves maturational changes in the already established structures. At term the TMJ is characterized by vascularization of all components and active bone formation in the condyle and glenoid fossa regions.

Postnatally, growth and maturational changes occur in the TMJ until approximately the end of the second decade of life (Wright and Moffett, 1974; Keith, 1982; Nickel et al., 1988). During this period several alterations in the morphology and composition of the condyle, articular eminence and disc are noted. The temporal articular fossa transforms from a shallow flat surface at birth, to one with marked anterior and posterior eminences and a deep fossa which is 50% the size of its adult configuration by 3 years of age (Wright and Moffett, 1974; Nickel et al., 1988). The condylar growth cartilage persists until late adolescence or early adulthood (Wright and Moffett, 1974; Keith, 1982). During this phase of development it comprises of zones of proliferating, mature and hypertrophic chondrocytes (Wright and Moffett, 1974; Livne and Silberman, 1983; Christensen and Ziebert, 1986; Luder et al., 1988). The growth of the condylar cartilage appears to contribute substantially to mandibular growth. With increasing age, the cartilaginous zones diminish in size and are replaced by bone (Keith, 1982; Wright and Moffett, 1974). The articular disc also undergoes maturational changes with age (Wright and Moffett, 1974; Christensen and Ziebert, 1986). During early postnatal life, the articular disc is primarily a fibrocollagenous tissue. With maturation it becomes progressively less cellular and more fibrous with the deposition of collagen (Wright and Moffett, 1974). Subsequently, probably in response to joint function and loading, the disc may undergo metaplastic change, becoming increasingly fibrocartilaginous (Fujita and Hoshino, 1989; also reviewed in Christensen and Ziebert, 1986). These changes have been demonstrated by the detection of cartilage-specific proteoglycans and differentiation of the fibroblastic cells into ones that display a greater chondrocyte-like phenotype (Mills et al, 1988; Milam et al., 1991; Nagy and Daniel, 1992). The collagenous content of the disc matrix, however, remains primarily type I collagen (Milam et al., 1991).

Microscopic Anatomy

(a). The synovium

Histologic examination of the TMJ capsule reveals that it is composed of two layers; an inner layer of thin synovial tissue, the synovial lining, and an outer fibrous layer, the synovial stroma. Very little other information is currently available on the specific organization of the TMJ synovium. However, findings from other joints indicate that the synovial lining is made up of 2 to 3 cell layers of elongated flattened cells, thought to be of two major types, the type A or macrophage-like cells and the type B or fibroblast like cell (Barland et al., 1962). Type A cells contain numerous vacuoles and a prominent golgi apparatus. Type B cells contain a large amount of endoplasmic reticulum and few vacuoles. A third type of cell, often called type C has also been described and appears to have features and function intermediate between the type A and B cells. Since cells with transitional forms between A and B cell types have been observed (Caulfield et al., 1982), it is presently uncertain whether these cells are two distinct cell lines or represent two phases in the differentiation of a single cell line (Barland et al., 1962; Ghadially and Roy, 1967; Fell et al., 1976). This synoviocyte lining serves very important functions by producing synovial fluid and also helps to remove products of degradation from the joint compartments. The synovial fluid lubricates the articular surfaces and transports metabolites and nutrients to the avascular articular surfaces. The synovial lining does not cover the articulating surfaces of the condyle, the superior articular component and the disc.

The normal synovial stroma is relatively well vascularized, has few fibroblasts and variable numbers of mononuclear cells, interdigitating dendritic cells, blood vessels, lymphatics and nerve fibers. It is a "loose connective tissue" largely made up of interspersed type I collagen fibers both in the knee and temporomandibular joints (Caulfield et al., 1982; Milam et al., 1991) (Table I). In an ultrastructural evaluation of the rat knee joint Caulfield et al. (1982) also noted that synovial lining cells attach to one another by desmosomes and to the extracellular matrix by hemidesmosomes (Caulfield et al., 1982). These cells contain a large nucleus, endoplasmic reticulum (ER), golgi apparatus, some mitochondria and a few vacuoles, and extend filipodia or processes into the joint space. Large synovial lining cells extend attenuated processes 10 to 15 μm in either direction from the cell body which loosely overlap other such processes.

The synovial capsule in the rabbit TMJ has two different matrix organizations; the portion of the capsule connecting the disc to the skull is largely composed of a fibrous outer layer of dense connective tissue of specifically oriented collagen fibers but few elastic fibers, while the part connecting the disc to the condyle contains oriented collagen and large amounts of elastic fibers (Savalle et al., 1990). This distribution of matrix molecules may

be related to the function of the capsule, permitting greater extensibility of the joint in the lower joint cavity.

(b). Temporomandibular joint disc

The TMJ disc is biconcave in shape and may be subdivided grossly into anterior and posterior bands separated by a thinner intermediate zone. The immature disc contains numerous fibroblasts or fibroblast-like cells surrounded by a matrix of type I collagen fibers and is often referred to as a fibrocollagenous tissue (summarized in Table I). Elastin and oxytalin fibers have also been demonstrated histochemically in the discs from several species of mammals (Christensen, 1975; Keith et al., 1979; Nagy and Daniel, 1991; Kino et al., 1993). Little else is known about the matrix composition of the TMJ disc in the growing animal.

With increasing age, cells variously identified as fibrocytes, chondrocytes, chondroid and "chondrocyte-like" differentiate, possibly from the fibroblastic precursors, and the fibrocollagenous matrix is gradually replaced by fibrocartilaginous matrix (Oberg and Carlsson., 1979; Christensen and Ziebert, 1986). This transformation is not well characterized, but probably entails tissue turnover in which the disc undergoes metaplastic change incorporating a greater proportion of cartilage specific matrix molecules. In the rabbit TMJ disc, cells identified phenotypically as chondrocytes first appear during the second postnatal week organizing in bands around which an extensive cartilaginous matrix is deposited by 3 to 4 weeks (Nagy and Daniel, 1992).

The fibrocartilaginous disc matrix of rats, rabbits, dogs, monkeys and man contains proteoglycans (PGs) with glycosaminoglycan (GAG) chains of keratan, chondroitin and dermatan sulfates, and hyaluronic acid (Mills et al., 1988; Nakano and Scott, 1989a and b; Milam et al., 1991). These GAGs are localized both as halos around the cells and as a heavy band in the central area of the disc (Granstorm and Linde, 1973; Kopp, 1976; Mills et al., 1988; Milam et al., 1991). Mills et al. (1988), using antibodies previously shown to be specific to rabbit cartilage chondroitin-6-sulfate and keratan sulfate, localized these GAGs largely around chondrocyte-like cells in the anterior and posterior band areas with less intense staining evident in the intermediate zone also. Keratan sulfate was also found around collagen fibers. Hyaluronic acid, link protein, chondroitin sulfate and keratan sulfate have also been localized around "chondrocyte-like" cells in the disc of adult baboons (Milam et al., 1991). Despite the presence of these cartilage-specific GAGs, type II collagen has not been detected either biochemically in the fetal bovine disc (Hirschmann and Shuttleworth, 1975), or immunohistochemically in the mature primate TMJ disc (Milam et al., 1991).

Table I: Cell types and the known matrix molecule composition of tissues of the mammalian TMJ.

Tissue	Tissue component and age	Cell type(s)	Matrix molecules	Sites found, species, age
Synovial capsule	Synovial lining	Phagocytic type A synoviocytes (macrophage-like cells), Fibroblastic type B synoviocytes	Not determined	Not determined
	Synovial stroma	Fibroblasts, dendritic cells, mononuclear cells	Collagen type I	Immunolocalized in synovium and capsule of adult baboons (13)
			Collagen (unspecified)	In fibrous outer layer of specifically oriented collagen fibers in rabbits (17)
			Elastic fibers	Portion of capsule from disc to condyle has large amounts of elastic fibers in rabbits (17)
			Laminin	Adventia of blood vessels in synovium, capsular ligament and attachment tissues of disc (13)
			Tenascin	Immunolocalized to synovium and capsule (13)
Disc	Young Fibrocollagen-ous	Fibrocytes (4), fibroblasts (3), "fibrochondrocytes", "chondrocyte-like" cells (16), or chondrocytes (15, 16)	Collagen type I	Detected biochemically in discs from fetal calf (5)
			Elastic fibers	In the antero-inferior and postero-inferior attachments prenatally and in intermediate zone and band areas in 2 week postnatal rabbits (15), histochemically detected throughout disc and retrodiscal areas of bovine TMJ (6)
			Oxytalin fibers	Detected histochemically in most areas of disc in Wistar rats (1)
	Adult Fibrocartilagin-ous	Fibrocytes (4,8), fibroblast-like (13), chondrocytes (4,14,15, 16), "chondrocyte-like" cells (13,14), or chondroid (8)	Collagen type I	Around "fibroblast-like" cells in the anterior and posterior attachment areas and "chondrocyte-like" cells throughout the body of the disc (13)
			Collagen type II	Immunohistochemically detected only in posterior region of experimental joints in adult rats (4)
			Proteoglycans	Histochemical staining in lacunae around disc cells (4)
			HA, KS and CS	Immunolocalized around "chondrocyte-like" cells (13)
			HA, KS, and CS/DS	Histochemical localization primarily in the central region of the human disc especially around chondroid cells (8)
			KS and C-6-S	Primarily immunolocalized to anterior and posterior band areas pericellular to chondrocyte-like cells in adult rabbits (14)
			Link protein	Found around "chondrocyte-like" cells in load bearing areas (13)
			Fibronectin	Immunolocalized around "chondrocyte-like" cells (13)
			Fn-α5 chain of integrin	Most cells in disc and attachment apparatus stained positive (13)

Disc (continued)	Adult Fibrocartilaginous		Elastic fibers	Most cells in disc and attachment apparatus stained positive (13)
				In retrodiscal areas of adult human joints (7), postero- and antero-inferior attachment area and intermediate zone of rabbit discs (15), mostly in the anterior and posterior regions of the disc in the miniature swine (2)
			Oxytalin fibers	Detected histochemically in most areas of disc in Wistar rats (1)
Mandibular condyle and articular fossa of temporal bone	Young fibrocartilage	Fibroblasts and fibrocytes, undifferentiated mesenchymal cells, chondroblasts, and chondrocytes found within layers from the articular surface to deeper zones forming cartilage (10,11,12)	Collagen I & III	Detected biochemically in fibrous layer in fetal calf TMJ (5)
			Collagen II	Detected biochemically in articular cartilage of fetal calf TMJ (5)
				Detected immunohistochemically in hypertrophic cell layer of condylar cartilage of Sherman rats (12)
			Elastic fibers	Detected histochemically running parallel to the articular surface in bovine TMJ (6)
			Oxytalin fibers	Detected histochemically in fibrous zone of articular surfaces in Wistar rats (1)
	Adult fibrocartilage	Fibroblasts and fibrocytes, chondroblasts, and chondrocytes found within layers from the articular surface to deeper zones forming cartilage (3)	Collagen type I	In the fibrous and prechondroblast zones, and near the subchondral bone area in the hypertrophic chondrocyte zone (13)
			Collagen type II	Around mature chondrocytes (13)
			Sulfated GAGs (KS, CS/DS, hyaluronate)	Localized in deep cartilage & mineralized cartilage layers mainly in anterior mandibular condyle and lateral temporal eminence (9)
			HA & KS	Around mature chondrocytes (13)
			Chondroitin sulfate	In deeper layer of articular cartilage (deep to mature chondrocytes) (13)
			Link protein	Present in all zones of the articular cartilage, but especially prominent in the prechondroblastic or mature chondrocyte zones (13)
			Fibronectin & tenasin	Prominent in the prechondroblastic zone and positive but less intense in cartilaginous zone (13)
			Fn-α5 integrin receptor	Cells in fibrous layer of articular surface of mandibular condyle, and in the zone of mineralization (13)
			Oxytalin fibers	Detected histochemically in fibrous zone of articular surfaces in Wistar rats (1)

Abbreviations:
HA=hyaluronic acid; KS=keratan sulfate; CS=chondroitin sulfate; DS=dermatan sulfate; C-6-S=chondroitin-6-sulfate; Fn=fibronectin

Table I (continued): Data from:

(1) Booij and Markens, 1983: Histochemical staining in young and adult Wistar rats
(2) Christensen, 1975: Histochemistry in the adult miniature swine
(3) Christensen and Ziebert (1986): Review article
(4) Fujita and Hoshino, 1989: Immunolocalization and histochemistry in adult rats
(5) Hirschmann and Shuttleworth, 1976: Biochemical assays on disc and cartilage of fetal calves
(6) Keith et al., 1979: Histochemical staining of bovine TMJ
(7) Kino et al., 1993: Histochemical staining of adult human TMJ
(8) Kopp, 1976: Histochemistry in young and adult human TMJs
(9) Kopp, 1978: Histochemistry in young and adult human TMJs
(10) Livne and Silbermann, 1983: Histologic and biochemical evaluations in young mice
(11) Livne et al., 1987a: Histologic and biochemical evaluations in young mice
(12) Luder et al., 1988: ^{3}H-thymidine autoradiography for cell proliferation and immunostaining for type II collagen in young Sherman rats
(13) Milam et al., (1991): Immunolocalization studies on adult male baboons (Papio cynocephalus)
(14) Mills et al., 1988: Immunolocalization and histochemistry in adult rabbits
(15) Nagy and Daniel, 1991: Histochemistry in prenatal, postnatal and adult rabbits
(16) Nagy and Daniel, 1992: Described in prenatal, postnatal and adult rabbits
(17) Savalle et al., 1990: Histochemistry on young and adult rabbits

Elastic fibers have also been noted in the anterior and posterior band areas as well as the intermediate zone of the disc in man (Keith, 1982) and rabbit (Nagy and Daniel, 1991), and are thought to provide resiliency to the disc. Developmentally, elastic fibers are first noted in the antero-inferior and postero-inferior attachment regions of rabbit disc 23 days into prenatal life, and are well established in the intermediate zone and band areas of the disc by 2 weeks postnatally (Nagy and Daniel, 1991). Elastic fibers have been noted in adult discs as well (Christensen, 1975; Nagy and Daniel, 1991; Kino et al., 1993). In man the disc may be vascularized up to about 5 years of age (Caltabiano et al., 1989), but is completely avascular in adults. This lack of vascularity together with its cartilage-like composition, may limit the discs' ability for repair following pathologic degradation of the matrix.

Although disputed by some investigators, several studies have reported insertions of fibers of the lateral pterygoid muscle (Wilkinson et al., 1989; Merida et al., 1993), and less often of the massetter and temporalis muscles (Klineberg, 1991; Merida et al., 1993) into antero-medial and antero-lateral boundaries of the disc, respectively. It is thought that these muscles help stabilize the disc to the condyle during mandibular movements (Klineberg, 1991). The disc contributes substantially to normal joint function permitting translatory and rotary movements of the mandibular condyle, and also serves a load absorbing and load distributing function (Gola et al., 1992). The disc may also serve a proprioceptive function since calcitonin gene-related peptide-containing nerve fibers, which are located around blood vessels, are found terminate as free nerve endings in the disc (Ichikawa et al., 1989). These nerve fibers may be sensory in nature.

The posterior attachment of the disc, or retrodiscal pad, is a highly vascularized and innervated loose connective tissue (Rees, 1954; Dixon, 1962; Wright and Moffett, 1974; Friedman et al., 1982; Savalle et al., 1990). The superior stratum of this attachment consists of loosely organized meshwork of collagen fibers and blood vessels, often in the form of a venous plexus. This superior component of the retrodiscal tissue is attached posteriorly to the anterior face of the postglenoid process, the bony auditory meatus of the temporal bone, the cartilaginous meatus, and fascia of the parotid gland. The inferior stratum is usually composed of a fairly compact, inelastic sheet of collagen that attaches to the posterior surface of the mandibular condyle. This tissue is composed of type I collagen (Savalle et al., 1990; Milam et al., 1991) and elastin fibers (Dixon, 1962; Keith, 1979; Savalle et al., 1990; Kino et al., 1993). The latter components of this tissue endow it with the ability to be extensible, thereby permitting the movement of the disc during mandibular movements (Dixon, 1962; Keith, 1979; Christensen and Ziebert, 1986; O'Dell et al., 1989; Kino et al., 1993).

(c). Condylar and temporal articulating surfaces

Histological examination of the condyle and articulating fossa demonstrates two relatively distinct regions, namely the superficial articular fibrous layer and its underlying cartilage. The superficial avascular layer of dense fibrous connective tissue consists of mainly type I collagen fibers (Milam et al., 1991) which primarily run parallel to the articulating surface (Christensen & Ziebert, 1986; Luder et al., 1988). Within the deeper zones of this fibrous layer, collagen fibers run perpendicular to the articular surfaces (Christensen and Ziebert, 1986). The articular fibrous layer also contains elastic fibers which are first seen as early as the 23 days in utero in the rabbit (Nagy and Daniel, 1991), and have also been demonstrated in the developing bovine articular fibrous layer (Keith et al., 1979). This layer of tissue contains cells that have been described as fibroblast-like in young and chondrocyte-like in mature monkeys (Luder and Schroeder, 1990).

The cartilaginous region beneath the fibrous articular surface in the growing mammal has a specific four layered structure having some organizational characteristics of the epiphyseal growth plates of long bones, and is thought to contribute to the growth of the condyle and mandible (Keith, 1982; Livne, et al., 1987a; Luder et al., 1988; also reviewed in Christensen & Ziebert, 1986). These zones contain cells in different stages of differentiation, namely (1) a polymorphic cell layer of resting undifferentiated mesenchymal cells or chondro-progenitor cells from which arise cells undergoing endochondral changes, (2) a flattened layer of proliferative cells which produce a precartilaginous matrix devoid of type II collagen while undergoing differentiation first into chondroblasts and then into chondrocytes when intracellular type II collagen expression begins, (3) the upper hypertrophic chondrocyte layer in which cells enlarge rapidly, and actively produce and deposit type II collagen into the matrix, and (4) the lower hypertrophic cell layer composed of chondrocytes at a terminal stage of enlargement where the cartilaginous matrix also begins to calcify. The most superficial layer containing undifferentiated mesenchymal cells is made up of thin collagen fibers oriented along the surface. The proliferating chondrocytes are embedded in PGs located in the interstices between randomly oriented collagen fibers. The principal PGs found here are chondroitin sulfate, keratan sulfate, dermatan sulfate and hyaluronate (Kopp, 1978; Milam et al., 1991). The third layer is made up of the same components as the second layer, except that collagen is oriented perpendicular to the joint surface. Chondrocytes in this layer form columns in growing animals and suggest the appearance of a continuously advancing mineralizing front similar to that seen in the epiphyseal plate of long bones (Keith, 1982; Luder and Schroeder, 1992). The final layer of calcified cartilage is fused to subchondral bone, from which osteoclasts resorb the calcified cartilage which is replaced by bone. With cessation of

growth, only the superficial cartilage remains as articular cartilage, the rest being replaced by compact bone. Cancellous bone is present beneath the layer of compact bone. The matrix in all the layers of the cartilage is composed of type II collagen, and proteoglycans including keratan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid (Kopp, 1978; Milam et al., 1991).

Matrix Composition and Properties

The mechanical properties and function of the articular cartilage and disc depend on the combined intrinsic properties and interactions of the collagen-PG network (Kempson et al., 1973; Armstrong and Mow, 1982; Christensen and Ziebert, 1986; Hardingham et al., 1986; Mills et al., 1988). Since GAGs on proteoglycans have a large number of fixed anionic charges, they readily imbibe water, producing an internal swelling pressure which is resisted by the tensile collagen fiber network (Maroudas, 1980; Myers et al., 1984). The net effect of this phenomenon is to provide a cushion against compressive forces (Caplan, 1984; also reviewed in Hascall and Hascall, 1981). The relatively inextensible collagen fibrils serve as a scaffolding for the PGs and endow the tissue with resistance to tensile forces (reviewed in Hascall and Hascall, 1981, Trelstad and Silver, 1981, and Birk et al., 1991). Elastin fibers are thought to provide resilience and resistance to distortion of tissues (Franzblau and Faris, 1981; Booij and Markens, 1983; Nagy and Daniel., 1991). When cartilage is loaded under compression or shear, the internal swelling pressure increases because of the increasing PG concentration, until it is in equilibrium with the externally applied force, when fluid movement ceases (Myers et al., 1984). For this reason, the physical properties of cartilage have often been described as visco-elastic and poro-elastic (McCuthcen, 1983). Such physical properties are also evident in the TMJ disc (Fontenot, 1985; Shengyi and Xu, 1991; Shengyi et al., 1991; Tanne, 1991).

Several investigations have attempted to characterize the matrix composition of various anatomic components of the TMJ using either histochemical or immunohistochemical techniques (see Table I for summary and references). In a relatively extensive study, Milam et al., (1991) immunostained for several matrix molecules including types I and II collagens, fibronectin, fibronectin-integrin receptor, tenascin, laminin, link protein and cartilage specific GAGs in the TMJs of adult baboon. Type I collagen, fibronectin and fibronectin-integrin receptor complex were immunolocalized throughout the joint, but were especially marked in the prechondroblastic and mineralized zones of the articular cartilages. Similarly tenascin was detected in the prechondroblastic and cartilaginous zones of the articular cartilages. Type II collagen and cartilage-specific GAGs were observed in cartilaginous zones of the articular cartilage. However, despite the presence of cartilage-specific GAGs in the disc, type II collagen was not localized to this

tissue. Similarly Fujita and Hashino (1989) noted in experiments in which unilateral malocclusion was induced in rats, that type II collagen could not be detected in control animals, but was found in the posterior area of the disc in experimental rats. This shift in matrix composition may be attributed to increased functional loads placed on the disc.

Besides the physical functions of the ECM in the articular cartilage and disc, these matrix molecules may also serve very important biologic roles. It has been demonstrated that the normal ECM is essential for the physiologic activity, attachment, proliferation, differentiation and migration of cells (Ruoslahti and Pierschbacher, 1987; Ruoslahti, 1988a and b; Werb et al., 1990; also reviewed in Hay, 1981 and 1991, and McDonald, 1988). Furthermore, products of matrix degradation may also have biologic activities. Fibronectin and collagen fragments, for example, have been shown to be proinflammatory and to induce increased expression of matrix degrading proteinases in various cells (Postlethwaite and Kang, 1976; Postlethwaite et al., 1978; Norris et al., 1982; Werb et al., 1989; Lohr et al., 1990; Homandberg et al., 1992). Loss of matrix molecules from tissues of the joint may, therefore, impact not only on the abilities of the joint to withstand normal functional forces, but may also disturb normal cell function which may in turn further aggravate the pathology.

Specialization of Anatomy and Function

Despite numerous similarities between the TMJ and other joints, several specific structural and functional differences exist between these joints (Symons, 1952; Baume, 1962; Baume and Holz, 1970; Friedman, 1982; Keith, 1982; Gall, 1988). These unique features of the TMJ include:

1. The TMJ develops from widely separated intramembranous bony elements which grow towards one another. This is unlike other synovial joints which develop by cavity formation within a single blastema (Symons, 1952; Baume, 1962; Baume and Holz, 1970; Keith, 1982).

2. The articular surfaces are covered with nonvascular fibrous tissue as opposed to the hyaline cartilage found in other joints (Miles and Dawson, 1962; Wilson and Gardener, 1974; Luder and Schroeder, 1990).

3. The articular surfaces are separated from each other by a fibrous or fibrocartilaginous meniscus, or disc.

4. This disc divides the joint cavity into two completely separate spaces, and permits both a rotational and translational movement of the mandibular condyle with respect to the lower and upper joint spaces, respectively.

5. Unlike other joints in which bilateral articulation can function independently of each other, the two condyles in the TMJ are connected by a rigid mandibular body, such that any movements in the joints have to be coordinated.

6. Unlike other joints involving long bones in which the epiphyseal growth plate is relatively distant from the joint, the growth site of the mandibular condyle is located within the joint capsule. This implies that inflammation within the TMJ may adversely affect growth of the mandible.

It is important to be cognizant of these differences in the anatomy and function of the TMJ and other joints, since these differences may impact upon the pathogenesis and progression of arthritis in this joint.

C. PATHOLOGIES OF THE TEMPOROMANDIBULAR JOINT

Much like other joints, most forms of arthritides can afflict the TMJ, although not necessarily with the same severity or incidence. The arthritides commonly afflicting the TMJ include internal joint derangement and temporomandibular joint dysfunction (TMD), osteoarthritis, and rheumatoid arthritis (McNeill et al., 1980; McNeill, 1993). Although he clinical, morphologic, and histopathologic manifestations (Carlsson et al., 1967; Scapino, 1983; Blaustein and Scapino, 1986; Isberg and Isacsson, 1986; Isberg et al., 1986) of internal joint derangement and its secondary degenerative joint disease have been relatively well studied, much less is known about RA of the TMJ. While the prevalence of signs and symptoms of temporomandibular joint dysfunction is reported as 75% and 33%, respectively (Rugh and Solberg, 1985; Schiffman and Fricton, 1988), little information is currently available on the relative prevalence of the other TMJ pathologies.

A relatively common and early developing arthropathy of the TMJ involves various types of internal joint derangements which result due to disturbances in disc-condyle relationships whose symptoms include joint sounds, limited or restricted movement of the mandible, and presence or absence of associated pain giving rise to the so-called pain-dysfunction syndrome (Rothwell, 1985; McNeill, 1993). These derangements include anterior displacement of the disc, which is frequently accompanied by changes in the morphology of the disc and less frequently by perforation of the disc. Osteoarthritis is often a common sequlae of internal joint derangement. Osteoarthrosis and osteoarthritis, occurring as primary conditions or secondary to internal joint derangement, are usually of late onset and often associated with changes in morphology of joint tissues including flattening of the condyle and formation of osteophytes.

Rheumatoid arthritis and JRA are autoimmune mediated diseases of unknown etiology which often have an acute onset followed by a chronic and progressive course. The inflammatory response in these arthropathies initially involves the synovium with subsequent destruction of osseous tissues, and ankylosis of the joint may ensue. Susceptibility to RA and JRA is to a large extent genetically determined but may be initiated by environmental factors (Stastny 1978; Winchester, 1981; Pope et al., 1982; Vaughan et al., 1983; Smith and Arnett, 1991). The prevalence of JRA in Sweden is reported as 82 per 100,000 children (Andersson-Gare and Fasth, 1991), and as approximately 113 of every 100,000 children in U.S.A. (Lawrence et al., 1989). The incidence of TMJ involvement in JRA has yet to be determined. However, studies on RA patients indicate that approximately 43% to 70% of these patients demonstrate TMJ involvement (Ericson and Lundberg, 1968; Franks, 1969; Crum and Loiselle, 1970; Ogus, 1975). In a prospective study on patients with JRA, it was noted that while only 1 out of 37 patients studied had TMJ symptoms at the onset of arthritis, about 50% of these individuals had developed TMJ symptoms such as joint noise, pain and limited motion within the first 5 years of the disease (Kaban, 1990).

The clinical features of RA of the TMJ include pain, limitations in mandibular movement, and crepitus (Franks, 1969; Chalmers and Blair, 1973). Patients often complain of pain on chewing and sometimes pain at rest. A clicking or crackling sensation is often noted on joint movements, and results due to displacement of the disc or destruction of disc and articular cartilage. Periarticular manifestations include swelling over the joint anterior to the ear and muscle spasm with resultant facial pain (Marbach, 1976-1977). Earaches may also be present. Flexion contractures of the muscles may eventually occur leading to an inability to open the jaw and ingest food with a resultant decreased nutritional intake (Marbach and Spiera, 1967). In some instances, retrognathism of the mandible develops over time giving rise secondarily to an increased overjet and other occlusal aberrations. These latter manifestations are more prevalent and dramatic in growing individuals with JRA than in adults with RA. Several investigators (Barriga et al., 1974; Larheim et al., 1981a and b; Larheim and Hannaes, 1981; Wenneberg, 1987; Borchorst et al., 1988) have demonstrated mandibular retrognathism, decreased dimensions of mandibular body and ramal height, increased gonial and mandibular plane angles, antegonial notching, anterior open-bite, and mandibular dental crowding in children with JRA of the TMJ. Although the mechanisms for these adverse effects of JRA on mandibulofacial development are not understood, it is probable that the degradation of the matrix of joint tissues, effects of inflammation on mandibular condylar growth, and hypofunction of the masticatory apparatus may contribute to the developmental

disturbances noted in these individuals (Larheim and Hannaes, 1981; Forsberg and Perrson, 1985). The resulting compromised function and esthetics make JRA of the temporomandibular joint both physically and psychologically debilitating.

Several clinical and radiologic changes have been documented in patients with JRA of the TMJ. In a study on 72 patients with JRA involving the TMJ, 69% had impalpable condyles, 51% had a mandibular asymmetry, 47% demonstrated deviation of the mandible towards the affected side on opening, 30% showed a palpable crepitus or click and 19% showed mandibular micrognathia (Blasberg et al., 1991). Radiographic abnormalities were found in 29 of 30 patients studied with 41% of these being unilateral and 51% being bilateral. Similarly, of 14 JRA patients evaluated in another study, 6 demonstrated asymmetric clinical findings of the TMJ, while the rest had symmetrical TMJ involvement (Taylor et al., 1991). MRI examination revealed cortical erosions or irregularities, flattening of the condylar head, articular fossa and disc, as well as disc perforation (Taylor et al., 1991). The range of motion was limited in about 65% of the joints examined.

Several disease entities, including rheumatoid factor positive RA, ankylosing spondylitis, psoriatic arthritis, ulcerative colitis (Crohn's syndrome) and Still's disease have features which are clinically identifiable as JRA (Bywaters, 1982). Most of these conditions also occur in adults. Although little is known about the specific histopathologic and biochemical changes that accompany JRA of the TMJ, findings from other similarly affected joints may provide insights into the potential pathogenesis of this disease. Histologically, JRA, like RA, is characterized by chronic synovitis, proliferation of synovial lining cells, villous hyperplasia, and presence of an inflammatory infiltrate composed of macrophages, lymphocytes and plasma cells (Hollister, 1979; Vischer, 1982; Krane et al., 1986; Harris 1989). Proliferative synovium often encroaches upon and wraps around the condyle, and subsequently results in destruction of the articular cartilage and disc (Gall, 1988). This invasive synovial tissue is referred to as the pannus, and is a highly vascularized complex tissue comprised of proliferative and hypertrophied synovial lining cells, and a variety of inflammatory cells including mononuclear cells and neutrophils in the subsynovium (Hollister, 1979; Vischer, 1982; Krane et al., 1986; Harris 1989). The pannus originates from the synovial reflection over cartilage and begins a circumfrential invasion of articular tissue and surrounding capsules and ligaments. The pannus may provide the optimal microenvironment for the destruction of the neighboring cartilage and bone probably via the actions of specific proteinases. Subsequently the chronic inflammation causes the disintegration of articular cartilage and exposure of the underlying bone, which may then articulate directly with the opposing bone (Hollister, 1979). In the case of the TMJ, this undesirable bone-to-bone articulation will occur only

after partial or complete disintegration of the intervening disc. This underscores the importance of understanding the mechanisms for loss of matrix from the TMJ disc.

In the arthritic TMJ, remodeling of the temporal fossa may be observed, while remodeling of the mandibular condyle head causes a flattening of its shape (Gall, 1988). As with other joints, the diagnosis of rheumatoid involvement of the TMJ is based on the diagnosis of RA by serological markers and presence of a systemic condition as well as clinical signs and symptoms. Other diagnostic aids include evaluation of aspirated synovial fluid, arthroscopy, and imaging by radiography, computerized tomography or magnetic resonance imaging.

D. PATHOGENESIS AND IMMUNOLOGY OF RA AND JRA

Since little information is currently available on the pathogenesis of JRA or RA of the TMJ, most of the information discussed in this section is from joints other than the TMJ. It is likely that, to a large extent, these findings also apply to the TMJ. However, differences related to the relatively distinct structural and functional organization of the TMJ as compared to other diarthrodial joints may contribute to a variable course in the pathogenesis of arthritis of the TMJ. Because of these unique features of the TMJ, including the presence of a disc within the joint and a condylar growth site in close proximity to the joint capsule, studies specific to the TMJ are indicated in order to better understand the pathogenesis of RA of this joint.

Although JRA and RA primarily affect diarthrodial joints manifesting as chronic recurrent synovitis, these pathologies are systemic disorders often associated with malaise, fatigue, weight loss, pericarditis, pleuritis, scleritis, sialadenitis, episcelritis, vasculitis and rheumatoid nodules (Bywaters, 1982; Pope and Talal, 1985). Diagnosis of RA and JRA is made with the aid of criteria characterizing the most typical signs and symptoms, since no one single manifestation or sign is diagnostic of RA. RA and JRA may be seropositive or seronegative, based on the presence or absence of circulating rheumatoid factor (RF), an autoantibody against the Fc portion of the IgG molecule (Miller, 1979; Bywaters, 1982).

The pathogenesis of RA may be divided into 3 stages, the initiation, destruction and self-perpetuation stages (Pope and Talal, 1985). Although not yet completely understood, the initiation stage may involve both an environmental factor, such as bacteria or virus or their products, and a genetic susceptibility of the host (Stastny, 1976 and 1978; Winchester, 1981; Pope et al., 1982; Vaughan et al., 1983; Bennet, 1989; Harris, 1989). The destructive phase is characterized by damage to bone, cartilage and supporting soft tissue structures which occurs due to a loss of matrix macromolecules (Hollister, 1979;

Krane et al., 1982 and 1986). A variety of events and agents are thought to cause this damage to tissues of the joint and are discussed in greater detail later. The destructive phase is followed by a phase of self-perpetuation which may result from aberrations in immunoregulation (Consden, 1971; Allen et al., 1985; also reviewed in Bennett, 1989), although proteolytic fragments from degraded matrices may also be partially responsible for the chronic progression of this arthropathy (Postlethwaite and Kang, 1976; Werb et al., 1989; Homandberg et al., 1992).

Although the etiology of RA remains unknown, two important factors have directly or indirectly been associated with this pathology, namely the immunogenetic characteristics of the patient and a challenge to the patient's immune system (Stastny, 1976 and 1978; Winchester, 1981; Pope et al., 1982; Vaughan et al., 1983; also reviewed in Bennet, 1989, Harris, 1989, and Winchester, 1989). In population studies, for example, RA has been associated with the human leucocyte antigen- (HLA-) DR4 major histocompatibility complex (MHC) genotype (reviewed in Ziff, 1985, Harris, 1989, and Winchester, 1989). Several triggering events including infectious agents such as pyogenic bacteria, mycobacteria, certain fungi and some viruses have also been implicated in the etiology of RA (reviewed in Bennet, 1989 and Harris, 1989). These organisms may be classified by their modality of action into (1) synovial thrivers which invade and multiply within the synovial capsule, (2) synovial inciters which trigger the immune response by entering the synovial cavity but do not multiply, (3) remote inciters which trigger an immune response from a distant site, whereby cross-reactivity is present between an inciting antigen from an organism and an autologous antigen in target tissues, and (4) toxicogenics in which some viruses directly and toxically attack the synovial tissue. Arthritis, therefore, may be caused by a variety of organisms in a number of different ways. The occurrence and consequence of this pathology, however, is dependent on the complex interaction between host and agent and are determined by the characteristics of an individuals' immune response to one particular infectious or triggering agent.

The destructive phase entails the loss of tissue components by a series of possible mechanisms mediated by a multitude of potential interactions between inflammatory cells, resident cells, and several bioactive agents such as cytokines (Hollister, 1979; Krane et al., 1982 and 1986; Pope and Talal, 1985). The cells implicated in this process include polymorphonuclear leucocytes, lymphocytes, macrophages, fibroblasts, chondrocytes and osteoclasts (reviewed in Vischer, 1982 and Krane et al., 1986). The activity of cells participating in synovial inflammation is modulated by other cells either by the release of hormone-like factors or by direct cell-to-cell contact, as well as through contacts with the extracellular matrix. The net effect of these interactions is the loss of matrix molecules

from cartilaginous and bony components of the joint. Several different mechanisms including cell death (Howson et al., 1986), a diminished or aberrant matrix synthesis (Gillard and Lowther, 1976; Jacoby and Jayson, 1976; Carmichael et al., 1977; Lowther et al., 1978; Reddy and Dhar, 1992), or an increased degradation of matrix macromolecules (Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans et al., 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein, 1992; Firestein and Paine, 1992 ; Hirose et al., 1992; Walakovits et al., 1992) are likely to contribute to these early changes in matrix composition and the later alterations in morphology of the tissues of the joint. The potential mechanisms for matrix loss are discussed in greater detail in a later part of this chapter (see page 51).

The mechanisms for chronicity of RA are also poorly understood, but the possible basis for this include a sustained stimulation or a failure of regulation of the immune system (Consden, 1971; Allen et al., 1985; also reviewed in Bennett, 1989). Sustained stimulation, in turn, may result either from a failure to eliminate heterologous antigen or due to a reaction to an autologous antigen. In antigen-induced arthritis, for example, prolonged retention of the antigen has been demonstrated in tissues of the joint (Consden et al., 1971; Webb et al., 1971; Cooke et al., 1972b; Hollister and Mannik, 1974) which may partially provide the persistent stimulation necessary for chronicity of RA. In contrast, in streptococcal cell wall- (SCW-) induced arthritis antigen retention is noted in the synovium and other tissues in both susceptible LEW/N and nonsusceptible F344/N strains of rats indicating that, at least in these animals, other phenomenon may be responsible for the chronic inflammation of arthritis (Wilder et al., 1983; Lehman et al., 1984). In experiments on SCW-induced arthritis in rnu/rnu athymic and NIH/rnu euthymic rats it was observed that, although both strains developed acute arthritis, chronic inflammation only persisted in the euthymic rats (Allen et al., 1985). Furthermore, even though both strains of rats demonstrated bacterial cell walls in tissues after 12 weeks, euthymic rats had a large T cell population and showed IL-2 production. These experiments suggest that normal T cell function may be necessary for chronic arthritis in this model.

The pathologic changes in soft tissues during RA and JRA begin as inflammation and progress to proliferation of the synovium, producing the characteristic hypertrophy of synovial lining cells in which these cell layers increase from 1 to 2 up to 10 layers in depth (Hollister, 1979; Pope and Talal, 1985). Studies done on early changes in RA indicate that inflammation begins around capillaries and venules in the subsynovium coincident with synovial effusion and joint swelling (Kulka et al., 1955; Schumacher and Kitridou, 1972). The initial perivascular infiltrate consists of polymorphonuclear leukocytes (PMNs) as well

as lymphocytes. The cellular infiltrate then spreads throughout the subsynovium and changes to a more chronic form containing small and large lymphocytes, plasma cells and macrophages. These changes may be related to focal and segmental vascular abnormalities permitting infiltration of the synovial stroma by mononuclear cells. Proliferative changes in the synovial lining coincide with the inflammation and the synovium becomes edematous with villous extensions projecting into the joint cavity.

Changes are also observed in the synovial fluid of RA and JRA patients (reviewed in Hollister, 1979; Hasselbacher, 1985, and Harris, 1989). The synovial fluid becomes more of an exudate while maintaining acute characteristics, despite the chronic nature of the synovial inflammation. PMNs comprise a majority of the cells, while a smaller proportion of the cells are lymphocytes. The synovial fluid becomes less viscid due to a decreased hyaluronic acid content, and the profile of its contents approaches that of serum. These changes in synovium and synovial fluid despite causing swelling, pain and limitations in function, do not in themselves result in permanent deformity unless the cartilaginous structures are irreparably damaged (reviewed in Hollister, 1979).

The irreversible changes in arthritis result as a consequence of a loss of tissue components of cartilage and bone. Some of the earlier changes observed in articular cartilage matrix is the loss of proteoglycans as assessed by decreased histochemical staining for these molecules often in regions remote from the pannus (Vischer et al., 1982; Hasty et al., 1990; Pettipher et al., 1990; Beesley et al., 1992). Ultrastructural studies reveal the presence of fibrin on the free surface and amorphous material in the superficial layers which may be products of degraded proteoglycans or cell organelles from PMNs or dying chondrocytes (Kimura et al., 1977; Caulfield et al., 1982). Although loss of proteoglycans may be reversible, at least in the earlier stages of the arthritis, the loss of collagen generally results in irreversible damage to cartilage. This disparity in reversible and irreversible damage to articular cartilage depending on the type of matrix molecule lost may be related in part to the limited ability of cells to resynthesize type II collagen, while portions of proteoglycans can be synthesized by chondrocytes (Krane et al., 1982). Progressive loss of articular cartilage leads to bone-to-bone articulation, which may further increase pain and limit joint function (reviewed in Hollister, 1979). In the case of the TMJ, this undesirable bone-to-bone articulation requires both the disintegration of the articular cartilage and also the articular disc, indicating the importance of understanding how the pathologic loss of disc tissue occurs.

Recent studies have also examined some of the more intricate mechanisms contributing to the histopathologic changes observed in RA and JRA. In both rheumatoid arthritis and experimental arthritis endothelial cells also play a substantial role (reviewed in

Wilder et al., 1991). At the molecular level, the synovial tissues display features of microvascular endothelial activation, such as enhanced expression of activation markers such as class II major histocompatibility complex, phosphotyrosine, leukocyte adhesion molecules, oncoproteins such as c-Fos and c-Myc, and metalloproteinases such as collagenase and stromelysin (Yocum et al., 1988; Case et al., 1989a and b; Wilder et al., 1989; Sano et al., 1990; also reviewed in Wilder et al., 1991). The development of severe, chronic, destructive arthritis is dependent upon thymic-derived lymphocytes and is accompanied by tumor-like proliferation of cells in the synovial connective tissue stroma, which results in resorptive destruction of bone and cartilage. Several distinct criteria support the analogy of this pathogenesis to a neoplastic process. Various paracrine and autocrine factors, including interleukin-1, platelet-derived growth factor, transforming growth factor-β, insulin-like growth factor, and epidermal growth factor probably contribute to the development of these lesions (Schmidt et al., 1982; Bunning et al., 1986; Remmers et al., 1988; Kumkumian et al., 1989; Lafyatis, 1989; Hiraoka et al., 1992; also reviewed in Harris, 1989).

The findings discussed above on the pathogenesis and immunology of RA have been derived largely from investigations on human specimens, and partly from studies on animal models of arthritis. While the two types of investigations serve to complement one another, each type of study has its distinct advantages and disadvantages. Although human studies provide the most relevant information for human pathologies and therapies, they often lack adequate or healthy controls, have a large number of known and unknown variables, such as previous therapy, are not amenable to invasive procedures, and may not be adequately followed for longitudinal information. Furthermore, insights into the various aspects of these arthropathies, especially those requiring invasive procedures, cannot be adequately obtained from human specimens. Specific to studies on RA, synovial and joint tissues and fluid are normally obtained at the end-stage of the disease from patients undergoing joint replacement or in patients demonstrating symptoms necessitating arthroscopy or withdrawal of joint effusion, and from patients receiving one or more potent drugs such as non-steroidal anti-inflammatory drugs (Wooley et al., 1977a and b; Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein and Paine, 1992). These tissues represent the extremes of the natural history of RA. Additionally, control tissues are difficult, if not impossible, to obtain. Due to the various unknowns in the pathogenesis of RA, and due to the limitations of human studies, several animal models have been devised in order to further understand this pathology. These models of experimental arthritis have been investigated for clinical,

radiologic and histologic characteristics, for cellular and molecular immune response mechanisms, matrix loss genetic-dependence, and environmental and behavioral factors which affect the development or progression of this pathology. (For a summary of recent studies and findings on experimental arthritis see Table II).

5. ANIMAL MODELS OF EXPERIMENTAL ARTHRITIS

In order to supplement the information derived from human studies, and because of the important insights into the pathogenesis of RA attainable from animal models there is a great need for well characterized animal models of arthritis. Such models may provide a better understanding on the genetic and environmental factors predisposing to arthritis, and also allow a clearer definition of the cellular and molecular basis for this pathology. On the basis of methods used for induction of arthritis, these models can be divided into five main categories, namely: a) antigen, b) adjuvant, c) immunologic agents, d) chemical, and e) infectious. A sixth category of spontaneously occurring arthritis has also been identified in specific strains of mice. With the exception of the last model, within each category, various agents have been utilized for inducing arthritis. Furthermore, numerous animal species, ranging from the chicken to non-human primates, have been studied. The susceptibility of the species to the specific modality of induction may vary, as may the immunologic mechanism and the type of arthritis induced (e.g. polyarthritis vs monoarthritis). The clinical and histopathologic features of most of these models are similar to each other and akin to those observed in human RA, although differences exist in the severity, progression and duration of onset of arthritis between the various models.

Antigen-induced Arthritis

Antigens of foreign proteins as well as homologous proteins, mixed in oil emulsions or Freund's adjuvant, have been shown to have arthritogenic activities in various species of animals (see pages 27 to 30 of Table II)). Several different antigens have been utilized for inducing these forms of arthritis, and include type II collagen, ovalbumin (OA) or bovine serum albumin (BSA), and streptococcal cell wall (SCW). Because of the different inductive agents used, these experimental arthritides have previously been classified as collagen-induced arthritis (CIA), antigen-induced arthritis (AIA) and streptococcal cell wall-induced arthritis, respectively. However, since all these methods utilize antigens administered to animals with hypersensitized immune systems for developing arthritis, for the purposes of the present discussion these methods have all been categorized as antigen-induced arthritis. Antigen-induced arthritis may be further subdivided into two classes; one which utilizes a homologous or heterologous antigen,

Table II: Animal models of experimental arthritis.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
Antigen-induced					
Type II collagen	Bakker et al., 1990	Bovine collagen II in Freund's adjuvant	Rhesus monkey	Polyarthritis Joints of the hand	Characterization of model by clinical, radiographic and immunological parameters, and histology. 50% of animals demonstrated arthritis with raised levels of IgG and IgA.
	Boissier et al., 1987; 1988	Homologous collagen II	DBA/1 mice	Polyarthritis Fore- and hind-paws	Characterization of model by clinical observations, histopathology, and autoantibodies to collagen II. Arthritis induced with greater susceptibility of males than females, IgG2a autoantibody predominance associated with onset of arthritis.
	Brahn et al., 1992	Native chick collagen II in Freund's adjuvant	Louvain rats	Polyarthritis All paws	Clinical and radiographic parameters, humoral and cellular immune response. TNF-α aggravates CIA.
	Breban et al., 1993	Native collagen II	Susceptible Dark Agouti & resistant Fisher rats	Polyarthritis	Histopathology? & humoral response in germ-free and conventional rats. Bacterial flora had a suppressive effect on CIA.
	Cathcart, et al., 1986	Bovine collagen II in CFA	Squirrel and cebus monkeys	Polyarthritis & pauciarticular arthritis	Clinical and histologic examination, anti CII antibodies. Squirrel monkeys were susceptible while cebus monkeys were resistant to developing arthritis. High serum levels of anti C II antibody associated with susceptibility. Pathology resembled acute arthropathy rather than RA.
	Caulfield et al., 1982	Chicken collagen II	Wistar or Sprague-Dawley rats	Polyarthritis Knee joints	Characterization of model by light, fluorescent and electron microscopy. Initial stages with fibrin deposition, followed by synovial hyperplasia and inflammatory infiltration, and finally granulation tissue and scarring noted.
	Drelon et al., 1992	Collagen II and muramyl dipeptide	Wistar Furth rats	Polyarthritis	Effects of IL-1β on clinical and radiologic evaluations of arthritis. Low (.02mg) and medium (.2mg) doses of IL-1-β ameliorated established arthritis, while high doses (2mg) accentuated the pathology.
	Gillet et al., 1989	Human collagen II	Wistar Furth rats	Polyarthritis	Radiologic and histopathology assessment of model. CIA may also serve as a model for axial ossifying enthesopathy.
	Hasty et al., 1990	Bovine Collagen II in Freund's adjuvant	Wistar rats	Polyarthritis Hind paws	Clinical and histopathologic determination of arthritis, immunolocalization and biochemical determination of stromelysin and collagenase. Stromelysin found in both the synovium & chondrocytes, while collagenase localized primarily to the cartilage-pannus junction.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
	Healy et al., 1989	Bovine collagen II	Rhesus monkeys & DBA/1 LAC J mice	Polyarthritis Joints from hind and fore-limbs	Clinical, histopathologic and anti CII antibodies evaluated following induction of arthritis by intraperitoneal implantation of collagen II on nitrocellulose filters. Successful induction of polyarthritis noted in both species.
	Holmdahl et al., 1987	Native rat collagen II	DBA/1 mice	Polyarthritis All paws	Effects of β-oestradial on CIA by clinical & histopathologic evaluations, anti-collagen II IgG and T cell responses. Low doses of β-oestradial suppresses development of CIA and T cell dependent immune reactivity.
	Kuruvilla, et al., 1991	Chicken collagen II in Freund's complete adjuvant	DBA/1 mice	Polyarthritis Carpal and tarsal joints	The preventive and therapeutic effects of TGF-β1 on development of CIA evaluated clinically and histopathologically. TGF-β1 protects against and also prevents the development of CIA.
	Novotna et al., 1989	Porcine native collagen II, IX and XI and denatured collagen XI in Freund's adjuvant	Wistar rats	Polyarthritis Interphalangeal, metatarsophala-ngeal & tarsal joints	Clinical, radiographic, thermovision and histologic characterization of model, and antibody response. Arthritis was induced in 60% of the animals injected with collagens II and XI only.
	Peacock et al., 1992	Collagen II from chicken	Louvian rats	Polyarthritis Paws	Clinical, radiographic and immunologic assessment of therapy with oligoclonal antibodies raised to a collagen II-specific T cell line. Therapy diminished severity and incidence of arthritis.
	Persson et al., 1992b	Native rat collagen II	DA rats	Polyarthritis	Levels of dynorphin-converting enzyme and substance P endopeptidase in cerebrospinal fluid measured. Levels of both enzymes were significantly reduced during the acute phase of arthritis.
	Rubin et al., 1987	Bovine collagen II in Freund's adjuvant	Rhesus monkeys	Polyarthritis Hip, knee, shoulder & elbow joints	Characterization of model by gross & histolologic examination. Chronic arthritis with synovial changes, cartilage destruction and loss of safranin-O staining noted.
	Watson et al., 1990	Human collagen II in Freund's adjuvant	Diabetic resistant (DR) subline of diabetic BB rats	Polyarthritis Ankle joint	Immunogenetic mechanisms of CIA in DR rats with a RA susceptibility sequence to CII or cyanogen bromide fragment-11 of CII determined by light & electron microscopy. Supports the role of antibody mechanisms in CIA and recognizes CB11 fragment to be an arthritogenic epitope.
Albumin	Beesley et al., 1992	Ovalbumin in Freund's adjuvant	New Zealand white rabbits	Monoarthritis Knee joints	Evaluation of histopathology and histochemical changes in GAG and collagen staining of joint tissues. Demonstrated decrease in GAG, but not collagen, staining in articular cartilage. Increased collagen and GAG staining noted in synovium.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
	Brackertz et al., 1977	Bovine serum albumin in Freund's adjuvant	Mice (6 strains)	Monoarthritis Knee joints	Histology and genetic studies on susceptibility associated with allotypes. Arthritis induced was chronic, antigen-specific and T-cell dependent, C57BL and BALB/c mice were susceptible.
	Consden et al., 1971	Ovalbumin in Freund's adjuvant	Old English rabbits	Monoarthritis Knee joint	Determination of dose, retention of antigen and histology of arthropathy evaluated. Antigen retained above normal levels for up to 100 days and substantial degree of arthritis induced with doses above 100μg.
	Edwards et al., 1988	Ovalbumin in Freund's adjuvant	New Zealand white rabbits	Monoarthritis Knee joint	Quantitative histologic evaluation of model for total and specific cell counts for 3 doses of antigen. PMNLs increase early followed by lymphoid cells, Severe arthritis only achieved with a dose of 5 mg of ovalbumin.
	Henderson et al., 1990	Ovalbumin in Freund's adjuvant or polycation poly-d-lysine	New Zealand white rabbits	Monoarthritis Knee joint	Clinical evaluation of arthritis, biochemical determination of proteoglycan loss from articular cartilage and metalloproteinases synthesis by synovium and cartilage. Joint swelling and kinetics of proteoglycan loss were similar in both models; Only synovial lining synthesized MMPs in OA-induced arthritis, while both synovial lining and articular cartilage synthesized these proteinases in polycation-arthritis.
	Hollister & Mannik, 1974	Human serum albumin in Freund's adjuvant	New Zealand white rabbits	Monoarthritis Knee joint	Duration and mechanism of antigen (Ag) retention determined by radioisotope labeling of Ag and antibody. Half-life for Ag retention was highest for meniscus followed by articular cartilage, ligaments, synovium and synovial fluid. Ag strongly bound to collagenous tissue by immune complex formation with specific antibody.
	Howson et al., 1986	Ovalbumin in Freund's adjuvant	New Zealand White rabbits	Monoarthritis Knee joints	Characterization of model by radiography, histology, radioautography and transmission electron microscopy. High doses of antigen induced acute cartilage necrosis while low doses mild to moderate inflammation.
	Lowther et al., 1978	Human serum albumin in Freund's adjuvant	Rabbits	Monoarthritis Knee joint	Histologic assessment of arthritis, and biochemical analysis for collagen and proteoglycan in cartilage. Acute to chronic arthritis induced with little change in collagen content but substantial decrease in proteoglycan content & synthesis.
	Pettipher et al., 1989	Ovalbumin in Freund's adjuvant	New Zealand white rabbits	Monoarthritis Knee joint	Clinical and histologic evaluation of arthritis, and biochemical determination of PGE_2 and proteoglycans following administration of indomethacin. Indomethacin reduced joint swelling and PGE_2 levels, but increased lymphocytes and loss of proteoglycans.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
	Pettipher et al., 1990	Bovine serum albumin in Freund's adjuvant	C57 and beige C57 mice	Monoarthritis Knee joint	Evaluation of the role of neutrophil elastase and cathepsin G in pathogenesis arthritis using mice deficient in these proteases by histology, histochemistry and protease assays. Both the histology and loss of safranin-O staining was similar in normal and elastase/cathepsin G deficient mice.
Streptoccocal antigens	Barridge et al., 1988	Whole cell sonicates, heat-killed cells & cell wall preparations of S. pyogenes	Sprague-Dawley rats	Monoarthritis Ankel and knee joints	Clinical, radiologic and histopathology evaluation of arthritis, immunofluorescence for immune complexes. Differences in severity of arthritis noted between typical and atypical strains of S. pyogenes. Levels of immune complexes increased up to 90 days and correlated with chronic arthritis.
	Case et al., 1989a	Streptococcal cell wall	Lewis/N and Lew.rnu/rnu rats	Polyarthritis Ankle joint	Synovial tissue evaluated by Northern blots and immunohistochemistry for transin/stromelysin and c-myc. Increased transin and c-myc expression in both early thymus-independent SCW arthritis & in T-cell dependent adjuvant arthritis. Transin not expressed in athymic mice.
	Sano et al., 1990	Streptococcal cell wall	Lewis/N and Lew.rnu/rnu rats	Polyarthritis Hind limb joints	Detection and localization of heparin binding growth factor-1 (HBGF-1 or aFGF) mRNA and protein. HBGF-1 detected in bone marrow, bone, cartilage, synovium, ligaments and tendinous structures in both T-cell competent and incompetent rats.
	Sano et al., 1992	Streptococcal cell wall and adjuvant arthritis	Euthymic & athymic Lewis rats	Polyarthritis Hind limb joints	*In vivo* expression of cyclogenase (COX) in RA, OA and experimental arthritis evaluated by immunostaining. High levels and sustained COX staining seen in arthritic joints from euthymic but not athymic rats
Others	Cambray et al., 1981	Hyaluronic acid and poly-D-lysine	Old English rabbits	Monoarthritis Knee joints	Evaluation of metalloproteinases and inhibitor expression by synovium. Increased levels of collagenase and other MMPs together with disappearance of free inhibitor observed during early and late stages of arthritis.
	Gondolf et al., 1991	Chemically cationized proteins & natural polycations including lysozyme	Male Wistar rats	Monoarthritis Knee joint	Influence of charge & size on antigen handling examined by immunofluorescence, 99m Technetium-pertechnetate scintigram and histology. Both pI and molecular size were important for long-term persistence of antigen in joint tissues.
	Mertz et al., 1991	Yersinia enterocolitica intracellular protein	Rats -IA challenge following immunization	Monoarthritis	Characterization of model by technetium-99m scintigraphy & histology. Acute inflammatory response was followed by chronic arthritis.
	Murphy et al., 1981	Hyaluronic acid and poly-D-lysine	Old English rabbits	Monoarthritis Knee joint	Evaluation of metalloproteinase and inhibitor synthesis by articular cartilage explants. A large increase in synthesis of latent collagenase and decrease in 28 kDa inhibitor observed.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
	Noyori et al., 1992	Lipopolysaccharides from E. coli	Sprague-Dawley rats	Monoarthritis Ankle joint	Immunolocalization of LPS and IL-1, ELISA for rheumatoid factor-like substance & anti-LPS IgM. Arthritis accompanied by localization of LPS and IL-1 in synovial cells and pannus.
	Rubin & Roberts, 1987	M. butryricum in Freund's adjuvant	New Zealand white rabbits	Monoarthritis Knee joint	Effects of prednisolone administration on histomorphometric parameters. A decrease in cellularity in articular cartilage, and increased cellularity in patella, femoral and tibial cartilages observed in prednisolone treated animals.
Adjuvant-induced					
	Calvino et al., 1987	Freund's adjuvant	Sprague Dawley rats	Radiocarpal & tibiotarsal joints	Clinical, radiologic and behavioral observations. 4 clinical & behavioral stages defined: preclinical, acute, post-acute & recovery.
	Calvino et al., 1991	Freund's adjuvant	Rats	Polyarthritis	Clinical & behavioral observations, plasma and cerebrospinal fluid substance P immunoreactivity. Plasma substance P elevated in acute & post-acute phases while levels in CSF increased in acute but not post-acute phase.
	Case et al., 1989a	Freund's adjuvant	Lewis/N and Lew.rnu/rnu rats	Polyarthritis Ankle joint	See streptococcal cell wall arthritis.
	Case et al., 1989c	M. butyricum in mineral oil	LEW/N rats	Polyarthritis Fore- and hind limbs	Therapeutic effects of chimeric cytotoxin, IL-2-PE40 evaluated by clinical, histologic and radiographic criteria. IL-2-PE40 delayed and mitigated the development of arthritis.
	Francis et al., 1989	Modified Freund's adjuvant	Sprague Dawley rats	Polyarthritis Paw	Clinical & histopathologic evaluation in response to prophylaxis or treatment with NE-58095, a disphosphonate. NE-58095 decreased the severity of arthritis.
	Godefroy et al., 1987	M. butyricum in Freund's adjuvant	Sprague Dawley rats	Polyarthritis	Levels of 5-hydroxytryptamine measured. Marked increase in 5-HT in serum, and initial increase in CNS which returned to normal levels.
	Greenwald et al., 1992	Freund's adjuvant	Lewis rats	Polyarthritis Paw	Effects of tetracycline and flurbiprofen clinical, radiographic and histologic signs of inflammation, and on metalloproteinase expression. These drugs administered together significantly reduced collagenase and gelatinase activity as well as joint damage.
	Issekutz & Issekutz, 1991	M. butyricum in oil	AO rats	Polyarthritis Hind- & forelimb joints	Clinical, histopathology, exudation of plasma proteins as well as migration of radioisotopically-labeled PMNLs and T-lymphocytes evaluated. Symmetrical arthritis was induced in most animals. PMN migration enhanced before accumulation of T-lymphocyte and onset of clinical disease; both cell types peaked at 2-3 weeks and then declined by 4 weeks.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
	Levine et al., 1988	M. butyricum in oil	Sprague-Dawley Rats	Polyarthritis	Clinical and radiographic evaluation of α- and β-adrenergic antagonists on joint injury. β2-adrenergic receptor antagonists, but not β1- or α–receptor antagonists, attenuate the onset and progression of joint injury.
	Reddy & Dhar, 1992	Freund's adjuvant with killed M. tuberculosis	Wistar rats	Polyarthritis Hind limbs	Clinical assessment of arthritis and evaluation of collagen metabolism in bone & tendon by measuring ^{3}H-hydroxyproline. Decreased total collagen content, decreased collagen synthesis and increased urinary ^{3}H-OHproline noted.
	Rubin & Roberts, 1987	Freund's adjuvant with killed M. butyricum	New Zealand White rabbits	Monoarthritis Knee joint	Evaluation of therapeutic effects of prednisolone by histology & histomorphometry. Increased cellularity of articular cartilages and retention of safranin-O staining observed in prednisolone treated animals.
	Sano et al., 1992	M. butyricum in paraffin oil and streptococcal cell wall	Euthymic & athymic Lewis rats	Polyarthritis Hind limb joints	See Antigen-induced Arthritis (Streptococcal Cell Wall Arthritis).
	Setnikar et al., 1991	Kaolin and M. butyricum in mineral oil	Sprague-Dawley rats	Monoarthritis and polyarthritis	See Chemical and Mechanical Irritants.
	Thompson et al., 1991a	Pristane	CBA/IgB mice	Polyarthritis Ankle joint	Immuonoprophylactic & immunotherapeutic potential of M. vaccae heat shock protein on pristane-induced arthritis determined clinically. Protection or exacerbation of arthritis dependent on dose and route of immunization of M. vaccae.
	Wooley et al., 1989	Pristane	Several strains of mice	Polyarthritis	Clinical, histologic and serologic characterization of model. Variable incidence of arthritis induced in susceptible strains; Association between arthritis susceptibility and RF seropositivity and incidence of anti-C II antibodies observed.
	Zamma, 1983	Mixed killed mycobacteria in squalene	Wistar, Sprague-Dawley & Lewis rats	Polyarthritis Temporomandi-bular joint	Development and characterization of model for routes of adjuvant delivery, and clinical & histologic parameters of arthropathy. Only Lewis rats developed severe disease following injection through parietal scalp.
Immunologically-induced arthritis (T-cell clones, cytokines, antibodies and passive transfer)					
Antibodies	Bjurstein et al., 1983	Immune complexes of BSA with antibodies retrieved from animals sensitized to BSA in Freund's adjuvant	Rabbits	Monoarthritis Knee joint	Leucocyte migration kinetics evaluated by morphologic and immunofluorescent labeling. Complement-activating immune complexes were located in leucocyte granulae, while complexes formed with antigen excess were associated with leucocyte membrane and caused pronounced leucocyte migration.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
	de Clerck et al., 1992	Monoclonal rat IgE	Sprague-Dawley rats	Monoarthritis Knee joint	Evaluation of histamine concentration in synovial fluid, permeability of synovial membrane and histopathology. IgE demonstrated to be arthritogenic.
	Fehr et al., 1982	Autologous Fab_2 from cathepsin D digested IgG	White Kunath rabbits	Monoarthritis Knee joint	Model characterized by immunohistochemistry, histology, and RF-like antibodies. Acute, subacute and chronic synovitis noted with increasing number of injections, Local formation of RF-like antibodies by synovial plasma cells.
	Yoo et al., 1988	Homologous rabbit IgG in Freund's adjuvant	New Zealand white rabbits	Monoarthritis Knee joint	Gross pathology and immunofluorescence with monoclonal antibodies to proteoglycans. Synovitis and pannus formation noted. Altered PG staining noted in arthritic cartilage.
Cytokines	Limb et al., 1989	Lymphokines & oil-elicited peritoneal exudate cells	Hartley strain guinea-pig	Monoarthritis Knee joint	Clinical and histopathologic characterization of model. Lymphokine induction demonstrated features of acute followed by chronic arthritis, which were less intense then with lymphokine and oil-elicited peritoneal exudate cells.
T cell clones and lines	Holmdahl et al., 1985	T-cell clones or lines derived from animals sensitized to C-II or OA in Freund's adjuvant	DBA/1 mice	Polyarthritis Hind foot joints	Effects of administration of T-cell clones / lines derived from sensitized animals on development of arthritis in non-radiated and irradiated syngeneic naive animals assessed histologically. Arthritis was induced in both groups of animals with C-II-specific T-cell lines and an autoreactive and C-II-specific T-cell clone. Fewer cells were needed to evoke arthritis in normal than in irradiated mice.
Chemical and Mechanical Irritants					
Carrageenan	Hansen et al., 1991	Carrageenan	Juvenile dogs	Monoarthritis Knee joints	Relationship between [^{99m}Tc] disphosphonate uptake and bone hemodynamics determined by measuring blood flow, plasma and red cell volumes. Disphosphonate uptake correlates positively with blood flow and plasma volume and negatively with red cell volume in a non-linear fashion.
	Hansen et al., 1992	Carrageenan	Juvenile dogs	Monoarthritis Unilateral gonarthritis	Effect of naproxen treatment on intraosseous vascular permeability, blood flow, plasma volume and bone metabolism. Naproxen reduced arthritic capsular hyperemia, and normalized blood flow & bone metabolism.
	Herlin et al., 1988	Carrageenan	Juvenile dogs	Monoarthritis Knee joint	Evaluation of lipoxygenase products of arachadonic acid. Increased leukotriene B4 and decreased 15-hydoxyeicosatetraenoic acid & 13-hydroxy-9,11-octadecadienoic acid noted in arthritic joints.
Kaolin	Setnikar et al., 1991	Kaolin and M. butyricum in mineral oil	Sprague-Dawley rats	Monoarthritis and polyarthritis	Therapeutic effects of glucosamine sulfate on clinical and histopathologic features of arthritis. Glucosamine sulfate was effective against experimental arthritis.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
Infectious					
Bacterial	Alderson & Nade, 1987	Staph. aureus	Chicken	Monoarthritis Hock joints	Histopathology, number of bacteria & number & type of leukocytes. Early increase in bacterial & leukocyte numbers. Gross destruction of articular cartilage observed by 4 days & associated with adherence of S. aureus to cartilage.
	Bremell et al., 1991; 1992	Staph. aureus LS-1	Mice	Polyarthritis Foe- & hindpaws and tail	Histopathology & serology (IL-6, TNF-α, IgG and autoantibodies). Arthritis apparent within 24 hours with bone and cartilage destruction obvious in 72 hours. Serum IL-6, TNF- α, antibodies to S. aureus and autoantibodies were elevated early and maintained at high levels.
	Linhart et al., 1990	Staph. aureus	Rabbits	Monoarthritis Knee joint	Effect of superoxide dismutase (SOD) on severity of arthritis evaluated by measuring lipid peroxide in plasma & synovial fluid, macroscopic & microscopic quantitation. SOD enhanced severity of arthritis.
	Patel et al., 1988	S. aureus	Chicken	Monoarthritis Hock joint	Therapeutic effects of cloxacillin assessed by growth rate, clinical & histologic evaluations, and bacterial & leucocyte counts. Regimen of cloxacillin given not adequate to prevent joint destruction.
	Renz et al., 1989	Erysipelothrix rhusiopathiae	Lewis and Han-rnu rats	Polyarthritis Hind limb joints	Evaluation of macrophage & lymphocyte stimulation during acute inflammation evaluated clinically clinically and biochemically. Temporally regulated synthesis of TNF-α, IL-1 and PGE_2 by macrophages and development of arthritis was dependent on T-lymphocytes.
	Schmitz et al., 1988	Borrelia burgdorferi	LSH/Ss hamster	Polyarthritis Hind paws	Characterization of lyme arthritis by histopathology. While non-irradiated hamsters develop only transient synovitis, irradiated animals showed neutrophilic infiltrate, granulation tissue and inflammation.
	Tissi et al., 1990	Type IV Strep. agalactiae	CD-1 mice	Polyarthritis	Characterization of model clinically and by histology, determination of antibody titers. Acute exudative synovitis was followed by chronic infection, and ankylosis. IgG and IgM classes of antibodies were detected.
Fungal	Nakamura et al., 1993	Candida albicans	Sprague-Dawley rats	Polyarthritis Limb joints	Characterization of model by radiographic and histologic examination. Abnormal formation of exostosis and bone resorption noted.

MODEL	REFERENCE	INDUCING AGENT	ANIMAL	TYPE & / OR JOINT	TECHNIQUES USED & FINDINGS
Spontaneous					
	Boissier et al., 1989	Homologous collagen II	MRL/1 mice	Polyarthritis Fore- & hind-paws	Clinical and histopathologic evaluation, anti C II antibodies. C II accelerated but did not increase the severity of spontaneous arthritis in MRL/1 mice.
	Bouvet, et al., 1990	N.A.	Biozzi H1 mice	Polyarthritis	Clinical and histopathologic assessment of arthritis, ELIZA for anti-C-II, RF and anti-nuclear (AN) antibodies. Features characteristic of RA observed. RF, AN, anti-C-II antibodies found in sera of arthritic and clinically normal mice.
	Hang et al., 1982	N.A.	MRL/1 mice	Polyarthritis Hind limb joints	Histologic and electron microscopic evaluation of arthritis, and serology for anti-single stranded DNA, anti-double stranded DNA and IgMRF. RA-like pathology observed with 75% incidence and close correlation between presence of circulating IgMRF and joint pathology.
	Holmdahl et al., 1992	N.A.	DBA/1 mice	Polyarthritis	Evaluation of clinical arthritis, anti C II antibodies and immunolocalization of immunologic markers. Spontaneous age-, sex- and behavior-dependent as well as genetically restricted arthritis develops in DBA/1 mice.
	Tarkowski, et al., 1987	N.A.	MRL/1 mice	Polyarthritis	Immunohistochemical characterization of synovial cells. Large numbers of macrophage-like cells in synovial lining and synovium, few Ia-expressing cells in synovial lining but present in deeper synovial tissue, and lymphocytes were absent.

N.A. = not applicable

such as type II collagen (CII), which following systemic administration results in polyarthritis in susceptible strains of animals, such as rats, mice and monkeys, and another in which systemic sensitization with an antigen, such as ovalbumin, has to be followed by an intra-articular challenge with the same antigen to cause a monoarthritis. The former type of model has often been referred to as experimental immune arthritis (EIA). In both categories of antigen-induced arthritis, the antigens are generally systemically delivered as an emulsion in complete Freund's or incomplete Freund's adjuvant or in oil, since many of these agents are arthritogenic only when administered in the form of an emulsion in an oily vehicle. These oily substances include squalene, mineral oil, and olive oil amongst others, and are mostly biologically inert, and not in themselves arthritogenic, but serve to modulate the intrinsic activity of the arthritogenic materials (Pearson and Wood, 1959). The inducing antigen is arthritogenic and it appears that, regardless of the antigen used, the arthritis induced is a progressive immune-mediated pathology dependent upon both cell and humoral immunity.

Collagen-induced arthritis was first described in 1977, when it was demonstrated that heterologous or homologous CII, in incomplete Freund's adjuvant, when injected intradermally, induced synovitis in 40% of rats of several strains (Trentham et al., 1977). Native types I and III collagen as well as denatured type II collagen were not arthritogenic. A similar protocol utilizing CII in complete, rather than incomplete Freund's adjuvant, was used to induce the disease in mice (Courtenay, et al., 1980) and non-human primates (Cathcart, 1986). The visible signs of arthritis are first apparent in the rat model after 2 to 3 weeks (Brahn et al., 1992; Drelon et al., 1992) whereas disease onset in mice occurs at 4 to 6 weeks (Boissier et al., 1988), and in monkeys at 3 to 9 weeks (Caulfield et al., 1982; Cathcart, 1986; also reviewed in Wooley and Chapedelaine, 1987). The disease pathology is basically similar in all three species. Affected animals develop an acute erythema and edema in one or more peripheral limbs, with metatarsal joints being the most frequent site of disease onset, and the disease usually progresses to a chronic, erosive arthritis. A high incidence of joint ankylosis is also noted in rodent collagen arthritis (Wooley and Chapedelaine, 1987). Histologically, the earliest signs of the disease include subsynovial infiltration by inflammatory cells and synovial hypertrophy, which appears to be maximal around synovial-cartilage junction in early arthritis (Caulfield et al., 1982; Healy et al., 1989). The rapid development of a cellular exudate in the synovial fluid and of pannus are followed by erosion of both cartilage and subchondral bone (Trentham et al., 1977; Caulfield et al., 1982; Cathcart et al., 1986; Bakker et al., 1990). Periostitis is also detected, and by the ankylosis phase of the disease, the joint architecture is disrupted with new bone and collagen formation evident.

The second method for inducing arthritis by antigens relies on a systemic sensitization of animals to an antigen, followed by an intra-articular challenge with the same antigen. The most commonly used proteins for this model include OA and BSA. The inflammatory stimulus in this type of antigen-induced arthritis involves a secondary immune response to locally administered antigen. This model, although not the same as the systemic autoimmune synovitis seen in RA, remains the model of choice for studying local cell and tissue changes because of its histopathologic similarities to RA and its chronicity (Edwards et al., 1988; Henderson et al., 1993). Dumonde and Glynn (1962) were the first to report the successful induction of arthritis by intra-articular injection of fibrin in rabbits previously sensitized with this antigen in complete Freund's adjuvant. Since then, the rabbit has been commonly used for this model, and has been considered an animal of choice over mice and rats because of its closer resemblance to human disease with respect to cellular infiltrate (Doble, 1986). The rational for using rabbits for the purposes of inducing TMJ arthritis in our studies is further strengthened because the rabbit TMJ is considered to be a suitable anatomic and functional analogue for the human TMJ (Weijs and Dantuma, 1981; Scapino, 1983), and because the TMJ of this animal has been well characterized (Weijs and Van der Weilen-Drent, 1982; Mills et al., 1988; Nagy and Daniel, 1991). In both CIA and arthritis induced by localized administration of antigen into the joint of previously sensitized animals, a loss of matrix macromolecules, primarily proteoglycans, from the articular cartilage is commonly observed (Lowther et al., 1978; Healy et al., 1989; Hasty et al., 1990; Pettipher et al., 1990; Beesley et al., 1992; Henderson et al., 1993) .

The immune mechanisms for CIA have been more extensively studied than those for other models of antigen-induced arthritis. Since many of these findings are also likely to hold true for other models of antigen-induced arthritis, such as that initiated by OA, a brief discussion is provided below. The immune responses in CIA or experimental immune arthritis implicate the role of both cell-mediated immunity and humoral immunity in this arthropathy. Boissier et al. (1988) demonstrated that a progressive and chronic polyarthritis was induced when homologous CII in complete Freund's adjuvant was injected into the footpad of DBA/1-susceptible mice. Although anti-CII autoantibodies were detected in all immunized animals, the onset of disease was associated largely with the predominance of IgG2a autoantibodies. Additionally, specific cellular autoreactivity to homologous CII occurred in lymphoid cells derived from the spleen, as well as peripheral and afferent popliteal lymph nodes of immunized animals. Further convincing evidence of the role of T cells in heterologous CIA is provided by experiments demonstrating that thymus-deficient rats do not develop arthritis (Klareskog, 1983), that CIA is prevented

after *in vivo* treatment with anti-T-cell autoantibodies (Ranges, 1985), or that disease can be transferred by administering mononuclear cells (Trentham et al., 1976) or serum (Stuart et al., 1982) from CII-sensitized donors or by giving CII-reactive T-cell lines (Holmdahl et al., 1985) to naive animals.

Both the histopathology and immune response in antigen-induced arthritis indicate its suitability as a model for RA. Synovial lining, stroma and villous hyperplasia together with pannus formation are commonly observed in this model (Dumonde and Glynn, 1962; Consden et al., 1971; Brackertz et al., 1977; Howson et al., 1986; Edwards et al., 1988; Beesley et al., 1992). Both lymphocyte and plasma cell infiltration of synovium are usually evident (Dumonde and Glynn, 1962; Consden et al., 1971; Edwards et al., 1988). Immunologic responses include the synthesis of specific antibodies against the immunizing antigen (Cooke et al., 1972a), and deposition of antigen-antibody complexes and complement C3 in the collagenous matrix of the joint (Cooke et al., 1972b). While the retention of antigen-antibody complexes may explain the chronicity of the arthropathy (Cooke et al., 1972b; Consden et al., 1971; Webb et al., 1971; Hollister & Mannik, 1974), it has also become apparent that cell-mediated immunity is a prerequisite for inducing chronic arthritis (Consden et al., 1971; Loewi, 1968; Menard and Dion, 1975), and that the persistence of immune complexes cannot alone explain the chronicity of the response (Fox and Glynn, 1975). Most of these observations are similar to those made in RA (Smiley et al., 1968; Slinwinski and Zvaifler, 1970)

Howson et al., (1986) examined the relevance of the antigen-induced arthritis model to human disease, by challenging rabbit knee joints with a high (7.5mg) and low (0.1 mg) dose of ovalbumin in previously sensitized animals. Severe and persistent synovitis with synoviocyte hyperplasia and polymorphonuclear infiltrate was evident as early as 5 to 7 days after challenge. By 14 days, thick villous synovium and a predominantly monocytic infiltrate had been established. The animals subjected to low challenging dose demonstrated a mild arthritis and synovitis persisted for 11 months. In both the high and low dose groups loss of really GAGs from articular cartilage, as evidenced by diminished Safranin-O and toluidine blue staining, was also observed. The investigators noted that the severity of this experimental arthropathy depended on the challenging dose, and suggested that the dose of antigen and its resulting pathology should be monitored by histologic examination in future studies utilizing this model for understanding the pathogenesis and mechanisms of RA.

Other studies indicate that both the retention of antigen and the arthritic response is specific to the sensitizing antigen (Consden et al., 1971; Hollister and Mannik, 1974). Using ^{125}I labeled ovalbumin for inducing arthritis in previously sensitized rabbits,

Consden et al. (1971) reported both an antigen-specific and sensitization-specific retention of ovalbumin in knee joints of these animals. Unsensitized animals as well animals sensitized with OA in incomplete Freund's adjuvant demonstrated very little long-term retention of OA in tissues of the joint. Similarly, animals sensitized to OA in CFA but challenged with radiolabeled bovine gamma globulin demonstrated a return of antigen levels to those of unimmunized controls 1 week following the challenging dose. In contrast, animals sensitized with OA in CFA and then challenged with OA showed prolonged periods of retention of the antigen in tissues of the joint and may partially explain the persistence of inflammation for up to 6 months.

In the case of the bacterial cell wall-induced arthritis the mechanism for development of arthritis is not clearly understood, but some evidence suggests an aberrant immune response involving primarily cell-mediated (delayed) hypersensitivity, although humoral immune response may play a role in early stages of disease development (Mackenzie et al., 1978; Trentham et al., 1978). It is not clear, however, whether the bacterial cell wall materials provide the necessary immunogen(s) or the necessary stimulus for the development of an aberrant immune response to some endogenous immunogen(s) within the host.

Adjuvant-induced Arthritis

Pearson (1956) first described the induction of aseptic experimental arthritis in rats by administration of adjuvant containing mycobacterial cell wall fragments. Subsequently this model of adjuvant-induced arthritis has been induced in several susceptible animals by a single injection of oil adjuvant that may or may not contain immunogenic bacterial components, usually a suspension of inactivated mycobacteria (Chang et al., 1980; Calvino et al., 1987 and 1991; Case et al., 1989a and c; Wooley et al., 1989; Thompson et al., 1991a; Reddy and Dhar, 1992). The challenge with a strong adjuvant induces an inflammatory response which for unknown reasons, manifests primarily, but not only, as arthritis. Usually a polyarthritic condition is induced whose histopathologic features include perivascular accumulation in the synovium of mononuclear cells, infiltration by PMNLs and pannus formation with synovial lining hyperplasia (Zamma, 1983; Rubin and Roberts, 1987; Issekutz and Issekutz, 1991). This form of arthritis involves the immune system, and the pivotal role of T-cell immunity has been well demonstrated (reviewed in Wilder, 1988a and b, and Wilder et al., 1991). The dominant inflammatory cells within the joint lesion are mononuclear cells, chiefly lymphocytes (Pearson and Wood, 1959; Jones and Ward, 1963); T helper/inducer cells dominate the lymphoid infiltrates (Larsson et al., 1985) and the arthropathy may be induced in heterozygous euthymic rnu/+ rats but not in homozygous nude athymic rats (Kohashi et al., 1982). Anti-lymphocyte globulin as well

as W3/13 (anti-pan T) monoclonal antibodies suppress the disease (Currey and Ziff, 1968; Larsson et al., 1985). Furthermore, the lesion may be passively transferred to naive animals by injection of T lymphocytes from adjuvant injected donors (Waksman and Wennersten, 1963; Taurag et al., 1983; Holoshitz et al., 1983). A few important differences exist between adjuvant-induced arthritis and antigen-induced arthritis. For example, while adjuvant-induced arthritis appears to rely upon an individuals' immune response, the immune mechanisms in antigen-induced arthritis seem to have a closer similarity to rheumatoid arthritis. Adjuvant arthritis is also believed to be a manifestation of delayed type hypersensitivity to disseminated mycobacterial fragments present in complete Freund's adjuvant (Waksman et al., 1960).

Immunologically-induced Arthritis

Several models of arthritis induced by cytokines, antibodies, and T-lymphocytes derived from sensitized animals have also been described (Fehr et al., 1982; Bjurstein et al., 1983; Holmdahl et al., 1985; Yoo et al., 1988; Limb et al., 1989; de Clerck et al., 1992). The purpose of most of these studies has been to understand the role of specific immunologic agents in progression of arthritis or the potential immune mechanisms of arthritis. For example it has been shown that transfer of CD4+ collagen II reactive T cells into naive animals induces arthritis (Holmdahl et al., 1985; Kakimoto et al., 1988), and that various T cell produced interleukins promote development of arthritis disease (Helfgott et al., 1985; Mauritz et al., 1988). Similarly, it has been shown that passive transfer of anti-CII immune serum (Stuart et al., 1982 and 1983) and possibly purified anti-CII antibodies (Kerwar et al., 1983) induce acute arthritis in naive animals. Such studies have enabled a better understanding of the complexities of immune interactions in arthritis. The histology observed in these models is not unlike that of RA with synovial lining and villous hyperplasia, mononuclear cell infiltration and pannus formation (Fehr et al., 1982; Yoo et al., 1988; Limb et al., 1989; de Clerck et al., 1992).

Chemical and Mechanical Irritants

Several chemical agents or mechanical irritants, including carrageenan and kaolin, have also been used for inducing arthritis (Bunger, 1987; Herlin et al., 1988; Setnikar et al., 1991; Hansen et al., 1991 and 1992). These agents are usually administered repeatedly into the joint in order to maintain a chronic pathology. Carrageenan is a sulfated polysaccharide form a cartilaginous seaweed, *Chondrus crispus.* Following intra-articular administration of this agent, the initial inflammatory event appears to involve the activation of the complement pathway, followed by an immediate release of histamine, serotonin, kinins and eicosanoids (Hansen et al., 1992). Carrageenan is readily ingested by synovial macrophages and stored in lysosomes, but is resistant to degradation. Subsequent rupture

of the lysosomes may release degradative enzymes which contribute to local tissue breakdown and further perpetuation of the inflammation. The specific immunopathogenesis of carrageenan-induced arthritis, therefore, does not mimic RA, but the local articular manifestations reflect chronic joint inflammation.

Infectious Arthritis

Several animal species, including chickens (Alderson and Nade, 1987), mice, rats, hamsters and rabbits have been used for this model of arthritis. Acute septic arthritis is induced by injecting bacteria (e.g. S. aureus) or fungi (C. albicans) into the joint. Many of the studies conducted to date on this type of experimental arthritis have been done to provide a clinical and histopathologic characterization of the model (Alderson and Nade, 1987; Schmitz et al., 1988; Tissi, 1990; Bremmel et al., 1991 and 1992; Nakamura et al., 1993). The findings generally indicate a rapid and gross destruction of joint tissues, including cartilage. In the chicken hock joint this gross destruction of articular cartilage occurs as early as 4 days, and is probably initiated by adherence of the staphylococci to the cartilage surface followed by their penetration and invasion into vascular tunnels in the epiphyseal cartilage (Alderson and Nade, 1987). Bacterial counts reveal early increases in numbers of bacteria with concomitant expansion of the leukocyte population in the joints. Although the arthritis is rapid and severe, this model is substantially different from RA, since the severity and progression of the arthritis appears to be largely determined by the infectious agent (Alderson and Nade, 1987). This arthropathy, therefore, better represents septic arthritis than RA.

Spontaneous Arthritis

Certain species of mice, including the MRL-lpr/lpr (MRL/I) (Eisenberg et al., 1979; Hang et al., 1982; Boissier et al., 1989), and DBA/1 mice (Holmdahl et al., 1992) are susceptible to developing spontaneous polyarthritis. Macroscopically, the involved joints often demonstrate swelling, while histology reveals infiltration by inflammatory macrophages and neutrophilic granulocytes, as well as pannus formation and destruction of bone and cartilage (Boissier et al., 1989; Holmdahl et al., 1992). However, in both these models no T lymphocytes are evident and the pathology does not correspond with autoimmune manifestations (Tarkowski, et al., 1987; Holmdahl, 1992). For example, immunization of the LPR/1 mice with homologous type II collagen increases the levels of circulating autoantibodies to mouse-CII and accelerates the appearance of the arthropathy but does not impact on its severity, indicating that spontaneous arthritis appears to occur independent of an autoimmune response to native CII (Boissier et al, 1989). The etiology and underlying mechanism for spontaneous arthritis are largely unknown, although the manifestations appear to be dependent upon age, sex, genetic, hormonal and behavioral-

mediated influences (Holmdahl et al., 1992). It has been shown that the MRL/1 strain of mice carry recessive genes promoting expansion of immature lymphocytes leading to lupus disease, and in later stages, a mild arthritis develops which does not correlate with autoimmune manifestations (Tarkowski, et al., 1986). Because of the differences in the presentation of spontaneous arthritis from that of other animal models of RA and from human RA in its predictable course of development, limited distribution to specific joints, and minimal immunologic manifestations, it has been suggested that this arthropathy may be more useful as a model for OA than RA (Holmdahl et al., 1992).

Experimental Arthritis of the Temporomandibular Joint

Despite the successful induction of arthritis in various joints, similar success has not been reported or attempts been made to induce arthritis in the TMJ. Only one partially successful attempt has been reported at inducing arthritis in the TMJ. Zamma (1983) developed a model of adjuvant-induced arthritis in Lewis rats by immunizing these animals with mycobacterial dilapidated cells in squalene through intradermal injections into the parietal scalp, rather than the footpad immunization normally used for inducing arthritis in the knee joint. Histologically evident incidence of TMJ arthritis ranged from 30% to 60% in rats immunized with 0.5 mg and 1 mg of Mycobacterium, respectively. All animals also demonstrated clinical signs of systemic polyarthritis. The TMJ arthritis was aggravated in animals in which teeth were extracted in one quadrant of the mouth, indicating the potential role of mechanical loading on the progression of experimental arthritis. Wistar and Sprague-Dawley rats were not susceptible to developing TMJ arthritis by this method. Furthermore, immunization of Lewis rats into other sites, including intradermal injection into the tail, posterior cervical region, or intra-inguinal lymphnodes did not induce TMJ arthritis. The systemic polyarthritis induced by immunization into the scalp was less severe than that produced by other routes of immunization. Although histologic evidence of TMJ arthritis is provided, the investigator reports a milder inflammatory response in the TMJ than in other joints. Furthermore, the data and information provided do not convincingly demonstrate a successful induction of arthritis. The findings of this study, therefore, raise questions about the severity, incidence and reproducibility of this technique for inducing TMJ arthritis.

Several important questions may be addressed with the development of a reliable and reproducible method for inducing arthritis in the TMJ. The issues to be addressed by such a model can be placed into two main categories as outlined below.

(1) Those related specifically to the TMJ: These include (a) elucidating the potential impact of the unique morphologic structures (e.g. the disc), organization and function of the TMJ on the pathophysiology of TMJ arthritis, (b) determining the effects of

TMJ arthritis in promoting aberrations in functions of specific tissues of the TMJ, such as the condylar growth cartilage, and (c) providing insights into the basis for other pathologies of TMJ, such as internal joint derangement and degenerative joint disease, which are more prevalent than RA and yet remain relatively poorly understood.

(2) Those of a more general nature which will complement or add to our existing knowledge on RA gained from other animal models and human studies, or will help bring about new knowledge on the pathogenesis of this disease. Therefore, this model may be as favorable as others for research in the pathogenesis and immunology of RA, in assessing the mechanisms for matrix loss during this arthropathy, and for evaluating the efficacy of anti-arthritic therapies.

Although the one previous model of TMJ arthritis was developed using adjuvant in susceptible strains of rats, for our studies we have opted to develop a model of antigen-induced arthritis in the rabbit TMJ for several reasons. First, the morphology and functional physiology of the rabbit TMJ is well characterized (Weijs and Dantuma, 1981; Weijs and Van der Wielen-Drent, 1982; Lund et al., 1984; Anapol et al., 1987; Mills et al., 1988; Nagy and Daniel, 1991; Savalle et al., 1990), providing much of the baseline information necessary for further use of the TMJ in this animal for development of models of arthritis. Second, the rabbit TMJ is considered to provide a suitable analogue to the human TMJ (Weijs and Dantuma, 1981; Scapino, 1983; Mills et al., 1988). The histology and histochemistry of the joints from the two species demonstrate numerous similarities including cellular organization, and distribution of collagen types I and II, proteoglycans, and elastin (Mills et al., 1988; Nagy and Daniel, 1992). The rabbit disc, for example, is biconcave, as in man, and has anterior and posterior band areas rich in PGs, separated by a narrow intermediate zone rich in antero-posteriorly directed collagen fibers (Mills et al., 1988). Furthermore, jaw movements in biting and chewing are similar in the two species (Weijs and Dantuma, 1981). Third, antigen-induced arthritis has been successfully and reliably induced in the rabbit knee by several investigators (Dumonde and Glynn, 1962; Consden et al., 1971; Lowther et al., 1978; Cambray et al., 1981; Murphy et al., 1981; Howson et al., 1986; Edwards et al., 1988; Henderson et al., 1990; Beesley et al., 1992), indicating a potential for similar success in the TMJ. Finally, in contrast to adjuvant-induced arthritis which requires susceptible strain of animals, antigen-induced arthritis produced by intra-articular challenge can be induced with a higher incidence, and in a non-susceptible animal such as the rabbit.

Despite these similarities, several differences also exist between rabbit and human TMJs. Firstly, the rabbit mandible has two parts joined at the symphysis by a fibrous joint as compared to the single bone in man. This fibrous union of the two halves of the

mandible in the rabbit, however, is stiff and the mandible essentially acts as a single functional unit (Weijs and Dantuma, 1981). Secondly, whereas the squamosal articular surface forming the glenoid fossa provides a complete roof over the disc and condyle in man, in the rabbit the temporal bone provides only a small articular surface such that the rabbit skull does not wall off the joint anteriorly and superiorly. This articular surface of the temporal bone in the rabbit is analogous only to a small part of the human glenoid fossa, namely the articular eminence in man (Nagy and Daniel, 1991). Due to this lack of a supero-posterior wall, the posterior band of the rabbit disc attaches with fibers of the temporalis muscle, as opposed to the bony attachment to the glenoid fossa in man (Mills et al., 1988). Thirdly, the rabbit condyle also differs from that in man in having a narrow projection extending posteriorly. As in man, however, the rabbit condyle is convex both antero-posteriorly and medio-laterally.

One important feature of the TMJ is its condylar cartilage which serves as a growth site for the mandible (Charlier et al., 1969; Petrovic et al., 1975; McNamara and Carlson, 1979; Whetten and Johnston, 1985; Groote, 1985), and as such, may be impacted upon by joint pathologies. Therefore, a model of TMJ arthritis in juvenile animals may offer substantial advantages in understanding the basis for aberrant mandibular growth in JRA. However, despite the availability of methods to induce arthritis in animals, these methods have largely been utilized in adult non-growing animals, and to our knowledge, only one such animal model has been described to date and used for studies evaluating the response to therapy (Hansen et al., 1992), and the expression of inflammatory agents during this arthropathy (Herlin et al., 1988). Such a model, if developed in the juvenile animal, may subsequently be useful in better understanding JRA of the TMJ, particularly with regard to the mechanisms for disturbed mandibulofacial development, evaluating the loss of extracellular matrix and the modalities for this matrix loss, and for comparing the differences in these mechanisms between juvenile and adult animals.

Limitations and Uses of Animal Models of Arthritis

Although animal models are likely to provide important information on the pathogenesis of RA, there are several specific limitations of these models. Two primary limitations should be considered when interpreting information derived from these models. Firstly, since the etiology of RA remains unknown, induction of arthritis in these animal models is based on procedures which cause joint inflammation in response to an antigen which may not be relevant to the initiation of RA, rather than inducing inflammation on the basis of any rational evidence of the etiology. Nevertheless, many of the post-induction events in several of these experimental models of arthritis closely mimic RA (Dumonde and Glynn, 1962; Edwards et al., 1988; Henderson et al., 1993). Secondly, substantial

differences exist in the organization and function of joints as well as the immune systems of humans and lower mammals. For this reason, the pathogenesis in the animal model may not accurately represent every aspect of the human disease. Such differences may introduce disparities between the observations made in RA and those made in experimental arthritis. For example, most rodent models of arthritis are seronegative and are rarely characterized by a course of remissions and exacerbations. Because of these reasons, findings from animal models cannot always be directly extrapolated to humans. However, since no better alternatives are currently available, experimental models of arthritis continue to provide important information about the biology of RA.

As discussed previously, several different models of experimental arthritis have been developed, each with its own limitations and advantages. The pathology of spontaneous arthritis is closer to osteoarthritis than RA because of minimal immunologic manifestations, limited distribution to specific joints and a predictable course of development (Holmdahl et al., 1992). Similarly, the immunopathogenesis of arthritis induced by mechanical or chemical irritants differs from that of RA, since the immune response in the latter pathology may be minimal. Arthritis induced by infectious agents mimics septic arthritis rather than RA with much of the manifestations resulting due to damage induced by the infecting organism or due to the immunologic response to the organism. Immunologically-induced arthritis using T-cell lines, antibodies or cytokines appears to initiate arthritis at a later stage of the inflammatory cascade and could, therefore, potentially miss some of the important earlier events. Although the histopathogenesis of both antigen and adjuvant-induced arthritis closely mimic RA, adjuvant arthritis relies on the individuals' immune response, while the immune response of antigen-induced arthritis has a closer semblance to RA.

Within the classification of antigen-induced arthritis, subtle but important differences exist between the various methods of induction of arthritis. The pathologies of collagen-induced and streptococcal cell wall-induced arthritis differ from RA because the inflammation in these models is largely based in the periosteum rather than the synovium (Henderson et al., 1993). The arthritis induced by systemic sensitization with an antigen and intra-articular challenge with the same antigen demonstrates a histopathology similar to RA, has inflammation largely confined to the synovium, and a synovitis which, in the rabbit, is chronic often lasting for years (Edwards et al., 1988; Henderson et al., 1993). This model of arthritis responds to therapy with second line drugs, such as pencillamine or gold, much like RA (Billingham, 1983; Hunneyball et al., 1986). A further advantage of the model of antigen-induced arthritis produced by an intra-articular challenge over adjuvant arthritis or over antigen-induced arthritis produced by systemic immunization in susceptible

animals is the greater reported incidence of successful induction of arthritis (reviewed in Henderson et al., 1993). Furthermore, of these models, antigen-induced arthritis produced by systemic immunization followed by intra-articular challenge with the antigen is relatively well understood and has been well studied and since its introduction by Dumonde and Glynn (1962). This model of arthritis has several characteristics similar to RA as outlined above, but differs from the human disease in that it is monoarticular while RA is polyarticular.

Previous studies on experimental arthritis have largely focused on characterizing the clinical, radiologic and histopathologic features of this disease (see summaries and references in Table II). However, recent studies are attempting to elucidate more detailed biologic mechanisms, such as those involved in the immune responses and mechanisms (Brackertz et al., 1977; Boissier et al., 1987 and 1988; Case et al., 1989a; Wooley et al., 1989; Bakker et al., 1990; Brahn, 1992; Drelon et al., 1992; Sano et al., 1992), factors contributing to genetic predisposition (Brackertz et al., 1977; Watson et al., 1990), evaluation of tissue loss (Lowther et al., 1978; Rubin et al., 1987; Rubin and Roberts, 1987; Yoo et al., 1988; Pettipher et al., 1989 and 1990; Henderson et al., 1990; Beesley et al., 1992; Reddy and Dhar, 1992), dissecting the cellular responses (Brackertz et al., 1977; Holmdahl et al., 1985; Case et al., 1989a; Issekutz and Issekutz, 1991), and assessing therapies based on biologically rational approaches (Rubin and Roberts, 1987; Pettipher et al., 1989; Case et al., 1989c; Francis et al., 1989; Kuruvilla et al., 1991; Setnikar et al., 1991; Peacock et al., 1992; Greenwald et al., 1992; Hansen et al., 1992). Since the subject of our studies is to understand modalities by which joint tissues are lost during arthritis, the discussion in the remainder of this section will focus on findings from the relatively few studies which have evaluated matrix loss in experimental arthritis.

Despite the apparent loss of matrix components in arthritis and the known clinical sequelae of tissue destruction, few studies on experimental arthritis have utilized such animal models for evaluating changes in matrix composition (Lowther et al., 1978; Rubin et al., 1987; Rubin and Roberts, 1987; Yoo et al., 1988; Pettipher et al., 1989 and 1990; Henderson et al., 1990; Beesley et al., 1992; Reddy and Dhar, 1992). Loss of GAG in experimental arthritis as assessed by decrease in Safranin O-staining has been reported in experimental antigen-induced arthritis (Rubin et al., 1987; Healy et al., 1989; Henderson et al., 1990; Pettipher et al., 1990; Beesley et al., 1992), and in adjuvant-induced arthritis (Rubin and Roberts, 1987). A reduction of as much as 50% to 60% in intensity of staining of arthritic cartilage compared with controls has been reported in the knee joints of arthritic mice by 1 to 2 weeks after antigen challenge (Pettipher et al., 1990). Furthermore, Yoo et al. (1988), using specific monoclonal antibodies to various epitopes of proteoglycans,

noted that loss of native proteoglycan monomer occurs in immunologically-induced synovitis and this decrease in PGs correlated well with the decreased metachromatic staining observed in this arthropathy (Kruijsen et al., 1985). Such loss in GAG staining was prevented by a therapeutic agent, prednisolone (Rubin and Roberts, 1987), while another commonly used anti-arthritic drug, indomethacin, decreases joint swelling but increases loss of PGs from cartilage (Pettipher et al., 1989). In contrast to these observable changes in GAG or proteoglycan content in arthritic articular cartilage, collagen loss has not been detected previously by histochemical or biochemical methods (Lowther et al., 1978; Beesley et al., 1992), but has been shown to occur by more sensitive radiolabeling techniques in tendon and bone around joints (Reddy and Dhar, 1992). Similarly, loss of matrix macromolecules has been reported in articular tissues retrieved from patients with RA and JRA (Hamerman, 1969; also reviewed in Hollister, 1979 and Harris, 1989). Matrix loss adversely likely impacts on both the physical and biologic functions of the joint as discussed below.

F. SEQUELAE OF MATRIX LOSS ON MATRIX INTEGRITY, AND CELL AND JOINT FUNCTION

The ECM of bone and cartilage serves two broad functions. Firstly, the matrix serves a physical role in which it provides the appropriate morphologic organization and optimal biomechanical properties for the normal function of the tissues and the joint (Kempson et al., 1970; Hascall and Hascall, 1981; Christensen and Ziebert, 1986; Muir and Carney, 1987; Mankin and Brandt, 1989). A second and equally important role of the ECM is its biologic interactions with the component cells of the tissues (reviewed in Toole, 1991). In this context, many matrix molecules and matrix-bound growth factors serve as receptors and mediators of cell function and regulation both in solid and soluble forms. These cell-matrix interactions contribute to several normal cellular functions including proliferation, differentiation, locomotion and metabolic activities (Gospodarowicz and Ill, 1980; Hauschka et al., 1985; also reviewed in Toole, 1991). For example, collagen has been shown to mediate the proliferation of vascular endothelial cells (Gospodarowicz and Ill, 1980) as well as support and promote differentiation of muscle cells in culture (Hauschka et al., 1985). Another matrix macromolecule, fibronectin is an adhesive multifunctional glycoprotein found in ECM of most cell types and exerts growth factor, differentiative and chemotactic activities towards many types of cells (reviewed in Ruoshalati, 1988a and b; McDonald, 1988; Hay, 1981 and 1991). Numerous growth factors bound to matrices of bone and cartilage also influence the proliferation, migration

and differentiation of cells either when bound to the matrix, or more often when released into solution (Pfeilschifter and Mundy, 1987; Hauschka et al., 1988; Centrella et al., 1988). Therefore, because of the dual physical and biologic roles of matrix, its loss adversely impacts on both the physical and biologic functions of joint tissues.

Impact on Biologic Functions

Loss of joint matrix possibly also impacts on cellular functions, including proliferation, differentiation and normal metabolic activities which likely adversely affects the tissue reparative capacity of the cells. Additionally, degradation of cartilage and bone matrices release various matrix-bound growth factors which, in turn, also impact on cellular functions. For example, release of growth factors such as transforming growth factor-β from resorbing bone matrices likely impacts on the activities and proliferation of osteoblasts and osteoclasts (Pfeilschifter and Mundy, 1987; Hauschka et al., 1988; Centrella et al., 1988). Lastly, fragments of several matrix molecules including collagen, elastin and fibronectin have bioactive properties to which cells may respond in a variety of ways. Collagen and collagen fragments have been shown to be chemotactic for fibroblasts and monocytes (Postlethwaite and Kang, 1976; Postlethwaite et al., 1978; Norris et al., 1982; Lohr et al., 1990). Collagen peptides also activate complement C3a with all its consequences on macrophages and other cells (Pontz et al., 1979). Xie et al. (1992) and others (Carsons et al., 1981; Scott, 1981 and 1982; Clemmensen, 1983; Shiozawa and Ziff, 1983; Carnemolla et al., 1984; Lavietes et al., 1985) have also demonstrated that the levels of fibronectin in synovial fluid, pannus and cartilage surface of rheumatoid and osteoarthritic joints are greatly increased, while other experiments reveal that fragments of fibronectin cause cartilage to release metalloproteinases, which is accompanied by severe proteoglycan depletion (Homandberg et al., 1992). Others investigators have also shown that these fragments also enhance MMP expression by synovial fibroblasts (Werb et al., 1989). By-products of matrix degradation may, therefore, in themselves be proinflammatory or enhance further matrix-degradative response of resident and immune cells. This may form an important area for future research both in further understanding the self-perpetuation of RA and also in designing therapies to prevent the resultant escalation of the arthropathy.

The effects of matrix loss on cell function may in turn affect matrix turnover in a variety of ways. Changes in cell activity due to matrix loss will likely impair both the quality and quantity of matrix synthesis, therefore affecting normal maintenance or repair of tissues. Reduced proliferation of cells will similarly impact on the total matrix synthesis because of the fewer cells involved in expressing matrix proteins. Finally, bioactive products such as growth factors or fragments of matrix molecules released from degraded

matrices may act as mediators of inflammation or further increase the expression of matrix degrading proteinases from resident or inflammatory cells, thereby perpetuating the pathology and contributing to its chronicity.

Impact on Physical Functions

The physical properties of cartilage, which acts as a hydrated, fiber-reinforced composite, are determined by a complex interaction between its major matrix components, namely collagen and proteoglycans, (Kempson et al., 1970; Hascall and Hascall, 1981, Christensen and Ziebert, 1986; Muir and Carney, 1987; Mankin and Brandt, 1989). Proteoglycans are highly sulfated molecules whose fixed anionic charge imbibes water into cartilage, thereby providing the capacity for the cartilage to resist deformation under mechanical forces and to regain the original form when the load is released (Kempson et al., 1970; Harris et al., 1972; Hascall and Hascall, 1981, Christensen and Ziebert, 1986; Muir and Carney, 1987; Mankin and Brandt, 1989). On the other hand, the relatively inextensible collagen fibers provide tensile strength and also serve as a scaffolding for the proteoglycans. The collagen also provides the thickness and form to the cartilage (Christensen and Ziebert, 1986; Muir and Carney, 1987; Mankin and Brandt, 1989). While, both collagen and proteoglycans serve important individual functions, it is the complex interaction between these two molecules and water that defines the net properties of cartilage. When cartilage is loaded under compression or shear, the internal swelling pressure increases because of the increasing PG concentration, until it is in equilibrium with the externally applied force, when fluid movement ceases (Myers et al., 1984). For this reason, the physical properties of cartilage have often been described as visco-elastic and poro-elastic (McCuthcen, 1983). Such physical properties are also evident in the TMJ disc (Fontenot, 1985; Tanne et al., 1991).

The initially detectable change in cartilage matrix in arthritis is that of loss of staining for glycosaminoglycans or proteoglycans (Hollister, 1979; Henderson et al., 1993). This results in increased hydration of articular cartilage and in its diminished capacity to resist deformation under mechanical loading (Harris et al., 1972; Muir and Carney, 1987; Harris, 1989; Mankin and Brandt, 1989) and also in its inability to withstand normal function. Abnormal hydration of cartilage also increases the tension on the collagen network in the absence of any external load (Kempson et al., 1973). If this is accompanied by a weakened collagen network, either due to changes in quality of collagen synthesized or due to its increased degradation, it may not withstand stresses of cyclical loading during normal movements, resulting in rupture of the fibers. In all, this loss of articular cartilage matrix results in compromised properties of the tissue. Continued loss of tissue matrix may lead to fibrillation of articular cartilage, and in the case of the TMJ,

perforation of the disc. In the later stages of the disease, osteophytes may be observed which probably represent an aggressive attempt at tissue repair.

The advanced stages of RA are accompanied by clinically obvious changes in joint morphology and function often as a direct result of aberrant matrix turnover. These late changes in chronic inflammation of the joint involve the disintegration of the articular cartilage with exposure of underlying bone and resultant bone to bone articulation (Hollister, 1979). In the TMJ, the undesirable bone-to-bone articulation occurs only after partial or complete destruction of the joint disc. Even in the absence of RA, progressive loss of disc matrix which leads to its perforation often results in secondary changes similar to those observed in OA in the articular surfaces of the mandibular condyle and the articulating fossa, as well as the synovium (Lekkas et al., 1988; Helmy et al., 1989; Taylor et al., 1991). In the advanced stages of RA subluxation of joints may occur probably due to a deterioration in the capsule and ligaments of the joint. These changes in composition and loss of integrity of joint matrix, therefore, are likely to contribute to some or several of the symptoms of arthritis, ranging from joint sounds to limitation in movement and pain. By retracing the events which lead to this end-point, it may be possible to gain insights into the mechanisms of joint damage, better define the stages of the pathology, and design new and rational therapies to alleviate or prevent joint destruction.

Summary

Little is currently known both about the loss of matrix and the mechanisms by which matrix is lost in JRA, and as such, there has been a tendency to extrapolate the findings on matrix degradation and pathogenesis of RA to JRA. However, important differences related to variations in the metabolism of the matrix and immunology in children versus those of adults indicate a potentially different course or pathogenesis of arthritis between these two age groups. For example, the differences in immunology and immune responsiveness of the child and adult (Dorai et al., 1987; Gahring and Weigle, 1990; Ho et al., 1990; McElhancy et al., 1990; Pross, 1990; Sironi et al., 1990) are also likely to contribute to varied progression of the disease in RA of juvenile versus adult-onset. Additionally, differences in matrix metabolism between adults and juvenile patients (Holmes et al., 1988; Fornieri, et al., 1989; Front et al., 1989; Millie et al., 1989; Martin et al., 1990; Termine, 1990; Mays et al., 1991), may have consequences on both the course of the pathology and the reparative capacities of the tissues in the two age groups. Although matrix loss in JRA remains uninvestigated, findings from RA, OA and experimental models of these pathologies indicate at least two mechanisms by which

matrices are lost from joint tissues, particularly the articular cartilage. These modalities of matrix loss include disturbances in matrix synthesis and increased matrix degradation.

G. POTENTIAL MECHANISMS FOR MATRIX LOSS IN ARTHRITIS

Aberrations in joint function in RA and JRA arise partly as a consequence of loss of joint tissue matrix, primarily that composing cartilage and bone. During arthritis early changes include an accelerated loss of proteoglycans, increased hydration of cartilage and a diminished ability of the tissues to withstand normal function (Harris et al., 1972; Hollister, 1979; Muir and Carney, 1987; Tammi et al., 1987; Henderson et al., 1993). Although a shift in PG content has also been noted with an change in the ratio of keratan sulfate (KSO_4) to chondroitin sulfate (CSO_4) (reviewed in Muir and Carney, 1987 and Tammi et al., 1987), the significance and mechanisms for this are not well understood. Subsequently, changes in collagen content are also likely to occur.

Two patterns of matrix loss are often observed in arthritis. Localized degradation of cartilage and bone are seen at sites where proliferative fibroblasts and inflammatory cells in the synovium invade the location at which the synovium is attached to cartilage and bone. The second pattern of matrix loss is more generalized which appears as a relatively uniformly distributed loss of cartilage matrix which is often observed in arthritic joints as a loss in staining for glycosaminoglycans (Lowther et al., 1978; Howson et al., 1986; Rubin and Roberts, 1987; Hasty et al., 1990; Beesley et al., 1992). Although the basis for this type of matrix loss is not well understood, at least two possible mechanisms may contribute to the decrease in quantity and change in quality of the matrix molecules in arthritic joint tissues, namely (1) a decrease or aberrant synthesis of matrix either due to a reduction in the number of viable cells or due to perturbed cell function, or (2) an increased degradation of the matrix or, most likely, (3) a combination of these two mechanisms. With regard to increased matrix degradation, members of specific families of proteinases, including matrix metalloproteinases, serine and cysteine proteinases have been detected within cells and matrices of rheumatoid cartilage and pannus (Morrison, et al., 1973; Sapolsky, et al., 1973; Wooley et al., 1977a and b; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990; McCachren, 1991; Garvallese et al., 1991; Firestein and Paine, 1992).

Disturbed Matrix Synthesis

There is some disparity in the findings of studies which have examined the matrix synthetic capability of cartilage and bone from arthritic joints. Several studies indicate that articular cartilage from animal models of OA show an increased synthesis of both collagen

and PGs (Eyre et al., 1980; Sandy et al., 1984). Furthermore, it has also been noted in both degenerative arthritis and rheumatoid arthritis, that chondrocytes are metabolically more active and produce increased amounts of GAGs (reviewed in Jacoby and Jayson, 1976 and Harris, 1989). Therefore, if this is indeed the case, the net loss of matrix molecules observed in arthritis would result from a greater degradation than synthesis of matrix. This also implies that if the degradation of PGs can be halted or reversed during early stages of arthritis, the integrity of cartilage could be restored by the synthetic ability of cartilage, especially in the younger individual. In contrast to the above findings, other investigators (Rubin and Roberts, 1987) have demonstrated a diminished ability of chondrocytes *in vivo* to incorporate radiolabeled sulfate into cartilage of joints with adjuvant-induced arthritis, indicating a reduced GAG synthetic capacity of these cells. Similarly in another model of adjuvant-induced arthritis in rats, Reddy and Dhar (1992) using ^{3}H-hydroxyproline, noted a decreased collagen content in bone and tendon attributable, at least in part, to decreased collagen synthesis. A decrease in matrix synthesis in experimental arthritis has also been reported by other investigators (Gillard and Lowther, 1976; Carmichael et al., 1977; Lowther et al., 1978).

Besides changes in the quantity of matrix synthesized by cartilage cells, a shift in the type of expressed matrix macromolecules may also accompany arthritis. Although little information is currently available on changes in quality or proportions of constituent cartilage matrix molecules in RA, this shift in matrix composition has been noted in experimental loading of joints and in experimental osteoarthritis. Articular cartilage in these models generally demonstrate a shift in galactosamine / glucosamine ratio in PG extracts (Oláh and Kostenszky, 1976; Säämänen et al., 1987; Jurvelin et al., 1989). This shift in aminosugar ratio reflects an increase in keratan sulfate relative to chondroitin sulfate. Furthermore, the hyaluronic acid content is increased in the rabbit joint cartilage subjected to increased loading (Säämänen et al., 1987). Similar age-related changes in matrix composition have also been noted in cartilage and meniscus from the knee joint, which show a general shift from the glucosamine containing keratan sulfate to galactosamine containing chondroitin sulfate with age (Bayliss and Ali, 1978; Inerot et al., 1978; Roughley and White, 1980; Garg and Swann, 1981; Zirn et al., 1984). Because of the analogous shift in matrix GAG composition in OA and aging, such changes in OA may reflect an accelerated aging process and a diminished reparative capacity of cartilage (Inerot et al., 1978; Säämänen et al., 1987). In contrast to studies done on compositional changes in cartilage matrix in OA and animal models of joint loading, similar studies evaluating changes in the quality of cartilaginous matrices have not been done in RA.

Enhanced Matrix Degradation

The other likely mechanism for tissue loss in arthritis is the increased or unregulated degradation of the matrix. Increased degradation could result due to an increased expression, or an enhanced activation, or a decreased extracellular inhibition of proteinases, or due to any combination of these events. Several classes of proteinases, including serine proteinases, such as elastase, cysteine proteinases, such as cathepsins, and matrix metalloproteinases, such as collagenase, have been implicated in these processes.

Serine proteinases probably contribute to degradation of tissue matrices by at least two different modalities. Firstly, serine proteinases can directly act on matrix molecules breaking them into smaller fragments. Elastase, for example, directly cleaves elastin, a relatively minor but important component of cartilage. Secondly, serine proteinases can enhance matrix degradation by two possible indirect mechanisms involving the activation of another family of proteinases, the MMPs, or by degradation of specific inhibitors of these MMPs. Activation of MMPs by serine proteinases such as the plasminogen activator-plasmin cascade have been demonstrated *in vitro* (Mignatti et al., 1986; He et al., 1989; Nagase et al., 1990; Suzuki et al., 1990; Birkedal-Hansen et al., 1992). Serine proteinases, such as neutrophil elastase, trypsin and α-chymotrypsin, may also reduce the inhibitory activity of TIMP towards MMP-3 by degrading this inhibitor into small fragments (Okada et al., 1988a).

Cathepsins, which are acid proteolytic enzymes, are capable of degrading the protein backbone to which sulfated sugars are attached in proteoglycans and also degrade collagen. However, a major objection to the role of these proteinases in matrix degradation during arthritis is that the pH optimum for many of these enzymes is 5, and they undergo a 5- to 10- fold decrease in proteolytic activity as the pH comes closer to the neutral environment of synovial fluid (Morrisson et al., 1973; Sapolsky, 1973). Nevertheless, it is possible that these proteinases contribute to intracellular degradation of matrix molecules in the acidic microenvironment of the lysosome. Further evidence indicating a relatively limited role of a specific cathepsin proteinase, cathepsin-G, as well as a serine proteinase, neutrophil elastase, in matrix degradation during experimental arthritis is provided by studies of Pettipher et al. (1990). These investigators demonstrated similar histopathology and loss of Safranin-O staining in the knee joints of wild type C57 mice and cathepsin G / neutrophil elastase deficient beige C57 mice following induction of antigen arthritis. These findings indicate that neutrophil elastase and cathepsin G may contribute minimally to loss of GAGs in immune arthritis and further suggest the role of other proteinases in this process.

The third group of proteinases implicated in matrix loss associated with rheumatoid arthritis is the family of MMPs. Although it is now known that a complex cascade of proteolytic events determines matrix degradation and that more than a single family of enzymes is involved in these processes, recent evidence indicates a central role for MMPs produced by endogenous cells as well as inflammatory cells. Several clinical studies have found elevated levels of MMPs in synovium and articular tissues, as well as in synovial fluid from patients with RA as compared to patients with OA, post-traumatic joint injury or normal control volunteers. (Cawston et al., 1984; Walakovits et al., 1992). However, the association between increased expression of MMPs by the synovium and matrix degradation remains largely conjectural since no study has demonstrated this relationship *in vivo*. Such an association between the enhanced levels of MMPs and increased loss of matrix molecules is further complicated by the mechanisms for extracellular regulation of MMP activity which is controlled by their secretion as inactive proenzymes requiring activation, and further by the inhibition of these MMPs by specific and non-specific inhibitors. This implies that for a clearer definition of the role of MMPs in matrix degradation during arthritis, one needs to demonstrate not only an increased expression of MMPs, but also of their increased extracellular activity either by increased activation or decreased inhibition or both. Since these proteinases are highly implicated in matrix degradation during RA, their biology, regulation and function are discussed in more detail in the next section of this chapter.

Several cellular sources of MMPs, namely synovial fibroblasts, articular chondrocytes and inflammatory cells, such as PMNLs, have been identified and implicated in RA. Synovial fibroblasts in culture constitutively synthesize the MMPs, collagenase, stromelysin, 72-kDa gelatinase and 92-kDa gelatinase, and at least two proteinase inhibitors (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987; Unemori and Werb, 1986; Okada et al., 1990a and b; MacNaul et al., 1990; Kolkenbrock et al., 1991; McCachren, 1991; Firestein et al., 1991; Unemori et al., 1991b; Firestein and Paine, 1992). Furthermore, the levels of several of these secreted proteinases are increased on exposing synovial fibroblasts to specific proinflammatory cytokines, such as interleukin-1, tumor necrosis factor-α and transforming growth factor-α (Kumkumian et al., 1989; Ahmadzadeh et al., 1990; Alvaro-Garcia, 1990; Case et al., 1990; MacNaul et al., 1990; Unemori et al., 1991b) implicating both these cytokines and MMPs of synovial fibroblast origin in the increased degradation of joint matrices in RA. Further *in vivo* evidence in support of the role of MMPs in matrix degradation during arthritis is provided by *in situ* localization studies using antibodies and probes to collagenase and stromelysin which have localized these proteinases or their message in cells of the synovial lining, the pannus and

articular chondrocytes, as well as in the matrix at the cartilage / pannus junction (Wooley et al., 1977b; Hasty et al., 1990; Henderson et al., 1990) from patients with RA or in experimental models of arthritis. The pattern of cartilage matrix degradation generally attributed to synovium-derived MMPs is that of the localized type in the vicinity of the pannus. Electron microscopic studies of Kobayashi and Ziff (1975) demonstrate the invasive potential of pannus and cartilage with resulting desolution of the ECM and chondrocytes. The intimate relationship between the pannus and the underlying degenerating cartilage may provide an optimal environment for the action of these proteinases.

Although some findings indicate that MMPs of synovial fibroblast and inflammatory cell origin secreted into the synovial fluid may also contribute to the generalized pattern of matrix loss in the articular cartilage, evidence contradicting the role of these MMPs in this type of matrix loss has been presented in some recent studies. Beesley et al., (1992), using an animal model of antigen-induced arthritis, have demonstrated that GAGs are lost from the deeper layers of articular cartilage while the superficial zones show normal staining for this matrix molecule, indicating that MMPs in synovial fluid may not be responsible for the loss of articular cartilage matrix. Furthermore, high levels of specific inhibitors, such as TIMP, and non-specific inhibitors, such as α2-macroglobulin, present in synovial fluid may readily inhibit MMPs secreted into the synovial fluid (Abe and Nagai, 1973; Cawston et al., 1984; Firestein et al., 1991; Osthues, 1992). These findings suggest that the generalized loss of matrix in cartilage may result from proteinases synthesized by chondrocytes within the cartilage. Indeed, articular chondrocytes constitutively express 92-kDa gelatinase in culture and may be stimulated by various cytokines to secrete other MMPs including collagenase and stromelysin (Lefebvre et al., 1990a, b, and 1991; Ogata et al., 1992). The potential involvement of chondrocytes in articular cartilage matrix loss in arthritis has been further demonstrated by immunohistochemistry in biopsied pathologic joints from animal models of arthritis (Henderson et al., 1990).

In the TMJ matrix loss from an additional and relatively unique morphologic structure, the disc, has been noted in individuals with degenerative joint disease (Kopp, 1976 and 1978), but not much else is known about the early matrix changes in this tissue. Although synovial fluid in arthritic joints contains MMPs, these may not be significant in matrix degradation of the joint disc for reasons similar to those discussed previously for articular cartilage matrix loss. In contrast, disc-derived MMPs may be of greater significance in matrix degradation in this tissue. Despite the prospect that MMPs of disc cell origin predispose to its increased matrix loss in arthritis, to date no study has been done

to evaluate if disc cells indeed synthesize these MMPs, and if so, whether the intra- and extracellular regulation of disc-derived MMPs is altered in arthritis.

In order to further comprehend the potential role of MMPs and PIs in tissue degradation associated with arthritis, an understanding of the biochemistry, intra- and extracellular regulation and matrix specificities of these proteins is essential. Such a background may offer clues as to how the loss of balance in matrix degradative activity in joints is dependent on the underlying biology of MMPs and PIs. Much of the information presently available on MMPs and PIs has been obtained largely from *in vitro* studies. Below is a review of some of these findings.

H. MATRIX METALLOPROTEINASES AND THEIR REGULATION

Several recent reviews provide information on our present knowledge on matrix metalloproteinases (reviewed in Matrisian, 1990; Alexander & Werb, 1989 and 1991; Murphy et al., 1991a; Woessner, 1991; Birkedal-Hansen et al., 1993). The MMP family encodes nine or more metal-dependent endopeptidases with activities against most if not all ECM molecules. Despite their several common structural and functional features, the activities of these enzymes differ somewhat in terms of substrate specificities and range of activities. These enzymes contain Zn^{2+} as an intrinsic component and require Ca^{2+} for conformational stability. Thus, the activities of these proteinases are inhibited by EDTA or 1,10-phenanthroline or by depletion of Ca^{2+}, but not by inhibitors of cysteine, serine or aspartic proteinases.

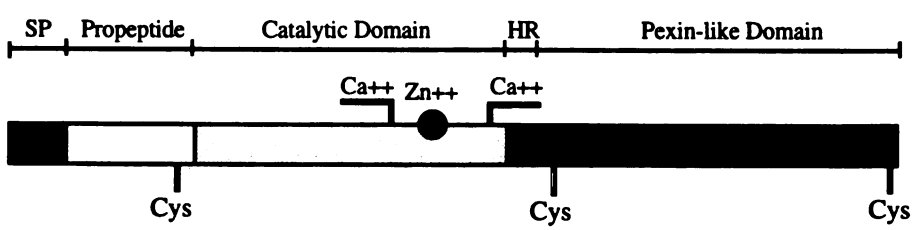
The nine members of this family of MMPs may be further classified into four categories, namely (1) interstitial collagenases, (2) stromelysins, (3) gelatinases and (4) other MMPs (Table III). Each of these categories has two or more members (see Table III). These enzymes are often regarded as derivatives of the five-domain modular structure characteristic of collagenases and stromelysins formed either by deletion or addition of domains (Figure 2). The 17 to 29 residue hydrophobic signal sequence is followed by a 77-87 residue propeptide that constitutes the NH_2 terminal domain of the secreted MMP precursor, and a catalytic domain that contains the catalytic machinery including the Zn^{2+} binding site. This sequence is followed by a 5 to 50 residue proline-rich hinge region that marks the transition to the approximately 200 residue hemopexin- or vitronectin-like COOH-terminal domain that appears to play a role in substrate specificity. The latter domain is not present in matrilysin (putative metalloproteinase-1 or PUMP-1) which is therefore considerably smaller than the other MMPs. The gelatinases contain a single insert in the catalytic domain consisting of three tandem repeats of fibronectin type II molecules

that endow both the latent and active enzymes with gelatin-binding properties (Goldberg et al., 1989; Collier et al., 1992). Each of the MMPs contains a putative tridentate Zn^{2+}-binding site believed to constitute the active site and play a role in the maintenance of catalytic latency of the precursor form (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). All these proteinases also contain a highly conserved Glu- and Asp-rich region between the Zn^{2+}-binding site and the hinge region that is likely to constitute a Ca^{2+}-binding site.

Table III: Members of the matrix metalloproteinase family and their substrate specificities.

Type	Enzyme Name	MMP#*	M_r	Extracellular Matrix Substrates
Collagenases	Fibroblast collagenase	MMP-1	52/57 doublet	Collagen I, II, III, VII, VIII, X; gelatin
	PMN-collagenase	MMP-8	75	Collagen I, II, III, VII, VIII, X; gelatin
Stromelysins	Stromelysin-1	MMP-3	55/60 doublet	Proteoglycan core protein; fibronectin; laminin; collagen IV, V, IX, X; elastin; procollagenase
	Stromelysin-2	MMP-10	55/60 doublet	Proteoglycan core protein; fibronectin; laminin; collagen IV, V, IX, X; elastin; procollagenase
	Matrilysin / Putative metalloproteinase-1	MMP-7	28	Fibronectin; laminin; collagen IV; gelatin; procollagenase; proteoglycan core protein
Gelatinases	72-kDa gelatinase	MMP-2	72	Gelatin; collagen IV, V, VII, X, XI; elastin; fibronectin; proteoglycan core protein
	92-kDa gelatinase	MMP-9	92	Gelatin; collagen IV, V; elastin; proteoglycan core protein
Others	Macrophage metallo-elastase	Non assigned	53	Elastin, proteoglycans, IgG, $\alpha 2$ macroglobulin
	Stromelysin-3	MMP-11	Not determined	Not determined

*MMP numbering according to Nagase et al., Matrix Special Supplement, 1992a; 1:421-424.



SP=Signal Peptide; HR=Hinge Region

Figure 2: General domain structure of matrix metalloproteinases.

In common with the peptide domain and sequence similarities between the various MMPs, their genes also show a highly conserved modular structure. For instance, the collagenase and stromelysin-1 and 2 genes each contain ten exons and nine introns in 8 to 12 kbp of DNA (Matrisian et al., 1985; Breathnach et al., 1987; Fini et al., 1987). The matrilysin gene lacks exons which encode for the hemopexin-like domains, while 72- and 92-kDa gelatinases contain three additional exons coding for the three fibronectin type II domains.

Activated MMPs demonstrate overlapping substrate specificities on both proteins and peptides, but also have unique activity profiles towards some proteins (Table III). For instance, interstitial collagenases of both fibroblast and PMN origins degrade only fibrilar collagens at specific sites, while other MMPs like stromelysin are broadly reactive on a wide range of ECM macromolecules (Table III). Most of the MMPs cleave gelatin and fibronectin although at varying rates, and also cleave type IV and V collagens at sufficiently high temperatures. On the other hand, the unique ability of fibroblast and PMN collagenases to degrade the triple helix of interstitial collagens is not shared by other members of the family, and suggests a potential *in vivo* biologic function of these proteinases. MMPs cleave a wide range of largely hydrophobic bonds in native proteins.

Regulation of Matrix Metalloproteinases

Due to the rather large repertoire of ECM substrates degraded by MMPs, these enzymes are well regulated both intracellularly at the transcriptional level, and extracellularly because they are secreted as proenzymes which require activation, and once activated can be inhibited by specific on non-specific inhibitors. The expression of MMPs is induced or repressed by many biologic and several chemical agents. The effects of the biochemical mediators on MMP expression can be categorized as largely catabolic, as that of interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), largely anabolic as that of transforming growth factor-β (TGF-β) or hybrid anabolic / catabolic effects attributable to epidermal growth factor (EGF), transforming growth factor-α (TGF-α), platelet derived growth factor (PDGF), interferon-γ (IFN-γ), and basic fibroblast growth factor (bFGF). Despite the redundancy, some level of specificity exists in the transcriptional regulation of MMPs in that (1) different growth factors and cytokines induce overlapping yet distinct repertoire of MMPs and inhibitors (MacNaul et al., 1990) and (2) different cell types respond to the same growth factors and cytokines by expression of unique and distinct combinations of MMPs and inhibitors (Petersen et al., 1987; Fini et al., 1990; MacNaul et al., 1990; Lefebvre et al., 1991; Ogata et al., 1992). The transcriptional regulation of MMPs has also been associated to physical events such as changes in cell shape due to disruption of the cytoskeleton (Werb and Reynolds, 1974; Brinckerhoff et al., 1982;

Aggeler et al., 1984; Unemori and Werb, 1986; Werb et al, 1986) and heat shock (Vance et al., 1989). Some studies also indicate that cell-substrate adhesion may modulate MMP expression (Werb et al., 1989; Emonard et al., 1990; Petersen et al., 1992).

Although a large body of information is available on factors affecting MMP and inhibitor expression, the signal transduction mechanisms remain poorly understood. Protein kinase-C has been implicated because of the effects of the protein kinase-C agonist, tetradecanoyl phorbol acetate (TPA), in the induction of MMPs by a large number of cell types (Aggeler et al., 1984; Chin et al., 1985; Herron et al., 1986a and b; Frisch et al., 1987; Welgus et al., 1990; Fini et al., 1990; Ogata et al., 1992). The role of 3'-5' cyclic adenosine monophosphate (cAMP) remains less clearly defined since a rise in cytoplasmic cAMP in some systems increases expression of MMPs, while in other systems it has the opposite effect (Matrisian et al., 1986; Kerr et al., 1988a; Civitelli et al., 1989; Takahashi et al., 1991).

The intermediary steps to gene transcription are also poorly understood. Many of the cytokine, growth factor and chemical stimulated regulatory pathways for MMPs converge at the AP-1 binding site, which also constitutes the phorbolester-responsive element (TRE) (Angel et al., 1987a and b). AP-1 complexes are heterodimers of proteins of two proto-oncogene families, Jun and Fos that bind to a ATGAGTCA consensus sequence in the 5' flanking regions. It has been shown that induction of fibroblast collagenase by oncogene and phorbol ester (Schonthal et al., 1988), and of stromelysin-1 by PDGF (Kerr et al., 1988b) occurs by *c-fos* dependent pathway. However, induction of fos does not necessarily result in expression of growth factor responsive MMPs, as shown by the finding that repression of MMP expression by TGF-β is also fos-dependent, but is independent to the AP-1 site (Kerr et al., 1990). The AP-1 binding sequences are present in the 5' flanking regions of fibroblast collagenase, stromelysin-1 and 92-kDa gelatinase genes, but are missing in the 72-kDa gelatinase gene (Angel et al., 1987b; Schonthal et al., 1988; Huhtala et al., 1991), implying potentially distinct regulatory mechanisms for 72-kDa gelatinase.

Once transcribed, the mRNA levels for most MMPs, with the possible exception of metalloproteinases of PMN origin, appear to correlate with the synthesis and secretion of proteins indicating that these enzymes, once translated, require no further stimulus for release of the zymogens into the extracellular milleu. To this category belong fibroblast collagenase, stromelysin-1 and 3 and 92-kDa gelatinase which are regulated at the transcriptional level largely in response to growth factors and cytokines (Kumkumian et al., 1989; Shinmei et al., 1989; Alvaro-Garcia, 1990; MacNaul et al., 1990; Lefebvre et al., 1990b and 1991; Martel-Pelletier et al., 1991; Smith et al., 1991; Unemori et al., 1991b;

Nguyen et al., 1992; Ogata et al., 1992; Quintavalla et al., 1993), and 72-kDa gelatinase which is constitutively expressed and appears to be only mildly induced or repressed by growth factors and cytokines (Overall et al., 1991; Salo et al., 1991). PMN collagenase and 92-kDa gelatinase, on the other hand, undergo a triggered release from prepackaged granules.

The extracellular regulation of MMPs is achieved in three ways: (1) Since these proteinases are secreted as inactive proenzymes, they have to be modified for biologic activity. (2) Once activated, these proteinases bind strongly and stochiometrically in a noncovalent manner to specific inhibitors of MMPs, called tissue inhibitors of metalloproteinases (TIMPs) which are often from the same cellular source as the proteinases. (3) The activity of the MMPs can be further spatially regulated by localization of the enzymes through binding to specific substrates (reviewed in Moscatelli and Rifkin, 1988 and Alexander and Werb, 1991).

Although little is known about the biologic activation of MMPs, *in vitro* studies have demonstrated several pathways for activation of these proteinases. The latency of MMPs seems to reside in part in the putative Cys-Zn^{2+} bond that links the propeptide Cys residue to the active Zn^{2+} and displaces the water molecule necessary for catalysis (Springman et al., 1990; Van Wart and Birkedal-Hansen, 1990). This Cys residue remains unavailable in the latent zymogen but is exposed in the active enzyme either by conformational change or by cleavage of the propeptide. Activation of the zymogens is achieved by physical (chaotropic agents), chemical (HOCl, organo-mercurials) and enzymatic (trypsin, plasmin) treatments that separate the cysteine residue from the zinc. Once stabilized in the open form, the enzyme catalyzes several autolytic cleavages to generate the fully processed form (Grant et al., 1987; Nagase et al., 1990; Suzuki et al., 1990).

Two categories of inhibitors further regulate the extracellular activity of MMPs. These are inhibitors belonging to the α-macroglobulin and tissue inhibitor of metalloproteinases (TIMP) families. The former inhibitors inactivate susceptible proteinases by entrapment followed by cleavage of the bait region (Sottrup-Jensen, 1989). The MMP cleaves one or more bonds in the 40 residue bait region of the α-macroglobulin, initiating a conformational change which causes the entrapment of the proteinase (Sottrup-Jensen, 1989). The rapidity of capture of MMPs, particularly of collagenase, by α-macroglobulin indicates their important role in regulation of MMP activity.

The functional activities of TIMPs are achieved by forming classic noncovalent bimolecular bonds with the active or, in some instances, the zymogen forms of MMPs. While bonding of TIMPs with the active proteinase inhibits the catalytic function of the

enzymes, combination of TIMP with the zymogen appears to prevent activation of the latter (DeClerck et al., 1991; Howard et al., 1991a). TIMPs are synthesized by a wide variety of cells including dermal (Hembrey and Ehrlich, 1986) and synovial fibroblasts (Aggeler et al., 1984; Chin et al., 1985; Unemori and Werb, 1986; Frisch et al., 1987), most of which also synthesize MMPs.

Many of the MMPs have matrix binding domains that may help localize matrix degradative activities. Localization of MMP activity may result by binding to appropriate molecules on the matrix or cell surface and / or inhibition by high concentrations of bound inhibitors. Such mechanisms have been fairly well described for serine proteinases (see Moscatelli and Rifkin, 1988), but have not yet been evaluated for MMPs. It is likely that little or no proteolysis by these proteinases occurs in the soluble phase.

Interstitial Collagenases

PMN collagenase is expressed only by PMNs, while several different cell types such as fibroblasts from various sources (Aggeler et al., 1984; Chin et al., 1985; Unemori and Werb, 1986; Frisch et al., 1987), osteoblasts (Otsuku et al., 1984; Quinn et al., 1990), chondrocytes (Lefebvre et al., 1990a and b), and macrophages and monocytes (Welgus et al., 1985; Campbell et al., 1987) produce fibroblast collagenase. Both collagenases have very similar core proteins, but the PMN-collagenase is highly glycosylated with an Mr of 75 kDa as compared to 52/57-kDa for fibroblast collagenase. The two collagenases differ substantially with regard to transcriptional regulation. While PMN-collagenase is released instantly from granule storage sites of triggered PMNs, fibroblast collagenase is not stored in cells but is released on demand by initiating transcription of the gene. Both types of collagenases primarily cleave fibrilar collagens at Gly-Ile or Gly-Leu bond producing the classic $^3/_4$ - $^1/_4$ fragments of the collagen molecule.

PMN collagenase further differs from fibroblast collagenase in that unlike the latter MMP, it cannot be activated by trypsin (reviewed in Birkedal-Hansen, 1993). Other potential physiologic activators of these collagenases include plasmin and stromelysin. APMA activation of fibroblast procollagenase converts the enzyme to Mr 42,000 which displays about 14-25% of maximal activity, but which is rapidly converted to a fully active Mr 41,000 molecule by stromelysin with a Phe81 amino terminus on the activated collagenase (Nagase et al., 1992b). Fibroblast procollagenase is also activated in vitro by stromelysin alone by cleavage of the Gln80-Phe81 bond, but this reaction is extremely slow suggesting that this bond is not readily available to stromelysin in the native configuration of procollagenase (Nagase et al., 1992b).

Stromelysins

At least three different stromelysins, and possibly a fourth, have been identified. Stromelysin-1 and 2 have identical substrate specificities cleaving a wide range of ECM molecules including proteoglycan core proteins, types IV, V and II collagens, fibronectin and laminin (Table II). Furthermore, type IX collagen is degraded by stromelysin but not by collagenase or 72-kDa gelatinase (Okada et al., 1989b). Stromelysin-1 is expressed by stromal cells, including synovial fibroblasts, either constitutively or after induction by IL-1, EGF, TNF-α and PDGF or phorbol esters (Chin et al., 1985; Herron et al., 1986a; Wilhelm et al., 1987). On the other hand stromelysin-2 is less abundantly expressed and cannot be substantially stimulated by growth factors or phorbol esters. Like collagenase prostromelysin-1 and 2 are doublets of 57/60 kDa. The higher molecular weight form is thought to be a product of post-translational glycosylation of the 57,000 kDa zymogen since it binds to concanavalin A (Okada et al., 1988b).

Sirum and Brinckerhoff (1989) have demonstrated that stromelysin-1 and 2 mRNA are differentially expressed in rheumatoid synovial cells and rabbit synovial fibroblasts. They also showed that the elements required for the TPA, EGF and IL-1β induction of stromelysin are contained on a 307 base pair fragment including approximately 270 base pairs of the 5'-flanking DNA. In the human, the sequences required for induction of transcription of the stromelysin promoter are contained on a 46 base pair fragment which contains a sequence with a high degree of similarity to the binding site for the transcription factor activator protein-1 (AP-1) (Sirum and Brinckerhoff, 1991). The AP-1 sequence of this fragment is necessary but not sufficient for the maximal response to TPA or IL-1. Maximal induction requires functional cooperation between the AP-1 sequence and a neighboring upstream regulatory sequence (URS). These investigators have demonstrated that both the AP-1 sequence and the URS bind phorbol or IL-1 induced nuclear proteins.

In vitro activation studies on stromelysin-1 have demonstrated that following treatment with 4-aminophenylmercuric acetate (APMA), the zymogen undergoes a sequential activation processing, first becoming active without a change in its apparent molecular mass, and then it is processed to low-Mr active species of 45,000 and 28,000. (Okada et al., 1988b). The kinetics of changes from the zymogen to its two active forms indicates that activation of this proteinase by an organomercurial compound is initiated by molecular perturbation of the zymogen that results in conversion into the 46,000-Mr intermediate by an intramolecular interaction; and the subsequent processing to Mr 28,000 species as a result of bimolecular reaction. The zymogen can also be activated by trypsin, chymotrypsin, plasma kallikrein and thermolysin, but not by thrombin (Okada et al., 1988b; Nagase et al., 1992b). It is stipulated that *in vivo* this zymogen is activated either

by direct limited proteolysis by tissue or plasma endopeptidases, or alternatively, by factors that cause certain conformational changes in the zymogen molecule.

Gelatinases

Both 72-kDa gelatinase and 92-kDa gelatinase are similar in many ways including their high affinity for gelatin in the latent and active forms. Some differences do however exist between the two MMPs. Structurally, the 92-kDa gelatinase has an extended hinge region of 54 amino acids (Wilhelm et al., 1989). Functionally, the pro-72-kDa gelatinase is tightly associated with TIMP-2, while pro-92-kDa gelatinase is more commonly found bound to TIMP (Goldberg et al., 1989; Wilhelm et al., 1989). The cells synthesizing these MMPs include dermal (Seltzer et al., 1981) and synovial fibroblasts (Aggeler et al., 1984; Unemori and Werb, 1986; Okada et al., 1990b; Kolkenbrock et al., 1991; Unemori et al., 1991b), chondrocytes (Lefebvre et al., 1991), osteoblasts (Overall and Sodek, 1987) and monocytes (Gabrisa et al., 1986) for 72-kDa gelatinase, and synovial fibroblasts (Aggeler et al., 1984; Unemori and Werb, 1986; Okada et al., 1990b; Kolkenbrock et al., 1991; Unemori et al., 1991b), monocytes and macrophages (Mainardi et al., 1984), and PMN leukocytes (Hibbs et al., 1985; Murphy et al., 1989) for 92-kDa gelatinase. 72-kDa gelatinase is expressed constitutively in culture but is only moderately responsive to TPA and growth factors that induce 92-kDa gelatinase (Salo et al., 1985; Templeton et al., 1990; Huhtala et al., 1991). The gelatinases cleave gelatin, types IV, V and VII collagens (Seltzer et al., 1989), cartilage type X collagen (Gadher et al., 1989; Welgus et al., 1990a), proteoglycan core protein (Nguyen et al., 1993), and elastin (Senior et al., 1991). In comparing degradation of type IV and V collagens by MMP-3 and MMP-2, it is noted that MMP-3 degrades type IV collagen more readily than MMP-2, while the latter proteinase is more effective against type V collagen (Okada et al., 1990b). 72-kDa gelatinase has optimal activity at pH 8.5, but retains 50% activity at pH 6.5 (Okada et al., 1990b). The activity of these gelatinases is inhibited by EDTA, 1,10-phenanthroline and TIMP, but not by inhibitors of serine, cysteine and aspartic proteinases.

The 72-kDa gelatinase zymogen is readily activated by organomercurial to an active form by removal of 8 kDa peptide at the Tyr85-Asn86 bond, eight residues downstream from the conserved peptide Cys residue (Stetler-Stevenson, et al., 1989a). In contrast, Nagase et al. (1992b), demonstrate slightly differing findings that 72-kDa gelatinase is activated rapidly by APMA to a fully active molecule of Mr 68,000 by cleavage at Asn80-Tyr81 bond. Subsequent to this activation this proteinase is gradually inactivated by autolysis (Okada et al., 1990b; Nagase et al., 1992b). Unlike procollagenase and prostromelysin, ProMMP-2 is not activated by endopeptidases trypsin, chymotrypsin, plasmin, plasma kallikrein, neutrophil elastase, cathepsin G, MMP-3, and thermolysin

(Okada et al., 1990a; Nagase et al., 1992). Activation of 92-kDa gelatinase by trypsin or auto-activation following organomercurial treatment results in cleavage of an amino terminal sequence with retention of the thiol-bearing sequence in the mature enzyme (Wilhelm et al., 1989; Tschesche et al., 1992).

Tissue Inhibitor of Metalloproteinases

At present three members of the TIMP family, namely TIMP, TIMP-2 and TIMP-3, have been cloned and sequenced (Docherty et al., 1985; Carmichael et al., 1986; Stetler-Stevenson et al., 1989b; Boone et al., 1990; Apte et al., 1994; Silbiger et al., 1994), but others are likely to exist (Herron et al., 1986b; Staskus et al., 1989). TIMP is a 28 kDa highly stable sialoglycoprotein with a 20 kDa core protein (Stricklin and Welgus, 1983; Carmichael et al., 1986; Williamson et al., 1990). It complexes in a 1:1 stochiometry with activated fibroblast collagenase but not with its zymogen form (Welgus et al., 1985). TIMP also complexes with the proenzyme form of 92-kDa gelatinase (Goldberg et al., 1989; Howard et al., 1991a and b). TIMP inhibits MMP-3 with a stochiometry of 1:1 on a molar basis (Okada et al., 1988a). This inhibitory activity of TIMP against MMP-3 is destroyed by serine proteinases, human neutrophil elastase, trypsin and alpha-chymotrypsin which degrade the inhibitor into small fragments (Okada et al., 1988a). It is therefore possible that in inflammatory conditions, neutrophils may contribute to increased matrix degradation by decreasing TIMP levels through the degradative activity of neutrophil elastase.

TIMP-2 is an unglycosylated protein of 22 kDa. In contrast to TIMP, TIMP-2 complexes with free 72-kDa progelatinase (Goldberg, et al., 1989; Stetler-Stevenson, 1989b; Kolkenbrock et al., 1991) from which it can be isolated in its fully active form (Ward et al., 1991; Moore et al., 1992). Progelatinase-TIMP-2 complex can also inhibit other metalloproteinases such as collagenase and gelatinase (Kolkenbrock et al., 1991). TIMP-2 is more effective than TIMP against 72- and 92-kDa gelatinases, while TIMP appears to inhibit fibroblast collagenase more effectively (Howard et al., 1991b). The overall homology between TIMP and TIMP-2 is 66% (Stetler-Stevenson, 1989b).

It is not yet known what constitutes the active site of TIMPs, but some evidence indicates that the inhibitory activity of TIMP may reside in its first 134 amino acid residues (Coulombe and Skup, 1988; Murphy et al., 1991b). Complexes between TIMPs and active MMPs appear to involve the active site of these proteinases since small complex formation is inhibited by small peptide inhibitors (Lelievre et al., 1990). On the other hand, complexes between TIMPs and the two progelatinases appear not to involve the active sites since the zymogens can be fully activated by organomercurials while complexed to the inhibitor (Goldberg et al., 1989; Howard et al., 1991a). The fully functional

inhibitor can be recovered from proteinase-inhibitor complexes suggesting that this interaction does not involve the cleavage of the inhibitor (Murphy et al., 1989b).

Summary

Although much has been learnt about MMPs and their regulation over the last one and a half decades, much of this information is based on *in vitro* studies. The findings of these studies are of extreme importance, but many have yet to be validated *in vivo*. For example, although the *in vitro* mechanisms for the transcriptional regulation, activation and inhibition of MMPs, the cleavage of collagen by collagenases, and the effects of several cytokines and growth factors in altering MMP and PI expression by specific types of cells are well understood, it is not yet quiet certain whether many of these mechanisms are also applicable *in vivo*. Furthermore, the effects of varied combinations and concentrations of cytokines on MMP and PI expression, as occurs in inflammatory disorders, can only be accurately evaluated *in vivo*, whereby these variables are not limited by the investigator. Although human studies would offer substantial insights into these mechanisms of matrix degradation *in vivo*, these types of studies have several limitations as discussed previously. Much of the intermediate information between the *in vitro* biochemical mechanisms of MMPs / PIs activities and their *in vivo* actions and implications can be obtained from animal studies. Therefore, in our studies we have utilized this approach to examine the role of MMPs and PIs in matrix degradation during arthritis of the TMJ.

I. RATIONALE, HYPOTHESIS AND SPECIFIC AIMS

Although the etiology of RA and JRA remain largely unknown, the intermediate and progressive changes in these pathologies involve the loss of tissue matrices, primarily from bone and cartilage. These events significantly affect cellular activities and matrix integrity and properties, thereby impacting on normal function of the joint. An understanding of the processes involved in matrix loss may provide a means for alleviating or treating these debilitating diseases. Recent evidence implicates the loss of matrix components in RA to an imbalance in regulation in the matrix degradative activity attributable to a family of enzymes, the MMPs, and their inhibitors.

Clinical studies on pathologic synovium and articular cartilage retrieved from arthritic patients demonstrate the presence of MMPs, including collagenase and stromelysin in these tissues (Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans, 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein, 1992; Firestein and Paine, 1992 ; Hirose et al.,

1992; Walakovits et al., 1992). These studies, although of extreme importance in our understanding of matrix loss in RA, lack adequate healthy controls, have predominantly examined tissues retrieved from advanced stages of the pathology requiring surgical intervention, and do not provide an insight into the alterations in MMP and PI profiles with progression of the pathology, or the association, if any, between MMP / PI levels and severity of arthritis. Several animal models of arthritis have been developed over the years and utilized for studying various aspects of this arthropathy including clinical and histologic characterization of the models, the effects of potential anti-arthritic therapeutic agents , as well as the molecular immune mechanisms of arthritis (reviewed in Table II). A few studies have also examined the loss of matrix molecules such as collagen, proteoglycans (PGs) and glycosaminoglycans (GAGs) accompanying experimental arthritis (Lowther et al., 1978; Rubin et al., 1987; Rubin and Roberts, 1987; Yoo et al., 1988; Pettipher et al., 1989 and 1990; Henderson et al., 1990; Beesley et al., 1992; Reddy and Dhar, 1992). Limited information, however, has been gathered from these animal models on the specific role of MMPs and PIs in the loss of these matrix molecules. Such information will enhance a more thorough understanding of the progressive mechanisms for matrix loss in arthritis, augment information currently available from human studies, examine the association between severity of arthritis and matrix degradative activities in tissues of the joint, and provide some insights into the *in vivo* role of the extracellular balance between MMPs and PIs associated with matrix degradation of arthritis.

Of further significance is the even greater lack of knowledge on matrix degradation in JRA. Although JRA may have similar modalities for matrix loss to those in RA, subtle but important differences may exist in the mechanisms for aberrant matrix turnover between these two disease entities. For example, the existing high rate of matrix turnover and remodeling as well as the greater reparative capacity of growing versus mature tissues (Holmes et al., 1988; Fornieri, et al., 1989; Front et al., 1989; Millie et al., 1989; Martin et al., 1990; Termine, 1990; Mays et al., 1991), are likely to impact on the progression of tissue destruction in the juvenile joint. Furthermore, differences also exist in the immunologic mechanisms and responses between the old and young mammal (Dorai et al., 1987; Gahring and Weigle, 1990; Ho et al., 1990; McElhancy et al., 1990; Pross, 1990; Sironi et al., 1990) which may impact on the progression and consequences of arthritis in the two age groups. It is, therefore, possible that disturbances in the expression of MMPs and PIs in the developing joint results in a progression of arthritis that differs from that in the adult joint. Substantial information on the pathogenesis of matrix degradation in JRA could be obtained from an animal model of this disease, but despite the logical step of adapting the established animal models of arthritis to younger animals, only one such

animal model has been described to date and used for studies evaluating the response to therapy (Hansen et al., 1992), and the expression of inflammatory agents during this arthropathy (Herlin et al., 1988).

With these reasons in mind, a series of studies were conducted whose underlying premise was to evaluate the matrix degradative activity in specific tissues of the joint during experimental arthritis of the juvenile rabbit temporomandibular joint (TMJ). This joint was selected for investigation for several reasons: Firstly, the TMJ has several unique anatomic and functional features not found in other joints. Therefore, a study of this joint would be of importance in comprehending the role of these unique tissues in the pathogenesis of TMJ arthritis. Secondly, of the various mammalian diarthrodial joints, the pathologies of the TMJ are least investigated and poorly understood. A detailed investigation on this joint would enhance a general understanding of TMJ pathologies. Finally, the condylar growth cartilage, which serves a similar function in mandibular growth as the epiphyseal growth plate does in growth of long bones, is located within the synovial capsule of the joint. The proximity of this mandibular growth site to the arthritic joint is likely to have a significant impact on growth of the mandibulofacial complex. Therefore, the development of a model of arthritis of the juvenile TMJ would be useful in understanding the mechanisms for developmental aberrations which accompany JRA of the TMJ .

The unifying hypothesis of these studies is that experimental arthritis of the juvenile temporomandibular joint is characterized by a change in the absolute and relative levels of MMPs and PIs synthesized by specific tissues of the joint. The specific aims to investigate this hypothesis were:

1. To develop a reliable and reproducible juvenile animal model of antigen-induced arthritis of the TMJ, and to provide a histopathologic characterization of this model of experimental arthritis.

2. To characterize and identify proteinases and inhibitors synthesized by TMJ disc cells, and further to evaluate the regulation of these proteins by disc cells.

3. To determine whether the synthesis of specific matrix degrading proteinases, the MMPs and their inhibitors, are altered in the synovium and the disc in antigen-induced arthritis of the TMJ.

4. To determine where the MMPs, collagenase and stromelysin, are localized to in arthritic and non-arthritic TMJs.

5. To determine, histochemically, the changes in glycosaminoglycan and collagen content of specific tissues of the TMJ in this model of antigen-induced arthritis.

The findings related to these specific aims are presented in the following 4 chapters and a synthesis of this information together with suggestions for directions for future

research are outlined in the final chapter. In the next chapter the development and histopathologic characterization of a new model of antigen-induced arthritis in the juvenile rabbit TMJ is described. In Chapter III this model of experimental arthritis is used to evaluate the temporal changes in the expression of MMPs and their PIs in the arthritic synovium retrieved from these animals. This tissue was selected for the first set of experiments on matrix degrading proteinases since previous studies have demonstrated that synovial fibroblasts in culture synthesize MMPs and PIs, and because high levels of these proteinases have been found in arthritic synovium, implicating synovial cells in joint matrix degradation during arthritis. In addition to synovial cells, *in situ* immunolocalization for collagenase and stromelysin in biopsied TMJs revealed staining in cells of the TMJ disc. A thorough literature search revealed no current information on MMP and PI expression by disc cells, or indeed by any analogous tissues, such as the knee meniscus. The studies discussed in Chapter IV demonstrate, for the first time, that explanted TMJ disc and cultured disc cells express several proteinases and inhibitors, which were characterized and identified as MMPs and their PIs. The regulation of these MMPs and PIs by disc cells in response to a protein kinase-C agonist was also partially characterized. In Chapter V, the disturbances in the levels of disc-cell derived MMPs and PIs in antigen-induced arthritis of the TMJ are assessed. In the final chapter a discussion of the implications of our findings and suggestions for directions for future research are provided. The findings of our studies provide an insight into the mechanisms for matrix loss in antigen-induced arthritis of the TMJ, and will be useful in defining rational therapeutic approaches in preventing or alleviating matrix degradation which is a prominent feature of both RA and JRA.

CHAPTER II

DEVELOPMENT AND CHARACTERIZATION OF AN ANIMAL MODEL OF ANTIGEN-INDUCED ARTHRITIS OF THE JUVENILE RABBIT TEMPOROMANDIBULAR JOINT

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A. INTRODUCTION

Juvenile rheumatoid arthritis (JRA) is a chronic inflammatory joint disease of unknown etiology occurring during childhood. This arthropathy frequently involves the temporomandibular joint resulting in pain and dysfunction, and is often accompanied by mandibular retrognathism, decreased dimensions of mandibular body and ramal height, increased gonial and mandibular plane angles, antegonial notching, anterior open-bite, and mandibular dental crowding (Barriga et al., 1974; Larheim et al., 1981a and b; Larheim and Hannaes, 1981; Wenneberg, 1987; Borchorst et al., 1988). Although the mechanisms for these adverse effects of JRA on mandibulofacial development are not understood, it is probable that the degradation of the matrix of joint tissues, effects of inflammation on mandibular condylar growth, and hypofunction of the masticatory apparatus may contribute to the developmental disturbances noted in these individuals (Larheim and Hannaes, 1981; Forsberg and Perrson, 1985; Tavakkoli, 1994). The resulting compromised function and esthetics make JRA of the temporomandibular joint both physically and psychologically debilitating.

Several clinical and radiologic changes have been documented in patients with JRA of the TMJ. In a study on 72 patients with JRA involving the TMJ, 69% had impalpable condyles, 51% had a mandibular asymmetry, 47% demonstrated deviation of the mandible towards the affected side on opening, 30% showed a palpable crepitus or click and 19% showed mandibular micrognathia (Blasberg et al., 1991). Radiographic abnormalities were found in 29 of 30 patients studied with 41% of these being unilateral and 51% being bilateral. Similarly, of 14 JRA patients evaluated in another study, 6 demonstrated asymmetric clinical findings of the TMJ, while the rest had symmetrical TMJ involvement (Taylor et al., 1991). MRI examination revealed cortical erosions or irregularities, flattening of the condylar head, articular fossa and disc, as well as disc perforation (Taylor et al., 1991). The range of motion was limited in about 65% of the joints examined.

Despite substantial amount of information available about the manifestations JRA of the TMJ both clinically and on imaging (Barriga et al., 1974; Larheim et al., 1981a and b; Larheim and Hannaes, 1981; Wenneberg, 1987; Borchorst et al., 1988; Blasberg et

al., 1991; Taylor et al., 1991), little is known about the histopathology of this arthropathy in this joint. However, studies from other joints with JRA or RA demonstrate intense mononuclear cell infiltration, synovial lining and villous hyperplasia and pannus formation (Schumacher and Kitridou, 1972; also reviewed by Hollister, 1979; Vischer et al., 1982; Krane et al., 1986; Harris, 1989). For obvious reasons, knowledge on the histopathology of these arthropathies has been obtained from clinical samples which are in the late stages of pathosis requiring surgical intervention, or which have either had previous treatment or invasive procedures, or both, and these studies also lack healthy controls for comparison. Although much information can be acquired from pathologic human joints, insights into several aspects of these arthropathies especially those requiring invasive procedures during its earlier stages can only be adequately gained from appropriate animal models of experimental arthritis. Indeed, such animal models have been developed over the years, and have provided insights into the pathogenesis (Caulfield et al., 1982; Edwards et al., 1988; Bakker et al., 1990; Henderson et al., 1990, Issekutz and Issekutz, 1991) the immune response and immune mechanisms (Brackertz et al., 1977; Boissier et al., 1987 and 1988; Case et al., 1989a; Wooley et al., 1989; Bakker et al., 1990; Brahn, 1992; Drelon et al., 1992; Sano et al., 1992), factors contributing to genetic predisposition (Brackertz et al., 1977; Watson et al., 1990), evaluation of tissue loss (Lowther et al., 1978; Rubin et al., 1987; Rubin and Roberts, 1987; Yoo et al., 1988; Pettipher et al., 1989 and 1990; Henderson et al., 1990; Beesley et al., 1992; Reddy and Dhar, 1992), and assessing the efficacy of anti-arthritic therapeutic regimens (Rubin and Roberts, 1987; Case et al., 1989c; Francis et al., 1989; Pettipher et al., 1989; Kuruvilla et al., 1991; Setnikar et al., 1991; Peacock et al., 1992; Greenwald et al., 1992; Hansen et al., 1992). However, despite the logical step of adapting the existing animal models of arthritis to growing animals, to date only one attempt has been made at devising such a model (Herlin et al., 1988; Hansen et al., 1991 and 1992). The use of this animal model, developed in the knee joints of juvenile dogs, was limited to an assessment of microvascular hemodynamics and response to therapy (Hansen et al., 1991 and 1992), and the expression of inflammatory agents (Herlin et al., 1988). Such a model, if developed in the TMJ of a juvenile animal, may be useful in better understanding pathogenesis of JRA of the TMJ, particularly with regard to the mechanisms for disturbed mandibulofacial development, evaluating the loss of extracellular matrices as well as the modalities for this matrix loss, and further, for evaluating the differences in the pathogenesis of RA and JRA.

Although several animal models of arthritis in the knee joint are currently available (summarized in Table II, Chapter I), only one study to date has attempted to induce arthritis in the TMJ (Zamma, 1983). In this investigation, Lewis, Wistar and Sprague-Dawley rats

were immunized with mycobacteria dilapidated cells in squalene administered into the parietal scalp, rather than into the footpad immunization used for inducing arthritis in the knee joint. Only Lewis rats developed TMJ arthritis which was evident histologically in 30% to 60% of the animals immunized with 0.5 mg and 1 mg of Mycobacterium, respectively. Because of the low incidence of TMJ involvement, this model as described, lacks adequate reproducibility and this report also provides only a partial histologic characterization of the model. For the reasons of a lack of a suitable animal model of TMJ arthritis particularly in a growing animal, we embarked on a study to define and characterize a model of experimental arthritis of the juvenile TMJ. The purpose of our study was to develop a juvenile animal model of antigen-induced arthritis of the TMJ, and further, to describe the histopathogenesis of this model. The specific aims of our study were to:

1. Describe the normal microscopic anatomy of the rabbit TMJ.
2. Determine the dose and the technique for inducing reproducible arthritis in the juvenile rabbit TMJ.
3. Characterize and quantitate the histopathologic changes which accompany this experimental arthritis.
4. Evaluate the histochemical changes in specific matrix molecules, namely glycosaminoglycans (GAGs) and collagen, in joint tissues during antigen-induced arthritis of the rabbit TMJ.

A preliminary study was done to determine if antigen-arthritis could be induced in the rabbit TMJ, and if so, what the inducing doses were, and at what time-points after induction were histopathologic changes evident. Once these studies had established the guidelines for antigen doses and time-points for retrieval of samples, a study was conducted for the histopathologic characterization of the model by an objective quantitative arthritic score, and for evaluating the loss of specific matrix molecules through histochemical techniques. Severe arthritis was evident as early as 5 days after challenge and maintained up to 55 days post-challenge. A decrease in the percent area of GAG staining in the TMJ disc was observed throughout the experimental period. The percent area of the TMJ disc staining positive for GAGs had a statistically significant inverse relationship to the severity of arthritis. The loss in collagen staining was primarily localized to sites where the pannus invaded the articular cartilage and bone, and in the disc at its junction with the synovium. The histopathology of this model of antigen-induced arthritis of the juvenile rabbit TMJ is similar to that observed previously in adult animal models of experimental arthritis (Dumonde and Glynn, 1962; Consden et al., 1971; Brackertz et al., 1977; Caulfield et al., 1982; Catchcart et al., 1986; Rubin et al., 1987; Edwards et al.,

1988; Case et al., 1989a; Bakker et al., 1990; Hasty et al., 1990; Beesley et al., 1992) and to RA (Schumacher and Kitridou, 1972; Vischer et al., 1982; Krane et al., 1986; Harris, 1989).

B. MATERIALS AND METHODS

Materials

Male New Zealand white rabbits (*Oryctolagus cuniculus*) were obtained from Nita Bell Laboratories (Hayward, CA.); ovalbumin (OA) grade III, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), paraformaldehyde, poly-l-lysine, Harris' and Weigert hematoxylin, eosin, Safranin-O, chromatrope 2R, phosphotungstic acid, and Bouin's solution from Sigma Chemicals (St. Louis, MO.), glycerol, ethylenediamine tetraacetic acid (EDTA), aniline blue and glacial acetic acid from Fisher Scientific (Pittsburgh, PA.), and OCT from Miles Scientific (Naperville, IL.)

Preliminary Studies

Initial studies were done to establish whether it would be possible to induce arthritis in the rabbit TMJ, and if so, to determine the sensitizing and challenging doses, and the time-points for evaluation of histopathologic changes. The rabbit was selected as the animal of choice for these studies since it provides a suitable anatomic, histochemical and functional analogue for the human TMJ (Weijs and Dantuma, 1981; Scapino, 1983; Mills et al., 1988). Furthermore, the rabbit TMJ is relatively well characterized (Weijs and Van der Weilen-Drent, 1982; Mills et al., 1988; Nagy and Daniel, 1991 and 1992). Having selected this animal species, a suitable method for induction of arthritis was sought. A review of the literature (Table II, Chapter I) revealed that antigen-induced arthritis using ovalbumin has been the most common, successful and reliable method used for inducing arthritis in the knee joints of rabbits (Dumonde and Glynn, 1962; Consden et al., 1971; Lowther et al., 1978; Howson et al., 1986; Edwards et al., 1988; Henderson et al., 1990; Beesley et al., 1992).

In the first preliminary study, 4 young adult animals, approximately 20 weeks of age, were sensitized with 1 ml of 10 mg/ml of OA in equal volumes of CFA and normal saline administered intradermally at 5 sites (approximately 200 µl per site) in the upper back between the scapulae. This was followed two weeks later by a second sensitizing dose of 10 mg/ml OA in equal volumes of IFA and normal saline administered as for the first sensitizing dose. One week after the second sensitizing dose, the animals were tested for sensitization by injecting 100 µl of 2 mg/ml of OA in normal saline administered subcutaneously on a shaved patch on the animals' back distant from the sites of

sensitization. Readings for sensitization were made at 4 and 24 hours of this injection by evaluating for erythema, induration and warmness. All animals showed mild to moderate signs of delayed hypersensitivity including erythema and induration. Both joints of all sensitized animals were challenged with 0.5 mls of 4 mg/ml of OA in normal saline (NS) administered intra-articularly after placing the animal under light anesthesia with 3-5 mg/kg of Xylazine (Rompan, Rugby Laboratories Inc., Rockville Centre, New York) given subcutaneously (SQ), and 25 mg/kg of Ketamine-hydrochloride (Ketalar, Parke-Davis, Morris Plains, New Jersey) given intramuscularly (IM). Two animals were lost when administering the challenging doses as detailed below. Therefore, one animal each was sacrificed at 7 and 30 days post-challenge and the right and left TMJs retrieved *en-bloc*, fixed with 10% formalin for 24 hours, decalcified in 10% EDTA until decalcification was complete (approximately 3 weeks), and processed for embedding in paraffin. Six-μm thick sections were cut, stained with hematoxylin and eosin, and examined microscopically. This preliminary study revealed that:

1) Challenging joints under light anesthesia with Xylazine and Ketamine-HCl frequently lead to a rapid respiratory arrest and death of animals probably because of the compounding effects of anaphylactic shock on an already centrally depressed respiration. This anesthetic regimen was changed following a similar response in the second preliminary study as described below. Following this change in the anesthetic protocol no other animals were lost.

2) The method used was successful in inducing arthritis in the rabbit TMJ. Three of the four joints injected demonstrated inflammatory changes within the joint, while the fourth had an inflammatory infiltrate within muscle fibers to the anterior of the disc. The latter finding may have resulted from the antigen inadvertently being administered extra-articularly. The features of arthritis included dense and widespread inflammatory infiltrate within the synovium, synovial lining and villous hyperplasia, variegated synovial lining cells, and an aggressive destruction of bone and cartilage. These features were observed at both the early and late time-points, but were especially marked at 30 days post-challenge. From these findings it was evident that, with the dose used, severe arthritis was successfully induced in the rabbit TMJ.

A second preliminary study was then conducted with five, 8-week-old rabbits in order to resolve the problems of animal death at challenge, to define the earliest possible time-point for evaluating observable histopathologic changes, and to further titer the dose of antigen for younger animals. In the experimental design for this study the right joint from each of four of animals served as experimental joints, while the left joints served as sham-treated controls. Both joints from the fifth animal were untreated controls. A similar

protocol for inducing arthritis as that used in the previous preliminary study was followed with the exception that the first and second sensitizing doses were reduced to 1 ml of 1 mg/ml of OA in CFA and NS and 0.5 mls of 1 mg/ml of OA in IFA and NS, respectively. The sensitivity test was done with 100 µl of 400 µg/ml of OA in normal saline injected subcutaneously on a shaved patch on the animals' back distant from the sites of sensitization. Readings for sensitization were made at 4 and 24 hours of this injection as before. All animals showed mild to moderate signs of delayed hypersensitivity. Despite the lower sensitizing dose and a lighter anesthetic dose (Ketamine HCl 10 mg/kg; Xylazine 2 mg/kg), the first two animals challenged died within a few seconds of administration of the challenging dose. The remaining two experimental animals were then anaesthetized with 0.1 ml of Acepromazine maleate (10 mg/ml; IM) and 0.8 ml Ketamine hydrochloride (10 mg/ml) and demonstrated no ill-effects during administration of the challenging dose. Two challenging doses were used in these animals; one animal received 0.2 mls of 10 mg/ml OA in NS, while the other animal was administered 0.2 mls of 1 mg/ml of OA in NS, both administered in the right joints. Both animals also received 0.2 mls of NS in the left TMJs. These rabbits together with the normal control animal were sacrificed 3 days after challenge, the TMJs retrieved *en bloc*, fixed, decalcified, processed, embedded in paraffin, sectioned and stained as described above. This study revealed histologically evident arthritis including inflammatory infiltrate and synovial lining hyperplasia which was mild in the low dose challenge joint and moderate in the high dose challenge joint at 3 days after induction of arthritis.

Induction of Arthritis

A total of twenty-seven ten-week-old male New Zealand white (NZW) rabbits were sensitized to ovalbumin by two systemic sensitizing doses of this antigen, prepared in NS and complete or incomplete Freund's adjuvant and given 14 days apart as described above and doses shown in Figure 1 (first sensitizing dose 1 ml of 1 mg/ml of OA in CFA/NS; second sensitizing dose of 1 ml of 0.5 mg/ml of OA in IFA/NS). Animals were tested for sensitization to the antigen 1 week later with 100 µl of 200 µg/ml OA in NS, and the TMJs challenged bilaterally with an intra-articular injection of 100 µl of 5 mg/ml of antigen (Total OA 500 µg per joint) in normal saline administered 2 weeks after the second sensitizing dose. Both sham-treated animals, which received both the sensitizing doses and an intra-articular administration of 100 µl of normal saline only, as well as untreated animals were used as controls. Three experimental, 1 or 2 sham-treated and one normal control animals each were sacrificed 5, 10, 15, 35 and 55 days post-challenge, the right TMJs were retrieved *en bloc* and fixed for histology and immunohistochemistry, while the left TMJ synovia and discs were retrieved under sterile conditions for culturing (Results reported in

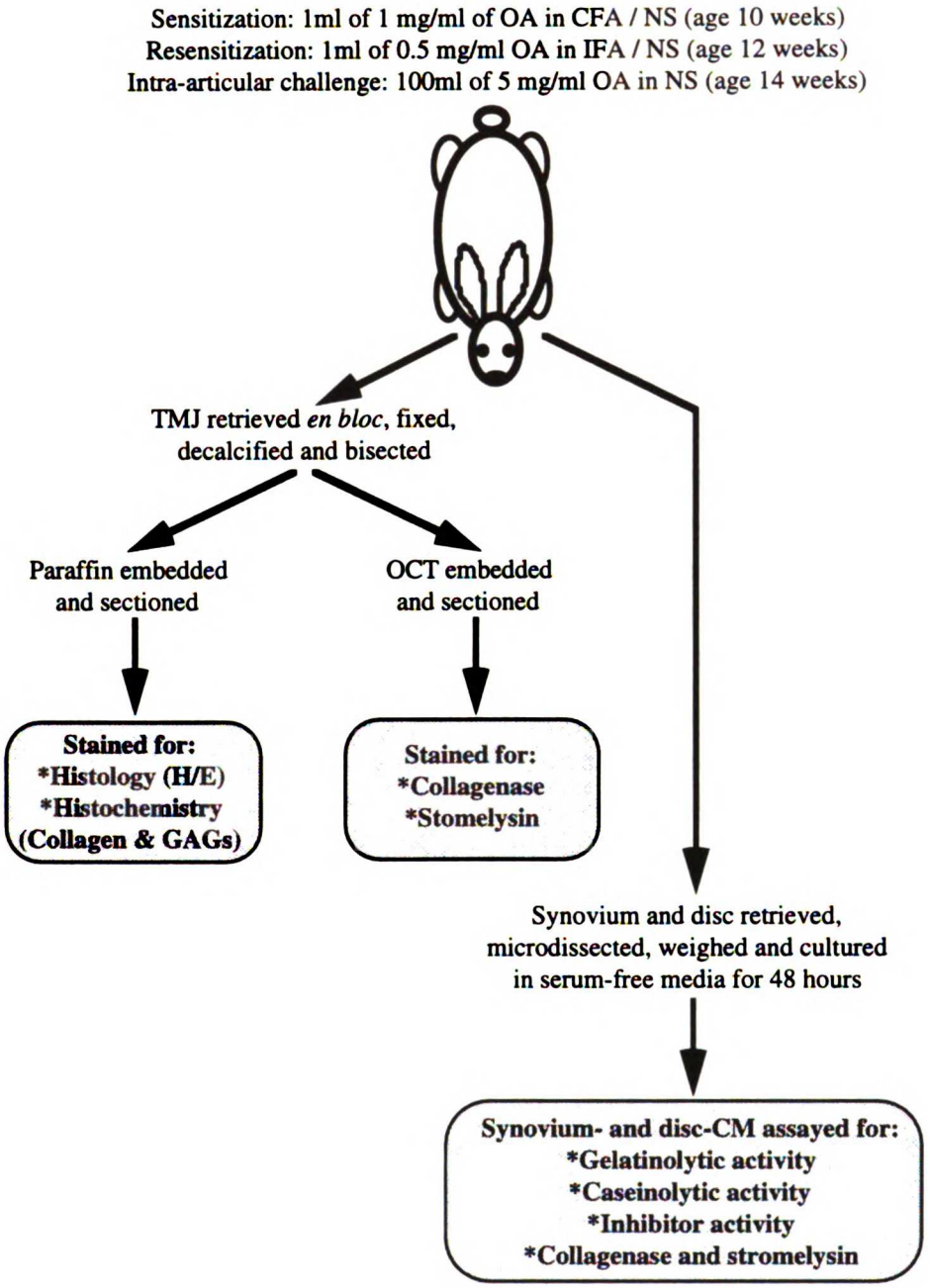

Figure 1: Experimental design (OA=ovalbumin; CFA=complete Freund's adjuvant; IFA=incomplete Freund's adjuvant; NS=normal saline; CM=conditioned media).

Chapters III and V, respectively).

Since the rabbit TMJ does not have a bony superior-posterior articulation, this was found to be the ideal site from which to approach the joint for the administration of the intra-articular challenging doses. After shaving the area over the TMJ, the joint was palpated approximately 5 to 10 mm. posterior to the posterior canthus of the eye while the

mandible was manipulated by opening and closing to provide movement of the condyle for a further positive identification of the joint. The needle was then inserted from a postero-superior direction until it contacted the mandibular condyle and could be moved by opening and closing the mandible. Every attempt was made to insert the needle directly into the inferior joint cavity so as to avoid passing through the disc. Once inside the joint, the antigen or normal saline was injected slowly over a period of approximately 5 seconds.

Retrieval, Processing and Sectioning of TMJs

The animals were anaesthetized with ketamine hydrochloride and Xylazine at the prespecified time-points after the challenging dose. The right TMJ was surgically exposed, dissected free of neighboring tissues, retrieved *en bloc* and immediately placed in 4% PBS-buffered paraformaldehyde for 24 hours. The joints were then decalcified by a procedure described by Mori et al. (1988) which provides a gentle decalcification process compatible with tissues to be used for immunohistochemistry. The tissues were washed in graded solutions of 5, 10 and 15% glycerol in PBS (pH=7.2) at 4°C for 15 minutes each, and then placed in 14.5% (w/v) dipotassium-EDTA, 1.25% (w/v) sodium hydroxide and 15% (v/v) glycerol. The decalcification end-point was determined chemically by sodium oxalate precipitation (AFIP Laboratory Methods in Histotechnology, pp:72-73). Each joint was then bisected parasagittally into two halves and the glycerol replaced by sucrose through graded washes at 4°C with PBS containing (a) 15% sucrose and 15% glycerol for 6 hours, (b) 20% sucrose and 10% glycerol for 1 hour, (c) 20% sucrose and 5% glycerol for 1 hour, and (d) 20% sucrose for 6 hours. One half of the joint was then embedded in OCT, while the other half was dehydrated through graded alcohol washes and embedded in paraffin. When embedding in paraffin or OCT, each half of the joint was oriented such that the midsagittal section was accessible for sectioning. Sections, 6 μm thick, were cut and placed on poly-l-lysine coated slides and let to air dry prior to staining.

Histology and Quantitative Scoring of Arthritis

Three randomly selected representative sections from each joint were deparaffinized and rehydrated through xylene and graded ethanol washes and stained with hematoxylin and eosin. The severity of arthritis was scored by two, previously calibrated, blinded and independent observers using a scoring criteria modified from that proposed by Pelletier et al. (1985) and Martel-Pelletier et al. (1986). Briefly, the severity of arthritis was graded from 0 to 12 by adding the scores of four histologic criteria: 1) synovial lining hyperplasia graded from 0 to 2 (0 = 1 to 3 layers of cells; 1 = 4 to 6 layers of cells; 2 = 7 or more layers of cells), the layers being counted from the surface of the membrane to the subsynovial tissue; 2) villous hyperplasia graded from 0 to 3 (0 = not present; 1 = few, scattered and short; 2 = marked and finger-like; 3 = marked and diffuse); 3) inflammatory

cell infiltrate as the degree of infiltration by mononuclear cells graded from 0 to 4 (0 = normal; 1 = mild; 2 = moderate; 3 = severe; 4 = marked cellular infiltration mixed with lymphoid follicles); 4) pannus formation graded from 0 to 3 (0 = absent; 1= mild to moderate synoviocyte proliferation and invasion into disc, cartilage or bone; 2 = severe synoviocyte and some inflammatory cell invasion into disc, cartilage or bone; 3 = marked synoviocyte and inflammatory cell invasion into disc, cartilage or bone).

Histochemical Staining and Quantitation of GAGs and Collagen

Safranin-O is a stochiometric stain of sulfated glycosaminoglycans, the oligosaccharide component of proteoglycans (Kiviranta et al., 1984). Its contrast from background is enhanced by observing with monochromatic light at 500 nm wavelength. Staining for GAGs was performed after deparaffinization and rehydration of tissues with 0.5% Safranin-O in 50% ethanol for 10 minutes as described by Kivranta et al. (1985). Excess stain was then rinsed off with 50% ethanol, and sections dehydrated and mounted with coverslips.

Collagen staining was done by the Gomori's trichrome method (AFIP Laboratory Methods in Histotechnology, pp:191-192). Briefly, the sections were rehydrated, placed in Bouin's solution for 1 hour at 60°C, washed until no yellow coloration was evident, stained with Weigert heme for 7 minutes, washed, stained with Gomori trichrome solution [chromatrope 2R, 0.54% (w/v); aniline blue, 0.27 % (w/v); phosphotungstic acid 0.72% (w/v); glacial acetic acid, 0.9% (v/v)] for 15 minutes, rinsed in 0.2% acetic acid for 1 minute, dehydrated through graded alcohol washes and mounted with coverslips.

Both the area and intensity of GAG staining were evaluated in a selected tissue of the joint, namely the TMJ disc. The disc was selected for these purposes since it is clearly demarcated from the attached synovia enabling the measurement of total disc area from which the changes in percent area of GAG staining can be derived with a degree of accuracy not possible with the less clearly defined condylar or articulating cartilages. Two randomly selected sections from each joint were stained with Safranin-O as described above, and the images video-digitized from a microscope (Nikon Microphot FX, Japan) onto a computer (Macintosh IICi, Apple Computers, Cupertino, CA.) using a CCD camera (NEC TI-24A, Japan) and imaging software (Image Version-1.45, NIH, Bethesda, Maryland) after initial calibration for linear measurements with a stage micrometer. The contrast between GAG and background was enhanced by using a filter which narrows the transmitted wavelength between 450 and 530 nm. Both the outline of the disc and area staining for GAGs were traced directly off the image on the computer monitor to obtain the total disc area and area as well as intensity of GAG staining. The two former measurements were used to derive the percent of the disc staining positive for GAGs. The

intensity of Safranin-O staining was measured by the software as the average density of staining measured by the software within the traced outline for GAGs in the disc.

Statistical Analyses

Descriptive statistics on means and standard deviations were determined for quantitative measures and displayed as histograms. Comparison of arthritic scores and GAG staining between antigen-challenged, sham-treated and untreated control animals were made by ANOVA and the intergroup differences further determined by Fisher's multiple comparisons test with the level of significance set at $P<0.05$. Relationships between measured variables were determined by regression analysis.

C. RESULTS

Histology of the Rabbit TMJ

The normal rabbit TMJ has an articulating surfaces comprised of the squamous part of the temporal bone superiorly, the mandibular condyle inferiorly, and the articular disc interposed between these two structures. Histology of the joint demonstrates that the disc is a dense connective tissue largely composed of collagen and GAGs and a well distributed cellular component (Fig. 2a). In the sagittal plane, the superior articular surface does not encompass the disc and condyle, but forms a roof over only the antero-superior portion of the joint disc and condyle. The mandibular condyle is a convex shaped structure whose articulating surface was found to be composed of collagenous fibrous tissue. Beneath this fibrous tissue is a cartilaginous layer which, in the growing animal demonstrated 4 distinct regions, namely the resting, proliferative, mature and hypertrophic zones named on the basis of the cellular phenotype. With age, it was noted that the proliferative and mature chondrocyte zones diminished in size as the end of the growth phase is reached. The condyle, disc and squamosal articular surface are encapsulated by the synovium, which demonstrated two histologically distinct regions, the inner synovial lining adjacent to the joint cavities, and the outer synovial stroma. The synovial lining was made up of 1 to 3 cell layers of synoviocytes (Fig. 2e). The lining of the superior joint cavity appeared especially delicate being composed of a dispersed single layer of cells. The underlying synovial stroma was noted to be primarily a loose connective tissue with sinusoids, blood vessels and a few scattered spindle shaped cells, probably fibroblasts. A few delicate villi composed of synovial lining and stroma were observed projecting into the joint cavity. Safranin-O staining for GAGs revealed intense staining of the mid-halves of the anterior and posterior bands as well as the entire intermediate zone of the TMJ disc (Fig. 3a and b). The zones of mature and hypertrophic chondrocytes of the condylar

cartilage, as well as small isolated areas of the underlying cancellous bone also stained positive for GAGs. Using Gomori's trichrome stain, intense blue staining, representative of collagen, was noted in the disc, the fibrous covering, and the subchondral bone, while lighter staining was evident in the mineralizing front of the subchondral bone, in proliferating and mature chondrocyte zones of the superior articular and condylar cartilages and in the synovial stroma (Fig. 4). Very little staining for collagen was noted in the hypertrophic zone of the condylar cartilage.

Histopathology of Antigen-induced Arthritis of the TMJ

Histologic examination and quantitation of arthritis revealed successful induction of arthritis as early as 5 days after challenge which persisted up to 55 days post-challenge (Fig. 2). Although the antigen-challenged animals demonstrated substantial amounts of individual variability in their arthritic response to the challenging antigen, all of them had positive histologic findings. Because of the individual variability in response, clear cut trends were not evident in the four scoring criteria over the 5 time-points. However, in general the predominant earlier features of the pathology were mononuclear cell infiltration, and some synovial lining and villous hyperplasia. The pannus at the 5 days post-challenge had a large component of inflammatory cells mixed with fewer fibroblasts, while in the later stages of the pathology fibroblasts were the predominant cells in the pannus (Fig. 2c and h). The magnitude of synovial lining and villous hyperplasia was more marked at 10 and 15 days post-challenge when compared to that observed at 5 days after challenge, while the severity of mononuclear cell infiltrate persisted at similar levels at these three time-points. The synovial lining cells often demonstrated mitotic figures, and frequently, the superficial cells were variegated.

The intensity of these histologic features diminished slightly over time (Fig. 5). Very mild levels of synovitis, primarily synovial lining and villous hyperplasia, were also noted in the sham-treated animals especially at the earlier time-points. Statistical analysis revealed a significant difference (ANOVA $P=0.0001$) in arthritis scores in joints from antigen-treated animals when compared to those from sham-treated animals ($P<0.0001$), or untreated control animals ($P<0.0001$) when data from all 5 time-points was combined. No statistically significant differences in arthritis scores were noted between sham-treated and normal control animals ($P=0.52$), and therefore, for the purposes of presenting descriptive statistics, the data from these two groups was combined (Fig. 5b). Furthermore, the duration of arthritis was found to have no significant effect on the arthritis score ($P>0.1$), further confirming the lack of any statistically detectable trends in the severity of arthritis over time.

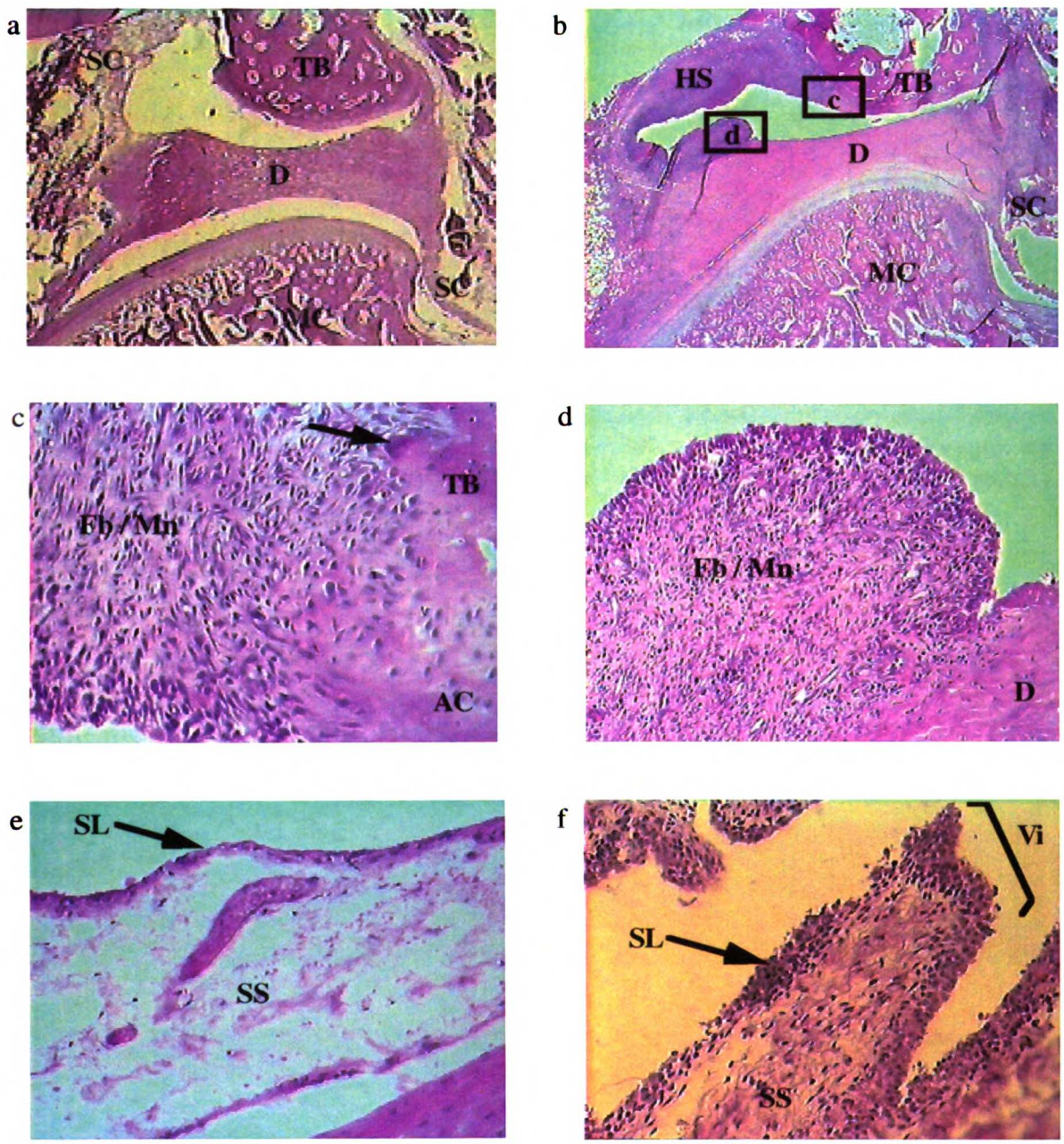

Figure 2: Histology of the normal and pathologic TMJ. Low power photomicrograph of the normal (a), and 5 day post-challenge (b) TMJ (original magnification x 10). (c) and (d) higher power photomicrographs of boxed areas in (b) showing inflammatory infiltrate at the periphery of the superior articular cartilage and bone (c), and the articular disc (d) (original magnification x 320 and x 200, respectively). (e) High power photomicrograph of synovium showing a delicate synovial lining (SL) and underlying synovial stroma (SS) of loose connective tissue in a normal joint (original magnification x 320). (f) 15 day post-challenge joint showing hypertrophic synovial lining and fibrous synovial stroma (original magnification x 200). TB=temporal bone; SC=synovial capsule; D=disc; MC=mandibular condyle; HS=hyperplastic synovium; AC=articular cartilage; Fb / Mn=fibroblast hyperplasia and mononuclear cell infiltration; Vi=villous. (continued on next page).

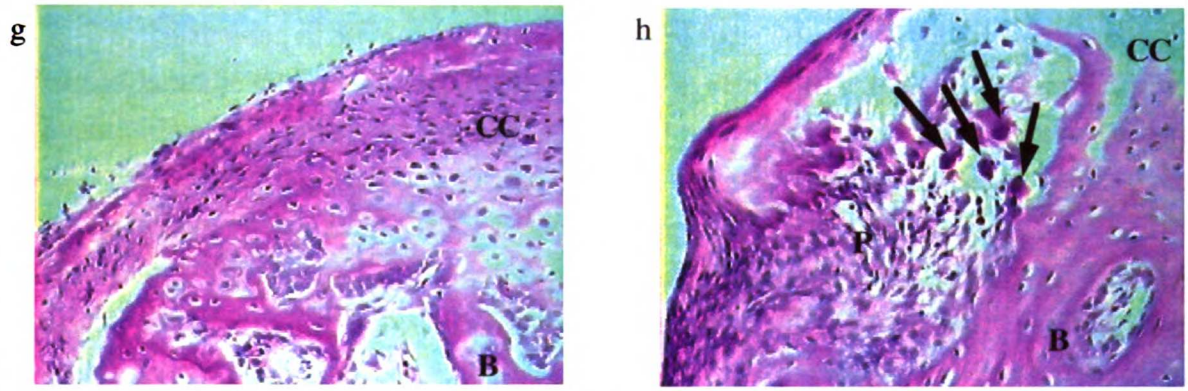

Figure 2 (continued): (g) Histology of the normal condyle. (h) 55 day post-challenge condyle showing infiltration of bone and cartilage by proliferative synoviocytes forming a pannus (P). (original magnification x 320). Arrows in (c) and (h) indicate multinucleated cells, probably osteoclasts, at the pannus-bone junction. CC=condylar cartilage; B=bone of mandibular condyle.

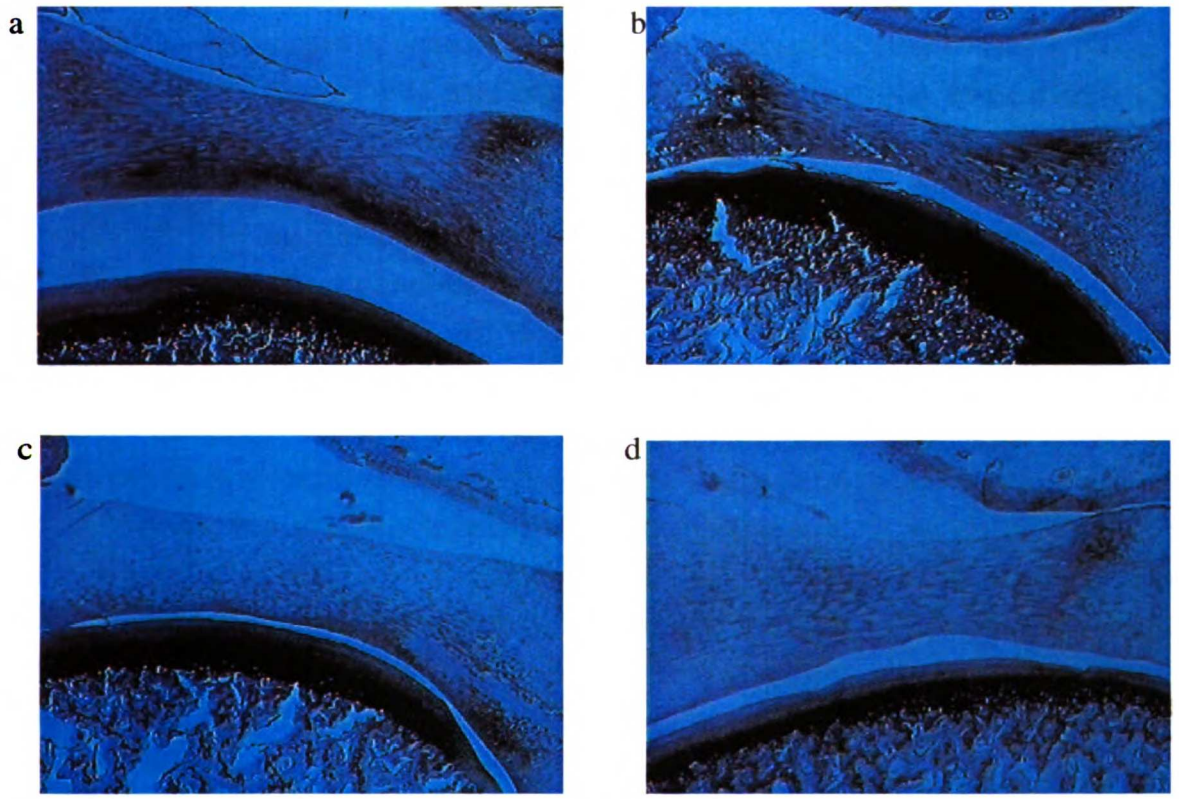

Figure 3: Representative sections of the TMJ demonstrating GAG staining in discs from untreated control (a), sham-treated control (b), and 5 (c) and 55 (d) day post-challenge joints. (original magnification x 60).

Histochemical Changes in Joint Matrices

Evaluation of Safranin-O staining revealed focal areas of GAG loss in the condylar cartilage, while in the disc, GAG loss was more generalized (Fig 3). Histomorphometric quantitation for Safranin-O staining in the TMJ disc demonstrated that, on the average, 49.07% (SD±5.29%) and 52.09% (±6.03%) of the disc area from untreated and sham-treated joints, respectively, stained positive (Figs. 3 and 6). In contrast, only 21.96% (±8.45%) of the area of discs from antigen-treated joints stained positive for GAGs. These findings reflect statistically significant differences (ANOVA $P<0.0001$) in Safranin-O staining when discs from all antigen-challenged joints are compared with those from sham-treated control ($P<0.0001$) or untreated control joints ($P<0.0001$). However, the differences in GAG staining between discs from sham-treated and untreated control joints were not significantly different ($P=0.49$), and the data from these two groups was combined for graphic presentation of the descriptive statistics (Fig. 6a).

Although the time-course changes in percent disc area staining for GAGs were not statistically significant ($P>0.05$), some trends were noted. By 5 days post-challenge, the percent area of the disc staining with Safranin-O in experimental joints was approximately half that in sham-treated or untreated control joints, decreased slightly further at 10 and 15 days post-challenge, and by 55 days after challenge was similar to the levels observed at 5 days after induction of arthritis. A statistically significant inverse correlation was found between the severity of arthritis and percent area of the disc staining for GAGs ($R^2= -0.79$; $p<0.0001$). The intensity of Safranin-O staining was slightly, but not significantly lower in discs from arthritic as compared to control animals (ANOVA $P=0.07$) (Fig. 6b).

Several areas of the normal TMJ stained intensely for collagen, primarily the disc, the articular fibrous layer of the condylar surface, and the subchondral trabecular bone of both the condyle and squamosal articulation, and the antero-superior and postero-inferior areas of the synovium. The mineralizing front of the subchondral bone also stained positively but less intensely than the areas described above. Even less stained is the condylar cartilage in the zone of mature chondrocytes. In the sagittal plane, the collagen fibers in the intermediate zone of the disc are oriented antero-posteriorly, while the anterior and posterior band areas demonstrate collagen fibers primarily lying perpendicular to the plane of the section, with fewer fibers oriented antero-posteriorly. The collagen fibers of the fibrous zone of the articular surfaces are oriented parallel to the articular surface and appear finer than collagen fibers in the disc.

The differences in collagen staining between joints from antigen-challenged and control animals were not as discernible as the differences in GAG staining between these groups of animals. Loss of collagen staining was mainly localized to areas where the

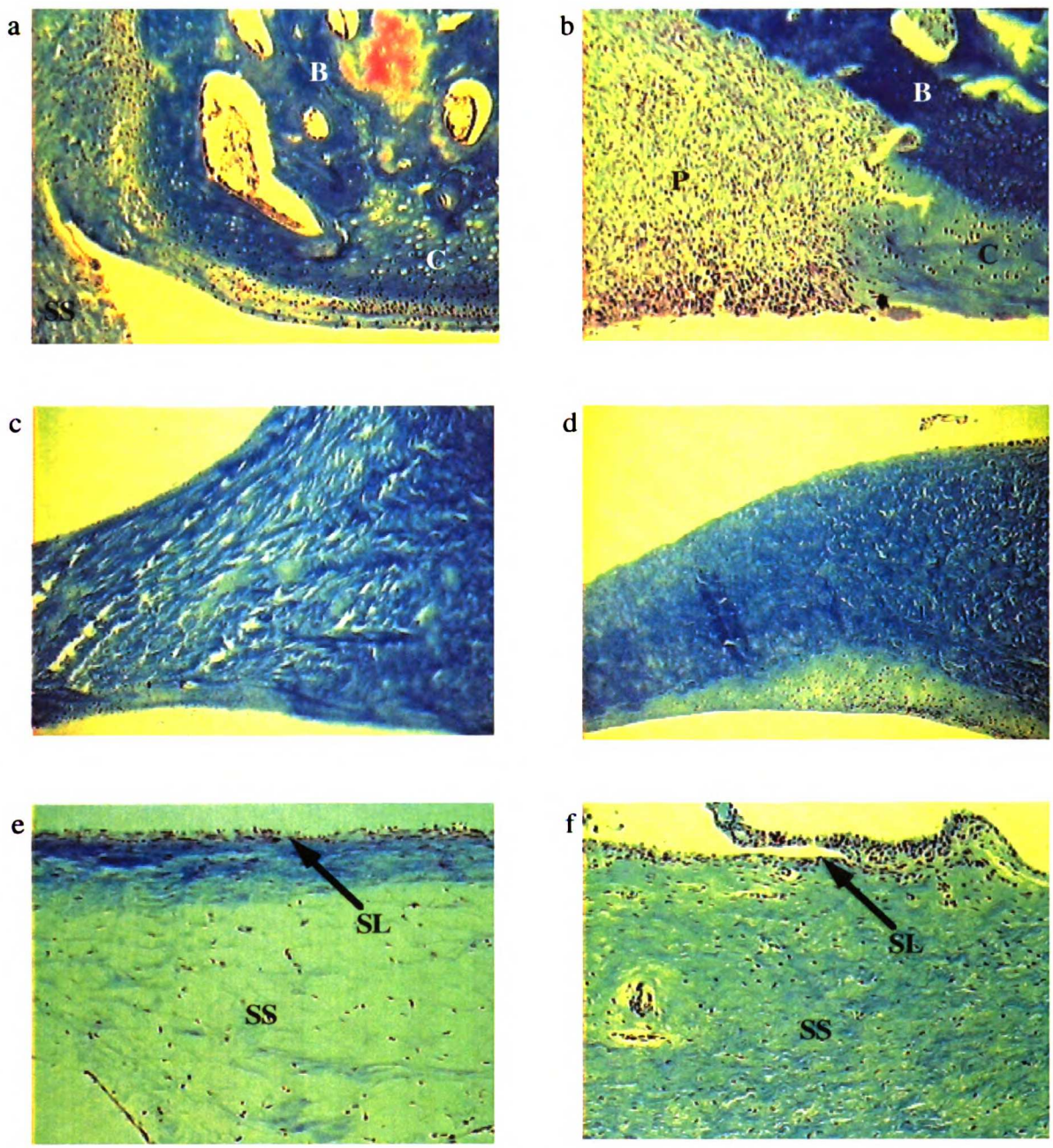

Figure 4: Gomori's trichrome staining in the postero-superior articular surface of a normal joint (a), and 5 day post-challenge joint (b), the latter showing loss of collagen at sites where pannus has invaded bone and cartilage (original magnification x 160). Normal disc (c), and 10 day post-challenge disc (d) with localized loss of collagen in the antero-inferior surface near the junction of the disc with the synovium (original magnification x 100). Normal (e) and 55 day post-challenge synovia (f). The synovial stroma (SS) from the arthritic joint demonstrates fibrosis (original magnification x 200). B=bone; C=cartilage; P=pannus; SL=synovial lining, SS=synovial stoma.

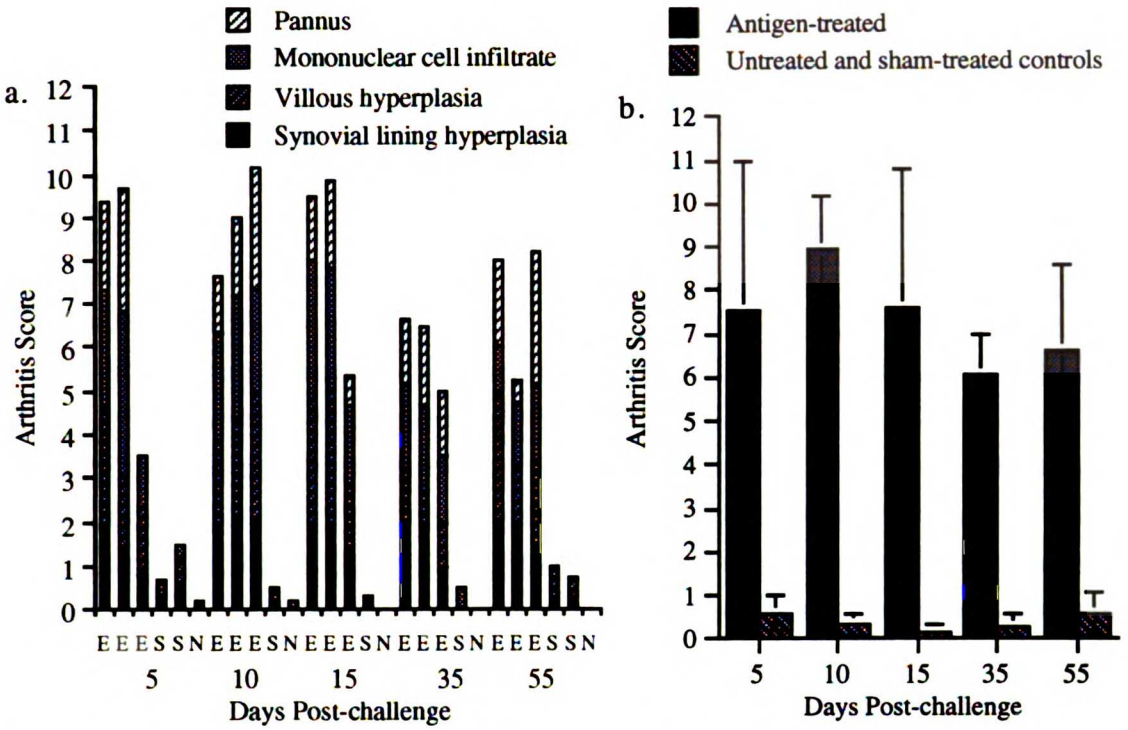

Figure 5: (a) Arthritis scores for individual animals (E=antigen-treated; S=sham-treated controls; N=untreated controls). (b) Means and standard deviations for arthritis scores in antigen-treated, as well as sham-treated and untreated control joints.

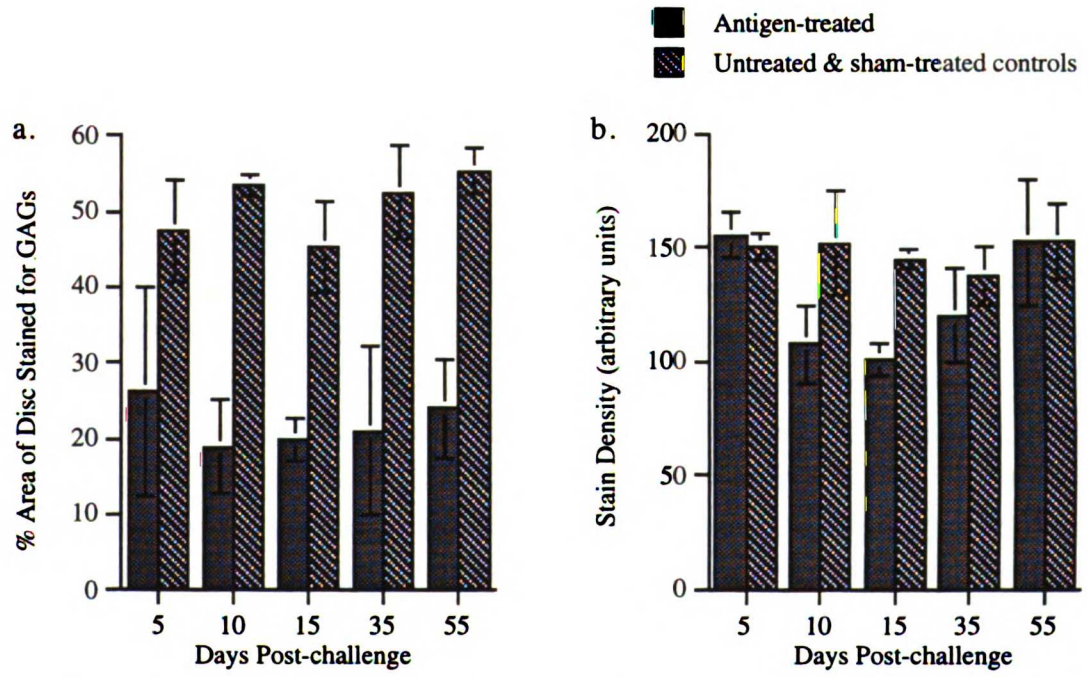

Figure 6: (a) Mean and standard deviations for percent area of disc staining positive with Safranin-O. (b) Mean and standard deviations for intensity of Safranin-O staining in the disc.

pannus invaded bone and cartilage, and in the disc near its border with the synovium (Fig. 4b and d). At the 35 and 55 day post-challenge time-points, a greater amount of staining for collagen was noted in synovial stroma from antigen-challenged versus control joints (Fig. 4e and f) a finding similar to that reported by Beesley et al. (1992). Few other discernible differences in collagen staining were noted between the 3 groups of animals.

D. DISCUSSION

In this study we describe the development of the first animal model of antigen-induced arthritis of the TMJ, and also provide the first histopathologic characterization of an arthritis model in a juvenile animal. Following induction of arthritis with systemic sensitization and intra-articular challenge with ovalbumin histologic features of arthritis were evident from 5 days after challenge to 55 days post-challenge. A generalized loss in GAG staining was noted in the disc, while localized loss in collagen staining was observed in sites where the pannus invaded into bone and cartilage. These histopathologic observations are similar to those made previously in other experimental models of arthritis (Dumonde and Glynn, 1962; Consden et al., 1971; Brackertz et al, 1977; Caulfield et al., 1982; Catchcart et al., 1986; Rubin et al., 1987; Edwards et al., 1988; Case et al., 1989a; Bakker et al., 1990; Hasty et al., 1990; Beesley et al., 1992), and in human RA (Schumacher and Kitridou, 1972; Vischer et al., 1982; Krane et al., 1986; Harris, 1989). The development and histopathologic characterization of this model will be useful for future studies to delineate the pathogenesis as well as define the cellular and molecular mechanisms for various other changes, such as the aberrant mandibulofacial development associated with JRA of the TMJ.

We opted to develop a model of antigen-induced arthritis in the rabbit TMJ for several reasons. Firstly, the morphology and physiology of the rabbit TMJ is well characterized (Weijs and Dantuma, 1981; Weijs and Van der Weilen-Drent, 1982; Lund et al., 1984; Anapol et al., 1987; Mills et al., 1988), providing much of the baseline information necessary for further use of the TMJ in this animal for development of models of arthritis. Secondly, the rabbit TMJ is considered to provide a suitable histologic and functional analogue to the human TMJ. The histology and histochemistry of the joints from the two species demonstrate numerous similarities including cellular organization, as well as distribution of collagen types I and II, proteoglycans, and elastin (Mills et al., 1988; Savalle et al., 1990; Nagy and Daniel, 1991 and 1992). The rabbit disc is biconcave, as in man, and has anterior and posterior band areas rich in PGs separated by a narrow intermediate zone (Mills et al., 1988). In the human TMJ disc, collagen fibers in the central

part run antero-posteriorly to interlace with transversely oriented fibers in the anterior and posterior bands (Scapino, 1983). We noted a similar arrangement of collagen fibers and GAG staining in the rabbit TMJ disc. Furthermore, jaw movements in biting and chewing are similar in the two species (Weijs and Dantuma, 1981). Thirdly, antigen-induced arthritis has been successfully and reliably induced in the rabbit knee by several investigators (Dumonde and Glynn, 1962; Consden et al., 1971; Lowther et al., 1978; Cambray et al., 1981; Murphy et al., 1981; Howson et al., 1986; Edwards et al., 1988; Henderson et al., 1990; Beesley et al., 1992), indicating a potential for similar success in the TMJ. A few differences, however, also exist between the human and rabbit TMJs The two most notable variations between the rabbit and human TMJs are (a) the lack of a glenoid fossa in the rabbit such that there is an incomplete superior and posterior articulation for the condyle, and (b) unlike humans, the rabbit condyle has a narrow projection extending posteriorly. Despite these differences in the TMJs of the two species, the similarities and previous characterization of this joint in the rabbit provide significant advantages for the use of this animal in our experiments.

As described previously (Chapter I), the TMJ condylar articular surface is made up of 5 histologically distinct zones; the fibrous articular surface with few cells, the resting zone of undifferentiated mesenchymal cells and chondro-progenitor cells, the proliferative zone, the hypertrophic zone with enlarged chondrocytes and unmineralized matrix and the zone of mineralized matrix. With age we noted that the latter four zones decreased in dimension being gradually replaced by bone. Similarly, previous studies have also demonstrated that very little condylar cartilage is left towards the end of growth (Livne et al., 1987b). The condylar cartilage of a growing animal, therefore, demonstrates histologic and organizational features, as well as maturational changes similar to those of the epiphyseal growth plate in long bones, and is thought to contribute significantly to the growth of the mandible (Charlier et al., 1969; Petrovic et al., 1975; McNamara and Carlson, 1979; Whetten and Johnston, 1985; Groote, 1985). This may make the condylar cartilage particularly susceptible to developmental perturbations during arthritis, thereby impacting on the normal growth of the mandible. The effects of TMJ arthritis on mandibulofacial development may be especially consequential due to the location of the condylar cartilage within the joint capsule, as opposed to the more distant epiphyseal growth plate of long bones. We are currently utilizing this juvenile animal model to characterize the gross morphologic alterations in mandibulofacial development during experimental arthritis of the TMJ.

Following the induction of arthritis in the TMJ, external clinical signs of inflammation were not usually evident in most of our animals, although a few rabbits

demonstrated erythema and induration for some days over the antigen-challenged joints. Similarly, in the one previous study on experimental arthritis of the TMJ, little or no externally discernible local clinical features of TMJ arthritis were noted (Zamma et al., 1983). These findings are in contrast to those made on experimental arthritis of the knee joint where swelling and erythema are frequently observed and are often used to grade the severity of arthritis (Cathcart et al., 1986; Barridge et al., 1988; Kuruvilla et al., 1991; Holmdahl et al., 1992). The lack of clinically evident swelling in the vicinity of the TMJ may be related to the anatomy of the joint which, unlike the knee or ankle joints, has several anatomic spaces medial, posterior and anterior to it (Clarke and Bueltmann, 1971) into which the inflammatory exudate may become distributed, thereby masking the characteristic clinical features of arthritis. Therefore, for the determination of successful induction of arthritis in the rabbit TMJ, confirmation by histologic examination is recommended.

In our study the arthritic joints demonstrated histologic signs of moderate to severe synovial inflammation including mononuclear cell infiltration, proliferation and hyperplasia of the synovial lining, hyperplastic villi, as well as invasion of bone and cartilage by synoviocytes and inflammatory cells. The histopathology of the TMJs in this study compares with the milder inflammatory features, including mild synovitis, villous hyperplasia and thinning of the articular fibrocartilage, reported in a previous study on adjuvant-induced arthritis of the rat TMJ (Zamma, 1983). Although not scored, we also observed increased fibrosis of the synovial stroma with increasing chronicity of arthritis. The chronic nature of the arthritis in our model together with findings of synovial hyperplasia, mononuclear cell infiltration, and pannus formation demonstrate histopathologic features akin to those seen in human RA (Schumacher and Kitridou., 1972; Vischer et al., 1982; Krane et al., 1986; Harris, 1989), adjuvant-induced arthritis (Case et al., 1989a), antigen-induced arthritis (Consden et al., 1971; Brackertz et al, 1977; Edwards et al., 1988; Beesley et al., 1992), and in collagen-induced arthritis (Caulfield et al., 1982; Catchcart et al., 1986; Rubin et al., 1987; Bakker et al., 1990; Hasty et al., 1990)

The histopathogenesis of various models of arthritis in the knee joint have been well documented previously. Edwards et al., (1988) noted in antigen-induced arthritis of the rabbit knee, that polymorphonuclear leucocytes appear in the tissue at 4 hours, then subside and reappear as the lymphoid cell infiltrate becomes prominent over a period of 20 days. In type II collagen-induced arthritis of the rat knee, Caulfield et al (1982) described four basic histopathologic stages of pathology. In the first stage, until 10 days post-immunization, all joint surfaces appear normal but fibrin deposition is present. In the

second stage from approximately 11 to 15 days after immunization, both proliferation of synovial cells over the fibrous synovium and their extension over cartilage are noted. Beginning from 16 days to 18 - 20 days, mononuclear and polymorphonuclear leucocyte infiltration of the synovium is observed. The final stage involves extensive scarring of the synovium, formation of granulomata and the persistence of the palisaded appearance of the synovium. The observations made in our study correspond with the last three stages described by Caulfield et al. suggesting that at 5 days post-challenge the arthropathy is relatively well established in our animal model. In addition, the findings of various studies taken together suggest that the time frame and severity of arthritic response may vary depending on the amount of challenging dose (Howson et al., 1986; Edwards et al., 1988), the site of sensitization (Zamma, 1983), as well as the joint being studied (Zamma, 1983).

Although all experimental animals demonstrated histologic signs of arthritis, a substantial amount of individual variability in response to the antigen was found between animals (Fig. 5a). Similarly, Edwards et al. (1988), using three different challenging doses of antigen of 0.05, 0.5 and 5 mg ovalbumin injected into the knee joint reported that the inflammatory reaction is related to dose, and also noted a variable arthritic response, particularly at low dose levels. A consistently aggressive synovitis was only achieved at a challenging dose of 5 mg of OA, a finding similar to that of Howson et al. (1986). Based on the findings of our preliminary studies, we introduced only 0.5 mg of OA into the TMJ. This dose was found to be adequate for inducing arthritis in this model probably because the TMJ is substantially smaller than the knee joint. Since a variable inflammatory response appears to be the rule rather than the exception in most models of experimental arthritis (Boissier et al., 1988; Edwards et al., 1988), the variability in severity of arthritis in our study may be related to the individual differences in immune responsiveness of animals rather than only to the small challenging dose used.

The synovial lining hyperplasia observed in this and other experimental models of arthritis is a common feature of RA, and manifests both as an increased number of lining synoviocytes as well as villi projecting into the joint cavity. Together with these manifestations, numerous mitotic figures are also observed in the synovial lining indicating a rapidly proliferating cell layer (Caulfield et al., 1982). Although the mechanisms for synovial lining hyperplasia remain poorly understood, several cytokines and growth factors, including interleukin-1, platelet derived growth factor, epidermal growth factor and insulin-like growth factors, have been shown to be mitogenic towards synovial fibroblasts in culture (Schmidt et al., 1982; Bunning et al., 1986; Remmers et al., 1988; Butler et al., 1989; Kumkumian et al., 1989; Lafyatis et al., 1989; Hiraoka et al., 1992). Specific

cytokines may therefore be implicated for inducing synovial lining hyperplasia because of the high levels of several cytokines in arthritic tissues and synovial fluid (Wood et al., 1983; Nouri et al., 1984; Hopkins and Meager, 1988; Remmers et al., 1991; Thornton et al. 1991), and because of their known synoviocyte-proliferative effects (Schmidt et al., 1982; Bunning et al., 1986; Remmers et al., 1988; Butler et al., 1989; Kumkumian et al., 1989; Lafyatis et al., 1989; Hiraoka et al., 1992).

At sites where the bone and cartilage fuse with the synovium, the highly proliferative and invasive synoviocytes form a pannus and cause destruction of bone and cartilage. This pannus has many features common to tumors, expressing high levels of oncoproteins such as c-myc and c-fos, of metalloproteinases and of cytoskeletal markers such as vimentin which characterize poorly differentiated mesenchymal cells (Yocum et al., 1988; Case et al., 1989a and b; Wilder et al., 1989; Sano et al., 1990; also reviewed in Wilder et al., 1991). Furthermore, early passage synovial fibroblasts from arthritic joints grow rapidly in culture, do not exhibit contact inhibition and can grow under anchorage-independent conditions in soft agarose (Yocum et al., 1988; Lafyatis, 1989). Nevertheless, although the pannus in RA and experimental arthritis behaves like a highly vascular, invasive tumor, it is not malignant, in that it is only locally invasive and its development is clearly dependent upon the immune system and factors generated in the inflammatory milieu of the arthritic joint (Yocum et al., 1988; Lafyatis, 1989; Wilder et al., 1989; Sano et al., 1990). It is possible that several bioactive agents present in arthritis alter the synoviocyte phenotype towards a more aggressive cell type. More work is needed to elucidate the basis for such a transformation of synoviocytes in arthritis.

Although little is known about the immune mechanisms of OA-induced arthritis, in a similar model using type II collagen, a synergy between delayed type hypersensitivity and immune complex mediated inflammatory mechanisms has been suggested (Holmdahl et al., 1989a and b). Several previous studies have correlated the emergence of humoral (Trentham et al., 1978; Stuart et al., 1979; Morgan et al., 1980) and cellular (Trentham et al., 1978; Stuart et al., 1979) immune responses to type II collagen with the onset of collagen-induced arthritis. Immunologic responses include the synthesis of specific antibodies against the immunizing antigen (Cooke et al., 1972a), and deposition of antigen-antibody complexes and complement C3 in the collagenous matrix of the joint (Cooke et al., 1972b). While the retention of antigen-antibody complexes may explain the chronicity of the arthropathy (Consden et al., 1971; Webb et al., 1971; Cooke et al., 1972b; Hollister & Mannik, 1974), some studies have shown that cell-mediated immunity appears to be a prerequisite for inducing chronic arthritis (Loewi, 1968; Consden et al., 1971; Menard and Dion, 1975). Furthermore, passive transfer of arthritis by spleen and

lymphnode cells from animals with collagen-induced arthritis provides more direct evidence that this model is an immunologically mediated disease (Trentham et al., 1978). Histologically both lymphocyte and plasma cell infiltration of synovium are usually evident (Edwards et al., 1988). Most of the observations on the immunopathogenesis of experimental arthritis are similar to those made in RA (Smiley et al., 1968; Slinwinski and Zvaifler, 1970; Edwards et al., 1988; Henderson et al., 1993). Therefore, antigen-induced arthritis appears to be a suitable model for RA on the basis of both its immune response mechanisms and histopathologic changes.

Since one of the characteristic features of arthritis is the loss of matrix molecules, we further evaluated the differences in collagen and GAG staining in arthritic joints as compared to control joints. The percent area, but not the intensity, of GAG staining was significantly lower in discs from arthritic joints than those from sham-treated and untreated control joints. Loss of GAGs in experimental arthritis, as assessed by a decrease in Safranin O-staining, has also been reported previously in antigen-induced arthritis, in some cases occurring as early as 48 hours after induction of arthritis (Rubin and Roberts, 1987; Healy et al., 1989; Hasty et al., 1990; Pettipher et al., 1989 and 1990; Beesley et al., 1992), and in human osteoarthritis (Bollet, 1963; Mankin et al., 1971; McDevitt et al., 1977; Inerot et al., 1978; Vasan, 1980; Inerot and Heinegard, 1982). A reduction of as much as 50% to 60% in intensity of staining of arthritic cartilage compared with controls has been reported in the knee joints of arthritic mice by 1 to 2 weeks after antigen challenge (Pettipher et al., 1990). Furthermore, Yoo et al. (1988), using specific monoclonal antibodies to various epitopes of proteoglycans, noted that loss of native proteoglycan monomer occurs in immunologically-induced synovitis and this decrease in PGs correlated well with the decreased metachromatic staining observed in this arthropathy (Kruijsen et al., 1985). Loss of GAGs and PGs may adversely affect joint function by decreasing the ability of the cartilage to resist loads (Muir and Carney, 1987; Tammi et al., 1987; Mankin and Brandt, 1989), and possibly also by impacting upon normal cellular function (Gospodarowicz and Ill, 1980; Hauschka et al., 1985; also reviewed in Toole, 1991).

In contrast to the generalized loss in GAG staining between antigen-treated and control joints, the loss in collagen staining was localized to the sites of invasion of cartilage and bone by pannus, and in the disc at its junction with the synovium. Similarly, several previous investigators, while noting early loss of proteoglycans, demonstrated little or no histochemically or biochemically detectable loss in collagen from arthritic joints (Lowther et al., 1978; Beesley et al., 1992). This lack of detectable generalized loss of collagen may result from the relative insensitivity of histochemical and biochemical techniques since the loss of this matrix molecule has been detected in tendon and bone in adjuvant-induced

arthritis using sensitive radiolabeling techniques (Reddy and Dhar, 1992). On the other hand, it is also possible that the differences in collagen and GAG loss in arthritic joints may result from the differences in their relative rates of metabolism (Mohamed, 1991) since collagenous components of the ECM have a relatively low rate of metabolism while PGs exhibit an active turnover. For instance, the half-life of a substantial portion of the proteoglycans is as short as 8 to 10 days (Gross et al., 1960; Mankin, 1967; Mankin and Lipiello et al., 1969), while that of collagen is approximately 180 days (Mankin, 1967; Repo and Mitchell, 1971). The rather specific nature of the initial cleavage of the major fibrilar collagens in joints, such as collagen types I and II, by only two known mammalian collagenases, and the degradation of PGs by a broad spectrum of enzymes may further explain the observed differences in GAG and collagen turnover in arthritis. The basis for the differences in rates of collagen and GAG loss in arthritis need to be determined.

The net loss of matrix molecules in arthritis may result from one or a combination of mechanisms including decreased or perturbed matrix synthesis, and increased matrix degradation. While in the past, several studies have examined the changes in matrix synthesis in various types of experimental arthropathies (Gillard & Lowther, 1976; Lowther and Gillard, 1976; Lowther et al., 1978; Reddy and Dhar, 1992), in recent years much attention has focused on increased matrix degradation during the clinical course of RA (Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans et al., 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein, 1992; Firestein and Paine, 1992 ; Hirose et al., 1992; Walakovits et al., 1992). Several classes of proteinases, including serine, cysteine and matrix metalloproteinases (MMPs), have been implicated in the increased degradation of joint matrices during arthritis. Of these proteinases, the family of MMPs appears to play a particularly significant role in arthritis-associated matrix loss. Our studies (see Chapters III and V) provide evidence of increased expression of MMPs and no change or a slight decrease in the synthesis of their naturally occurring inhibitors in tissues and cells of the joint which may contribute substantially to enhanced matrix loss during arthritis as observed in the present study.

In summary, we have developed and characterized a juvenile animal model of TMJ arthritis. This model of experimental TMJ arthritis, developed using methods described previously, is relatively unique because it is the first to describe a reliable method of inducing arthritis in the TMJ, and also because this study provides the first histopathologic characterization of experimental arthritis in a joint of a juvenile animal. This model resembles RA in its chronicity, histopathology and changes in matrix composition, and

CHAPTER III

SYNOVIUM-DERIVED MATRIX METALLOPROTEINASES AND INHIBITORS IN ANTIGEN-INDUCED ARTHRITIS OF THE TEMPOROMANDIBULAR JOINT

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A. INTRODUCTION

In Chapter II the development and histologic characterization of a model of antigen-induced arthritis of the juvenile rabbit temporomandibular joint (TMJ) was described. Besides histopathologic features akin to those observed in RA, this model of arthritis also demonstrated a loss of glycosaminoglycans (GAGs) in the cartilaginous tissues of the joint, confirming previous findings on tissues biopsied from human osteoarthritic joints (Bollet, 1963; Mankin et al., 1971, McDevitt et al., 1977; Vasan, 1980; Inerot and Heinegard 1982) or retrieved from other animal models of experimental arthritis (Yoo et al., 1988; Healy et al., 1989; Pettipher et al., 1990; Hasty et al., 1990; Beelsey et al., 1992). Since previous studies (Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans et al., 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein, 1992; Firestein and Paine, 1992 ; Hirose et al., 1992; Walakovits et al., 1992) on arthritic tissues, have attributed the loss of joint matrix macromolecules to the increased expression of matrix metalloproteinases (MMPs), particularly by the synovium, the purpose of the present investigation was to further characterize the changes in levels of MMPs and their inhibitors expressed by the synovium in this model of antigen-induced arthritis of the TMJ.

The organization and histology of the synovium has been described in Chapter I. Briefly, the TMJ synovium, like those of other joints, is composed of two distinct layers; an inner layer of thin synovial tissue or synovial lining, and an outer fibrous layer, the synovial stroma (Caulfield et al., 1982). The synovial lining approximates the joint space and is made up of 2 to 3 layers of flattened elongated cells thought to be of two major types, the type A or macrophage-like cells, and the type B or fibroblast like cells. Type A cells contain numerous vacuoles and a prominent golgi apparatus. Type B cells contain a large amount of endoplasmic reticulum and few vacuoles (Barland et al. 1962; Caulfield et al., 1982). A third type of cell, often called type C, has also been described and appears to have features and function intermediate between the type A and B cells. The outer layer of the synovium, the synovial stroma, is relatively well vascularized, has few fibroblasts and

is composed of loose connective tissue largely made up of interspersed type I collagen fibers (Caulfield et al., 1982; Milam et al., 1991). The synovial stroma also contains mononuclear cells, interdigitating dendritic cells, lymphatics and nerve fibers.

During RA and JRA the synovium becomes inflamed and is characterized histologically by infiltration by mononuclear cells, extensive hyperplasia of the synovial lining, and formation of hyperplastic villi projecting into the joint space (Schumacher and Kitridou., 1972; Harris, 1989). At its periphery, where the synovium fuses with cartilaginous and bony tissues of the joint, the proliferative synoviocytes form a pannus, and contribute to the circumfrential destruction of the tissue matrices. *In situ* and immunolocalization for specific MMPs in joints and tissues retrieved from patients with RA have demonstrated that synovial lining cells and cells within the pannus express collagenase and stromelysin, indicating a potential role of these proteinases in matrix loss during arthritis (Wooley et al., 1977a and b; Case et al., 1989b; Okada et al., 1989a and 1990a; Firestein et al., 1991; Garvallese et al., 1991; McCachren et al., 1991; Firestein and Paine, 1992). However, while most studies (Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans et al., 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein, 1992; Firestein and Paine, 1992; Hirose et al., 1992; Walakovits et al., 1992) agree that increased expression of MMPs by synoviocytes and inflammatory cells within the synovium probably contribute to the aberrant degradation of joint cartilage and bone, these studies do not explain whether the extracellular activity of the MMPs is also enhanced by a decrease in levels of specific inhibitors, such as the tissue inhibitors of metalloproteinases (TIMPs). Indeed studies on proteinase inhibitors (PI) expressed by arthritic synovium provide conflicting findings, demonstrating either an increased (Cawston et al., 1984 and 1990; Firestein et al., 1991) or decreased levels (McCachren, 1991) of inhibitors synthesized by inflamed synovium. Many of the disparities of these findings can be attributed to the lack of suitable controls in these studies. Conclusive evidence on changes in inhibitor levels in the arthritic synovium would contribute further to the understanding of the mechanisms for tissue loss in RA.

Much of our knowledge on matrix degradative activity in arthritic joints has been obtained from human studies, which although providing the information most pertinent to human disease, lack adequate or healthy controls, represent advanced stages of the pathology, have a large number of known and unknown variables, such as previous therapy, are not amenable to invasive procedures, and may not be adequately followed for longitudinal information (Wooley et al., 1977a and b; Cawston et al., 1984; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990; Firestein et al., 1991;

Garvallese et al., 1991; McCachren, 1991; Firestein and Paine, 1992; Henderson et al., 1993). Because of these limitations, and in order to supplement information derived from human studies, several animal models of arthritis have been developed previously and have been used to gain insights into the clinical- and histopathogenesis of arthritis, the immune mechanisms and responses in RA, as well as the efficacy of potential anti-arthritic therapeutic agents (reviewed in Table II, Chapter I). Limited information, however, has been gathered from these animal models of the role of MMPs and PIs in the loss of joint matrix macromolecules. In this investigation, an animal model of antigen-induced arthritis of the TMJ was utilized for the purposes of determining the alterations in levels of synovium-derived MMPs and PIs in the arthritic TMJ. The specific aims of this study were:

1. To evaluate the differences in expression of MMPs and PIs between explanted synovium retrieved from animals with antigen-induced arthritis of the TMJ with those from control joints.

2. To examine the temporal expression of MMPs and PIs by synovium explanted from arthritic and non-arthritic control joints.

3. To determine the cellular sources and sites of *in situ* immunolocalization of specific MMPs, collagenase and stromelysin, in arthritic and non-arthritic TMJs.

This study demonstrated an increased synthesis of procollagenase, prostromelysin, 72-kDa gelatinase, and 92-kDa gelatinase in synovium retrieved from animals with antigen-induced arthritis when compared to synovium from sham-treated and untreated control joints from 5 to 55 days after induction of arthritis. The findings also indicate that the expression of 30- and 20-kDa PIs is not significantly different between control and inflamed synovium. Immunolocalization for collagenase and stromelysin revealed positive staining primarily in synovial lining and stromal cells, articular chondrocytes, disc cells and cells within the pannus in arthritic joints. The findings of this study provide further characterization of this model of antigen-induced arthritis of the TMJ in a juvenile animal, and suggest a mechanism for joint matrix loss observed during experimental arthritis.

B. MATERIALS AND METHODS

Materials

Ten-week-old male New Zealand white rabbits (*Oryctolagus cuniculus*) were obtained from Nita Bell Laboratories (Hayward, CA.); 12-O-tetradecanoylphorbol-13-acetate (TPA), lacto albumin hydrolysate (LAH), sodium dodecyl sulfate (SDS), α-casein, 1,10-phenanthroline, 4-aminophenylmercuric acetate (4-APMA), Tris-base, glycine, non-

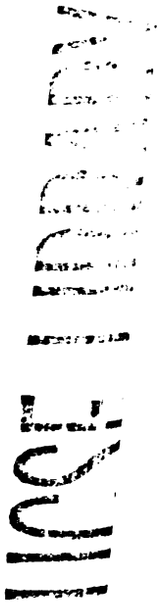
specific mouse IgG and biotinylated goat anti-mouse antibody from Sigma (St. Louis, MO.); Dulbeccos minimum essential medium (DMEM), Triton X-100, Commassie blue, acetic acid, and methanol from Fisher (Pittsburgh, PA.); gelatin (EIA grade), nitrocellulose membrane, nitroblue tetrazolium (NBT), and bromochloro-indolyl phosphate (BCIP) from Biorad (Hercules, CA.); trypsin, penicillin and streptomycin from Gibco (Gaithersburg, MD.); streptavidin-alkaline phosphatase from Boeringer Mannheim (Indianapolis, IN.); fetal bovine serum (FBS) from Hyclone (Logan, UT.); acrylamide and bis-acrylamide from Promega (Madison, WI.); OCT from Miles Scientific (Naperville, IL.); Vectastain ABC kit from Vector Laboratories Inc. (Burlinghame, CA.). Synovial fibroblasts and mouse anti-rabbit collagenase monoclonal antibody was the gift of Dr. Zena Werb (Werb et al., 1989), and mouse anti-human stromelysin monoclonal antibody was the gift of Dr. Scott Wilhelm (Wilhelm et al., 1992).

Induction of Arthritis

Arthritis was induced as described in Chapter II (page 75). Briefly, 22 ten-week-old male New Zealand white (NZW) rabbits were sensitized to ovalbumin by two systemic sensitizing doses of this antigen, prepared in normal saline and complete or incomplete Freund's adjuvant and given 14 days apart (see Fig. 1 in Chapter II). Animals were tested for sensitization to the antigen 1 week later, and the TMJs challenged bilaterally with an intra-articular injection of 100 µl of 5 mg/ml of antigen (total OA 500 µg) in normal saline administered 2 weeks after the second sensitizing dose. Both sham-treated animals and untreated animals were used as controls. Three experimental, 1 to 2 sham-treated and one normal control animals were sacrificed 5, 15, 35 and 55 days post-challenge, the right TMJs were retrieved *en bloc* and fixed for histology and immunohistochemistry (see Chapter II), while the left TMJ synovia and discs were retrieved under sterile conditions for culture.

Retrieval, Processing and Sectioning of TMJs

The retrieval of TMJs for histology and immunohistochemistry was described in Chapter II (page 77). Briefly, the animals were anaesthetized at prespecified time-points following the challenging dose, and the right TMJ was surgically exposed, dissected free of neighboring tissues, retrieved *en bloc* and immediately placed in 4% PBS-buffered paraformaldehyde for 24 hours. The joints were decalcified as described by Mori et al. (1988), following which each joint was bisected parasagittally into two halves; one half of the joint was embedded in OCT for immunohistochemistry, while the other half was dehydrated and embedded in paraffin. Sections, 5 to 6 µm-thick, were cut and placed on poly-l-lysine coated slides and air dried prior to staining.



Retrieval of Synovium and Tissue Culture Techniques

We chose to study the synthesis of MMPs and PIs by explanted tissues since this helps to enhance the levels of these proteins to detectable levels. The observations made by these *ex vivo* techniques were confirmed by immunolocalization of collagenase and stromelysin in contralateral joints. The synovium and disc were retrieved from the left joints of experimental, sham-treated and untreated control animals under sterile conditions, and immediately placed in medium (DMEM) containing 10% FBS and antibiotics (100 U/ml penicillin and 100 mg/ml of streptomycin). The synovium was microdissected from the disc and weighed. The mean weight of antigen-treated synovia was 72.4 (±SD 39.9) mg, sham-treated control synovia was 101.4 (±35.6) mg, and untreated control synovia was 92.2 (±56.1) mg (ANOVA P=0.37). The synovium was then cultured in 400 µl of serum-free medium (DMEM with 0.2% LAH) containing antibiotics for 48 hours after which the synovium-conditioned media (CM) was retrieved and stored at -70 °C until further analysis.

Substrate Zymography and Densitometry

Gelatin zymography was used for evaluating secreted proteinase levels in experimental, sham-treated and normal control synovia. Synovium-CM was standardized by tissue weight (75 mg/ml), 4x sample buffer (0.25M Tris-base, 0.8% SDS, 10% glycerol and 0.05% bromophenol blue) added, and electrophoresed at 15°C on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) containing 2 mg/ml of gelatin as described previously (Heussen and Dowdle, 1980; Chin et al., 1985; Fisher and Werb, 1991). After electrophoresis, the SDS was removed by washing the gels in 2.5 % Triton X-100 for 30 minutes with one change of washing media. The gelatin gels were incubated at 37°C for 20 hours in incubation buffer (50 mM Tris-HCl buffer, pH 8, 5 mM $CaCl_2$, 0.02% NaN_3). The optimum time-of-incubation was determined from enzyme-substrate kinetic studies as described below. The gels were stained with 0.5% Commassie blue and destained in 10% acetic acid and 40% methanol until proteinase bands were clearly visible. Proteinase bands were further characterized by incubating zymograms in incubation buffer containing 0.3 mM 1,10-phenanthroline, a metalloproteinase inhibitor.

Enzyme-kinetic profiles were obtained by running gelatin gels with serial dilutions of a sample demonstrating high proteinase activity. This was done to determine the optimum time-of-incubation for the levels of gelatinolytic activities observed in our samples so that quantitative comparisons of gelatinolytic activities could be made between conditioned media from control and arthritic synovium. Serial dilutions of conditioned media from TPA-treated synovial fibroblasts (cells courtesy of Dr. Zena Werb), which demonstrated high proteinase activity, were electrophoresed in 10% gelatin-impregnated

SDS-PAGE gel as described above, and incubated for 6, 20 or 44 hours, and stained and destained in a standardized manner. Images of the gels were video-digitized by a CCD camera (NEC T1-24A, Japan) and image software (Image Version 1.42, NIH, Bethesda, MD). The imaging of the gels were standardized by capturing them at the same focal length and exposure, and the intensity and area of gelatinolytic activity was quantified. Video-densitometric analysis revealed that, for the level of proteinase activities in our samples, 20 hours of incubation provided a log-linear gelatin degradative activity profile (Fig. 1b), indicating this as the optimum incubation time for quantitation of gelatin gels. This undiluted TPA-treated synovial fibroblast-conditioned media was run in one lane of every gel done subsequently on arthritic and control synovium-conditioned media to ensure that none of the samples exceeded this proteinase activity, and to help standardize for inherent variabilities between gels. The gelatinolytic activity of these samples was determined as described above by digitization followed by densitometry of proteinase activities.

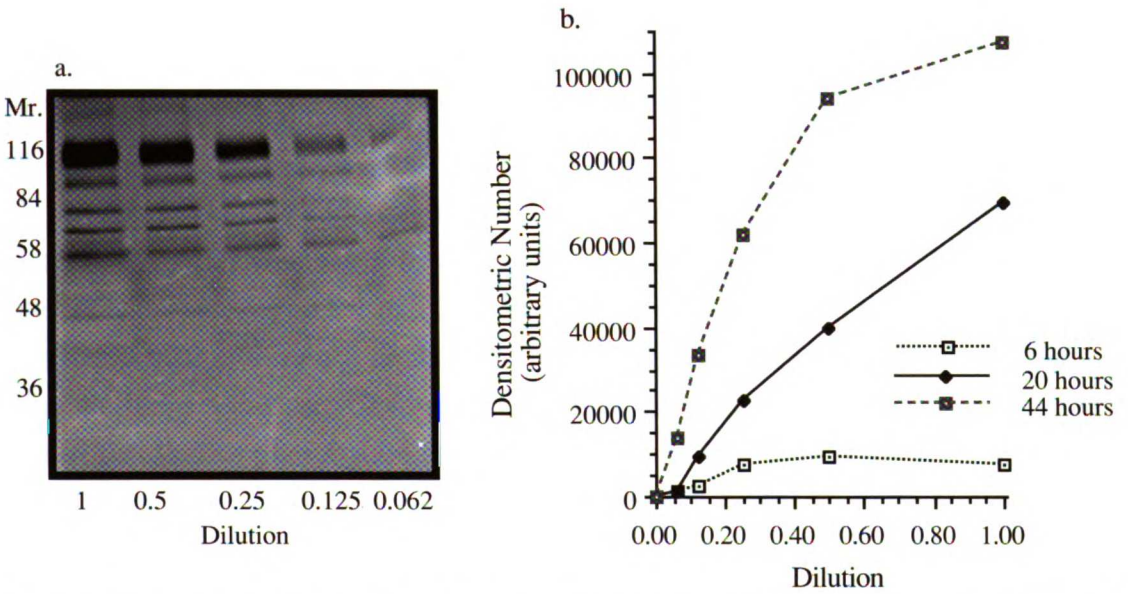


Figure 1: (a) Negative image of gelatin zymogram demonstrating gelatinolytic activities of 5 serial dilutions of TPA-treated synovial fibroblast-conditioned media following 20 hours of incubation. (b) Proteinase-substrate kinetics for total gelatinolytic activities at 6, 20 and 44 hours of incubation in substrate buffer. The data incorporates densitometric values for gelatinolytic activities at 92 kDa, 72 kDa, 53/58 kDa, as well as intermediate bands which probably represent activated proteinases.

Reverse Zymograms

Proteinase inhibitors present in synovium-CM were visualized by reverse zymography on 15% polyacrylamide gels impregnated with 1 mg/ml of gelatin as described by Herron et al. (1986b) and Fisher and Werb (1991). Briefly, the synovium-conditioned

media was standardized by tissue weight (75 mg/ml) as described above, mixed with 4x sample buffer and electrophoresed at 15°C. The technique used for reverse zymography was identical to that utilized for substrate zymograms, except that after washing with Triton X-100, the gels were incubated for 20 minutes in 4-aminophenylmercuric acetate-activated CM from rabbit synovial fibroblasts treated with 50 ng/ml of TPA. This activated CM contains several proteinases that partially degrade the gelatin, except where inhibitors are present. Gels were then incubated in substrate buffer at 37°C for 20 hours, stained and destained as described above. Inhibitor activity was quantitated by video-densitometry as described above.

Western Immunoblots

To identify and further quantify collagenase and stromelysin in synovium-CM, samples were standardized by tissue weight (75 mg/ml), mixed with 4x sample buffer, and electrophoretically resolved on 10% SDS-polyacrylamide gels. The proteins were transferred to nitrocellulose membrane in a semi-dry transfer unit (Semi-Phor TE-70, Hoeffer) using Towbin buffer (0.025 M Tris base, 0.0192 M glycine and 20% methanol). The membranes were blocked for non-specific binding of antibodies with 5% dry low-fat milk in tris-buffered saline (TBS) for 1 hour. The membranes were washed once with TBS (0.2% milk), and incubated for 1 hour with mouse monoclonal antibodies recognizing rabbit collagenase (1:500 dilution in TBS) or stromelysin (1:150), or non-specific mouse IgG (1:100). After washing 5 times for 5 minutes each with TBS or TBS with 0.2% Tween (TBST) containing 5% milk, the membranes were incubated with 1:250 dilution of biotinylated goat anti-mouse antibody in TBS with 0.2% milk for 1 hour, followed by further washings as before. The membranes were then incubated with 1:1000 dilution of streptavidin-alkaline phosphatase conjugate for 1 hour, followed by 1 wash each in TBS, TBST and alkaline phosphatase substrate buffer (100 mM Tris-base, 100 mM NaCl, 50mM $MgCl_2.6H_20$, pH=9.5). The bands were visualized by incubating the blots in 50 µg/ml NBT and 25 µg/ml of BCIP. To compare collagenase and stromelysin levels between arthritic, sham-treated and normal control animals, the immunoblots were quantified by video densitometry as described previously.

Immunohistochemistry

Five to 6 µm-thick sections of the frozen tissue were cut and subjected to immunocytochemistry to detect collagenase and stromelysin. These procedures were conducted in a wet chamber at room temperature unless otherwise indicated either with alkaline phosphatase or immunoperoxidase chromogen development using the avidin-biotin-complex technique (Vectastain ABC). For sections developed with alkaline phosphatase, the tissues were permeabilized with 0.1% Triton X-100 in TBS for 20

minutes at 4°C, blocked with horse serum for 20 minutes, and incubated with antibodies recognizing rabbit collagenase (1:100 to 1:500 in PBS), or with non-specific IgG (1:100) for 1 hour. Tissue sections were washed 3 times for 5 minutes each in PBS, and incubated in secondary biotinylated horse anti-mouse antibody . The sections were washed with PBS as before, incubated with streptavidin-biotin-alkaline phosphatase for 1 hour, followed by 1 wash with PBS and a 5 minute wash with 0.1 M TBS (pH=8.2) containing 5 mM levamisole to block endogenous alkaline phosphatase activity, then incubated with the substrate solution (made 1 drop each of reagent 1, 2 and 3 in 0.1 M TBS, pH 8.2 and 1 mM levamisole) until staining was observed (approximately 10 to 15 minutes). Sections were washed twice in double distilled water, counterstained with methyl green for 3 minutes, air-dried and then dehydrated through graded alcohol and xylene washes and mounted with coverslips.

For immunoperoxidase development, the sections were rinsed in PBS, quenched for endogenous peroxidase activity with 0.1% H_2O_2 for 30 minutes, rinsed three times in PBS, and blocked for endogenous biotin for 30 minutes with avidin / biotin blocking kit. The sections were then incubated with 0.5% casein in PBS for 15 minutes to block for non-specific binding, washed briefly in PBS, and blocked for endogenous immunoglobulin binding sites with 10% normal goat serum for 30 minutes which was then blotted off. Sections were then incubated either with 1:100 dilution of primary antibodies to collagenase or stromelysin or with non-immune IgG for 30 minutes, rinsed three times with PBS, incubated with 1:200 dilution of biotinylated goat anti-mouse secondary antibody for 30 minutes and washed with PBS as before. Tissues were incubated with diluted ABC reagent for 30 minutes, rinsed three times with PBS, developed with diaminobenzidine, counterstained with Harris's hematoxylin, dehydrated through graded alcohol washes and mounted with a coverslip.

Statistical Analyses

Means and standard-deviations were derived for each quantitative measure in order to provide a descriptive analysis of the findings. Measurements from densitometric scans of gels and immunoblots from antigen-challenged, sham-treated and untreated control groups were compared by analysis of variance and Fisher's (PLSD) multiple comparisons with the level of significance set at $P<0.05$.

C. RESULTS

Enzyme-substrate Kinetics for Quantitation of Substrate Zymograms

Quantitative assessments from substrate zymograms are complicated because of the nature of enzyme-substrate kinetics which have been controlled for only in a few studies to date (Overall et al., 1989; Fini et al., 1990). In order to overcome these limitations of substrate zymograms, we ran preliminary experiments to determine the optimum time-of-incubation for the range of activity observed in our samples. Serial dilutions of a sample with high proteinase activity indicated that the optimal incubation time for gelatin zymograms was 20 hours (Fig. 1). The sample shown in Figure 1 was used undiluted in one lane of every gel to ensure that it indeed had higher activity than any of the experimental and control synovium-CM, and was also used for inter-gel standardization to compensate for inherent variables between gels.

MMP and PI Expression in Synovium from Control and Arthritic TMJs

Explanted TMJ synovium synthesized 3 gelatinolytic proteinases at 92-kDa, 72-kDa and 53/58-kDa, as well as 2 caseinolytic proteinases at 92-kDa and 51/54-kDa which, because of their inhibition by 1,10-phenanthroline, were characterized as matrix metalloproteinases (Fig. 2). The 53/58-kDa gelatinolytic activity and the 51/54 caseinolytic activities were identified by Western immunoblots as procollagenase and prostromelysin, respectively (Fig. 3). Additional gelatinolytic bands at 62 kDa and 43/48-kDa were also observed in many samples, and were especially marked in synovium-CM from antigen-challenged joints (Fig. 2a). Two PIs of approximate 30-kDa and 20-kDa were also noted in most synovium-CM (Fig. 4).

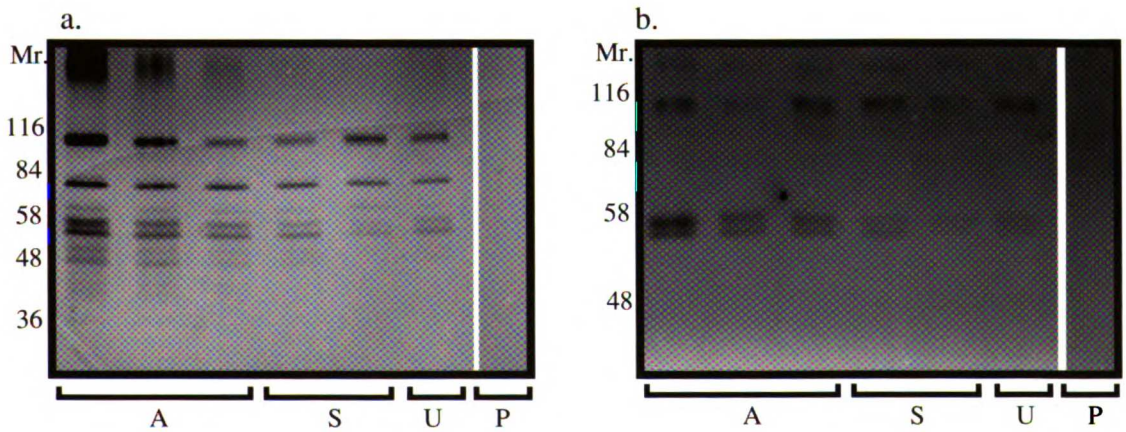


Figure 2: Negative images of gelatin zymogram (a), and casein zymogram (b) of synovium-conditioned media from 55-day post challenge antigen-treated (A), sham-treated control (S), and untreated control (U) joints. All bands were inhibited by 1, 10-phenanthroline (P), a metalloproteinase inhibitor.

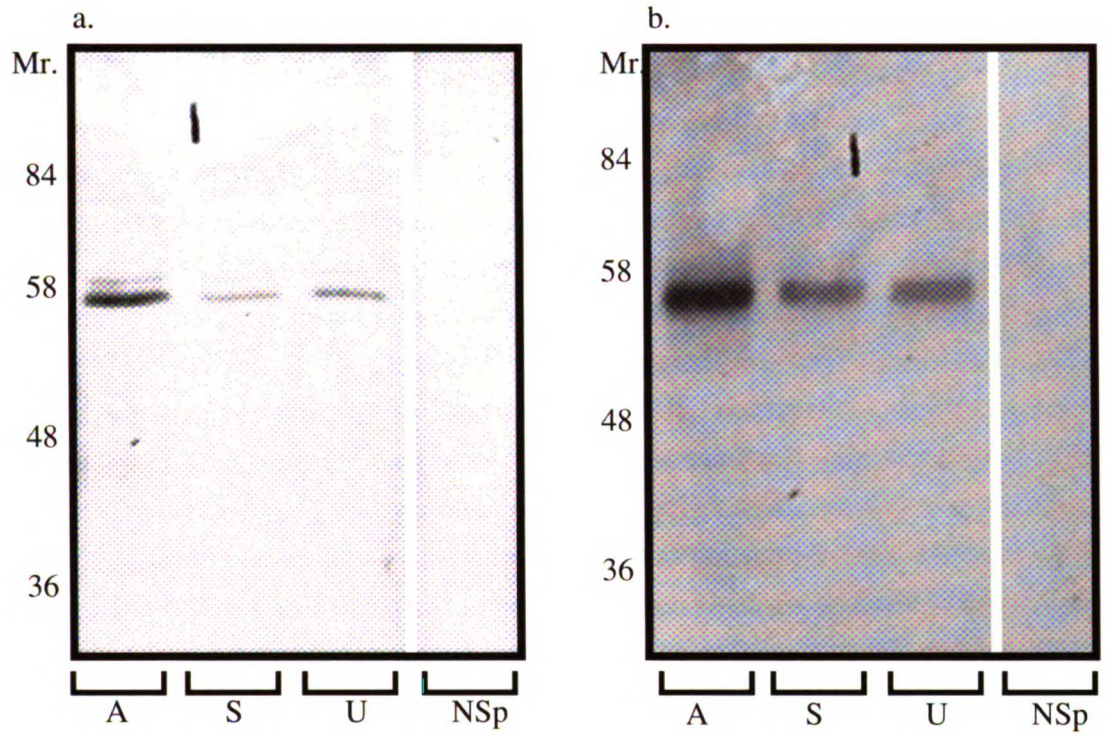


Figure 3: Western immunoblots for collagenase (a) and stromelysin (b) with 55-day post-challenge antigen-treated (A), sham-treated control (S), and untreated control (U) synovium-conditioned media. NSp=non-specific IgG.

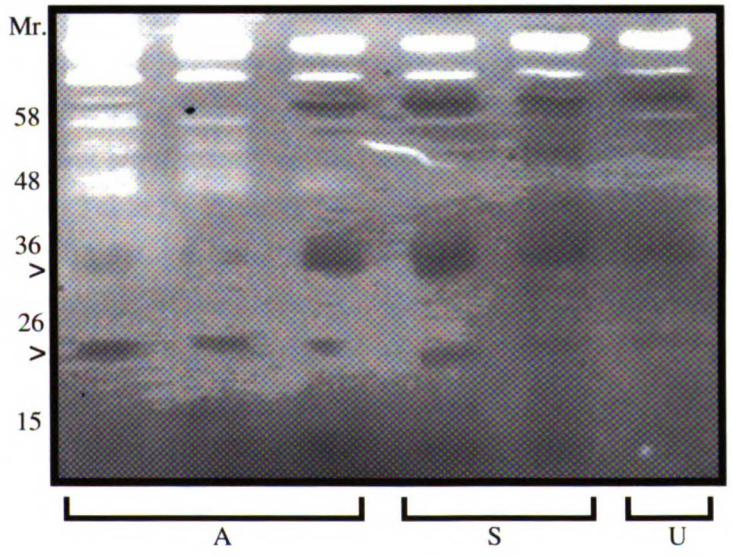


Figure 4: Reverse zymogram of synovium-conditioned media from 55-day post-challenge antigen-treated (A), sham-treated control (S), and untreated control (U) joints.

Densitometric measures from gelatin zymograms, and immunoblots revealed that the levels of all MMPs were increased in synovium-CM from antigen-challenged joints compared with those from sham-treated and untreated joints as early as 5 days after induction of arthritis and persisted until 55 days post-challenge (Figs. 5a and 6). However, no statistically obvious temporal trends were noted in the levels of proteinases expressed by the arthritic synovium (P=0.393). Excluding the effects of time, the levels of 72-kDa and 53/58-kDa gelatinolytic activities, as well as total gelatinolytic proteinases were significantly greater in synovium from arthritic joints than those from sham-treated or untreated joints (Table I). The levels of these proteinases were not significantly different between synovia from sham-treated and untreated joints, and therefore, for the purposes of presentation of data, the proteinase activity for sham-treated and untreated control synovia was combined (Fig. 5a). The expression of 92-kDa gelatinolytic activity was significantly greater in arthritic than sham-treated synovia, but was not significantly different between synovia from arthritic and untreated joints or between sham-treated and untreated joints. Although not quantitated, the caseinolytic activities reflected trends similar to those observed in gelatin zymograms (Fig. 2b). Western immunoblots for collagenase and stromelysin (Fig. 3) also revealed statistically higher levels of these proteinases in arthritic than in both control groups, but no statistical differences were noted between the two control groups (Fig. 6), further confirming the findings of the substrate zymograms.

In contrast to the differences in levels of proteinases between arthritic and sham-treated or untreated control synovia, the levels of total inhibitors expressed by synovia and assayed on reverse zymograms were not significantly different between the three groups of animals (Fig. 5b and Table I). Because of the higher levels of proteinases but little difference in levels of PIs in arthritic versus control synovium-CM, the net proteinase-to-inhibitor (P:I) activity was greater in synovia from inflamed than sham-treated or untreated control joints, but not significantly different between sham-treated and untreated control groups (Fig. 5c and Table I). Although a trend of decreasing P:I ratio was observed from 5 to 35 days post-challenge followed by a sharp increase at 55 days after challenge, because of the variability of the data, the effects of time after induction of arthritis on P:I ratios were not statistically significant (P=0.385).

Immunolocalization of Collagenase and Stromelysin in TMJs

In general, collagenase and stromelysin demonstrated a similar pattern of distribution in tissues of the TMJ. Both proteinases were localized to cells and matrices of the synovial stroma, synovial lining, disc, in and around hypertrophic chondrocytes of the condylar cartilage and in the pannus in arthritic joints (Fig. 7). In arthritic joints, collagenase and stromelysin staining was especially pronounced in the synoviocytes of the

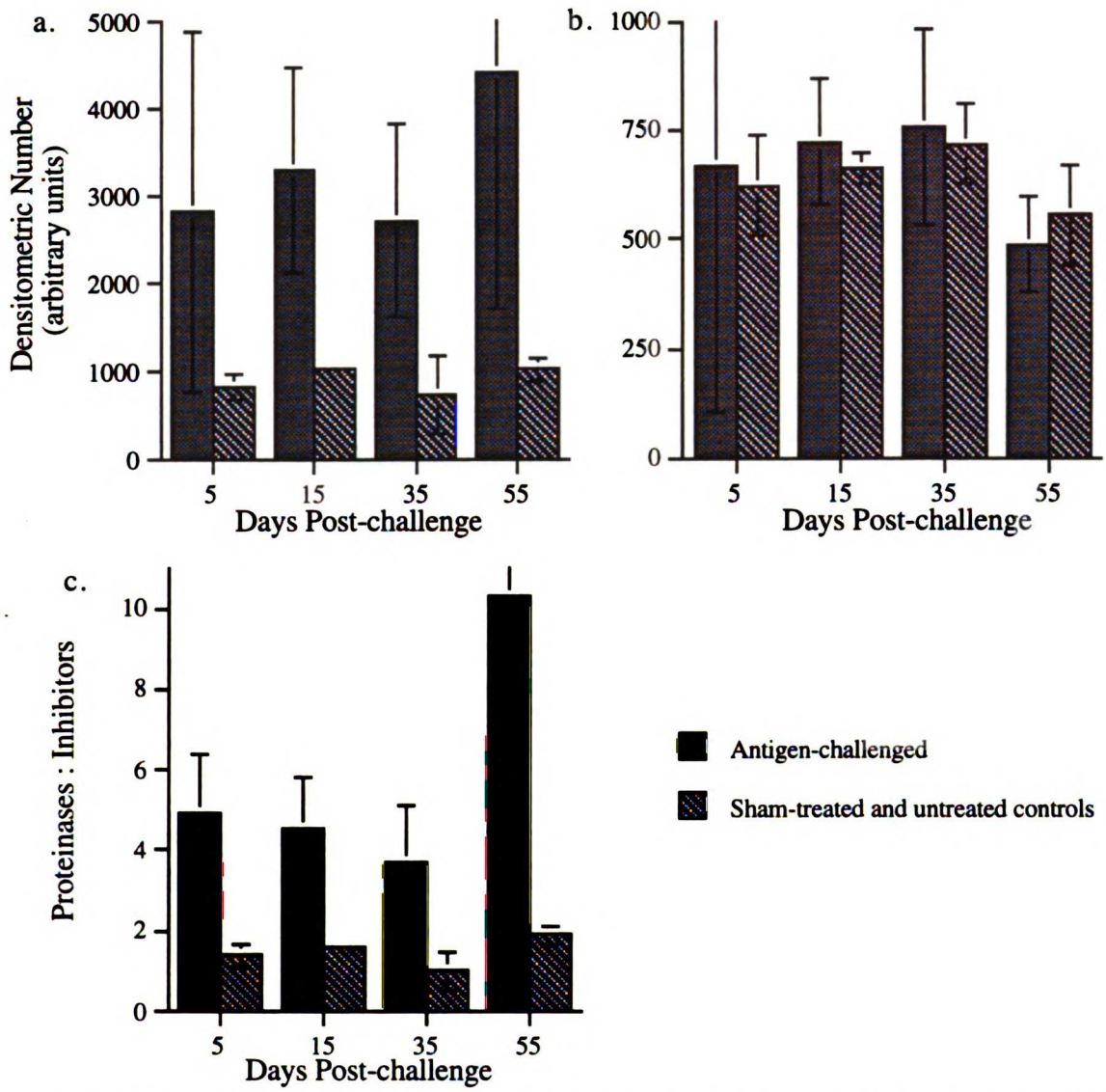


Figure 5: Means and standard deviations for densitometric measurements of gelatinolytic proteinases (a), total inhibitor activities (b), and proteinases-to-inhibitor ratios (c) at 5, 15, 35 and 55 days after induction of arthritis.

Table I: Statistical comparisons of the levels of gelatinolytic proteinases, total inhibitors and proteinase-to-inhibitor ratios expressed by synovium from antigen-challenged (A), sham-treated control (S), and untreated control (U) joints.

Test		92-kDa gelatinase	72-kDa gelatinase	Procollag-enase	Total gelatinolytic proteinases	Total inhibitors	Proteinases : Inhibitors
ANOVA		0.045*	<0.001*	0.022*	0.002*	0.957	0.026*
Fisher's PLSD:	A vs S	0.037*	<0.004*	0.023*	0.002*	0.818	0.021*
	A vs U	0.058	<0.001*	0.027*	0.004*	0.811	0.034*
	S vs U	0.946	0.775	0.844	0.822	0.971	0.915

* = statistically significant P-value.

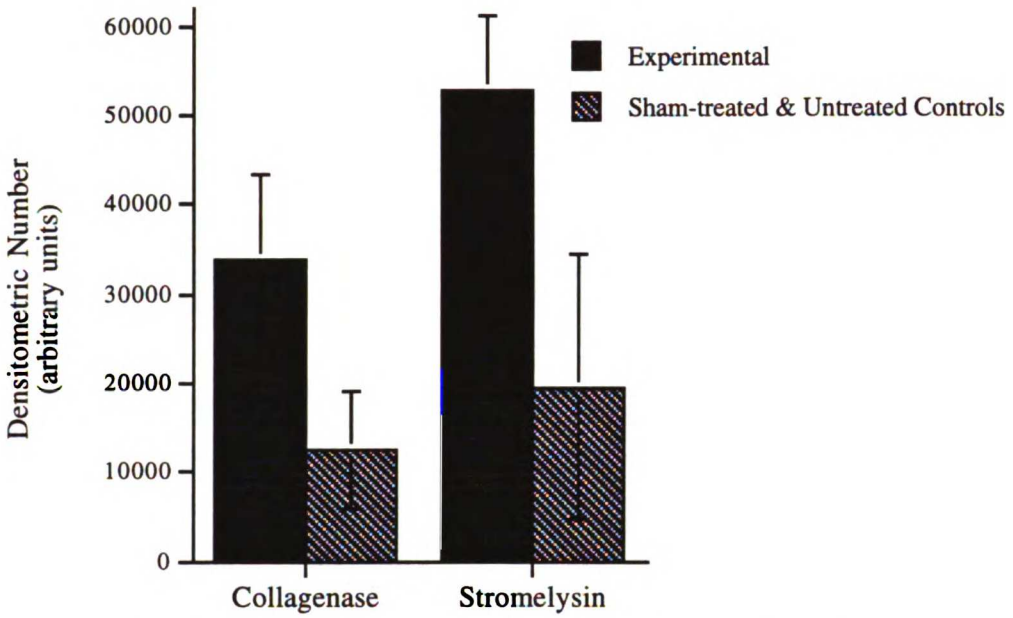


Figure 6: Means and standard deviations for densitometric measurements from collagenase and stromelysin immunoblots for synovium-conditioned media from antigen-challenged, sham-treated control and untreated control joints combined for the four post-challenge time-points.

hypertrophic synovial lining as well in cells of the pannus of arthritic joints. Control joints also demonstrated milder levels of staining for collagenase and stromelysin in the synovial lining and disc cells, as well as in mature chondrocytes.

D. DISCUSSION

In this investigation the expression of MMPs and PIs by synovial explants retrieved from TMJs with antigen-induced arthritis was compared with those from sham-treated and untreated control joints. The *in situ* localization of the MMPs, collagenase and stromelysin, in these joints was also examined. The findings demonstrate that both control and arthritic synovia express at least 4 MMPs, including 92- and 72-kDa gelatinolytic proteinases, procollagenase and prostromelysin. The explanted synovium also synthesized 30-kDa and 20-kDa PIs. These synovium-derived 92- and 72-kDa gelatinolytic proteinases have previously been identified as 92-kDa gelatinase and 72-kDa gelatinase, respectively (Okada et al., 1990b; Kolkenbrock et al., 1991; Unemori et al., 1991b), while the inhibitors have been identified as TIMP and TIMP-2 (MacNaul et al., 1990; Okada et al., 1990a; Kolkenbrock et al., 1991; Firestein et al., 1991; McCachren, 1991; Firestein and Paine, 1992). This study also demonstrated an increased

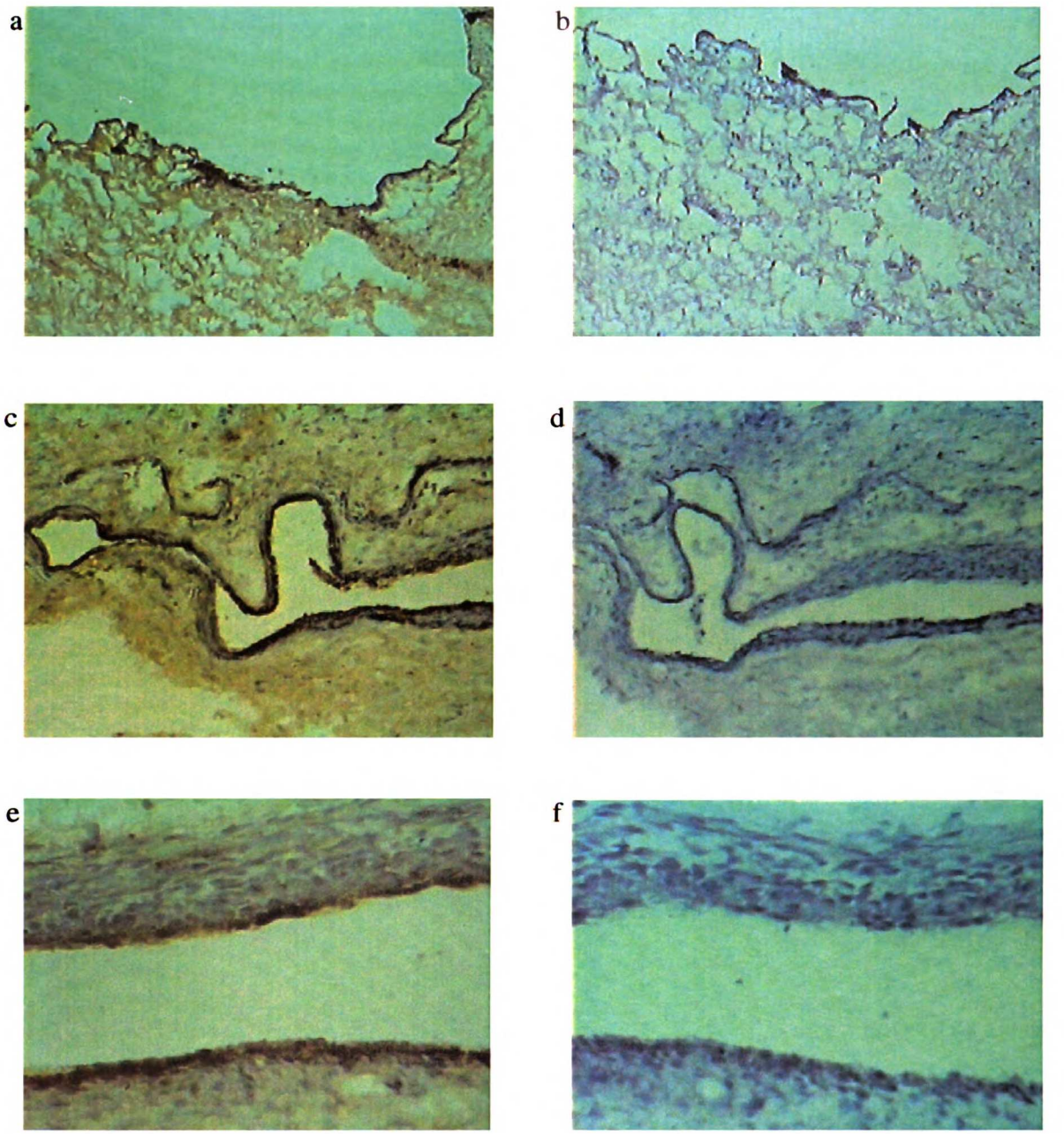


Figure 7: Immunolocalization of stromelysin (a to f) and collagenase (g to l) in TMJs. Tissues were incubated with monoclonal antibodies recognizing rabbit stromelysin (a, c, and e), or collagenase (g, i, j and k), or non-immune IgG (b, d, f, h, and l). Stromelysin staining in normal synovia (a), and arthritic synovia (c and e). Original magnification is x 160 for (a) and (b), x 200 for (c) and (d), and x 400 for (e) and (f). (continued on next page).

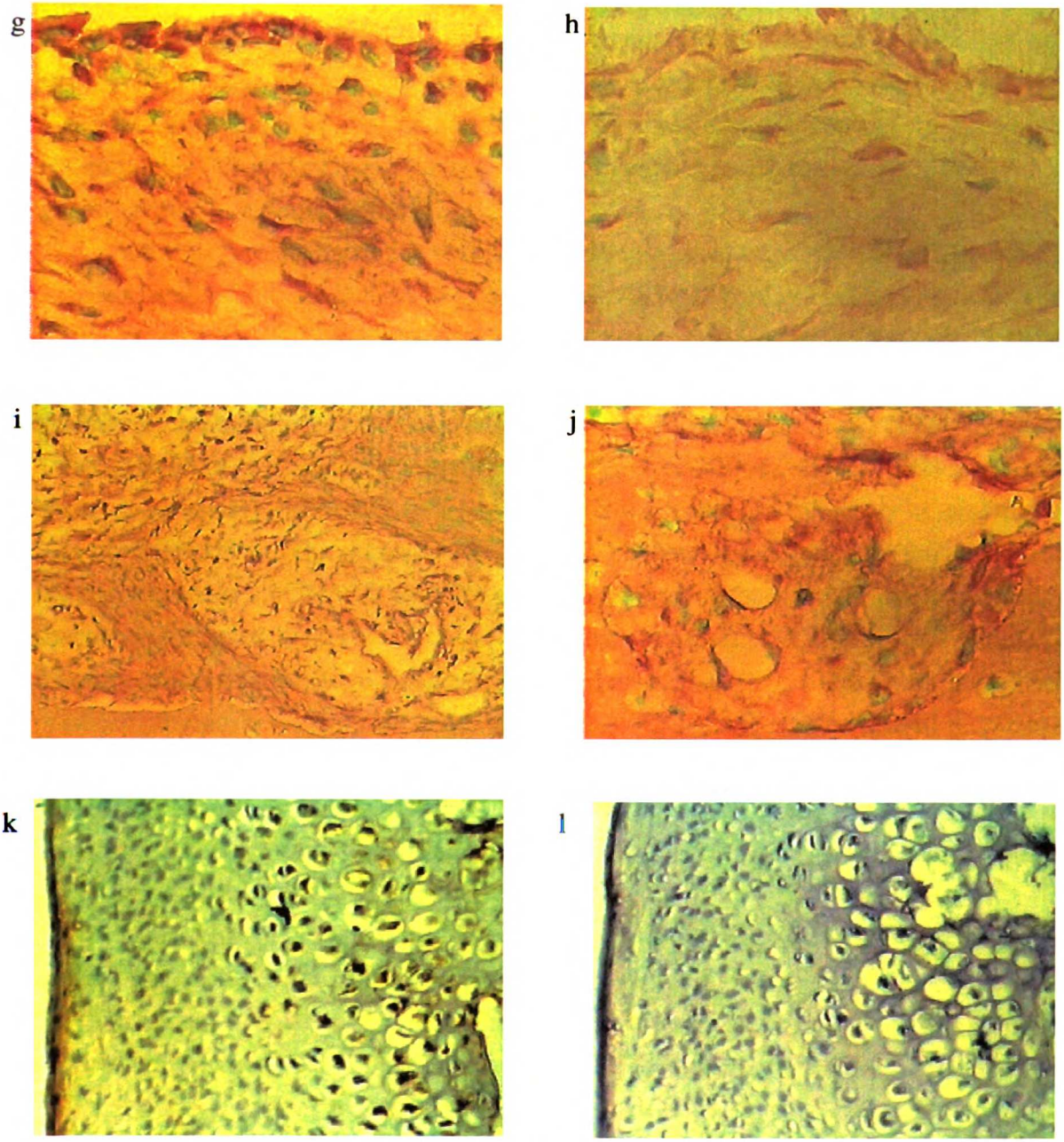


Figure 7 (continued): Collagenase staining in synovial lining (g), cellular and matrix localization in the pannus (i and j), and condylar cartilage (k) from arthritic joints. Original magnification is x 460 for (g), (h) and (j), x 250 for (i), and x 320 for (k) and (l).

expression of these 4 MMPs and little discernible difference in PI expression by arthritic synovia as compared with control synovia. Immunolocalization of collagenase and stromelysin in arthritic joints revealed positive staining of cells of the synovial lining, synovial stroma, the pannus and the disc, as well as mature and hypertrophic chondrocytes. These findings have important implications on the role of MMPs and PIs in matrix degradation during arthritis.

Several investigations have evaluated the constitutive expression of MMPs and PIs by rheumatoid synovial fibroblasts (Dayer et al., 1976; Wooley et al., 1978 and 1979; Krane et al., 1982; Aggeler et al., 1984; Chin et al., 1985; Unemori and Werb, 1986; Frisch et al., 1987; Unemori et al., 1991a and b), as well as the regulation of these proteins by proinflammatory cytokines (Mizel et al., 1981; Dayer et al., 1985; Ito et al., 1988; MacNaul et al., 1990; Firestein et al., 1992; Firestein and Paine, 1992) in order to better understand the role of synoviocyte-derived proteinases and inhibitors in joint matrix loss during arthritis. The present study, on the other hand, has focused on the changes in *ex vivo* expression of MMPs and PIs by inflamed synovium to better comprehend the matrix degradative response of these cells during synovitis. Furthermore, the study examines matrix degradative changes during inflammation of the TMJ, a joint which has not been previously investigated in this regard, and which demonstrates several functional, developmental and morphologic differences from other joints (Symons, 1952; Baume et al., 1962; Baume and Holz, 1970; Friedman, 1982; Gall, 1988; Keith, 1982). The findings suggest an early and sustained increase in the 4 MMPs synthesis by inflamed synovium.

These changes in MMP expression by explanted inflamed synovium may result for various reasons. Firstly, proinflammatory cytokines such as interleukin-1α, interleukin-1β, tumor necrosis factor-α and transforming growth factor-α (Mizel et al., 1981; Dayer et al., 1985; Ito et al., 1988; MacNaul et al., 1990; Firestein et al., 1992; Firestein and Paine, 1992), as well as other bioactive agents such as proteolytic fragments of matrix molecules (Werb et al., 1989; Homandbergh et al., 1992) enhance the expression of MMPs by synovial fibroblasts. An increase in concentrations of these agents has been demonstrated in tissues (reviewed in Dayer and Demczuck, 1984; Firestein et al., 1990) and synovial fluid (Carsons et al., 1981; Scott, 1981 and 1982; Wood et al., 1983; Clemmensen, 1983; Shiozawa and Ziff, 1983; Carnemolla et al., 1984; Lavietes et al., 1985; Xie et al., 1992) from arthritic joints, which could trigger increases in transcription of MMPs. Secondly, the increased mass of synovial tissue, especially that due to the hyperplasia of the synovial lining, would result in an increase in the total number of cells expressing these MMPs (Garvallese et al., 1991; Firestein and Paine, 1992; Firestein et

al., 1992; Hiraoka et al., 1992). While in one study Firestein and Paine (1992) report a positive relationship between MMP expression and cellularity as assessed by in situ hybridization for stromelysin and actin, respectively, in another investigation (Firestein et al., 1991), the authors report no correlation between synovial lining thickness and expression of collagenase. In the latter study, however, a strong relationship of TIMP and collagenase gene expression with the degree of sublining mononuclear infiltration and the presence of lymphoid aggregates was noted, further implicating a role of factors secreted by these cells in altered gene expression for these proteins. In the present study, the effect of increased cellularity on the expression of MMPs by arthritic and non-arthritic synovia may have been minimized since the samples were standardized by tissue weight. Thirdly, proliferative synovial lining cells appear to undergo a neoplast-like transformation with an associated increase in synthesis of proto-oncogenes of the Fos, Jun and Myc families, as well as other markers of transformation such as increased MMP expression (Matrisian et al., 1985; Matrisian et al. 1986; Case et al., 1989a and b; also reviewed in Wilder et al., 1991). Case et al. (1989a) suggest that the early expression of transin, the homologue for human stromelysin, in synovium of rats with streptococcal cell wall-induced arthritis may indicate that this enzyme is possibly one of the first markers of synoviocyte activation and transformation in invasive arthropathies. The relative contributions of each of these mechanisms, together with other as yet unknown mechanisms, to the altered expression of MMPs in inflamed synovium remain to be determined.

The net ECM degradative activity of MMPs is determined by the transcription, synthesis, and secretion of proteinases and their inhibitors, as well as by the activation of secreted zymogens (reviewed in Alexander and Werb, 1989 and 1991; Birkedal-Hansen et al., 1993). Our experiments indicate that when data from all time-points are combined, the expression of MMPs is significantly greater in synovia from arthritic as compared with sham-treated or untreated control joints. On the other hand, control and arthritic synovia expressed similar levels of PIs. Furthermore, a limited number of samples demonstrated additional bands on the zymograms reflecting activation of proteinases, which is considered to be a prerequisite for the effective degradation of matrix macromolecules. Gelatinolytic bands at 62-kDa, probably attributable to the activated form of 72-kDa gelatinase, and a doublet at 43/48-kDa, likely to be activated collagenase, were observed primarily in synovium-conditioned media from antigen-challenged joints (Fig. 2). The greater expression and activation of MMPs together with the little discernible change in PI expression in arthritic over control synovia reflect a net increase in extracellular matrix degradative activity resulting from an altered intracellular and extracellular regulation of MMPs.

The assessment of P:I ratios derived from gelatin and reverse zymograms, however, provides only an estimate of potential changes in matrix degradation attributable to the arthritic synovia for two reasons; This assessment is derived from semi-quantitative assays which lack the accuracy and sensitivity of quantitative functional or antibody based methods, and such an assessment does not take into account the rather specific nature of proteinase / inhibitor interactions. For example, it is known that TIMP-2 has a greater affinity for 72-kDa progelatinase over other MMPs (Goldberg et al., 1989; Stetler-Stevenson, 1989b; Kolkenbrock, 1991) and is more effective than TIMP against 72-kDa and 92-kDa gelatinase, while TIMP appears to inhibit fibroblast collagenase more effectively (Howard et al., 1991b). Despite these limitations our findings indicate an increase in net matrix degradative activity in inflamed over control synovia.

The consistently high levels of proteinases and P:I ratios evident in synovia from antigen treated joints from 5 to 55 days after challenge, together with the persistent histologic evidence of inflammation observed previously (see Chapter II), attest to the chronic nature of the pathology in this model of experimental arthritis. Since the levels of these enzymes were high as early as 5 days after intra-articular challenge with the antigen and persisted throughout the period of the experiment, no significant changes in proteinase expression could be related to time after induction of arthritis . Such a lack of definitive time-related trends in MMP expression may result from the large individual variations in responses to antigen challenge and the consequent variations in severity of inflammation as noted previously in our study (Chapter II) and those of other investigators (Boissier et al., 1988; Edwards et al., 1988). The individual variability in severity of arthritis together with a lack of temporal trends in both arthritic scores and MMP levels indicate that post-challenge time-points do not provide an accurate representation of disease levels. In contrast, histopathologic criteria may provide a more precise assessment of disease severity (Chapter II), and indeed, as discussed later (Chapter VI), these parameters also show a significantly strong positive correlation (R^2=0.69; P<0.0001) to the levels of one of the MMPs, 72-kDa gelatinase. The histopathology, the chronic nature of the arthritis and the increased expression of MMPs in this model show similarities to RA. Our findings also provide new information not available from human studies, which although demonstrating high levels of MMPs in synovia of patients with RA, have evaluated tissues from advanced stages of the disease and that lack healthy controls for appropriate comparisons (Case et al., 1989b; Okada et al., 1989a and 1990a; Garvallese et al., 1991; Firestein et al., 1991; McCachren., 1991; Firestein and Paine, 1992).

Previous studies on changes in PI expression by inflamed synovia have not been conclusive. For example, while Firestein et al. (1991) and Cawston et al. (1984) show

increased levels of TIMP in synovial fluid of patients with RA as compared to patients with OA, other investigations (Cambray et al., 1981; McCachren, 1991) report decreased expression of this PI or its mRNA by arthritic synovia. The findings of our study indicate that in contrast to the greater expression of MMPs by arthritic as compared to control synovia, PI levels were not significantly different between these samples. The lack of change in levels of PIs despite an increased expression of MMPs by inflamed synovia are likely to be related to the independent regulation of these proteins by various cytokines present during inflammation (MacNaul et al., 1990; Circolo et al., 1991; Overall et al., 1991; Firestein et al., 1992; Ito et al., 1992). It is also possible that increased levels of inhibitors were not detected in CM from arthritic synovia because these complexed with the greater amounts of MMPs synthesized by inflamed versus normal tissues. However, high molecular weight inhibitor activities indicative of such complexes were not observed on reverse zymograms in most of our samples, and indeed, when seen were mainly confined to synovium-conditioned media from untreated or sham-treated joints which had lower levels of MMPs (Fig. 5). It is also possible that more sensitive techniques for quantitating inhibitors may have detected subtle differences in levels of PIs expressed by control and inflamed synovia.

Collagenase, stromelysin, 72-kDa gelatinase and 92-kDa gelatinase have among them the ability to degrade all the major and most of the minor components of the joint tissue matrices. Stromelysin, and 92-kDa and 72-kDa gelatinase degrade proteoglycan core proteins, fibronectin, laminin, gelatin, collagen types V, VII, IX, X and XI and elastin while collagenase degrades most forms of fibrilar collagens including types I, II, III, VII, VIII and X (reviewed in Alexander et al., 1989 and 1991; Birkedal-Hansen et al., 1993). The broad spectrum of matrix macromolecules degraded by these 4 MMPs, therefore implicate them in degradation of joint tissues. Furthermore, in addition to the role of MMPs in degrading specific ECM components, these proteinase also have synergistic effects on each other (Mohamed, 1991; Birkedal-Hansen et al., 1993). Gelatinases act synergistically with collagenase in degrading interstitial collagens, while stromelysin activates procollagenase at least *in vitro* (Vater et al., 1983; Treadwell et al., 1986; Ishihashi et al., 1987; Murphy et al., 1987; Ito and Nagase, 1988; Unemori et al., 1991a). Therefore, the activity of these enzymes in degrading matrices may be initiated and transpire through a cascade of interactions.

Although, synovium-derived MMPs secreted into the synovial fluid may degrade matrices exposed to the fluid, recent experimental evidence seems to indicate that their role in generalized matrix loss of the joint may be less extensive than previously thought. Beesley et al. (1992), for instance, have demonstrated that rabbits with antigen-induced

arthritis of the knee joint show a rapid and significant loss of proteoglycans primarily from the intermediate zones, but not from superficial zones of the articular cartilage. The authors therefore suggest that cartilage matrix degradation is not due to action of proteases in the synovial fluid. These findings are further supported by studies which show increased levels of inhibitors, including TIMP and α2-macroglobulin, in the synovial fluid of patients with RA (Abe and Nagai, 1973; Cawston et al., 1984; Firestein et al., 1991), indicating that the increased levels of MMPs in synovial fluid may be partly balanced by increased levels of inhibitors. Although the role of synovium-derived MMPs in the generalized pattern of degradation of articular cartilage and disc requires further study, their contribution to localized matrix degradation in the pannus is likely as demonstrated by the biochemical and immunohistochemical findings of our studies (Chapter V).

That cells besides those of the synovial lining and stroma stained positive for collagenase and stromelysin further emphasizes the notion that a substantial amount of disc and articular cartilage matrix loss probably results from disc cell- and chondrocyte-derived MMPs, respectively. Other studies have similarly shown staining for collagenase and stromelysin proteinases or mRNA in chondrocytes of the articular cartilage (Case et al., 1989a; Hasty et al., 1990). The present study is the first to demonstrate localization of these MMPs in the disc. Since inflammatory cells are not observed within articular cartilage and the disc, the increased synthesis of MMPs by disc cells could result from an paracrine stimulation of these cells by cytokines expressed by synovial and inflammatory cells as has been suggested for articular chondrocytes (Quintavalla et al., 1993). Because the induction of chondrocytes and disc cells has important implications to cartilage and disc matrix loss as well as to the perpetuation of the disease process, this concept should be examined further.

Staining for collagenase and stromelysin in mature and hypertrophic chondrocytes was also observed in control joints and probably reflects high rates of matrix turnover in this tissue, associated with replacement of cartilage by cancellous bone in these rapidly growing animals. Also, some evidence of immunohistochemical staining for these proteinases in disc cells from normal joints suggests a similar remodeling activity in the disc.

With respect to the synovium, collagenase and stromelysin were predominantly localized in cells of synovial lining and pannus, with some sublining cells also demonstrating positive staining for these enzymes. Using immunohistochemistry or in situ hybridization, several investigators have reported similar results previously (Wooley et al., 1977a and b; Case et al., 1989a and b; Okada et al., 1989a and 1990a; McCachren et al., 1990; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein and

Paine, 1992). Although in the present study no attempt was made to characterize which of the two types of synovial lining cells express collagenase and stromelysin, McCachren (1991), using the HAM56 antibody recognizing monocyte / macrophage phagocyte antigen, reported that both HAM56 positive and negative cells in the lining synthesize these proteinases. Furthermore, cells corresponding to those expressing type I procollagen, representing the fibroblast type B synoviocytes, hybridized to collagenase and stromelysin probes. On the other hand, Okada et al. (1989a) demonstrated collagenase synthesis only in the type B fibroblast-like synoviocytes. In the present study, collagenase and stromelysin immunostaining was noted along long stretches of the synovial lining indicating that both types of synoviocytes may be secreting these enzymes. Besides the cellular localization of collagenase and stromelysin, these proteinases were also found in the matrix particularly at the junction of the pannus and the resorbing bone or cartilage, suggesting an ongoing matrix resorptive activity. These localization studies, however, do not indicate whether the proteinases localized in matrices are active or inactive. Future studies using antibodies specific to activated proteinases would provide further insights into the temporal and spatial dynamics of joint matrix degradation in arthritis.

The findings of increased levels of synovium-derived MMPs in this model of experimental TMJ arthritis in a juvenile animal concur with similar findings in adult animal models of RA (Cambray et al., 1981; Case et al., 1989a; Hasty et al., 1990). Future studies done concurrently on adult and juvenile animals are recommended in order to compare the expression of MMPs and PIs and the pathogenesis of these diseases, and to detect any subtle differences that may exist in the severity and timing as well as the mechanisms for matrix loss in these two age groups. Any differences in matrix loss should also be correlated with differences in baseline matrix turnover of the tissues and with the immune responsiveness of adult and juvenile animals.

In this study we provide the first characterization of changes in MMP and PI expression in explanted TMJ synovium from juvenile animals with antigen-induced arthritis. The increased expression of 92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin and the small differences in PI levels in arthritic as compared to control synovia suggest an increased net matrix degradative activity in inflamed synovia. Besides synovial lining and stromal cells, condylar chondrocytes and disc cells stained positive for collagenase and stromelysin. These findings provide a further characterization of this animal model of TMJ arthritis, complement previous human and animal studies, and give further insights into joint matrix loss in arthritis, particularly that associated with the pannus. The study also provides baseline information for future evaluations on the mechanisms for alterations in the levels of synovium-derived MMPs and

CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF PROTEINASES AND PROTEINASE INHIBITORS SYNTHESIZED BY TEMPOROMANDIBULAR JOINT DISC CELLS

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IDENTIFICATION AND CHARACTERIZATION OF PROTEINASES AND PROTEINASE INHIBITORS SYNTHESIZED BY TEMPOROMANDIBULAR JOINT DISC CELLS

A. INTRODUCTION

The adult mammalian temporomandibular joint (TMJ) disc is a fibrocartilaginous tissue that separates the articulating surface of the glenoid fossa and eminence from that of the mandibular condyle, dividing the joint cavity into two compartments. The disc is of considerable functional significance, permitting rotational and translational movements of the condyle in its fossa, and also serves to absorb and distribute loads placed on the joint (Christensen and Ziebert, 1986; Gola et al., 1992). The functional importance of the disc is underscored in various arthropathies of the TMJ, in which aberrations in its position, morphology and composition are accompanied by symptoms which range from joint crepitus and clicking to limited range of mandibular movement and pain (Rothwell, 1985; Isberg et al., 1986). Progression of pathologic changes may eventually lead to a displaced or perforated disc and secondary degenerative changes, akin to those observed in osteoarthritis, often ensue in the mandibular condyle and articular fossa (Westesson and Rohlin, 1984; Lekkas et al., 1988; Helmy et al., 1989).

Both the biochemical composition and structural organization of the extracellular matrix (ECM) of the TMJ disc provide it with the optimal biomechanical properties to withstand tensile and compressive forces and distribute loads during function (Christensen and Ziebert, 1986; Mills et al., 1988; Nakano and Scott, 1989a and b; Nagy and Daniel, 1991; Berkovitz, 1992). The mammalian TMJ disc ECM is composed mainly of fibrilar type I collagen, with smaller quantities of type III collagen (Gage et al., 1990; Milam et al., 1991). Proteoglycans (PGs), namely chondroitin sulfate, keratan sulfate, dermatan sulfate and hyaluronic acid, many of which have cartilage-specific characteristics, make up most of the remainder of the matrix and are primarily found as halos around disc cells (Mills et al., 1988; Nakano and Scott, 1989a and b; Scott et al., 1989; Milam et al., 1991). These highly sulfated PGs allow the disc to resist compressive forces, while the collagen provides tensile strength and also serves as a scaffolding for the proteoglycans (reviewed in Christensen and Ziebert, 1986). Other minor components of the joint disc include elastin, fibronectin, tenascin and oxytalin (Christensen, 1975; Booij and Markens, 1983; Milam et al., 1991; Nagy and Daniel., 1991 and 1992). The cells that synthesize

this matrix have been described as fibroblasts, fibrocytes, chondroid cells, chondrocytes, "chondrocyte-like", or fibrochondrocytes (Kopp, 1976; Christensen and Ziebert, 1986; Mills et al., 1988; Fujita and Hoshino, 1989; Milam et al., 1991; Nagy and Daniel, 1991 and 1992), reflecting their relative lack of characterization. Although, these cells in culture have been shown to resemble chondrocytes in shape, and synthesize PGs similar to those of cartilage in hydrodynamic volume and which are localized by antibodies specific to cartilage PGs (Mills et al., 1988; Milam et al., 1991), they have otherwise not been well characterized. The further characterization of the disc cell phenotype and expression of cell-specific proteins will help in understanding the behavior of the cells and the discal tissue in normal function and in disease.

Disc cells are also likely to play a substantial role in matrix turnover of the disc. Several studies indicate that developmental changes in the TMJ disc entail the progressive differentiation of the disc from a fibrocollagenous tissue to one which is primarily fibrocartilaginous, probably in response to normal joint function and loading (Christensen and Ziebert, 1986; Nagy and Daniel, 1992). Apparently such changes in tissue composition require a turnover of the matrix involving both the degradation of the existing matrix, and its replacement by new matrix. Although not yet demonstrated in the TMJ disc, development and function related changes in matrix composition and synthesis have been noted in other cartilaginous tissues (Palmoski et al., 1980; Palmoski and Brandt, 1984; Knets, 1987; Muir and Carney, 1987; Tammi et al., 1987). Similarly, although no information is currently available on how the disc matrix is degraded during remodeling, findings on the developmental remodeling and morphogenesis of other tissues and systems, indicate that a family of matrix degrading enzymes, the matrix metalloproteinases (MMPs), play a central role in these processes (Fisher et al., 1989; Brenner et al., 1989; Nomura et al., 1989; Flenniken and Williams, 1990; Gershan et al., 1994). For example, during mouse embryogenesis, MMPs are expressed in a temporally-regulated and tissue-specific manner, indicating their significance in development (Brenner et al., 1989). Similarly, the expression of collagenase, a MMP, is noted to be temporally-determined during the pre- and postnatal development of the rat calvaria (Gershan et al., 1994). Although, it is likely that a similarly regulated expression of MMPs and PIs may be involved in the developmental transformation of the joint disc, no study to date has demonstrated that TMJ disc cells can indeed express these proteinases or their inhibitors.

The MMPs are a family of enzymes which are characterized by their ECM substrate specificity, zinc-dependent activity, extracellular inhibition by tissue inhibitor of metalloproteinases (TIMP), secretion as zymogen, and sequence similarities (reviewed in Matrisian, 1990; Alexander and Werb, 1989 and 1991; Birkedal-Hansen et al., 1993).

The MMP family includes enzymes such as interstitial collagenase (MMP-1), stromelysin (MMP-3), 72-kDa gelatinase (MMP-2) and 92-kDa gelatinase (MMP-9), whose activities range from specific to broadly reactive. Because of their substantial capabilities for ECM degradation, the net activity of these proteinases is regulated intracellularly, at the transcriptional level, and extracellularly, by their synthesis as inactive proenzymes and their binding to specific proteinase inhibitors (PIs).

In this study we tested the hypothesis that TMJ disc cells synthesize specific matrix degrading proteinases and proteinase inhibitors. The purpose of our study was two-fold; firstly to characterize and identify matrix degrading proteinases and their inhibitors synthesized by explanted TMJ discs and isolated disc cells in culture, and secondly, to characterize the regulation of synthesis of these proteinases and inhibitors by disc cells in response to a protein kinase-C agonist, 12-O-tetradecanoylphorbol-13-acetate (TPA).

Our studies demonstrate, for the first time, that both cultured TMJ discs and isolated disc cells synthesize 92-kDa and 72-kDa gelatinolytic proteinases, procollagenase and prostromelysin, as well as two proteinase inhibitors of Mr 30-kDa and 20-kDa. TPA increased the synthesis of 92-kDa gelatinase and 30-kDa inhibitor, but had little discernible effect on procollagenase and prostromelysin expression in both cell and tissue cultures. The findings of this study provide important baseline information for future research on matrix turnover and tissue remodeling of the normal TMJ disc, as well as on the potential contribution of MMPs and PIs to the pathologic degradation of the disc during various arthritides.

B. MATERIALS AND METHODS

Materials

Sixteen-week-old male New Zealand white rabbits (*Oryctolagus cuniculus*) were obtained from Nita Bell Laboratories (Hayward, CA.); 12-O-tetradecanoylphorbol-13-acetate (TPA), lact albumin hydrolysate (LAH), sodium dodecyl sulfate (SDS), α-casein, 1,10-phenanthroline, 4-aminophenylmercuric acetate (4-APMA), Tris-base, glycine, non-specific mouse IgG and biotinylated goat anti-mouse antibody from Sigma (St. Louis, MO.); Dulbeccos minimum essential medium (DMEM), Triton X-100, Commassie blue, acetic acid, and methanol from Fisher (Pittsburgh, PA.); gelatin (EIA grade), nitrocellulose membrane, nitroblue tetrazolium (NBT), and bromochloro-indolyl phosphate (BCIP) from Biorad (Hercules, CA.); trypsin, penicillin and streptomycin from Gibco (Gaithersburg, MD.); collagenase / dispase, streptavidin-alkaline phosphatase and streptavidin Texas red from Boeringer Mannheim (Indianapolis, IN.); fetal bovine serum (FBS) from Hyclone

(Logan, UT.); acrylamide and bis-acrylamide from Promega (Madison, WI.); OCT from Miles Scientific (Naperville, IL.); Vectastain ABC kit from Vector Laboratories Inc. (Burlinghame, CA.). Mouse anti-rabbit collagenase monoclonal antibody was the gift of Dr. Zena Werb (Werb et al., 1989), and mouse anti-human stromelysin monoclonal antibody was the gift of Dr. Scott Wilhelm (Wilhelm et al., 1992).

Retrieval of Discs, Cell Isolation, Cell and Tissue Culture Techniques

In order to identify and characterize proteinases and inhibitors synthesized by disc cells, both cell and tissue cultures studies were done. For tissue explant studies, intact temporomandibular joint discs were retrieved bilaterally from three, 16-week old male NZW rabbits, and cultured in serum-free medium (DMEM with 0.2% LAH) containing 1x antibiotics (100 U/ml penicillin and 100 mg/ml of streptomycin) with or without 50 ng/ml of TPA (Fig. 1) at 37°C in 5% CO_2 in air. Three discs comprised each of the subsamples. Eighty µl of media was retrieved at 12, 24, and 36 hours of culture and the same volume of fresh media added at these time-points. The remaining media was collected after 48 hours of culture. The disc-conditioned media (CM) was stored at -70°C until further analysis, and the disc fixed in 2% PBS-buffered paraformaldehyde for immunohistochemistry.

For cell culture studies, TMJ discs were minced and digested in bacterial collagenase / dispase for 4 hours (Fig. 1). The digested residue was cultured at 37°C in 5% CO_2 in air in DMEM with 10% fetal bovine serum (FBS) and 1x antibiotics (100 U/ml penicillin and 100 mg/ml of streptomycin) for a period of approximately 2 weeks, after which the explanted cells were trypsinized into 100-mm plates and grown until 90% confluent. Twenty-thousand cells were seeded per well into 24-well plates containing coverslips. Once 75% confluent, the cells were rinsed with phosphate-buffered saline (PBS), and the media was replaced with serum-free medium (DMEM, 0.2% LAH with 1x antibiotics). Cultures were rinsed again after 24 hours and, 350 µl of fresh serum-free medium, with or without 50 ng/ml of TPA, was added. All the cell-CM from 3 wells each was collected at 12, 24, 36 and 48 hours. The disc-cell CM was stored as described above, and the cells on the coverslips were fixed with 2% PBS-buffered paraformaldehyde for immunocytochemistry.

Substrate Zymography and Densitometry

Gelatin and casein substrate zymography were used for evaluation and characterization of disc and disc cell secreted proteinases and proteinase inhibitors. The methods used for substrate zymograms, as well as the characterization and densitometric quantitation of proteinases were as described previously (Chapter III, page 99).

Reverse Zymograms

Proteinase inhibitors present in disc-CM and disc cell-CM were visualized by reverse zymography as described in Chapter III (page 100).

Western Immunoblots

The procedures used for further identifying the proteinases, collagenase and stromelysin, by Western immunoblots were as detailed in Chapter III (page 101).

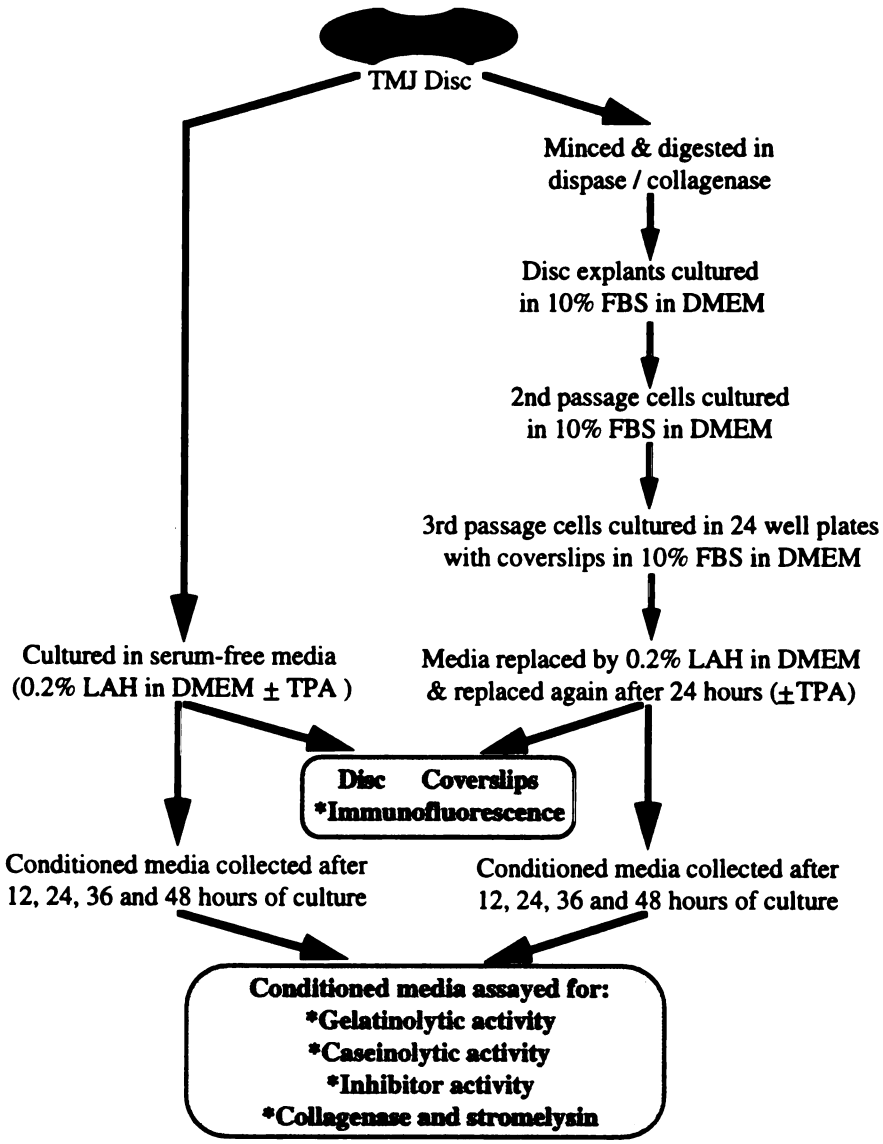

Figure 1: Experimental design for the identification and characterization of proteinases and inhibitors synthesized by TMJ discs and disc cells.

Immunocytochemistry

TMJ discs were fixed with 2% PBS-buffered paraformaldehyde, snap frozen in OCT, and 6 μm thick cryosections were cut for immunolocalization of collagenase. Coverslips with disc cells were fixed with 2% PBS-buffered paraformaldehyde for 10 minutes at 4°C, permeabilized in 0.1% Triton X-100 for 15 minutes at 4°C, and subjected to indirect immunofluorescence for collagenase and stromelysin. After blocking for non-specific binding with horse serum (Vectastain ABC), the cells or tissues were incubated for one hour with antibodies recognizing rabbit collagenase (1:250 in PBS), or stromelysin (1:100 in PBS), or non-specific antibody (1:100 in PBS) raised in mice. After three washes with PBS, the tissues or cells were incubated with 1:1000 dilution of biotinylated horse anti-mouse antibody for 1 hour, washed three times with PBS, and incubated with 1:250 dilution streptavidin Texas red in PBS for 1 hour. The tissue or cells were washed three times and mounted with gelvitol before observation and photographing on a fluorescence microscope (Zeiss Axiophot).

C. RESULTS

Identification and Characterization of Proteinases and Proteinase Inhibitors Synthesized by TMJ Discs and Disc Cells in Culture

Both disc- and disc cell-conditioned media demonstrated 92-kDa, 72-kDa and 53/57-kDa gelatinolytic activities (Fig. 2), as well as 51/54-kDa caseinolytic activities (Fig. 3). Additional gelatinolytic bands were also observed at approximately 68- and 62-kDas primarily in disc cell-CM. All proteinase activities were completely inhibited when control gels were incubated in substrate buffer containing 1, 10-phenanthroline, characterizing these activities as metalloproteinases. Since the 53/57-kDa gelatinolytic and 51/54-kDa caseinolytic activities and their inhibition by 1, 10-phenanthroline suggested the presence of procollagenase and prostromelysin, Western immunoblots using monoclonal antibodies to these proteins were performed on disc-CM and disc cell-CM for further identification of these activities. The molecular weights of the bands observed on the immunoblots corresponded to those for procollagenase and prostromelysin, respectively (Aggeler et al., 1984; Chin et al., 1985; Unemori and Werb, 1986) (Fig. 4). All proteinase activities were evident as early as 24 hours of culture, and a time-related increase in the number and intensity of these bands was noted (Figs. 2 to 4). The reverse zymograms demonstrated two inhibitory activities at 30-kDa and 20-kDa (Fig. 5).

Regulation of Metalloproteinase and Inhibitor Synthesis by TPA in TMJ Discs and Disc Cells

TPA enhanced the synthesis of the 92-kDa gelatinase, as well as 30-kDa inhibitor in both discs and disc cell cultures (Figs. 2, 5 and 6). In contrast, this protein-kinase C agonist decreased the expression of 72-kDa gelatinase by disc cells, and had little effect on its synthesis by explanted discs (Figs. 2 and 6). TPA stimulation of discs and disc cells also appeared to have little discernible effect on procollagenase and prostromelysin synthesis (Figs. 2, 3, 4 and 6).

Immunolocalization of Collagenase and Stromelysin in Disc and Disc Cells

Several cells both in disc explants and in cell culture stained positive for collagenase and stromelysin (Fig. 7). Obvious differences in the cytoplasmic distribution of collagenase and stromelysin were noted in cell culture, with the former largely localized in the perinuclear region, while the latter was found both around the nucleus and distributed throughout the cytoplasm in small granules.

D. DISCUSSION

The role of MMPs and PIs in tissue development, morphogenesis, differentiation and remodeling is well documented (Brenner et al., 1989; Fisher et al., 1989; Nomura et al., 1989; Flenniken and Williams, 1990; Gershan et al., 1994; also reviewed in Alexander and Werb, 1991 and Birkedal-Hansen, 1993). Tissues subjected to loading, such as articular components of joints, are particularly susceptible to adaptive remodeling which likely entails the degradation of the existing ECM and its replacement by new matrix that is more capable of sustaining the new stresses that the tissues must bear (Knets, 1987). Changes in quantity and quality of matrix have been noted in articular cartilage subjected to loading both *in vivo* and *in vitro* (Palmoski et al., 1980; Palmoski and Brandt, 1984; Knets, 1987; Muir and Carney, 1987; Tammi et al., 1987). Similarly, age-related changes have been reported in the matrix of the load-bearing TMJ disc in which the fibrocollagenous matrix of the young disc is gradually replaced by fibrocartilaginous tissue in the adult (Christensen and Ziebert, 1986; Nagy and Daniel, 1992). The degradation of the disc matrix necessary for these differentiation and remodeling events may be accomplished by MMPs. Our findings on the constitutive expression of MMPs and PIs by normal discs and disc cells suggest a potential role of these proteinases in normal turnover of disc matrix macromolecules.

This investigation provides the first characterization of disc cells with respect to their matrix-degradative proteinase and inhibitor profiles. These cells are poorly

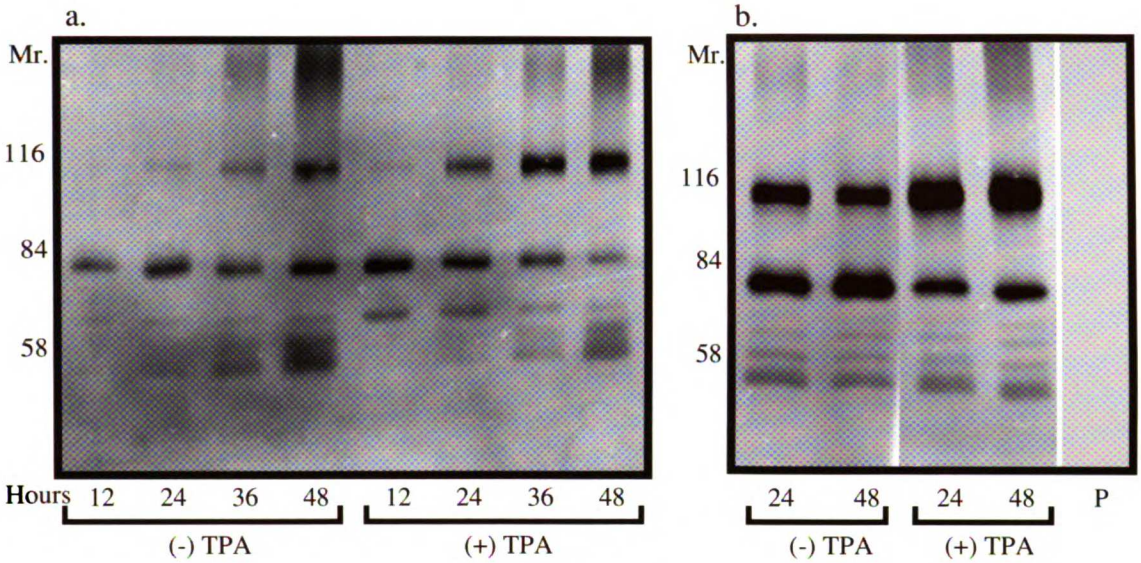

Figure 2: Negative images of gelatin zymograms of conditioned media from explanted discs (a) and disc cells (b) cultured in the absence (-) or presence (+) of 50 ng/ml TPA. For disc cells, only data from conditioned media retrieved after 24 and 48 hours of culture is shown here. All bands were inhibited by 1, 10-phenanthroline (P), a metalloproteinase inhibitor.

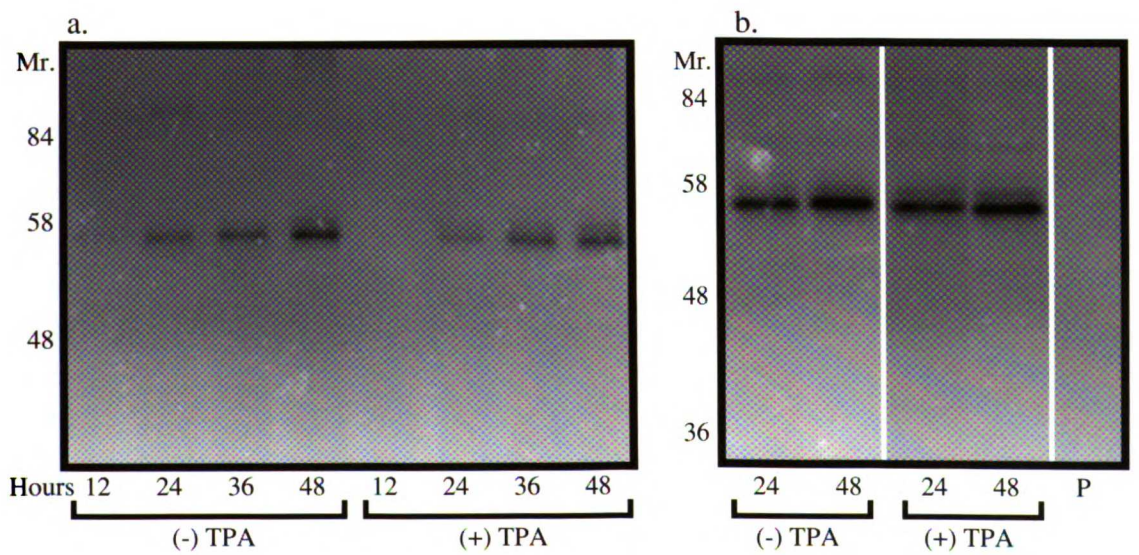

Figure 3: Negative images of casein zymograms of conditioned media from explanted discs (a) and disc cells (b) cultured in the absence (-) or presence (+) of 50 ng/ml TPA. For disc cells, only data from conditioned media retrieved after 24 and 48 hours of culture is shown here. All bands were inhibited by 1, 10-phenanthroline (P), a metalloproteinase inhibitor.

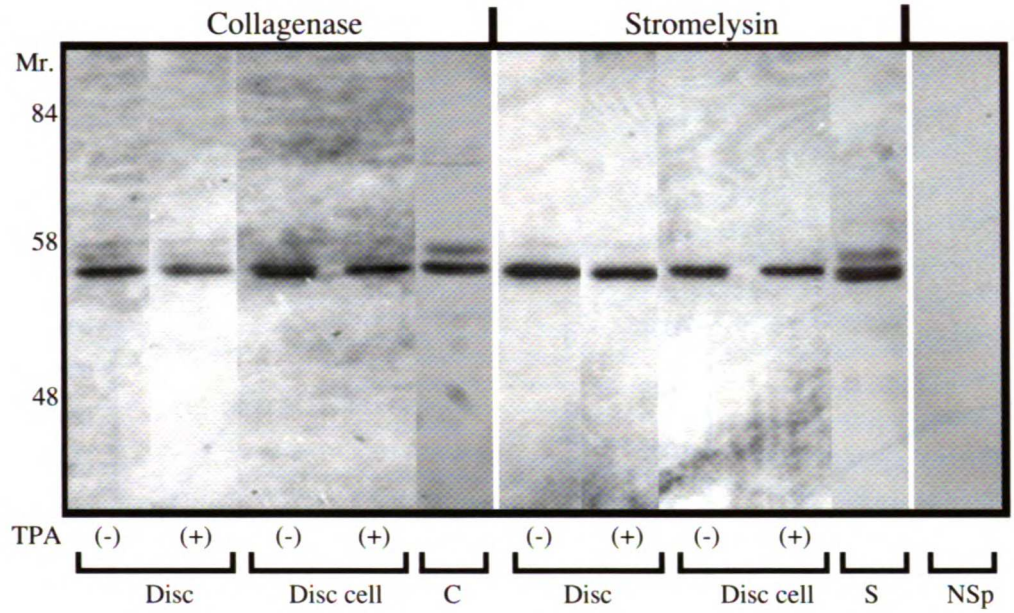

Figure 4: Immunoblots using monoclonal antibodies recognizing rabbit collagenase or stromelysin demonstrate bands corresponding in molecular weight to procollagenase and prostromelysin, respectively, in both disc- and disc cell-conditioned media. Only data from 48 hour conditioned media is provided here. (-)=no TPA; (+)=with TPA; C=positive control for collagenase; S=positive control for stromelysin; NSp=non-specific IgG.

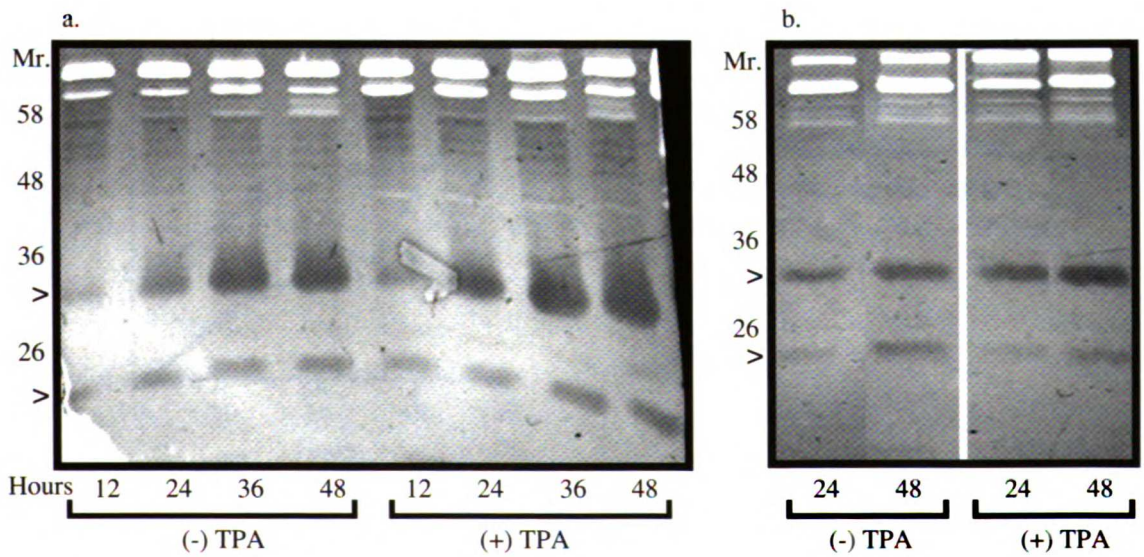

Figure 5: Reverse zymograms of conditioned media from explanted discs (a) and disc cells (b) cultured in the absence (-) or presence (+) of 50 ng/ml TPA. For disc cells, only data from conditioned media retrieved after 24 and 48 hours of culture is shown here.

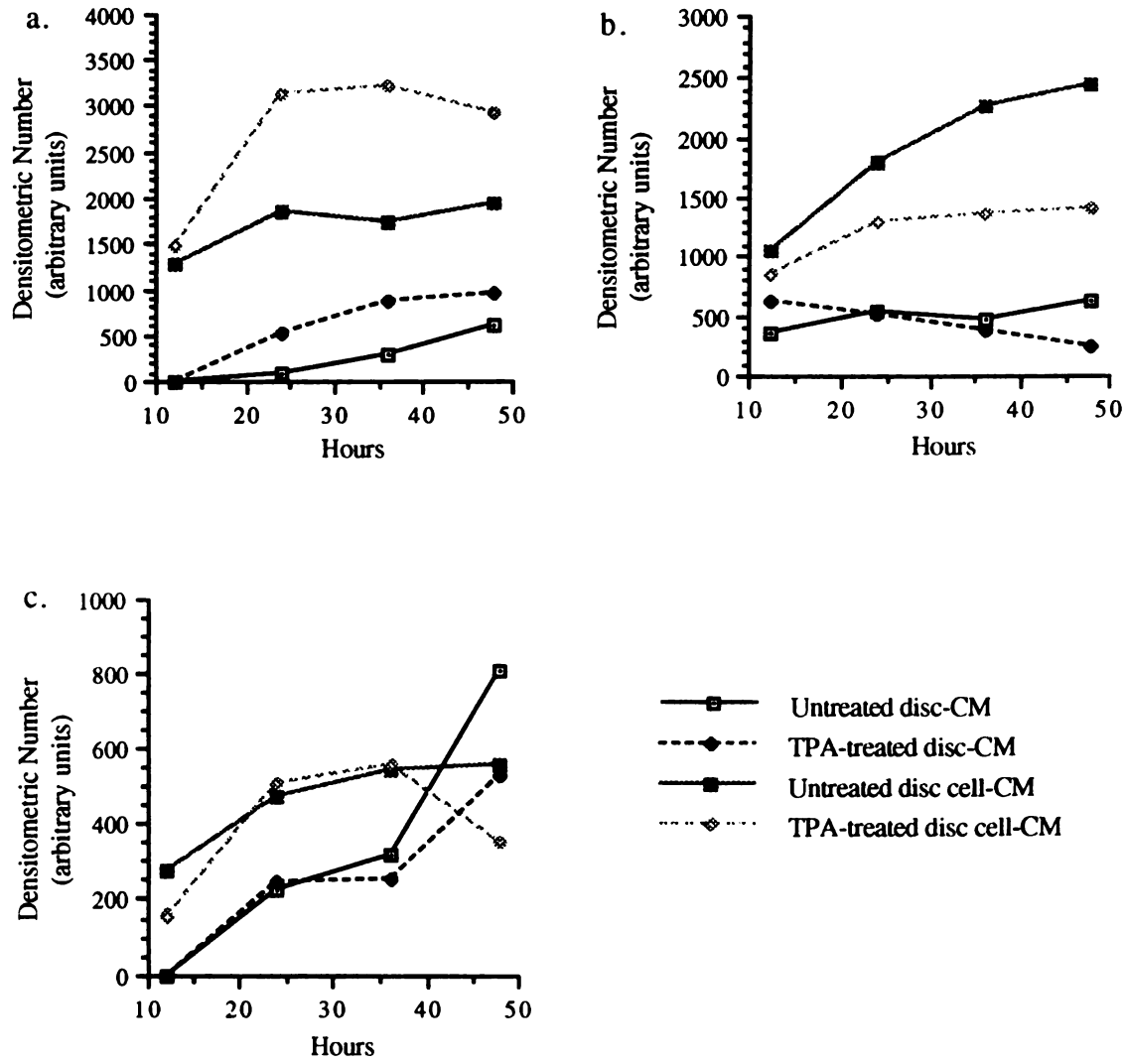

Figure 6: Time-related expression of 92-kDa gelatinase (a), 72-kDa gelatinase (b), and procollagenase (c) by untreated and TPA-treated TMJ disc explants and isolated disc cells.

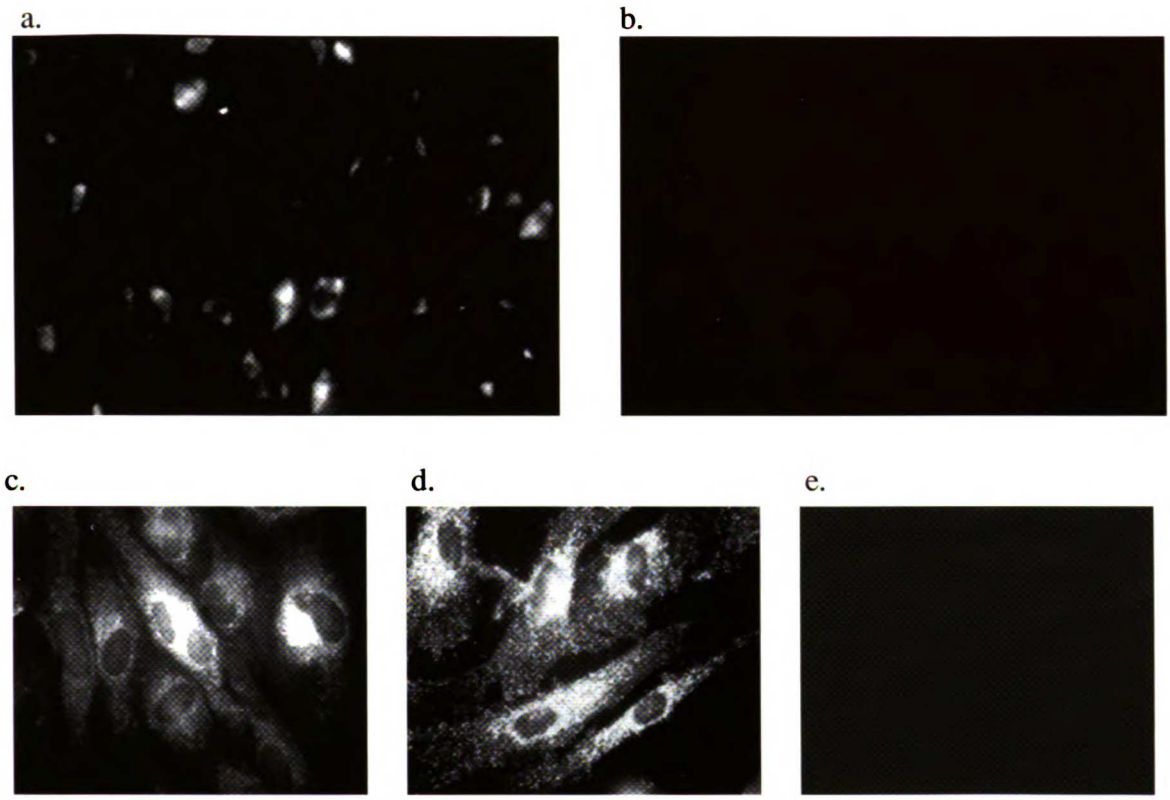

Figure 7: Immunolocalization of collagenase (a) in TMJ disc, and of collagenase (c) and stromelysin (d) in cultured disc cells. Negative controls of disc (b) and disc-cells (e), respectively, incubated with non-specific IgG.

characterized, and have frequently been referred to by various names ranging from fibroblasts to chondroid cells (Kopp, 1976; Christensen and Ziebert, 1986; Mills et al., 1988; Fujita and Hoshino, 1989; Milam et al., 1991; Nagy and Daniel, 1991 and 1992). In contrast to these descriptors, we demonstrate that the profile of constitutively expressed MMPs from disc cells differs from that of articular chondrocytes, which only express 72-kDa gelatinase (Lefebvre et al., 1991; Ogata et al., 1992). Furthermore, although disc cells and synovial fibroblasts have similar patterns of constitutively expressed MMPs and PIs, (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987; Unemori and Werb, 1986), disc cells differ from synovial fibroblasts in their TPA stimulated regulation of MMPs. Whereas, treatment of synovial fibroblasts with TPA induces increased synthesis of procollagenase and prostromelysin (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987), we noted no discernible increase in the expression of these proteinases on exposing disc explants or cells to TPA. These findings suggest that disc cells may be a unique type of cell which differ significantly from chondrocytes in their constitutively expressed MMPs, and from synovial fibroblasts in the regulation of MMP synthesis. The

differences between TMJ disc cells and fibroblasts or chondrocytes are further emphasized by the unique ability of the disc cells to synthesize a matrix composed of cartilage-specific proteoglycans together with type I, rather than type II collagen (Hirschmann and Shuttleworth, 1976; Mills et al., 1988; Milam et al., 1991). Our findings provide only a partial characterization of these cells. Further work is needed to define the matrix-degradative and synthetic characteristics of disc cells during development, in normal function, and in disease. The differential regulation of MMPs and PIs by disc cells, synovial fibroblasts, and articular chondrocytes may also have important implications in specifically targeting each of these cell types in preventing or alleviating matrix loss during arthritis.

Both the constitutive and phorbol ester-mediated regulation of MMPs can also be affected by the differentiational status of the cells and the number of passages in culture (Brown et al., 1989; Millis et al., 1989; Sottile et al., 1989; Fini et al., 1990; Campbell et al., 1991; Lefebvre et al., 1991; Nguyen et al., 1992). Primary cultures of corneal fibroblasts respond to TPA by increasing their synthesis of the 72-kDa gelatinase, the only constitutively expressed MMP in these cells, but show only minimal changes in their expression of 92-kDa gelatinase, collagenase or stromelysin (Fini et al., 1990). However, when passaged only once, these fibroblasts respond to TPA by substantially increasing the synthesis of the latter three MMPs, but not the 72-kDa gelatinase. Findings of various investigations (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987; Fini et al., 1990; MacNaul et al., 1990; Lefebvre et al., 1991; Ogata et al., 1992), taken together with ours, support an emerging body of evidence indicating that divergently differentiated mesenchymal cells regulate the synthesis of their MMPs and PIs differently, and further that both the constitutive and stimulated expression of MMPs may be altered by the number of cell passages. In our experiments, however, the effect of time in culture on the expression of MMPs and PIs by disc cells appears negligible since both the unstimulated and TPA stimulated MMP / PI patterns observed within disc explants and in third passage cultured disc cells were very similar.

The TPA stimulation studies also indicate that the synthesis of MMPs and PIs in disc cells is not coordinately regulated, a finding similar to that made previously on corneal fibroblasts and synovial fibroblasts from arthritic joints (Fini et al., 1990; MacNaul et al., 1990; Unemori et al., 1991b). Unemori et al. demonstrated that the expression of 68-kDa gelatinase (MMP-2) is independent from that of 92-kDa gelatinase in dermal fibroblasts both at the protein and mRNA levels on stimulation of these cells by several cytokines. Furthermore, these investigators noted that collagenase and 92-kDa gelatinase also show discordant regulation in these cells. Although the intermediary steps to gene transcription

of these MMPs are poorly understood, many of the cytokine, growth factor and chemical stimulated regulatory pathways for MMPs converge at the activation protein-1 (AP-1) binding site, which also constitutes the phorbol ester-responsive element (TRE) (Angel et al., 1987a and b). AP-1 complexes are heterodimers of proteins of two proto-oncogene families, Jun and Fos whose synthesis is induced by TPA. TPA and other protein kinase-C agonists appear to stimulate MMP mRNA transcription by the binding of AP-1 complexes to a ATGAGTCA consensus sequence in the 5' flanking regions of several of these proteinases. AP-1 binding sequences have been shown to be present in the 5' flanking regions of fibroblast collagenase, stromelysin-1 and 92-kDa gelatinase genes, but are missing in the 72-kDa gelatinase gene (Angel et al., 1987b; Schonthal et al., 1988; Huhtala et al., 1991), which may explain the distinct regulatory mechanisms for 72-kDa gelatinase. Our observations on the differences in the regulation of 72-kDa gelatinase and 92-kDa gelatinase in disc cells are, therefore, further supported by the known molecular mechanisms for induction of these enzymes. However, the non-responsiveness of collagenase and stromelysin expression by disc cells to TPA can not be explained by any presently known mechanisms, although these proteinases are also not appreciably induced in chondrocytes (Ogata et al., 1992) and corneal fibroblasts (Fini et al., 1990) on stimulation with phorbol esters.

Additional gelatinolytic bands, indicative of the activation of proteinases, were observed infrequently, and were largely limited to Mr between 72- and 57-kDas. These bands are probably attributable to activated forms of the 72-kDa gelatinolytic proteinase. Activation of proMMPs is not often observed in cell culture systems without the addition of exogenous activators such as plasminogen and trypsin (reviewed in Birkedal-Hansen et al., 1993). Although the reasons for this lack of activation are not well understood, it may be related to the co-expression of metalloproteinase inhibitors, TIMP and TIMP-2, which bind to the proMMPs, thereby retarding their activation. On the other hand, some evidence has been presented demonstrating that the activation of pro 72-kDa gelatinase may be mediated by a cell surface bound "activator" (Overall and Sodek, 1990; Ward et al., 1991a), which may explain the additional 62-kDa gelatinolytic activity observed in some of our samples.

Immunocytochemistry for collagenase and stromelysin demonstrated differences in cytoplasmic distribution of collagenase and stromelysin in cultured disc cells. While collagenase was localized to the perinuclear region, probably in the golgi as demonstrated previously (Trabandt et al., 1990), stromelysin was detected throughout the cytoplasm. These differences in cytoplasmic distribution between collagenase and stromelysin may be related to differences in post-translational modifications between these two enzymes, or to differences in modalities of secretion, or both, and remain to be determined. With regard to

modalities of secretion, Okada et al. (1989b) noted that stromelysin could only be localized in macrophage-conditioned media stimulated synovial fibroblasts if they were first treated with a monovalent ionophore, monensin. Their results seem to suggest that in stimulated rheumatoid synovioblasts, stromelysin is synthesized and secreted continuously without storage.

In the one previous study of cultured disc cells, Mills et al. (1988) identified two phenotypes of cells derived from distinct sites of the adult rabbit TMJ disc. The posterior attachments generated cells that had a fibroblastic phenotype, while the cells from the anterior and posterior bands were polygonal in shape and resembled chondrocytes in appearance. Our findings from disc explants and isolated cells possibly reflect the net matrix-degradative profile of the latter cell type since the surrounding attached synovial tissue together with a part of the periphery of the disc was carefully dissected away for both studies. However, since immunohistochemistry for collagenase in disc tissue showed that all cells did not stain positive for this proteinase, further studies are recommended to determine the repertoire of MMPs and PIs expressed by cells cloned from different sites of the disc. Additionally, although our investigations have primarily focused on the MMP family of proteinases, other classes of proteinases important in matrix degradation, including serine proteinases, may also be expressed by these cells and requires further elucidation. The synthesis of serine proteinases, such as plasminogen activators, by disc cells may provide insights into the possible mechanisms for the activation of the proMMPs.

In summary, we demonstrate, for the first time, the constitutive expression of 92-kDa and 72-kDa gelatinolytic proteinases, procollagenase, prostromelysin and two proteinase inhibitors by cultured TMJ discs and disc cells. Due to their electrophoretic mobility and inhibition by 1, 10-phenanthroline, the 92-kDa and 72-kDa gelatinolytic activities are likely to be 92-kDa gelatinase (proMMP-9) and 72-kDa gelatinase (proMMP-2), respectively. The 30-kDa and 20-kDa inhibitors are probably TIMP and TIMP-2, respectively. Our studies on the regulation of MMPs and PIs in response to the protein kinase-C agonist, TPA, revealed differences in the regulation of some of these proteinases between disc cells and those observed previously in synovial fibroblasts, chondrocytes, and corneal fibroblasts (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987; Fini et al., 1990; Lefebvre et al., 1991; Ogata et al., 1992). These studies provide the first characterization of disc cells with respect to their expression of proteinases and PIs. The findings implicate MMPs and PIs in normal tissue remodeling of the TMJ disc and also suggest their potential role in the pathologic degradation of disc matrix in various arthropathies such as osteoarthritis and rheumatoid arthritis. The role of MMPs and PIs of

CHAPTER V

MODALITIES FOR MATRIX DEGRADATION IN THE ARTHRITIC TEMPOROMANDIBULAR JOINT DISC

CHAPTER V

MODALITIES FOR MATRIX DEGRADATION IN THE ARTHRITIC TEMPOROMANDIBULAR JOINT DISC

A. INTRODUCTION

In a previous study (Chapter IV) we identified and characterized proteinases and inhibitors synthesized by explanted temporomandibular (TMJ) discs and disc cells. We found that disc cells constitutively synthesize at least 4 matrix metalloproteinases (MMPs), namely 92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin, as well as two proteinase inhibitors (PIs). Because of the known changes in matrix composition during development of the TMJ disc, our findings suggest a potential role of these MMPs and PIs in normal tissue turnover of the disc. It is, however, also likely that disturbances in the regulation of disc-derived MMPs and PIs mediate degenerative changes in the disc during various arthropathies of the TMJ. In the present study we examine the potential role of MMPs and PIs synthesized by disc cells in matrix degradation during TMJ arthritis.

The temporomandibular joint (TMJ) disc is a fibrocollagenous or fibrocartilaginous tissue that separates the articulating surface of the glenoid fossa and eminence from that of the mandibular condyle, and permits rotational and translational movements of the condyle. Both the biochemical composition and structural organization of the extracellular matrix (ECM) of the TMJ disc provide it with the optimal biomechanical properties to withstand tensile and compressive forces and distribute loads during function (Christensen and Ziebert, 1986; Mills et al., 1988; Nakano and Scott, 1989a; Nagy and Daniel, 1991; Berkovitz, 1992). The ECM is composed mainly of fibrilar type I collagen, with smaller quantities of type III collagen (Gage et al., 1990; Milam et al., 1991). Proteoglycans, namely chondroitin sulfate, keratan sulfate, dermatan sulfate and hyaluronic acid, make up most of the remainder and are primarily found as halos around disc cells (Mills et al., 1988; Nakano and Scott, 1989a and b; Scott et al., 1989; Milam et al., 1991). Other minor components of the joint disc include elastin, fibronectin, tenascin and oxytalin (Christensen, 1975; Booij and Markens, 1983; Milam et al., 1991; Nagy and Daniel, 1991 and 1992). The highly sulfated proteoglycans allow the disc to resist compressive forces, while the collagen provides tensile strength and also serves as a scaffolding for the proteoglycans (reviewed in Christensen and Ziebert, 1986). Since extracellular matrices in various tissues have been shown to affect several cellular functions, including cell migration, proliferation and differentiation, the disc matrix is also likely to contribute

substantially to the normal biologic function of its cells (reviewed in Hay, 1981 and 1991; Ruoshlati, 1988a and b; McDonald, 1988). For these reasons, the loss of disc matrix in arthritis may impact on both the physical and biologic functions of the disc.

Although changes in matrix composition have been widely reported for cartilage and other tissue components in several joints during various arthropathies (Lowther et al., 1978; Rubin and Roberts, 1987; Yoo et al., 1988; Pettipher et al., 1989 and 1990; Henderson et al., 1990; Beesley et al., 1992; Reddy and Dhar, 1992; also reviewed in Muir and Carney, 1987; Harris et al., 1989; Mankin and Brandt, 1989), only limited information is presently available on the loss of matrix molecules in the TMJ disc in such pathologies (Blaustein et al., 1986; de Bont et al., 1986; Fujita and Hoshino, 1989; Kapila et al., 1994 and Chapter II). Fujita and Hoshino (1989) reported a change in organization and reduction in number of collagen fibers of the rat TMJ disc following experimentally-induced malocclusion. Additionally, although there was no immunoreactivity of the fibers with an antibody to type II collagen in control animals, experimental animals demonstrated the presence of type II collagen in the disc. In experiments on antigen-induced arthritis of the rabbit TMJ, we noted that the percent area of the disc staining for glycosaminoglycans was significantly lower ($P<0.0001$) in experimental joints ($21.96 \pm$ SD 8.45%) than in sham-treated ($52.09 \pm 6.03\%$) or untreated controls ($49.07 \pm 5.29\%$) (Chapter II). These changes in the composition of the disc matrix are likely to impair the normal function of the joint. Progressive loss of the matrix components can eventually lead to perforation of the disc, and secondary degenerative changes, akin to those observed in osteoarthritis, often ensue in the mandibular condyle, articular fossa and synovium (Westesson and Rohlin, 1984; Lekkas et al., 1988; Helmy et al., 1989).

To a large extent, studies on other joints have implicated the loss of the joint matrix in osteoarthritis and rheumatoid arthritis to the increased expression of a family of matrix-degrading enzymes, known as matrix metalloproteinases (MMPs) (Wooley et al., 1977a and b; Cawston et al., 1984; Martel-Pelletier et al., 1986; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein and Paine, 1992; Henderson et al., 1993). This family of MMPs includes enzymes such as interstitial collagenase (MMP-1), stromelysin (MMP-3), 72-kDa gelatinase (MMP-2) and 92-kDa gelatinase (MMP-9), whose activities range from specific to broadly reactive. The MMPs are characterized by their ECM substrate specificity, zinc-dependent activity, extracellular inhibition by tissue inhibitor of metalloproteinases (TIMP), secretion as zymogen, and sequence similarities (reviewed in Matrisian, 1990; Alexander and Werb, 1989 and 1991; Birkedal-Hansen et al., 1993).

Because of their substantial capabilities for ECM degradation, the activity of these proteinases is well regulated both intracellularly, at the transcriptional level, and extracellularly, by their synthesis as inactive proenzymes and their binding to specific proteinase inhibitors (PIs).

In most studies on arthritic human and animal joints the synovium and its cells have largely been held responsible for the increased matrix-degradative activity associated with these arthropathies (Wooley et al., 1977a and b; Cawston et al., 1984; Martel-Pelletier et al., 1986; Case et al., 1989b; Okada et al., 1989a and 1990a; McCachren et al., 1990; Firestein et al., 1991; Garvallese et al., 1991; McCachren, 1991; Firestein and Paine, 1992; Henderson et al., 1993). However, any degradation of the TMJ disc by MMPs synthesized by synovial fibroblasts and inflammatory cells may be limited to areas where the synovial capsule fuses with the disc, since secreted MMPs have relatively localized effects because of their immobilization by binding to the matrix or to cell surface receptors (reviewed in Alexander and Werb, 1991 and Moscatelli and Rifkin, 1988). Furthermore, the activity of MMPs secreted into synovial fluid may be limited by the presence of specific and non-specific proteinase inhibitors in synovial fluid (Abe and Nagai, 1973; Cawston et al., 1984 and 1990; Firestein et al., 1991). It is, therefore, likely that MMPs of disc cell origins may contribute to the degradation of the TMJ disc matrix in arthritis. In the present study we tested the hypothesis that the levels of disc-derived MMPs and PIs are altered in experimental arthritis of the TMJ. The aims of this investigation were to determine whether the *ex vivo* expression of specific MMPs and PIs by discs is altered in antigen-induced arthritis of the TMJ using a rabbit model previously developed by our group (Kapila et al., 1992c and Chapter II).

Our studies demonstrate that while the levels of disc-derived MMPs are substantially elevated, those of PIs are slightly decreased in antigen-induced arthritis of the rabbit TMJ. Together with the histochemical and immunohistochemical findings of our previous study (Kapila et al., 1994 and Chapter II), these findings implicate MMPs and their PIs in the increased degradation of disc matrix during experimental arthritis of the TMJ.

B. MATERIALS AND METHODS

Materials

Ten-week-old male New Zealand white rabbits (*Oryctolagus cuniculus*) were obtained from Nita Bell Laboratories (Hayward, CA.); 12-O-tetradecanoylphorbol-13-acetate (TPA), lact albumin hydrolysate (LAH), sodium dodecyl sulfate (SDS), α-casein,

1,10-phenanthroline, 4-aminophenylmercuric acetate (4-APMA), Tris-base, glycine, non-specific mouse IgG and biotinylated goat anti-mouse antibody from Sigma (St. Louis, MO.); Dulbeccos minimum essential medium (DMEM), Triton X-100, Commassie blue, acetic acid, and methanol from Fisher (Pittsburgh, PA.); gelatin (EIA grade), nitrocellulose membrane, nitroblue tetrazolium (NBT), and bromochloro-indolyl phosphate (BCIP) from Biorad (Hercules, CA.); trypsin, penicillin and streptomycin from Gibco (Gaithersburg, MD.); streptavidin-alkaline phosphatase from Boeringer Mannheim (Indianapolis, IN.); fetal bovine serum (FBS) from Hyclone (Logan, UT.); transforming growth factor-α, acrylamide and bis-acrylamide from Promega (Madison, WI.); OCT from Miles Scientific (Naperville, IL.); Vectastain ABC kit from Vector Laboratories Inc. (Burlinghame, CA.). Mouse anti-rabbit collagenase monoclonal antibody was the gift of Dr. Zena Werb (Werb et al., 1989), and mouse anti-human stromelysin monoclonal antibody was the gift of Dr. Scott Wilhelm (Wilhelm et al., 1992).

Induction of Arthritis

Arthritis was induced as described previously (Kapila et al., 1992c and Chapter II, page 75). Briefly, ten-week-old male New Zealand white (NZW) rabbits were sensitized to ovalbumin by two systemic sensitizing doses of this antigen, prepared in normal saline and complete or incomplete Freund's adjuvant and given 14 days apart (see Fig. 1 in Chapter II). Animals were tested for sensitization to the antigen 1 week later, and the TMJs challenged bilaterally with an intra-articular injection of 100 μl of 5 mg/ml of antigen (total OA 500 μg) in normal saline administered 2 weeks after the second sensitizing dose. Both sham-treated animals and untreated animals were used as controls. Three experimental, 1 to 2 sham-treated and one normal control animals were sacrificed 5, 15 and 55 days post-challenge, the right TMJs were retrieved *en bloc* and fixed for histology and immunohistochemistry (see Chapter II), while the left TMJ synovia and discs were retrieved under sterile conditions for culturing.

Retrieval, Processing and Sectioning of TMJs

The retrieval of TMJs for histology and immunohistochemistry has been described previously (Chapter II, page 77). Briefly, the animals were anaesthetized at prespecified time-points following the challenging dose, and the right TMJ was surgically exposed, dissected free of neighboring tissues, retrieved *en bloc* and immediately placed in 4% PBS-buffered paraformaldehyde for 24 hours. The joints were then decalcified by a procedure described by Mori et al. (1988). Following decalcification, each joint was bisected parasagittally into two halves; one half of the joint was embedded in OCT for immunohistochemistry, while the other half was dehydrated and embedded in paraffin for histology and histochemistry (see Chapter II, page 77). When embedding, each half of the

joint was oriented such that the midsagittal section was accessible for subsequent sectioning. For immunohistochemistry, 5 to 6 μm-thick sections were cut from frozen tissues and placed on poly-l-lysine coated slides and air dried prior to staining.

Retrieval of Discs and Tissue Culture Techniques

Intact TMJ discs were retrieved from the left joints of experimental, sham-treated and untreated control animals under sterile conditions, and immediately placed in medium (DMEM) containing 10% FBS and 1x antibiotics (100 U/ml penicillin and 100 mg/ml of streptomycin). The tissue was microdissected to remove any attached synovium, and the discs weighed. The mean weight of discs from antigen-challenged joints was 38.8 (±SD 10.5) mg, sham-treated joints was 30.6 (±11.5) mg, and untreated control joints was 29.0 (±1.0) mg (ANOVA P=0.23). The discs were cultured in 300 μl of serum-free medium (DMEM with 0.2% LAH) containing 1x antibiotics for 48 hours after which the disc-conditioned media was retrieved and stored at -70 °C until further analysis.

Substrate Zymography and Densitometry

Gelatin zymography and densitometric quantitation of disc-CM was done as described previously (Chapter III, page 99), with the exception that samples were standardized by tissue weight of 60 mg/ml.

Reverse Zymograms

Proteinase inhibitors present in disc-CM were visualized and quantitated by reverse zymography as described in Chapter III (page 100), with the exception that samples were standardized by tissue weight of 60 mg/ml.

Western Immunoblots

To identify and further quantify collagenase and stromelysin in disc-CM, Western immunoblots were done and quantitated as described previously (Chapter III, page 101), with the samples were standardized by tissue weight of 85 mg/ml.

Immunohistochemistry

TMJs retrieved *en bloc* from experimental and control animals were fixed, decalcified and processed as described above (Mori et al., 1988). Five to 6 μm-thick sections of the frozen tissue were cut and subjected to immunocytochemistry to localize collagenase and stromelysin as described in Chapter III (page 101).

Statistical Analyses

Means and standard-deviations were derived for each quantitative measure in order to provide a descriptive analysis of the findings. Measurements from densitometric scans of gels and immunoblots from antigen-challenged, sham-treated and untreated control groups were compared by an analysis of variance and Fisher's multiple comparisons test at a 95% confidence level.

C. RESULTS

MMP and PI Synthesis by Discs from Control and Arthritic TMJs

As described previously, explanted TMJ discs synthesized 4 matrix metalloproteinases, namely 92-kDa and 72-kDa gelatinolytic proteinases, procollagenase and prostromelysin in addition to 2 PIs of approximate 30 kDa and 20 kDa. An additional gelatinolytic band at 62 kDa was also observed in most samples. No statistically significant temporal trends were observed for changes in levels of gelatinolytic MMPs, PIs and proteinase to inhibitor ratios. However, when data from all time-points were combined, discs retrieved from antigen-challenged animals demonstrated statistically higher levels (ANOVA P=0.003) of total gelatinolytic proteinase activities than discs from sham-treated (P=0.0015) and untreated (P=0.0163) control animals (Figs. 1 and 3a). Levels of PIs expressed by discs from antigen-challenged joints were lower than those secreted by discs from sham-treated and untreated joints. However, these differences were not statistically significant (ANOVA P=0.081) (Figs. 2 and 3b). The ratio of the sum of all gelatinolytic activities to that of inhibitors was consistently and statistically, greater (ANOVA P=0.018), in discs from antigen-challenged animals than in those from sham-treated (P=0.009) or untreated (P=0.047) control animals (Fig. 3c). Densitometric evaluation for procollagenase and prostromelysin on immunoblots revealed similar differences between experimental and control samples as those observed for total gelatinolytic proteinases (ANOVA P=0.013 and 0.005, respectively) (Figs. 4 and 5). For all quantitative measures of MMPs and PIs, no statistically significant differences were noted between sham-treated and untreated controls. As such, the data from these two groups was combined for presentation of descriptive statistics (Fig. 3).

In Situ Immunolocalization of Collagenase and Stromelysin in TMJ Discs

Immunohistochemical localization for collagenase and stromelysin in TMJs revealed staining of cells on the surface layer and within discs from experimental animals (Fig. 6). In general, similar patterns of staining were observed for both collagenase and stromelysin. In arthritic joints, synoviocyte and inflammatory cells encroaching the surface of the disc also stained positive for these two proteinases. Few discs from control animals demonstrated staining for these proteinases.

D. DISCUSSION

In these studies we examined the levels of MMPs and PIs secreted by discs retrieved from rabbits with antigen-induced arthritis of the TMJ, and compared these to those from sham-treated and untreated controls. We found that arthritic discs expressed

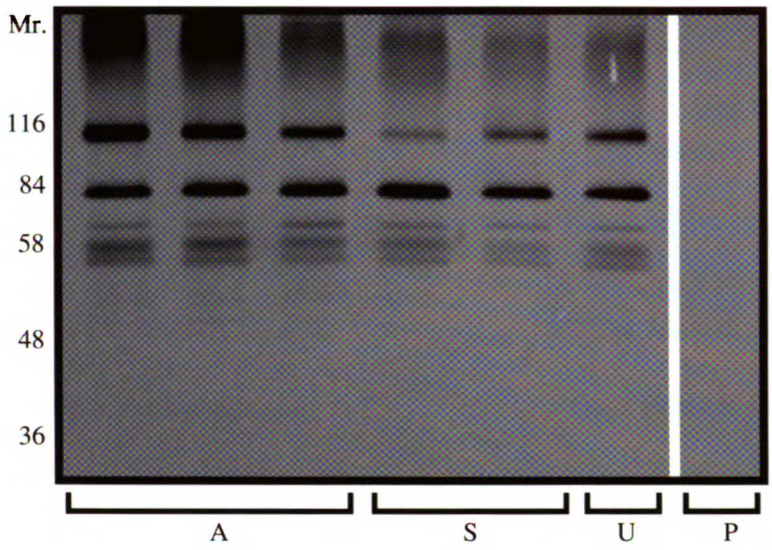

Figure 1: Negative image of gelatin zymogram of disc-conditioned media from 55-day post challenge antigen-treated (A), sham-treated control (S), and untreated control (U) joints. All bands were inhibited by 1, 10-phenanthroline (P), a metalloproteinase inhibitor.

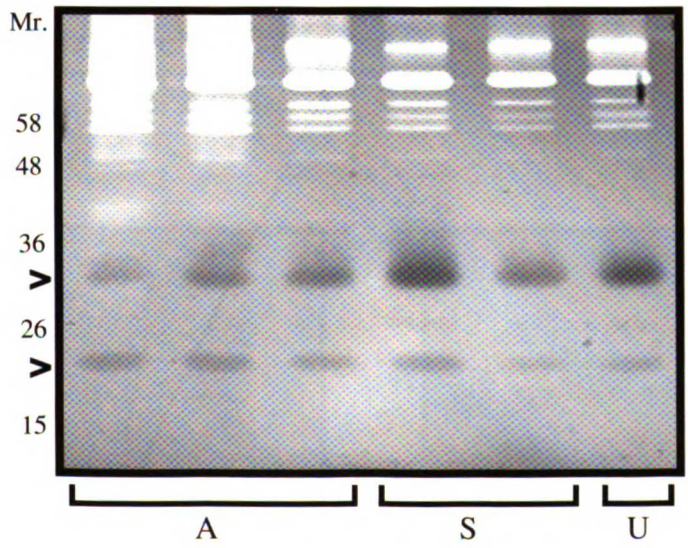

Figure 2: Reverse zymogram of disc-conditioned media from 55-day post-challenge antigen-treated (A), sham-treated control (S), and untreated control (U) joints.

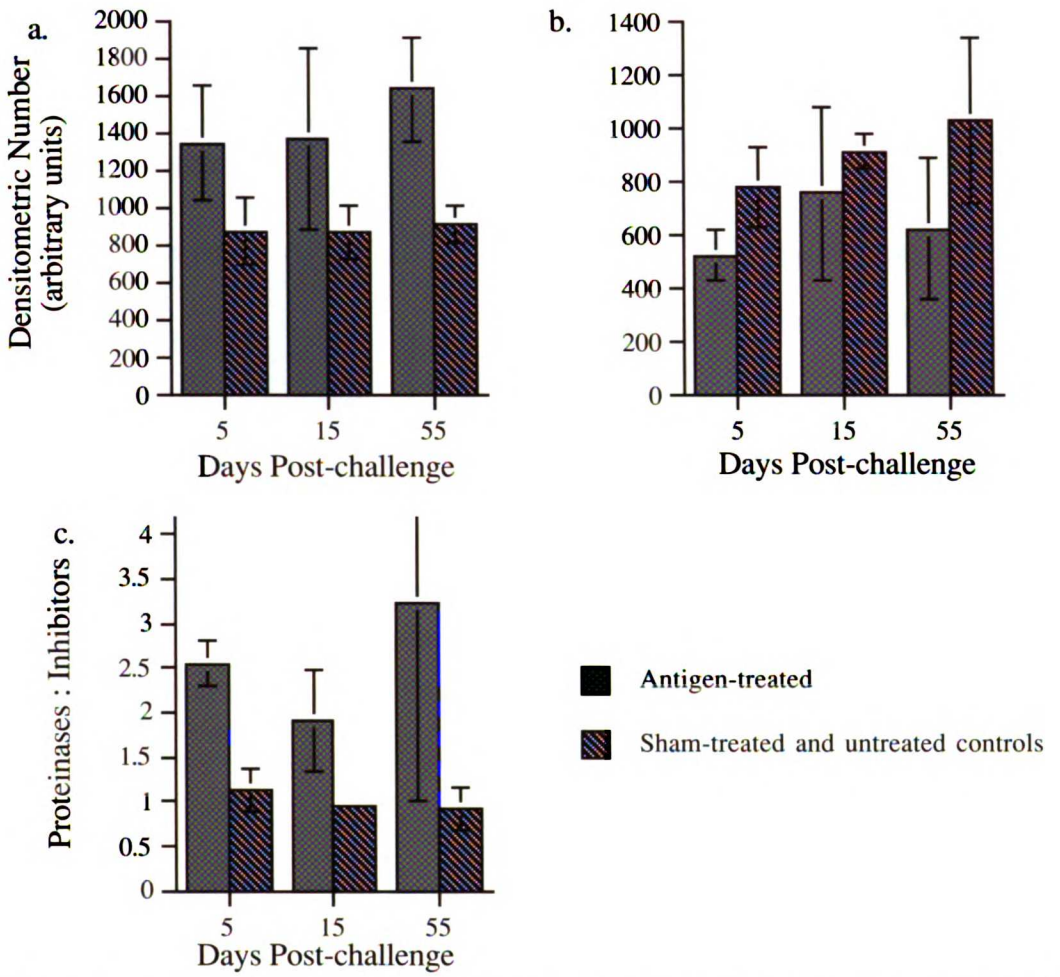

Figure 3: Means and standard deviations for densitometric measurements of gelatinolytic proteinases (a), total inhibitor activities (b), and proteinases-to-inhibitor ratios (c) at 5, 15 and 55 days after induction of arthritis.

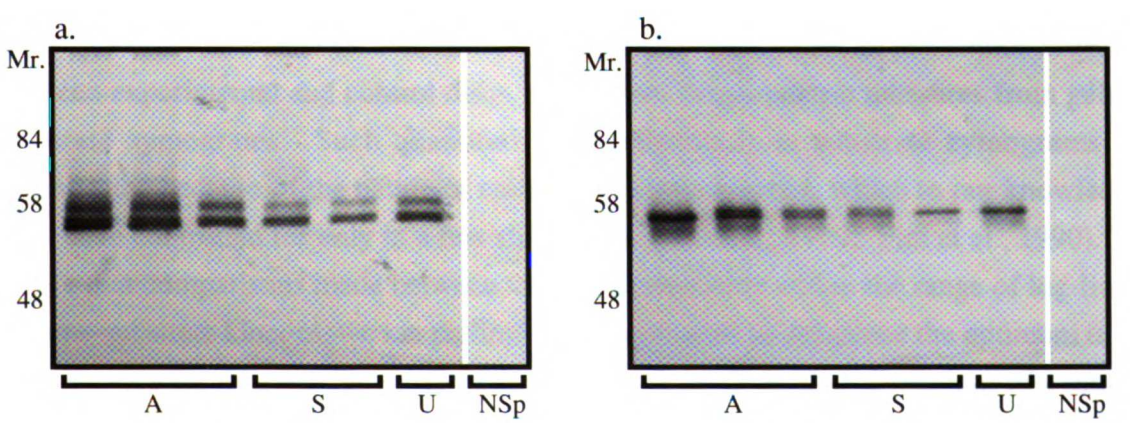

Figure 4: Western immunoblots for collagenase (a), and stromelysin (b) with 55-day post-challenge antigen-treated (A), sham-treated control (S), and untreated control (U) disc-conditioned media. NSp=non-specific IgG.

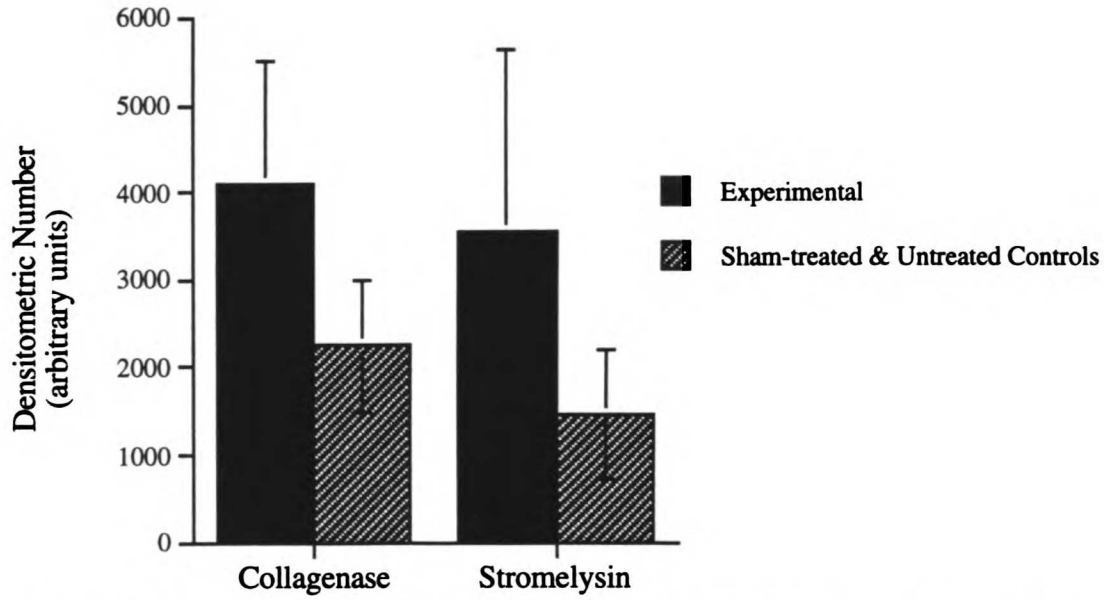

Figure 5: Means and standard deviations for densitometric measurements from collagenase and stromelysin immunoblots for disc-conditioned media from antigen-challenged, sham-treated control and untreated control joints combined for the three post-challenge time-points.

significantly higher levels of prostromelysin and all gelatinolytic proteinases, including procollagenase, as well as slightly, but not significantly, lower levels of inhibitors when compared with control discs. These findings are the first to implicate MMPs and PIs of TMJ disc-cell origin in the pathologic degradation of the disc in inflammatory arthritis, and may have important implications to other arthropathies such as osteoarthritis and internal joint derangement, which commonly affect this joint.

In order to obtain an overview of quantitative differences in proteinase activities between experimental and control discs, we utilized densitometric measures from gelatin substrate zymograms. Such quantitative assessments from substrate zymograms are complicated because of the nature of enzyme-substrate kinetics, which to our knowledge, have been controlled for only in a few studies (Overall et al., 1989; Fini et al., 1990). To ensure that comparisons made between various groups were within the range of log-linear enzyme-substrate kinetics, we ran preliminary experiments to determine the optimum time-of-incubation for the range of activity observed in our samples. Serial dilutions of a sample with high proteinase activity provided the necessary information on the appropriate incubation time for the gels (Chapter III, page 99). Subsequently this sample was used undiluted in one lane of every gel to ensure that it indeed had higher activity than any of the

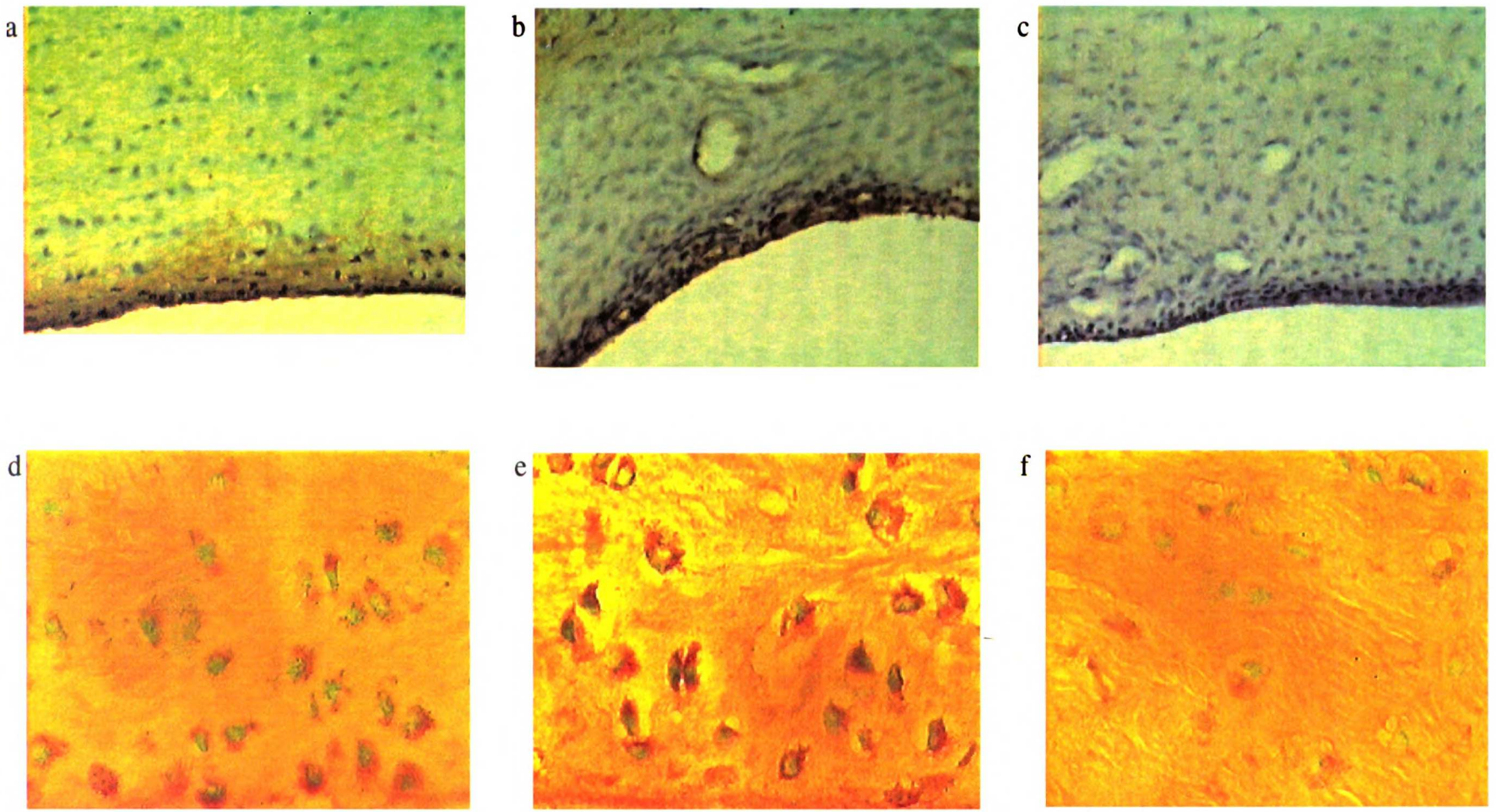

Figure 6: Immunolocalization of stromelysin (a to c) and collagenase (d to e) in TMJ discs. Tissues were incubated with monoclonal antibodies recognizing rabbit stromelysin (a and b), or collagenase (d and e), or non-immune IgG (c and f). Stromelysin and collagenase staining in discs from normal (a and d) and arthritic (b and e) joints. Original magnification is x 320 for (a) to (c), and x 500 for (d) to (f).

experimental and control disc-CM, and also for standardizing for inherent variabilities between gels. Such a method is recommended if quantitative assessments are desired from gelatin substrate gels.

The net ECM degradative activity of MMPs is determined by the transcription, synthesis, and secretion of proteinases and their inhibitors, as well as by the activation of secreted zymogens (reviewed in Alexander and Werb, 1989 and 1991; Birkedal-Hansen et al., 1993). Our experiments indicate that when data from all time-points are combined, the secretion of MMPs and the resultant proteinase-to-inhibitor ratios are significantly greater in arthritic discs as compared with sham-treated and untreated control discs. These differences reflect a net change in both the intracellular and extracellular regulation of MMP activity. Our data (Fig. 3) also suggests an early and sustained increase in total gelatinolytic activity in experimental over control discs. Although these data provide a general impression of these time-related trends in MMP levels with increasing duration of the pathology, the differences in the levels of these proteins was not significantly different between the 3 groups at any of the 3 time-points. This lack of statistically significant differences in levels of MMPs over time may be attributed to the small sample size of the three groups of animals, and the relatively large variation in inflammatory response in the animals (Chapter II, page 85). Further studies are recommended with bigger sample sizes in order to better define if any such time-related changes do indeed exist in disc MMP and PI levels in experimental arthritis.

A finding in common with others observed previously in cell culture systems is the relative lack of additional bands on the zymograms reflecting activation of proteinases, which is considered to be a prerequisite for the effective degradation of matrix molecules. A gelatinolytic band at 62-kDa, probably attributable to the activated form of 72-kDa gelatinase, was the only additional band consistently observed in most of our samples. Activated MMPs are not often observed in cell cultures without the addition of exogenous activators such as plasminogen and trypsin (reviewed in Birkedal-Hansen et al., 1993). Although the reasons for this lack of activation remain to be determined, they may be related to the co-expression of metalloproteinase inhibitors, TIMP and TIMP-2, which bind to the proMMPs, thereby retarding their activation (Birkedal-Hansen et al., 1993). On the other hand, some evidence has been presented demonstrating that the activation of pro-72-kDa gelatinase may be mediated by a cell surface bound "activator" (Overall and Sodek, 1990; Ward et al., 1991a), which may explain why the activation observed in our samples was limited to this proteinase. Despite the lack of activation, the increased proMMP and decreased PI levels in arthritic as compared to control discs does reflect a net increase in the matrix-degradative potential within discs from inflamed joints.

Immunohistochemistry for collagenase on TMJs retrieved from animals provided subjective confirmation of the biochemically detected differences between experimental, sham-treated control and untreated control joints, and also confirmed that proteinase secretion from explanted discs was not a result of culture. These proteinases were localized to outer layer of the disc neighboring the joint space as well as in sites within the disc primarily in antigen-challenged joints. Additional staining was also observed at the periphery of the discs in antigen-challenged joints where the proliferative synoviocytes and inflammatory cells approximated the disc. While double staining for collagenase and stromelysin was not done, the general areas of localization of these two proteins were the same, indicating that cells may be concurrently secreting these enzymes.

The MMPs synthesized by TMJ disc cells have the capacity to degrade all of the major and most of the minor ECM components of the joint disc. Interstitial collagenase degrades collagen types I, II and III, whereas stromelysin, 72-kDa gelatinase and 92-kDa gelatinase degrade proteoglycans, some collagens, fibronectin, elastin and gelatins (reviewed in Alexander and Werb, 1989 and 1991; Birkedal-Hansen et al., 1993). Furthermore, stromelysin has been shown to activate procollagenase *in vitro* (Vater et al., 1983; Treadwell et al., 1986; Ishihashi et al., 1987; Murphy et al., 1987; Ito and Nagase, 1988; Unemori et al., 1991a). Thus, the likely effect of the increase in total degradative activity in the arthritic disc is the loss of disc matrix, as demonstrated in our previous study (Chapter II). This loss of matrix would impact not only the biomechanical properties of the disc and on its ability to withstand normal function (reviewed in Muir and Carney, 1987 and Mankin and Brandt, 1989), but possibly also on the normal proliferation and differentiation of cells within the disc (reviewed in Toole, 1991). Additionally, degradation products of ECM components, such as peptide fragments of collagen, fibronectin, and elastin, may in turn induce increased synthesis of MMPs by disc cells (Werb et al., 1989; Homandberg et al., 1992), thereby making the matrix-degradative response, previously initiated by inflammatory mediators, self-perpetuating.

In summary, we demonstrate that disc-derived MMP levels are increased and PI levels slightly, but not significantly, decreased in antigen-induced arthritis of the rabbit TMJ. These findings implicate MMPs and PIs of disc-cell origin in the increased matrix degradation of the TMJ disc during inflammatory arthropathies. The findings of this study may be useful in defining potential therapeutic approaches for preventing or alleviating TMJ disc degeneration in various arthropathies.

CHAPTER VI

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER VI

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

A. INTRODUCTION

In the preceding chapters, a series of studies describing the development of a juvenile animal model of antigen-induced arthritis of the TMJ, identifying and characterizing proteinases and proteinase inhibitors expressed by disc cells, and evaluating the alterations in levels of synovium- and disc-derived MMPs and PIs in this model of experimental arthritis were presented. These studies were undertaken because of the relatively poor understanding of the pathogenesis of TMJ arthritis, specifically with regard to the role of its anatomically unique and functionally important structure, the disc, in this disease process. The unifying hypothesis of these studies was that antigen-induced arthritis of the juvenile TMJ is accompanied by an increase in the absolute and relative levels of MMPs to their inhibitors expressed by specific tissues of the joint. The purpose was to evaluate the alterations in synovium- and disc- derived MMPs and PIs in antigen-induced arthritis of the TMJ in order to provide greater insights into the possible mechanisms for loss of tissue matrices during this arthropathy. In this chapter, a synthesis and synopsis of the findings of these studies are outlined, a critique of the studies is provided, the clinical and scientific implications are discussed, and suggestions for areas of future research are described.

B. SUMMARY

Our studies describe the first animal model of antigen-induced arthritis of the TMJ, and the first such model in a juvenile animal. The histopathology of this model demonstrates features characteristic of RA, including synovial lining and villous hyperplasia, mononuclear cell infiltration, pannus formation, and the loss of GAGs and collagen from condylar cartilage and the joint disc. The loss of matrix macromolecules in antigen-challenged joints was accompanied by marked immunohistochemical staining for collagenase and stromelysin in cells of the synovial lining, synovial stroma, and disc as well as in articular chondrocytes. This model of experimental arthritis of the TMJ has several potential applications, both of a general nature, as well as those specifically related to the TMJ. The general applications of this model include its use in the further understanding of the pathogenesis and immunology of JRA, evaluating for possible

biologic markers of disease severity, and assessing the efficacy of biologically rational anti-arthritic therapies. Applications of this model which are specific to the TMJ include (a) a further determination of the specific role of the disc in the pathogenesis of TMJ arthritis, as well as the mechanisms for, and the effects of TMJ disc matrix loss in the progression of this disease and on the function of the joint, and (b) because of the proximity of the condylar growth cartilage to the joint, assessing the effects of TMJ arthritis in the growing animal on the development of craniofacial structures (Tavakkoli, 1994).

These studies provide the first characterization and identification of proteinases and PIs synthesized by TMJ discs and disc cells as 92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin, as well as a 30-kDa and a 20-kDa PI, probably TIMP and TIMP-2. Further studies on the regulation of MMPs by disc cells suggest that the profile of MMPs constitutively synthesized by disc cells is different from that of chondrocytes (Lefebvre et al., 1991; Ogata et al., 1992), while their phorbol ester-stimulated regulation differs from that of synovial fibroblasts (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987) and articular chondrocytes (Bandara et al., 1992; Ogata et al., 1992). These findings suggest a rich area for future research in understanding the mechanisms for regulation of MMPs by different cell types. An understanding of the unique MMP regulatory mechanisms may be important in designing anti-arthritic drugs aimed specifically at distinct cell types in order to independently limit their matrix degradative responses in arthritis.

Finally, these studies demonstrate an increased expression of MMPs by synovium and disc cells retrieved from joints with antigen-induced arthritis of the TMJ. The studies on the synovium confirm similar findings from studies done previously on adult animal models of arthritis (Cambray et al., 1981; Hasty et al., 1990; Pettipher et al., 1990), and supplement information for the less conclusive human studies (Wooley et al., 1977a and b; Cawston et al., 1984; Dieppe et al., 1988; Case et al., 1989a and b; Okada et al., 1989a and 1990c; Hasty et al., 1990; Henderson et al., 1990; Pettipher et al., 1990; Brinkerhoff, 1991; Evans et al., 1991; Firestein et al., 1991; Garvallese et al., 1991; McCachren et al., 1991; Firestein, 1992; Firestein and Paine, 1992 ; Hirose et al., 1992; Walakovits et al., 1992). On the other hand, the constitutive synthesis and regulation of MMPs and PIs by disc cells, as well as their altered expression in inflammatory joint disease has not been characterized previously. Our studies demonstrate an increased expression of prostromelysin and all gelatinolytic proteinases, including procollagenase, in arthritic versus non-arthritic synovia and discs. Levels of synovium-derived PIs do not show differences between experimental and control tissues, while discs from arthritic joints expressed slightly, but not significantly, lower levels of PIs than control discs. These

observations are consistent with the hypothesis that tissue loss associated with antigen-induced arthritis of the TMJ results, at least in part, from an increase in MMPs and a net increase in MMP to PI ratios.

C. CELLULAR INTERACTIONS AND PATHOPHYSIOLOGY OF MATRIX DEGRADATION IN TMJ ARTHRITIS

Utilizing the findings of previous studies on other joints, and those of the present investigations, a synthesis of known and potential cellular interactions and basis for matrix loss in arthritis is presented in Figure 1. Although this flowchart may be applicable to other joints, an attempt has been made to focus specifically on the TMJ by emphasizing the interactions of disc cells with cytokines and their potential role in the matrix degradative cascade. While many of the cellular interactions and events have previously been determined by *in vitro* or *in vivo* studies, several other aspects of the pathogenesis of this disease, particularly those pertaining to the TMJ disc, still remain to be elucidated. Furthermore, this figure is by no means all inclusive since it does not take into account the role of altered matrix synthesis or other classes of proteinases or possibly other as yet unknown interactions between resident and inflammatory cells, cytokines, matrix degradation products, the effects of altered matrix environment on cellular activities, as well as the extracellular activation of MMPs in the net loss of matrix macromolecules from the tissues of the joint.

Although the etiology of RA and JRA remain unknown, it has been proposed that these pathologies may be initiated by an environmental factor, such as bacteria or viruses or their products, acting in a genetically susceptible host (Stastny, 1976 and 1978; Winchester, 1981; Pope et al, 1982; Vaughan et al., 1983). The ensuing inflammatory response, via both cellular immune and humoral mechanisms, leads to the localization, differentiation and activation of immune cells and resident cells, induces the expression of proinflammatory cytokines and other bioactive agents, and initiates the cascade of cell and cytokine interactions. Since both the etiology and immunopathogenesis of RA are beyond the scope of the present discussion, the rest of this discussion focuses on the proposed cascade of matrix degradative events that occur in inflamed joints secondary to the cytokine-induced alterations in expression of MMPs and PIs.

Substantial evidence indicates that alterations in the expression of MMPs and PIs by fibroblasts and chondrocytes in inflammation result primarily in response to cytokines, with the modulating effects of growth factors and eicanosoids (Bandara et al., 1989; Kumkumian et al., 1989; Shinmei et al., 1989; Ahmadzadeh et al., 1990; Alvaro-Garcia,

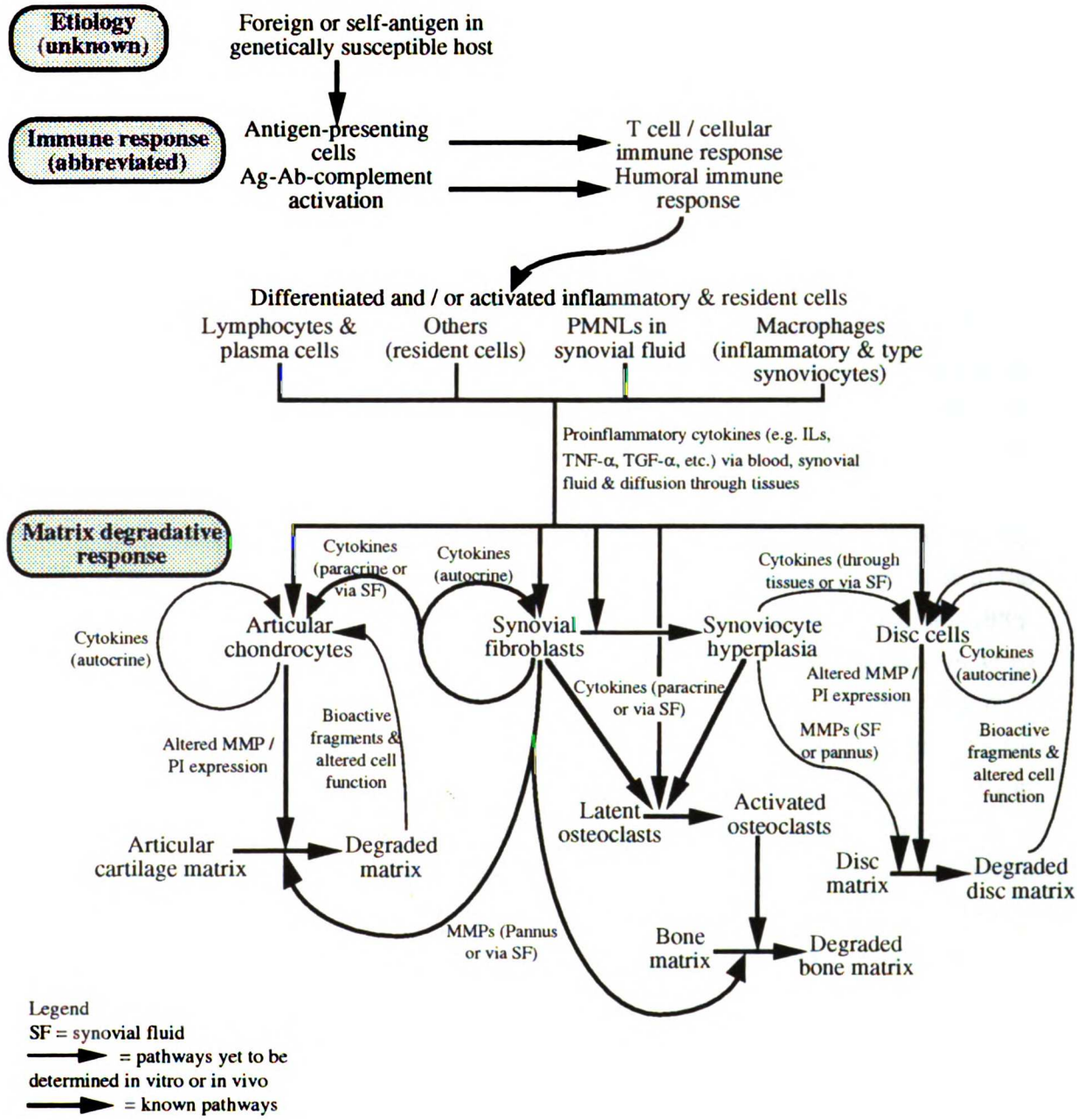

Figure 1: Pathophysiology of matrix degradation in TMJ arthritis.

1990; Case et al., 1990; MacNaul et al., 1990; Lefebvre et al., 1990b and 1991; Martel-Pelletier et al., 1991; Smith et al., 1991; Unemori et al., 1991b; Wright et al., 1991; Hardingham et al., 1992; Nguyen et al., 1992; Ogata et al., 1992; Quintavalla et al., 1993), although other factors, such as aberrant loading of tissues and cells (Kapila and Rao, unpublished data) may also be important. It is generally accepted that in arthritis, following the initiation of the pathology, a cascade of events largely mediated by these agents eventually results in degradation of joint matrices from bone, articular cartilage and disc as outlined in Figure 1 (Harris 1989; Gall, 1988).

Unlike cells in the avascular disc and condylar cartilage, the type A and B synoviocytes and stromal fibroblasts in the vascular synovium are probably the first resident cells of the joint to demonstrate alterations in the expression of MMPs and PIs since they are likely the first cells of the joint exposed to cytokines and other bioactive agents. However, evidence for such temporally differentiated involvement of tissues of joints has not been presented. In our investigations, the pathology is relatively advanced by 5 days after induction of arthritis (Chapters II, III and V), such that these early temporal events may have been missed. An evaluation of the time-related involvement of different tissues in TMJ degeneration therefore requires an earlier assessment of the disease process.

Other changes, including the neoplast-like transformation and the relatively uncontrolled proliferation of synovial lining cells probably occur largely in response to the inflammatory milieu in the synovium and neighboring synovial fluid (Yocum et al., 1988; Lafyatis et al., 1989 Wilder et al., 1989; Sano et al., 1990). Indeed, several *in vitro* studies have demonstrated enhanced proliferation of cultured synoviocytes on exposure to cytokines, such as interleukin-1, platelet derived growth factor, epidermal growth factor and insulin-like growth factor (Schmidt et al., 1982; Bunning et al., 1986; Remmers et al., 1988; Kumkumian et al., 1989; Lafyatis, 1989; Hiraoka et al., 1992). These changes are evident histologically as synovial lining and villous hyperplasia in which mitotic figures, indicative of rapidly proliferating cells, are also commonly observed (Chapter II; Caulfield et al., 1982). Both synovial stromal fibroblasts and proliferative lining synoviocytes may possibly further aggravate the pathology not only because of their altered expression of MMPs and PIs, but also by their synthesis of cytokines and growth factors (Pilsworth and Saklatvala et al., 1983; Duff et al., 1985; Guerne et al., 1989; Remmers et al., 1991; Bandara et al., 1992). The cytokines of synoviocyte origin likely impact on these synovial cells in an autocrine modality, and also affect other resident and inflammatory cells in the joint in a paracrine manner either by diffusion through tissues or via the synovial fluid. It has been shown, for example, that cultured chondrocytes or cartilage when exposed to synovial fluid from arthritic joints or to conditioned media from

synovia retrieved from arthritic joints demonstrate an increased expression of MMPs and breakdown of tissue matrices (reviewed in Steinberg et al., 1986). Although a similar mechanism for altered MMP / PI expression is likely to also hold true for the TMJ disc, the exact contribution of synoviocyte-secreted cytokines to the perturbations in disc MMP / PI synthesis by their paracrine stimulation of disc cells, via the synovial attachment to the disc or via the synovial fluid, remains to be determined. Such studies can be done by exposing cultured discs or disc cells to synovial fluid or synovium-conditioned media from arthritic joints. Our preliminary studies (Kapila, unpublished data), however, do indicate that specific proinflammatory cytokines, particularly IL-1α, TGF-α and TNF-α, induce the synthesis of procollagenase and prostromelysin by cultured disc cells, and indicate a potential for similar responses *in vivo*.

Once stimulated, both articular chondrocytes and disc cells probably continue the inflammatory cascade and the matrix degradation previously initiated by synoviocytes and inflammatory cells, both by the secretion of more bioactive agents (Shinmei et al., 1989; May et al., 1991; also reviewed in Steinberg et al., 1986), as well as increase in their matrix degradative activities (Andrews et al., 1989; Bandara et al., 1989 and 1992; Shinmei et al., 1989; Ahmadzadeh et al., 1990; Lefebvre et al., 1990b; Martel-Pelletier et al., 1991; Smith et al., 1991; Nguyen et al., 1992; Ogata et al., 1992; Quintavala et al., 1993; Kapila et al., 1993 and Chapter V). The chondrocyte- and disc cell-secreted proinflammatory agents likely affect the activities of these cells in an autocrine manner, and probably also those of other cells in the vicinity. Furthermore, several proteolytic fragments of matrix degradation have also been shown to have both proinflammatory and chemo-attractive properties, and also enhance the expression of MMPs by chondrocytes and fibroblasts (Pontz et al., 1979; Postlethwaite and Kang, 1976; Postlethwaite et al., 1978; Norris et al., 1982; Werb et al., 1989; Lohr et al., 1990; Homandberg et al., 1992). Additionally, the alterations in the matrix microenvironment may perturb normal cellular activities (Gospodarowicz and Ill, 1980; Hauschka et al., 1985; also reviewed in Toole, 1991). As a result, these consequences of matrix degradation not only sustain the ongoing matrix degradative activities, but may indeed further aggravate the arthropathy through the several positive feedback loops. The role of both fibroblast, disc cell and chondrocyte secreted cytokines, the effects of proteolytic fragments of matrix macromolecules, as well as the alterations in cell-matrix interaction on the perpetuation of arthritis requires further study.

The sequence of events leading to the degradation of mineralized matrix in arthritis is likely to be complex, since it requires the loss of organic matrix, the induction of osteoclasts, and further the activation of these cells by appropriate stimulation and contact

with calcified matrix (Chambers et al., 1985; Chambers and Fuller, 1985; Delaisse et al., 1985). Despite these relatively stringent requirements, the milieu of the pannus appears to afford a suitable environment for osteoclastic activity. Indeed, several multinucleated cells, likely to be osteoclasts, are frequently observed in the pannus at the surfaces of bone spicules (see Figure 2c and h, Chapter II). It is probable that the stimulation of osteoclast precursors by proinflammatory mediators, the degradation of the osteoid by matrix metalloproteinases in the pannus, and the consequent contact of the inactive osteoclasts with mineralized matrix provide the appropriate microenvironment for the resorption of bone. The exact mechanisms for, and the sequence of events in pannus related bone resorption needs detailed investigation.

Cytokines and other bioactive agents also likely mediate several histologically obvious alterations in the tissues of the joint. These agents may directly cause synovial lining and villous hyperplasia by modulating the neoplast-like transformation and uncontrolled proliferation of lining synoviocytes (Schmidt et al., 1982; Bunning et al., 1986; Remmers et al., 1988; Kumkumian et al., 1989; Lafyatis, 1989; Hiraoka et al., 1992). These agents also indirectly cause the histochemically observed loss of GAGs and collagen from articular tissues by mediating the upregulation of MMPs by resident and inflammatory cells in the joint (Welgus et al., 1985; Campbell et al., 1987; Bandara et al., 1989; Kumkumian et al., 1989; Shinmei et al., 1989; Ahmadzadeh et al., 1990; Alvaro-Garcia, 1990; Case et al., 1990; MacNaul et al., 1990; Lefebvre et al., 1990b and 1991; Martel-Pelletier et al., 1991; Smith et al., 1991; Unemori et al., 1991b; Wright et al., 1991; Hardingham et al., 1992; Nguyen et al., 1992; Ogata et al., 1992; Quintavalla et al., 1993; Kapila et al., 1993 and Chapter V). The pattern of matrix loss, however, varies at different sites of the joint. Localized loss of collagen and proteoglycans is seen where the pannus invades into bone, cartilage and disc (see Fig. 4, Chapter II). On the other hand, a generalized pattern of matrix loss occurs in cartilage and disc where substantial areas demonstrate lack of staining for GAGs and PGs (Lowther et al., 1978; Rubin and Roberts, 1987; Yoo et al., 1988; Healy et al., 1989; Hasty et al., 1990; Pettipher et al., 1990; Beesley et al., 1992; Kapila et al., 1994 and Chapter II). This loss of PGs and GAGs occurs early, is sustained during the pathology, and the degradation of these macromolecules may be attributed to two distinct sources of MMPs, namely (1) the increased levels of MMPs synthesized and secreted by synoviocytes into the synovial fluid, and (2) the stimulation of chondrocytes and disc cells by proinflammatory agents resulting in their increased matrix degradative response. Although both these mechanisms probably contribute to the generalized pattern of matrix degradation, the former modality has been called to question by the findings of some investigations demonstrating high levels of MMP

inhibitors in arthritic synovial fluid (Abe and Nagai, 1973; Cawston et al., 1984 and 1987; Osthues, 1992), and others showing that matrix macromolecules are lost from the deeper regions of articular cartilage rather than its superficial surface which is in contact with the synovial fluid (Beesley et al., 1992).

For reasons of simplification, several aspects of the pathophysiology of matrix degradation in arthritis are not represented in Figure 1. For example, besides synoviocytes, chondrocytes and disc cells, MMPs are also synthesized by inflammatory cells, such as polymorphonuclear leucocytes (PMNs) and macrophages. While PMNs are not found in any large amounts in arthritic tissues, substantial numbers of these cells are often present in the synovial fluid (reviewed by Harris, 1989). PMNs secrete the only other known mammalian interstitial collagenase, PMN collagenase. This proteinase, unlike its counterpart in fibroblasts and chondrocytes whose synthesis and secretion is largely regulated at the transcriptional level, is stored in granules and released on appropriate stimulation. Macrophages constitutively synthesize 92-kDa gelatinase, and smaller amounts of collagenase and stromelysin (Campbell et al., 1987; Welgus et al., 1985 and 1990b). Therefore, in addition to MMPs expressed by resident cells of the joint, those synthesized by PMNs and macrophages probably also contribute to arthritis-associated degradation of joint matrix macromolecules.

Since the focus of our studies is limited to MMPs and PIs, several other aspects of the pathophysiology of joint matrix degradation are not included in Figure 1. The role of other proteinases, such as serine and cysteine proteinases in both matrix degradation and in the activation of MMPs during arthritis is not discussed. Serine-, cysteine- and matrix metalloproteinases together probably all contribute to the cascade of proteinase activations and matrix degradative events. Also not presented in Figure 1 are the potential effects of proinflammatory cytokines and other bioactive agents on the proliferation, differentiation, metabolic and matrix synthetic activities, or premature death of chondrocytes (Andrews et al., 1989; Kato and Iwamoto, 1990; Thompson et al., 1991; Suzuki, 1992; also reviewed in Hardingham et al., 1992) and possibly also on disc cells, all of which, if adverse, would result in a net decrease in matrix content of tissues of the joint. If such alterations in matrix synthesis, resulting either due to reduced number of cells or their diminished matrix synthetic activity, are superimposed on an increased matrix degradative activity the consequences could be a rapid deterioration of joint tissues.

As indicated in Figure 1, several of the proposed pathways still require to be elucidated, specifically with regard to cytokine stimulation of disc cells which are located in an avascular environment bathed in synovial fluid and attached only at the periphery to the synovium. Some of the questions that require further study include how cytokines reach

cells within the disc, can disc cells, once activated, further stimulate themselves in an autocrine modality, what, if any, is the role of matrix degradation products in stimulating MMP synthesis by disc cells, and what effect does the altered matrix environment have on disc cell function and their expression of MMPs? Many of the same questions have also yet to be addressed with regard to articular chondrocytes. Another important inquiry would be in determining the exact contribution of synoviocyte-derived MMPs in the pannus or secreted into synovial fluid on disc matrix degradation.

D. LIMITATIONS OF STUDIES

Although the findings of the studies described in this dissertation provide several novel and important insights into the pathophysiology of matrix loss in experimental arthritis, some caution should be exercised in extrapolating these findings *en masse* to RA and JRA in humans. These cautions in interpretation of the findings are recommended partially because animal models of arthritis do not necessarily represent every aspect of the human disease, and partially because of the limitations of the experimental design and of some of the assays used in these investigations as detailed below.

Although animal models are likely to provide important information on the pathogenesis of RA, there are several specific limitations of these models. Two primary limitations should be considered when interpreting information derived from these models. First, substantial differences exist in the organization and function of joints as well as the immune systems between humans and lower mammals, and as a consequence the arthropathy in the animal model may not accurately represent every aspect of the human disease. Second, since the etiology of RA remains unknown, induction of arthritis in these animal models is based on procedures which cause joint inflammation in response to a specific antigen that may not be relevant to the initiation of RA. Nevertheless, many of the post-induction events in several of these experimental models of arthritis closely mimic RA (Dumonde and Glynn, 1962; Edwards et al., 1988; Henderson et al., 1993).

The specific limitations of the present study are largely related to the variability in the inflammatory and matrix degradative responses in the TMJs of antigen challenged joints. Although such variability in response is commonly observed in *in vivo* studies (Boissier et al., 1988; Edwards et al., 1988), this, combined with the small numbers of animals at each time-point diminished the ability to discern clear-cut temporal trends both in the severity of arthritis and in the expression of MMPs and PIs. A larger sample size, combined with sensitive quantitative assays for MMPs and PIs, may have demonstrated substantially greater differences in the expression of these proteins between experimental

and control tissues and between the different time-points. However, since findings of previous studies indicate that post-challenge temporal trends may be masked by the large individual variabilities in response between animals (Boissier et al., 1988; Edwards et al., 1988), the determination of disease activity and the associated matrix degradative responses of the tissues based on time after induction of arthritis may not provide an accurate assessment of the severity of arthritis. Instead our findings suggest that histopathologic criteria, rather than post-challenge time-points, provide a more accurate assessment of the severity of arthritis in such experimental models.

The histochemical staining did not reveal generalized loss of collagen, despite its localized loss where the pannus invaded into bone, cartilage and disc, and despite the generalized pattern of loss of GAGs. Although similar findings have been reported by other investigators (Lowther et al., 1978; Beesley et al., 1992) using histochemical techniques, it is likely that generalized loss of collagen does occur in experimental arthritis, but requires more sensitive techniques for detection. Indeed, Reddy and Dhar (1992) using radioactive labeling methods have demonstrated loss of collagen from tendon and bone of rats with adjuvant-induced arthritis. Therefore, more sensitive techniques for collagen staining, such as immunohistochemistry, may have provided a better understanding of generalized loss of this matrix macromolecule from tissues of the TMJ.

Since the manifestation of a net decrease in matrix macromolecules may result from one or more of a variety of reasons, including disturbances in cell proliferation and differentiation, as well as matrix synthetic and matrix degradative activities, the findings of these studies do not prove a cause and effect relationship between increased matrix degradative activity and loss of matrix macromolecules. However, our work does indicate a positive correlation between GAG loss from the TMJ disc and levels of MMPs expressed by this tissue (Chapter V). In order to obtain more conclusive evidence on the contribution of MMPs in the net loss of joint matrix macromolecules, studies which utilize MMP inhibitors in such experimental models of arthritis are indicated. Further work is also suggested to evaluate the relative contribution of the cell proliferative, differentiation and metabolic responses to the net loss of matrix macromolecules in joint tissues in arthritis.

E. SCIENTIFIC AND CLINICAL IMPLICATIONS, AND FUTURE DIRECTIONS

The findings of the studies described in Chapters II to V provide both important new information, particularly with regard to MMP and PI expression by normal and arthritic discs, as well as confirm some findings of previous studies on the role of MMPs

and PIs on joint matrix turnover in arthritis. These findings have implications to several areas ranging from the regulation of MMPs and PIs to the diagnosis and treatment of arthritis. They also suggest areas of future research as outlined below.

Constitutive Expression and Regulation of MMPs and PIs by Resident Cells of the TMJ

The TMJ primarily contains four types of resident cells, namely type A or macrophage-like synoviocytes, type B or fibroblast-like synoviocytes, chondrocytes, and the as yet poorly characterized disc cells. Although little is known about the specifics of MMP profiles from synovial fibroblasts and chondrocytes from the TMJ, the MMP profile of these cells from other joints has been well characterized (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987; Unemori and Werb, 1986; Lefebvre et al., 1990a and b and 1991; Ogata et al., 1992). Our investigations and those of others (Aggeler et al., 1984; Chin et al., 1985; Welgus et al., 1985 and 1990b; Frisch et al., 1987; Unemori and Werb, 1986; Campbell et al., 1987; Bandara et al., 1989; Lefebvre et al., 1990a and b and 1991; Ogata et al., 1992) demonstrate that disc cells, chondrocytes, synovial fibroblasts, as well as macrophages have different constitutive and stimulated profiles of MMP expression (Table I). Thus, synovial fibroblasts, like disc cells, constitutively express 92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin, while chondrocytes constitutively synthesize only 72-kDa gelatinase. However, despite the similarities in the profile of constitutively synthesized MMPs by synovial fibroblasts and disc cells, the TPA-stimulated MMP secretion differs between the two cell types. Thus, while TPA treatment induces increased expression of 92-kDa gelatinase, procollagenase and prostromelysin in synovial fibroblasts (Aggeler et al., 1984; Chin et al., 1985; Frisch, et al., 1987), in disc cells this phorbol ester only increases the synthesis of 92-kDa gelatinase, and has little discernible effect on the expression of procollagenase and prostromelysin expression (Chapter IV). Therefore, disc cells, which demonstrate both fibroblast and chondrocyte phenotypes expressing type I collagen and cartilage-specific PGs (Mills et al., 1988; Milam et al., 1991), also show a different constitutive pattern of MMPs relative to chondrocytes (Lefebvre et al., 1990a, b and 1991; Ogata et al., 1992), and a divergent pattern of regulation of MMPs from synovial fibroblasts (Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987). These findings indicate the unique nature of MMP regulation in different types of cells. The cellular and molecular basis for these differences should be examined further.

With regard to macrophages, although little is known about type A synoviocytes, lung alveolar macrophages constitutively secrete 92-kDa gelatinase, and smaller quantities of procollagenase and prostromelysin. TPA increases the expression of 92-kDa gelatinase

and procollagenase, but not of 72-kDa gelatinase and prostromelysin by these cells. Clinically, the findings on the differential regulation of MMPs by joint cells may imply that therapies aimed at decreasing matrix degradative activity within the joint may have to be targeted to specific cells present in the joint.

Table I: The phenotypic characteristics of the three primary resident cell types of joints and of macrophages.

Cell	*In vivo* cell morphology	Matrix macromolecules	Constitutively expressed MMPs	MMP expression on stimulation by phorbol esters
Synovial fibroblasts	Spindle shaped cells	Type I collagen	92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin[1]	Increased 92-kDa gelatinase, procollagenase and prostromelysin, no change in 72-kDa gelatinase[1]
Chondrocytes	Large round cells with large nucleus	Type II collagen, cartilage-specific proteoglycans	72-kDa gelatinase[2]	Slight increase in 92-kDa gelatinase, no change in 72-kDa gelatinase, procollagenase and prostromelysin[2]
Disc cells	Small round cells	Type I collagen, cartilage-specific proteoglycans	92-kDa gelatinase, 72-kDa gelatinase, procollagenase and prostromelysin[3]	Increased 92-kDa gelatinase, no change in procollagenase and prostromelysin, decreased 72-kDa gelatinase[3]
Macrophages	Irregular or ovoid cells, reniform or ovoid nucleus	N/A	92-kDa gelatinase, small amounts of procollagenase and prostromelysin[4]	Increased 92-kDa gelatinase & procollagenase, no change in prostromelysin and 72-kDa gelatinase[4]

Data from:
[1] Aggeler et al., 1984; Chin et al., 1985; Frisch et al., 1987; Unemori and Werb, 1986
[2] Bandara et al., 1989; Lefebvre et al., 1990a and b and 1991; Ogata et al., 1992
[3] Kapila et al., 1992a and Chapter IV
[4] Welgus et al., 1990 from lung alveolar macrophages

Implications to the Progression and Self-perpetuation of Arthritis

The mechanisms for chronicity of arthritis are poorly understood, but the possible basis for this include a sustained stimulation or a failure of regulation of the immune system (Consden, 1971; Allen et al., 1985; also reviewed in Bennet, 1989). Sustained stimulation of the immune system may in turn result either from a failure to eliminate heterologous antigen or due to a consistent reaction to an autologous antigen. However, the findings of several *in vitro* studies suggest yet other mechanisms for the chronicity of arthritis. For instance, the ability of joint resident cells to synthesize cytokines in response to mediators from inflammatory cells may initiate positive feedback loops contributing to the perpetuation of arthritis (Shinmei et al., 1989; May et al., 1991; also reviewed in Steinberg et al., 1986). Additionally, proteolytic fragments of matrix macromolecules have been shown to be chemotactic to inflammatory cells, and can induce cytokine and MMP synthesis by resident and inflammatory cells (Pontz et al., 1979; Postlethwaite and Kang,

1976; Postlethwaite et al., 1978; Norris et al., 1982; Werb et al., 1989; Lohr et al., 1990; Homandberg et al., 1992). Such activities of matrix macromolecule fragments may contribute substantially to the progression and aggravation of arthritis. Investigations, both *in vitro* and *in vivo*, on the potential role of proteolytic fragments of matrix macromolecules in the maintenance or perpetuation of arthritis are indicated, and will add further to our understanding of the basis for chronicity of arthritis.

Implications to the Diagnosis of Arthritis

Currently, the diagnosis of RA is derived from a series of clinical signs and symptoms complemented by some imaging and laboratory diagnostic criteria. While these approaches provide arbitrary determinations of the presence and approximate severity of disease, they lack accuracy in classifying the diseases into precise categories, in evaluating with great precision the severity of the on-going processes in the joint, and in defining the prognosis. An important role of studies like those discussed in Chapters II to V are to provide greater insights into the pathogenesis of RA, such that more precise diagnostic tests and biologically rational therapies can be devised. Although the present studies do not specifically suggest new methods for diagnosing arthritis, the findings may offer methods for more precisely determining the severity of the disease. Regression analysis on the levels of different synovium-derived proteinases with arthritic score revealed that the levels of total gelatinolytic proteinases and 72-kDa gelatinase are strongly correlated with the severity of arthritis (R^2=0.53, P=0.0001 and R^2=0.69, P<0.0001, respectively) (also see Figure 2). These findings need to be tested further in order to determine their validity and reliability for applications in diagnostic procedures.

In contrast to the strong correlations between levels of 72-kDa gelatinase and disease severity, the levels of both procollagenase and 92-kDa gelatinase did not correlate well with disease severity (R^2=0.26, P=0.015 and R^2=0.36, P=0.003, respectively). The reason for the strong correlation between the levels of 72-kDa gelatinase, rather than other MMPs traditionally thought to be upregulated by inflammation, with disease severity is not yet clear and requires further evaluation. However, it is appealing to conjecture that it is precisely for the reason that 92-kDa gelatinase and procollagenase transcription is rapidly modulated by proinflammatory cytokines (Kumkumian et al., 1989; Shinmei et al., 1989; Alvaro-Garcia, 1990; MacNaul et al., 1990; Lefebvre et al., 1990b and 1991; Martel-Pelletier et al., 1991; Smith et al., 1991; Unemori et al., 1991b; Nguyen et al., 1992; Ogata et al., 1992; Quintavalla et al., 1993), while 72-kDa gelatinase is under a tighter transcriptional control (Overall et al., 1991; Salo et al., 1991), that the levels of 72-kDa gelatinase correlate strongly with disease severity during the chronic stages of arthritis in our studies. During these phases of the disease, the levels of cytokines present may be

maximally inducing 92-kDa gelatinase and procollagenase such that correlations between disease severity and the levels of these MMPs may be obviated. For the same reasons, it is possible that both procollagenase and 92-kDa gelatinase may correlate well with disease activity in the very early stages of the pathology as the levels of cytokines increase, but not once the disease has become chronic and these MMPs are potentially being expressed at their peak levels. These concepts need to be examined further by studies during the very early stages of inflammatory response in such animal models of arthritis.

Since proteoglycans are depleted early in experimental arthritis (Pettipher et al., 1989 and 1990; Hasty et al., 1990; Beesley et al., 1992; Kapila et al., 1994 and Chapter II), proteolytic fragments of PGs in synovial fluid may be important early makers of disease activity. If specific fragments can be detected and found to correlate with disease activity, these may provide a good determination of matrix loss and severity of arthritis. Similarly, since synovial lining hyperplasia is a characteristic feature of RA, markers specific to this change, such as the increased levels of protoncogenes, myc, jun and fos, (Yocum et al., 1988; Case et al., 1989a and b; Wilder et al., 1989; Sano et al., 1990; also reviewed in Wilder et al., 1991) may be beneficial in detecting disease activity.

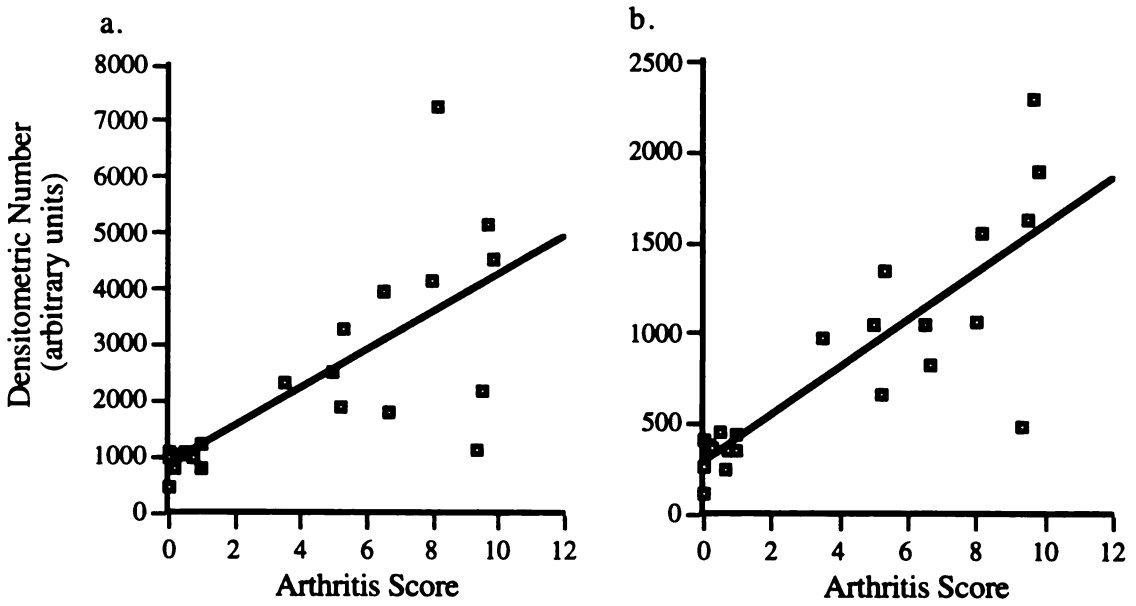

Figure 2: Regression analyses of total gelatinolytic proteinases (a), and 72-kDa gelatinase (b) with arthritic score.

Implications to Potential Anti-arthritic Therapies

Since the etiology of RA and JRA remains unknown, most of the present day therapies are directed towards alleviation of symptoms and slowing the destructive

processes of this disease. While many of these therapies are based on empirical clinical observations of response, ongoing research on treatments are directed towards known biologic mechanisms of the disease process (Greenwald et al., 1987; Case et al., 1989b; Francis et al., 1989; Kuruvilla et al., 1991; Greenwald et al., 1992; Peacock et al., 1992), and is based on findings of studies such as the ones discussed in this dissertation. Such rationally based therapies are likely to offer more positive long-term outcomes with fewer local and systemic adverse effects than previous empirical treatments.

Although it is likely that matrix loss in arthritis results from the combined effects of diminished matrix synthesis and increased matrix degradation, there is some controversy on the effects of inflammation on matrix synthesis by joint cells. While some investigators have demonstrated decreased matrix synthetic activities of cells from inflamed joints (Lowther et al., 1978; Ostensen et al., 1991; Reddy and Dhar, 1991), others have shown just the opposite effects of inflammation and proinflammatory cytokines on synthesis of matrix macromolecules such as GAGs (Jacoby and Jayson, 1976; Daireaux et al., 1990; reviewed in Harris, 1989). If the latter findings are true, it implies that matrix degradation must exceed matrix synthesis, since a net loss of matrix molecules is characteristically observed in arthritis. In such a scenario, if degradation of PGs and collagen can be halted or reversed in early stages of the arthritis, the integrity of cartilage could be restored by the synthetic ability of cartilage, especially in the younger individual. Since our findings implicate an imbalance in extracellular levels of MMPs and PIs in the increased degradation of tissues of the TMJ, potential therapies specifically targeted either intracellularly at regulating MMPs and PIs at the transcriptional level, or extracellularly at preventing the activation, or enhancing the deactivation or inhibition of MMPs may be beneficial in arthritis-associated matrix loss as outlined below.

(a) Transcriptional regulation of MMPs and PIs: Several agents that alter the expression of MMPs and PIs may be useful for this type of therapy. Our studies on disc cells (Chapter V) and those of previous investigators on synovial fibroblasts and chondrocytes (Bandara et al., 1992) have demonstrated that both TGF-β and INF-γ decrease the synthesis of MMPs by these cells. Furthermore, TGF-β1 also increases the synthesis of TIMP and collagen (Daireaux et al., 1990; Wright et al., 1991), thereby producing a net matrix anabolic effect. Indeed, the efficacy of TGF-β1 in preventing the development of arthritis in a mouse model of collagen-induced arthritis has recently been demonstrated (Kuruvilla et al., 1991). Although the authors suggest that the mechanisms for this anti-arthritic efficacy of TGF-β1 is dependent on its modulation of the immune response, it is also possible that the anti-arthritic effects of TGF-β also reside in its ability to downregulate MMPs and upregulate PI and collagen synthesis. This latter mechanism for

the anti-arthritic effects of TGF-β needs to be evaluated. Some fundamental problems with this approach to anti-arthritic treatment is the inability, at present, to target transcriptional regulation of MMPs and PIs specifically to the cells of the joint.

(b) Decreased activation of MMPs: Although several pathways, including the plasmin / plasminogen activator cascade and stromelysin have been shown to activate MMPs, these findings are largely based on *in vitro* studies. For therapeutic approaches designed along these lines to be successful, an understanding of the exact *in vivo* mechanisms for activation of MMPs is necessary. However, this approach to anti-arthritic therapy may be complicated if numerous redundancies in activation pathways exist *in vivo* as has been shown in *in vitro* experiments (Grant et al., 1987; Okada et al., 1988b; Nagase et al., 1990; Suzuki et al., 1990; Nagase et al., 1992b).

(c) Use of exogenous MMP inhibitors: Several recombinant and synthetic analog MMP inhibitors may have potential uses in alleviating joint damage in arthritis. Tetracyclines, for example, have been shown to have anti-collagenase properties independent of their antimicrobial activities. The mechanism of the anti-collagenase function of these drugs is not yet known but may be related to the drugs' metal ion (Zn^{2+}, Ca^{2+}) binding capacity (reviewed by Golub et al., 1991, 1992 and 1994). Recently tetracycline molecules have been modified in multiple ways, generating a new family of compounds called chemically modified tetracyclines (CMTs) which lack antimicrobial activity but retain anti-collagenase activities (reviewed by Golub et al., 1992 and 1994). In light of findings of our studies (Kapila et al., 1992b, 1993 and Chapters III and V) and those of others (Cambray et al., 1981; Murphy et al., 1981; Case et al., 1989a; Henderson et al., 1990), such drugs may have potential uses in alleviating or preventing matrix loss and subsequent tissue destruction of arthritis with minimal or little side effects. The anti-arthritic effects of tetracycline analogs have been tested both in an animal model of adjuvant arthritis (Greenwald et al., 1992), experimental osteoarthritis (Yu et al., 1992), and in human trials (Greenwald et al., 1987) with good success.

Some of the current research is also directed towards overexpressing MMPs in mammalian cells such that larger amounts of these proteins can be used for biophysical and X-ray crystallographic analyses (reviewed in Murphy et al., 1991). These studies, coupled with protein engineering studies to modify the structure of individual MMPs using recombinant DNA techniques, are attempting to analyze the role of the protein structure in defining the function of the enzymes. This should provide vital information for the design of specific inhibitors as potential therapeutic agents in degenerative diseases. Similar to these developments in the understanding of MMPs, biochemical, biophysical and mutagenesis studies on their naturally occurring inhibitors, the TIMPs, will permit a better

understanding of the largely uncharacterized mechanism of MMP inhibition and provide a basis for the design of low molecular weight analogs as potential drugs.

Some studies have also been done to understand the biologic basis for the efficacy of several drugs commonly used in arthritis (Rubin and Roberts, 1987; Pettipher et al., 1989; DiBattista et al., 1991; Firestein et al., 1991). Firestein et al. (1991), for example, have demonstrated that intra-articular corticosteroid therapy appears to decrease the levels of synovial collagenase and TIMP mRNA as observed by *in situ* hybridization in 3 patients with RA. Additionally, DiBattista et al. (1991) have shown that glucocorticoid receptor binding by the glucocorticoid, dexamethasone, inhibits the IL-1-stimulated release of stromelysin and collagenase by normal human chondrocytes. Such findings may provide a biochemical rational for the therapeutic effects of glucocorticoids in arthritis. Besides the therapies designed to alleviate the progression of the pathology by suppression of matrix degradative activities, those aimed at suppressing the immune responses by use of arthritogenic oligoclonal T cell line may also prove successful in preventing arthritis (Peacock et al, 1992).

Other Future Studies

(a). *In vivo* mechanisms for matrix degradation

One of the current issues in MMP research is the inability to define the mechanisms for their activation *in vivo*. With specific regard to our studies and those of previous investigators (Wooley et al., 1977a and b; Case et al., 1989a and b; Okada et al., 1989a and 1990a; Hasty et al., 1990; Henderson et al., 1990), it is not possible to deduce whether the proteinases localized in the matrices of the joint are active or inactive. Antibodies directed to specific epitopes of MMP proenzymes, activated MMPs as well as MMP / PI complexes will provide further insights into the extracellular regulation of their function *in vivo*. An analyses of the cell types associated with matrix degradation, and the spatial and temporal relationship between MMP localization, matrix degradation and synthesis, as well as cytokine production will build a more precise picture of the physiologic and pathologic matrix turnover in tissues of the joint.

(b). Temporal expression of MMPs and PIs during embryogenesis and post-natal development of the TMJ

Unlike the case of stimulated cultured cells where simultaneous induction of several MMPs and PIs is frequently observed in many cells, unique and often non-coordinated patterns of MMP expression occur in developing tissues (Brown et al., 1989; Brenner et al., 1989; Fisher et al., 1989; Nomura et al., 1989; Flenniken and Williams, 1990; Breckon et al., 1990 and 1991; Gershan et al., 1994). Such site- and time-specific expression of MMPs is probably consistent with the specific roles of the individual

proteinases. Although little is known about the developmental expression of MMPs and PIs in the TMJ, a temporally-related expression of matrix macromolecules, elastin and collagen, has been demonstrated in the developing fetal rabbit TMJ (Nagy and Daniel, 1991 and 1992). Similarly, it is likely that both developmental modeling and maintenance-associated remodeling of joint tissues occurs by a well regulated and coordinated expression of MMPs and PIs. Furthermore, the pattern of developmental expression of MMPs, PIs and matrix macromolecules in the TMJ may differ from that of other joints since, unlike most joints which form by cavitation within a single blastema, the TMJ arises from two separate blastemas. Because investigations on the developmental expression of MMPs and PIs will provide useful baseline information for understanding the TMJ in health and in disease, further study on the expression of these proteins in the developing TMJ is recommended.

(c). Further characterization of disc cells

As discussed previously, disc cells display characteristics of both fibroblasts and chondrocytes (Table II), but have otherwise not been well characterized. At present it is not known whether these cells demonstrate these different characteristics based on their location in the tissue, or due to a developmental transformation with age, or both, or whether these cells do indeed represent a unique cell type which has characteristics of both chondrocytes and fibroblasts. Whatever the reason for these characteristics of disc cells, this is an important area for future research because a cell type showing both fibroblast and chondrocyte characteristics, or which undergoes such a transformation has not been described previously, and because of the important role the TMJ disc has in joint function. A further determination of characteristics such as the site- and age-specific expression of matrix macromolecules, cell surface markers, integrin expression, synthesis of other classes of proteinases, as well as a more detailed evaluation of the regulation of MMPs and PIs by these cells is indicated. Since the TMJ disc is a load bearing structure, the potential role of functional loads in the phenotypic characteristics of these cells, and of joint loading on matrix synthesis and degradation by these cells should also be evaluated. An in-depth characterization of these cells will help us predict their behavior in health and in disease.

(d). Mechanisms for altered MMP and PI expression by disc cells during arthritis

The TMJ disc is a non-vascular tissue surrounded by synovial fluid and attached at its periphery to the synovium. How does it receive signals that induce an increase in alterations in its expression of MMPs and PI? The two possible scenarios involve the paracrine effects of proinflammatory cytokines and bioactive agents secreted by synoviocytes and inflammatory cells reaching the disc either via the synovial fluid or by diffusing through the synovium into the disc. These may not only alter the expression of

MMPs and PIs by disc cells, but may also induce disc cells to secrete their own cytokines, thereby initiating an autocrine positive feedback loop. Since no evidence is currently available to support these concepts, these potential mechanisms for induction of disc matrix degradative activity need to be examined.

In several more common pathologies of the TMJ, such as internal joint derangement and osteoarthrosis, degeneration of joint tissues occurs in the absence of overt joint inflammation. Although it is likely that subclinical inflammatory processes may be involved in predisposing to destruction of joint tissues, it is also possible that the perturbed pattern of loading of joint tissues in these arthropathies leads directly to their degeneration by alterations in matrix turnover by the cells. Our preliminary studies indicate that cyclic loading of TMJ disc cells at 30 cycles a minute and 20% elongation over a period of 48 hours significantly increases their expression of 92-kDa gelatinase, procollagenase and prostromelysin, but not of 72-kDa gelatinase and the 30-kDa and 20-kDa inhibitors (Kapila and Rao, unpublished data). Further work will be done to determine the mechanism for this load-related induction of specific MMPs.

(e). Histopathogenesis of RA and antigen-induced arthritis

The mechanisms for several histopathologic changes in arthritis still need to be determined. For example, although several cytokines and growth factors have been implicated in inducing hyperplasia of the synovial lining cells (Schmidt et al., 1982; Bunning et al., 1986; Remmers et al., 1988; Kumkumian et al., 1989; Lafyatis, 1989; Hiraoka et al., 1992), both the mechanisms for this uncontrolled proliferation and the transformation of these cells is not well understood. Furthermore, synovial lining hyperplasia is also observed in osteoarthritis, and animal models of internal joint derangement, presumably in the absence of histologic signs of inflammation. Since a substantial amount of damage to the tissues of arthritic joints results from their invasion by proliferative synoviocytes and by the increased expression of MMPs by proliferative synoviocytes, a better understanding of this phenomenon may provide some insights into therapies for managing these arthropathies. Similarly, it is also important to investigate the relative contributions of cytokine stimulation, increased cell numbers and the neoplast-like transformation of synoviocytes to the increased expression of MMPs by arthritic synovium.

Another histologically characteristic feature of arthritis is the loss of GAGs and collagen from cartilage and bone. The loss of matrix macromolecules from joint tissues may result from one or a combination of factors, including a decrease in number or activity of viable cells, decreased matrix synthesis and increased matrix degradation. Although our studies provide evidence for the role increased matrix degradation in this process, the role

of the other two mechanisms in joint matrix loss need to be elucidated further. Furthermore, studies should also be done to determine the temporal pattern of loss of the various matrix macromolecules from the TMJ.

(f). Applications for experimental model of antigen-induced arthritis of the TMJ

Future research applications of the model of antigen-induced arthritis described previously (Chapter II) fall into two categories, namely those of a general nature which will complement or add to our existing knowledge on RA gained from other animal models, and those, because of the unique structure and organization of the TMJ, related specifically to the TMJ. In the former category fall studies such as those involving the development of drugs and efficacy trials on anti-arthritic therapies, for determining the immunopathogenesis of arthritis, and for correlating histopathology with non-invasive diagnostic tools such as magnetic resonance imaging. These investigations will provide biologically rational approaches to diagnosis and therapy of arthritis. Furthermore, since both the immunologic mechanisms and responses (Dorai et al., 1987; Gahring and Weigle, 1990; Ho et al., 1990; McElhancy et al., 1990; Pross, 1990; Sironi et al., 1990), as well as the composition and rate of matrix turnover differs between juvenile and adult mammals (Holmes et al., 1988; Fornieri, et al., 1989; Front et al., 1989; Martin et al., 1990; Mays et al., 1991; Millie et al., 1989; Termine, 1990), models such as the one described in this dissertation, can also be used to further evaluate the role of these differences in the pathogenesis of arthritis between these two age groups.

Two important areas of research are indicated with regard to the application of this animal model to address specific questions related to the unique organization of the TMJ. The first involves evaluating the normal and pathologic turnover of TMJ disc matrices and the impact of this on the overall progression of arthritis and the function of the TMJ. The second area relates to the intra-articular location of the growth cartilage of the mandible and the related potential for aberrant development of the mandibulofacial skeleton. In this regard we are currently characterizing, by serial cephalometric studies, the disturbances in the growth of the mandible and maxilla in this animal model of antigen-induced arthritis. Findings of this study reveal significantly smaller dimensions of the mandibular ramus and body length in antigen-challenged as compared to control animals, an observation similar to that in children with JRA of the TMJ (Tavakolli, 1994). Future studies will be done to examine the potential mechanisms for the aberrant mandibular development in this experimental model of arthritis.

Future research to examine the exact role of specific proteins, such as MMPs and PIs in the matrix loss associated with arthritis are likely to involve the use of transgenic animals which over- or under-express these proteins. Such approaches will provide an

important tool in further detailed dissection of the mechanisms of immunopathogenesis and joint destruction during arthritis.

F. CONCLUSIONS

The series of investigations described in this dissertation provide insights into the histopathologic changes, histochemical loss of collagen and GAGs, and the localization and changes in MMPs and PIs during chronic phases of antigen-induced arthritis of the juvenile TMJ. These studies provide the first animal model of antigen-induced arthritis of the TMJ, and the first such animal model in a juvenile animal. These studies are also the first to identify and characterize disc cell-derived proteinases as the MMPs, 92-kDa gelatinase, 72-kDa gelatinase, procollagenase, prostromelysin and two of their PIs. Further studies also demonstrate an increase in net matrix degradative activity of synovia and discs retrieved from inflamed joints relative to those from control joints. The knowledge gained from these studies will not only help in the understanding of the biology of joint matrix loss in inflammatory arthropathies, but will also provide a background for a better understanding of other more common pathologies such as internal joint derangement and osteoarthritis of the TMJ. Although important insights on the mechanisms for matrix loss in antigen-induced arthritis have been gained from these studies, several areas still remain to be investigated as discussed in this chapter. These range from the basic understanding of the differential regulation of MMPs and PIs by divergently differentiated mesenchymal cells to clinical trials on potential anti-arthritic drugs targeted at preventing or alleviating tissue destruction mediated by the altered expression of MMPs and PIs by tissues of the inflamed joint.

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