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Hormonal modulation of collagen and elastin metabolism in cultured human breast tumor cells

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HORMONAL MODULATION OF COLLAGEN AND ELASTIN METABOLISM IN CULTURED HUMAN BREAST TUMOR CELLS

by

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ABBREVIATIONS

ConA-Sepharose	: Concanavalin A-Sepharose 4B
CPK	: creatine kinase
dcFBS	: dextran charcoal treated fetal bovine serum
Dex	: dexamethasone
E_2	: estradiol
ECM	: extracellular matrix
ER	: estrogen receptor
ER+	: estrogen receptor positive
ER-	: estrogen receptor negative
FBS	: fetal bovine serum
HFBA	: n-heptafluorobutyric acids
HO-C	: hydroxycortisone
HPLC	: high performance liquid chromatography
IP	: (estrogen) inducible protein
MCF 7	: human breast cancer cell line from Michigan Cancer Foundation
MDA 435, 436	: human breast cancer cell lines from M.D. Anderson Hospital
NBF	: newborn (foreskin) fibroblast cultures
P	: progesterone
PA	: plasminogen activator
PgR	: progesterone receptor
Pro	: proline
PTF	: peritumor fibroblast cell cultures
RMF	: reduced mammoplasty fibroblast cell cultures

SDS-PAGE : SDS - polyacrylamide gel electrophoresis

T : testosterone

Tam : tamoxifen

TCA : trichloroacetic acid

Val-pro : valine-proline dipeptide

ZR75-1, -30, -31A: human breast cancer cell lines from NCI, NIH

ABSTRACT

A common clinical presentation of human breast cancer is a palpable tumor mass in the breast. The predominent component of this tumor mass is its connective tissue stroma. With hormone treatment, dissolution of the fibrous tissue occurs. It is not known whether this accumulation of scleroproteins and their subsequent removal is the result of altered stromal synthesis, altered degradation, or due to a combination of these two processes. This thesis has examined both the synthesis and degradation of stromal proteins in cultured human breast tumor and stromal cells treated with hormones to resolve this question.

The synthesis and deposition of scleroproteins in response to human tumors is termed the desmoplastic reaction. The most profound response is found in human breast cancer. The extracellular matrix of human breast tumor cells has been shown to be both mitogenic and capable of modulating collagen and elastin synthesis in fibroblasts. By increasing the number of stroma producing cells and the rate of scleroproteins synthesis, a dense fibrotic response is formed. This process, however, as examined in these studies does not appear to be under hormonal control.

Scleroprotein degradation was found to be the key event in the dissolution of fibrotic response with hormone treatment. A collagenase and two elastases were found to be produced by the breast tumor cells themselves. These enzymes exist as proenzymes and require proteolytic treatment for the activation of their catalytic potential. Though the biological mechanism for elastase activation is unknown at this time, the conversion of procollagenase appears to be mediated by an estradiol- and progesterone- responsive

plasminogen activator. This activity was purified from cultured breast tumor cells.

In summary, the breast tumor cells themselves modulate the turnover of stromal cells and scleroproteins, their accumulation through tumor matrix-induced mitogenesis of stromal cells and stimulation of stromal cell synthesis, and the removal of scleroproteins, in part, by elastinolytic and by tumor cell plasminogen activator control of collagenolytic activity. If the tissue culture situation described herein can be extrapolated to in situ events, these may be key events in the modulation of the desmoplastic response to human breast cancer.

CHAPTER I. BACKGROUND AND THESIS HYPOTHESIS

A. INTRODUCTION

Human breast cancer is a devastating illness which will effect 1 in every 10 women living today (Kelsey, 1979; American Cancer Society, 1981). It is the most common cancer in women, accounting for 27 percent of all malignancies, and is also the largest killer, causing 19 percent of all deaths from malignant disease. Each year, 1 million cases of breast cancer in women will be reported worldwide and more than 100,000 in the United States alone.

The incidence of breast cancer is increasing in the world (Cutler et al., 1976). Data from various cancer registries show that in Western countries, there has been a significant increase in the incidence of breast cancer in the last 25 years. This means that the problem of breast cancer may become even more important during the next decade as the number of developing countries experience modernization.

In planning an overall strategy for controlling any type of cancer, the basic biology of the cancer must be understood, in order to establish an effective mode of prevention, detection, and treatment. This thesis represents an attempt to delineate certain key aspects of breast cancer biology, the regulation of the stromal reaction to the tumor.

B. OBJECTIVES

Beneficial effects may be achieved for some patients with breast cancer by altering the hormonal environment of the tumor. Despite the fact that the underlying mechanism is not understood, endocrine therapy is often used as an adjuvant for patients with breast cancer. Clinically, the response to endocrine therapy is measured by the decrease in the size of the tumor mass.

The tumor mass of human breast cancer consists of the breast malignant epithelial cells and the surrounding normal stromal cells, and their scleroprotein products. Most of the tumor mass consists of connective tissue stroma (Groniowski et al., 1975; Meissner and Diamandopoulos, 1977; Azzopardi, 1979). It is the stroma of the human breast tumor which is felt when a clinician palpates a mass (Barsky et al., 1982). The stroma of the human breast tumor is mainly composed of fibrous proteins such as collagen (Gullino et al., 1962, 1963; Groniowski et al., 1975), and elastin (Adnet et al., 1976; Azzopardi, 1969) in a bed of proteoglycans and glycoproteins.

With the decrease in tumor mass in respone to treatment, in addition to tumor cell death, there is a loss of stroma. It is not known whether this connective tissue loss is the consequence of decreased stromal synthesis, an increase in stromal degradation, or is perhaps dependent on an interaction between biosynthesis and degradation. This dissertation specifically analyzes the molecular mechanism by which the human breast tumor mass first accumulates and then can decreases with hormonal modulation.

B. BACKGROUND

1. Hormonal Considerations in Breast Cancer Treatment

Steroid hormone receptors play a major role in the endocrine therapy of patients with breast cancer. Hormonal treatments have been particularly effective in patients with disseminated forms of breast cancer. Until recently, the response rate to hormonal modulation was only about 30% (Osborne et al., 1980a). Recent findings have made it possible to select patients who are more likely to respond to hormone modulation. A cytoplasmic protein, estrogen receptor (ER), is responsible for the binding of estrogen in target cells. Its presence is necessary for estrogen action. The presence of ER is useful

in detecting tumor that will respond to estrogen. The validity of this hypothesis has been only partially confirmed. Remission can be achieved through endocrine treatment in 50-60% of patients who are ER+ (McGuire et al., 1975; Osborne et al., 1980b).

Breast tumors that are ER- rarely respond to endocrine therapy with a regression rate of only about 8%. It should be emphasized, however, that though a tumor is ER+, it may be deficient in some other step required for steroid hormone action, such as binding, activation, translocation, or processing. This may account for the 40-50% non-responsive population in ER+ patients. Thus, there are two classes of ER+ patients, responders and non-responders. The ability to distinguish these two groups is important in establishing an appropriate treatment protocol for each patient.

To predict response to endocrine therapy in ER+ patients, progesterone receptor (PgR) assays are used. The rationale comes from the observation that PgR is induced by estrogens in normal reproductive tissues in situ and in cultured breast cancer cells (Horowitz et al., 1975). It is reasoned that ER+ tumors that do not respond to estrogen have a defect in the estrogen response pathway distal to the binding step. PgR, therefore, may be a better marker than ER for an intact estrogen response pathway since it is an end product of estrogen action.

The use of PgR assays have improved the ability for selecting patients likely to respond to endocrine therapy. As predicted, tumors lacking both ER and PgR receptors are usually endocrine independent. ER-, PgR+ tumors occur rarely but a positive response rate of 46% has been observed. The highest response rate (77% overall) is observed in the group of tumors containing both ER and PgR. However, the response rate of ER+, PgR- group is perturbing. These tumors should not regress with endocrine therapy, yet, 27% do respond.

Possible explainations may be that PgR synthesis may not be linked sufficiently close to the pathway of tumor growth to an be an accurate predictor in all situations. Alternatively, the lack of PgR may be the result of "false" negative in some cases. In certain phases of the menstrual cycle and in situations in which there is a lack of sufficient circulating estrogens to stimulate PgR synthesis, there may be a high endogenous progesterone level already at the receptor site. Nonetheless, the PgR assay does improve the ability to predict these patients who will respond to endocrine therapy.

It should be emphasized that the ER and PgR status of a patient is rarely used to select the particular hormonal therapy modality. The choice of hormone treatment is based on clinical criteria and physician preference. Traditionally, ovarectomy has been used for initial endocrine therapy in younger women, whereas estrogen administration has been used in post-menopausal women. In the past decade, endocrine treatment has been categorized as either hormonal addition or hormonal reduction. Four types of hormonal addition procedures are administered: estrogen, progesterone, androgen, and corticosteroid therapy. At the present time, reduction is through surgical ablative procedures or the use of anti-estrogen drugs such as tamoxifen, nafoxidine, and clonidine. These non-steroidal antiestrogens compete with the natural estradiol for the ER and antagonize estrogenic activity by a mechanism that is presently not understood (Horwitz and Hance, 1977). Response rates to two antiestrogens, tamoxifen, and nafoxidine, range from 30-50% in all age groups (Heuson et al., 1975; Ingle et al., 1981).

In summary, beneficial effects can be achieved through the use of hormonal modulation based on the presence of ER and PgR. Understanding the molecular basis for hormonal therapy may improve the effectiveness of treatment for breast cancer patients. The experiments described herein

specifically address this question. They examine the effect of hormones on cultured stromal cell and tumor cell interactions, in particular their modulation of matrix deposition.

2. The Nature of the Breast Tumor Mass

Many human tumors have a profound connective tissue response or desmoplastic reaction (Parsa and March, 1976; Brownstein and Shapiro, 1977; Meissner amd Diamandopoulos, 1977; Fornieri et al., 1978; Azzopardi, 1979; Valensi, 1979). The most profound reaction is elicited by human breast tumors, particularly infiltrating ductal carcinomas.

The extracellular matrix (ECM) components in tumor desmoplasia consist of a complex of collagen and elastin interwoven in a bed of proteoglycans and glycoproteins (Hay, 1982). The matrix exists as a three-dimensional scaffold which influences the attachment, proliferation, differentiation, and morphogenesis of the cells situated upon it (Bernfield et al., 1972; Gospodarowicz et al., 1978; Murray et al., 1979, 1980; Carlsson et al., 1981; Ekblom, 1981; Bissell et al., 1982; Couchman et al., 1983). There are several genetically distinct collagens, types I through VIII (Bornstein and Sage, 1980; Bissell et al., 1982); elastin (Sandberg, 1977; Rucker and Tinker, 1977; Burke and Ross, 1979); and proteoglycans, a complex macromolecule composed of a core protein to which at least one class of glycosaminoglycan is covalently bound (Roden, 1980; Hascall, 1981), and glycoproteins (Yamada, 1981) in the matrix.

3. Thesis Hypothesis

The decrease in breast cancer mass with endocrine therapy must be caused by a net loss of scleroproteins. In response to hormones, several possible combination of biochemical changes may result in the net loss of breast tumor stroma as shown in table 1-1.

Clinical and animal studies have failed to define the mechanism by which endocrine modulation leads to regression of the tumor stroma. Additionally, it is not known whether these scleroproteins and their degradative enzymes are elaborated by the malignant epithelial cells, the stromal cells, or perhaps result from an interaction between the two cell types. In the present studies, the influence of hormones on collagen and elastin biosynthesis and degradation have been studied utilizing breast tumor and stromal cells grown in vitro. These cells grown singularly, in mixed culture, and on preformed extracellular matrices in various combinations were examined for their ability to synthesize elastin, collagen, elastases, and collagenase in the presence and absence of various hormones.

Table 1-1: Possible Effects of Hormone on Breast Tumor Mass

Biochemical Process	Effect	Effect on Tumor Mass
Matrix Biosynthesis Matrix Degradation	Decrease No Change	Decreased Stroma
Matrix Biosynthesis Matrix Degradation	No Change Increase	Decreased Stroma
Matrix Biosynthesis Matrix Degradation	Decrease Increase	Decreased Stroma
Matrix Biosynthesis Matrix Degradation	Slight Increase Greater Increase	Decreased Stroma

CHAPTER II. CHARACTERIZATION OF CELLS

A. INTRODUCTION

Clinical and animal studies have failed to define the mechanism of the desmoplastic response and to formulate a theoretical basis for treatment modalities such as endocrine manipulation. An alternate approach to the study of the desmoplastic reaction is to study these phenomenon with human breast cancer cells in continous tissue culture. Even though there are several studies concerning the hormonal regulation of cultured human breast cancer cells (for review see McGuire, 1977, Monaco, 1979), there have been few studies of stromal synthesis and hormone-responsive proteases in these cells.

The use of tissue culture to study any phenomenon requires precautions. First, all breast cell lines used in such studies have been established uniformly from malignant effusions. As such, they represent a subset of the original tumor population, and perhaps not at all representative of the original tumor. These cells must possess metastatic potential to have been present in effusions and resistance to the several therapeutic modalities that may had been implemented prior to culturing. Second, the media used in any culture situation may lack factors necessary for the full phenotypic expression of these cells. Third, mycoplasmic or viral contamination of cultures can occur which also alters the phenotypic expression of these cells. Finally, it is difficult to extrapolate the responsiveness of cultured cells to hormones to the in vivo situation. It cannot be certain whether the level and manner by which hormones are introduced to the culture is physiologically relevant. For example, MCF-7 cells will grow optimally in insulin and charcoal-treated serum in the

absence of estradiol. If one adds estrogen to the system, no further stimulation of growth is observed and the cells might therefore be considered hormone independent. On the other hand, the progesterone receptor is inducible under these same conditions (Osborne et al., 1979), suggesting that an estrogen response is taking place.

Despite these difficulties, there are many advantages to the cell culture approach. First, the use of cells in culture eliminate many difficulties of working with animal systems. The study of hormone interactions is possible without the constant dynamic changes that exist under physiological situations. Problems such as drug and hormone metabolism, tissue redistribution, tissue binding, and excretion are eliminated. Cell culture permits the precise manipulation of hormones on the breast cancer cells. Second, the use of a single cloned subculture of the tumor decreases variability in response compared to the heterogenous population in the original neoplasm. Finally, it is possible to perform many experiments in defined or modified medium. In such situations, one can be certain that the hormonal effect studied is not mediated by additional unknown or extraneous factors.

With these considerations in mind, the properties of the human breast cancer cell lines used in these studies will be reviewed. A comprehensive review of the phenotypic properties of some of the human breast cancer cell lines in continous culture is available (Engel and Young, 1978b). The criteria used for defining human mammary cancer cells is based on histological characterization, karotypic analysis, and the characterization of organ specificity as supported by morphological evidence of epithelial structures and secretory activity. The secretory activities most often

examined include sex steroid hormone receptors, fatty acids, and production of milk proteins and milk-specific antigens.

There are several studies of the hormonal regulation of human breast cancer cells in continous culture. The major focus has been on the influence of estrogen, androgen, and glucocorticoids on MCF-7 and ZR75-1 breast cancer cells.

The first cultured human breast cancer cell line, MCF-7, was established and described by Soule et al. (1973). Brooks et al. (1973) demonstrated that these cells contain estrogen receptors (ER). Several subsequent studies demonstrated that these cells show a variety of growth responses to physiologically relevant concentrations of estrogen and of growth inhibitions to antiestrogens (Lippman et al., 1976b; Lippman, a,b, 1976, 1977). Synthesis of a variety of proteins was also demonstrated to be under the influence of estrogen including thymidine kinase (Bronzert et al., 1981), progesterone receptors (Horwitz et al., 1978), lactic dehydrogenase isoenzyme (Burke et al., 1978), creatine kinase (Westley and Rochefort, 1980), and plasminogen activator (Butler et al., 1979). The failure to obtain an estrogenic response is often due to the inadequate removal of endogenous hormones, or the failure to remove insulin from the medium. It is important to deprive the cells of insulin since this often serves as an alternative growth factor (Butler et al., 1981).

It has been observed clinically that some human breast cancers will respond to androgen therapy (Stoll, 1972). Several human breast cancer cell lines contain androgen receptors (Engel and Young, 1978b). Androgen addition to MCF-7 cells cultures increases thymidine incorporation and protein synthesis (Lippman et al., 1975a, 1976a). The level of dihydrotestosterone used in these studies are higher than physiological

concentrations. Lippman has proposed that since MCF-7 cells are capable of metabolizing dihydrotestosterone to androstanediols and more polar conjugates, a non-physiological concentration may be required before an androgen effect can be observed. Zava and McGuire (1978) have also demonstated that high concentrations may be required because of the ability of androgens to occupy estrogen receptors. Due to the metabolism and cellular compartmentalization of androgens, androgen responsiveness of human cancer cells often requires non-physiological concentrations of this hormone.

Therapeutic effects have been possible in certain cases of metastatic breast cancer using glucocorticoid administration. In tissue culture, glucocorticoid hormones exert a variety of inhibitory effects on breast cancer cell growth (Lippman, 1976c) and have been demonstrated to antagonize the trophic effects of insulin (Osborne et al., 1979). These effects are mediated by glucocorticoid receptors (Allegra et al., 1979). The mechanism of these direct inhibitory effects are not clear and requires further elucidation.

Biological responses to hormones can be assessed by monitoring either cell growth or by measuring the synthesis of newly synthesized proteins. Cell growth is often monitored by cell growth kinetics and thymidine incorporation. More recently, the focus has been to study synthesis of specific proteins in response to certain hormones. One example is the synthesis of inducible protein (IP) in response to estrogen addition. In 1966, Notides and Gorski (1966) noted the appearance of an inducible protein in rat uterus after estrogen treatment. It was believed this IP might serve as a marker for the estrogen response. The presence of estrogen receptors (ER) in human breast cancer is useful in predicting the sensitivity of tumors to estrogens. However, only 60% of the ER+ tumors will respond to endocrine

manipulations. As mentioned in the preceding chapter, the progesterone receptor (PgR) is regulated by estrogens and it is postulated to be a marker for tumor sensitivity to estrogens (Horwitz et al., 1975, 1978). The simultaneous measurement of ER and PgR has clearly improved the reliability of predicting tumor responsiveness, but it is far from satisfactory. Therefore, other biochemical markers of estrogenic action are needed.

The identity of the estrogen IP was unknown until recently. Reiss and Kaye (1981) have shown that the IP in rat uterus is the BB-isozyme of creatine kinase (E.C. 2.7.3.2), the brain type of isozyme. In surveying the occurance of the BB creatine kinase (CPK-BB), Kaye and his collegues have observed its increase over basal levels in several estrogen sensitive tissues including breast, uterus, and placenta (personal communication).

Clinical studies have indicated the presence of creatine kinase-BB in the serum of women with breast cancer (Thompson et al., 1980). It was found that elevated serum CPK-BB occurs in a high proportion of patients with metastatic breast cancer. Approximately 20% of breast cancer patients do not have the marker. It was proposed the high level of CPK-BB was produced by the tumor itself. In women with breast cancer, serum CPK-BB correlates with clinical response to hormonal treatment. If elevated levels of serum CPK-BB serve as a valuble indicator of response to therapy, it is crucial to examine the hormonal regulation of creatine kinase in detail. Tissue culture techniques may prove useful for such purposes.

Recently, estrogen IP has been detected in cultured MCF-7 human breast cancer cells (Edward et al., 1980). After estrogen starvation, the addition of estrogen induces the synthesis of two specific proteins with the molecular weight of 24K and 54K. Translation studies has revealed the 24K and 54K mRNA increase 1.9 and 1.7 fold respectively (Adams et al., 1980).

Evidence for transcriptional control of this protein make it an attractive probe for the study of hormonally regulated gene expression in human breast cancer. The IP reported by Edwards and by Adams have been identified by Kayes and coworker to be creatine kinase, but the exact isozyme form is not known (personal communication).

Prior to any studies of hormonal regulation, characterization of the breast cancer cell is necessary. Since most breast cancer cell lines are derived from tumor effusions consisting of tumor cells and contaminating stromal fibroblasts, it is necessary to determine if the cell lines used are truly of epithelial origin. In this chapter, the characterization of four different breast cancer cell lines will be described. These cell lines have been characterized as to morphology, lack of stromal contamination, and steroid hormone receptor status, to hormone responsiveness, and to the profile of secretory protein products, and it has been established that they are indeed malignant epithelial cells.

B. MATERIALS AND METHODS

1. Source of Cells

MCF-7 cells were first established and described by Soule et al. (1973). The present subline was obtained from the Michigan Cancer Foundation. The ZR75-1, ZR75-30, and ZR75-31A cell lines were first obtained from pleural effusions by Engel et al. (1978a). The ZR75-31A, a subclone of the ZR75-30, was found to possess different phenotypic patterns (Engel and Young, 1978b). These ZR75 cell lines were given to us by Ms. L. Engel and are presently available from the American Type Culture Collection. Breast fibroblasts from normal patients undergoing reduction mammoplasties (RMF) and from breast cancer patients, peritumor fibroblasts (PTF), were

obtained from Dr. Helene Smith, Peralta Cancer Research Institute, Oakland, CA. Newborn foreskin fiborblasts (NBF) were obtained from UCSF tissue culture facility.

2. Culture Medium

All cells were maintained in RPMI 1640 with 4% fetal bovine serum (FBS). Cells were grown at 37°C in an atmosphere of 95% air and 5% CO_2. For studies of hormonal manipulation, FBS was first treated with dextran charcoal (dcFBS) as described by Lippman and Bolan (1975) to remove endogenous steroidal hormones. For creatine kinase studies, no serum was added to the media.

3. Creatine Kinase Assay

Cells were cultured in the presence of FBS. After 24 hr, the media was changed to RPMI-1640 supplemented with only antibiotics and tamoxifen, an antiestrogen. Another 24 hr later, the media was changed to hormone supplemented media. The media was conditioned for a total of 24 hr and subsequently used in the creatine kinase assay.

The supernatant was assayed for total creatine kinse by the method of Rosalki (1966). This method is based on the measurement of NADP reduction in the following reation:

$$\text{ADP + creatine phosphate (CP)} \longrightarrow \text{ATP + creatine}$$

$$\text{ATP + glucose} \xrightarrow{\text{hexokinase}} \text{ADP + glucose-6-phosphate}$$

$$\text{glucose-6-phosphate + NADP} \xrightarrow{\text{G6P dehydrogenase}} \text{NADPH +}$$

$$\text{6 phosphogluconate}$$

A solution of ADP (1 mM), creatine phosphate (10 mM), glucose (20 mM), $MgCl_2$ (30 mM), hexokinase (0.6 IU/ml), glucose-6-phosphate dehydrogenase (0.3 IU/ml), and NADP (0.8 mM) was made in Tris buffer (0.05 M, pH 6.8). A 2 ml volume of this substrate mixture was added to 1.0 ml of cell sample. Measurements are made at 340 nm O.D. against distilled water blank. One unit of creatine kinase activity is the amount of enzyme that will utilize 1 µmol of creatine phosphate per min at 25°C.

4. Growth Kinetics

The growth kinetics were determined by obtaining cell counts at various intervals. Triplicate cell counts from three different 35-mm dishes were made by light microscopy with the use of hemocytometer. Viability was determined by the trypan blue exclusion test. Cell viability was always greater than 97%.

5. Thymidine Incorporation

Confluent cultures were suspended with 0.25% trypsin and trypsinization was terminated by the addition of 7% dcFBS. The cell suspension was plated in 35mm dishes containing 4 ml of RPMI-1640 with 7% dcFBS to a final density of 5×10^6 cells per dish. After 48 hr, the medium was removed and the cells were rinsed with Hank's balance salt solution. These cells were incubated in 4 ml of medium containing either 0.5% or 7.0% dcFBS and the various hormones to be tested. After 48 hr, 2.5 µCi/ml of methyl-^{3}H-thymidine was added to the culture and labeling was allowed to proceed for 4 hr. The labeling period was terminated by the addition of 0.10 ml of cold 0.8% bovine serum albumin, which also served as carrier. After three cycles of cold 10% TCA wash, the amount of ^{3}H-thymidine incorporated into TCA precipitable material was determined. Pellets were solubilized in 0.20 ml of 88% formic acid and 7 ml of Aquasol (New England

Nuclear) was added. Samples were counted in a Beckman LS 3133T scintillation counter. The data are reported as counts per min (cpm) per 10^6 viable cells. Cells were counted in duplicate cultures at the beginning of the labeling period.

C. RESULTS

1. Morphology

Buehting and Hackett (1974) have reported that the morphology of cells in culture may reflect the tissue of origin. The cell lines used in our studies revealed characteristic epithelial morphology under light microscopy (fig. 2-1). These cells grew in flat cuboidal sheets resembling epithelial cells in vivo. Ultrastructural studies by Soule et al. (1973) and Engel et al. (1978a) confirm that these cell lines possess cell organelles characteristic of epithelial cells. These include desmosomes, tonofibrils, and intracytoplasmic duct-like vacuoles. Histochemical analyses have been performed by Dr. D. Fujii (UCSF) and Mr. T. Yasumura (U. Maryland) confirming the homogenity of these cultures. Cells were free of stromal contamination.

2. α-Lactalbumin and Casein Synthesis

α-lactablbumin and casein synthesis in breast cancer cells were examined by Mr. T. Yasumura (Dept. Anatomy, U. Maryland). Cell layer and medium were harvested from cells grown in culture and analyzed for the presence of α-lactalbumin and casein by enzyme linked immunosorbent assay (ELISA). α-lactalbumin was present in all breast tumor cells examined. Casein, however, was not present in any of the cells examined. All stromal cells revealed an absence of both α-lactalbumin and casein (table 2-1).

3. Estrogen Receptor and Inducible Protein

The presence of steroid hormone receptors and inducible proteins have been studied in several of the cell lines used. The presence of estrogen receptors have been reported for the MCF-7 (Brooks et al., 1973) and in some of the ZR75 cell lines (Engel et al., 1978a). Assays for estrogen receptors were performed by Dr. P. Hoffman (table 2-1). The MCF-7, ZR75-1, and ZR75-30 cell lines were ER+ while the ZR75-31A was ER- . It is not clear whether stromal fibroblasts possess ER. The failure of some ER+ patient to respond to hormone modulation may due in part to the binding of estrogen by host stromal cells. To eliminate this possibility, ER status in stromal cells were examined. No receptors could be detected in NBF, RMF, and PTF stromal cell lines (table 2-1).

The appearance of specific estrogen-inducible proteins (IP) is one of the earliest effects of estrogen (Walker et al., 1979). This estrogen IP has been identified to be the CPK-BB-isozyme of creatine kinase. IP was measured by determining the total creatine kinase activity in these cells. Following estrogen addition, increased creatine kinase activity was observed in the MCF-7 and ZR75-1 cell lines. No change in the basal level of creatine kinase was observed when estrogen was added to the ZR75-30 or ZR75-31A cell lines (table 2-1), whereas IP in MCF-7 appears 30 min after estrogen addition (Westley and Rochefort, 1980). Creatine kinase activity in the ZR75-1 cell did not increase until 1 hr after estrogen addition. The simultaneous additon of tamoxifen and estrogen resulted in the suppression of the creatine kinase effect (fig. 2-2). After 12 hr, the cells were washed and fresh medium containing only 10^{-8}M estradiol was added. After 60 min, increased creatine kinase activity was again observed (fig. 2-2). Dr. M. DeLuca has analyzed creatine kinase isozymes in ZR75-1 cells.

The following isozyme was observed: 20% mitochondria, 15-20% MM, 2-3% MB, and 60% BB CPK.

4. Hormone Responsiveness

The growth of MCF-7 cells in response to estrogen addition has been characterized (Lippman et al., 1975a; Lippman 1975). Addition of physiological levels of estrogen resulted in an increased growth rate as measured by cell kinetics and thymidine incorportion. The effect of other steroid hormones on either the MCF-7 and ZR75-1 cells are not well documented. To characterize further the hormone reponsiveness of MCF-7 and ZR75-1 cells, physiological levels of steroid hormones were added. Fig. 2-3 shows the change in cell number in response to various hormone additons. Increased cell number, an index of cell growth, occured only with ZR75-1 and MCF-7 cells grown in response to estradiol. The growth response of MCF-7 cells was comparable to those levels reported by Lippman et al. (1975a,b). To study further the properties of estrogen-induced tumor cell growth, ZR75-1 cells were cultured in the presence of 10^{-8}, 10^{-10}, and 10^{-12} M estradiol. Increased estradiol resulted in higher final cell densities. The final cell density also correlated with the concentration of estradiol present (fig. 2-4a). When 10^{-12}M estradiol and 10^{-7}M tamoxifen were simultaneously present, there was a significant change in cell growth rate (fig. 2-4b). When the estradiol level was increased to 10^{-8}M, tamoxifen inhibition no longer occured and increased growth rate was again observed.

Cell growth can also be monitored by thymidine incorporation (table 2-2). With the additon of estradiol, there was an increase in thymidine incorportation. The simultaneous additon of estradiol and tamoxifen resulted in a decreased level of thymidine incorporation over the estradiol group. These data confirm cell growth kinetic studies.

D. DISCUSSION

I have characterized MCF-7 and ZR75-1 cell lines. The MCF-7 and ZR75 cell lines were epithelial-like in morphology and produced α-lactoalbumin. Only certain cell lines of those examined, were hormone responsive.

Morphologiclly, MCF-7 and ZR75 cells appeared epithelial-like under light microscopy. Ultrastructure studies by Soule et al. (1973) and Engel et al. (1978a) have indicated the presence of desmosomes, tonofibrils, and intracytoplasmic vacuoles. Intracytoplasmic vacuoles are structures frequently found in mammary epithelial cells in vivo (Buehring and Hackett, 1974; Battifora,1975). The presence of desmosomes, tonofibrils, and intracytoplasmic duct-like vacuoles suggested that the cells originated from mammary epithelium.

α-lactalbumin is a protein believed to be the specific secretory product of functionally differentiated mammary epithelial cells (Palmitter, 1969). This protein has also been identified as the β-subunit of lactose synthetase, an enzyme involved in lactose formation from UDP-galactose and glucose. Recently, Kleinberg et al. (1977) have found that α-lactalbumin can be detected in some non-breast tissues including amniotic fluid, cord blood, and uterus. Though lactalbumin may be found in extramammary tissue, the level is quite low. Thus, the presence of α-lactalbumin is suggestive of mammary origin. α-lactalbumin has been detected in a number of breast cell lines (Engel et al., 1978b). We have confirmed previous reports of α-lactalbumin in the MCF-7 and ZR75 cell lines.

The presence of hormone receptors is essential for hormone action. Upon entering the cellular cytoplasm, the estrogen molecule bind to a cytoplasmic protein receptor to form a steroid-receptor complex. This steroid receptor complex is translocated from the cytoplasm to the nucleus.

In the nucleus, interaction between the steroid-receptor complex and chromatin occurs. This results in the stimulation of transcription of certain classes of mRNA. It is the translation of these newly formed mRNA that results in the specific proteins that accounts for the various hormone-responsive biologic activities. The presence of estrogen receptors have been previously described in both MCF-7 and ZR75 cell lines. Since cells are known to lose their phenotypic expression of receptor synthesis, cells used in these studies were analyzed for their ER status. MCF-7, ZR75-1, and ZR75-30 were all estrogen receptor positive and ZR75-31A was estrogen receptor negative, confirming previous studies.

The biologic responses to steroid hormones have been only partially characterized in the MCF-7 and ZR75 cell lines. Biologic responses to steroid hormones was studied using estrogen-inducible protein (IP) and growth analysis.

Increased cell growth of MCF-7 occurs in the presence of estradiol (Lippman et al., 1975a; Lippman, 1975a). This stimulation of cell growth can be inhibited by tamoxifen. With higher concentractions of estradiol, tamoxifen inhibition can be overcome. Westley and Rochefort (1980) have previously described the appearance of IP upon estradiol additon. The appearance of IP is inhibited by the addition of the antiestrogen, tamoxifen. Our observation of MCF-7 growth response and IP synthesis was consistent with these earlier studies.

The growth response of ZR75 cell lines to estradiol addition has not been thoroughly studied. The ZR75-1 cell line was previously described to be both estrogen responsive and susceptible to tamoxifen inhibition (Osborne et al., 1979). Our results were consistent with this earlier study. The ZR75-30 and ZR75-31A cell lines have been shown to be estrogen

independent. The results of growth response in these cells correlated with the appearance of IP. With estradiol addition, the appearance of IP was observed only with the ZR75-1 cells. No increase in IP synthesis was observed with either the ZR75-30 or the ZR75-31A cell lines.

Tamoxifen has been previously shown to be capable of inhibiting the trophic effects of estrogen (Lippman, 1975, 1976a). We have confirmed these observation in our estrogen-responsive cells (ZR75-1 and MCF-7). Tamoxifen inhibition can be reversed by estradiol. Simultaneous addition of tamoxifen and estradiol leads to stimulation when tamoxifen is less than 2 logs higher in concentration that estradiol. This may be explained by the 1000 fold lower affinity of tamoxifen for the estrogen receptor. Thus, a greater concentration of tamoxifen is needed to inhibit estrogen-induced growth response. Confirming previous studies (Lippman, 1976a), tamoxifen had no effect on cells that did not respond to estradiol (ZR75-30, ZR75-31A).

The MCF-7 and ZR75 cell lines have been characterized in terms of morphology, protein synthesis, hormone receptor status, and hormone responsiveness. These results are summarized in table 2-2. The morphologic appearance and α-lactalbumin biosynthesis confirmed that these cells are of mammary epithelial origin.

The ZR75-1 and MCF-7 cells are analogous to patients with ER+ breast tumor that respond to endocrine therapy. For those patients with ER+ breast tumors that do not respond to hormonal modulation, the ZR75-30 cells are the ideal paradigm. Lastly, the ZR75-31A cells are similar to those patient that have ER- tumors which do not respond to therapy. These cells may thus serve as suitable <u>in vitro</u> culture models for the various

TABLE 2-1: PROPERTIES OF BREAST CANCER AND STROMAL CELL LINES

Cells	α-lactalbumin (ng/mg protein)	Casein (pos/neg)	Estrogen Receptor (pos/neg)	Creatine Kinase¶ (mU/mg protein)	
				- Estrogen	+ Estrogen§
Breast Tumor Cells					
ZR75-1	176	neg	pos	51	237 *
ZR75-30	148	neg	pos	37	38
ZR75-31A	81	neg	neg	46	50
MCF-7	124	neg	pos	89	327 *
Stromal Cells					
NBF	neg #	neg	neg	31	33
RMF	neg	neg	neg	22	21
PTF	neg	neg	neg	28	30

¶ Creatine kinase is the estrogen-induced protein (Walker, 1979).

§ Cells cultured in the presence of 10^{-8} M estradiol.

* $p < 0.005\%$ as calculated by t-test.

negative α-lactalbumin = < 20 ng/mg.

Table 2-2: Thymidine incorporation in cultured human breast tumor cells using various hormones. Hormone supplementing either 7.0% dcFBS (table 2-2a) or 0.5% dcFBS (table 2-2b) was introduced 48 hr prior to labeling. Labeling was performed as described in MATERIALS AND METHODS. Data are presented as the mean of triplicate assays ± S.D.

TABLE 2-2a: Thymidine Incorporation in Cultured Human Breast Cancer Cells with Various Hormones

Culture Condition	ZR75-1 (cpm/10^{-6} cells)	ZR75-30 (cpm/10^{-6} cells)	ZR75-31A (cpm/10^{-6} cells)
+ 7.0% dcFBS	19,300 ± 127	15,210 ± 175	20,405 ± 164
+ E_2 (10^{-8} M)	23,105 ± 193	15,820 ± 185	18,735 ± 171
+ E_2 (10^{-10}M)	20,646 ± 197	15,795 ± 178	19,763 ± 185
+ E_2 (10^{-12}M)	20,732 ± 183	15,859 ± 182	20,660 ± 121
+ Tam (10^{-7} M)	16,010 ± 102	15,975 ± 161	20,105 ± 197
+ Tam (10^{-7}M) + E_2 (10^{-10}M)	19,900 ± 191	15,510 ± 193	20,719 ± 89
+ Tam (10^{-7} M) + E_2 (10^{-8} M)	22,799 ± 128	15,715 ± 89	21,325 ± 198
+ P (10^{-8} M)	20,800 ± 103	15,010 ± 95	21,005 ± 186
+ T (10^{-8} M)	15,305 ± 128	14,880 ± 187	21,110 ± 141
+ HO-C (10^{-8} M)	15,780 ± 143	14,080 ± 187	21,030 ± 183
+ Dex (10^{-8} M)	13,475 ± 196	15,220 ± 175	21,380 ± 172
+ Ins (1 µg/ml)	28,252 ± 157	21,728 ± 151	20,723 ± 82

TABLE 2-2b: Thymidine Incorporation in Cultured Human Breast Cancer Cells with Various Hormones

Culture Condition	ZR75-1 (cpm/10^{-6} cells)	ZR75-30 (cpm/10^{-6} cells)	ZR75-31A (cpm/10^{-6} cells)
+ 0.5% dcFBS	1,076 ± 227	728 ± 170	1,455 ± 288
+ E_2 (10^{-8} M)	15,736 ± 128	1,188 ± 156	1,598 ± 179
+ E_2 (10^{-10}M)	14,379 ± 136	1,756 ± 149	1,034 ± 165
+ E_2 (10^{-12}M)	14,079 ± 142	1,364 ± 189	1,383 ± 133
+ Tam (10^{-7} M)	2,867 ± 145	1,694 ± 123	1,875 ± 187
+ Tam (10^{-7}M) E_2 (10^{-10}M)	3,489 ± 148	1,948 ± 179	2,394 ± 192
+ Tam (10^{-7} M) E_2 (10^{-8} M)	13,868 ± 173	1,389 ± 98	1,923 ± 107
+ Ins (1 µg/ml)	17,845 ± 163	18,736 ± 126	18,679 ± 198

Abbreviations: dcFBS (dextran-charcoal treated fetal calf serum); E_2 (estradiol); Tam (tamoxifen); P (progesterone); T^2(testosterone); HO-C (hydroxycortisone); Dex (dexamethasone); and Ins (insulin).

Fig. 2-1: The morphologic apppearance of human breast carcinoma cells by light microscopy. Typical epithelial-like morphology is observed with a) ZR75-1; b) ZR75-30; c) ZR75-31A; and d) MCF-7.

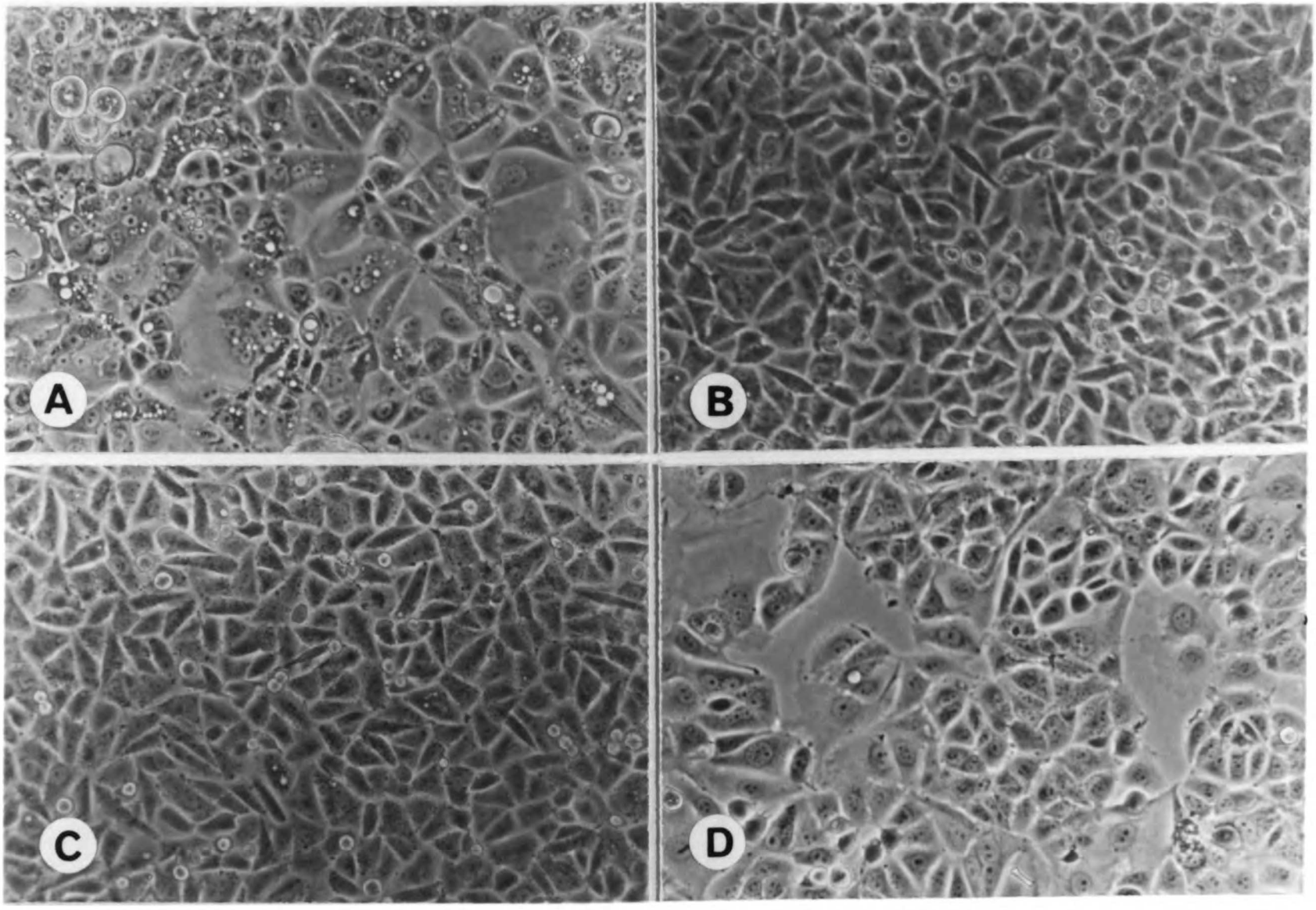

A
B
C
D

Fig. 2-2: Creatine kinase activity in cultured ZR75-1 human breast tumor cells. Panal A depict the timecourse of CPK detected after 10^{-8}M estradiol addition. Panel B depicts the inhibition of 10^{-7}M tamoxifen when added simultaneously with 10^{-10}M estradiol. After 12 hr, the media was changed to 10^{-10}M estradiol without tamoxifen. Estradiol and tamoxifen were added to media containing 0.5% dcFBS. Data are presented as the mean of triplicated assays ± S.D. One unit is defined as 1 μmol creatine phosphate converted per min at 25°C.

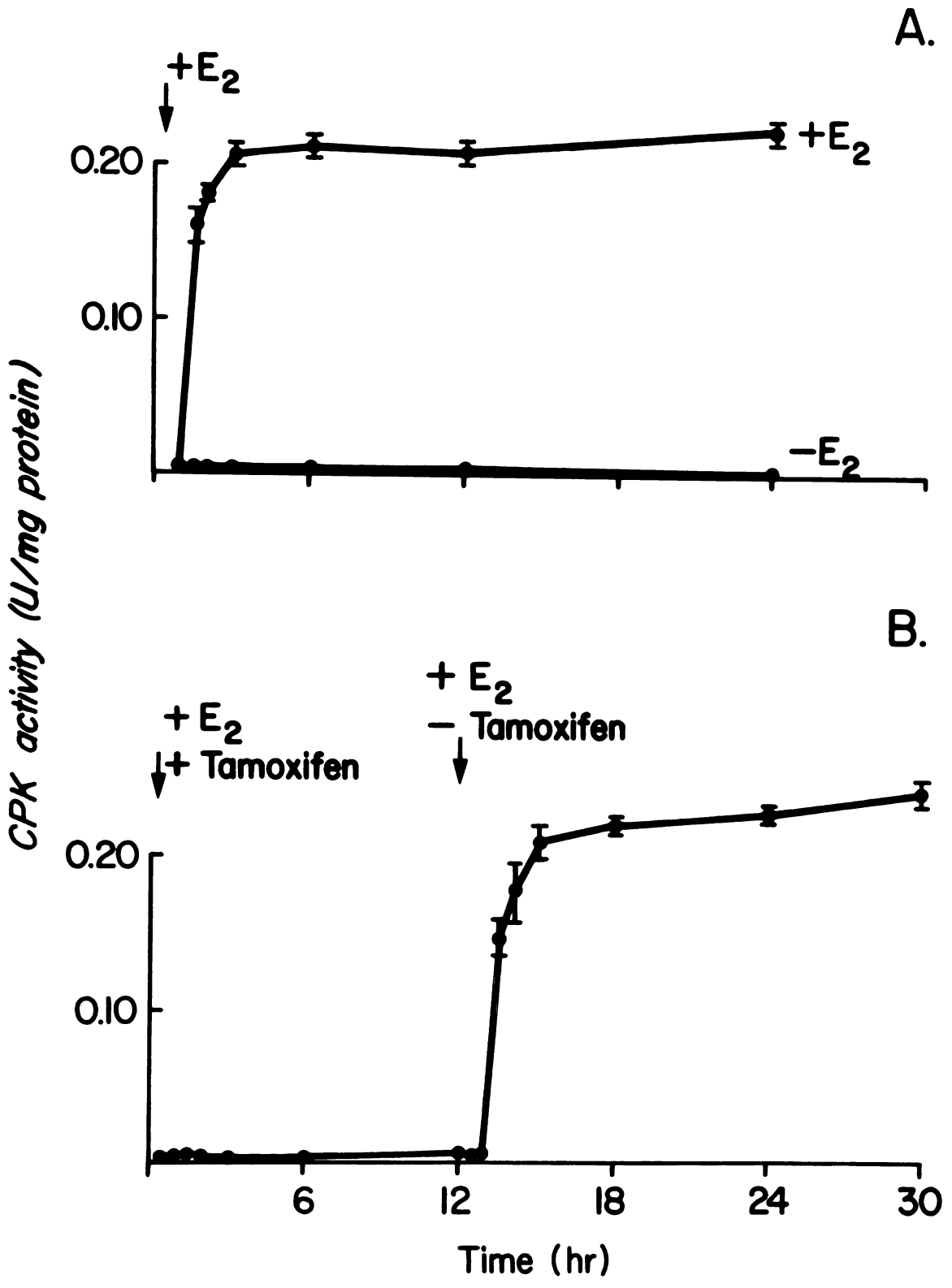

A.
+E2
+E2
0.20
0.10
−E2
B.
+ E2
− Tamoxifen
+ E2
+ Tamoxifen
0.20
0.10
6
12
18
24
30
Time (hr)
CPK activity (U/mg protein)

Fig. 2-3: Effect of hormones on the cell density of human breast cancer cells. ZR75-1, ZR75-30, ZR75-31A, and MCF-7 cells (5×10^4) were plated onto 35 mm dishes. The media was changes after 24 hr to 7% dcFBS supplemented with hormones. Twelve days after hormone addition, cells were trypsinized and counted. Data are presented as the mean of triplicate plates ± S.D. Asterik labelled bars indicate major changes.

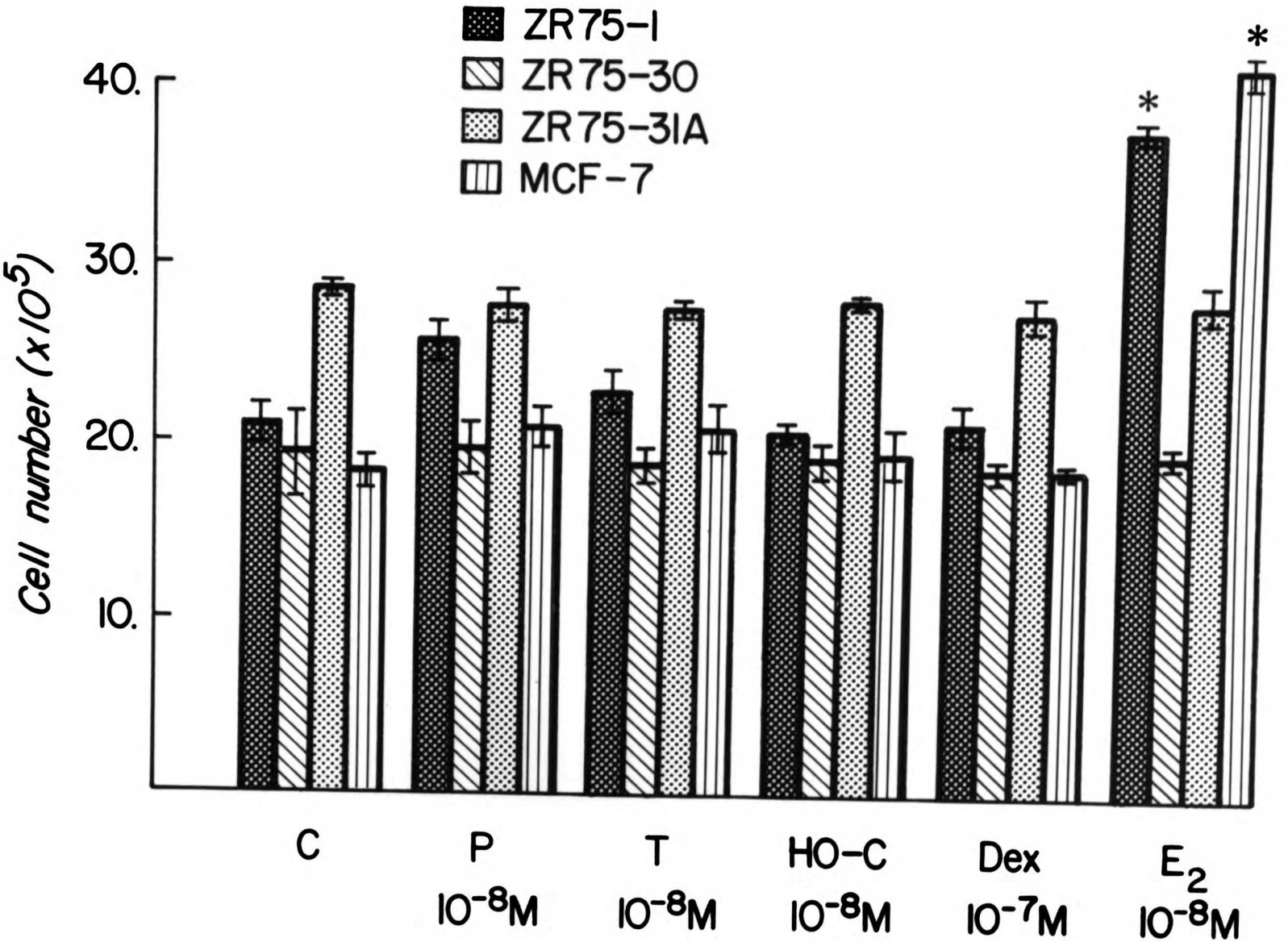
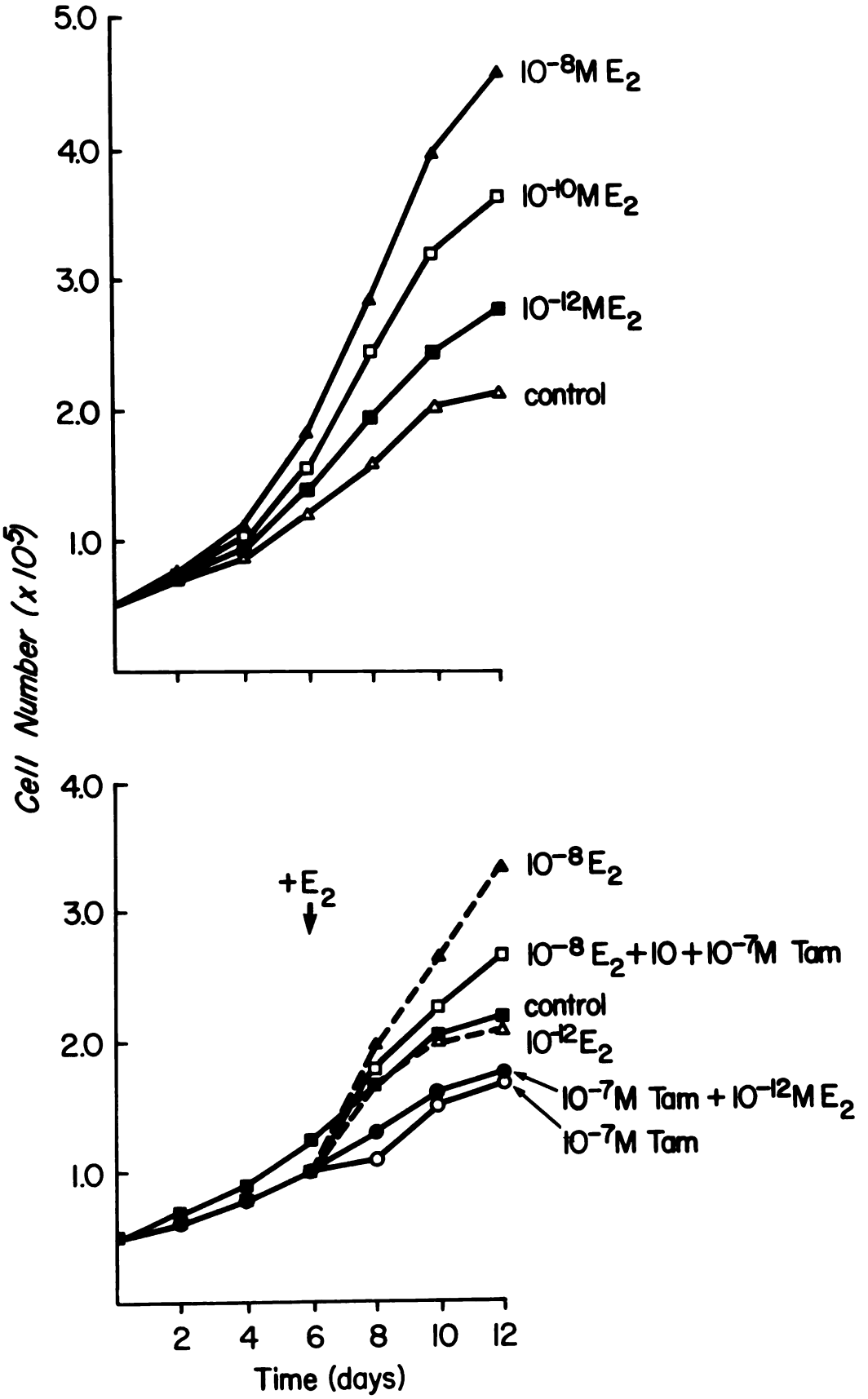

ZR75-1
ZR75-30
ZR75-31A
MCF-7
Cell number (x10^5)
40.
30.
20.
10.
*
*
C
P
10^-8M
T
10^-8M
HO-C
10^-8M
Dex
10^-7M
E2
10^-8M

Fig. 2-4: Cell growth kinetics of cultured ZR75-1 cells. Panel A, influence of estradiol. Panel B, the influence of tamoxifen. Day 6 following tamoxifen addition, the culture medium was changed to include estradiol in all except under the control condition. Tamoxifen was either removed (----), or left with the estradiol supplemented medium (——). Data presented are the the mean of triplicate experiments ± S.D.


5.0
4.0
3.0
2.0
1.0
Cell Number (x 10^5)
10^-8M E2
10^-10M E2
10^-12M E2
control
4.0
3.0
2.0
1.0
+E2
10^-8 E2
10^-8 E2+10+10^-7M Tam
control
10^-12 E2
10^-7M Tam + 10^-12M E2
10^-7M Tam
2
4
6
8
10
12
Time (days)

CHAPTER III. STROMAL BIOSYNTHESIS

A. INTRODUCTION

The desmoplastic reaction is a poorly understood phenomenon. This increase in ECM may be the product of the collapse of preexisting matrix (Jackson and Orr, 1957); the synthesis of ECM by tumor cells in the breast carcin- oma (Al-Adnani et al., 1975); the synthesis of ECM by host stromal cells (Barsky et al., 1982; Iozzo et al., 1982); or an interaction between the tumor and stromal cells (Kao et al., 1984), or some combination of the above.

Jackson and Orr (1957) argue that the desmoplastic response is not newly synthezized matrix but is the result of the collapse of pre-existing tissue. Microscopic examination of 400 clinical specimens has led Jackson and Orr to propose the dense stromal change in breast cancer is due to the collapse of a "field" of multiple ductal tumorigenic foci. They suggest it is this collapse of the ductal system which accounts for the presence of the dense stroma.

Alternatively, Al-Adnani et al. (1975) proposed the desmoplastic reaction is the product of the tumor cells. Employing immmunohistochemical techniques, they showed that collagen biosynthesis and a collagen biosynthetic enzyme, prolyl hydroxylase, are associated only with tumor cells in the scirrhous reaction. Neither collagen biosynthesis nor the enzyme is associated with the host spindle cells in the stroma of these tumors. These results suggest that the collagenous component of the stroma in scirrhous breast cancer is produced by the malignant epithelium itself.

Alternatively, the increase in ECM is the consequence of matrix synthesized by host cells. Barsky et al. (1982) concluded through

immunofluorescence studies that there is increased collagen synthesis in breast tumor desmoplasia. Type V colagen, a collagen type not normally detected in normal breast or fibrocystic disease, is found in increased amounts adjacent to the stroma cells and away from the invasive tumor cells. In the study by Iozzo et al. (1982), the synthesis of proteoglycans in the desmoplastic reaction to colon carcinoma was analyzed autoradiographically. Increased chondroitin 4- and 6- sulfate glycosaminoglycans are synthesized by host stromal cells and not by the tumor cells.

Alterations in the cellular population of the desmoplastic reaction has also been reported. Schurch et al. (1981) and Seemayer et al. (1979) observe an increase in the number of myofibroblasts. The myofibroblast is a cell type not normally observed in normal breast stroma. The simulataneous appearance of type V collagen and myofibroblasts have led Liotta et al. (1983) to propose that invasive tumors may elicits factors that stimulate the formation and recruitment of myofibroblasts. These myofibroblasts may be the cell type responsible for the synthesis of type V collagen, elastin, and proteoglycans found in breast tumor desmoplasia.

In the present studies, I have proposed the increased ECM in breast tumor is dependent on an interaction between the breast tumor and host stromal cells. The ECM deposited by human breast tumor cells was demonstrated to be mitogenic for fibroblasts. This mitogenic effect was mediated through the tumor matrix or some factors associated with the matrix. The matrix was also shown to be capable of transforming the morphology of the stromal cell. These transformed cells resembled myofibroblasts in culture. Since collagen is the major synthetic product of stromal cells,

the stimulation of cell proliferation by the adjacent breast tumor matrix may be the basis of the desmoplastic reaction.

Although the etiology of the desmoplastic reaction is not known, the contents of that matrix are well studied. The ECM contain high levels of collagen and elastin. Collagen is the most abundant protein of the animal world, constituting the major proportion of mammalian body protein. This protein is the predominent component of skin, tendon, bone, cartilage, and extracellular matrix. There are presently eight collagen types. The best known and most characterized are the three interstitial collagens, types I, II, and III,and have been reviewed (Bornstein and Sage, 1980; Burgeson, 1982; Glanville, 1982). Type I, the main constituent of skin, tendon, bone, and vessel walls, is synthesized by fibroblasts, smooth muscle cells, and osteoblasts. Other stromal cells also produce type III collagen, which is often found together with type I. Type II collagen, the only collagen constituent of hyaline cartilage, is produced by chondrocytes. Type IV, a network-forming collagen, appears to be present in all basement membranes, regardless of embryonic origin. This molecule is characterized by having both collagenous and non-collagenous domains resulting in a flexible molecule (Timpl and Martin, 1982; Duncan et al., 1983). Type V collagen is present at low levels (less than 10%) in all connective tissues except hyaline cartilage. Its distribution includes the pericellular matrix (Martinez-Hernandez et al., 1982) and in the ECM of various tissues including cornea, blood vessels, and the perimysium of skeletal muscle (Linsenmayer et al., 1983). However, the present functional role of type V collagen is unclear. Several new classes of collagen have recently been identified but their structure and functions are unknown. These include: type VI collagen from uterus and placental villi (Odermatt et al., 1983;

Furthmayer et al., 1983); type VII collagen from human amnion (Bentz et al., 1983); and EC (endothelial) collagen from cultures of endothelial cells and other cell types (Sage et al., 1980). This is now termed type VIII collagen (Sage, 1984).

Elastin is a connective tissue component found in a variety of tissues and organs. It is present in skin, yellow tendons, and in blood vessels (Sandberg, 1981). These structures are believed to be the consequence of elastin biosynthesis by several cell types including smooth muscle cells, fibroblasts, chondrocytes, and endothelial cells (Carnes et al., 1979; Quintarelli et al., 1979; Mecham, 1980). Elastin is also a prominent component of breast carcinoma (Cheate and Cutler, 1961; Azzopardi, 1974). The elastic tissue is associated with the desmoplastic rection to the tumor and is often referred to as the elastosis or the "chalky streaks" in the tumor. The source and etiology of the elastin component of desmoplasia is unclear. Similar to the collagen, it is not known whether this stromal protein is the product of fibroblasts in the invaded host tissue, is synthesized by the malignant epithelial cells of the tumor, or is perhaps dependent on an interaction between the two cell types.

B. Materials and Methods

1. Tissue Culture and Metabolic Labeling

ZR75-1, -30, and -31A, human breast tumor cell lines and newborn fibroblasts (NBF) were cultured as described in the previous chapter (CHAPTER II, Materials and Methods). For collagen and elastin biosynthesis studies, cells were grown in Costar T-35 flasks in RPMI 1640 in the presence of 7% dextran charcoal-treated FBS, penicillin and streptomycin, fungizone 2.5 µg/ml, and 2 mM glutamine. Ascorbic acid (25 µg/ml) was

added 24 hr prior to metabolic labeling. One hr prior to labeling, the media was changed to modified RPMI 1640 (without proline, hydroxyproline, or glutamine) which contained FBS, antibiotics, antimycotics, and ascorbic acid in the concentrations described above. At the time of labeling the media was once again changed to 6 ml of the same medium. Isotopic labels were added at this time. For collagen and elastin synthesis, [^{3}H]proline or [^{14}C]lysine at 5 μCi/ml was used. Metabolic labeling was performed for a period of 24 hr.

2. Sample Processing

The culture medium was removed from the cultures and washed three times with cold (4°C) PBS. The culture medium and the first wash were combined and centrifuged at 800 xg, 10 min to remove cellular debris. The cell layers which contained the cells and the ECM were scraped into a hypotonic solution containing 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100 (pH 7.5), and homogenized. At this stage, the media and cell homogenate was combined and dialyzed against 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5 to remove traces of free radiolabeled amino acids. Dialysis was continued against distilled water for 2 changes over a 48 hr period. Concentrated KOH was added to a final concentration of 2 N hydrolysis and incubated in a vacuum over for 24 hr at 110° in a sealed tube. The solution was neutralized with perchloric acid and centrifuged at 2000 rpm at 4° C. The supernatent was lyophilized to dryness.

3. HPLC Procedures

Separation of the hydrolysate was performed in a 250 x 4.6 mm I.D. partisil PXS 10/25 ODS HPLC column (Whatman, Pierce Chemical Co.). The HPLC system was composed of a high pressure Spectra-Physic (SP 8700) and a

sample injection valve with a 1.0 ml loop (Spectra-Physic SP 8750). In all experiments, glass distilled water and organic solvents were used.

Samples were dissolved in distilled water and centrifuged at 800 xg for 10 min to remove insoluble substances. The supernatant was passed through a Nylon 66 filter (Rainin) and adjusted to pH 3 with n-heptafluorobutyric acid (HFBA, Pierce Chemical Co.). No loss of hydroxyproline or val-pro was detected during this manipulation.

Sample was injected into a column equilibrated with 50 mM HFBA (pH 3.0) and eluted according to the following steps: isocratic condition at 0% 1-propanol in 50 mM HFBA (pH 3.0) for 60 min followed by an 80 min linear gradient from 0 to 10% 1-propanol. The flow rate was 0.5 ml/min in all experiments.

Eluted samples were counted in a Beckman LS-3133T scintillation counter with a counting efficiency of 96% for ^{14}C and 76% for ^{3}H.

C. RESULTS

Human breast tumor and stromal subconfluent cell cultures were labeled with [^{3}H]proline and [^{14}C]lysine for 24 hr. As shown in table 3-1a, the amount of [^{3}H]proline present in stromal and breast cell lines was nearly the same for all cell lines examined. However, the radioactivity recovered in the form of hydroxyproline in stromal cell lines (NBF and RMF) was 3.8 times that of human breast tumor cell lines. By calculation (Green and Goldberg, 1965), more of the protein synthesis in stromal cells were devoted to collagen biosynthesis. Examination of val-pro dipeptide (table 3-1b) content indicated relatively little difference in elastin synthesis between stromal and breast tumor cell lines. By calculation, approximately

0.79-1.49% of total protein synthesis was devoted to tropoelastin and elastin biosynthesis.

The ECM plays a key role in the regulation of cellular developemnt and synthesis of extracellular connective tissue. The influence of the ECM on collagen (Reddi and Ramachandran, 1976; Slavkin and Greulich, 1977) and elastin (Mecham et al., 1981) biosynthesis has been demonstrated. Recently, I have shown that the ECM of human breast tumor cells is mitogenic for host stromal cells (Kao et al., 1984). The ability of breast tumor cell ECM to influence collagen and elastin biosynthesis was also examined. Collagen biosynthesis of ZR75-1 breast tumor cells was not influenced by the matrix of human stromal cells (NBF). However, the synthesis of collagen increased by almost 50% when stromal cells were cultured on the breast tumor ECM on a per cell basis(table 3-2a). Elastin biosynthesis was similarly influenced, with a 70% increase in stromal cultures grown on breast tumor ECM on a per cell basis (table 3-2b).

The ability of hormones to influence collagen and elastin synthesis was examined. Human breast tumor cells (ZR75-1), stromal cells (NBF), and NBF cells cultured on breast tumor ECM were exposed to a cocktail of various hormones (estradiol, progesterone, testosterone, and hydroxycortisone) at physiological levels, and at higher pharmacological levels of estradiol and progesterone. Collagen and elastin biosynthesis was examined. Synthesis by ZR75-1, NBF, and NBF cells on ZR75-1 ECM was not influenced by hormonal addition (table 3-3). The increased collagen and elastin biosynthesis observed with NBF cultured on breast tumor preformed matrix versus plastic was also unaffected by hormone addition.

The influence of ECM and hormones on the cross-linking of elastin was examined (table 3-4). Val-pro dipeptide is a measure of synthesis of both

tropoelastin and cross-linked elastin. Elastin biosynthesis, however, may be analyzed independent of tropoelastin synthesis by measuring the levels desmosine and isodesmosine crosslinks. By labeling cells with [^{14}C]lysine, radiolabeled desmosine and isodesmosine can be measured by the HPLC technique. Breast tumor and stromal cells were exposed to various substrates and to the hormonal environment. The level of cross-linked elastin corresponded to that of tropoelastin synthesis, indicating there was not a differential affect by hormones on either synthesis or cross-linking.

D. Discussion

Many human tumors have a dense fibrotic reaction called the desmoplastic or scirrhous response. Breast tumors have the most profound reaction followed by prostate, pancreatic, gastric, and colon carcinomas (Robbins and Cotran, 1982). The source and etiology of desmoplasia in human breast cancer is not known. It is not clear whether this connective tissue reaction is the product of fibroblasts in the invaded host tissue, the malignant epithelial cells, or is perhaps dependent upon an interaction between these two cell types. The synthesis of the scleroproteins, collagen and elastin, in human breast and stromal cell lines was examined. Collagen is a major synthetic product of stromal fibroblasts. Approximately 4% of the total protein synthesis in fibroblasts is devoted to collagen biosynthesis (Green and Goldberg, 1965). In breast tumor cells, however, only 0.9-1.16% of the total protein synthesis was devoted to collagen synthesis. Though these data suggest the stromal cells are the predominant collagen producing cells, the levels of collagen synthesized cannot account for the profound fibrotic reaction seen in breast tumor desmoplasia.

Grobstein proposed originally a role for the cell matrix in the control of cell proliferation and morphogenesis (Grobstein, 1955). Since then, there have been many examples described in which the proliferative capacity of cells is modulated by the substrate upon which the cells are maintained (Vlodavsky et al., 1980; Wicha et al., 1982). The synthesis and deposition of collagen and glycosaminoglycans by corneal epithelial cells are stimulated when cells are grown on killed lens capsule (Meir and Hay, 1974a, 1974b). Here the lens capsule represents the matrix provided in situ by the adjacent stromal tissue. Madri and coworkers (1983) have demonstrated endothelial cells may be influenced by the qualitative composition of the substrata upon which they are grown. When the cells are grown on interstitial collagens, they undergo proliferation. In contrast, when cells are grown on basement membrane collagens, they do not proliferate but form tube-like aggregates. Not only are the phenotype and proliferative capacity different, the cells on the basement membrane collagen also express more basement membrane constituents compared to cells grown on interstitial matrices. These studies indicate that the ECM may not only modulate cell proliferation but also stromal synthesis, both in quantity and in quality.

The mechanism by which the ECM can influence fibroblast cellular behavior is not clear. The matrix of cells is composed of a complex of type-specific collagens, elastin, glycoproteins, many of which are adhesion proteins, and a spectrum of proteoglycans and their attendant glycosaminoglycans, in a highly ordered three-dimensional structure (Hay, 1981). The components of the breast tumor ECM are now being characterized. It contains type IV basement membrane specific collagen, as well as type I and III interstitial collagens. Laminin and fibronectin have been observed

by immunofluorscence microscopy. Elastin is present. There are also high concentration of proteases, including collagenases and elastases produced by breast tumor cells onto their surfaces as well as into the tissue culture medium (Kao et al., 1982a,b). Trypsin and other proteases are known to stimulate division of cells in culture (Sefton et al., 1980; Greene et al., 1971). From these observations, one mechanism for the mitogenic activity of the tumor matrix can be suggested. Fibroblasts may be stimulated to divide by proteases imbedded in or on the surface of the tumor ECM. These rapidly dividing cells may also have altered levels of stromal proteins synthesis.

Another possible model is that a structural component of the ECM is directly responsible for the mitogenic stimulation. Receptors for soluble collagen (Goldberg, 1979, 1982) and for other ECM components (Rao et al., 1983; Lesot et al., 1983) have been demonstrated. Some structural moiety within the matrix may thus be responsible for the stimulation, and some as yet unidentified plasma membrane receptor of the stromal cell perhaps perceives that material and relays the signal to divide and to synthesize stromal proteins.

In the present experiments, observations on the influence of breast tumor ECM on stromal cells has been extended. Collagen and elastin synthesis increased by 47% and 69%, respectively, when fibroblasts were cultured on breast tumor ECM compared to plastic. The fibroblast ECM had no influence on stromal biosynthesis in breast tumor cells. This situation was analogous to the corneal epithelial cells studies discussed previously, except here the stromal cells are responding to the matrix provided by the adjacent epithelial cells. A general biologic principal can be recognized,

that the ECM is informational not only for the cells that produce the matrix, but also for adjacent cell populations.

The substrate upon which cultured cells are maintained has been shown to be capable of phenotypic modification of cells (Gospodarowicz et al., 1978; Folkman and Moscona, 1978; Vlodavsky et al., 1980). When fibroblasts were seeded on the breast tumor ECM, changes in phenotypic characteristics were observed. Instead of the usual elongated spindle shape, an elliptical cell shape was adopted. In surgical pathology specimens, the spindle-shaped fibroblast cell population is not conspicuous by light microscopy in the stroma of breast tumors. A change in shape may account for the scarcity of spindle-shaped cells in scirrhous carcinomas of the breast. The desmoplasia of human breast carcinoma contains a 10-fold increase in type V collagen (Barsky et al., 1982). This increase is attributed to host myofibroblsts that are recruited by the tumor. The origin of such myofibroblasts is not clear. I speculate the morphologic changes observed when resident fibroblasts are transformed to elliptical shaped cells corresponds to transformation to myofibroblasts, and this may be the actual origin of such cells in vivo (Kao et al., 1984).

When these spindle shaped fibroblasts were transformed into elliptical shaped cells, there was also an increase in collagen synthesis. The nature of the collagen types produced is under investigation. Additionally, it is not clear whether morphologic transformation is coupled to increased collagen biosynthesis, or whether this increased collagen synthesis is simply a reflection of cells being grown on ECM. Additional qualitative and quantitative work is needed to ascertain whether these cells are truely myofibroblasts and to establish the types of collagen produced by these cells.

Clinically, a decrease in tumor mass occurs following hormonal treatment. In addition to the dissolution of scleroproteins, a decrease in stromal synthesis may be invoked. This was examined in the present experiment by exposing cultures to a cocktail of hormones which included estradiol, progesterone, testosterone, and hydroxycortisone at physiological levels. No alteration in levels of either collagen or elastin synthesis was observed. This observation assumed the hormone cocktail contained hormones whose action were not antagnostic. No evidence for such an effect by the hormone cocktail have been reported using breast tumor and stromal cells (Liotta et al., 1979a). Increased levels or pharmacological levels of hormones were also found to have little influence on stromal synthesis. Thus, it is unlikely decreased synthesis plays a role clinically in a decrease of tumor mass following hormone treatment.

In summary, the intense fibrotic response observed with human breast tumors is the result, in part, of the influence of human breast tumor ECM on the host stromal cells. This matrix influenced not only the phenotypic expression of host stromal cells, but also regulated their rate of growth (Kao et al., 1984). In this chapter, I have also demonstrated that this matrix also influenced levels of collagen and elastin biosynthesis. Hormones had no apparent influence on such matrix-induced stromal protein synthesis.

Table 3-1: Collagen (3-1a) and elastin (3-1b) biosynthesis by various cultured breast tumor and stromal cell lines. Breast tumor (ZR75-1, -30, and -31A) and stromal (NBF and RMF) cells were labeled with ^{3}H-Pro for collagen and elastin biosynthesis as described in Materials and Methods.

¶ Total elastin = tropoelastin + cross-linked elastin.

+ Collagen / total protein ratio is calculated assuming that hydroxyproline residue content of collagen is 12.2% and that the average proline content in total cellular protein is 4.1% (Green, 1965).

§ Tropoelastin & cross-linked elastin / total protein ratio is calculated assuming that val-pro dipeptide is 5.75% and that the average proline content in total cellular protein is 4.1% (Keith, 1979).

Table 3-1a: Collagen Biosynthesis in Breast Tumor and Stromal Cells

Cells	HO-Pro	Total Incorporation	HO-Pro / Total Incorporation	Collagen + / Total Protein
	$\times 10^4$ cpm	$\times 10^5$ cpm		
	10^6 cells	10^6 cells	(%)	(%)
ZR75-1	2.29 ± 0.04	84.27 ± 0.07	2.72	0.91
ZR75-30	3.87 ± 0.03	82.48 ± 0.07	4.63	1.58
ZR75-31A	3.44 ± 0.05	86.10 ± 0.07	3.99	1.34
NBF	9.90 ± 0.05	85.58 ± 0.09	11.57	3.89
RMF	9.83 ± 0.06	75.63 ± 0.08	13.00	4.37

Table 3-1b: Elastin Biosynthesis in Breast Tumor and Stromal Cells

Cells	Val-Pro	Total Incorporation	Val-Pro / Total Incorporation	Total Elastin¶§ / Total Protein
	$\times 10^4$ cpm	$\times 10^5$ cpm		
	10^6 cells	10^6 cells	(%)	(%)
ZR75-1	1.24 ± 0.05	84.27 ± 0.07	1.47	1.05
ZR75-30	1.13 ± 0.04	82.48 ± 0.07	1.37	0.98
ZR75-31A	0.95 ± 0.03	86.10 ± 0.07	1.10	0.79
NBF	1.15 ± 0.06	85.58 ± 0.09	1.34	0.96
RMF	1.58 ± 0.03	75.63 ± 0.08	2.09	1.49

Table 3-2: The influence of the substratum on collagen (3-2a) and elastin (3-2b) biosynthesis in the ZR75-1 and NBF cell lines. Statistic for probability value calculated based on t-test.

Table 3-2a: The Influence of Substratum on Collagen Biosynthesis

Cells (Substratum)	HO-Pro $\frac{x10^4 \text{ cpm}}{10^6 \text{ cells}}$	Total Incorporation $\frac{x10^5 \text{ cpm}}{10^6 \text{ cells}}$	$\frac{\text{HO-Pro}}{\text{Total Incorporation}}$ (%)	$\frac{\text{Collagen +}}{\text{Total Incorporation}}$ (%)
ZR75-1 (plastic)	2.29 ± 0.03	84.27 ± 0.06	2.72	0.92
ZR75-1 (ECM:NBF)	2.31 ± 0.03	86.17 ± 0.07	2.68	0.90
NBF (plastic)	9.90 ± 0.04	85.58 ± 0.07	11.57	3.89
NBF (ECM:ZR75-1)	* 13.73 ± 0.04	80.89 ± 0.07	16.97	5.70

Table 3-2b: The Influence of Substratum on Elastin Biosynthesis

Cells (Substratum)	Val-Pro $\frac{x10^4 \text{ cpm}}{10^6 \text{ cells}}$	Total Incorporation $\frac{x10^5 \text{ cpm}}{10^6 \text{ cells}}$	$\frac{\text{HO-Pro}}{\text{Total Incorporation}}$ (%)	$\frac{\text{Total Elastin¶+}}{\text{Total Incorporation}}$ (%)
ZR75-1 (plastic)	1.24 ± 0.03	84.27 ± 0.06	1.47	1.05
ZR75-1 (ECM:NBF)	1.25 ± 0.04	86.17 ± 0.07	1.45	1.03
NBF (plastic)	1.15 ± 0.03	85.58 ± 0.07	1.34	0.96
NBF (ECM:ZR75-1)	* 1.83 ± 0.03	80.89 ± 0.07	2.26	1.61

¶ Total elastin = tropoelastin and cross-linked elastin.
+ See table 3-1 for amino acid residue content in collagen & elastin.
* $p < 0.005\%$

Table 3-3a: The Influence of Hormone on Collagen Biosynthesis

Cells (Substratum) *Hormone	HO-Pro $\times 10^4$ cpm / 10^6 cells	Total Incorporation $\times 10^5$ cpm / 10^6 cells	HO-Pro / Total Incorporation (%)	Collagen / Total Incorporation (%)
ZR75-1 (plastic)				
*Control §	2.29 ± 0.03	84.27 ± 0.06	2.72	0.91
*Hormone Cocktail#	2.31 ± 0.03	81.02 ± 0.07	2.85	0.96
*E_2 (10^{-6}M)	2.17 ± 0.03	82.62 ± 0.08	2.62	0.88
*P (10^{-6}M)	2.34 ± 0.04	81.32 ± 0.08	2.88	0.97
NBF (plastic)				
*Control	9.90 ± 0.04	85.58 ± 0.07	11.57	3.89
*Hormone Cocktail	9.21 ± 0.04	84.37 ± 0.07	10.92	3.67
*E_2 (10^{-6}M)	9.93 ± 0.05	85.02 ± 0.08	11.68	3.92
*P (10^{-6}M)	9.76 ± 0.05	84.12 ± 0.09	11.60	3.90
NBF (ECM:ZR75-1)				
*Control	13.73 ± 0.02	80.89 ± 0.07	16.97	5.71
*Hormone Cocktail	13.34 ± 0.03	82.12 ± 0.08	16.24	5.46
*E_2 (10^{-6}M)	13.96 ± 0.05	79.97 ± 0.09	16.20	5.87
*P (10^{-6}M)	13.26 ± 0.04	80.14 ± 0.08	16.55	5.56

§ Control: culture medium contain 7% dcFBS. All hormone addition was to medium containing 7% dcFBS.

Hormone Cocktail: E_2 (10^{-8}M); P (10^{-8}M); T (10^{-8}M); and HO-C (10^{-8} M).

Table 3-3b: The Influence of Hormone on Elastin Biosynthesis

Cells (Substratum) *Hormone	Val-Pro	Total Incorporation	Val-Pro / Total Incorporation	Total Elastin¶ / Total Incorporation
	$x10^4$ cpm / 10^6 cells	$x10^5$ cpm / 10^6 cells	(%)	(%)
ZR75-1 (plastic)				
*Control §	1.24 ± 0.03	84.27 ± 0.06	1.47	1.05
*Hormone # Cocktail	1.39 ± 0.04	81.02 ± 0.07	1.71	1.22
*E_2 (10^{-6}M)	1.32 ± 0.03	82.62 ± 0.08	1.60	1.14
*P (10^{-6}M)	1.17 ± 0.04	81.32 ± 0.08	1.44	1.03
NBF (plastic)				
*Control	1.15 ± 0.03	85.58 ± 0.07	1.34	0.96
*Hormone Cocktail	1.20 ± 0.03	84.37 ± 0.07	1.42	1.01
*E_2 (10^{-6}M)	1.09 ± 0.04	85.02 ± 0.08	1.28	0.91
*P (10^{-6}M)	1.23 ± 0.05	84.12 ± 0.09	1.46	1.04
NBF (ECM:ZR75-1)				
*Control	1.83 ± 0.03	80.09 ± 0.07	2.26	1.61
*Hormone Cocktail	1.66 ± 0.04	82.12 ± 0.07	2.05	1.44
*E_2 (10^{-6}M)	1.86 ± 0.04	79.97 ± 0.09	2.33	1.66
*P (10^{-6}M)	1.72 ± 0.04	80.14 ± 0.08	2.15	1.53

¶ Total elastin: tropoelastin and elastin.
§ Control: culture medium contain 7% dcFBS. All hormone addition was to medium containing 7% dcFBS.
# Hormone cocktail: as described in table 3-3a.

Table 3-4: Correlation of total elastin synthesis (tropoelastin and elastin) with cross-linked elastin synthesis in breast tumor cell line, ZR75-1, and stromal cell line, NBF. Total elastin synthesis was measured by val-pro dipeptide and cross-linked by isodesmosine and desmosine content.

Table 3-4: Correlation of Total Elastin Synthesis with Cross-linked Elastin Synthesis

Cells (Substratum) *Hormone	Isodes +Des	Total Incorporation	Isodes + Des[2] / Total Incorporation	Val-Pro[3] / Total Incorporation
	$\times 10^{-4}$ cpm / 10^6 cells	$\times 10^{-5}$ cpm / 10^6 cells	(%)	(%)
ZR75-1 (plastic) *Control §	4.48 ± 0.03	77.72 ± 0.08	5.76	1.47
ZR75-1 (ECM:NBF) *Control	4.32 ± 0.05	75.81 ± 0.06	6.43	1.44
NBF (plastic) *Control	5.03 ± 0.04	78.17 ± 0.09	6.43	1.34
NBF (ECM:ZR75-1) *Control	8.77 ± 0.06	73.85 ± 0.12	11.87	2.26
*Hormone Cocktail +	8.73 ± 0.03	72.79 ± 0.09	11.99	2.05
*E_2 (10^{-6}M)	8.48 ± 0.05	72.27 ± 0.10	11.73	2.33
*P (10^{-6}M)	8.71 ± 0.05	73.67 ± 0.09	11.82	2.15

§ Control: culture medium contain 7% dcFBS. All hormone addition was to medium containing 7% dcFBS.
[2] Isodes + Des: reflective of cross-linked elastin.
[3] Val-pro : reflective of cross-linked elastin and tropoelastin.
+ Hormone cocktail: as described in table 3-3a.

CHAPTER IV. COLLAGEN AND ELASTIN DEGRADATION

A. Introduction

The stroma of human breast cancer consists predominantly of fibrous proteins such as collagen (Gullino et al., 1962, 1963; Groniowski et al., 1975), elastin (Adnet et al., 1976; Jeffrey and Gross, 1970), and non-collagenous glycoproteins. A decrease in the size of the tumor mass in response to treatment suggests that in additon to breast tumor cell death, tumor stromal degradation occurs. Collagenolytic, elastinolytic, and non-specific proteolytic activities must be invoked to account for this stromal degradation.

Mammalian collagenases have been identified in many cell and tissue extracts since the original description by Gross and coworkers (reviewed by Gross, 1981; Harper, 1980; Harris and Krane, 1974). Collagenases have also been shown in a variety of tumor tissue. McCroskery et al. (1975) and Biswas (1978) have reported a collagenase activity in the rabbit V2 carcinoma. Wirl and coworkers (1977; 1979) have reported an increase in collagenolytic activity associated with chemical carcinogenesis in mouse skin. Abramson et al. (1975), Hashimoto et al. (1972, 1973), Kuettner et al. (1977), Paranjpe et al. (1980), and Yamanishi et al. (1972, 1973) have identified collagenases in cultured human tumor cells.

Vertebrate collagenase is an unique class of enzyme with the capacity to cleave native collagen in the triple helical conformation. The cleavage of collagen types I, II, and III has been well characterized (Gross et al., 1974; Miller et al., 1976). The cleavage site of these collagen chains by vertebrate collagenase is at a Gly-Ile bond. The products of this single cleavage, at 25°C, are 3/4 and 1/4 triple helical fragments. Purification

and characterization of mammalian collagenase by a number of investigators (Eisen et al., 1968; Gross and Nagai, 1965; Woolley et al., 1975; Riley and Peacock, 1977) have demonstrated activities that degrade collagens I, II, and III. They are calcium- and zinc- dependent enzymes which function at neutral pH.

The degradation of basement membrane collagens IV and V by collagenase have been reported by several investigators (Liotta et al, 1979a, 1981a,b; Mainardi et al., 1980, 1981; Murphy 1981). Collagen types IV and V are not susceptible to attack by classical vertebrate collagenase (Liotta et al., 1979; Sage et al., 1979; Bornstein and Sage, 1980). A separate group of collagenolytic proteases have been identified that degrade basement membrane collagens. Type IV collagenolytic metalloproteinase has been isolated from leukocytes (Mainardi et al., 1980) and a murine tumor cell line (Liotta et al., 1981). The latter enzyme has been characterized by Liotta and coworkers to be a metalloproteinase of 62K. This enzyme exhibits a latent and an active form with an inhibitory profile and physiochemical properties similar to classical vertebrate collagenases. The specific cleavage reaction of type IV is also a single clip in a region approximately one-third of the way from the N-terminal. Some regions of the type IV collagen are susceptible to degradation by serine proteases under various conditons (Sage et al., 1979; Mainardi et al., 1980; Uitto et al., 1980).

A type V degrading enzyme has been chacterized in tumor cells (Liotta et al., 1981). This 80K metalloproteinase is collagen type-specific and is not capable of degrading other collagen types. Degradation of type V collagen produces specific high molecular weight products that await further characterization. Type IV and V degrading metalloproteinases have been demonstrated in cultured normal involuting epithelial duct cells

(Liotta et al., 1979). These proteases appear to play a role in the selective physiologic turnover of basement membrane during normal hormonally regulated tissue remodeling.

There are several lines of evidence that suggest hormones can modulate tumor stromal degradation. Heuson et al. (1975) observed that human breast cells in the presence of 17β-estradiol lyse the stroma surrounding islands of tumor cells. Similar observations have been made in basal cell carinomas by Bauer and coworker(1977). It is not known whether these enzymes are elaborated by the malignant mammary epithelial cells, the breast fibroblasts, or are somehow due to an interaction between the two cell types. Also unclear is whether the elaboration of these degradative enzymes represents hormonally dependent events. This is a particularly provocative question for breast and prostate tumors in which the tissue of origin as well as their tumors are often hormone-responsive.

Elastin-degrading enzymes from mammalian tissue have been reported by several investigators (for review see Bieth, 1978; Werb et al., 1982). The presence of an elastase in porcine pancreas was first established by Balo and Banga (1949). Two types of pancreatic elastases have been described (Barret and Baron, 1980). The best characterized is the porcine pancreatic elastase I, which is a serine proteinase secreted in a zymogen form by the pancreatic β-cells. Several isozymes of elastase have been reported in the granules of human polymorphonuclear leukocytes (Baumstark, 1967; Janoff and Scherer, 1968; Janoff, 1973; Ohlsson and Olsson, 1974; Fernstein and Janoff, 1976). A latent elastase has been reported in human blood platelets (Robert et al., 1970). The presence of elastinolytic activities in other mammalian tissues have been demonstrated including human aorta (Robert et al., 1974; Hornebeck and Robert, 1975); macrophages (Janoff et

al., 1971; Werb, 1978); human skin (Malemud and Janoff, 1976), and in tumor cells (Gilfillan, 1968). An elastinolytic activity has also been demonstrated previously by Hornebeck and Robert in extracts of human breast carcinomas (1977). The source and the nature of the hormone-dependency of breast tumor elastases remains to be established.

Collagenolytic and elastinolytic activities elaborated by cultured human breast cancer cells is described herein. These activities have been partially purified and characterized. Hormonal effects on these activities have also been studied.

B. Materials and Methods

1. Cell Culture

The source of cells and the culture technique were similar to those previously described in Chapter II. All cells were maintained in RPMI 1640 with 4% FBS. For hormonal studies, the cells were washed three times with HBSS and cultured in serum free medium supplemented with hormones. The medium was harvested and changed every 48 hrs. HeLa cells were obtained from the UCSF tissue culture facility.

2. Enzymatic Assay

Collagenolytic activity was assayed by the method of Johnson-Wint (1978). For the collagenase assay, trypsin activation was required and performed as described for the elastinolytic samples. A unit of collagenolytic activity is defined as 1 µg of collagen degraded per min.

Elastin was radiolabeled using ^{3}H-$NaBH_4$, according to the method described by Banda and Werb (1982). For each assay, 175 µl of the conditioned media was trypsin activated. Activation was effected by using 12.5

µl of 0.025% trypsin for 5 min and terminated by the addition of 12.5 µl of 0.125% soybean trypsin inhibitor. The 200 µl sample was then added to 100 µl of a 2 mg/ml suspension of ^{3}H-elastin. The reaction was performed at 37° C for 24 hrs. A unit of elastinolytic activity is defined as 1 µg of elastin degraded per hour. The combination of trypsin and soybean trypsin inhibitor results in less than 2-3% collagen and elastin degradation. The data presented has been corrected for the trypsin-soybean trypsin inhibitor background. Inhibition studies were performed by incubating the activated samples with inhibitor for 10 min at 37°C. The substrate was then added and incubation continued as described.

3. DEAE Cellulose Chromatography

DE-52 (Whatman) was packed into a 1 X 25 cm column and equilibrated with 50mM Tris-HCl containing 5mM $CaCl_2$ and 0.02% NaN_3, pH 8.3, at 4°C. A 40 ml sample media conditioned by confluent cell layer was used for partial purification. The sample was dialyzed against the equilibration buffer overnight and applied with a flow rate of 60 ml/hr. The bound proteins were eluted with a linear gradient from 0.0 to 0.15 M NaCl in the same buffer, and 5 ml fractions were collected. The total gradient was 800 ml. This was followed by a subsequent wash of 1 M NaCl.

4. Ultragel Chromatography

Elastinolytic fractions were lyophilized separately and dissolved in 2 ml of the equilibration buffer (50 mM Tris/150 mM NaCl/50 mM $CaCl_2$ pH 7.6). Each sample was applied to an Ultragel AcA 54 column (1.5 X 100 cm) and eluted with the equilibration buffer at a flow rate of 15 ml/hr. Fractions (2.0 ml) were collected and assayed for protein concentration and

elastinolytic activity. The Ultragel AcA 54 column was caliberated with Blue Dextran, bovine serum albumin, ovalbumin, myoglobin, cytochrome C, and DNP-glycine.

5. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Neville (1971). A discontinous sytem with a 4% stacking gel and a 7% separating gel was used for collagen degradation analysis.

C. RESULTS

1. Collagenolytic Protease: Partial Purification and Characterization

Cultured human breast cancer and stromal cell lines were grown and the conditioned media examined for collagenolytic activities (table 4-1). Such activities were present in all breast epithelial tumor cell lines examined. Relatively low activities were found in the media of stromal cells from newborn foreskins and from reduction mammoplasties. HeLa cells, another human malignant epithelial cell line, had relatively high collagenolytic activity. As reported by previous workers (Harris, 1972), collagenolytic activities could be detected only when cells were grown in the absence of FCS.

The three ZR75 human breast tumor cells were selected for further studies. The period of culture when maximal collagenolytic activity occurs was determined. Cells were cultured and the medium was assayed for collagenolytic activity. The medium was either pretreated with or without 0.1% trypsin. Collagenolytic activity was consistently highest between day 2 and 4 under serum-free conditons in all of the ZR75 cell lines examined (fig. 4-1). Activity decreased during subsequent culture periods.

Partial purification of ZR75-31A collagenolytic protease was accomplished. Dialyzed culture medium was concentrated 20-fold and applied to a column of DEAE-cellulose (fig. 4-2). Three major peaks of collagenolytic activity were observed. Collagenolytic protease peaks I and II were not stable for further purification. Fractions of collagenolytic protease peak III were pooled and lyophilized. This protease fraction was reconstituted in 4 ml of distilled water and subsequently dialyzed against 50 mM Tris, 150 mM NaCl, 50 mM CaCl at pH 7.4. The sample was applied to an Ultragel AcA 54 column (fig. 4-3). One major peak and three minor peaks of collagenolytic activities were separated from the DEAE-cellulose peak III. The major band of collagenolytic activity had a molecular weight of approximately 60K. Throughout the purification, collagenolytic activity was observed only after each fraction had been trypsin-activated prior to the assay. The partial purification of ZR75-31A collagenolytic proteases is summarized in table 4-2. A 13-fold total purification was achieved.

Classical mammalian collagenases are unique in that they make a single scission in triple helical collagen (Gross, 1981). The cleavage site has been identified as a single Gly-Ile in collagen chain (position 775-776 in the amino acid sequence). Excision products of breast tumor collagenolytic protease were examined using a type I collagen substrate. A classic 3/4 and 1/4 product indicate this is a true collagenase(fig. 4-4).

Collagenolytic proteases are classically metalloproteinases. The partially purified fraction of the collagenolytic protease activity was subjected to inhibition studies (table 4-3). No significant inhibition was observed with serine and thiol protease inhibitors. However, metalloprotease inhibitors, serum, and serum components were effective collagenolytic protease inhibitors.

The substrate specificity of the cultured human breast tumor was determined. Degradation analysis of collagen type I, III, IV, and V was examined. Only collagen type I and III were degraded (table 4-4). SDS-polyacrylamide analysis revealed the degradation of type I and III into the multiple cleavage products after an incubation of 24 hrs (fig. 4-5). Multiple cleavage products were observed due to subsequent proteolytic digest of the 3/4 and 1/4 collagen fragments by contaminating neutral protease. No significant cleavage of collagen types IV and V was observed.

2. Elastinolytic Protease: Partial Purification and Characterization

Several lines of human breast cancer and stromal cell lines were grown and the conditioned media examined for elastinolytic activities (table 4-5). Elastinolytic activities were found in the media of all the human breast tumor cell lines examined. Elastinolytic activities were present only when cells were cultured in the absence of FBS. Very low activity was found in the media of fibroblasts. Fibroblasts from a variety of sources were examined. The RMF cells, fibroblasts obtained from normal breast tissues had low activity as did foreskin fibroblasts. HeLa cells, another human malignant epithelial cell line, was examined and also found to have low activity. The ZR75-31A cells produced the highest activity and was selected for further study.

The period of culture when maximal elastinolytic activity occurs was determined. Cells were cultured in the presence and absence of 4% FBS. Elastinolytic activity was highest between day 0 and 2 under serum free conditions for all ZR75 cell lines (fig. 4-6). Activity decreased during subsequent culture periods. We have observed that cells also have vacuolization and granularity of their cytoplasm by day 6 of growth under

serum free conditons. Activity collected between day 0 and 2 were selected for purification and further studies.

Elastinolytic enzymes fall into all classes of proteases, cysteine, serine, and metalloproteases (Werb et al., 1982). To characterize the present activities futher, partial purification was undertaken. Dialyzed culture medium was applied to a column of DEAE-cellulose and the proteins eluted with a shallow gradient of buffered NaCl. Each fraction was trypsin activated and assayed for elastinolytic activity. Three activities were separated (fig. 4-7) and designated protease I, II, and III. Since these cells also produce collagen-degrading activity (Kao et al., 1982b), collagenolytic assays were performed (fig. 4-2). Most of the collagenolytic activity was found in the column void volume and in the eluate following application of the high-salt wash. It cannot be concluded from these observations whether protease III also had a collagenolytic effect or was a mixture of more than one enzyme. These experiments suggested that breast tumor elastinolytic activity was the result of three separate enzymes, at least two of which had no associated collagenolytic activity. The latter two proteases were further purified.

Elastinolytic protease fractions I and II were lyophilized, reconstituted in 4 ml of distilled water, and dialyzed overnight against 50 mM Tris, 150 mM NaCl, 50 mM $CaCl_2$, pH 7.4. Each of the samples were then applied to an Ultragel AcA 54 column (fig. 4-8). Both protease I and II were eluted as a single major band of elastinolytic activity with a molecular weight of 24K and 31K, respectively. A summary of the purification of elastinolytic proteases I and II are shown in table 4-5. A 42-fold and 14-fold purification were achieved for proteases I and II respectively (table 4-6).

The two proteases were compared by inhibition studies (table 4-7). A number of inhibitors specific for cysteine, serine, and metalloprotease were used. Serum contains a number of protease inhibitors. Serum and serum components were also examined in the inhibition studies. Protease peak I was inhibited by serine protease inhibitors. Protease II activity decreased in the presence of metalloprotease inhibitors. Both protease I and II were sensitive to inhibition by serum and a inhibitor cocktail consisting of serine and metalloprotease inhibitors. Protease III gave mixed results suggesting the possiblity of a complex mixture (data not shown).

C. Effect of Hormones on Proteolytic Activities

In surveying the biosynthesis of elastinolytic and collagenolytic proteases, only the human breast cancer cells produced significant levels. The ability of hormones at physiological levels to influence the levels of proteolytic activity was determined. Physiological levels of estradiol (10^{-8}M), tamoxifen (10^{-6}M), progesterone (10^{-8}M), testosterone (10^{-8}M), hydroxycortisone (10^{-8}), or dexamethasone (10^{-8}M) was added to cultured breast tumor cells. Collagenolytic and elastinolytic activities were asssessed.

Alteration in collagenolytic activity was observed in the media of ZR75-1 cells grown in the presence of certain hormones (fig. 4-9). In the presence of estradiol and progesterone, a significant increase in enzymatic activities was observed. The addition of tamoxifen with estradiol resulted in a decreased level of collagenolytic activity. With trypsin pretreatment, the hormonal modulation effect was no longer observed. No hormonal modulation of collageolytic activity was observed with ZR75-30 and ZR75-31A cells, either with or without trypsin pretreatment.

The regulation of collagenase activity by estradiol and progesterone was further studied. Instead of utilizing only physiological levels of estradiol and progesterone (10^{-8}M), higher, pharmacological levels were used (table 4-8). No difference in collagenase activity was observed with trypsin pretreatment regardless of hormone concentration compared to controls. Hormonal modulation was observed only in the medium not pretreated with trypsin. With the addition of either estradiol or progesterone, the level of non-trypsin treated collagenolytic activity was proportional to the level of hormone present. Simultaneous addition of estradiol and progesterone resulted in neither an additive nor synergistic effect.

With hormones, an alteration of elastinolytic activites was observed in the ZR75-1 cells but not in either the ZR75-30 or ZR75-31A cells (table 4-9). Decreases in elastinolytic activities was observed when ZR75-1 cells were grown in the presence of a combination of all hormones.

D. Discussion

Collagenase activity in human breast tumor cells was previously reported by Paranjpe et al. (1980). In this chapter, we have confirmed and extended those observations and have characterized the collagenase synthesized by human breast tumor cells.

Cultured in the absence of FBS, collagenolytic activities were detected in all breast epithelial tumor cell lines examined. The levels of collagen degradation were 4-10 fold higher than in stromal cell lines, and were comparable to levels reported by Paranjpe et al. (1980). Cultures grown in the presence of FBS possessed relatively low collagenolytic activity. This is consistent with reports that collagenolytic activity in rabbit synovial fibroblasts (Harris, 1972) can be detected only when cultured

in serum-free medium. It is not clear whether the decreased collagenolytic activity was due to the suppression of collagenolytic protease synthesis or the presence of collagenolytic protease inhibitors. Inhibition experiments with breast tumor collagenolytic proteases indicated that the addition of FBS can inhibit collagenolytic activity. This inhibition accounted for the difference observed between cells grown in the presence and absence of FBS, This suggested indirectly that collagenolytic protease inhibitors may mask the presence of collagenolytic proteases.

A number of serum inhibitors may regulate collagenolytic proteases. Woolley et al. (1975) has found a 40K molecular weight β_1-serum protein that specifically inhibits collagenase. Whole serum also contain α_2-macroglobulin (725K) which also inhibits collagenase (Werb, 1974).

Collagenolytic activity in all breast tumor cells was dependent on enzymatic activation. Without trypsin activation, less than 5% of the total possible collagenolytic activity was observed. Trypsin activation has been shown by Strickland et al. (1983) to be caused by destabilization of the cleaved proenzyme. It is this destabilized intermediate form which converts into the catalytic form. The conversion of collagenase proenzyme can be mediated by thermolysin, chymotrypsin, papain, and plasmin (Vaes, 1972; Eeckhout and Vaes, 1977; Werb et al., 1977). Without this conversion, little collagenolytic activity can be observed. The activity observed with trypsin activation is reflective of the total collagenase produced by the cell. The collagenolytic activity that one observes without enzymatic activation is reflective of the system's ability to convert procollagenase to collagenase. These previous findings apparently are applicable to the breast tumor collagenolytic protease system.

In order to characterize the breast tumor cell collagenolytic protease, this enzyme was partially purified from the medium of ZR75-31A cells. A purification of only 13-fold was accomplished. A single major peak with three minor peaks resulted from the gel filtration chromatography. The major collagenolytic band had a calculated molecular weight of 60K. This is consistent with the molecular weight range reported for collagenase purified from rat uterus (Woessner et al., 1977) and human skin fibroblasts (Stricklin et al., 1977).

A unique feature of mammalian collagenases is the ability to make a single proteolytic scission of collagen into two reaction products. The larger peptide (75% of the molecule) is called TC^A and the smaller peptide (25% of the molecule) is called TC^B. A molecular sieve chromatogram of the human breast tumor protease digests demonstrated the appropriate reaction products were generated, and that a true collagenase-type cleavage was occurring. Minor bands were also detected. These are probably due to subsequent proteolysis of the TC^A and TC^B fragments by contaminating proteases such as neutral proteases (Harper, 1970; Harris , 1972), cathepsins (Etherington, 1976; Gibson et al., 1978), gelatinase (Sopata and Dancewicz, 1974; Sellers et al., 1978), or collagen peptidases (Strauch, 1968) present in the preparation.

Inhibition studies indicated that, similar to other mammalian collagenases, the human breast tumor collagenase is a metalloprotease. Addition of either serine and thiol protease inhibitors were ineffective. Inhibition was observed with serum and has been discussed previously.

Substrate specificity of the human breast tumor collagenase was analyzed. Enzymatic assay and digest analysis indicated that only type I and III collagens were susceptible to collagenolytic degradation. No activity

was observed with type IV and V collagen. There appears to be no difference in preference of the human breast collagenase for either type I or type III collagen. This is consistent with the findings by McCroskery and coworkers (1975). There are collagenases, however, in which rate of cleavage of the various types of collagen can differ. Horwitz et al. (1977) have shown that human leukocytes preferentially cleave type III collagen over type I. Thus, it appears that there are two groups of classical mammalian collagenases, those that digest collagen with no preference for type I or III, such as the human breast tumor collagenase, and those that demonstrate preference for a particular collagen type, such as the human leukocyte collagenase.

In the present chapter, I also present evidence that human breast tumor cells in culture secrete into the media elastase-like enzymes. This type of elastinolytic activity has been demonstrated previously by Hornebeck and Robert (1977) in extracts of human breast carcinomas. The source of the elastinolytic protease in those experiments was not clear. Experiments presented here demonstrate that the elastinolytic protease is elaborated by the malignant epithelial cells. Relatively little elastinolytic activity was present in either host fibroblasts obtained from the malignant tumor mass (PTF) or in mammary fibroblasts obtained from reduction mammoplasty (RMF). This observation corroborates the work of Gullino and coworkers (1962, 1963), that it is the neoplastic cell population that regulates the overall scleroprotein produced in the neoplastic mass and not the stromal cells.

A partial purification of the elastinolytic activities revealed that there are at least two proteases. One, a serine protease with a molecular weight of 24K, and the other a metalloprotease with a molecular weight of

31K. Each exists in an inactive form and requires trypsin pretreatment for activity.

Cultures grown in the presence of FCS possessed relatively low elastinolytic activity. Once again, it is not certain whether this is a reflection of protease inhibition or the suppression of protease synthesis. The level of inhibition was proportional to the amount of serum present. It must be emphasized that other serum components such as α_2-macroglobulin may inhibit elastinolytic activities. α_2-macroglobulins have been shown to be effective inhibitors of pancreastic, leukocyte, and macrophage elastase (Banda and Werb, 1981). This may account for some of the inhibition observed with addition of serum.

The regulation of collagenase by hormones has been demonstrated in several laboratories. Jeffrey et al. (1971) found that a pharmacological dose of progesterone (10^{-6}M) when added to the media of cultured uterine explants inhibits collagenase synthesis. Estrogen, however, appears to potentiate collagenase synthesis. Contrary to Jeffrey's work, Woessner (1969) and Ryan and Woessner(1974) were able to inhibit collagenase synthesis by uterine explants by injection of estrogen. The synthesis of collagenase by macrophages has been shown to be inhibited by estradiol, progesterone (Wahl et al., 1974), and dexamethasone (Werb, 1978), and stimulated by prostaglandin and cyclic-AMP (Dayer, 1976).

The regulation of elastase by hormones has not been examined previously. The only study in this area is the regulation of macrophage elastase by glucocorticoids (Werb, 1978). The production and the release of elastase by mature macrophages have been shown to be inhibited by physiological concentration of glucocorticoids.

For this thesis, no consistent pattern of hormonal modulation of elastinolytic activities was observed. Hormones also had little influence on collagenolytic activities observed with the ZR75-30 and ZR75-31A cells. Hormone addition had no effect on collagenase activity in the ZR75-1 cells when medium was pretreated with trypsin but without trypsin pretreatment, the addition of either estradiol or progesterone to ZR75-1 cultures resulted in increased collagenolytic activities. The level of activity present was dependent on the concentration of the hormone. No additive or synergistic effect was observed with the simultaneous addition of estradiol and progesterone.

Three possible mechanisms can explain the hormone-dependent increase in collagenase activity. First, the hormone may regulate the synthesis or release of collagenase. Second, the hormone may decrease the levels of collagenase inhibitors. Lastly, the enzyme that converts procollagenase to collagenase may be the hormone dependent reaction.

Studies by various investigators suggest that hormones may directly affect the synthesis or release of collagenase. Koob and Jeffrey (1974) found that prostaglandins and cyclic-AMP inhibit collagenase production in post-partum rat uterine tissue culture. Ryan and Woessner (1971, 1974) have shown that estradiol and progesterone inhibit the production or release of collagenase present in involuting uterus. Newsome and Gross (1977) have shown that Provera decreases the activity of corneal collagenase via the inhibition of enzyme release by tissue. Despite several lines of evidence suggesting that hormones may influence collagenase production or release, our data does not favor this possibility. Trypsin treatment activated the total collagenase pool present in the medium. In the presence of estradiol or progesterone, no increase was observed with

trypsin pretreatment. This suggested that the total collagenase pool did not change. Thus estradiol and progesterone probably had little influence on the synthesis and secretion of collagenase. It was the collagenolytic activities observed without trypsin pretreatment that increased. As mentioned previously, this is reflective of procollagenase activation. Thus, the addition of these two hormones resulted in the activation of the procollagenase pool.

Several inhibitors may regulate collagenase in vivo. Serum protease inhibitors such as β_1-microglobulin, and α_2-macroglobulin have been previously discussed as inhibitors of collagenolytic activities. It is conceivable that the tumor epithelial cells synthesize these inhibitors to regulate the activity of newly synthesized collagenase. The tumor cells may also produce inhibitors yet to be characterized. Collagenase inhibitors have been observed in several tissue including human rheumatoid synovium (Harris, 1978), human skin (Welgus et al., 1978), rabbit tissue (Murphy et al., 1981), and tumors (McCroskery et al., 1975). The presence of a collagenase inhibitor that decrease in the presence of estradiol and progesterone is a hypothesis that remains to be tested.

Another possiblity is that hormones may modulate collagenase activators. Recent work by Vater and coworkers (1983) indicated that synovial cell procollagenase exists in both a high and low M_r form. The low M_r form corresponds to that of collagenase after proteolytic or organic mercurial compound treatment. These workers demonstrated that the high M_r procollagenase may be proteolytically converted to the low M_r form without activation of its catalytic potential. Only when an activator is added to the procollagenase of either M_r form does the enzymes become capable of degrading the collagen substrate. This activator can be purified in the

latent form (M_r=53.4K) from the culture medium of monosodium urate monohydrate- or phorbol- stimulated rabbit synovial cells. Activation of this latent activator with either trypsin or organic mercurial compounds results in a lower M_r form (M_r=51.9K). This activator, itself, has no collagenolytic activity but is capable of converting the procollagenase into collagenase. Hormones may influence either the synthesis of this activator or its conversion from the latent to the active form.

Alternatively, estradiol and progesterone may influence the conversion of the latent collagenase activators to the active form. Werb et al. (1977) have proposed plasmin as one possible activator of the latent form of collagenase in human rheumatoid synovium. Gross and coworkers (Gross et al., 1982, 1983) have demonstrated the modulation of both endothelial cell plasminogen activator and collagenase in the presence of an angiogenic factor. The purpose of inducing elevated levels of plasminogen activator is to activate the plasminogen pool. It is the activation of this plasminogen pool by plasminogen activator which accounts for the conversion of procollagenase to collagenase. This endothelial cell plasminogen activator has been characterized as being both urokinase-like and capable of activating endothelial cell collagenase. The presence of urokinase-like plasminogen activator has been demonstrated in breast tissue (Evers et al., 1982). Plasminogen activators have also been reported in cultured breast tumor cells (Paranjpe et al., 1980). The possiblity that estradiol and progesterone may regulate procollagenase activation by influencing the level of plasminogen activator, is investigated further in the following chapter.

Table 4-1: Survey of Collagenolytic Activities in Cultured Cells

Cell Type	Collagen Degradation (Cells Cultured + 4% FBS) mU / 5 x 10^6 cells	Collagen Degradation (Cells Cultured - FBS) mU / 5 x 10^6 cells
ZR75-1	12 ± 6	281 ± 14
ZR75-30	8 ± 6	196 ± 14
ZR75-31A	18 ± 11	496 ± 12
MCF-7	14 ± 8	268 ± 16
NBF	16 ± 10	48 ± 12
RMF	13 ± 12	42 ± 14
HeLa	28 ± 14	526 ± 24

Table 4-1: Survey of collagenolytic activities in various human cell cultures. Cells were plated at a density of 5 x 10^6 cells per well. After 24 hrs, medium with or without FBS was used. The media was pretreated with 0.025% trypsin for 30 min followed by 0.125% SBTI treatment. The activities represent the mean of experiments in triplicate ± S.D.

Table 4-2: Summary of Breast Tumor Collagenase Purification

Method	Volume (ml)	Total Protein (µg)	Total Activity (U)	Specific Activity (U/mg)	Yield	x fold Purification
Culture Medium	200	6450	1127.8	173.6	100%	1.0
Amicon P10	10	4761	969.4	203.6	86%	1.2
DEAE Chromatography	28	761	703.9	924.9	62%	5.3
Ultragel AcA 54	6	284	642.4	2261.9	57%	13.0

Table 4-3: Inhibition profile of collagenase activity separated by Ultragel chromatography. The collagenase was trypsin activated and incubated with the inhibitor at the final concentration shown for 15 min. PBS was added for the control. Collagenase assay was performed for 4 hr. The data depicted represents the mean of triplicate assays ± S.D.

Table 4-3: Inhibition Profile of Breast Tumor Collagenase

Inhibitor		% of Control
*Serine & Thiol Protease Inhibitors		
iPr_2P-F	1 mM	97.8
PMSF	1 mM	99.3
SBTI	1 mg/ml	94.7
4-chloromercuribenzoate	1 mM	97.6
NEM	1 mM	97.9
Mersalyl	1 mM	96.1
*Metalloprotease Inhibitors		
EDTA	0.1 mM	35.6
	1.0 mM	11.1
EGTA	0.1 mM	42.5
	1.0 mM	17.5
1,10 Phenanthroline	0.1 mM	27.5
	1.0 mM	20.1
*Serum		
Human Serum	0.5 %	30.3
Fetal Bovine Serum	0.5 %	29.1
	5.0 %	11.4
	10.0 %	3.2

Table 4-4: Substrate Specificity of Breast Tumor Collagenase

Collagen Type (source)	Bacterial Collagenase collagen degradation (cpm)	Breast Tumor Collagenase collagen degradation (cpm)
Type I (rat skin)	107,035 ± 278	43,675 ± 156
Type III (human fetal skin)	104,895 ± 318	34,665 ± 148
Type IV (bovine anterior lens)	124,455 ± 298	4,435 ± 158
Type V (human placenta)	100,740 ± 286	4,850 ± 147

Table 4-4: Survey of human breast tumor collagenase substrate specificity. Sources for type I, III, IV, and V collagen were rat skin, human fetal skin, bovine anterior lens, and placenta, respectively. Collagen was labeled exogenously with ^{3}H-acetic anhydride and the specific activity adjusted to to 1,000 - 1,250 kcpm/mg of collagen. Degradation is presented as cpm collagen degraded. The mean of triplicate assays ± S.D. is shown.

Table 4-5: Survey of Elastinolytic Activities in Cultured Cells

Cell Type	Elastin Degradation (Cells Cultured + 4% FBS) mU / 5 x 10^6 cells	Elastin Degradation (Cells Cultured - FBS) mU / 5 x 10^6 cells
ZR75-1	317 ± 14	3, 263 ± 18
ZR75-30	508 ± 14	2, 806 ± 13
ZR75-31A	455 ± 16	5, 731 ± 16
MCF-7	186 ± 13	1, 426 ± 14
NBF	131 ± 11	578 ± 18
PTF	74 ± 13	328 ± 18
RMF	69 ± 12	142 ± 8
HeLa	138 ± 14	476 ± 16

Table 4-5: Survey of elastinolytic activities in various human cell cultures. Cells were plated at a density of 5 x 10^6 cells/well. After 24 hrs, medium with or without FBS was used. The activities seen here represent the mean of experiments in triplicate, from three separate cultures. Activities are presented ± S.D.

Table 4-6: Summary of Breast Tumor Elastase Purification

*Breast Tumor Elastase I

Method	Volume (ml)	Total Protein (μg)	Total Activity (U)	Specific Activity (U/mg)	Yield	x-fold Purification
Culture Medium	200	6450	1,150	178	100%	1.0
Amicon P10	10	4761	989	208	86%	1.2
DEAE Chromatograph	20	328	621	1894	54%	10.6
Ultragel AcA 54	8	79	592	7484	51%	42.0

*Breast Tumor Elastase II

Method	Volume (ml)	Total Protein (μg)	Total Activity (U)	Specific Activity (U/mg)	Yield	x-fold Purification
Culture Medium	200	6450	1,150	178	100%	1.0
Amicon P10	10	4761	988	207	87%	1.1
DEAE Chromatograph	12	379	372	981	32%	5.5
Ultragel AcA 54	8	148	358	2418	31%	13.6

Table 4-7: Inhibition profile of elastinolytic proteases I and II separated by Ultragel chromatography. The elastase was trypsin-activated and incubated with the inhibitor at the final concentration shown for 15 min. PBS was added for the control. Elastase assay was performed for 16 hr. The data depicted represents the mean of triplicate assay. The data are expressed in percentage observed relative to control.

Table 4-7: Inhibition Profile of Elastinolytic Protease I and II

Inhibition		Protease I (% of control)	Protease II (% of control)
*Serine Protease Inhibitors			
iPr_2P-F	0.1 mM	23.0	100.4
	1.0 mM	8.8	99.3
PMSF	0.1 mM	18.9	93.9
	1.0 mM	7.3	101.3
SBTI	0.1 mg/ml	38.1	100.0
	1.0 mg/ml	18.9	103.1
*Thiol Protease Inhibitors			
4-Chloromercuribenzoate	0.5 mM	95.5	105.2
	1.0 mM	100.2	103.8
NEM	0.5 mM	101.5	97.1
	1.0 mM	97.6	97.4
Mersalyl	0.5 mM	97.6	97.7
	1.0 mM	91.5	98.5
*Metalloprotease Inhibitors			
EDTA	1.0 mM	99.3	10.8
	10.0 mM	99.9	6.3
EGTA	1.0 mM	97.6	23.0
	10.0 mM	98.7	3.3
1,10 Phenanthroline	1.0 mM	98.9	31.5
	10.0 mM	99.5	18.3
*Serum			
Human Serum	0.5 %	40.7	41.2
Fetal Bovine Serum	0.5 %	40.7	42.0
	5.0 %	12.4	9.7
	10.0 %	4.3	1.2

Table 4-8: Estrogen and Progesterone Effect on Breast Tumor Collagenase Activity

Hormone Concentration	Collagenase Activity (% of control)		
	+ Estradiol	+ Progesterone	+ Estradiol Progesterone
10^{-6} M	1, 299 %	1, 092 %	1, 247 %
10^{-8} M	1, 144 %	989 %	1, 097 %
10^{-10} M	967 %	821 %	941 %
10^{-12} M	744 %	689 %	807 %

Table 4-8: Estrogen and progesterone effect on ZR75-1 collagenase activity. Cells were exposed to either estradiol, progesterone, or both at various concentrations including physiological (10^{-8} M) and pharmacological (10^{-6} M) levels. Samples were not trypsin activated. The data are presented in terms of percentage of collagenolytic activities observed relative to control cultures. Control cultures were cells grown in the absence of hormones. The mean of triplicate assays are presented.

Table 4-9: Hormonal Effects on Breast Tumor Elastinolytic Activity

Hormones	ZR75-1	ZR75-30	ZR75-31A
*Control	2.24 ± 0.72	2.84 ± 0.19	5.57 ± 0.58
*Estradiol (10^{-8}M)	0.82 ± 0.25	2.43 ± 0.18	5.21 ± 0.31
*Tamoxifen (10^{-8}M)	1.32 ± 0.26	2.21 ± 0.16	4.62 ± 0.51
*Estradiol (10^{-8}M) plus Tamoxifen (10^{-8}M)	1.87 ± 0.11	2.52 ± 0.10	5.01 ± 0.41
*Progesterone (10^{-8}M)	0.89 ± 0.13	2.37 ± 0.25	4.83 ± 0.42
*Testerone (10^{-8}M)	1.56 ± 0.26	2.34 ± 0.24	5.27 ± 0.31
*HO-cortisone (10^{-8}M)	2.00 ± 0.26	1.83 ± 0.26	4.81 ± 0.12
*Dexamethasone(10^{-8}M)	0.70 ± 0.13	1.21 ± 0.29	4.21 ± 0.16

Table 4-9: Hormonal effects on breast tumor cell elastinolytic activity. Physiological levels of hormones were used as described in fig. 4-9. The mean of triplicate assays and S.D. are depicted.

Fig. 4-1: Timecourse of collagenolytic protease synthesis. Cells were plated with 5 X 10^6 cells per 35 mm plates. After 24 hrs, medium without FCS was used for culturing. After every 48 hrs, the medium was harvested and fresh medium replaced. Collagenolytic activities were determined with and without trypsin pretreatment. The mean of experiments in triplicate from three separate cultures are presented ± S.D.

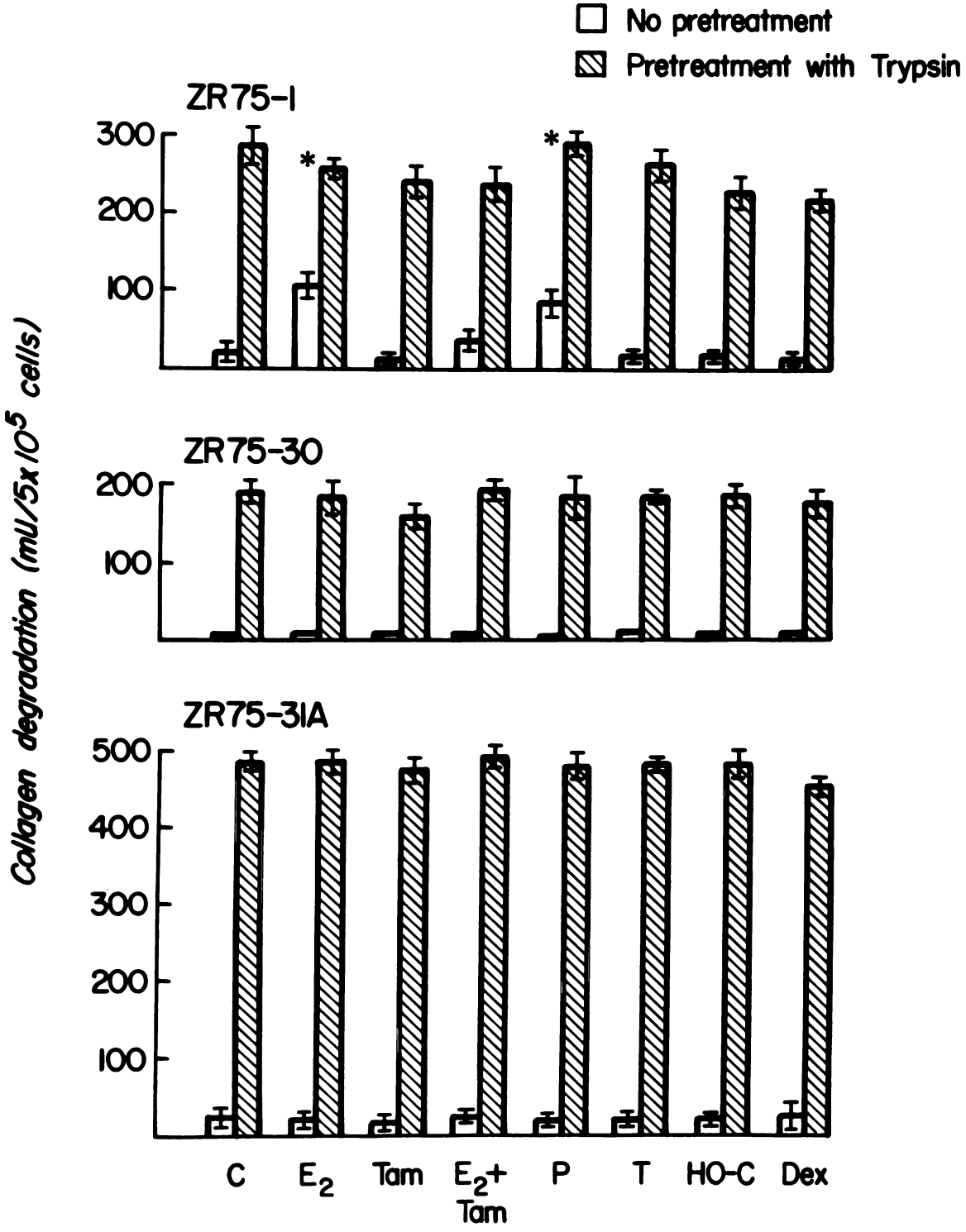

No pretreatment
Pretreatment with Trypsin
ZR75-1
ZR75-30
ZR75-31A
Collagen degradation (mU/5x10^5 cells)
300
200
100
500
400
C
E2
Tam
E2+ Tam
P
T
HO-C
Dex
*

Fig. 4-2: DEAE-cellulose chromatography of collagenolytic activity. Sample was applied to a 1 x 25 cm column. The NaCl gradient is depicted in the inset panel. Collagenolytic activity is shown as a solid line and absorbance at 280 nm is represented by the dotted line.

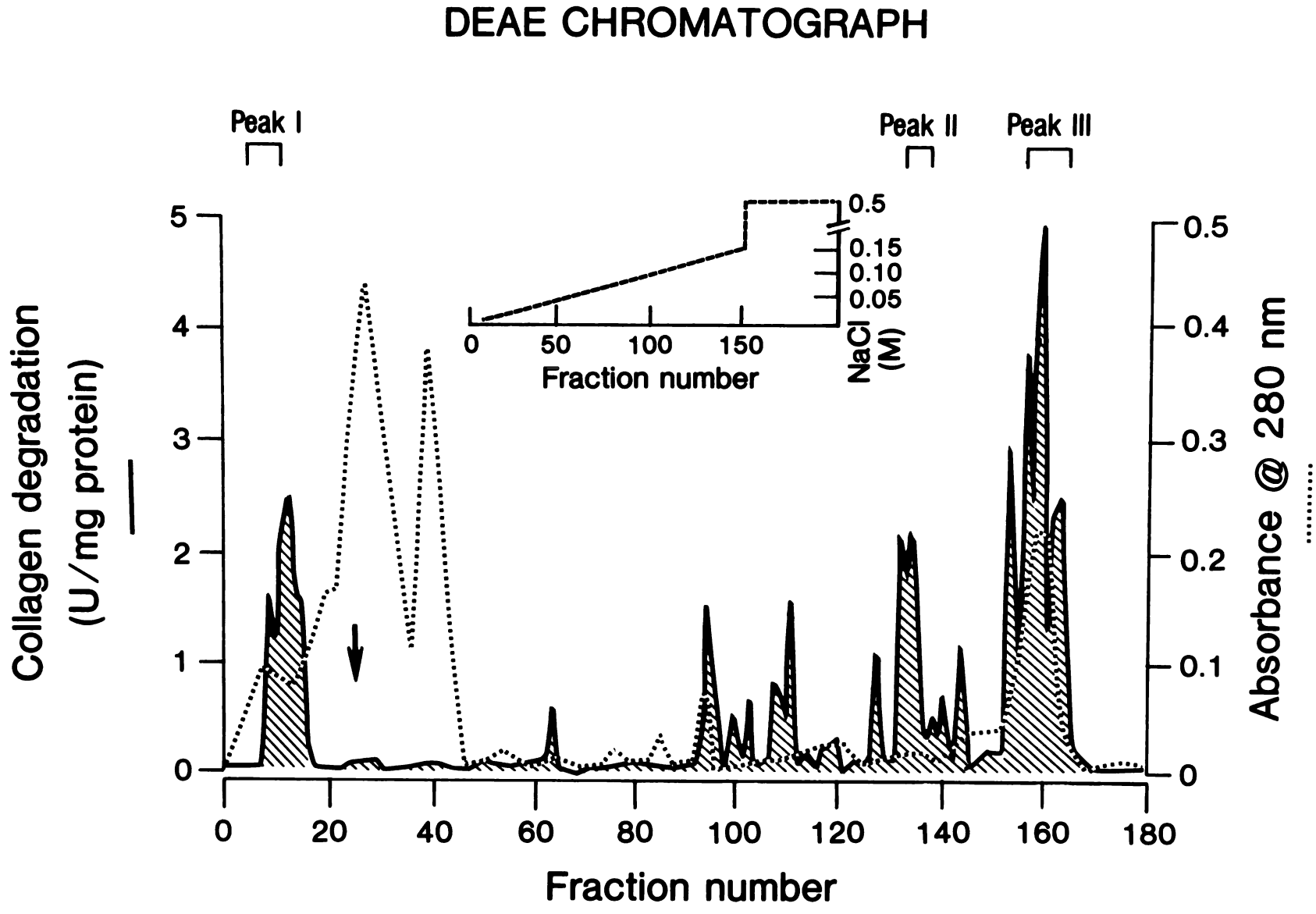

DEAE CHROMATOGRAPH
Peak I
Peak II
Peak III
Collagen degradation
(U/mg protein)
Absorbance @ 280 nm
Fraction number
NaCl (M)
0.5
0.15
0.10
0.05
0
50
100
150
0
20
40
60
80
100
120
140
160
180
1
2
3
4
5
0.1
0.2
0.3
0.4

Fig. 4-3: Ultragel AcA 54 chromatograph of collagenolytic activity. Sample was applied to a 1.5 x 100 cm column. Collagenolytic activity is shown as a solid line and absorpance at 280 nm is represented by the dotted line.

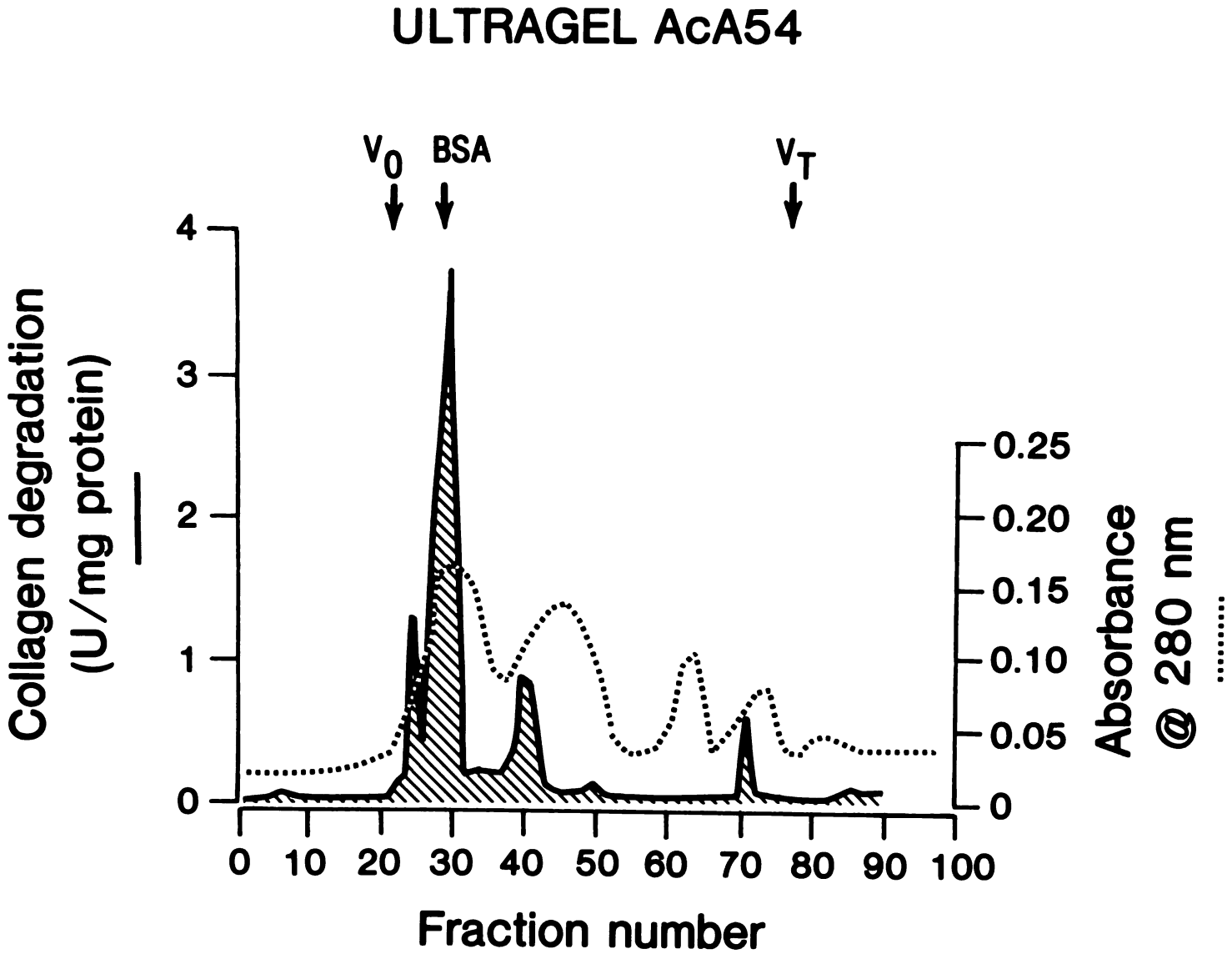

ULTRAGEL AcA54
V_0
BSA
V_T
Collagen degradation
(U/mg protein)
Absorbance
@ 280 nm
0
1
2
3
4
0.25
0.20
0.15
0.10
0.05
0
0 10 20 30 40 50 60 70 80 90 100
Fraction number

Fig. 4-4: SDS-PAGE profile of human breast tumor collagenase digest product. Lane A: type I collagen without digestion (15 μg); Lane B: collagen after 3 hr of digestion by breast tumor collagenase (30 μg); and Lane C: collagen after 3 hr of digestion by breast tumor collagenase (15 μg).

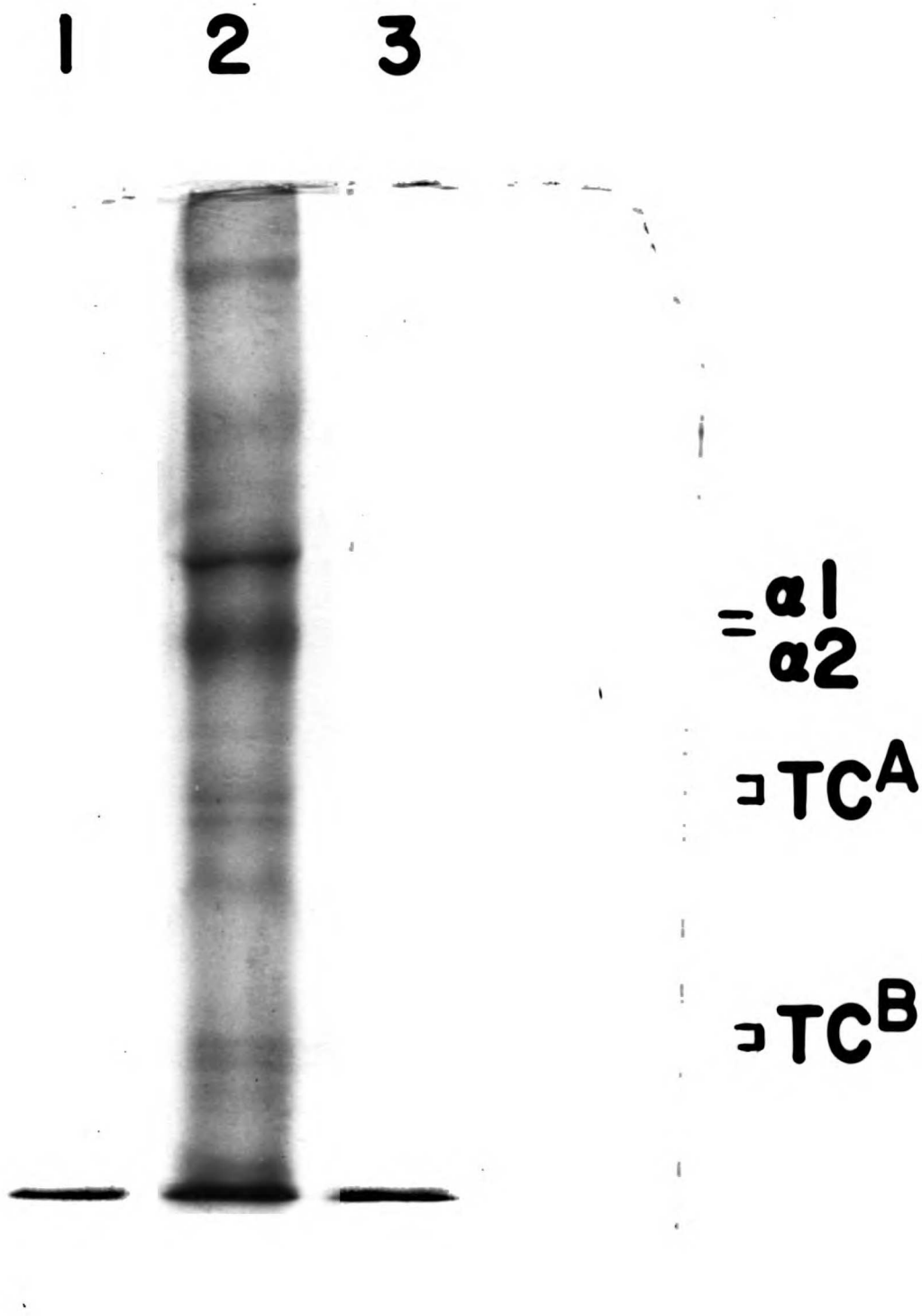

1
2
3
α1
α2
TC^A
TC^B

Fig. 4-5: SDS-PAGE profile of human breast tumor collagenase digest. Source for collagen have been previously described in the legend for Table 4-4. Lane 1: type I collagen digest; Lane 2: type III collagen digest; Lane 3: type IV collagen untreated; Lane 4: type IV collagen digest; Lane 5: type V collagen untreated; and Lane 6: type V collagen digest.

1 23456

Fig. 4-6: Timecourse of elastinolytic protease synthesis. Cells were plated 5 x 10^6 cells / 35 mm plates. After 24 hrs, medium without FBS was used for culturing. After every 48 hr, the medium was harvested and fresh medium replaced. Elastinolytic activities were determined with and without trypsin pretreatment. The mean of triplicate from three separate cultures are presented ± S.D.

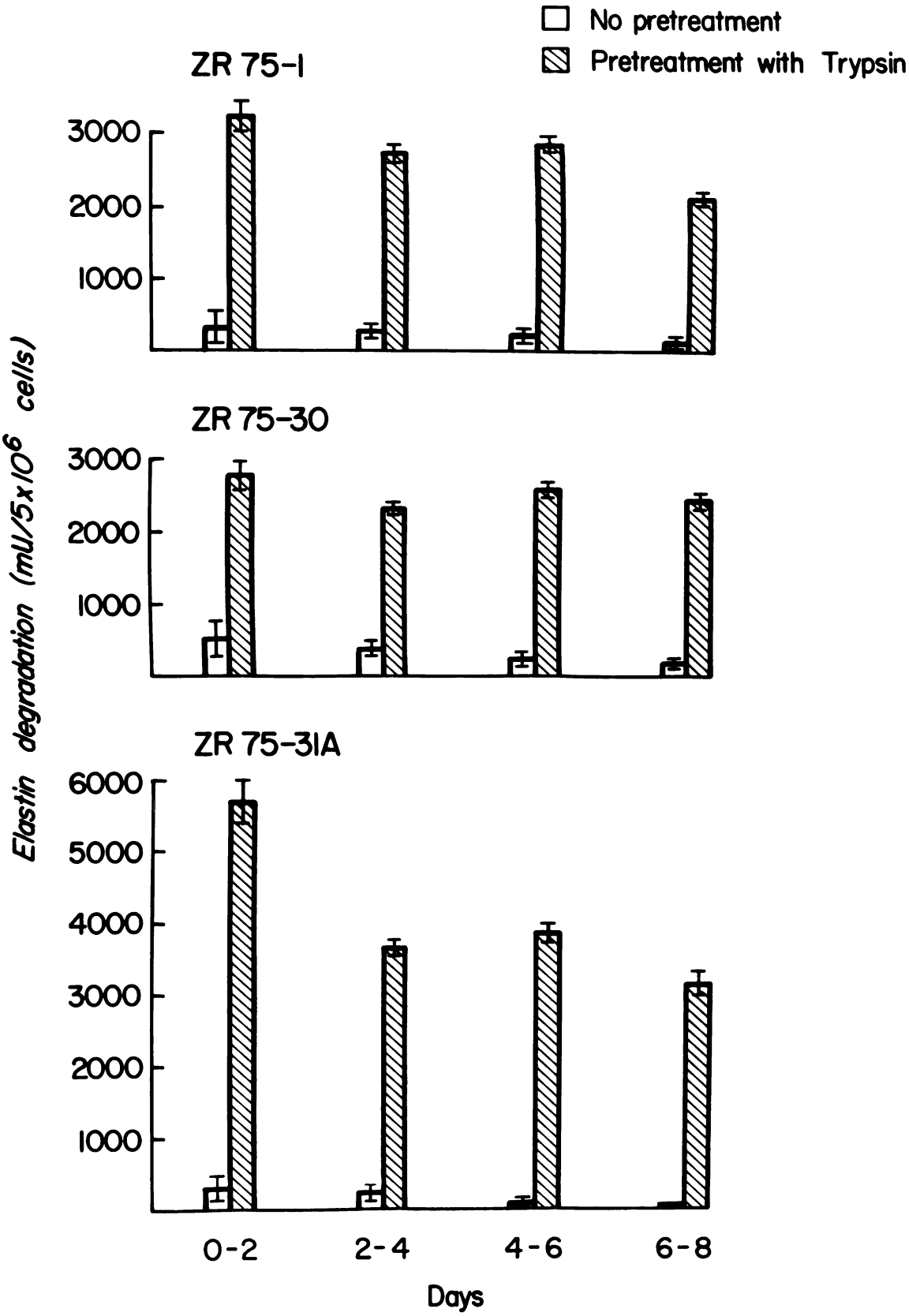

No pretreatment
Pretreatment with Trypsin
ZR 75-1
3000
2000
1000
ZR 75-30
3000
2000
1000
ZR 75-31A
6000
5000
4000
3000
2000
1000
Elastin degradation (mU/5x10^6 cells)
0-2
2-4
4-6
6-8
Days

Fig. 4-7: DEAE chromatograph of elastinolytic activity. Sample was applied to a 1 x 25 cm column. The NaCl gradient is depicted in the inset. Elastinolytic activity is shown as a solid line and absorpance at 280 nm is represented by dotted line.

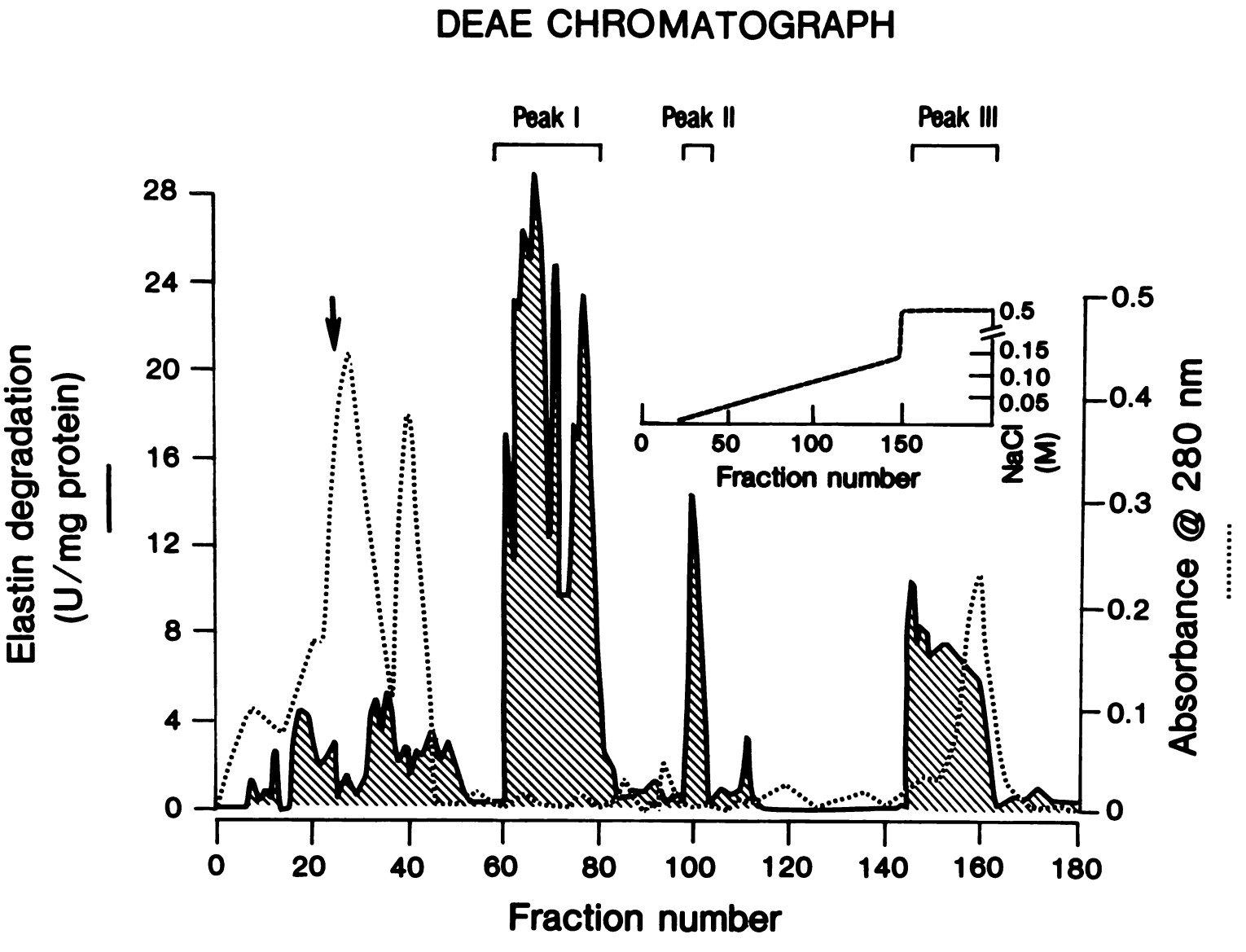

DEAE CHROMATOGRAPH
Peak I
Peak II
Peak III
Elastin degradation
(U/mg protein)
Absorbance @ 280 nm
Fraction number
NaCl
(M)
0
4
8
12
16
20
24
28
0
20
40
60
80
100
120
140
160
180
0.1
0.2
0.3
0.4
0.5
0.15
0.10
0.05
50
150

Fig. 4-8: Ultragel AcA 54 chromatograph of elastinolytic protease I and II. Sample were applied to separate 1.5 X 100 cm columns. Elastinolytic activity is shown as a solid line and absorpance at 280 nm is represented by the dotted line.

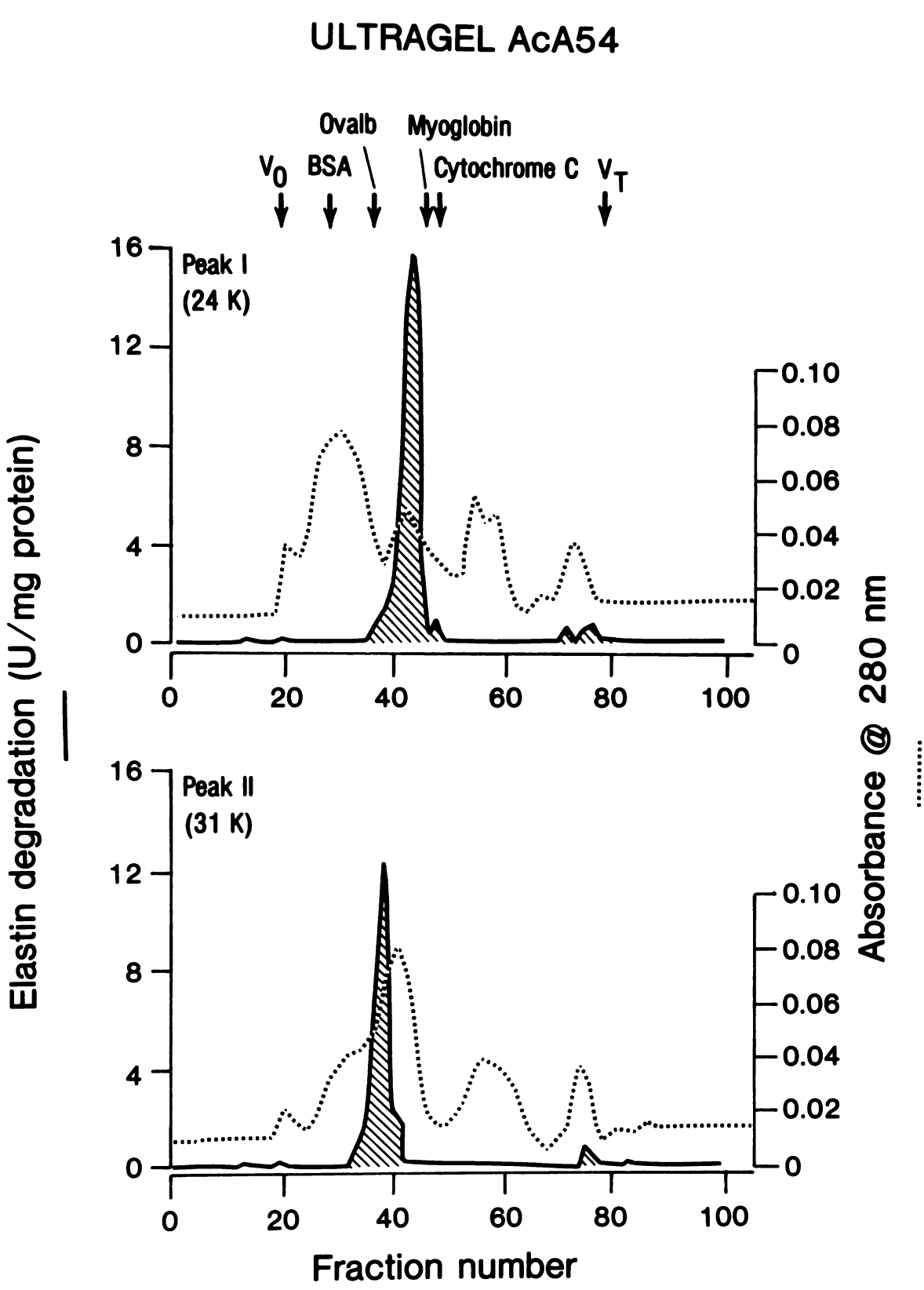
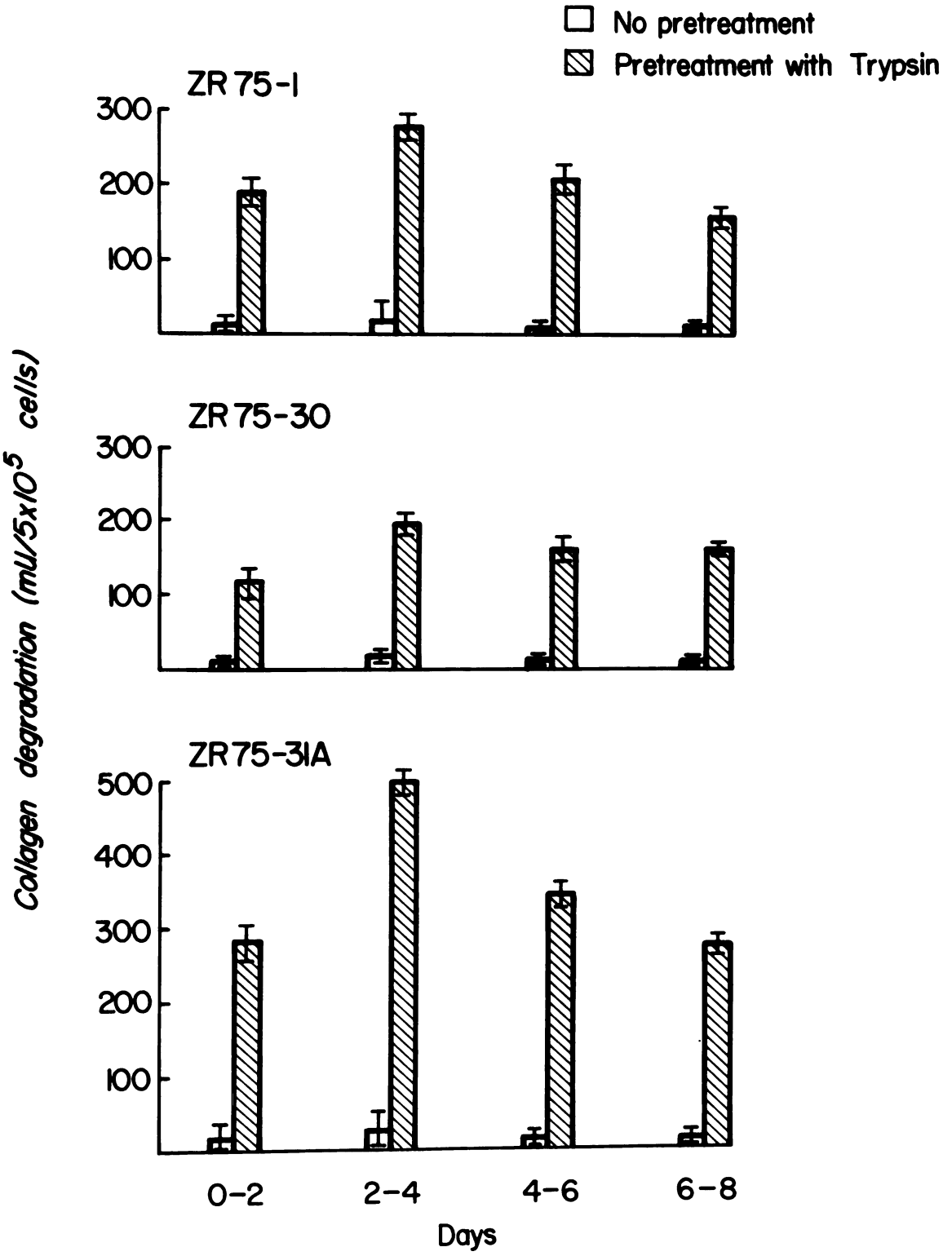

ULTRAGEL AcA54
Ovalb
Myoglobin
V_0
BSA
Cytochrome C
V_T
Peak I
(24 K)
Peak II
(31 K)
Elastin degradation (U/mg protein)
Absorbance @ 280 nm
Fraction number
0
4
8
12
16
0
20
40
60
80
100
0
0.02
0.04
0.06
0.08
0.10

Fig. 4-9: Hormonal effect on breast tumor cell collagenolytic activity. Medium were either pretreated with trypsin for 30 min or not treated. Levels of hormones used were estradiol (10^{-8} M); tamoxifen (10^{-8} M); progesterone (10^{-8} M); hydroxycortisone (10^{-8} M); and dexamethasone (10^{-8} M). All hormones used were physiological levels. The mean of triplicate assays ± S.D. is depicted. * $p < 0.01\%$.


No pretreatment
Pretreatment with Trypsin
ZR 75-1
ZR 75-30
ZR 75-31A
300
200
100
500
400
0-2
2-4
4-6
6-8
Days
Collagen degradation (mU/5x10^5 cells)

CHAPTER V. HORMONAL MODULATION OF PLASMINOGEN ACTIVATOR

A. INTRODUCTION

Various aspects of cell behavior can be attributed to plasminogen activator activity (PA). This enzyme activates several serum zymogens including plasminogen (for review, see Reddy and Kline, 1980). Differentiation of tissue and the expression of the transformed state in neoplasia may be dependent on plasminogen (Ossowski et al., 1973, 1974; Reich, 1973; Pollack et al., 1974, 1975). These putative plasminogen-mediated changes in phenotype are thus regulated by various plasminogen activators.

The enzyme plasmin, responsible for the dissolution of the fibrin clot, circulates in blood as the inactive zymogen plasminogen. The mechanism of conversion of inactive plasminogen to active plasmin involves proteolytic cleavage of plasminogen. This conversion is mediated by proteases that are present in many tissues (tissue activators), in blood (blood activators), in urine (urokinase), and in other body fluids.

Blood obtained from victims of sudden or violent death either does not clot at all, or if it does, the clot dissolves very rapidly, This activity is due to the release of a plasminogen activator from the walls of blood vessels. Fearnley and Tween (1953) report that fibrinolytic activity appears in the blood of healthy individuals following various physiological and pharmacological stimuli. The activator is present in exceedingly low levels in normal individuals. This may be the reason that the blood activator has not yet been isolated and characterized.

Another activator which appears in the circulation is the Hageman Factor-dependent plasminogen activator. Ratnoff and co-workers (1955, 1958) report that during the activation of Hageman Factor which results in the clotting of

blood, there is also the generation of fibrinolytic activity (Niewiarowski and Prou-Wartelle, 1959; Iatrides and Ferguson, 1961,1962). Ogston et al. (1969) found that activated Hageman Factor is capable of activating the conversion of prekallikrein to kallikrein, an enzyme in plasma which possesses plasminogen activating activity. Activation of Hageman Factor involves proteolytic cleavage of the native molecule into one or more active fragments. The resulting fragments can activate prekallikrein to kallikrein which in turn convert plasminogen to plasmin (Ogston et al., 1969; Laake and Vennerod, 1974; Saito et al., 1974; Weiss et al., 1974).

Plasminogen activators may be found in various tissues of the body and have been categorized as tissue activators. To date, tissue activators have been found in human uterus, adrenals, lymph nodes, prostate, thyroid, lung, ovary, pituitary, kidney, and skeletal muscle. The activator activity of tissues from any organ in one species is found to be the same molecule which is bound to cellular elements. Todd (1959) found that vascular endothelial cells are the source of plasminogen activator in tissues.

MacFarlane and Pilling (1947) were the first to report the presence of fibrinolytic activity in normal urine. This activity is named urokinase. The existence of different molecular forms of urokinase has been established by various purification techniques (White et al., 1966; Ogawa et al., 1975; Lesuk et al., 1965), and by immunological methods (Ball and Day, 1970). Two forms of urokinase has been established with a M_r=47-54K and 31-34K. The lower molecular weight form is believed to be the result of proteolytic degradation during purification. The 54K urokinase consist of two polypeptide chains linked by a disulfide bond. Proteolytic cleavage or autocatalysis results in the formation of a 33K form of urokinase (Soberano et al., 1976).

Kwan and coworkers (Barlow and Lazer, 1972; Bernik et al., 1974) demonstrated that the kidney is the major source of urokinase. The activation of human plasminogen by urokinase involves the cleavage of an arg-val peptide bond in the plasminogen molecule. This cleavage, though trypsin-like in specificity, is not inhibited by pancreatic trypsin inhibitor or soybean trypsin inhibitor (Walton, 1965).

The presence of plasminogen activator in the medium of cultured cells has been reported by several investigators. Barrett and Baron (1959) and Painter and Charles (1962), have shown that the activator obtained from kidney cell cultures is closely related if not identical to urokinase. Studies on the plasminogen activator from cultured human fetal lung, ureter, and renal blood vessels indicate that the same urokinase may be produced by many different tissues of the body (Bernik et al., 1969). Malignant cells in culture possess both intracellular and extracellular activators of plasminogen (Reich, 1975). The levels of plasminogen activator in malignant cells are higher than their normal counterparts. Plasmin may play an important role in several cellular processes (Reich, 1975; Christman et al., 1975; Goldberg, 1975; Roblin and Young, 1975; Pollack et al., 1975). Plasminogen activators are found in Rous sarcoma virus-transformed chick embryo fibroblasts, SV40-transformed hamster cells, R2426 rat breast carcinoma cells, human pancreatic carcinoma cells, murine sarcoma virus-transformed 3T3 cells, human melanoma cells, as well as in normal human stromal fibroblasts (Reddy and Kline, 1980).

Hormonal modulation of plasminogen activator activity have been demonstrated by several investigators. Mak et al. (1976) has reported the rapid induction of fibrinolytic activity in a murine mammary carcinoma (Shinogi SC-115) cell culture in the presence of physiological concentrations of dihydroxytestosterone. Vassali et al.(1976) have studied the regulation of

plasminogen activator in activated macrophages. Glucocorticoids, cholera toxin, cyclic-AMP, and prostaglandins E_1 and E_2 all inhibited the production of macrophage plasminogen activators. Another example of hormonal modulation of plasminogen activator occurs in the process of ovulation (Strickland et al., 1977). Rat ovarian granulosa cells produce high levels of plasminogen activator at the time of ovulation. It is produced only by those cells obtained from follicles destined to ovulate. Inactive granulosa cells may be stimulated to elaborate activators only upon exposure to gonadotropins.

Recently, the breast tumor cell line, MCF-7, has been demonstrated to secrete plasminogen activators. In the presence of estradiol, the level of synthesis is enhanced, whereas the subsequent addition of antiestrogens resulted in an inhibition of activator synthesis (Butler et al., 1979). These studies suggest that the level of plasminogen activators may play an important role in many cellular activities by responding to hormonal modulation.

The stroma of human breast cancer contains high levels of both collagen and elastin. The presence of collagenolytic and elastinolytic proteases has been demonstrated in the ZR75 human breast tumor cell lines (see Chapter IV). These enzymes may be involved in the modulation of the desmoplastic reaction found in human breast cancers. Both of these enzymes exist in a zymogen form. Activity is detectable only after mild tryptic pretreatment of the media of cultured tumor cells. The activation system for these enzymes could control the level of the desmoplastic reaction, and may be invoked as an important regulatory mechanism for that response. I propose that PA is the activation system involved for collagenase in human breast cancer and the hormone dependent step in that process.

I describe herein PA activity elaborated by cultured human breast cancer cell lines. This activity was hormone-dependent and urokinase-like. I also

demonstrate that the PA was able to activate the collagenase but not the elastase synthesized by the same tumor cells. Hormonal modulation of PA may be the key event in the turnover of the collagenous component of the desmoplastic reaction in human breast cancer and possibly in other malignancies.

B. MATERIALS AND METHODS

1. Cells and Culture Conditions

Cells were previously described in Chapter II. MDA 435 and MDA 436 breast tumor cell lines were generously provided by Dr. Dolphine Oda, Indiana University. For these experiments, cells were plated at $5X10^5$ cells/ml. After 24 hr the cells were washed three times with Hank's buffer. All media were supplemented with 7% dextran charcoal-treated FBS. Dextran charcoal-treated FBS was prepared as described by Lippman (1975b). After 8 hr of pre-incubation, the medium was changed to either hormone-free medium or medium supplemented with hormones as indicated.

2. Plasminogen Preparation

Plasminogen was prepared from human plasma by affinity chromatography on columns of L-lysine-Sepharose 4B based on the method of Deutsch and Mertz (1970). A sample of 30 ml of outdated serum was applied to a 2.0 X 1.0 cm column of lysine-Sepharose 4B with a flow rate of 24 ml/hr. Steps up to this point were performed at room temperature, and at 4°C hereafter. Following a 0.5 M NaCl wash, the plasminogen was specifically eluted with a 0.2 M α-aminocaproic acid. The plasminogen was separated from α-aminocaproic acid on a 20 X 1.5 cm Sephadex G-25 column equilibrated with 5 mM phosphate buffer (pH 7.4). A typical preparation of 100 ml outdated plasma produced 8-10 mg of plasminogen.

3. Plasminogen Activator Assay

Fibrinogen II was prepared from fibrinogen I (Miles Lab) by the method of Laki (1951) and stored frozen at 10 mg/ml in calcium and magnesium free PBS (pH 7.4).

Acetylation of fibrinogen II was performed as described by Gisslow and MacBride (1975). Stock fibrinogen was diluted to a concentration of 2 mg/ml in 0.2 M borate buffer (pH 9.2). With gentle stirring, 0.1 mCi of ^{3}H-acetic anhydride (500 mCi/mmol, Amersham) was added for each 2 mg of fibrinogen. After 1 hr the pH was returned to pH 7.4 with 0.1 N HCl. The specific activity of the labeled fibrinogen was approximately 200,000 cpm/100 ug.

^{3}H-fibrin coated plates was prepared by the method of Barrett et al. (1977). Costar plastic petri dishes (35-mm) were evenly coated with 100 ug of ^{3}H-fibrinogen. The plates were dried overnight at 45° C. Fibrin formation was achieved by incubation with 2 ml of Eagle's MEM with 5% FCS for 2 hr at 30°C. The medium was then aspirated and washed twice with PBS.

To test for fibrinolytic activity, 2 ml of 0.1 M Tris-HCl (pH 8.0) and 10 ug plasminogen were added immediately before use. Conditioned medium or cell lysate at a volume of 0.1 ml was added to the assay system. The plates were incubated at 30°C for 2 hr. An aliquot (0.4 ml) of the resulting digest was counted in a Beckman scintillation counter with Aquasol (New England Nuclear).

4. Concanavalin A Sepharose Chromatography

A column of 1.0 X 1.0 cm of Concanavalin A Sepharose (Pharmacia) was equilibrated with 3.4 M phosphate buffer, 1 M NaCl, and 0.01% Triton X-100, pH 7.4 at 4° C. Samples consisted of 0.5 ml of conditioned medium and were dialyzed against the equilibration buffer. Elution of the sample from the affinity column was carried out with 0.6 M α-methylmannoside in the same buffer. Fractions of 0.6 ml were collected and 0.1 ml aliquots were assayed. Human

urokinase was obtained from Calbiochem and tissue plasminogen activator was from Dr. C. Hoyn (Genetech Corp.). The urokinase and tissue plasminogen activator was dissolved in equilibration buffer.

5. Sephadex G-100 Chromatography

Fractions containing plasminogen activator from concanavalin A chromatography were pooled and lyophilized. The PA sample was applied to a column of Sephadex G-100 (1.0 X 100 cm) after suspension in 3.0 ml of elution buffer consisting of 0.05 M Tris, 0.5 M NaCl, 0.01% Triton X-100, pH7.4. Chromatography was at an elution rate of 30 ml/hr at 4° C. Fractions (3.0 ml) were assayed for PA activity.

6. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed as described in Chapter IV. A discontinous system with a 4% stacking gel and 10% separating gel was used as described by Neville (1971).

7. Enzymatic Assays

Elastin and collagenase assays were performed as described in Chapter IV.

C. RESULTS

The several lines of human breast cancer cell lines were examined for PA activities. The cell layer and the conditioned media were examined separately (table 1). PA activities were found in the media and cell lysate of all six of the breast tumor cell lines examined. However, the three ZR75 cell lines produced the highest levels of activity and were selected for further study.

The period of culture when the maximal PA production occurred was determined (fig. 5-1). Cells were cultured in the presence of charcoal-treated FBS. Medium and cell layers were harvested every 48 hr and PA activities

determined. Though there were no consistent pattern for PA production between the three ZR75 cell lines, levels were generally higher between day 4 and 8. This correlated with the days in culture when collagenolytic acitivity was also the highest (fig. 4-1).

Two classes of PA have been described in tissue culture systems, the tissue-type and the urokinase type (Kucinski et al., 1968; Reddy and Kline, 1980). The urokinase-type differs in having a higher molecular weight, lower carbohydrate content, and the ability to bind fibrin. The tissue type PA is a glycoprotein whereas the urokinase is not. Differentiation of these two types is possible by ConA-Sepharose affinity column chromatography and by the difference in molecular weight. In order to characterize better the breast tumor cell PA, the enzyme was purified. The culture supernatent was dialyzed (M_r cutoff < 2K) at 4° C. The medium was lyophilized and resuspended in 4 ml of the ConA chromatography elution buffer. The elution pattern of breast tumor cell PA is depicted in fig. 5-2a. Commercially available urokinase and tissue PA were chromatographied on similar columns (fig. 5-2b,c). The PA produced by the ZR75-1 cell line was not retained by the ConA-Sepharose column, similar to the human urokinase standard. The tissue PA was retained on the column due to its glycosylated nature. ZR75-1 cells secreted predominantly the non-glycoprotein form of PA. Its comigration form in the ConA-Sepharose column with the human urokinase standard suggested that this PA was of the urokinase type.

Peak fractions of breast tumor cell PA from the ConA-Sepharose chromatographic run were pooled, dialyzed, and lyophilized. The breast cancer PA was resuspended in the gel filtration running buffer and applied to a Sephadex G-100 column. The breast tumor cell PA was eluted as a single band with a calculated molecular weight of 56K (fig. 5-3). This molecular weight

was close to the 52-56K values reported for urokinase (Reddy and Kline, 1980). A summary of the purification of PA is shown in table 5-2. A 60-fold purification was achieved using sequentially the two chromatographic procedures, with an 86% recovery of enzyme activity.

The influence of hormonal modulation on the production of PA was studied using ZR75 cells grown in the presence of charcoal-treated FBS. Charcoal treatment removes the endogenous hormones usually present in serum and particularly in the FBS. Hormones were added on day 1 and PA activity was determined from conditioned media and cell lysates of cells cultured between day 4 and 6.

As shown in table 5-3, the production of PA by ZR75-1 was increased by 283% in the presence of estradiol. Likewise, a 63% increase in PA production was observed with progesterone addition. In both ZR75-30 and ZR75-31A cell lines, no significant increase in PA production was observed with either estradiol or progesterone addition. All three cell lines exhibited no significant stimulation with testosterone but inhibition was observed with hydroxycortisone and dexamethasone addition.

To elucidate further the estrogen regulation of PA activity in the ZR75-1 cells, a range of levels of estradiol and estrogen inhibitors in various combinations were used (table 5-4). Maximal stimulation of PA activity was observed in cells grown in the presence of 10^{-8} or 10^{-10} M estradiol. This stimulation of PA activity by estradiol was observed in both the media and in the cell lysate. The additon of tamoxifen, an antiestrogen, in the presence of estradiol suppressed control levels by 34%. With higher levels of estradiol, however, the inhibition by tamoxifen was largely overcome. There apparently was also a slight inhibitory effect of the tamoxifen. Lastly, the stimulation by estradiol was blocked by cycloheximide.

I next wished to established whether the increased breast cancer PA was of the urokinase type. The supernatent of cells treated with estradiol was chromatographed on ConA-Sepharose column. With estradiol, the peak of PA activity increased (fig. 5-4b). This increase correlated with the elution of urokinase activity (fig. 5-4d). Addition of estradiol and tamoxifen together reversed the effect (fig. 5-4c).

Collagenolytic and elastinolytic proteases in breast tumor cells have been described in the previous chapter. Both of these activities were detected only after mild tryptic treatment, suggesting that both enzymes exist as zymogens that require proteolytic activation. To characterize the role of PA in the activation of these zymogens, medium from the ZR75-1 cell lines was harvested. Upon exposure to trypsin treatment, there was an increase in both collagenolytic and elastinolytic activities (table 5-5). With the addition of either breast PA or urokinase and plasminogen, collagenolytic activation was observed. Degradation of collagen can also be confirmed by SDS-PAGE (fig. 5-5). The increase in collagenolytic activity was at a level comparable to that observed for tryptic pretreatment. With the inhibition of breast cancer PA by anti-urokinase antibody, there was no collagenolytic activation . Protease nexin, an inhibitor similar to antithrombin III, has been shown to be an effective inhibitor of urokinase-like PA and urokinase (Scott, 1983). Protease nexin was generously provided by Dr. R. Scott (U. Kansas) and was capable of inhibiting the PA-dependent activation of collagenase. No effect on the activation of elastinolytic activity could be observed with either PA, plasminogen, or with plasmin.

D. DISCUSSION

An increase in collagenase activity may account for the disappearance of the fibrous reaction in human breast cancer associated with hormone treatment. I have attempted to identify such a hormone-responsive collagenolytic activity in tumor epithelial cells, in stromal cells surrounding the tumor, and in cell combinations. I was able to document the existence of a collagenase in breast epithelial cells confirming the earlier result of Paranjpe et al. (1980). However, I was unable to detect any such activity that was estrogen-responsive. The collagenase synthesized by these tumor cells required trypsin pretreatment in order for the collagenolytic activity to be manifest. I postulated that it was perhaps this proteolytic processing and activation step that was under hormonal control. I have now characterized an estrogen-responsive urokinase-like PA that appeared to participate in this extracellular conversion of procollagenase to collagenase, thus fulfilling the requirement for at least one hormonally modulated step in the cascade of events that lead to collagen dissolution.

The presence of PA in breast tumor cell lines has been previous described (Butler et al., 1979; Evers et al., 1982). To characterize further the modulation of PA by estrogen in breast tumor cells, I examined three ZR75 human breast tumor cell lines. Though all three cell lines produced high levels of PA relative to the other lines examined, only PA synthesis by ZR75-1 was regulated by estrogen. Previous studies described in Chapter II indicated that the ZR75-1 cell line was the only cell line both estrogen receptor positive as well as estrogen responsive. Both the ZR75-30 and ZR75-31A cell lines were estrogen receptor positive but estrogen nonresponsive. This probably underlies the inability to demonstrate an estrogen effect on the PA activity of the latter two ZR75 cell lines.

Estradiol addition to the ZR75-1 cell line caused a 280% increase in the level of PA activity. The addition of tamoxifen inhibited the level of PA activity. With increased levels of estradiol, however, this inhibition was overcome. This observation is consistent with MCF-7 cell growth kinetic studies (Lippman, 1976). The present studies indicated that the inhibitory effect of antiestrogen can be reversed in a dose-dependent response with excess estrogen addition.

Glucocorticoid treatment of various cells in culture including human polymorphonuclear leukocytes, embryonic lung cells, melanocytes (Roblin and Young, 1980), macrophages (Werb, 1978), and rat hepatoma cells (Siefert and Gelehrter, 1978; Wigler et al., 1975), decreases their PA activity. Several laboratories have reported that the hormonal changes in PA activity may be associated with the regulation of inhbitory substances (Loskutoff and Edgington, 1977 Siefert and Gelehrter, 1978). The incubation of rat hepatoma cells with dexamethasone, a synthetic glucocorticoid, decreases the PA activity of these cells (Siefert and Gelehrter, 1978). In mixing experiments using cell extracts, a decrease in urokinase-directed fibrinolytic activity is attributed to the presence of inhibitors in the extracts. The regulation of PA activity in response to hormones has been proposed to be modulated by these inhibitors. My studies indicated that the stimulation of PA activity by estradiol can be blocked by the addition of cycloheximide. Apparently the stimulation of PA activity was dependent on protein synthesis, and was not merely the release of pre-existing PA.

The PA produced by the tumor cells was of the urokinase-type as determined by its inability to bind to ConA-Sepharose column, from the estimated molecular weight of 56K, from the comigration studies with a human urokinase, from urokinase-specific inhibitors studies, and from the antibody effects.

This coroborates histopathological studies demonstrating that the PA in various breast tumor tissue reacts with antibody formed against human urinary urokinase (Evers et al., 1982).

With the use of ConA-Sepharose column chromatography, I have demonstrated increased synthesis of urokinase-type PA by the ZR75-1 breast tumor cell line following estrogen addition. With tamoxifen, the estrogen-induced PA synthesis was inhibited. This histopathological studies of Evers et al. (1982) indicate no correlation between PA and the amount of estrogen-binding protein in the cytosol of the tumors. This apparent lack of correlation may reflect variations in estrogen responsiveness. These tissue samples may contain cell populations which are similar to the ZR75-31A cell lines, cells that possess estrogen receptors but are estrogen unresponsive. Thus, all hormonal studies necessitate the determination not only of hormone receptor status but also responsiveness to those hormones.

I have observed latent collagenase and elastase activities in ZR75 cell lines (Kao et al., 1982a,b). In these studies, the latent forms of both enzymes could be activated by brief protease pretreatment. Since plasmin is one of the proteases capable of activating collagenase, I invoked the possiblity tumor cells could activate the latent collagenase and elastase through PA secretion. The PA could then convert plasminogen into plasmin which in turn activates the latent enzymes. Utilizing both partially purified PA from ZR75-1 cells and human plasminogen, the plasmin produced was capable of activating the collagenolytic proteases in our breast tumor culture system. However, plasmin was not able to convert the elastase zymogen. Estrogens may regulate the turnover of collagen specifically in the desmoplastic reaction of breast cancers. An as yet undetermined system may be responsible for the turnover of elastin.

In the present studies, a urokinase-like PA produced by human breast cancer cells in culture has been demonstrated. This PA activity was estrogen dependent and regulated the activation of collagenolytic proteases produced by these same cells *in vivo*.

Table 5-1: Survey of PA Activity Produced by Various Cultured Human Breast Tumor Cells

Cell Types	Conditioned Medium ($mU/10^{-6}$ cells)	Cell Lysate ($mU/10^{-6}$ cells)
ZR75-1	68.2 ± 2.8	36.3 ± 1.6
ZR75-30	74.4 ± 2.4	22.7 ± 1.6
ZR75-31A	67.3 ± 1.8	41.2 ± 1.4
MCF-7	43.4 ± 2.0	30.8 ± 1.9
MDA 435	34.4 ± 2.1	24.8 ± 2.0
MDA 436	50.9 ± 1.9	31.7 ± 1.6

Table 5-1. A survey of PA activity produced by various cultured human breast tumor cells into the culture medium. Cells were plated at a density of 5×10^{-5} cells/well in 7% FBS-supplemented culture medium. After 24 hr, 7% charcoal treated FBS-supplemented medium was used. PA activity was examined in the conditioned medium and in the cell lysate following a 48 hr exposure. An example of low PA activity is in newborn fibroblasts where conditioned medium and cell lysate had 16.4 ± 2.5 and 7.9 ± 1.4 $mU/10^{-6}$ cells, respectively. The PA assay was performed as described in Materials and Methods. The mean of experiments in triplicate are presented here. A unit of activity is expressed in terms of urokinase equivalents per 10^{-6} cells.

Table 5-2: Summary of Breast Tumor PA Purification

Procedure	Vol. (ml)	Total Protein (ug)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	X-fold Purification
Culture Medium	200	4,866	3,300	678	100	1.0
Con A-Sepharose	10	2,872	3,129	1,089	95	1.6
Sephadex G-100	6	69	2,846	41,246	86	60.8

Table 5-2: Summary of breast tumor PA purification from the culture supernates of human breast cancer cell line ZR75-1.

Table 5-3: Influence of Hormones on Breast Tumor PA Activities

Hormone Addition	ZR75-1 ($mU/10^{-6}$ cells)	ZR75-30 ($mU/10^{-6}$ cells)	ZR75-31A ($mU/10^{-6}$ cells)
Control	66.0 ± 2.4	67.9 ± 3.5	67.9 ± 4.1
Estradiol	* 188.1 ± 3.5	82.2 ± 4.3	71.4 ± 2.3
Progesterone	* 108.3 ± 4.3	72.0 ± 4.7	74.2 ± 3.4
Testosterone	66.9 ± 4.2	68.3 ± 2.9	73.3 ± 3.1
HO-cortisone	* 35.1 ± 3.2	32.4 ± 3.7	49.5 ± 3.5
Dexamathasone	* 21.8 ± 3.5	13.5 ± 2.1	38.2 ± 3.8

Table 5-3: Hormonal modulation of PA activities in the conditioned medium of breast tumor cell lines. Cells were cultured in the presence of hormone-supplemented 7% charcoal-treated FBS culture medium. Hormones were added at a final concentration of 10^{-8} M. The 48 hr period between day 4 and 6, medium was harvested and PA activities determined. The mean of experiments in triplicate from three separate cultures are presented here. $p < 0.005\%$.

Table 5-4: Estradiol Regulation of PA Activity in ZR75-1 Cells

Treatment	Medium ($mU/10^{-6}$ cells)	Cell Lysate ($mU/10^{-6}$ cells)
Control	66.0 ± 2.4	33.6 ± 2.4
Estradiol (10^{-6} M)	* 94.2 ± 2.0	** 48.3 ± 4.8
Estradiol (10^{-8} M)	* 188.0 ± 2.4	* 79.5 ± 2.8
Estradiol (10^{-10}M)	* 127.6 ± 3.4	* 66.3 ± 3.2
Tamoxifen (10^{-6} M)	54.5 ± 4.2	25.5 ± 2.6
Estradiol (10^{-10}M) Tamoxifen (10^{-6} M)	43.8 ± 4.5	24.1 ± 3.1
Estradiol (10^{-8} M) Tamoxifen (10^{-6} M)	* 149.8 ± 3.5	* 48.2 ± 3.7
Estradiol (10^{-8} M) Cycloheximide(10 ug/ml)	31.7 ± 3.2	33.8 ± 3.8

Table 5-4: Estradiol regulation of PA activity in ZR75-1 cells. Cells were cultured in the presence of estradiol supplemented with 7% charcoal treated FBS culture medium. Tamoxifen and cycloheximide was added with media change on day 4. The medium and cell lysate were prepared from cells between day 4 and 6. The mean of experiments in triplicate from three separate cultures are presented here. * $p < 0.005\%$ ** $p < 0.01\%$.

Table 5-5: PA Activation of Breast Tumor
Collagenolytic and Elastinolytic Activities

Medium Pretreatment	Collagenolytic Activity (cpm)	Elastinolytic Activity (cpm)
Control (no treatment)	946 ± 218	486 ± 63
Trypsin Activation	29,460 ± 263	4,980 ± 126
Breast Tumor PA (100 mU) Plasminogen (10 ug/ml)	21,071 ± 257	680 ± 73
Urokinase (100 mU) Plasminogen (10 ug/ml)	22,113 ± 305	445 ± 57
Anti-urokinase Antibody Breast Tumor PA (100 mU) Plasminogen (10 ug/ml)	1,247 ± 206	572 ± 81
Protease Nexin (1 ug) Breast Tumor PA (100 mU) Plasminogen (10 ug/ml)	7,278 ± 208	497 ± 94

Table 5-5: The ability of PA to activate collagenolytic and elastinolytic activity in the medium of ZR75-1 cells. Medium between day 4 and 6 was harvested. For control studies, trypsin activation (0.025% v/v) was used. After 30 min of tryptic treatment, soybean trypsin inhibitor (0.125%) was added. This was subsequently added to the PA enzymatic assay as described in Materials and Methods. For PA and plasminogen treatement, the incubation period was for 30 min prior to addition to the enzymatic assay. the mean of triplicate assay are presented here.

Fig. 5-1: Time course of PA production by ZR75 cells lines. Medium was conditioned for 48 hr period. On the day indicated, the medium was harvested and PA activity examined, as described in Methods and Materials. The mean of experiments in triplicate from three separate cultures are presented. S.D. < 0.05%.

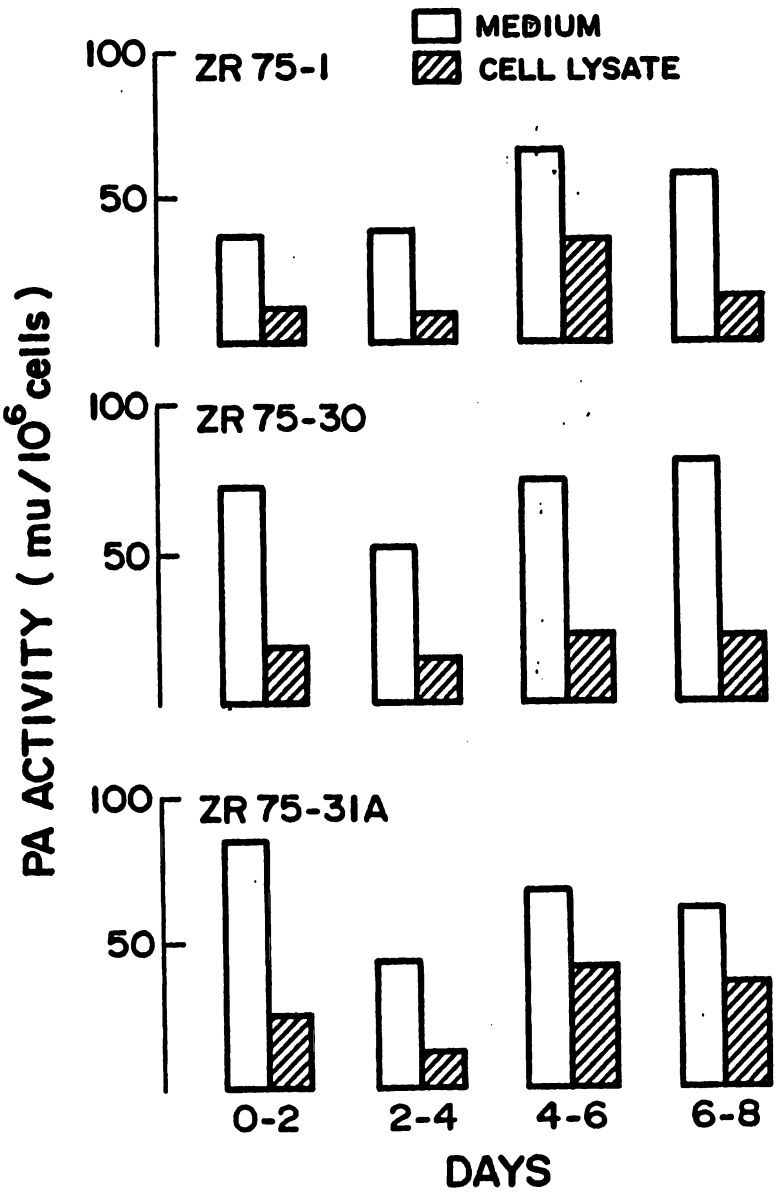

MEDIUM
CELL LYSATE
ZR 75-1
ZR 75-30
ZR 75-31A
100
50
PA ACTIVITY (mu/10^6 cells)
0-2
2-4
4-6
6-8
DAYS

Fig. 5-2: Chromatographs of breast tumor culture medium, urokinase, and tissue plasminogen activator on Concanavalin A - sepharose 4B column. Panel A depicts the chromatography of the culture supernate after lyophilization, reconstitution and dialysis against the equilibration buffer (see Materials and Methods). Panel B depicts the chromatography of urokinase. Panel C depicts the chromatography of tissue plasminogen activator. Urokinase and tissue plasminogen activator sample were reconstituted with the equilibration buffer. All samples were applied and eluted with equilibration buffer (3.4M phosphate buffer, 1 M NaCl, 0.01% Triton X-100, pH 7.4). The arrow indicates the start of α-methymannoside elutions. Aliquots of each fraction were assayed for plasminogen activator activity.

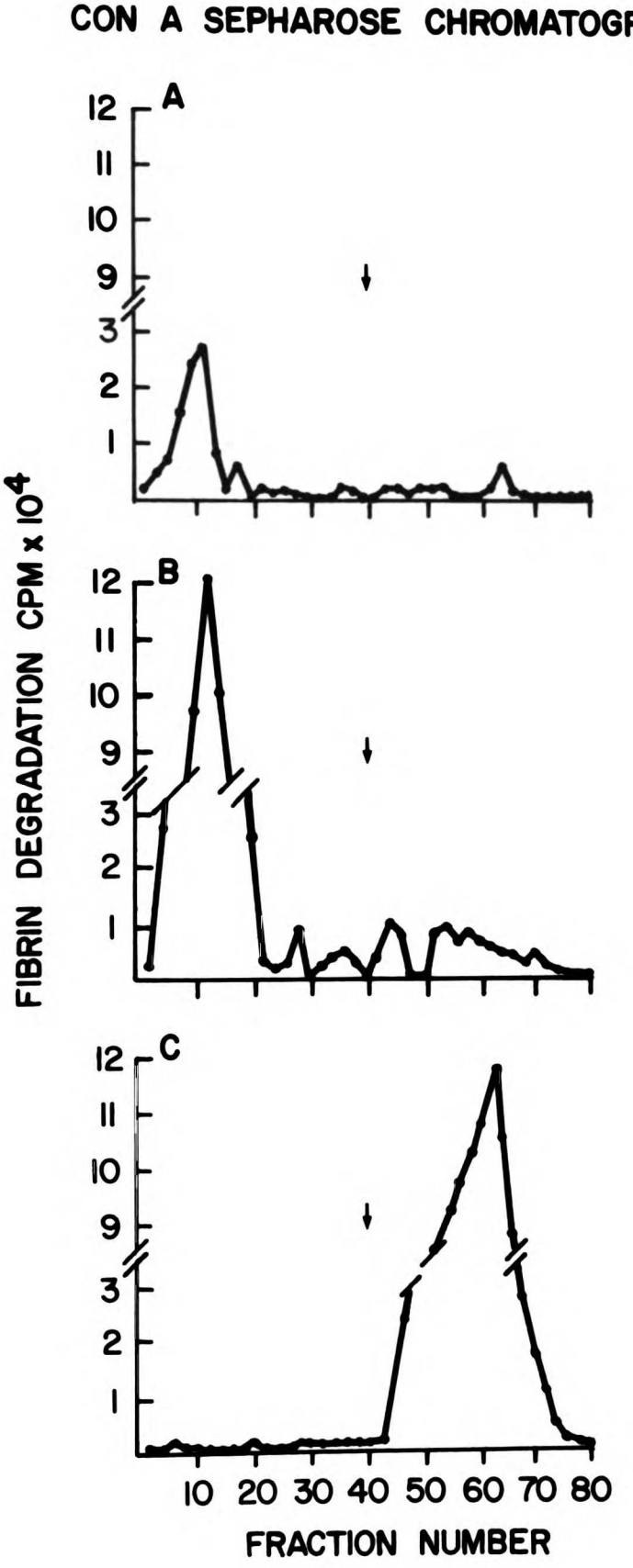

CON A SEPHAROSE CHROMATOGRAPHY
A
B
C
12
11
10
9
3
2
1
FIBRIN DEGRADATION CPM x 10⁴
10 20 30 40 50 60 70 80
FRACTION NUMBER

Fig. 5-3: Sephadex G-100 chromatography of breast tumor plasminogen activator. Fractions from the Con A column were pooled and lyophilized and applied to a 1 X 100 cm Sephadex G-100 column equilibrated with 50 mM Tris, 500 mM NaCl, 0.01% Triton X-100, pH 7.4. The column was eluted at a flow rate of 30 ml/hr and 3 ml fractions were collected. Aliquots from each fraction were assayed for plasminogen activator activity and for protein content.

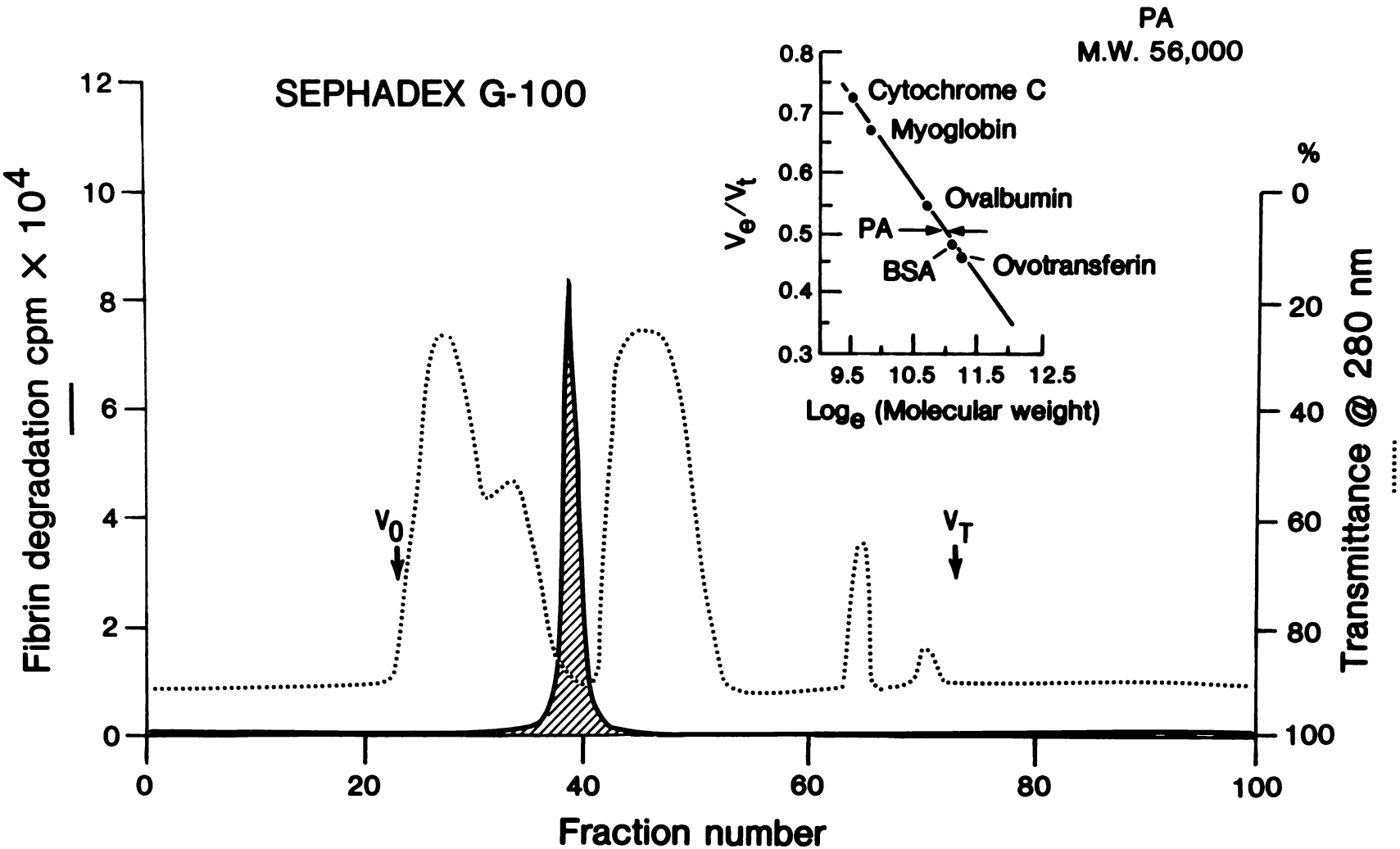

SEPHADEX G-100
PA
M.W. 56,000
Cytochrome C
Myoglobin
Ovalbumin
PA
BSA
Ovotransferin
V_e/V_t
Log_e (Molecular weight)
Fibrin degradation cpm X 10^4
Transmittance @ 280 nm
%
V_0
V_T
Fraction number

Fig. 5-4: Concanavalin A - Sepharose chromatography of PA. Samples of medium were applied to a 1.0 x 1.0 cm Con A - Sepharose column. The arrow indicates the start of α-methylmannoside elutions. Fractions were assayed for PA and expressed as fibrinolytic activity (cpm). Panel A: control ZR75-1 medium; Panel B: estradiol (10^{-10}M) treated ZR75-1 medium; Panel C: estradiol (10^{-10}M) and tamoxifen (10^{-6}M) treated ZR75-1 medium; and Panel D: human urokinase.

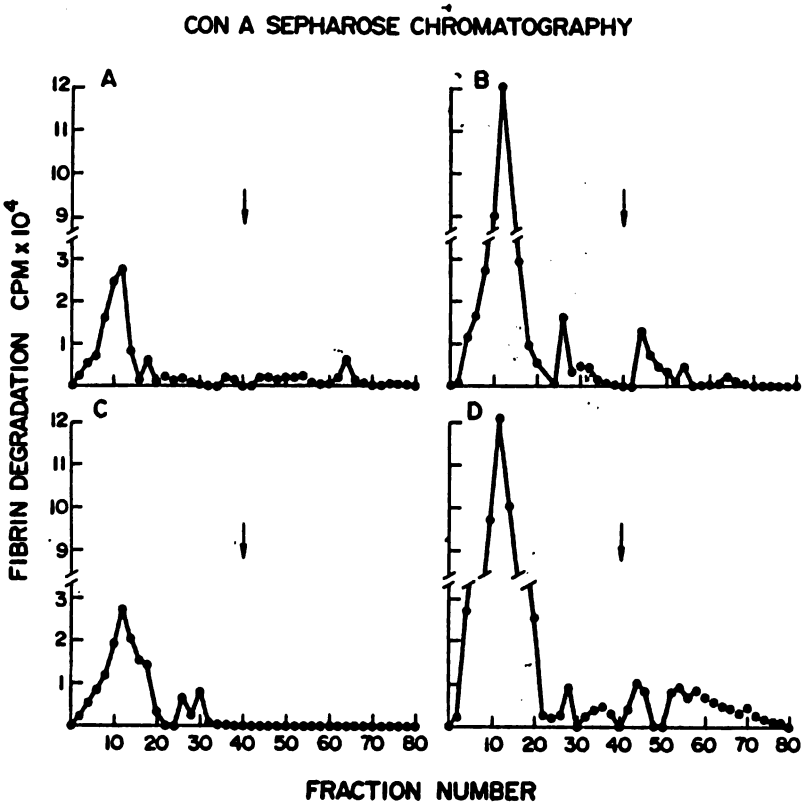
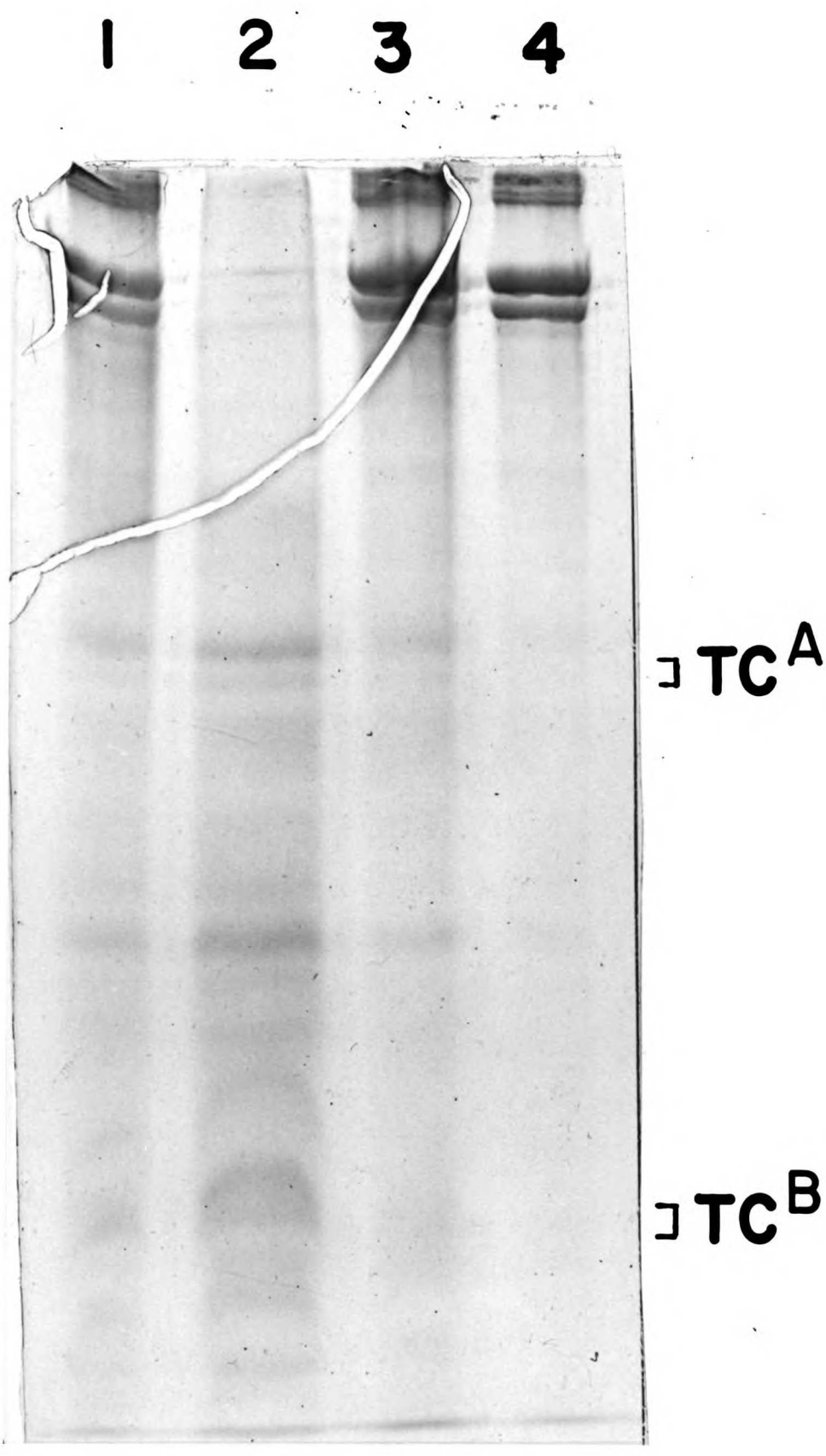

CON A SEPHAROSE CHROMATOGRAPHY
A
B
C
D
FIBRIN DEGRADATION CPM x 10⁴
FRACTION NUMBER
12
11
10
9
3
2
1
10 20 30 40 50 60 70 80

Fig. 5-5: Collagenolytic activity of PA-treated collagenase. Lane 1: 10 min pretreatment of collagenase with PA and plasminogen resuled in collagenase activation as shown by type I collagen degradation observed; Lane 2: 1 hr treatment resulted in increased level of collagenase activation as suggested by the observed increase collagen degradation; Lane 3: activation of collagenase by urokinase and plasminogen at 10 min of pretreatment; and Lane 4: type I collagen untreated.


1
2
3
4
TC A
TC B

CHAPTER VI SUMMARY

In most human breast tumors, particularly in infiltrating ductal and lobular carcinoma, desmoplasia is so marked that it comprises much of the tumor mass. Clinally, dissolution of the fibrous response occurs together with a decrease in the tumor mass, following treatment. Hormonal modulations are an important component of the treatment modalities for human breast cancer. The molecular basis for the effectiveness of this treatment modality is not clear. An experimental model system has been designed here in which breast cancer and stromal cell lines in culture are used to clarify the basis of the hormonal dissolution of the tumor mass in breast cancer.

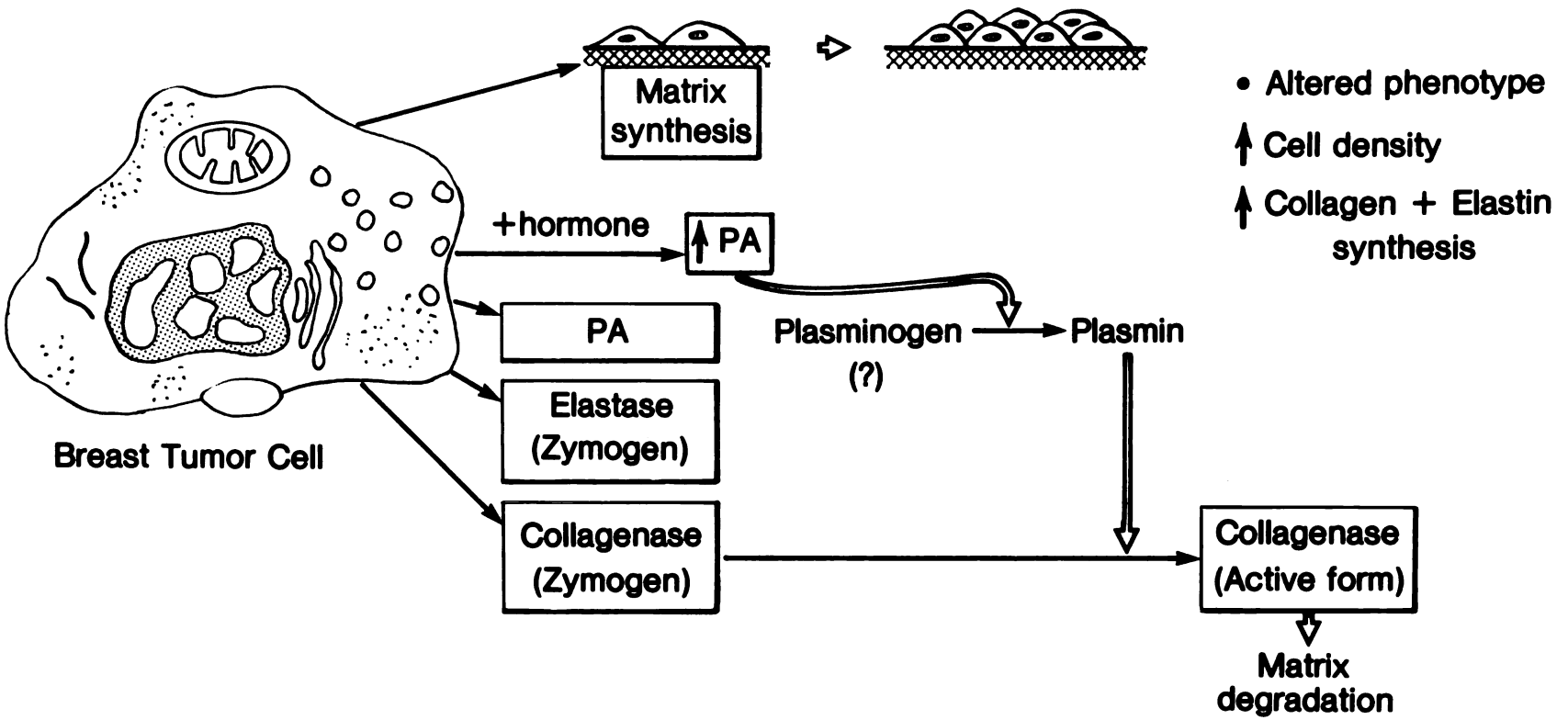
The fibrous reaction to human breast cancer is comprised mainly of stromal proteins, most notably, collagen and elastin. I invoked the assumption that since there is a decrease in tumor mass with hormone modulation, there must be a net loss of stromal proteins. In Chapter I, I proposed three possible interactions between stromal synthesis and degradation which would result in a loss of stroma. To test these possiblities, I have examined collagen and elastin biosynthesis and degradation in cultured breast cancer and stromal cell lines.

In the present studies, I have demonstrated that the origin of the desmoplastic reaction to breast cancer is due to the regulation of phenotypic expression of host stromal cell by the tumor ECM. Not only is the breast tumor ECM mitogenic for the host stromal cells (Kao, 1984), but it also modulates increased collagen and elastin synthesis in these same cells. The combination of increase cell population and increased rate of scleroprotein synthesis may account for the profound fibrotic response observed in these

tumors. I have demonstrated that these properties of the breast tumor ECM is however not influenced by such hormones.

These studies also suggest that the regression of tumor stroma is due solely to increased degradation. Stromal degrading enzymes have been partially purified and characterized from breast tumor cells. Hormones such as estrogen and progesterone were capable of regulating the activation of at least one class of these enzymes. It is proposed that this mechanism may in part explain for the loss of tumor mass observed with endocrine treatment. This process is summarized in fig. 6-1.

These observations corroborate the pioneering work of Gullino and coworkers (Gullino et al., 1962, 1963), that in the scirrhous reaction to cancer the neoplastic cell population itself regulates the level of the stromal reaction. These studies have confirmed this concept and expand this concept further that the stromal regulation is also modulated by the hormonal environment of the tumor.



BREAST TUMOR CELL INTERACTION WITH THE EXTRACELLULAR MATRIX

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