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The distribution of fibronectin and laminin in human oral leukoplakia and squamous cell carcinoma

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THE DISTRIBUTION OF FIBRONECTIN AND LAMININ IN HUMAN ORAL LEUKOPLAKIA AND SQUAMOUS CELL CARCINOMA

by

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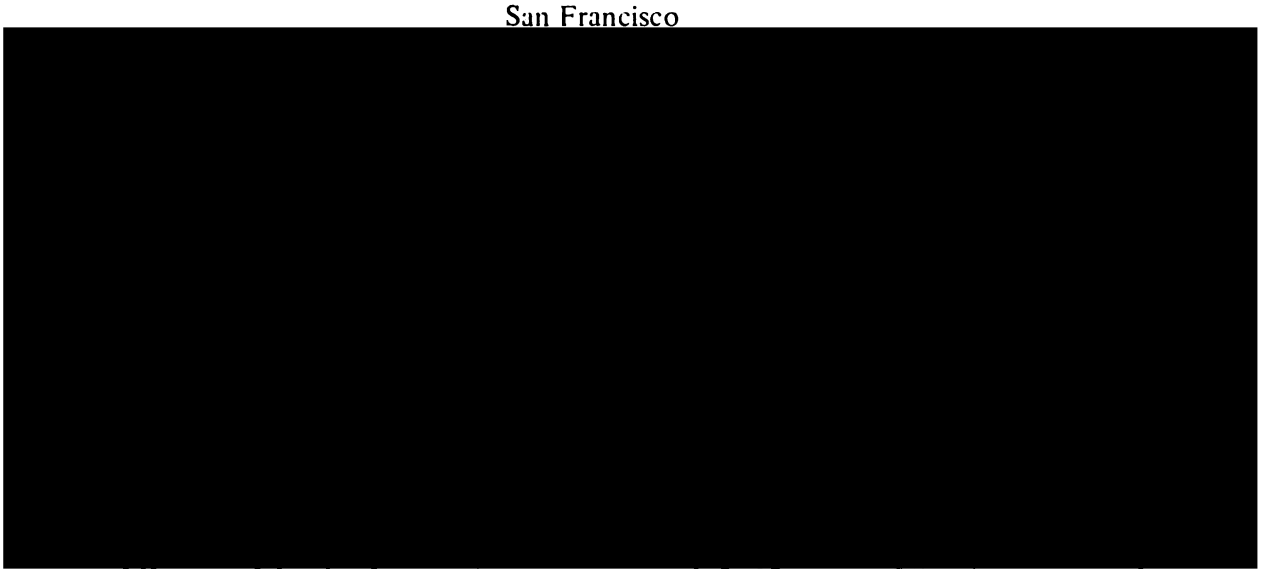

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INTRODUCTION

Cancer is a group of potentially fatal diseases destined to afflict one of every four Americans in their lifetime. In the United States, cancer of the oral cavity accounts for 3.4 percent of new cancers at all sites (except for skin) and 2.4 percent of all cancer deaths (1). The best measure to minimize the morbidity and mortality resulting from oral cancer is early detection and appropriate treatment. The clinical signs and symptoms of oral cancer can often be confused with those due to traumatic ulceration, aphthous ulcers, and drug-induced lesions. In addition, early carcinoma of the oral cavity may be painless. Some patients do not seek consultation until severe or persistent pain are present. Squamous cell carcinoma is the most prevalent type of oral cancer accounting for approximately 90 percent of all oral malignancies.

Carcinoma of the oral mucosa may be associated with other lesions taking the form of white patches or plaques. These lesions may precede the carcinoma or be present in association with the cancerous lesion. Therefore, the assumption has been made that white lesions are etiologically associated with oral carcinoma. To many, the diagnosis of leukoplakia has carried connotations of premalignancy. However, many oral carcinomas are not preceded by white plaques or associated with them (2). Also, in the older literature, the prevalence of malignant transformation of leukoplakia has varied considerably. In some reports, transformation rates of up to 30 percent in cases of "leukoplakia" were given (2). Recent studies indicate the malignant transformation rate in leukoplakia is between 3 and 6 percent (2). The definition of leukoplakia is the

most important factor influencing the reported prevalence of malignant transformation in oral leukoplakia. If the term, leukoplakia, is applied to white patches with epithelial dysplasia, a higher risk of malignant transformation is obtained in comparison to the transformation rate of all white lesions. Defining oral leukoplakia as a clinical white patch is confusing because it includes a variety of pathologic entities not associated with premalignancy. Lichen planus, candidiasis, white sponge nevus, and other lesions have a superficially similar appearance but are not premalignant. Therefore, the World Health Organization now defines oral leukoplakia as "a white patch or plaque that cannot be characterized clinically or pathologically as any other disease" (2). The use of the term, leukoplakia, is still unrelated to the presence or absence of epithelial dysplasia. However, this definition serves to form a group of white lesions distinct from other classifiable lesions known not to be premalignant. The prevalence of oral leukoplakia in the general population is unknown and the transformation rate has not been firmly established (6). The reported transformation rate in previous studies has varied according to geographic location, follow-up period and management. Despite the variation in reported incidence and risk of transformation, oral leukoplakia is clearly a precancerous lesion and prudent follow-up of these patients is indicated (3-6).

Correlation of the clinical appearance of leukoplakia with the presence of dysplasia is not reliable. Lesions that clinically appear dangerous may exhibit only hyperkeratosis on microscopic examination. Conversely, leukoplakia without ulceration, erythema or pain can be early infiltrating squamous cell carcinoma (7). In addition, the morphologic changes seen by the histopathologist are subject to variations in interpretation. There are a number of histopathological features

that are considered components of epithelial dysplasia. Obvious abnormalities are easily agreed upon, but in many instances the degree of dysplasia is the subject of dispute among observers and prediction of future behavior of the lesion from these appearances is unreliable. Attempts have been made to apply computer-aided analyses, using a number of recognizable features in assessing the prognosis of premalignant lesions (5,8,9,10). However, at the present time, it is not possible to predict which premalignant lesions will eventually become cancer. A better understanding of biological events that accompany the development of oral squamous cell carcinoma in humans would be beneficial.

Fibronectin is a cell surface and blood plasma glycoprotein that can mediate the attachment of certain cells to specific components of the extracellular matrix. Fibronectin has both collagen and heparin binding domains as well as a site for hyaluronic acid binding. It has been known by a number of names since it was first described by Morrison et al (11) in 1948. At that time, it was recognized as a component of the fibrinogen-containing fraction that was cold-insoluble, hence its first name - "cold-insoluble globulin" or CIg. Since its initial discovery, it has been designated by a variety of terms including: large external transformation-sensitive protein (LETS) (12), soluble fibroblast antigen (SF-antigen) (13), cell surface protein (CSP) (14), cell adhesion factor (CAF) (15), galactoprotein a (16), Z (17), opsonic protein (18), cell-spreading factor (19), and anti-gelatin factor (20). Fibronectin is the only designation that has received general acceptance (21). Fibronectin is distributed throughout all connective tissues and is associated with most basement membranes (22,23,24,25,26). Most blood vessels contain fibronectin in the basement membrane beneath endothelial cells. Most epithelial cells are attached to basement membranes that contain

fibronectin. These include the small and large intestine, stomach, bronchi, lung, and kidney. Fibronectin is also located in the sheaths surrounding muscles, nerves, cartilage, and lymph nodes (22,23,26,27,28). In plasma, it is normally present at concentrations of 300 ug/ml (28). Platelets contain fibronectin which is released when activated (29). Fibronectin can mediate the attachment and spreading of platelets on collagen-coated surfaces (30). It has been suggested that fibronectin mediates platelet aggregation induced by collagen (31) and also may serve as a factor necessary for adhesion and migration of cells into a wound. The cellular form is believed to function as an adhesive protein involved in cell-cell aggregation, cell-substratum adhesiveness, cell morphology and motility (32). Intracellular fibronectin appears to function in the maintenance of normal cell morphology by means of microfilament and actin interaction (33,34). Cells in culture require fibronectin to bind to collagen (35) and for the attachment and spreading of cells on collagenous surfaces (35,36). It also promotes the binding of phagocytic material to monocytes (37) and thus may serve as an opsonin. Fibronectin thus appears to serve an organizing role in the extracellular matrix to promote orderly growth and cell positioning as well as maintenance of differentiated connective tissue structure. The word fibronectin (fibra-fiber and nectare-to bind) emphasizes its propensity to bind to fibrous proteins such as collagen and fibrin.

Fibroblast fibronectin is a disulfide-linked dimer of 450,000 molecular weight (Figure 1). There appear to be distinct sites on the molecule responsible for collagen-binding, heparin-binding, cell-binding, and possibly for cell-spreading as well (39,40,41). Fibronectin contains about 5 percent carbohydrate which enables it to react with various lectins (42). The functional importance of this

lectin-binding capacity, if any, is not known. Fibronectin is strongly antigenic and antibodies to it, prepared by immunization with fibroblast or plasma fibronectin preparations, show identical reactivity against both the plasma and cellular forms (43). Fibronectin is present in a variety of vertebrate species including avian. Anti-human fibronectin antibodies produced in sheep have been shown to react not only with human fibronectin, but also with plasma and cellular fibronectin in rabbit, rat, mouse, and chicken (21). It is unusual for proteins from widely separated species to show such degrees of cross-reactivity. This suggests that fibronectin serves a very basic function, common to many organisms.

Fibronectin has been shown to be an extracellular matrix molecule and it is presently believed to be a peripheral membrane protein. A current hypothesis regarding the receptor for fibronectin is that glycolipids of the plasma membrane bind fibronectin to the cell (44) (Figure 2). Fibronectin also binds directly to actin (35,45) and appears to affect the cytoskeleton via microtubules and microfilaments. In the presence of fibronectin, functional alterations can be induced ranging from cellular shape changes (46,47,48) to the enhanced synthesis of collagen in liver epithelial cells (49).

Fibronectin can be synthesized by fibroblasts (43,50,51), astroglial cells (52), endothelial cells (53,54), and myoblasts (55,56). Some epithelial cells are capable of producing fibronectin, at least when cultured in vitro (57,58). Fibronectin is present in early mesenchymal cells and is subsequently lost with differentiation (48). Fibronectin binds to both collagen (59,60) and glycosaminoglycans (61,62). It binds collagen types I-V (59,60), but binds type III collagen better than type I (60,64). Fibronectin has different binding affinities to glycosaminoglycans, it is evident that fibronectin binds well to hyaluronic acid and

to a lesser extent to heparin sulfate (62) when determined by ^{3}H-labeled glycosaminoglycans and glycopeptides. The fraction of total radioactivity that was recovered after a 60 minute incubation of chick embryo fibroblasts is depicted in figure 3. These findings indicate that large macromolecular complexes may be formed by the binding of hyaluronic acid and other glycosaminoglycans to fibronectin-collagen complexes, stabilizing the latter (Figure 4) (63,64). These findings suggest that fibronectin is important in the maintenance of extracellular matrix integrity. In vitro, the attachment and spreading of many cells is mediated by fibronectin. When freshly dissociated fibroblasts are cultured on plastic or collagen substrates, fibroblast attachment is stimulated by fibronectin.

Fibronectin affects cell differentiation in several in vitro cell lines. Myogenic and chondrogenic cell cultures lose fibronectin with differentiation. Cultured myoblasts have fibronectin when cultured but subsequently lose it as myotubes differentiate and fusion occurs (65). In a similar manner, when chondrocytes differentiate in vitro and synthesize their characteristic extracellular matrix, they lose fibronectin (27). In contrast, fibroblasts in cell culture do not lose fibronectin as they differentiate. In fact, fibroblasts have a high level of fibronectin associated with them. Special interest in fibronectin stems from experiments that demonstrated a reduction in amount of this glycoprotein on the surface of malignantly transformed cells (50,51,52,66,67). In experimental systems where mutant viruses (temperature-sensitive with respect to transforming capacity) were used (68), loss of surface fibronectin was concomitant with transformation. It has been postulated that the loss of surface fibronectin would increase the freedom of movement of malignant cells and

possibly aid tumor invasion. Chen et al (69) have suggested that the loss of surface fibronectin correlates well with the capacity of tumor cells to metastasize. Transformed but nonmetastatic murine tumor cells retain the ability to attach to several collagen isotypes by means of fibronectin, but the metastatic line attaches preferentially to type IV collagen without fibronectin assistance (70). Metastatic cells may possess a unique attachment capability to attach to type IV collagen. Normal and spontaneously transformed adult connective tissue fibroblasts were shown to have similar attachment ability to type I and type IV collagen. However, a metastatic cell line, derived from a pulmonary metastasis arising after a subcutaneous T241 fibrosarcoma injection, adhered spontaneously to type IV collagen without fibronectin assistance (70).

There are several references reporting the presence of fibronectin on epithelial cells (58,69,71,72,73,74,75,76,77). In cell culture, fibronectin is associated with the extracellular matrix. However, there does seem to be a time factor of importance; in one study, fibronectin was present during the first 8 culture passages but was subsequently absent (69). When a basement membrane was present, fibronectin was present on the epithelial cell surfaces abutting the basement membrane and in the underlying connective tissue (76,77). Reports have demonstrated fibronectin on epithelial cell surfaces from oral epithelium and skin in inflammatory diseases such as lichen planus, lupus erythematosis, pemphigoid and dermatitis herpetiformis (73,75).

Laminin is a recently discovered glycoprotein that appears to be a structural constituent of the basement membrane. Laminin is present in a variety of normal tissues including the basement membranes of the placenta and skin (78), glomerular and tubular basement membranes and Bowman's capsule of the kidney,

alveolar basement membranes of the lung, the choroid plexus of the brain, large and small blood vessels, and in the lens capsule and Descemet's membrane of the cornea (79). It is chemically and immunologically distinct from fibronectin, and is present in the basement membrane separating epithelial cells from underlying connective tissue (79,80,81,82,83). Timpl and colleagues (84) isolated laminin from the mouse Engelbreth-Holm-Swarm (EHS) sarcoma and found it to be composed of two polypeptide chains, one 220,000 daltons, and the other 440,000 daltons. Studies of murine tumor cell lines demonstrated that the synthesis of laminin by tumor cells is transient and occurs prior to the establishment of a tumor mass. A limiting basement membrane was synthesized by the tumor cells and laminin was shown to be a constituent (79,82,85).

Like laminin, type IV collagen is confined to basement membranes and synthesized by basal epithelial cells (78). Both occur in similar amounts in basement membranes (79), but differ in ultrastructural location. Laminin is located in the lamina lucida and type IV collagen in the lamina densa (78). Epithelial cells attach preferentially to type IV collagen (86) and laminin mediates epithelial cell attachment to this collagen isotype(87,88,89). Recently, two sulphated glycoproteins have been observed in the basement membrane of normal parietal endodermal cells from mouse embryos (90). The smaller glycoprotein (M_r=150,000) is probably entactin (91) which appears to form a noncovalent complex with laminin. Several recent reports (90,92,93) suggest that both laminin and entactin are synthesized by mesenchymal tissues.

The purpose of this study was to determine the distribution of fibronectin and laminin in human normal oral mucosa, leukoplakia, and squamous cell carcinoma. Labeling of fibronectin was done using indirect immunofluorescence

and an avidin-biotin complex (ABC)/peroxidase technique. Identification of laminin was done using only indirect immunofluorescence. Leukoplakia with histologic changes of epithelial dysplasia are generally considered to carry a "higher risk" of malignant transformation (2). Therefore, we determined if the presence or absence of fibronectin or laminin differed in leukoplakia with or without epithelial dysplasia. Alteration of the distribution of fibronectin or laminin in leukoplakia that transformed to carcinoma would be a valuable diagnostic and prognostic finding. It would provide a means of identifying oral leukoplakia lesions which have a greater potential of malignant transformation. More aggressive patient management in such cases would improve the clinical care for these patients.

We also observed whether fibronectin or laminin distribution was different in squamous cell carcinoma from that seen in normal oral mucosa or leukoplakia. TNM tumor staging was done for each patient with carcinoma (94). This system reports the size of the primary tumor, the evidence of spread to cervical lymph nodes and the presence or absence of distant metastatic sites. The classification and staging of tumors generally correlates with patient survival. The pretreatment size of a particular type of tumor is related to the length of time it has been present. Therefore, a larger primary tumor that has been present longer than a small lesion, has a greater opportunity for local extension, nodal involvement or metastatic dissemination (1). We determined if there was any correlation between the staging of the carcinomas and the presence and distribution of fibronectin or laminin. In addition, we observed the distribution of basement membrane laminin in normal oral mucosa and squamous cell carcinoma to determine if laminin distribution changed in carcinoma.

MATERIALS AND METHODS

A. Specimen Selection

Patients who presented at the Oral Medicine Clinic of the School of Dentistry, University of California, San Francisco with suspected precancerous or cancerous lesions comprised the study group. Five mm punch biopsy specimens were collected from twenty nine patients, a total of thirty specimens were obtained. In each case, the diagnostic specimen was bisected, one specimen was placed in 10 percent formalin and the other was quick-frozen in liquid nitrogen and stored at -70 degrees C. Of the specimens collected, 3 were diagnosed as nonspecific inflammation, 14 as epithelial hyperplasia, 4 as atypical verrucoid hyperplasia, 2 as epithelial dysplasia and 7 were carcinoma. The diagnostic groups studied and the location of the lesions studied are shown in Table I. Attached gingiva (in excess of that required for tissue adaption following third molar removal), and normal oral mucosa from the hard palate and buccal mucosa (adjacent to diagnostic biopsy sites), served as normal control tissue. Hematoxylin and eosin slides were prepared from the bisected biopsy specimens and hispathology reports procurred from the Division of Oral Pathology.

B. Specimen Preparation

Frozen specimens were cut as 5 micron sections with a Slee cryostat (South London Electrical Equipment Co., Ltd.) at -20 degrees C. The sections were mounted on formol-gelatin coated slides. The specimens labeled by indirect immunofluorescence (IIF) were air-dried without fixation. These specimens were stored at -70 degrees C. for not more than two days prior to IIF reaction. The specimens labeled by the avidin-biotin complex(ABC)/peroxidase technique were fixed in cold acetone for 10 minutes and air-dried prior to storage at -70 degrees C. for not more than two days.

C. Antisera and Conjugates

The antisera and conjugates used in this project were purchased from commercial vendors and are listed below. The only exception was antibody to laminin which was made available by Dr. R. Kramer (originally donated by G.R. Martin, NIDR).

1. Rabbit Anti-Human Fibronectin IgG fraction-15 mg/ml
   Cappel Laboratories; Cochranville, PA. 19330
2. Human Fibronectin (fibrinogen fraction)-1 mg/ml
   Collaborative Research, Inc.; Waltham, MA. 02154

3. Fluorescein conjugated IgG fraction Goat Anti-Rabbit IgG (H & L chains)-10mg/ml. Cappel Laboratories; Cochranville, PA. 19330
4. Rhodamine conjugated IgG fraction Goat Anti-Rabbit IgG (H & L chains)-10mg/ml. Cappel Laboratories; Cochranville, PA. 19330.
5. Biotinylated Goat Anti-Rabbit IgG (H & L chains)-0.5mg/ml Vector Laboratories; Burlingame, CA. 94010
6. Rabbit IgG (NRS)-21.7 mg/ml Cappel Laboratories; Cochranville, PA. 19330
7. Normal Goat Serum (NGS)-1.5mg/ml Vector Laboratories; Burlingame, CA. 94010

D. Fibronectin Antiserum Specificity

1. Immunodiffusion (ID)

Two sources of purified human plasma fibronection were used in all of the control procedures. FN_1 was purchased from Collaborative Research, Inc. (Waltham, MA 02154) and FN_2 was donated by R. Kramer (made available by E. Ruoslahti; LaJolla, CA).

To determine the equivalent concentrations or optimal ratio (OR) of antigen and antibody to use in the immunodiffusion and immunoelectrophoresis experiments, an Ouchterlony immunodiffusion method was performed with pure fibronectin antigen (FN_1) and different antiserum concentrations. Establishing the appropriate optimal ratio (OR) ensured the production of stationary, strong precipitate band formation.

Agarose containing 0.85% electrolyte and 0.5% sodium azide was poured into two petri plates and allowed to cool to room temperature. After gelation, 4.0mm diameter reactant wells were cut and 10 microliters of pure fibronectin (FN_1) was placed in the central well and three different antiserum dilutions were placed in the surrounding wells. Anti-fibronectin, diluted with PBS 1:4, 1:2, and 1:1, were used in the latter.

At 48 h, precipitate bands were present between the central well and all three peripheral wells. All the bands appeared to be single and proved to be stationary after an additional 48 h. No precipitate migration or band duplication was evident indicating that the antiserum dilutions were all acceptable for further immunodiffusion and immunoelectrophoresis experiments.

Figure 6(a & b) shows single, stationary, precipitate bands that formed when FN_1 and FN_2 were allowed to diffuse against rabbit anti-human fibronectin. 10 microliters of the test FN reactant were placed in each of 2 wells and rabbit anti-human fibronectin was placed in the remaining single well. Diffusion of the reactants took place for 24 h in total darkness. The petri plates were rinsed in 0.9% NaCl for 15 hours, rinsed in distilled H_2O for 2.5 h and dried overnight. Precipitate bands were stained by immersion in Coomassie Blue for 5 min. followed by a distilled H_2O rinse, destained for 15 min. on a shaker, rinsed in distilled water and dried. The absence of duplicate or migrating bands indicated the antiserum to human fibronectin does specifically bind to purified human fibronectin. Figure 6_c shows the results of an experiment in which both FN_1 and FN_2 were placed in wells and allowed to diffuse against fibronectin antiserum. The resulting identity arc formed is further evidence that the antiserum is monospecific.

2. Immunoelectrophoresis (IEP)

This procedure was performed with each fibronectin antigen (FN_1 & FN_2). The procedure outlined by Weir (77) was used in all immunoelectrophoresis tests. In one experiment, the melted agarose solution was poured onto 2 glass plates (84 x 94 mm.) and allowed to gel. A telescopic punch was used to cut 3 circular reactant wells (4mm. in diameter) on each plate. Horizontal troughs were cut with a scalpel and the gel removed prior to electrophoresis. On plate #1 (Figure 7_a) 10 microliters of Bromthymol Blue, FN_1 and FN_2 were placed; the FN antigens were undiluted. On plate #2 (Figure 11_b) 10 microliters of FN was placed in each

well, undiluted in the first well, diluted with PBS 1:2 in the second and 1:4 in the third. Electrophoresis was completed in 45 min. at 135 mV/20 mA; the plates were then placed into a humidity chamber at room temperature for 24 hours. Precipitation bands were evident after 24 hours and Coomassie Blue protein staining was done in the manner previously described. Figure 7_a demonstrates the precipitation arcs formed with this technique. Single precipitation arcs were formed when FN_1 was used in the assay. FN_2 showed residual staining in the reaction well which could be eliminated by using higher dilutions of the antigen preparation (Figure 7_b). The purified molecule does readily self-aggregate (38,78) which may explain why this type of result was obtained. Due to fibronectin's heterogeneous carbohydrate structure and charge, several classes of fibronectin may have been identified on electrophoresis. The results of the immunoelectrophoresis (IEP) experiments indicated the fibronectin antiserum binds specifically at one antigenic site or region of the fibronectin molecule.

3. Primary Antiserum Working Concentration - Fibronectin Antiserum

The optimum working concentration for anti-human fibronectin antiserum was determined as follows:

Five micron cryostat sections of normal gingiva were placed on formol-gelatin coated slides. After warming to room temperature, the unfixed sections were reacted with antiserum in the manner described in the appendix. The following primary antisera concentrations were used: 1:5, 1:10, 1:20, 1:40, 1:60, 1:80, 1:100, 1:160, 1:320, and 1:640. Goat anti-rabbit IgG conjugated with FITC

was used at a 1:20 dilution. All dilutions were done with phosphate buffered saline (PBS). For the avidin-biotin complex (ABC)/peroxidase technique, a similar procedure was performed to verify the optimum primary antiserum concentration. The biotinylated goat anti-rabbit IgG was purchased as a kit in which a 1:200 dilution was utilized.

Rabbit antiserum to laminin was a gift from G.R. Martin (NIDR). The specificity and working concentration of this antiserum has been previously established (49,78). A dilution of 1:40 was used and incubation times were increased from 30 to 60 minutes to ensure bright fluorescence patterns. The secondary antiserum, rhodamine conjugated goat anti-rabbit IgG, was used at a 1:20 dilution. Normal rabbit IgG was substituted for antilaminin to serve as a control.

4. Fibronectin Antigen Absorption Control

Normal gingiva served as substrate tissue to identify the optimum antigen concentration for antigen absorption controls. One-tenth ml of fibronectin antigen diluted 1:5, 1:25, 1:50, 1:250 and 1:500 in PBS was mixed with 0.1 ml of rabbit anti-human fibronectin (1:50) resulting in a fibronectin antiserum dilution of 1:100 and fibronectin antigen dilutions of 1:10, 1:50, 1:100, 1:500 and 1:1,000. Each mixture was incubated for 20 minutes at room temperature before placing on sections. The sections were stained using one of the five prepared dilutions and comparing them to a section stained with rabbit anti-human fibronectin 1:100 without antigen absorption. A section stained with normal rabbit serum (NRS) instead of anti-human fibronectin served as another control. Goat anti-rabbit IgG

conjugated with FITC completed the reaction sequence for all sections. For the ABC method, a similar procedure was done to determine the proper fibronectin antigen concentration to serve as an antigen absorption control.

*A more detailed discussion of immunohistochemical labeling and antiserum specificity can be found in the Appendix.

5. Positive Controls

Positive controls for both fibronectin and laminin were done by using normal gingiva as substrate tissue. Utilizing the optimum primary antisera concentrations determined previously, substrate tissue was labeled for fibronectin and laminin. Indirect immunofluorescence with rabbit anti-human fibronectin (1:100) and goat anti-rabbit IgG/FITC (1:20) stained fibronectin. Rabbit antiserum to laminin (1:40) and goat anti-rabbit IgG/Rhodamine (1:20) stained laminin.

E. Indirect Immunofluorescence with FITC - Fibronectin Labeling

Slides holding specimen sections were washed for 30 minutes in two changes of phosphate buffered saline (PBS) at pH 7.2. Specimens were rinsed in distilled water and placed in a moist chamber. Incubation with rabbit IgG anti-human fibronectin (1:100) was done at room temperature for 30 minutes. A PBS rinse was followed by two 15 minute PBS washes. After a distilled water rinse, each specimen was incubated with goat anti-rabbit IgG/FITC for 30 minutes in total darkness at room temperature. Following a PBS rinse and two 15 minute PBS washes, specimens were dipped in distilled water and cover slips mounted with PBS/glycerin solution at pH 8.6. In all cases, primary antiserum was substituted with the corresponding antibody absorbed by antigen and by normal rabbit serum (NRS) as controls. Observation and photography of the tissue specimens was performed with a Wild photomicroscope equipped for transmission fluorescence with a high pressure mercury light source (HBO 200). The pattern and intensity of staining in both epithelium and connective tissue was recorded. Any specimens with positive reactions in the epithelium were photographed using Ektachrome 400 film (ASA 400).

F. Indirect Immunofluorescence with Rhodamine - Laminin Labeling

Specimens were stained for laminin in the same manner as described for fibronectin labeling with FITC except that a rhodamine conjugated antibody was used to visualize the primary antiserum bound to laminin. Incubation with rabbit antiserum to laminin (1:40) was done at room temperature for 60 minutes. Following PBS rinsing, goat anti-rabbit IgG/Rh (1:20) incubation was done for 30 minutes in total darkness at room temperature. Slide cover slips were mounted with PBS/glycerin at pH 8.6. Normal rabbit serum (NRS) served as the control. Observation and photography of the labeled tissue specimens was done as described for FITC fibronectin labeling.

G. Avidin-Biotin Complex (ABC)/Peroxidase Labeling

Biotinylated anti-rabbit IgG was purchased from Vector Laboratories, Inc. (Burlingame, CA 94010). Five micron cryostat sections were mounted on formol-gelatin coated slides and fixed in cold acetone for 10 minutes. The slides were air dried for 10 to 15 minutes and washed in Tris HCl buffer, pH 7.6 for 10 minutes x 2. Slides were dried and placed in a moist chamber and the sections incubated with 3% goat serum for 20 minutes. Unconjugated rabbit anti-human fibronectin IgG (1:200) was placed on each section and incubated for 20 minutes. A Tris HCl buffer wash was followed by a 20 minute incubation with biotin-conjugated goat anti-rabbit IgG. Following another Tris HCl wash, the sections were incubated for 60 minutes with the avidin-biotin complex (ABC)/peroxidase solution. The

specimens were again washed and incubated with freshly prepared 3,3 - diaminobenzidine tetrahydrochloride (DAB) for 5 minutes, washed in buffer for 10 minutes and counterstained with 2% methyl green for 6 minutes, dehydrated and cover slips mounted with permount. Observation and photography were done with a Zeiss photomicroscope and Kodachrome 25 film (ASA 25).

H. Classification and Staging of Carcinoma

Of the seven patients with oral squamous cell carcinoma, complete clinical records were available from six describing the stage of the lesion, its response to treatment and patient survival. Table V lists this information for each patient. The classification and staging of the tumors was done by using the TNM classification system of malignant tumors with T representing the size of the primary tumor, N indicating the extent of lymph node involvement and M the existence of distant metastasis. The mode of treatment and tumor response to treatment as well as patient survival were also recorded.

RESULTS

A. Primary Antiserum Concentration for Fibronectin Antiserum

The results of the primary antiserum working concentration experiments appear in Tables II and III. In normal epithelium, fibronectin was not anticipated to be strongly evident. Therefore, any cell staining was interpreted as nonspecific background stain. However, the lamina propria was expected to react rather strongly because of the presence of abundant collagen which fibronectin readily binds. The level of epithelial background fluorescence was negligible at 1:80 and the specific fluorescence end-point was at 1:160. Therefore, the primary antiserum dilution selected was 1:100. This assured good fibronectin-specific staining and minimal background reactivity. For the avidin-biotin complex (ABC)/peroxidase method, a chessboard titration was used to verify optimal dilutions of the unlabeled primary antiserum and the biotin labeled secondary antiserum (Table III). The upper number in each box represents stain intensity in the epithelium and the lower number, stain intensity present in the lamina propria. To assure reliable and consistent staining, a 1:200 dilution was selected for experimental use.

B. Fibronectin Antigen Absorption Control

The results of the optimum antigen absorption control experiment appears in Table IV. The concentration selected for use was fibronectin (1:10). For the avidin-biotin complex(ABC)/peroxidase method, the same fibronectin concentration was found to serve as a good control.

C. Positive Controls for Fibronectin and Laminin

Substrate tissue labeled for fibronectin by IIF with FITC resulted in positive staining in the basement membrane and several connective tissue structures. Muscle and blood vessel basement membranes were positive and other interstitial connective tissue components were reactive for fibronectin. Substrate tissues labeled for laminin by IIF with rhodamine showed positive staining in muscle and blood vessel basement membranes and in the basement membrane separating epithelium from the underlying lamina propria.

D. Normal Oral Mucosa

In sections of normal oral mucosa from 3 different regions (buccal mucosa, attached gingiva and hard palate), there was no epithelial fibronectin present. Fibronectin was present only in the basement membrane and underlying connective tissue. Figure 8 demonstrates the pattern of fibronectin distribution

within the basement membrane and underlying connective tissue in normal oral mucosa. Substituting normal rabbit serum for antifibronectin totally abolished both staining patterns.

Laminin was observed in the basement membranes of blood vessels and skeletal muscle, it was absent from the epithelium while strongly reactive along the basement membrane.

E. Nonspecific Inflammation

There was no discernible change in fibronectin distribution in sections showing nonspecific inflammation when compared to normal oral mucosa.

F. Leukoplakia

There were no discernible changes in the distribution of fibronectin in the basement membrane or underlying connective tissue in oral leukoplakia when compared with normal oral mucosa. Several specimens demonstrated significant epithelial hyperplasia and elongated rete peg formation. However, in none of these sections was the fibronectin distribution altered. Figure 9(a,b,c,d) are photomicrographs of a specimen of leukoplakia adjacent to an area of squamous cell carcinoma; epithelial hyperplasia adjacent to the region of carcinoma was striking but the distribution of fibronectin was not altered as detected by both the avidin-biotin complex(ABC)/peroxidase and indirect immunofluorescence (IIF)

methods. Figure 10(a,b) shows the absence of fibronectin staining in epithelial cells with the exception of a uniform surface stain considered as artifact. Figure 11(a,b,c) shows a specimen of verrucous hyperplasia labeled for fibronectin using both the avidin-biotin complex (ABC)/peroxidase and indirect immunofluorescence (IIF) techniques. Fibronectin appeared to be present in superficial keratinocytes. Figure 12(a,b,c) shows the reaction obtained using the ABC method to label fibronectin. There was intense staining in the interstitial connective tissue and within the keratin pearl.

In the single specimen of severe dysplasia there was no evidence of fibronectin in the epithelial component and no alteration in the distribution of fibronectin in the basement membrane or underlying connective tissue.

G. Squamous Cell Carcinoma

There were a variety of histomorphologic patterns in the 7 specimens of oral squamous cell carcinoma. Two specimens demonstrated a significant amount of keratin pearl formation. In one specimen, there was a streaming or pallisading pattern of epithelial invasion into the adjacent connective tissue. In several specimens, large islands of cancerous epithelial cells were only separated by thin connective tissue. In one specimen, the tumor epithelium extended to the skeletal muscle located in the deep connective tissue.

At the tumor margin in the area of transition from normal oral epithelium to cancerous epithelium, fibronectin was present in both the basement membrane and adjacent connective tissue. There was no fibronectin present in the epithelial component of the tumor. Figure 13_a shows reactivity of FITC-labeled antiserum to fibronectin, there was intense staining for fibronectin in the connective tissue and within a keratin pearl. Figure 13_b is a photomicrograph of the same specimen reacted with antiserum directed against laminin. The keratin pearl reactivity was similar to that observed for fibronectin, but the absence of interstitial connective tissue reactivity was striking. Basement membrane and perivascular structures positive for laminin were quite distinctive, a broad continuous band of laminin reactivity surrounded blood vessels and a narrow continuous pattern of staining was present in the basement membrane.

Figure 14(a,b,c) shows the positive reaction for fibronectin in the connective tissue between islands of cancerous epithelium. Fibronectin was not present in tumor epithelial cells and loss of fibronectin in the surrounding connective tissue stroma was never seen. In contrast, figure 15(a,b) shows laminin

reactivity present as a narrow, continuous band interfacing between islands of cancerous epithelium. Figure 16(a,b) shows the morphologic pattern of tumor cell islands in a patient with squamous cell carcinoma. Figure 16_a is an H & E photomicrograph that illustrates the streaming growth pattern of the lesion and the thin intervening connective tissue present. Figure 16_b shows fibronectin present within a continous basement membrane structure surrounding tumor islands and in the adjacent connective tissue. Figure 17(a,b) are photomicrographs of a specimen of squamous cell carcinoma in which the tumor mass extended up to the region of striated skeletal muscle. Figure 17b shows the persistence of laminin surrounding the invasive tumor mass and a laminin-positive basement membrane in the muscle perimysium.

In the seven patients with oral squamous cell carcinoma, clinical records were available for six. A clinical evaluation of each patient was performed as outlined in the Materials and Methods section. The stage of the tumor, mode of treatment, response to treatment and survival of each patient was recorded (Table V).

Table VI summarizes in tabular form the distribution of both fibronectin and laminin in human normal oral mucosa, leukoplakia and squamous cell carcinoma. The histologic diagnosis of the specimens studied and the location of fibronectin and laminin are listed.

DISCUSSION

In this study, the distribution of the glycoproteins fibronectin and laminin was determined in normal and pathologic oral mucosa. The purpose of this study was to observe the distribution of both fibronectin and laminin in human normal oral mucosa, leukoplakia, and squamous cell carcinoma. The presence and location of fibronectin and laminin was compared with the histologic appearances in each specimen. We observed the distribution of both glycoproteins in normal oral mucosa from 3 oral sites; attached gingiva, buccal mucosa and hard palate. Identification of the structures that normally contain fibronectin and laminin was done. We determined if the distribution of fibronectin or laminin differed in leukoplakia with or without epithelial dysplasia, also whether an alteration of either glycoprotein occurred in leukoplakia that later transformed to carcinoma. Such a finding would be of great prognostic value to help identify lesions of leukoplakia that are truely premalignant. The stage of the lesion, the tumor response to treatment, and patient survival were recorded for these patients. We compared the staining patterns for fibronectin and laminin for each of the tumors, any differences in glycoprotein distribution among them might indicate the behavior of the cancer and the most appropriate treatment necessary.

Fibronectin and laminin are located in distinct structures of normal oral mucosa. In normal oral mucosa, fibronectin was present in blood vessel and muscle basement membranes and in basement membranes separating epithelium from the lamina propria. Other connective tissue components were also reactive for fibronectin; these probably included interstitial collagens and glycosaminoglycans (GAG's). Fibronectin was not present within or between

epithelial cells. Our findings corraborate with others which indicate that fibronectin serves as a general binding protein maintaining normal mesenchymal-epithelial form and function. Fibronectin is an adhesive protein in fibroblast cell aggregation, and cell-substratum attachment (32), it mediates fibroblast attachment to collagen (35) and the attachment and spreading of cells on collagenous matrices (35,36). Fibronectin also binds to other extracellular matrix components including hyaluronic acid and heparin sulfate (62) to form stable macromolecular complexes. Although cultured epithelial cells do synthesize fibronectin (58,69,72,74), it appears that stratified epithelia do not contain it (95). Other cell membrane structures are apparently responsible for cell-cell adhesion in epithelia. Among these epithelial cell membrane specializations are the zonula occludens (tight junction), zonula adherens (intermediate junction), and macula adherens (desmosome) (96). Laminin was also absent in the epithelium of normal oral mucosa and was found in basement membrane structures of blood vessels, muscle, and at the epithelial-connective tissue interface. This was consistent with the the findings of numerous other authors in a wide variety of tissues (78,79,80,81,82,83).

In nonspecific inflammation, there was no discernible alteration in the distribution of either fibronectin or laminin. One might anticipate an increase of fibronectin in inflammed mucosa due to the release of blood plasma fibronectin from the serum. Also, because fibronectin binds to certain bacteria (97,98) and promotes macrophage phagocytosis (99,100,101,102), an increase of macrophage-associated fibronectin could occur. However, such changes were not seen and may be attributable to the limited sensitivity of the immunolabeling methods used, or, in vivo, fibronectin does not play a significant opsonic function.

In leukoplakia with epithelial hyperplasia or verrucoid hyperplasia, fibronectin and laminin were located in the same regions they occupy in normal oral mucosa. An occasional positive reaction for fibronectin was seen on epithelial surfaces but was probably spurious. Such immunoreactivity appears attributable to slight epithelial folding or edge effect. In 3 or 4 specimens, mild fibronectin staining was present in intracellular and extracellular regions of hyperkeratinized epithelium. However, the strong immunoreactivity of both fibronectin and laminin antisera for keratin pearls suggested that strong nonspecific binding affinity to keratin was present. Therefore, the faint reactivity for fibronectin in hyperkeratosis was considered to be nonspecific binding. There was no evidence of either loss of laminin or de novo laminin synthesis by basal epithelial cells. The latter might have occurred considering the increase in epithelial cell proliferation present in these lesions. These results indicate that new epithelial basal cell attachment to the basement membrane via laminin was not detectable by the methods used. Similarly, there was no change in fibronectin or laminin distribution in leukoplakia with epithelial dysplasia. In addition, none of the patients with leukoplakia have subsequently developed carcinoma during the course of this investigation. Therefore, the distribution pattern of fibronectin and laminin in leukoplakia may not afford a means to identify lesions with a high risk of later transformation.

A number of authors suggest that alteration of the connective tissue extracellular matrix accompanies the evolution of epithelial cell neoplasia. It is understood that epithelial-mesenchymal interactions play an important role in embryogenesis and maintenance of tissue structure and function. In fact, an interplay between the two has become an active area of research. Bornstein (103)

first used the term "dynamic reciprocity" to describe the intimate interplay between epithelial cells and their normal stroma. The mesenchyme often plays a cardinal role in directing the differentiation of epithelia. Mesenchyme appears to serve an inducing function to elicit functional differentiation of epithelial cells. As an example, mesenchyme of the urogenital sinus induces urinary bladder epithelium to form prostatic acini (104). Conversely, the removal of the extracellular matrix leads to loss of differentiated functions (105).

Epithelial neoplasms can progress through a series of abnormal states prior to invasive behavior. This is especially evident in cervical and endometrial carcinogenesis in which a progression of epithelial atypias have been described (106,107). A preliminary alteration in these lesions is vaginal hyperplasia and hyperkeratinization (108). This hyperplasia and keratinization has been induced by estrogen injection in perinatal mice and is maintained into adulthood even in the absence of estrogen. Induction of this change requires the maintenance of the epithelial-mesenchymal unit at the time of estrogen exposure (109). The continued expression of estrogen-independent vaginal hyperplasia is maintained into adulthood by an interaction between the abnormal epithelium and mesenchyme (109).

These findings support the view that neoplasia is an abnormal form of differentiation (110,111). In neoplasms, transforming genes may be activated that are related to normal differentiation. If this is correct, an embryonic inductor should exist that may regulate the neoplasm. The appropriate extracellular environment could have a regulatory influence on the induction, progression, and maintenance of carcinomas. Evidence for this concept exists in bladder (112), mammary (113), skin (114), oral (115) and salivary tumors (116). The results in this

study suggest that mesenchymal changes responsible for the development of epithelial neoplasms in oral mucosa do not directly involve alterations of fibronectin or laminin.

In oral squamous cell carcinoma, there was surprisingly little change in distribution of either glycoprotein. In transitional regions of normal and neoplastic epithelium (i.e. the tumor margin), fibronectin and laminin were located in their normal tissue sites and at apparently normal concentration. Therefore, it was impossible to correlate the clinical course of the disease with an alteration of either fibronectin or laminin distribution. Despite the fact that oral cancer survival data correlates with stage grouping according to tumor size and evidence of metastatic spread (1), alterations of fibronectin or laminin do not appear helpful as a prognostic aid according to the results we obtained. Most of the tumors studied were either Stage I or II according to the TNM classification (5/6). Of this group of lesions, 4/5 responded well to either surgical or radiation treatment while one patient currently has recurrent disease. The single patient with Stage III disease developed recurrent carcinoma that could not be controlled and resulted in death. However, the presence and distribution of both fibronectin and laminin was not detectably different between these 6 carcinomas. Therefore, prediction of treatment response or mortality by means of fibronectin or laminin distribution is not feasible. From previous studies (50,51,52,66,67,69), it was hypothesized that loss of connective tissue fibronectin would signal the transition of premalignant epithelium to neoplastic epithelium. However, in all instances, fibronectin was present as both a continuous band enveloping neoplastic cells and uniformly present in the surrounding stroma. Neoplastic epithelia were always negative for fibronectin indicating neoplastic cell fibronectin synthesis did not

occur. Other events probably occur in the tumor extracellular matrix that are responsible for the cleavage of fibronectin. The events observed in this study were probably the result of a number of molecular events culminating in neoplastic cell proliferation, connective tissue destruction, and tumor migration or invasion.

Information presently available suggests that transformation is associated with changes in fibronectin, collagen and sulfated glycosaminoglycans (117). A decrease in the amount of surface-associated fibronectin is a consistent finding in virus transformation models (52). Therefore, a number of possible mechanisms could explain the loss of fibronectin from the extracellular matrix. Among these are: decrease in fibronectin synthesis or release of an altered fibronectin molecule, proteolytic cleavage of fibronectin, altered microfilament function, or loss of fibronectin receptors.

Human transformed cells continue to synthesize fibronectin but fail to retain the molecule on their cell surfaces (52,118,119). In addition, the fibronectin molecule synthesized by transformed human cells appears to be identical to normal fibronectin in subunit size (118) and in immunologic characteristics (52,119). Both tumor and stromal proteases may play a role in degradation of the extracellular matrix. In basal cell carcinoma, collagenase is present only in the stromal elements surrounding tumor islands and none was present in the epithelial component of such tumors (120). However, Timpl (121) and Woolley (122) have reported that skin collagenase fails to degrade type IV collagen under conditions in which type I, II, and III collagen are cleaved. A separate enzyme might be involved in the turnover of basement membrane collagen. In well characterized cell transformation models, there is an enhanced secretion of plasminogen

activator (123). Plasminogen activator converts serum plasminogen to the proteolytic enzyme plasmin. It is well known that fibronectin is highly sensitive to plasmin because of its flexible structure (124). In addition, many connective tissue cells can synthesize procollagenase which can be activated by serine proteases to the active protease, collagenase (125,126). Removal of serum plasminogen or addition of protease (plasmin) inhibitors fails to restore surface fibronectin to transformed cells (55). Although until recently only intact fibronectin molecules have been isolated from the media of transformed cultures (118), a melanoma metastatic cell line can degrade fibronectin. B16 melanoma cells are able to invade subendothelial matrix and degrade matrix polypeptides and sulfated proteoglycans (127). Subendothelial fibronectin was cleaved into an unmodified alpha form (M_r=230,000) and a tumor-cell modified beta form (M_r=225,000). The results obtained in experiments of cocultures of normal and transformed cells are at variance as well. One study determined that normal and transformed cells when cultured together retain their respective fibronectin-positive and fibronectin-negative phenotypes (128). A similar study reported a significant loss of surface fibronectin from normal cells when transformed cells were layered over the previously cultured normal cells (129). The latter result suggests that degradative enzymes from the neoplastic cells were responsible for cleavage of surface fibronectin from their normal counterparts. These findings indicate that plasmin proteolysis of extracellular matrix components is not responsible for matrix component degradation or that other as yet unidentified proteases are also involved. Such proteases may be synthesized by the tumor cells (127), by fibroblasts (125) or macrophages (130). Indeed, many forms of connective tissue invasion occur in normal development and in other biologic

events. For example, plasminogen activator is present in trophoblasts and probably mediates tissue remodeling during embryogenesis (131). Ovarian granulosa cells also contain plasminogen activator and its release triggers follicular wall degradation to allow ovulation (132).

Transformed cells usually have less well-ordered microfilaments and readdition of fibronectin causes changes in the organization of intracellular microfilaments (133). This disruption of microfilament bundles and their importance for fibronectin retention is not well understood. It is interesting to note that during mitosis, fibronectin loss and microfilament disorganization occur (12). Cell adherence or anchorage to the extracellular matrix may not be required during cell division and proliferation (134). In fact, malignantly transformed cells have escaped normal anchorage dependence (135) and are able to grow in suspension. Therefore, altered microfilament function and loss of fibronectin interaction may allow neoplastic cells to proliferate dramatically.

Proteases are mitogenic for fibroblasts and diminish the amounts of fibronectin and alter microfilament organization (17). Trypsin, collagenase, plasmin, alpha-chymotrypsin and thrombin significantly stimulate chick embryo fibroblast cell division (17). It has recently been determined that human breast tumor matrix is mitogenic for fibroblasts (136). The newly acquired fibroblasts showed a marked change in cell morphology, growth pattern, and loss of contact inhibition. Previously, the same authors discovered a 400 to 500 percent increase in collagen synthesis when human fibroblasts were plated on the extracellular matrix of breast tumor cells.

Therefore, the release of proteases by tumor cells or elicited release from

protease-containing cells in the stroma may explain, in part, how a tumor induces modulation of its surrounding extracellualr matrix. An apparent increase of connective tissue components (e.g collagen and fibroblasts) may actually be the production of a debilitated extracellular matrix incapable of preventing tumor cell migration. Such a series of events may also explain why in this study there was no apparent diminution of stromal fibronectin. Theoretically, this reactive stromal matrix could continue to elicit further neoplastic proliferation by liberation of large amounts of "embryonic" inducer molecules.

A similar curious finding was the uniformily continuous laminin-positive basement membrane structure enveloping all neoplastic epithelial islands. This indicated that laminin was present during tumor migration and invasion. Perhaps it continues to serve its normal function as an epithelial attachment glycoprotein. Laminin-mediated neoplastic epithelial cell attachment to a continually elaborated basement membrane may afford a mechanism for tumor migration. Highly malignant cell lines attach preferentially to type IV collagen rather than to other collagen types (70). Tumor cells do readily adhere to exposed areas of the basement membrane (137) and B16 melanoma cells attach firmly and spread on subendothelial matrix with no evidence of matrix degradation prior to invasion (138).

It is often believed that the transition from in situ to invasive carcinoma is accompanied by degradation of the underlying basement membrane (139). Local dissolution of the basement membrane was observed with tumor cells entering or exiting vascular channels (140). Focal breaks in the basement membrane were observed in cervical carcinoma (141) and experimentally induced carcinoma of the skin (142). However, some areas of invasive cervical carcinoma do form basal

lamina (141) and some epithelial tumors produce large quantities of basal lamina material (143). In fact, the EHS sarcoma has been a convenient source of both laminin (84) and type IV collagen (85). Pierce (144) concluded that basement membrane formation by tumors represents retention of normal epithelial secretory function and the basement membrane does not act as a barrier to growth.

An alteration of the extracellular matrix beneath epithelial cells appears to be a prerequisite for the evolution and maintenance of neoplasia. Tissue recombination studies have demonstrated that epithelial tumors recombined with normal Extracellualar matrix regress (116,145). Just as epithelial-mesenchymal interactions are necessary in normal tissue development, an alteration of both epithelium and connective tissue may be required for progression of neoplasia. If this proves to be true, an abnormal epithelial and stromal component can synergistically promote neoplastic progression and invasion.

The variety of types of neoplasia and their different biologic and clinical activities suggests differences exist in both the neoplastic epithelial cell population and its associated connective tissue extracellular matrix. The gene pool of different cell types may equip them with varying capacities for proliferation and metastatic dissemination. Perhaps highly metastatic tumor cells such as the EHS sarcoma or the B16 melanoma can gain access to vascular structures because they contain unique proteases capable of solubilizing the limiting subendothelial matrix. The pulmonary metastasis of the T241 fibrosarcoma sarcoma liberates a neutral protease that specifically cleaves type IV collagen (146) and highly invasive and metastatic B16 melanoma cell lines readily solubilize sulfated glycosaminoglycans (147).

The findings of this study support the view that neoplasia is an abnormal form of differentiation. In oral squamous cell carcinoma, loss of fibronectin or laminin was not observed. Transformation of both epithelial cells and fibroblasts may be required for progression of neoplasia with subsequent tumor migration. Transforming genes located in each cell population may be required to enhance neoplastic cell proliferation and production of an abnormal extracellular matrix. Proteases liberated by both stromal and neoplastic cells can serve as a means to modulate the extracellular matrix and enhance tumor cell proliferation. Laminin may, in fact, assist neoplastic cell attachment to the connective tissue extracellular matrix via type IV collagen. Highly metastatic cells may possess proteases capable of degrading type IV collagen in endothelial basement membranes to offer neoplastic cells access to vascular channels promoting tumor cell dissemination. Neoplasms with limited metastatic potential may be deficient in the proteases required for endothelial basement membrane degradation.

SUMMARY

Lesions such as leukoplakia present difficulties in diagnosis and proper treatment because the significance of clinical and histopathological features of these lesions are disputed among observers and prediction of their future behavior is not reliable. Fibronectin is a ubiquitous blood plasma glycoprotein which mediates a variety of cell functions and numerous studies have documented the loss of fibroblast-associated fibronectin in malignantly transformed cells. Such findings have led to the hypothesis that the loss of surface fibronectin may allow tumor cells to metastasize because of the altered interactions between tumor cells and their surrounding extracellular matrix. Laminin is another recently discovered glycoprotein that is only present in basement membranes. At the present time, laminin appears to serve as the attachment factor for epithelial cells to the basement membrane. In fact, laminin assists epithelial attachment to a specific component of basement membranes, that being type IV collagen.

We observed whether human oral lesions of leukoplakia and squamous cell carcinoma differed with respect to the distribution of fibronectin when compared to normal oral mucosa. We also observed the distribution of laminin in human normal oral mucosa, leukoplakia and squamous cell carcinoma. We determined if fibronectin or laminin was lost in some leukoplakia lesions and whether those lesions eventually converted to neoplasia. Also, we determined if basement membrane laminin was present surrounding oral squamous cell carcinoma.

We obtained biopsy specimens from patients who presented at the Oral

Medicine Clinic of the School of Dentistry, University of California, San Francisco with suspected precancerous or cancerous lesions. We used both an indirect immunofluorescence and an avidin-biotin complex (ABC)/peroxidase technique to label fibronectin. Specificity of the human fibronectin antiserum was verified by immunodiffusion (ID), immunoelectrophoresis (IEP) and antiserum absorption prior to tissue incubation. Antiserum to laminin was obtained from a donor and rhodamine-labeled antiserum was used to visualize the antiserum-bound laminin.

The results obtained indicate that fibronectin and laminin are located in distinct structures of human normal oral mucosa. Fibronectin was present within basement membranes of blood vessels, muscles and separating epithelium from lamina propria. It was also present in other interstitial connective tissue components not identifiable by these methods. In no case was fibronectin located in the epithelium. Laminin was present in the same basement membranes as described for fibronectin, but was never seen in epithelium or interstitial connective tissue with the exception of blood vessel and muscle basement membranes. In nonspecific inflammation, and all lesions of leukoplakia, fibronectin and laminin distribution was no different than that seen in normal oral mucosa. Unfortunately, this does not offer a means of classifying the truely premalignant leukoplakia lesion. In oral squamous cell carcinoma, fibronectin was always present in the stroma surrounding neoplastic epithelium and was not synthesized by the neoplastic epithelium. Laminin was always present as a continuous structure enveloping neoplastic islands.

This study indicates that both fibronectin and laminin continue to serve a functional role in tissue interactions occurring in neoplastic oral mucosa. The

persistence of both glycoproteins in their normal tissue sites lends support to the hypothesis that neoplasia is an abnormal form of differentiation. The extracellular environment could play an important regulatory role in neoplastic induction, progression and maintenance. The alterations of the epithelial and mesenchymal elements in carcinogenesis of oral mucosa must involve more basic molecular events than those observed in this investigation. The end result may be an enhanced but abnormal form of differentiation characterized by significant but poorly coordinated epithelial and stromal growth patterns and quite rapid turnover of basement membrane components. Fibronectin may well be present in normal amounts but be unable to function properly due to the derangement in other extracellular matrix components. Likewise, new basal lamina components, laminin and type IV collagen may be synthesized by the neoplasm or components of the stroma to aid tumor invasion.

Although the results of this study do not offer immediate benefits in the management of patients presenting with leukoplakia or squamous cell carcinoma, it does suggest that other molecular changes must occur to trigger the abnormal growth patterns present in these lesions. In particular, it emphasizes that alteration of the basement membrane may occur in only a limited or selective fashion. Retention of normal growth functions of basement membrane components may be required for tumor induction, progression, and invasion. The traditional conception of the basement membrane acting as a barrier to growth and tumor invasion may require modification.

APPENDIX

The method of detection of fibronectin and laminin in this study was by indirect immunofluorescence and the avidin-biotin complex (ABC)/peroxidase technique.

A. Immunohistochemical Labeling

Immunohistochemical labeling is the localization of specific substances (antigens) in tissue by means of specific antibody binding. Visualization of this antigen-antibody binding in tissue sections is achieved by the covalent coupling of fluorescent molecules or enzymes to substances that bind directly or indirectly to the tissue substance of interest.

Direct immunofluorescence (DIF) is the attachment of fluorescent antibody to a tissue antigen. A separate fluorescent antibody is required for each antigen studied and thus this method lacks the versatility available with the indirect immunofluorescent technique. Indirect immunofluorescence (IIF) is a sandwich technique in which a fluorescent IgG is used to detect the presence of a specific antibody previously attached to the antigen of interest. This method is valuable for two major reasons: a) antigens that are difficult to obtain preclude the production of large quantities of fluorescent specific antibodies and b) the indirect technique allows amplification of fluorescence by a factor of 3 or 4. Another popular technique for immunolabeling are the various immunoenzyme methods. In these techniques a marker enzyme rather than a fluorescent molecule is used to visualize the location of the antigen of interest. Three commonly used enzymes

are E. coli alkaline phosphatase, Aspergillis niger glucose oxidase, and horseradish peroxidase. These enzymes have been frequently used in an indirect antibody labeling procedure by enzyme attachment to a second stage antibody that binds to a primary antibody previously bound to a specific tissue antigen. The substrate most frequently used for demonstration of peroxidases has been 3,3'-diaminobenzidine (DAB) (148). This reagent is a carcinogen, so alternative substrates are being developed. Mason, et.al. (149,150) and Sternberger (151) developed an unlabeled antibody enzyme technique, the Peroxidase-Antiperoxidase (PAP) method. This immunoperoxidase procedure increases the sensitivity 4 to 5 times that possible with the simple indirect immunoperoxidase technique. The sensitivity of the technique is dependent upon the formation of a stable peroxidase-antiperoxidase complex that increases the number of peroxidase enzymes available for reaction.

The most recent step in the evolution of these immunoenzyme techniques has been the development of the Avidin-Biotin Complex (ABC)/Peroxidase method as an alternative to the unlabeled antibody (PAP) procedure (152). Guedson, et. al. (153) developed two procedures using avidin-biotin interaction in quantitative enzyme immunoassay. Avidin is an egg white glycoprotein with an extraordinary affinity for the vitamin, biotin. Covalently coupling biotin to IgG or peroxidase molecules gives them the ability to bind avidin molecules. The indirect bridged avidin-biotin technique therefore couples the affinity of specific antibody for their respective antigen in tissue with the strong affinity of avidin for biotin to attach peroxidase molecules to tissue-labeled antigens. Following the addition of biotin-labeled secondary antibody, a preformed avidin-biotin-peroxidase complex is added. Visualization by addition of DAB reveals the peroxidase molecules.

The affinity of avidin to biotin is quite remarkable, the dissociation constant is 10^{-15}M. (154). Most antigen-binding sites of antibodies have binding (dissociation) constants that range from 10^{-4} to 10^{-10}M. (154). Therefore, the improved sensitivity and specificity of this technique over the PAP method may, in part, be attributable to this difference in binding affinity.

B. Antiserum Specificity

Specificity of antiserum can be verified by several methods. Gel diffusion, immunoelectrophoresis, and specific antigen preabsorption are the most reliable methods. Gel diffusion techniques have been used since 1897 to create immunoprecipitates by the interaction of soluble antigen and the corresponding antiserum. Immune complexes formed after antigen-antibody interaction rapidly exceed the gel pore size and precipitate in the gel. An equivalent ratio of antigen and antibody is best employed to ensure a balanced gel diffusion system. The ratio of reactants present at the shortest time of precipitation is called the optimal ratio (OR). If the optimal ratio is not predetermined, precipitate formation can be partially or totally inhibited.

Electrophoresis is the migration of charged particles in a conductive solvent under the influence of an electric field. The amount of charge and the size and shape of molecules placed in the medium affect the rate of migration. More rapid separation can be achieved by using an increased voltage; a cooling system is employed to avoid excessive heat production with resulting dehydration and deformation of the gel. Immunoelectrophoresis (IEP) combines gel

electrophoresis and gel diffusion to determine antiserum specificity. Following the separation of distinct particles by electrophoresis, antiserum specific for the test antigen is placed in the medium and diffusion of both the separated antigens and the antiserum takes place. If a single precipitation arc is formed, the antiserum is considered to be monospecific for the antigens evaluated.

A schematic representation of the IEP technique is shown in Figure 9. In the first step, gel electrophoresis is performed which would separate the distinct antigens present (i.e. a-b-c). In the second step, immunodiffusion of the corresponding antiserum (A-B-C) causes specific precipitate arcs to form.

Immunodiffusion and immunoelectrophoresis can be used to identify an antiserum that is not monospecific. However, monospecificity for a particular tissue antigen can be best proven by the admixture of pure antigen and antiserum prior to tissue staining (155). If this antigen absorption prevents antiserum binding, the specificity of the antiserum can be assured. Optimum absorbing antigen concentration must be determined by experimentation.

The immunochemical reagents should stain only by means of specific immunological reactions. Electrostatic and hydrophobic binding of stain reagents is conceivable but with proper methods can be adequately eliminated and confirmed by control reactions. The net positive (+) charge of tissues in relation to immunoglobulins could cause nonspecific staining. Therefore, conjugates of low fluorochrome/protein ratio are advisable; a low F/P ratio minimizes the negative charge of the conjugate (155). Following fluorochrome conjugation, macromolecular protein aggregates can form and adhere to tissue nonspecifically by means of hydrophobic bonding. Centrifugation of fluorochrome conjugates prior to specimen staining will effectively remove these aggregates. The final

possible source of nonspecificity is binding of conjugated antiserum to F_c receptors located in tissue. However, the affinity of these receptors is generally low and a moderate dilution of the conjugate will significantly diminish this type of staining (76).

The proof of specificity of antigen localization in tissue differs dependent upon the method used, i.e. DIF or IIF. Regardless of the technique employed, both method specificity and antibody specificity must be verified. The former requires that no staining should be evident in the absence of the primary antiserum. The second requirement is that staining should be abolished by the absorption of the antiserum with the primary antigen. When using direct immunofluorescence (DIF), staining should be inhibited by preincubation of the tissue section with unconjugated specific immune serum but not by nonimmune serum. This is commonly referred to as a Blocking Test. When using the indirect immunofluorescent (IIF) method, staining should not occur when the conjugated antiserum is first absorbed with the antigen. This is an absorption control. Staining should also be eliminated when either nonimmune primary antiserum or no primary antiserum is used.

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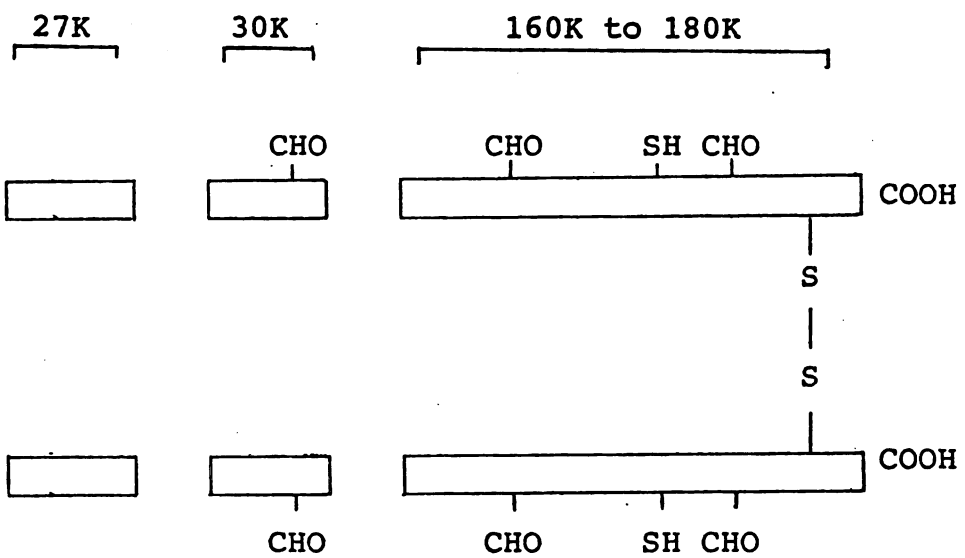
FIGURE 1 STRUCTURE OF FIBRONECTIN



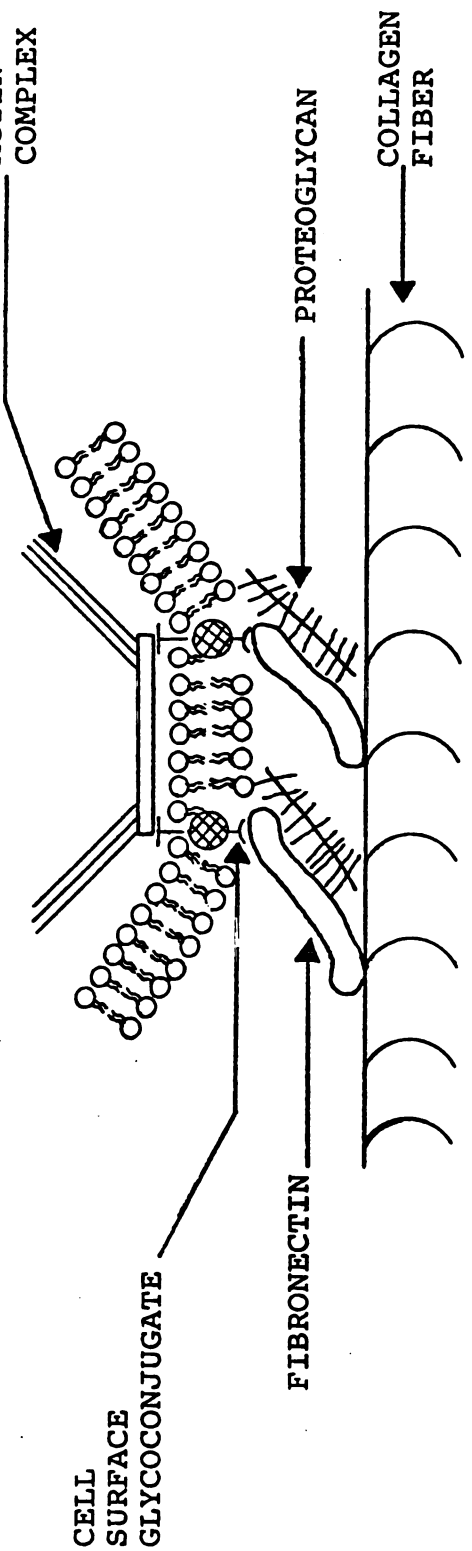
Collagen Binding Cell Binding

Schematic representation of structure of fibronectin*

* Adapted from Ruoslahti, E, J Oral Path 10: 3, 1981

FIGURE 2 SCHEMATIC MODEL OF FIBRONECTIN ADHESION SITE

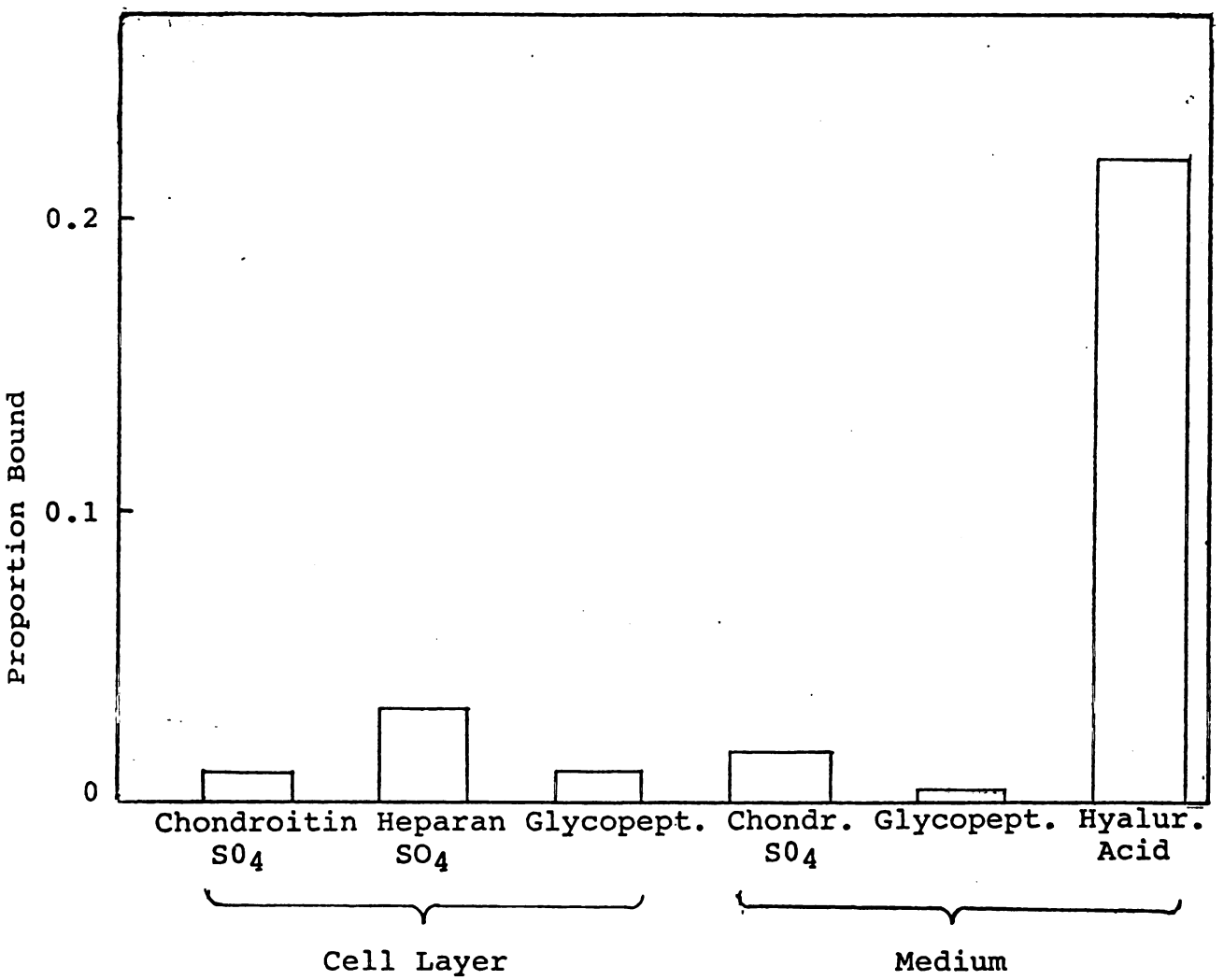
SCHEMATIC MODEL OF FIBRONECTIN ADHESION SITE



Schematic model of an adhesion site*

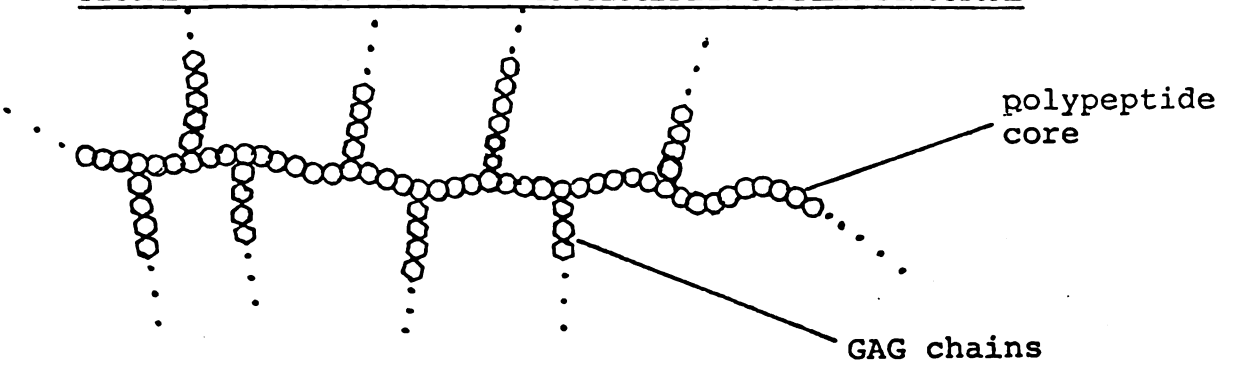
* Adapted from Kleinman, HK, Klebe, RJ, & Martin, GR; In: J. Cell. Biol. 88:473, 1981

FIGURE 3 SPECIFICITY OF GLYCOSAMINOGLYCAN BINDING TO FIBRONECTIN

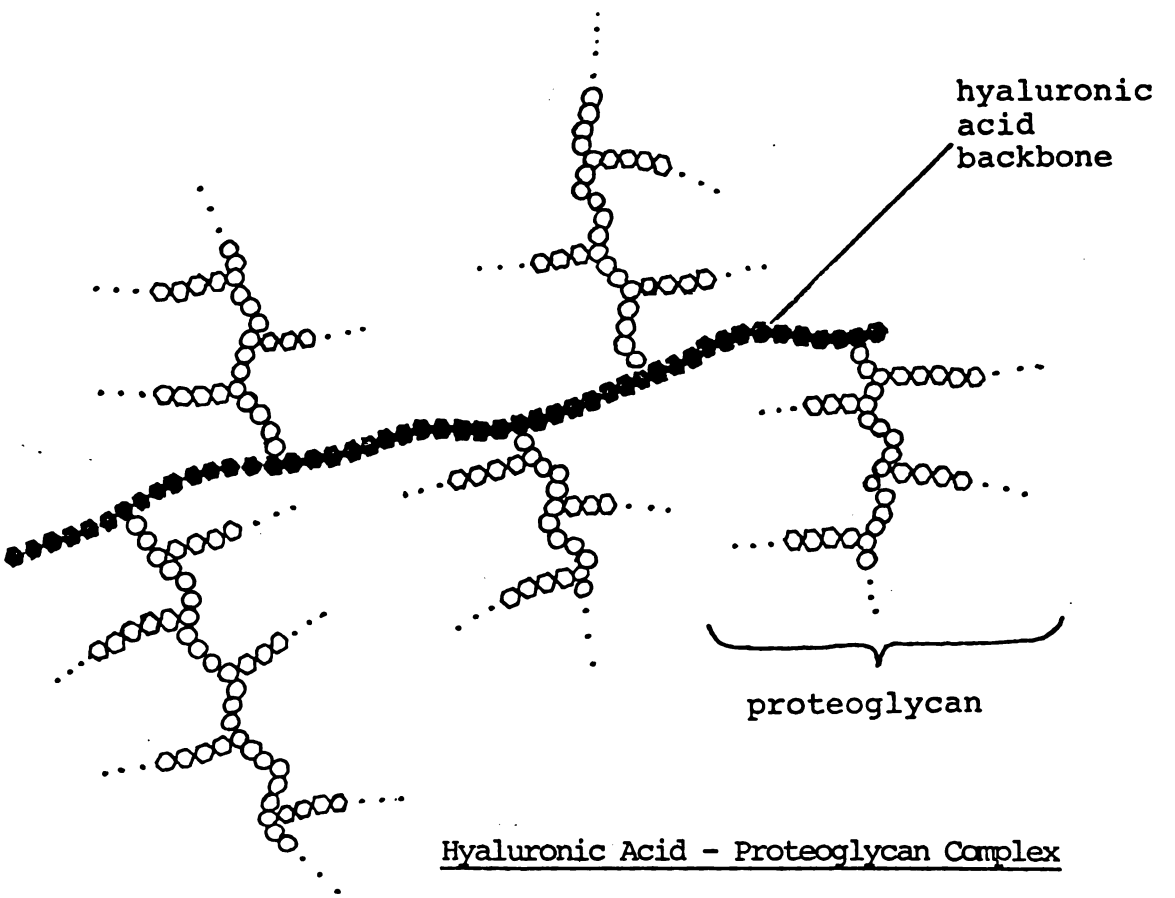


From Yamada, KM, Kennedy, DW, Kimata, K, Pratt, RM
J. Biol. Chem. 255:6055, 1980

FIGURE 4 HYALURONIC ACID - PROTEOGLYCAN COMPLEX STRUCTURE



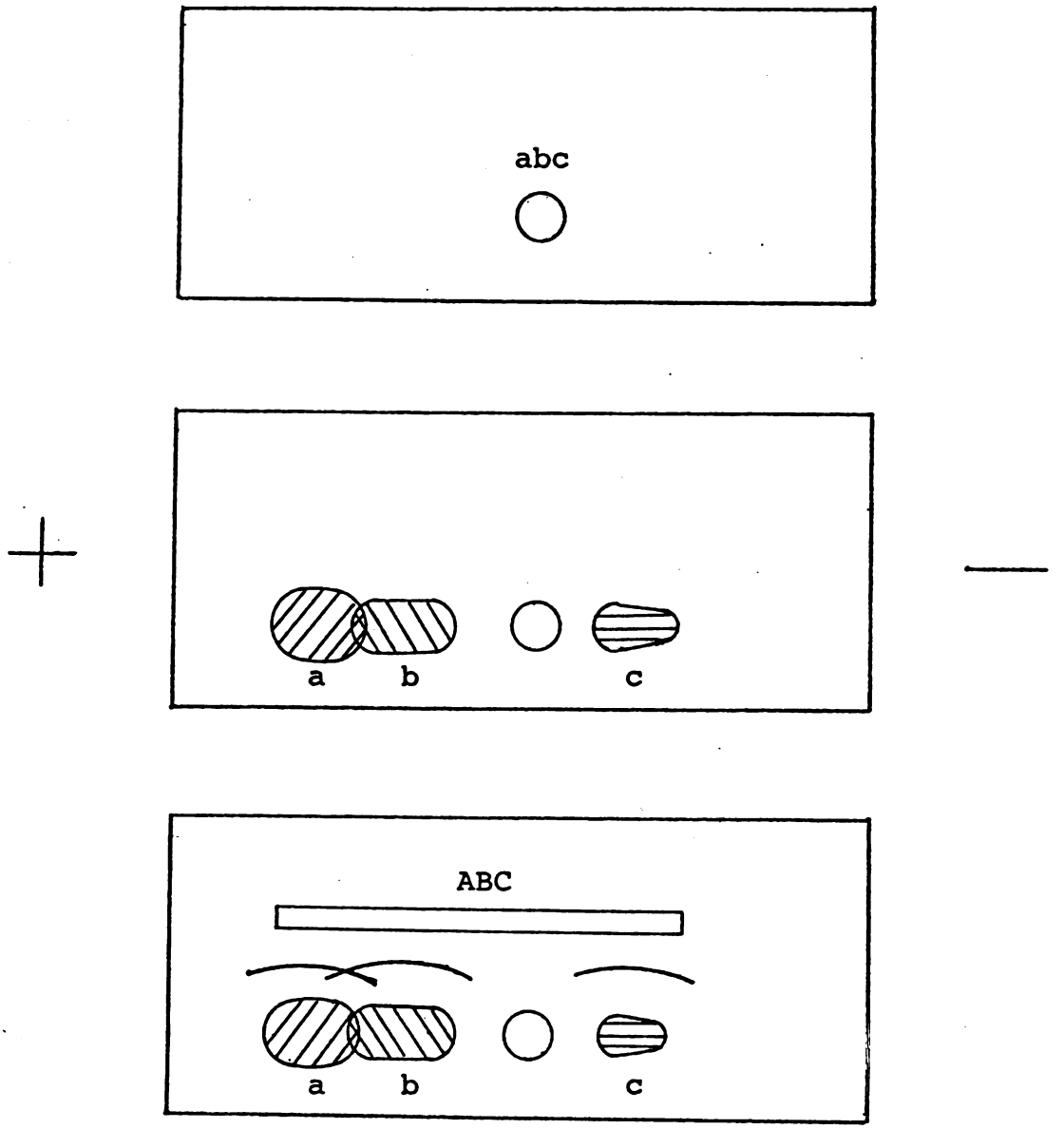
Proteoglycan Structure



Hyaluronic Acid - Proteoglycan Complex

Adapted from Wolfe, SL; In: Biology of the Cell, 2nd Ed., 1981

FIGURE 5 SCHEMATIC REPRESENTATION OF IMMUNOELECTROPHORESIS (IEP)



Schematic Representation of Immunoelectrophoresis

FIGURE 6 IMMUNODIFFUSION (ID)

Figure 6a Single precipitate arcs formed when FN_1 (1) was allowed to diffuse against rabbit anti-human fibronectin (3) for 24 h. Coomassie blue protein staining was used to reveal the precipitate bands.

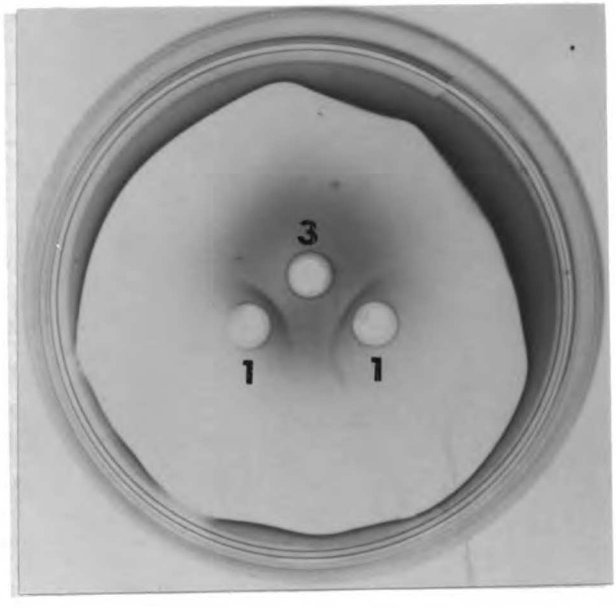


Figure 6b Single precipitate arcs formed when FN_2 (2) was allowed to diffuse against rabbit anti-human fibronectin (3) for 24 h.

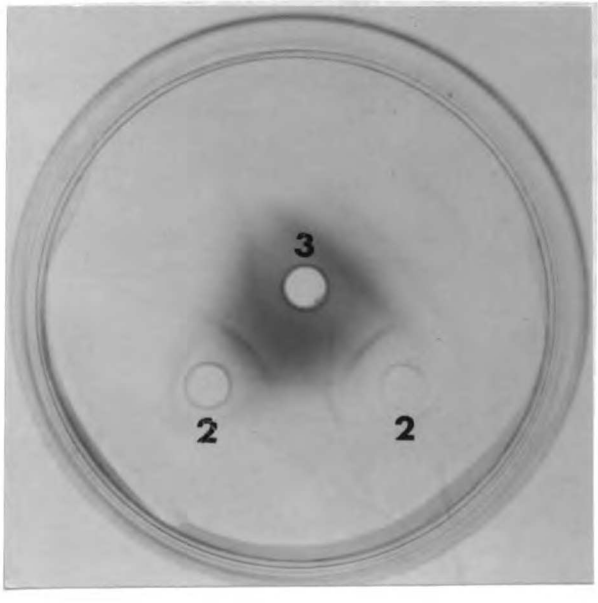


FIGURE 6 IMMUNODIFFUSION (ID)

Figure 6c Precipitate arcs formed when FN_1 (1) and FN_2 (2) were allowed to diffuse against rabbit anti-human fibronectin (3). The single identity arcs formed indicate the antiserum is monospecific.

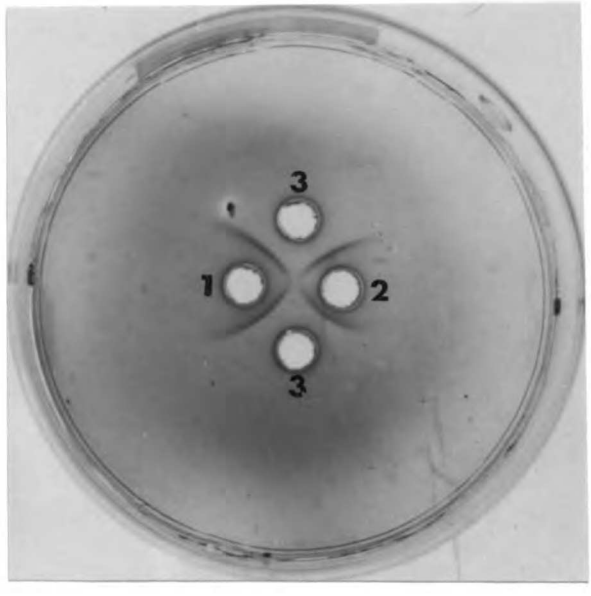


FIGURE 7 IMMUNOELECTROPHORESIS (IEP)

Figure 7a IEP Plate #1 - 10 microliters of Bromthymol blue (B), FN_1 (1) and FN_2 (2) were placed in the agarose wells and electric current applied for 45 min. at 135 mV/20mA. 100 microliters of FN antiserum was placed in each horizontal trough and incubated for 24 h, precipitate arcs were revealed by immersion in Coomassie blue for 5 min. A single arc was formed against FN_1 and FN_2, residual staining occurred in the well itself.

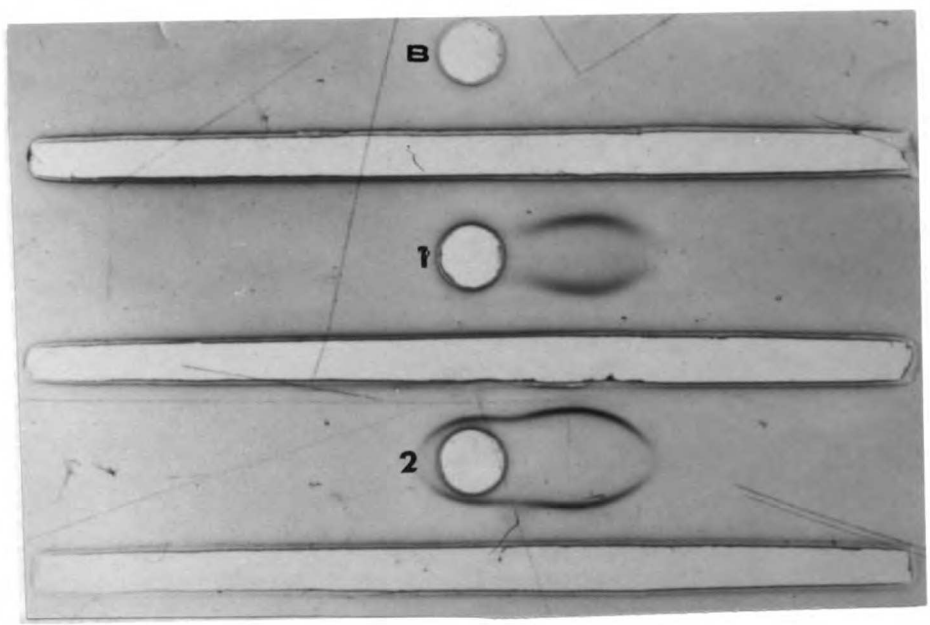


FIGURE 7 IMMUNOELECTROPHORESIS (IEP)

Figure 7b IEP Plate #2 - 10 microliters of FN_2 was placed in each well at undiluted (U), 1:2 and 1:4 concentrations. Electric current and protein staining were done as with IEP Plate #1. Residual staining in the reactant well was eliminated at a higher FN dilution (i.e. 1:4).

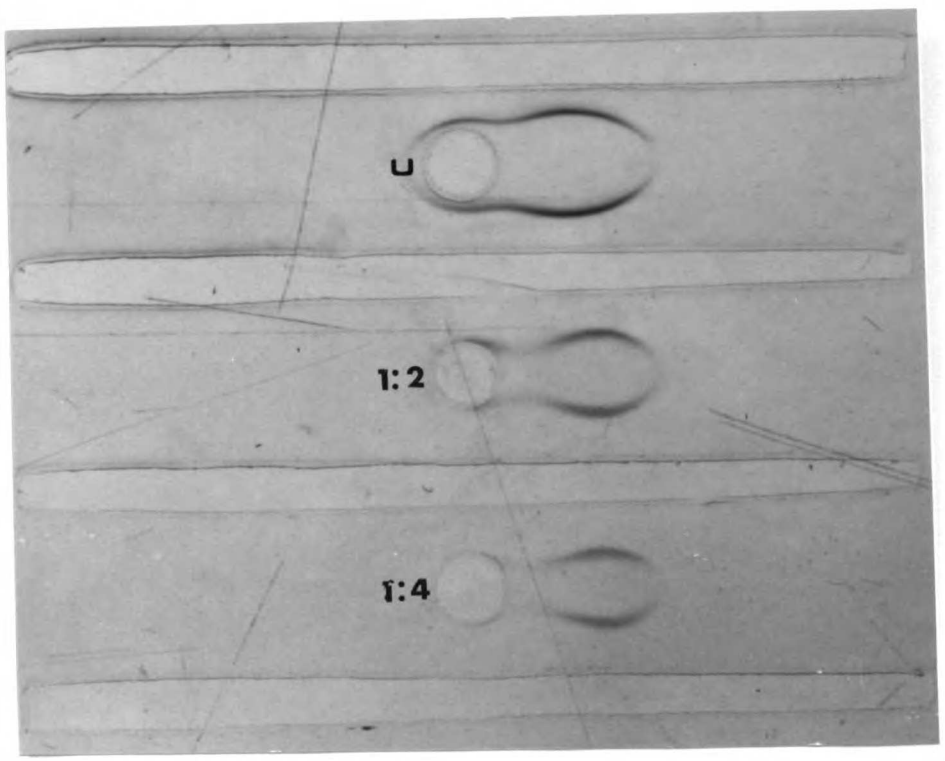


FIGURE 8

Figure 8 Distribution of fibronectin in normal oral buccal mucosa. Indirect immunofluorescence labeled fibronectin localized in the basement membrane (arrows) and the underlying connective tissue (CT). Notice the negative reaction in the overlying epithelium. IgG fraction anti-human fibronectin (1:100) and FITC conjugated antiserum (1:20); X156.

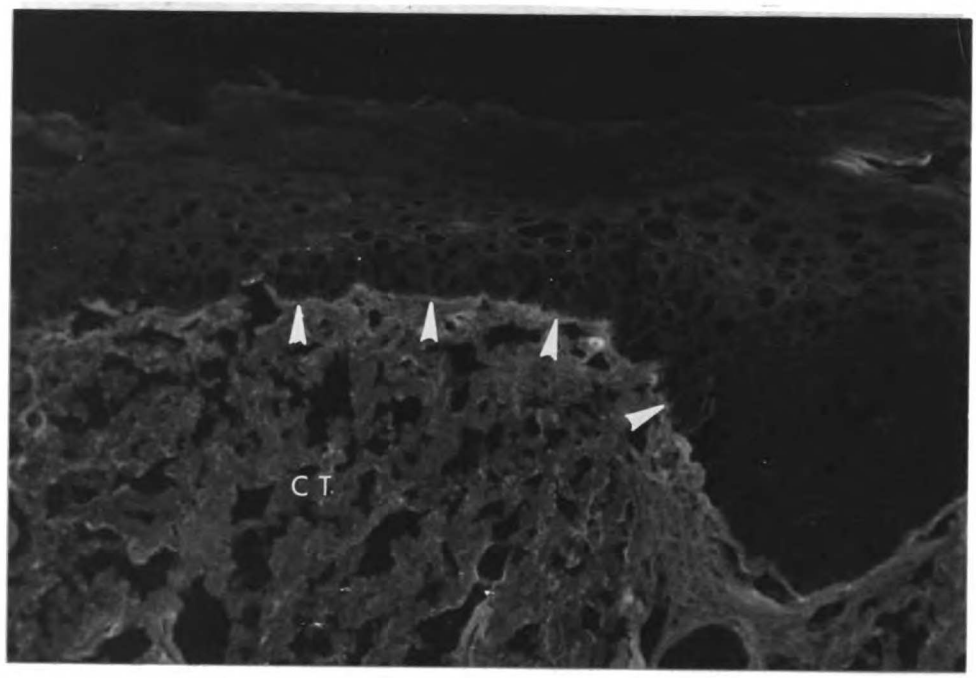


FIGURE 9

Figure 9a Hematoxylin and eosin photomicrograph of poorly differentiated squamous cell carcinoma that was located on the lateral border of the tongue. There is a region of hyperplastic stratified squamous epithelium (E) adjacent to invasive neoplastic squamous epithelial cells (CA); keratin pearl (K). X16.

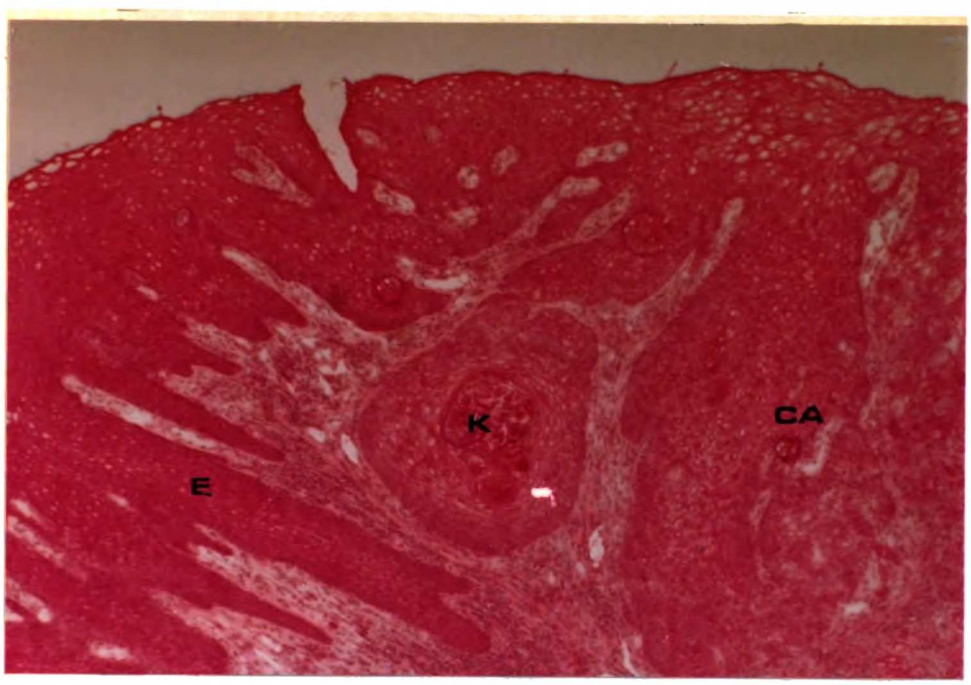


FIGURE 9

Figure 9b Avidin-biotin complex labeled fibronectin in the same region as shown in Figure 9b. Notice extensive reactivity in the connective tissue (CT) and absence of staining in the stratified squamous epithelium (E). X40.

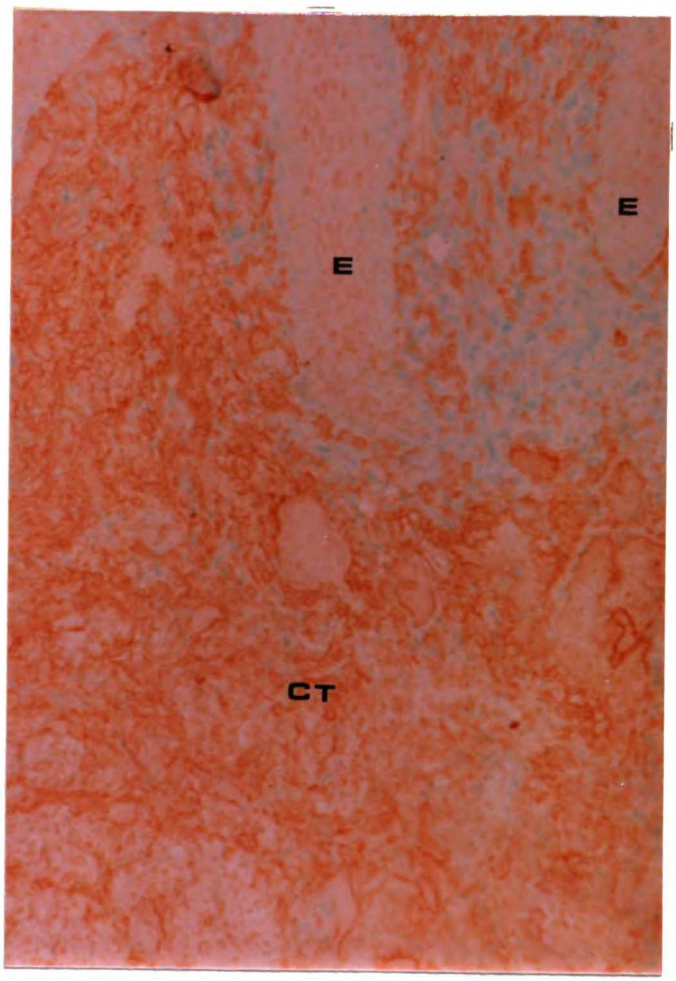


FIGURE 9

Figure 9c Indirect immunofluorescence labeling of fibronectin in the same region of hyperplastic squamous epithelium. Generalized staining in the connective tissue (CT) and no staining in the squamous epithelium (E) is noted.

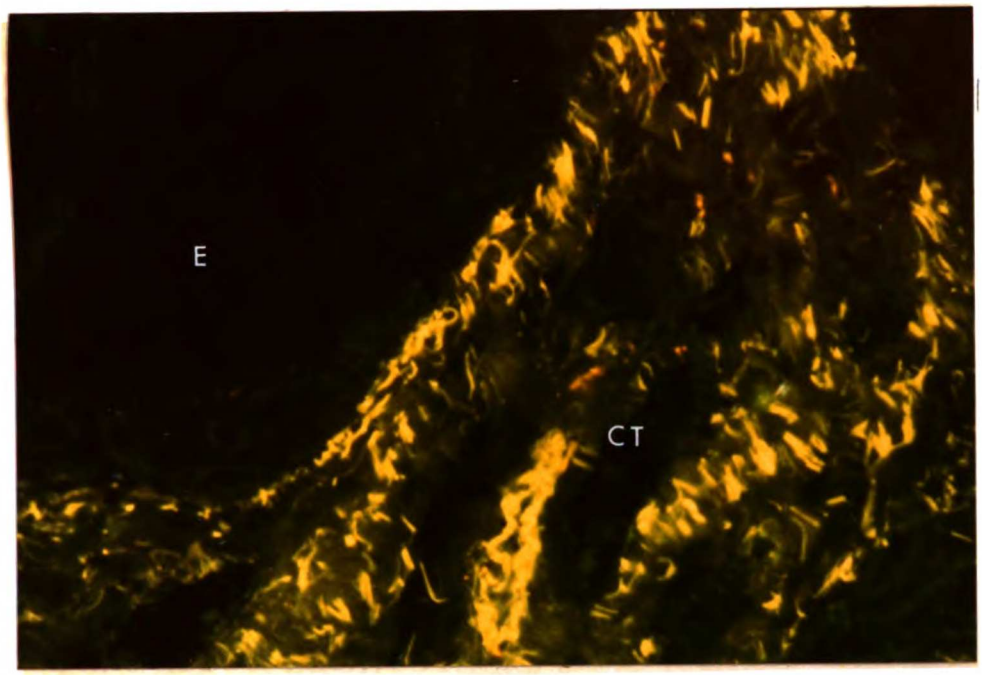


Figure 9d Fibronectin preabsorbed control shows diminished connective tissue (CT) staining for fibronectin. X156.

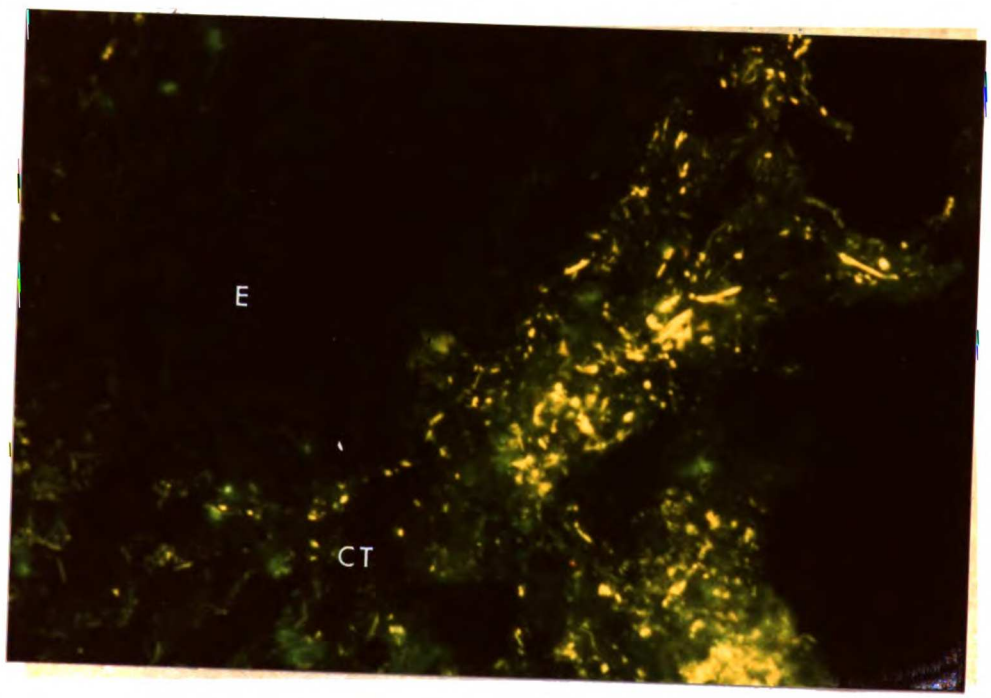


FIGURE 10

Figure 10a Photomicrograph of specimen of leukoplakia from the floor of the mouth. The extensive amount of hyperkeratosis (K) is quite evident. X16.

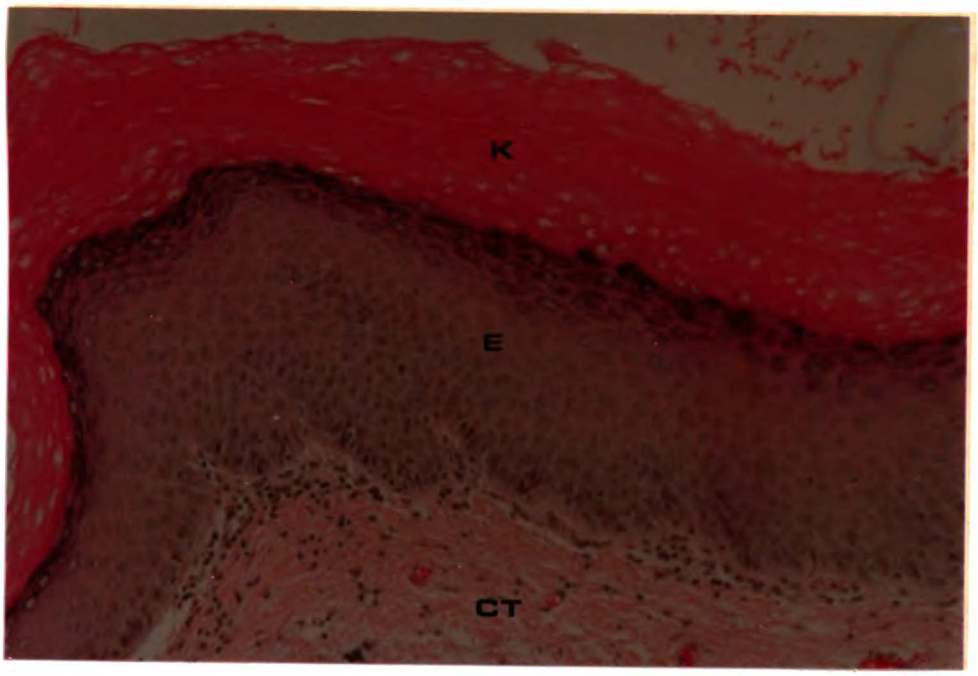


Figure 10b Photomicrograph of a specimen of leukoplakia obtained from the buccal mucosa, fibronectin was labeled as described previously. Note the connective tissue (CT) staining and absence of epithelial (E) staining.

FIGURE 11

Figure 11a Photomicrograph of leukoplakia from labial mucosa diagnosed as atypical verrucoid hyerplasia. Notice the significant amount of hyperkeratosis. X16.

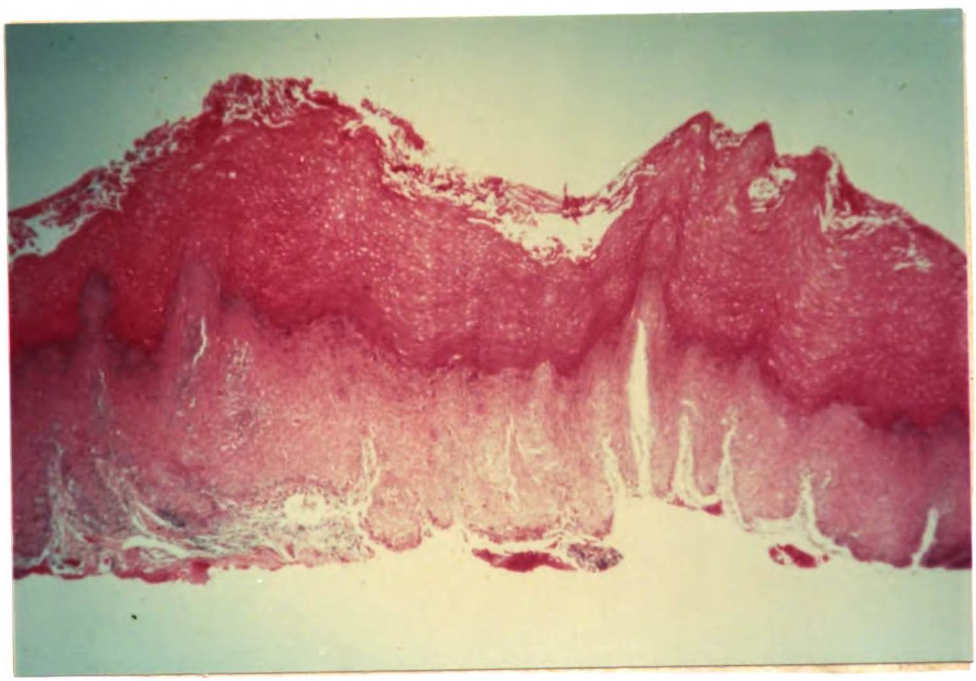


FIGURE 11

Figure 11b Indirect immunofluorescence staining of the same specimen, intracellular staining appears to be present in keratinocytes (arrows).

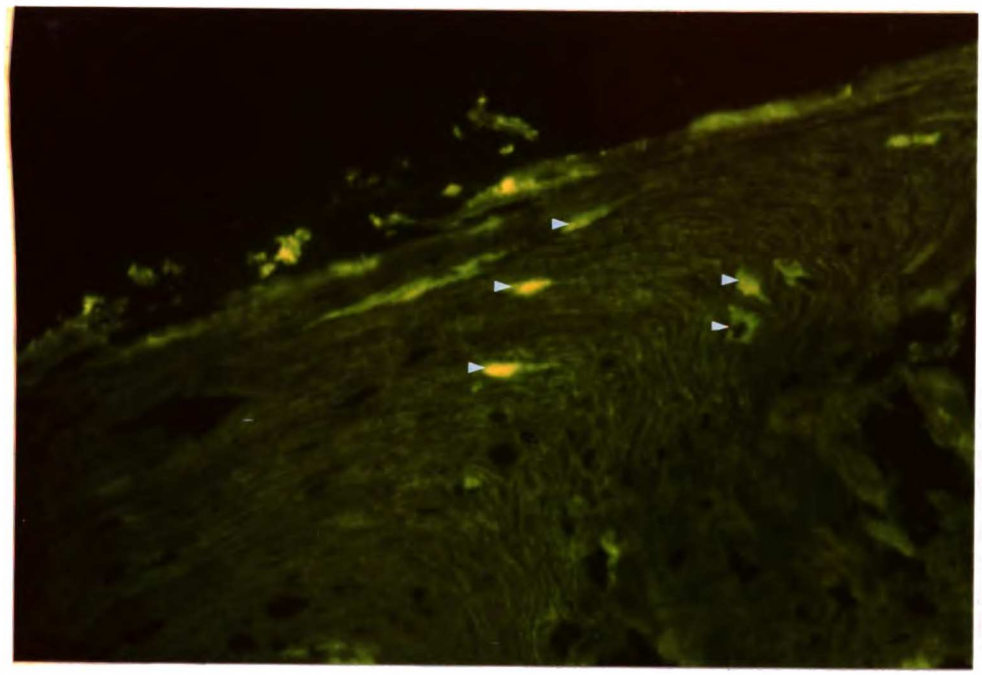


Figure 11c Avidin-biotin complex reaction in same specimen, again highlighting apparent fibronectin in keratinized epithelial cells (arrows). As described in the text, varying results were obtained with fibronectin preabsorbed controls. Therefore, the actual presence of fibronectin in these epithelial cells was questionable.

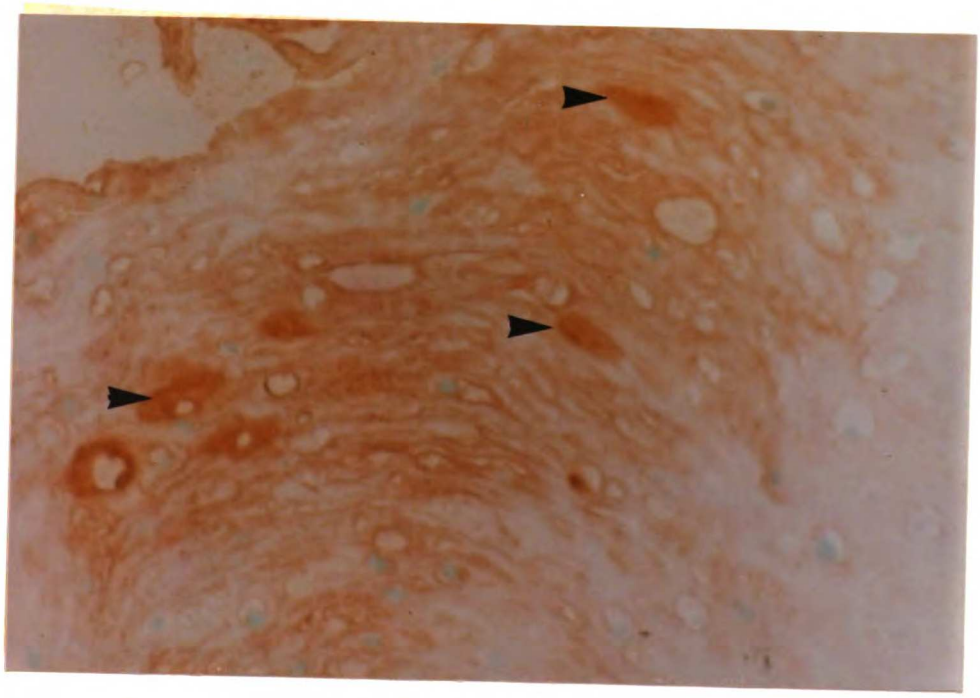


FIGURE 12

Figure 12a H & E photomicrograph of infiltrating squamous cell carcinoma (CA) and keratin pearl (K). X40.

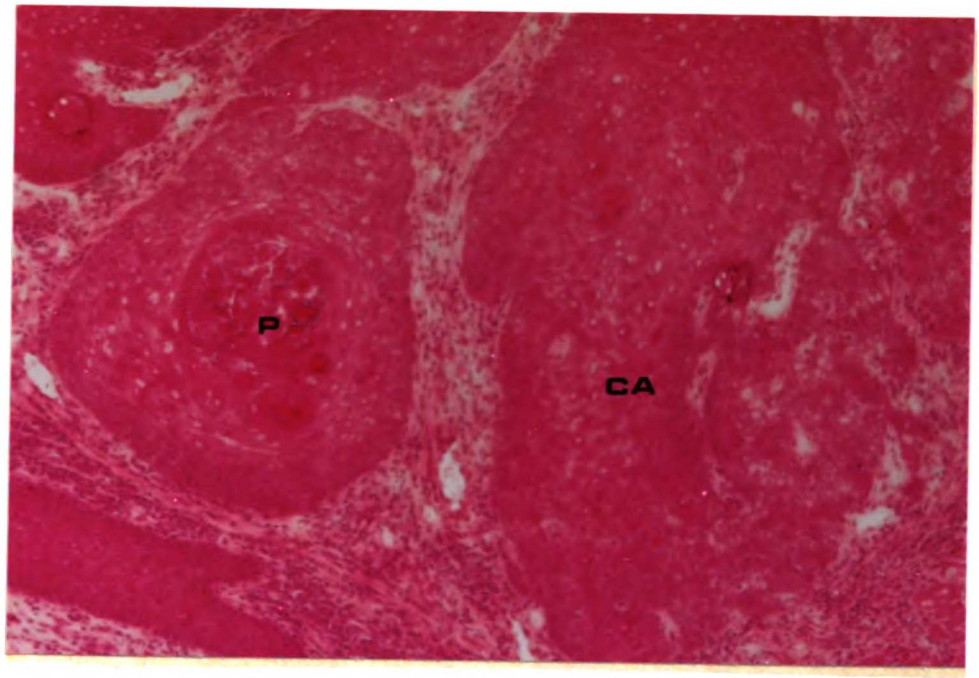


FIGURE 12

Figure 12b The same specimen labeled for fibronectin using the avidin-biotin complex technique. Notice the positive reactivity for fibronectin in the central region of the keratin pearl (P) and its absence in peripherally located epithelial cells (E). The interstitial connective tissue (CT) also contains fibronectin but the neoplastic epithelium does not (CA).

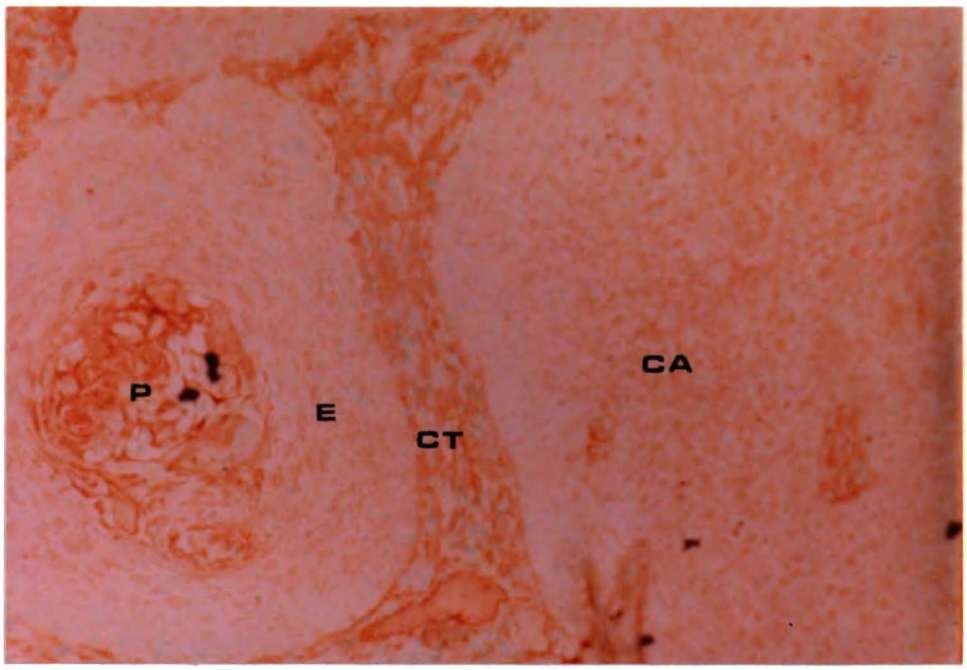


Figure 12c Fibronectin preabsorbed ABC reaction for fibronectin, staining was totally abolished.

FIGURE 13

Figure 13a Photomicrograph of keratin pearl and adjacent connective tissue (CT). Fibronectin was labeled by the FITC indirect immunofluorescence method as described elsewhere. Notice the positive reaction in the central region of the pearl (P) and in the connective tissue (CT).

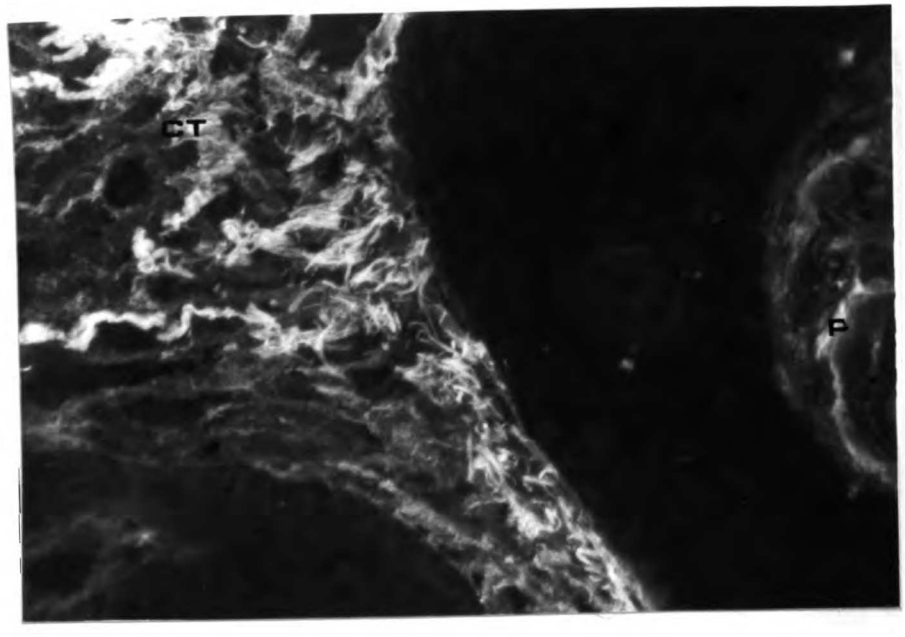


FIGURE 13

Figure 13b The same specimen labeled for laminin using monospecific primary antiserum at 1:40 dilution and secondary rhodamine labeled antiserum at 1:20 dilution. Notice the positive reaction in the keratin pearl (P), the basement membrane (BM) and the blood vessel basement membranes (V). No reaction was evident in the interstitial connective tissue.

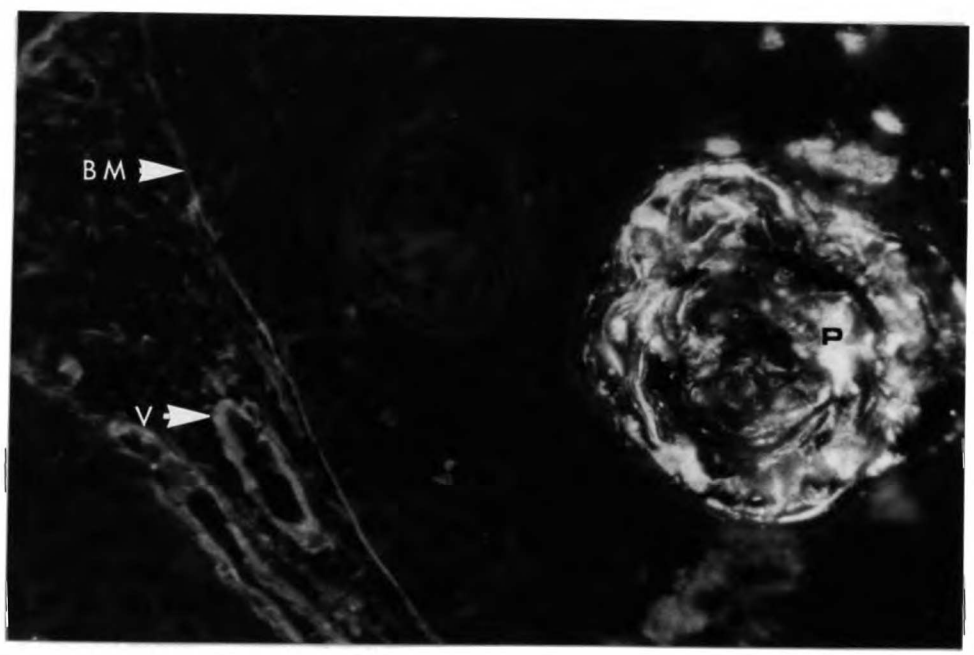


FIGURE 14

Figure 14a H & E photomicrograph of a specimen of oral squamous cell carcinoma located on the lateral margin of the tongue. In this specimen there are islands of neoplastic epithelium that closely approximate one another with thin intervening connective tissue (arrows). X20.

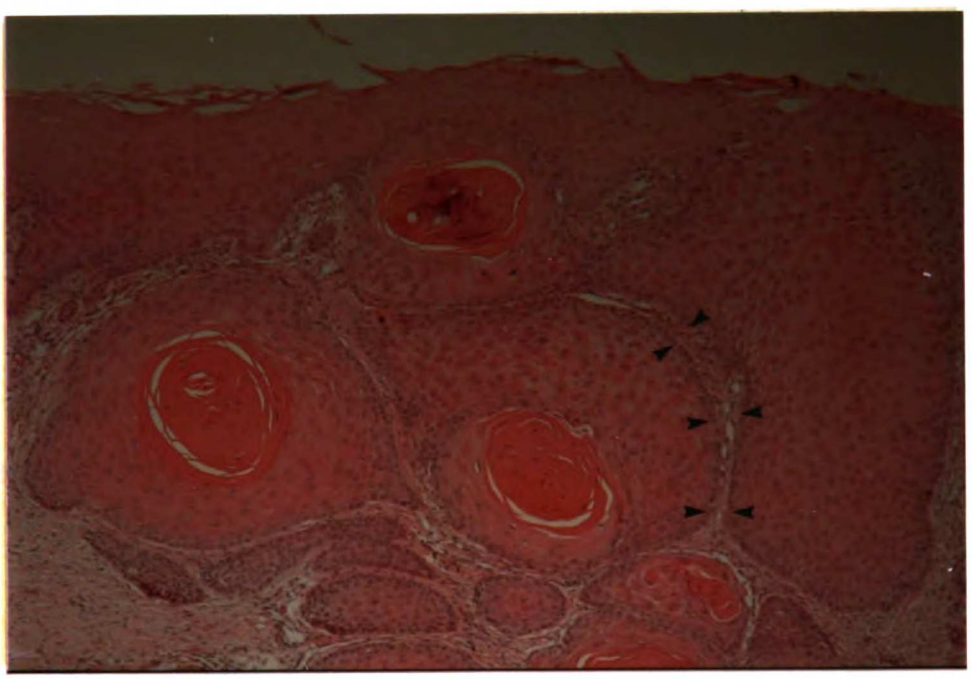


FIGURE 14

Figure 14b Fibronectin labeled specimen showing no fibronectin synthesis by the neoplastic epithelial cells (N) and positive reactivity for fibronectin in the connective tissue (arrows).

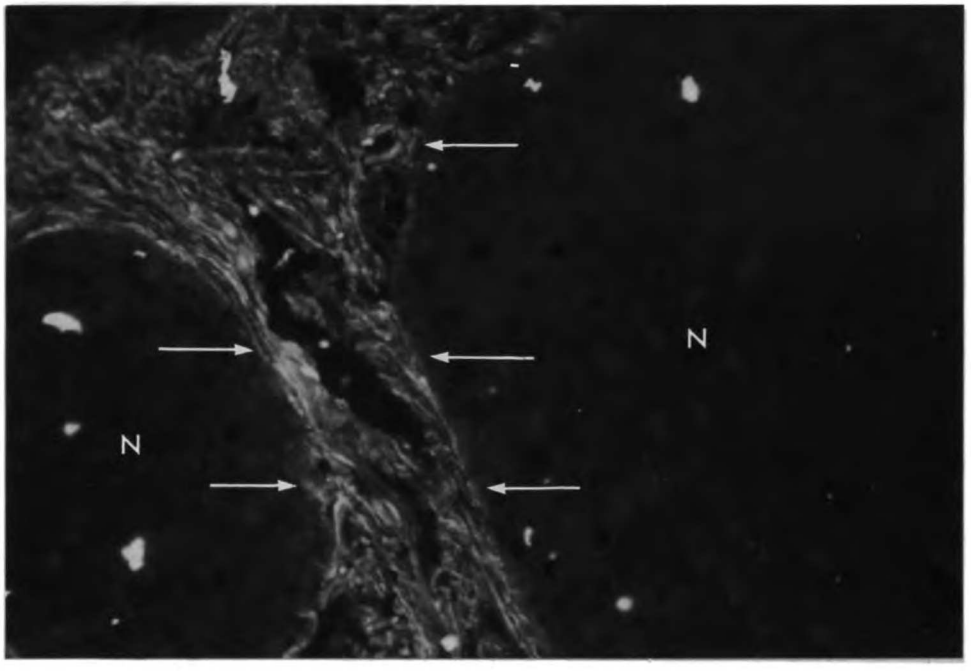


Figure 14c Fibronectin preabsorbed control specimen, fluorescence reactivity was totally abolished.

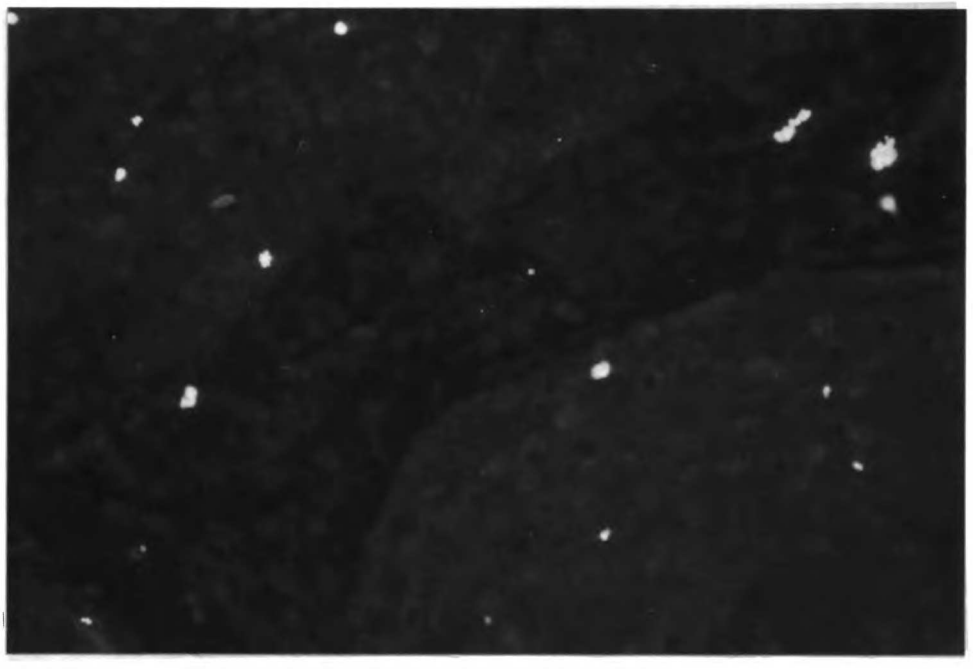


FIGURE 15

Figure 15a High power H & E photomicrograph illustrating another instance of neoplastic island approximation (arrows). X100.

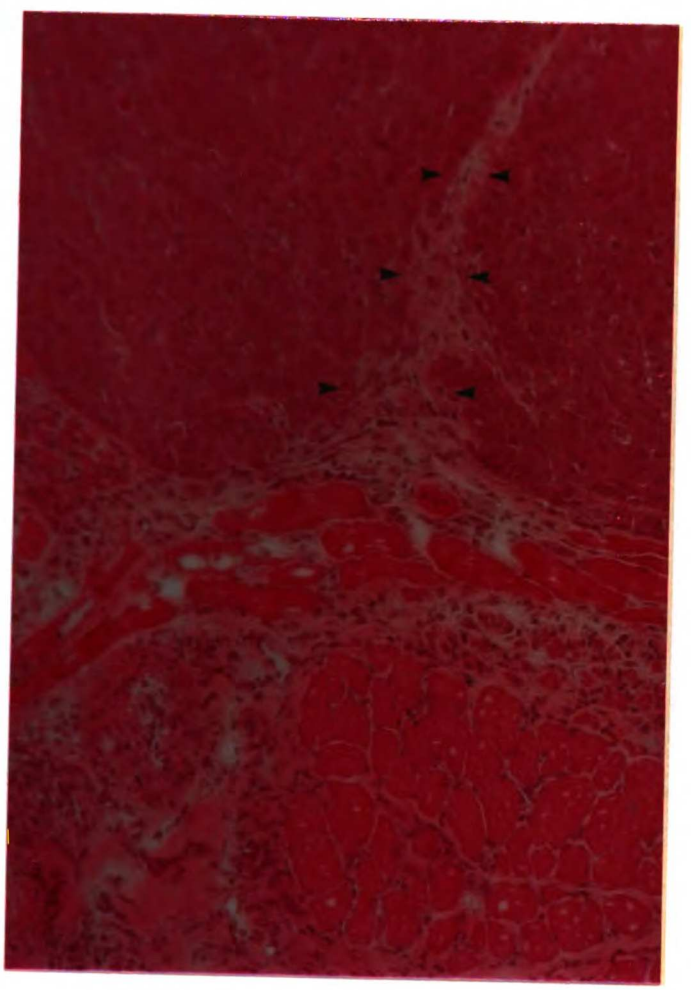


FIGURE 15

Figure 15b The same specimen stained for laminin (L) using rhodamine labeled secondary antiserum. Notice the distinct, linear staining pattern separating the two neoplastic islands.

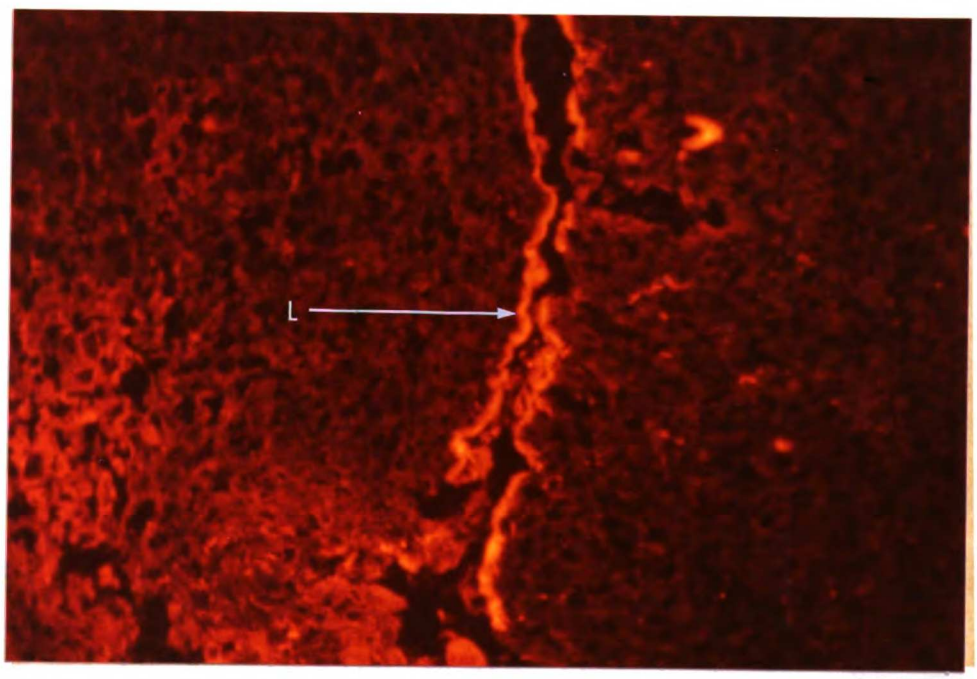


FIGURE 16

Figure 16a H & E photomicrograph of streaming epithelial growth pattern of oral squamous cell carcinoma located at the base of the tongue. LP - lamina propria, N - neoplastic epithelium, arrows - invasive front of neoplasm.

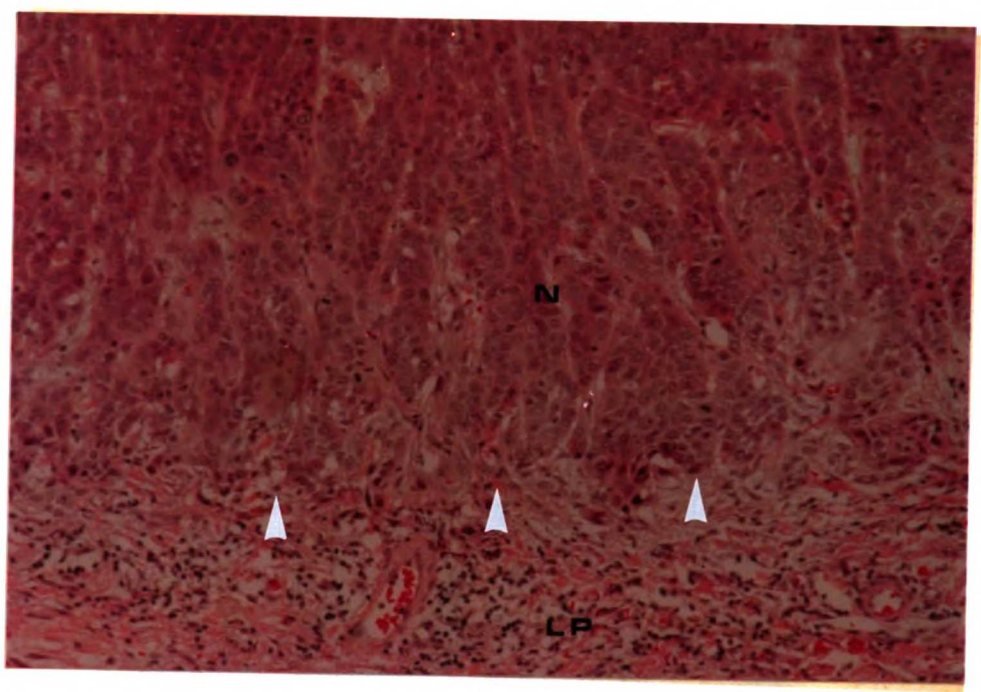


FIGURE 16

Figure 16b FITC labeled fibronectin in the same specimen. Note the absence of FN in the neoplastic epithelium (N) and its persistence in the intervening connective tissue stroma (arrows).

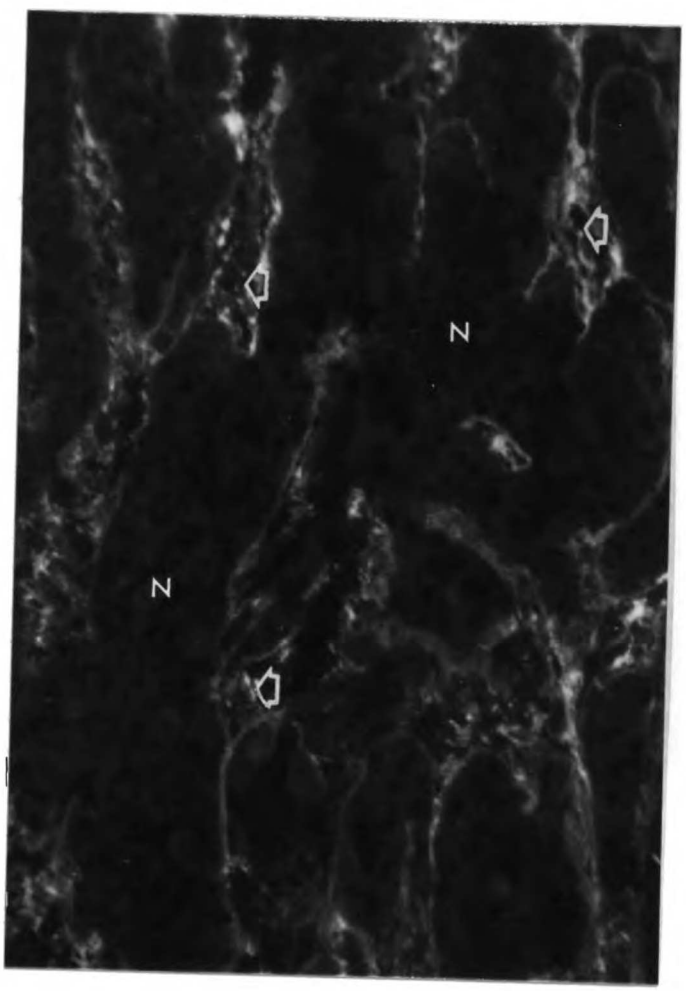


FIGURE 17

Figure 17a H & E photomicrograph showing infiltrative squamous cell carcinoma surrounding skeletal muscle; neoplastic epithelium (e), muscle (m). X40.

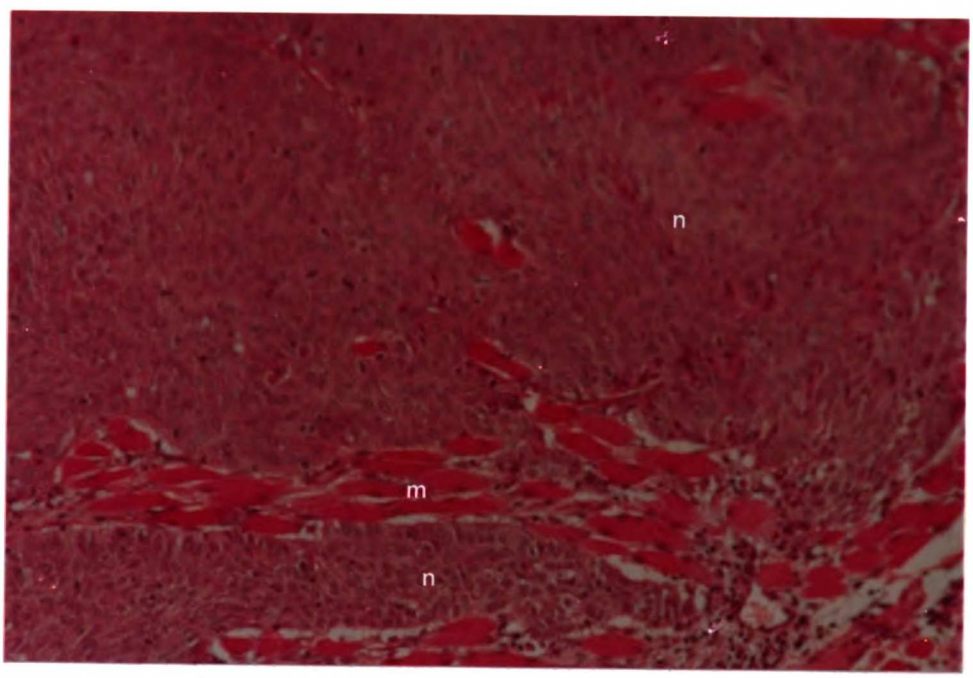


FIGURE 17

Figure 17b Laminin localization in the same specimen clearly shows a uniformly laminin-positive invasive front (open arrows) and adjacent muscle basement membrane (closed arrows).

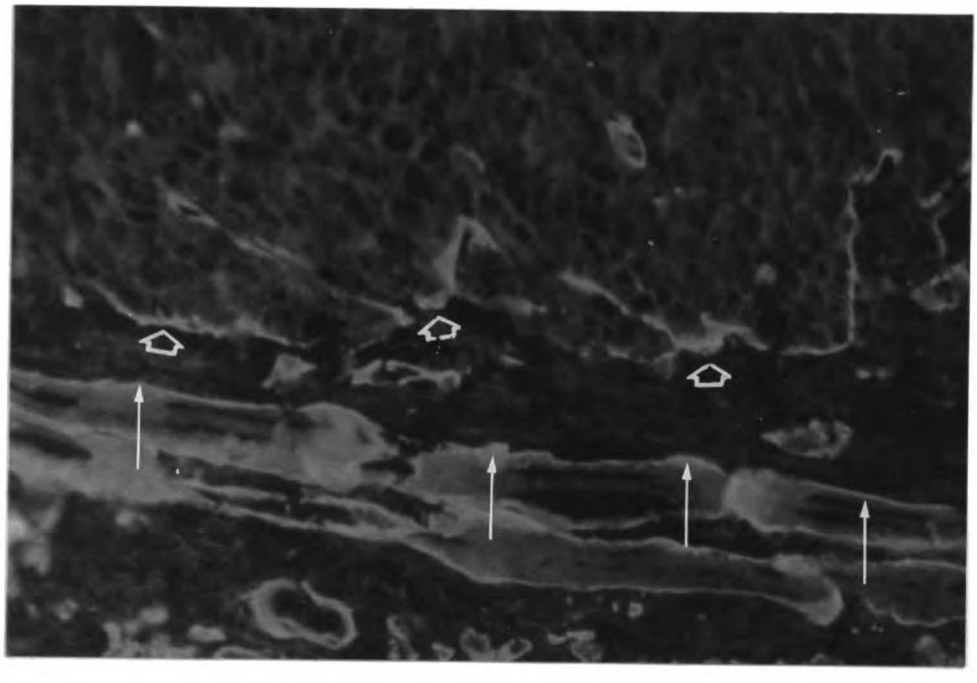


TABLE I. DIAGNOSTIC GROUPS STUDIED

Histologic Diagnosis	Total # of Specimens	Buc. Muc.	Ventral Fl. of Tongue	Mouth	Ging.	Palate	Labial Mucosa
Focal Keratosis	6	4	2	0	0	0	0
Inflammation	2	0	0	0	1	1	0
Hyperkeratosis	7	3	1	0	1	0	2
Leukoplakia	5	1	0	1	0	3	0
Mild Dysplasia	1	0	1	0	0	0	0
Severe Dysplasia	1	0	0	1	0	0	0
Carcinoma, well-diff.	1	0	1	0	0	0	0
Carcinoma, mod. well-d.	4	0	1	0	2	0	1
Carcinoma, poorly diff.	2	0	1	0	1	0	0
Spindle cell prolif.	1	1	0	0	0	0	0

TABLE II. PRIMARY ANTISERUM DILUTION DETERMINATION

Rabbit IgG Anti-human Fibronectin	Specific Stain (Lamina propria)	Background Stain (Epithelium)
1:5	+1	0
1:10	+4	+4
1:20	+4	+4
1:40	+3/+4	+2
1:60	+3	+1
1:80	+2/+3	0
1:100	+2/+3	0
1:160	+2	0
1:320	+/-1	0
1:640	0	0

Stain Intensity:
0 - None
+1 - Slight
+2 - Mild
+3 - Moderate
+4 - Intense

TABLE III

AVIDIN-BIOTIN-PEROXIDASE (ABC)

ANTISERUM TITRATION

Goat Anti-Rabbit IgG (Biotinylated)

bit Anti-an FN	1:100	1:200	1:400	1:800	$1:10^3$	$1:10^4$	$1:10^5$
1:10	3/4	=	=	=	1/2	0/0	0/0
1:100	3/4	3/4	3/4	2/4	2/4	0/0	0/0
1:200	3/4	3/4	3/4	2/4	3/4	0/0	0/0
1:400	2/4	3/4	3/4	2/4	2/4	0/0	0/0
1:800	2/4	2/4	2/4	0/3	0/3	0/0	0/0
$1:10^3$	1/3	1/3	0/3	0/3	0/3	0/0	0/0
$1:10^4$	0/3	0/1	0/0	0/0	0/0	0/0	0/0
$1:10^5$	0/2	=	=	=	=	0/0	0/0

TABLE IV

TABLE IV. ANTIGEN PREABSORPTION

Fibronectin Dilution	Stain Intensity
0	+3
1:10	0
1:50	+2
1:100	+3
1:500	+4
1:1,000	+3

TABLE V

TUMOR STAGING AND RESPONSE TO TREATMENT

RESEARCH #	HISTOLOGIC DIAGNOSIS	LOCATION	TNM CLASSIF.	STAGING	RESPONSE TO TREATMENT
R80-011	Moderately well Differentiated Carcinoma	Labial mucosa	$T_2N_0M_0$	II	Good resp. to Rad. Tx. No evidence of recurrent disease
R80-176	Poorly Differentiated Carcinoma	Gingiva & Max. sinus	$T_3N_0M_0$	III	Tumor recurred & was resistant to Rad. Tx. Patient died with Dz.
R80-010	Well Differentiated Carcinoma	Ventral Tongue	$T_1N_0M_0$	I	Tumor recurred & is present at this time
R80-066	Information Not Available	Base of Tongue	$T_2M_0N_0$	II	Good resp. to Rad. Tx. Osteoradionecrosis developed but no recurrent Dz.
R80-072	Moderately well Differentiated Carcinoma	Gingiva	$T_1N_0M_0$	I	Good resp. to Surgical Tx. No evidence of recurrent Dz.
R82-093	Early, moderately Well Differentiated Carcinoma	Gingiva	$T_1N_0M_0$	I	Good resp. to Surgical Tx. No evidence of recurrent Dz.

TABLE VI

TABLE VI. LOCALIZATION OF FIBRONECTIN AND LAMININ IN ORAL MUCOSA

SITE / HISTOLOGIC DIAGNOSIS		SURFACE	PRICKLE CELL LAYER	BASEMENT MEMBRANE ZONE	CONNECTIVE TISSUE
Normal	FN	-	-	+	+
	LN	-	-	+	-
Nonspecific Inflammation	FN	-	-	+	+
	LN	-	-	+	-
Epithelial Hyperplasia	FN	-	-	+	+
	LN	-	-	+	-
Atypical Verrucoid Hyperplasia	FN	-	-	+	+
	LN	-	-	+	-
Epithelial Dysplasia	FN	-	-	+	+
	LN	-	-	+	-
Carcinoma	FN	-	-	+	+
	LN	-	-	+	-