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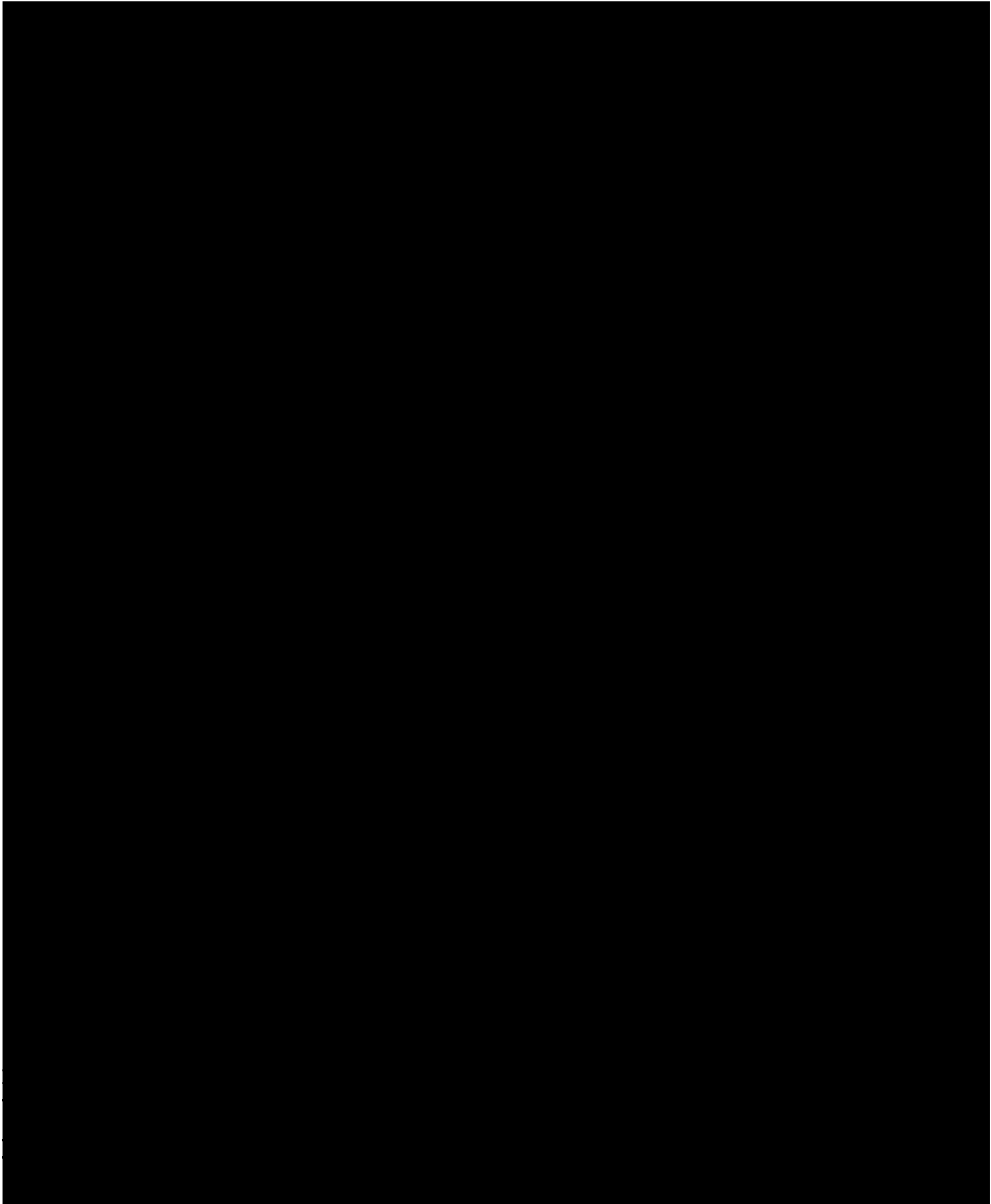**Author**

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UROKINASE PLASMINOGEN ACTIVATOR AND INTEGRIN
EXPRESSION IN MIGRATING ORAL EPITHELIUM

by

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D.M.D., University of California, San Francisco, 1994

ACKNOWLEDGEMENTS

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ABSTRACT

Migration of junctional epithelium is important in two phases of periodontal physiology. During pathogenesis, it is the event that renders the disease irreversible and, after surgery, it determines the epithelial position of the attachment. Numerous studies have shown a correlation between increased expression of urokinase plasminogen activator (uPA) and cell migration. Also of interest is the reorganization of molecules used to attach cells to the extracellular matrix during migration. To test the hypothesis that uPA is necessary for the migration of oral epithelium during the healing phase of periodontal therapy and to investigate integrin expression, human gingival explants (about 1x1x2 mm) were placed on Nucleopore membranes (8-um pore size) and incubated in the presence of serum for five days. The cut edge of the epithelium and the connective tissue was placed on the membrane and epithelial cells migrated to form a sheet between the explant and the membrane surface as well as across the membrane and across the cut surface of the connective tissue (Salonen, 1983; JPR 18:311). Immunocytochemistry with monoclonal antibodies against uPA, the α5, αv, α6, and β4 integrin subunits, the k-type laminin kalinin, and the matrix-degrading proteinase collagenase was performed. The uPA enzyme was located in the basal epithelial cells of the explant and in the cells at the leading edge of the migrating sheet where it appeared to be the most intense. Media from explants also showed the presence of 55 kDa activity capable of activating plasminogen in an zymogram. Function perturbing antibodies to uPA, when incubated with the explants, were capable of inhibiting the quantifiable migration of epithelium between tissue and membrane. Results of the integrin experiments showed that α5 was consistently expressed by the migrating epithelial sheets but was negative on the basal layer of stationary epithelium. Alpha 6, αv, and β4 were all present in basal epithelium, as well as in the migrating sheets of epithelium. Kalinin was found in all epithelial- connective tissue interfaces, as well as under the epithelium next to the membrane. Collagenase was found only between migrating epithelium and connective tissue.

INTRODUCTION

The junctional epithelium is a segment of the oral sulcular epithelium which forms the epithelial attachment of gingiva to the tooth surface. This specialized epithelium is a stratified non-differentiating epithelium that attaches to the tooth via hemidesmosomes and a basal lamina. It is widest (20-30 cell layers) at its most coronal aspect and tapers to only a few cells at the cementoenamel junction. The basal cells are cuboidal or flattened relative to the cells of the oral epithelium. Upon leaving the basal layer, the cells become flattened, and the nuclei elongate in a direction parallel to the long axis of the tooth surface (Schroeder and Listgarten, 1971)

Migration of junctional epithelium is important in two phases of periodontal physiology. During the destructive phase of periodontitis, there is loss of both epithelial and connective tissue attachment to the tooth surface. It is believed the migration of the junctional epithelium proceeds after degradation of the adjacent collagenous connective tissue attachment. This is thought to be due to the secretion and extracellular action of matrix metalloproteinases (MMP's) on these collagen fibers by either epithelial or fibroblast cells. The second phase of periodontal disease in which the migration of the epithelium plays a key role is wound healing following periodontal treatment. During the healing phase, the migration and subsequent attachment of the junctional epithelium to a specific point to the tooth surface is an important aspect of periodontal therapy. With therapy, the re-adaptation of junctional epithelium to the tooth is a function of the type of therapy provided and the repair response of the periodontium. This repair response is, in part, a function of the rate of migration and reformation of the hemidesmosomal attachment to the tooth. Rapid rate of migration and hemidesmosome formation results in the formation of long junctional epithelium. Slower rates permit the formation of a minimal zone of epithelial attachment. The biological factors which influences epithelial migration during this healing process are not so clear.

Cell migration requires degradation of the macromolecular components of the connective tissues and granulation tissue. Plasminogin activators are a family of serine proteases that convert the proenzyme plasminogen into the active enzyme plasmin. Plasmin is responsible for the degradation of

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insoluble fibrin and many extracellular matrix components such as fibronectin and laminin. Two types of plasminogin activators have been identified in mammals. Tissue plasminogen activator is a 72 Kd serine protease that has been identified as the major thrombolytic enzyme. Urokinase plasminogen activator is a smaller enzyme of about 55 Kd which is involved in tissue remodeling and cell migration under normal and pathological conditions.

The presence or absence of specific ECM components is very important during migration because when cells migrate they need to change from a stationary to a motile phenotype. This action is characterized by cell mediated changes in matrix components via matrix degradation by changes in cell membrane receptors. The integrins are a major group of extracellular matrix (ECM) receptors that mediate cell-cell and cell-ECM attachments and numerous studies have demonstrated their importance in migration and wound healing. Characterizing the integrin profiles that are involved in the migratory process may lead to a greater understanding of the mechanisms involved in epithelial migration and, ultimately to therapy directed at inhibiting undesired apical migration of junctional epithelium.

REVIEW OF THE LITERATURE

Biological role of plasminogen activators

Migration of epithelium involves the partial dissolution of intracellular and extracellular attachments. Plasminogen activators (PA's) are a family of serine proteases, which, along with their classic role in fibrinolysis, have been shown to be involved with a variety of processes including cell migration (Ossowski et al. 1983), epithelial differentiation (Isserhoff et al. 1983), and tissue remodeling (Kalderon, 1984).

Two enzymes have been identified: urokinase-type PA (uPA) and tissue-type PA (tPA). They are products of two distinct genes and are secreted as single chain proteins. Upon secretion, tPA is active, whereas single chain uPA is secreted in an inactive form (pro-uPA) and is bound to specific cellular receptors (Petersen et al. 1980). Cleavage of pro-uPA at lysine 158 results in a two-chained disulfide-linked active enzyme. The activation event is still not known and may not always involve proteolysis.

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In vitro, plasmin can activate pro-uPA both in solution and on the cell surface (Nielsen, et al. 1982; Wun et al. 1982; Cubellis et al. 1986), although, activation of receptor-bound pro-uPA by plasmin is at least twenty-fold higher than in solution (Ellis et al. 1989). However, Quigley et al. 1991, showed an arginine specific protease distinct from plasmin and detectable in the membrane fraction of RSV-transformed chicken embryo fibroblasts that is capable of converting receptor-bound pro-uPA to uPA (Berkenpas and Quigley, 1991). Other known activators of pro-uPA include kallikrein, factor XIIa and cathepsin B.

Numerous *in vitro* studies of cultured cells have shown that production of active uPA occurs almost exclusively on the cell surface (Del Rosso et al. 1990; Stephens et al. 1989). Current models suggest that uPA is secreted as single-chain inactive form, binds to a uPA receptor on the cell surface and that this receptor binding initiates plasminogen activation in the absence of proteases. Subsequent activation of pro-uPA by plasmin or other proteases would sustain plasminogen activation. Active uPA can then convert receptor bound plasminogen to plasmin which can itself degrade many ECM components including fibronectin and laminin but not collagen (Werb, 1976). Plasmin can directly activate procollagenase (Werb et al. 1987), and can also convert prostromelysin to stromelysin which activates procollagenase (He et al. 1989; Thompson et al. 1987). Thus the uPA system, which is characterized by one enzyme (uPA), one substrate (plasminogen), and two receptors (uPAR and the plasminogen receptor) is a very powerful proteolytic system that is capable of degrading ECM components both directly and indirectly via activation of latent metalloproteinases (Blasi, 1993).

Cell types expressing uPA receptors include monocytes/macrophages, neutrophils, fibroblasts, endothelial cells, and keratinocytes (Vassalli et al. 1991). The uPA receptor is a heavily glycosylated protein of about 55 kD and is expressed at 10,000 to 250,000 per cell (Blasi, 1987). Cells possessing specific receptors for uPA bind pro-uPA and uPA at the epidermal growth factor (EGF)-like terminal domain found within the amino terminal fragment of the A chain of the molecule. Removal of the amino terminal fragment yields a low molecular weight form of the enzyme that can activate plasminogen, but can no longer bind to the cell surface receptor (Testa and Quigley, 1990).

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In the extracellular environment, uPA activity is regulated by its protein inhibitors, plasminogen activator inhibitors 1, 2, & 3 (PAI-1, PAI-2, PAI-3), and protease nexin (PN). PAI-1 is the major inhibitor of uPA activity and is secreted by a variety of cell types. It is synthesized in an active form, however, when in serum or secreted into cell conditioned medium, it rapidly becomes inactive. Recent evidence suggests that both active and latent PAI-1 bind to ECM and the putative matrix binding protein is vitronectin (Salonen et al. 1989). PAI-1 does not interact with other matrix proteins such as fibronectin, type IV collagen, or laminin (Seiffert, et al. 1990). Binding to vitronectin stabilizes and maintains PAI-1 activity (Sigurdardottir, et al. 1990).

PAI-2 was originally described in extracts prepared from placenta and as such was called placental PA inhibitor (Kawano et al. 1968). It exists in both glycosylated and non-glycosylated form, both of which are functionally identical and can inhibit uPA. However, PAI-2 reacts more slowly with active uPA compared to PAI-1 (Testa and Quigley, 1990). The main physiologic function of PAI-2 is not known although, it is interesting to note that bacterial lipopolysaccharide (LPS) significantly increases production of this inhibitor (Webb, 1987).

Both PAI-3 and PN-1 are not major factors in inhibiting PA activity. PAI-3 has only recently been isolated from human urine and very little information exists concerning its expression and regulation. The protease nexins are a family of secreted proteins that can inhibit certain serine proteases. PN-1 has been found in fibroblast conditioned media and can inhibit uPA, although a recent study has demonstrated that PN-1 forms complexes with thrombin and not uPA suggesting a primary role as a thrombin inhibitor (Wagner, et al. 1989).

Several growth factors have been shown to regulate the levels of uPA in cultured cells. Lee and Weinstein demonstrated increased uPA activity by epidermal growth factor (EGF) in HeLa cells (Lee and Weinstein, 1978). EGF also increases uPA mRNA levels in the human carcinoma cell line A431, which has a high number of EGF receptors. In a recent study by Jensen and Rodeck, they studied the effect of transforming growth factor -α (TGF-α) and EGF on keratinocyte uPA. They found that exogenous TGF-α increased uPA in the cultured human keratinocytes and, in the absence of exogenous TGF-α and EGF, the basal levels of uPA were decreased by the

addition of a function perturbing antibody against the TGF-α and EGF receptor. They suggest that activation of the TGF-a/EGF receptor may positively regulate keratinocyte response to wounding by enhanced uPA expression (Jensen and Rodeck, 1993).

Platelet derived growth factor (PDGF) stimulates uPA activity expressed by carcinoma cells by increasing uPA mRNA levels (Stoppelli et al. 1986). Basic fibroblast growth factor (bFGF) is also a potent stimulator of uPA activity, especially in capillary endothelial cells (Moscatelli et at. 1988).

Members of the transforming growth factor-β superfamily (TGF-β) can exert both positive and negative influence on uPA activity depending on the cell type influenced. TGF-β decreases the secretion of uPA in bovine endothelial cell cultures and increases the expression of uPA activity in normal human lung fibroblasts (Laiho, et al. 1986; Sakelsa, et al. 1987).

Urokinase plasminogen activator in tumor cell invasion and cell migration

Invasive behavior of various tumor cells has been related to uPA. In a histochemical study done by Skiriver et al., they showed in Lewis lung carcinoma, uPA was consistently present in areas with histological signs of tumor invasion and tissue degradation (Skriver et al. 1984). Hearing et al. found B16 melanoma cells express single chain pro-uPA on their cell surface and that these cells are capable of plasminogen-dependent fibronectin degradation. When they incubated the B16 cells with anti-uPA antibody, they found decreased uPA activity and decreased numbers of metastasis as compared to controls. On the other hand, pre-treatment of the tumor cells with plasmin, which converts pro-uPA to uPA, significantly increased the number of metastasis. (Hearing et al. 1988). In a study with colon carcinoma cells and their effect on laminin degradation, Schlechete et al showed pre-treatment of carcinoma cells with the peptide UK12-32, which inhibits binding of uPA to its receptor, resulted in a 60%-80% reduction in laminin cleavage (Schlechte et al. 1989). Ossowski showed that when Wish cells were pre-incubated with exogenous high molecular weight uPA, cell surface plasminogen-activating activity increased and invasiveness was significantly enhanced. In addition, when HEp-3 cells were pretreated with anti-uPA antibody, there was a 75% decrease in the number of cells found to have invaded a wounded chick chorioallantoic membrane (Ossowski, 1988). The results from these and many other

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experiments have clearly demonstrated uPA involvement in tumor cell invasion.

An extensive body of evidence indicates uPA is overproduced during cell movement and is an important early component of the reparative process. Morioka et al. studied healing in keratinocyte cultures wounded with a blade. Using an anti-uPA antibody, they localized uPA at the leading edge of the migrating cultures and, also found an increase in uPA activity from the supernatant of the wounded cultures (Morioka et al. 1987). Del Rosso et al. have published evidence suggesting epithelial migration is associated with, and perhaps dependent upon uPA. Using radio-ligand binding experiments and TEM of a gold uPA conjugate, they demonstrated specific receptors for the A chain of uPA on the surface of human keratinocytes. They also found that uPA was chemotactic for NCTC keratinocytes, and the authors suggest that the cell migration is dependent upon the interaction between the uPA molecule and the receptor on the cell surface (Del Rosso et al. 1990). McNeil et al. have reported that enhanced expression of uPA receptor is characteristic of migrating keratinocytes in culture. They used audioradiographic as well as immunocytochemical techniques to demonstrate uPA binding sites selectively on the plasma membrane of cells at the leading edge of the migrating epithelial sheet. (McNeil et al. 1990). These studies are supported by the finding of Grondahl-Hansen et al. who have localized uPA to the epithelial outgrowth of cutaneous wounds (Grondahl-Hansen et al. 1988).

The involvement of uPA in cell migration has been studied in many other cell types and systems. Migrating embryonic cells, such as hemopoietic cells colonizing the *bursa of fabricius*, and neural crest cells have been found to produce uPA (Valinsky, et al. 1981). Similarly, Pepper, et al. found that if endothelial cells in confluent cultures are wounded by mechanically removing some of the cells from the dish, the adjacent cells will migrate into the denuded area, and as they migrate, they secrete increased amounts of uPA (Pepper, et al. 1987).

The above experiments certainly suggest a strong role for uPA in the movement of cells through the ECM. It seems logical then, to presume that oral epithelial cells may also utilize the PA system to facilitate migration through a wound matrix following periodontal therapy.

Plasminogen activators within the periodontium

To date, little is known about the role of plasminogen activators in the periodontium. Lucas et al. reported on the presence of plasminogen activator in the normal gingiva from beagle dogs. Microscopic examination of fibrin coated slides revealed lytic areas associated with activation of plasminogen (Lucas et al. 1975). Plasminogen activators have also been demonstrated in normal human oral epithelium (Southam, et al. 1981), saliva (Moody, 1982; Schmid and Chambers, 1989), and gingival crevicular fluid (Watanabe, et al. 1987). These early studies however, failed to identify the significant contribution of either tPA or uPA to the overall plasminogen activator activity. In a recent study by Barlow and Southam, both uPA and tPA were found in sulcus epithelium from human gingival mucosa by fibrolytic autography, however they failed to demonstrate the presence of either uPA or tPA in sulcular or gingival epithelium by immunofloresence (Barlow and Southam, 1992).

The source of plasminogen activators within the periodontium has been postulated to come from junctional epithelial cells, although definitive support for this claim is lacking (Schmid, et al. 1991). A recent study looked at biopsies of human gingiva taken from around dental implants that were placed on fibrin coated slides and assayed for PA activity. Anti-uPA or anti-tPA antibodies were added to the fibrin coated slides and the authors report that the addition of the anti-tPA IgG to the fibrin film limited the zone of lysis on the fibrin slide, whereas the addition of anti-uPA IgG had no effect on the lysis of fibrin. The authors concluded that tPA was the predominant PA within the gingival sulcus and that the source of the PA activity comes from "the top of the JE". They also noted that no PA activity was observed in oral epithelium (Schmid, et al. 1992).

Epithelial migration

Epithelial migration involves a complex array of biological events that include dissolution of intercellular and extracellular attachments mediated, in part by, proteolytic enzymes. In addition to the role of PA's in migration, evidence exists that neutral metalloproteinases such as collagenase and gelatinase may also involved in the process. Woodley, et al. demonstrated that skin keratinocytes migrating over dermal tissue produce type I collagenase (Woodley, et al. 1986), and the 72-kD

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gelatinase, has been shown to degrade basal lamina components (Liotta, 1988).

Another important aspect in migration is the relationship of the migrating cells to the substratum. Migratory cells interact with the ECM through cell surface adhesion proteins called integrins. Integrins are composed of non-covalently linked α and β subunits and are divided into subfamilies according to the β subunits that are shared by different α subunits. The $\beta1$ integrin, which can be linked with at least 7 different α peptides, form a group of receptors that is a major matrix binding integrin group (Uitto and Larjava, 1991). Both $\alpha5\beta1$ and $\alpha v\beta1$ integrins bind to fibronectin, and the αv can also bind collagen and vitronectin. Alpha6 can be associated with either the $\beta1$ or $\beta4$ subunits, both having affinity to laminin.

During the early stages of epithelial migration, it has been demonstrated in skin keratinocytes that $\beta1$ integrins are redistributed from a uniform cell surface location in stationary cells to the leading edge of activated cells (Guo, et al. 1990). Activation also results in the increased expression of the $\alpha5\beta1$ integrin complex, which has been shown to mediate the migration of keratinocytes over fibronectin (Guo, et al. 1991). A recent study supporting these results demonstrated inhibition of retinal pigment epithelial cell migration with a monoclonal antibody to the $\beta1$ integrin subunit (Hergott, et al. 1993). Another integrin subunit that may be important in the initiation of epithelial migration is the recently described $\beta4$. Beta4 is mainly localized in basal epithelial cell surface facing the basement membrane and in this location is probably associated with $\alpha6$ (Kajiki, et al. 1989). There is some evidence that the $\alpha6\beta4$ integrin complex is a component of the hemidesmosome structure (Kurpakus, et al. 1991).

Two reports have described integrin localization in human gingiva and oral mucosa (Hormia, et al. 1990; Larjava, et al. 1993). The study by Hormia et al. showed that the $\beta1$ subunit was expressed at the cell membrane of basal cells of gingival epithelium, throughout the cells of the junctional epithelium, and in the cells of the connective tissue. The $\alpha2$ and $\alpha3$ subunits were expressed in basal cells of gingival epithelium and cells of the JE, corresponding to the location of the $\beta1$ subunit. The $\alpha6$ subunit was polarized to the basal aspects of basal epithelial cells and was also

present in an overall cell surface distribution in cells of the JE. The β4 integrin subunit was mainly expressed at the basal aspects of basal cells in gingival epithelium and JE. These results imply that the α6β4 complex is the major candidate for mediation of the attachment of epithelial cells both to the basement membrane adjacent to the connective tissue and facing the tooth.

Recently, Larjava, et al. looked at the integrin expression in migrating oral keratinocytes and demonstrated β1 integrin expression at the leading front of the migrating epithelium as early as 24 hours, and that cells in contact with the fibrin clot substratum were found to express more β1 integrins than the resting cells at the wound margin. The migrating cells however, contained strong staining for β1 integrins at the basal aspect of the cells, which was similar to what was seen in the non-motile epithelium, in which β1 integrins were detected all around the basal cells. The β1 integrin staining pattern returned to normal in the zone where the two epithelia were joining after 7 days, although staining still appeared above the basal layer. The α2, α3, and α6 subunits were detected in keratinocytes at the wound margin and at the leading front, whereas the α1 or α4 were not detected in keratinocytes at any stage of migration. The α5 and the αv subunits, which were not found in the non-motile epithelium, were localized at the leading front of the migrating epithelium. Beta 3 was found close to some migrating cells. They also found fibronectin present under the migrating front of epithelial cells, and interestingly, epiligrin detected under the migrating keratinocytes at all stages of wound healing (Larjava, et al. 1993)

Study of junctional epithelial migration

The regeneration and migration phases of junctional epithelium following periodontal surgical therapy are critical to successful reattachment of gingiva to the root surface (Listgarten, 1972). Mechanisms leading to junctional epithelial detachment and migration are essentially unknown. Salonen et al. have published a series of experiments that characterize human junctional epithelium in an organ culture model (Salonen, et al. 1983, 1986, 1989, 1990, 1994). In this model, human oral gingival samples are cultured on a substratum to produce a migrating epithelium that moves between the substratum and connective tissue

surface similar to the way junctional epithelium interposes itself between the root surface and connective tissue. Support for this model being a reasonable representation of the *in vivo* situation comes from at least two sources. First, it is recognized that cells other than embryological reduced ameloblasts and stratum intermedium can differentiate into junctional epithelial cells. In the wound healing that follows gingivectomy procedures, a new junctional epithelium is formed that is functionally identical to the one prior to surgery. The cells of the newly formed junctional epithelium originate from basal cells of the remaining oral epithelium or the oral sulcular epithelium (Schroeder, Listgarten, 1977). Second, Salonen, et al. have shown that with time in culture, the cells of the migrating epibolus produced an electron dense material with hemidesmosomes resembling a basal lamina (Salonen, et al. 1983) and increase in reactivity with antibodies to cytokeratin 19, a known marker for JE cells (Salonen, et al. 1989).

My hypothesis is that uPA is needed for migration of oral epithelial cells during the healing phase of periodontal therapy. I intend to show that uPA and the uPAR are expressed at the leading edge of migrating oral epithelium and that migration of oral epithelium can be quantitatively altered with antibody against uPA.

OBJECTIVE

The objectives of this study were: (1) to show that uPA and the uPAR are expressed at the leading edge of migrating epithelium and to demonstrate that uPA is present in conditioned media from migrating epithelium; (2) that treatment with function perturbing antibodies can inhibit uPA activity and thereby quantitatively alter epithelial migration; and (3) to characterize the distribution pattern of various integrin molecules within this model system of migration.

The working hypothesis is that uPA is necessary for the migration of oral epithelium during the healing phase of periodontal therapy and that α6 and β4 integrins are downregulated in migrating epithelium and replaced by α5 and αv.

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MATERIALS AND METHODS

Tissue samples

Specimens of palatal mucosa were taken from normal human gingiva adjacent to maxillary molars and bicuspids during routine periodontal surgery in the post-graduate periodontics clinic at UCSF. Following a palatal flap procedure, the collar of tissue adjacent to the palatal aspect of the tooth was removed and stored in keratinocyte basal medium (KBM) supported with 10% Penicillin-Streptomycin. The tissue was stored at 4 C for no longer than 6 hours prior to culture.

Tissue culture

Tissue specimens were washed three times in PBS with 1% penicillin-streptomycin and fungizone and maintained in this during dissection. Only normal-appearing, non-inflamed tissue was used for culture. The organ culture model used for this study was described by Salonen, et al. 1983. Tissue was cut under a dissecting microscope into approximately 1 x 1 x 2 mm pieces and placed on 9 mm microporous filters (Falcon, pore size 8.0 um) to get the cut edge of the connective tissue and epithelium in direct contact with the filter. In this way, the epithelium is able to move along three areas of the cut piece of tissue: (1) along the cut edge of the connective tissue alone; (2) between the connective tissue and the membrane; and (3) along the membrane only (Fig. 1).

The inserts containing the gingival explants were placed in 24 well plates (Corning 24 well flat bottom, Corning Glass Works, USA) and 0.5 ml of medium was added to each well just sufficient enough to saturate the membrane. The medium was added very carefully to each well so as not to disturb the tissue on the membrane. The samples were cultured for 5 days at 37 C under the following media conditions:

1. Alpha Minimum Essential Media (α-MEM) + 10% Fetal Bovine Serum (FBS)
2. Lactalbumin Hydrosylate (0.2% LAH)
3. Alpha MEM + 10% FBS and 10 ug of serum goat anti-uPA (American Diagnostica Inc.,USA)
4. 0.2% LAH and 10 ug of serum goat anti uPA

5. Alpha MEM + 10% FBS and 10 ug of Melanoma PAI-1 (American Diagnostica Inc.,USA)

6. 0.2% LAH and 10 ug of Melanoma PAI-1

In addition, a fibrin adhesive (American Red Cross, Rockville MD, USA) was added to some of the gingival specimens cultured in Alpha MEM with 10 % FBS . The gingival samples were placed on the membranes and overlayed with 20 ul of a 12 ug/ml solution of Topical Fibrinogen Complex (TFC). The fibrinogen was reacted with 20 ul of thrombin to form a fibrin network over the gingival specimens.

Tissue processing

After 5 days in culture, tissue samples were fixed in 10% formalin and either frozen or embedded in paraffin. For frozen sections, the insert containing the tissue specimen was immersed in OCT. and frozen in liquid nitrogen. The frozen tissue sample and membrane were carefully extracted from the insert and were cut on a microtome at 8μm sections perpendicular to the membrane and mounted on frosted microscope slides (Fisherbrand, Fisher Scientific, USA). The slides were stored at -70 C^{o} until used for immunocytochemical experiments.

For preparation of paraffin processing, the tissue samples were removed from the inserts by carefully cutting the membrane away from the insert, leaving only the tissue sample adherent to the membrane. These were then placed in small containers, dehydrated in 70%, 95%, 100% ETOH and xylene, and embedded in paraffin. Eight micron sections were cut in either a step serial fashion, or consecutively until the entire block was cut, and the sections were processed for hematoxalin & eosin (H & E) stain or immunocytochemistry.

Immunocytochemistry and antibodies

Paraffin embedded slides were baked in 60-70° C oven for 30 minutes, following which paraffin was removed in xylene and ETOH. After washing 2X in distilled H$_2$0 (dH$_2$0) for 5 minutes, the slides were incubated in 3% H$_2$0$_2$ to remove endogenous peroxidase, washed in phosphate buffered saline (PBS), and incubated for 5 minutes in 0.4% pepsin. Following incubation with 3% serum to block non-specific binding, the primary antibody was added and the samples were incubated at 4° C overnight.

After at least 12 hours of incubation, the samples were washed in PBS, then incubated in a biotinylated secondary antibody solution for 30 minutes. This was followed by reaction with the avidin/biotinylated enzyme complex (ABC, Vectastain Kit, Vector Laboratories, USA). The slides were washed in PBS and 3-amino-9 ethyl carbazole chromagen (AEC substrate, DAKO Kit) was used to visualize the enzyme complex. The slides were then counterstained in Meyer's hematoxalin, dried, mounted in crystal mount, and coverslipped.

Immunocytochemical procedures on cryosections were essentially the same as the paraffin embedded sections except that the frozen sections were first fixed in cold acetone for 10 minutes, and Gill's, instead of Meyer's hematoxalin was used to counterstain the sections.

A list of the primary antibodies used for the immunocytochemical experiments is found in Table 1. All staining procedures were performed with negative control experiments by omitting the primary antibody in the staining process. In addition, certain antibodies were used to test the specificity of the staining procedure.

Immuno-gold labeling and TEM

Tissue was fixed for 1 hr. in 1.5% glutaldehyde in 1% PFA in 0.067 M sodium cacodylate buffer at pH 7.4. For conventional sections, the tissue was post fixed with 1% OsO4 in veronal buffer and dehydrated in graded ethanol and embedded in Epon-812 with degassing. The specimens were cured overnight at 60° C, sectioned at 0.5 μm, and stained with 1% toluidine blue.

The tissue used for the immuno-gold labeling was embedded in Lowicryl. Following primary fixation, the tissue was dehydrated in 70% ethanol and submerged into a -30° C freezer, then, dehydrated in 95% and 100 % ethanol at -30° C. The Lowicryl was changed 4 times with light degassing and the sections were cured by long wave UV-light (365 nm) at 16 cm distance for 24 - 48 hours at -30° C.

Thin sections, approximately 850A, were placed on formvar coated nickel grids and blocked 20 minutes with 0.25% BSA/PBS. The grids were incubated with a 1:5 dilution (0.2 ug/ul) of anti-uPAR MAb (American Diagnostica) at room temperature for 1 hour. Following incubation in the primary antibody, the grids were rinsed three times with PBS and

incubated for 1 hour at room temperature with a 1:15 dilution of goat anti-mouse IgG-gold 5nm (Janssen). The specimens were rinsed 4 times each in PBS and dH2O, fixed for 10 minutes in 1% OsO4 in veronal buffer, and contrasted with uryral acetate and Reynolds lead citrate. Screening was done on a Jeol 1200 Ex microscope at 80kv.

Gel electrophoresis and zymography

Samples of organ culture conditioned media with and without anti-uPA antibody treatment and with and without serum were electrophoresed in 10% polyacrylamide gels impregnated with casein and lys-plasminogen. 15 ul of media sample + 5 μl of 4X sample buffer was added to each well. The sample buffer consisted of 40 ml glycerol, 20 ml dH2O, 3.0 gm. TRIS-Base, and 4 ml. of 20% SDS. The buffer was adjusted to a pH of 6.8 with addition of concentrated HCl. Prestained BRL-Gibco Hi molecular weight markers were used. Gels were run for approximately 1.5 hrs., removed from plates, and SDS was washed from the gel in 2.5% Triton-Tris-HCl. The gels were incubated overnight at 37^0 C, stained with Coomassie Blue, and destained to reveal clear bands of plasminogen activator activity. Gels were visualized with a high resolution video camera into NCSA-Image program on a Macintosh computer.

Quantitative analysis

Histologic specimens were examined under a light microscope and captured with a video camera into Image Analysis Software on a Macintosh computer. Each sample was calibrated with a stage micrometer and measurements of epithelial migration were made from a fixed reference point, (basal layer of epithelium), Fig. 2, and from the point where the migrating epithelium contacted the membrane (between membrane and connective tissue) Fig. 3. Three measurements were made in each specimen: 1) along the connective tissue and membrane on the left side of the tissue from the fixed reference point, 2) along the connective tissue and the membrane on the right side of the tissue from the fixed reference point, and 3) between the membrane and the connective tissue from where the epithelium contacted the membrane. A fourth (4) measurement was made in those samples that also exhibited migration

along the membrane itself Fig. 3. All measurements were done three times and the average was used for statistical analysis.

Statistical analysis

Statistical analysis was performed only on the measurement of migration between the tissue and the membrane as measured from the point at which the epithelium contacted the membrane. Epithelial migration was compared between control versus antibody treated tissue samples using a paired t-test from Statview Statisical Analysis Software.

RESULTS

Histology

Most of the samples cultured in media supplemented with 10% serum presented a similar appearance to those specimens shown in Figs. 4 & 5. These are 8 um sections of gingival tissue cultured in media containing serum for 5 days. The membrane is clearly visible and the epithelium has migrated between the cut surface of the connective tissue and the membrane. The left of Fig. 4 (above solid arrow) shows the epithelium along the membrane itself. In addition, epithelial tissue can be seen within the pores in the membrane and migrating along its undersurface (clear arrow, Fig. 5).

Histochemistry

Results of immunocytochemical assays are shown in Figs. 6 through 21, and Table 1 lists the primary antibodies used in these experiments. All of the immunohistochemistry experiments were performed with negative controls by excluding the primary antibody, and none of these specimens showed any staining (Fig. 6). Figure 7 shows a 5-day gingival sample reacted with GB3, an antibody used to detect the presence of the k-type laminin, kalinin, which is found localized to the basement membrane. This was used as a positive control, as it always detected the interface of epithelium when the detection system was working. Interestingly, kalinin was also found under migrating epithelium along the membrane (Fig.8)

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The pattern of stain associated with the α-v integrin subunit is shown in Fig. 9. Intense stain was seen under the migrating epithelium and throughout the connective tissue. The basal epithelium also stained positive for a-v, however, the intensity of the stain was less than that observed under the migrating cells. Results from experiments using antibodies against the α-5 and α-6 integrin subunits are shown in Figs. 10 - 13. The α-5 integrin subunit was found along the migrating epithelial cells and associated with blood vessels and fibroblasts in the connective tissue (Figs. 10 & 11). Non-migrating basal epithelial cells were essentially negative for α-5. Heavy staining for α-6 was found throughout the basal epithelium, as well as along all the migrating cells (Figs. 12 & 13).

The localization of the β-4 integrin subunit was very similar to α-6 (Figs. 14 & 15). Here again, heavy stain was seen more in the basal layers of non-migrating epithelium, and under the migrating epithelium.

Figures 16 & 17 depict results from experiments for localization of collagenase performed on frozen tissue samples. Essentially, most of the epithelium was negative, however, very light positive stain could be observed in the epithelium migrating along the membrane between the connective tissue.

In general, uPA was found in both the basal cell layer of the oral epithelium and at the leading edge of the migrating cells, where the staining was most intense. Fig. 18 shows the leading edge of migrating epithelium along the cut connective tissue stained positive for uPA. Also evident in this section is a blood-vessel which stained positive for uPA. The leading edge of the migrating epithelium along the membrane is shown in fig. 19. Here again, intense staining is seen in the flattened epithelium on both sides of the membrane.

Histochemical results using antibody against the uPA receptor (uPAR) can be seen in Figs. 20 and 21. Staining was intense at the leading edge of the migrating cells, both along the cut connective tissue, and the membrane. These results were similar to those observed for uPA, but slightly more positive stain was observed in the basal cells than in the specimens reacted with the anti-uPA antibody. However, the amount of stain seen in the basal cell layer was much less than that observed at the leading edge.

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TEM and immuno-gold labeling

Results from the u-PAR receptor labeling experiments are shown in Figs. 34 through 41. Figure 34 and Fig. 35 show the leading edge of the epibolus migrating along the membrane (membrane is the darker material in the left of the figure). UPAR-gold complexes can be seen distributed throughout the cell surface in an irregular manner, often arranged in small clusters. Figure 36 shows part of a cell moving through one of the pores in the membrane which also demonstrates u-PAR-gold conjugates distributed in clusters throughout the cell surface. In addition, some gold particles were found in small clusters along the membrane, most of which occurred near the cell-membrane interface.

Negative control experiments were performed by omitting the antibody against the uPA receptor and these results are depicted in Figs. 38 and 39. Fig. 38 shows part of an epithelial cell along the membrane (membrane at lower left corner). No gold-uPAR complexes can be seen on the epithelial cell surface or the on membrane. Fig. 39 shows the leading extension of the migrating epithelium, also negative for any gold labeling.

In an additional attempt to test the specificity and validity of the antibody-gold labeling, wounds from biopsies of intraoral wart tissue was reacted with the conjugate. Figure's 40 and 41 show scattered gold particles irregularly arranged in clusters present in localized areas of the wound tissue.

Function perturbing antibody experiments

Gingival samples were cultured with or without serum and with or without the addition of anti uPA or PAI-1. Ten ug/ml of antibody was added to the culture media and the samples were incubated for 5 days. The tissue was processed for H & E stain and subjected to qualitative and quantitative analysis while the media was collected and run on a plasminogen substrate gel. The zymogram is shown in Fig. 43. Lanes 3 and 4 represent media from the explants cultured in serum. Clear bands of plasminogen activator activity can be observed at about 55 kDa, near the molecular weight of uPA. In addition, plasminogen activator activity corresponding to uPA can also be demonstrated in lanes 2 and 6, which are media from the cultures void of both serum and anti-uPA antibody. When anti-uPA antibody was added to the media, and the gingival samples were

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cultured, (either with or without serum), no plasminogen activator activity could be detected (lanes 1 and 5). The control is shown in lane 7 and represents media containing serum alone (i.e. no cultured tissue sample). No plasminogen activator activity was found in media void of tissue samples, suggesting the tissue was the source of plasminogen activator. More importantly, these experiments show that a molecule capable of activating plasminogen can be demonstrated in culture media from 5-day gingival explants, and not in the presence of an antibody against u-PA.

The H & E stained sections from these function perturbing antibody experiments can be seen in Figs. 22 through 29. Figures 22 & 23 show specimens cultured for 5 days in media containing serum. The epithelium (E) can be seen migrating along the membrane to the right and to the left, and in between the membrane and the connective tissue (compare with figs. 4 and 5). Sections of gingival samples cultured with anti-uPA antibody are seen in Figs. 24 through 27. In general, the migration of the epithelium along the membrane itself, (Fig. 26- left) and along the cut surface of the connective tissue, (Fig. 24-E) was similar to the migration seen in samples cultured without the addition of anti-uPA antibody. There was, however, a striking difference between the two in the migration of the epithelium along the membrane between the connective tissue (Figs. 25, 26, and 27.). Here, the epithelial migration was much less, as it appeared as though the epithelium could not migrate between connective tissue and the membrane. Higher power views of the epithelial cells migrating along the membrane can be seen in Figs. 30 through 33. Figure 33 shows a tissue sample cultured with anti-uPA antibody and it appears as though the cells do not move as easily between the connective tissue and the membrane compared to the controls (Figs. 30-32).

In addition to treatment with anti-uPA antibody, some gingival samples were also cultured with PAI-1 or overlayed with a fibrin adhesive (Figs. 28 & 29). The results from the addition of PAI-1 were extremely variable, but in general, it appeared as though the inhibitor had very little effect on migration. There appeared to be no difference in migration in each area measured compared to control, and epithelium was readily seen migrating between connective tissue and the membrane (Fig. 28). The addition of a topical fibrinogen complex overlayed with thrombin produced a fibrin matrix surrounding the gingival tissue (Fig. 29). The addition of

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the fibrin adhesive had little effect on epithelial migration as these samples were very similar to the controls.

Quantitative analysis

In an attempt to quantitate the migration of epithelium in this model, and to compare control samples with those treated with the anti-uPA antibody, slides of gingival samples were captured with a video camera into Image Analysis software. The program was calibrated with a stage micrometer and measurements were made in three different areas of each tissue sample with reference to a line drawn through the basal layer of the stationary epithelium (Salonen, et al. 1990). A fourth measurement was also made along the membrane, between the connective tissue, from the point where the epithelium contacted the membrane. All measurements were done three times and the averages from the control and experimental tissues are listed in tables 2 and 3. Table 4 represents results of migration experiments between control and experimental tissues as measured between the membrane and the tissue from the point at which the epithelium contacted the membrane. A highly statistical difference was observed between the two groups in the migration of epithelium along the membrane, in-between connective tissue (P < 0.001).

Figure 40 shows the results from the migration experiments from the 10 control and 10 experimental samples listed in tables 2 and 3. In general, the migration seen in the antibody treated specimens was somewhat less than that seen in the controls for the migration along the tissue right, tissue left, and the membrane only. However, there was a large difference in the migration between the membrane and the connective tissue between the control tissue and the tissue cultured with the anti-uPA antibody.

integrin/enzyme	antibody	concentration used	source	reference
uPA	Mab #3689	1:30 (25ug/ml)	mouse	American Diagnostica
uPAR	Mab #3936	1:30 (25 ug/ml)	mouse	American Diagnostica
α5	Mab BIIG2	1:250	rat, mouse	Werb, Z JCB 109:877
α6	Mab GoH3	1:250	rat	Sonnenberg, J Cell Sci 207
αv	Mab VNR147	1:500	mouse	Telios
β4	Mab 3E1	1:250	mouse	Telios
collagenase	Mab MαR	1:10	mouse	Werb, JCB 109: 877
gelatinase-92K	Mab MH1	1:100	rabbit	Unemori, J Clin Invest 88:1656
gelatinase-72K	Mab J1.1	1:100	rabbit	Unemori, J Clin Invest 88:1656

Table 1.   Antibodies and concentrations used for immunocytochemical experiments

Organ Culture of Gingiva

Epithelium

Connective Tissue

Membrane

Movement along connective tissue
Movement along the membrane
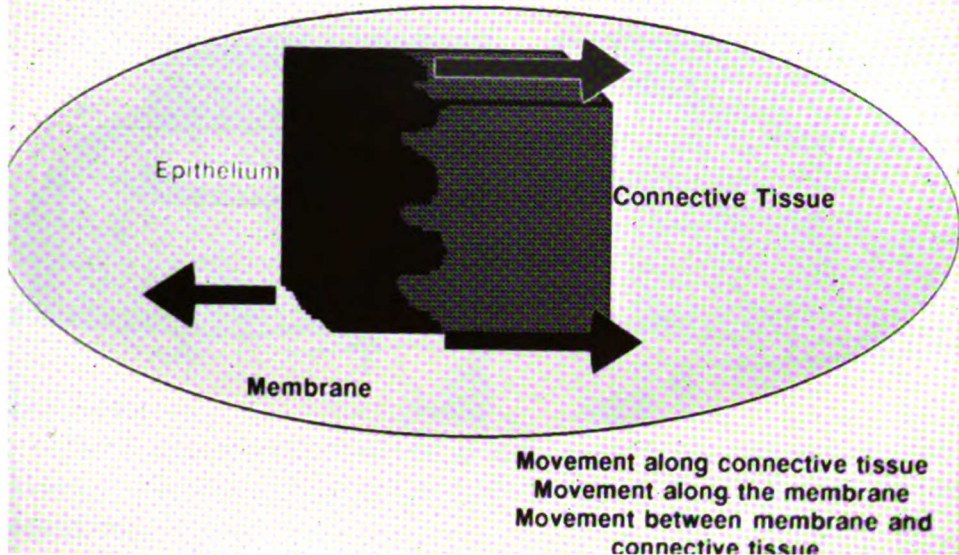**Movement between membrane and**
connective tissue

Fig 1. Schematic representation of organotypic model to study epithelial migration, Tissue samples are placed on microporus filters and cultured in serum for 5 days. The epithelium can migrate in three directions: 1. along the connective tissue; 2. along the membrane; and 3. between the membrane and connective tissue.


Fig. 2

Fig. 3

Measurement of
Migration over tissue
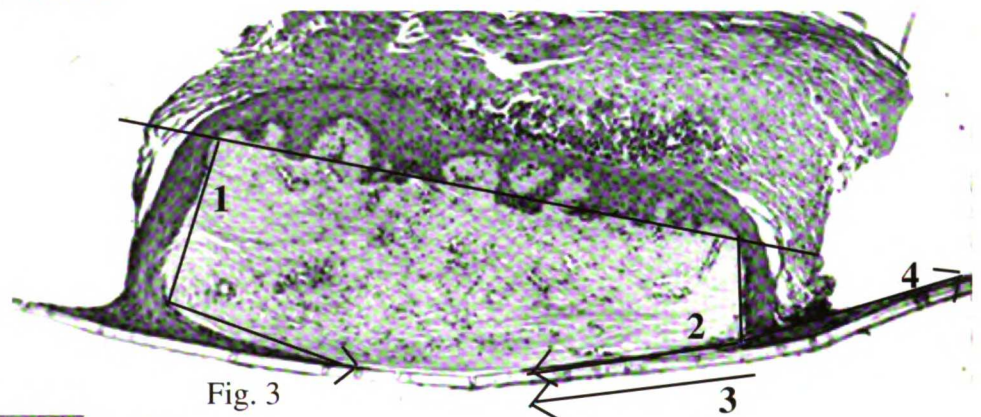
1-migration left, from reference
2-migration right, from reference
3-migration between membrane and CT
(measured from where epithelium meets
the membrane)
4-migration, membrane from reference
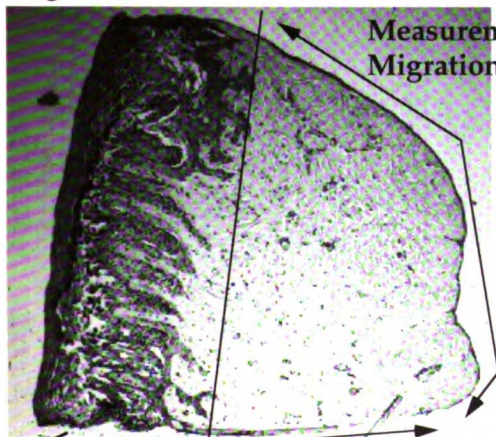
Migration along Measurement of Migration
membrane only between membrane and tissue

Figs. 2 & 3 -Quantitative measurement. Images were obtained by a video and calibrated measurements were made with Image Analysis software with reference to the basal cell layer in the directions indicated above. An additional measurement was made just between the membrane and tissue only, not using the basal cell layer as a reference point (#3 in Fig. 3).

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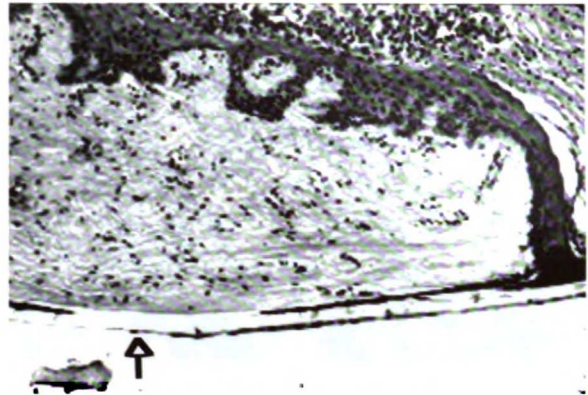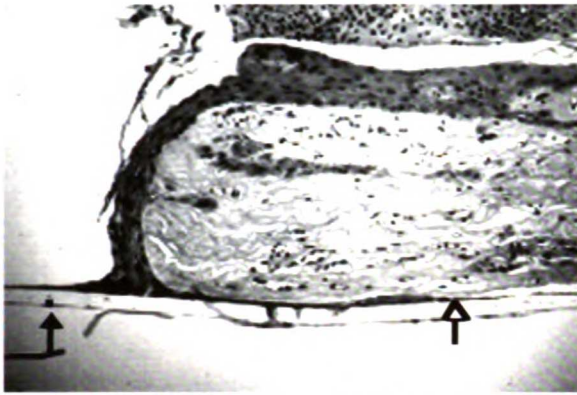
Figs. 4 & 5- H& E stained sections of oral gingival samples cultured in serum for 5 days (solid arrow -membrane; clear arrow -leading edge of migrating epithelium between CT and membrane). (10x).
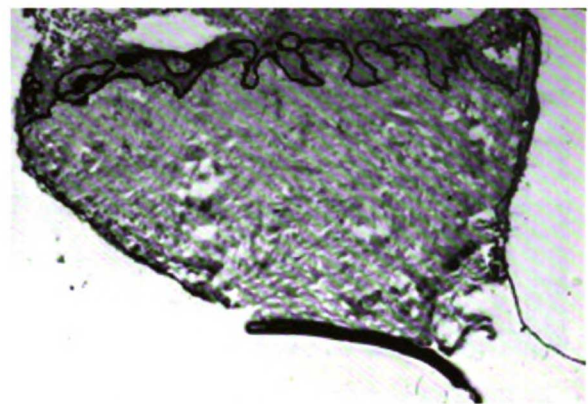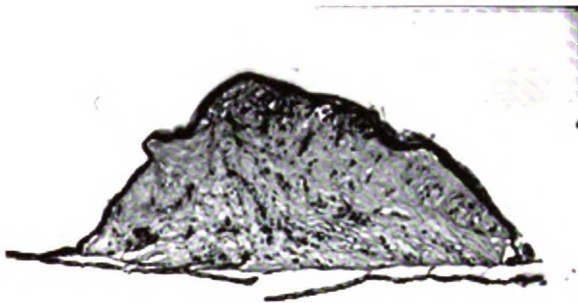

Fig. 6- Negative control. Immunohistochemistry on 5-day gingival sample not reacted with primary antibody. (4x)

Fig. 7- Immunohistochemistry of 5 day gingival sample reacted with anti-kalanin. (arrow- basal cells show positive stain). (4x)
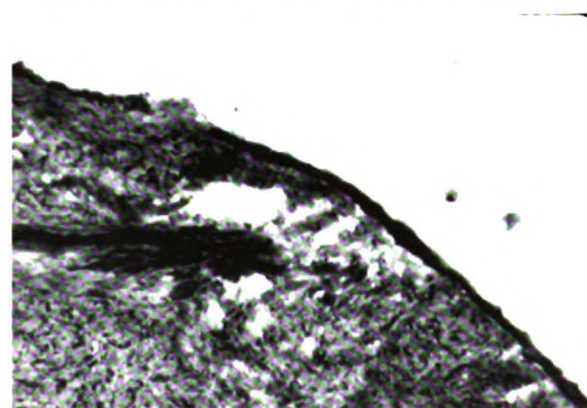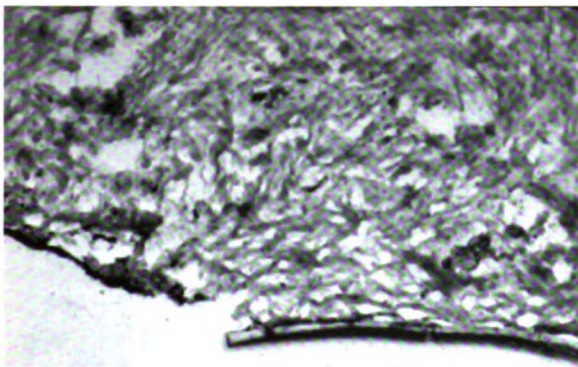

Fig. 8- Histochemical results on 5-day gingival sample reacted with k-type laminin kalinin; leading edge of migrating epithelium along the membrane (10x).

Fig. 9- Immunohistochemistry on 5-day gingival sample reacted with the α-v integrin subunit; leading edge of migrating epithelium. Results show heavy staining under the migrating epithelium and throughout the connective tissue (10x).

Fig. 10- Immunohistochemistry on 5-day gingival specimen reacted with anti-α5 integrin subunit. (4x).

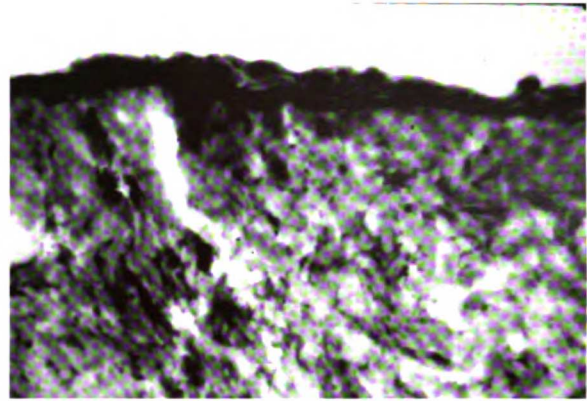
Fig. 11- High power view of leading edge of migrating epithelium in specimen seen at left. Arrows show staining for α5 under migrating epithelium. (10x)
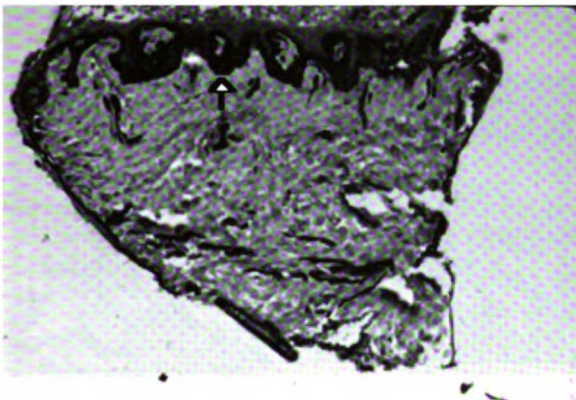

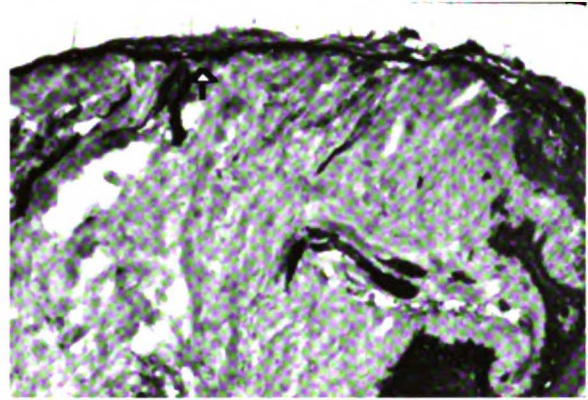Fig. 12- Immunohistochemistry on 5-day gingival specimen reacted with anti-α6 integrin subunit. (4x)

Fig 13.-High power view of leading edge of migrating epithelium in specimen seen at left. Arrows show heavy staining for α6 in both the stationary basal cells and under the migrating cells at the leading edge. (10x)
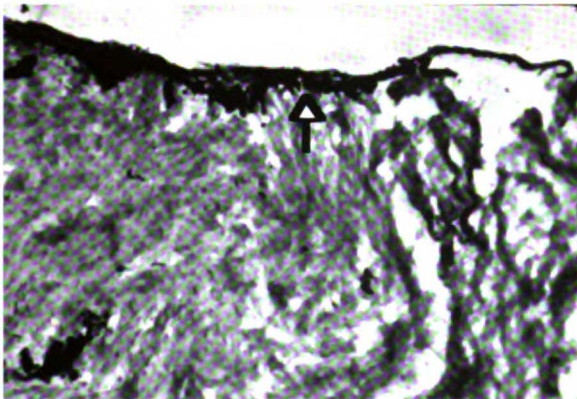

Fig. 14- Immunohistochemistry on 5-day gingival sample reacted with the β-4 integrin subunit; sample leading edge of migrating epithelium. (10x)

Fig. 15- 5-day gingival reacted with the β-4 integrin subunit; basal cell layer. Arrows depicting heavy staining in both the basal cells and under the migrating cells at the leading edge. (10x)
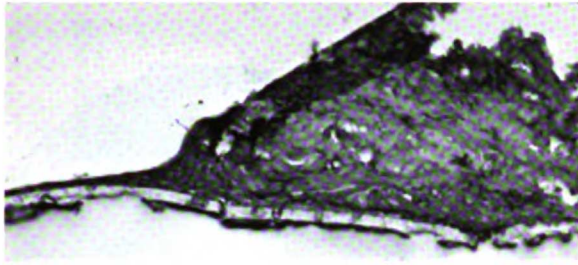
Fig. 16- Histochemistry on frozen tissue specimen reacted with the metalloproteinase collagenase. Mild staining was observed in epithelium along the membrane. (4x)
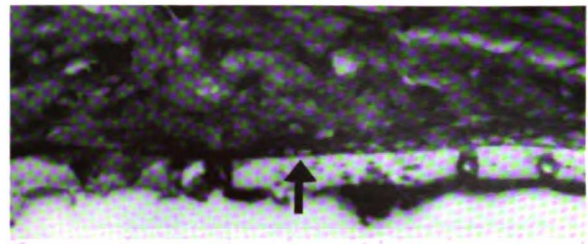
Fig.17- High power view of the specimen seen at left in area of migrating epithelium along the. membrane. Mild staining for collagenase seen in epithelium migrating along the membrane in between the connective tissue (arrow). (25x)
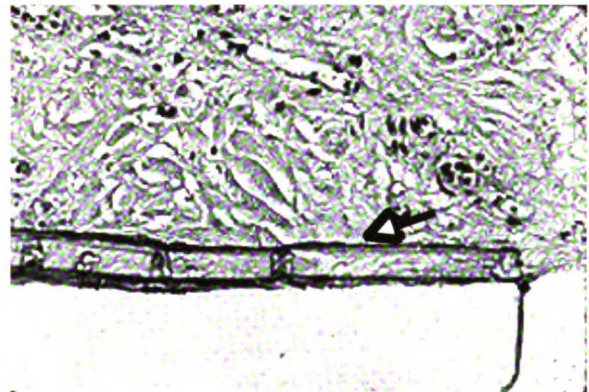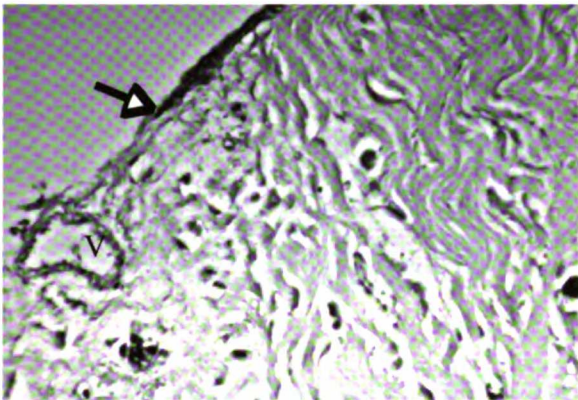
Figs. 18 & 19- Immunohistochemical results of gingival samples grown in serum for 5 days, reacted with anti-uPA antibody and counterstained with Hematoxalin. Arrows repre sent leading edge of migrating epithelium stained positive for uPA. V=vessel. (25x)
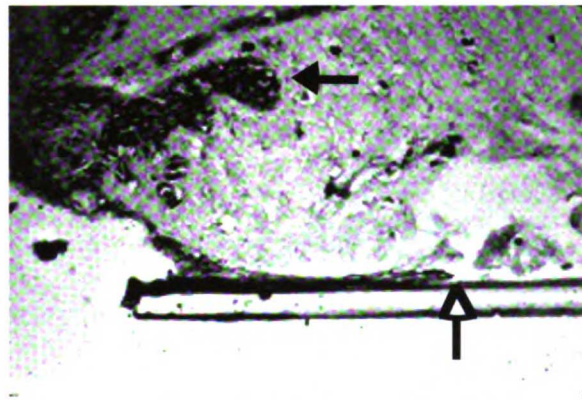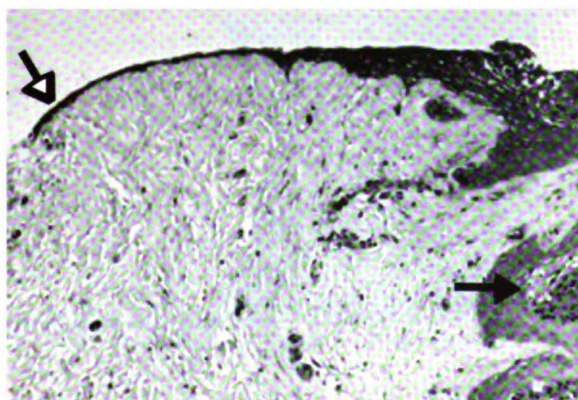
Figs. 20 & 21- Immunohistochemical results of gingival samples grown in serum for 5 days and reacted with anti-uPAR antibody and counterstained with hematoxalin. Solid arrows show basal cells with less intense stain that that seen in cells at the leading edge (clear ar rows) (Left - 10x, Right - 25x)
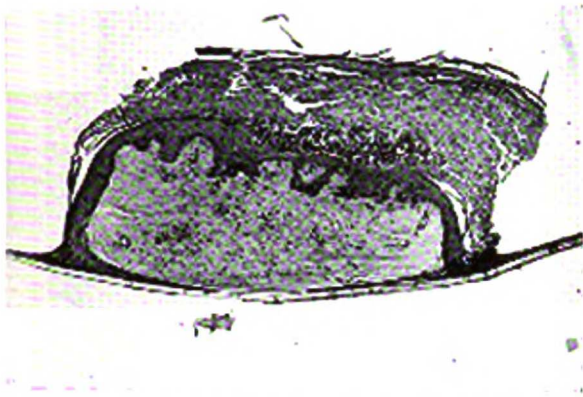
Fig. 22- H & E stained section of control specimen cultured in serum for 5 days. (4x)
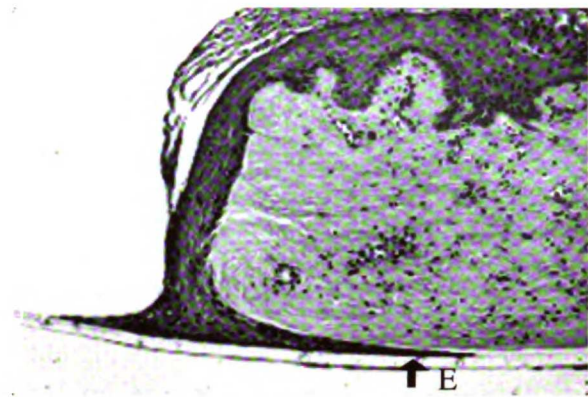
Fig. 23- Higher power view of tissue section seen at left. (10x)   E=epithelium
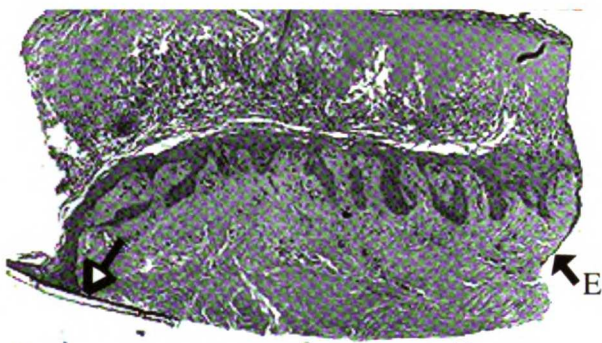
Fig. 24 -H & E stained specimen cultured in serum and 10 ug/ml of anti-uPA antibody for 5 days. (4x)
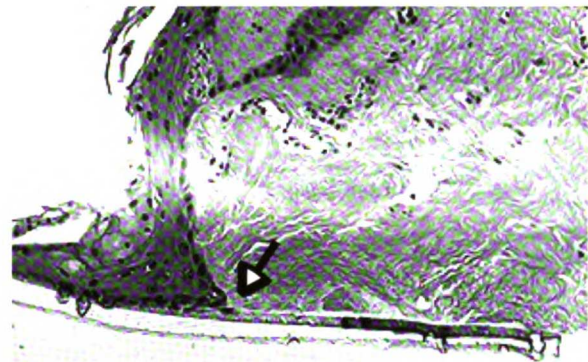
Fig. 25 -High power view of specimen seen at left. Arrow shows lack of epithelial migration between the connective tissue and the membrane. (10x)
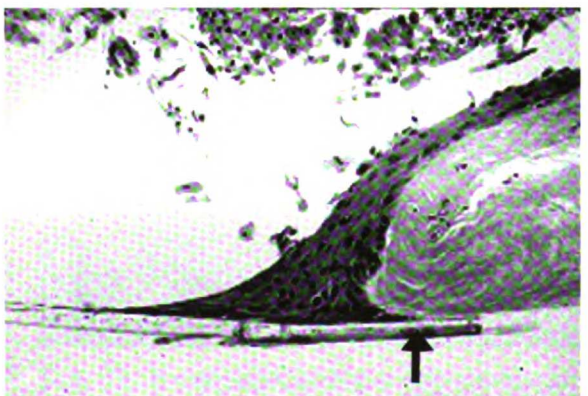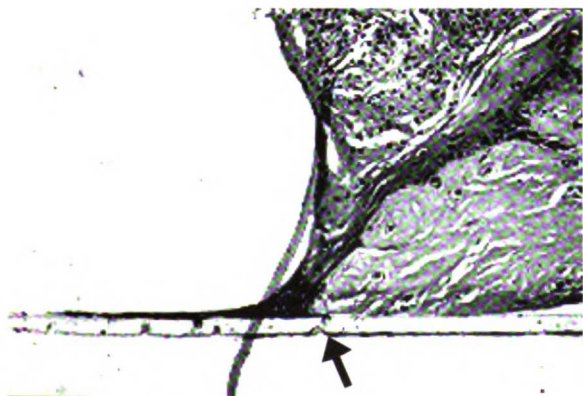
Figs. 26 & 27- H& E stained sections of experimental specimens treated with 10 ug/ml of anti-uPA antibody and cultured in serum for 5 days. The scans are taken from different tissue samples and demonstrate migration of epithelium along the membrane itself, however, very little migration between the membrane and connective tissue (arrow). (10x)
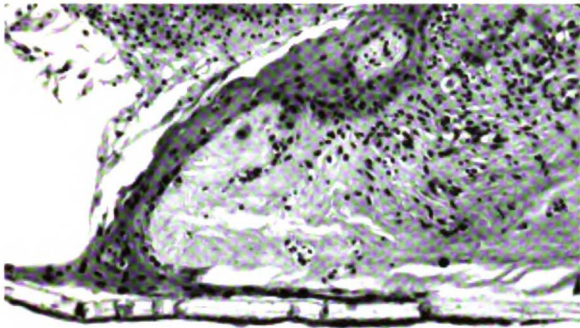
Fig. 28- H & E stained specimen cultured in serum and 10 ug/ml of PAI-1 for 5 days. (10x)
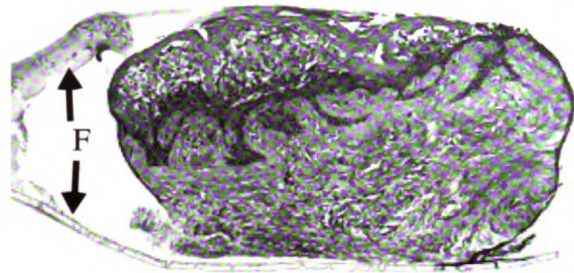
Fig. 29- H & E stained specimen cultured in serum and fibrin adhesive (F). (4x)
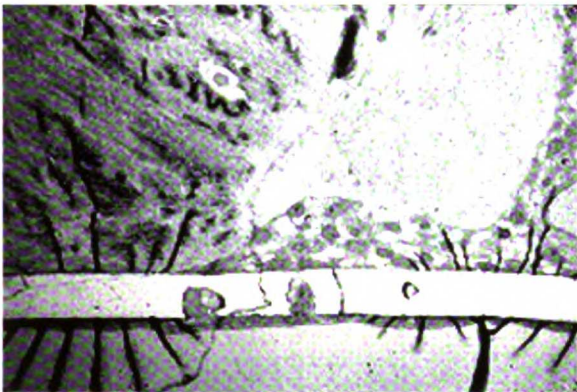
Fig. 30- Plastic embedded control tissue sectioned at 0.5 um. Epithelium can be seen migrating along the membrane in between the connective tissue. (25x)
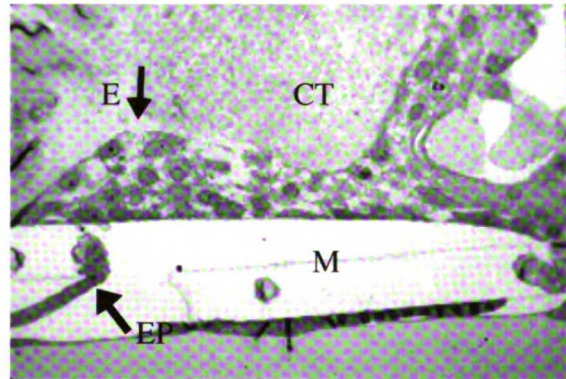
Fig. 31- Higher power view of specimen seen at left. (E-epithelium, CT-connective tissue M-membrane, EP-epithelium in pores of membrane. (40x)

Fig. 32- Plastic embedded non-antibody treated tissue specimen. Epithelium seen migrating between the membrane and connective tissue. (40x)
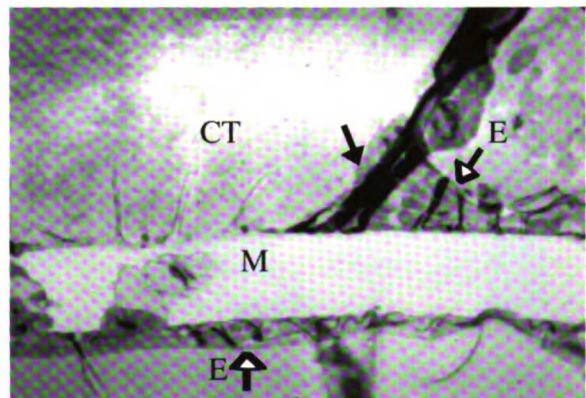
Fig. 33- Experimenal tissue specimen cultured with 10 ug/ml anti uPA antibody. Very little epithelial migration between the membrane(M) and connective tissue (CT). Solid arrow= artifact. (40x)
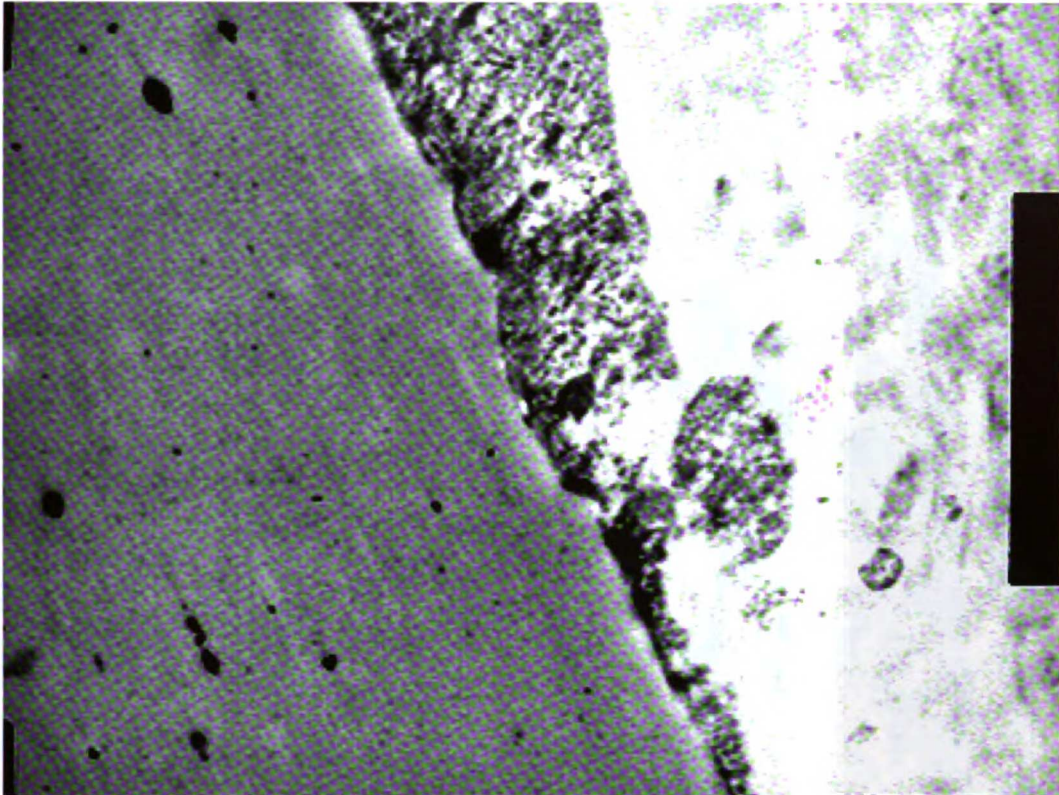
Fig.34 - TEM photomicrograph of leading edge of migrating epithelium reacted with anti-uPAR antibody and labled with gold particles. (X12K 500N)
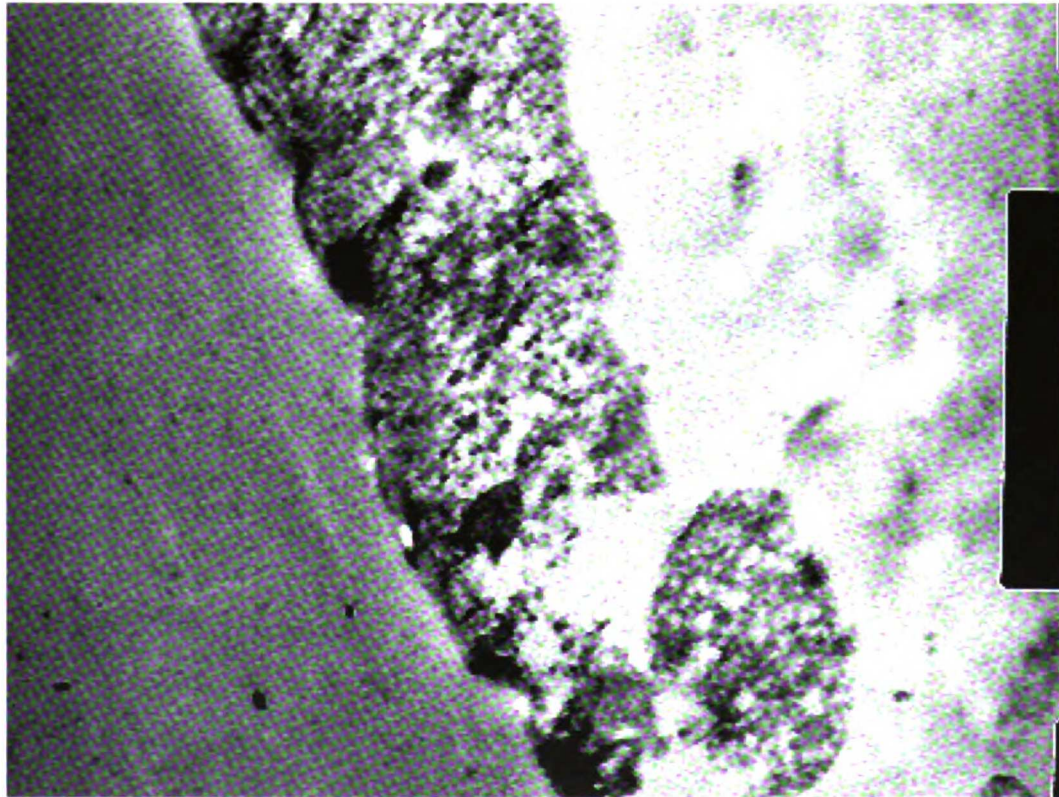
Fig. 35 - Higher power view of section seen above. (X20K 200N)
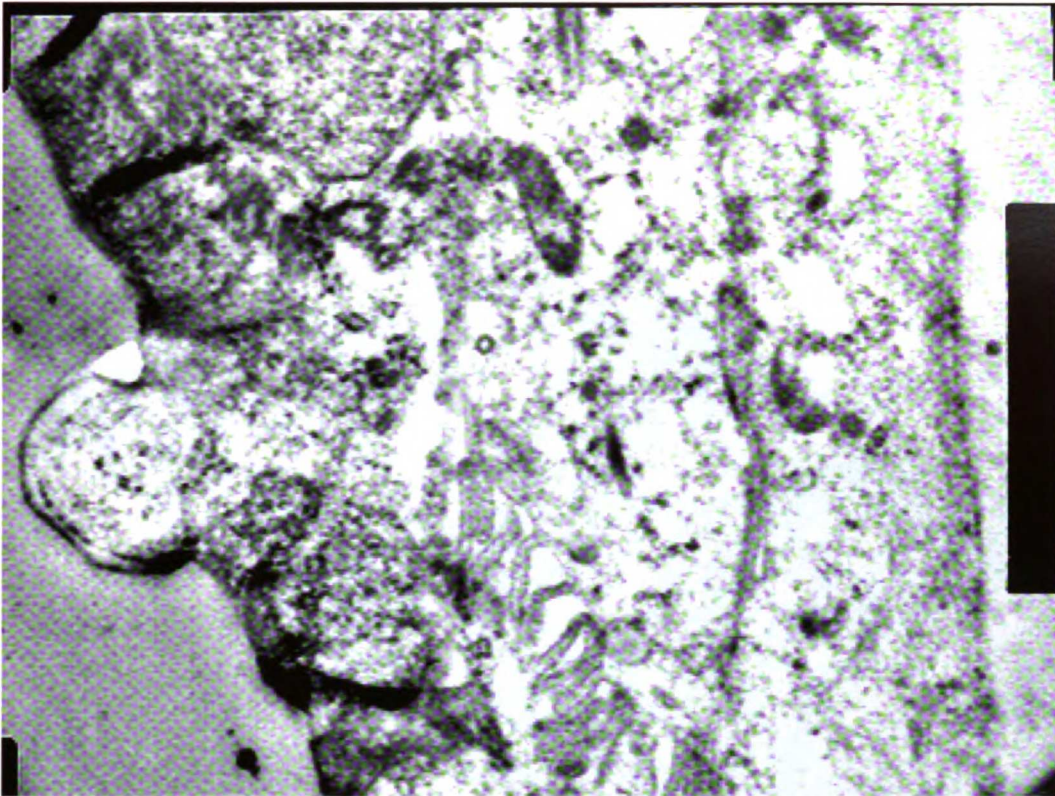
Fig. 36- TEM photomicrograph of migrating epithelium reacted
with anti-uPAR antibody labled with gold particles. (X20K 200N)
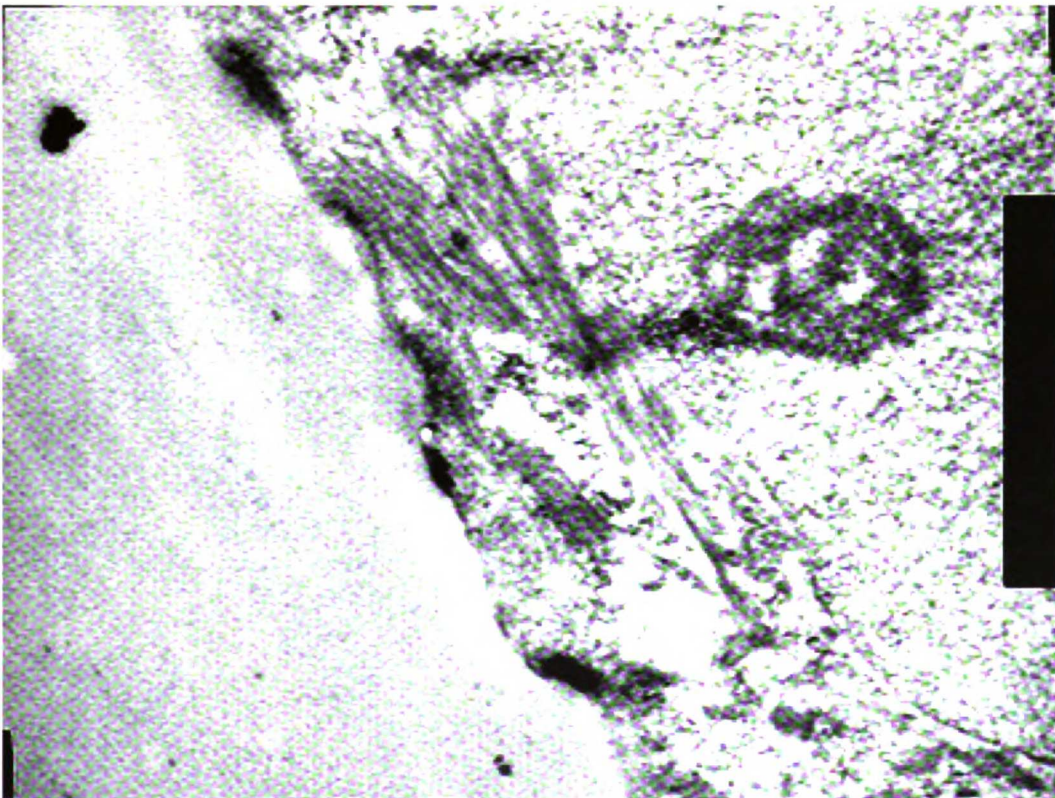
Fig. 37- TEM photomicrograph of area of migrating epithelium reacted with anti-
u-PAR antibody and labeled with gold particles. (X20K 200N)
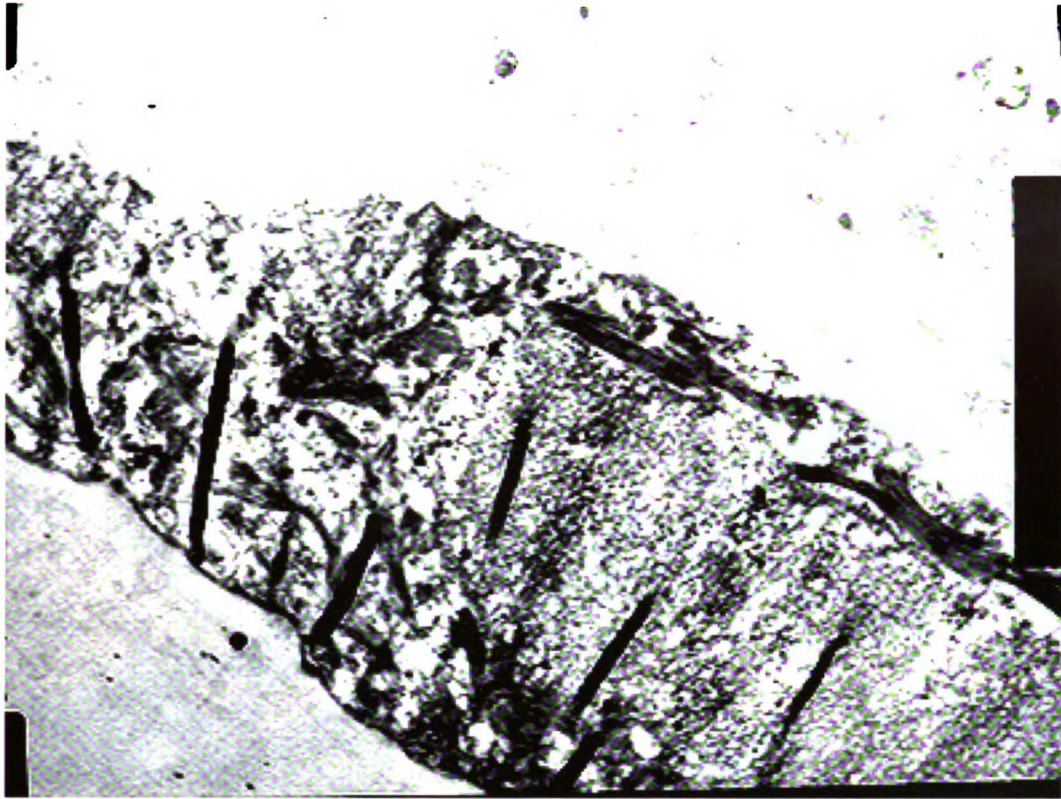
Fig. 38- Negative control. TEM photomicrograph of migrating epithelium not reactedwith the anti-uPAR antibody and labeled with gold particles. (X12K 500N)
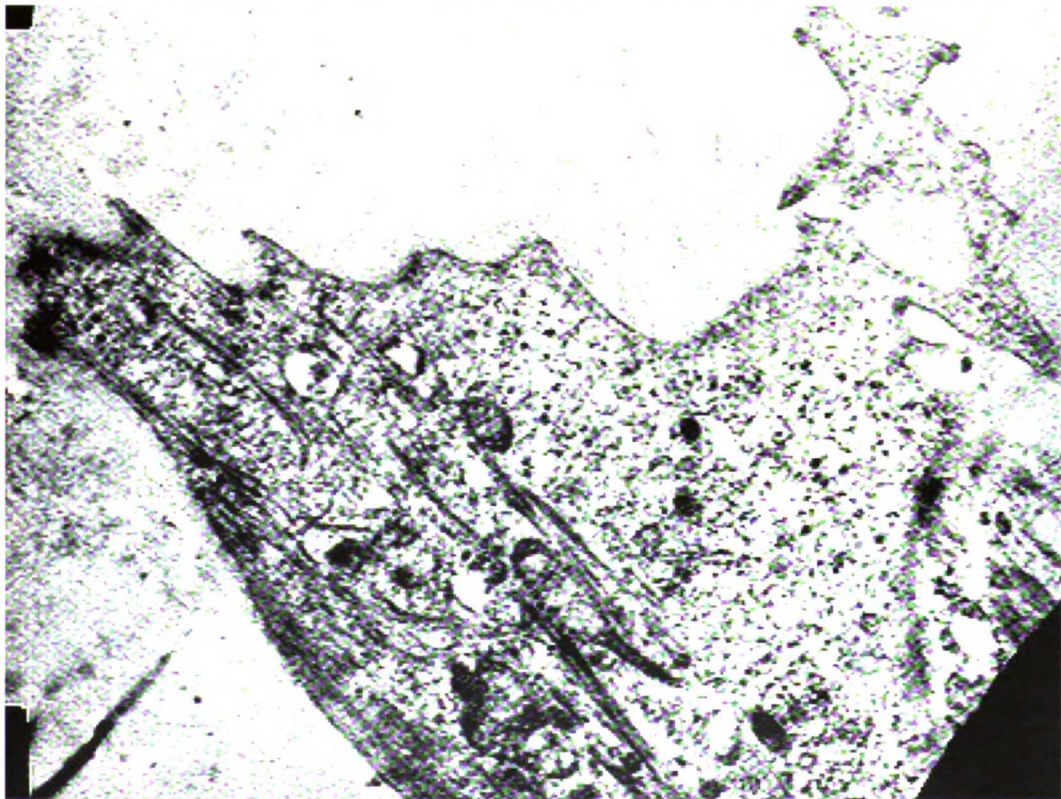
Fig. 39- Negative control. TEM photomicrograph of leading migrating epibolus not reacted with anti-uPAR and labeled with gold particles. (X12K 500N)
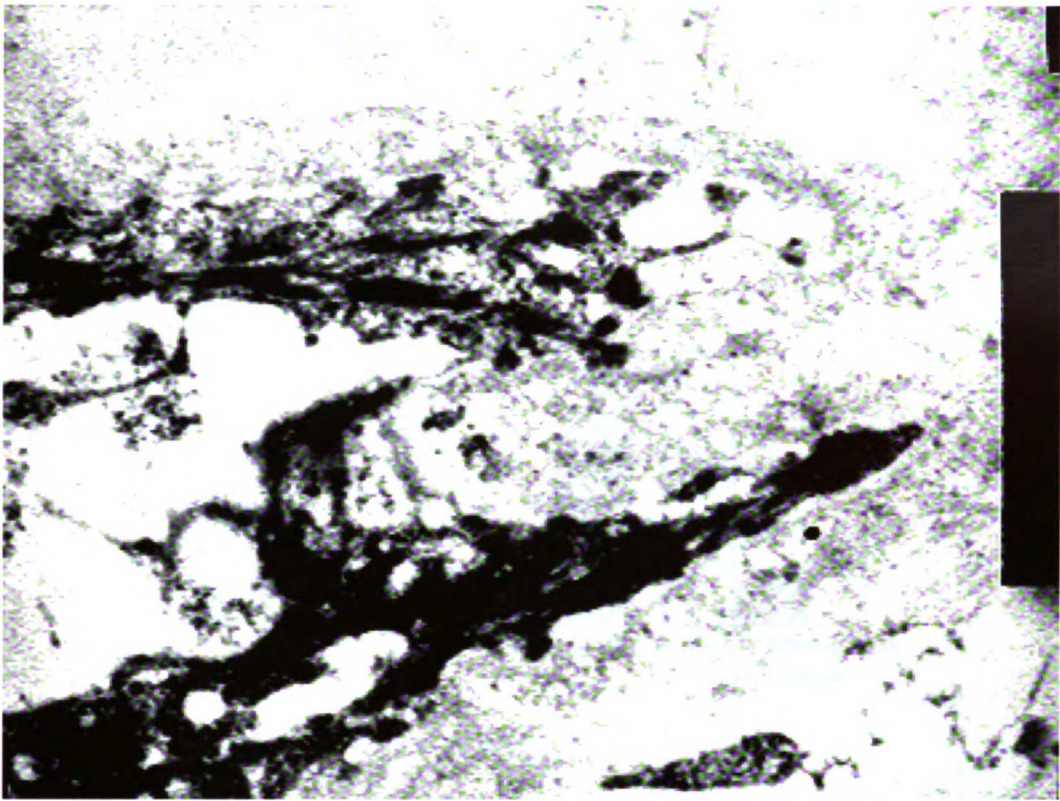
Fig. 40- TEM photomicrograph of oral mucosal tissue taken
from a biopsy of an intraoral wart and reacted with anti-uPAR antibody and
labeled with gold particles. (15k 500N)
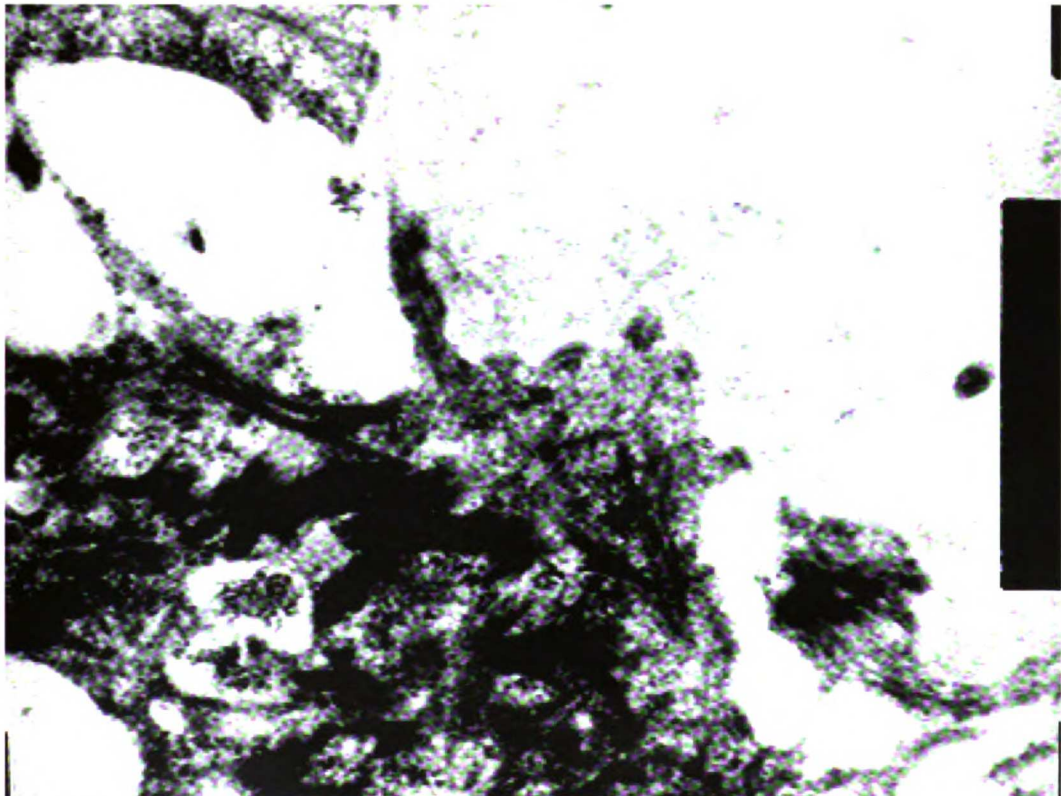
Fig. 41 - High power view of specimen seen above. (25K 200N)

Epithelial Migration

Legend:
- ◻ Control
- ▨ Antibody Treated

Y-axis: μm of migration

X-axis categories: Tissue left, Tissue right, Membrane only, Between Membrane and Tissue
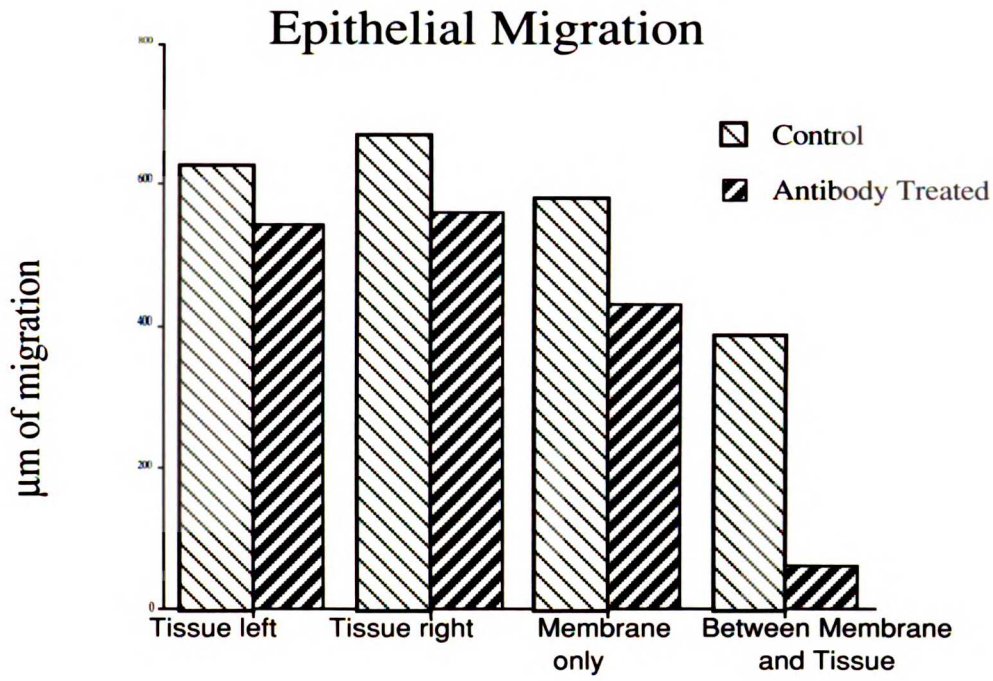
Areas of Migration

Fig. 42 -Mean results of migration from the 10 control and 10 antibody treated specimens in tables 2 and 3. Each area of migration was measured three times from each sample and the average was used.

Plasminogen Activator Zymogram

1	2	3	4	5	6	7
+Ab	-Ab	-Ab	-Ab	+Ab	-Ab	Media

Molecular weight markers: 205, 105, 68, 43

-Serum   +Serum   -Serum +Serum
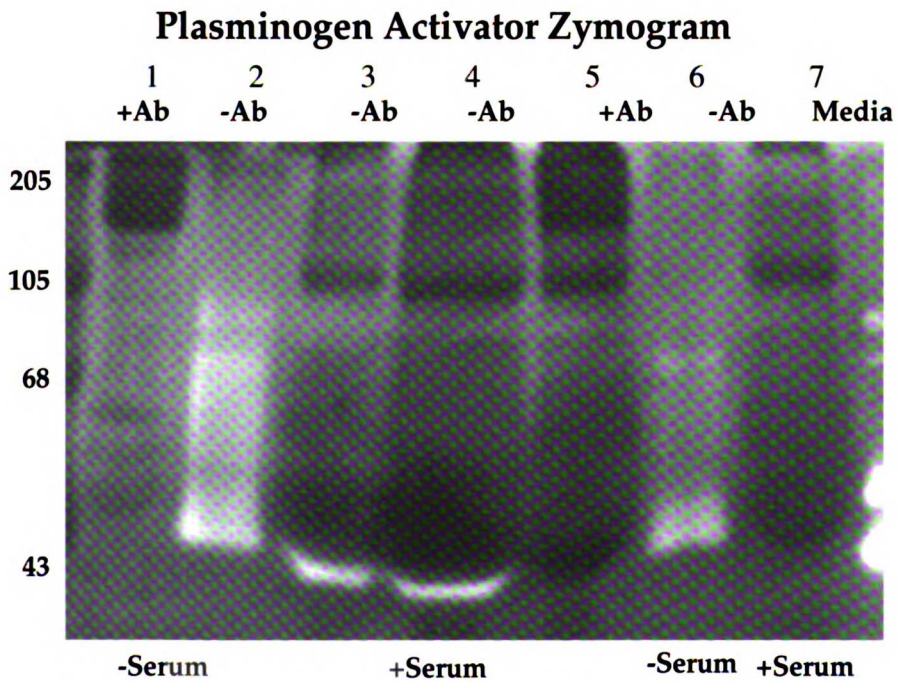
Fig. 43

Plasminogen substrate gel of conditioned media of 5-day gingival samples.

MIGRATION OF EPITHELIUM (um) FROM CONTROL TISSUE

left	std dev	right	std dev	mem-brane	std dev	b-t mem & tis	std dev
478.23	3.58	1030.68	29.04	371.74	3.59	271.75	0.76
1109.12	7.17	800.58	9.65			296.39	1.30
752.95	3.65	600.83	2.17	603.33	3.38	513.32	2.23
481.56	4.99	272.10	1.10			161.44	2.05
644.83	1.64	813.69	1.75	922.75	2.12	380.69	1.75
442.93	3.94	748.63	4.48	499.73	4.71	748.63	4.48
891.69	5.16	989.27	2.24			430.06	1.27
486.64	1.05	556.56	1.83	501.28	6.02	464.94	3.77
329.20	2.77	200.35	0.38			200.35	0.38
536.86	1.59	708.01	1.99	566.68	2.00	356.73	0.47
MEANS 624.13	251.04	672.07	273.63	577.59	208.59	382.43	170.82

Table 2. Data from migration experiments from control tissue (in um). The numbers are expressed as means of three measurements for each sample. Not all samples exhibited migration from all four areas.

MIGRATION OF EPITHELIUM (um) FROM EXPERIMENTAL TISSUE

left	std dev	right	std dev	mem-brane	std dev	b-t mem & tis	std dev
244.76	1.87	866.13	20.68			46.64	1.42
541.11	10.86	113.39	3.31	362.32	1.18	148.02	2.32
334.09	3.43	907.2	0.89	496.63	1.48	58.68	0.13
399.02	3.15	254.93	2.09	434.72	4.41	8.65	1.13
1494	68.15	976.18	11.43			60.65	10.1
251.84	3.6	248.58	2.71			54.5	1.06
657.87	3.54	404.15	5.97	471.12	5.67	69.14	2.6
486.75	2.98	352.68	9.35	387.14	3.12	78.76	2.17
802.3	9.63	623.11	4.45	307.83	4.05	27.84	1.89
316.43	3.1	298.24	3.76			56.1	1.69
MEANS 552.82	376.60	504.46	313.74	409.96	70.83	60.90	36.62

Table 3. Data from migration experiments from tissue incubated with 10ug/ml anti-uPA antibody. The numbers are expressed as means of three measurements for each sample.

control b-t mem & tis	exp b-t mem & tis
271.75	46.64
296.39	148.02
513.32	58.68
161.44	8.65
380.69	60.65
748.63	54.5
430.06	69.14
464.94	78.76
200.35	27.84
356.73	56.1
MEANS 382.43	60.90

	df	t-Value	P-value
control			
ab treated	9	6.068	0.0002

Table 4. Data from migration of epithelium between membrane and tissue (as measured along the membrane) and paired t-test between control tissue (cultured in serum only) and experimental tissue (cultured in serum and 10 ug/ml anti-uPA antibody)

DISCUSSION

This study characterized oral epithelial migration using an organ culture model similar to the one Salonen, et al. 1983, introduced as an attempt to simulate junctional epithelium. Using this model, oral epithelium was cultured in media with and without serum for up to 14 days. Migration of the oral epithelium occurred along the cut connective tissue, and between the tissue and the microporus filter. This migration was greatest during the first 4 to 6 days and occurred only when the tissue was cultured with serum. In subsequent investigations, they found that once epithelium stopped migrating, its phenotype changed from a flattened to a more cuboidal shape. This change in phenotype was associated with a decrease in reactivity with antibodies to ψ-3 antigen, which is associated with epithelial migration, and an increase in reactivity with antibodies to cytokeratin 19, which is a marker for junctional epithelial cells (Salonen, et al. 1989).

The present study agrees with the work of Salonen, et al. with respect to the phenotype of the migrating epithelium. These cells were elongated and the migrating epithelium was very thin. The non-migrating epithelium resembled keratinized oral mucosa with cuboidal shaped basal cells and stratified squamous superficial layers. The migrating epithelium appeared to originate from the basal layer and the cells were elongated and thin with flattened nuclei, often only one cell layer. Some of the cells appeared to move over one another as they migrated along the connective tissue and between the connective tissue and the membrane. This supports the "leap-frog hypothesis" of reepthelialization of dermal wounds where suprabasal cells roll over cells adjacent to them to reach the wound surface (Winter, 1972). Generally, no mitotic figures were observed in the migrating epithelium in this study, indicating that epithelial migration was not due to cell proliferation of the advancing cells, but rather to cell migration as cells moved over one another. These results were similar to the findings of Salonen, et al, who did not see mitotic figures until the sixth day in culture. This is consistent with the literature on healing of epidermal wounds. Migration is thought to be the initial event following epidermal wounding as suprabasal cells move over basal cells to form an advancing cell sheet (Krawczyk, 1971). Proliferation and differentiation

are thought to be delayed somewhat as cells are lost from a suprabasal position (Odland, et al.1968; Winter, 1972). In this study, mitotic figures were only seen proximal to the advancing edge of cells.

A third area of epithelial migration observed in these cultures, was that of epithelial cells along the membrane itself. The membrane used contained 8 μm pores which allowed easy passage of cells to the underside of the membrane where they also migrated. These cells had the same essential appearance as the advancing cells between tissue and membrane and over tissue.

The work presented in this thesis is the first to demonstrate integrin expression using this organ culture model of migrating epithelium and the results are consistent with what has been reported in the literature (Guo, et al. 1991; Larjava, et al. 1993). Larjava, et al. examined the expression of different integrins and their ligands during human mucosal wound healing They performed immunofloresence studies on biopsies from human palatal wounds and found that the migrating keratinocytes continually expressed kalinin during all phases of wound healing. The expression of the $\beta 1$ subunit was increased in keratinocytes during migration and the $\alpha 5$ and αv integrin subunits were expressed only by the migrating epithelial sheet. The αv subunit was localized to the basal aspect of the migrating keratinocytes. The $\alpha 6$ and $\beta 4$ integrin subunits were colocalized at the basal surface of basal keratinocytes in the non-wounded epithelium and, during migration, staining for both the $\alpha 6$ and $\beta 4$ surrounded keratinocytes at the wound margin in 1 day old wounds.

In this study, kalinin was found associated with the basement membrane zone under the surface epithelium, and was also detected along the membrane associated with all migrating epithelium. These results agree with the observations of Larjava, et al. who suggested that kalinin may serve as an extracellular ligand for migrating epithelium (Larjava, et al. 1993). The $\alpha 5$ and αv subunits were expressed by the migrating epithelium and were not detected in the basal cells. Migration of keratinocytes over fibronectin has been shown to be mediated by the $\alpha 5 \beta 1$ integrin (Clark, et al. 1990; Guo, et al. 1990, 1991), thus the epithelium in this model is probably migrating over a matrix rich in fibronectin.

The $\alpha 6 \beta 4$ integrin has been shown to localize in hemidesmosomes, suggesting a role for this molecule in cell-basement membrane adhesion

(Jones, et al. 1991; Sonnenberg, et al. 1991). Results from the localization of α6 and β4 showed both to be present in the basal epithelium and along the length of the migrating cells, thus suggesting a role for the α6β4 integrin in cell migration. These results agree with the work of Kurpakus, et al. 1991, where using an in vitro model of wound healing, they found that α6β4 appeared along the entire surface of migrating keratinocytes. In addition, Larjava, et al. 1993, also found α6 and β4 surrounding keratinocytes in 1 and 3 day-old wounds. They proposed that kalinin may be the ligand for α6β4 in migrating keratinocytes. The results from the present study support this contention, however, the β1 integrin could also be the β subunit pair of α6 and this receptor might recognize an unidentified matrix component.

The αv integrin subunit can complex with either β1, β5 or β6. The αvβ1 integrin has been reported to bind fibronectin (Hynes. 1992), whereas the αvβ5 integrin mediates keratinocyte adhesion to vitronectin (Adams, et al. 1991). Alpha v subunits can also serve as receptors for collagen if RDG containing peptides are present (Agrez, et al. 1991). Larjava, et al. found αv expression only in keratinocytes not in contact with the basement membrane and has recently been reported that β6 is present in healing wounds. It is uncertain from the results of this study what the ligand is for αv.

Interpretation of these results imply that these migrating cells could be using the 5, 6 or v alpha subunits and the β4 and by inference the β1 subunits for attaching and migrating along substratum. It also suggests that kalinin and by inference fibronectin are used for migration. However, it does not prove which of these integrins or components is necessary for migratory behavior.

Urokinase plasminogen activator has been found to be a key enzyme in the breakdown of extracellular matrix proteins in a variety of processes involving cell migration and degradation of tissues (Liotta, et al. 1981; Grondahl-Hansen, et al. 1988). Given this, we formulated the hypothesis that uPA is necessary for migration of oral epithelium. uPA and the uPAR were densely localized at the leading edge of migrating epithelium using immunohistochemical and immuno-gold labeling with monoclonal antibodies. These findings agree with previous studies that have demonstrated uPA production by keratinocytes in culture, and by

wounded keratinocytes, (Jensen, et al. 1990; Grondahl-Hansen, et al. 1988; Morioka, et al. 1987). Additionally, these studies demonstrate the presence of uPA & uPAR in migrating oral epithelium. Romer et al. have suggested that a similar demonstration of uPA and uPAR at the leading edge of healing mouse skin implicate these molecules in reepithelialization (Romer, et al. 1991).

In addition, uPA activity was found in the culture media from 5-day gingival samples. Since there was little uPA detected in the connective tissue, the most likely source of this activity would be the epithelium. This finding of PA activity, and specific immunohistochemical localization of uPA, supports the thesis that uPA is present in migrating epithelium.

The tissue was cultured with or without serum added to the media and the epithelium consistently migrated only in the presence of serum. This was especially evident between the connective tissue and the membrane, where migration occurred only in media containing serum. When tissue was cultured without serum, the samples were easily dislodged from the membrane, possibly due to the lack of epithelial movement through the pores in the membrane, or lack of migration between membrane and connective tissue. Salonen et al. 1983, also found that epithelium only migrated in media containing serum, although they gave no explanation as to why this occurred. The requirement for serum could be due to a variety of molecules, including growth factors, fibronectin, vitronectin and plasminogen among others. It is possible that the plasminogen in serum is being converted to plasmin by uPA secreted by the migrating epithelium. The plasmin is able to degrade fibrin and fibronectin and to activate latent metalloproteinases which can degrade collagen and other matrix components. Epithelium from samples cultured in media without serum produce uPA, however, the media contains no plasminogen, therefore, the ECM-degrading proteolytic cascade is not activated. This may explain why no migration was seen between the connective tissue and the membrane in samples cultured in media without serum.

When gingival samples were cultured with the anti-uPA antibody, no uPA activity was detected in the media and migration of epithelium was altered. Epithelial migration along the connective tissue and along the membrane were similar to the control tissue, however, migration was significantly reduced between the connective tissue and the membrane in

the in the tissue cultured with the uPA antibody. This suggests that uPA activity is necessary for epithelial migration between the connective tissue and the membrane. It is hypothesized that migrating cells secrete proteolytic enzymes to facilitate movement through ECM and it is very likely that the oral epithelium in the present investigation secrete uPA to facilitate movement through the connective tissue and the membrane, although it is not clear what components are adherent to the membrane.

It is also possible that the antibody prevents uPA from binding to its cell surface receptor. Quigley et al have shown that receptor binding is capable of activating uPAR in the absence of other enzymes. In addition binding uPA on the cell surface may have other effects on cell behavior.

The results from the gingival tissues cultured with PAI-1 were somewhat surprising. Incubation of the gingival samples with PAI-1 did not inhibit uPA activity as media from these samples did not show activity on zymography. Although the inhibitor could have been separated form the enzyme in this assay, the addition of PAI-1 to the culture media also had no effect on epithelial migration. In these samples, epithelium was consistently seen migrating between the connective tissue and the membrane and the results were very similar to the control tissue. The only explanation of these findings is that the PAI-1 may not have been active. Although synthesized in an active form, PAI-1 in serum or secreted into cell conditioned medium rapidly becomes inactive (Vassalli, et al. 1991). Loskutoff, et al. feel that PAI-1 is released from cells in an active form and can be maintained in the active form when bound to ECM or a binding protein present in the plasma. Free PAI-1 in conditioned medium spontaneously decays into the latent form due to a conformational change that masks its reactive center (Loskutoff, et al. 1989). Denaturing agents such as SDS and guanidinium hydrochloride can activate latent PAI-1, since these reagents disrupt the secondary structures of proteins

The results from plasminogen activator experiments in this study differ from the results reported by others who have studied plasminogen activators in oral mucosa. Barlow and Southam, 1992, were unable to demonstrate plasminogen activators in either normal sulcus or gingival epithelium by immunofloresence. However, using a fibrin overlay lysis technique, they were able to detect PA activity and found that the majority of lysis over sulcular epithelium was due to tPA. They used

polyclonal antibodies to inhibit PA activity in their autographs which may not have been specific enough to distinguish between uPA and tPA. However, they did find uPA activity at the leading edge in cell cultures from normal gingival epithelium which compares favorably with the results from the present investigation. It is not clear as to why they were unable to detect PA activity by immunofloresence, although the author's speculate that there may have been a problem with the technique.

Studies by Schmid, et al. 1991, 1992 also demonstrated tPA activity in diseased pocket epithelium and epithelium adjacent to endosseous dental implants. They also used the fibrinolytic autographic technique to demonstrate lysis along diseased epithelium taken from periodontitis subjects, and in the coronal portion of epithelium adjacent to dental implants. Using polyclonal antibodies against uPA and tPA, they found that the anti-tPA antibody inhibited the zone of lysis and concluded that tPA was the major activator in these tissues. This model however, fails to specifically localize the PA activity to the epithelium. In addition, tissue-type plasminogen activator is activated by fibrin whereas uPA is not. It is possible that the uPA produced was not active and thus was unable to lyse the fibrin. In both of these studies, experiments were performed on stationary sectioned mucosa, whereas the present investigation was primarily interested in investigating PA activity in migrating cultured epithelium.

Significance to therapy

Following periodontal therapy, the most aggressive tissue in the initial phases of wound healing is gingival epithelium (Listgarten, 1967). Epithelium migrates apically along connective tissue between the tooth and the flap, and forms a long junctional or sulcular epithelium. This type of healing prevents formation of new connective tissue attachment by preventing repopulation of the root surface by cells derived from the periodontal ligament (Karring, et al. 1985; Caton, et al. 1980). A technique to inhibit gingival epithelium using barriers was introduced by Nyman, et al 1982 directed at excluding the undesired apical migration of gingival epithelium on the tooth aspect of the flap between the connective tissue and the root surface resulting in the formation of a long junctional epithelial attachment (Listgarten, et al).

My interest is to use these concepts, coupled with an understanding of epithelial migration and biology for new approaches to surgical regeneration without the use of physical barriers. If gingival epithelium can be inhibited from migrating along the gingival connective tissue and it still forms a junctional attachment, then a barrier may not be needed to allow repopulation of root surface by periodontal cells.

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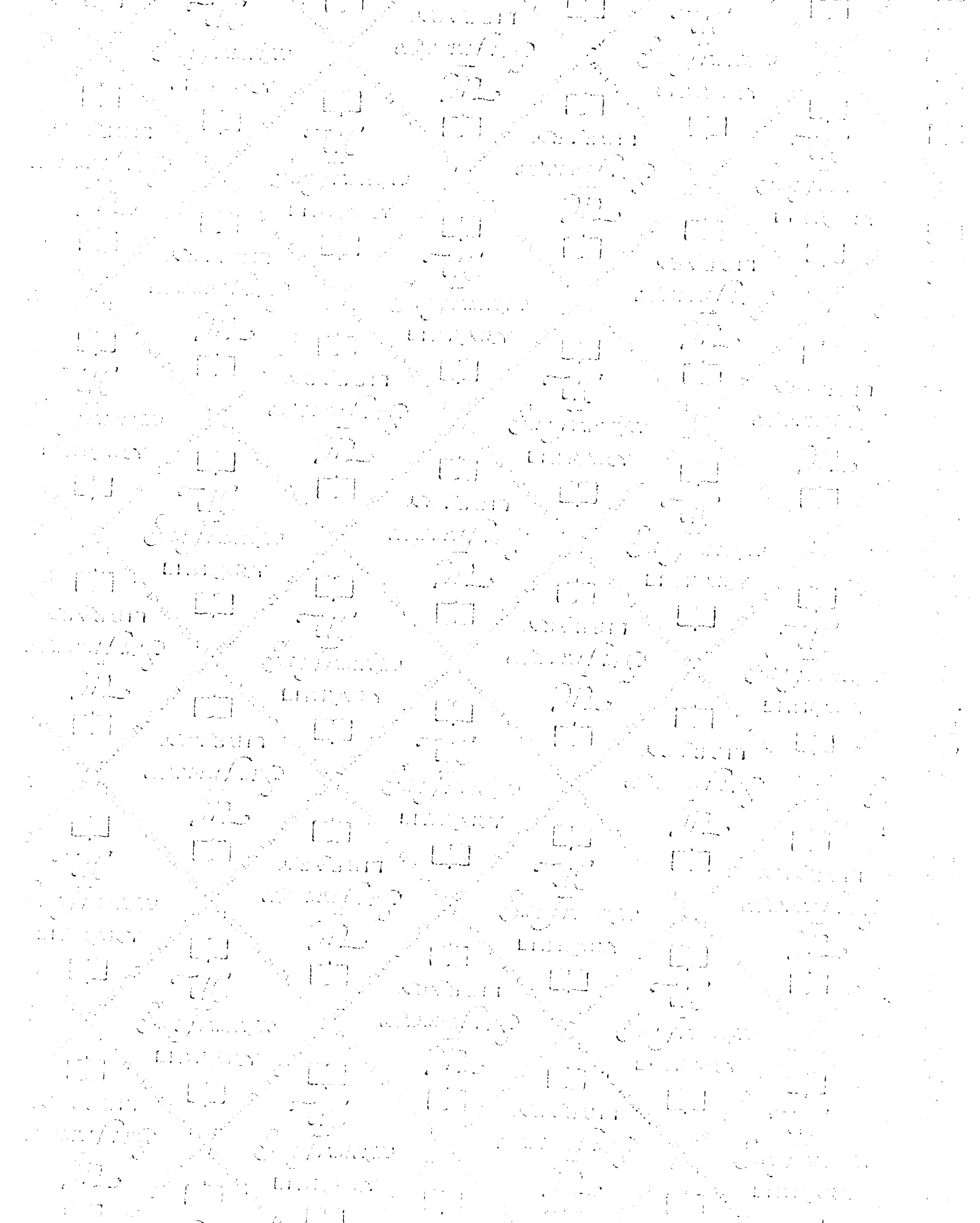
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