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The requirement of the u-PA receptor in an in vitro model for epithelial migration

Author
Loo, Kevin Scott

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The requirement of the u-PA receptor in an in vitro model for epithelial migration.

by

Kevin Scott Loo

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INTRODUCTION

Epithelial cell migration plays a critical role during embryogenesis, wound healing, tumor invasion, and periodontal disease. The process of epithelial migration is a complex event dependent upon several factors. Epithelial migration depends on the organization of a cell's cytoskeleton which consists of actin filaments, microtubules, intermediate filaments and their associated proteins (Alberts, 1989). The directed migration of an epithelial cell is also the result of external signals from its environment, such as growth factors or cytokines (Alberts, 1989). And epithelial cell migration through tissues relies on the breakdown of the extracellular matrix by proteases produced by the cell itself. For example, urokinase-type plasminogen activator (u-PA), which is a serine protease, plays an important role in extracellular proteolysis in processes involving tissue destruction and cell migration (Blasi, *et al.*, 1987).

The gingival epithelium of the periodontium consists of three components: oral, sulcular, and junctional. The oral gingival epithelium extends from the alveolar mucosa to the coronal aspect of the free gingival margin, while the sulcular epithelium extends from the base of the gingival sulcus to the free gingival margin. The junctional epithelium has as its most coronal border the base of the gingival sulcus and is adjoined to the tooth surface. The junctional epithelium attaches to the surface of the tooth via hemidesmosomes and a basal lamina, and acts as a physical barrier between the subjacent connective tissue and the oral environment.

Periodontitis is a inflammatory disease process of the oral periodontium which occurs as a consequence of the presence in the gingival sulcus of pathogenic bacteria and their harmful by-products. Their presence invokes an immune response by the host, which results in the destruction of the connective tissue attachment and resorption of the bone that surround and support the teeth. Also during periodontitis,

the epithelial attachment is disrupted. This process is then followed by the apical migration of the junctional epithelium. The final result is a deepening of the gingival sulcus with the subsequent formation of a periodontal pocket (Armitage, 1980).

After removal of the causative factors and resolution of the inflammatory process through conventional periodontal therapy, an epithelial lininig against the tooth forms without the restoration of the original dento-epithelial complex (Caton and Zander, 1979; Listgarten, *et al.*, 1979). This represents a repair process, which unlike the process of periodontal regeneration, does not result in the reformation of the prediseased periodontal state. This may be explained by the fact that the repair of connective tissue to the tooth takes more time than does the regeneration of junctional epithelium to the tooth (Armitage, 1980). Consequently, by inhibiting epithelial migration, the potential for the dento-gingival complex to reform its normal architecture will be enhanced.

Urokinase-type plasminogen activator has been implicated in the process of epithelial migration and tumor invasion. Migrating epithelial cells have been shown to express the mRNA for the u-PA receptor in vivo, while protease inhibitors and antibodies against the u-PA enzyme itself have been shown to retard epithelial migration in vitro (Blasi, 1993).

Various experimental models have been used to test the necessity of the u-PA receptor in cell migration. In one experimental model, the noncatalytic amino terminal chain of u-PA was added to keratinocytes in a Boyden chamber, and the amount of cell migration that occurred was then measured (Del Rosso, *et al.*, 1990). A related experiment that has been used was to expose cells to a chemotactic gradient and determine if their membrane bound u-PA receptors localize to the leading edge of cell migration (Gyetko, *et al.*, 1994). Localization of the u-PA receptor to the cells at wound edges has also been done with the use of radioactively labeled u-PA

(McNeill and Jensen, 1990). And an additional model that has been used dealt with the invasion of tumor cells in a matrix, either in the presence or absence of antibodies to the u-PA receptor (Stahl and Mueller, 1994).

Explants of oral epithelium have been used for studying epithelial migration in vitro (Salonen and Santti, 1983). Explants placed on microporous filters and grown in culture for up to fourteen days have remained viable and maintained morphological characteristics of oral mucosa. In this in vitro model, epithelial cells also migrated along the filter while simultaneously maintaining contact with the connective tissue of the explant.

My first hypothesis would be to determine if the u-PA receptor is involved or necessary in the process of epithelial migration in vitro. A second related hypothesis would be to determine if the protease inhibitor, plasminogen activator inhibitor (PAI-1), is able to prevent epithelial migration in vitro.

LITERATURE BACKGROUND

EPITHELIAL MIGRATION

Epithelial cells migrate in response to signals from their surrounding environment. These signals may be cytokines or other inflammatory mediators that are chemotactic for epithelial cells, and are released during such events as wound healing. Thus during wound repair, epithelial cells will begin to migrate and proliferate in order to cover the denuded area (Alberts, 1989). Migration and proliferation of epithelial cells are further regulated by polypeptide growth factors, like fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), or epidermal growth factor (EGF). In vitro, EGF has been shown to stimulate the DNA synthesis and cell growth of epithelial cells (Caffesse and Quinones, 1993).

A critical event related to the ability of epithelial cells to migrate is the production of proteases (Blasi, *et*

al., 1987). Proteases degrade the extracellular matrix and so allow epithelial cells to migrate through tissues. This is especially important in the process of tumor invasion. Antibodies or other perturbing agents that block the activity of type IV collagenase have been shown to result in a retardation of metastasis in vivo (Alberts, 1989). Thus, an increase in expression of proteases appears necessary for cell migration to occur.

u-PA AND CELL MIGRATION

Plasminogen activators are a family of serine proteases of which two types have been isolated in mammals: urokinase-type plasminogen activator and tissue-type plasminogen activator. Several studies have shown the importance of u-PA for cell migration. Epithelial cells at wound edges display an increased expression in u-PA activity indicating its involvement in keratinocyte migration. The enhanced levels of u-PA activity, however, later decrease to background levels when the wound closes and epithelial migration is stopped (Pepper *et al.*, 1987).

In another experiment, similar results were found in which u-PA activity was present only in keratinocytes at the edge of a wound after twelve hours, while it was present in all keratinocytes of the epithelial outgrowth that covered the wound at two to ten days. At day fourteen, it was observed that the epidermis appeared normal and no u-PA was detectable (Grondahal-Hansen *et al.*, 1988). Taken together, these studies indicate the importance of u-PA for epithelial migration.

u-PA RECEPTOR AND CELL MIGRATION

The u-PA receptor has further been implicated as necessary in the process of cell migration. During wound repair, u-PA binds to sites selectively on the plasma membrane of cells at the leading edge of migrating epithelium (McNeill and Jensen, 1990). It was also observed that the u-

PA receptor on monocytes exposed to a chemotactic gradient strongly localized to the leading edge of cell migration, and that chemotaxis was abolished when the expression or activation of the u-PA receptor was affected but not when the u-PA molecule was affected (Gyetko *et al.*, 1994). And it was found that melanoma cells showed enhanced migration of thirty-two percent when u-PA bound its receptor and seventy-two percent when the nonenzymatic amino terminal fragment of u-PA bound to the receptor (Stahl and Mueller, 1994). The amino terminal fragment of the u-PA molecule has also been shown to stimulate keratinocyte and endothelial cell migration (Del Rosso *et al.*, 1990; Fibbi *et al.*, 1988). Therefore, these results indicate the importance of the u-PA receptor in cell migration, as well as suggest that the activation of the u-PA receptor alone is sufficient to enhance cell migration and may be independent of plasmin generation.

u-PA AND u-PA RECEPTOR IN TUMOR INVASION AND METASTASIS

The plasminogen activator system has been implicated in playing a role in the tumor process. Several studies have found that tumor cells, versus normal cells, show a significant increase in u-PA production. When compared to noncancer cells, cell lines from squamous cell carcinomas produce significantly higher levels of both total plasminogen activator activity and u-PA (Petruzzelli *et al.*, 1994). Also epithelial ovarian carcinomas produce both u-PA receptor and u-PA, while normal ovarian epithelium express u-PA receptor but no u-PA (Young *et al.*, 1994). Thus, u-PA is expressed in significantly higher amounts by tumor cells.

The invasiveness or ability of tumor cells to metastasize is partially dependent on the plasminogen activator system. It has been shown that the inhibition of u-PA decreases the invasiveness and metastasis of tumor cells. B16 melanoma cells treated with an antibody against u-PA demonstrated a significant decrease in the number of

cells metastasizing when compared with controls (Hearing *et al.*, 1988). Tumor invasion was further shown to be dependent on u-PA by the observation that a forty-six percent inhibition of Matrigel invasion by melanoma cells occurred with the addition of the u-PA inhibitor PAI-2 (Stahl and Mueller, 1994). And by completely abolishing the binding of single chain u-PA to ovarian cancer cells, invasion of Matrigel was shown to be inhibited by up to seventy-five percent (Wilhelm *et al.*, 1994).

Because u-PA must first bind to its receptor to become activated, the expression by tumor cells of the u-PA receptor is important in the tumor process. With the use of immunohistochemistry, it was found that the signal for the presence of u-PA receptor in colonic adenocarcinomas, as compared with normal tissue, was more intense and predominated in the invasive foci on tumor infiltrating monocytes and neutrophils (Pyke *et al.*, 1994). Similar results were obtained from breast tissue in which the u-PA receptor was present in human breast cancer and absent in nonmalignant breast tissue (Bianchi *et al.*, 1994). And when looking at tumor invasiveness, it was observed that a decreased expression of surface u-PA receptor resulted in a decrease in tumor cell invasion, while an antagonist to the u-PA receptor reduced colon cancer cell invasion up to seventy-eight percent (Kook *et al.*, 1994).

The levels of u-PA and u-PA receptor expressed by tumor cells are different between benign and malignant tumor cells. Unlike what occurs in benign melanocytes, u-PA and PAI-1 mRNAs accumulate in atypical nevocytes and melanoma cells. Active u-PA is mainly located in melanoma cells too (Delbaldo *et al.*, 1994). It was additionally observed that the invasive part of lung carcinoma expressed large amounts of single chain u-PA compared to other parts, and that u-PA, u-PA receptor, PAI-1, and PAI-2 could only be detected in advanced primary melanoma and melanoma metastatic lesions but not in benign lesions (Skriver *et al.*, 1984; de Vries *et al.*,

1994). And it was shown that invasive colon cancer cells possessed ten fold more u-PA receptor than noninvasive colon cancer and that this difference was a result of differences in transcription rates (Wang *et al.*, 1994).

CELL MIGRATION AND PERIODONTITIS

During and following the pathogenesis of periodontal disease, junctional epithelium lengthens and travels apically along the root of the tooth to form a periodontal pocket (Armitage, 1980). Periodontal pockets potentially predispose a tooth's attachment to further loss of connective tissue attachment due to the creation of an environment conducive to the growth of harmful bacteria as well as the difficult and limited accessibility for their removal by both patients and clinicians. The typical healing response of the periodontal pocket to conventional nonsurgical and flap surgical therapy is the formation of an epithelial lining which attaches to the root of the tooth at a more apical level via hemidesmosomes and a basement membrane. With the use of the surgical modified Widman flap procedure, it was observed that healing resulted in the formation of an epithelial lining along the treated root surface without any evidence of new connective tissue attachment (Caton *et al.*, 1980).

The presence of an epithelial lining against the tooth surface following conventional periodontal therapy represents a repair process. Therefore, clinicians have sought alternative treatment modalities to reconstitute the bone and connective tissue attachment destroyed by periodontal disease so as to reestablish the normal architecture of the periodontium. And even though a healthy situation can exist around a tooth as a result of a reparative process, periodontal regeneration can offer the patient the potential for the regaining of lost support around teeth as well as a return to a prediseased state.

In order to potentiate the regeneration of the periodontium, clinicians have used several different

treatment modalities. Clinicians have used such treatments options as synthetic or natural bone grafts, polypeptide growth factors, root surface demineralization, and guided tissue regeneration (GTR). Currently, GTR relies on the use of a membrane barrier to allow specific cells to selectively populate the periodontal wound in order to enhance regeneration.

In the periodontium, there are four distinct connective tissue components: the periodontal ligament, cementum, bone, and gingival corium which include both the epithelium and connective tissue (Melcher, 1976). The connective tissue cells of each compartment possess different phenotypes, and the specific connective tissue cells repopulating a wound would ultimately determine the regenerative response. Therefore, only periodontal ligament cells will regenerate the periodontal ligament, while epithelial cells will only produce an epithelial lining along the tooth surface that precludes connective tissue attachment. In a study where periodontal ligament cells and cementum were entirely removed from the roots of teeth by curettage, periodontal wound healing resulted in an epithelial lining extending down the length of the roots of teeth with no new formation of cementum or functionally oriented fibers (Aukhil *et al.*, 1986).

Several additional studies have shown the importance of excluding epithelium during wound healing in order for periodontal regeneration to occur. After resecting the crowns of teeth and completely covering the roots with a mucosal flap to exclude epithelium, it was found that all roots demonstrated formation of new cementum with inserting collagen fibers (Karring *et al.*, 1985). Thus, by excluding epithelium, new attachment was formed presumably by coronal migration of cells originating from the periodontal ligament.

The potential for periodontal regeneration to occur in the presence of epithelial exclusion was further investigated using a similar approach of resecting the crowns of teeth and

completely covering the roots with a mucosal flap. This experimental approach also placed membrane barriers over certain roots to further exclude connective tissue and epithelial cells (Gottlow *et al.*, 1984). In this situation, it was observed that roots with membranes exhibited significantly more new attachment than roots without membranes, thus indicating that the placement of a membrane barrier favored selective population by cells from the periodontal ligament. Taken together, these studies indicate the importance of epithelial exclusion during periodontal wound healing.

UROKINASE PLASMINOGEN ACTIVATOR AND THE ORAL CAVITY

The presence of plasminogen activator activity in the oral cavity has been studied by several investigators. Junctional epithelial cells of the oral periodontium express differential levels of plasminongen activator activity in diseased and healthy conditions. From human gingival biopsies, it was found that a local or focal zone of plasminogen activator activity was present in the most superficial cells of the junctional epithelium from periodontally healthy tissues. This contrasted to a diseased state in which all the epithelium lining the periodontal pocket demonstrated plasminogen activator activity (Schmid *et al.*, 1991).

Similarly, it was observed that the junctional epithelium from periodontally healthy tissues around oral dental implants exhibited a distinct focal spot of plasminogen activator activity along the epithelium lining the implant crevice (Schmid *et al.*, 1992). And it has been demonstrated that plasminogen activity is present in both the saliva and gingival crevicular fluid (Verrusio *et al.*, 1987; Watanabe *et al.*, 1987).

u-PA STRUCTURE AND ACTIVATION

u-PA has been shown to exist in both a one and two chain form. The single chain, or proenzyme, form is initially secreted by cells and has a molecular weight of 52,000 - 54,000 daltons (Nielsen *et al.*, 1982; Fibbi *et al.*, 1990). The single chain form of u-PA has little enzymatic activity when compared with the two chain form of u-PA, and is unable to activate plasminogen (Petersen *et al.*, 1988; Nielsen *et al.*, 1982).

In contrast to the single chain u-PA, the two chain u-PA is considered the active form with enzymatic activity. The single chain form of u-PA is converted by proteolysis at lysine 158 into the active enzyme consisting of two chains bonded together by a disulfide bond, and having a molecular weight of about 20,000 and 32,000 daltons (Blasi *et al.*, 1986; Nielsen *et al.*, 1982). It was also observed that the enzymatic activity was located on the 32,000 dalton fragment, while the 20,000 dalton, or amino terminal, fragment binds to the u-PA receptor (Nielsen *et al.*, 1982; Del Rosso *et al.*, 1990).

One way that single chain u-PA can be converted into an active enzyme is through the catalytic action of plasmin. When single chain u-PA is incubated with plasminogen, plasminogen activator activity is not detectable. But when single chain u-PA is incubated in the presence of plasmin, plasminogen activator activity is observed (Nielsen *et al.*, 1982). It has also been demonstrated that a mast cell tryptase, which is a trypsin-like serine proteinase, is able to activate single chain u-PA into the two chain form. This activation is accomplished by cleavage of single chain u-PA at Lys^{158}-Ile^{159} residues which agrees with what others have found (Stack and Johnson, 1994). The resultant molecular weight of the two chains were 22,000 and 33,000 daltons, which is consistent with the amino terminal and carboxy terminal fragments that others have isolated.

An additional way that u-PA can become activated is by binding to its receptor. Once single chain u-PA is secreted, it can bind to the u-PA receptor on the cell surface in an autocrine fashion (Fibbi *et al.*, 1990; Stoppelli *et al.*, 1986). It was further shown that single chain u-PA is progressively converted into an active form and this process requires the presence of intact cells or a plasma membrane enriched fraction (Berkenpas and Quigley, 1991). This result suggests that single chain u-PA is proteolytically converted to the active two chain u-PA by a catalytic mechanism present on the cell surface. It has also been demonstrated that the melanoma cell line M24met secretes u-PA but is unable to use it efficiently for invasion unless it is bound first to the u-PA receptor (Stahl and Mueller, 1994).

Additional evidence indicating the requirement for the binding of u-PA to its receptor for activation has been observed. The cocultivation of two cell lines, one that only expresses the u-PA recptor and the other that only expresses single chain u-PA, caused an increase in both the conversion of inactive u-PA to active u-PA as well as the rate of matrix degradation. These increases were dependent on the binding of single chain u-PA to its receptor, which also needed to be cell surface bound to catalyze this conversion (Quax *et al.*, 1991). Furthermore, the binding of u-PA to its receptor resulted in it acting as a membrane protease (Vassalli *et al.*, 1985).

The specificity and kinetic binding properties of both the one and two chain forms of u-PA to its receptor are not appreciably different, and the conversion of inactivated to activated u-PA does not lead to its dissociation from the membrane (Cubellis *et al.*, 1986). In fact, when u-PA binds to its receptor, it remains on the membrane surface for activation of plasminogen to plasmin and is not released or internalized. After incubating keratinocytes with ^{125}I-u-PA for 180 minutes, it was found that seventy-five percent of the total cell-associated radioactivity stayed on the cell

surface (McNeill and Jensen, 1990). This suggests that the interaction of u-PA with its receptor does not result in a rapid ligand internalization.

AMINO TERMINAL FRAGMENT OF u-PA

Only a specific part of the inactivated and activated u-PA molecule binds to the u-PA receptor. Keratinocytes have receptors for u-PA distributed on the cell surface as singlets or clusters, and the 17,500 dalton molecular weight amino terminal fragment, or A chain, of u-PA specifically binds to the u-PA receptor. The low molecular weight 33,000 dalton fragment of u-PA retains the enzymatic activity and does not compete for receptor binding, thus indicating that it is not specific for the u-PA receptor (Del Rosso *et al.*, 1990). Using a monoclonal antibody against the 17,500 dalton fragment of the A chain of u-PA, binding to the u-PA receptor was impaired thereby providing further evidence for an interaction of the A chain with the u-PA receptor (Fibbi *et al.*, 1988).

With the use of synthetic peptides that corresponded to different areas of the amino terminal part of u-PA, the specific area within the amino terminal fragment that was responsible for binding to the u-PA receptor was investigated. It was found that residues twenty to thirty of u-PA provided receptor binding specificity, while residues thirteen to nineteen gave the proper conformation to the adjacent binding region. Peptides one to fourteen of u-PA did not bind to the u-PA receptor but did share partial sequence homology to epidermal growth factor. This region has thus been referred to as the growth factor module or domain (Appella *et al.*, 1987).

The amino terminal fragment of u-PA possesses a growth factor domain which promotes cell proliferation. When active urokinase was added to human renal glomerular epithelial cells, it caused a dose dependent enhancement in 3H-thymidine incorporation and a doubling of cell number after forty-eight

hours. This increase in cell proliferation was dependent on both u-PA binding to its receptor as well as being enzymatically active. The growth promoting effect of u-PA, however, was not as strong as fibroblast growth factor or epidermal growth factor (He *et al.*, 1991). Thus, u-PA was shown to possess a growth promoting activity on normal renal epithelial cells by stimulating DNA synthesis and increasing cell number. And when looking at the growth promoting effects of both inactived u-PA and the amino terminal chain of u-PA on SaOS-2 osteosarcoma cells, it was observed that both molecules were mitogenic in SaOS cells and increased cell numbers (Rabbani *et al.*, 1992).

PLASMINOGEN ACTIVATION BY u-PA

Once activated, u-PA converts plasminogen into plasmin, which is a serine protease that can degrade circulating and tissue proteins, and activate zymogens or growth factor precursors (Blasi *et al.*, 1987). Plasminogen binds to cells in a specific and saturable manner, and this binding requires an unoccupied high affinity lysine site on plasminogen. Inactive and active u-PA also enhance plasminogen binding to cells by 1.4 to 3.3 fold, and in the presence of activated u-PA, plasmin is the predominant form of the bound ligand (Plow *et al.*, 1986). These results suggest that cells have a separate and specific binding site for both plasminogen and u-PA.

The binding of u-PA and plasminogen to their specific cell surface receptor is important for the conversion of plasminogen to plasmin. Single chain u-PA when bound to its receptor on cell surfaces, potentiated plasmin generation. This potentiation, however, is not observed when single chain u-PA is bound to its receptor but not attached to the cell surface. This enhancement is additionally inhibited by the presence of 6-aminohexanoic acid, which is a lysine analog and prevents plasminogen from binding to its receptor (Lee *et al.*, 1994). These results suggest that plasmin generation

through receptor bound u-PA is dependent on plasminogen binding to its cell surface receptor as well.

Furthermore, it was demonstrated that activation of plasminogen was enhanced by sixteen fold in the presence of optimal concentrations of single chain u-PA, but was abolished by the addition of either 6-aminohexanoic acid or the amino terminal fragment of u-PA (Ellis *et al.*, 1989). This activation of plasminogen to plasmin was shown to occur on the cell surface and was dependent on u-PA being bound to the cell surface to mediate this conversion (Campbell *et al.*, 1994). And it was found that an antibody against the u-PA receptor completely abolished the potentiation of plasmin generation seen during the incubation of single chain u-PA and plasminogen with U937 cells (Ronne *et al.*, 1991).

Single chain u-PA and plasminogen activation on human HT-1080 fibrosarcoma cells was additionally investigated. It was found that plasmin was bound to the cell surface after the addition of plasminogen, and that this was the result of plasminogen activation that occurred on the cell surface. The conversion of plasminogen to plasmin was also dependent on u-PA being bound to the cell surface, and the single chain form of u-PA predominated in the absence of plasminogen, while the addition of plasminogen leads to a predominance of the two chain form of u-PA. This process was catalyzed by plasmin which implies a feedback mechanism (Stephens *et al.*, 1989).

The existence of a feedback activation loop was further studied. Active u-PA is generated more rapidly from single chain u-PA and plasminogen in the presence, versus the absence, of U937 promonocytic cells. This enhanced activation in the presence of U937 promonocytic cells is also significantly less susceptible to plasmin inhibitors (Duval-Jobe and Parmely, 1994). Thus this again points to the dependence of plasmin for the positive feedback amplification of plasminogen activator activation.

FACTORS AFFECTING u-PA EXPRESSION

Cell differentiation influences the intracellular level of plasminogen activator. As keratinocytes differentiate their intracellular levels of plasminogen activator increase (Isseroff *et al.*, 1983). Also the most differentiated keratinocytes in culture contained both t-PA and u-PA, but when compared to less differentiated cells, they possess a higher ratio of t-PA:u-PA (Jensen *et al.*, 1990). Thus plasminogen activator systems depend to some extent on cell maturity. These results further indicate that plasminogen activators are first stored intracellularly before being secreted. When looking at the availability of plasminogen activators, the initial increase of plasminogen activator activity was primarily due to secretion of preformed enzyme, while the secretion of newly made plasminogen activator accounted for late secretion events (Fibbi *et al.*, 1990).

The availability of plasminogen activator and its inhibitors are influenced by several exogenous factors. Transforming growth factor-alpha increases u-PA expression while antibodies against transforming growth factor-alpha and epidermal growth factor decrease it (Jensen and Rodeck, 1993). Epidermal growth factor also stimulates the enzyme production of plasminogen activator in HeLa cells (Lee and Weinstein, 1978). And basic fibroblast growth factor appears to enhance plasminogen activator activity expression while transforming growth factor-beta retards plasminogen activator synthesis (Sakela *et al.*, 1987).

u-PA RECEPTOR STRUCTURE

For cell surface activation, the interaction of the single chain form of u-PA with its receptor is an essential step. The receptor for u-PA has been isolated on various cell types, including T lymphocytes, mononuclear cells, neutrophils, and endothelial and epithelial cells. The u-PA receptor is a 55,000 to 60,000 dalton cell surface protein which is heavily glycosylated and contains both N-acetyl-D-

glucosamine and sialic acid but no N-acetyl-D-galactosamine. The deglycosylated part of the polypeptide in contrast comprises only 35,000 daltons, and the protein receptor also possesses a high content of cysteine residues (Behrendt *et al.*, 1990).

Furthermore, the u-PA receptor consists of 313 amino acid residues, and has an amino acid sequence organized into three repeats of approximately ninety residues each (Roldan *et al.*, 1990; Behrendt *et al.*, 1991). The first repeat additionally corresponds to the ligand binding fragment (Behrendt *et al.*, 1991). Therefore, the ligand binding capacity is located on the amino terminal part of the intact u-PA receptor.

The u-PA receptor is linked to the plasma membrane of cells through a glycosylphosphatidylinositol (GPI) membrane anchor. The GPI anchor consists of a complex oligoglycan linked to a phosphatidylinositol molecule located in the lipid bilayer. Proteins bind to the GPI through their C-terminal amino acid, and this binding is specifically mediated by an amide linkage of the alpha carboxyl group of the C-terminal amino acid to the amino group of a phosphoethanolamine moiety. The phosphoethanolamine is next linked to a glycan consisting of mannose and glucosamine, and the glucosamine is then glycosidically linked to an inositol containing phospholipid. GPI anchored proteins have also been shown to be cleaved and released from the membrane surface by phosphatidylinositol-specific phospholipase C (PI-PLC). Thus, phosphatidylinositol is responsible for the anchorage of the protein to the membrane (Low *et al.*, 1989).

By analyzing the amino acid sequence of the u-PA receptor and by the in vivo incorporation of [^{3}H] ethanolamine, it was found that the u-PA receptor contained two to three mol of ethanolamine per mol of protein. It was also shown that an inositol residue participated in the membrane attachment of the u-PA receptor by the release of the u-PA receptor from the cell surface after treatment with

PI-PLC. After its cleavage from the cell surface, the u-PA receptor still retained its binding specificity for u-PA. And the presence of inositol in the u-PA receptor was directly demonstrated by the in vivo incorporation of myo-[^{3}H]inositol (Ploug *et al.*, 1991). Taken together, these results strongly indicate that the u-PA receptor is anchored to the cell membrane by a GPI moiety.

u-PA RECEPTOR AND ACTIVATION OF SECOND MESSENGER

Cell migration via u-PA receptor stimulation has been shown to involve a second messenger. Both the intact u-PA molecule and its amino terminal fragment stimulate migration of fibroblasts. This, however, is impaired by the down regulation of protein kinase C. It was also found that both u-PA and its amino terminal fragment stimulated the formation of diacylglycerol (DAG) but not inositol lipid metabolism or intracellular calcium content (Anichini *et al.*, 1994). Because u-PA only increased intacellular levels of DAG, and not inositol or calcium, the u-PA receptor may somehow activate phospholipase D which then cleaves membrane phosphatidylcholine to produce DAG. This DAG can subsequently activate protein kinase C.

It was further shown that the u-PA receptor forms a complex with protein kinase C, and that parts of the complex undergo chemical changes as a result of ligand and receptor interaction (Busso *et al.*, 1994). And increased cell proliferation of human renal glomerular epithelial cells by activated and bound urokinase was inhibited by either down regulation of protein kinase C or by H7, an inhibitor of protein kinase C (He *et al.*, 1991).

FACTORS INFLUENCING u-PA RECEPTOR EXPRESSION

The expression of the u-PA receptor can be regulated exogenously. In mononuclear phagocytes, interferon-gamma increases the amount of surface bound by u-PA 2.2 fold and u-PA receptor by 2.1 fold in comparison to unstimulated

controls. This contrasted with tumor necrosis factor-alpha which had no effect. Interferon-gamma additionally induced an increase in u-PA receptor mRNA and intracellular levels of u-PA receptor too (Sitrin *et al.*, 1994). Also in neutrophils, the expression of u-PA receptor was enhanced by phorbol myristate acetate (PMA), FMLP, or tumor necrosis factor-alpha (Plesner *et al.*, 1994).

INTERACTION OF u-PA:PAI-1 COMPLEX WITH u-PA RECEPTOR

PAI-1, PAI-2, and protease nexin-1 are high affinity plasminogen activator inhibitors that react with active, but not inactive, u-PA and are members of the serine protease inhibitor family (Blasi *et al.*, 1987). PAI-1 is a single chain glycoprotein with a molecular weight of 50,000 daltons and inhibits both u-PA and t-PA. Initially PAI-1 is made in an active form, but after secretion, it is quickly converted into an inactive latent form. The stabilization of the active conformation of PAI-1 appears to be partly dependent on its binding with the extracellular matrix and vitronectin (Seiffert *et al.*, 1990). Vitronectin also forms a complex with PAI-1 in a stoichiometry of a 1:1 ratio between the two molecules (Sigurdardottir and Wiman, 1990).

In contrast to when u-PA alone binds to its receptor, the binding of the u-PA:PAI complex to the u-PA receptor enhances ligand internalization and eventual degradation of this complex. In the two chain form of u-PA, the amino terminal domain interacts with the u-PA receptor, while the carboxy terminal protease domain interacts with PAI (Bu *et al.*, 1994). Looking at complex internalization, only LB6 cells which express a transfected human u-PA receptor are able to degrade u-PA:PAI-1 complexes. This internalization was inhibited by blocking the binding of u-PA:PAI-1 complex to the u-PA receptor, thus suggesting the dependence of internalization on the u-PA receptor (Olson *et al.*, 1992). The u-PA:PAI-2 complex was also found to be rapidly cleared from the surface of monocytes which involved the process of

endocytosis and its eventual degradation (Estreicher *et al.*, 1990).

The internalization of the u-PA:PAI complex with the u-PA receptor has been shown to be specifically mediated by the surface bound protein receptor alpha-two-macroglobulin. With the recombinant protease nexin-one (rPN-1), which is an inhibitor of u-PA, it was shown that the u-PA:rPN-1 complex specifically bound to both the u-PA and alpha-two-macroglobulin receptors. The internalization of this complex was additionally dependent upon binding to both of these receptors too (Conese *et al.*, 1994). The rate of internalization, however, is dependent primarily on the u-PA receptor, while the GPI anchor may enhance complex internalization and recycling of u-PA receptor when binding sites on alpha-two-macroglobulin receptor are limited (Li *et al.*, 1994). In addition, it was observed that the alpha-two-macroglobulin receptor and epithelial glycoprotein 330 (gp330) mediate endocytosis of the u-PA:PAI complex when bound to the cell surface u-PA receptor (Andreasen *et al.*, 1994).

Purified alpha-two-macroglobulin receptor/low density lipoprotein receptor related protein binds only to the u-PA:PAI-1 complex and not to u-PA alone. This binding was blocked by both an alpha-two-macroglobulin associated protein and a monoclonal antibody to PAI-1. The degradation of this bound complex was also enhanced three to four fold as compared with only bound u-PA alone (Nykjar *et al.*, 1994). In addition, it was demonstrated that the low density lipoprotein receptor related protein mediated the internalization and degradation of the u-PA:PAI-1 complex in mouse embryos (Herz *et al.*, 1992).

I propose that the u-PA receptor is necessary for the activation of the single chain form of u-PA and its subsequent enhancement of epithelial migration in vitro. I additionally submit that PAI-1 will retard epithelial

migration in vitro by interacting with u-PA and inhibiting its action.

MATERIALS AND METHODS

TISSUE

Gingival tissue samples, which appeared clinically healthy and scheduled to be discarded, were obtained from patients at the Postgraduate Periodontology clinic at the University of California at San Francisco School of Dentistry. Tissue samples were initially placed into keratinocyte basal medium (KBM) for two to six hours. KBM was supplemented with penicillin-streptomycin at 1000 ug/ml of streptomycin and 1000 u/ml of penicillin. Afterwards, tissue samples were washed three times in PBS containing penicillin-streptomycin at 1000 ug/ml of streptomycin and 1000 u/ml of penicillin. Tissue samples were then cut into approximately 1 X 1 X 2 mm rectangular block sections under a dissecting microscope. All surfaces of the rectangular tissue samples were connective tissue except for one surface which was epithelium.

TISSUE CULTURE

Tissue samples were placed on the membrane surface of 8.0 um Falcon inserts in such a way that the epithelial surface was on top and a connective tissue surface was against the membrane. The Falcon inserts with the tissue samples were then placed into the individuals wells of a twenty-four well plate (Corning 24 well flat bottom, Corning Glass Works; Corning, NY). Five hundred microliters of alpha modified eagles media (aplha MEM) were added to each well to just saturate the insert's membrane and tissue sample. The following perturbing agents were added to the media at various concentrations ranging from five to twenty-five micrograms per mililiter: melanoma PAI-1 (#105, American Diagnostica; Greenwich, CT), rabbit antihuman urokinase receptor IgG (#399R, American Diagnostica; Greenwich, CT), or

murine monoclonal antibody against urokinase receptor (#3936, American Diagnostica; Greenwich, CT). All media also contained either 5% or 10% fetal bovine serum (FBS). Plates were then incubated at 37°C for five days. In addition, one experiment was performed that contained tissue samples incubated with alpha MEM containing 5% FBS for 10 days.

After incubation, conditioned media was collected and frozen until further use, and the membrane with the attached tissue sample were separated from the insert with a scapel blade. Both membrane and tissue sample were then placed in ten percent formalin for one day, transferred to seventy percent alcohol for an additional day, and processed for histological sectioning. Membrane and tissue sample were sequentially dehydrated in an ascending series of alcohol rinses and then embedded in paraffin. Tissue samples were embedded in such a way that when sectioned they were cut perpendicular to both the membrane and tissue sample surface. Tissue samples were cut at a thickness of 7 um, mounted on Superfrost/Plus microscope slides (Fisher Scientific), and stained with hematoxylin and eosin (H & E).

QUANTIFICATION

With the use of both a video camera attached to a light microscope and Image analysis software, histological images of both tissue sample and membrane were captured on a Macintosh computer and analyzed for the amount of epithelial migration. All measurements were first standardized with the use of a micrometer staging device. To quantify the amount of epithelial migration, a fixed reference point was identified by a line drawn along the basal cell layer of the epithelium (Figure 1, Line A). This line depicted the demarcation between the original epithelium and migratory epithelium. Another line was drawn that extended from the first line to the distance the epithelium traveled along the right and left sides of the tissue sample (Figure 1, Lines B

& C). This represented the amount of epithelial migration that occurred on the connective tissue surface.

The next two lines spanned from the point where the epithelium first made contact with the membrane to the point where migration stopped between the membrane and the connective tissue surface (Figure 1, Lines D & E). This represented the distance that the epithelium traveled in-between the membrane and connective tissue. The distance that the epithelium migrated on the membrane surface alone was also measured (Figure 1, Lines F & G).

All measurements for each histological tissue sample sections were recorded three times and averaged. The measurements for epithelial migration on both the right and left sides of the connective tissue were then added together to obtain one value (B + C). The value was obtained for the amount of epithelial migration between the membrane by adding together Lines D + E as well as migration along the membrane alone by adding together Lines F + G.

STATISTICAL ANALYSIS

Epithelial migration measurements are reported in milimeters, and expressed as mean and standard deviation. The three different migration measurements reflected the amount of epithelial migration on the connective tissue surface, in-between the membrane and connective tissue surface, and on the membrane surface alone. An analysis of the migration measurements was first performed between the serum controls with 5% and 10% FBS using a *t* test statistical analysis. This was done to determine if the concentration of FBS affected the migration measurements.

All subsequent analyses were done using a one-way analysis of variance (ANOVA). In these analyses, the three different epithelial migration measurements were compared between serum controls and tissue samples with perturbing agents present. If a statistically significant result was obtained, a Dunnett's test was then performed to determine

which migration measurement was statistically different from the serum control.

ZYMOGRAMS

From the collected conditioned media, zymograms containing three different substrates were used: 0.2% gelatin, 0.2% casein, or 0.2% casein-plasminogen. All gels contained ten percent acrylamide and were .75 mm in thickness. Gels were attached to a mini-gel apparatus and SDS electrode buffer (20% SDS and 10X electrode buffer which contained 15.1 g of tris-base and 72.1 g of glycine per 500 ml of 10X electrode buffer; pH 8.1-8.5) was added to it. 15 ul of conditioned media and 5 ul of 4X sample buffer were then loaded to all wells of the substate gels except one. This well had 6 ul of a prestained BRL-Gibco Hi molecular weight marker added to it.

Gels were electrophoresed for 45 minutes with 60 mAmps constant current at 15°C. After electrophoresis, gels were removed and washed twice in tris with triton X-100 (2.5% triton x-100 and 50 mM tris base; pH 7.5) for fifteen minutes each. Gels were next rinsed twice with incubation medium (Tris without Triton) for five minutes each, and then incubated at 37°C in a water bath for either 6 hours (casein-plasminogen), 18-20 hours (gelatin), or 40 hours (casein). Gels were stained with Coomassie Blue for one hour after incubation and then destained with a Coomassie Blue Destaining Stock solution (0.5% coomassie blue R250, 30% isopropyl alcohol, and 10% acetic acid) until bands of interest were visible. Lastly, pictures of gels were taken using a video camera and Image analysis software.

IMMUNOBLOT

An immunoblot experiment was performed on conditioned media to determine the presence of u-PA in the tissue cultures. All gels contained 10% acrylamide, and were 1.5 mm in thickness. Gels were attached to a mini-gel apparatus

which had SDS electrode buffer added to it. 30 ul of conditioned media and 10 ul of 4X sample buffer were loaded to all wells of the gels except one which had 12 ul of a prestained BRL-Gibco Hi molecular weight marker added to it.

Gels were electrophoresed for 45 minutes using a constant current of 80 mAmps at 15°C. After electrophoresis, gels were removed from the apparatus and loaded in a cassette with blotter paper and a reinforced nitrocellulose membrane for transfer. Cassettes were next placed in a tank electrophoresis unit with Towbin transfer buffer (.025 M Tris base, .192 M glycine, 20% methanol, pH=8.2-8.4), and transfered for 1.5 hours at a constant 100 volts.

Membranes were next incubated in a 50 ml test tube on a rotary mixer with 20 ml of 5% BlottoT (5% non-fat dry milk, .01% thimerosal, and wash detergent which contained 50mM Tris base pH 7.5, 150 mM NaCl, and 0.1% Tween 20). After one hour, the BlottoT was discarded and a mixture of 8 ul of serum goat anti-u-PA (#398, American Diagnostica; Greenwich, CT) plus 4 ml of BlottoT were added to the membranes. Membranes were incubated for two hours on a rotary mixer, and then were rinsed once for fifteen minutes and twice for five minutes each with wash detergent to remove unbound primary antibody. 8 ul of biotin-labled secondary antibody with 4 ml of BlottoT were added to membranes and incubated on a rotary mixer for one hour, and then washed again to remove unbound secondary antibody. Four microliters of streptavidin labled with horse radish peroxidase and 4 ml of BlottoT were finally added to the membranes for thirty minutes and later washed. Lastly, membranes were reacted with ECL chemoluminescence reagent (Amersham, NY) for 30 seconds, covered and wraped in plastic wrap, and placed on Kodak-Xomat-AR5 film in a darkroom. Films were exposed for one minute and then developed in an automatic x-ray film developing machine. Images were also captured using a video camera and Image analysis software on a Macintosh computer.

UROKINASE ACTIVITY ASSAY

A urokinase activity assay was developed to determine the amount of available urokinase activity present in the tissue conditioned media. The assay involved the use of a buffer solution with a chomogenic substrate, Spect UK (#244, American Diagnostica; Greenwich, CT). Spect UK is composed of the compound carbobenzoxy-L-gamma-glutamyl-(alpha-t-butoxy)-glycyl-arginine-p-nitroanilide diacetate salt. When urokinase interacts with Spect UK, p-Nitroanilide is cleaved and released into solution producing a color change. This change in buffer color would then be detected with the use of a spectrophotometer at a wavelength of 405 nm.

Initially the assay involved the use of a urokinase standard (#124, American Diagnostica; Greenwich, CT) at various concentrations ranging from 1-15 international units (iu). This was done in order to detect the presence of activated urokinase. This step was also used as a positive control or reference point to which samples of conditioned media could be compared. A 5X concentrated buffer was made which contained Tris pH 8.8, 0.01% Tween 80, and 10 KIU/ml aprotinin. 10 ul of buffer, 90 ul of urokinase standard, and 50 ul of Spect UK were added to each well in a microtiter plate. Plates were then read in a spectrophotometer at 405 nm using the Softmax program version 2.1. The spectrophotometer was programmed to read the plates every 30 minutes over a 10 hour time period.

The urokinase activity assay measures the level of activated urokinase in a spectrophotometer. Therefore, the presence of substances within the conditioned media, such as FBS or phenol red, may alter the results of the assay. To determine if the presence of FBS would affect the results, experiments were done where either alpha MEM alone or alpha MEM supplemented with 10% FBS were added to the urokinase standards. 90 ul of this sample, along with 10 ul of buffer and 50 ul of Spect UK, were then added to each well used in a microtiter plate and read as before using a

spectrophotometer. The affect of the presence or absence of phenol red in alpha MEM was studied using a similar design protocol as well.

Activated urokinase is important to the migratory process. Thus, measuring the presence and levels of activated urokinase in conditioned media would be useful in determing the affect of the different perturbing agents on migration inhibition. The following experiments involved the use of conditioned media instead of urokinase standards. 10 ul of buffer, 90 ul of conditioned media, and 50 ul of Spect UK were added to each well in a microtiter plate. Plates were then read in a spectrophotometer.

By inhibiting the activity of the activated urokinase, results from the plasminogen activity assay should be altered. Therefore, an experiment was performed in which various amounts of serum goat antibody to u-PA (#398, American Diagnostica; Greenwich, CT) was added to the urokinase standards to determine its affect on the activity of available urokinase. 90 ul of this sample, 10 ul of buffer, and 50 ul of Spect UK were added to a microtiter plate and then read as before in a spectrophotometer at 405 nm.

RESULTS

TISSUE CULTURE

In the tissue culture model, epithelium was seen to migrate along the connective tissue surface, in-between the connective tissue surface and membrane, and along the membrane surface. Figure 2 represents a tissue sample where only a little amount of epithelial migration occurred. This tissue sample was cultured in the presence of 20 ug of PAI-1, and showed epithelial migration mostly along the connective tissue surface. In this tissue sample there was also minimal migration in-between the connective tissue surface and membrane or along the membrane surface.

A tissue sample where maximal epithelial migration occurred is seen in Figure 3. This tissue sample was cultured in 5% FBS, and demonstrated epithelium completely encapsulating the tissue sample. In this tissue sample, epithelium migrated along the entire connective tissue surface as well as in-between the connective tissue and membrane. Epithelium was also observed migrating along the membrane surface.

QUANTIFICATION AND STATISTICAL ANALYSIS

The amount of epithelial migration that was observed in the tissue samples cultured in either 5% or 10% FBS appeared similar with the difference between measurements small. (Table 1). Using the *t* test analysis, the only statistically significant difference between the 5% and 10% FBS tissue samples was when comparing the migration measurements along the membrane surface alone ($p=0.000$). A statistically significant difference was not found for the migration measurements between the 5% or 10% FBS tissue samples either in-between the membrane and connective tissue surface ($p=0.215$) or along the connective tissue surface ($p=0.081$). This suggested that the use of either 5% or 10% FBS was not a critical factor in affecting the amount of epithelial migration. Consequently, migration results of tissue samples cultured in conditioned media containing either 5% or 10% FBS were combined.

Table 2 shows the migration results for the various tissue samples cultured in the presence of the different perturbing agents. Tissue samples cultured in the presence of PAI-1 showed a direct relationship to the concentration of PAI-1 present and the amount of epithelial migration that occurred. Thus, tissue samples grown in the presence of 25 *ug* of PAI-1 showed more epithelial migration than tissue samples grown in 10 *ug* of PAI-1. Using an ANOVA, a statistical difference between serum controls and tissue samples cultured in the presence of PAI-1 was found only for

the measurement of epithelial migration along the connective tissue surface alone ($p=0.021$). The migration measurements for both the membrane surface alone ($p=0.109$) and in-between the connective tissue surface and membrane ($p=0.636$) were not statistically significant between the tissues samples with PAI-1 and serum control.

When analyzing the migration measurements along the connective tissue surface alone with the use of a Dunnett's test, only the migration measurement for tissue samples cultured in 25 *ug* of PAI-1 differed from serum control ($p<0.05$). Tissue samples cultured in either 10 *ug* or 20 *ug* of PAI-1 did not statistically differ from serum controls for the amount of epithelial migration that occurred on the connective tissue surface alone ($p>0.05$).

For the tissue samples cultured with anti-u-PA receptor 3936, there appeared a weak inverse relationship between the amount of epithelial migration and the concentration of the perturbing agent present (Table 2). Tissue samples cultured in 10 *ug* of anti-u-PA receptor 3936 demonstrated more epithelial migration than tissue samples cultured in either 20 *ug* or 25 *ug* of anti-u-PA receptor 3936. When using an ANOVA, migration measurements for both the membrane surface alone ($p=0.705$) and connective tissue surface alone ($p=0.103$) were not statistically different between serum controls and tissue samples cultured with the anti-u-PA receptor 3936. Only the migration measurements for in-between the connective tissue surface and membrane differed between tissue samples with anti-u-PA receptor 3936 and serum controls ($p=0.002$).

With the use of a Dunnett's test, only tissue samples cultured in the presence of 10 *ug* of anti-u-PA receptor 3936 differed from serum controls in the amount of epithelial migration that occurred in-between the connective tissue surface and membrane ($p<0.05$). Migration measurements for in-between the connective surface and membrane, however, did not statistically differ between serum controls and tissue

samples cultured in the presence of either 20 *ug* or 25 *ug* of anti-u-PA receptor 3936 ($p>0.05$).

No consistent relationship existed between the amount of epithelial migration and the concentration of anti-u-PA receptor 399R present (Table 2). Tissue samples cultured in the presence of either 5 *ug* or 25 *ug* of anti-u-PA receptor 399R showed similar migration results. With the use of an ANOVA, no statistical difference was observed between serum controls and tissue samples cultured in the presence of anti-u-PA receptor 399R for the amount of epithelial migration that occurred along the connective tissue surface ($p=0.121$), in-between the connective tissue surface and membrane ($p=0.305$), or along the membrane surface alone ($p=0.575$).

In general, epithelium migrated more on the free connective tissue surface than in-between the connective tissue surface and membrane or the membrane surface alone (Table 1 & 2). This trend was consistently observed for all tissue samples cultured in the presence of serum or perturbing agents. And when comparing the amount of epithelial migration that occurred in-between the connective tissue surface and membrane to that along the membrane surface, migration results appeared similar with differences small.

ZYMOGRAMS

The various zymograms were performed to determine the different types of enzymes present in the conditioned media. Enzymes present in the condtioned media will degrade the substrate present in the polyacrylamide gel, and produce a cleared band in the stained gel. The position of this band will also reflect the molecular weight of the enzyme.

All zymograms demonstrated a displacement or distortion of bands at between 71 kD to 44 kD (Figures 4-7). It appeared that the presence of some molecule in the conditioned media was altering the position of these bands in all the different zymograms. Thus, protein bands at 71 kD

were being displaced slightly higher, whereas bands at 44 to 60 kD were being displaced slightly lower.

All samples of conditioned media in the casein-plasminogen zymogram consistently showed three to four bands present at between molecular weights of 105 kD to 71 kD, and two to three bands at 44 kD (Figures 4 & 5). Occasionally bands were observed that migrated at 200 kD and/or 30 kD. There appeared no significant difference in the bands observed between all the various samples of conditioned media used.

For the casein zymogram, all samples of conditioned media consistently produced three bands at between 105 kD to 71 kD, and two to three bands at 44 kD (Figure 6). When comparing the casein and casein-plasminogen zymograms (Figures 4-6), the width and intensity of the bands at 44 kD in the casein-plasminogen zymogram were significantly wider and stronger than the bands at 44 kD in the casein zymogram. And for the gelatin zymogram, conditioned media consistently demonstrated two to three bands at between 105 kD to 71 kD, two bands at 71 kD, and two to three bands at 44 kD (Figure 7).

IMMUNOBLOT

From the immunoblots (Figure 8) probed for the presence of u-PA, all samples of the conditioned media showed single bands appearing between the molecular weights of 44 kD and 28 kD. It appeared that there also existed an inverse relationship between the intensity of the signal and the dose of the inhibitor to the plasminogen activator system, which included PAI-1 and antibodies to the u-PA receptor. Thus, conditioned media that was collected after ten days of tissue growth and that did not contain any type of inhibitor produced the most intense signal (Figure 8, Lane H).

When comparing the intensity of the bands for conditioned media that had anti-u-PA receptor (399R) added to it, lane D on the immuno blot, which contained conditioned

media supplemented with five micrograms of anti-u-PA receptor (399R), showed the most intense band. The second most intense signal was from lane E which had ten micrograms of anti-u-PA receptor (399R). The least intense signal for the conditioned media supplemented with anti-u-PA receptor (399R) were lanes F and G both of which contained twenty-five micrograms each. The signal for lane C which contained conditioned media with twenty-five micrograms of anti-u-PA receptor (3936) appeared similar to lanes F and G, whereas lane B's signal which had conditioned media with ten micrograms of anti-u-PA receptor (3936) was slightly more intense than lane E.

UROKINASE ACTIVITY ASSAY

An assay was developed to determine the presence and levels of activated urokinase present in the conditioned media and relate this to the migration results. The development of the urokinase activity assay first began with obtaining a positive control or reference point to which all subsequent experiments could be compared with. Using different concentrations of urokinase, there was a direct correlation of urokinase activity with increasing amounts of added urokinase standard (Figure 9). 5 iu of urokinase showed the greatest amount of urokinase activity, whereas 0 iu of urokinase showed the least.

The presence of FBS did not appear to affect the results obtained from the urokinase activity assay (Figure 10 & 11). Urokinase with and without FBS showed similar levels of urokinase activity, with the only exception being controls with no urokinase added. FBS in this situation slightly enhanced the levels of urokinase.

In addition, the effect of the presence of phenol red in alpha MEM on the urokinase activity assay was determined by comparing it with alpha MEM without phenol red. From figure 10, the urokinase levels for alpha MEM with phenol red only very slightly differed from those for alpha MEM without

phenol red. Thus, the affect of phenol red on the plasminogen activity assay was minimal.

The results of the urokinase activity assay for conditioned media supplemented with anti-u-PA receptor 399R can be seen in figure 12. Conditioned media supplemented with anti-u-PA receptor 399R showed less urokinase activity than 2.5 iu of urokinase standard, but more activity than 0 iu of urokinase standard.

In addition, there was no consistent relationship to the amount of available urokinase activity and the concentration of the perturbing agent (Figure 12). Conditioned media supplemented with 5 *ug* of anti-u-PA receptor 399R demonstrated higher urokinase activity than conditioned media supplemented with either 10 *ug* or 20 *ug* of anti-u-PA receptor 399R. Levels of urokinase activity were similar for conditioned media supplemented with either 10 *ug* or 20 *ug* of anti-u-PA receptor 399R too.

From Figure 13, conditioned media supplemented with anti-u-PA receptor 3936 showed less urokinase activity than 2.5 iu of urokinase standards, but more urokinase activity than 0 iu of urokinase standards. Also conditioned media containing 25 *ug* of anti-u-PA receptor 3936 showed higher urokinase activity than conditioned media containing 10 *ug* of anti-u-PA receptor 3936. This reflected an inverse relationship between the amount of urokinase activity and the presence of anti-u-PA receptor 3936.

All conditioned media supplemented with PAI-1 showed less urokinase activity than 2.5 iu of urokinase standards (Figure 10). Conditioned media supplemented with 5% FBS and either 10 *ug* or 20 *ug* of PAI-1 showed similar levels of urokinase activity as the urokinase standard with 0 iu. Conditioned media supplemented with 10% FBS and 20 *ug* of PAI-1, however, showed more urokinase activity than 0 iu of urokinase standard.

From figures 15 and 16, the addition of various amounts of the u-PA antibody to the controls with 1 and 2.5 iu of

urokinase standards appeared to decrease the amount of urokinase activity available. Both the 1 and 2.5 iu urokinase standards showed more urokinase activity than the other samples which had the u-PA antibody added to it.

When comparing the amount of urokinase activity with the concentration of anti-u-PA present, there existed a direct correlation (Figure 15). Thus, 1 iu of urokinase standards supplemented with 10 *ug* of anti-u-PA showed higher urokinase activity than 1 iu of urokinase standards supplemented with either 2.5 *ug* or 5 *ug* of anti-u-PA. This relationship, however, did not exist for the 2.5 iu of urokinase standards supplemented with anti-u-PA (Figure 16). In this situation, there existed no consistent pattern between amount of urokinase activity and the concentration of the anti-u-PA present.

DISCUSSION

By disrupting the dentogingival complex, periodontal disease results in the detachment and apical migration of junctional epithelium (Armitage, 1980). The process of epithelial migration has been shown to be partially dependent on the presence and activity of u-PA (Pepper *et al.*, 1987). Several investigators have concluded that in order to be enzymatically active, pro-u-PA must first bind to its receptor (Berkenpas and Quigley, 1991). Thus, an in vitro tissue culture model was used to determine the importance of the u-PA receptor in epithelial migration.

The tissue culture model used in this experiment was similar to the one described by Salonen and Santti (1983). The tissue culture model I used provided a suitable environment for the maintenance and viability of epithelial cells for at least a ten day period. This was demonstrated by the healthy appearance and extensive migration of epithelial cells in tissue samples at ten days. The perturbing agents used in these experiments also did not have a negative affect on cell viability. This was again seen by

the viability of epithelial cells at five and ten days. Therefore, epithelial cells were able to survive in this tissue culture model for periods up to ten days.

The proposed hypothesis was that the u-PA receptor was necessary for the activation of single chain u-PA and its subsequent enhancement of epithelial migration. A second hypothesis was that PAI-1 will retard epithelial migration by interacting with u-PA and inhibiting its action. In a ideal situation, the amount of activated u-PA present in the conditioned media would decrease with the addition of PAI-1 or anti-u-PA receptor in a direct proportional manner. This would then be reflected by the inhibition or lack of epithlial migration. The tissue culture experiments, however, did not support the ability of PAI-1 or antibodies to the u-PA receptor to significantly inhibit epithelial migration. This was further demonstrated by the inconsistent levels of u-PA present in the conditioned media as observed in both the casein-plasminogen substrate gel and the plasminogen activity assay.

No consistent pattern to the migration results was present when comparing the amount of epithelial migration that occurred to the presence of the different perturbing agents. The concentration of the perturbing agents further failed to show any relationship to the inhibition of epithelial migration. Thus, low concentrations of perturbing agent showed a similar inability to inhibit migration as high concentrations of perturbing agents. The inability of the perturbing agents to inhibit epithelial migration was also seen when the migration results of serum controls were compared to those of the different perturbing agents. In general, epithelial migration occurred equally well in tissue samples grown in serum controls or perturbing agents.

A statistical difference for migratory results was found between serum controls and tissue samples grown in either 25 *ug* of PAI-1 or 10 *ug* of anti-u-PA 3936. This may be a result of the small number of tissue samples present. A larger

number of tissue samples may have shown that no statistical difference existed between serum controls and these tissue samples with either 25 *ug* PAI-1 or 10 *ug* of anti-u-PA 3936. Also dilution of the perturbing agents may have been incorrectly done and thus could account for this statistical difference too.

Several factors could account for this lack of epithelial migration inhibition. First is that the model itself allows for the introduction of several addditional or extraneous variables. The presence of activated inflammatory cells, like macrophages, PMNs, or lymphocytes, in the tissue sample could be a source of either proteases involved in matrix degradation or other additional factors, such as cytokines or growth factors. Growth factors and cytokines have been shown to regulate a cell's expression of u-PA and u-PA receptor. Thus additional factors, like transforming growth factor-alpha or basic-fibroblast growth factor, may increase the expression or activation of u-PA by epithelial cells if present.

Another potential area for the introduction of outside variables or error is in the preparation of the tissue samples. The preparation of the tissue samples for culture is a tedious process and subject to operator error. For instance, each tissue sample may have been cut differently and thus differ in size or in the number of epithelial cells initially present. The handling of the tissue sample during culture preparation may further have traumatized or destoyed a significant portion of the epithelial cells.

Furthermore, the placement of the tissue sample on the membrane of the Falcon insert is another source for operator error. The tissue sample may not have been placed with the epithlial surface on top, or if it was initially placed correctly, the tissue sample may later have fallen over on its side during the addition of the media or the handling of the twenty-four well plate. Thus, the lack of continuous

contact of the tissue sample with the membrane may have introduced further complications into the model.

An additional problem with the model may involve the histological processing of the tissue samples. When cutting away the membrane from the insert, the tissue sample sometimes disengaged from the membrane and so was lost. The tissue sample may have further separated from the membrane during either the paraffin embedding process or the histological sectioning. This was a possibility, because on inspection of several slides under the light microscope, the membrane was not present. Also occasionally the membrane was present in the histological section but separated from the tissue. Thus, I was unable to use the tissue sample because the membrane's original location to the tissue sample could not be determined.

Furthermore, the inability to retard epithelial migration may be explained by the perturbing agents themselves. Antibodies to the u-PA receptor should prevent the binding of pro-u-PA and its subsequent activation. The perturbing agents, however, may have been defective and so unable to function or work properly. The perturbing agents may also not be effective or specific enough to fully inhibit pro-u-PA from binding to its receptor. In other words, the epitope that the antibody recognizes on the u-PA receptor may not be the part that is necessary to prevent pro-u-PA from binding to and becoming activated. Thus, complications with the perturbing agents themselves may result in the inability to inhibit epithelial migration. This also could possibly explain why epithelium migrated equally well in either a high or low concentration of perturbing agents.

Epithelium appeared to migrate more on the connective tissue surface than on either the membrane surface alone or in between the connective tissue surface and the membrane. Epithelial migration depends not only on u-PA, but also on cell attachments, such as integrins, glycoproteins, or proteoglycans. The extracellular matrix of connective tissue

has been shown to contain high quantities of these molecules as well as act as a reservoir for many different growth factors. Taken together, these may have enhanced the amount of epithelial migration seen along the connective tissue surface in comparison to the other two surfaces.

An additional reason why epithelium was observed to migrate more on the connective tissue surface versus the other two surfaces is that this was the first or initial surface that it made contact with. In other words, epithelium had to first migrate on the connective tissue surface before it reached the membrane. After reaching the membrane, it could then migrate on either the membrane surface or in between the connective tissue surface and membrane. This also implied that the epithelium had a longer time to migrate on the connective tissue surface than on either of the other two surfaces.

In order to determine what types of enzymes were present in the conditioned media and potentially involved in the process of epithelial migration, a gelatin, casein, and casein-plasminogen zymogram were performed. In general, the three different zymograms showed bands at similar molecular weights. Thus, all zymograms showed three to four bands present at between 105 to 71 kDa and two to three bands at 44 kDa. This suggests that not only were the enzymes present in all three zymograms the same based on similar molecular weights, but that they were active on all substrates as well.

Furthermore, in all zymograms it was observed that a displacement or distortion of bands occurred at between 71 kD to 44 kD. It appeared that the distortion was due to the presence in FBS of albumin, which has a molecular weight of around 66,000 (Hirayama *et al.*, 1990). Consequently, the migration of albumin during electrophoresis could have displaced the protein bands slightly above and below it in all three zymograms, and so cause the distortion observed.

The degradation of the substrate in the zymograms, with the resultant production of clear bands, are the result of

enzymes present in the conditioned media. These enzymes may be members of the matrix metalloproteinase family which include collagenase, gelatinase, and stromelysins. These enzymes are important in the breakdown of extracellular matrix and may affect the process of epithelial migration. Taking into account the affect of albumin, the bands seen at between 105 kD to 71 kD may be the result of 72 kD and 92 kD gelatinases. The clear bands detected at 44 kD may also be due to either collagenase or stromelysin which both have a molecular weight of about 54 kD.

Matrix metalloproteinases can degrade both gelatin and casein. Using zymograms, both gelatinolytic and caseinolytic activities have been identified in human prostatic secretions (Wilson *et al.*, 1993). These protease activities were the result of metalloproteinases.

In comparison to the casein zymogram, the casein-plasminogen zymogram demonstrated a band at 44 kD that was wider and more intense. The increased width and intensity of the band can be explained by the presence of an additional enzyme that was not detected by the casein zymogram. This enzyme may be u-PA which has a molecular weight of around 52 kD and would only be detected on a casein-plasminogen zymogram and not a casein zymogram. The presence of albumin would also displace this enzyme downward, thus causing it to merge with other enzymes around 44 kD and so account for the wider and more intense band.

In order to confirm the presence of u-PA in the conditioned media, an immunoblot was performed. The immunoblot was probed for the presence of u-PA using an antibody to this enzyme. From the results of the immunoblot, u-PA was shown to be present in all samples of conditioned media. Single bands appeared between the molecular weight of 28 kD and 44 kD in the immunoblot. The downward displacement of the bands was again the result of the presence of albumin. Thus, the results from the immunoblot suggest that u-PA was

present in the conditioned media of all tissue samples, which further explains the lack of migration inhibition.

The intensity of the signal in an immunoblot reflects the amount of protein present. The more intense the signal, the higher the amount of protein present. From the immunoblot experiment, an inverse relationship existed between the intensity of the signal for u-PA and the dose of the perturbing agent present. Therefore, the higher the concentration of the perturbing agent present, the less the amount of u-PA present in the conditioned media. This indicated that the perturbing agents had a partial, although not complete, affect on altering the levels of u-PA.

The intensity of the immunoblot's signal additionally appeared to be dependent on the length of time in culture. Thus, conditioned media collected after ten days in culture demonstrated the most intense signal. This indicated that the amount of u-PA expressed by cells increased over time in culture.

A urokinase activity assay was developed to help determine the presence and levels of activated u-PA in the samples of conditioned media, and further relate this to the migration results. Using different amounts of urokinase, urokinase activity was observed to increase in direct proportion to the amount of urokinase added. Consequently, this assay was able to detect the presence of activated u-PA as well as to show that activity was proportional to concentration.

Because the urokinase activity assay requires the measuring of absorbance by a spectrophotometer, the presence of FBS and phenol red in the conditioned media may alter the results. In general, the results showed that neither FBS nor phenol red affected the urokinase activity assay. This implied that the presence of substances within the conditioned media did not significantly alter the results of the urokinase activity assay. The only exception was the observance of a slight increase in urokinase levels when

comparing the presence to the absence of FBS in controls with no urokinase standard added. This difference, however, was small and not of significance.

From the results of the urokinase activity assay, all samples of conditioned media contained activated u-PA. The amount or level of u-PA present in each sample, however, showed no consistent relationship with the concentration or type of perturbing agent that was added to it. All samples of conditioned media showed urokinase activity that was less than 2.5 iu of urokinase standards, but greater than 0 iu of urokinase standards. This lack of affect of perturbing agents on the amount of activated u-PA present partly explains the migration results obtained from the tissue samples. Thus, both of these results suggest that u-PA was present, and that the perturbing agents were ineffective in inhibiting epithelial migration.

Because the urokinase activity assay detects the amount of activated urokinase available, the affects of anti-u-PA on urokinase was investigated. It was observed that the addition of anti-u-PA decreased the amount of activated urokinase. This suggests that the anti-u-PA was able to interact with urokinase and inactivate or inhibit its activity.

The ability to inactivate the urokinase, however, did not appear to be related to the amount of anti-u-PA present. This may be a result of operator error in which the dilution of anti-u-PA was incorrectly done. By incorrectly making the wrong dilution, the amount or level of urokinase activity inhibition would subsequently be altered.

In conclusion, the results of this in vitro epithelial migration model do not support the hypothesis that PAI-1 or antibodies to the u-PA receptor are sufficient to either retard the levels or activation of u-PA, thus inhibiting epithelial migration. The inability of these perturbing agents to alter the level of activated u-PA was further demonstrated with the results from both the casein-

plasminogen substrate gel and the urokinase activity assay. In both of these experiments, the presence and amount of activated u-PA in all the various conditioned media was independent of the addition of inhibitors to the plasminogen activator system. These lack of consistencies, however, may be explained by the model itself as previously stated.

5% controls	TOT CT	TOT BET	TOT MEMB
A	0.08	0.8	0.19
B	0.56	0.06	0
C	0.61	0	0.34
D	0.38	0.35	0.28
E	0.61	0.62	0.4
F	0.28	0.45	0.43
TOTAL	.42 +/- .21	.38 +/- .31	.27 +/- .16
10% controls	TOT CT	TOT BET	TOT MEMB
A	0.67	0.2	0
B	1	0.28	0.06
C	0.74	0.25	0
D	0.39	0.12	0
E	0.53	0.3	0
F	0.79	0.22	0.05
G	0.73	0	0
H	0	0	0
I	0.52	0.19	0.08
TOTAL	.6 +/- .29	.17 +/- .11	.02 +/- .03

TABLE 1. EPITHELIAL MIGRATION MEASUREMENTS IN MILIMETERS FOR TISSUE SAMPLES CULTURED IN aMEM AND EITHER 5% OR 10% FBS. TOT CT = TOTAL CONNECTIVE TISSUE. TOT BET = TOTAL BETWEEN. TOT MEMB = TOTAL MEMBRANE.

MEDIA	SMPL #S	TOT CT	TOT BET	TOT MEMB
SERUM CTL	N = 15	.53 +/- .27	.26 +/- .23	.12 +/- .16
10 ug PAI-1	N = 5	.51 +/- .2	.12 +/- .14	.02 +/- .02
20 ug PAI-1	N = 3	.39 +/- .48	.16 +/- .23	.18 +/- .18
25 ug PAI-1	N = 4	1.1 +/- .51	.27 +/- .33	.3 +/- .26
10 ug 3936	N = 2	1.15 +/- .64	.79 +/- .16	0 +/- 0
20 ug 3936	N = 2	.56 +/- .06	.03 +/- .04	.09 +/- .12
25 ug 3936	N = 6	.6 +/- .35	.1 +/- .13	.08 +/- .1
5 ug 399R	N = 3	.62 +/- .13	.1 +/- .09	.16 +/- .28
10 ug 399R	N = 2	.12 +/- .17	0 +/- 0	0 +/- 0
25 ug 399R	N = 4	.67 +/- .29	.23 +/- .17	.21 +/- .19

TABLE 2. EPITHELIAL MIGRATION MEASUREMENTS IN MILIMETERS FOR TISSUE SAMPLES CULTURED IN THE PRESENCE OF VARIOUS CONCENTRATIONS OF PERTURBING AGENTS. TOT CT = TOTAL CONNECTIVE TISSUE. TOT BET = TOTAL BETWEEN. TOT MEMB = TOTAL MEMBRANE.

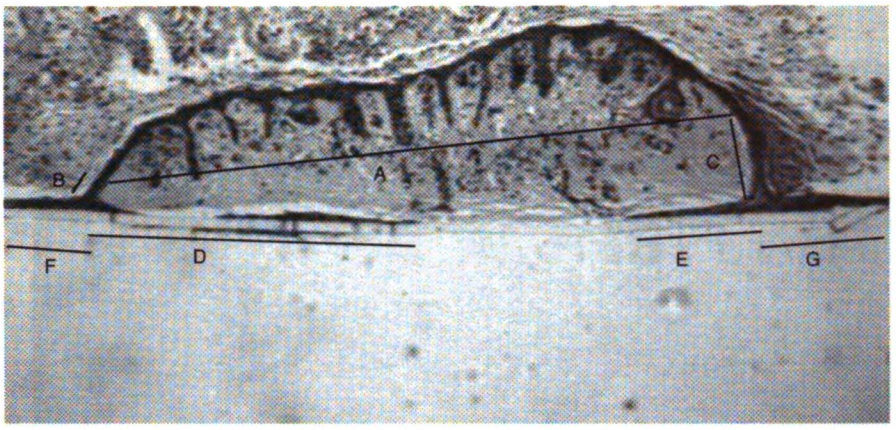


FIGURE 1. 5 DAY TISSUE SAMPLE IN aMEM & 5%FBS. LINE A REPRESENTS REFERENCE LINE ALONG BASAL CELL LAYER. LINES B & C REPRESENT EPITHELIAL MIGRATION ALONG CONNECTIVE TISSUE SURFACE. LINES D & E REPRESENT EPITHELIAL MIGRATION IN BETWEEN THE CONNECTIVE TISSUE AND MEMBRANE SURFACE. LINES F & G REPRESENT EPITHELIAL MIGRATION ALONG THE MEMBRANE SURFACE.

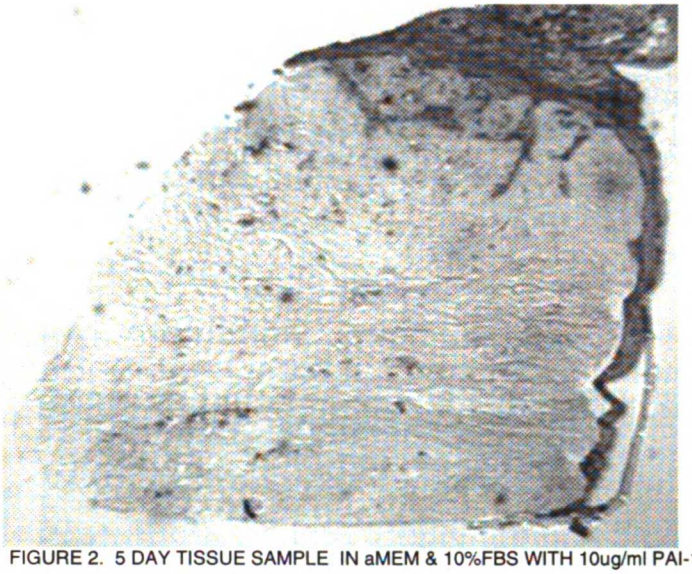


FIGURE 2. 5 DAY TISSUE SAMPLE IN aMEM & 10%FBS WITH 10ug/ml PAI-1.

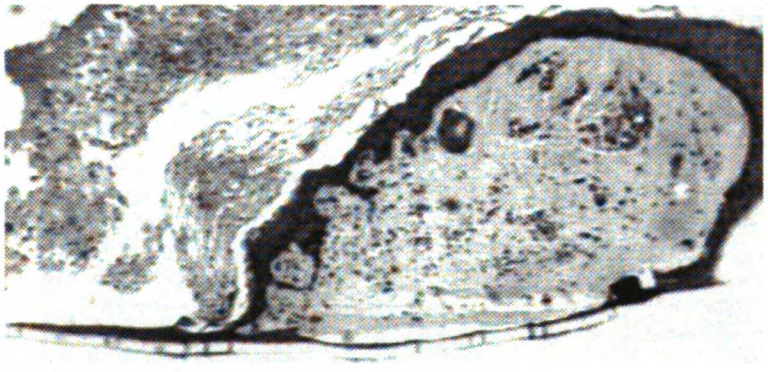


FIGURE 3. 5 DAY TISSUE SAMPLE IN aMEM & 5%FBS.

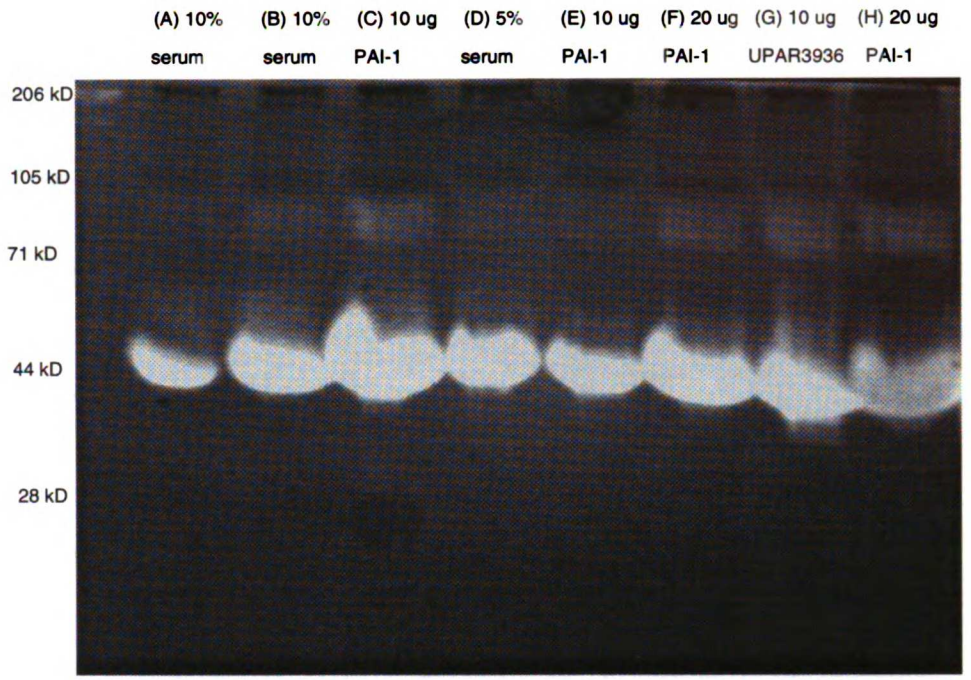


FIGURE 4. CASEIN-PLASMINOGEN ZYMOGRAM OF CONDITIONED MEDIA FROM TISSUE SAMPLES CULTURED FOR 5 DAYS. (C) & (H) CONTAINED 10%FBS. (E) - (G) CONTAINED 5% FBS.

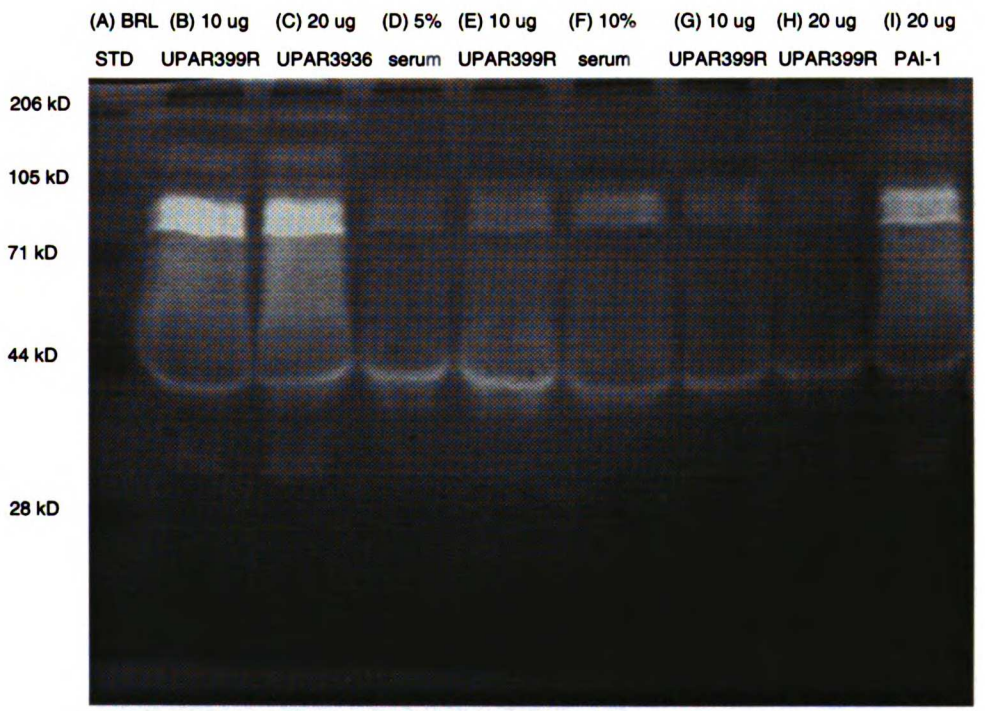


FIGURE 5. CASEIN-PLASMINOGEN ZYMOGRAM OF CONDITIONED MEDIA FROM TISSUE SAMPLES CULTURED FOR 5 DAYS. (B), (C), & (I) ALL CONTAINED 10% FBS. (E) - (H) CONTAINED 5% FBS.

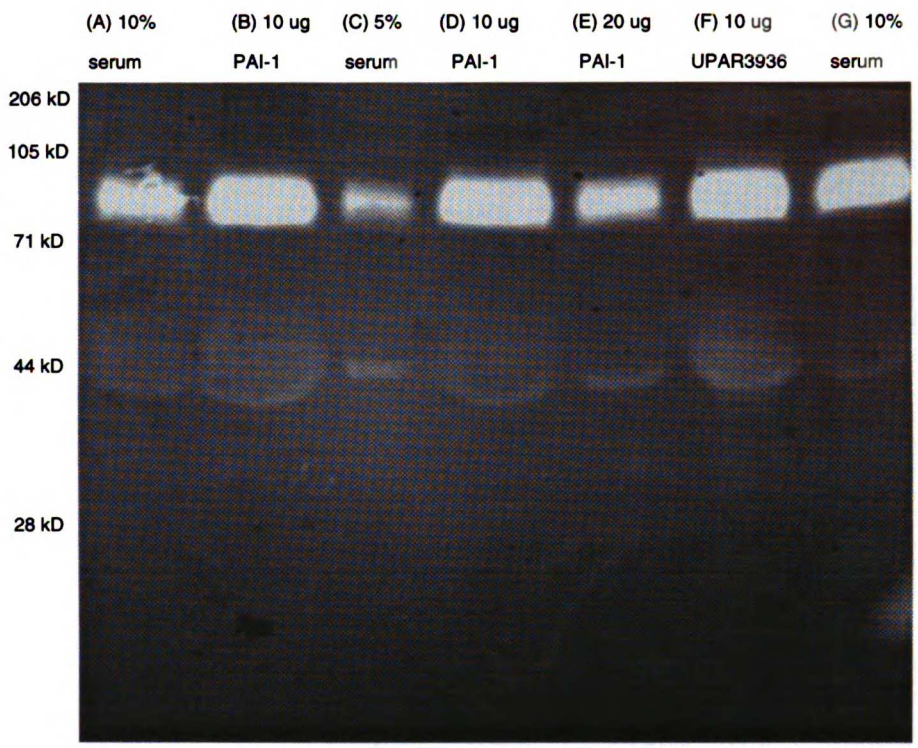


FIGURE 6. CASEIN ZYMOGRAM OF CONDITIONED MEDIA FROM TISSUE SAMPLES CULTURED FOR 5 DAYS. (B) CONTAINED 10% FBS. (D) - (F) CONTAINED 5% FBS.

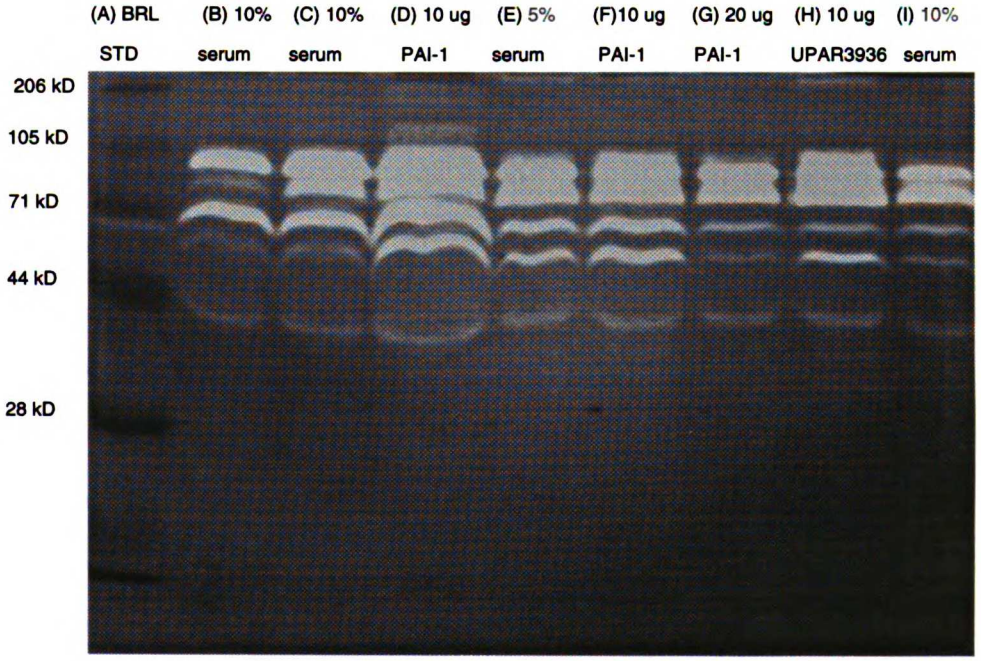


FIGURE 7. GELATIN ZYMOGRAM OF CONDITIONED MEDIA FROM TISSUE SAMPLES CULTURED FOR 5 DAYS. (D) CONTAINED 10% FBS. (F) - (H) CONTAINED 5% FBS.

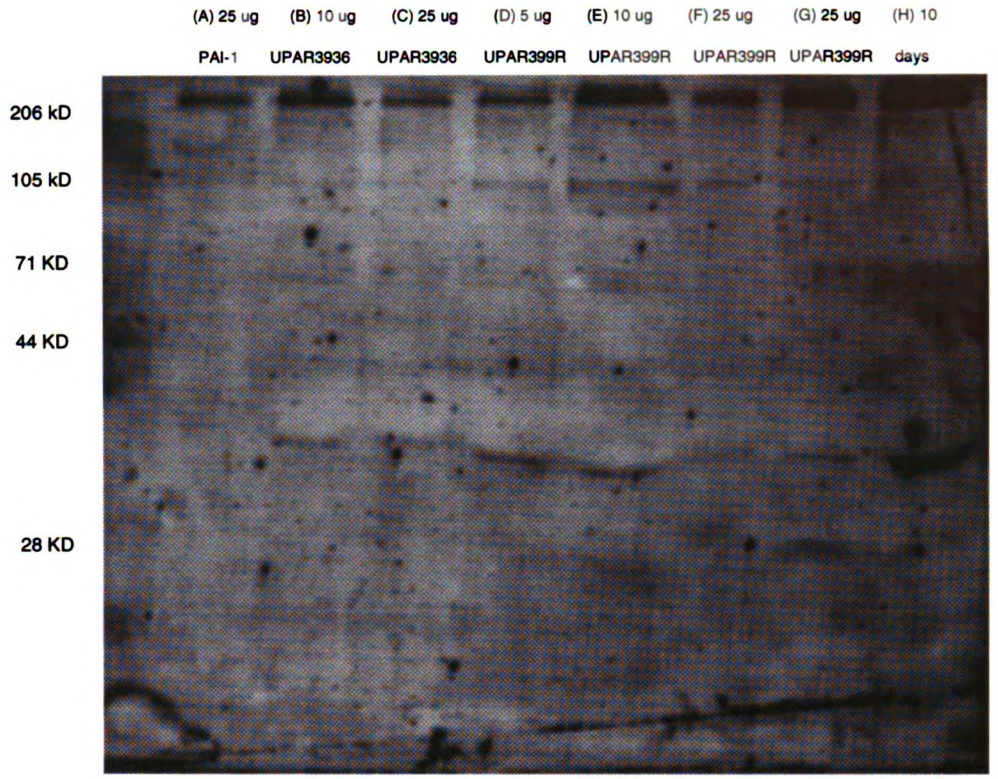


FIGURE 8. IMMUNOBLOT OF CONDIONED MEDIA FROM TISSUE SAMPLES CULTURED FOR EITHER 5 OR 10 DAYS.

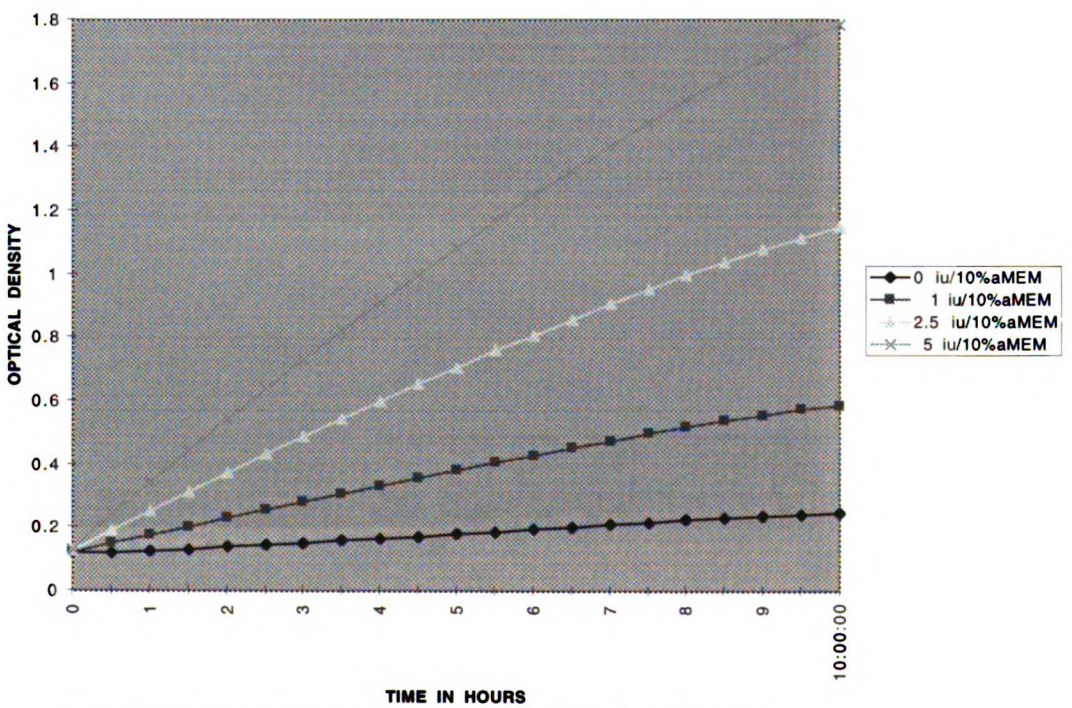


FIGURE 9. UROKINASE ACTIVITY OF 0 - 5 IU OF UROKINASE STANDARDS.

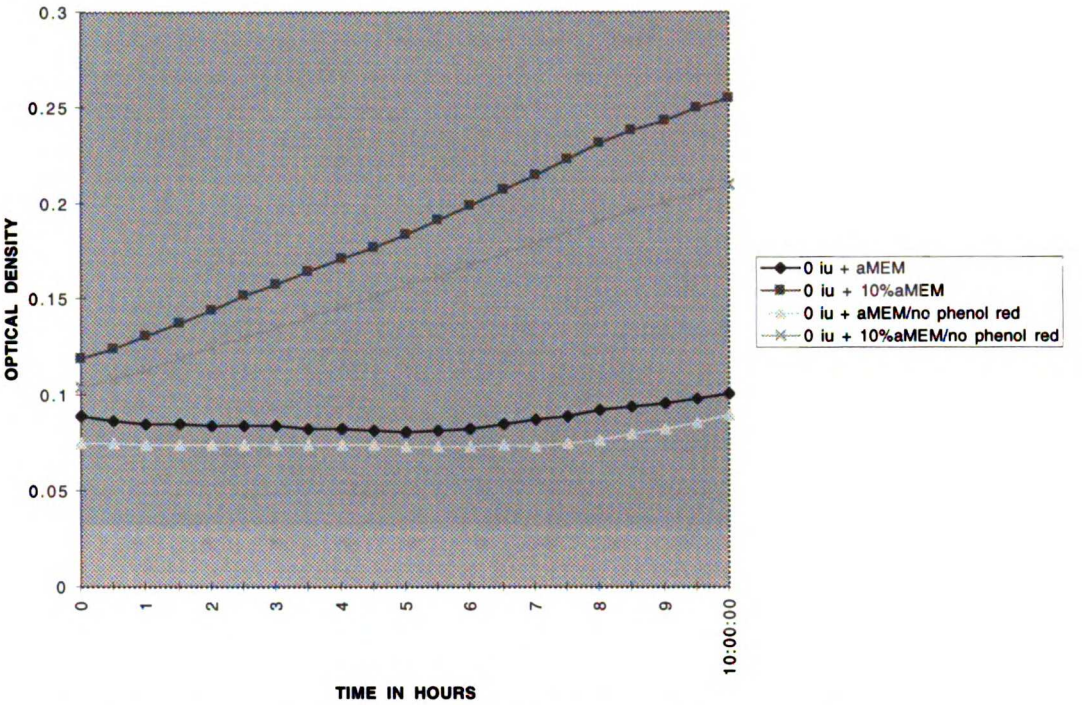


FIGURE 10. UROKINASE ACTIVITY OF CONDITIONED MEDIA IN THE PRESENCE OR ABSENCE OF FBS OR PHENOL RED.

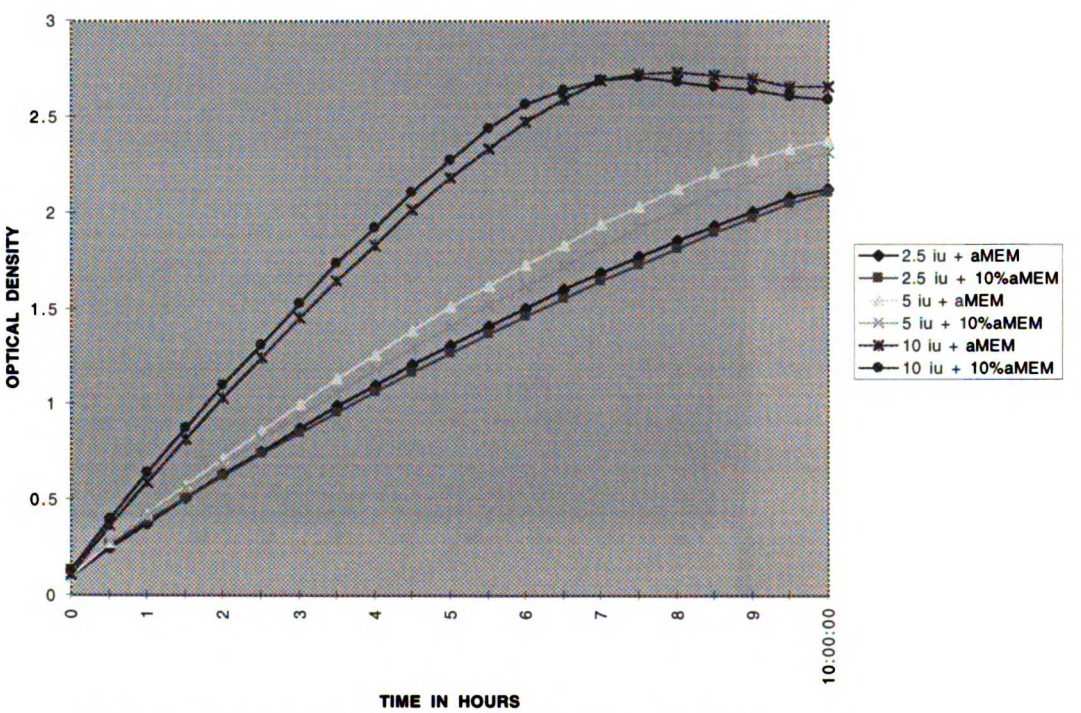


FIGURE 11. 2.5 - 10 IU OF UROKINASE STANDARDS IN THE PRESENCE OR ABSENCE OF FBS.

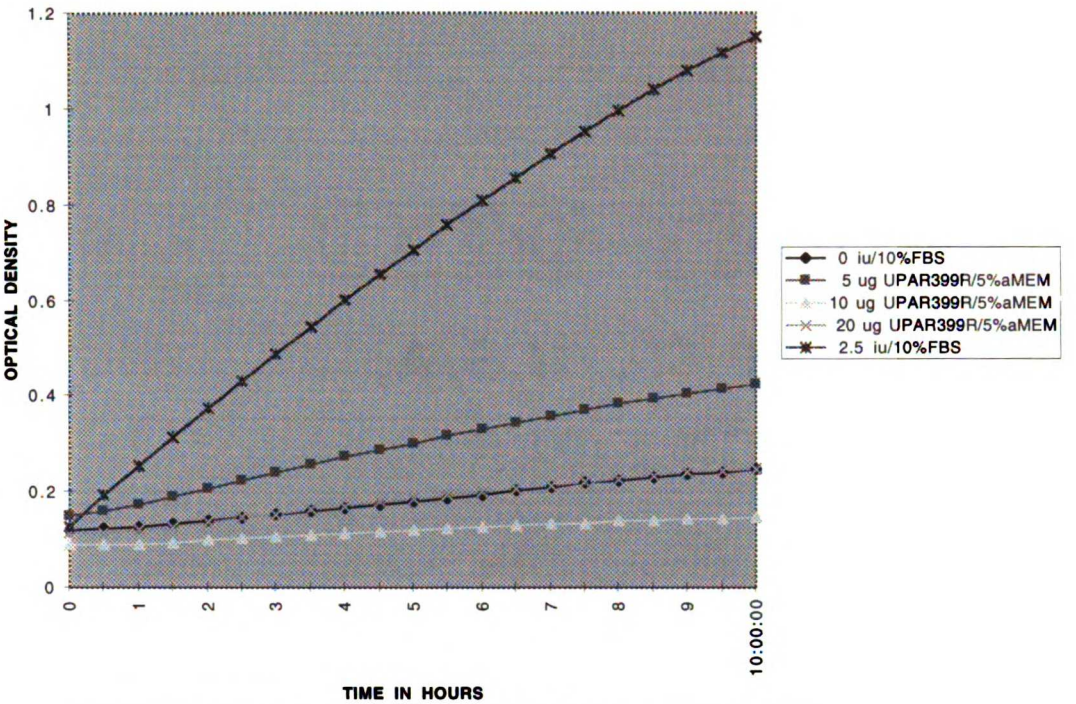


FIGURE 12. UROKINASE ACTIVITY OF CONDITIONED MEDIA WITH UPAR 399R.

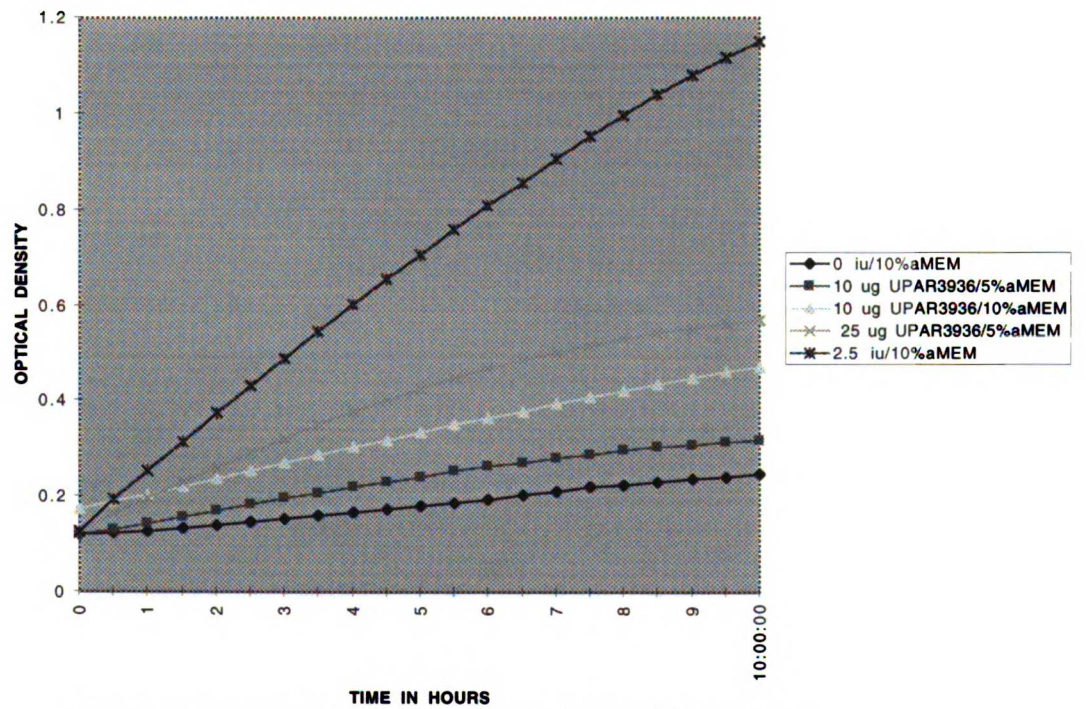


FIGURE 13. UROKINASE ACTIVITY OF CONDITIONED MEDIA WITH UPAR 3936.

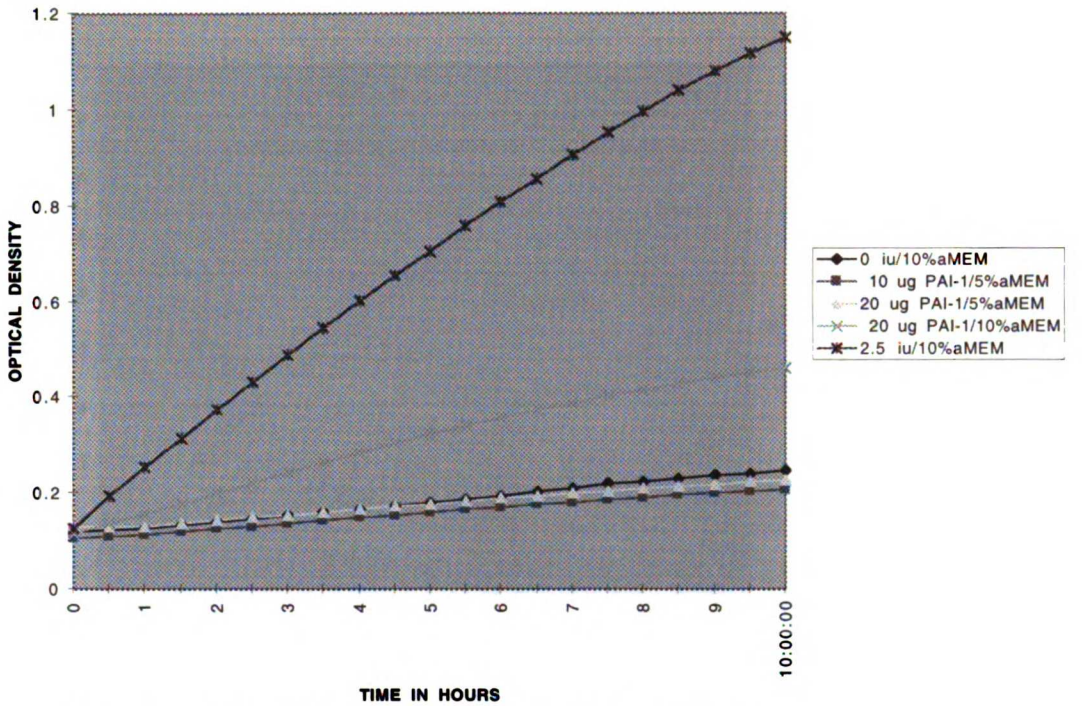


FIGURE 14. UROKINASE ACTIVITY OF CONDITIONED MEDIA WITH PAI-1.

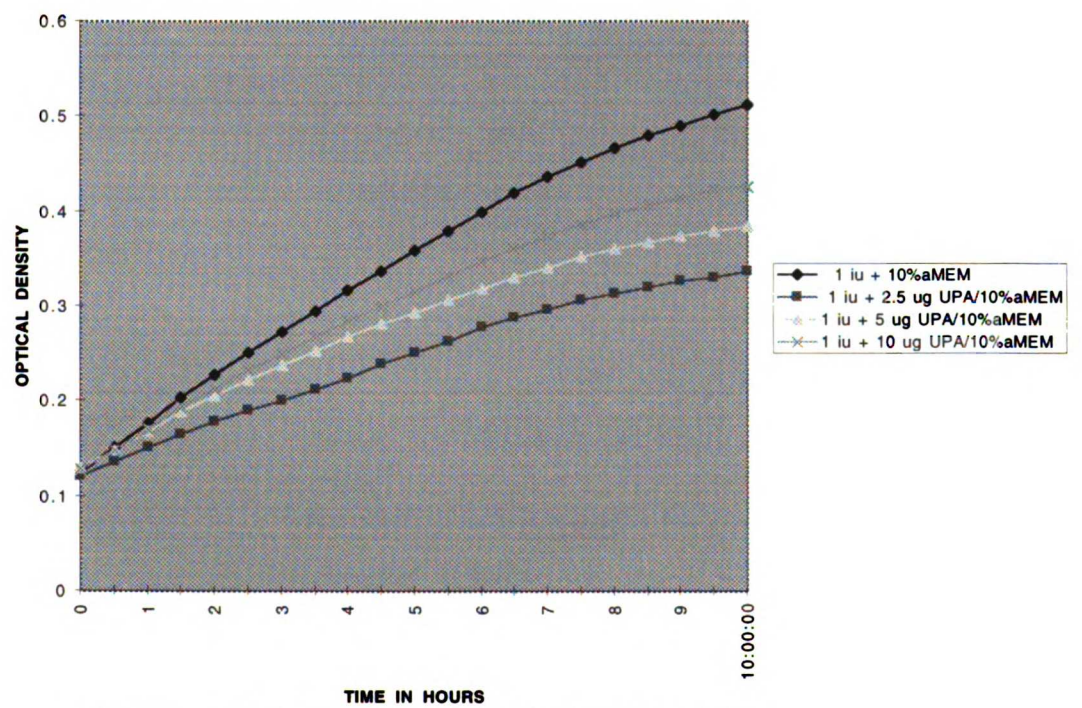


FIGURE 15. UROKINASE ACTIVITY OF CONDITIONED MEDIA WITH UPA.

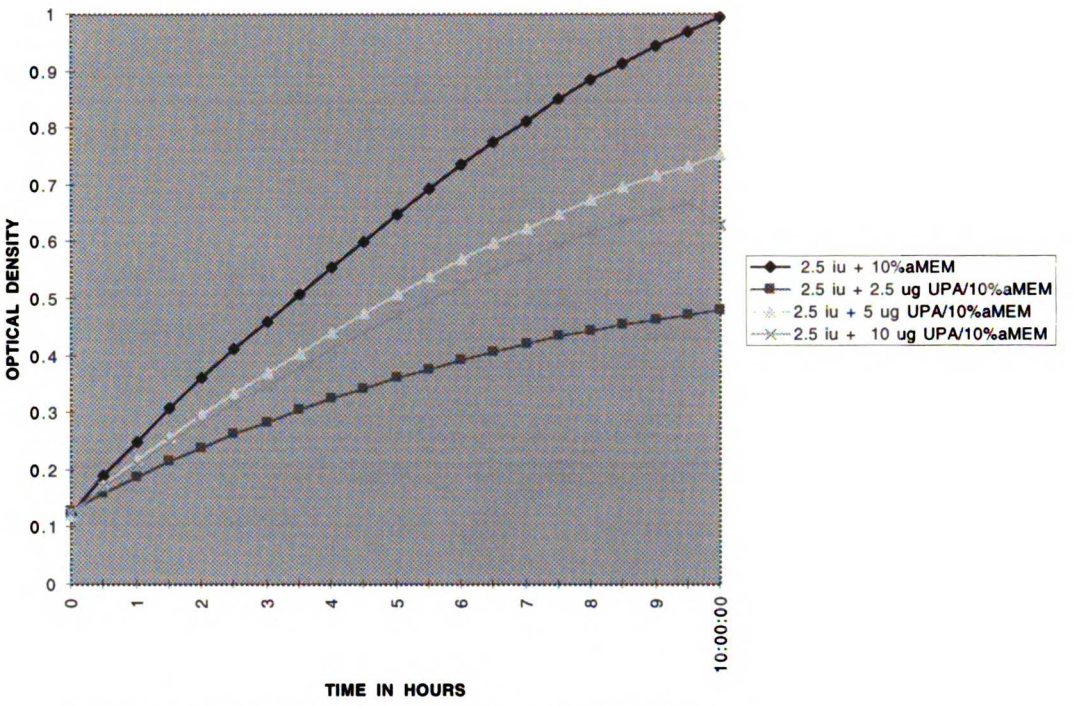


FIGURE 16. UROKINASE ACTIVITY OF CONDITIONED MEDIA WITH UPA.

BIBLIOGRAPHY

Alberts, B. *et al.*, Molecular biology of the cell. 2nd Ed., New York, Garland Publishing, Inc., 1989, p. 676, 745, 824 & 1201.

Andreasen, PA. *et al.* Receptor-mediated endocytosis of plasminogen activators and activator/inhibitor complexes. Febs Letters. 338(3): 239-245, 1994.

Anichini, E. *et al.* Production of second messengers following chemotactic and mitogenic urokinase-receptor interaction in human fibroblasts and mouse fibroblasts transfected with human urokinase receptor. Exp Cell Res. 213: 438-448, 1994.

Appella, E. *et al.* The receptor-binding sequence of urokinase. A biological function for the growth-factor module of proteases. J Bio Chem. 262: 4437, 1987.

Armitage, GC. Biological basis of periodontal maintenance therapy. Berkely, Praxis Publishing Company, 1980, p. 4 & 103.

Aukhil, I. *et al.* Periodontal wound healing in the absence of periodontal ligament cells. J Perio 58(2): 71-77, 1987.

Bastholm, L. *et al.* Confocal fluorescence microscopy of urokinase plasminogen activator receptor and cathepsin D in human MDA-MB-231 breast cancer cells migrating in reconstituted basement membrane. Biotech and Histochem. 69(2): 61-67, 1994.

Bastholm, L. *et al.* Immunoelectron microscopy of the receptor for urokinase plasminogen activator and cathepsin D in the human breast cancer cell line MDA-MB-231. Apmis 102(4): 279-286, 1994.

Becquemont, L. *et al.* Expression of plaminogen/plasmin receptors on human glomerular epithelial cells. Am J Physiol. 267: F303-F310, 1994.

Behrendt, N. *et al.* The human receptor for urokinase plasminogen activator. Amino terminal amino acid sequence and glycosylation variants. J Bio Chem. 265(11): 6453-6460, 1990.

Behrendt, N. *et al.* The ligand-binding domain of the cell surface receptor for urokinase-type plasminogen activator. J Bio Chem. 266(12): 7842-7847, 1991.

Berkenpas, MB. and Quigley, JP. Transformation-dependent activation of urokinase-type plasminogen activator by a plasmin-independent mechanism: involvement of cell surface membranes. Proc Natl Acad Sci. 88:7768-7772, 1991.

Bianchi, E. *et al.* The urokinase receptor is expressed in invasive breast cancer but not in normal breast tissue. Cancer Research. 54: 861-866, 1994.

Blasi, F. *et al.* The receptor for urokinase-plasminogen activator. J Cell Biom. 32: 179-186, 1986.

Blasi, F. Urokinase and urokinase receptor: a paracrine/autocrine system regulating cell migration and invasiveness. BioEssays. 15(2): 105-111, 1993.

Blasi, F. *et al.* Urokinase-type plasminogen activator: proenzyme, receptor, and inhibitors. J Cell Bio. 104: 801-804, 1987.

Bu, G. *et al.* Cellular receptors for the plasminogen activators. Blood. 83(12): 3427-3436, 1994.

Busso, N. *et al.* Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J Cell Biol. 126: 259-270, 1994.

Caffesse, RG. and Quinones, CR. Polypeptide growth factors and attachment proteins in periodontal wound healing and regeneration. Perio 2000. 1: 69-79, 1993.

Camani, C. *et al.* The role of plasminogen activator inghibitor type 1 in the clearance of tissue-type plasminogen activator by rat hepatoma cells. J Bio Chem. 269(8): 5770-5775, 1994.

Campbell, P. *et al.* Binding and activation of plasminogen on the surface of osteosarcoma cells. J Cell Physio. 159: 1-10, 1994.

Casey, JR. *et al.* The structure of the urokinase-type plasminogen activator receptor gene. Blood 84(4): 1151-1156, 1994.

Caton, J. and Zander, H. The attachment between tooth and gingival tissues after periodic root planing and soft tissue curettage. J. Perio. 50(9): 462-466, 1979.

Caton, J. *et al.* Histometric evaluation of periodontal surgery. II. Connective tissue attachment levels after four regenerative procedures. J Clin Perio 7: 224-231, 1980.

Cesarman, GM. *et al.* An endothelial cell receptor for plasminogen/tissue plasminogen activator (t-PA). II. Annexin II-mediated enhancement of t-PA dependent plasminogen activation. J Bio Chem. 269(33): 21198-21203, 1994.

Conese, M. *et al.* Protease nexin-1-urokinase complexes are internalized and degraded through a mechanism that requires both urokinase receptor and alpha-two-macroglobulin receptor. 269(27): 17886-17892, 1994.

Cubellis, MV. *et al.* Accesibility of receptor-bound urokinase to type-1 plasminogen activator inhibitor. Proc Natl Acad Sci USA 86: 4828, 1989.

Cubellis, MV. *et al.* Binding of single-chain prourokinase to the urokinase receptor of human U937 cells. J Bio Chem. 261(34): 15819-15822, 1986.

Cubellis, MV. *et al.* Receptor-mediated internalization and degradation of urokinase is caused by its specific inhibitor PAI-1. EMBO J. 9: 1079, 1990.

Dano, K. *et al.* Plasminogen activators, tissue degradation, and cancer. Adv Cancer Res 44: 136-266, 1985.

Del Rosso, M. *et al.* Interaction of urokinase with specific receptors abolishes the time of commitment to terminal differentiation of murine erythroleukaemia (Friend) cells. Brit J Haemat. 66: 289-294, 1987.

Del Rosso, M. *et al.* Role of specific membane receptors in urokinase dependent migration of human keratinocytes. J Invest Derm. 94: 310-316, 1990.

Delbaldo, C. *et al.* Plasminogen activation in melanocytic neoplasia. Cancer Res. 54: 4547-4552, 1994.

de Vries, TJ. *et al.* Plasminogen activators, their inhibitors, and urokinase receptor emerge in late stages of melanocytic tumor progression. Am J Path. 144(1): 70-81, 1994.

Duval-Jobe, C. and Parmely, MJ. Regulation of plasminogen activation by human U937 promonocytic cells. J Bio Chem. 269(33): 21353-21357, 1994.

Ellis, V. *et al.* Inhibition of receptor-bound urokinase by plasminogen-activator inhibitors. J Bio Chem. 265: 9904, 1990.

Ellis, V. *et al.* Plasminogen activation initiated by single-chain urokinase-type plasminogen activator. J Bio Chem. 264(4): 2185-2188, 1989.

Estreicher, A. *et al.* Characterization of the cellular binding site for the urokinase-type plasminogen activator. J Bio Chem 264: 1180, 1989.

Estreicher, A. *et al.* The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J Cell Biol. 111: 783, 1990.

Fibbi, G. *et al.* Interaction of urokinase A chain with the receptro of human keratinocytes stimulates relase of urokinase-like plasminogen activator. Exp Cell Res. 187: 33-38, 1990.

Fibbi, G. *et al.* Interaction of urokinase with specific receptors stimulates mobilization of bovine adrenal capillary endothelial cells. Exp Cell Res. 179: 385-395, 1988.

Fibbi, G. *et al.* The Mr 17500 region of the A chain of urokinase is required for interaction with a specific receptor in A431 cells. Biochim Biophys Acta. 885: 301-308, 1986.

Gibson, P. *et al.* Secretion of urokinase and plasminogen activator inhibitor-1 by normal colonic epithelium in vitro. Gut 35: 969-975, 1994.

Gottlow, J. *et al.* New attachment formation as the result of controlled tissue regeneration. J Clin Perio 11: 494-503, 1984.

Grondahl-Hansen, J. *et al.* Urokinase- and tissue-type plasminogen activators in keratinocytes during wound reepithelialization in vivo. J Invest Derm. 90: 790-795, 1988.

Gunzler, WA. *et al.* The primary structure of high molecular mass urokinase from human urine. The complete amino acid sequence of the A chain. Hoppe-Seyler's Z. Physiol Chem. 363: 1155-1165, 1982.

Gyetko, MR. *et al.* The urokinase receptor is required for human monocyte chemotaxis in vitro. J Clin Invest. 93: 1380-1387, 1994.

Hajjar, KA. *et al.* An endothelial cell receptor for plasminogen/tissue plasminogen activator. I. Identity with annexin II. J Bio Chem. 269(33): 21191-21197, 1994.

Harvey, SR. *et al.* Secretion of plasminogen activators by human colorectal and gastric tumor explants. Clin Expl Metastasis 6(6): 431-450, 1988.

He, C. *et al.* Growth factor-like effect of urokinase type plasminogen activator in human renal cells. Biochem and Biophys Res Comm. 176(3): 1408-1416, 1991.

Hearing, VJ. *et al.* Modulatin of metastatic potential by cell surface urokinase of murine melanoma cells. Cancer Research. 48: 1270-1278, 1988.

Heegaard, CW. *et al.* The plasminogen activation system in bovine milk: differential localization of tissue-type plasminogen activator and urokinase in milk fractions is cause by binding to casein and urokinase receptor. Biochim et Biophys Acta 1222: 45-55, 1994.

Herz, J. *et al.* LDL receptor-related protein internalizes and dgrades u-PA:PAI-1 complexes and is essential for embryo implantation. Cell 71: 411, 1992.

Hirayama, K. *et al.* Rapid confirmation and revision of the primary structure of bovine serum albumin by ESIMS and Frit-FAB LC/MS. Biochem and Biophysi Res Commun. 173(2): 639-646, 1990.

Isseroff, RR. *et al.* Plasminogen activator in differentiating mouse keratinocytes. J Invest Derm. 80: 217-222, 1983.

Jensen, PJ. and Rodeck, U. Autocrine/paracrine regulation of keratinocyte urokinase plasminogen activator through the TGF-alpha/EGF receptor. J Cell Physio 155: 333-339, 1993.

Jensen, PJ. and Wheelock, MJ. Regulation of urokinase plasminogen activator localization in keratinocytes by calcium ion and E-cadherin. Exp Cell Res. 202: 190-198, 1992.

Jensen, PJ. *et al.* Urokinase and tissue type plasminogen activators in human keratinocyte culture. Exp Cell Res. 187: 162-169, 1990.

Karring, T. *et al.* New attachment formation on teeth with a reduced but healthy periodontal ligament. J Clin Perio 12: 51-60, 1985.

Kook, YH. *et al.* The effect of antisense inhibition of urokinase receptor in human squamous cell carcinoma on malignancy. EMBO. 13(17): 3983-3991, 1994.

Laiho, M. *et al.* Transforming growth factor-beta induction of type-1 plasminogen activator inhibitor. J Bio Chem. 262(36): 17467-17474, 1987.

Lee, LS. and Weinstein, IB. Epidermal growth factor, like phorbol esters, induces plasminogen activator in HeLa cells. Nature. 274: 696-697, 1978.

Lee, SW. *et al.* Characterization of plasminogen activation by glycosylphosphatidylinositol-anchored urokinase. J Bio Chem. 269(4): 2411-2418, 1994.

Li, H. *et al.* Endocytosis of urokinase-plasminogen activator inhibitor type 1 complexes bound to a chimeric transmembrane urokinase receptor. J Bio Chem. 269(11): 8153-8158, 1994.

Listgarten, MA. Electron microscopic features of the newly formed epithelial attachment after gingival surgery. J Perio Res 2: 46-52, 1967.

Listgarten, MA. and Rosenberg, MM. Histological study of repair following new attachment procedures in human periodontal lesions. J Perio 50(7): 333-344, 1979.

Low, MC. The glycoyl-phosphatidylinositol anchor of membrane proteins. Biochim Biophys Acta 988: 427, 1989.

Lucas, ON. *et al.* Plasminogen activator in the normal gingiva of the dog. J Perio Res. 10: 203-210, 1975.

McNeill, H. and Jensen, PJ. A high-affinity receptor for urokinase plasminogen activator on human keratinocytes: characteriaztion and potential modulaion during migration. Cell Regul. 1:843-852, 1990.

Melcher, AH. On the repair potential of periodontal tissues. J Perio 47(5): 256-260, 1976.

Mizukami, IF. *et al.* Immunologic detection of the cellular receptor for urokinase plasminogen activator. Clin Immun and Immunopath. 71(1): 96-104, 1994.

Morioka, S. *et al.* Involvement of urokinase-type plasminogen activator in acantholysis induced by pemphigus IgG. J Invest Derm. 89: 474-477, 1987.

Morioka, S. *et al.* Migrating keratinocytes express urokinase-type plasminogen activator. J Invest Derm. 88: 418-423, 1987.

Nakao, H. *et al.* A new function of calphobindin I (annexin V). Promotion of both migration and urokinase-type plasminogen activator activity in normal human keratinocytes. Eur J Biochem 223: 901-908, 1994.

Nielsen, LS. *et al.* A 55,000-60,000 Mr receptor protein for urokinase-type plasminogen activator. J Bio Chem. 263: 2358, 1988.

Nielsen, LS. *et al.* Purification of zymogen to plasminogen activator from human glioblastoma cells by affinity chromatography with monoclonal antibody. Biochem. 21: 6410-6415, 1982.

Nowak, UK. *et al.* Unfolding studies of the protease domain of urokinase-type plasminogen activator: the existence of partly folded states and stable subdomains. Biochem. 33: 2951-2960, 1994.

Nykjaer, A. *et al.* Purified alpha-2-macroglobulin receptor/LDL receptor related protein binds urokinase:plasminogen activator inhibitor type-1 complex. J Biol Chem 267: 14543, 1992.

Nykjaer, A. *et al.* Urokinase receptor. An activation antigen in human T lymphocytes. J Immuno. 152: 505, 1994.

Odekon, LE. *et al.* Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta. J Cell Physio. 158: 398-407, 1994.

Olson, D. *et al.* Internalization of the urokinase-plasminogen activator inhibitor type-1 complex is mediated by the urokinase receptor. J Bio Chem. 267: 9129, 1992.

Ossowski, L. and Reich, E. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell 35: 611-619, 1983.

Patthy, L. Evolution of the proteases of blood coagulation and fibrinolysis by assembly from modules. Cell 41: 657-663, 1985.

Pepper, MS. *et al.* Urokinase-type plasminogen activator is induced in migrating capillary endothelial cells. J Cell Biol 105(6): 2535-2541, 1987.

Petersen, LC. *et al.* One-chain urokinase-type plasminogen activator from human sarcoma cells is a proenzyme with little or no intrinsic activity. J Bio Chem. 263(23): 11189-11195, 1988.

Petruzzelli, G. *et al.* In vitro urokinase type plasminogen activator levels and total plasminogen activator activity in squamous cell carcinomas of the head and neck. Arch Otolaryngol Head Neck Surg. 120: 989-992, 1994.

Plesner, T. *et al.* The receptor for urokinase-type plasminogen activator and urokinase is translocated from two distinct intracellular compartments to the plasma membrane on stimulation of human neutrophils. Blood 83(3): 808-815, 1994.

Ploug, M. *et al.* Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. J Bio Chem. 266(3): 1926-1933, 1991.

Ploug, M. *et al.* Ligand interaction between urokinase-type plasminogen activator and its receptor probed with 8-anilino-1-naphthalensulfonate. Evidence for a hydrophobic binding site exposed only on the intact receptor. Biochem. 33: 8991-8997, 1994.

Ploug, M. and Ellis, V. Structure-function relationships in the receptor for urokinase-type plasminogen activator. Comparison to other members of the Ly-6 family and snake venom alpha-neurotoxin. Febs Letters 349(2): 163-168, 1994.

Plow, EF. *et al.* The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. J Cell Biol. 103(6): 2411-2420, 1986.

Pyke, C. *et al.* Immunohistochemical detection of the receptor for urokinase plasminogen activator in human colon cancer. Histopath. 24: 131-138, 1994.

Quax, PHA. *et al.* Complementation between urokinase-producing and receptor-producing cells in extracellular matrix degradation. Cell Regul. 2: 793-803, 1991.

Quigley, JP. *et al.* Limited cleavage of cellular fibronectin by plasminogen activator purified from transformed cells. Proc Natl Acad Sci. 84: 2776-2780, 1987.

Rabbani, SA. *et al.* Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. J Biol Chem. 267(20): 14151-14156, 1992.

Roldan, AL. *et al.* Cloning and expression of the receptor for human urokinase plasminogen activator, a central molecule in cell surface, plamin dependent proteolysis. EMBO 9(2): 467-474, 1990.

Romer, J. *et al.* Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. J Invest Derm. 97: 803-811, 1991.

Romer, J. *et al.* Expression of uPA and its receptor by both neoplastic and stromal cells during xenograft invasion. Int J Cancer 57: 553-560, 1994.

Ronne, E. *et al.* Cell-induced potentiation of the plasminogen activation system is abolished by a monoclonal antibody that recognizes the NH2-terminal domain of the urokinase receptor. FEBS 288(1): 233-236, 1991.

Saksela, O. *et al.* The opposing effects of basic fibroblast growth factor and transforming growth factor beta on the regulation of plasminogen activator activity in capillary endothelial cells. J Cell Biol. 105: 957-963, 1987.

Salonen, J. and Santti, R. An attempt to simulate junctional epithelium of human gingiva in vitro. J Perio Res. 18: 311-317, 1983.

Salonen, J. Basal cell characteristics in human oral epithelium adhered to the tooth in vivo and to an inert substrate in vitro. J Perio Res. 21: 22-29, 1986.

Salonen, J. Changes in cell phenotype during regeneration of junctional epithelium of human gingiva in vitro. J Perio Res. 24: 370-377, 1989.

Salonen, JI. and Persson, RG. Migration of epithelial cells on materials used in guided tissue regeneration. J Perio Res. 25: 215-221, 1990.

Salonen, JI. Proliferative potential of the attached cells of human junctional epithelium. J Perio Res. 29: 41-45, 1994.

Schafer, BM. *et al.* Plasminogen activation in healing human wounds. Am J Path. 144(6): 1269-1280, 1994.

Schlechte, W. *et al.* Invasion of extracellular matrix by cultured colon cancer cells: dependence on urokinase receptor display. Cancer Comm. 2(5): 173-179, 1990.

Schmid, B. *et al.* Plasminogen activator in human gingival tissue adjacent to dental implants. Clin Oral Impl Res. 3: 85-89, 1992.

Schmid, J. and Chambers, DA. Molecular characterization of plasminogen activator in human supragingival plaque. Archs Oral Biol. 34: 17-21, 1989.

Schmid, J. *et al.* Plasminogen activator in human periodontal health and disease. Archs oral Biol. 36(3): 245-250, 1991.

Schmid, J. *et al.* The presence of plasminogen activator (PA) in human supagingival plaque. J Dent Res. 66: 172, 1987.

Schwartz, BS. Differential inhibition of soluble and cell surface receptor-bound single-chain urokinase by plasminogen activator inhibitor type 2. J Bio Chem. 269(11): 8319-8323, 1994.

Sigurdardottir, O. and Wiman, B. Complex formation between plasminogen activator inhibitor 1 and vitronectin in purified systems and in plasma. Biochim et Biophys Acta 1035: 56-61, 1990.

Sitrin, RG. *et al.* Cytokine-specific regulation of urokinase receptor (CD87) expression by U937 mononuclear phagocytes. Blood 84(4): 1268-1275, 1994.

Skriver, L. *et al.* Immunocytochemical localization of urokinase-type plasminogen activator in Lewis lung carcinoma. J Cell Bio. 99: 753-757, 1984.

Stack, MS. and Johnson, DA. Human mast cell tryptase activates single-chain urinary-type plasminogen activator (pro-urokinase). J Bio Chem. 269(13): 9416-9419, 1994.

Stahl, A. and Mueller, BM. Binding of urokinase to its receptor promotes migration and invasion of human melanoma cells in vitro. Cancer Res. 54: 3066-3071, 1994.

Stahl, SS. Repair potential of the soft tissue-root interface. J Perio 48(9): 545-552, 1977.

Stephens, RW. *et al.* Activation of pro-urokinase and plasminogen on human sarcoma cells: a proteolytic system with surface-bound reactants. J Cell Biol. 108: 1987-1995, 1989.

Stoppelli, MP. *et al.* Autocrine saturation of pro-urokinase receptors on human A431 cells. Cell 45: 675-684, 1986.

Stoppelli, MP. *et al.* Differentiation-enhanced binding of the amino-terminal fragment of human urokinase plasminogen activator to a specific receptor on U937 monocytes. Proc Natl Acad Sci USA 82: 4939, 1985.

Vassalli, JD. *et al.* A cellular binding site for the M_r 55,000 form of the human plasminogen activator, urokinase. J Cell Biol. 100: 86-92, 1985.

Vassalli, JD. *et al.* The plasminogen activator/plasmin system. J Clin Invest. 88: 1067-1072, 1991.

Verrusio, E. *et al.* Plasminogen activator and nonspecific fibrinolytic activity in human saliva. J Dent Res. 66: 172, 1987.

Wagner, OF. *et al.* Interaction between plasminogen activator inhibitor type 1 (PAI-1) bound to fibrin and either tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator(u-PA). J Clin Invest. 84: 647-655, 1989.

Wang, H. *et al.* Transcriptional activation of the urokinase receptor gene in invasive colon cancer. Int J Cancer 58: 650-657, 1994.

Watanabe, K. *et al.* Molecular and antigenic characterization of plasminogen activators in human crevicular fluid. J Dent Res. 66: 172, 1987.

Wilhelm, O. *et al.* Recombinant soluble urokinase receptor as a acavenger for urokinase-type plasminogen activator (uPA). Inhibition of proliferation and invasion of human ovarian cancer cells. Febs Letters, 337(2): 131-134, 1994.

Wilson, MJ. *et al.* Gelatinolytic and caseinolytic proteinase activites in human prostatic secretions. J Urology 149(3): 653-658, 1993.

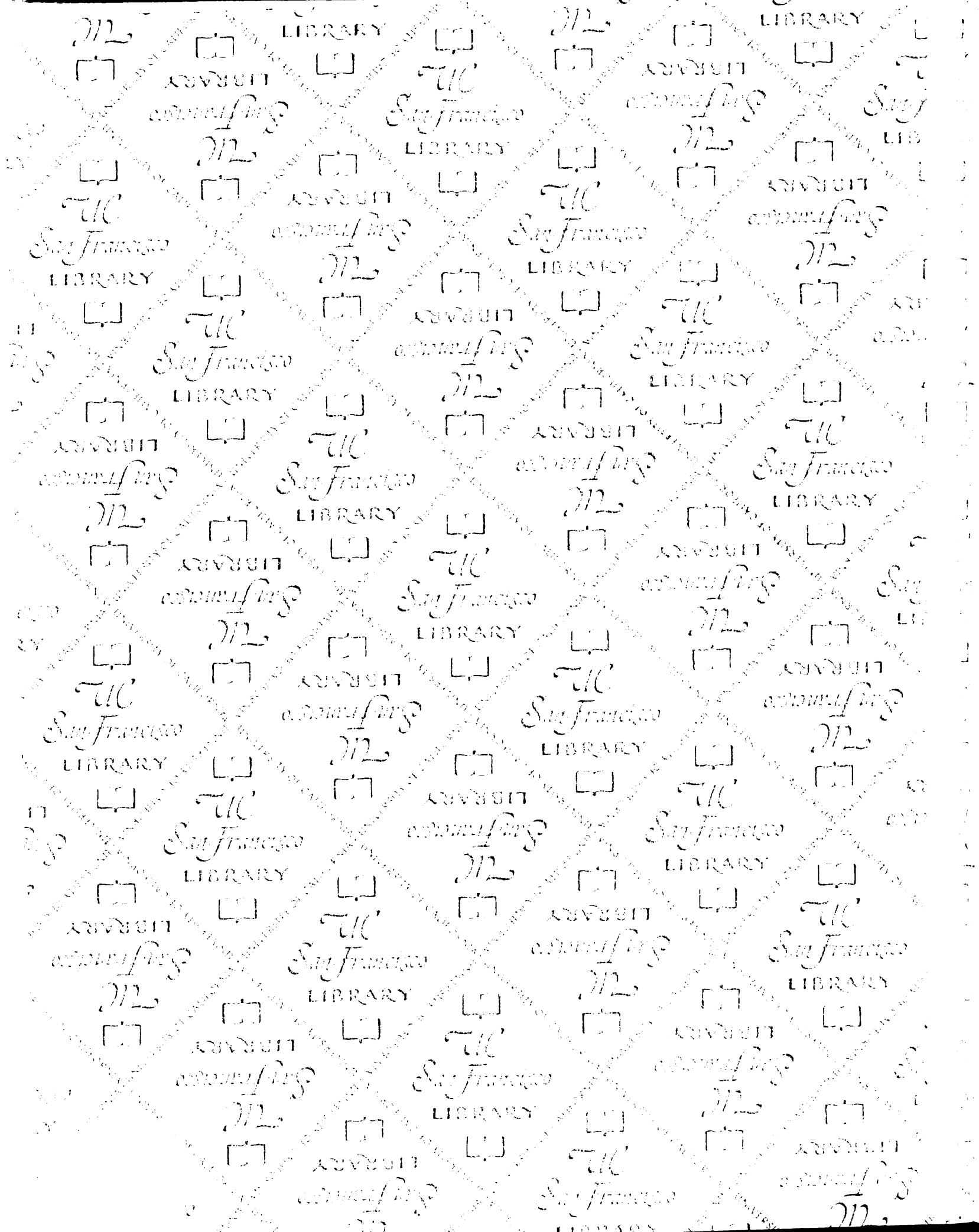
Wun, TC. *et al.* A proenzyme form of human urokinase. J Bio Chem. 257(12): 7262-7268, 1982.

Xue, W. *et al.* Physical association of complement receptor type 3 and urokinase-type plasminogen activator receptor in neutrophil membranes. J Immuno. 152: 4630, 1994.

Yamamoto, M. *et al.* Expression and localization of urokinase-type plasminogen activator receptor in human gliomas. Cancer Res. 54: 5016-5020, 1994.

Young, TN. *et al.* Coordinate expression of urinary-type plasminogen activator and its receptor accompanies malignant transformation of the ovarian surface epithelium. Am J Obstet Gynecol. 170: 1285-1296, 1994.

Zander, HA. *et al.* Goals of periodontal therapy. J Perio. 47(5): 261-266, 1976.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