Plasminogen Activator Inhibitor Type 2 in Human Corneal Epithelium

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PURPOSE. To examine normal human corneal epithelium in vivo and in vitro for expression and status of plasminogen activator inhibitor type 2 (PAI-2).

METHODS. Normal human corneas were prepared for frozen sections and for culture of corneal keratinocytes. PAI-2 was analyzed by immunohistochemistry and western blot analysis using antibodies that recognize all forms of PAI-2.

RESULTS. In vivo and in vitro, PAI-2 was immunohistochemically localized to the superficial corneal keratinocytes. Immunostaining also revealed the presence of PAI-2 in its relaxed (i.e., cleaved) conformation. In vivo, the staining pattern of the relaxed form was identical with that of total PAI-2, but in vitro the relaxed form was detected in a smaller subpopulation of superficial cells. In vitro, the staining pattern indicated a cytoplasmic localization for PAI-2. Western blot analysis revealed that most of the PAI-2 was cell associated and functionally active.

CONCLUSIONS. The present results are the first to show that PAI-2 is found in normal human corneal epithelium in vivo and in vitro, where it can be considered as a differentiation product. At least in vitro, all detectable PAI-2 is cell associated, with a cytoplasmic distribution. A subpopulation of keratinocytes also contains PAI-2 in its relaxed (i.e., cleaved) conformation. Cleavage by an as yet unidentified cytoplasmic proteinase may constitute a crucial aspect of the function of corneal epithelial PAI-2, which may be relevant to terminal differentiation and death of the corneal keratinocyte. (Invest Ophthalmol Vis Sci. 1999;40:1669–1675)

Cells within the cornea and conjunctiva have the ability to synthesize many proteinases and their inhibitors, which have been implicated in physiological and pathologic ocular processes.1,2 One such proteolytic system is the plasminogen activator (PA) cascade.3–6 The PA system consists of the serine proteinases urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA), each of which converts plasminogen to plasmin, a serine proteinase able to cleave many extracellular matrix proteins, including its classic substrate fibrin.7 Several inhibitors in the class known as serpine inhibitors (serpins) regulate uPA and tPA. These include PA inhibitors type 1 and type 2 (PAI-1 and PAI-2) and protease nexin 1.8 The PA cascade has been implicated in many physiological and pathologic events such as ovulation, inflammation, wound repair, angiogenesis, neuronal plasticity, and neoplasia.7,9–15 Within epithelial tissues, uPA has been associated with cell migration and proliferation, whereas tPA has been correlated with differentiation.14–21 PAI-2 (but not PAI-1) has been detected constitutively in several normal murine and human stratified squamous epithelia.22–25 The high levels of PAI-2 and its concentration in the superficial layers of stratified squamous epithelia have led to the hypothesis that this inhibitor plays a role during differentiation of at least some epithelia.

PAI-2 is a member of the ovalbumin family of serpins26 and was first identified in human placenta and macrophages.27–31 Although early reports suggest that PAI-2 may have a very limited tissue distribution, it is now appreciated that PAI-2 is present in stimulated human endothelial cells,32 cultured vascular smooth muscle cells,33 fibroblasts from fetal lung and foreskin,34 human gingival cervical fluid and whole saliva,35,36 normal human epidermis and cultured epidermal keratinocytes,22,23,37 epithelial cells of the murine hair follicle and nail apparatus,24 and numerous other stratified squamous epithelia.25 PAI-2 has been associated with pregnancy, inflammation, apoptosis, and epidermal differentiation (reviewed in Ref. 31). In many cell types, including the epidermal keratinocyte, a large proportion of PAI-2 is found in the cytoplasm.29,37 Given the vast evidence that both plasminogen activators (the only known proteinase targets for PAI-2) function extracellularly, the intracellular localization of PAI-2 is very unexpected and raises the possibility that PAI-2 interacts with an unidentified cytoplasmic proteinase.

Very recently, with the generation of a novel antibody,58 it has become possible to detect PAI-2 that has previously interacted with a proteinase as either a substrate or inhibitor in vivo.59 Similar to other serpins, PAI-2 contains an exposed

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reactive loop near the C terminus which, on proteolytic cleavage, is inserted into the central β-sheet of the inhibitor, resulting in a conformational change to a stabilized, or relaxed, state. When PAI-2 interacts with target proteinase, the inhibitor is cleaved in its reactive loop, a stable enzyme-inhibitor complex is formed, and the proteinase is, for all practical purposes, irreversibly inhibited. PAI-2 may also be cleaved by a proteinase that rapidly escapes from the inhibitor after cleavage, thus retaining its proteolytic activity while generating an inactive inhibitor; in this case PAI-2 is acting not as an inhibitor but as a substrate. In either case, the cleaved PAI-2 assumes a similar stabilized, that is, relaxed, conformation. We have used an antibody that specifically detects a conformational determinant unique to the relaxed form of PAI-2\(^1\) to examine the state of PAI-2 in human corneal keratinocytes in vivo and in vitro. Our data show for the first time not only that PAI-2 is synthesized by corneal keratinocytes, but also that this inhibitor is constitutively cleaved by an endogenous corneal proteinase.

**MATERIALS AND METHODS**

**Cell Culture**

Normal human corneas were obtained from the Delaware Valley Lions Eye Bank, and the epithelium was recovered by incubation overnight at 4°C in phosphate-buffered saline (PBS) containing dispase (50 caseinolytic units/ml; catalog 40235; Collaborative Biomedical Products, Becton Dickinson, Bedford, MA). Epithelium was pulled from the stroma, suspended in PBS with 20% fetal bovine serum, pipetted up and down several times to disrupt the epithelium further, centrifuged, and resuspended in culture medium (Dulbecco’s minimal essential medium: Ham’s F12 medium 3:1, 10% fetal bovine serum, 0.4 μg/ml hydrocortisone, 10 μg/ml epidermal growth factor, 10\(^{-10}\) M cholaer toxin, 5 μg/ml insulin, 24 μg/ml adenine, and 2 × 10\(^{-9}\) M 3,3′,5-triiodo-L-thyronine), according to Lindberg et al.\(^1\) Cells were plated onto mitomycin C-treated 3T3 feeder layers, which gradually detach as the epithelial colonies expand. Nearly confluent cultures were split into six-well plates, and cells between the first and sixth passages were used for experiments. Anti-keratin antibody stained at least 99% of the cells, conclusively identifying them as epithelial.

**Immunohistochemistry**

Frozen sections (5 μm) of normal human cornea were fixed in acetone for 20 minutes at −20°C. After washing in PBS and blocking in PBS containing 10% normal horse serum, sections were incubated for 45 minutes at room temperature with 4 μg/ml primary antibody: mouse monoclonal anti-human PAI-2 antibody (3750; American Diagnostica, Greenwich, CT), which recognizes all known forms of PAI-2, or mouse monoclonal anti-relaxed PAI-2 antibody 2H5, which is specific for a conformational determinant found only on the relaxed form of PAI-2 (produced and purified by Dr. Baker and Dr. Saunders).\(^1\) As negative controls, mouse monoclonal antibodies against chlamydia antigen or hepatitis antigen were used. After washing, sections were sequentially incubated with biotinylated secondary horse anti-mouse IgG, avidin-biotin-peroxidase (Vector, Burlingame, CA), and diaminobenzidine tetrahydrochloride substrate (Sigma, St. Louis, MO).

Corneal keratinocyte cultures grown on glass coverslips were immunohistochemically stained using similar procedures, except that fixation and permeabilization were carried out by sequential treatments with acetone and then methanol for 10 minutes each at −20°C. In some experiments, detection was with the peroxidase method just described; in other cases, fluorescein isothiocyanate (FITC)-labeled goat anti-mouse secondary antibody (Pierce, Rockford, IL) was used.

In one series of experiments, we tested for the presence of PAI-2 on the cell surface by incubating live cells with antibodies under conditions in which internalization of immune complexes was blocked. Briefly, cultures on coverslips were preincubated in medium containing 0.05% sodium azide for 30 minutes at 37°C and then incubated with primary antibody on ice for 45 minutes. After washing, the cultures were fixed in 1% paraformaldehyde at room temperature for 10 minutes, blocked in PBS containing 10% normal goat serum and 0.1 M glycine (pH 7.6) and incubated with FITC-labeled goat anti-mouse IgG for 45 minutes. No staining was detectable on the cells using this method with either anti-PAI-2 or anti-relaxed PAI-2 antibodies.

**Western Blot Analysis**

Extracts of cultured human corneal keratinocytes were prepared by scraping the cells directly into 0.1 M Tris-HCl (pH 8.1) with 0.2% Triton X-100, (200 μl per 35-mm well). Western blot analysis was performed as previously described.\(^1\) Briefly, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed under reducing conditions, and proteins were transferred onto a 0.45-μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Two anti-PAI-2 antibodies were used: 3 μg/ml mouse monoclonal or 5 μg/ml goat polyclonal antibody (3750 and 375G, respectively, from American Diagnostica). Secondary, horseradish peroxidase-labeled antibodies were sheep anti-mouse (Amersham Life Sciences, Arlington Heights, IL) or mouse anti-goat (Pierce). Finally, detection was performed with enhanced chemiluminescence reagents (Amersham) and recorded on film (XOMAT; Kodak, Rochester, NY). The anti-relaxed PAI-2 antibody 2H5\(^1\) recognizes a conformational determinant and thus was unsatisfactory for western blot analysis.

**RESULTS**

**PAI-2 Localization in Normal Human Cornea**

In normal human corneal epithelium, immunocytochemical staining for PAI-2 revealed a distribution that was concentrated around the periphery of the uppermost wing and most of the superficial cells (Figs. 1A, 1C). There was also a fainter, focal, and more diffuse staining in the lower cell layers. At the corneal-limbal junction, there was a gradual change in the localization of PAI-2 until, in the limbal region, all the cell layers exhibited peripheral staining. When experiments were performed with antibody 2H5, which specifically recognizes the relaxed form of PAI-2,\(^1\) an identical staining pattern was observed (Fig. 1B). Staining for PAI-2 was not detectable in the corneal stroma.
PAI-2 Localization in Human Corneal Keratinocytes In Vitro

Normal human corneal keratinocytes were propagated in vitro under conditions that led to formation of a differentiated, multilayered epithelial sheet and then were examined for expression and distribution of PAI-2. Immunofluorescent staining revealed that PAI-2 was concentrated in the more superficial layers of the culture, which are the larger, more elongated cells (Figs. 2A, 2B). The basal layer, which comprises small cells arranged in a cobblestone pattern, had little or no detectable PAI-2 staining (Figs. 2A, 2B). The immunofluorescent staining pattern was cytoplasmic, with a sparing of the cell-cell border regions. In many cells the PAI-2 staining pattern was filamentous throughout the cytoplasm (Figs. 2C, 2D).

When replicate culture wells were processed for immunocytochemistry with antibody to relaxed PAI-2, a subpopulation of superficial cells revealed positive staining (Figs. 2E, 2F). Fewer cells were stained with antibody to relaxed PAI-2 than were detected on staining with antibody that recognizes all forms of PAI-2. The staining pattern was generally diffuse and cytoplasmic, but in many fields there was also a concentration around the cell periphery. Nonspecific staining with irrelevant monoclonal antibodies was very faint and mostly limited to the basal cells.

Western Blot Analysis for PAI-2

To investigate further the biochemical nature of PAI-2 in corneal keratinocytes, culture extracts were subjected to western blot analysis with antibodies against PAI-2. As shown in Figures 3A, 3B, and 3C (lane 1 in each case), the predominant band detected in the cell extracts, using either mouse or goat anti-PAI-2 antibodies, had an apparent molecular weight of 45 kDa. This is consistent with the molecular weight of the nonglycosylated form of PAI-2, previously reported in human macrophages and epidermal keratinocytes. The additional bands at approximately 60 kDa and 100 kDa in Figure 3B are nonspecific; they were also observed on immunoblots with nonimmune goat IgG. No PAI-2 was detected in the conditioned media, even when they were concentrated 10-fold before western blot analysis.

To test whether corneal keratinocyte PAI-2 was functionally active, its ability to complex with 33-kDa uPA was determined. As shown in Figure 3, incubation of cell ex-
tracts with uPA before loading the gel for western blot analysis led to a significant loss in intensity of the 45-kDa band and appearance of a band at 78 kDa, indicative of complex formation. Such an assay is possible because PAI-2, similar to other serpins, forms an SDS-resistant complex with its target proteinases.\(^2\) Incubation with increasingly greater amounts of uPA revealed a plateau in intensity of the complex band, as expected (Fig. 3B). After incubation with uPA, a weak band appeared that had a mobility slightly greater than that of the 45-kDa PAI-2 band. We believe that this represents a small amount of PAI-2 that has interacted with and been cleaved by uPA and then has dissociated from the proteinase; PAI-2 that has complexed with uPA is known to have a slightly lower molecular weight than active PAI-2, because of cleavage of the C-terminal 35 amino acids.\(^3\) To consider further this possibility, we deliberately dissociated

**Figure 2.** PAI-2 localization in human corneal keratinocytes in culture. (A, B) Human corneal keratinocytes propagated on glass slides were fixed, permeabilized, and then incubated with monoclonal antibody #3750 that recognizes PAI-2. Detection was performed using an FITC-labeled secondary antibody. Fluorescence (A) and phase-contrast (B) views are shown. Note staining in the superficial cells (which are larger and more elongated) but not in the underlying basal cells (which have a cobblestone appearance). (C, D) Similar experiments were performed using an avidin-biotin-peroxidase and DAB detection system. Note the filamentous staining pattern in many cells. (E, F) In cultures stained with antibody to the relaxed form of PAI-2, a subpopulation of superficial cells was positive in a more diffuse pattern with frequent concentration along the cell–cell borders. Scale bar, (A, B) 200 μm; (C) 75 μm; (D, F) 24 μm; (E) 38 μm.

\(^2\) Incubation with increasing amounts of plasminogen activator inhibitor 2, similar to other serpins, forms an SDS-resistant complex with its target proteinases.

\(^3\) To consider further this possibility, we deliberately dissociated.
the PAI-2–uPA complexes by incubation with NH$_4$OH.$^{29,43}$ After this dissociation, the complex band was greatly diminished in intensity, and most of the PAI-2 traveled with an apparent molecular weight of slightly less than 45 kDa (Fig. 3C).

**DISCUSSION**

In the present report we show that PAI-2 was present in normal human corneal epithelial cells. Both in vivo and in vitro, PAI-2 was concentrated in the more superficial cell layers, suggesting that it is a product of differentiating corneal keratinocytes. Furthermore, in our findings, corneal keratinocyte PAI-2 appeared to be primarily cytoplasmic rather than secreted. Evidence for this comes from several experimental approaches: (1) We were unable to detect any PAI-2 in the conditioned media, even if they were concentrated 10-fold. (2) The immunocytochemical staining pattern for PAI-2 in cultured corneal keratinocytes appeared cytoplasmic (Fig. 2). In agreement with this interpretation, live cells were not stained with anti-PAI-2 antibody, suggesting that PAI-2 was not on the cell surface (data not shown). (3) By western blot analysis, we observed only the 45-kDa, nonglycosylated form of PAI-2. We never obtained any evidence for the presence of a higher

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**FIGURE 3.** PAI-2 western blot analysis of human corneal keratinocyte extracts. Human corneal keratinocyte cultures from donor 1 at second passage (A, B) or donor 2 at third passage (C) were extracted in Tris/Triton buffer. (A, B) Aliquots of each extract were incubated for 10 minutes on ice with the indicated amounts of standard 33-kDa uPA to allow complex formation. (C) The indicated aliquots were incubated with 1 µg uPA for 10 minutes on ice and then with 0.5 M NH$_4$OH for 10 minutes at room temperature. All extracts were then resolved with SDS-PAGE and transferred to nitrocellulose membranes for immunodetection with mouse monoclonal antibody #3750 that recognizes PAI-2 (A, C) or goat polyclonal antibody against PAI-2 (B), followed by enhanced chemiluminescence detection. Lane 1 in each blot shows control extract; note intense band at 45 kDa. When extracts were incubated with uPA, there was a diminution in the intensity of this band and the appearance of a band at 78 kDa, the approximate size of a PAI-2–uPA complex. The complex was dissociated by incubation with NH$_4$OH.

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molecular weight, glycosylated form that, at least in macrophages, is the predominant secreted species. Because there is strong evidence that known target proteinases for most serpins, including PAI-2, act in the extracellular milieu, the cytoplasmic localization is unusual and suggests the possibility of an alternative, intracellular function.

Our use of a novel antibody that recognizes specifically the relaxed form of PAI-2 provided further evidence for and some insight into a cytoplasmic role for this inhibitor in normal human corneal epithelium. The relaxed form of PAI-2 is generated through proteolytic cleavage of the inhibitor within its reactive loop, followed by insertion of the new C-terminus into the central β-sheet of the inhibitor. Our finding that relaxed PAI-2 was immunocytochemically detectable not only in corneal epithelium in vivo but also in cultured corneal keratinocytes in vitro strongly indicated the presence of an endogenous corneal keratinocyte proteinase that cleaved PAI-2. Although at present we do not know the nature of the cleaving proteinase, the staining patterns with both anti-PAI-2 antibodies make it highly likely that the proteinase is cytoplasmic. Our data thus suggest that constitutive, cytoplasmic cleavage of PAI-2 to its relaxed state represents one aspect of its role in normal human corneal epithelium. Our western blot analysis did not provide evidence of a cleaved form of PAI-2 constitutively present in the keratinocyte extracts, perhaps indicating that the cleaved form was present at relatively low levels, not detectable by western blot analysis. It should be noted that relaxed PAI-2 has not previously been reported in any stratified squamous epithelium in vivo or in vitro; the only published report of relaxed PAI-2 in any tissue deals exclusively with gestational tissues and shows that relaxed PAI-2 is present in amnion epithelium.

Using immunolocalization and in situ hybridization, we have previously shown that PAI-2 is synthesized by the more differentiated cells of numerous stratified squamous epithelia (e.g., epidermis, vagina, oral mucosa, esophagus, hair, and nail). This similar expression pattern suggests that PAI-2 may play a role in a process that is common to these very diverse epithelia. One possible common function is to protect the superficial epithelial cells, which contain high levels of PAI-2, from dying prematurely during the later stages of terminal differentiation. Terminal differentiation of corneal keratinocytes, similar to that of other stratified squamous epithelia, can be considered a highly specialized form of programmed cell death. Several published studies using tumor cell lines have revealed that high levels of cytoplasmic PAI-2 confer resistance to programmed cell death, probably through inhibition of unknown cytoplasmic proteinases. We speculate that PAI-2 may act similarly in keratinocytes of stratified squamous epithelia, which must remain viable and metabolically active for a significant period after their assumption of the postmitotic state and detachment from the basement membrane. Protective functions of stratified squamous epithelia depend on the highly regulated formation and temporary retention of the dead, terminally differentiated cells that constitute the outermost layers of these tissues. Premature demise of the keratinocytes, before they have had time to synthesize all the products necessary for terminal differentiation, could have disastrous effects on tissue function. We hypothesize that PAI-2 may protect the keratinocytes from dying too soon, through its constitutive interaction with and cleavage by an intracellular proteinase.

An alternative possibility is that PAI-2 functions primarily as part of the epithelial defense mechanism against microbial infection in the eye. Proteinase activity has been implicated in the pathogenic mechanisms of a variety of infectious agents. For example, the parasite *Acanthamoeba castellanii*, which produces a blinding inflammatory disease of the cornea, synthesizes a plasminogen activator, the activity of which has been correlated with pathogenic potential. In addition, there is recent evidence that intracellular PAI-2 can protect macrophages against the cytotoxic effects of certain viruses. Further investigation of PAI-2 in the cornea and in other ocular epithelia is needed to understand the potential multifaceted roles of this inhibitor.

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**References**


