Effect of Protease Inhibitors on Corneal Epithelial Migration

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Recently protease inhibitors were used to treat corneal ulceration and persistent epithelial defect. An organ culture system was used in this study to examine the effect of four protease inhibitors on rat corneal epithelial migration and on incorporation of ^3^H-leucine into trichloroacetic acid-precipitable material as an indicator of protein synthesis. The effect of the protein synthesis inhibitor, verrucarin A, on both parameters also was examined. These results showed that epithelial migration was dependent linearly on protein synthesis with inhibition ranging from 100% at 10^{-5} M to 0% at 10^{-6} M. Pepstatin A, an acid protease inhibitor, had no effect on migration at concentrations of 10^{-5} M and 10^{-6} M. At 0.5 mM, 1,10-phenanthroline, a metalloprotease inhibitor, blocked epithelial migration at 100% in a dose-dependent manner with an equivalent reduction in ^3^H-leucine incorporation. Aprotinin blocked migration 74% and 71% at 500 U/ml and 50 U/ml, respectively, whereas protein synthesis decreased only 41% and 21%, respectively. At 1.0 mM and 0.5 mM, phenylmethylsulfonyl fluoride (PMSF) blocked migration 92% and 71% and lowered protein synthesis 74% and 55%, both respectively. These data suggest that the serine proteases may be involved directly in epithelial migration because their inhibitors, aprotinin and PMSF, blocked it to such an extent that this finding cannot be explained by inhibition of protein synthesis alone. Invest Ophthalmol Vis Sci 32:2073-2078, 1991

Corneal injury, subsequent epithelial migration, and wound repair involve many biochemical events including the release of proteolytic enzymes (proteases) to degrade specific molecules, such as the digestion of type I collagen by collagenase during corneal ulceration. Proteases are involved in corneal ulceration and hypothetically associated with persistent epithelial defect and recurrent corneal erosion. The overproduction of proteases may be responsible, in part, for these conditions, leading to the destruction of matrix and basement membrane components. Clinically, much attention has been devoted to identifying specific inhibitors of the proteases to help minimize tissue damage after corneal wounds.

Aprotinin, a naturally occurring protease inhibitor first discovered in bovine tissue, specifically inhibits serine proteases such as plasmin, plasminogen activator, and trypsin. Clinically, aprotinin has been used since 1953 to treat delayed coagulation, acute pancreatitis, and hypovolemic shock. Recently, aprotinin was suggested as therapy to prevent corneal ulceration in patients with high plasmin levels in their tear fluid and used experimentally to prevent lesions after alkali burns to rabbit eyes. In both studies, aprotinin appeared to accelerate the rate of epithelial wound healing. This finding prompted us to examine the effect of protease inhibitors on epithelial migration.

We assayed inhibitors that block the action of three types of proteases for the effect of these inhibitors on epithelial migration rates in an organ culture wound model. Those tested were aprotinin and phenylmethylsulfonyl fluoride (PMSF), which inhibit serine proteases including plasmin and plasminogen activator; 1,10-phenanthroline, which inhibits metalloproteases including collagenases, gelatinases, and stromelysin; and pepstatin A, which inhibits acid proteases.

Materials and Methods

Abras ion Wounds and Organ Culture

The organ culture system used in these studies has been described. Briefly, Sprague-Dawley rats with clear, healthy corneas were killed by an intraperitoneal overdose of sodium pentobarbital. An epithelial wound, 3 mm in diameter, was created in situ by marking an area on the central cornea with a 3-mm trephine and removing the encircled epithelium with a small scalpel. The eyes were then excised, and the corneas removed and allowed to heal in culture for 18 hr in a completely defined medium. Four corneas...
were cultured in each 60 × 15-mm Petri dish. The corneas were pinned to rounded paraffin posts for support during harvesting of epithelium. Four or more corneas were used for each concentration at each time.

Protease and Protein Synthesis Inhibitors

Verrucarin A (also called muconomycin A) is a toxin that inhibits eukaryotic protein synthesis. This compound was solubilized in dimethyl sulfoxide (DMSO) at a stock concentration of 0.02 M and added to culture medium at final concentrations of 10⁻⁵⁻⁻¹⁰⁻⁸ M. Verrucarin A was present throughout the entire culture period.

Aprotinin (Mobay, New York, NY) was added to cultures at concentrations ranging from 0.05–1000 U/ml. The PMSF, an inhibitor of serine proteases, was dissolved in ethanol at a stock concentration of 0.2 M and added to cultures at concentrations ranging from 0.1–1 mM. Pepstatin A, an inhibitor of acid proteases, was dissolved in water and used at concentrations of 10⁻⁵⁻⁻¹⁰⁻⁶ M. The 1,10-phenanthroline, an inhibitor of metalloproteases, was dissolved in ethanol to a stock solution of 1 M and used in cultures at concentrations of 0.1, 0.5, and 1.0 mM. All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. As controls, corneas with 3-mm wounds were allowed to heal in culture with no inhibitors added. However, the vehicle (DMSO or ethanol) was added in amounts corresponding to those used with the inhibitor to ensure that the vehicle was not affecting the migration rate or 3H-leucine incorporation. All inhibitors were present throughout the entire culture period.

Healing Rates and Protein Synthesis

Healing rates were determined by staining the remaining epithelial defect with Richardson's stain, photographing the cornea, and measuring the area on the photograph with a Zeiss Videoplan 2 (Rainin, Woburn, MA).

To measure the rate of protein synthesis, incorporation of ³H-leucine into trichloroacetic acid (TCA)-precipitable material was determined. In the protein synthesis-inhibitor experiments, ³H-leucine (5 μCi/ml) was added to the culture medium 3 hr before harvesting of epithelium. In all other inhibitor studies, ³H-leucine (2 μCi/ml) was added. At 18 hr after wounding, the remaining epithelial defect was detected with Richardson's stain. The original 3-mm area concentric to the defect was marked with the trephine, and the epithelium in that area was removed with a small scalpel. The epithelial proteins were precipitated in 7.5% TCA and washed three times in 7.5% TCA. The TCA-precipitable material was then digested in 0.2 M NaOH, its radioactivity was determined using Aquasol II in a scintillation counter (Beckman, Palo Alto, CA), and the amount of epithelial protein was determined with the bicinchoninic acid assay (Pierce, Rockford, IL). All experimental techniques adhered to the ARVO Resolution on the Use of Animals in Research.

Results

Aprotinin inhibited epithelial migration in a dose-dependent manner (Fig. 1A) as follows: 77.3% (1000 U/ml), 76.2% (500 U/ml), 70.7% (50 U/ml), 49.8% (5 U/ml), 09.1% (0.5 U/ml), and 03.9% (0.05 U/ml). This inhibition was statistically significant (P ≤ 0.01)

Fig. 1. The effect of the following protease inhibitors on corneal epithelial migration: (A) aprotinin, (B) phenylmethylsulfonyl fluoride (PMSF), and (C) 1,10-phenanthroline. Total culture time for all three inhibitors = 18 hr; number of corneas = 4 for all time points; values represent mean ± standard error of the mean (SEM). Original 3-mm wound area = 7.07 mm².

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at 5 U/ml and higher. The inhibition at 0.05 U/ml was not statistically significant ($P \leq 0.05$). The corneal epithelium treated with aprotinin was not stained by Richardson's stain and showed the same degree of adherence to the underlying stroma as did the migratory, untreated corneas.

To determine if aprotinin slowed epithelial healing rates through its effect as a serine-protease inhibitor—or rather through a secondary, unknown effect—a second inhibitor of serine proteases, PMSF, was tested in wounded corneas (Fig. 1B). The PMSF similarly inhibited epithelial migration in a dose-dependent manner: 92% at 1.0 mM and 71% at 0.5 mM. No inhibition was observed at 0.1 mM. The inhibition at 1.0 mM and 0.5 mM was statistically significant ($P \leq 0.01$) compared with that of corneas treated with vehicle alone. As with aprotinin, PMSF did not appear to alter the adhesion of the epithelium or increase the degree of Richardson's staining compared with the untreated control.

Inhibitors of two other categories of proteases also were tested for their effect on epithelial migration. Pepstatin A, an inhibitor of acid proteases and generally effective at a concentration of $10^{-7}$ M,12 had no effect on migration at $10^{-5}$ M and $10^{-6}$ M (Table 1). At a concentration of 0.1 mM, 1,10-phenanthroline blocked migration by 60%. At 0.5 and 1.0 mM, after 18 hr in culture, the remaining epithelial defects were larger (8.75 mm$^2$ and 11.58 mm$^2$, respectively) than the original 3-mm wound area (7.07 mm$^2$) (Fig. 1C). The remaining epithelium was loosely adherent and stained heavily by Richardson's stain.

To determine if the protease inhibitors affected protein synthesis, $^3$H-leucine incorporation was measured. Aprotinin lowered protein synthesis 41% and 21% at concentrations of 500 and 50 U/ml, respectively (Fig. 2A). The PMSF reduced protein synthesis 55% and 74% at concentrations of 0.5 and 1.0 mM, respectively (Fig. 2B). The 1,10-phenanthroline lowered synthesis by 65% at 0.1 mM and by 100% at 0.5 mM and 1.0 mM (Fig. 2C).

The relationship between protein synthesis and epithelial migration was determined by measuring the inhibition of both in the presence of verrucarin A (Fig. 3, Table 1). The results indicated a linear relationship between epithelial migration and $^3$H-leucine incorporation ($r = 0.99$) Similar data were obtained using the protein-synthesis inhibitor cycloheximide (data not shown.)

### Discussion

Recently, Salonen et al10 proposed the use of the serine protease inhibitor aprotinin to prevent chronic corneal ulceration in 18 patients with high levels of plasmin present in their tear fluid. These researchers postulated that aprotinin would inhibit plasmin and allow wound healing. Their results indicated that topical applications of aprotinin, consisting of 1–2 drops (20 or 40 U/ml) every 3 hr, except during sleep, led to rapid reepithelialization. Others also found that aprotinin blocked corneal ulceration after alkali burns in rabbits and reduced the number of inflammatory cells infiltrating the burn area.11 In these experiments, aprotinin (5000 U/ml) was applied at 12-hr intervals for 2 weeks, and then at 2500 U/ml at 12-hr intervals for 2 additional weeks.

In this study, aprotinin inhibited epithelial migration at concentrations of 0.5 U/ml and higher. This

### Table 1. Comparison of inhibition rates of protein synthesis and epithelial migration*

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>Type of inhibitor</th>
<th>Inhibition of protein synthesis† (%)</th>
<th>Inhibition of migration‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aprotinin</td>
<td>500 U/ml</td>
<td>Serine</td>
<td>41</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>50 U/ml</td>
<td></td>
<td>21</td>
<td>71</td>
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<tr>
<td>Phenylmethylsulfonylfluoride (PMSF)</td>
<td>1.0 mM</td>
<td>Serine</td>
<td>74</td>
<td>92</td>
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<tr>
<td></td>
<td>0.5 mM</td>
<td></td>
<td>55</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pepstatin A</td>
<td>$10^{-6}$ M</td>
<td>Acid</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-5}$ M</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>1.0 mM</td>
<td>Metalloprotease</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.5 mM</td>
<td></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>0.1 mM</td>
<td></td>
<td>65</td>
<td>60</td>
</tr>
<tr>
<td>Verrucarin A</td>
<td>$10^{-7}$ M</td>
<td>Protein synthesis</td>
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<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-6}$ M</td>
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<td>20.7</td>
<td>19.9</td>
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<td></td>
<td>$5 \times 10^{-7}$ M</td>
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<td>68.1</td>
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<td></td>
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<td>90.5</td>
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<tr>
<td></td>
<td>$10^{-4}$ M</td>
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<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Compilation of data presented in Figures 1 and 2 allows comparison of inhibition rates of protein synthesis and of migration following treatment with various protease inhibitors.

† Inhibition of protein synthesis represents the decrease in $^3$H-leucine incorporation compared with that of cultures containing vehicle only.

‡ Inhibition of migration is calculated by dividing the sizes of the remaining wound areas in the presence of inhibitors versus vehicle alone.
suggests that the acceleration of wound healing seen by Salonen et al.\textsuperscript{10} did not result from aprotinin directly increasing the rate of epithelial migration. Our results suggest, however, that high concentrations of protease inhibitors should be avoided so that epithelial migration can proceed normally. Aprotinin inhibited epithelial migration in our system at concentrations lower than those used by others.\textsuperscript{10,11} However, dilution of the aprotinin solution by tear fluid makes direct comparison of concentrations difficult.

Since aprotinin slowed epithelial migration in our model, we further examined the effect of protease inhibitors, and by inference the role of proteases, in epithelial migration. The role that proteases play in the normal reepithelialization of the cornea is unclear. Plasminogen activator and plasmin may degrade temporary attachments of the epithelial cells to extracellular matrix, thus, possibly leading to epithelial migration.\textsuperscript{18} If this hypothesis is correct, protease inhibitors would be expected to slow migration. To test this hypothesis, three other protease inhibitors were assayed for their effect on epithelial healing. In addition, we assayed each inhibitor for its effect on protein synthesis, as indicated by $[^3]$H-leucine incorporation, to determine if the protease inhibitors affected the proteases directly or influenced migration through a secondary “toxic” effect. To determine whether an inhibitor was directly affecting migration, we investigated the relationship between the rate of epithelial wound closure and protein synthesis. We previously showed that protein synthesis levels increase dramatically during epithelial migration.\textsuperscript{13,15} Most of the proteins present in unwounded corneal epithelium are synthesized at increased levels during epithelial migration. However, vinculin appears to be synthesized more than the other proteins.\textsuperscript{19} This increased level suggests that vinculin is necessary for migration, perhaps as a component of focal contacts. In the current study, verrucarin A was used at varying concentrations to examine the effect of blocking protein synthesis during wound healing. This drug inhibits eukaryotic protein synthesis by binding to the 60S ribosomal subunit and blocking peptidyltransferase activity; it has few
other toxic side effects.\textsuperscript{16,20,21} Our results indicate that there may be a linear relationship between the rate of epithelial migration and the level of protein synthesis. This linear relationship may not be precise, particularly at the extremes of drug concentrations, because wound measurements and tissue harvesting involve inherent inaccuracies. However, $r = 0.99$ whether epithelial migration was expressed as the remaining wound area (Fig. 3) or as the remaining wound radius (data not shown).

This linear correlation enabled us to determine whether an agent affected epithelial migration directly or indirectly. If inhibition of migration exceeds that of protein synthesis, it strongly suggests that the agent alters migration directly. If migration and protein synthesis levels are blocked equally, then it cannot be determined if the agent is working directly (blocking a protease) or indirectly (exerting a toxic effect).

Protease inhibitors of three categories of proteases were examined for their effects on epithelial migration. Acid protease inhibitors had no apparent effect on migration. However, both metalloprotease inhibitors and serine-protease inhibitors slowed epithelial migration in a dose-dependent manner. The inhibition shown by $1,10$-phenanthroline probably resulted from its toxic effect on corneal epithelial cells. This conclusion was supported by the observation that the degree of epithelial migration inhibition was nearly identical to that of protein synthesis inhibition (Table 1), with $1.0$ mM and $0.5$ mM inhibiting both synthesis and migration by $100\%$, and $0.1$ mM inhibiting migration by $65\%$ and synthesis by $60\%$. Additionally, the epithelial tissue was loosely adherent and stained heavily by Richardson’s stain after incubation with $1,10$-phenanthroline.

By contrast, the inhibition of migration by apro tin and PMSF appears to result partly from their direct effect on serine proteases. With both inhibitors, inhibition of epithelial migration was greater than that of protein synthesis (Table 1). For example, apro tin ($50$ U/ml) slowed migration by $71\%$ but lowered protein synthesis by only $21\%$; this finding suggests a possible role for serine proteases in epithelial migration. A certain level of serine proteases may be necessary for migration, whereas their overabundance may slow migration and lead to corneal ulceration. The inhibition of migration by apro tin appeared to reach a plateau around the $80\%$ level, but the mechanism involved here was unclear.

Three possible mechanisms may explain the involvement of serine proteases in epithelial migration. First, the corneal epithelium normally is secured in place by the hemidesmosome-anchoring fibril network.\textsuperscript{22,23} During epithelial migration, the hemidesmosomes are lost. One potential mode of action for serine proteases is that they may degrade a component necessary for maintenance of the hemidesmosomal complex. However, we observed that apro tin also blocks migration if added to organ cultures $4$ hr after wounding; this observation implies that apro tin may have an effect during active migration. Second, serine proteases may cause the breakdown of provisional adhesion junctions. We observed that vinculin, a component of focal contacts, was localized along the basal membrane of cells adjacent to the basement membrane after the loss of hemidesmosomes.\textsuperscript{19} We postulated that provisional cell-substrate attachments were made and subsequently broken by serine proteases to allow cell movement while still maintaining epithelial-sheet adherence. This hypothesis was supported by the observation that plasminogen activator (a serine protease) was localized in focal contacts.\textsuperscript{24} The component of the focal contact-like structure that might be degraded was not identified; the plasminogen activator/plasmin system may lead to extracellular matrix degradation.\textsuperscript{19} The inhibition of mitosis also could explain the inhibition of migration. However, this mechanism was unlikely since several investigators showed that inhibition of mitosis had little or no effect on the rate of epithelial wound closure.\textsuperscript{25-27}

In summary, we showed that (1) epithelial migration is dependent on protein synthesis; (2) metalloproteases and acid proteases do not appear to be directly involved in epithelial migration—metalloprotease inhibitors block epithelial migration only with a corresponding block in protein synthesis; and (3) serine proteases may be involved directly in epithelial migration since their inhibitors, PMSF and apro tin, blocked migration to a greater extent than can be explained by inhibition of protein synthesis alone.

\textbf{Key words:} apro tin, protease inhibitors, corneal epithelial migration, protein synthesis inhibitors, verrucarin A

\textbf{References}


