Plasminogen activator (urokinase) causes vascularization of the cornea

M. Berman, S. Winthrop, D. Ausprunk, J. Rose, R. Langer, and J. Gage

The presence of a peripheral zone of (presumed intracellular) plasminogen activator in the normal rabbit cornea has suggested that activator, once released, might regulate the permeability of limbal vessels and angiogenesis, by plasmin-dependent pathways. Plasminogen activator (urokinase [UK]) in rabbit serum albumin (RSA) was injected once (20 µl, 3.7 CTA U) into the corneal stroma, 2 mm from the limbus. Sprouts arose from the engorged circumlimbal vessels (16 of 20 corneas) beginning on the third day and grew into the cornea over the next several days. Histologically, PMNs were observed in association with growing vessels. Contralateral corneas injected with UK (in RSA) previously inactivated by 99.7% with the specific active site inhibitor, Phe-Ala-Arg-chloromethyl ketone showed minimal vessel engorgement or stromal edema and no vascularization (0 to 20 corneas). Injuries to the so-called (plasminogen activator-containing) "critical zone" of the cornea which elicit neovascularization possibly do so by causing extracellular release of endogenous plasminogen activator. Thus, in addition to initiating the destructive events of ulceration, activator might initiate increases in vessel permeability and also neovascularization, which would result in the eventual arrest of ulceration. (INVEST OPHTHALMOL VIS SCI 22:191-199, 1982.)

Key words: collagenase, plasminogen activator, vascularization, cornea, ulceration

Vascularization of the cornea occurs in many pathological conditions and, with scarring, can result in loss of visual acuity. Vascularization is also thought to predispose the cornea to rejection of grafts by facilitating the detection of foreign antigens in donor material. Yet despite these negative aspects, vascularization is also thought on clinical and experimental grounds to prevent ulceration after injury, presumably by introducing antiproteases and metabolites for wound repair.

The mechanisms of corneal vascularization have been studied by many investigators, and diverse mediators have been implicated as involved in the process; thus prostaglandins, vasoactive amines, epithelial angiogenic factors, and component(s) of leukocyte extracts have all been reported to cause vessel growth into the cornea. Such mediators, it has been proposed, are released after injury to the cornea. In addition, epidermal growth...
factor\(^8\) and fibroblast growth factor\(^8\) have also been reported recently to stimulate vessel growth into the cornea.

The recent observation that the normal rabbit cornea contains a peripheral zone of plasminogen activator in the superficial stroma\(^9\) suggests that corneal plasminogen activator might have an important role in regulating both the permeability of limbal vessels, through known plasmin-dependent pathways,\(^10\) and the neovascularization of the cornea. This report demonstrates that human plasminogen activator, urokinase (UK), is able to cause neovascularization of the rabbit cornea.

Materials and methods

Crude human UK was obtained from Calbiochem (cat. no. 672123) and purified UK was obtained from Collaborative Research (cat. no. 40110). The pure enzyme has a specific activity of 238,000 CTA U/mg, is a single band on sodium dodecyl sulfate gel electrophoresis, and has a molecular weight of 35,000 daltons. Purified rabbit serum albumin (RSA) for use as carrier of UK was obtained from Cappel Laboratories. The active site inhibitor (I) of UK, the chloromethyl ketone Phe-Ala-ArgCH\(_2\)Cl \(\cdot 2\) HCl,\(^11\) was obtained through the courtesy of Dr. C. Kettner and Dr. E. Shaw of the Brookhaven National Laboratory.

Preliminary studies indicated that the purified UK requires carrier to prevent its adsorption to glass and/or surface denaturation. Serial dilutions of UK and RSA demonstrated that 0.2% to 0.5% RSA as carrier gives reproducible UK enzyme activities. Plasminogen activator activity was measured by a modification of the method of Astrup and Kok,\(^12\) with a plasminogen-containing fibrin substrate in a capillary tube as described previously.\(^9\) In this assay, there is a linear relationship between amount of activator and millimeters of lysis of the fibrin clot, when millimeters of lysis are expressed vs. activator on semi-log paper. UK (129 CTA U) was inhibited by incubation with the chloromethyl ketone in Pipes buffer, pH 7, at 25\(^\circ\)C for up to 8 hr. Typically, several aliquots of the inhibitor (10\(^{-6}\)M) were added at 2 or 3 hr intervals. Inhibition was determined after extensive dialysis of the inhibited preparation vs. 0.15M NaCl in comparison to UK that was incubated in Pipes buffer plus the diluent of the inhibitor, 10\(^{-6}\)M HCl, and also by dialysis vs. 0.15M NaCl. Tests of angiogenic activity were made by injecting purified active or inhibited UK plus carrier intrastromally, 20 \(\mu\)l per injection, as a bleb about 2 mm on center from the limbus into the corneas of 2 to 3.5 kg New Zealand albino rabbits. Each of 20 rabbits received active UK in one cornea and inhibited UK in the contralateral cornea. In some studies, pellets of ethylene-vinyl acetate\(^13\) containing active or inhibited UK and carrier were implanted 2 mm from the limbus by methods previously described.\(^14\) Eyes were examined daily by slit lamp, and photographs were made as appropriate.

Corneas were prepared for histologic study by fixing the tissues in 4% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 24 hr. Tissues were postfixed in 1% osmium tetroxide in the same buffer at 4\(^\circ\)C for 2 hr. Tissues were dehydrated in ethanol, treated with propylene oxide, and embedded in Epon-Araldite. One micrometer- thick sections were stained with methylene blue and azure II for light microscopy. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

Results

Intrastromal injections (four eyes) of pure UK alone (i.e., without carrier) caused local vessel engorgement, conjunctival and corneal stromal edema, and vessel ingrowth. Injection of RSA carrier alone (four eyes) caused no vessel engorgement, edema, vessel ingrowth, or iritis. In subsequent studies, 20 \(\mu\)l aliquots of active (3.7 CTA U) or inhibited (0.01 CTA U residual activity) pure UK in RSA carrier were placed by syringe and 30-gauge needle as intrastromal blebs approximately 2 mm on center from the limbus of contralateral corneas. As shown in Table I, vessels grew into the stromas of 16 of 20 corneas injected with active UK whereas 0 of 20 corneas injected with inhibited UK showed vessel ingrowth.

By 24 hr after the intrastromal injection of active or inhibited UK, the adjacent cir-

<table>
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<th>Treatment</th>
<th>No. of eyes</th>
<th>Vascularized</th>
<th>Not vascularized</th>
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<tr>
<td>UK</td>
<td>20</td>
<td>16</td>
<td>4</td>
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<tr>
<td>Inhibited UK</td>
<td>20</td>
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Table I

192 Berman et al.
Fig. 1. Macroscopic views of the response to injected plasminogen activator (UK). a, Sprouts first arose from the circumlimbal vessels beginning on the third day. (×4.) b, Higher magnification of limbal region showing sprouts. (×27.) c, New vessels grew into the cornea over the next several days, with growth perpendicular to the limbus. (×4.) d, Higher magnification showing progressing vessels. (×27.) e, Vessel regression was evident by the seventh day. (×4.) f, Higher magnification of indistinct, regressing vessels. (×27.) g, Corneas injected with inhibited UK showed no persistent vessel engorgement, conjunctival or stroma edema, or neovascularization. (×4.) h, Higher magnification of the limbal region of cornea that had received inhibited UK. (×27.) Arrows in paired pictures identify the same structures.
cumlimbal vessels had become engorged. By 3 to 4 days after the intrastromal injection, engorgement of the circumlimbal vessels had subsided in the eyes injected with inhibited UK. In the case of eyes injected with the active UK, however, the vessels remained engorged through day 5, after which vascular engorgement began to subside. In addition, conjunctival edema and mild stromal edema were observed in UK-treated eyes. Vascular sprouts (Fig. 1, a and b) in UK-treated eyes arose opposite the bleb site from the circumlimbal vessels (16 of 20 corneas), usually beginning on the third day. The new vessels (Fig. 1, c and d) grew into the cornea over the next several days, with growth perpendicular to the limbus and toward the region of UK injection. Subsequent vessel regression (Fig. 1, e and f) was evident clinically by the seventh day. It is not known why four of 20 corneas injected with active UK did not vascularize. Intrastromal blebs of UK were observed to form and resolve over the same time course as in corneas that responded to UK; so it would seem that the nonresponsive eyes had received the enzyme. Corneas that did not vascularize did not demonstrate persistent vessel engorgement or stromal edema either. None (0 of 20) of the corneas injected with inhibited UK (and carrier) showed persistent vessel engorgement or showed conjunctival or stromal edema, or neovascularization (Fig. 1, g and h). Clinically, neither corneas injected with active UK nor those injected with inhibited UK showed an inflammatory infiltrate.

Histologically, corneas that had developed vascular sprouts contained lymphocytes, plasma cells, mononuclear cells, and polymorphonuclear leukocytes (PMNs) in the superficial stroma, between the bleb site and the adjacent limbus. PMNs were observed just in advance of the vessel tips (Fig. 2) and also in the more central stroma. The corneal stroma itself was edematous, and the epithelium demonstrated both intraepithelial and interepithelial cell edematous changes. With increasing vessel ingrowth, the cellular infiltrate diminished. Progressing stromal vessels (Fig. 3, a and b) were, however, still preceded by PMNs in the stroma. Vessels that had begun to involute were plugged with erythrocytes, and macrophages appeared to be phagocytizing degenerating vascular endothelial cells and erythrocytes. Fibroplasia was observed where vessels had been. Corneas that had received inhibited UK contained no histologically detectable blood vessels (Fig. 3, c) in agreement with the clinical observations (Fig. 1, g and h). Inflammatory cells were more numerous in the limbus of some corneas than others, but no inflammatory cells were seen within the corneal stroma proper (Fig. 3, c).

Initially, attempts were made to evaluate the ability of active UK to elicit vessel in-
growth into the corneal stroma by delivering UK from slow-release pellets. In preliminary experiments crude UK (1.5 CTA U/pellet; four eyes) caused a denser ingrowth of vessels than did UK previously inhibited by the specific chloromethyl ketone (0.09 residual CTA U/pellet; four eyes). Subsequently, pure active UK (3.7 CTA U/pellet; eight eyes) or inhibited UK (0.01 residual CTA U/pellet; eight eyes) in RSA carrier were incorporated into pellets and tested for angiogenic activity. Pellets of both types elicited weak vessel ingrowth, and it was not possible to discriminate between the effects of the active and the inhibited preparations. Thus it would seem that very low levels of
Fig. 4. Hypothetical events in neovascularization after the release of plasminogen activator in the cornea. Stage 1, Thermal burns of the “critical zone” of the cornea are thought to release plasminogen activator, (1) which like injected or implanted UK, diffuses to the circumlimbal vessels. As it diffuses peripherally, activator generates plasmin from plasminogen in the stroma (2), and plasmin activates Hageman factor (3), which, in turn, generates kallikrein from pre-kallikrein (4). Kallikrein, in turn, generates kinins from kininogens (5). Vessel engorgement, permeability leak, and stromal edema are thought to result from the actions of the engendered mediators on the vascular endothelium. Endothelial cell replication itself is thought to result directly from the mitogenic activity of plasminogen activator (6) or plasmin (7) or from kinins (5). Stage 2, Plasmin is thought to generate fragments chemotactic for PMNs from the third component of complement (8). As vessel ingrowth occurs to the top of the initial gradient of corneal plasminogen activator (or its more distal products), PMNs extravasate (9) and set up a second gradient of plasminogen activator–plasmin–dependent mediators in advance of the growing tip of the vessel (10).

active UK are still able to cause vessel ingrowth when released slowly into the stroma despite the fact (see above) that the same total units of activity (0.01 CTA U) given by injection are not angiogenic. The reasons why the results depend on the mode of delivery are not, as yet, understood.

Despite the current inability to discriminate clearly between the effects of active and inhibited UK delivered by pellets, the use of such slow-release devices to deliver UK might still be useful in the study of corneal vascularization. The observation in the current work, however, that a level of active UK (3.7 CTA U/pellet) which is only weakly angiogenic causes vessel ingrowth no greater than that caused by 0.3% that level of activity (0.01 CTA U/pellet), prompted the delivery of UK by injection in subsequent experiments, in attempts to demonstrate the de-
dependence of corneal neovascularization on active UK (see above).

Discussion

The observation in previous work\textsuperscript{9} that the normal rabbit cornea contains a zone of (presumed intracellular) plasminogen activator near the periphery suggested the possibility that the plasminogen activator–plasmin system has important roles in the cornea, including the regulation of vessel permeability and angiogenesis and the initiation of PMN chemotaxis.\textsuperscript{9} Injury to the cornea is thought, then, to result in the release of plasminogen activator into the extracellular space, with significant biological consequences. The results of the present study demonstrate that UK, the plasminogen activator derived from human kidney cells, is able to cause stromal edema and vessel ingrowth into the rabbit cornea as well as PMN infiltration of the corneal stroma after injection of activator into the cornea. The fact that edema, ingrowth, and PMN infiltration were prevented when the UK had been first inhibited irreversibly by a specific, small-molecular-weight, active site inhibitor indicates that the UK must be active enzymatically in order to cause the observed responses. Moreover, even though the relatively quick vascular ingrowth response (3 days) to UK would appear to preclude the requirement for an immune response to heterologous activator, the fact that potentially antigenic sites were probably still present in the inhibited enzyme (despite no vessel ingrowth) indicates that ingrowth is not in response to foreign antigens. The present work would suggest also the possibility that specific, active-site–directed, irreversible inhibitors can be used to prevent biologically important, plasminogen activator–dependent events in the cornea.

Plasminogen activators, including UK, appear quite specific in their known activities in that they activate plasminogen by cleaving a single arginine-valine bond in the plasminogen molecule.\textsuperscript{15} It is assumed that the ability of UK in the present work to cause vessel ingrowth is due either to the direct mitogenic effect of UK on vascular endothelial cells or to the effects of plasmin generated from plasminogen in the stroma and/or to other active molecules, e.g., kallikrein or kinin, which can be activated after plasmin is generated. Indeed, the long-held clinical view that edema is required for corneal vascularization\textsuperscript{16} might reflect the fact that plasmin, once generated by activator, in turn activates Hageman factor, which then activates prekallikrein to kallikrein and that the latter generates, from kininogens, kinins that are vasoactive and produce vessel engorgement and edema.\textsuperscript{10} The actual ingrowth of vessels, however, is thought to reflect the mitogenic activity of one or more of the mediators of the plasminogen activator–plasmin system. Thus, although neither plasmin nor more distal mediators have been shown to be generated in the cornea after UK injection, a hypothetical explanation for the clinical observations can be suggested on the basis of known, plasmin-dependent pathways\textsuperscript{10} (Fig. 4).

Although plasminogen activator (UK) was injected into the corneal periphery in the current study, it is thought possible that injuries to the peripheral cornea might cause the release of endogenous activator and thus initiate activator-dependent effects on the limbal vessels (Fig. 4). Indeed, Campbell and Michaelson\textsuperscript{17} have reported the presence of a "critical zone" near the limbus within which thermal burns must be administered for vessel ingrowth to occur. Maurice et al.\textsuperscript{18} have provided further evidence that a diffusible substance, vasostimulating factor (VSF), is released from the burned critical zone, and Eliason\textsuperscript{6} has reported that angiogenic factor activity can be obtained from cultures of corneal epithelium. UK itself is stable to boiling (M. Berman, unpublished observations), possibly reflecting, in part, its high cystine content\textsuperscript{15}, and it would seem possible that thermal burns to the critical zone release a heat-stable plasminogen activator that causes subsequent neovascularization.

It is noteworthy that peripheral thermal burns 2 mm from the limbus, but not central burns, also cause PMN infiltration, a high incidence of corneal ulceration, and the release of increased collagenase from fibro-
blasts as well as subsequent neovascularization.\textsuperscript{5,12} Perhaps thermal burns within the critical zone also result in the generation of factors chemotactic for PMNs which are thought to take part in ulceration.\textsuperscript{20} Plasmin is reported to generate fragments chemotactic for PMNs from the third component of complement\textsuperscript{21} and also to stimulate corneal fibroblasts in alkali-burned corneas to degrade corneal collagen.\textsuperscript{9} It would seem possible therefore that injury-induced release of plasminogen activator from the peripheral cornea (Fig. 4) (i.e., critical zone) could result in both PMN infiltration and ulceration as well as in subsequent vascularization and the arrest of ulceration.

The reports that neovascularization of the cornea is normally associated with an inflammatory cell infiltrate\textsuperscript{22} and that injected leukocytes\textsuperscript{7} or leukocyte extracts\textsuperscript{23} cause neovascularization have given rise to the hypothesis that leukocytes are necessary for neovascularization. Other studies in leukopenic animals\textsuperscript{6,24} have been interpreted to mean that leukocytes are not required for initial vessel ingrowth but do augment vessel ingrowth elicited by trauma to the cornea. In this regard, it is possible that it is the plasminogen activator of PMNs\textsuperscript{25} or activated macrophages\textsuperscript{26,27} which is responsible for the reported ability of those cells\textsuperscript{28} or their extracts\textsuperscript{7} to cause vessel ingrowth into the cornea.

The observation in other studies that initial rates of neovascularization are comparable in normal (control) and leukopenic animals but that vessel ingrowth rates then decrease relative to normal in leukopenic animals\textsuperscript{6,24} would suggest that neovascularization after injury to the cornea has two stages (Fig. 4): (1) leukocyte-independent and (2) leukocyte-dependent. In the initial stage released corneal plasminogen activator (VSF?) (or more distal mediators) could initiate vessel ingrowth by acting as mitogen directly on vascular endothelial cells. In leukopenic animals, the initial vessel ingrowth (stage 1) would still occur in response to the released activator. In control animals (nonleukopenic), leukocytes would enter the stroma in advance of the growing vessel (as observed in the current UK experiments) and would set up a new gradient of plasminogen activator–plasmin–dependent mediators (stage 2). In leukopenic animals, the second leukocyte-dependent phase would not occur, and vessel ingrowth would diminish.

Previous work has demonstrated that the plasminogen activator–plasmin system can initiate collagen degradation in corneal ulceration after alkali burns\textsuperscript{9,20} (Fig. 16 of ref. 9). The current study indicates that this same system can also initiate reparative events in the cornea. In this regard, the system is similar to that in plasma,\textsuperscript{10,30} in which activated Hageman factor initiates both events that lead to clot formation and events that lead to clot lysis.

\textbf{Note added in proof}

It has recently been observed independently that human plasminogen activator (urokinase) is angiogenic in the rabbit cornea. (R. H. Goldfarb: in Biology of Tumor Cell Invasion and Metastasis, Liotta L and Hart I, editors. Amsterdam, Martinus Nijhoff, Publishers [in press].)

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