Extrahepatic synthesis of plasminogen in the human cornea is up-regulated by interleukins-1 α and -1 β

Sally S. TWINING¹, Patricia M. WILSON and Chatri NGAMKITIDECHAKUL

Departments of Biochemistry and Ophthalmology, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI 53226, U.S.A.

The avascular cornea has limited access to plasma proteins, including plasminogen, a protein that is synthesized by the liver and supplied to most tissues via the blood. Recent experiments by others using plasminogen-deficient mice revealed the importance of plasmin, the active form of plasminogen, for the maintenance of the normal cornea and for corneal wound healing [Kao, Kao, Bugge, Kaufman, Kombrinck, Converse, Good and Degan (1998) *Invest. Ophthalmol. Vis. Sci.* **39**, 502–508; Drew, Kaufman, Kombrinck, Danton, Daugherty, Degen and Bugge (1998) *Blood* **91**, 1616–1624]. In the present experiments, plasmin was identified as a major serine proteinase in the human cornea. The major plasminogen and plasmin forms on non-reducing zymograms and Western blots had M_r values of 76×10^3 and 85×10^3 , with minor forms of M_r 200×10^3 , 135×10^3 , 68×10^3 and 45×10^3 . Angiostatin-like peptides with M_r s of 48×10^3 , 45×10^3 and 38×10^3 were observed which bound to lysine-Sepharose and reacted with anti-plasminogen monoclonal antibodies directed towards kringle domains 1–3 of plasminogen. The cornea contained $1.1 \pm 0.15 \mu\text{g}$ of plasminogen + plasmin/cornea, or $0.54 \pm 0.05 \mu\text{g}$ of plasminogen + plasmin/mg of pro-

tein. Cornea conditioned medium contained nine times the amount of plasminogen + plasmin that could be extracted from the cornea. These data suggested that corneal cells, unlike most extrahepatic cells, synthesize plasminogen. The synthesis of plasminogen by the cornea was confirmed by immunoprecipitation of metabolically labelled plasminogen, sequencing of its cDNA obtained by reverse transcriptase-PCR and inhibition of protein synthesis. Interleukins-1 α and -1 β stimulated corneal plasminogen synthesis 2–3-fold; however, interleukin-6 decreased corneal plasminogen synthesis by approx. 40% at early times after addition of the cytokine. By 24 h of culture, no differences were noted in the presence and absence of interleukin-6. Thus the cornea can synthesize plasminogen and regulate its synthesis in response to its environment, including cytokines induced in the cornea by injury and inflammation. Therefore the cornea can control the amount of plasminogen, the precursor of both plasmin and angiostatin.

Key words: angiostatin, inhibitors, interleukin-6, plasmin, serine proteinase.

INTRODUCTION

The cornea is a transparent, avascular tissue with no lymphatics and few resident immune cells [1]. This tissue is composed of three cellular layers: a multicellular epithelial layer on the anterior surface, a thick stromal layer composed of a sparse population of stromal cells plus extracellular matrix, and a monocellular endothelial layer. In the human cornea, the epithelial and stromal layers are separated by Bowman's layer, and the stromal and endothelial layers by Descemet's membrane. A regular arrangement of the collagen and proteoglycan molecules in the stroma allows light to pass freely through the extracellular matrix. In addition, the crystalline properties of transketolase and aldehyde dehydrogenase 3 found in corneal cells are important for corneal transparency [2,3]. Because diffusion of proteins in the stromal matrix is slow ($1.0 \times 10^{-1} \text{ mm}^2/\text{day}$ for albumin and $2.0 \times 10^{-2} \text{ mm}^2/\text{day}$ for IgG) [4,5], the limbal vessels at the cornea-scleral junction are probably not major sources of corneal proteins. Uptake of proteins from the tear film and aqueous humour does not occur in the normal cornea because of tight junctions located between epithelial cells on the anterior surface and between the endothelial cells on the posterior surface of the normal cornea [6,7]. Based on these facts, we hypothesized that corneal cells synthesize proteins that are normally synthesized by the liver and distributed to extrahepatic tissues by the circulatory system [8].

Plasminogen is one of the molecules that the liver synthesizes for export to other tissues. Not only is this molecule the precursor of the fibrinolytic proteinase plasmin, but it is also the precursor of the potent inhibitor of angiogenesis, angiostatin. This latter molecule is composed of the first four kringle domains of plasminogen [9]. The only extrahepatic cell reported to synthesize plasminogen is the rat brain microglia cell [10]. Because of the avascular nature of the cornea, we hypothesized that the cornea synthesizes plasminogen. Further, we hypothesized that this synthesis is altered by cytokines synthesized by human corneal cells in response to injury and infection.

The importance of the activated form of plasminogen, i.e. plasmin, in the cornea was demonstrated in experiments utilizing plasminogen-deficient mice [11,12]. The most prominent ocular effect of plasminogen deficiency was the occurrence of ligneous conjunctivitis; however, the corneas of these mice were also affected by the deficiency [11]. With increasing age of the plasminogen-deficient mice, vascularization, inflammatory infiltrates, corneal lesions and deposits of fibrin and periodic acid/Schiff-staining materials were noted. Healing of experimental corneal epithelial wounds was impaired in these mice, with severe fibrin deposition, persistent inflammatory responses, neovascularization and the formation of scars [12]. These defects observed in the non-wounded and wounded corneas of the plasminogen-deficient mice were dependent on the presence of fibrin. The

Abbreviations used: E-64, epoxysuccinyl-leucylamido-4-guanidinobutane; ϵ -AHA, ϵ -aminohexanoic acid; HMT, hexadecyltrimethylammonium bromide; IL, interleukin; IL-1ra, IL-1 receptor antagonist; MMP, matrix metalloproteinase; RT-PCR, reverse transcriptase-PCR; Tos-Lys-CH₂Cl, tosyl-lysylchloromethane ('TLCK'); Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator.

¹ To whom correspondence should be addressed (e-mail stwining@mcw.edu).

corneal abnormalities were not observed in mice deficient in both plasminogen and fibrinogen.

Little is known about the control of plasminogen synthesis. Liver specificity for plasminogen synthesis is associated with a recognition site for hepatic nuclear factor 1 within the 5' untranslated region and a nuclear factor similar to activator protein 3 in a region 2 kb from the mRNA cap site [13]. Examination of putative transcription factor binding sites in the promoter of plasminogen led to experiments showing that interleukin-6 (IL-6) up-regulates plasminogen mRNA synthesis and the expression of a reporter gene fused to the plasminogen promoter in hepatocarcinoma cells [8,14,15]. In addition, potential transcription factor binding sites are present in the plasminogen promoter for two types of IL-1-inducible transcription factors that are immediate-early response genes [8]: four activator protein 1 and three C/EBP (CCAAT enhancer binding protein) binding sites. This suggested that IL-1 α and IL-1 β , as well as IL-6, may be regulatory molecules for plasminogen synthesis. In rabbit corneal stromal cells, IL-1 α has been shown to be an autocrine regulator of corneal collagenase synthesis [16]. IL-1 α , but not IL-1 β , is synthesized in the normal mouse cornea; however, under ulcerative conditions, IL-1 β is found at much higher levels than IL-1 α [17].

In the present study, we show that plasminogen is a major serine proteinase present in the human cornea, that the human cornea synthesizes plasminogen, and that the synthesis of plasminogen by the human cornea is increased in response to IL-1 α and IL-1 β , but not IL-6.

EXPERIMENTAL

Human cornea extraction

Human corneas were obtained from the Eastern and South-eastern Wisconsin Lions Eye Bank (Milwaukee, WI, U.S.A.) and were used for the extraction of proteins and RNA and for organ culture. Experiments with these corneas followed the tenets of the Declaration of Helsinki (1989) of the World Medical Association, and were approved by the Human Research Committee of the Medical College of Wisconsin. The corneas were dissected from the globe, and the central 8 mm region was obtained using a trephine. This region did not include the limbal vessels. In some experiments, the corneas were further dissected into epithelial, stromal and endothelial layers. The epithelial layer was removed using a gill knife and the endothelial/Descemet's layer was removed by tear stripping, leaving the stromal layer.

Whole corneas and corneal stromal layers were frozen in liquid nitrogen and freeze-fractured by percussion. The resulting powder from the whole cornea was placed into 1 ml of 50 mM Tris buffer, pH 7.6, per cornea and homogenized with a ground glass tissue grinder. In some cases, hexadecyltrimethylammonium bromide (HTM) was added to the Tris buffer extracts at a final concentration of 0.5%. The corneal extracts were centrifuged at 13 000 *g* for 15 min to remove particulate matter. The supernatant fractions were stored at -80 °C until used. In some experiments 1 ml of TRI Reagent (Molecular Resources Center, Cincinnati, OH, U.S.A.) was added to the powder from the stroma, and 200 ml of TRI Reagent was added to the endothelial and epithelial layers. The tissue was then homogenized and the proteins isolated according to the manufacturer's protocol.

Human corneal organ culture

Whole human corneas were cut into eight pieces, weighed and then placed into 8 ml of minimum essential medium prepared with endotoxin-free water and containing 100 units/ml penicillin

and 100 μ g/ml streptomycin (Life Technologies, Gaithersburg, MD, U.S.A.). In some experiments, individual pieces were incubated in 1 ml of the medium. The corneas were pre-incubated for 2 h and then cultured for 0, 2, 4, 5, 8, 15 or 24 h in the presence of 5 mg/ml lipopolysaccharide-free BSA (Pierce, Rockford, IL, U.S.A.) without or with one or more of the following: IL-1 α or IL-1 β at 1, 5 or 10 ng/ml, IL-6 at 5 ng/ml, and IL-1 receptor antagonist (IL-1ra) at 50 ng/ml (Promega, Madison, WI, U.S.A.).

Some corneas were cultured in methionine-deficient minimal essential medium (Life Technologies) containing 100 μ Ci of [³⁵S]methionine (Amersham, Arlington Heights, IL, U.S.A.). Selected corneas were pre-incubated for 2 h and then cultured for an additional 0, 2, 4, 8, 15 or 24 h in the absence or presence of IL-1 β . Other corneas were cultured for 36 h in the presence of *Pseudomonas* exotoxin A at 1 μ g/ml (generously donated by Dr. Barbara Iglewski, University of Rochester Medical School, Rochester, NY, U.S.A.) to stop protein synthesis. Corneas from the same individuals were paired as experimental and controls.

SDS/PAGE and zymography

SDS/8% (w/v)-PAGE was carried out using the method of Laemmli [18] without a reducing agent and without boiling. Zymography was performed using SDS/8% (w/v)-polyacrylamide gels containing 0.1% (w/v) casein, 0.1% casein plus 13 μ g of plasminogen, or 0.2% (w/v) gelatin, as previously described [19]. For visualization of both plasmin and plasminogen, 17.5 μ -units of urokinase-type plasminogen was added per ml of casein gel incubation buffer. Human plasminogen, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) were gifts from Richard Marlar (University of Colorado Health Sciences Center, Denver, CO, U.S.A.). Casein, gelatin and *M_r* standards were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Gelatinase A was purified from cornea conditioned medium using affinity chromatography on gelatin-Sepharose [20]. Standard curves of plasminogen (5–50 ng/well), uPA (2–25 μ -units/well), tPA (2–25 μ -units/well) and gelatinase A (2–25 ng) were included on the appropriate gels. The bands on the gels were digitized using a scanner (Microtek, E6, Redondo Beach, CA, U.S.A.), and the molecular sizes and density of the bands were calculated relative to *M_r* standards and the standard concentration curves for plasminogen, uPA, tPA and gelatinase A, using SigmaGel (Jandel, San Rafael, CA, U.S.A.). These plotted standard curves had *r* values of 0.94–0.99. All experimental samples were in the linear portion of the standard curves.

To identify the classes of the proteinases observed by zymography, the following proteinase inhibitors (Sigma Chemical Co.), at the given final concentrations, were pre-incubated with the corneal samples for 15 min prior to electrophoresis and were added to the gel incubation solutions used for digestion of the substrates following electrophoresis: PMSF at 10 mM, aprotinin at 1 μ g/ml, ϵ -aminohexanoic acid (ϵ -AHA) at 100 mM, tosyl-lysylchloromethane (Tos-Lys-CH₂Cl; 'TLCK') at 1 mM, tosylphenylalanylchloromethane (Tos-Phe-CH₂Cl; 'TPCK') at 1 mM, 1,10-phenanthroline at 5 mM, EDTA at 10 mM, pepstatin at 1 μ M, epoxysuccinyl-leucinylamido-4-guanidinobutane (E-64) at 10 μ M and chymostatin at 100 μ M. Control samples were incubated with an equivalent amount of solvent (water, DMSO, ethanol) used to dissolve the inhibitors.

To visualize metabolically labelled proteins, gels containing [³⁵S]methionine-labelled proteins were incubated in the presence of Amplify (Amersham) according to the manufacturer's instruc-

tions, dried and exposed to X-ray film (Kodak, Rochester, NY, U.S.A.).

Plasminogen/plasmin isolation from human cornea conditioned medium

Serum-free 24 h human cornea conditioned medium (10, 30, 40 or 50 ml) was passed over a 5 ml lysine-Sepharose column (Sigma). The column was washed sequentially with 20 ml of 50 mM sodium phosphate buffer, pH 7.4, containing 5 mM EDTA, 1 mM benzamidine and 1 μ M E-64, 10 ml of the same buffer with inhibitors plus 50 mM NaCl, 10 ml of the same buffer with inhibitors plus 500 mM NaCl, and then 5 ml of the initial buffer. The specifically bound proteins were eluted with 3–5 ml aliquots of the initial buffer containing 200 mM ϵ -AHA. The eluted proteins were separated further on a 1.5 mm SDS/polyacrylamide gel and the proteolytic bands were excised with a razor blade and electroeluted using a Bio-Rad electroeluter (Bio-Rad, Hercules, CA, U.S.A.). The electroeluted samples were concentrated using a 30×10^3 - M_r spin concentrator (Amicon Inc., Beverly, MA, U.S.A.).

Protein determination

The amounts of protein in the corneal extracts and the cornea conditioned medium were determined by the Lowry method [21].

Western blots

Human cornea conditioned medium, the supernatant fractions from corneal extracts and eluant fractions from the lysine-Sepharose column were electrophoresed on 8% (w/v) polyacrylamide gels. The proteins were transblotted to nitrocellulose membranes (0.2 μ m; Schleicher and Schuell, Keene, NH, U.S.A.). The blots were blocked with 1% (w/v) non-fat dried milk (Nestle USA, Inc., Solon, OH, U.S.A.), washed and probed as previously reported [22]. The primary antibody was one of the following: rabbit anti-(human plasminogen) IgG (CalBiochem, Hercules, CA, U.S.A.), mouse monoclonal anti-(human plasminogen kringle 1–3) (Enzyme Research Lab, South Bend, IN, U.S.A.), rabbit anti-(human α_2 -antiplasmin) (Athens Research Technology, Athens, GA, U.S.A.) or mouse monoclonal anti-(human α_2 -macroglobulin) (Medix Biotech, Foster City, CA, U.S.A.). The secondary antibody was horseradish peroxidase conjugated to either goat anti-(rabbit IgG) (Bio-Rad) or goat anti-(mouse IgG) (Kirkegaard and Perry Lab., Gaithersburg, MD, U.S.A.). Control blots were incubated with either rabbit IgG (Sigma Chemical Co.) or mouse IgG (Serotec, Inc., Raleigh, NC, U.S.A.) rather than the primary antibody. The immunospecific bands were visualized using a luminol-based chemiluminescent ECL* detection system (Amersham) and Biomax film (Kodak).

Immunodot blots

Human corneal supernatant fractions and cornea conditioned media were loaded on to nitrocellulose membranes using a 96-well dot-blot apparatus (Schleicher and Schuell). Each sample was assayed in triplicate. A plasminogen standard curve from 1 ng to 100 ng in minimum essential medium was also applied in triplicate. The dot blots were probed in the same manner and with the same antibodies as the Western blots. The bound horseradish peroxidase-labelled antibodies were reacted with luminol (ECL* detection system, Amersham). The luminescence of the dots was recorded on preflashed Biomax film (Kodak). Non-specific control blots were treated with non-specific rabbit

IgG (Sigma Chemical Co.). The labelled dots were quantified using an ELISA plate reader (BioTek Instruments, Winooski, VT, U.S.A.) with a 530 nm filter. The values for the samples were averaged and the S.D. was determined. The standard curves for plasminogen were linear, with $r = 0.95$ – 0.99 . All experimental samples were within the linear range of the standard curves.

Immunoprecipitation of plasminogen

Plasminogen was immunoprecipitated from human cornea conditioned medium as described previously for α_1 -proteinase inhibitor, with the following modifications [23]. Rabbit anti-(human plasminogen) antibodies (5 μ g/200 μ l of 50 mM Tris buffer, pH 7.5, containing 150 mM NaCl) were bound to the wells of 96-well Durapore plates (Millipore, Bedford, MA, U.S.A.). Control wells received non-specific rabbit IgG at the same concentration as the specific IgG. Then, 100 μ l of [35 S]-methionine-labelled cornea conditioned medium was added per well, followed by incubation for 2 h at room temperature.

Reverse transcriptase-PCR (RT-PCR), cloning and sequencing of corneal plasminogen cDNA

Total RNA was isolated from human cornea epithelial cells scraped from human corneas using TRI Reagent (Molecular Research Center) according to the manufacturer's procedure. Because of the high similarity of the kringle regions of plasminogen to apolipoprotein (a), primers in the less similar proteinase domain region of human plasminogen [positions 1656–1673 (forward; 5'-TGATGGTGATGTAGGTGG-3') and 2383–2400 (reverse; 5'-AAGACCCCAAGAAGTGAC-3')] [24] were obtained from Life Technologies. RT-PCR was performed using GeneAmp kit (Perkin Elmer, Foster City, CA, U.S.A.). The reverse transcriptase reaction was carried out by incubation at room temperature for 10 min, at 42 °C for 20 min and at 99 °C for 5 min. PCR was performed using five cycles of 94 °C for 1 min, 42 °C for 1 min and 72 °C for 1 min, and then 30 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min. The PCR product was cloned into pGEM-Easy vector (Promega) and sequenced completely in both directions using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit and an ABI PRISM™ Sequencer (Perkin-Elmer).

Statistical analysis

SigmaStat (Jandel Scientific) programs for ANOVA and Student–Newman–Keuls Test were used for comparison of proteinase activity in the presence and absence of cytokines.

RESULTS

Plasminogen is found in human corneas

The major proteinase bands in extracts of whole human corneas, individual layers of the cornea and whole cornea conditioned medium were observed by casein zymography as bands of M_r (76–85) $\times 10^3$ (Figure 1). Less prominent proteinase bands of M_r 135 $\times 10^3$ and 68 $\times 10^3$ were observed when corneas were extracted using the detergent HTM or the organic solvent mixture in TRI Reagent (Figure 1). A minor band with an M_r of 45 $\times 10^3$ was observed in some extracts (Figures 1 and 2). Because these proteins were electrophoresed under non-reducing conditions and were not boiled, the M_r values are not true molecular masses.

The bands with M_r s of 135 $\times 10^3$, 85 $\times 10^3$, 76 $\times 10^3$ and 68 $\times 10^3$ were identified as serine proteinases with a specificity for positively charged amino acids, based on inhibitor studies using casein zymography (Figure 3). These bands were inhibited by the

Figure 1 Casein-degrading proteinases in human corneal extracts and whole cornea conditioned medium

Whole human corneas or separated layers (Epi, epithelium; Str, stroma; Endo, endothelium) were extracted directly in 50 mM Tris buffer, pH 7.6, without or with 0.5% (w/v) HTM or TRI Reagent. Whole human corneas were cultured for 24 h under serum-free conditions. The 27 000 g supernatant fractions of all extracts and conditioned medium were electrophoresed under non-reducing conditions on 8% (w/v) polyacrylamide gels containing 0.1% (w/v) casein and then processed for casein zymography. The gels were overloaded with respect to the $76/85 \times 10^3$ - M_r bands in order to visualize the 135×10^3 - and 45×10^3 - M_r bands.

Figure 2 Activation of corneal serine proteinases by uPA

Samples of plasminogen (20 ng), 5 h serum-free cornea conditioned minimal essential medium (10 μ l) and corneal extract in 50 mM Tris buffer, pH 7.6/0.5% HTM (45 μ g of protein) were separated on 8% (w/v) polyacrylamide gels containing 0.1% (w/v) casein. Following electrophoresis, the gels were incubated in casein gel incubation buffer without (b) or with (a) 17.5 μ -units of uPA/ml for 16 h. The proteinases were visualized by staining the gels with Coomassie Brilliant Blue.

serine proteinase inhibitors PMSF and aprotinin, but not by the metalloproteinase inhibitors 1,10-phenanthroline (Figure 3) or EDTA (results not shown), the aspartic proteinase inhibitor pepstatin or the cysteine proteinase inhibitor E-64 (Figure 3). In addition, these bands were inhibited by the lysine derivative inhibitors ϵ -AHA and Tos-Lys-CH₂Cl (Figure 3), but not by the aromatic amino acid derivative inhibitors Tos-Phe-CH₂Cl and chymostatin (results not shown). The same inhibitory pattern was observed for the proteinases present in the cornea conditioned medium (results not shown). Inhibition of the $68/76/85 \times 10^3$ - and 135×10^3 - M_r proteinases by the lysine derivative inhibitors suggested the possibility that at least one of the proteinase bands was plasminogen and/or plasmin.

If plasminogen were present in one or more of the active bands on the zymogram gels, it would be expected that incubation of these gels with uPA would increase the activity of the proteolytic bands. The 135×10^3 - and 45×10^3 - M_r bands were not enhanced

Figure 3 Characterization by inhibitor studies of the casein-degrading enzymes in corneal extracts and cornea conditioned medium

Corneas were extracted with 50 mM Tris buffer, pH 7.6, containing 0.5% HTM. These extracts were incubated for 15 min in the absence or presence of PMSF at 5 mM, aprotinin (Aprot.) at 1 μ g/ml, ϵ -AHA at 100 mM, Tos-Lys-CH₂Cl at 1 mM, 1,10-phenanthroline (Phen) at 5 mM, pepstatin (Pepst) at 1 μ M or E-64 at 10 μ M. Control samples were incubated with the solvents of the inhibitors. The mixtures were subjected to electrophoresis on SDS/polyacrylamide gels containing 0.1% (w/v) casein. Following electrophoresis, the Triton X-100 incubation solution and the casein incubation buffer also contained the inhibitors or their solvents alone. Propan-2-ol, DMSO or ethanol solvent control gels were similar to that with water (None).

upon uPA treatment of the gels, which indicated that these bands did not represent plasminogen, but were due to plasmin or other proteinases. The proteolytic activities of the bands with M_r s of $68/76/85 \times 10^3$ were increased upon incubation with uPA (compare Figures 2a and 2b), suggesting that they contained various forms of plasminogen. Subsequent gels were incubated with uPA to activate plasminogen.

The proteolytic activity with M_r s of $68/76/85 \times 10^3$ observed without uPA activation (Figure 2b) was due either to plasmin activated prior to electrophoresis or to activation of plasminogen by a small amount of uPA or tPA present in the electrophoresed samples that leached into the gel incubation buffer. Either of these scenarios are possible because the cornea contains both uPA and tPA; in addition, plasmin, produced by uPA activation of plasminogen, migrated as a triplet of bands with M_r s of $68/76/85 \times 10^3$. In this case, more of the 76×10^3 - M_r band and less of the 85×10^3 - M_r band were observed than for plasminogen activated following electrophoresis on the casein zymogram gels (results not shown).

If the observed active proteinase bands on the zymogram gels contained the first four kringle domains of plasminogen and plasmin, the proteinases would be expected to bind to lysine-Sepharose. When cornea conditioned medium was passed over a lysine-Sepharose column, the casein-cleaving enzymes bound specifically to the column (Figure 4b), whereas gelatinase A was eluted during the initial washes of the column (results not shown). The active bands with M_r values of $76/85/68 \times 10^3$ and 135×10^3 that were present in the conditioned medium, plus an additional active band of M_r 200×10^3 , were eluted with ϵ -AHA (Figure 4b). These bands on non-reducing Western blots, as well as the bands with M_r s of 38×10^3 and $43/45/48 \times 10^3$, reacted with rabbit polyclonal antibodies to human plasminogen (Figure 4a) and mouse monoclonal antibodies to kringle domains 1–3 of human plasminogen (results not shown). This suggests that the lower bands ($38/43/45/48 \times 10^3$) contain various forms of angiotensin. The antibodies used do not react with the homologous protein hepatocyte growth factor, nor with apolipoprotein (a), a high- M_r protein that contains multiple kringles which are homologous to plasminogen kringle 4 (80% identity) and a homologous non-proteolytically active proteinase domain (94% identity) [25].

Figure 4 Identification of the corneal serine proteinases as plasminogen/plasmin

Serum-free, 24 h cornea conditioned minimal essential medium (30 ml) was passed over a lysine-Sepharose column (5 ml). The column was washed with 50 mM phosphate buffer, pH 7.4, containing 5 mM EDTA, 1 mM benzamidine and 1 μ M E-64 (20 ml), and eluted sequentially with the same buffer containing inhibitors plus 50 mM NaCl, 500 mM NaCl (10 ml), and then 200 mM *e*-AHA (3 $\times$ 5 ml). Aliquots (20 μ l) of cornea conditioned (Cond.) medium and fractions eluted from the column were separated on SDS/polyacrylamide gels without or with 0.1% (w/v) casein (b). Following electrophoresis, the gels were developed by Western blot techniques using rabbit antibodies to human plasminogen (a), or for the detection of proteolytic activity (b). Results similar to those in (a) were obtained using mouse monoclonal antibodies to kringle 1–3 (results not shown). Control blots incubated with non-immune IgG instead of the specific antibody were negative (results not shown). An aliquot (95 μ l) of a 100 $\times$ concentrate of the *e*-AHA-eluted fraction was separated on preparative SDS/polyacrylamide gels. The 200 $\times$ 10³, 135 $\times$ 10³ and 76/85 $\times$ 10³ *M_r* bands were excised, electroeluted and concentrated 10 $\times$, and 20 μ l samples were separated on SDS/polyacrylamide gels containing 0.1% (w/v) casein (c).

The association of proteolytic activity with immunoreactive bands with *M_r*s of 200 $\times$ 10³, 135 $\times$ 10³, 85 $\times$ 10³ and 76 $\times$ 10³ suggests that these bands are probably different forms of plasminogen and plasmin. To explore this possibility, these bands were cut from a preparative SDS/PAGE gel, and the proteinases were electroeluted and re-electrophoresed on casein zymogram gels (Figure 4c). The 200 $\times$ 10³-*M_r* eluted proteinase band separated into three bands of proteolytic activity; one equal to the initial size, plus bands with *M_r*s of 135 $\times$ 10³, 76 $\times$ 10³ and 68 $\times$ 10³. The 135 $\times$ 10³-*M_r* proteinase band gave a band of the original size and a doublet with *M_r*s of 65 $\times$ 10³ and 68 $\times$ 10³. The 76/85 $\times$ 10³-*M_r* eluted bands produced bands at the initial *M_r*s of 76 $\times$ 10³ and 85 $\times$ 10³, a small amount of the 135 $\times$ 10³-*M_r* proteinase band and a band at an *M_r* of 68 $\times$ 10³. The bands of 135 $\times$ 10³ and 200 $\times$ 10³ did not increase in intensity on the zymogram gels upon incubation with uPA (results not shown). These results suggest that the 200 $\times$ 10³- and 135 $\times$ 10³-*M_r* bands are either plasmin dimers and trimers or complexes of plasmin with the major plasmin proteinase inhibitors found in the cornea, i.e. α_2 -macroglobulin and α_2 -antiplasmin [22,26]. To explore this latter possibility, the isolated bands were subjected to Western analysis using antibodies to the two inhibitors. Antibodies to the inhibitors did not bind to the proteinase bands with *M_r*s of 135 $\times$ 10³ and 200 $\times$ 10³, nor to potentially separated inhibitor bands (results not shown). The antibodies did bind to the inhibitors found in the initial cornea conditioned medium (results not shown). Active bands with *M_r*s of 200 $\times$ 10³ and 135 $\times$ 10³ were not observed upon electrophoresis of plasmin- α_2 -antiplasmin or plasmin- α_2 -macroglobulin complexes (results not shown). These results suggest that the larger-*M_r* proteinase bands at 135 $\times$ 10³ and 200 $\times$ 10³ are complexes that contain plasmin as dimers and trimers.

Quantification of plasminogen plus plasmin in human cornea and cornea conditioned medium

The amounts of plasminogen plus plasmin were quantified in the human cornea and human cornea organ culture medium by

immunodot-blot assays. The 76/85 $\times$ 10³-*M_r* forms accounted for about 95% of the plasminogen + plasmin activity present in the cornea and cornea conditioned medium (Figures 1 and 4a). The total immunoreactive plasminogen + plasmin was present at 1.1 ± 0.15 μ g/human cornea, or 0.54 ± 0.05 μ g/mg of protein (*n* = 10). Thus plasminogen plus plasmin represent approx. 0.05% of the extractable proteins. Corneas cut into eight pieces and placed into organ culture produced 9.6 ± 2.1 μ g of plasminogen + plasmin/24 h per cornea (*n* = 10). The 9-fold greater amount of plasminogen plus plasmin present in cornea conditioned medium compared with that extracted from the cornea suggests that most of the plasminogen and plasmin molecules in the cornea conditioned medium are newly synthesized.

Plasminogen is synthesized by the human cornea

To determine if plasminogen is synthesized by the human cornea, corneal proteins were metabolically labelled with [³⁵S]methionine. Antibodies to human plasminogen specifically immunoprecipitated [³⁵S]methionine-labelled bands from cornea conditioned medium that migrated with the same mobility as plasminogen at *M_r*s of 76 $\times$ 10³ and 85 $\times$ 10³ (Figure 5a). The distribution of labelled products between these two bands varied between samples, as observed in Figure 5(a) (compare lanes 2 and 4).

To explore further the synthesis of plasminogen, RT-PCR was used to produce a 745 bp cDNA of the proteinase domain of plasminogen from human corneal epithelial RNA. This plasminogen fragment was chosen for amplification because the primers hybridized with sequences with low sequence identity with other serine proteinases and the related proteins hepatic growth factor and apolipoprotein (a). In addition, the sequence of the plasminogen fragment diverged significantly from those of the related proteins. The amplified cDNA fragment was cloned and sequenced. The sequence of the cornea-derived protein was identical with that of the reported sequence of human plasminogen [24].

If the plasminogen and plasmin detected by zymography in the cornea conditioned medium are newly synthesized, it would be

Figure 5 Synthesis of [^{35}S]methionine-labelled plasminogen by human corneas (a), and inhibition of plasminogen synthesis by *Pseudomonas* exotoxin A (b)

(a) Plasminogen, metabolically labelled with [^{35}S]methionine, in 24 h cornea organ culture medium was immunoprecipitated using anti-plasminogen antibodies bound to Durapore membranes. Bound proteins were eluted with SDS sample buffer and then separated on SDS/polyacrylamide gels. The gels were incubated with Amplify, dried and exposed to X-ray film. (b) Whole human corneas were incubated in serum-free minimum essential medium without or with 1 $\mu\text{g}/\text{ml}$ *Pseudomonas* exotoxin A for 36 h. The medium was separated on 8% (w/v) SDS/polyacrylamide gels containing 0.1% (w/v) casein or 0.2% (w/v) gelatin. Following electrophoresis, the casein and gelatin gels were processed for proteolytic activity. Abbreviations: plgn, plasminogen; pln, plasmin; Ab, antibody.

expected that agents that inhibit protein synthesis should prevent the appearance of these molecules in the cornea organ culture medium. Plasminogen and plasmin were not found in the conditioned medium when *Pseudomonas* exotoxin A at 1 $\mu\text{g}/\text{ml}$ was added to corneal organ cultures (Figure 5b, lanes 1 and 2).

As expected, the inducible matrix metalloproteinase gelatinase B [27] was also absent from the culture medium of corneas cultured in the presence of *Pseudomonas* exotoxin A (Figure 5b, lanes 3 and 4). In contrast, gelatinase A, a matrix metalloproteinase that is constitutively synthesized by corneal stromal cells [27], was present in the medium of corneas cultured in the presence of the protein synthesis inhibitor (Figure 5b, lanes 3 and 4). Exotoxin A totally inhibited new protein synthesis, as determined by metabolic labelling of the corneal proteins with [^{35}S]methionine, with no significant loss of previously synthesized proteins, as determined by Coomassie Brilliant Blue staining of proteins separated on analytical polyacrylamide gels (results not shown). These data showed that the plasminogen in the bands of proteolytic activity with M_r s of 76/85 $\times 10^3$ observed by casein zymography represents newly synthesized plasminogen.

IL-1 α and IL-1 β , but not IL-6, modulate corneal plasminogen synthesis

Because plasminogen is required for the degradation of fibrin deposited in the cornea in response to corneal wounds and infections [12], inflammatory cytokines such as IL-1 and IL-6 would be expected to increase the synthesis of plasminogen by the cornea. Incubation of human corneas in the presence of either IL-1 α or IL-1 β increased the concentration of plasminogen in the conditioned medium, with a maximal response at 5 ng of IL-1 α (Figure 6a) or IL-1 β (results not shown). With 5 ng of IL-1 α , both the 85 $\times 10^3$ - and 76 $\times 10^3$ - M_r forms of plasminogen/plasmin were present, but were not observed on this gel as separate bands. At 1 and 10 ng of IL-1, the major band had an M_r of 76 $\times 10^3$. IL-1 β increased the concentration of plasminogen in the medium by 2–3 times over background levels, with the maximal increase at 8 h (Figures 6b and 6e). In contrast with its

Figure 6 Effects of IL-1 α (a), IL-1 β (b), IL-1ra (c) and IL-6 (d) on the levels of plasminogen/plasmin in whole human cornea conditioned medium

Whole corneas were cut into eight pieces, pre-incubated in serum-free minimum essential medium for 2 h and then further incubated as follows: (a) in the absence or presence of increasing amounts of IL-1 α for 5 h; (b) with 5 ng/ml IL-1 β for 0–24 h; (c) for 8 h in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of IL-1ra at 50 ng/ml, without (lanes 1 and 3) or with (lanes 2 and 4) IL-1 β at 5 ng/ml; and (d) with 5 ng/ml IL-6 for 0–24 h. The proteinases in the cornea conditioned media were separated on 8% (w/v) polyacrylamide gels containing 0.1% (w/v) casein and subjected to zymography. Similar results were obtained with three additional independent sets of corneas treated in the same manner. (e) The 68/76/85 $\times 10^3$ - M_r proteolytic activity of the plasminogen/plasmin bands in the presence or absence of IL-1 β (∇ , $n = 5$) or IL-6 ($\bullet$, $n = 4$) at 2, 4, 8 and 24 h in the presence and absence of IL-1 β were digitized and the levels determined relative to a standard curve for the individual enzymes. The levels in the presence of IL-1 β or IL-6 are expressed as percentages of control levels. The bars represent S.D. * $P < 0.01$ compared with control.

Figure 7 Stimulation of the synthesis of plasminogen by IL-1 β

[³⁵S]Methionine-labelled plasminogen was immunoprecipitated from serum-free cornea conditioned medium by rabbit anti-(human plasminogen) antibodies immobilized on Durapore membranes. Bound proteins were eluted with SDS sample buffer and then separated on SDS/polyacrylamide gels without (a) or with (b) 0.1% (w/v) casein. For detection of the radiolabel, the gel without casein was incubated with Amplify, dried and exposed to X-ray film. The casein-containing gel was processed for the detection of proteinase activity.

effects on plasminogen levels, IL-1 β did not alter the levels of either plasminogen activator (uPA or tPA) in the culture medium (results not shown). The effect of IL-1 β on the plasminogen concentration was much more rapid than that on the inducible proteinase gelatinase B. Gelatinase B was not observed until 24 h following the addition of IL-1 β into the cornea organ culture medium (results not shown), compared with 2 h for plasminogen (Figure 7).

The stimulation of plasminogen synthesis by IL-1 β was confirmed by immunoprecipitation experiments. Increased amounts of immunoprecipitated plasminogen/plasmin were observed at 2, 5 and 15 h compared with that in the control cornea conditioned medium (Figure 7a). Companion casein zymogram gels showed that the immunoprecipitated bands contained proteinase activity (Figure 7b). Similar results were observed for three additional sets of corneas.

To confirm the specificity of the effect of IL-1 β on corneal plasminogen levels, corneas were cultured in the presence and absence of IL-1 α . IL-1 α inhibited the increase in plasminogen and plasmin concentrations in the conditioned medium in the presence of IL-1 β (Figure 6c). In addition, this receptor antagonist inhibited the increase in plasminogen/plasmin in the control cultures, suggesting that IL-1 α and/or IL-1 β synthesized in the cornea is involved in controlling corneal synthesis of plasminogen under non-stimulated conditions.

In contrast with the effects of IL-1 α and IL-1 β on plasminogen concentrations in the cornea conditioned medium, IL-6 did not increase the amount of the 76/85 $\times 10^3$ - M_r forms at any time point over the 24 h culture period. The amount of plasminogen + plasmin in the IL-6-treated corneas was decreased by 40% at 2 and 8 h relative to control cultures, but not at 24 h (Figures 6d and 6e). This suggests that control of plasminogen levels in corneal cells differs from that in liver cells.

DISCUSSION

The major forms of plasminogen and plasmin present in the human cornea, or produced by the human cornea and released into the culture medium, were observed on casein non-reducing polyacrylamide gels with M_r s of 76 $\times 10^3$ and 85 $\times 10^3$. Plas-

minogen has an M_r of 93 $\times 10^3$ [28], but migrates under non-reducing conditions with a lower apparent M_r . Upon cleavage of the plasminogen activation site (Arg-561-Val-562) in the polypeptide that joins kringle 5 and the proteinase domain, the propeptide remains attached to the proteinase domain through a disulphide bond. This form migrates on casein zymography gels with the same mobility as plasminogen. This is probably because the samples are not boiled and are electrophoresed under non-reducing conditions. Under these conditions, the multiple disulphide bonds present in the four kringle domains of plasmin and plasminogen are intact. Plasmin can cleave both Glu-plasminogen and Glu-plasmin between Lys-77 and Lys-78 to release the pre-activation peptide. The corneal proteinase bands with M_r s of 85 $\times 10^3$ and 76 $\times 10^3$ are probably the Glu and Lys forms of plasminogen and plasmin.

Additional plasminogen/plasmin bands were detected with M_r s of 200 $\times 10^3$, 135 $\times 10^3$, 68 $\times 10^3$ and 45 $\times 10^3$. The reaction of the 68 $\times 10^3$ - M_r band with antibodies to plasminogen, its binding to the lysine-Sepharose column and the increase in activity upon incubation of the zymogen gels with uPA suggest that this proteinase band contains a degradation product of plasminogen missing the propeptide and kringle 1 regions. The 200 $\times 10^3$ - and 135 $\times 10^3$ - M_r bands are complexes that contain plasmin rather than plasminogen, since these bands did not increase in intensity upon reaction of casein gels with uPA. Upon re-electrophoresis, the isolated 200 $\times 10^3$ - M_r proteinase band produced bands with M_r s of 68 $\times 10^3$, 76 $\times 10^3$ and 135 $\times 10^3$, and the 135 $\times 10^3$ - M_r proteinase band produced bands with M_r s of 65 $\times 10^3$ and 68 $\times 10^3$. Antibodies to α_2 -antiplasmin or α_2 -macroglobulin did not react with the proteinase bands with M_r s of 200 $\times 10^3$ and 135 $\times 10^3$, or with other bands separated upon re-electrophoresis. Neither did plasmin complexed with either of these inhibitors generate active bands with M_r s of 200 $\times 10^3$ and 135 $\times 10^3$. These results suggest the 200 $\times 10^3$ - and 135 $\times 10^3$ - M_r forms are plasmin trimers and dimers.

The smaller proteolytically active and inactive bands that reacted with anti-(human plasminogen) antibodies are plasminogen degradation products. uPA did not alter the intensity of the 45 $\times 10^3$ - M_r band of activity, suggesting that this band is due to mini-plasmin. This form is a cleavage product of plasmin in which the peptide connecting kringle 5 and the proteinase domain is cleaved outside the double bond that links peptides A and B [29]. The binding of the proteolytically inactive small protein bands (M_r s of 38 $\times 10^3$, 45 $\times 10^3$ and 48 $\times 10^3$) that were eluted from the lysine-Sepharose column and the reaction of these bands with anti-plasminogen antibodies suggests that they are angiostatin-like plasminogen cleavage products containing kringles 1–3. These angiostatin-like molecules may play a role in the control of growth of vessels into the cornea.

The ability of cells to respond to injuries and infection is important to any tissue, but the response of the corneal cells is crucial in order to minimize the loss of transparency. Because of the avascular nature of the cornea, this tissue does not have normal access to blood proteins such as plasminogen. The present studies show that plasmin is a major serine proteinase in the human cornea and that the human cornea can synthesize the precursor, plasminogen. Although some mRNA for plasminogen or a related gene has been shown to be present in kidneys and testes [30], the only extrahepatic tissue shown previously to synthesize plasminogen is the brain; more specifically, microglia cells [10]. Because of the blood-brain barrier, the brain also synthesizes proteins that are normally synthesized by the liver for export to other tissues.

The regulation of corneal plasminogen synthesis by IL-1 α and IL-1 β is physiologically important in the cornea. First, in the

present experiments, plasminogen synthesis in organ culture is inhibited by IL-1 α . This suggests that IL-1 α and/or IL-1 β is involved in the induction of plasminogen synthesis when no exogenous cytokine is added. Secondly, the synthesis of IL-1 α and IL-1 β by corneal cells, as well as synthesis of IL-6 and other cytokines, is increased in response to experimental alkali burns, *Pseudomonas* keratitis and acute UV irradiation [17,31–33]. Although mRNAs for IL- α and IL-1 β are increased in response to infection, IL-1 α is increased marginally at the protein level, but IL-1 β is increased dramatically [17,34]. In response to alkali burns, both IL-1 α and IL-1 β are increased at both the mRNA and protein levels [32]. Thirdly, IL-1 regulates the levels of the potential target molecules of plasmin. IL-1 induces the synthesis of procollagenase [matrix metalloproteinase (MMP)-1], progelatinase B (MMP-9) and prostromelysin (MMP-3) by corneal stromal cells in culture [35]. These three proenzymes are activated by plasmin [29,36]. IL-1 α is required in an autocrine fashion for collagenase synthesis in corneal stromal cells [16].

IL-1 β induced the synthesis of plasminogen (2 h) much more rapidly than its effect on gelatinase B synthesis (24 h) in the human cornea organ culture system, suggesting an earlier role for plasminogen than for gelatinase B in wound healing. In plasma, fibrinogen circulates in a complex with four molecules of plasminogen, four molecules of α_2 -antiplasmin, four molecules of factor XIII (fibrin's cross-linking enzyme) and tPA [37]. The deposition of fibrin in the cornea occurs within 8 h of corneal wounding *in vivo* [38]. Because the synthesis of plasminogen is induced within 2 h of cornea organ culture, the co-deposition of plasminogen and fibrin is feasible. tPA or uPA was present in the cornea conditioned medium by 2 h of culture, suggesting that these plasminogen activators may also be associated with the fibrin deposits.

IL-6 partially inhibited the synthesis of plasminogen by the cornea at early time points, but by 24 h no difference was noted. This was in contrast with the up-regulation of the synthesis of plasminogen by hepatocytes in response to this cytokine [8,15]. Another protein normally synthesized by the liver for export to the tissues, α_1 -proteinase inhibitor, is also synthesized by the cornea [39]. Differences in the control of the synthesis of this protein are also observed between liver and cornea. IL-6 increases the synthesis of α_1 -proteinase inhibitor in liver, but not in cornea. IL-1 β increases α_1 -proteinase inhibitor synthesis in the cornea, but not in the liver. The effect of IL-1 β on plasminogen synthesis by the liver is not known.

The present studies show that the human cornea can regulate the level of plasminogen through local synthesis. This synthesis is stimulated by IL-1 α and IL-1 β , but not by IL-6. Through the control of plasminogen synthesis, the cornea can control its supply of plasmin and angiostatin. The actual concentrations of these bioactive molecules depend upon the presence of the appropriate activating enzymes. In addition, the active plasmin concentration depends upon the levels of α_2 -antiplasmin, α_1 -proteinase inhibitor and α_2 -macroglobulin.

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REFERENCES

- Scheiffarth, O. F., Stefani, F. H., Gabriel, N. and Lund, O. E. (1987) *Br. J. Ophthalmol.* **71**, 384–386
- Sax, C. M., Salamon, C., Kays, W. T., Guo, J., Yu, F. X., Cuthbertson, R. and Piatigorsky, J. (1996) *J. Biol. Chem.* **271**, 33568–33574
- Kays, W. T. and Piatigorsky, J. (1997) *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13594–13599
- Allansmith, M., de Ramus, A. and Maurice, D. (1979) *Invest. Ophthalmol. Vis. Sci.* **18**, 947–955
- Maurice, D. M. and Watson, P. G. (1965) *Exp. Eye Res.* **4**, 355–363
- Sugrue, S. P. and Zieske, J. D. (1997) *Exp. Eye Res.* **64**, 11–20
- Barry, P. A., Petroll, W. M., Andrews, P. M., Cavanagh, H. D. and Jester, J. V. (1995) *Invest. Ophthalmol. Vis. Sci.* **36**, 1115–1124
- Kida, M., Wakabayashi, S. and Ichinose, A. (1997) *Biochem. Biophys. Res. Commun.* **230**, 129–132
- Cao, Y., Ji, R. W., Davidson, D., Schaller, J., Marti, D., Sohndel, S., McCance, S. G., O'Reilly, M. S., Llinas, M. and Folkman, J. (1996) *J. Biol. Chem.* **271**, 29461–29467
- Nakajima, K., Tsuzaki, N., Takemoto, N. and Kohsaka, S. (1992) *FEBS Lett.* **308**, 179–182
- Drew, A. F., Kaufman, A. H., Kombrinck, K. W., Danton, M. J. S., Daugherty, C. C., Degen, J. L. and Bugge, T. H. (1998) *Blood* **91**, 1616–1624
- Kao, W. W. Y., Kao, C. W. C., Bugge, T. H., Kaufman, A. H., Kombrinck, K. W., Converse, R. L., Good, W. V. and Degan, J. L. (1998) *Invest. Ophthalmol. Vis. Sci.* **39**, 502–508
- Meroni, G., Buraggi, G., Mantovani, R. and Taramelli, R. (1996) *Eur. J. Biochem.* **236**, 373–382
- Malgaretti, N., Bruno, L., Pontoglio, M., Candiani, G., Meroni, G., Ottolenghi, S. and Taramelli, R. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1013–1018
- Jenkins, R. G., Seiffert, D., Farmer, R. J. and Miles, L. A. (1997) *Blood* **89**, 2394–2403
- West-Mays, J. A., Strissel, K. J., Sadow, P. M. and Fini, M. E. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6768–6772
- Hobden, J. A., Masinick, S. A., Barrett, R. P. and Hazlett, L. D. (1997) *Infect. Immun.* **65**, 2754–2758
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Twining, S. S., Schulte, D. P., Wilson, P. M., Zhou, X., Fish, B. L. and Moulder, J. E. (1996) *Biochim. Biophys. Acta* **1317**, 112–118
- Kjeldsen, L., Bjerrum, O. W., Hovgaard, D., Johnsen, A. H., Sehested, M. and Borregaard, N. (1992) *Eur. J. Haematol.* **49**, 180–191
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Twining, S. S., Fukuchi, T., Yue, B. Y. J. T., Wilson, P. M., Zhou, X. and Loushin, G. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**, 3226–3233
- Twining, S. S., Fukuchi, T., Yue, B. Y. J. T., Wilson, P. M. and Boskovic, G. (1994) *Invest. Ophthalmol. Vis. Sci.* **35**, 458–462
- Forsgren, M., Råden, B., Israelsson, M., Larsson, K. and Hedén, L. O. (1987) *FEBS Lett.* **213**, 254–260
- Byrne, C. D., Schwartz, K., Meer, K., Cheng, J. F. and Lawn, R. M. (1994) *Atherosclerosis Thromb.* **14**, 534–541
- Zhou, L., Sawaguchi, S., Twining, S. S., Sugar, J., Feder, F. S. and Yue, B. Y. J. T. (1998) *Invest. Ophthalmol. Vis. Sci.* **39**, 1117–1124
- Fini, M. E. and Girard, M. T. (1990) *Invest. Ophthalmol. Vis. Sci.* **31**, 1779–1788
- Petersen, T. E., Martzen, M. R., Ichinose, A. and Davie, E. W. (1990) *J. Biol. Chem.* **265**, 6104–6111
- Andreasen, P., Kjoller, L., Christensen, L. and Duffy, M. J. (1997) *Int. J. Cancer* **72**, 1–22
- Tomlinson, J. E., McLean, J. W. and Lawn, R. M. (1989) *J. Biol. Chem.* **264**, 5957–5965
- Hobden, J. A., Masinick, S. A., Barrett, R. P. and Hazlett, L. D. (1995) *Invest. Ophthalmol. Vis. Sci.* **36**, 1107–1114
- Kennedy, M., Kim, K. H., Harten, B., Brown, J., Planck, S., Meshul, C., Edelhauser, H., Rosenbaum, J. T., Armstrong, C. A. and Ansel, J. C. (1997) *Invest. Ophthalmol. Vis. Sci.* **38**, 2483–2491
- Fukuda, M., Mishima, H. and Otori, T. (1997) *Jpn. J. Ophthalmol.* **41**, 63–66
- Kernacki, K. A., Goebel, D. J., Poosch, M. S. and Hazlett, L. D. (1998) *Infect. Immun.* **66**, 376–379
- Girard, M. T., Matsubara, M. and Fini, M. E. (1991) *Invest. Ophthalmol. Vis. Sci.* **32**, 2441–2454
- Murphy, G., Ward, R., Gavrilovic, J. and Atkinson, S. (1992) *Matrix Suppl.* **1**, 224–230
- Chung, S. I., Lee, S. Y., Uchino, R. and Carmeliet, P. (1996) *Semin. Thromb. Hemostasis* **22**, 479–488
- Phan, T. M., Foster, C. S., Wasson, P. J., Fujikawa, L. S., Zagachin, L. M. and Colvin, R. B. (1989) *Invest. Ophthalmol. Vis. Sci.* **30**, 377–385
- Boskovic, G. and Twining, S. S. (1998) *Biochim. Biophys. Acta* **1403**, 37–46