
OVER-EXPRESSION OF A GELATINASE A ACTIVITY IN KERATOCONUS

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SUMMARY

Keratoconus is an ocular disorder in which the central cornea becomes thin, conical and frequently scarred. We are exploring the possibility that this condition is induced and maintained by proteases that exist in the corneal matrix in an activated form. In this study, the activities of the proteases secreted *in vitro* and *in vivo* by keratocytes of normal, clear keratoconic, scarred keratoconic and traumatically scarred corneas have been compared and partially characterised. Data obtained by assaying acyl transferase activity showed that the matrix metalloproteinases account for a minimum of 95% of the total protease secreted by cultured keratocytes. Their summated specific activity was consistently and significantly higher in the culture medium of keratoconic keratocytes than in the medium of other keratocyte cultures. Analysis of the individual protease activities secreted by these corneal keratocytes *in vitro* and *in vivo* by SDS-gelatin polyacrylamide gel electrophoresis showed that a gelatinase of molecular weight 65 000 is the major protease secreted by normal keratocytes. Whereas clear keratoconic and traumatically scarred corneal keratocytes secrete an additional activity of molecular weight 61 000, scarred keratoconic corneal keratocytes generally produced little or none of this gelatinase activity. Both activities may be ascribed to gelatinase A, and although the 61 000 molecular weight form may be a significant feature of keratoconus, neither appears to be active as secreted.

Keratoconus is a non-inflammatory corneal disorder that particularly affects young adults at a frequency of 1 per 1000 individuals¹ and is characterised by progressive thinning and scarring of the central cornea. At the onset of keratoconus, the induced irregular astigmatism may be corrected with contact lenses. Ultimately, a large proportion of keratoconic patients require corneal grafts to restore vision and

they represent a major group (approximately 20%) of patients who receive donor corneas.²

Ultrastructural characteristics of keratoconic corneas include fragmentation of Bowman's layer, fragmentation of the epithelial cell basement membrane and fibrillation of the anterior stroma.³⁻⁵ The possibility that these pathological features are a result of enhanced matrix metalloproteinase (MMP) activity has been investigated previously, but the published results are conflicting.⁶⁻⁹ Furthermore, in the studies that have found MMP activity to be greater in keratoconic corneas than in normal corneas, the possibility that this increase relates to the wound-healing and repair mechanisms, rather than a direct effect of the primary disease, was not clarified.

The aim of the present investigation was, therefore, to partially characterise the complement of proteases secreted by corneal keratocytes *in vitro* and *in vivo* and to assess their status in normal, keratoconic and scarred corneas.

METHODS

Experimental Material

Specimen corneas were either donated by Mr A. Tullo, Manchester Eye Hospital, or obtained from Bristol Eye Hospital Theatre and Eye Bank. To distinguish between inductive and secondary biochemical processes that correlate with the pathology of keratoconus, specimen keratoconic corneal buttons were divided into two groups comprised of either non-scarred (KCS) or scarred [KCS_(scar)] tissue. In addition, for comparative purposes, normal and traumatically scarred [Scar_(trauma)] corneal tissues were also utilised.

Cell Culture

Epithelial cell layers were stripped from their underlying corneal stroma prior to dicing these tissues in small quantities of Dulbecco's MEM growth medium supplemented with fetal calf serum

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(FCS, 10%), glutamine, glucose (Gibco) and antibiotic, antimycotic mixture (Sigma). The explants were seeded into 25-ml culture flasks and grown to confluence in the same medium (3 ml). On achieving confluence, the FCS was omitted from the culture medium for intermittent periods of 4 days. These media samples were collected for further processing.

Protease Preparations from Keratocyte Culture Media

Proteins secreted during the 4-day incubation period in serum-free media were precipitated with ammonium sulphate, added as a saturated solution in 10 mM potassium phosphate buffer, pH 7.2, to a final concentration of 70% (w/v). All solutions were left at 4 °C for a minimum period of 24 hours before centrifuging at 20 000 g for 1 hour at 4 °C. After decanting the supernatants, the pelleted proteins were redissolved in 0.01 M potassium phosphate, pH 7.2 containing 0.2 M NaCl and 10% (v/v) glycerol, and stored at either -20 °C (short term) or -85 °C (long term).

Protease Extraction from Corneal Stromal Tissue

Prior to extraction, the specimen corneal tissues were stored in liquid nitrogen. They were then pulverised in liquid nitrogen using a small ceramic mortar and pestle, and homogenised in 0.01 M potassium phosphate buffer, pH 7.4, containing 0.2 M sodium chloride. Homogenates were transferred to Eppendorf tubes and centrifuged for 10 minutes to pellet insoluble material. The supernatants were removed and, after the addition of glycerol (10% v/v, final concentration), were stored for assaying at -20 °C.

Enzyme Assays

Acyl transferase activity. All proteases possess acyl transferase activity and will catalyse the liberation of 4-nitrophenol from 4-nitrophenyl acetate. Substrate stocks (0.5 mM) were prepared in dimethyl sulphoxide (DMSO). The enzyme preparations were assayed against this substrate (0.025 mM) in 0.01 M potassium phosphate buffer, pH 7.2 containing 0.2 M sodium chloride in a total volume of 1 ml. The kinetics of nitrophenol production (calculated $E_{340} = 7620$) was followed spectrophotometrically at 37 °C.

Substrate gel electrophoresis. The gelatinases secreted by the various keratocyte cultures were separated and visualised after electrophoresis on polyacrylamide gels (8.5%) containing gelatin (1 mg/ml), as described by Unemori and Werb.¹⁰ The sample solutions contained the ionic detergent SDS (1% w/v) and glycerol (10% v/v), and were applied to the gels without boiling or reduction. After electrophoresis, the gels were incubated in Triton X-100 (2.5% v/v) for 30 minutes at 37 °C, rinsed in distilled water and then incubated for approximately 16 hours in 50 mM Tris HCl, pH 7.4, containing

5 mM calcium chloride and 0.02% (w/v) sodium azide. After rinsing with distilled water, they were then stained with Coomassie Blue [2.5% w/v in an aqueous solution of methanol (45%) and glacial acetic acid (10%)], and destained with aqueous methanol/acetic acid (5% and 7.5% v/v, respectively).

Protein Estimation

Protein concentration was estimated spectrophotometrically from the relationship $OD_{225} 9.18 = 1.0 \text{ mg/ml}$.¹¹

RESULTS

For the purposes of this comparative investigation of the proteases that are secreted by cultured corneal keratocytes and are present in intact corneal tissue, keratocyte cultures were prepared from 8 normal (age range 17–84 years), 4 non-scarred keratoconic (28–34 years), 4 scarred keratoconic (22–48 years) and 4 traumatically scarred (27–66 years) corneal buttons. In addition, the tissue used for direct enzyme extraction also included 8 normal (age range 52–83 years), 4 non-scarred keratoconic (22–46 years), 4 scarred keratoconic (18–25 years) and 4 traumatically scarred corneas (28–73 years). The proteases present in soluble protein preparations from the media of the cultured keratocytes and from intact corneal tissues were analysed and quantified by assaying against nitrophenyl acetate as substrate and by substrate (gelatin) gel electrophoresis.

The results obtained by assaying aliquots of the soluble protein extracts of either keratocyte culture medium or corneal stromal tissue, indicated that total protease activity was significantly higher in non-scarred keratoconic samples than in either normal or traumatically scarred samples (Figs. 1, 2). Furthermore, since activity was inhibited by metal ion chelators such as ethylenediaminetetraacetic acid (EDTA) to the extent of 95% at a concentration of 2.5 mM (Fig. 3) it was tentatively concluded that the majority of the secreted proteases belonged to the MMP family.

This possibility was supported by visualising individual activities on SDS-gelatin polyacrylamide gels. A typical zymogram showing the predominant gelatinase activities secreted by normal, KCS, KCS_(scar) and Scar_(trauma) corneal keratocytes *in vitro* is presented in Fig. 4. By incubating SDS-gelatin polyacrylamide gels of similar samples in assay buffer containing EDTA (1 mM) after electrophoresis, cleared bands failed to develop (data not shown).

From the summated activity data (Tables I, II), it was concluded that the predominant gelatinase activity secreted by normal corneal keratocytes was of molecular weight (M_r) 65 000, that KCS and

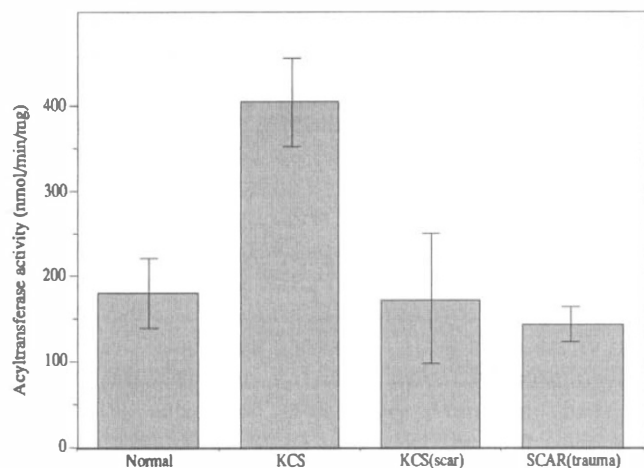


Fig. 1. Histogram of acyl transferase activity in protein preparations from serum-free media of confluent primary cultures of keratocytes derived from normal, KCS, KCS_(scar) and Scar_(trauma) corneal tissue.

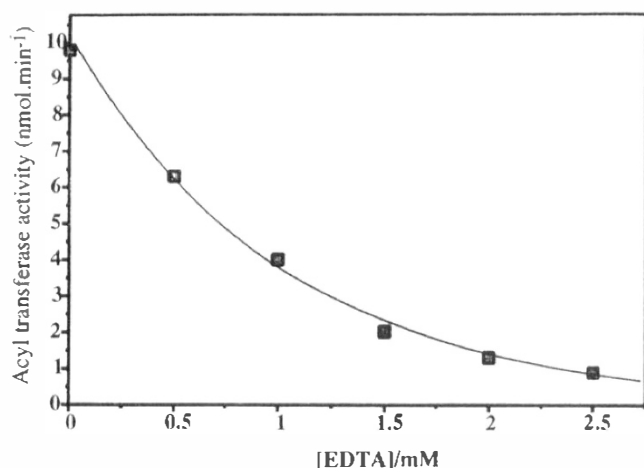


Fig. 3. Plot of acyl transferase activity against EDTA concentration. The relationship is logarithmic and the protein preparation assayed was obtained from the culture medium of KCS corneal keratocytes.

Scar_(trauma) corneal keratocytes produced an additional activity of M_r 61 000, and that KCS_(scar) keratocytes generally produced little or none of the latter gelatinase activity. Attempts to locate the gelatinase activities on gelatin polyacrylamide gels prepared and run in the absence of SDS, in various buffer systems, failed. The proteolytic enzymes, as secreted, apparently exist in an inactivated form.

Although the acyl transferase activity in all protein preparations was inhibited by EDTA, it was also found to be independent of added Ca^{2+} salts. By contrast, the gelatinase activities in these protein preparations, as detected after SDS-PAGE (polyacrylamide gel electrophoresis), required the addition of Ca^{2+} . Maximum activity was observed at calcium chloride concentrations above 1 mM. These observations support the suggestion that the proteases secreted by corneal keratocytes are predominantly MMPs. The metal ion that is held at the catalytic centre of all MMPs is Zn^{2+} . A second Zn^{2+}

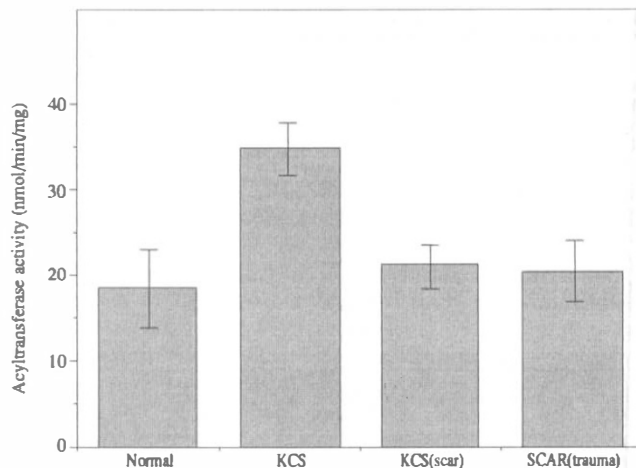


Fig. 2. Histogram of acyl transferase activity in the soluble protein fractions of stromal tissue obtained from normal, KCS, KCS_(scar) and Scar_(trauma) corneal buttons.

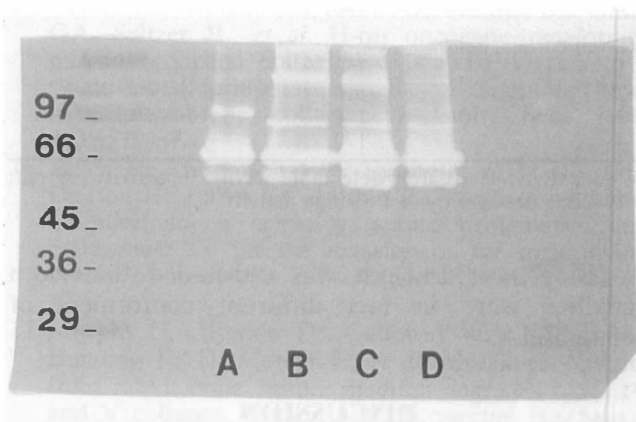


Fig. 4. Zymogram of gelatinase activities secreted by normal (lane A), KCS_(scar) (lane B), KCS (lane C) and Scar_(trauma) (lane D) keratocytes cultured in vitro. The running positions of the molecular weight ($\times 10^{-3}$) standards are marked. The protein samples were of similar concentration.

ion and Ca^{2+} are also integral components of the active protein.¹² Presumably, therefore, EDTA effectively displaces both Zn^{2+} and Ca^{2+} from these enzymes. Whereas Zn^{2+} appears to be involved in acyl transfer, Ca^{2+} either functions in a subsequent reaction in the catalytic mechanism or plays an important role in maintaining the active conformation of the enzyme.

Thiol reagents rapidly catalyse the decomposition of nitrophenyl acetate. It was not possible, therefore, to assess the effect of such reagents on acyl transferase activity. However, both mercaptoethanol and dithiothreitol (DTT) were found to be potent inhibitors of the 65 000 and 61 000 M_r gelatinase activities visualised after SDS-PAGE. Since thiol sensitivity¹³ is a property of gelatinase A (MMP-2) that is not shared by collagenase (MMP-1) or stromelysin (MMP-3), and since the 65 000 and 61 000 M_r activity bands co-migrated with major bands produced by an authentic sample of gelatinase

Table I. Protease activities secreted by keratocytes

Samples	Transferase activity (nmol/min/mg)	Gelatinase activity	
		65 000 kDa	61 000 kDa
Normal			
1	240	++	+/-
2	130	++	+/-
3	120	++	+/-
4	190	++	+/-
5	150	++	-
6	160	++	-
7	200	++	+
8	230	+	-
Keratoconus: KCS			
1	550	+++	+++
2	360	+++	+++
3	310	+	+++
4	400	+++	++
Scarred keratoconus: KCS _(scar)			
1	260	++	+
2	240	+/-	+/-
3	100	+/-	+/-
4	90	+/-	+/-
Scarred cornea: Scar _(trauma)			
1	150	+++	+++
2	110	++	+
3	144	+++	+++
4	170	+++	+++

-, no activity; +/- barely detectable activity; +, activity present; ++, moderate activity; +++, high activity.

A (Biogenesis Ltd), it was concluded that both activities were in fact different conformers of gelatinase A.

DISCUSSION

The results presented in this paper indicate that a single gelatinase of M_r 65 000 and characterised as gelatinase A (MMP-2) is the major protease secreted by normal corneal keratocytes both *in vitro* and *in vivo*. This finding is essentially in agreement with the observations of previous authors, with the exception of some variance in the estimated molecular weight of the enzyme from and between published data.^{7,14}

In initiating this investigation of the role that proteases, particularly the MMPs, may play in inducing keratoconus, the possibility was considered that the protease activities detected may relate to wound-healing processes rather than to the primary disease. Thus keratoconic corneal samples were divided into two groups, dependent upon whether they were clear or scarred. Additionally, traumatically scarred corneal samples were included in the study.

By comparison with the total protease activity secreted by normal keratocytes, the total protease activity secreted by KCS keratocytes, as determined by acyl transferase specific activity measurements, was high. This enhanced activity correlated with the appearance of an additional band of gelatinase activity of approximate M_r 61 000. This gelatinase species was also a component of the proteases

Table II. Protease activities in homogenised corneas

Samples	Transferase activity (nmol/min/mg)	Gelatinase activity	
		65 000 kDa	61 000 kDa
Normal			
1	19	+	—
2	16	+	—
3	19	+	—
4	11	+	—
5	22	+	—
6	23	+	—
7	15	+	—
8	20	+	—
Keratoconus: KCS			
1	37	+	+
2	38	+	++
3	32	+	+
4	31	+	+
Scarred keratoconus: KCS _(scar)			
1	21	+/-	+/-
2	24	+/-	+/-
3	22	+	++
4	17	+	+/-
Scarred cornea: Scar _(trauma)			
1	18	+	+
2	22	+	+
3	15	+	++
4	24	+	++

-, no activity; +/- barely detectable activity; +, activity present; ++, moderate activity; +++, high activity.

produced by Scar_(trauma) keratocytes but not, surprisingly, by KCS_(scar) keratocytes. Although the appearance of the 61 000 gelatinase activity in the KCS and Scar_(trauma) samples represents an abnormal situation as concerns corneal metabolism, it is, in fact, a well-documented form of gelatinase A.^{15,16} That this particular gelatinase activity may play a significant role in inducing keratoconus is an attractive proposition. Though it will not cleave interstitial collagens (types I, III), activated gelatinase A hydrolyses type IV collagen,^{17,18} the major structural element of all basement membranes; disruption of the epithelial cell basement membrane and of Descemet's membrane are pronounced pathological features in keratoconic corneas. The mechanism for generating the 61 000 M_r gelatinase A in keratoconic corneas is unknown. A number of previous authors have suggested that it represents the active form of gelatinase A generated from the higher molecular weight species by autocatalytic cleavage of the N-terminal domain upon removal of the specific MMP inhibitors known as TIMP-1 and TIMP-2.¹⁹⁻²² Although it has also been reported that both these inhibitors exist in culture medium of keratocytes derived from normal corneas, in association with a single gelatinase activity of M_r 68 000,¹⁴ and that their modification gives rise to increased gelatinolytic activity in keratoconic corneas,⁹ we have found no evidence for such an activation mechanism. Both the 65 000 and 61 000 M_r gelatinase A species appear to be secreted in corneal stromal tissue in inactive

forms; if the latter species were produced through lack of TIMP, then it is considered unlikely that it would be held in an inactive form by TIMP. Furthermore, both gelatinases are activated by SDS treatment followed by electrophoresis on SDS-gelatin polyacrylamide gels. While this treatment dissociates hydrogen-bonded proteins, it will not dissociate TIMP/MMP that is complexed by the formation of disulphide bridges. In the context of keratoconus, therefore, the presence of the 61 000 gelatinase A species does not signify necessarily that this enzyme species is responsible for inducing the condition, but the possibility remains that an endogenous component of the corneal stroma acts in a similar manner to SDS in selectively activating this enzyme.

In conclusion, the mechanism by which gelatinase A is activated and inactivated in keratoconic corneas is poorly understood and is the subject of current research endeavours.

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Key words: Cornea, Gelatinase, Keratoconus, Keratocyte, Matrix metalloproteinase (MMP)

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