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# Matrix metalloproteinase 2: involvement in keratoconus

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PURPOSE. *The activation of matrix metalloproteinase-2 (MMP-2) is postulated to be a crucial pathogenic factor behind progressive and chronic diseases in which basement membranes are disrupted. An ocular example is keratoconus. The purpose of the present enquiry was therefore to investigate and compare the activities of the MMP-2 secreted by keratocytes of normal and keratoconic corneas.*

METHODS. *The spectrum of MMP-2 activities secreted by cultures of keratocytes derived from normal and keratoconic corneas was analysed by zymography. Subsequently, selected preparations were assayed for peptidase activity, using Type I, Type III, Type IV and Type V collagen as substrate, under native conditions and after treatment with a variety of putative activating reagents.*

RESULTS. *Although MMP-2 of $M_r$ 65,000 on SDS gelatin polyacrylamide gels is the major protease secreted by keratocytes of normal corneas, the keratocytes of early-phase keratoconic corneas secrete an additional zymographic activity of $M_r$ 61,000. From their N-terminal amino acid sequences, both these proteins were shown to be conformers of proMMP-2. Treatment with SDS followed by protein fractionation was required to achieve* in vitro *activation of the MMP-2 secreted by normal corneal keratocytes. Treatment with SDS alone partially activated the enzyme produced by early-phase keratoconic corneal keratocytes. This procedure and autocatalysis, yielded an enzyme of $M_r$ 43,000 that selectively hydrolysed Type IV and denatured Type 1 collagen.*

CONCLUSIONS. *The zymographic gelatinase activities of apparent Mr 65,000 and 61,000 are conformers of corneal proMMP-2. Activated enzyme, of $M_r$ 43,000, is more readily generated from protein preparations of the culture media of early phase keratoconic corneal keratocytes than from protein preparations of the culture media of normal corneal keratocytes. (Eur J Ophthalmol 2000; 10: 215-26)*

KEY WORDS. *Gelatinase, Keratoconus, Corneal keratocyte, Matrix metalloproteinase (MMP)*

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## INTRODUCTION

The matrix - metalloproteinases (MMP's) are a family of closely related enzymes that exhibit differential substrate specificity and together are capable of completely degrading all extracellular matrices. Common features of these enzymes include cleavage of collagenous helices at Gly - Leu or Gly - Ileu loci (1), inclusion of $Zn^{2+}$at the catalytic centre, and a requirement for $Ca^{2+}$ for activity (2,3). Additionally, the production and activities of these enzymes are under stringent control: They are all secreted as proenzymes that are activated by cleavage of an N-terminal peptide (4,5) and simultaneously or subsequently inhibited by co-secreted specific protein ligands known as the Tissue Inhibitors of the Matrix - metalloproteinases or TIMPS (6,7) MMP-2 is the matrix - metalloproteinase that will cleave Type IV basement membrane colla-

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gen, denatured Type I collagen (gelatin), Type V collagen, Type VI collagen and elastin (8). This enzyme is therefore postulated to be a crucial factor behind tumour cell invasion and metastasis (9,10) and a number of other chronic, progressive diseases that involve disruption of extracellular matrices. Such diseases may include the ocular disorder known as keratoconus. This is a non-inflammatory condition in which the central cornea of predominantly young adults becomes thin, conical and ultimately scarred. The primary pathology includes disruption of the epithelial basement membrane (11) and MMP-2 is known to be the major protease that is secreted by corneal keratocytes (12,13).

Despite the potential pathological importance of MMP-2, its mechanism of activation is not fully understood. In recent years, membrane bound MMPs (MT-MMPs) have been implicated as facilitators of MMP-2 activation either in conjunction with TIMP-2 (14) or with TIMP-2 acting as a regulator (15). However, with respect to activation *in vitro*, the methods that work reliably for MMP-I (collagenase) and MMP-3 (stromelysin) and include limited proteolysis by serine proteases or treatment with aminophenylmercuric acetate (APMA), p-chloromethoxy benzoate (p-CMB), sodium dodecyl sulphate (SDS) or oxidised glutathione, apparently do not work well for MMP-2 (16). There also exists conflicting data concerning the *in vivo* activity status of the MMP-2 activities visualised by zymography. Although the original and frequently quoted molecular weights of pro MMP-2 and activated MMP-2 are 72,000 and 66,000 respectively (1,17,18), there are many reports in which their given molecular weights are 66,000 and 62,000 respectively (19, 20). Since the discovery of MT-MMP, it has also been reported that proMMP-2 of $M_r$ 66,000 generates an intermediary proMMP-2 of $M_r$ 64,000 by cleaving the $Asp^{37}$ $Leu^{38}$ bond. In the absence of TIMP, this apparently facilitates the subsequent production of fully activated MMP-2 of $M_r$ 62,000 (14).

The primary aim of the present investigation is to determine whether MMP-2 is directly or indirectly involved in the induction of keratoconus or its pathological progression. To this end, in this paper, the activity status of the MMP-2 produced in culture by normal and keratoconic corneal keratocytes has been explored.

## MATERIALS AND METHODS

### *Experimental material*

Normal corneas that were unsuitable for transplant because of minor endothelial defects were obtained from the Bristol Eye Hospital CTS Eye Bank. Specimen keratoconic corneal buttons were obtained from the Bristol Eye Hospital Theatre. The latter tissue was classified as 'early-phase' or 'late-phase' according to the degree of scarring. This was assessed visually or taken from the patients' clinical records.

### *Tissue culture*

The following procedure was followed to prepare keratocyte cultures from normal and keratoconic corneas: Stromal tissue explants were obtained by dicing the corneas, stripped of epithelial cells, in small quantities of Dulbecco's MEM Growth Medium supplemented with foetal calf serum (FCS; 10% v/v), glutamine, (Gibco Ltd) and a penicillin, streptomycin, amphotericin B antibiotic antimycotic mixture (Sigma). They were then seeded into 25 ml. culture flasks and incubated at 36°C under 5% $CO_2$ / 95% air in the same medium (3 ml). Cell growth usually became apparent after 1 week. When appropriate, the keratocytes were subcultured from the primary cultures by trypsinisation. Routinely, this was carried out once only to obtain fully confluent keratocyte cultures of similar cell density. The method involved washing the cells with sterile, $Ca^{2+}$-free phosphate buffered saline and incubating with 0.8 ml Trypsin solution (Gibco Ltd) for 3-5 min at 37°C. The cells were then collected by centrifugation at 1,500 rpm for 3 min at room temperature, resuspended in the MEM Growth Medium and aliquoted into 4 - 6 culture flasks. Once the cells had attached to the flasks (approx 1h at 36°C) additional medium was added and changed at regular intervals (3 - 4 days).

### *Methods of harvesting the keratocyte secreted proteins*

Keratocytes that had achieved confluence were incubated alternatively in FCS containing medium and FCS - free medium. Both were collected and stored at -80°C before processing. The soluble proteins produced by the keratocytes in serum free medium were routinely collected by precipitation with $(NH_4)_2SO_4$ (fi-

nal concentration 70% w/v), followed by centrifugation and resuspension in 0.01M potassium phosphate buffer pH 7.2 containing 0.2M NaCl and 10% (v/v) glycerol. Alternatively, the harvested culture media were diluted 6 - fold with 0.01M potassium phosphate buffer pH 7.2 and eluted through a 20 x 2.0 cm column of the CM-cellulose (Whatman CM 52) packed and equilibrated in the same buffer at a flow rate of 30 ml. $h^{-1}$. Fractions were collected, monitored for protein at OD 254 nm and assayed for MMP activity using 4 nitrophenyl acetate as substrate. Most of the proteins constituting FCS washed through the column with the equilibration buffer. The bound proteins, eluted with 0.01M potassium phosphate buffer, pH 7.2 containing 2M NaCl, included the soluble, non crosslinked collagens and MMP-2.

### *Protein estimation*

For comparative purposes, all MMP samples were routinely electrophoresed or assayed at similar protein concentration. Because of the small quantities of sample, protein concentration was estimated spectrophotometrically from the relationship $OD_{225}$ 9.18 = 1mg $ml^{-1}$ (21). Briefly, the samples were diluted 20 - 40 fold with distilled water and read in reduced volume lcm path-length quartz cuvettes against similarly diluted growth medium or the buffer in which the proteins were dissolved.

### *SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-Polyacrylamide gels, for protein analysis, were cast and run according to the methods described by Laemmli (22). For separating and analysing the MMP-2 species secreted by the keratocytes, the acrylamide concentration in the gels was 8.5% (w/v). For analysing the collagens used to assay the keratocyte secreted MMP-2 preparations, the acrylamide concentration was decreased to 6% (w/v). All samples were loaded in SDS-gel electrophoresis buffer (22), and generally alongside marker proteins of known molecular weight (Sigma). Electrophoresis was performed at 75v. for approximately 2.5h. using the Biorad mini protein gel equipment. After removing the gels from their casts, they were stained with Coomassie Blue [2.5%w/v in an aqueous solution of methanol (45%) and glacial acetic acid (10%)] for a minimum period of 30 min and destained with aqueous methanol / acetic acid (5% and 7.5% v/v respectively).

### *Enzyme assays*

To assay and investigate the activity status of the MMP-2 secreted by keratocyte cultures derived from normal and keratoconic corneas, three different procedures were used: (i) Hydrolysis of 4-nitrophenyl acetate. All proteases possess acyl transferase activity and will therefore catalyse the production of nitrophenol from 4-nitrophenyl acetate. Since the reaction proceeds in the absence of peptidase activity, it was used to detect MMP-2 after eluting the keratocyte culture media through the CM-cellulose columns. The assay protocol has been described previously (13).

(ii) Substrate gel electrophoresis. The keratocyte secreted gelatinases were separated and visualised after electrophoresis at 75v for approximately 2.5 h. on 8.5% (w/v) polyacrylamide gels containing gelatin (1 mg $ml^{-1}$), as described by Unemori and Werb (23). 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 min at 37°C, rinsed in distilled water and then incubated for approximately 16 h in 50mM Tris HCl, pH 7.4, containing 5mM $CaCl_2$ and 0.02% (w/v) $NaN_3$. After rinsing with distilled water, they were then stained with Coomassie Blue and destained as described above. The MMP-2 activities appeared as cleared bands in a blue background.

(iii) Collagen hydrolysis: The hydrolysis of standard collagen preparations [Type I, (native and denatured), III, IV and V] was used to determine the specificity or action spectrum of the various keratocyte secreted MMP-2 samples. The collagens were dissolved in 0.01M acetic acid (4 mg $ml^{-1}$) at 37°C before adding an equal volume of 0.1M Tris HCl, pH 7.8 containing 5mM $CaCl_2$. Denatured Type I collagen was obtained by heating a sample of the solution of Type I collagen at 60°C for 30min.These collagens were assayed as possible substrates for the keratocyte secreted proteases by incubating aliquots (50 µl) with an equal volume of similarly buffered enzyme solution. Samples (15 µl) were removed with time and added to SDS-gel electrophoresis buffer (20 µl) containing mercaptoethanol (1% v/v). These were temporarily stored at -20°C and

analysed by SDS-PAGE subsequent to thawing and heating at 60°C for 30 min.

### *Amino acid sequence analysis*

This was carried out to determine the relationship between the $M_r$ 65,000/66,000 and $M_r$ 61,000/62,000 zymographic activities of MMP-2. For the purposes of this investigation these have been designated band A and band B respectively. The MMP-2 samples, obtained from the media of early-phase keratoconic corneal keratocytes, were electrophoresed under standard conditions without reduction with mercaptoethanol. As reference, rainbow coloured protein molecular weight markers (Amersham LifeScience) were included on these gels. The resolved proteins were subsequently blotted onto Immobilon psq membranes (Millipore), using 25mM Tris Glycine buffer, pH 8.3 containing 0.05% SDS and the Biorad Trans -blot SD semi dry transfer cell.

The Western blots were stained for 1 min with Coomassie Blue R-250 (0.1% in 40% methanol, 1% glacial acetic acid in $H_2O$), destained with 50% aqueous methanol, rinsed with distilled $H_2O$ and dried before excising the protein bands that corresponded to the zymographic 65/66,000 and 61/62,000 molecular weight species of MMP-2. The N terminal sequences of these proteins was obtained using an Applied Biosystems 477A Protein Sequencer.

## RESULTS

Unless otherwise stated, the experiments described were carried out using concentrated protein fractions of normal and keratoconic corneal keratocyte culture media. These were obtained by ammonium sulphate precipitation and obviously contain both MMP and TIMP (TIMP- 1 and TIMP-2). The relative amounts of the inhibitory ligands in these protein preparations therefore dictates the extent of MMP-2 activation possible in these preparations and the ability to measure the activity of the activated form, if present.

### *Characterisation of the zymographic MMP-2 activities secreted by normal and keratoconic corneal keratocytes in culture*

The major protease secreted *in vitro* and *in vivo* by keratocytes of normal corneas has been classified previously as inactive MMP-2 of apparent molecular weight 65,000 on SDS gelatin polyacrylarnide gels (13). The data presented in Figure 1 show the zymographic distribution of gelatinase activities secreted by representative

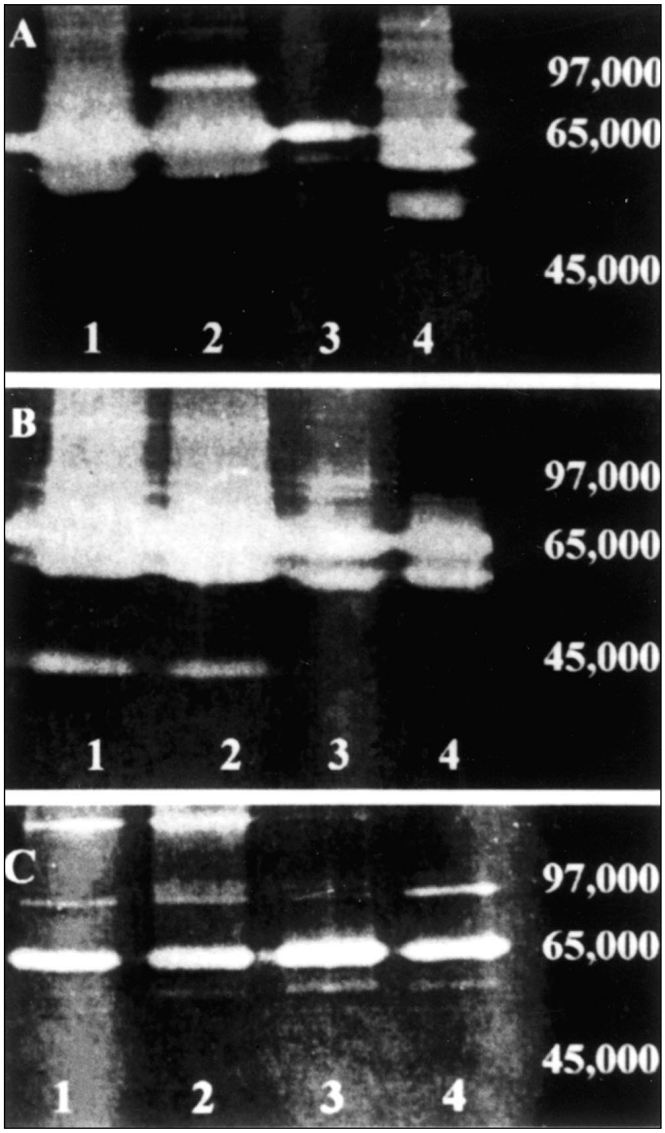


**Fig. 1 -** *Zymographic activity profiles of the gelatinases secreted by corneal keratocyte cultures. Samples of the following cultures were applied to the gels at similar protein concentrations: Normal primary keratocytes (Gel A lanes 2,3 and Gel C lanes 3,4), primary early-phase keratoconic keratocytes (Gel A lane 1 and Gel B lanes 3,4), primary late-phase keratoconic keratocytes (Gel C lanes 1,2), secondary cultures of normal and early-phase keratoconic corneal keratocytes (Gel A lane 4 and Gel B lanes 1, 2 respectively). The high molecular weight MMP activities (>65,000) may represent enzyme that has not dissociated from other proteins prior to electrophoresis.*

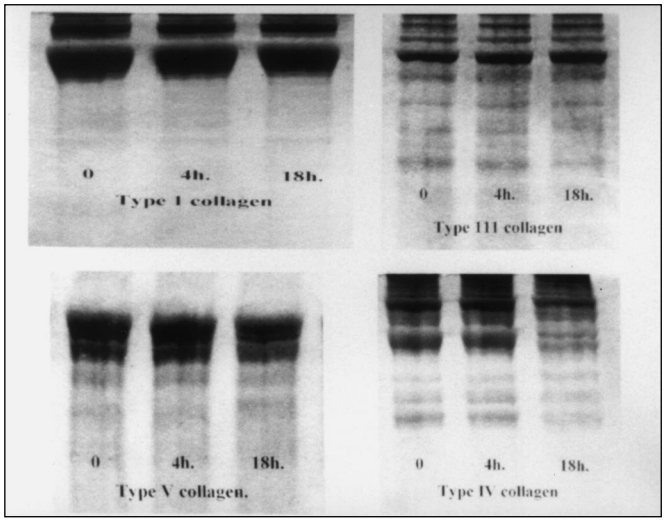


**Fig. 2 -** *SDS - PAGE analysis of Types I, III, IV and V collagens assayed against the MMP-2 secreted by a secondary culture of early-phase keratoconic keratocytes. The control Type IV collagen incubated for 18 h in the absence of enzyme was not degraded.*

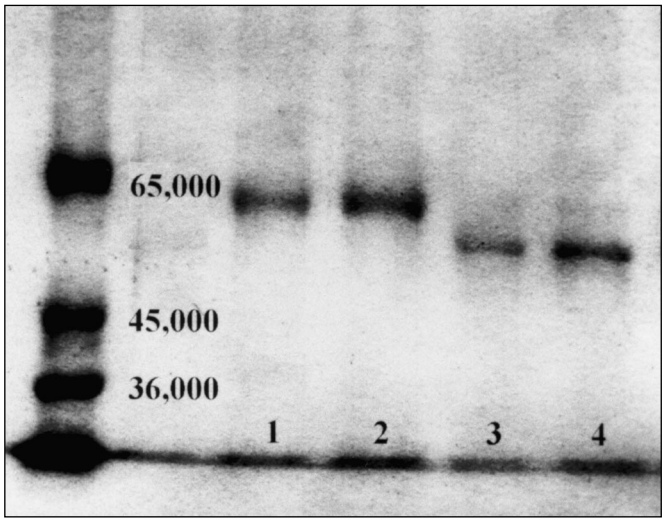


**Fig. 3 -** *Effect of mercaptoethanol on the electrophoretic mobility of corneal MMP 2 protein. In lanes 1 and 2 concentrated samples of an MMP-2 preparation obtained from the culture medium of an early-phase keratoconic keratocyte culture were heated in sample buffer containing SDS (0.1%w/v) and MSH (1.0%v/v) prior to electrophoresis. In lanes 3 and 4, samples of the same preparation were preheated in sample buffer containing SDS (0.1%w/v) only.*

primary cultures of keratocytes derived from normal and keratoconic corneas and also so-called secondary cultures of normal or keratoconic corneal keratocytes that had been subjected to metabolic stress by maintaining the cells in the same growth medium for several weeks.

An observation that early-phase keratoconic keratocytes produce an MMP-2 activity of apparent $M_r$ 61,000 in addition to the $M_r$ 65,000 activity has been reported previously (13). In addition to these activities, it was also found that the secondary keratocyte cultures produced activity bands corresponding to $M_r$ 55,000 and 43,000 (gel A, lane 4, gel B, lanes 1 and 2). Significantly, although all the enzyme preparations shown in Figure 1 were assayed against Types I, III, IV and V collagen, only those containing the activity bands corresponding to either $M_r$ 55,000 or 43,000 exhibited activity. Typical results are presented in Figure 2. Activity was directed against Type IV collagen specifically. Over an assay period of 18 h, Types I, III and V collagens were not detectably hydrolysed by these enzyme preparations.

### *Analysis of the corneal MMP-2 activities resolved by zymography*

Experiments in which samples of the keratocyte secreted protein preparations were preincubated with dithiothreitol (DTT) prior to electrophoresis on SDS gelatin polyacrylamide gels showed that this reagent completely inhibited visualisation of both the 65/66,000 (band A) and 61/62,000 (band B) activities at concentrations exceeding 2 mM (data not shown). Similar results were obtained using mercaptoethanol (MSH). Furthermore, when a particular sample of an early-phase keratococonic enzyme preparation, obtained by chromatography on CM-cellulose and expressing band B only on substrate gelatin gels, was boiled for 2 min in SDS-PAGE sample buffer (1:3 v/v) prior to electrophoresis on standard SDS-polyacrylamide gels, the protein bands detected depended upon whether or not MSH (1% v/v) was also included in the sample buffer. In the absence of this reagent, a single Coomassie blue stained band, corresponding to MMP-2 activity band B, was apparent. In its presence, a protein band corresponding to the apparently higher molecular weight activity, band A, was observed (Fig. 3). To resolve this apparently anomalous situation, it was postulated that bands A and B differed in conformation but not molecular mass. In support of this possibility, further experiments were carried out in which other keratoconic keratocyte enzyme preparations were electrophoresed in the absence or presence of MSH on both standard and gelatin polyacrylamide gels. After staining the resolved proteins and developing the zymographic gelatinase activities,

the gels were scanned with a densitometer (Biorad GS 156) to determine the relative intensities of the MMP-2 band A and band B protein stain and gelatinase activities. These data are presented in Table I. In addition to corroborating the initial observation that the protein migrating as band B activity migrated as band A under reducing conditions, they also showed that band A has intrinsically more gelatinase activity than band B which is putatively the activated form of MMP-2, freed of TIMP.

### *Attempts to activate the MMP-2 secreted by cultured corneal keratocytes*

*(i) By SDS Gelatin polyacrylamide gel electrophoresis (zymography):-* Information pertaining to the MMP-2 activity status *in vivo* is not provided by zymography. The procedure that was developed to visualise MMPs on gelatin-polyacrylamide gels effects separation of MMP-TIMP complexes and *in situ* activation of the applied proMMP-2. To achieve the latter, the appropriate use of Triton X-100 is essential. To determine which species of MMP-2 actually generates the zynographic activities, samples of an early-phase keratoconic corneal gelatinase preparation were subject to SDS-gelatin polyacrylamide gel electrophoresis. Without developing the gelatinase activities, excised sections of the gel, corresponding to the band A and band B activities of MMP 2, were incubated in the presence or absence of Triton X-100 for 30 min before electrophoresing again, under the same conditions, and developing the activity. The data obtained showed that without pretreating the gel slices with Triton X-100, the activity profiles did not change significantly. Band A remained as band A and band B as band B. By contrast, pretreatment with Triton X-100 not only enhanced the visualised activity considerably but effected a change in the activity profiles. Whereas the enzyme originally associated with band A appeared both to aggregate and to generate band B and the $M_r$ 43,000 gelatinase activity, the enzyme originally associated with the band B activity produced the $M_r$ 43,000 gelatinase activity without any indication of aggregation. Although these data are not shown, the results of similar experiments in which the Triton X-100 treated gel slices were further incubated for 2h at 37°C in 0.05M Tris.HCl, pH 7.3, or in the same buffer containing either iodoacetamide (1mM), dithiothreitol (1mM), phenanthroline (1mM) or EDTA (1mM) are given in Figure 4. These zymograms show that the original band A enzyme, when rerun on a second SDS gelatin polyacrylamide gel (Gel A) both aggregated and resolved into 5 other lower molecular weight activities ($M_r$ 65,000, 63,000, 61,000, 55,000 and 43,000). DTT inhibited the breakdown of band A, iodoacetamide prevented enzyme aggregation and EDTA and phenanthroline limited aggregation and the production of the $M_r$ 55,000 / 43,000 activities. By contrast when the isolated band B enzyme was rerun on a second SDS gelatin polyacrylamide gel, it generated the $M_r$ 43,000 activity predominantly (Gel B). While DTT inhibited the band B enzyme activity, all other treatments failed to inhibit

**TABLE I -** RELATIVE PROTEIN CONCENTRATIONS AND GELATINOLYTIC ACTIVITIES OF THE BAND A AND BAND B CONFORMERS OF CORNEAL MMP-2

| **SDS-PAGE** | **Corneal Enzyme Source** | | | | | |
|---|---|---|---|---|---|---|
| **Gel** | **Normal** | | **KCS (clear)** | | **KCS (scar)** | |
| **Scanned** | **-MSH** | **+MSH** | **-MSH** | **+MSH** | **-MSH** | **+MSH** |
| Protein Band A | 0.06 | 0.20 | 0.05 | 0.3 | 0.05 | 0.2 |
| Band B | 0.09 | - | 0.16 | - | 0.18 | - |
| Ratio A/B | 0.67 | | 0.31 | | 0.27 | |
| Gelatin Band A | 5.20 | ND | 5.60 | ND | 4.80 | ND |
| Band B | 0.60 | ND | 2.60 | ND | 0.50 | ND |
| Ratio A/B | 8.70 | ND | 2.20 | ND | 9.60 | ND |

ND: Activity not detectable in the presence of mercaptoethanol

the production of the $M_r$ 43,000 activity from band B.

*(ii) Using known MMP activating reagents:-* An accepted and standard procedure for dissociating MMP / TIMP complexes is to sequentially treat such protein preparations with 1mM DTT to reduce disulphide bridges, with 5mM iodoacetic acid (IA) to carboxymethylate free sulphydryl groups and hence prevent the MMP/ TIMP complexes from reforming, and finally with 1.5mM aminophenylmercuric acetate (APMA) to attack the bridged N-terminal cysteine residue and so induce cleavage of the inactivating peptide (24). The MMP-2 activity profiles produced by incubating an early phase keratoconic keratocyte - secreted enzyme preparations with these reagents sequentially and in various combinations, are presented in Figure 5. They show that treatment with DTT produced an activity band of $M_r$ 68,000, apparently at the expense of the $M_r$ 61,000 activity, that IAA alone had little effect but in combination with DTT restored the $M_r$ 61,000 activity, and that APMA alone or in combination with DTT and/or IAA produced an additional activity band of $M_r$ 63,000. All the variously treated enzyme samples were subsequently assayed against Type IV collagen. Though the results are not shown, none exhibited detectable peptidase activity. In the absence of a chemical mechanism for peptide bond cleavage by APMA (25) and in consideration of data already presented, it is suggested that these reagents had caused conformational changes in MMP-2 rather than peptide bond cleavage and activation following TIMP inactivation. TIMP-I and TIMP-2 are both secreted by corneal keratocytes (12, unpublished data,) and thus the failure to observe activity after treatment may indicate that the zymographically visualised activities are of the proenzyme. Evidence that this was the case was obtained by excising the protein bands corresponding to the band A and band B gelatinase activities from Western Blots of the MMP-2 obtained from the culture medium of clear keratoconic keratocytes, and determining their N-terminal amino acid sequences. Nine and ten sequencing rounds were carried out on the protein bands corresponding to the A and B gelatinase activities respectively. In both cases the data obtained fitted the sequence APSPIIKFP(G), which is that of the proenzyme (17).

*(iii) Using SDS, Triton X- 100 and chondroitin sulphate*: Experiments were carried out in which either SDS (0.5%w/v), Triton X-100 (0.5%v/v) or chondroitin sulphate (0.5%) was added to normal and early-phase

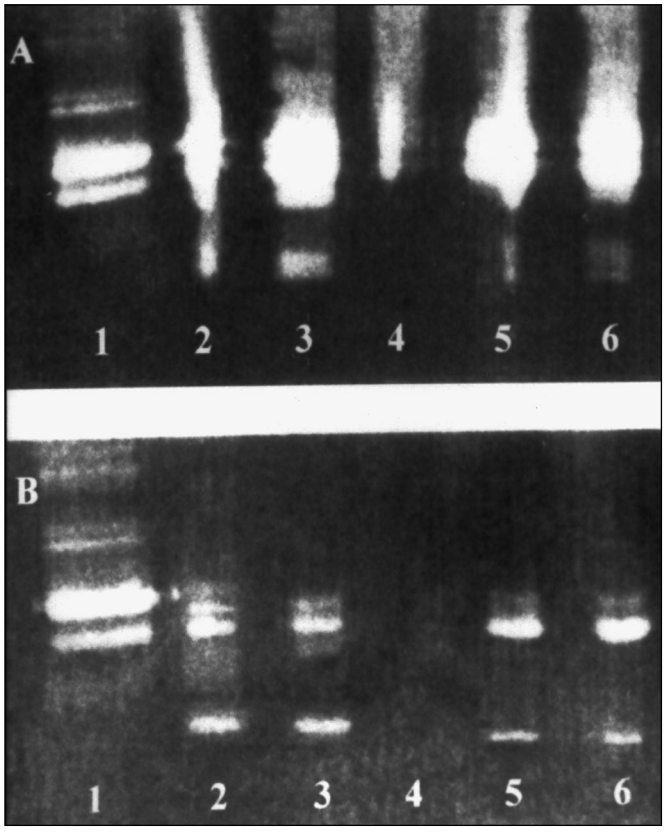


**Fig. 4 -** *SDS-gelatin PAGE analysis of the band A and band B MMP-2 activities produced by early-phase keratoconic corneal keratocytes. The zymographic activity profiles shown are of the original MMP-2 sample (Gels A and B, lane 1) and of those generated from the electrophoretically separated band A and band B proteins (Gel A and Gel B respectively) after incubation with Triton X-100 followed by Tris.HCl buffer alone (lane 2), by the same buffer containing iodoacetamide (lane 3), DTT (lane 4), phenanthroline (lane 5) or EDTA (lane 6).*

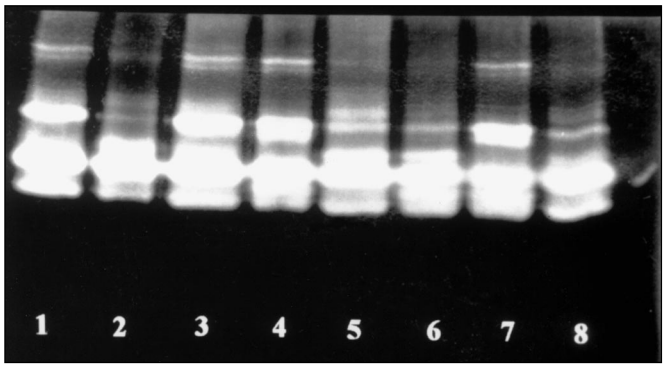


**Fig. 5 -** *Zymographic activity profiles of an early-phase keratoconic corneal MMP-2 preparation following treatment with MMP activating reagents. Lane 1 control; lane 2 - with DTT; lane 3 - with IA; lane 4 - with APMA; lane 5 - with DTT and IA; lane 6 - with DTT, IA and APMA; lane 7 - with IA and APMA; lane 8 - with DTT and APMA.*

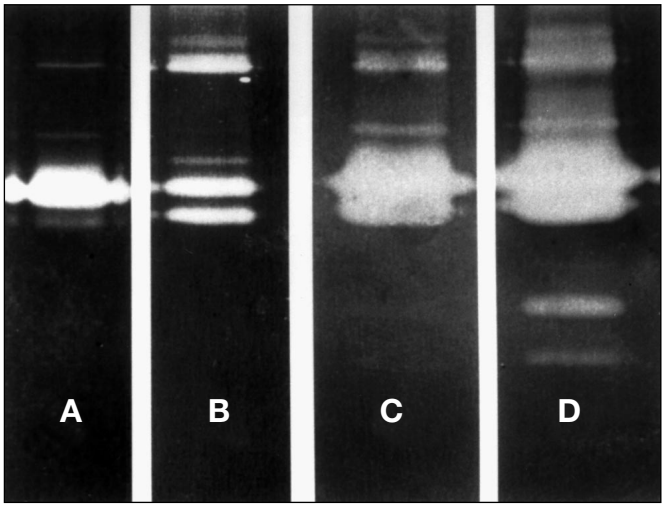


**Fig. 6 -** *Zymographic activity profiles of normal and early-phase keratoconic corneal MMP-2 preparations before and after treatment with SDS. Lanes A and B normal corneal keratocyte secreted MMP-2 preincubated in the absence and presence of SDS respectively. Lanes C and D - early-phase keratoconic keratocyte secreted MMP-2 preincubated in the absence and presence of SDS respectively. In the latter case only, gelatinase activities of $M_r$ 43.000 and 36,000 have been generated.*

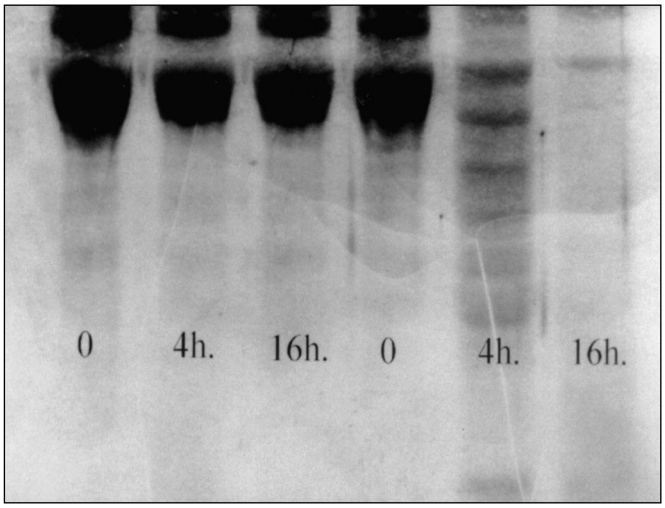


**Fig. 7 -** *SDS-PAGE analysis of native and denatured Type I collagen assayed against SDS - activated corneal MMP-2. LHS of Gel - Native Type I collagen. RHS of Gel - Denatured Type I collagen (gelatin).*

keratoconic corneal keratocyte MMP-2 preparations in 0.05M Tris HCl buffer, pH 7.2, containing $Ca^{2+}$ (5mM) and incubated for 60 min before centrifugation at 20°C in centricon concentrators (10,000 $M_r$ cut off). This was repeated twice and between washes, the samples were resuspended in 0.05M Tris HCl, pH 7.2 containing 5mM $CaCl_2$. The recovered proteins were then analysed by SDS-gelatin PAGE. The resultant zymograms showed that the gelatinase activity profiles of both enzyme preparations were unaffected by treatment with Triton X-100 and chondroitin sulphate (data not shown). In the case of the enzyme produced by normal corneal keratocytes, the SDS treatment resulted only in the production of band B from band A. In the case of the early-phase keratoconic keratocyte secreted enzyme preparation, in addition to the original bands A and B, the SDS treatment generated two extra activities of approximate $M_r$ 43,000 and 36,000 (Fig. 6). A sample of this preparation was subsequently boiled for 2 min with additional SDS (1% w/v) and mercaptoethanol (1% v/v) and run on an SDS-polyacrylamide protein gel. A protein corresponding to the band A activity and two other Coomassie Blue bands of low staining intensity but matching the $M_r$ 43,000 and 36,000 gelatinase activity bands were present in the resultant electrophoretogram (data not shown). A protein band corresponding to the band B activity was however, absent. From these observations it was again concluded that the appearance of the lower $M_r$ bands is associated with MMP-2 activation: When assayed against Type I and denatured Type I collagen, the SDS-treated enzyme preparation from early-phase keratoconic corneal keratocytes selectively hydrolysed the latter substrate (Fig. 7).

*(iv) By gel filtration on Sephadex G-75:* The protein and zymographic gelatinase activity elution profiles obtained by sequentially eluting a sample (0.6ml) of normal MMP-2 through a column of Sephadex G-75 (30 x 1.5cm) packed, equilibrated and eluted in 0.05M Tris HCl, pH 7.6, containing 5mM $CaCl_2$ and in the same buffer containing SDS (0.1%w/v), are shown in Figures 8 and 9 respectively. Under non-denaturing conditions, the enzyme was excluded from the column matrix and was thus either bound to other proteins (collagen, TIMP) or self-aggregated.The summated molecular weight of these aggregates was $>10^5$. Such aggregates were apparently disrupted by SDS. When chromatographed in the presence of this detergent, most of the gelatinase activity became included in the matrix and eluted within the apparent $M_r$ range of 65,000 - 40,000. This would be expected from the $M_r$ values determined by SDS-PAGE.

*(v) By autoactivation:* An MMP-2 preparation that was obtained from the medium of keratocytes derived from

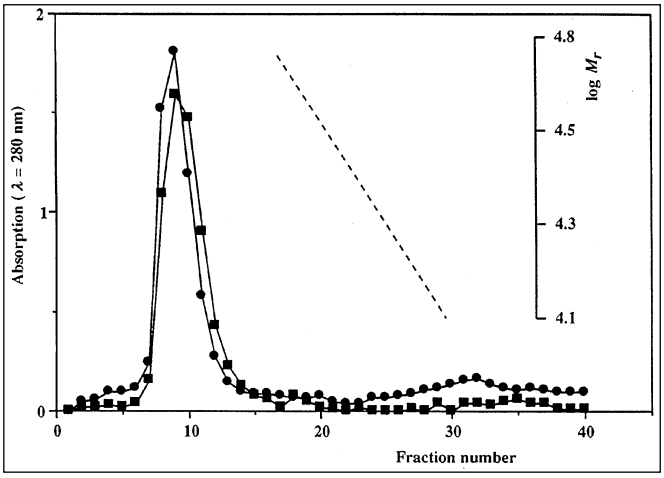


**Fig. 8 -** *Protein elution profile of a normal corneal MMP-2 preparation after chromatography on Sephadex G-75: The enzyme was eluted with Tris HCl buffer, pH 7.5, 5mM $CaCl_2$ (▪-▪) or with the sarne buffer containing 0.1% (w/v) SDS (•- •).*

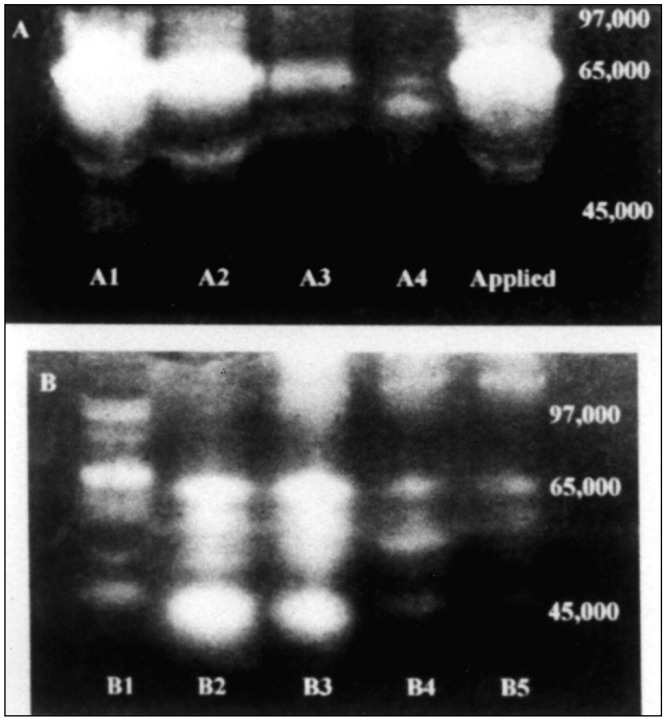


**Fig. 9 -** *Zymographic activity profiles of a normal corneal MMP-2 preparation fractionated on Sephadex G-75 in the absence (Gel A) or presence of SDS(Gel B). Lanes A1/ B1: excluded protein, fractions 7-11; lanes A2/ B2: fractions 14-16; lanes A3/B3: fractions 17-20; lanes A4/B4: fractions 28-34; lane B5: fractions 36-40.*

early-phase keratoconic corneas by elution through CM-cellulose was stored for approximately 6 months at -20°C. Before storage, this particular enzyme preparation exhibited predominantly the gelatinase activity band B on substrate polyacrylamide gels but was inactive when assayed against Type IV collagen. After storage, the preparation cleaved Type IV collagen (Fig. 10) but, instead of band B, exhibited predominantly the gelatinase activity of approximate $M_r$ 43,000 (Fig. 11).

Small quantities of this enzyme preparation were subsequently incubated at 37°C with aliquots of other normal and keratoconic keratocyte enzyme preparations in 0.05M Tris HCl buffer, pH 7.2 containing 5mM $CaCl_2$ (1: 50 ratio), and sampled over a period of 18h. These samples were added to SDS (final concentration 1% w/v) and snap frozen in liquid $N_2$ prior to analysis by zymography. The results obtained are shown in Figure 12. In the case of the preparations of MMP-2

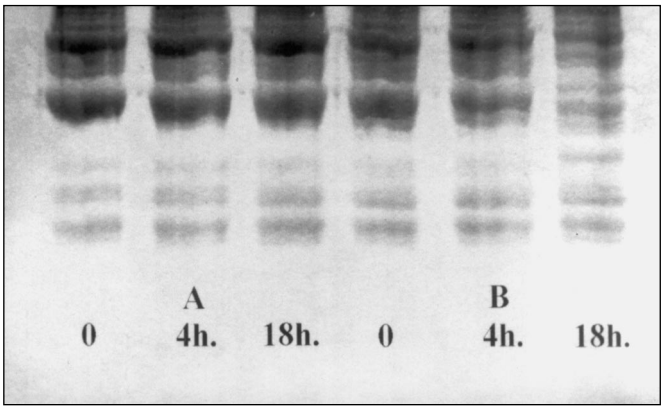


**Fig. 10 -** *SDS-PAGE analysis of Type IV collagen assayed against the MMP-2 produced by early-phase keratoconic corneal keratocytes. The enzymes assayed were of $M_r$ 61,000 (original preparation; A) and of $M_r$ 43,000 (preparation after storage at -20°C; B). In the latter case only was Type IV collagen degradation apparent.*

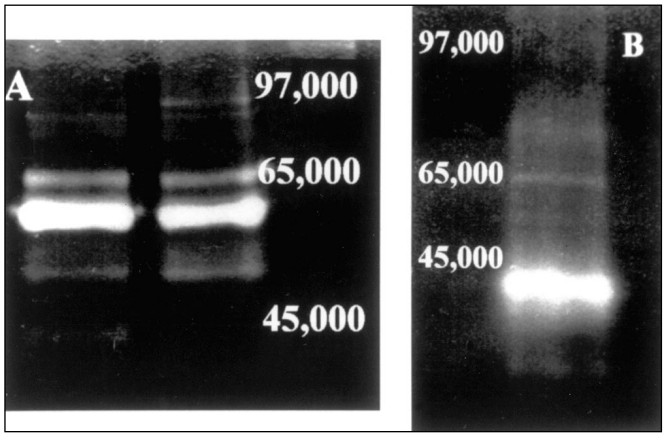


**Fig. 11 -** *Zymographic activity profiles of an MMP-2 preparation obtained from the medium of an early-phase keratoconic corneal keratocyte culture. These were analysed before (Gel A, in duplicate) and after (Gel B) prolonged storage at -20°C.*

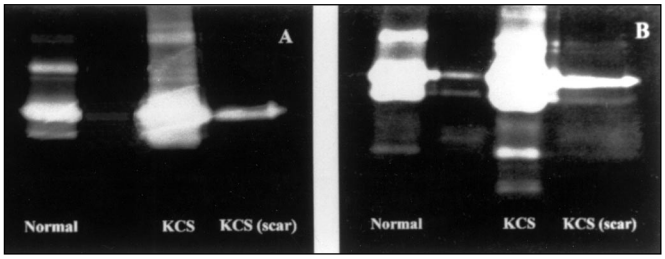


**Fig. 12 -** *Zymographic activity profile of MMP-2 produced by normal, early-phase keratoconic and late-phase keratoconic corneal keratocytes before (Gel A) and after (Gel B) incubation with activated MMP-2 of apparent $M_r$ 43,000.*

secreted by keratocytes of normal corneas, the incubation with activated enzyme generated the band B activity predominantly. In the case of the early-phase keratoconic keratocyte enzyme preparation particularly, the $M_r$ 43,000 gelatinase activity became very apparent. This finding suggested that to a limited extent, the unpurified enzyme produced by the keratocytes of early-phase keratoconic corneas is capable of self-activation *in vivo*.

## DISCUSSION

The major protease secreted by normal, healthy corneal keratocytes is inactive MMP-2, of apparent $M_r$ 65,000/66,000 on SDS-gelatin polyacrylamide gels (12,13,19). In addition, results presented here and in a previous publication have indicated that the keratocytes of early-phase keratoconic corneas produce a second MMP-2 activity of $M_r$ 61,000 (13), that is reputably the activated form of the enzyme (1, 17, 18, 26, 27) generated by the action of MT-MMPs (20). Although enzyme preparations that exhibited this activity zymographically did not possess detectable activity *in vitro*, the most obvious explanation was that MMP-2, once activated, was maintained in an inactive state by the TIMP ligands. In support of this hypothesis, it is known that corneal keratocytes secrete TIMP-I and TIMP-2 (12) and it has been suggested that the keratoconic condition may result from an inbalance in the ratio of TIMP-I / TIMP-2 to MMP-2 production (26). Despite this, attempts to activate corneal MMP-2 by chemically dissociating MMP-2 / TIMP complexes were not successful. Moreover, the zymographic specific activity of the conventionally inactive form of MMP-2 ($M_r$ 65 / 66,000, band A) was found to be considerably higher than that of the putatively activated form of $M_r$ 61 / 62,000 (band B) and after reducing early-phase keratoconic MMP-2 samples with mercaptoethanol or DTT, band B migrated as band A. These data indicated that the proteins of band A and band B gelatinase activities differed only in conformation and inferred that the apparently lower $M_r$ species, with its intramolecular S-S bonds intact, was spherical whereas the reduced protein was elongate, and hence subject to drag during electrophoresis. Confirmation of this proposition was obtained by sequencing the N-terminal amino acids of the proteins corresponding to band A and band B activities. They proved to be the same and corresponded to the published N-terminal sequence of proMMP-2 (17).

Proteolytic cleavage of an N-terminal inactivating peptide is the favoured mechanism of MMP activation (4,5). An alternative mechanism has been proposed that involves the dissociation of a cysteine residue, ($Cys^{73}$ in the case of human fibroblast collagenase), from the active site zinc atom and its replacement by water, and is known as the 'cysteine switch' (28,29). In this context it is considered likely that, if TIMP is limiting or absent, the reduced form of MMP-2 secreted by keratocytes of normal corneas (band A) would exhibit some activity *in vivo*. However, by assaying a number of MMP-2 preparations produced by keratocytes of normal and keratoconic corneas against various collagenous substrates, and by zymography, it was deduced that ability to selectively hydrolyse Type IV collagen and gelatin *in vitro* correlated with the zymographic visualisation of activity bands of apparent $M_r$ 55,000, 36,000 and predominantly, 43,000 on SDS-gelatin polyacrylamide gels. On the basis of these data, and a subsequent finding that SDS treatment both activated early-phase keratoconic MMP-2 preparations and generated activity bands of 43,000 and 36,000 on SDS-gelatin polyacrylamide gels, it was tentatively postulated that the main form of MMP-2 that was capable of proteolysis was of $M_r$ 43,000. More conclusive evidence for this hypothesis was provided by a subsequent observation that an early-phase keratoconic MMP-2 preparation, initially inactive and comprised of band B only, was completely transformed into the $M_r$ 43,000 activity during storage at -20°C and as such, efficiently and selectively catalysed the hydrolysis of Type IV collagen and gelatin and the production of this activity in other corneal MMP-2 prepa-

rations. Although this finding may be contrary to current supposition, the appearance of activated MMP-2, of similar molecular weight, has been noted by other authors (19,26,27) and the reported loss of a carboxy-terminal peptide in this entity (7), may signify a loss in ability to bind TIMP and account for the fact that it retains activity in the presence of TIMP.

To date, the only method that has been found to reliably activate MMP-2 *in vitro* is to treat the protein with SDS, a strong ionic detergent that separates proteins/ polypeptides electrostatically. Based on the chromatographic behavior of MMP-2 on CM-cellulose and on Sephadex G-75 run in the absence and presence of SDS, it was concluded that the proenzyme, in addition to TIMP, associated with co-secreted high molecular weight proteins. By dissociating these complexes, activated enzyme was generated subsequent to fractionation and presumably by autoactivation. Although, as mentioned previously, SDS-treatment followed by dialysis generated activated enzyme in the case of unpurified MMP-preparations obtained from early-phase keratoconic keratocyte cultures, this did not work for similar MMP-2 preparations obtained from normal corneal keratocyte cultures: SDS-treatment generated band B from band A but there was no gelatinase of $M_r$ 43,000 visualised by zymography, and activity against Type IV collagen or gelatin *in vitro* could not be detected. The chromatographic data presented indicated that peptide cleavage, by autocatalysis, followed MMP-2 dissociation and separation from TIMP and other matrix proteins. The most plausible explanation for the observed difference in ability to cleave and hence activate the normal and early-phase keratoconic MMP-2 may therefore lie in the relative amounts of inhibitor (TIMP) present in the respective enzyme preparations. This possibility is currently being explored.

Although it has been shown that band A and band B are conformers of proMMP-2, the data obtained from experiments in which the band A and band B proteins were excised from SDS-polyacrylamide gels and after Triton X- 100 treatment rerun under the same conditions, indicated that the band B activity expressed by keratocytes of keratoconic corneas, represented the form of MMP-2 that is proteolytically cleaved to yield the activated enzyme. This could be achieved by autocatalysis as observed *in vitro*, or by the action of other enzymes.

In conclusion, the results presented indicate that the MMP-2 that is of apparent $M_r$ 61,000 and abnormally secreted by early-phase keratocytes of keratoconic corneas is simply a conformer of the proenzyme normally detected in corneal stromal tissue and in the culture medium of corneal keratocytes. It is also tentatively suggested that the proMMP-2 cannot be cleaved and hence activated except when it exists in this conformation. Whether the conformational switch is an intracellular or extracellular event is currently under investigation but may well depend upon whether the pathological signs are genetically or environmentally induced.

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