Mooren's ulcer

Histopathology and proteolytic enzymes of adjacent conjunctiva

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It was reported in 1971 that the conjunctiva adjacent to Mooren's ulcers exhibited collagenolytic activity (Brown, 1972). More recently, both normal and rabbit conjunctiva inflamed by Freund adjuvant was shown to produce a collagenase that was typical of earlier reported mammalian collagenase (Bloomfield and Brown, 1975).

The limbal conjunctiva adjacent to Mooren's ulcers has been excised, both as a treatment and in order to examine the histopathology, ultrastructure, and proteolytic enzymes of the tissue (Brown, 1975). The results of the pathological examination are presented in this paper.

Methods

FIXATION FOR EXAMINATION WITH LIGHT AND ELECTRON MICROSCOPE

The corneal and conjunctival specimens were first fixed in 3 per cent glutaraldehyde in 0.2M sodium cacodylate buffer (pH 7.4) and then fixed with 2 per cent osmium tetroxide in 0.2M sodium cacodylate and embedded in Epon. Thick sections of the epoxy-embedded tissue were stained with basic fuchsin and alkalized methylene blue and then examined under the light microscope. Thin sections were cut with an LKB-3 microtome using a diamond knife, mounted on coated copper grids, stained with uranyl acetate and lead citrate, and examined with an AEI EM-6B at 60 kV. Excised conjunctiva from three additional eyes from two patients were also examined.

HARVEST OF PROTEOLYTIC ENZYMES

The excised conjunctiva was cut into approximately 2 × 2 mm pieces, washed with a mammalian Tyrode's streptomycin solution, and placed in sterile organ culture dishes. The culture media were prepared from equal amounts of Tyrode's solution and Dulbecco's modified Eagle's medium with 100 mg/l streptomycin sulphate,

and were equilibrated with 95 per cent oxygen and 5 per cent carbon dioxide before use. The tissues were incubated at 37°C in a humidified chamber for 6 days with a replacement of medium every 2 days. The unconcentrated, harvested media were frozen and stored.

RADIOACTIVE COLLAGEN PREPARATION

Radioactive collagen was prepared from the skin of guinea-pigs after intraperitoneal injection of 100 µCi glycine 1-C¹⁴, 6 and 12 h before death (Gross, 1958). Solubilized collagen was eluted from the skin with 0.4M NaCl and purified by repeated salt precipitation. After dialysis, the collagen was lyophilized and stored at -20°C. The specific activity of the labelled collagen was 20 000 disintegrations per min/mg protein.

TISSUE CULTURE ASSAY FOR COLLAGENOLYTIC ACTIVITY

The excised pieces of conjunctiva and cornea were cut into pieces approximately 2 × 2 mm. They were then dipped in a tissue culture medium and placed on rigid opalescent collagen gels in tissue culture plates with an inside diameter of 3.5 cm. Eight similarly prepared pieces of normal human conjunctiva were removed with donor eyes from deceased humans within 4 h of death. The plates were incubated at 37°C. The method and preparation of the collagen has previously been described (Brown, Weller, and Wasserman, 1969).

COLLAGENASE ASSAY

Plastic conical tubes containing 0.2 ml 0.2 per cent glycine C¹⁴ collagen solution which was trypsin resistant pH 7.6, were incubated at 37°C for 15 h to facilitate gelling. The fractions to be assayed for collagenase activity were applied to the gels. The tubes were then incubated for 24 or 48 h in a moist atmosphere at 37°C. After incubation, 0.1 ml distilled water was added and the tubes were centrifuged at 44 000 g for 10 min. Aliquots of the supernatant (0.25 ml) were pipetted into vials containing glass filter papers and dried overnight. The radioactivity was counted in a liquid scintillation counter in 1.0 ml POPOP-1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, PPO-2, 5-di-phenyloxazole, and toluene mixture.

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INHIBITION OF THE HARVESTED COLLAGENASE

Cysteine 10^{-2} M, Na_2EDTA 10^{-3} M, and freshly prepared rabbit serum 1:100 were separately added to the crude collagenase harvest preparation and assayed for collagenase activity.

PROTEOGLYCAN PREPARATION

Proteoglycans were extracted from rabbit corneas with 4.0M guanidine chloride. (Fractions high in uronic acid content were obtained through the courtesy of Dr John D. Gregory of Rockefeller University, New York, New York.)

VISCOMETRY

Viscometry was used to indicate both collagen and proteoglycan breakdown. The viscometry measurements were made in Ostwald viscometers with water flow time of 49 and 53 s at 37°C .

The preparation of proteoglycan and the viscosity measurements were carried out as previously described (Brown, Hook, and Tragakis, 1972; Brown, Bloomfield, and Tam, 1974). In brief, the proteoglycan substrate was prepared by adding 0.05M acetic acid containing 0.15M NaCl, pH 3.5, or mixed with 0.05M Tris-HCl buffer, with 0.15M NaCl, pH 7.4. Measurements of proteoglycan breakdown from crude and NH_4SO_4 precipitated harvest media were made over a 3-h period. The initial viscosity (N_{sp}) was approximately 4.0.

In order to relate the breakdown of proteoglycan with changes in viscosity, a diffusion apparatus was constructed consisting of two plastic chambers separated by a millipore filter of 0.2 micron size. This is similar to recently reported diffusion vessels which permitted diffusion of chondroitin sulphate but not the larger proteoglycan across the millipore filter (Brown and others, 1974; Weissmann and Spilberg, 1968). Measurements of uronic acid (hexuronate) in the 'output' chamber indicated the amounts of proteoglycan degraded (Brown and others, 1972; 1974).

Corneal proteoglycan 0.9 per cent in 2 ml of 0.05M Tris-HCl with 0.15M NaCl and $5\mu\text{g}$ of the harvest media were placed in the input chamber. In the output chamber were placed 7 ml of 0.05M Tris-HCl with 0.15M NaCl. The diffusion vessel was then incubated at 37°C for 4 h. The uronic acid content of the output chamber was estimated hourly by the carbazole reaction. The controls for this were two additional diffusion vessels with the same contents except that one contained only Tyrode's medium and was dialysed against 0.05M Tris-HCl, pH 7.1, with 5mM CaCl_2 , and in the other, the harvest media were added after it had been warmed at 80°C for 15 min. This technique was identical to that used in an earlier study of the enzyme of *Pseudomonas aeruginosa* (Brown and others, 1974).

The collagen substrate was prepared and optical rotation and viscosity measurements carried out as previously reported (Brown and others, 1972; Hook, Brown, Iwanij, and Nakanishi, 1971). Control viscosity of only the collagen solution was run simultaneously

with the viscosity of the reaction mixture. The collagen breakdown products were analysed by polyacrylamide gel electrophoresis (Nagai, Gross, and Piez, 1969).

Results

LIGHT AND ELECTRON MICROSCOPY

Histological examination of the host's excised, ulcerated cornea revealed many lymphocytes and polymorphonuclear leucocytes. The conjunctival epithelium was intact in most sections while the subepithelial tissues were packed with plasma cells and only an occasional polymorphonuclear leucocyte or monocyte (Fig. 1). Examination of the conjunctiva with the electron microscope corroborated the cell types and showed substantial amounts of endoplasmic reticulum (Fig. 2). Histological examination of the remaining three specimens of conjunctiva showed similar findings—that is, concentrations of plasma cells and varying amounts of lymphocytes. The specimens examined from the eye that had multiple excisions of conjunctiva showed relatively fewer cells.

TISSUE CULTURE ASSAY FOR COLLAGENASE

Growth of the excised conjunctiva from the eyes with Mooren's ulcers on collagen gels resulted in total lysis of the whole gel within 48 h in all specimens. When 10 similar sized pieces of human conjunctiva excised from essentially normal eyes within 6 h of death of the donor were grown on similar gels, gel lysis of 6.0–10.0 was noted in three specimens.

Growth of the excised conjunctiva from eyes with Mooren's ulcer on collagen gels containing either cysteine, NaEDTA, or serum did not prevent gel lysis.

COLLAGENASE ASSAY WITH RADIOACTIVE COLLAGEN

The crude, unconcentrated harvest media completely lysed the radioactive collagen gels. This gel breakdown was inhibited 50 per cent by NaEDTA 10^{-3} M and not at all by cysteine 10^{-2} M or serum 1:100. When the volume of the harvest media added to the gels was halved, its collagenolytic activity was completely inhibited by serum 1:100, 91 per cent NaEDTA, and 72 per cent by cysteine (Table).

VISCOMETRY

The specific viscosity of a solution of collagen and undiluted crude harvest media was reduced to 60 per cent after 50 min. A control run simultaneously without the harvest media did not show any signifi-

FIG. 1 *Histological appearance of excised conjunctiva adjacent to Mooren's ulcer. Stroma almost filled with plasma cells*

cant reduction in viscosity for up to 90 min (Fig. 3). The optical rotation readings were all between -425 and -430 .

PROTEOGLYCAN

The viscosity of the reaction mixture of proteoglycan and the harvest media fell quickly and at a uniform rate to almost zero within 30 min.

At the end of the 4-h incubation period there was $7 \mu\text{g/ml}$ hexuronate released into the output chamber of the control diffusion vessel without the

active fraction, $10 \mu\text{g/ml}$ hexuronate in the output chamber of the control vessel in the pre-heated fraction, and $57 \mu\text{g/ml}$ hexuronate released into the output chamber in the vessel with the active fraction. This shows that corneal proteoglycan is degraded by the harvest media, that heat inactivates

Table *Inhibitors of collagenase of Mooren's ulcer*

	Collagen lysis (cpm)	Inhibition (%)
Control	$1090 \pm 52^*$	0
Cysteine 10^{-2}M	414 ± 15	72
Cysteine 10^{-3}M	545 ± 30	50
$\text{Na}_2\text{EDTA } 10^{-3}\text{M}$	51 ± 10	91
Serum 1:100	0	100

*Standard error of mean

FIG. 3 *Viscosity measurements of reaction of mixture of conjunctival harvest media with solution of collagen*

FIG. 2 Electron microscopic appearance of tissue in Fig. 1, showing plasma cells with abundance of endoplasmic reticulum

the enzymatic activity of the media, and substantiates the relationship of fall in viscosity to substrate degradation.

ANALYSIS OF COLLAGEN BREAKDOWN PRODUCTS

Polyacrylamide gel electrophoresis of the reaction mixture showed collagen breakdown products that were typical of mammalian collagenase (Fig. 4).

Discussion

The results of the investigation of the excised human tissues with Mooren's ulcer showed that tissue culture of the corneal epithelium, the ulcerated corneal stroma, and the limbal conjunctiva on collagen gels resulted in breakdown of these gels. The collagenolytic activity by the conjunctiva

was further indicated to be the result of a true collagenase since the conjunctival harvest media caused the characteristic limited reduction in viscosity of a collagen solution without a change in its optical density. The typical collagen breakdown products exhibited by polyacrylamide gel electrophoresis was the final proof for the presence of a collagenase.

The collagen gel breakdown by the conjunctiva surrounding Mooren's ulcer was at least 10 times more than the breakdown caused by a similar quantity of any of the diseased corneal tissues previously investigated by the author. Further indications of the relatively large amounts of the collagenase produced by growth of the conjunctiva was that the crude harvest media did not have to be concentrated to effect significant collagen breakdown. In fact, they had to be diluted before they

FIG. 4 Polyacrylamide gel electrophoretic patterns of collagen degraded by conjunctival collagenase.

A. Collagen control

B. Effect of conjunctival collagenase :

single polypeptide chain(s) ;
cross-linked dimers of chains
(β) at 18 h

could be inhibited. Once diluted, the crude enzyme was completely inhibited by NaEDTA and serum. The enzyme was almost, but not completely inhibited by cysteine, probably because the enzyme was still too concentrated to be inhibited by cysteine. This hypothesis was not tested because of the limited supply of the enzyme. For the same reason the enzyme was not further characterized and compared with rabbit conjunctiva collagenase.

The harvest media from Mooren's ulcers also quickly degraded corneal proteoglycan which is a

major component of the ground substance of the corneal stroma. This is significant since a recent study showed that corneal proteoglycan protects collagen from breakdown by collagenase indicating that, in order for collagenase to attack collagen fibrils *in vivo*, the proteoglycan must first be degraded (Nagai and others, 1969). Currently, the only cell known to contain a protease capable of degrading proteoglycan at a neutral pH is the polymorphonuclear leucocyte PMN (Brown and others, 1972; Weissmann and Spilberg, 1968). However, there were very few PMN found in the excised specimens of conjunctiva.

It was interesting that each of the four specimens of conjunctiva examined histologically had unusually large numbers of plasma cells. The significance of this finding is not clear at present. It may be that these cells are the source of proteolytic enzymes, activators of precursors of enzymes, or finally and more probably, the source of substances which induce other cells to produce enzymes. The presence of these cells in the conjunctiva suggested that they were part of an immune or an autoimmune phenomenon. However, repeated attempts by the author to identify immune globulins in the conjunctiva *via* fluorescent antibody staining were negative.

In conclusion, this and the following study, showing that the conjunctiva adjacent to Mooren's ulcers contains large numbers of plasma cells, that it produces a collagenolytic enzyme and probably a proteoglycanolytic enzyme, and that the ulcers heal after excising this tissue, indicate that the conjunctiva is intimately involved in the pathogenesis of Mooren's ulcers.

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