Presence, significance, and inhibition of lysosomal proteoglycanases

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Lysosomal lysates of polymorphonuclear leukocytes (PMNL) were found to reduce the viscosity of a solution of corneal proteoglycan at pH 7.3 and 3.5. This indicates that PMNL contain proteoglycanases that can operate inside and outside the cell. The effect of the lysates on the proteoglycan can be inhibited by the α_2-macroglobulin fraction of serum.

Key words: Lysosomal lysates, polymorphonuclear leukocytes, viscosity, corneal proteoglycan

Recent studies by Hook and associates[1] showed that both corneal and cartilage proteoglycan protects collagen from breakdown by corneal collagenase. These studies may have explained why injection of corneal collagenase into the alkali-burned cornea which is almost devoid of proteoglycogen results in an immediate ulceration while injection of a similar quantity of collagenase into the normal cornea does not result in ulceration.[2] This indicates that in the normal cornea the proteoglycan or ground substance must be destroyed before the collagen framework can be attacked by collagenase. Partly because of these results, Brown hypothesized that in order for an ulceration to occur in a normal cornea, there must be an epithelial defect and inflammation of the underlying stroma. Stromal inflammation will induce the epithelium to produce collagenase. In addition, it seemed likely that stromal inflammatory cells contained enzymes which could destroy the stromal proteoglycan, thereby exposing corneal collagen to attack by collagenase.[3] This theory of selective enzymatic degradation would be meaningful if, in fact, there was an enzyme that could break down corneal proteoglycan.

In 1968, Weissmann and Spilberg[4] reported that lysates of lysosomal fractions of polymorphonuclear leukocytes (PMNL) contained enzymes which could break down the proteoglycan from nasal cartilage at both acid and neutral pH values. This proteoglycanase activity could be inhibited by epsilon aminocaproic acid.

In 1970, Dziewiatkowski and associates[5] showed that the reduction in viscosity of a solution of proteoglycans after exposure to cathepsins was evidence for enzymatic breakdown of the proteoglycans by the cathepsins. The present report utilizes a viscosity technique to study enzymatic breakdown of corneal proteoglycan and the inhibition of this breakdown.

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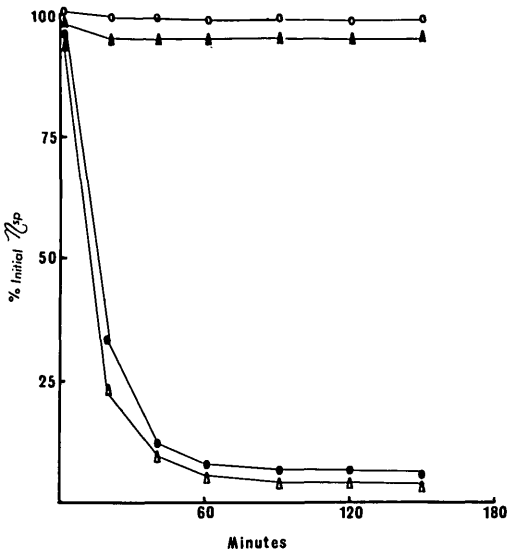

Fig. 1. This graph shows that the specific viscosity of corneal proteoglycans at pH values of 3.5 and 7.3 was not significantly reduced. However, addition of lysosomal lysates from PMNL reduced the specific viscosity to almost zero at pH 3.5 and 7.3. ○, Specific viscosity of proteoglycan at pH 3.5; ▲, specific viscosity of proteoglycan at pH 7.3; ●, specific viscosity of proteoglycan with lysosomal lysates at pH 7.3; △, specific viscosity of proteoglycan with lysosomal lysates at pH 3.5.

Material and methods

Preparation of lysosomal lysates of PMNL. PMNL were harvested from the peritoneal exudates of albino rabbits according to the method of Cohn and Hirsch.[6] In brief, four hours after intraperitoneal injection of a mixture of 0.9 per cent NaCl and glycogen, peritoneal exudates were collected, filtered, and centrifuged. Ninety eight per cent of the cells in the centrifuged pellet were PMNL.

The lysosomes from the PMNL were prepared by a modification of the method of Weissmann and associates.[7] The PMNL pellet as suspended in cold 0.34M sucrose and the cells were homogenized with a ground glass homogenizer. The resultant suspension was then repeatly frozen with Dry Ice and thawed and divided into two samples.

The first sample was centrifuged at 18,000 g for 15 minutes, and the supernatant was dialyzed at 4° C. for 18 hours against 0.9 per cent NaCl. The second sample was exposed to two drops of 0.1 per cent Triton X-100 for one hour at 4° C. and centrifuged at 18,000 g for 15 minutes. The supernatant was dialyzed at 4° C. for 18 hours against 0.9 per cent NaCl. The protein content of the supernatant of both samples was determined by the method of Lowry and associates.[8] Readings were made at 760 nm. on a Beckman DB-G spectrophotometer.

The presence of a cytoplasmic inhibitor was tested for in the supernatant of the Triton X-100 sample following centrifugation at 50,000 × g for one hour.

Proteoglycan preparation. Proteoglycans were extracted from rabbit corneas with 4.0M guanidinium chloride.[9] Fractions high in uronic acid content obtained through the courtesy of Dr. John D. Gregory of Rockefeller University, New York, New York.

Viscometry. Viscometry measurements were made in Ostwald viscometers with water flow time of 49 and 53 seconds at 37° C. The proteoglycan solutions were prepared for viscometry 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 7.3. Measurements of proteoglycan breakdown with lysosomal lysate protein (3 mg.) prepared with or without Triton-X, bacterial collagenase (1 μg),* corneal collagenase, or only Triton-X were taken over a three hour period. Na_2 ethylenediaminetetaacetic acid (EDTA) 10^{-3}M, L-cysteine 10^{-2}M, aminocaproic acid 0.03M, freshly prepared rabbit serum diluted 1:10, or the α_2-macroglobulin's fraction of human serum 600 μg were each mixed with 2 ml. of the proteoglycan solution. The initial viscosity ($\eta_o sp$) was approximately 4.0. Lysosomal lysate protein or bacterial collagenase was then added to the proteoglycan mixture.

Results

The specific viscosity of a solution of corneal proteoglycan was not altered significantly by time, by changes to 7.3 and 3.5 or addition of Triton-X. Addition of the first sample of lysosomal lysates to the proteoglycan solution also did not change the specific viscosity. However, when the second sample of lysosomal lysates, that had been exposed to Triton-X, was mixed with the corneal proteoglycan, the specific viscosity abruptly fell to almost zero (Fig. 1). This drop in specific viscosity was not effected by addition of disodium EDTA or cysteine. The drop in viscosity caused by the second fraction was completely inhibited by serum or the α_2-macroglobulin fraction of serum. Epsilon aminocaproic acid slowed the reduction in the specific

*Schwarz Bioresearch, Inc.

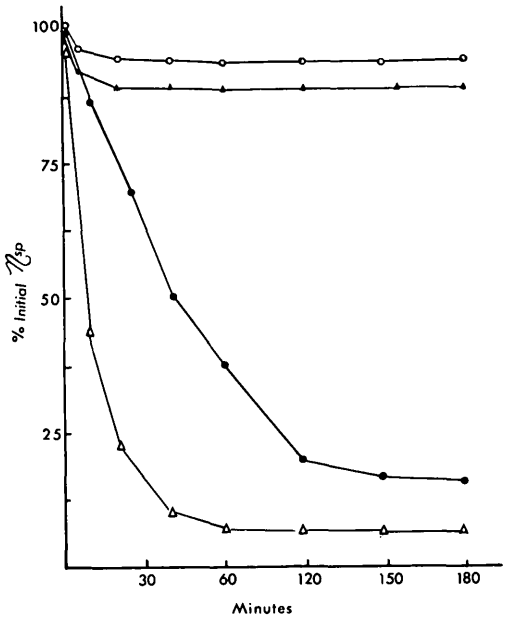

Fig. 2. Graph shows that neither cysteine nor Na_2EDTA pH 7.3 inhibits the reduction of the specific viscosity of corneal proteoglycan by lysosomal lysates. Epsilon aminocaproic acid pH 7.3 slightly inhibits the effect of the lysosomal lysates. Serum and α_2-macroglobulins at pH 7.3 almost completely inhibit the reduction in specific viscosity of corneal proteoglycan by lysosomal lysates. ○, Specific viscosity of corneal proteoglycan lysosomal lysates and serum pH 7.3; ▲, specific viscosity of corneal proteoglycan lysosomal lysates and α_2-macroglobulins pH 7.3; ●, specific viscosity of corneal proteoglycan lysosomal lysates and epsilon aminocaproic acid; △, specific viscosity of proteoglycan, lysosomal lysates, and either cysteine or Na_2EDTA pH 7.3.

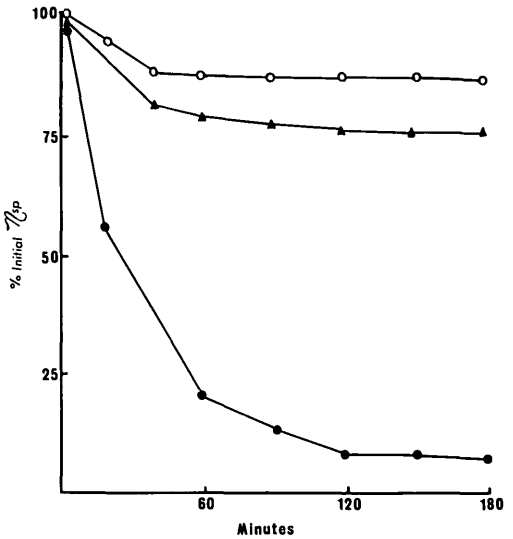

Fig. 3. Graph shows that bacterial collagenase reduces the viscosity of corneal proteoglycan pH 7.3. Na_2EDTA almost completely inhibits the effect of bacterial collagenase. Cysteine also reduced the effect of bacterial collagenase but not as completely as Na_2EDTA. ●, Specific viscosity of a solution of corneal proteoglycan and bacterial collagenase; ○, specific viscosity of a solution of corneal proteoglycan, bacterial collagenase, and Na_2EDTA; ▲, specific viscosity of a solution of corneal proteoglycan, bacterial collagenase and cysteine.

viscosity and also limited the final reduction to ten per cent of the initial specific viscosity (Fig. 2). A PMNL cytoplasmic inhibitor was detected which decreases proteoglycanase activity by approximately 50 per cent.

Although corneal collagenase did not have any effect on the specific viscosity of corneal proteoglycan, the addition of bacterial collagenase resulted in an immediate reduction of the specific viscosity to almost zero. The effect of bacterial collagenase on corneal proteoglycan could be almost completely reversed by the addition of disodium EDTA. Cysteine also reduced the effect of the bacterial collagenase but not as completely as did disodium EDTA (Fig. 3).

Comment

The decrease in viscosity that was repeatedly observed after exposure of corneal proteoglycan to lysosomal lysates indicates an enzymatic degradation of the proteoglycan. The breakdown of the proteoglycan occurred at both acid and neutral pH values. The fact that the proteoglycanase activity was observed only in those lysate fractions that had been pretested with Triton-X indicates that the proteoglycanases are probably located on the lysosomal membranes.

The findings that PMNL's contain proteoglycanases and corneal colagenase does not breakdown corneal proteoglycan support the concept of selective enzymatic tissue destruction in the non-alkali-burned, noninfected corneal ulcers. This concept

doesn't apply to infected corneas since bacterial collagenase was found to degrade proteoglycan in addition to collagen. These results explain why injection of corneal collagenase into a normal cornea produced little if any effect while injection of bacterial collagenase into the normal cornea resulted in a full-thickness corneal ulcer.[10]

Both cysteine and EDTA which inhibit the collagenase activity of bacterial collagenase also inhibit its proteoglycanase activity. However, neither cysteine nor the EDTA inhibits the proteoglycanase activity of the lysosomal lysates. These proteoglycanases are inhibited by dilute concentrations of homologous serum and by the α_2-macroglobin fraction of serum by a PMNL intracellular substance and are slightly inhibited by epsilon aminocaproic acid. The former findings are significant in two ways: The inhibition of both collagenase and the proteoglycanases by components of serum again indicates that corneal vascularization is important in limiting enzymatic destruction of the cornea.[3] Finally, the clinical use of inhibitors of both the proteoglycanases and the collagenase may offer a more complete way to prevent and treat corneal destruction.

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