SERINE PROTEINASE AND SERINE PROTEINASE INHIBITORS OF NORMAL AND DEGENERATE KNEE JOINT MENISCI

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ABSTRACT

Knee joint menisci were obtained at the time of autopsy from Caucasians of ages greater than 40 years. Menisci were extracted using 6 M urea containing 10 mM mercaptoethanol. Fractions obtained by gel filtration on Sephadex G-100 superfine were shown to contain a distinct gelatinolytic enzyme which also could degrade fibrinogen. By means of specific inhibitors this enzyme was assigned to the serine proteinase classification. Fractionation of extracts on Sephadex G-50 superfine were found to possess trypsin and plasmin inhibitory activity, but did not inhibit porcine pancreatic elastase. Degenerated menisci contained enhanced gelatinolytic activity but no detectable trypsin inhibitory activity. The meniscal proteinase(s) were completely inhibited by the trypsin inhibitor also present in the normal tissues. On SDS-polyacrylamide gel electrophoresis meniscal serine proteinase had an apparent molecular weight of 24,000 daltons, whereas the molecular weight of meniscal trypsin inhibitor was approximately 7,000 daltons.

It is proposed that the elevated levels of serine proteinase activity and absence of appropriate inhibitors are important factors in degeneration of knee joint menisci.

The knee joint semilunar cartilage (meniscus) is now well established as a weight bearing structure (9, 11, 22, 23, 30), which increases tibiofemoral congruence (12, 24) and facilitates joint rotation during the flexion-extension translation (5, 15). The composition and structure of the meniscus have been investigated for a number of years (4, 7, 16, 27), but it is only recently that the proteoglycans have been characterised in some detail (13, 18). These

studies have demonstrated the presence within normal human menisci of cartilage-like proteoglycans, together with a non-aggregating population containing dermatan sulphate. Traumatic injury to the meniscus is a relatively common sporting injury in the young adult, but horizontal tears arising from localised tissue degeneration occur more frequently, particularly in the older age groups (25). While our earlier studies (4, 7) showed that the degenerative area of the adult meniscus contained less collagen and more hexosamine than the non-degenerative area of age matched controls, there is still limited information (7, 23) on the mechanisms responsible for these compositional changes. In the present investigation we have addressed ourselves to this question and report on the presence within degenerative menisci of serine proteinases, in excess of endogenous inhibitors, which could be responsible for degradation of matrix components including proteoglycans.

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Abbreviations: EDTA, ethylenediaminetetraacetate; NPGb, *p*-nitrophenylguanidinobenzoate and Tris-NaCl-CaCl₂, 0.05 M Tris-HCl buffer, pH 7.8, containing 0.15 M NaCl and 5 mM CaCl₂

MATERIALS AND METHODS

Porcine plasmin and pancreatic elastase, bovine pancreatic trypsin inhibitor, human α -1 antitrypsin, soybean trypsin inhibitor, bovine pancreatic trypsin (type XI), 6-aminocaproic acid, and *p*-nitrophenylguanidinobenzoate (NPGB) were all purchased from Sigma Chemical Co., St. Louis, MO. Aquacide II-A was obtained from Calbiochem. Aust., and Sephadex G-50 superfine, Sephadex G-100 superfine, lysine-Sepharose 4B, and Sepharose 4B were obtained from Pharmacia (South Sea Division, Australia). [$1\text{-}^{14}\text{C}$]Acetic anhydride was purchased from the Radiochemical Centre, Amersham, England. Sodium lauryl sulphate, 2-mercaptoethanol, and glycine were products of BDH Chemicals, Australia. Acrylamide and *N,N'*-methylene-bis-acrylamide were purchased from Eastman Kodak Co., Rochester, NY, and urea, analytical grade, from AJAX Chemicals, Sydney, Australia.

Human menisci were obtained at the time of autopsy at the Royal North Shore Hospital and classified into normal (medial and lateral) menisci and degenerated or torn menisci, on the basis of their macroscopic appearances. The autopsy cases were 45–73 years old (Table 1). Menisci from osteoarthrotic joints with normal appearances were not used in this study. Synovial and ligamentous tissues were dissected from the menisci, which were then finely diced with surgical blades and scissors.

Extraction of Proteinases and Proteinase Inhibitors

The diced menisci were extracted using a magnetic stirrer at 4°C with 6 M urea in 0.05 M Tris-HCl buffer, pH 7.8, containing 0.15 M NaCl, 5 mM CaCl_2 and 0.01 M mercaptoethanol. After 48 h, the decanted solution was clarified by centrifugation at 30,000 *g* for 30 min. The supernatant was then filtrated through an

Amicon XM-100A membrane (Amicon, Sydney) to remove macromolecules over 100,000 M.W. The filtrate was concentrated by Aquacide II-A using acetylated Visking tubing and then applied to a Sephadex G-50 superfine or Sephadex G-100 superfine column (1.6 × 80 cm) equilibrated with 0.05 M Tris-HCl, pH 7.8, containing 0.15 M NaCl and 5 mM CaCl_2 (Tris-NaCl- CaCl_2) buffer at a flow rate of 6 ml per h.

Assay of Proteinase and Proteinase Inhibitor

Assay for gelatinolytic activity was carried out on the Sephadex G-100 column fractions, using [^{14}C]-labelled heat-denatured polymeric guinea pig skin collagen substrate as described by Sunada and Nagai (28). Fibrinogenolytic activity was also assayed using ^{125}I -labelled fibrinogen as a substrate. Two hundred μg /0.1 ml of ^{125}I -labelled fibrinogen in 0.05 M Tris-HCl buffer, pH 7.8, containing 5 mM EDTA, 0.15 M NaCl, and 0.5 M D-glucose was incubated with 90 μl of test fraction for an appropriate time at 37°C. The enzyme reaction was stopped by adding 10 μl of 2 mM NPGB solution and then cooled on an ice bath. After addition of 0.2 ml dioxane and vigorous shaking, the resulting solution was centrifuged at 30,000 *g* for 10 min to precipitate undigested fibrinogen. One half of the supernatant was assayed for radioactivity. Inhibition of gelatinolytic activity of column fractions by ϵ -aminocaproic acid (final concentration 25 mM), dithiothreitol (5 mM), *N*-ethylmaleimide (5 mM), soybean trypsin inhibitor (50 μg /0.2 ml), NPGB (0.5 mM), and EDTA (10 mM) was also investigated using the same method.

Trypsin inhibition of column fractions was determined using [^{14}C]-labelled gelatin as a substrate. Trypsin (10 ng) was preincubated with 90 μl of test fraction for 30 min at room temperature and then further incubated at 37°C for 6 h after adding 0.1 ml of [^{14}C]-labelled gelatin substrate (200 μg , 10,000 dpm). The reaction was stopped by the addition of two volumes of dioxane at 0–2°C and the mixture was centrifuged at 30,000 *g* for 10 min. Two-thirds of the resulting supernatant were added to 5 ml of Toluene-TX100 scintillant and radioactivity (dpm) was determined with a Searle Mark III Scintillation Counter.

Inhibition of fibrinolysis by column fractions was also performed using the fibrin plate assay of Unkeless *et al.* (29). An inhibition test of plasmin with column fractions was also performed by using [^{14}C]-labelled gelatin as a

Table 1 Age and Sex Distribution of Meniscal Samples Examined

Sex	Age				Total
	40–49	50–59	60–69	70–	
Male	1	1	4	2	8
Female	1	3	1	1	6
Total	2	4	5	3	14

Normal menisci (19 joints): Medial 19, Lateral 19.
Degenerated or torn menisci (9 joints)

substrate.

Degradation of proteoglycan by proteinases isolated from menisci was determined by Sepharose 4B chromatography. Using the method of Rosenberg (17), [^{35}S]-labelled proteoglycan was prepared from costal cartilage of young rabbits prelabelled *in vivo* with $\text{Na}_2^{35}\text{SO}_4$. [^{35}S]-Labelled proteoglycan (1 mg; 10,000 dpm) in Tris-NaCl- CaCl_2 buffer was incubated with or without one of the effluent fractions from the G-100 column showing gelatinolytic activity. The reaction was stopped by the addition of NPGB (final concentration 0.1 mM). After centrifugation at 30,000 *g* for 10 min, the supernatant was applied to a Sepharose 4B column (1.6 $\times$ 80 cm) equilibrated with 0.5 M acetate buffer, pH 6.4, and eluted with the same buffer at a flow rate of 6 ml/h. Aliquot (0.5 ml) of each fraction (1.8 ml) was counted using a Searle Mark III Scintillation Counter.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using 10.8% gels impregnated with 0.1% gelatin according to the general method of Heussen and Dowdle (6). Native plasmin and mini-plasmin were also run on the same gel. Mini-plasmin was prepared from porcine pancreatic elastase-treated plasminogen as described by Nagamatsu and Soeda (14).

RESULTS

Fig. 1a shows the G-100 protein profile for amicon filtered extracts of degenerative and normal menisci applied to the column. As can be seen, extracts of degenerate menisci contain two major protein fractions eluting just before bovine serum albumin and after cytochrome C. The larger component was not present in normal menisci but the smaller was present in normal medial menisci, although to a lesser extent. Normal lateral menisci did not contain the same low molecular weight protein.

Fractionation of normal lateral menisci extracts on Sephadex G-100 superfine gave rise to three peaks of gelatinolytic activity eluting with apparent molecular weights of 75,000, 25,000, and 12,000, each peak showing approximately the same activity (Fig. 1b). From normal medial menisci gelatinolytic activity was found in two peaks; the first peak showed level of activity similar to that of the lateral meniscus but the second eluted midway between the second and third peaks of those present in the lateral

tissue. The activity in this peak was eight times higher than in the lateral meniscus. This profile was more exaggerated in degenerated meniscus, the first and second peaks forming one peak,

Fig. 1 Sephadex G-100 superfine chromatography of gelatinolytic and fibrinogenolytic enzymes in normal and degenerated human meniscal extracts. Concentrated extracts (1 ml) of 5 g each of normal lateral (broken line) and medial (dotted line) and degenerated (solid line) menisci were separately applied on a column (1.6 $\times$ 80 cm) equilibrated with Tris-NaCl- CaCl_2 buffer (2.5 ml fractions; flow rate 7.5 ml/h) and aliquots (0.1 ml each) of the effluent fractions were incubated with an equal volume of [^{14}C]-labelled gelatin substrate (200 μg , 16,000 dpm, b) or [^{125}I]-labelled fibrinogen (200 μg , c) at 37°C for 16 h. a: Absorbance at 280 nm

the activity of which was eighty times that of the second and third peak of normal lateral meniscus. The molecular weight of the gelatinolytic enzyme present in degenerated meniscus was within the range 75,000 to 10,000 daltons, and corresponded to the protein profile through the first to second peak shown in Fig. 1a. The gelatinolytic activity was inhibited by ϵ -aminocaproic acid, NPGB and soybean trypsin inhibitor, but not by dithiothreitol, *N*-ethylmaleimide and EDTA (Table 2).

In contrast to the gelatinolytic activity profiles from these tissues was the fibrinogenolytic activity which was found to be present only in extracts from degenerate menisci (Fig. 1c). This fibrinogenolytic activity eluted in the same position as the major peak for gelatinolytic activity. Fractions 22 and 37 which contained the two major peaks with gelatinolytic activity from G-100 Sephadex chromatography of degenerative menisci extracts were concentrated by amicon dialysis at 4°C and subjected to SDS-polyacrylamide gel electrophoresis containing 0.1% gelatin against standard proteins, bovine trypsin, native plasmin, and 'mini-plasmin' prepared according to Nagamatsu and Soeda (14). Under the dissociative conditions used, the gelatinolytic activity which appeared in fraction 22 with molecular weights greater than 45,000 showed similar migration in the gel to those of native plasmin (Fig. 2, columns 4 and 6). In addition, both fractions 22 and 37 showed a molecular weight similar to that of bovine trypsin, *viz.*, 23,500.

Column fractions containing the major

gelatinolytic activity from degenerative menisci were then examined for their ability to degrade

Fig. 2 SDS-Polyacrylamide gel electrophoresis patterns of human meniscal enzymes. Electrophoresis was performed on a 10.8% slab gel (0.2 × 10 × 10 cm) containing 0.1% gelatin using 0.1% SDS in Tris-HCl buffer, pH 8.8, for 3.5 h. After the electrophoretic run, the resulting gel plate was rinsed with distilled water and then incubated with 2.5% Triton X-100 solution for 2 h at room temperature to remove the SDS. The gel was further incubated in 0.1 M glycine-NaOH buffer, pH 8.3, for 8 h, stained with 0.1% Amido Black 10B in methanol-acetic acid-H₂O (30:10:60), and finally destained with the same solvent with no dye.

Table 2 Effects of Various Reagents on Meniscal Gelatinolytic Enzyme

Reagent	Concentration mM	Gelatinolytic activity dpm	Inhibition %
Buffer alone	—	6,430	—
ϵ -Aminocaproic acid	25	88	99
NPGB	0.5	0	100
Dithiothreitol	5	6,910	0
<i>N</i> -Ethylmaleimide	5	6,200	4
EDTA	1	6,310	2
	10	6,660	0
SBTI	250 μ g/ml	0	100

Gelatinolytic enzyme from degenerative menisci (50 μ l, tube No. 35 in Fig. 1) was preincubated with 50 μ l of each reagent for 30 min at 37°C and enzyme reaction was performed by incubating with 100 μ l of [¹⁴C]-labelled gelatin substrate solution (24,000 dpm, 200 μ g) at pH 7.8 and 37°C for 24 h. SBTI: soybean trypsin inhibitor

Fig. 3 Elution profiles on a Sepharose 4B gel column of proteoglycan with and without treatment with meniscal enzyme. [^{35}S]-Labelled rabbit costal cartilage proteoglycan (1 mg/0.5 ml, 10,000 dpm) was incubated with or without 0.2 ml of an effluent fraction (tube No. 37) of degenerated meniscal extract (Fig. 1) in Tris-NaCl-CaCl₂ buffer at 37°C for 16 h. After stopping the enzyme reaction by adding 10 μl of 2 mM NPGB, the reaction mixture was applied on a column (1.6 $\times$ 50 cm) equilibrated with 0.5 M acetate buffer, pH 6.4 (1.8 ml fractions; flow rate 10 ml/h). Aliquots (0.5 ml each) of effluent fractions were counted for radioactivity. PG: proteoglycan

purified proteoglycan substrate. From the profiles shown in Fig. 3, it can be seen that after 16 h incubation with the meniscal enzyme, the [^{35}S]-labelled proteoglycan was extensively degraded. This degradation was inhibited by ϵ -aminocaproic acid, NPGB, and soybean trypsin inhibitor, but not 10 mM EDTA.

The fractions obtained from normal lateral menisci extracts after Sephadex G-50 chromatography showed strong inhibition against trypsin using gelatin substrate with a molecular weight of 7,000 (see Fig. 4a). Normal medial menisci also contained inhibitory activity against this proteinase and eluted at the same position from the column but possessed only one-eighth the activity of the corresponding lateral tissues. In contrast, fractionated extracts of degenerated menisci failed to demonstrate significant inhibitory activity against trypsin (see Fig. 4a).

As can be seen from Fig. 4b, a significant inhibition was obtained for normal medial and lateral menisci against plasmin using gelatin as a substrate. No plasmin-inhibitory activity was observed with fractions from the degenerate menisci.

The major trypsin inhibitor fraction isolated from normal lateral menisci was able to abolish the gelatinolytic activity present in the fractionated extracts of both normal and degenerate menisci.

Fig. 4 Profiles of meniscal trypsin- and plasmin-inhibitory activity on a Sephadex G-50 superfine column. Concentrated extracts (1 ml) of 5 g each of normal lateral (broken line) and medial (dotted line) and degenerated (solid line) menisci were separately applied on a column (1.6 $\times$ 80 cm) equilibrated with Tris-NaCl-CaCl₂ buffer (3.0 ml fractions; flow rate 9 ml/h). Purified TPCK-trypsin (10 ng/10 μl , a) or porcine plasmin (1.25 μg /10 μl , 3.8 units/mg protein, b) was preincubated with 90 μl of each test fraction for 30 min at room temperature and then further incubated at 37°C for 6 and 48 h respectively after adding 0.1 ml of [^{14}C]-labelled gelatin substrate (200 μg , 10,000 dpm).

DISCUSSION

Although degeneration and cleavage of the knee joint meniscus is an extremely common phenomenon, little is known of the factors responsible. Previous studies (4, 7) have demonstrated that degenerate areas of adult menisci contained less collagen than corresponding areas of normal menisci, but hexosamine values were found to be elevated suggesting enhanced biosynthesis and thus turnover of proteoglycans

in these areas. Wegelius *et al.* (33) described collagenolytic activity in menisci from joints of patients with rheumatoid arthritis, but there have been no reports of corresponding studies in normal or degenerate menisci. In the present investigation, we have clearly demonstrated the presence within macroscopically degenerative menisci of serine proteinase(s) which possess the capacity to degrade proteoglycan, denatured collagen, and fibrin (data not shown). While the gelatinolytic activity was also present in normal tissue, but at a much reduced level, the fibrinogenolytic activity was only apparent in degenerative menisci. Since the gelatinolytic and proteoglycanolytic activity of this enzyme was inhibited by ϵ -aminocaproic acid, soybean trypsin inhibitor and NPGB but not EDTA, dithiothreitol and *N*-ethylmaleimide, we consider the proteinases detected to be of the serine type.

Under the reducing (mercaptoethanol) extraction conditions used, the gelatinolytic activity from normal medial and degenerate menisci eluted in two peaks corresponding to molecular sizes of 75,000 and 25,000 daltons. Using SDS-gel electrophoresis (Fig. 2) containing gelatin substrate to detect protease activity, only a single band from both these fractions was identified, suggesting that the 75,000 form could be disaggregated to a fundamental unit with a molecular size similar to trypsin (M.W. 23,500). While the origin and complete characterization of the enzyme(s) has yet to be established, we consider that it is not an elastase of the pancreatic type, or mini plasmin (see Fig. 2) derived from plasminogen as described by Nagamatsu and Soeda (14). The reasons for this conclusion are described later. Serine proteinases of similar activity and molecular weight have been described in human osteoarthritic articular cartilage (31, 32), and rheumatoid synovium (14). Saklatvala and Barrett (21) consider that the serine proteinase from rheumatoid synovium may arise from macrophage or lymphocytes, cells present in quantities in inflamed tissues. Our menisci were derived from noninflamed joints and suggest that normal synovial tissues may also provide sources of certain serine proteinases.

It is conceivable that the cells of the meniscus may respond like articular cartilage to exogenous factors such as catabolin (20) or macrophage factors (2), thereby releasing serine proteinase. It is not unreasonable to assume that a torn or degenerate meniscus may provoke a low grade synovitis which would stimulate macrophage or

synovial cells to release these activation factors. During such episodes, tissue matrix components would normally be protected from the degradative effects of released proteinase by endogenous polypeptide inhibitors. Such inhibitors have been identified in human cartilage and intervertebral discs (8), as well as in other connective tissues (3, 19), and from the present study we can now add human fibrocartilage as another source. The size of the meniscus polypeptide trypsin inhibitor(s) was established by gel filtration chromatography after dissociative reductive extraction and estimated to be in the order of 7,000 daltons; however, this is probably an underestimation of its native size. Those fractions active against trypsin (Fig. 4a) also inhibited the serine proteinase(s) isolated from the normal and degenerate menisci but were found to have no effect against chymotrypsin and human granulocyte elastase (unpublished data).

Of particular significance was our finding that the degenerated meniscus, while rich in serine proteinase activity, was essentially devoid of the appropriate inhibitors of these enzymes, probably due to the accumulation of an excessive amount of proteinase(s). Even though the macroscopic appearance of the menisci was normal, medial menisci contained more gelatinolytic activity (Fig. 1b) and less inhibitor than lateral menisci (Fig. 4a). From the biomechanical aspect, the medial meniscus is more important in weight bearing than the lateral meniscus and clinically the osteoarthritic knee shows degeneration in the medial meniscus, whereas the lateral meniscus remains intact. These clinical observations may be well explained by our findings in this study. Such an imbalance could undoubtedly account for the state of degradation in those tissues classified by appearance as degenerate. Since the meniscus is a weight bearing tissue (*loc. cit.*), loss of proteoglycans from its matrix by the mechanism proposed would render the collagenous framework more susceptible to mechanical attrition and subsequent fragmentation. While such a mechanism may not be exclusive, it is compatible with clinical experience (1, 10, 26) and as such is worthy of further investigation.

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