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PARTIAL CHARACTERIZATION OF A LOW MOLECULAR WEIGHT PROTEOGLYCAN ISOLATED FROM BOVINE PARIETAL PERICARDIUM

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Knowledge of the nature of pericardial connective tissue components is incomplete. To gain a better understanding of the composition of this tissue, bovine parietal pericardium was extracted with 4 M guanidine hydrochloride yielding a proteoglycan-containing protein mixture. This was fractionated by a three-step chromatographic procedure with the resultant purification of a 75-110 Kd proteoglycan. The purified proteoglycan was susceptible to chondroitinase ABC digestion but resistant to chondroitinase AC and nitrous acid degradation suggesting the presence of dermatan sulfate glycosaminoglycan(s). This is the first reported isolation of a proteoglycan from parietal pericardium.

The pericardial sac is an important functional component of the heart. Adhesive or constrictive pericarditis may cause impairment of cardiac function by restricting distension of the ventricles or aorta (1).

In recent years, interest in the structure and biochemical composition of pericardium has grown due to the extensive use of aldehyde-pretreated bovine pericardium as a bioprosthetic material in heart valve replacements (2,3,4). The results are promising but such complications as calcification and fibrosis

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Abbreviations used: PG, proteoglycan; GAG, glycosaminoglycan; GU, guanidine hydrochloride; BEA, benzamidine hydrochloride; NEM, N-ethylmaleimide; PMSF, phenylmethanesulfonylfluoride; EDTA, ethylenediaminetetraacetate disodium salt; KAc, potassium acetate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HexU, hexuronic acid; LMW, low molecular weight; HNO_2, nitrous acid; Kd, kilodaltons.

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of the pericardial leaflets limit the durability of the bioprosthetic valves.

Studies to date show that bovine parietal pericardium is a dense fibrous connective tissue composed mostly (≃ 90%) of type I collagen (2). Knowledge of the nature of other connective tissue components in the pericardium is incomplete. Although the presence of proteoglycans (PG) in the pericardium has been suggested from ultrastructural studies (5), the precise nature of these components and their role in the pathobiology of pericardium have not been defined.

The present study provides evidence of the presence in bovine parietal pericardium of a low molecular weight, chondroitinase ABC sensitive PG which is resistant to nitrous acid and to chondroitinase AC.

MATERIALS AND METHODS

Materials: Fresh parietal pericardia from 16-18-month-old calves were obtained at a local slaughterhouse. Ultrapure guanidine hydrochloride (GU) was obtained from Schwarz-Mann Biochem., Cleveland, OH.; benzamidine hydrochloride (BEA), N-ethylmaleimide (NEM), phenylmethanesulphonyl fluoride (PMSF), ethylene- diaminetetraacetate disodium salt (EDTA), bovine serum albumin (BSA)-fraction V, chondroitinase ABC and AC were obtained from Sigma, St. Louis, MO; m-hydroxydiphenyl was obtained from ICN Biochem., Cleveland, OH; DE-52 (DEAE-cellulose) was purchased from Whatman, Kent, England; Sepharose CL-4B, columns and accessories were obtained from Pharmacia, Uppsala, Sweden; D-glucuronolactone was obtained from Calbiochem, Los Angeles, CA.

Extraction Procedure: The pericardia were brought to the laboratory on ice, rinsed in cold saline containing protease inhibitors (1.56 gr/l BEA, 3.2 gr/l EDTA, 1.25 gr/l NEM, 0.17 gr/l PMSF), carefully cleaned of excess fat and residual blood and cut into small (=2x2mm) fragments. The fragments were rinsed in saline inhibitors solution and extracted twice for 24 h, with (50 ml/gr wet tissue) 4 M GU in 0.05 M acetate buffer, pH 5.8 with inhibitors (same concentrations as above) at 4°C with stirring. The filtered extracts were combined and precipitated with 3 volumes of 1.3% potassium acetate (KAc) in 95% ethanol overnight with stirring at 4°C. The precipitate was separated by centrifugation at 10,000 rpm, redissolved in 3 M GU in 0.05 M acetate buffer, pH 5.8 and frozen in 5 ml aliquots.

Gel Filtration of either the initial extract, DEAE purified or the enzyme digested PG samples was performed on a 1.5x135cm Sepharose CL-4B column equilibrated with 3 M GU in 0.05 M acetate buffer, pH 5.8 at a flow rate of 6 ml/h at room temperature. Blue Dextran 2000, (a marker for void volume), acid soluble rat tail tendon type I collagen beta component (molecular weight 192 Kd) and alpha-chains (96 Kd), BSA (66 Kd), cytochrome C (12.4 Kd) and phenol red (a marker for total volume), were used for calibration of the column.

DEAE-cellulose ion-exchange chromatography was performed on a 1.6 x 4cm (bed height) column of DE-52 equilibrated with 7 M deionised urea in 0.02 M Tris-HCl, 0.02 M NaCl buffer, pH 7. After applying the sample, the column was washed with 60 ml equilibrating buffer and was eluted at 20 ml/h with a linear gradient of 0.02 to 1 M NaCl in the above buffer.

Monitoring of the effluents was done continuously at 230 nm using a Beckman 25 Spectrophotometer. Hexuronic acids (HexU) were determined colorimetrically (6) with D-glucuronolactone as standard. In all cases 3 ml fractions were

collected using a Gilson fraction collector. HexU containing peaks were pooled and concentrated by ethanol/KAc precipitation (see above). The purified PG was dialyzed extensively against water and freeze dried.

Enzymatic and Chemical Degradation: 1.5 mg purified PG was digested with 0.5 U chondroitinase ABC or AC (7) in 1.5 ml 0.05 M Tris-acetate buffer pH 8 with protease inhibitors (0.01 M EDTA, 0.01 M NEM, 0.005 M PMSF, 0.01 M BEA) for 3.5 h at 37°C. To the mixture, solid GU was added to make a 3 M solution and after 10 min. at 45°C the mixture was applied to the Sepharose CL-4B column. Enzyme activities were tested against chondroitin sulfate by gel filtration of the reaction products. Nitrous acid degradation (HNO_2) was performed (8) by dissolving 1 mg PG in 1.5 ml 0.2 M $NaNO_2$ in 1.8 M acetic acid and stirring at room temperature for 2 h. The mixture was neutralized with NaOH, solid GU was added to make a 3 M solution and the sample applied to the same Sepharose column.

Electrophoretic Studies: Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (9) using a 7.5% separating gel and a 3% stacking gel. Staining was done with 0.25% Coomassie Blue R in 20% trichloroacetic acid. To the enzymatically digested samples SDS was added to a final concentration of 0.2%; the samples were boiled for 5 min. dialyzed against sample buffer (0.0625 M Tris-HCl, 4 M urea, 10% glycerol, 2% SDS, 0.025% Bromphenol Blue, pH 6.8) overnight at 4°C and applied to the gel.

RESULTS

Extraction and Purification of PG

Fresh bovine parietal pericardia were extracted in 4 M GU at pH 5.8 in the presence of protease inhibitors (10). Upon precipitation with ethanol/KAc, the extract was fractionated by Sepharose CL-4B gel filtration into four fractions (Fig. 1). One broad, HexU containing fractions (peak 3 in Fig. 1)

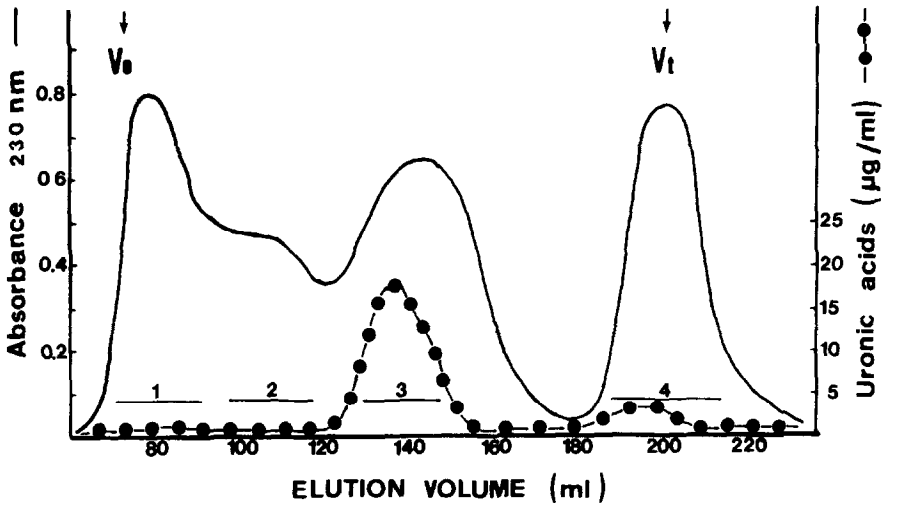

Fig 1. Sepharose CL-4B gel filtration pattern of pericardial GU extract. Effluent was monitored at 230nm (———) for protein. Uronic acids were determined (-●-●-) in each fraction as described under "Materials and Methods". The uronate-rich fraction 3 was pooled for further analysis. Vo=void volume, Vt=total volume.

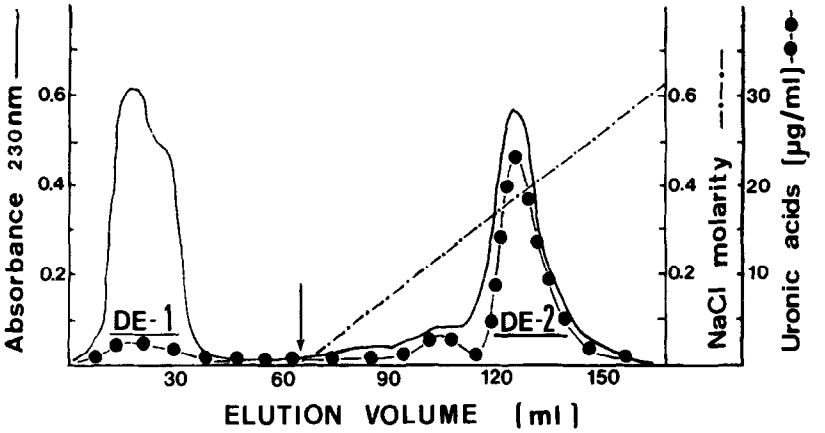

Fig. 2. DEAE-cellulose chromatography of fraction 3 from the Sepharose CL-4B column (Fig. 1). After a 60 ml wash with the equilibrating buffer, a linear NaCl gradient (arrow) was started. Fraction DE-2 was pooled for further purification.

was eluted at a Kav of 0.47. The pooled peak was precipitated as described above, dissolved in 7 M urea and chromatographed on DEAE-cellulose (Fig. 2). The washing step produced a large peak of HexU-poor, unbound protein(s). A major HexU-rich protein peak eluted at about 0.35 M NaCl (DE-2 in Fig. 2). Peak DE-2 was pooled, precipitated with ethanol/KAc and rechromatographed on a Sepharose CL-4B column (closed circles in Fig. 3). The PG eluted as a single, sharp peak at a Kav of 0.47 and on SDS-PAGE overloaded lanes did not reveal any other stainable bands (Fig. 4 lane 2).

Partial Characterization of the PG

Molecular Weight: from the gel filtration pattern a value of 75-80 Kd was estimated for the PG, while SDS-PAGE analysis suggested a molecular weight

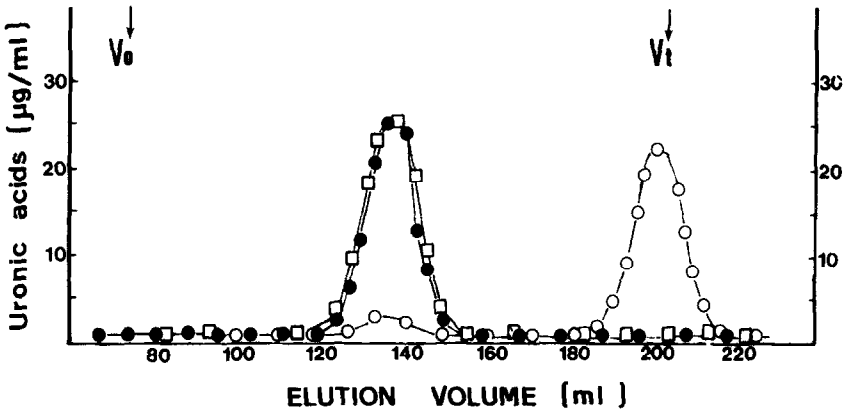

Fig. 3. Combined profile of gel filtration on the Sepharose CL-4B column of DEAE-cellulose purified PG (DE-2 from Fig. 2) before (-●-●-) and after (-○-○-) chondroitinase ABC digestion or nitrous acid degradation (-□-□-). Only uronic acid profiles are shown for simplification. Vo=void volume, Vt=total volume.

Fig. 4. SDS-PAGE on a 7.5% gel of the purified PG before and after chondroitinase ABC digestion. Lane 1 - 0.1 U enzyme alone; lane 2 - 125 µg PG alone; lane 3 - 60 µg PG digested with 0.1 U chondroitinase; lane 4 - 130 µg PG digested with 0.1 U chondroitinase. * - BSA from the enzyme preparation. Molecular weight standards were: myosin (200 Kd), beta-galactosidase (116 Kd), phosphorylase b (92 Kd), BSA (66 Kd) and ovalbumin (45 Kd).

distribution between 92.5 Kd and 116 Kd (Fig. 4). This anomalous behavior of PGs has been well recognized (11).

Chemical and Enzymatic Degradation: chondroitinase ABC digestion of the PG caused a shift of about 90% of the HexU peak to the Vt of the column (open circles in Fig. 3). Analysis by SDS-PAGE of the digestion products revealed a core protein of approx. 48 Kd (Fig. 4). HNO_2 degradation did not alter the gel filtration pattern of the purified PG (open squares in Fig. 3). The elution pattern of the PG did not change after chondroitinase AC digestion (data not shown).

DISCUSSION

This study has shown that a low molecular weight PG (LMW-PG) could be extracted from bovine parietal pericardium by 4 M GU and purified using a three-step chromatographic procedure. This PG has an apparent molecular weight of between 75-110 Kd by gel filtration and SDS-PAGE. The purified PG is not degraded by chondroitinase AC and is depolymerised by more than 90% by chondroitinase ABC suggesting a glycosaminoglycan (GAG) comprised of dermatan sulfate. The resultant core protein had an apparent Mr of 48 Kd. Nitrous acid failed to degrade the PG indicating low or absent amounts of heparan sulfate GAGs.

LMW-PGs, as classified by Heinegard (11) are considered a distinct group of PGs, different from the high molecular weight PGs. The common characteristics of the LMW-PGs are: molecular weight of up to 150 Kd, a core protein of 45-48 Kd with 1-5 GAG chains attached (chondroitin or dermatan sulfate) and a decreased ability to self-aggregate or to interact with hyaluronic acid. LMW-PGs have been found and characterized in tendon (12), sclera (13), cornea (14), bone (15), skin (10), articular cartilage (13), nasal cartilage (13), and aortic intima (11). Intermediate molecular weight PGs (between 150 and 800 Kd) bearing heparan sulfate GAGs have been identified in glomerular basement membranes (16) and at the surface of cultured liver cells (17). The functions of the LMW-PGs are as yet unknown although maintenance of corneal transparency (18), control of mineralization processes (19), control of fibrillogenesis (20) and resistance to compressive forces (11) have been suggested.

The present studies may prove of value in attempting to understand the mechanisms of pericardial-related cardiac complications (adherence, fibrosis, calcification) associated with surgically-induced trauma (1).

Aldehyde-pretreated bovine parietal pericardium has been extensively used in cardiovascular surgery as patches, conduits and artificial heart valves with promising results (2,3,4,19,21,23). The interaction of PGs with collagen and/or other noncollagenous proteins and their role in the function and pathobiology of the parietal pericardium as well as of pericardial-derived bioprostheses require additional study.

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