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The role of proteoglycans in maintaining collagen fibril morphology

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Summary. The aortic wall contains various heterogeneous proteoglycan populations which interact in different ways with other components of extracellular matrix. Proteoglycans (PGs) are known to provide structural support to the vessel wall as well as to influence specific physiological functions of the tissues. The aim of the present study was to investigate the effects of Chondroitinase AC (Chase), Streptococcal Hyaluronidase (Hyase) and Heparanase on human aortic wall collagen which had been treated previously with 4M GuHCl, in order to verify the effects of selective glycanolytic treatment on type I collagen fibril ultrastructure. Following 4M GuHCl treatment, collagen fibrils are seen to have a clearly visible period. Subsequent to GuHCl and Streptococcal Hyase treatment all collagen fibrils appear to be completely swollen in thin aperiodic filaments; the typical 64 nm collagen period is completely undetectable. After GuHCl and Chase treatment a small number of collagen fibrils are seen to be swollen in thin fibrils which are mainly localized at some distance from elastic fibres. Following GuHCl and Heparanase/Heparitinase III treatment a considerable number of collagen fibrils appear to be swollen in thin fibrils; the majority of which are situated in the vicinity of elastic fibrils. The swelling of collagen fibrils underlines the fundamental role of proteoglycans in maintaining collagen fibril integrity and periodicity. It is as yet impossible to precisely map interactions between these proteoglycans and collagen fibres. The role of Hyaluronic acid requires further investigation, although the nature of this interaction is undoubtedly a matter of considerable interest.

Key words: Collagen fibrils, Ultrastructure, Glycanolytic digestion

Introduction

Proteoglycans are known to provide both structural support to the vessel wall and to influence specific physiological tissue functions.

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Recent observations have demonstrated the capacity of the latter to interact with various families of lipoproteins and their involvement in the pathogenesis of atherosclerosis as been reported (Vijayagopal et al., 1983; Berenson et al., 1985, 1988; Wagner et al., 1986; Dalferes et al., 1987; Bihari-Varga et al., 1988; Camejo et al., 1989; Cherchi et al., 1990; Chang et al., 1991).

It has been demonstrated that treatment with extractive agents such as $MgCl_2$ 3.0M, GuHCl 4.0M (Hascall and Sajdera, 1969), Urea 8M (Hardingham and Muir, 1974), salt solutions (Malawista and Shubert, 1958) or distilled water alone (Toole and Lowther, 1968) facilitates the removal of proteoglycans from fresh tissue fragments and solubilization in the medium.

Proteoglycan extraction may also be obtained using histochemical methods; this finding is supported by the progressive disappearance of positive reaction from tissues treated over longer time intervals (Petkov, 1978a,b).

In the case of the human aortic wall, most majority of the available data concerning proteoglycans deal with the molecules which can be extracted from tissue using solvents (Salisbury and Wagner, 1981; Wagner et al., 1981, 1986; Berenson et al., 1985, 1988; Wagner, 1985; Dalferes et al., 1987; Wight et al., 1987; Cherchi et al., 1990; Register and Wagner, 1990; Chang et al., 1991; Stocker et al., 1991).

With regard to proteoglycans extraction however, the above mentioned methods do not allow a complete solubilization to occur: to demonstrate the latter, the treatment of bovine aorta with extractive agents allows a total solubilization of galactosaminoglycan-containing PGs whereas heparansulphate-containing PGs is only marginally affected (Oegema et al., 1979). Subsequent to 4M GuHCl treatment of the tissue, heparansulphate-containing PGs were obtained in a higher yield from bovine and pig aorta by controlled digestion of the residues with the collagenase/elastase (Vijayagopal et al., 1983) and bacterial collagenase (Wegrowski et al., 1986) respectively.

Likewise, in normal human aorta, treatment of intima and media with 4M GuHCl did not allow complete recovery of proteoglycans. In all samples treated in this way no more than 69% of the total hexuronate content was solubilized. Collagenase

digestion of the extraction residue allowed a further significant recovery hexuronate, accounting for almost a third of total tissue content. Therefore, these molecules, at least from a quantitative point of view represent an important component of human aorta proteoglycans (Bardoni et al., 1994).

Nevertheless the ultrastructure of collagen fibrils was not affected by 4M GuHCl treatment (Bardoni et al., 1994).

Proteoglycans obtained after collagenase digestion showed a different composition of galactosaminoglycans when compared to proteoglycans extracted using 4M GuHCl. In agreement with results obtained in pig aorta (Wegrowski et al., 1986), only chondroitinsulphate and heparansulphate chains were detected and no trace of dermatansulphate was found. Selective digestion of galactosaminoglycans by Streptomyces hyaluronidase prior to electrophoresis, clearly indicated the presence of significant amounts of hyaluronic acid (Bardoni et al., 1994).

A previous study demonstrated that performing Chasi AC and streptococcal Hyase (glycanolytic digestions) on nasal septum cartilage strongly affects collagen (type II) fibril morphology (Quacci et al., 1992). The latter treatment evidentiated a longitudinal cleft along collagen fibrils and disruption into subfibrils.

The aim of the present study was to investigate the effects of Chasi AC, streptococcal Hyase and Heparanase on human aortic wall collagen which had previously been treated with 4M GuHCl, in order to verify the effects of selective glycanolytic treatment on type I collagen fibril ultrastructure.

Materials and methods

Preparation of the samples

Human thoracic aortas were obtained within 24 h of death from the autopsy department of Varese University, Italy. Samples obtained from subjects with clinical case histories of connective tissue disorders, diabetes mellitus, chronic hypertension or obstructive pulmonary disease were excluded.

Aortas from subjects of both sexes aged from 34 to 87 years were used and informed consent was obtained from relatives.

The tissue was kept chilled on ice throughout preparation.

After removal of the adventitia and outer media, the normal areas were accurately separated, using a magnifying glass, from tissue showing atherosclerotic involvement.

Areas with dubious lesions were discarded. In many cases samples were pooled when donors of similar ages were available at the same time.

Extractive process (GuHCl treatment)

Aorta minces were gently shaken for 24 h at 2 ºC and extracted twice using 5 and 2.5 volumes respectively of 4M GuHCl in 59 mM sodium acetate buffer, pH 6,0, containing protease inhibitors (10 mM EDTA, 5mM benzamidine hydrochloride, 100 mM 6-aminocaproic acid, 100 mM PMSF).

Glycanolitic digestions

Following extractive treatment, tissue residues were washed with distilled water until free of Cl^- and subsequently digested as follows:

a) Streptococcal hyaluronase (St. Hyaluroliticus) (Seikagaku Koguko Co. Ltd. E1512) was dissolved in acetate buffer 0.05M pH 5.0 (100 IU/ml). Aorta fragments, immersed in this solution were digested for six hours at 37 ºC (Poole et al., 1980);

b) Flavobacter chondroitinase ABC (Seikagaku Koguko Co. Ltd. E8805) was used as suggested by Saito et al. (1968). Digestion of aorta minces was performed at pH 8.0 and 37 ºC for six hours in a Tris buffer containing 0.13 IU/ml.

c) Heparanase/heparatinase III (Seikagu Koguko Co.) was used as suggested by Turnbull and Gallagher (1991). Digestion of aorta minces was performed at pH 7.0 and 37 ºC for sixteen hours in 100 mM sodium acetate buffer containing 50 mIU/ml.

Protease inhibitors: 0.1M 6-aminocapronic acid (Aldrich Chem. Co.); 0.01M EDTA (Aldrich Chem. Co.); 0.05M benzamidine HCl (Sigma) and 0.36 mM pepstatin were added to all enzyme solutions to avoid matrix digestion due to protease impurities (Oegema et al., 1975).

Controls were carried out by treating the fragments in the relative solutions (with protease inhibitors) free of enzymes

Electron microscopy

Minces of aorta intima and media obtained both prior and following 4M GuHCl extraction, as well as fragments of residual tissue obtained after enzyme digestion, were fixed in either 4% (v/v) glutaraldehyde in 0.1M phosphate buffer pH 7.4, in 0.2M sodium acetate buffer, pH 5.6, or in 0.1M cacodylate buffer, pH 7.2.

After a short preliminary fixation, the aorta minces were fragmented to allow better penetration of fixative solutions. The fragments were fixed for 24 h at 4 ºC.

Due to the particular ultrastructural histochemistry of proteoglycans, the samples were fixed in 2% glutaraldehyde in 0.2M sodium acetate buffer, pH 5.6, containing 1% alcian blue and 0.3M $MgCl_2$. After a few minutes primary fixation, the specimens were dissected longitudinally in order to obtain smaller fragments and allow better penetration of fixative. Treatment was prolonged for 7 h in order to stain PGs (Ruggeri et al., 1975). The specimens were repeatedly washed in the same solution without alcian blue, in acid solution (0.01M HCl) and in distilled water, in order to remove the excess cationic dye.

Subsequently all specimens were postfixed in 1%

(v/v) OsO_4 solution in 0.1M acetate buffer (pH 7.4), dehydrated in increasing concentrations of cold ethanol, passed through cold propyleneoxide and embedded in Epoxy resin.

Ultrathin sections cut with Reichert OM12 ultramicrotome were collected on 300 mesh copper grids.

All grids were stained using either uranyl acetate-lead citrate (Reynolds, 1963) or uranyl acetate-sodium bismuth (Riva, 1974) and examined with a Philips 300 electron microscope.

Results

Human aorta specimens were characterized by the typical structure of an elastic artery.

The tunaca intima was composed of endothelium and a thin subendothelial loose connective layer.

The tunaca media accounted for eight ninths of total aorta thickness; it was characterized by a low cellularity and was made up of numerous concentric fenestrated elastic laminae.

Under the electron microscope, the D period of collagen fibrils is clearly visible and is comparable to data published previously. The axial period measures 64 nm and is invariably visible when fibrils are observed longitudinally.

In transverse section collagen fibrils are observed as round homogeneous electrondense areas. The borderline is regular and continuous and no holes are present in the area.

Elastic fibers appear to be completely unstained by uranyl acetate and lead citrate. A thin electronopaque layer is evident around elastic fibers.

In specimens fixed in glutaraldehyde and osmium, PGs appeared as thin electrondense granules (no more than 40 nm in diameter) and were observed prevalently in spaces between elastic fibers and collagen bundles (Fig. 1).

Following treatment with 1% alcian blue 0.3M $MgCl_2$ the proteoglycans were detected either as 10-12 nm wide filaments or as 20-25 nm wide rod-like particles. Thin alcianophylic filaments were visible in the areas occupied by bundles of collagen fibrils; they were usually perpendicular to collagen period and spaced at one period. Alcianophylic rod-like particles were evident in both the pericellular areas and around elastic fibers (Fig. 2).

Extractive process (GuHC1 treatment)

Following 4M GuHCl treatment of aorta specimens,

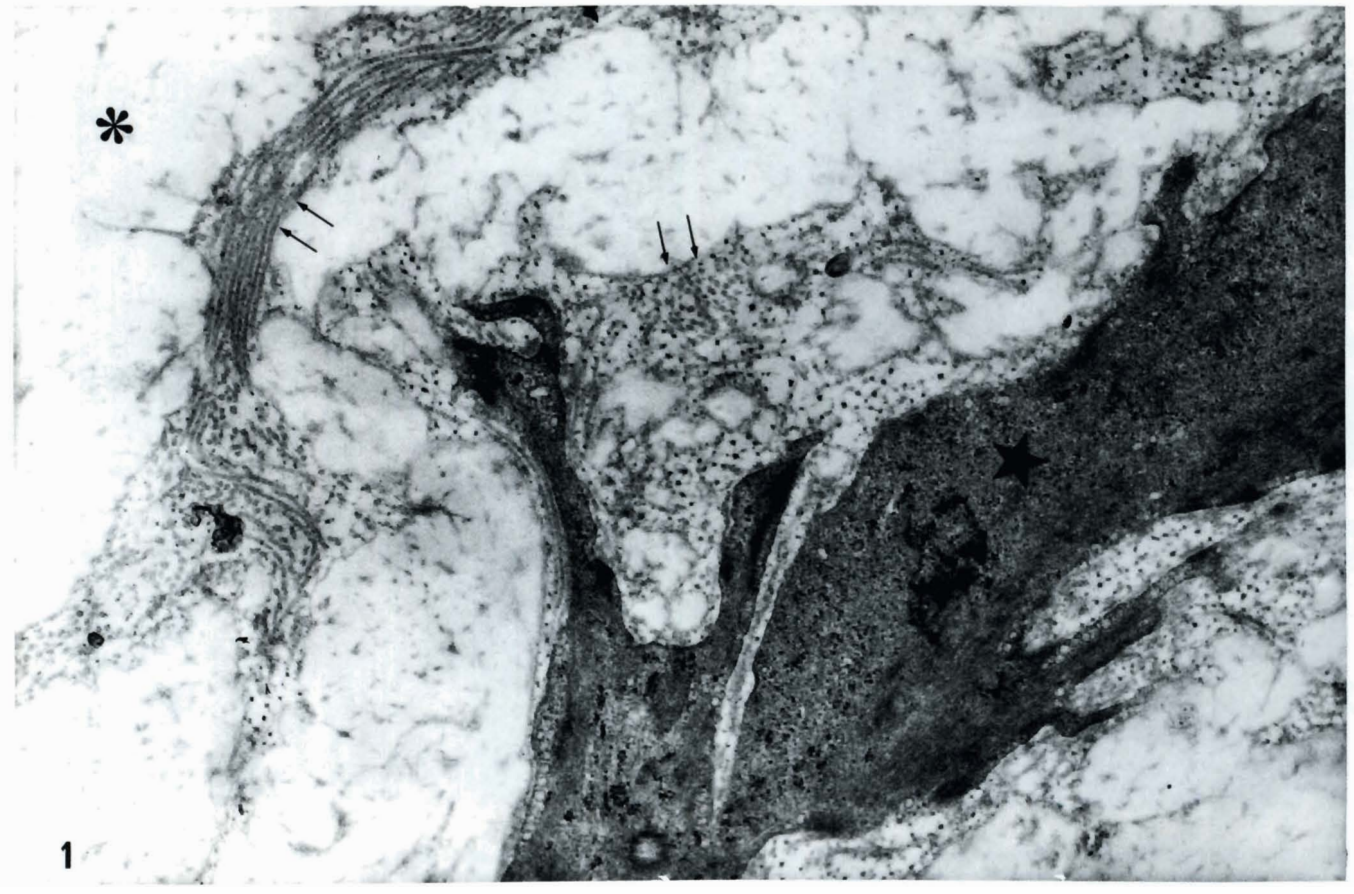


Fig. 1. Glutaraldehyde-osmium fixation. Between smooth muscle cells (stars), elastic fibers (asterisk) and periodic collagen fibril bundles (arrow), some electrondense matrix granules, morphological expression of PGs, are present. x 12,000

the alcianophylic material (both thin electronopaque filaments and electron opaque rod-like particles) could not longer be detected (Fig. 3). Collagen fibrils had been demasked and their period was particularly evident.

4M GuHCl treatment had no effect on morphology of elastic fibers although it damaged the fragment cells.

Extractive process followed by glycanolytic digestion

Subsequent to GuHCl and Streptococcal hyaluronidase treatment all collagen fibrils were completely swollen in thin aperiodic filaments 7-10 nm in diameter. The characteristic 64 nm collagen period could no longer be detected. Elastic fibers did not seem to be affected by such treatment (Fig. 4a).

In control specimens all collagen fibrils were periodic and perifibrillar space appeared to be empty.

Following GuHCl and chondroitinase treatment a small number of collagen fibrils were swollen in thin fibrils which were localized at some distance from elastic fibers. The latter did not seem to be affected by treatment (Fig. 4b).

In control specimens all collagen fibrils were periodic and perifibrillar space appeared to be empty.

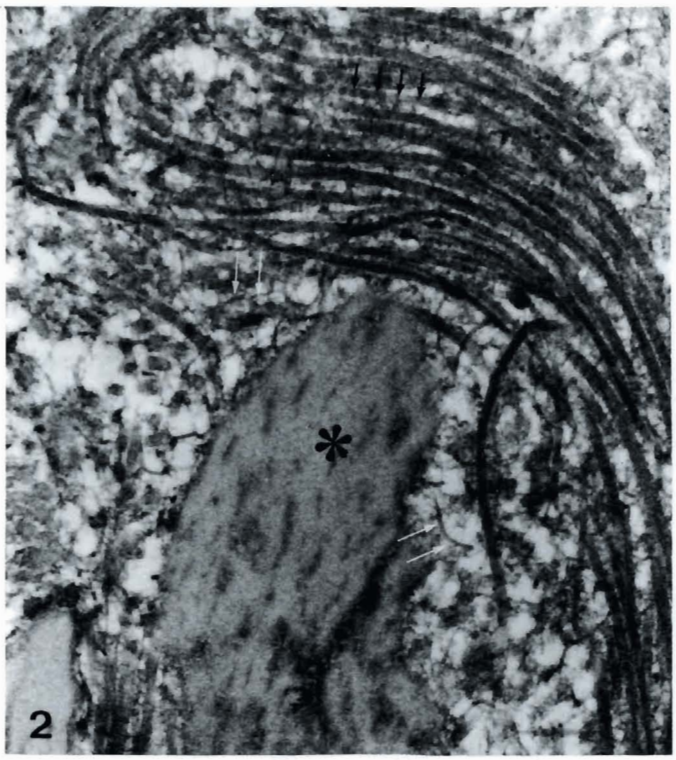
After GuHCl and heparanase/heparitinase III treatment a considerable number of collagen fibrils were swollen in thin fibrils, the majority of which were situated in vicinity of elastic fibrils. Elastic fibers did not seem to be affected by such treatment (Fig. 4c).

In control specimens all collagen fibrils were periodic and perifibrillar space appeared to be empty.

Discussion

The aortic wall contains various heterogeneous proteoglycan populations which interact in different ways with other components of extracellular matrix. Dermatansulphate-containing PGs may be extracted from tissue using dissociative solvents whereas complete solubilization of chondroitinsulphate- and heparan sulphate-containing PGs is obtained following digestion of collagen fibrous component (Oegema et al., 1979; Vijayagopal et al., 1983; Wegrowski et al., 1986; Bardoni et al., 1994).

Transmission electron microscopy investigation showed that proteoglycans extracted by 4M GuHCl invariably occupied the spaces between collagen and elastic fibers. In particular, thin alcinaophilic filaments were strictly correlated with collagen fibrils suggesting the existence of a periodical repetitive interaction site along the axis of collagen fibrils. These findings are in agreement with results obtained by Wight et al. (1987), who reported a periodic association of small proteoglycan matrix granules with blood vessel collagen.



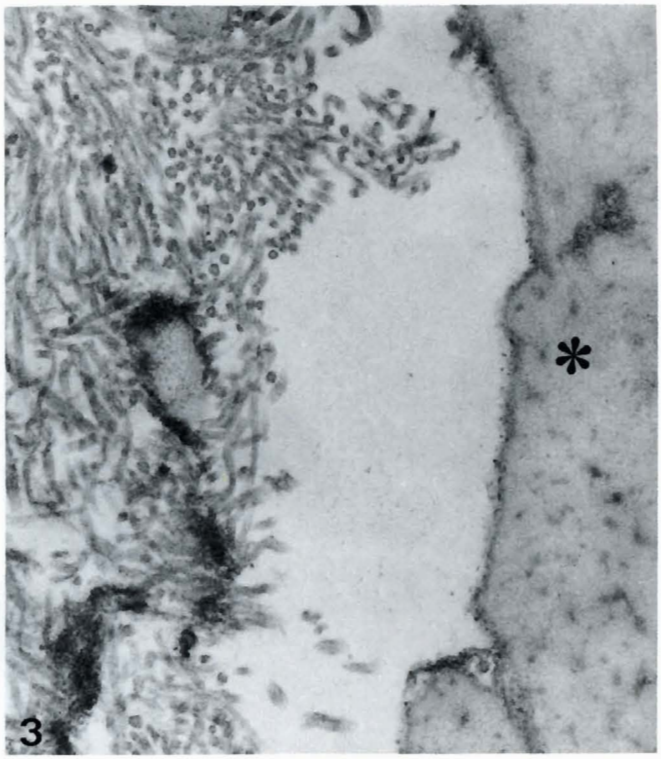


Fig. 2. Alcian blue-glutaraldehyde-osmium fixation. Alcianophylic PGs appear as thin filaments in close relation to collagen fibrils (arrow) or as rod-like particles (white arrows) distributed in the large areas between collagen bundles, elastic fibers (asterisk). x 29,000

Fig. 3. 4M GuHCl. Alcian blue-glutaraldehyde-osmium fixation. The area between collagen bundles, elastic fibers (asterisk), is completely empty and around collagen fibrils no alcianophylic segment could be detected. The period of collagen fibrils is easily detectable. x 29,000

The alcianophilic rod-like particles were visible prevalently around the elastic fibers. The latter observations suggested that these proteoglycans may act connect the different components of arterial wall, acting as bridges in the spaces between smooth muscle cells, elastic fibers and collagen fibrils. It is likely that this connecting system consists mainly of chondroitin-sulphate- and dermatansulphate-containing PGs, extracted using extractive solvent and hence no longer visible at ultrastructural level. 4M GuHCl extraction did not appreciably affect the structure of collagen fibrils, which maintained a clearly visible period.

The second family of proteoglycans, resistant to prolonged treatment with 4M GuHCl, were not detected using ultrastructural histochemical techniques performed subsequent to GuHCl treatment. Possibly the former might either be concealed inside the collagen fibrils or connected to collagen fibrils by non saline links. Although the latter hypothesis cannot be completely excluded, it is not very probable due to the fact that collagenase digestion solubilizes proteoglycan residues into the medium (Bardoni et al., 1994).

Further digestion of GuHCl-extracted by means of Streptococcal hyaluronidase, chondroitinase or heparanase/heparitinase III caused a disruption and subsequent swelling of collagen into microfibrils with a consequent loss of periodicity. Streptococcal hyaluronidase completely disrupted all collagen fibrils while chondroitinase caused swelling particularly in collagen fibrils sited far from elastic fibers; heparanase/heparitinase digestion disorganized collagen fibril sites in the vicinity of elastic fibers.

Selective enzymatic digestion of any of the proteoglycan components of aortic wall is sufficient to swell collagen fibrils; the varying activity of these enzymes would suggest the contribution of different proteoglycans towards the morphological integrity of collagen fibrils in different areas of human aorta tunica media.

Wegrowski et al. (1986) suggested a probable basement membrane nature of chondroitinsulphate-PGs and heparansulphate-PGs obtained from pig aorta which had been previously treated with GuHCl and subsequently collagenase digested. This suggestion could also be applied to chondroitinsulphate-PGs and heparansulphate-PGs isolated from human aorta using the same procedure, owing to the strong similarity of collagen, with regard to galactosaminoglycan composition and hydrodynamic size.

The phenomenon of collagen fibril swelling demonstrates the fundamental role of these proteoglycans in maintaining the integrity and periodicity of

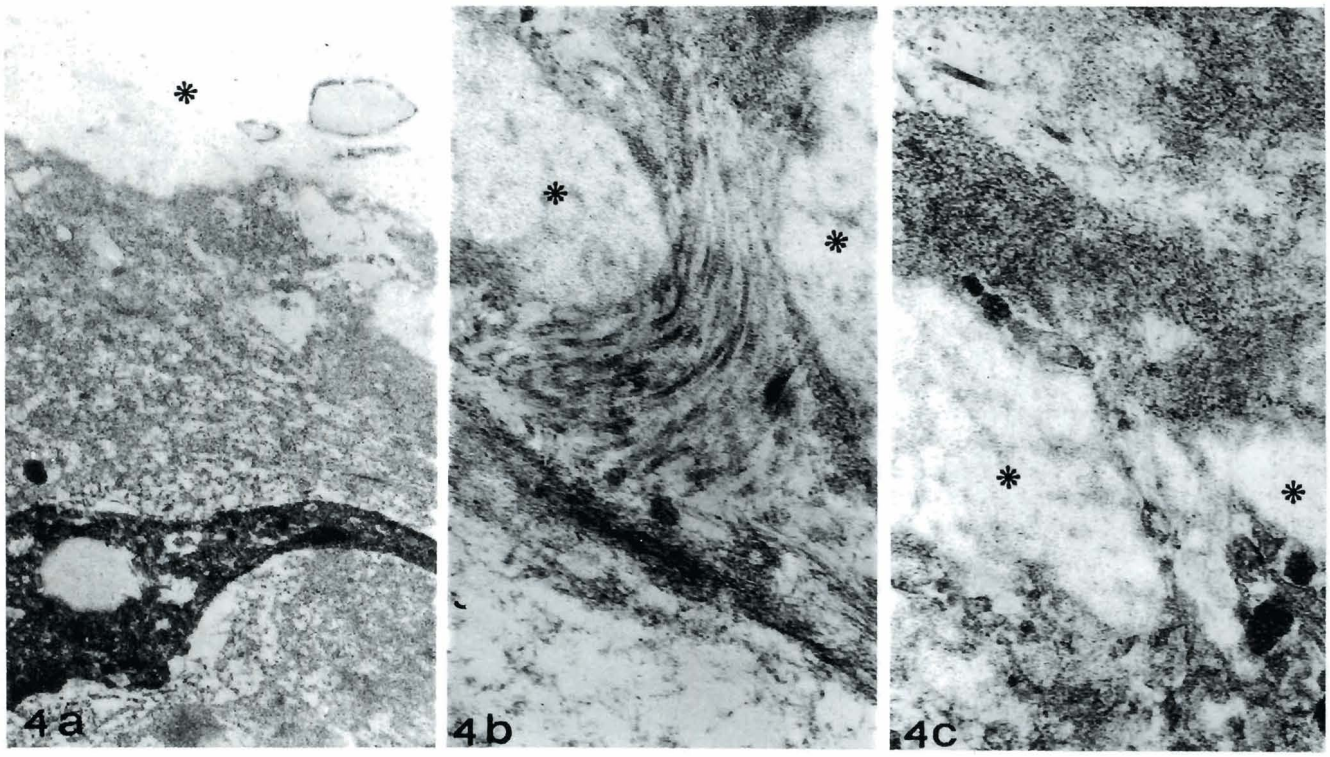


Fig. 4. 4M GuHCl followed by enzymatic treatment. Alcian blue-glutaraldehyde-osmium fixation. **a.** Streptococcal hyaluronase digestion. The area between elastic fibers (asterisk) is completely occupied by thin aperiodic filaments resulting from collagen fibril swelling. No alcianophylic segment is evident. x 16,000. **b.** Flavobacter chondroitinase ABC digestion. A small number of collagen fibrils appear swollen in thin fibrils. The swollen fibrils are localized prevalently far from elastic fibers (asterisk). No alcianophylic segment is evident. Elastic fibers do not seem affected by such treatment. x 29,000. **c.** Heparanase/heparitinase III digestion. A great number of collagen fibrils appear swollen in thin fibrils. The fibrils prevalently swollen are near to elastic fibrils (asterisk). No alcianophylic segment is evident. Elastic fibers do not seem affected by such treatment. x 29,000

collagen fibrils.

However, it is as yet impossible to precisely map possible interactions between proteoglycans and collagen fibres. Moreover the role of hyaluronic acid should undergo further investigation although undoubtedly, the nature of this interaction is a matter of considerable interest.

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