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Synthesis of proteoglycans in tissue explants derived from aortas of rabbits with experimentally induced atherosclerosis

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Proteoglycans and their constituent glycosaminoglycans are present in blood vessel walls and form important structural links between fibrous components of the intercellular matrix (collagen and elastic fibres) and the arterial endothelial and smooth muscle cells. Proteoglycans are involved in the processes essential for the physiological function of blood vessels, as well as in pathogenetic changes leading to formation of atherosclerotic plaques [1].

Our previous investigations were aimed at quantitative and qualitative evaluation of proteoglycan fractions isolated from rabbit aorta in experimentally induced atherosclerosis [2]. The presented experiments concerned the synthesis of proteoglycans in aortic tissue explants derived from rabbits with the diet-induced atherosclerosis and control animals, and were based on studying the incorporation of labelled precursors (leucine and sulfate) [3, 4].

New Zealand white male rabbits (2.5 - 3.0 kg) were fed standard feed mixture *ad libitum* for 3 months. Then the experimental group (n = 20) was maintained for 12 weeks on rabbit chow supplemented with 0.5% cholesterol and the control group (n = 10), on regular rabbit chow. The rabbits weight and blood cholesterol were controlled periodically.

Immediately after killing of the animals, by injection of Morbital solution, the entire aorta was removed, stripped off all extraneous tissue, slit longitudinally and rinsed with cold 0.15 M NaCl supplemented with fungizone and doxycyclinum. Aorta was cut into 2 - 3 mm segments which were placed in Eagle solution supplemented with Hepes buffer (20 mM, pH 7.4), penicillin and streptomycin but no fungizone or doxycyclinum. After preincubation (1 h, air atmosphere, 37°C) the medium was replaced by a new portion which contained in addition 50 μCi [^{3}H]leucine and 100 μCi $Na_2{}^{35}SO_4$ and the tissue was incubated at 37°C. At time intervals, the tissue was separated by centrifugation (6000 × *g*, 30 min at 4°C), washed twice with cold Eagle balanced salt solution and extensively dialyzed against 0.1 M NaCl containing nonlabelled leucine and nonspecific protease inhibitors (phenylmethylsulfonyl fluoride, 0.5 M; aminocaproic acid, 0.1 M; *N*-ethylmaleimide, 5 mM; EDTA, 10 mM; benzamidine•HCl, 1 mM). Then the tissue was transferred to cold (4°C) acetone, dry-defatted, weighed and stored.

Proteoglycans were isolated from the culture incubation media and the tissues derived from both atherosclerotic and control groups.

Proteoglycans of the aortic tissue were isolated at 4°C by extraction for 48 h with guanidine hydrochloride (GdnHCl) supplemented with 0.5% Chaps in 0.05 M Tris/HCl, pH 8, and the protease inhibitors as above. After centrifugation at 6000 × *g*, to the supernatant and culture medium (the latter supplemented with solid GdnHCl to 4 M concn.), solid CsCl was added (final density, 1.35 g/ml) and the samples were centrifuged for 30 h at 10°C in a Beckman L5W75 centrifuge at 130000 × *g*.

Fractions containing proteoglycan materials were pooled, the density raised to 1.45 g/ml by adding solid CsCl and the samples centrifuged again in dissociative density CsCl gradients for 20 h.

The specific gravity of each fraction was determined by weighing 1 ml aliquots. The radioactivity of ^{35}S and ^{3}H in macromolecular fractions was measured in an LKB Scintillation

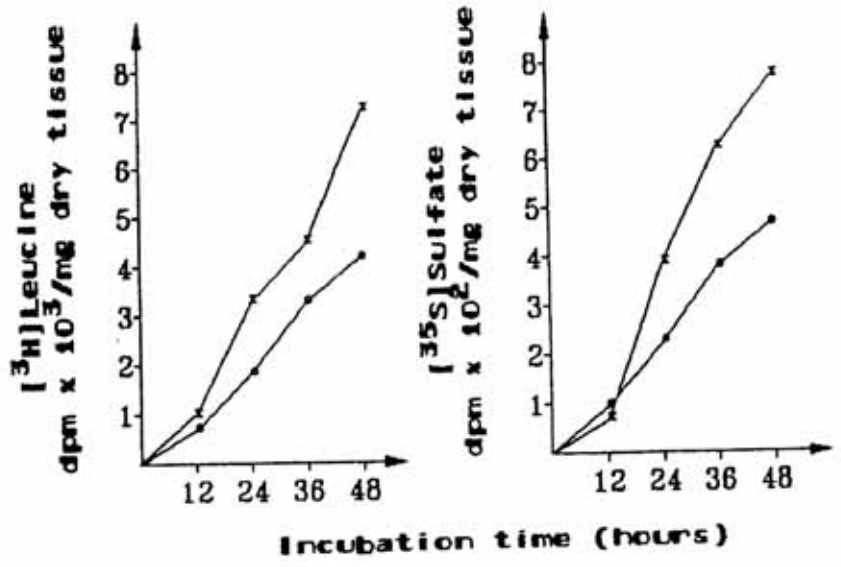

Fig. 1. [^{3}H]Leucine and [^{35}S]sulfate incorporation into macromolecules of control and atherosclerotic rabbit aorta explants.
Note the difference in ^{3}H- and ^{35}S-radioactivity scales. O, Control; x, experimental

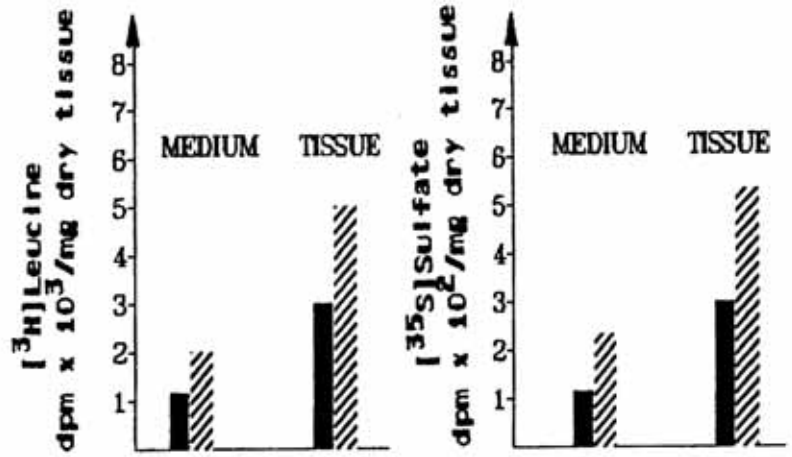

Fig. 2. Differences in ^{3}H- and ^{35}S-radioactivity in macromolecular material extracted by 4 M guanidine HCl from the culture medium and aortic tissue explants after 48 h of labelling.
Note the difference in ^{3}H- and ^{35}S-radioactivity scales. ■, Control; □, experimental

Spectrophotometer (1211 Rackbete). The content of protein was determined by the Lowry *et al.* [5].

The incorporation of [^{3}H]leucine and [^{35}S]sulfate into proteins of the whole 4 M Gdn HCl extracts from aortic explants, both from the animals with atherosclerosis and control ones (Fig. 1) increased sharply with time. After 12 h the results for the two groups were practically the same but later the incorporation of either precursor was higher for the experimental group. After 48 h the incorporation of [^{3}H]leucine was higher in this group by about 88% and that of [^{35}S]sulfate, by about 79%. Therefore, in further experiments the tissue explants were labelled for 48 h.

Both in the control and experimental group, about 30% of the total radioactivity (^{3}H or ^{35}S) was recovered in the GdnHCl extracts from the culture medium, whereas about 70%, in the tissue extracts (Fig. 2). The ^{3}H/^{35}S ratio (about 10) was consistently the same for proteins ex-

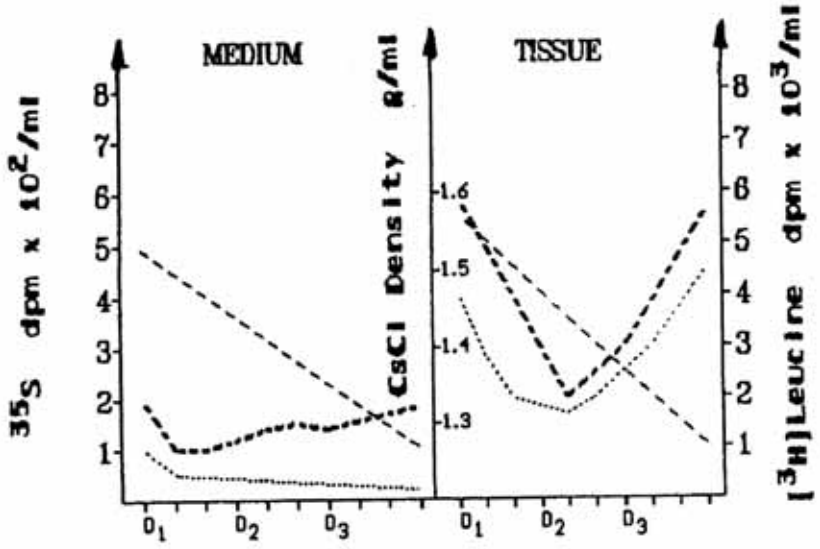

Fig. 3. CsCl isopycnic centrifugation of the guanidine•HCl extracted proteoglycans from the culture medium and aortic tissue.

Centrifugation was performed under double dissociative conditions at $\rho_0 = 1.45$. Thirty fractions, starting from the bottom, were collected from each tube. Basing on their ^{35}S- or ^{3}H-radioactivity they were pooled, for further studies, into three fractions: D_1, D_2 and D_3. —, ^{3}H-Activity; ···, ^{35}S-activity; ---, density CsCl

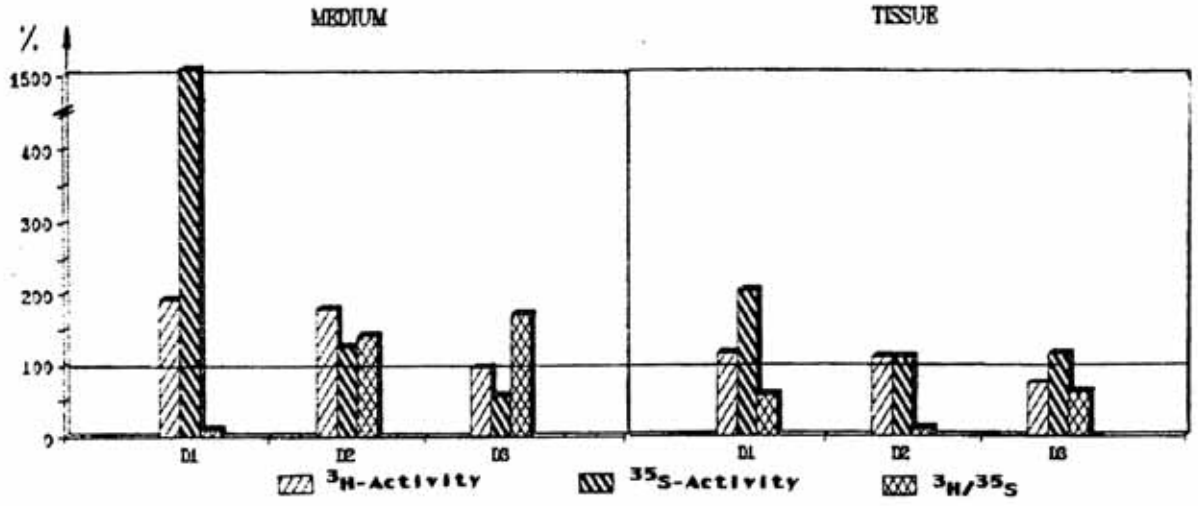

Fig. 4. Differences in ^{3}H- and ^{35}S-radioactivity of fractions D_1, D_2 and D_3 obtained by CsCl isopycnic centrifugation of the GdnHCl extracts of culture medium and aortic tissue explants derived from rabbits with experimental artherosclerosis.

The results are expressed as percentage of the respective controls. On the Figure the control level is shown as 100%.

tracted from culture medium and aortic explants. The results obtained for the whole Gdn HCl extracts indicate that the incorporation of one sulfate residue was accompanied by incorporation of about 10 residues of leucine.

The extracted material was fractionated by double dissociative CsCl gradient centrifugation, and the three fractions obtained, D_1, D_2 and D_3, were further studied (Fig. 3). It appeared that, in agreement with the data of other workers [3, 4], the proteoglycans were present in fraction D_1.

The proteoglycan fraction D_1, (Fig. 4) isolated from the culture medium of atherosclerotic tissue showed increased incorporation of [^{3}H]leucine (by about 90%) and [^{35}S]sulfate (by

1500%) in relation to the incorporation found in the same fraction from control culture medium (Fig. 4). In consequence of such disproportional increase in incorporation of the two precursors, the ratio of $^{3}H/^{35}S$ label in the proteoglycans of fraction D_1 from the atherosclerotic tissue culture medium was only about 4.1. This value was much lower (by about 88%) in relation to the control, and lower from the ratio found for the whole extract. The results indicate that in the proteoglycans which were secreted into the culture medium by atherosclerotic tissue, incorporation of one sulfate residue was accompanied by incorporation of only 4 leucine residues, in contrast to the control, where as much as 33 leucine residues were incorporated along with one sulfate.

The results for the proteoglycans recovered in fraction D_1 from the atherosclerotic tissue after 48 h of ^{3}H or ^{35}S labelling proved similar: the ratio of $^{3}H/^{35}S$ equalled 4.4. However, in comparison with fraction D_1 of the control tissue [^{3}H]leucine incorporation was increased only by 10% whereas the increase in [^{35}S]sulfate incorporation reached about 100%.

The distribution of ^{3}H and ^{35}S radioactivity fractions D_2 and D_3, was also different, both in the culture medium and in tissue.

An increase in incorporation of leucine (by about 80%) and sulfate (by about 25%) in fraction D_2 obtained from culture medium of the atherosclerotic tissue, was observed. Values of the $^{3}H/^{35}S$ ratio were higher both in fraction D_2 and D_3 than in D_1 (proteoglycans); they were also higher (by 40 and 70%, respectively) than for the appropriate fractions of the control medium. This suggests reduced sulfatation of macromolecules isolated in fraction D_2 and D_3 from the culture medium of atherosclerotic tissue.

The label incorporation into the corresponding fractions, D_2 and D_3, isolated from aortic tissue explants was not significantly different. An about 10% increase in both ^{3}H and ^{35}S incorporation into fraction D_2 was observed, but the $^{3}H/^{35}S$ ratio (11) was the same for the control and experimental group and was equal to that for the whole GdnHCl extracted proteins from the culture medium and aortic explants (cf. Fig. 2). These results indicate that experimental atherosclerosis did not affect either synthesis or sulfatation of those proteins which have been recovered from cultured aortic tissue as fraction D_2.

In this study it was found that incorporation of [^{3}H]leucine and [^{35}S]sulfate into proteoglycan fractions, after 48 h labelling of aortic explants from rabbits fed a cholesterol-rich diet, was increased in comparison to that in the explants from control animals. The results suggest an increase in synthesis and sulfatation of the proteoglycans secreted into the culture medium by atherosclerotic aortic tissue, as well as of other proteoglycans that were strongly bound to the connective tissue matrix of the explants. However, no differences were observed in the synthesis of other proteins of this matrix between the experimental and control groups.

REFERENCES

1. Wight, T.N. (1989) *Artherosclerosis* **9**, 1 - 20.
2. Skop, B., Dróżdż, M., & Żak, I. (1991) *Acta Biochim. Polon.* **38**, 219 - 227.
3. Mazzicato, P., Faris, B., Hollender, W., Franzblau, C. & Schmid, K. (1982) *Atherosclerosis* **45**, 359 - 363.
4. Radhakrishnamurthy, B., Srinivasan, S.R., Eberle, K., Ruiz, H., Dalferes, E.R., Shrama, Ch. & Berenson, G.S. (1988) *Biochim. Biophys. Acta* **964**, 231 - 243.
5. Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265 - 275.