Diabetologia (1993) 36: 316–322

Diabetologia

Distribution of glycosaminoglycans in the intima of human aortas: changes in atherosclerosis and diabetes mellitus

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Summary. Arterial glycosaminoglycans are considered to be important in atherogenesis due to their ability to trap lipid inside the vessel wall and to influence cellular migration and proliferation. Atherosclerotic lesions have displayed an altered glycosaminoglycan content and distribution. Diabetes is a recognized risk factor for atherosclerosis, but no information is available on the arterial glycosaminoglycans in human diabetes. We examined glycosaminoglycans in normal and atherosclerotic intima of non-diabetic and Type 2 (non-insulin-dependent) diabetic patients. Intima was stripped from autopsy samples of thoracic aortas; normal and plaque areas were separated. Glycosaminoglycans were isolated by delipidation, proteolytic digestion, and precipitation and characterized by quantitation of total glycosaminoglycan and evaluation of glycosaminoglycan distribution by electrophoresis and densitometry. Results indicate a significant decrease in total glycosaminoglycan and significant changes in their distribution in atherosclerotic plaques: a relative decrease in heparan sulphate, a relative increase in dermatan sulphate and thus a decrease in the ratio of heparan sulphate to dermatan sulphate. A similar but less marked change in the ratio was found in normal intima of diabetic subjects, while in their plaques this change was more pronounced. This suggests that changes in arterial glycosaminoglycans (especially the ratio of heparan sulphate to dermatan sulphate) precede the development of lesions in diabetes and may be important in atherogenesis.

Key words: Glycosaminoglycans, atherosclerosis, diabetes mellitus, proteoglycans.

Glycosaminoglycans (GAGs) have been found to be actively involved in a number of biological processes. Their role in atherosclerosis is mainly due to their property of interacting with lipoproteins (LP) which might contribute to both extracellular and intracellular lipid accumulation in the arterial wall [1]. Recently, studies have implicated proteoglycans (PG) in adhesion, migration and proliferation of endothelium and smooth muscle cells (SMC), which are important events in atherogenesis [2, 3]. Since different types of PGs have different and often opposing actions, it is important to detect the exact nature of changes in GAG components in atherosclerosis and their relation to risk factors.

For the last 30 years many investigators have been studying GAGs in human atherosclerosis and have reported both qualitative and quantitative changes [4–14]. These studies have indicated that GAGs increase during early atherogenesis, but decrease as the lesion advances. Various changes in GAG distribution have been reported; the more common findings are an increase in dermatan sulphate (DS) and chondroitin sulphate (CS) and a decrease in heparan sulphate (HS) and hyaluronic acid (HA) proportions. Changes similar to these have also been found in experimental atherosclerosis using either injury or hypercholesterolaemic models [15–17].

The prevalence of atherosclerosis and its clinical manifestations are markedly higher in diabetic individuals [18, 19]. Its cause and mechanism are not clearly known, although many potentially atherogenic factors have been implicated [20, 21]. It seems logical to raise the question of the role of GAGs in this process. Changes in arterial GAGs prior to or concomitant with the development of lesions may be an important factor in accelerated atherogenesis [22]. Up to now all of the studies have been performed in experimental models of Type 1 diabetes and the changes reported in arterial GAGs have been diverse [23–27]. No study has yet been undertaken on Type 2 diabetes in humans or animals.

Our study was initiated to study changes in GAG concentration and distribution in human atherosclerotic plaques and intima of Type 2 diabetic subjects, and to find whether a link existed between these two.

Subjects and methods

Subjects

Fresh human aortas were received at the time of autopsy and immediately frozen at −20 °C until processed. A maximum time interval of 24 h between death and autopsy was allowed in this study. Most of the aortas were from elderly patients, no specimen was included from young individuals. Tables 1 and 2 show the characteristics of subjects whose aortas were used. The mean age of the non-diabetic and diabetic groups was 69.5 ± 7 and 71.2 ± 11.5 years, respectively and the difference was not statistically significant. Groups were not segregated according to gender, nor on the basis of concomitant diseases. All of the diabetic subjects had Type 2 disease and had been treated with oral hypoglycaemic agents (subject no. 1 often required insulin). Their mean blood glucose levels were calculated from the values obtained during the last 3 years.

Tissues

Tissues for the isolation of GAGs were selected from the arch and descending thoracic part of the aorta. Only those aortas were included which had sufficient amounts of both normal looking and uncomplicated plaque areas. The outer adventitial layer was removed and the innermost layer (intima) was carefully separated from the underlying media. Normal and plaque areas were selected from the intimal layer. Normal areas were those which were grossly normal, thin, translucent and with no visible elevations. Areas selected as lesion contained definitely elevated white to yellow opaque plaques with no gross ulcers or calcification. No differentiation was made on the basis of the amount of lipid in the lesion or the ratio of fibrous to fatty tissues. Selection of tissues was confirmed by histological examination following staining with haematoxylin and eosin. This also confirmed that tissues used for isolation of GAGs contained mainly intima with little or no media attached.

Four types of tissues were obtained: 1. Normal tissues from non-diabetic subjects (control); 2. plaques from non-diabetic subjects (plaque); 3. normal tissues from diabetic subjects (diabetic); 4. plaques from diabetic subjects (diabetic-plaque).

Isolation of GAGs

The methodology used for isolation of GAGs has been successfully employed by many investigators with some minor variations and includes delipidation, proteolytic digestion, trichloroacetic acid (TCA) precipitation and ethanol precipitation [28]. One to three aortas were processed at the same time depending upon the availability of tissues. After peeling and segregating the two types of tissues, their wet weight was determined. Tissues were delipidated in chloroform:methanol (2:1, volume/volume) using an electric homogenizer, as described by Folch et al. [29]. Lipid solvents were removed by passing through a Whatman filter paper while tissues were separated from the filter paper and allowed to dry for a few hours. The final weight, called dried defatted tissue (DDT) weight, was about 15–20% of the wet weight. DDTs were digested with papain in a buffer containing 0.1 mol/l Na acetate, 10 mmol/l cystein, 10 mmol/l EDTA and pH adjusted to 6.5 with 1 mol/l NaOH. Each 50 mg of tissue was incubated with 0.5 mg of papain in 1.5 ml tubes at 60 °C in a shaking water bath for about 20 h. An extra 0.25 mg papain and buffer was added during the incubation. Following incubation, the tubes were centrifuged and washed once. Undigested protein and nucleic acids in the collected supernatant and washings were precipitated with 5% TCA. For precipitation of GAGs 4 volumes of ethanol and 1 volume of a saturated solution of sodium acetate were added to the tubes and left overnight at 4 °C. Precipitates were dissolved in distilled water and the process of ethanol precipitation was repeated. Finally, precipitates containing purified GAGs were allowed to dry and dissolved in 200 µl of distilled water.

Confirmation of losses during GAG isolation

To ascertain that extraction of GAG from tissues was complete and to detect losses during the isolation process the following procedures were performed:

(i) In some cases (four non-diabetic and two diabetic samples) digested tissues were further digested with papain for 24 h to determine whether GAGs were still incorporated. Redigested materials were further processed in a similar manner; however, only an additional 1.5% of total GAG was recovered.

(ii) Since incompletely digested PGs may be precipitated by TCA, we tried to extract GAGs from TCA precipitates. TCA precipitates (four non-diabetic and two diabetic samples) were dissolved in 0.1 mol/l NaOH, neutralized with 0.1 mol/l HCl and then digested with papain as already described. A loss of 1.8% was detected.

(iii) To detect losses during the isolation process other than those due to incomplete digestion, a known quantity of standard GAGs was aliquoted and the process of GAG isolation was performed starting from the papain digestion. Losses were calculated by comparing GAG weight before and after the process. Values of 95–105% recovery were obtained with a mean of 99.7%. Since all the losses combined were still less than 4% no corrections were made for them.

Table 1. Characteristics of the non-diabetic group

No.	Sex	Age (years)	Major chronic problem	Immediate cause of death
1	F	61	Metastatic parotid carcinoma	Respiratory failure
2	F	68	Motor neuron disease	Aspiration pneumonia
3	F	79	Liver and breast carcinoma	Hypovolaemic shock
4	F	70	Primary biliary cirrhosis	Hypovolaemic shock
5	M	78	Adenocarcinoma stomach	Bronchopneumonia
6	F	67	Squamous cell carcinoma lung	Bronchopneumonia
7	M	66	Carcinoma colon	Myocardial infarction
8	F	76	Parkinsonism	Bronchopneumonia
9	F	58	Metastatic uterine sarcoma	Hypovolaemic shock
10	F	72	Metastatic adenocarcinoma lung	Bronchopneumonia

Table 2. Characteristics of diabetic group

No	Sex	Age (years)	Duration of diabetes (years)	Mean blood glucose (mmol/l)	Other chronic problem(s)	Immediate cause of death
1	F	92	> 15	10.0 ± 3.5	Adenocarcinoma caecum Chronic cholecystitis	Bronchopneumonia
2	F	67	3	12.1 ± 3.4	–	Myocardial infarction
3	F	73	> 15	12.7 ± 6.0	Hypertension	Cardiac failure
4	M	68	> 15	10.0 ± 4.0	Peripheral vascular disease	Myocardial infarction
5	M	70	12	12.2 ± 5.3	Carcinoma rectum Peripheral vascular disease	Myocardial infarction
6	M	57	6	10.3 ± 2.9	Idiopathic cirrhosis Peripheral vascular disease	Hepatic failure

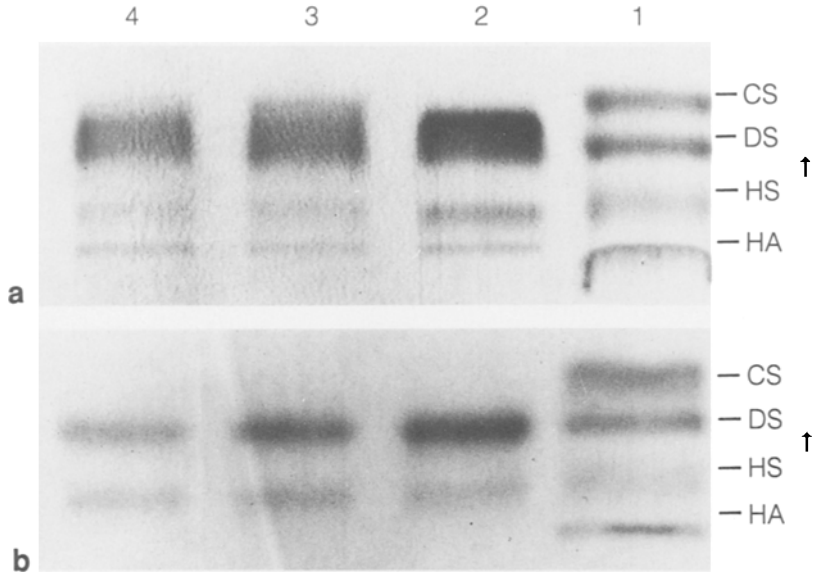

Fig. 1. Separation of glycosaminoglycans (GAGs) after electrophoresis on cellulose acetate membranes in 0.3 mol/l cadmium acetate, pH 4.1 at a current of 4.5 mA/strip for 1 h. Membranes were stained in 0.2% alcian blue. Lane 1: a mixture of standard GAGs (both panels); Lanes 2–4: Samples, before (panel a) and after (panel b) digestion with chondroitinase AC. Arrows depict the direction of migration. HA, Hyaluronic acid; HS, heparan sulphate; DS, dermatan sulphate; CS, chondroitin sulphate

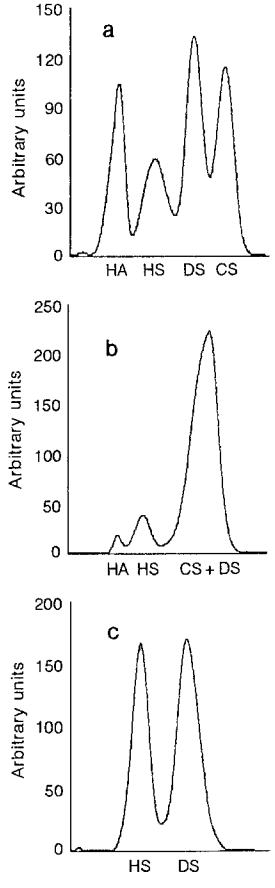

Fig. 2a–c. Densitometric scans of glycosaminoglycan (GAG) bands after electrophoretic separation (Fig. 1)
a A mixture of equal quantities of four standard GAGs
b A sample
c Same sample after chondroitinase AC digestion. HA, Hyaluronic acid; HS, heparan sulphate; DS, dermatan sulphate; CS, chondroitin sulphate

Characterization of GAGs

GAGs were measured by a method involving complex formation with alcian blue on cellulose acetate membrane [30]. Samples (4.5 µl) were spotted inside the marked areas on the membrane, allowed to dry, and stained in a solution of 50% ethanol containing 0.2% alcian blue 8 GX (Sigma, St. Louis, Mo., USA), 0.03 mol/l $MgCl_2$, and 0.1% glacial acetic acid for 30 min followed by destaining in a similar solution but lacking alcian blue. Later, the membrane was dried; pieces were cut out and dissolved in 1 ml of dimethyl sulphoxide containing 0.5% concentrated sulphuric acid. Absorbance was measured at 678 nm 20 min later. A blank containing distilled water and standards containing 0.2–1 mg/ml solution of CS-6 (shark cartilage) were included in every run. All measurements were made in triplicate. The assay was repeated in a few samples to ensure precision; a mean value of 3.8% was obtained for the coefficient of variation. Comparisons were made between other standard GAGs (DS, porcine kidney; HS, bovine kidney; HA, human umbilical cord: purchased from Sigma) and CS-6 to find out their relative binding capacities for alcian blue. Values of 65%, 90%, and 79% were obtained for HA, HS and DS, respectively. These values were used for the final adjustment of GAG weight.

To further validate the results, GAGs were also measured by a uronic acid assay using a method developed by Blumenkrantz and Asboe-Hansen [31]. o-Hydroxydiphenyl (Sigma) was used as the reagent.

To evaluate GAG distribution, electrophoresis was performed on cellulose acetate membrane followed by staining with alcian blue and densitometry. Initially a calcium acetate buffer was tested but later cadmium acetate was found to give better results. With cadmium acetate buffer a good separation of HA and HS was obtained. Although standards of DS showed a distinct band, resolution of DS from the CS band was not complete in the samples. To overcome this problem every sample was digested with chondroitinase AC which resulted in digestion of CS (and HA) leaving behind a distinct DS band (Fig. 1). However, some DS chains which are poor in iduronic acid could be digested by the enzyme. The bands were further identified with the help of other glycolytic enzymes. Electrophoresis was routinely performed in 0.3 mol/l cadmium acetate, pH 4.1 at a current of 4.5 mA/strip for 1 h in a Gelman Semi-Micro electrophoresis chamber (Ann Arbor, Mich., USA). Bands were stained with alcian blue following the run. We used a solution similar to that in total GAG assay but the concentration of ethanol was reduced from 50% to 10% to avoid dye precipitation following introduction of the membrane. Staining was performed for 30 min and was followed by destaining for 45 min. Finally, membranes were made transparent in glycerol for densitometry. These results were reproducible.

For densitometry, GS 300 densitometer (Hoefer Scientific Instruments, San Francisco, Calif., USA) and its software was used. Each band was scanned in triplicate. Chondroitinase AC digested samples were used to compare the relative proportion of DS with HS (Fig. 2). Differential binding capacity of GAGs for alcian blue was separately determined for this part of the GAG characterization. Compared with CS, values of 57%, 76%, and 108% were obtained for HA, HS and DS respectively. The distribution of GAGs in the samples was adjusted accordingly.

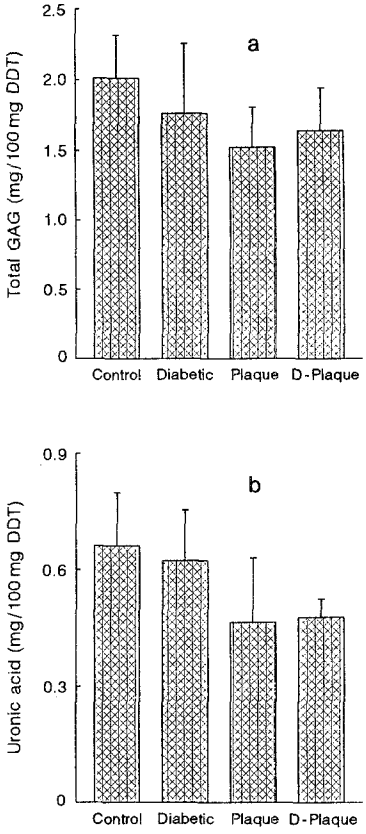

Fig. 3. **a** Total glycosaminoglycan (GAG) values (mean ± SD) from normal intima and plaques of ten non-diabetic and six diabetic samples. Assay was performed by complexing GAGs with alcian blue on cellulose acetate membrane followed by spectrophotometry at 678 nm. **b** Uronic acid values from the same samples. Control, normal area from non-diabetic subjects; Diabetic, normal areas from diabetic subjects; Plaque, plaques of non-diabetic subjects; D-Plaque, plaques of diabetic subjects; DDT, dry defatted tissue

Statistical analysis

Data from all groups were compared by one way analysis of variance and pairwise comparison was performed by Tukey post-hoc HSD test using the software Systat (Systat Inc., Evanston, Ill., USA).

Results

The results are depicted in Figures 3–5 and the significance of statistical comparisons are given in Table 3. These results represent the values which have been adjusted for the difference in alcian blue binding capacity of different GAGs. We obtained a value of about 2 mg/100 mg DDT for total GAG in the control group. Lower values were detected in plaques (Fig. 3a). A similar trend was observed when uronic acid was assayed. On the average the uronic acid values were about one-third of GAG values with a range of 29–35% for different groups. This agrees well with the values previously detected in our laboratory using different assay techniques [32]. To get an accurate representation of GAG distribution, data are expressed as percent of total GAG values (Fig. 4). We found values of 62.5%, 19.5%, 12.0%, 6.0% for CS, HS, DS, and HA, respectively for the control tissues. A change in distribution can be noticed in the plaque and diabetic tissues, especially a trend to lower HS values and higher DS values. This led us to express results in the form of a ratio of HS to DS. A ratio of 1.65 was calculated for the control tissues while lower values were obtained from the other groups (Fig. 5). It is evident that changes in GAG distribution in the diabetic group are similar to those found in the plaque but are of lesser magnitude. Interestingly, diabetic-plaque tissues clearly show similar changes but to a greater extent.

The results of statistical comparisons (Table 3) indicate that there is a significant decrease in total GAG and uronic acid along with highly significant changes in GAG distribution in atherosclerotic plaques (plaque vs control). There is a decrease in the proportion of HS, an increase in the proportion of DS, and a decrease in the ratio of HS to DS. Comparison of the diabetic with the control group shows no significant change in total GAG but a significant change in GAG distribution: an increase in DS and a decrease in HS-to-DS ratio. Diabetic-plaque tissues show more significant changes in GAG distribution. No significant difference was found between the diabetic and plaque groups.

The ratio of female to male was much higher in the non-diabetic group as compared with the diabetic group (4 vs 1). In order to rule out any potential effect of gender on GAG values we compared HS/DS ratio values from males and females of the control and diabetic groups. Both diabetic males and females showed significantly lower values than control females ($p < 0.05$). No other comparison was significant. It is difficult to draw any conclusion from comparison of males due to the small sample size, and for a similar reason, lack of any significant difference between males and females within each group does not completely rule out the possibility of a gender difference. However, the fact that HS/DS values of diabetic females are significantly lower than control females, suggests that the difference is independent of any effect due to gender.

To conclude, the most significant change observed in atherosclerosis was a decrease in the ratio of HS to DS. A similar but less pronounced change was present in the intima with normal appearance of diabetic humans that became more marked after the appearance of atherosclerotic plaques.

Table 3. Statistical significance

	Total GAG	Uronic acid	HA	HS	DS	CS	HS:DS
Plaque vs control	0.02	0.01	0.9	0.0005	0.002	0.8	0.0002
Diabetic vs control	0.5	0.9	0.3	0.3	0.03	0.3	0.001
D-Plaque vs control	0.2	0.06	0.8	0.0002	0.0002	0.3	0.0002
Diabetic vs plaque	0.5	0.1	0.6	0.2	0.9	0.7	0.4
D-Plaque vs plaque	0.9	1.0	1.0	0.2	0.05	0.7	0.5
D-Plaque vs diabetic	0.9	0.3	0.8	0.004	0.03	1.0	0.04

Control, normal intima from non-diabetic subjects; Diabetic, normal intima from diabetic subjects; Plaque, plaques of non-diabetic subjects; D-Plaque, plaques of diabetic subjects; GAG, glycosaminoglycans; HA, hyaluronic acid; HS, heparan sulphate; DS, dermatan sulphate; CS, chondroitin sulphate

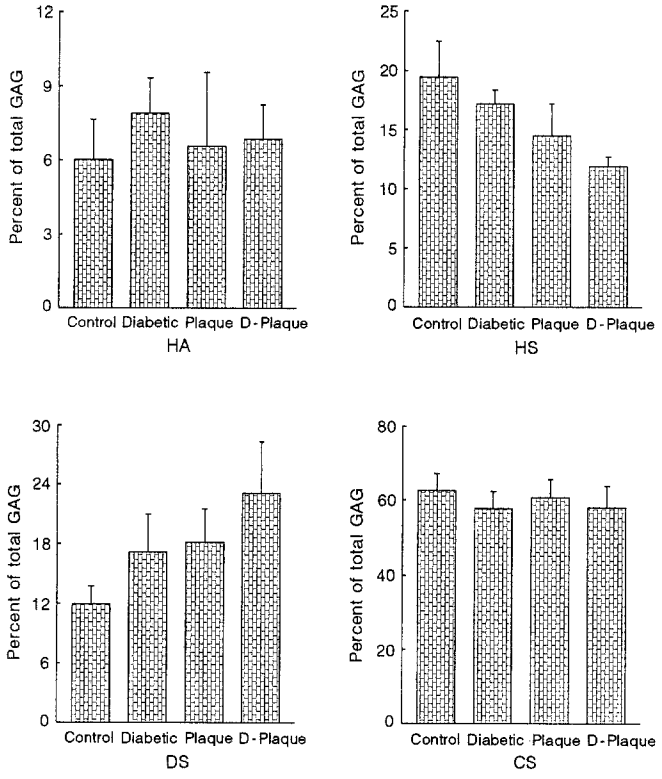

Fig. 4. Glycosaminoglycan distribution in normal and atherosclerotic intima from ten non-diabetic and six diabetic samples from human subjects. Values were obtained by densitometric evaluation of glycosaminoglycan bands after electrophoretic separation. Bars represent mean ± SD of *n* samples. Control, normal intima from non-diabetic subjects; Diabetic, normal intima from diabetic subjects; Plaque, plaques of non-diabetic subjects; D-Plaque, plaques of diabetic subjects; HA, hyaluronic acid; HS, heparan sulphate; DS, dermatan sulphate; CS, chondroitin sulphate

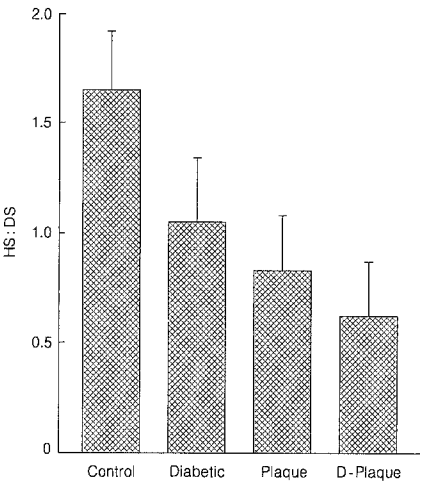

Fig. 5. Values (mean ± SD) for the ratio of heparan sulphate (HS) to dermatan sulphate (DS) obtained from the data presented in Figure 4

Discussion

We found a significant decrease in total GAG in fully developed atherosclerotic lesions. This is in full agreement with the results of most of the studies which have described an increase in GAG concentration in fatty streaks and a progressive decrease with the progression of lesions [5–13]. This appears contradictory to the common concept of an increase in matrix components in atherosclerosis. In fact, due to a concomitant increase in the major constituents of DDT, such as collagen, the accumulation of GAGs is overshadowed and despite the increased synthesis by smooth muscle cells of the lesion their concentration is decreased. However, since the intima of plaques is greatly thickened, the overall GAG content is actually increased: this has been documented by Wagner et al. [15].

We detected highly significant changes in GAG distribution in atherosclerotic plaques, i.e. a decrease in HS proportion and an increase in DS proportion. Results were more significant when we used the HS-to-DS ratio for comparison. Our results of increased DS and decreased HS distribution are in good agreement with other studies [10–14]. Some of these studies have also reported an increase in CS with or without a decrease in HA but our results differ in this respect. Studies involving PG extraction have also reported results agreeing with our findings [33–35]. Studies in experimental animals have also described very similar results [15–17]; these changes have been found to be the result of changes in synthetic capabilities of intimal tissues [36].

It is unknown how these changes in GAG distribution develop nor do we know the significance of these changes in atherosclerosis. However, it is known that intimal GAGs are produced by both endothelium and SMC. Endothelium provides predominantly HSPG while PGs from SMC are rich in CS and DS [2]. Endothelial injury has been described as a key event in atherosclerosis [37, 38]; with disturbed endothelial behaviour, synthesis of HSPG may also be affected. On the other hand SMC proliferation, which is an important feature in atherosclerosis, has been found to be associated with increased synthesis of the CS group of GAGs [39]. A selective increase in DS could be the result of the intimate relationship-morphologically and biologically between DS and collagen [2]. An increase in collagen content of lesions may be associated with an increase in DS in a cause-effect fashion or as an expression of some other underlying mechanism. In any case, the increase in DS may be necessary for the maintenance of the extracellular matrix of the lesions.

The GAG changes in injury-induced atherosclerosis have been found to be associated with increase avidity of LP-PG interactions [40]. The same phenomenon may be present in human lesions also but we cannot conclude that the change in HS-DS distribution is responsible. However, the decrease in HS is expected to have a bearing on SMC proliferation. Heparin and HS have been found to be antiproliferative for SMC and a decrease in HS may result in increased proliferation of SMC [41]. In addition HS and heparin have been found to be associated with cell adhesion and inhibition of cell migration [42]. A decrease in HS may therefore cause/enhance endothelial detachment and facilitate SMC migration from media to intima.

In this study we present the first report of the changes in arterial GAGs in human diabetes and also the first in Type 2 diabetes. Changes in GAG distribution were simi-

lar to those in atherosclerosis, particularly a significant decrease in the ratio of HS to DS. Changes detected in atherosclerotic intima of diabetic subjects were also similar but more marked. These results indicate that changes in GAGs are present in diabetic intima before the appearance of lesions and become pronounced at the same time or after their appearance. These findings emphasize that GAG changes might have implications for atherogenesis.

These changes, along with widespread alterations found in other matrix components, may be a direct effect of hormonal imbalance in diabetes [23]. In fact, insulin has been found to alter the metabolism of GAGs in cultured arterial SMC [43]. The changes in GAGs can also be explained from another point of view. Since we found that changes in diabetic intima are similar to those found in atherosclerosis, it can be speculated that a similar mechanism, such as endothelial injury, might be operative in both cases. Indeed, a number of studies, employing both morphological and functional indicators, have established the presence of endothelial injury in diabetes [44, 45]. A number of factors have been suggested to be involved in this process in diabetes and include hyperlipoproteinaemia, hypertension, polyol pathway disturbance, lipid peroxides, and insulin, among others [20, 21]. The changes in arterial GAGs in diabetes may then be viewed as the result of continued and widespread endothelial dysfunction that is milder than in atherosclerosis.

In conclusion we detected significant changes in intimal GAGs in atherosclerosis and diabetes. We found a decrease in the HS-to-DS ratio in atherosclerosis and a similar but less marked decrease in diabetes. The significance of this ratio is not fully known but, extrapolating our findings in diabetes, we can speculate that a lesser ratio of HS to DS may be an indicator of the increased risk of atherosclerosis. This finding agrees well with that of Ying-Shan et al. [35] who detected similar changes in arterial GAGs in a population at increased risk of atherosclerosis.

Acknowledgements. This study was supported by a grant from the Medical Research Council of Canada (MT-10590).

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Received: 7 September 1992
and in revised form: 16 November 1992

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