Acta Biochimica Polonica

Vol. 43 No. 3/1996

567–574

QUARTERLY

Glycosaminoglycans of human serum and their alterations in diabetes mellitus

Ewa M. Koźma[a], Krystyna Olczyk[a], Andrzej Głowacki[a], Katarzyna Komosińska[a], Piotr Sonecki[a], Tomasz Najmiec[b] and Magdalena Jaźwiec[b]

[a]*Department of Clinical Chemistry and Laboratory Diagnostics, Silesian Academy of Medicine, Jagiellońska 4, 41-200 Sosnowiec, Poland,* [b]*First Municipal Hospital, Sosnowiec, Poland*

Received: 23 February, 1996; accepted: 30 July, 1996

Key words: diabetes mellitus, glycosaminoglycans, serum

Human serum contains several glycosaminoglycans (GAGs), mainly chondroitin sulphates and significantly less of heparan sulphate + heparin and dermatan sulphate. The non-insulin-dependent diabetes mellitus (with vascular complications) was associated with a significant increase in total serum GAG concentration, mainly of chondroitin sulphates and dermatan sulphate, with a simultaneous decrease in heparan sulphate + heparin level. These alterations were much more evident in patients with poor metabolic control. Hyaluronic acid (undetectable in healthy subjects and in patients with good metabolic control) appeared only in trace amounts in poorly controlled diabetic individuals. The obtained data allow to conclude that the diabetes mellitus-associated disturbances in tissue GAG metabolism lead to significant alterations in serum GAG composition.

Glycosaminoglycans (GAGs) are heteropolysaccharides commonly present in variety of tissues. Chondroitin sulphates (ChS), dermatan sulphate (DS), heparan sulphate (HS) and heparin (H), keratan sulphate (KS) and hyaluronic acid (HA) constitute the most common GAG structures. In most cases the GAG chains are attached to core proteins, forming proteoglycans [1]. These components are present mainly in extracellular matrix, consisting of basement membranes and classical interstitium, which occupies intercellular spaces in various tissues [2]. GAGs perform a wide range of biological functions. They are involved in: cell adhesion, migration and proliferation, protein secretion and gene expression. Thus, GAGs contribute to the general architecture and permeability properties of connective tissue. In addition, they serve as an anchor for cell-specific growth factors and enzymes in extracellular matrix and at the cell surface (for review see [3]).

It is known that diabetes mellitus affects the metabolism of several components of extracellular matrix, including glycosaminoglycans [4]. Changes in GAG metabolism are believed to play an important role in development of diabetic complications [2], being one of the main causes of diabetes-associated vascular pathology [5, 6].

Most studies on pathobiochemistry of GAGs in human diabetes were performed on various organs and cell cultures [7, 8–10]. Urinary excretion of these polysaccharides has been also investigated [11, 12]. Very few information concerning serum GAGs and their alterations in diabetes mellitus have been published. Moreover, only a small number of diabetic patients without symptoms of vasculopathy was inves-

Abbreviations: ChS, chondroitin sulphates; DS, dermatan sulphate; GAGs, glycosaminoglycans; H, heparin; HA, hyaluronic acid; HS, heparan sulphate; KS, keratan sulphate.

tigated and the relationship to patient's metabolic control was not fully evaluated [13, 14].

The present study was undertaken in order to determine the contents of various glycosaminoglycans in human serum and their alterations in non insulin-dependent diabetes mellitus, accompanied by the symptoms of macro- and microangiopathy.

MATERIALS AND METHODS

Studies were carried out on 21 control subjects of either sex, 62–74 years old and 35 patients of either sex, with non insulin-dependent diabetes mellitus, 64–79 years old, hospitalized in the First Municipal Hospital (Sosnowiec, Poland).

The control subjects were selected after clinical examinations to exclude metabolic or cardiovascular diseases. Control subjects had normal fasting and postprandial blood and urine glucose level and negative record of familial diabetes.

The patients had diabetes from 9 to 11 years. They were divided into two groups: those with good or poor metabolic control, according to the criteria described by Lorenzi [15]. The diabetic patients of both groups were treated with Tolbutamid (Diabetol, Polfa) in daily doses of 100–1000 mg.

Metabolic control was evaluated using conventional parameters such as serum and urine glucose concentrations, serum fructosamine concentration and serum cholesterol level. Patients with good metabolic control showed elevated blood glucose but no glucosuria. The blood level of fructosamine and cholesterol was normal. Patients with poor metabolic control showed distinctly higher blood glucose and glucosuria. The blood level of fructosamine and cholesterol was significantly higher than in the patients with good metabolic control. Control subjects had no symptoms of the disease nor abnormal physiological characteristics.

Peripheral venous blood samples were taken after an overnight fast and allowed to clot at room temperature. Serum was collected for GAG analysis.

Extraction and determination of serum GAGs

Glycosaminoglycans were isolated by the method of van Amerongen *et al.* [16]. GAGs were extracted from serum by extensive papain digestion. To 100 mg of dried and defatted serum 40 mg papain in 3 ml of 0.1 M phosphate buffer (pH 6.5) containing 5 mM EDTA-Na_2 and 5 mM cysteine hydrochloride was added. After papain digestion (24 h at 65°C) proteins were precipitated with cold 40% (w/v) trichloroacetic acid to a final concentration of 5% and were discarded. To the supernatant 3 vol. of 5% (w/v) CH_3COOK in 96% ethanol were added and GAGs were allowed to precipitate for 24 h at 4°C. After centrifugation (20000 × *g*, 0°C, 20 min.) 1 ml of 0.5 M aqueous CH_3COOK was added and GAGs were reprecipitated with 3 vol. of 96% ethanol for 24 h at 4°C. The final precipitate was stored at –10°C until used for biochemical analysis. Total amount of GAGs was quantified by the hexuronic acid assay according to Blumenkrantz and Asboe-Hansen [17] as modified by Slim *et al.* [18].

Assay of the serum GAGs

Total GAGs. Sample of serum GAGs (10 μg of hexuronic acids) was directly subjected to electrophoresis on cellulose acetate (Cellogel, Serva, Germany).

Heparan sulphate/heparin. To separate heparan sulphate + heparin, serum GAGs were treated with chondroitinase ABC in order to remove chondroitin-4- and 6-sulphates, hyaluronic acid and dermatan sulphate. The GAG sample, containing 10 μg of hexuronic acids was incubated with this enzyme (0.1 U) in a buffer composed of 50 mM Tris/HCl, 1 mM CH_3COONa, pH 8.0, at 37°C for 8 h. The reaction was stopped by addition of 100% (w/v) trichloroacetic acid to a final concentration of 5% (w/v) and the mixture was centrifuged at 10000 × *g*, for 30 min. The supernatant was collected and the sediment was discarded. To the supernatant 3 vol. of 96% ethanol were added and HS + H were sedimented at –10°C, for 16 h. The precipitate was centrifuged, dissolved in water and subjected to electrophoresis.

Dermatan sulphate. To separate DS, serum GAGs were treated successively with heparitinase, nitrous acid and chondroitinase AC in order to remove heparan sulphate + heparin, chondroitin-4- and 6-sulphates and hyaluronic acid. The GAG solution, containing 10 μg of hexuronic acids was incubated with heparitinase (0.01 U) in a buffer composed of 50 mM Tris/HCl and 6.5 mM CH_3COONa, pH 7.5, at 25°C, for 5 h. The reaction was stopped by

adding 100% (w/v) trichloroacteic acid to a final concentration of 5% (w/v). The precipitated products were removed by centrifugation and GAGs remaining in the supernatant were precipitated with ethanol as described above for separation HS + H.

Heparan sulphate + heparin, which was not digested with heparitinase, was treated additionally with nitrous acid according to Lagunoff & Warren [19]. To a 5 µl aliquot of GAGs solution 5 µl of aqueous 5% (w/v) $NaNO_2$ and 5 µl of 33% (w/v) aqueous CH_3COOH were added. The reaction was run for 80 min, at room temperature and excess of nitrous acid was then decomposed by the addition of 10 µl of aqueous solution of ammonium sulphamate (20 mg/ml).

The remaining GAGs: chondroitin-4- and 6-sulphates, dermatan sulphate and hyaluronic acid were precipitated with 3 vol. of 96% ethanol and digested with chondroitinase AC (0.125 U) in a buffer composed of 50 mM Tris/HCl and 50 mM NaCl, pH 7.3, for 8 h, at 37°C. The reaction was stopped by adding 100% (w/v) trichloroacetic acid to a final concentration of 5% (w/v). The precipitated products were removed by centrifugation. From supernatant DS was precipitated with ethanol as described above and after dissolving in water, subjected to electrophoresis.

Electrophoresis of GAGs. Total serum GAGs, HS + H and DS were subjected to electrophoresis on cellulose acetate by the method elaborated in our laboratory, as follows: 0.044 M $Al_2(SO_4)_3$ (pH 4.3) was used as an electrode solution at voltage of 150 V per strip applied. Electrophoresis was run at 18°C, for 1.5 h. The strips were stained with 0.2% (w/v) Alcian Blue 8GS (Aldrich-Chemie, Germany), in aqueous solution containing 10% ethanol (v/v), 0.1% glacial acetic acid (v/v) and 0.03 M $MgCl_2$ according to Hronowski & Anastassiades [20]. The excess of dye was rinsed off with the same solvent mixture (without Alcian Blue). The fragments of strips containing the stained GAG bands and unstained fragments of the same size (blank) were air-dried and dissolved in dimethyl sulfoxide containing 0.5 ml concentrated H_2SO_4 per 100 ml of the solvent. Full dissolution was achieved by incubation of the strips with the solvent in a water bath for 30 min, at 37°C. The absorbance was measured at 677 nm with the use of Hewlett Packard spectrophotometer. The calibration curves for standard GAGs (Ch-4-S from whale cartilage, HS from bovine kidney and DS from porcine skin, Serva, Germany) were performed over the concentration range from 0.125 to 4.0 mg hexuronic acids/ml. ChS content in serum GAGs samples was estimated as the difference between the total GAGs content and the sum of HS + H and DS contents. Densitometric reading were made with the use of GS 300 densitometer (Hoefer Scientific Instruments, U.S.A.). Because, HA shows slight heterogeneity and the lowest electrophoretic mobility, its identification was based on comparing its electrophoretic mobility with the mobility of standard HA from rooster comb.

Statistical analysis. Samples representing the population of diabetic patients with good metabolic control (n = 16), poor metabolic control (n = 19) and non-diabetic subjects (n = 21) were evaluated with Shapiro-Wilk test as described by Domański [21], in order to verify the assumption of normal distribution. Results were expressed as mean ±SD. Statistical evaluation was performed with the Student's *t*-test, accepting $P < 0.05$ as significant.

RESULTS

A report on blood and urine glucose level and serum fructosamine and cholesterol levels in the diabetic patients and healthy subjects is given in Table 1.

As can be seen from Fig. 1, total GAG concentration increased in serum of diabetic patients, particularly of those with poor metabolic control.

Electrophoretic patterns of total serum GAGs from control subjects and diabetic patients are presented in Figs. 2 and 3. It can be seen that GAGs isolated from the serum of control subjects separated on electrophoresis into two fractions: a fast and a slowly migrating one. Densitometric evaluations showed that in control material about ⅔ of GAGs were found in the slowly migrating fraction and ⅓ in the fast migrating one (Fig. 3A). In contrast, in the serum of diabetic patients (both with good and poor metabolic control) practically total amount of GAGs was found in the slowly migrating fraction (Fig. 2 and Fig. 3B and C). Only traces of material were found in the fast and in the very slowly migrating fractions. Electrophoretic mobility of

Table 1
Clinical data of control subjects and patients with non-insulin-dependent diabetes mellitus. Mean values ±SD.

	Number of subjects (n)	Sex (female /male)	Age mean (range)	Serum glucose (mmol/l)	Urinary glucose (μmol/24 h)	Serum fructosamine (μmol/l)	Serum cholesterol (mmol/l)
Control, non-diabetic subjects	21	15/6	68 (62–74)	4.53 ± 0.49	0	226.70 ± 39.66	4.24 ± 0.18
Diabetic, patients with good metabolic control	16	12/4	71 (64–79)	7.12 ± 2.65	0	243.00 ± 21.00	4.37 ± 0.17
Diabetic, patients with poor metabolic control	19	14/5	70.5 (64–79)	11.29 ± 5.83	64.94 ± 23.18	376.10 ± 42.17	5.36 ± 0.61

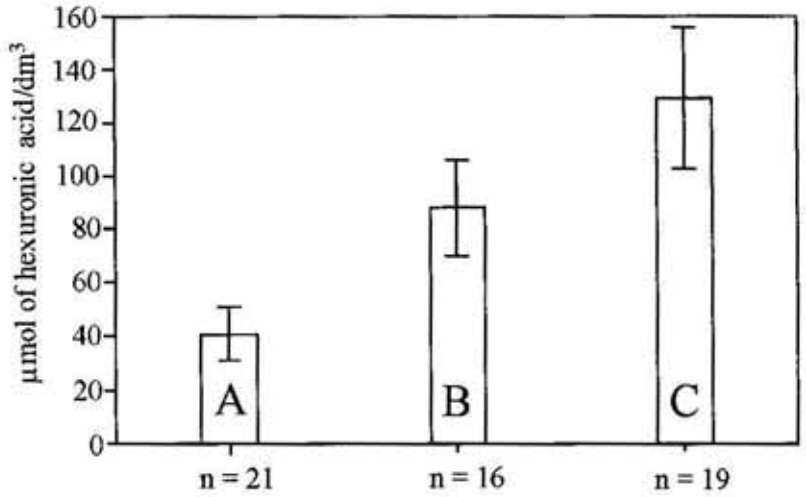

Fig. 1. Total glycosaminoglycans concentration in the serum of control subjects (A) and diabetic patients with good (B) and poor (C) metabolic control.

the fast and the very slow fractions from both diabetic groups was a little higher than that of GAGs fractions of control group.

The comparison of electrophoretic patterns of serum GAGs allowed to localize ChS, DS, HS + H and HA in electrophoregrams and to estimate them quantitatively (Figs. 3 and 4). Chondroitin sulphates (as judged from the difference between total GAGs and the remaining GAG components) were found to constitute the main fraction of serum GAGs both in control and in two diabetic groups. The levels of dermatan sulphate and heparan sulphate + heparin were distinctly lower (Figs. 3 and 4).

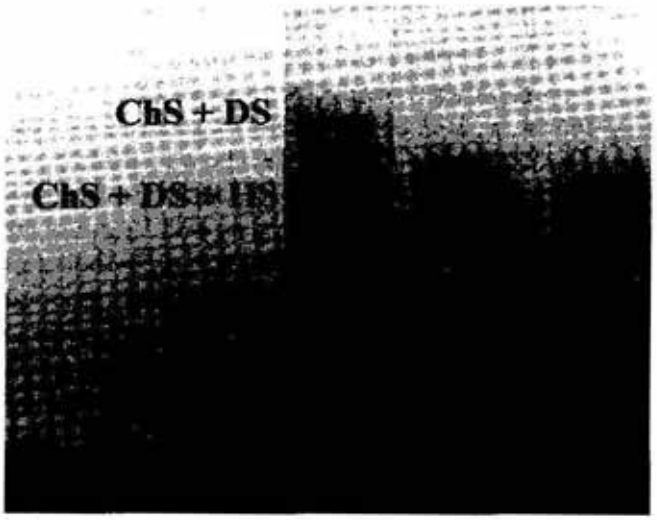

Fig. 2. Electrophoresis of glycosaminoglycans isolated from the serum of control subjects (A) and those of diabetic patients with good (B) and poor (C) metabolic control.

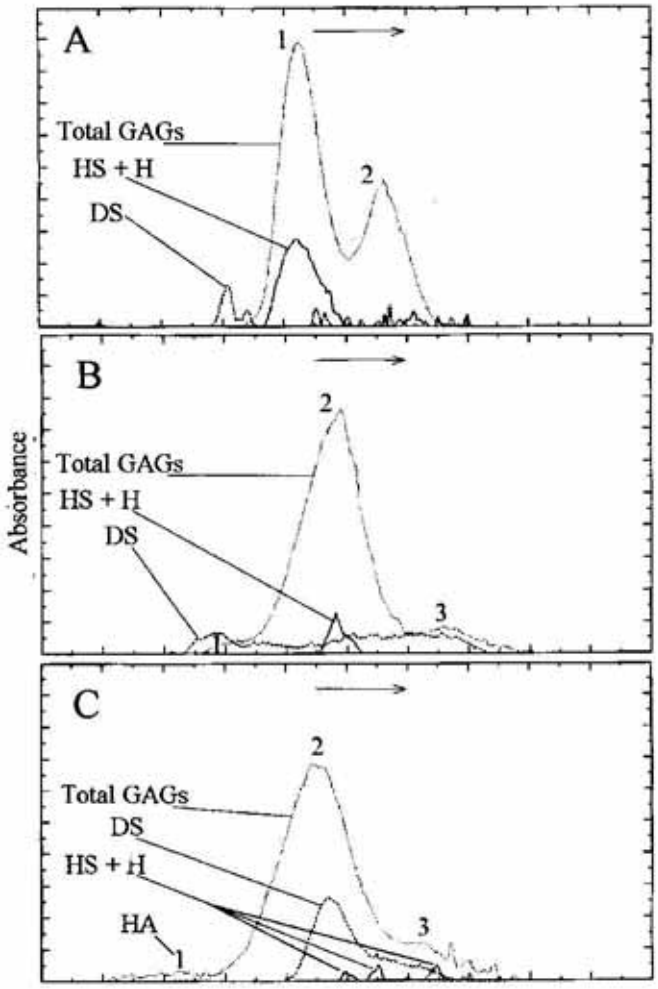

Fig. 3. Densitograms of serum glycosaminoglycans (GAGs) subjected to electrophoresis on cellulose acetate.
(A), Control subjects; (B), diabetic patients with good metabolic control; (C), diabetic patients with poor metabolic control. Bands of total GAGs are composed of the following glycans: (A) band 1: ChS, DS, HS; band 2: ChS, DS; (B) band 1: DS; band 2: ChS, DS, HS; band 3: ChS, DS; (C) band 1: HA; band 2: ChS, DS; band 3: ChS, DS. Arrow depicts the direction of the migration.

Chondroitin sulphates demonstrated electrophoretic heterogeneity in sera of all investigated groups. Two ChS pools, migrating within the GAG fractions with the fast and the slow electrophoretic mobility were found. In this group the presence of the very slowly migrating DS fraction was detected. In control group contribution of the slow and the fast migrating ChS fraction was similar. In both diabetic groups the slow migrating ChS fraction dominated. Dermatan sulphate also demonstrated electrophoretic heterogeneity, most evident in DS fraction, isolated from the serum of patients with good metabolic control. In contrast to the above mentioned GAGs, heparan sulphate + heparin obtained from sera of control subjects and patients with good metabolic control, migrate as single bands in slow migrating fractions (Fig. 3A and B). However, in patients with poor metabolic control trace amounts of HS + H were found both in the slow and in the fast migrating GAGs fraction (Fig. 3C).

Patients with non insulin-dependent diabetes mellitus showed significant quantitative changes in all the investigated serum GAGs (Fig. 4). First of all, concentration of ChS and DS distinctly increased, especially in the patients with poor metabolic control. In contrast, concentration of HS + H was insignificantly higher in patients with a good metabolic control but it decreased to trace amounts in those with poor metabolic control.

Hyaluronic acid was found only in trace amounts in the serum of some patients with poor metabolic control (Fig. 3C). Furthermore, KS was not found neither in serum of control subjects nor in sera of two diabetic groups.

DISCUSSION

Very little is known about the serum GAGs and their alterations in diabetic patients. The origin of serum GAGs is not fully elucidated. However, it is believed that the serum GAG level reflects active metabolic processes taking place at the tissue level [14]. This suggestion has been supported by studies on hyaluronic acid

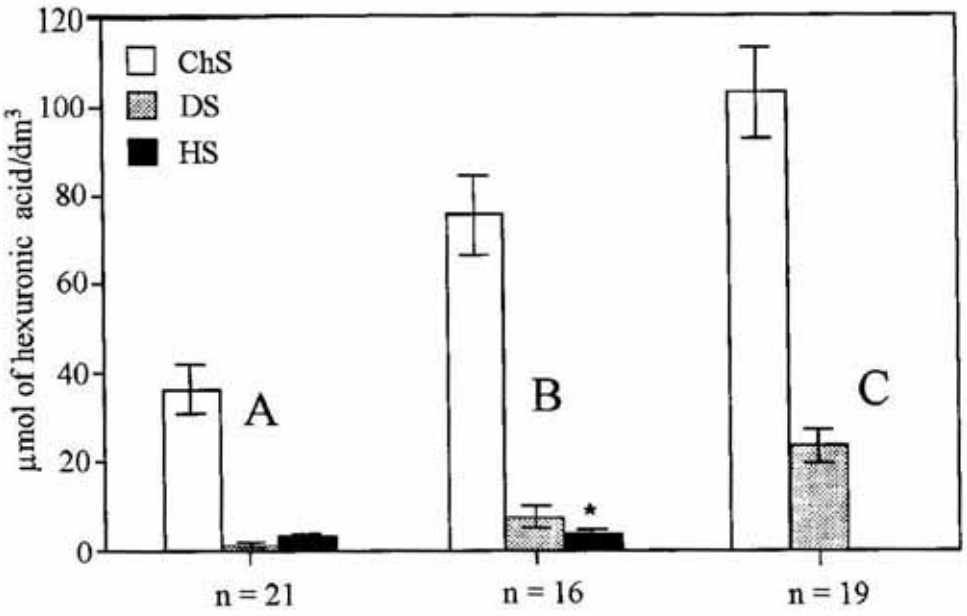

Fig. 4. The concentration of individual glycosaminoglycans in the serum of control subjects (A) and diabetic patients with good (B) and poor (C) metabolic control.
All differences statistically significant at $P < 0.05$ except for HS + H marked by *.

turnover which have shown that the circulating polymer comes from the peripheral tissues *via* the lymph [22]. First studies on serum GAGs in diabetic patients carried out by Malathy & Kurup [13] showed a decrease in chondroitin-4- and 6-sulphates and heparan sulphate and an increase in hyaluronic acid concentrations. Similar results were obtained by Ceriello *et al.* [14].

Our studies showed that in diabetic patients total serum GAG concentration was increased, particularly distinct GAG elevation was observed in the patients with poor blood glucose control. Similar results were reported by Ceriello *et al.* [14]. The increase in total serum GAG concentration, observed in our investigations, is expected to bear on the altered glucose metabolism in diabetic patients. In the course of diabetes mellitus, particularly in the case of poor glucose control, a number of factors, e.g. hyperlipoproteinaemia, polyol pathway disturbance and lipid peroxides lead to the damage of the vessel walls [23, 24]. The vessel impairment causes a release of some growth factors which induce alterations of GAG synthesis [25]. Transforming growth factor β was described to participate in atherogenesis and in diabetic nephropathy by stimulating the DS and ChS synthesis [26]. The observed elevation of DS/ChS ratio in serum of the patients with poor metabolic control might also be related with this phenomenon.

In both groups of patients we also found a distinct increase in chondroitin sulphates level, particularly significant in patients with poor metabolic control. The differences between the values of chondroitin sulphate concentration observed in our studies and those described by Malathy & Kurup [13] as well as Ceriello *et al.* [14] could result from different methods of GAGs determination as we have used a more efficient method of GAG isolation and fractionation and more sensitive and specific technique of individual GAG determination.

It is worth noting that serum chondroitin sulphates concentration found in our studies was significantly higher than that of dermatan sulphate, heparan sulphate or hyaluronic acid. This finding is in agreement with that of van de Lest *et al.* [27] who have found that ChS (mainly Ch-4-S) constitute the major GAGs in serum of healthy individuals. According to these authors DS is the second major serum GAG. However, it is inconsistent with our results, which are compatible with those described by Murata & Horiuchi [28]. Higher level of serum DS reported by van de Lest *et al.* [27] could results from age differences of blood donors. It is known that proportions of individual GAGs change during development and ageing processes [29, 30]. Serum dermatan sulphate concentration was very low in healthy subjects but distinctly higher in patients of both diabetic groups, especially in patients with poor metabolic control. This tendency is in agreement with that described by Wasty *et al.* [23] who have found a marked rise in DS level in the intima of diabetic individuals with and without macrovascular complications.

Our studies have also demonstrated that DS isolated from the serum of control subjects

showed very low electrophoretic mobility. This fraction appearing after heparitinase, nitrous acid and chondroitinase AC hydrolysis is probably composed of the aggregated DS iduronic domains. It is noteworthy that DS isolated from serum of diabetic patients with good metabolic control showed higher electrophoretic heterogeneity than that of poorly controlled diabetics. This might indicate differences in DS structure confirmed recently (unpublished).

As regards heparan sulphate and heparin, we found insignificant differences between healthy subjects and diabetic patients with good metabolic control. In the patients with poor metabolic control only trace amounts of HS + H were recorded. These results are in agreement with those described by Malathy & Kurup [13] and Ceriello *et al.* [14] who reported on the decrease in serum HS in diabetic subjects. Also the studies at the tissue level carried out by Wasty *et al.* [23] pointed to a distinctly decreased HS level in diabetic subjects with macroangiopathy. Simultaneously, these authors found a considerably reduced HS to DS ratio. The lower HS/DS ratio may be an index of the increased risk of atherosclerosis [23]. The same tendency was observed in our studies, particularly in the patients with poor metabolic control.

We did not find any amount of hyaluronic acid in the serum, neither in healthy individuals nor diabetic subjects with good metabolic control, whereas the trace amounts of hyaluronan appeared in patients with poor metabolic control. This might be due to its low concentrations in blood [31]. According to Lebel *et al.* [32] the normal concentration range of hyaluronic acid in the serum of healthy subjects varies from 10 to 100 μg/l. Also it can not be excluded that such low amount of hyaluronic acid is not detectable by the method applied by us. On the other hand, one can take into consideration a hypothesis on hepatic degradation of hyaluronan [31, 32]. This mechanism may be not sufficiently effective in diabetic patients with poor metabolic control. Furthermore, the slightly increased level of serum hyaluronan in the mentioned diabetic patients group may be related to the changes observed by Wasty *et al.* [23] in the intima of diabetic individuals with and without atherosclerotic plaques.

It is known that diabetes is accompanied by increased proteolytic activity in various tissues [33, 34]. This may lead to the cleavage of protein cores of proteoglycans releasing the GAGs from these complexes. The products of proteoglycan degradation may penetrate into circulating blood and elevate the concentration of glycosaminoglycans in the serum.

REFERENCES

1. Yanagishita, M. (1993) Function of proteoglycans in the extracellular matrix. *Acta Pathol. Japon.* **43**, 283–293.
2. Danne, T., Weber, B., Spiro, M.J. & Spiro, R.G. (1992) Changes of extracellular matrix metabolism in early diabetic angiopathy; in *Structural and Functional Abnormalities in Subclinical Diabetic Angiopathy. Pediatric and Adolescent Endocrinology* (Weber, B., Burger, T. & Danne, T., eds.) vol. 22, pp.11–22, Karger, Basel.
3. Wight, T.N., Heinegard, D.K. & Hascall, V.C. (1991) Proteoglycans: Structure and function; in *Cell Biology of Extracellular Matrix* (Hay, E.D., ed.) pp. 45–78, 2nd edn., Plenum Press, New York-London.
4. Sternberg, M., Cohen-Forterre, L. & Peyroux, J. (1985) Connective tissue in diabetes mellitus: Biochemical alterations of the intercellular matrix with special reference to proteoglycans, collagens and basement membranes. *Diabete Metabol. (Paris)* **11**, 27–50.
5. Makino, H., Ikeda, S., Haramoto, T. & Ota, Z. (1992) Heparan sulfate proteoglycans are lost in patients with diabetic nephropathy. *Nephron* **61**, 415–421.
6. Silbiger, S., Schlondorff, D., Crowley, S., Rosenberg, L., Choi, H., Hatcher, V. & Gordon, P. (1993) The effect of glucose on proteoglycans produced by cultured mesangial cells. *Diabetes* **42**, 1815–1822.
7. Silbert, C.K. & Kleinman, H.K. (1979) Studies on cultured human fibroblasts in diabetes mellitus. Changes in heparan sulfate. *Diabetes* **28**, 61–64.
8. Makino, H., Yamasaki, Y., Haramoto, T., Shikata, K., Hironaka, K., Ota, Z. & Kanwar, Y.S. (1993) Ultrastructural changes of extracellular matrix in diabetic nephropathy revealed by high resolution scanning and immunoelectron microscopy. *Lab. Invest.* **68**, 45–55.
9. Tamsma, J.T., van der Born, J., Bruijn, J.A., Assmann, K.J.M., Hermans, J., Veerkamp, J.H., Lemkes, H.H.P.J. & van der Woude, F.J. (1994) Expression of glomerular extracellular matrix components in human diabetic nephropathy: decrease of heparan sulphate in glomerular basement membrane. *Diabetologia* **37**, 313–320.

10. Hagedorn, M., Esser, P., Wiedemann, P. & Heimann, K. (1993) Tenascin and decorin in epiretinal membranes of proliferative vitreoretinopathy and proliferative diabetic retinopathy. *German J. Ophthalmol.* **2**, 28–31.

11. Colette, C., Etienne, P., Percheron, C., Bancel, E., Boniface, H., Lapiński, H. & Monnier, L. (1994) Glycosaminoglycan excretion in diabetes mellitus: Relation with atherosclerosis and glomerulosclerosis. *Diab. Nutr. Metab.* **7**, 295–303.

12. Gambero, G., Cicerello, E., Mastrosimone, S., Lavaginni, T. & Baggio, B. (1989) High urinary excretion of glycosaminoglycans: A possible marker of glomerular involvement in diabetes. *Metabolism* **38**, 419–420.

13. Malathy, K. & Kurup, P.A. (1973) Levels of glycosaminoglycans in the serum of diabetic patients. *Indian J. Biochem. Biophys.* **10**, 132–133.

14. Ceriello, A., Giungliano, D., Dello Russo, P., Pasariello, N., Saccomanno, F. & Sgambato, S. (1983) Glycosaminoglycans in human diabetes. *Diabete Metabol.* **9**, 32–34.

15. Lorenzi, M. (1986) Diabetes mellitus; in *Clinical Endocrinology* (Fitzgerald, P.A., ed.) pp. 337–409, Jones Medical Publications, Chicago.

16. van Amerongen, J.P., Lemmens, A.G. & Tonino, G.J.M. (1990) Glycosaminoglycans in dental pulp; in *Dynamic Aspects of Dental Pulp. Molecular Biology, Pharmacology and Pathophysiology* (Inoki, M., Kudo, K. & Olgart, T., eds.) pp. 259–276, Chapman and Hall, London-New York-Tokyo- Melbourne-Madras.

17. Blumenkrantz, N. & Asboe-Hansen, G. (1973) New method for quantitative determination of uronic acids. *Anal. Biochem.* **54**, 484–489.

18. Slim, G.C., Furneaux, R.H. & Yorke, S.C. (1994) A procedure for analysis of glycosaminoglycan mixtures based on digestion by specific enzymes. *Carbohydr. Res.* **255**, 285–293.

19. Lagunoff, D. & Warren, G. (1962) Determination of 2-deoxy-2-sulpho-aminohexose content of mucopolysaccharides. *Arch. Biochem. Biophys.* **99**, 396–400.

20. Hronowski, L. & Anastassiades, T.P. (1979) Quantitation and interaction of glycosaminoglycans with Alcian Blue in dimethyl sulfoxide solutions. *Anal. Biochem.* **93**, 60–72.

21. Domański, C. (1979) Modyfikacje testu Kołmogorowa. Test Shapiro-Wilka; in *Statystyczne testy nieparametryczne*; pp. 120–121, PWE, Warszawa (in Polish).

22. Laurent, U.B.G. & Laurent, T.C. (1981) On the origin of hyaluronate in blood. *Biochem. Int.* **2**, 195–199.

23. Wasty, F., Alavi, M.Z. & Moore, S. (1993) Distribution of glycosaminoglycans in the intima of human aortas: changes in atherosclerosis and diabetes mellitus. *Diabetologia* **36**, 316–322.

24. Olczyk, K., Kościelniak-Kocurek, E., Sonecki, P. & Zdenkowski, W. (1994) The lipid peroxidation products and the enzymes of antioxidant system in patients with diabetes mellitus. *Ann. Acad. Med. Bialostoc.* **39**, 93–99.

25. Ross, R., Bowen-Pope, D.F. & Raines, E.W. (1985) Platelets, macrophages, endothelium and growth factors: their effect upon cells and their possible role in atherogenesis. *Ann. N.Y. Acad. Sci.* **451**, 254–260.

26. Yamamoto, T., Nakamura, T., Noble, N.A., Ruoslahti, E. & Border, W.A. (1993) Expression of transforming growth factor β is elevated in human and experimental diabetic nephropathy. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1814–1818.

27. van de Lest, C.H.A., Versteeg, E.M.M., Veerkamp, J.H. & Van Kuppevelt, T.H. (1994) Quantification and characterisation of glycosaminoglycans at the nanogram level by a combined Azure A-silver staining in agarose gels. *Anal. Biochem.* **221**, 356–361.

28. Murata, K. & Horiuchi, Y. (1977) Molecular weight-dependent distribution of acidic glycosaminoglycans in human plasma. *Clin. Chim. Acta* **75**, 59–69.

29. Sames, K. (1994) *The Role of Proteoglycans and Glycosaminoglycans in Aging* (von Hahn, H.P., ed.) vol. 28, Karger, Basel.

30. Bańkowski, E., Sobolewski, K., Romanowicz, L., Chyczewski, L. & Jaworski, S. (1996) Collagen and glycosaminoglycans of Wharton's jelly and their alterations in EPH-gestosis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **66**, 109–117.

31. Laurent, T.C. & Fraser, J.R.E. (1992) Hyaluronan. *FASEB J.* **6**, 2397–2404.

32. Lebel, L., Gabrielsson, J., Laurent, T.C. & Gerdin, B. (1994) Kinetics of circulating hyaluronan in humans. *Eur. J. Clin. Invest.* **24**, 621–626.

33. Bohley, P. (1987) Intracellular proteolysis; in *Hydrolytic Enzymes* (Neuberger, A. & Brocklehurst, K., eds.) pp. 307–331, Elsevier Science Publication BV.

34. Pałka, J., Bańkowski, E. & Wolańska, M. (1993) Changes in IGF-binding proteins in rats with experimental diabetes. *Ann. Biol. Clin.* **50**, 701–706.