ELSEVIER

Manganese deficiency alters arterial glycosaminoglycan structure in the Sprague-Dawley rat

Peiying Yang and Dorothy J. Klimis-Tavantzis

Department of Food Science and Human Nutrition, University of Maine, Orono, ME USA

This study was designed to investigate the effect of dietary manganese on rat arterial glycosaminoglycan structure. Weanling male Sprague-Dawley rats were randomly assigned to two groups and were fed either a manganese-deficient or a manganese-sufficient diet. After 15 weeks, proteoglycans and glycosaminoglycans were extracted from the aorta and isolated by DEAE-Sephacel chromatography. The disaccharide composition of glycosaminoglycans was determined by high performance liquid chromatography following chondroitinase ABC digestion. Manganese deficiency significantly ($P \leq 0.01$*) reduced the total amount of arterial proteoglycans. The molecular size of chondroitin sulfate in both the manganese-deficient and the manganese-sufficient group ranged between* 3×10^4 *and* 6×10^4*. The size of chondroitin sulfate of the manganese-deficient groups was slightly smaller than that of the manganese-sufficient group as analyzed by Sepharose CL-6B column chromatography. Results on the disaccharide composition of glycosaminoglycans showed that* Δ*Di-OS,* Δ*Di-4S, and* Δ*Di-6S accounted for 90% of the disaccharides. There was a significant increase in the ratio of* Δ*Di-6S to* Δ*Di-4S disaccharides in chondroitin sulfate in the manganese-deficient group (*Δ*Di-6S:*Δ*Di-4S, 2.0) compared with the manganese-sufficient group (1.2). Our results demonstrate for the first time that dietary manganese deficiency not only reduces the total proteoglycan content of the aorta, but also alters the molecular weight and sulfation pattern of chondroitin sulfate in that tissue. This alteration may change the composition of the extracellular matrix and consequently affect the structural properties of the vascular wall.* (J. Nutr. Biochem. 9:324–331, 1998)

Keywords: manganese; glycosaminoglycan; sulfation pattern; aorta; rats; chondroitin sulfate

Introduction

Manganese has been demonstrated to be essential for many species of animals and presumably for humans. Among the numerous functions of manganese, the most significant is its effect on proteoglycan or glycosaminoglycan (GAG) metabolism.[1] Manganese deficiency affects the biosynthesis of GAGs and results in decreases in total and individual GAGs, especially chondroitin sulfate in chick cartilage and rat skin.[2–4] In our laboratory, we observed that manganese deficiency decreased total arterial GAG concentration and more specifically affected heparan and chondroitin sulfate concentrations.[5] Investigators found a chondroitin sulfate proteoglycan with smaller molecular weight from manganese-deficient bird cartilage.[6] It is well known that manganese is a specific activator of glycosyltransferases, enzymes that are involved in the polymerization and elongation of the GAG chain in connective tissue.[7] Manganese has also been reported to be the most effective divalent metal ion for the activation of the sulfotransferases, enzymes involved in the sulfation of GAG and its synthesis.[8] Thus far, there have been several studies conducted to investigate the role of manganese in the metabolism of cartilage GAG.[3,7,8] However, it has not been elucidated whether manganese deficiency influences the structure of arterial GAG. The structure of GAG is important because it is directly related to proteoglycan properties as well as to the biological function of the proteoglycan.[9,10]

Proteoglycans are a heterogeneous group of macromolecules that contain GAG side chains as a common feature.[11] To date, chondroitin sulfate, heparan sulfate, and dermatan

Address correspondence and reprint requests to Dr. Dorothy J. Klimis-Tavantzis, Department of Food Science and Human Nutrition, University of Maine, Orono, ME 04469–5497 USA.
Received September 24, 1997; accepted February 10, 1998.
Supported by a Strengthening Grant, project number 5535249, from the United States Department of Agriculture.

655 Avenue of the Americas, New York, NY 10010
0955-2863/98/$19.00
PII S0955-2863(98)00010-2

sulfate proteoglycans are three major proteoglycans that have been identified in blood vessels. Chondroitin sulfate glycosaminoglycan chains have a linkage region and repeating dissacharide unit containing uronic acid and 4- and/or 6-sulfated hexosamine. Although they are only minor components in the arterial wall, they can interact with a number of different macromolecules through their core protein as well as their GAG.[12] These interactions influence arterial properties such as viscoelasticity, permeability, lipid metabolism, homeostasis, and thrombosis.[12] These properties are all related to vascular function. Quantitative and qualitative determinations of arterial proteoglycans and GAGs in atherosclerosis have been reported,[12–16] but the results remain controversial. Among a number of properties of proteoglycans, the hydrodynamic size of molecules, their state of aggregation, and their sulfation pattern are most likely to be altered in atherosclerosis.[15,16] Sulfation of GAG is an important posttranslational modification in GAG biosynthesis. The defective sulfation of GAGs results in defective growth or severe pathologic conditions.[17,18] The sulfation pattern of proteoglycan affects the binding between low density lipoproteins (LDL) and proteoglycans.[19] Changes of sulfation pattern have also been observed in human atherosclerotic aortas[14,20] and in tumor cells of rectal cancer.[21]

Because there is no information available about the effect of dietary manganese deficiency on the structure of GAGs in rat aorta, we therefore determined and compared the molecular size of chondroitin sulfate and analyzed the sulfation pattern of proteoglycan and chondroitin sulfate isolated from manganese-deficient (MnD) and manganese-sufficient (MnS) rat aortas.

Materials and methods

Animals and diets

Three-week-old weanling male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were randomly placed on either a MnD (0.42 mg/kg) or a MnS (105 mg/kg) diet. Both diets contained 691 g/kg dextrose, 200 g/kg egg white solids, 4 g/kg D-L methionine, 2 mg/kg biotin (Tekland Test Diets, Madison, WI), 60 g/kg corn oil ("Mazola" Best Foods, Englewood Cliffs, NJ), 10 g/kg vitamin mix (A.O.A.C. Special Vitamin Mixture, Purina Mills, Richmond, IN), and 35 g/kg trace mineral mix (ICN Biochemicals, Cleveland, OH) containing 115.5 g/kg glucose and 6.02 g/kg manganese carbonate (MnS) or 121.5 g/kg glucose without manganese carbonate (MnD). The animals were housed in individual steel mesh cages in an environmentally-controlled room (22°C ± 2°C, 50% relative humidity, dark period from 1800 to 0600) and allowed free access to food and tap water (Mn $<$ 0.1 g/kg) for 15 weeks. The rats were weighed weekly.

Tissue sampling

At the end of the feeding period, food was withheld from the rats for 12 hours. All animals were anesthetized with diethyl ether, and the thoracic-abdominal aortas and livers were carefully removed from the animals. Aortas were washed with cold physiologic saline and immediately frozen in liquid nitrogen and stored at −70°C until used for the following assays. Livers were washed with physiologic saline, blotted with paper, weighed, frozen (−70°C), lyophilized, and pulverized. Liver manganese was determined by the Maine Forest and Agriculture Experiment Station Analytical Laboratory using atomic absorption spectroscopy.

Isolation and purification of proteoglycans

Four aortas from each group were pooled and finely minced and proteoglycans were extracted from minced tissue by the modification of procedures described by Radhakrishnamurthy et al.[22] and Hascall et al.[23] Briefly, the tissues were extracted with 4 M guanidine hydrochloride (Gdn-HCl)/50 mM sodium acetate (pH 5.8) containing protease inhibitors[23] at 4°C for 48 h with constant shaking. The extract was collected by centrifugation at 3000 × g and dialyzed against 8 M urea, 0.05 M sodium acetate, 0.15 M NaCl (pH 6.0) at 4°C for 24 h.[24] After dialysis, detergent CHAPS was added to the dialysate to give a final concentration of 0.5% (w/v). The proteoglycans in the extract were purified using anion exchange resin DEAE-Sephacel (Sigma Chemical Company, St. Louis, MI).[23] The proteoglycans purified by ion-exchange resin were further precipitated with 4 volumes of ethanol[25] at 4°C overnight. The uronic acid content in the proteoglycans was measured by the method of Blumenbrantz and Asboe-Hansen.[26]

Extraction of glycosaminoglycans in the tissue residue

The tissue residue remaining after Gdn-HCl extraction was washed with a versene buffer of 0.1 mM sodium acetate:0.05 M cysteine hydrochloride:0.01 M disodium ethylenediaminetetraacetic acid (7:1:1, v/v/v)[27] and digested with 2× crystallized papain at a concentration of 5 μg/mg of uronic acid at 65°C for 5 h. The digestion was dialyzed against 20 mM NaCl buffer and precipitated with trichloroacetic acid. GAG in the supernatant was precipitated with 0.5% cetylpyridinium chloride (CPC) containing 30 mM sodium sulfate. The GAG-CPC complex was dissociated with 2 M NaCl/absolute ethanol (100:15 v/v) and GAGs were precipitated again by the addition of 3 volumes of absolute ethanol at 4°C overnight. GAGs were collected and washed with ethanol again as described previously.[5] The total GAGs were separated with cellulose acetate electrophoresis with 0.3 M calcium acetate at 50 v/strip for 3 h. Individual GAGs were identified by their similar electrophoretic mobility as authentic GAG standard chondroitin sulfate, dermatan sulfate, and heparan sulfate.

Isolation and purification of chondroitin sulfate

Chondroitin sulfate in rat aortas was isolated from the Gdn-HCl unextractable total GAG as described above by DEAE-Sephacel ion exchange chromatography.[28–30] The total GAG water solution was applied to a column (0.7 x 4 cm; Bio-Rad, Richmond, CA) of DEAE-Sephacel (Pharmacia Biotech Inc., Piscataway, NJ) that had been preequilibrated with 0.15 M NaCl, 0.05 M sodium acetate, pH 4.5. The column was initially washed with 6 volumes of the above buffer.[30] The bound species were then eluted at a flow rate of 10 ml/h, with a linear gradient of sodium chloride (0.15 M to 1.2 M)[29] in 0.05 M sodium acetate buffer, pH 4.5. The fraction 0.4 ml was collected and analyzed for the content of sulfated GAGs using the 1,9 dimethyl-methylene blue (DMMB) (Aldrich, Milwaukee, WI) dye binding assay.[31] In brief, an aliquot (30 μl) of each fraction was mixed with 0.05 M sodium acetate buffer (pH 6.9; 70 μl) and 400 μl of DMMB reagent in a disposable 1 ml curvette (Bio-Rad, Richmond, CA) and the absorbance was immediately measured at 530 nm. Chondroitin sulfate from shark cartilage was used as the standard (0–4 μg/ml).

Gel permeation chromatography

The average molecular weight of chondroitin sulfate was measured by gel permeation chromatography.[32] Gel permeation chromatography on Sepharose CL-6B (Pharmacia Biotech Inc.) (60 × 1.0 cm i.d.) was performed at room temperature in 4M Gdn-HCl, 50 mM

Table 1 Alterations in body weight, liver weight, and hepatic manganese in manganese-deficient and manganese-sufficient rats[1]

Diet group	Body weight (g)	Liver weight (g)	Liver weight as % of body weight	Hepatic manganese (ppm)
Manganese deficient	524.0 ± 11.2[2]	12.0 ± 0.2[3]	2.41 ± 0.12	2.65 ± 0.42[2]
Manganese sufficient	582.0 ± 12.6	14.6 ± 0.3	2.52 ± 0.18	7.25 ± 0.53

[1]Values are expressed as means ± standard error of 12 rats per treatment group.
[2]Statistically significant at $P \leq 0.0001$.
[3]Statistically significant at $P \leq 0.01$.

sodium acetate, pH 5.8. The void (V_o) and total volumes (V_t) of the column were determined by chromatography of Dextran blue 2000 and glucuronic acid lactone, respectively. The average molecular weight of chondroitin sulfate was determined by chromatography on Sepharose CL-6B calibrated with standard chondroitin sulfates with average molecular weights of 6×10^4 (ICN Biochemical, Cleveland, OH) and 3×10^4 (Sigma, St. Louis, MI), respectively.

Enzymatic digestion

Digestion of the proteoglycans, total GAGs, and chondroitin sulfate with chondroitinase ABC (Sigma) was performed at 37°C for 8 h in 50 mM Tris/HCl (pH 7.5),[33] using 0.01 units/10 μg of uronic acid and 50 μl solution. After the digestion of proteoglycans and total GAGs, 4 volumes of ethanol was added and the mixture was precipitated overnight.[25] The supernatant was collected by centrifugation at 10,000 x g for 10 min and analyzed for disaccharides. In the case of digestion of chondroitin sulfate, the digestion was terminated by boiling in a water bath for 2 min, and the mixture was centrifuged in a microcentrifuge at 10,000 x g for 10 min. The supernatant was then used for disaccharide analysis.

Analysis of disaccharides by high performance liquid chromatography (HPLC)

The disaccharide composition of the GAGs was analyzed with high performance liquid chromatography (HPLC) by the modifications of the procedure described by Karamanos et al.[25] Chromatography was performed on a HPLC system with Waters Associate Pump 6000 using a 250 × 4.6 mm i.d. Econosphere NH_2 5U (Alltech Associates, Inc., Deerfield, IL) column connected with a 30 × 2.1 mm i.d. amino precolumn (Applied Biosystems, Foster City, CA). The column was eluted with 50 mM sodium sulfate-10 mM sodium acetate buffered with acetic acid (pH 5.0) at a flow rate of 1.2 ml/min. The absorbance of the column eluate was monitored by a Beckman spectrophotometer at 231 nm (Beckman, Fullerton, CA) and the peak height was quantitated by a HP integrator 3396A following calibration with known amounts of individual standard disaccharides. Standards of disaccharides (Oxford Glycosciences Inc., United Kingdom) including ΔDi-OS [2-acetamido-2-deoxy-3-O-(α-L-threo-enopyranosyluronic acid)-D-galactose], ΔDi-4S [2-acetamido-2-deoxy-3-O-(α-L-threo-enopyranosyluronic acid)-D-galactose 4-sulfate], ΔDi-6S [2-acetamido-2-deoxy-3-O-(α-L-threo-enopyranosyluronic acid)-D-galactose 6-sulfate], ΔDi-di(4,6)S [2-acetamido-2-deoxy-3-O-(α-L-threo-enopyranosyluronic acid)-D-galactose 4,6-disulfate], ΔDi-di(2,4)S [2-acetamido-2-deoxy-3-O-(2-sulfo- α-L-threo-enopyranosyluronic acid)-D-galactose 4-sulfate], ΔDi-di(2,6)S [2-acetamido-2-deoxy-3-O-(2-sulfo-α-L-threo-enopyranosyluronic acid)-D-galactose 6-sulfate], and ΔDi-tri(2,4,6)S [2-acetamido-2-deoxy-3-O-(2-sulfo-α-L-threo-enopyranosyluronic acid)-D-galactose 4,6-disulfate] were used to set up the separation pattern of those disaccharides and to determine the linear range for the analysis of disaccharides obtained from the above digestion.

Statistical analysis

Data of uronic acid content are expressed as means ± S.E. Student's two tailed *t*-test was used to assess differences between the MnD and MnS groups. The significance level was set at a *P*-value of 0.05 or less.

Results

Animals fed either a MnD or a MnS diet all gained weight during the 15-week period. However, the rate of growth between the two groups showed a significant ($P \leq 0.001$) difference from the fourth week. *Table 1* presents body weights, liver weights, and hepatic manganese concentration. The final mean body weights were 582 ± 12.6 g for the MnS rats and 524 ± 11.2 g for the MnD rats. Liver weights (12.0 ± 0.2 g) of the MnD rats were significantly lower ($P \leq 0.01$) than those of the MnS rats (14.6 ± 0.3 g), but when expressed as the percentage of body weight, there was no significant difference. Manganese deficiency was confirmed by significantly ($P \leq 0.0001$) lower hepatic manganese concentration (2.65 ± 0.42 ppm) in the MnD rats compared with the liver manganese concentration of MnS rats (7.25 ± 0.53 ppm).

Table 2 shows the fresh aorta weight and uronic acid content in MnD and MnS rat aortas. The fresh aorta weight (91.3 ± 4.3 mg) of the MnD rats was significantly ($P \leq 0.01$) lower than that of MnS rat aortas (127.7 ± 6.6 mg). The uronic acid content in Gdn-HCl extractable proteoglycans (0.34 ± 0.12 μg/mg) in the MnD group was significantly ($P \leq 0.01$) lower than the uronic acid content on the MnS proteoglycan preparation (0.64 ± 0.10 μg/mg). The GAGs that were not extractable by 4M Gdn-HCl were completely recovered by the papain digestion and ethanol precipitation. Electrophoretic analysis of GAGs on cellulose acetate membrane showed that Gdn-HCl unextractable GAG extraction contained predominantly heparan sulfate

Table 2 Alterations in the wet weight and the amount of uronic acid of the aorta from manganese-deficient and manganese-sufficient rats[1]

Diet group	Wet weight (mg/aorta)	Uronic acid (μg/mg aorta)
Manganese-deficient	91.3 ± 4.3*	0.34 ± 0.12*
Manganese-sufficient	127.7 ± 6.6	0.64 ± 0.10

[1]The amount of uronic acid refers to total uronic acid concentration (mass/mg wet weight aorta). The values are expressed as means ± standard error of 12 rats per treatment group.
*Statistically significant at $P \leq 0.01$.

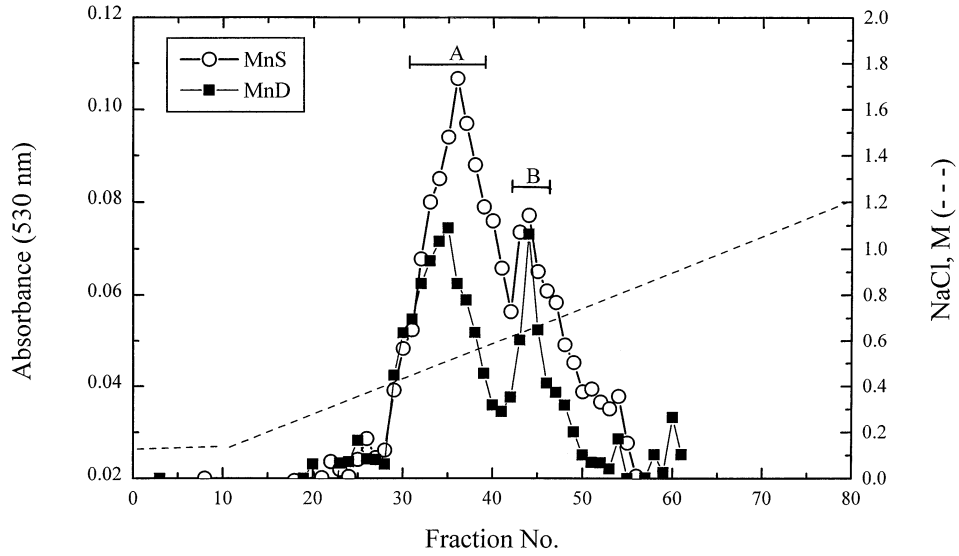

Figure 1 Ion exchange chromatography of guanidine hydrochloride unextractable glycosaminoglycans in manganase-deficient (MnD) and manganase-sufficient (MnS) rat aortas. The glycosaminoglycan prepared by papain digestion was chromatographed on DEAE-Sephacel (4 x 0.7 cm) eluted with a NaCl linear gradient from 0.15 to 1.2 M in 0.05 M sodium acetate buffer (pH 4.5). The collected fractions A and B were analyzed by a dimethyl-methylene blue binding assay.

and chondroitin sulfate in both MnD and MnS groups. No detectable amount of dermatan sulfate was observed with cellulose acetate electrophoresis (data not shown). No GAG was detected in the ethanol supernatant by cellulose acetate membrane electrophoresis.

Chromatography of the total GAG on DEAE-Sephacel, eluted with NaCl linear gradient systems, showed the presence of two DMMB binding assay positive fractions (*Figure 1*). Fraction A, which was eluted with 0.57M NaCl, was identified as heparan sulfate by cellulose acetate membrane electrophoresis. Fraction B eluted out at concentration of 0.7M NaCl showed the same migration distance as the authentic chondroitin sulfate in cellulose acetate membrane electrophoresis. Heparan sulfate and chondroitin sulfate were further confirmed by comparing the digestion capability of heparintinase III and chondroitinase AC. This result was consistent with the results we saw in the cellulose acetate membrane electrophoresis on total GAGs.

Gdn-HCl unextractable chondroitin sulfates from both MnD and MnS groups purified by the DEAE-Sephacel column chromatography were rechromatographed on Sepharose CL-6B with 4M Gdn-HCl, 50 mM sodium acetate (pH 5.8) as eluant. Analysis of the eluted fractions for a DMMB binding assay showed the presence of a single peak in both MnD and MnS samples (*Figure 2*), respectively, with k_d = 0.31 and 0.29. Comparing those k_d values with the ones obtained for the reference GAGs, the average molecular weights of chondroitin sulfate from both MnD and MnS groups were estimated in the range between 3×10^4 and 6×10^4. The electrophoretic analysis of GAGs extracted from Gdn-HCl unextractable tissue shows that there were two sulfated GAGs—heparan sulfate and chondroitin sulfate—in both the MnD and MnS groups. Between these two GAGs, heparan sulfate was the predominant one. Separation of individual GAGs can be achieved by using ion-exchange chromatography with NaCl or lithium chloride linear gradient elution.[28] We selected the DEAE-Sephacel ion-exchange chromatography to separate heparan sulfate and chondroitin sulfate in rat aortas. DEAE-Sephacel chromatogram showed similar elution profiles for heparan sulfate and chondroitin sulfate in both MnD and MnS groups. However, during our experiments, we always observed that the separation of chondroitin sulfate and heparan sulfate in the MnS sample was not as complete as the separation in the MnD sample.

The sulfation pattern of GAG and chondroitin sulfate was analyzed by HPLC after digestion with chondroitinase ABC. The standard disaccharides were completely sepa-

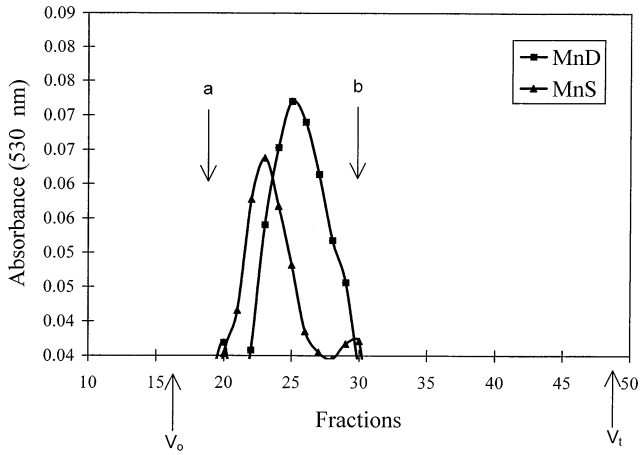

Figure 2 Gel permeation chromatography of chondroitin sulfate from manganase-deficient (MnD) and manganase-sufficient (MnS) rat aortas. Chromatography was performed on Sepharose CL-6B (60 x 1 cm) using as eluent 4M Gdn-HCl/50 mM sodium acetate (pH 5.8) and fractions of 1.0 ml were collected and analyzed by DMMB assay. a and b indicate the elution positions of reference proteoglycans of M_r 6.0 x 10^4 and 3.0 x 10^4, respectively.

Table 3 Sulfation pattern of arterial proteoglycan, glycosaminoglycan, and chondroitin sulfate in manganese-deficient (MnD) and manganese-sufficient (MnS) rats determined by HPLC[1]

	Proteoglycan extract		GAG extract		Chondroitin sulfate	
Δ-Disaccharides	MnD	MnS	MnD	MnS	MnD	MnS
ΔDi-OS	12	15	11	12	27	24
ΔDi-4S	50	55	31	36	21	29
ΔDi-6S	31	22	47	40	40	35
ΔDi-dis	7	8	10	12	12	12

[1]The sulfation pattern was determined by high performance liquid chromatography (HPLC) after digestion with chondroitinase ABC. Proteoglycan extract and GAG extract represent the materials extracted by Gdn-HCl and unextracted by Gdn-HCl, respectively. The results are expressed as percent of the digested material.

rated on a Econosphere NH_2 column eluted with 50 mM Na_2SO_4/10 mM sodium acetate (pH 5.0). The detector response obtained for all the standard disaccharides was linear up to 500 ng. The detection limits for all the disaccharides were as low as 25 ng. Analysis of the sulfation pattern performed on Gdn-HCl extractable proteoglycan, Gdn-HCl nonextractable GAG, and chondroitin sulfate alone, after digestion with chondroitinase ABC, showed that there were remarkable differences in the sulfation pattern of the GAG chain, specifically, chondroitin sulfate between the MnD and MnS groups. *Table 3* shows the disaccharide composition in the GAG chain and chondroitin sulfate from the MnD and MnS rat aortas. The Gdn-HCl extractable and unextractable GAG chain and pure chondroitin sulfate were all composed of more than 90% of the repeating disaccharide units ΔDi-4S, ΔDi-6S and ΔDi-OS disaccharides in both MnD and MnS groups (*Table 3*). Only approximately 10% disulfated disaccharide was observed in all the extractions. In the case of Gdn-HCl extractable proteoglycans, the ΔDi-4S disaccharide was the predominant disaccharide, whereas the Gdn-HCl unextractable GAG mainly contained ΔDi-6S disaccharides in both MnD and MnS rat aortas (*Table 3*). The position of the sulfate groups in disulfate disaccharides was not able to be confirmed due to the limitation of the amount of the material used for the analysis. The trisulfated disacchachrides were not detected in any of the extracts from both MnD and MnS rat aortas.

When the ratio of ΔDi-6S to ΔDi-4S was analyzed, the effect of dietary manganese on the sulfation pattern of the GAG chain or chondroitin sulfate in rat aortas was remarkable (*Figure 3*). Manganese deficiency increased the ratio of ΔDi-6S to ΔDi-4S to 49% compared with the ratio in the MnS group in all the extractions. In addition to that, the chondrotin sulfate in MnD aortas was composed of 50% more 6-sulfated chondroitin than 4-sulfated chondroitin. However, in MnS rat aortas chondroitin was sulfated almost evenly in position 4 or 6 (*Figure 3*). This result demonstrates that manganese deficiency changes the sulfation pattern of chondroitin sulfate by increasing the sulfation of chondroitin in position 6, which may indicate that there may be more chondroitin-6-sulfate than chondroitin-4-sulfate in MnD rat aortas compared with those in the MnS groups.

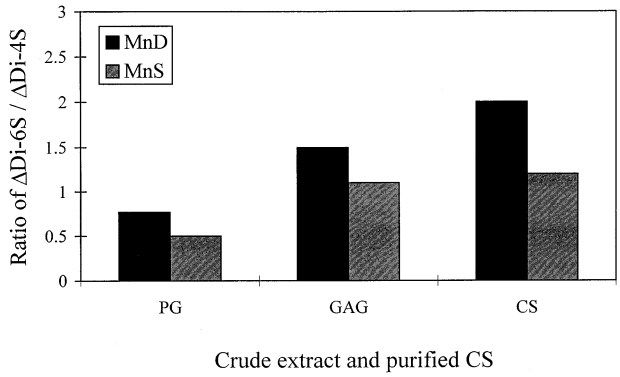

Figure 3 The effect of dietary manganese deficiency on the disaccharide composition of glycosaminoglycans (GAGs) and chondroitin sulfate (CS) in rat aortas. Proteoglycans (PG) were isolated by ion-exchange chromatography. GAGs were isolated by papain digestion and chondroitin sulfate was purified by DEAE-Sephacel ion-exchange chromatography. The disaccharides were obtained by digestion with chondroitinase ABC followed by high performance liquid chromatography analysis.

Discussion

In our experiment, we have observed that the MnD animals exhibited significantly lower body weight gain compared with the MnS rats. The retardation of growth of rats or mice by dietary manganese deficiency had been previously reported by Paynter[34] and Fahim et al.[35] They explained that the retarded-growth effect associated with manganese deficiency in an animal model was not a function of reduced food intake; instead, it was a result of reduced efficiency of food conversion with the depletion of nutritional manganese. Significantly lower level of hepatic manganese indicated development of manganese deficiency in these animals. Although the liver weight of the MnD rats was lower than that of MnS rats, the decrease in liver weight was proportional to the decrease of body weight. Even though the hepatic manganese concentration decreased by 63% in the MnD rats, at this time we cannot specify the level of manganese deficiency (mild, medium, or severe) because there are no established levels available as yet in the literature.

Total Gdn-HCl extractable proteoglycan content which is represented by the total uronic acid content in the MnS rat aortas was 46% greater than that of MnD rat aortas. This finding is in good agreement with Liu et al.'s[6] reports that there was a significantly lower amount of proteoglycan in MnD bird cartilage than in the control group. It is not surprising to observe this difference because we had previously determined that the total GAGs in MnD rat aortas decreased significantly.[5] The Gdn-HCl extractable arterial proteoglycan includes exclusively large amounts of DS-PG and some CS-PG, whereas only 10% of HS-PG can be extracted by a dissociative solvent.[13] As previously mentioned, we observed that in the MnS sample separation of chondroitin sulfate and heparan sulfate was not as complete as the separation in the MnD sample. As described previously by Hallen[28] the separation of an individual GAG mainly depended on the degree of sulfation, molecular size, and backbone structure. One explanation for the incomplete

separation of heparan sulfate and chondroitin sulfate in the MnS group may be due to a larger molecular weight of heparan sulfate, which could increase the retention time of heparan sulfate in the column and consequently reduce the separation of heparan sulfate and chondroitin sulfate. Another explanation may be the different sulfation pattern of chondroitin sulfate between MnD rats and MnS groups, which also may have some effect on the separation between heparan sulfate and chondroitin sulfate. Because there were significantly lower amounts of chondroitin sulfate in the MnD rat aorta, the overlapping of chondroitin sulfate and heparan sulfate also could be due to column overload in the case of the MnS group. Nevertheless, the heparan sulfate and chondroitin sulfate in the MnD and MnS animals showed similar behavior in the DEAE-Sephacel column.

To identify the heterogeneity and molecular size of chondroitin sulfate in the MnD and MnS groups, the chondroitin sulfate purified from the ion-exchange column was further chromatographed on Sepharose CL-6B in 4M Gdn-HCl. Chondroitin sulfate from both the MnD and MnS groups had an estimated molecular weight ranging between 3×10^4 and 6×10^4. However, the chondroitin sulfate in the MnD group eluted later than that in the MnS group. This demonstrates that the molecular weight of chondroitin sulfate, which was bound tightly to elastin in the MnD rats, was smaller than the molecular weight of chondroitin sulfate in the MnS group, suggesting shorter chondroitin sulfate chain in the MnD rat aortas. This result is similar to that obtained by Liu et al.[6] who reported a shorter chondroitin sulfate chain in CS-PG molecules in MnD bird cartilage. Because manganese is required for optimal activity of glycosyltransferases, which are responsible for the elongation of the GAG chain,[1,3] this phenomenon could be a result of the impaired synthesis of chondroitin sulfate due to lack of manganese in the diet.

When we analyzed the disaccharide composition in Gdn-HCl extractable, unextractable GAG, and chondroitin sulfate, we observed that the GAG chains in Gdn-HCl extractable and Gdn-HCl unextractable PG were all predominantly (more than 90%) composed of three disaccharide repeat structures: ΔDi-OS, ΔDi-4S, and ΔDi-6S. Only a small portion of disulfated disaccharides were detected in the above samples. The ratio of ΔDi-6S to ΔDi-4S disaccharide in all types of extract from MnD rats significantly increased compared with the one in MnS groups. The exact mechanism whereby dietary manganese status influences the sulfation pattern of chondroitin sulfate is not yet understood. It seems likely that the control occurs through its effect on the activity of specific glycosyltransferases and sulfotransferase involved in its biosynthesis. It has been reported that manganese is the most effective metal ion for the activation of the sulfotransferases that are involved in the formation of the sulfate group in GAG chain synthesis.[8] However, unlike its specific function in glycosyltransferase, sulfotransferase activity can also be stimulated by other divalent metal ions such as magnesium, zinc, and copper.[36] In comparison with manganese, which can activate both 4-sulfotransferase and 6-sulfotransferase, zinc and copper can activate 6-sulfotransferase and inhibit the 4-sulfotransferase.[8] Therefore, in the case of insufficient manganese in the diet, other metal ions such as zinc, copper, or magnesium may play crucial roles in the sulfation of the GAG chain and consequently cause a marked rise in the ratio of 6-sulfation to 4-sulfation of chondroitin sulfate. The factors determining the position of sulfation remain unknown. Whether it is controlled by the relative activity of sulfotransferase or by the nature of acceptors is unclear. Another mechanism for explaining how the sulfation position is determined relates to the primer molecule on which the chondroitin chain is built.[37] The structural factors of oligosaccharide acceptors, such as the number of sugars in the oligosaccharide or the length of the oligosaccharide chain, were reported to affect the sulfation pattern. It has been shown that for the even number oligomer or shorter chain the specificity for 6-sulfation of the nonreducing terminal GlcUA-GalNAc disaccharide was maximum and no 4-sulfation was produced, whereas the nonreducing terminal GalNAc residues of the odd number oligomers were good acceptors for 4-sulfation. It is clear that manganese is a specific activator of glycosyltransferases that are involved in the polymerization and elongation of the GAG chain. Therefore, depletion of manganese may alter the structure of the primer molecule for the chondroitin chain by reducing the chain length and causing the sulfation to be favored in this 6 position for chondroitin.

The nutritional or biological significance of the alteration of the sulfation pattern in manganese deficient aorta is not well known. The sulfation pattern of chondroitin sulfate in tissues is affected by many factors and involves important biological implications.[38] A number of studies indicate that the increased ratio of 6-sulfated chondroitin to 4-sulfated chondroitin of the chondroitin sulfate chain has been related to cellular events and some diseases. For example, Schonherr et al.[39] found that with smooth muscle cells, the proliferative response to platelet-derived growth factor was accompanied by the synthesis of versican-like proteoglycans bearing chondroitin sulfate chains of increased 6-sulfation. Tsara et al.[21] observed significant alterations on the sulfation pattern presented by marked increases in ΔDi-6S disaccharide of chondroitin sulfate in tumor cells. Additionally, it is interesting that a relationship between the sulfation pattern of chondroitin sulfate and development of atherosclerosis has been observed by many investigators. Braunstein[40] discovered histochemical evidence that metachromatic material in the ground substance of atheromatic vessels was primarily composed of chondroitin 6-sulfate. Srinivasan et al.[20] reported that chondroitin-6-sulfate was the predominant GAG present in the LDL-proteoglycan complex isolated from human atherosclerosis. Moreover, there was a loss of chondroitin 4-sulfate and a concomitant increase of chondroitin 6-sulfate in human atherosclerotic aortas (ΔDi6S/ΔDi4S ratio, 5.0).[14,41] Other studies have shown that proteoglycan containing the chondroitin 6-sulfate isomer exhibits the highest binding preference for LDL.[42] Thus, the accumulation of chondroitin 6-sulfate in the microenvironment surrounding arterial smooth muscle cells may bind and trap the low density lipoprotein, setting the stage for probable lipid accumulation in the blood vessel during atherosclerosis.

In contrast, chondroitin 4-sulfate (but not chondroitin 6-sulfate) has been found to reduce the deposition and accumulation of lipoprotein by preventing the formation of

complexes between LDL and highly sulfated GAGs.[43] More recently, Albertini et al.[44] reported that chondrotin 4-sulfate inhibited copper induced LDL oxidation by prolonging the lag time, reducing the rate of propagation, and protecting tryptophan residues of Apo-B-100 in the early stage of LDL oxidation, whereas chondroitin 6-sulfate had no effect on LDL oxidation. Thus, it is obvious that increases in the ratio of chondroitin-6-sulfate to chondroitin-4-sulfate in the MnD rat aortas may change the conformational properties of the arterial wall, impact arterial integrity, and lead to arterial disease.

It has been reported that manganese is involved in lipoprotein metabolism in that it alters apoprotein E concentration in the high density lipoprotein (HDL) particle and the structure of HDL_1 and changes the rotation of the HDL_2 proteins.[45] Furthermore, manganese deficiency was found to cause significant damage of the endothelial cells in the aorta of the Sprague-Dawley rats.[46] Results from our laboratory utilizing scanning electron microscopy revealed significantly higher surface damage of the aorta of rats fed an MnD diet. Transmission electron microscopy of the arterial wall revealed less dense extracellular matrix around smooth muscle cells especially in the medial layers of the MnD rats.[46] Thus, results demonstrated that manganese is essential in maintaining the integrity of the arterial wall.

In conclusion, this study is the first to show that manganese is involved not only in the polymerization and elongation but also in the sulfation of the GAG chain of proteoglycan biosynthesis in rat aortas. Manganese deficiency leads to a slightly reduced molecular size of arterial chondroitin sulfate. More interestingly, lack of manganese in the diet alters the sulfation pattern of chondroitin sulfate, increasing the ratio of chondroitin 6-sulfate to chondroitin 4-sulfate. This alteration of the sulfation pattern may affect the extracellular matrix composition during blood vessel development, which may cause the loss of the integrity of the arterial wall and predispose the aorta to lipid deposition and lipoprotein oxidation. Further studies are necessary to determine whether these structural alterations in proteoglycan concentration, composition, and metabolism influence the functional properties of the arterial wall and subsequently affect cellular events involved in the development of arterial diseases.

Acknowledgments

We would like to thank Dr. D. Croall and Dr. R. Bushway for their technical consultations and support.

References

1 Leach, R.M. (1986). Mn (II) and glycosyltransferase essential for skeletal development. In *Manganese in Metabolism and Enzyme Function* (B.L. Schramm, ed.), pp. 81–91, Academic Press, New York, NY

2 Bolze, M.S., Reeves, R.D., Lindbeck, F.E., Kemp, S.F., and Elders, M.J. (1985). Influence of manganese on growth somatomedin and glycosaminoglycan metabolism. *J. Nutr.* **54,** 352–362

3 Leach, R.M., Muenster, A.M., and Wien, E.M. (1969). Studies on the role of manganese in bone formation. II. Effect upon chondroitin sulfate synthesis in chick epiphyseal cartilage. *Arch. Biochem. Biophy.* **133,** 22–28

4 Shetlar, M.R. and Shetlar, C.L. (1994). The role of manganese in wound healing. In *Manganese in Health and Disease* (D.J. Klimis-Tavantzis, ed.), pp. 146–157, CRC Press, Boca Raton, FL

5 Yang, P. and Klimis-Tavantzis, D.J. (1995). Effect of dietary manganese on arterial glycosaminoglycan metabolism. *FASEB J.* **9,** A576 (Abstr.)

6 Liu, A.C-H., Heinrichs, B.S., and Leach, R.M. (1994). Influence of manganese deficiency on the characteristics of proteoglycans of avian epiphyseal growth plate cartilage. *Poultry Sci.* **73,** 663–669

7 Leach, R.M. (1971). Role of manganese in mucopolysaccharide metabolism. *Federation Pro.* **30,** 991–994

8 Gundlach, M.W. and Conrad, H.E. (1985). Glycosyltransferases in chondroitin sulfate biosynthesis—effect of acceptor structure on activity. *Biochem. J.* **226,** 705–714

9 Kjellen, L. and Lindahl, U. (1991). Proteoglycans: structures and interactions. *Ann. Rev. Biochem.* **60,** 443–475

10 Rouslahti, E. (1988). Structure and biology of proteoglycans. *Ann. Rev. Cell Biol.* **4,** 229–255

11 Hascall, V.C. and Hascall, G.K. (1981). Proteoglycans. In *Cell Biology of Extracellular Matrix* (E.D. Hay, ed.), pp. 39–63, Plenum Publishing Co., New York, NY

12 Wight, T.N. (1989). Cell biology of arterial proteoglycans. *Arteriosclerosis* **9,** 1–20

13 Radhakrishnamurthy, B., Srinivasan, S.R., Vijayagopal, P., and Berenson, G.S. (1990). Arterial wall proteoglycans-biological properties related to pathogenesis of atherosclerosis. *Europ. Heart J.* **11** (supplement E), 148–157

14 Robinson, R.W., Likar, I.N., and Likar L.J. (1975). Glycosaminglycans and arterial disease. In *Monographs on Atherosclerosis* (J.E. Kirk, et al., ed.), vol. 5, pp. 1–115. S. Karger, Basel, Switzerland

15 Srinivasan, S.R., Radhakrishnamuthy, B., Vijayagopal, P., and Berenson, G.S. (1990). Proteoglycans, lipoproteins, and atherosclerosis. In *Hypercholesterolemia, Hypocholesterolemia, Hypertriglyceridemia* (C.L. Malmendies, ed.), pp. 373–381, Plenum Press, New York, NY

16 Wagner, W.D. (1985). Proteoglycan structure and function as related to atherosclerosis. *Ann. New York Acad. Sci.* **454,** 52–68

17 Nakazawa, K., Hassel, J.R., Hascall, V.C., Lohmander, S., Newsome, D.A., and Krachmer, J. (1984). Defective processing of keratan sulfate in macular corneal dystrophy. *J. Biol. Chem.* **259,** 13751–13757

18 Sugahara, K. and Schwartz, N.B. (1979). Defect in 3′-phosphoadenosine 5′-phophosulfate formation in branchymorphic mice. *Proc. Natl. Acad. Sci. U.S.* **76,** 6615–6618

19 Olsson, U., Camejo, G., Olofsson, S., and Bondjers, G. (1991). Molecular parameters that control the association of lipoprotein apo B-100 with chondroitin sulfate. *Biochim. Biophys. Acta* **1097,** 37–44

20 Srinivasan, S.R., Dollan, P., Radhakrishnamurthy, B., Pargaonkar, P.S., and Berenson, G.S. (1975). Lipoprotein-acid mucopolysaccharide complexes of human atherosclerotic lesions. *Biochim. Biophys. Acta.* **338,** 58–64

21 Tsara, M.E., Papageorgacopoulou, N., Karavias, D.D., and Theocharis, D.A. (1995). Distribution and changes of glycosaminoglycans in neoplasias of rectum. *Anticancer Res.* **15,** 2107–2112

22 Radhakrishnamurthy, B., Ruiz, H., Dalferes, E.R., Klevay, L.M., and Berenson, G.S. (1989). Composition of proteoglycans in the aortas of copper-deficient rats. *Proc. Soc. Exp. Biol. Med.* **190,** 98–104

23 Hascall, V.C., Calabro, A., Midura, R.J., and Yanagishita, M. (1994). Isolation and characterization of proteoglycans. *Methods Enzymol.* **230**, 390–417

24 Hronowski, L.J. and Anastassides, T.P. (1988). Detection and quantitation of proteoglycans extracted from cell culture medium and cultured cartilage slices. *Anal. Biochem.* **174,** 501–511

25 Karamanos, N.K., Syroukou, A., Vanky, P., Nurminen, M., and Hjerpe, A. (1994). Determination of 24 variously sulfated galactosaminoglycan- and hyaluronan-derived disaccharides by high performance liquid chromatography. *Anal. Biochem.* **221,** 189–199

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

27 Wagner, W.D. and Salisbury, B.G. (1978). Aortic total glycosaminoglycan and dermatan sulfate changes in atherosclerotic Rhesus monkeys. *Lab. Invest.* **39,** 322–328

28 Hallen, A. (1972). Chromatography of acidic glycosaminoglycans on DEAE-cellulose. *J. Chromatogr.* **71,** 83–91

29 Karamanos, N.K., Manouras, A., Politou, D., Gritsoni, P., and Tsegenisis, T. (1991). Isolation and high-performance liquid chromatographic analysis of ray (raja clavata) skin glycosaminoglycans. *Comp. Biochem. Physiol.* **100B,** 827–832

30 Ramasmy, S. Boissonneault, G.A., Lipke, D.W., and Hennig, B. (1993). Proteoglycans and endothelial barrier function: effect of linoleic acid exposure to porcine pulmonary artery endothelial cells. *Atherosclerosis* **103,** 279–290

31 Chandrasekhar, S., Esterman, M.A., and Hoffman, H.A. (1987). Microdetermination of proteoglycans and glycosaminoglycans in the presence of guanidine hydrochloride. *Anal. Biochem.* **161,** 103–108

32 Karamanos, N.K., Manouras, A., Tsegenidis, T., and Antonopoulos, C.A. (1991). Isolation and chemical study of the glycosaminglycans from squid cornea. *Int. J. Biochem.* **23,** 67–72

33 Yamagata, T., Saito, H., Habuchi, O., and Suzuki, S. (1968). Purification and properties of bacterial chondroitinases and chondrosulphatases. *J. Biol. Chem.* **243,** 1523–1535

34 Paynter, D. (1980). Changes in activity of manganese superoxide dismutase enzyme in tissues of the rat with changes in dietary manganese. *J. Nutr.* **110,** 437–447

35 Fahim, F.A., Morcos, N.Y.S., and Esmat, A.Y. (1990). Effects of dietary magnesium and/or manganese variables on the growth rate and metabolism of mice. *Ann. Nutr. Metab.* **34,** 183–192

36 Delfer, D.M. and Conrad, H.E. (1985). Sulfation of chondroitin oligosaccharides in vitro-analysis of sulfation ratio. *J. Biol. Chem.* **260,** 14446–14451

37 Deluca, V., Richmond, M.E., and Silbert, J.E. (1973). Synthesis of chondroitin sulfate. Sulfation of the polysaccharide chain. *Biochem.* **12,** 3911–3915

38 Poole, A.R. (1986). Proteoglycans in health and disease: structures and functions. *Biochem. J.* **236,** 1–14

39 Schonherr, E., Jarvelainen, H.T., Sandell, L.J., and Wight, T.N. (1991). Effects of platelet-derived grwoth factor and transforming growth factor-1 on the synthesis of a large versican-like chondroitin sulfate proteoglycan by arterial smooth muscle cells. *J. Biol. Chem.* **266,** 17640–17647

40 Braunstein, H. (1960). Histochemical study of the adult aorta. *Arch. Path.* **69,** 617–622

41 Toledo, O.M.S. and Mourao, P.A.S. (1979). Sulfated glycosaminoglycans of human aorta: chondroitin 6-sulfate increase with age. *Biochem. Biophys. Res. Com.* **89,** 50–55

42 Christner, J.E. and Baker, J.R. (1990). A competitive assay of lipoprotein: proteoglycan interaction using a 96-well microtitration plate. *Anal. Biochem.* **184,** 388–394

43 Nakashima, Y., Ferrante, N.D., Jackson, R.L., and Pownall, H.J. (1975). The interaction of human plasma glycosaminoglycans with plasma lipoproteins. *J. Biol. Chem.* **250,** 5386–5392

44 Albertini, R., Ramos, P., Giessauf, A., Passi, A., Deluca, G., and Esterbauer, H. (1997). Chondroitin 4-sulfate exhibits inhibitory effect during Cu^{2+}-mediated LDL oxidation. *FESB Letters* **403,** 154–158

45 Taylor, P.N., Patterson, H.H., and Klimis-Tavantzis, D. J. (1996). Manganese deficiency alters high-density lipoprotein subclass structure in the Sprague-Dawley rat. *J. Nutr. Biochem.* **7,** 392–396

46 Ekanayake, R.P. (1995). Ultrastructural evidence for adverse effects on aorta and liver in rats fed a short term manganese-deficient diet. FASEB J. 9, A535