Effect of Endothelium on Glycosaminoglycan Accumulation in Injured Rabbit Aorta

THOMAS N. WIGHT, PhD,
KATHLEEN D. CURWEN, PhD,
MARIA M. LITRENTA, DANIEL R. ALONSO,
and C. RICHARD MINICK, MD

From the Department of Pathology, University of Washington School of Medicine, Seattle, Washington, and the Department of Pathology, New York Hospital, Cornell Medical Center, New York, New York

Previous studies have indicated that reendothelialized regions of injured rabbit aortas are more susceptible to diet-induced atherosclerosis than persistently deendothelialized regions or uninjured aortas. However, the mechanism responsible for this selective lipid deposition is not understood. One possibility is that these regions differ with respect to the quantity and type of glycosaminoglycan-containing proteoglycans which are known to interact with lipoproteins. To determine whether these regions differed with respect to their glycosaminoglycan composition, the authors divided 53 rabbits into four groups. Groups IA and IB were fed a regular diet beginning 5 weeks prior to aortic deendothelialization; Groups IIA and IIB were fed the same diet supplemented with 0.5% cholesterol. The rabbits were continued on these diets following aortic deendothelialization with a balloon catheter. Those in Groups IA and IIA were sacrificed either at 2–5 weeks or 6–8 weeks following deendothelialization; proteoglycans were assessed morphometrically following staining with alcian blue. Groups IB and IIB were sacrificed at 10 weeks following injury; glycosaminoglycans were extracted from deendothelialized and reendothelialized aortas, separated by electrophoresis, and quantitated by scanning densitometry. Morphometric analysis of stained aortic sections revealed significantly increased quantities of alcianophilic material in the neointima of reendothelialized aortas as compared with deendothelialized aortas in both diet groups. Chemical analysis revealed significantly more of each glycosaminoglycan in reendothelialized aortas when compared with deendothelialized or uninjured aortas. The major glycosaminoglycans present in all regions were heparan sulfate and chondroitin sulfate; and although absolute quantities of these particular glycosaminoglycans increased in the reendothelialized region, their relative percentages remained the same for each area analyzed. Cholesterol feeding did not appear to influence glycosaminoglycan concentration and composition in reendothelialized and deendothelialized regions when compared with normal diets, but cholesterol feeding alone did increase aortic glycosaminoglycans in uninjured aortas. The results suggest that the presence of endothelium influences the quantity and type of glycosaminoglycans accumulating in the neointima, and that the differences in proteoglycans in the reendothelialized artery may account at least in part for the propensity of this area to accumulate lipid and evolve as atherosclerosis. (Am J Pathol 1983, 113:156–164)

PROTEOGLYCANS are high-molecular-weight protein polysaccharides that consist of carbohydrate polymers, glycosaminoglycans, covalently linked to a protein backbone or core.[1] These macromolecules form important structural links between fibrous and cellular components of the arterial wall and are believed to contribute to the viscoelasticity and selective permeability of this tissue.[2] Proteoglycans or their glycosaminoglycan constituents have been shown to affect hemostasis,[3] to influence the growth of vascular smooth-muscle cells,[4] and to bind low-density lipoproteins both *in vitro* and *in vivo*.[5,6] Thus, proteoglycans have been implicated in many of the processes that contribute to atherogenesis.[2,7]

Mechanical injury to the aorta induced by a balloon catheter has been a useful experimental system in which to study the pathogenesis of atherosclerosis.[8–17] This form of injury leads to arterial lesions

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Dr. Curwen's present address is Department of Animal Sciences, University of New Hampshire, Durham, NH 03824.

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Address reprint requests to Thomas N. Wight, PhD, Department of Pathology SM-30, School of Medicine, University of Washington, Seattle, WA 98195.

that resemble the atherosclerotic lesions of man. Some experiments in the rabbit indicate that the neointima of injured aortas covered by regenerating endothelium preferentially accumulates lipid and lipoprotein, while regions devoid of endothelium show minimal to no lipid deposition.[8-12] Recent reports indicate that a similar phenomenon may also occur in man.[18] The mechanism for this selective lipid deposition is not understood. One possibility is that the lipid is being trapped in reendothelialized neointima by intercellular glycosaminoglycan-containing proteoglycans.[5,6] However, little is known about the types and quantities of glycosaminoglycans in these different areas, which appear to differ in their susceptibility to diet-induced atherosclerosis. To determine whether these particular areas differ with respect to glycosaminoglycan composition and concentration, we have morphologically and biochemically analyzed vessels characterized by reendothelialized and deendothelialized neointima in animals on normal and cholesterol-supplemented diets. The results of our experiments indicate that marked glycosaminoglycan deposition occurs in the reendothelialized neointima as compared with the deendothelialized regions. These findings suggest, but in no way prove, that these macromolecular differences may be important in the preferential lipid accumulation that occurs in the reendothelialized regions of blood vessels.

Materials and Methods

Animals and Experimental Groups

A total of 53 young New Zealand white male rabbits weighing from 2000 to 3500 g were divided into two groups and fed either commercial rabbit ration relatively low in lipid (Groups IA and IB) or the same diet supplemented with 0.5% cholesterol (Groups IIA and IIB) (Purina Rabbit Chow, total lipid approximately 2% by weight; cholesterol, Certified Fischer). Aortic tissues from Groups IA and IIA were used for morphometric estimations of the quantity of glycosaminoglycan in deendothelialized and reendothelialized areas, while intima-media preparations from aortas of rabbits from Groups IB and IIB were used for chemical analysis of glycosaminoglycans. Rabbits were fed the regular diet *ad libitum* or 100 g/day of the cholesterol-supplemented diet beginning 5 weeks prior to balloon-catheter injury. This cholesterol-supplemented diet has proved effective in generating lipid-rich atherosclerotic lesions in the period of time used in this study.[9]

Experimental Injury

After the initial 5 weeks of feeding, a portion of the thoracic and abdominal aorta of all rabbits was deendothelialized with a balloon catheter by using a modification of the technique of Baumgartner et al.[17] Briefly, a 4F thin-walled Fogarty embolectomy catheter (Edwards Laboratory, Santa Ana, Calif) was inserted into the right femoral artery of anesthetized rabbits and advanced to the level of the aortic arch. The balloon was inflated to a pressure of 450–500 mm Hg and pulled through the aorta three times. The catheter was then removed, the wound was sutured, and the animals were continued on their respective diets for an additional 2–14 weeks.

Serum Cholesterol

Serum samples were taken from rabbits of all experimental groups prior to the initiation of the experiment. In addition, serum samples were taken from rabbits receiving the cholesterol-supplemented diet at 2–3-week intervals following endothelial injury. In all instances, serum samples were taken after a 12–18-hour fast. Serum was stored at −20 C until assayed for total cholesterol by the method of Levine and Zak.[19]

Autopsy Procedures

To distinguish reendothelialized and deendothelialized areas of the aorta, rabbits were injected with 3 ml/kg of a 0.5% solution of the protein-binding azo dye Evans blue (wt/vol) in physiologic saline 1 hour prior to sacrifice. As previously described, areas of aortic wall devoid of endothelium were stained blue. Areas of aorta covered by endothelium that restricted or excluded passage of the dye remained unstained and appeared as nonblue islands or bands surrounded by deendothelialized blue-staining areas.[8-12] Previous studies have shown that deendothelialization is complete at the time of injury.[9]

Rabbits used for morphometric analysis (Groups IA and IIA) were sacrificed while under pentobarbital anesthesia by perfusion with glutaraldehyde. The vessels were perfused at 100 mm Hg via the left ventricle with efflux from a vena cava catheter with the use of Ringer's solution for 2 minutes, followed by 1% glutaraldehyde in Sorenson's phosphate buffer, pH 7.4, for 40 minutes, with all solutions at 37 C. Following perfusion, while continuing fixation in 1.5% glutaraldehyde, the aortas were opened along the anterior wall to expose the luminal surface and were photographed. The aortas were fixed for a total

period of 2 hours. Following fixation in glutaraldehyde, portions of the aorta to be used for light microscopy were postfixed in neutral buffered formalin.

The animals in which aortas were to be used for chemical analysis were not perfused. After the animals were sacrificed, the aortas were dissected from the adjacent tissues and maintained in cold saline at 4 C until frozen. Periadventitial debris and adherent blood were removed, and the adventitia was stripped. The artery was opened by a midventral incision extending from the arch to the iliac bifurcation and photographed. Representative tissue samples from the midportion of the thoracic and distal abdominal aortas were dissected and fixed in 10% neutral buffered formalin for confirmation of the absence of adventitia.

After removal of tissue samples for microscopy, remaining portions of thoracic and abdominal segments were dissected into nonblue islands and blue areas. Blue and nonblue areas were blotted dry and frozen at −70 C until analysis. Uninjured aortas were also removed for analysis.

Morphometric Analysis

For morphometric analysis, 5-μ sections were cut from paraffin blocks of abdominal portions of the thoracic aorta and stained with hematoxylin and eosin (H&E) or with alcian blue, pH 2.7.[20] Sections from thoracic aorta were taken at the level of the eighth to eleventh intercostal branch, while sections from the abdominal aorta were taken distal to the origin of the renal arteries. In other instances, 3-μ frozen sections were cut with a cryostat and stained with alcian blue or oil red O. Specificity of the alcian blue staining was controlled by digestion with testicular hyaluronidase and chondroitinase ABC prior to staining. To accomplish this, we incubated paraffin or frozen sections in either 0.1% testicular hyaluronidase (Sigma Chemical Co., St. Louis, Mo) in sodium acetate buffer, pH 5.4, or 0.5 units/ml of chondroitinase ABC (Miles Laboratories, Inc., Kankakee, Ill), in enriched Tris-buffer, pH 8.0, for 2–4 hours at 37 C before staining.[21,22] Testicular hyaluronidase degrades chondroitin sulfates A and C as well as hyaluronic acid. Chondroitinase ABC degrades dermatan sulfate in addition to chondroitin sulfates A and C and hyaluronic acid. Control slides were incubated without enzyme in buffer for the same period of time. The relative degree of alcian-blue-positive staining in the intima of control and injured (blue versus white) vessels was estimated by a grading system as follows: 0, no staining; 0.5, equivocal staining; 1, faint perceptible staining; 2, small patchy areas of intense staining; 3, large patchy areas of intense staining; and 4, large patchy areas of staining with confluence. In order to obtain mean scores for thoracic and abdominal segments of aorta, we summed the scores for a given group and divided by the number of animals in the groups. Only blue (deendothelialized) and white (reendothelialized) areas were microscopically analyzed. Zones projecting from the ostia and colored "gray"[9] were excluded from analysis, since these regions did not contain a thickened intima.

Aortic cellularity was analyzed morphometrically in the intima and media of white zones of nonblue areas and adjacent blue areas of animals of Groups IA and IIA. This was accomplished with the use of a ×40 objective and a 12.5 ocular reticle (each square of the net micrometer was 144 sq μ). Each intersection falling on a nucleus was counted as one cell. An average of 175 intersections was counted for each section. The data was expressed as the number of intersections lying over nuclei divided by the total number of intersections.

Glycosaminoglycan Analysis

Glycosaminoglycans were isolated from frozen aortic segments of approximately 250 mg wet weight, using the procedure of Mankin and Lipiello,[23] as modified by Curwen and Smith.[24] Whole tissue was finely minced, defatted in ethanol overnight, and lyophilized. Following lyophilization, the samples were resuspended in water, boiled for 20 minutes to inactivate hydrolytic enzymes, and subjected to extensive proteolysis by initial incubation with 2.5% pronase (Calbiochem, San Diego, Calif) in 0.2 M Tris buffer for 48 hours at 37 C, followed by 0.3% papain (Sigma Chemical Co., St. Louis, Mo) in 0.1 M phosphate buffer, pH 7.0, for 18 hours at 65 C. Ice-cold trichloroacetic acid (TCA) was added for a final concentration of 5%, and the precipitate was removed by centrifugation. The supernatant was exhaustively dialyzed at 4 C against tap water, and the glycosaminoglycans were precipitated with three volumes of cold 5% (wt/vol) potassium acetate in absolute ethanol.

The percentage of recovery of glycosaminoglycans for each sample was determined by adding a known standard mixture of glycosaminoglycans (15 μg/mg dry tissue) directly to the dry defatted sample. The difference between total uronic acid of the sample plus the glycosaminoglycan standard and the uronic acid content of the sample alone was calculated after extraction. Uronic acid content was determined by

the method of Blumenkrantz and Asboe-Hansen.[25] The percentage of recoveries exceeded 85% for all samples analyzed.

Samples of extracted glycosaminoglycans were separated by cellulose acetate electrophoresis in 0.3 M cadmium acetate buffer, pH 4.1, for 4 hours at 1.2 mamp/strip at 20 C[24] and analyzed in triplicate. Following electrophoresis, the strips were stained with alcian blue (1% wt/vol in glacial acetic acid) for 10 minutes and subsequently destained with 7% acetic acid. Electrophoresis in cadmium acetate buffer separates hyaluronic acid, heparan sulfate, dermatan sulfate, and chondroitin 4- and 6-sulfate into four distinct bands. To confirm the identity of each band, 15-μl aliquots of the samples were subjected to each of the following treatments: 1) 40 μg leech hyaluronidase (Biotrics, Inc., Boston, Mass) in McIlvaine's standard buffer, pH 5.4, every hour for 5 hours[26]; 2) 0.02 units of chondroitinase ABC (Miles Laboratories, Inc., Kankakee, Ill) in enriched Tris HCl, pH 8.0, for 15 hours at 37 C[21]; 3) nitrous acid (prepared by mixing equal volumes of concentrated acetic acid and 5% sodium nitrate) degradation at 37 C for 18 hours.[27] We used glycosaminoglycan standards to determine the specificity and optimal conditions for the degradative treatments by quantitating the amount of standard glycosaminoglycans before and after digestion using the microanalytic procedure of Curwen and Smith.[24] Standard glycosaminoglycans consisted of beef-lung heparan sulfate, which was the generous gift of Dr. J. A. Cifonelli, of the University of Chicago, human umbilical-cord hyaluronic acid, pigskin dermatan sulfate, and shark-cartilage chondroitin 6-sulfate, which were all obtained from Miles Laboratories.

Quantitation of the glycosaminoglycans was accomplished by densitometric scans (Helena Scanning Densitometer, Beaumont, Tex) of the stained cellulose acetate strips performed in triplicate. Samples were quantitated by reference to standard curves prepared by plotting known standard glycosaminoglycan concentrations versus the area under the corresponding densitometric curves.[24] Areas were determined by curve resolution and by the use of a Hewlett-Packard Digitizer. Glycosaminoglycan concentration was expressed as micrograms of glycosaminoglycans per milligram of dry defatted tissues.

The central portion of the nonblue island or gray area does not undergo intimal hyperplasia[9] and therefore does not accumulate appreciable quantities of glycosaminoglycans. Therefore, a more accurate assessment of glycosaminoglycans in the nonblue island must account for any dilutional factor contributed by the relatively glycosaminoglycan-deficient gray area. In the present study, this was accomplished by projecting photographs of the ballooned aorta onto tracing paper and determining the relative percentage of the nonblue islands that were gray for the thoracic and abdominal segments of the aorta of each animal. The weight of the nonblue island was then reduced by the percent of the surface area that was gray. The proportion of gray to white was variable, and at times the gray zones were two to three times more extensive than the white zones.

Statistical Analysis

Serum cholesterol concentrations for various treatment groups were compared by use of a *t* test. The extent of alcianophilia in reendothelialized and deendothelialized areas of the abdominal and thoracic segments of Group IA and IIA rabbits was compared with the use of a two-factor (mixed design) analysis of variance. If the analysis of variance demonstrated a significant difference between areas, these were then compared with the use of paired *t* tests. Since morphometric analysis of cellularity indicated that the findings in the abdominal and thoracic aortas were similar, data were collapsed across segments and then analyzed in a manner similar to that for data for alcianophilia. The mean quantities of individual glycosaminoglycans measured in the reendothelialized and deendothelialized and uninjured areas of aortas of Group IB and IIB animals were then compared with the use of two-factor (mixed design) analysis of variance with repeated measures on one factor. If the analysis of variance demonstrated significant differences among groups and/or areas, subsequent analysis was performed with the use of paired or independent *t* tests as indicated.

Results

Serum Cholesterol Concentrations

The overall mean serum cholesterol concentrations were within the normal range for rabbits prior to the initiation of the experiment. Following feeding of the cholesterol-supplemented diet, the overall mean serum cholesterol concentration for animals in Group IIA sacrificed at 2–5 weeks was 672 ± 54 mg/dl, and for those sacrificed at 6–8 weeks, 718 ± 74 mg/dl. The overall mean serum cholesterol concentrations in animals of Group IIB were 722 ± 77 mg/dl and were not significantly different from those animals in Group IIA. The mean serum cholesterol concentra-

Table 1—Amount of Alcianophilia in Intima*

	Regular diet		Cholesterol-supplemented diet	
	Reendothelialized region	Deendothelialized region	Reendothelialized region	Deendothelialized region
Thoracic segment				
2-5 weeks	2.8 ± 0.20[a†]	1.0 ± 0.32[a]	2.9 ± 0.32[c]	1.07 ± 0.25[c]
6-8 weeks	3.0 ± 0.20[b]	0.5 ± 0.18[b]	3.2 ± 0.29[d]	1.1 ± 0.21[d]
Abdominal segment				
2-5 weeks	2.8 ± 0.25[e]	0.75 ± 0.25[e]	2.8 ± 0.24[h]	0.64 ± 0.12[h]
6-8 weeks	2.5 ± 0.35[g]	0.21 ± 0.13[g]	3.3 ± 0.13[i]	0.85 ± 0.20[i]

* Quantity of glycosaminoglycan determined morphometrically with a 0-4 grading system (see text).
† Mean ± SEM.
[a-i] Numbers with the same letters are significantly different ($P < 0.05$).

tions for Groups IA (65 ± 5) and IIA (69 ± 5) were within the normal range for total serum cholesterol of the rabbit,[9] and they were not significantly different.

Microscopic Observations

The pattern of endothelial regeneration following denudations of the thoracic and abdominal aorta of rabbits has been previously described in detail by Minick et al.[9] The results of the present experiments were similar. Briefly, when animals with previously deendothelialized aortas were given injected Evans blue dye, persistently deendothelialized areas stained blue, while reendothelialized areas excluded the passage of the dye, remained unstained, and appeared nonblue. At the time of sacrifice of these animals, the luminal surface of the aorta was characterized by blue deendothelialized areas that surrounded nonblue islands covered by regenerated endothelium. Each of these nonblue islands had a complex structure composed of a central gray zone and an elevated peripheral white zone.[9]

Morphometric Estimation of Glycosaminoglycans

As summarized in Table 1, the extent of intimal alcianophilia was consistently related to the zone of the injured aorta. The amount of alcianophilic material was significantly greater in the neointima of the reendothelialized area of the injured aorta than in the neointima of the deendothelialized area. In contrast, the extent of alcianophilia did not correlate with the periods of time (2-5 weeks or 6-8 weeks) in the experiment, the segment of the aorta examined, or the presence of hypercholesterolemia. In hypercholesterolemic animals, the distribution of the intimal fatty change, as assessed by oil red O stains, appeared to correlate with the distribution of alcianophilic material. However, we made no attempt to quantitate this correlation morphometrically.

As summarized in Table 2, the cell density in the intima varied significantly with the zone of the injured aorta examined. Persistently deendothelialized areas consistently exhibited more cells, as compared with adjacent reendothelialized areas. In contrast, diet or length of interval between injury or sacrifice did not appear to influence the degree of hyperplasia.

Table 2—Intimal and Medial Hyperplasia*

	Regular diet		Cholesterol-supplemented diet	
	Reendothelialized region	Deendothelialized region	Reendothelialized region	Deendothelialized region
Intimal cellularity				
2-5 weeks	0.15 ± 0.03[a†]	0.22 ± 0.03[a]	0.19 ± 0.02[c]	0.27 ± 0.03[c]
6-8 weeks	0.12 ± 0.01[b]	0.21 ± 0.03[b]	0.12 ± 0.01[d]	0.18 ± 0.01[d]
Medial cellularity				
2-5 weeks	0.13 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.02
6-8 weeks	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01

* Number of intersections overlying nuclei divided by the total number of intersections.
† Mean ± SEM.
[a-d] Numbers with same letters are significantly different ($P < 0.05$).

Hyperplasia of the underlying media could not be shown to relate to the zone of injured aorta, the length of interval between injury and sacrifice, or the serum-cholesterol concentration.

Chemical Analysis of Glycosaminoglycans

Glycosaminoglycans in reendothelialized, deendothelialized, and uninjured portions of individual rabbit aortas from the two diet groups (IIA,IIB) were quantitated by densitometry of cellulose acetate electrophoretograms.[24] In the animals on regular diets, each type of glycosaminoglycan (hyaluronic acid [HA], heparan sulfate [HS], dermatan sulfate [DS], and chondroitin sulfate [CS] was found to be significantly greater in the reendothelialized zone as compared with the deendothelialized zone or uninjured aorta (Figure 1A). Glycosaminoglycan concentrations in the deendothelialized zones and uninjured areas were approximately the same, with the exception that deendothelialized regions contained significantly more chondroitin sulfate (CS) than uninjured aortas (P = <0.005). Although the absolute quantities of each glycosaminoglycan increased significantly in the reendothelialized zones, compared with the deendothelialized regions and uninjured aortas, the relative percentages of each type of glycosaminoglycan in these regions were quite similar (HA, 15-20%; HS, 25-33%; DS, 12-17%; CS, 27-38%). These percentages demonstrate that CS and HS represent the major glycosaminoglycans in normal as well as in injured rabbit aortas. In animals placed on cholesterol-supplemented diets, the reendothelialized regions also contained significantly more of each glycosaminoglycan as compared with deendothelialized and uninjured regions of aorta (Figure 1B). However, there was no difference in the absolute or relative concentration of each glycosaminoglycan in the reendothelialized zones in animals fed regular or cholesterol-supplemented diets. Likewise, the concentrations of individual glycosaminoglycans in the deendothelialized zones in both diet groups were also not different. However, cholesterol feeding alone (ie, without mechanical injury) did appear to increase aortic glycosaminoglycan (P = <0.005) when compared with uninjured aortas in animals on regular diets.

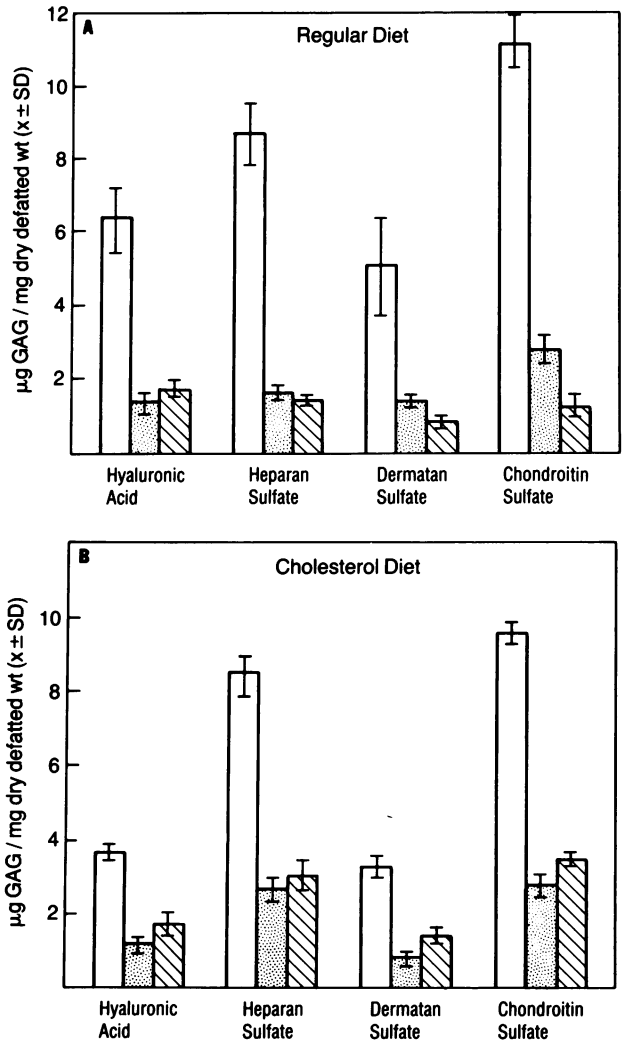

Figure 1 — Amount of glycosminoglycans, expressed as micrograms per gram of dry defatted weight of tissue in reendothelialized (▭), deendothelialized (▩), and uninjured (▨) regions of rabbit aortas on **A** regular and **B** cholesterol-supplemented diets. Amounts are means of triplicate samples quantitated by scanning densitometry of electrophoretograms[24] with reference to standard curves.

Discussion

The present study has used morphologic and chemical analysis to demonstrate quantitative and qualitative differences in the glycosaminoglycans present in regions of the rabbit aortic wall that have reendothelialized after injury, as compared with regions that persistently remain deendothelialized or regions of aorta that are not mechanically injured. Previous morphologic studies have demonstrated increases in glycosaminoglycans in rabbit reendothelialized intimas, compared with adjacent deendothelialized intimas, following balloon injury.[28-30] In addition, biochemical studies have demonstrated increased glycosaminoglycans in aortic intimas following mechanical injury[31,32] in the rabbit, but no attempt was made in these studies to separate regions of the vasculature that had "healed" (reendothelialized) from regions completely devoid of endothelium (deendothelialized). These changes in the glycosaminoglycan content of injured vessels were interpreted as a nonspe-

cific process of repair of vascular tissue, because other forms of injury such as systemic hypoxia[33,34] and cholesterol feeding[35-42] have also been shown to increase aortic glycosaminoglycans in this species. However, the results of our study, plus the ultrastructural studies of Collatz-Christensen et al.[28] and more recently Richardson et al.,[30] indicate that this nonspecific process of injury and repair, which results in increased aortic glycosaminoglycan content, is markedly influenced by the presence or absence of endothelium.

The exact mechanism by which proteoglycans and their constituent glycosaminoglycans preferentially increase in this region is not understood. Both the major cell types of the arterial wall, endothelium and smooth muscle, are capable of synthesizing and secreting proteoglycans.[43-54] Furthermore, the pattern of proteoglycan synthesis by each of these cell types *in vitro* is unique, in that endothelial cells synthesize and secrete large quantities of HS-containing proteoglycan,[51-54] while smooth-muscle cells synthesize and secrete primarily a CS containing proteoglycan, moderate amounts of DS proteoglycan, and small amounts of a HS proteoglycan.[43,44] The preferential accumulation of glycosaminoglycans in hyperplastic arterial intimas that are reendothelialized compared with regions that are deendothelialized, even though both regions were initially injured by the same mechanism, may indicate that endothelial cells exhibit metabolic influences on the smooth-muscle cells' ability to produce glycosaminoglycans, or vice versa. Examples for this type of interaction include the ability of corneal fibroblasts to produce markedly more glycosaminoglycans if maintained in the presence of corneal epithelium or endothelium than when the epithelium or endothelium is removed from the mesenchyme.[55-57] Similar results have been obtained when arterial smooth muscle cells are co-cultured with endothelial cells.[58] In addition, recent studies in our laboratory have indicated that heparin, a highly sulfated subclass of HS, markedly stimulates the synthesis of glycosaminoglycans by human skin fibroblasts and arterial smooth-muscle cells *in vitro*,[59] while markedly decreasing the proliferation of these cells, as shown by others.[60] These studies are relevant, because endothelial cells have been shown to synthesize and secrete a large amount of a heparin-like molecule,[4,51] which inhibits the proliferative capacity of arterial smooth-muscle cells. However, it is not known whether the HS from endothelial cells influences the synthesis of glycosaminoglycans by arterial smooth-muscle cells, as has been demonstrated for heparin. The relationship between arterial cell proliferation and glycosaminoglycan synthesis needs to be clarified.

The presence of increased quantities of glycosaminoglycans in the neointima covered by regeneration endothelium has several functional ramifications. Morphologic studies demonstrate disorganization of connective tissue elements in deendothelialized zones, while reendothelialized regions contain organized elastic fibers, increased basement-membrane-like material, and granules resembling glycosaminoglycan-containing proteoglycans.[28,30] The increased organization of the connective tissue elements within the reendothelialized zones could be due to the increased amount of proteoglycans present in this tissue because these macromolecules are thought to form important structural links between the fibrous components of the intercellular matrix and cells of the artery wall.[2] This increase in glycosaminoglycans may result in altered charge density of the intercellular matrix, which would in turn influence the binding of salts such as CA^{2+}, as well as water within the walls of the affected arteries. These events would lead to alteration of tissue turgor and viscoelasticity of the arterial wall.[61] Increased glycosaminoglycans within the reendothelialized intima could also influence the permeability of this region to small and large molecules. Perhaps the best example of this possibility is demonstrated by the interaction of glycosaminoglycans with lipoproteins. Several studies have demonstrated that low-density lipoproteins and very low-density lipoproteins bind ionically to glycosaminoglycans (see Wight[2] for review), and the strength of this ionic binding is greatest with L-iduronic acid, containing glycosaminoglycans in order of affinities of heparin > DS > HS > CS > HA.[5] The large amounts of HS present in the reendothelialized neointimas in animals on regular diets suggest that this region may be preferentially susceptible to lipid accumulation, compared with regions that contain significantly less glycosaminoglycans of this type. Intact aortic lipoprotein-glycosaminoglycan complexes have been demonstrated in the rabbit[62] as well as other species,[6,63] and the major glycosaminoglycan present in the rabbit aortic lipoprotein-glycosaminoglycan complex is heparan sulfate. The finding of a soluble pool of HS–lipoprotein complex appears unique for rabbit aorta, since other species contain principally CS in this 0.15 NaCl-extractable pool.[63] This may partially explain the high degree of susceptibility of the rabbit to accumulate lipid within the aorta when challenged with a hyperlipemic diet, a finding confirmed in this study. Whether the nature of the arterial wall injury caused by cholesterol feed-

ing is the same as observed for mechanical injury is not known, and it will be important to determine whether the lesion created by cholesterol feeding is characterized by endothelial damage and regrowth. It is clear that both forms of trauma to the arterial wall lead to altered glycosaminoglycan composition. It remains to be demonstrated whether altered glycosaminoglycan content of the arterial wall is a prerequisite for the genesis of atherosclerotic lesions.

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