Fibronectin Synthesis by Aorta Explants from Rabbits Fed High Cholesterol Diets

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Abstract. **Fibronectin synthesis was studied in aorta explants in culture from rabbits fed a high fat-high cholesterol diet. [^{3}H]Mannose and [^{14}C]leucine were used to label oligosaccharide side chains and the protein core, respectively. The synthesis was followed by monitoring immunoprecipitable fibronectin from the culture medium using polyclonal goat anti-rabbit fibronectin antibody. Synthesis of fibronectin increased by [^{14}C]leucine (81%) and [^{3}H]mannose (29%) incorporation over controls. On gel filtration, fibronectin synthesized by controls and cholesterol-fed rabbit resolved into four fractions. Pulse-chase experiment with [^{3}H]glucosamine or [^{3}H]leucine showed that fibronectin secreted by the aorta explants from rabbits fed high fat-high cholesterol diets incorporated an increased amount of radioactivity. Pulsing with [^{3}H]mannose showed decreased incorporation of the label. During the chase period, the rate of secretion of fibronectin into the media by the hypercholesterolemic rabbit aorta explants was increased. The fibronectin that bound to the gelatin or heparin columns from cholesterol-fed rabbit aorta media had lower levels of [^{3}H]mannose incorporated into the glycoprotein than the control. These results indicate that there is an alteration in carbohydrate composition of the fibronectin synthesized by the aorta explants from rabbits fed a high cholesterol diet. High fat-cholesterol intake could play a causative role in matrix dysfunction during atherogenesis by altering glycoprotein synthesis.**

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Fibronectin is a pericellular matrix glycoprotein with adhesive properties to extracellular matrix components. It is a high molecular weight glycoprotein that is found in a soluble form in blood and other body fluids and also in an insoluble form in connective tissue (1). It has multiple binding sites for cells and for extracellular matrix components, such as collagen, proteoglycans, and also heparin and fibrin (2). Fibronectin, by its functional nature, may have an important organizational role in the formation of extracellular matrix and thus act as an adhesive protein for an orderly growth and placement of cells (2). Although not precisely known, the carbohydrate side chains of fibronectin, as in other glycoproteins, may have one or more of the following functions: (i) transport of metabolites across membranes (3, 4); (ii) the insertion and proper orientation of glycoproteins in the cell membrane (5, 6); (iii) the secretion of these proteins (5, 7) and (iv) the protection of these proteins from proteolytic degradation (6, 8). Alteration in glycosylation of N- and O-linked oligosaccharide side chains in glycoproteins have been noted in tumor cells and have been suggested as a predictor of metastatic potential of tumors (9–13).

Injury to the intima of the arterial wall plays a major role in the atherosclerotic process. The presence of a high lipid-lipoprotein environment not only stimulates the proliferation of smooth muscle cells in the aorta but also enhances the interaction of lipids with extracellular matrix components such as, collagen, proteoglycans, elastin, and glycoproteins. This interaction

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of lipids and matrix materials leads to an impairment in repair of the arterial wall and development of disease (14). Cholesterol-fed experimental models used to study atherosclerosis show closely related changes in arterial wall lipids, various matrix proteins, and complex carbohydrate macromolecules (15–17).

Although many studies have dealt with the synthesis of extracellular matrix components such as collagen and proteoglycans (18–21) during atherosclerosis, little is known about glycoprotein biosynthesis in atherosclerosis.

The present study focuses on the biosynthesis of fibronectin in aorta explants from rabbits fed high fat-cholesterol diets.

Materials and Methods

Animals and Diets. Twenty-five New Zealand White male rabbits (1–1.5 kg) were fed atherogenic rabbit chow containing 4% peanut oil and cholesterol (1 mg/kcal) *ad libitum.* After 12 weeks of feeding, rabbits were fasted overnight and serum total cholesterol was assessed in blood samples collected from the ear vein. Eighteen rabbits with high cholesterol levels (over 1000 mg/dl) were used in the experiment. Twelve age-matched rabbits fed on regular rabbit chow were used as controls.

Animals were anesthetized with pentabarbitol and a laparotomy was performed. Rabbit aortas (thoracic and abdominal) were dissected free of adhering tissues, and then washed in saline, blotted, and weighed. On gross visual examination, 90% of the surface area of the hypercholesterolemic rabbit aortas had atherosclerotic lesions. None of the control rabbit aortas had any visible lesions.

Incubation Conditions. Each of the atherosclerotic and control rabbit aorta tissue explants were cut into small segments, and these explants were incubated in duplicates (weighing 0.5 g each) in 5 ml of Hanks' balanced solution containing 20 m*M* HEPES buffer, pH 7.4, at 37°C as per the method of Mills and Adamany (22). The synthesis of glycoprotein was followed in the incubation media using [^{14}C]leucine and [^{3}H]-mannose as markers for protein and oligosaccharide moieties of the fibronectin, respectively. Aorta tissue slices were incubated with the medium containing 1 m*M* mannose and [2-^{3}H]mannose (1 μCi/ml; sp act 27.2 μCi/mmol; New England Nuclear Corp., Boston, MA) or 2 m*M* cold leucine and [1-^{14}C]leucine (2 μCi/ml; sp act 140.8 μCi/mmol; New England Nuclear). At the end of the incubation period, the flasks were immediately chilled and the medium was removed. The tissue explants were washed twice with 2.5 ml of ice-cold Hanks' medium. The medium with the washings was then processed for immunoprecipitation of fibronectin and for total glycoproteins. Total glycoproteins in the medium was determined as per the method of Lucas *et al.* (23). Briefly, proteins in the media were precipitated by the addition of 2 ml of 12% trichloroacetic acid solution for every milliliter of the medium. The precipitate was then washed with trichloroacetic acid and dissolved in Protosol and counted in Instagel in a Beckman LS250 liquid scintillation counter.

The residual tissue was defatted with chloroform:methanol (2:1), dried, and weighed; a part of the tissue was dissolved in Protosol and counted in Instagel in a Beckman LS250 liquid scintillation counter. The incorporation of [^{3}H]mannose and [^{14}C]leucine in the defatted tissue was taken to represent matrix-associated insoluble glycoproteins.

Trichloroacetic acid precitable proteins and defatted tissue proteins were determined by the method of Lowry *et al.* (24).

Immunoprecipitation of Fibronectin. Fibronectin was immunoprecipitated from the medium by the method of Hayman *et al.* (25). Briefly, 1 ml of radioactive sample was incubated with 20 μl of polyclonal goat anti-rabbit fibronectin antibody (Cooper Biochemical, Inc., Malvern, PA) and 100 μl of 50% suspension of protein A-Sepharose (Sigma Chemical Co., St. Louis, MO) for 3 to 5 hr at room temperature with gentle rocking. Preliminary experiments showed that the amounts of antibody and protein A added are in excess of what is needed to precipitate glycoprotein and the anti-rabbit IgG, respectively. After washing the Sepharose beads four times with 0.9% saline, containing 0.5% Tween-20 (Sigma), bound material was eluted with 500 μl of saline containing 2% sodium dodecyl sulfate (SDS). The eluate, with the addition of 10 ml of Instagel, was counted as ^{3}H and ^{14}C in a Beckman LS250 liquid scintillation counter.

Purity of the immunoprecipitate fibronectin was checked by SDS gel electrophoresis and affinity column chromatography. Immunoprecipitated fibronectin was exhaustively dialyzed against saline solution to get rid of SDS and samples were pooled and lypholyzed; SDS gel electrophoresis of the control and experimental lypholyzed samples gave rise to two bands with molecular weights similar to those of fibronectin (M_r 400,000 and 250,000). The lypholyzed control in experimental samples were eluted from gelatin Sepharose and heparin Sepharose columns with 4 *M* urea and 2 *M* NaCl and counted. Results show that 75–80% immunoprecipitable fibronectin bind to these affinity columns (Table III).

Gel Filtration by High Performance Liquid Chromatography. Dialyzed and lypholyzed immunoprecipitated fibronectin was dissolved in 300 μl of acetate buffer 0.05 *M*, pH 5.8, with 10% ethanol. Aliquots (150 μl) were chromatographed with a Perkin-Elmer Series 3 high performance liquid chromatography system equipped with a 7.5 × 300 mm Biogel-TSK G 5000 PW column (BioRad Laboratories). The protein from

the column was eluted at a flow rate, 0.5 ml/min, with 0.05 *M* sodium-acetate buffer, pH 5.8. The eluent was monitored at 252 nm.

Pulse-Chase of Labels in Fibronectin. The rate of secretion of fibronectin into medium was examined by a pulse chase protocol. The tissue explants were pulsed with the radiolabeled *N*-acetylglucosamine (acetyl-^{3}H) (2 μCi/ml; sp act 40 Ci/mmol), in 100 μM cold *N*-acetylglucosamine, or [2-^{3}H]mannose (1 μCi/ml in 1 m*M* cold mannose) or [1-^{14}C]leucine (2 μCi/ml, containing 2 mM cold leucine) for 1 hr. After 1 hr, incubation tissue slices were washed twice with the respective isotope-free medium and chased; at different time periods of chase, the medium was changed with medium containing cold *N*-acetylglucosamine (100 μM) or mannose (1 m*M*) or leucine (2 m*M*). Immunoprecipitable radiolabeled fibronectin was isolated from the medium, dialyzed, and counted.

Binding of Fibronectin to Gelatin or Heparin Affinity Column. Immunoprecipitated fibronectin from the control and experimental culture media was dialyzed and lypholyzed. This sample was then dissolved in 0.154 NaCl and then passed through gelatin or heparin Sepharose columns; bound and unbound fibronectin were separated by the method of Ruoslahti *et al.* (26). Sample (300 μl) containing equal amounts of fibronectin protein was applied on a heparin- or gelatin-Sepharose column and the column was washed with 2 ml of 0.154 *M* NaCl. The wash containing unbound fibronectin fractions was counted for [^{3}H]mannose and [^{14}C]leucine. The bound fibronectin was then eluted from the column by 2 ml of 4 *M* urea containing 2 *M* NaCl and the effluent was counted for radiolabel.

Results

Incorporation of [^{3}H]Mannose and [^{14}C]Leucine into Glycoproteins. Table I summarizes the results of the radioactive mannose and leucine incorporation into the glycoproteins in the medium and matrix-associated insoluble glycoproteins of control and cholesterol-fed rabbit aorta explants in culture. A significant increase in [^{3}H]mannose incorporation into glycoproteins was noted in the medium in the cholesterol-fed rabbit aorta explant culture over controls ($P < 0.01$), while leucine incorporation in proteins did not differ significantly. The radiolabeled mannose and leucine incorporation into matrix-associated insoluble glycoproteins in the experimental aorta showed a significant increase in mannose and leucine incorporation.

Fibronectin Synthesis. Results of the radioactive mannose and leucine incorporation into the immunoprecipitable fibronectin from the incubation media of the control and atherosclerotic rabbit aorta explants are summarized in Table II. Although marked increases in both leucine and mannose incorporation in the fibronectin from the incubation medium of the cholesterol-fed rabbit aorta explants over the control were noted, there was a difference in the level of incorporation of [^{3}H]mannose and [^{14}C]leucine in the fibronectin synthesized by the atherosclerotic rabbit aorta explants. An 80% increase in the incorporation of leucine into fibronectin was noted from the incubation medium of the atherosclerotic aorta explants over controls, while there was only a 30% increase in mannose incorporation over the controls into this glycoprotein. The difference in the degree of mannose and leucine incorporation is reflected in the ^{3}H to ^{14}C ratio, which is low in the atherosclerotic animals in comparison to that of controls.

Gel Filtration Profile of the Immunoprecipitable Fibronectin. The elution profile of immunoprecipitable fibronectin from the incubation media of control and atherosclerotic aortas is shown in Figure 1. Fibronectin from both media on gel filtration by high performance liquid chromatography separated into four different molecular weight species. Fraction 2 is the major fraction in both groups, having an approximate molecular weight of 440,000. Fraction 2 was the predominant fraction in hypercholesterolemic aorta incubation medium.

Fibronectin Binding to Gelatin and Heparin Affinity Columns. Table III summarizes the binding affinity of fibronectin preparation from the media on to gelatin Sepharose and heparin Sepharose columns.

The percentages of incorporation of [^{14}C]leucine label into the fibronectin that was bound to the gelatin and heparin columns from atherosclerotic and the control aorta explants cultures were similar; however, the

Table I. Radiolabeled Mannose and Leucine Incorporation in Media and Membrane Glycoproteins from Control and Atherosclerotic Rabbit Aortas in Culture

Rabbit aortas	dpm × 10^3/mg protein			
	Media		Membrane	
	[^{3}H]Mannose	[^{14}C]Leucine	[^{3}H]Mannose	[^{14}C]Leucine
Control	42.9 ± 1.6^a	306.9 ± 2.9	26.1 ± 0.3^c	511.0 ± 4.0^e
Atherosclerotic	48.3 ± 2.1^b	295.3 ± 3.8	29.2 ± 0.9^d	552.6 ± 2.3^f

Note. Mean ± SE of 14 animals from the experimental group and eight animals from the control group. $b > a$, $d > c$, $f > e$ at $P < 0.05$ level.

Table II. Radiolabeled Mannose and Leucine Incorporation in Immunoprecipitable Fibronectin in the Media from Control and Hypercholesterolemic Rabbit Aorta Cultures

Rabbit aortas	dpm/ml of the incubation media		Mannose:leucine ratio
	[^{3}H]Mannose	[^{14}C]Leucine	
Control	2,917 ± 229^{a}	17,693 ± 421^{c}	0.16 ± 0.05
Atherosclerotic	3,768 ± 185^{b}	32,010 ± 568^{d}	0.12 ± 0.01
Percent change	29	81	

Note. Aortic explants from normal and atherosclerotic rabbits were incubated for 3 hr in Hanks' balanced salt medium containing either [^{3}H] mannose or [^{14}C]leucine. Fibronectin was immunoprecipitated from the medium and counted for radiolabeled mannose and leucine. Mean ± SE of 14 animals in the experimental group and eight animals in the control group. $b > a$, $d > c$ at $P < 0.01$ level.

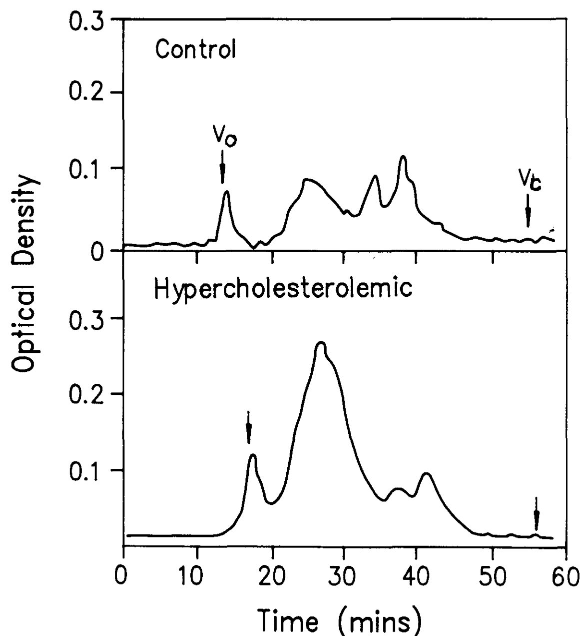

Figure 1. Gel filtration profiles of control and hypercholesterolemic rabbit aorta fibronectin. Immunoprecipitable fibronectin from the media after the incubation of control and hypercholesterolemic rabbit aorta explants were chromatographed by high performance liquid chromatography using a TSK G 5000 PW column.

fibronectin that bound to the gelatin or heparin columns from the experimental group had less mannose than the control (Table III).

The ratio of radiolabeled mannose to leucine counts in the bound fibronectin from cholesterolemic rabbit aorta was smaller than that of controls (Table III).

Kinetics of Fibronectin Synthesis. On pulsing with radiolabeled leucine and *N*-acetylglucosamine for 1 hr, the fibronectin synthesized by the atherosclerotic rabbit aorta had incorporated radioactive compounds in increments of 50% and 100%, respectively, over controls; during the same period of pulsing, incorporation of [^{3}H]mannose into the fibronectin from the cholesterol-fed rabbit tissue was less than controls (Fig. 2). During the initial period of chase (during the first 30 min), most of the radiolabeled fibronectin secreted into the media and the rate of secretion (the slope of the curve, dpm/30 min) were greater from atherosclerotic rabbit aorta explants.

Discussion

It is evident from observations made in this study that aortic tissue explants from rabbits fed high cholesterol diet synthesize increased levels of fibronectin and perhaps other glycoproteins. Marked increases in the incorporation of radiolabeled leucine and mannose into the newly synthesized fibronectin by the experimental aorta tissue explants over controls suggest an increased synthesis of this glycoprotein. This finding is in agreement with the earlier studies, wherein it was noted that during development of atherosclerotic lesions there is a rapid proliferation of arterial wall smooth muscle cells. The latter are the chief cell lines that elaborate extra-

Table III. Percentage of [^{3}H]Mannose- and [^{14}C]Leucine-Labeled Fibronectin that Bound to Heparin-Sepharose 6 B Column from Control and Hypercholesterolemic Rabbit Aorta Explant Cultures

Tissue Explants	Percentage of the total counts (dpm) bound*					
	Gelatin-sepharose			Heparin-sepharose		
	[^{3}H]Mannose	[^{14}C]Leucine	^{3}H/^{14}C	[^{3}H]Mannose	[^{14}C]Leucine	^{3}H/^{14}C
Control	65 ± 5^{a}	80 ± 3	0.37 ± 0.03^{c}	69 ± 6^{e}	88 ± 5	0.4 ± 0.05^{g}
Atherosclerosis	40 ± 4^{b}	75 ± 2	0.17 ± 0.01^{d}	47 ± 1^{f}	74 ± 1	0.23 ± 0.03^{h}

Note. Equal amounts of immunoprecipitable fibronectin protein from control or experimental samples were loaded onto a gelatin or heparin sepharose affinity column and were then eluted with a solution containing 4 *M* urea in 2 *M* NaCl. Mean ± SE of four samples from experimental and normal groups. $a > b$, $c > d$, $e > f$, $g > h$ at $P < 0.01$ level.

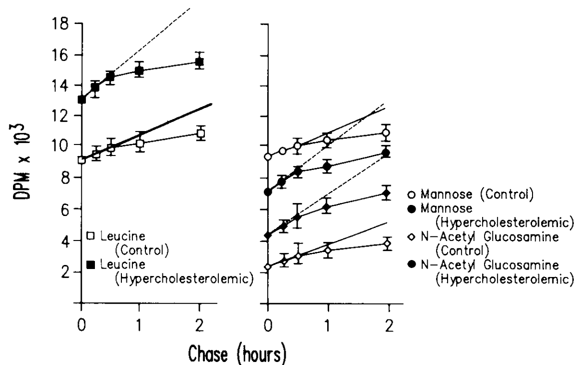

Figure 2. Kinetics of fibronectin secretion into the incubation medium of the control and hypercholesterolemic rabbit aorta explants. The rate of synthesis of fibronectin into medium was examined by a pulse-chase protocol. The tissue explants were pulsed with the radiolabeled *N*-acetylglucosamine or mannose or leucine for 1 hr and chased in media containing cold *N*-acetylglucosamine, or mannose or leucine immunoprecipitable radiolabeled fibronectin was isolated from the media and counted. These results represent mean ± SE of four animals.

cellular matrix components, including glycoproteins (17–19).

It is of particular interest to note the ratio of radiolabeled mannose to leucine incorporation in the fibronectin synthesized by atherosclerotic aorta was reduced. This observation suggests that the newly synthesized fibronectin by the experimental tissue is mannosylated to a lesser degree. Alteration by enzymatic glycosylation of proteins has been noted in other pathologic conditions, such as malignant transformation (9–13).

Decreased mannosylation of the oligosaccharide side chains of the fibronectin synthesized by atherosclerotic aorta could be due to (i) a decreased number of oligosaccharide side chains attached to the fibronectin, or (ii) the number of mannose residues attached to the side chains may be decreased in fat-cholesterol-induced atherosclerosis. Yet, on pulse labeling with *N*-acetylglucosamine and leucine, the incorporation of the radiolabeled compounds increased in the fibronectin synthesized by the atherosclerotic aorta. Hence, it is less likely that the number of oligosaccharide chains attached to the protein is reduced. However, the pulse-chase experiments with mannose strongly suggest that the fibronectin synthesized by the atherosclerotic aorta is mannosylated to a lesser degree than the control, thus altering the composition of the oligosaccharides of the fibronectin synthesized by the atherosclerotic tissue.

An important biological function of fibronectin is its ability to specifically bind to collagen (27–29). Procedures for purification of this glycoprotein by affinity chromatography using this characteristic of fibronectin have been devised (26). Studies have identified the various binding domains within the fibronectin molecule through the use of appropriate proteolytic enzymes (29). Each of these functional binding domains of this glycoprotein contain N-linked oligosaccharide chains (2). In this study, we were interested mainly in identifying the comparative amounts of fibronectin that are bound to gelatin and heparin affinity columns. Hence, the fibronectin was used in its preparative state without further hydrolysis. Within the limitation of these binding studies, these results further suggest that the fibronectin synthesized by the atherosclerotic aorta have different amounts of mannose incorporated in the oligosaccharide of the fibronectin. It is possible that the different amounts of mannose in the N-linked oligosaccharide side chains of this glycoprotein may play a crucial role in their ability to bind with the extracellular components and the cells of the arterial wall.

The present results also indicate that there is an accelerated rate of secretion of the fibronectin in the hypercholesterolemic rabbit aorta, suggesting that synthesis, sorting, and the passage through endoplasmic reticulum of this glycoprotein are enhanced. Earlier results have suggested that carbohydrate side chains of glycoproteins play a major role in signaling and sorting of secretory and membrane glycoproteins (8). These results suggest that the newly synthesized fibronectin by the hypercholesterolemic rabbit aorta may have been transported more rapidly than the control because of altered carbohydrate composition.

These studies illustrate the potential dysfunction in glycosylation of fibronectin that can occur in the aorta. Further studies are needed to identify the regulatory mechanisms involved in arterial wall glycoprotein synthesis. The interaction suggests that fibronectin of altered composition could play a role in the pathogenesis of atherosclerosis.

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