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Dieter M. Kramtsch, ... , Carl Franzblau, William Hollander

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Research Article

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The Protein and Lipid Composition of Arterial Elastin and Its Relationship to Lipid Accumulation in the Atherosclerotic Plaque

DIETER M. KRAMSCH, CARL FRANZBLAU, and WILLIAM HOLLANDER

From the Department of Hypertension and Atherosclerosis, University Hospital, and from the Department of Biochemistry, Boston University Medical Center, Boston, Massachusetts 02118

ABSTRACT Elastin preparations from intimal layers and the media of normal and atherosclerotic human aortae were analyzed for protein and lipid content. In atherosclerotic aortae, elastin from plaques was compared with elastin from adjacent normal appearing areas of the same aorta.

Arterial elastin purified by alkaline extraction appeared to be a protein-lipid complex containing free and ester cholesterol, phospholipids, and triglycerides. The lipid component of normal arterial elastin was small (1-2%). With increasing severity of atherosclerosis, there was a progressive accumulation of lipid in intimal elastin from plaques, reaching a mean lipid content of 37% in severe plaques. The increase in the lipid content of plaque elastin preparations was mainly due to large increases in cholesterol, over 80% of which was cholesteryl ester. This deposition of cholesterol in plaque elastin accounted for 20-34% of the total cholesterol content of the plaque. The increased lipid deposition in plaque elastin was associated with alterations in the amino acid composition of plaque elastin. In elastin from plaque intima, the following polar amino acids were increased significantly: aspartic acid, threonine, serine, glutamic acid, lysine, histidine, and arginine; whereas, cross-linking amino acids: desmosine, isodesmosine, and lysinonorleucine were decreased significantly. The amino acid and lipid composition of elastin from normal appearing aortic areas was comparable to that of normal arterial elastin except for intimal elastin directly adjacent to and medial elastin directly below the most severe plaques.

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The data indicate that the focal lipid deposition in early atherosclerotic plaques is due to a large extent to lipid accumulations in altered elastin protein of localized intimal areas. Continued lipid deposition in altered elastin appears to contribute substantially to the progressive lipid accumulation in the plaque. The study suggests that elastin of intimal elastic membranes may play an important role in the pathogenesis and progression of atherosclerosis.

INTRODUCTION

Atherosclerosis is associated with focal accumulations of lipid in the intima of arteries. The areas of lipid accumulation commonly show marked structural alterations of the connective tissue elements of the intima and subintima. While the changes of intimal lipids in atherosclerosis have been studied extensively with histological and biochemical methods, the alterations of the intimal connective tissue usually have been examined only morphologically.

One of the most consistent structural changes of connective tissue elements in atherosclerotic lesions is the splitting and fragmentation of the intimo-medial elastic membranes which may be found in early as well as in advanced plaques. There is histological evidence that structurally altered portions of the intimo-medial elastic membranes may play a role in the focal accumulation of lipid in the arterial intima. Accumulation of stainable lipids on the split and/or fragmented internal elastica has been regarded by many investigators as one of the earliest manifestations of atherosclerosis (1-7). Recent radioautographic studies after intravenous injection of tritium-labeled cholesterol, have shown that cholesterol is closely associated with arterial elastic membranes of normal arteries (8) and is present in dense accumula-

tions on the split and fragmented intimo-medial elastic lamellae in all stages of atherosclerosis (9).

The structural changes of elastic membranes in atherosclerotic lesions suggest that the arterial elastic tissue may have undergone biochemical changes, possibly involving the elastin core itself. There has been some evidence that purified elastin from ligamentum nuchae contains small amounts of lipids even after delipidation with methanol acetone (10) and ethanol ether (11). It seemed possible, therefore, that the lipid deposited on arterial elastic membranes was tightly associated with elastin protein.

The present study was undertaken to determine the protein and lipid composition of arterial elastin from normal intima and plaque intima in an attempt to clarify the relationship of arterial elastin to the lipid accumulation in atherosclerotic lesions.

METHODS

21 human aortae were obtained at autopsy within 8 hr after death. Pertinent data on the patients from whom the aortae were taken are summarized in Table I. In each aorta the severity of atherosclerosis was graded according to the prevailing type of plaques as follows: Grade 0, no grossly visible lesions; grade I, fatty streaks and dots; grade II, large confluent plaques; grade III, severe fibrous-fatty plaques with ulcerations and calcifications. Small segments of normal aortae, plaques, and adjacent normal-appearing aortic areas were processed for histology as previously described (8).

For biochemical studies, the aortae were cleaned of contaminating blood with a moist saline gauze and stripped of their adventitia. Aortae with no visible atherosclerosis were separated into an intimal layer and a medial layer. In atherosclerotic aortae, plaque areas and adjacent normal areas of equal size were cut out and pooled separately for each aorta. The aortic segments then were separated into intimal layers and medial layers. The intimal layers of normal and atherosclerotic aortae, by microscopic examination, were found to be comprised of the entire intima and a small portion of adherent media. Small samples from each set of intimal tissue were taken to determine dry weight and total intimal lipid as well as calcium content by ashing. The calculated total intimal calcium content was subtracted from the dry weight of the intima and all figures referring to intimal tissue are given in terms of dry, calcium-free intima.

Intima of normal aorta as well as plaque intima and adjacent normal intima of atherosclerotic aorta were processed separately for elastin extraction. The intimal tissue was cut into small pieces of about 2 × 2 mm and minced in a Virtis blender (Virtis Company, New York) in a 0.25 M sucrose solution. The mincing procedure was done 1 min at a time with a 1-min rest period for a total mincing time of 10 min. The Virtis flask was cooled in crushed ice in order to avoid overheating of the tissue. The minced intima were homogenized in a Potter-Elvehjem homogenizer and then were spun at 600 g for 20 min in a Lourdes centrifuge. The resultant supernatant was decanted leaving a sediment of intimal debris which was found microscopically to consist of fragmented nuclear material, collagen fibers, and elastic membranes with adherent acid mucopolysaccharides, glycoprotein, and collagen.

TABLE I
Summary Data on Donors of Aortae

Patient	Age	Sex		Grades of atherosclerosis
C. R.*	17	M	Chronic glomerulonephritis	0
J. B.	21	M	Sarcoma	0
W. H.*	24	M	Hodgkin's disease	0
G. R.	24	F	Pulmonary embolus	0
F. L.*†	27	M	Fatal accident	0
O. T.*	32	F	Myocardopathy	I
S. B.	46	M	Virus encephalitis	I
S. W.	48	M	Fatal accident	I
E. L.*	48	F	Endothelioma	I
M. K.*	51	F	Myocardial infarction	I
R. P.§	44	M	Bronchogenic carcinoma	II
C. C.§	47	F	Lupus erythematosus	II
G. S.*†	53	F	Myocardial infarction	II
R. F.*	62	M	Hypertension, CHF	II
G. T.	64	M	Hypertension, uremia	II
P. R.*	89	M	Cerebral vascular accident	II
M. V.§	48	M	Adrenal carcinoma	III
R. B.*	54	M	Hypertension, uremia	III
E. T.*	65	F	Diabetes mellitus	III
M. M.	76	F	Cerebral vascular accident	III
E. M.*	82	F	Mushroom poisoning	III

* Elastin of aortic media included in analysis.

† Elastin of normal lung included in analysis.

§ Injected intravenously with cholesterol-³H.

Elastin was extracted from each intimal debris fraction with boiling 0.1 N NaOH for 45 min, according to the method of Lansing, Rosenthal, Alex, and Dempsey (10). The extracted elastin was spun at 3000 rpm for 20 min. The resultant supernatant was decanted and the sedimented elastin was washed three times with normal saline for 10 min and three times with distilled water for 10 min with centrifugation of the elastin after each washing step. The elastin preparation was then dehydrated for a total of 2 hr with three changes of absolute ethanol and defatted for 1 hr (in total) with three changes of chloroform-methanol 2:1 (v/v). After chloroform-methanol, the elastin was extracted with two changes of acetone and two changes of ether with the extraction time being 10 min for each solvent change. Delipidation of the elastin was complete after this procedure as indicated by the absence of lipids in portions of the defatted elastin after hydrolysis with elastase.¹

A dry weight of the defatted elastin was established, and its total protein content was measured from a portion by the Kjeldahl method. Other portions were analyzed for hexosamine by the method of Boas (12), glucuronic acid by the method of Dische (13), and neuraminic acid by the methods of Aminoff (14) and Svennerholm (15). The defatted elastin preparation was hydrolyzed in a vacuum oven

¹ Obtained from Worthington Biochemical Corp., Freehold, N. J.

with 6 N HCl at 110°C for 24 hr, and its amino acid composition was determined with a Technicon amino acid auto-analyzer (Technicon Corp., Ardsley, N. Y.), according to the method of Hamilton (16). The lipid solvents used in the dehydration and delipidation of each set of intimal elastin (ethanol, chloroform-methanol, acetone and ether) were pooled and analyzed for total lipid content and composition. Free and ester cholesterol was determined by the method of Schoenheimer and Sperry (17); triglycerides by the method of Van Handel and Zilversmit (18); and phospholipids by the method of Youngburg and Youngburg (19). The significance of the data was determined by statistical analysis of unpaired data.

Three of the patients, two with grade II and one with grade III atherosclerosis of the aorta, were injected intravenously with 1 mCi 7- α - 3 H-cholesterol² (specific activity 4.66 Ci/mM) 8 to 16 wk before death from a fatal illness. The intravenously administered cholesterol- 3 H was complexed to human serum lipoprotein of density 1.006-1.063 according to the method of Whereat and Staple (20). All radioactive material extracted from intimal elastin of these patients was digitonin precipitable and hence was recovered as sterol. The digitonin-precipitable radioactivities were counted in a liquid scintillation spectrometer using a methanol-toluene system as described by Chobanian and Hollander (21). The extracted elastin of normal and plaque intima from patients who had been injected intravenously with cholesterol- 3 H also was smeared on glass slides and processed for radioautography as previously described (8).

The composition of elastin in the aortic media also was analyzed. The elastin was extracted from the medial layers of normal aortae and from the medial layers immediately below plaque areas and from adjacent normal areas of atherosclerotic aortae. Likewise, elastin was extracted from two normal human lungs and the amino acid and lipid composition was determined.

The extraction procedure with hot alkali was controlled by the following studies:

1. The possibility that extraelastin lipids were transferred and bound to the elastin during the tissue homogenization and extraction procedures was tested. For this purpose 10 ml of serum from a hypercholesterolemic patient containing 58.3 mg cholesterol was added to 1.0 g (calculated dry weight) of normal intima as well as plaque intima before homogenization and alkali extraction of the tissues. The lipid content of this elastin preparation was not higher than that of elastin prepared from portions of the same tissues without addition of lipids.

In addition, plaque tissue from each grade of atherosclerosis was mixed with an equal amount (by weight) of normal intimal tissue. The mixture then was homogenized and extracted with hot alkali. The elastin extracted from these mixed tissues showed a protein and lipid composition which was intermediate between that of normal elastin and that of elastin from the type of plaque used in the mixture (see Results).

2. To test the purity of the extracted elastin, several sets of normal and plaque elastin were treated in succession with collagenase, hyaluronidase, and trypsin after extraction of the elastin with hot alkali. The amino acid and lipid composition of the alkali extracted and enzyme-treated elastin was comparable to that of elastin isolated by alkali extraction alone.

3. The capacity of hot alkali to hydrolyze lipids was also tested. Pieces of fatty liver containing cholesteryl ester, triglycerides, and phospholipids were homogenized using the same techniques as described for the homogenization of intima. The liver homogenates then were also subjected to boiling in 0.1 N NaOH for 45 min. After the alkali hydrolysis, no cholesteryl ester, triglycerides, and phospholipids were demonstrable; 97 per cent of the total cholesterol was recovered as free cholesterol. Similarly, the lipid and protein moieties of ultracentrifugally isolated low density lipoproteins from hyperlipidemic human serum also were completely hydrolyzed after treatment with hot NaOH in the same manner.

It seemed possible that some of the lipids found to be associated with arterial elastin were only loosely attached to the elastin protein. An attempt was made, therefore, to mobilize these lipids, especially cholesterol and cholesteryl esters, by centrifugation of the extracted elastin preparation at 105,000 *g* for 22 hr in saline of density 1.063 and 1.210 at 25°C. The densities of these salt solutions are considerably higher than those of cholesterol or its esters. The density of free cholesterol is reported as 1.040 (22). The density of the two major cholesteryl esters in atherosclerotic plaques, cholesteryl oleate, and cholesteryl linoleate, as measured in our laboratory, was found to be 0.9646 and 0.9383, respectively, with reference to water at 25°C. Less than 5% of the free and ester cholesterol contained in the elastin preparation could be floated from the elastin in the hypertonic salt solutions. Further treatment of the elastin preparation with two changes of petroleum ether for 1 hr removed less than 20% of the lipids.

To explore the manner in which the elastin lipids are associated with the elastin protein, alkali-extracted intimal elastin containing the lipids was digested with elastase. Duplicate samples of the elastin hydrolysate were separated into peptide moieties by disc electrophoresis in polyacrylamide gel (7.5%, 11.25%, and 15%) using a modification of the method of Davis (23). After electrophoresis, one of the paired gel columns was stained with amido-Schwarz by the method of Nagai, Gross, and Piez (24) while the other column was stained with Oil Red O according to the method of Smithies (25).

RESULTS

1. Intimal elastin

(a) CONTENTS OF ELASTIN IN AORTIC INTIMAE

Table II shows the contents of elastin per gram dry calcium-free intima in intimal layers of normal and atherosclerotic human aortae before and after delipidation of the extracted elastin preparation.

Intima of normal aortae. The mean content of non-delipidated elastin in intima from aortae without atherosclerosis (grade 0) was 20.5%. After removal of the small amount of lipid contained in normal intimal elastin, the mean content of delipidated elastin protein in normal aortic intima was 20.3%. On microscopic examination, the intimal elastic membranes of normal aortae were not structurally altered.

Plaque intimae. As compared to the elastin content of intimae from normal aortae, the mean elastin content of grade II and grade III plaques was reduced strikingly

² Obtained from New England Nuclear Corp., Boston, Mass.

TABLE II

Contents of Nondefatted and Defatted Elastin in Intimal Layers of Normal and Atherosclerotic Human Aortae

Grade 0 atherosclerosis* (Normal intima)				Grade I atherosclerosis* Plaque intima Adjacent "normal" intima			
Nondefatted elastin		Defatted elastin		Nondefatted elastin		Defatted elastin	
<i>mg/g dry calcium-free tissue (Mean ±SD)</i>				<i>mg/g dry calcium-free tissue (Mean ±SD)</i>			
204.9		203.1		189.2		199.5	
±28.3		±27.9		±26.2		±29.1	
Grade II atherosclerosis‡ Plaque intima Adjacent "normal" intima				Grade III atherosclerosis* Plaque intima Adjacent "normal" intima			
Nondefatted elastin		Defatted elastin		Nondefatted elastin		Defatted elastin	
<i>mg/g dry calcium-free tissue (Mean ±SD)</i>				<i>mg/g dry calcium-free tissue (Mean ±SD)</i>			
122.0		100.0		185.7		165.3	
±19.5		±18.4		±29.1		±22.4	
182.0		182.0		107.4		146.6	
±25.5		±25.5		±5.7		±24.9	

* 5 patients.

‡ 6 patients.

with the reduction being highly significant ($P < 0.01$), for both nondelipidated and delipidated elastin. The extent of the reduction in elastin protein content becomes especially apparent when the delipidated elastin of grade II and grade III plaques is compared with the delipidated elastin of intimae from normal aortae. Although grade I plaques also showed some decrease in mean elastin content, the reduction of both nondelipidated and delipidated elastin was not statistically significant ($P = 0.4$ and 0.1 , respectively). On microscopic examination, grade I plaques usually showed moderate splitting and fragmentation of the intimal elastic membranes. In grade II and grade III plaques these structural alterations of the intimal elastic membranes were severe; frequently large sections of the intimal elastica were missing.

"Normal" intimae adjacent to plaques. There was no statistically significant difference in the mean elastin content between intimae of normal aortae (grade 0) and normal intimae adjacent to grade I and grade II plaques ($P = 0.6$ and 0.4 , respectively). The elastic membranes of these intimae were histologically normal. The variations occurring in the elastin content of normal intimae appeared to be due to a variable thickness of the cellular layers of normal intimae. The grossly normal appearing intima adjacent to grade III plaques, however, did show a significant reduction in the mean content of delipidated elastin ($P < 0.05$); a decrease in nondelipidated elastin content also occurred but was not statistically significant ($P = 0.07$). Histologically, the "normal" intima adjacent to grade III plaques revealed considerable fragmentation and splitting of the arterial elastic membranes.

(b) PROTEIN AND LIPID COMPOSITION OF INTIMAL ELASTIN

The elastin of normal and plaque intimae contained both protein and lipid, with the lipid component consisting of free and ester cholesterol, phospholipids, and triglycerides (Table III). When elastase-digested arterial elastin was electrophoresed in polyacrylamide gel, staining of the gel with amido-Schwarz demonstrated two distinct peptide moieties of the elastin hydrolysate under the conditions employed. These peptide moieties also stained with Oil Red O, indicating that neutral lipids had migrated with the peptide moieties of elastin protein.

Intima of normal aortae. Elastin of intimae from normal aortae (grade 0) consisted of 98.7% protein (mean) and 1.3% lipid (mean) (Table III). In histological sections of normal aortae, no lipids were demonstrable on the intimal elastic membranes by staining. However, the isolated intimal elastin of these aortae did stain weakly with Oil Red O.

Plaque intimae. Elastin from atherosclerotic plaques contained a significantly greater percentage of lipids than did elastin of normal intima. The lipid content of plaque elastin increased with increasing severity of atherosclerosis (Table III). In grade III plaques the elastin contained 37.4% lipid and 62.6% protein. The increase in the lipid content of plaque elastin was mainly due to large increases in cholesterol, especially ester cholesterol, with minor changes in phospholipid and triglyceride content (Table III). Over 80% of the lipid contained in plaque elastin was cholesterol regardless of the severity of atherosclerosis. In histological

TABLE III
Protein and Lipid Composition of Elastin from Intimal Layers of Normal and Atherosclerotic Human Aortae

Intimal atherosclerosis	Protein	Lipid	Ester cholesterol	Free cholesterol	Phospho-lipid	Trigly- ceride
<i>mg/g elastin (Mean ±SD)</i>						
Grade 0*						
"Normal"	987.2 ±2.4	12.8 ±2.4	7.9 ±1.7	1.3 ±0.7	1.0 ±0.3	2.6 ±0.8
Grade I*						
(a) Plaque	895.6 ±40.1	104.4 ±40.1	75.2 ±30.2	11.8 ±5.6	4.8 ±2.3	12.6 ±3.0
(b) Adjacent "normal"	986.9 ±4.7	13.1 ±4.7	8.2 ±3.3	1.2 ±0.9	0.9 ±0.4	2.8 ±1.2
Grade II‡						
(a) Plaque	787.2 ±16.9	212.8 ±16.9	166.5 ±6.9	31.2 ±12.6	10.4 ±0.8	4.7 ±0.5
(b) Adjacent "normal"	984.0 ±2.4	16.0 ±2.4	8.6 ±2.7	1.7 ±0.2	1.8 ±0.7	3.9 ±1.6
Grade III*						
(a) Plaque	625.6 ±52.2	374.4 ±52.2	222.0 ±15.8	89.1 ±23.5	17.4 ±5.1	45.2 ±23.6
(b) Adjacent "normal"	881.4 ±36.2	118.5 ±36.2	82.7 ±24.4	14.4 ±5.6	8.8 ±6.5	11.5 ±1.0

* 5 patients.

‡ 6 patients.

sections of plaques of all grades, depositions of stainable lipids were often demonstrable on the degenerating intimal elastic membranes. Isolated plaque elastin always stained intensely with Oil Red O.

"Normal" intimae adjacent to plaques. In contrast to plaque elastin, the protein and lipid composition of elastin from the histologically normal intimae adjacent to grade I and grade II plaques was not appreciably different from that of intimal elastin from normal aortae (Table III). But elastin from relatively normal intima adjacent to grade III plaques did show a highly significant increase in its lipid component ($P < 0.01$) with the major lipid increase being cholesteryl ester (Table III). The increases in the lipid content of elastin from relatively normal intimae adjacent to grade III plaques were comparable to the lipid increases of elastin in grade I plaques. On microscopic examination, the split and fragmented elastic membranes of relatively normal intima adjacent to grade III plaques frequently stained very intensely with Oil Red O.

Mixtures of plaque intima and intima of normal aorta. Elastin extracted from mixtures of normal and plaque intima of equal weight had the following intermediate lipid and protein content per gram of elastin (average of three determinations for each mixture). Mixture with grade I plaque intima: 45.6 mg lipid and 954.4 mg pro-

tein; mixture with grade II plaque intima: 118.3 mg lipid and 881.7 mg protein; mixture with grade III plaque intima: 182.7 mg lipid and 817.3 mg protein (compare with lipid and protein content of normal and plaque elastin, Table III). As with normal and plaque elastin about 80% of the lipid from elastin of these mixed tissues was cholesterol, mainly in the form of cholesteryl ester.

(c) INCORPORATION OF LABELED CHOLESTEROL INTO INTIMAL ELASTIN

Table IV shows the incorporation of intravenously administered cholesterol-³H into elastin of plaques and adjacent normal intima, 2-4 months after injection. The total radioactivity of free and ester cholesterol was about six times higher in plaque elastin than in adjacent normal elastin. The specific activity of ester cholesterol was similar in normal and plaque elastin, whereas the specific activity of free cholesterol was strikingly reduced in plaque elastin. Radioautographs of smears of the extracted normal and plaque elastin also revealed the presence of radioactive cholesterol in the isolated elastin with the cholesterol radioactivity being much denser in plaque elastin than in normal elastin.

(d) TOTAL INTIMAL CHOLESTEROL CONTENT AS COMPARED WITH ELASTIN CHOLESTEROL CONTENT IN AORTIC INTIMAE

Fig. 1 compares the total intimal cholesterol content with the cholesterol content of the elastin contained in the same portion of the intima.

Intima of normal aortae. In intimae of normal aortae, about 7.9% (mean) of the total intimal cholesterol was contained in the intimal elastin. Histologically, no stainable lipids were demonstrable in normal intimae.

Plaque intimae. As the total intimal cholesterol rose in the plaque with increasing severity of atherosclerosis, there was a progressive and highly significant rise in the intimal cholesterol contained in plaque elastin ($P < 0.01$ for grade I, II, and III plaques). In grade I and grade II plaques about one-third of the total intimal cholesterol was contained in plaque elastin whereas in grade III plaques only about 20% of the intimal cholesterol was elastin cholesterol. Microscopic examination revealed an abundance of stainable lipids in the raised intima of plaques in addition to lipid depositions on degenerating intimal elastic membranes. In grade I and grade II plaques, the lipid was mainly contained in intimal cells but frequently also was found extracellularly in necrotic areas in the center of the plaque. In grade III plaques the stainable lipid was mainly extracellular and was contained, together with calcium deposits, in a thick fibrous capsule which was frequently ulcerated.

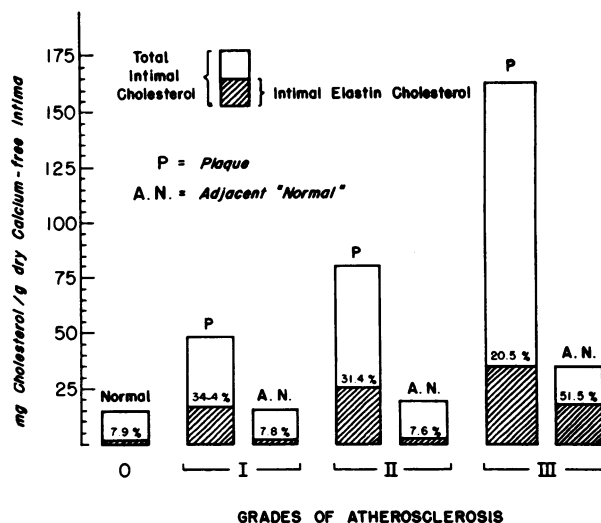


FIGURE 1 Total intimal cholesterol content as compared to elastin cholesterol content of normal and atherosclerotic human intimae in milligrams per gram dry calcium-free intima (calcium content subtracted from dry weight of tissue). The per cent values given refer to the percentage of the total intimal cholesterol which was associated with intimal elastin.

"Normal" intima adjacent to plaques. In the histologically normal intimae adjacent to grade I and grade II plaques, the total intimal cholesterol content as well as the elastin cholesterol content was comparable to the values obtained in intimae of normal aortae. However, in relatively normal intimae adjacent to grade III plaques, there was a highly significant rise in both total and elastin-bound intimal cholesterol ($P < 0.01$ for both) with about 52% of the total intimal cholesterol being contained in the intimal elastin. On microscopic examination, the intimae adjacent to grade III plaques were not raised. Although lipid was frequently demonstrable on the split and fragmented elastic membranes in these intimal areas, there was usually no stainable lipid present in intimal cells. Macroscopically, these intimae appeared to be normal.

(e) AMINO ACID COMPOSITION OF INTIMAL ELASTIN PROTEIN

Table V shows the mean amino acid composition of elastin protein of normal and atherosclerotic human aorta.

Plaque intima. As compared to elastin of intimae from normal aortae (grade O), the elastin protein of atherosclerotic plaques showed striking increases in the following polar amino acids: aspartic acid, threonine, serine, glutamic acid, lysine, histidine, and arginine. These increases in polar amino acids were statistically significant in elastin from plaques of all grades of atherosclerosis except for threonine and arginine in

TABLE IV

Incorporation of Intravenously Injected Cholesterol-³H into Elastin of "Normal" Intima and Adjacent Plaque Intima of Human Atherosclerotic Aortae

Patient	Free cholesterol		Ester cholesterol	
	"Normal" intima	Plaque intima	"Normal" intima	Plaque intima
	<i>cpm/g elastin</i>			
R. P.	532	2260	1072	6114
C. C.	380	1839	769	2595
M. V.	284	1680	1375	9632
Average	399	1926	1072	6113
	Elastin cholesterol specific activity			
	Free cholesterol		Ester cholesterol	
Patient	"Normal" intima	Plaque intima	"Normal" intima	Plaque intima
	<i>cpm/g elastin</i>			
R. P.	622	249	531	417
C. C.	636	265	535	421
M. V.	606	231	526	412
Average	621	248	530	416

TABLE V
Amino Acid Composition of Elastin Protein from Intimal Layers of Normal and Atherosclerotic Human Aortae

Amino acid	Grade 0 atherosclerosis*	Grade I atherosclerosis*		Grade II atherosclerosis:‡		Grade III atherosclerosis*	
	(Normal)	Plaque	Adjacent "normal"	Plaque	Adjacent "normal"	Plaque	Adjacent "normal"
	<i>mean§ residues/1000 residues ±SD</i>						
Cysteic acid	0.8 ±0.4	0.7 ±0.5	0.8 ±0.5	1.2 ±0.8	1.2 ±0.5	1.2 ±0.7	0.8 ±0.5
Hydroxyproline	12.5 ±1.5	12.2 ±4.0	11.2 ±2.2	12.5 ±1.6	12.9 ±3.6	15.2 ±0.6	12.8 ±1.9
Aspartic acid	5.6 ±1.6	14.6 ±4.7	5.2 ±1.3	21.5 ±2.7¶	7.8 ±1.6	22.7 ±3.9¶	13.6 ±3.9
Threonine	12.2 ±2.4	16.8 ±2.2	12.1 ±1.9	16.9 ±1.6	10.9 ±2.8	18.0 ±1.8¶	13.6 ±1.5
Serine	9.5 ±1.1	14.8 ±3.1	9.7 ±1.3	16.9 ±2.6¶	9.4 ±0.5	18.1 ±1.8¶	14.8 ±4.1
Glutamic acid	21.2 ±2.3	33.1 ±5.2¶	22.6 ±1.8	39.2 ±6.2¶	23.2 ±3.6	40.7 ±2.7¶	26.0 ±2.3¶
Proline	127.5 ±9.5	130.8 ±12.0	138.4 ±7.8	124.5 ±12.7	127.5 ±12.2	122.0 ±17.6	124.0 ±5.1
Glycine	297.6 ±21.9	261.1 ±21.9	271.2 ±17.0	260.2 ±8.5	270.6 ±15.0	272.7 ±10.4	278.0 ±11.7
Alanine	210.6 ±25.3	207.6 ±14.7	222.5 ±15.1	197.2 ±5.4	226.1 ±9.3	193.8 ±6.6	211.4 ±13.2
Valine	130.6 ±8.3	135.1 ±22.7	132.8 ±1.1	123.7 ±9.0	139.7 ±25.3	119.8 ±5.7	123.4 ±8.8
Isoleucine	27.3 ±2.0	25.2 ±1.7	26.2 ±2.2	26.9 ±1.7	26.7 ±2.3	28.1 ±3.4	27.3 ±1.5
Leucine	64.5 ±3.9	61.7 ±3.2	65.1 ±1.9	64.7 ±2.4	64.0 ±3.7	61.0 ±4.6	62.7 ±3.6
Tyrosine	23.6 ±2.1	24.8 ±4.2	24.3 ±1.3	25.0 ±3.4	24.8 ±3.6	23.3 ±1.3	27.9 ±2.9
Phenylalanine	26.2 ±1.5	25.3 ±3.3	26.5 ±1.4	27.1 ±1.7	25.8 ±3.5	25.1 ±2.6	27.2 ±1.9
‡ Isodesmosine	5.0 ±0.3	3.8 ±0.7	5.2 ±0.3	3.0 ±0.5¶	4.2 ±1.2	2.7 ±0.2¶	3.9 ±0.4¶
‡ Desmosine	7.3 ±0.7	3.8 ±0.6¶	6.9 ±0.5	3.4 ±0.2¶	6.4 ±0.4	3.3 ±0.2¶	3.9 ±0.6¶
‡ Lysinonorleucine	1.3 ±0.3	0.1 ±0.2¶	1.3 ±0.3	0.1 ±0.1¶	1.0 ±0.3	0¶	0.7 ±0.3
Lysine	6.9 ±1.2	12.2 ±2.3¶	6.8 ±0.7	14.9 ±2.2¶	8.0 ±2.2	12.4 ±1.2¶	12.3 ±2.8
Histidine	1.9 ±1.1	4.4 ±1.1	1.7 ±0.3	5.4 ±0.9¶	1.9 ±0.3	5.0 ±0.5¶	4.0 ±1.0
Arginine	7.9 ±0.9	11.5 ±3.6	8.1 ±1.0	14.7 ±1.1¶	7.7 ±1.1	14.2 ±1.2¶	12.3 ±2.7

* Five patients.

‡ Six patients.

§ Recovery of amino acids for each sample = 1000.0 ±1.0.

|| Significant change from values of normal aorta ($P < 0.05$).

¶ Highly significant change from values of normal aorta ($P < 0.01$).

grade I, and threonine in grade II plaques. On the other hand, the cross-linking amino acids: isodesmosine, desmosine, and lysinonorleucine, were reduced markedly in plaque elastin with the reduction in cross-links being statistically significant in all grades of atherosclerosis. In grade III plaques lysinonorleucine was absent. There was no statistically significant difference in the mean contents of all other amino acids between normal and plaque elastin.

"Normal" intimae adjacent to plaques. There was no statistically significant difference in the amino acid composition between intimal elastin from normal aortae and elastin from normal intima adjacent to grade I and grade II plaques. However, elastin from relatively normal intima adjacent to grade III plaques showed statistically significant increases in polar amino acids and highly significant reductions in cross-linking amino acids. These changes were comparable to those of elastin from grade I plaques.

2. Medial elastin

(a) PROTEIN AND LIPID COMPOSITION OF AORTIC MEDIAL ELASTIN

The protein and lipid composition of elastin extracted from the media of normal and atherosclerotic human aortae is listed in Table VI.

Media of normal aortae and of normal aortic areas adjacent to plaques. The protein and lipid composition of medial elastin from aortae without atherosclerosis (grade 0) was similar to that of intimal elastin from the same normal aortae (compare Table III). Likewise, medial elastin from normal appearing aortic areas adjacent to plaques of all grades was comparable in its composition to intimal or medial elastin of normal aortae. On microscopic examination, these media were structurally normal.

Media below plaques. Elastin from the media located below grade I plaques as well as elastin from the media below two of the grade II plaque samples also had the protein and lipid composition of normal arterial elastin. Histologically, these media also revealed no abnormalities. However, the elastin from the media of one of the grade II plaques as well as the medial elastin below all grade III plaques showed increases in lipid content and decreases in protein content which were similar to those of intimal elastin from grade I plaques (compare Table III). The changes in lipid and protein content of elastin from the media of one of the grade II plaques accounts for the higher mean lipid content and the lower mean protein content of medial elastin below grade II plaques. As with intimal elastin from plaques, the increase in the lipid content of medial elastin below grade II and grade III plaques was mainly due to large in-

TABLE VI

Protein and Lipid Composition of Elastin from Medial Layers of Normal and Atherosclerotic Human Aortae

Medial area of aorta	Protein	Lipid	Ester cholesterol	Free cholesterol	Phospho-lipid	Triglyceride
<i>mg/g elastin</i>						
Grade 0						
Below normal intima	988.1 ±2.0	11.9 ±2.0	6.3 ±0.6	1.3 ±0.6	1.1 ±0.4	3.2 ±0.9
Grade I						
(a) Below plaque intima	987.1 ±1.9	12.9 ±1.9	6.1 ±0.8	1.6 ±0.4	1.2 ±0.2	4.0 ±1.2
(b) Below adjacent "normal" intima	987.1 ±2.4	12.9 ±2.4	6.2 ±0.9	1.5 ±0.4	1.1 ±0.3	4.0 ±1.5
Grade II						
(a) Below plaque intima	949.6 ±70.1	53.4 ±70.1	36.5 ±50.4	6.5 ±9.6	3.0 ±2.8	7.4 ±7.2
(b) Below adjacent "normal" intima	987.0 ±0.8	13.0 ±0.8	7.4 ±0.9	1.3 ±0.7	1.2 ±0.2	3.1 ±0.8
Grade III						
(a) Below plaque intima*	866.2 ±25.8	133.8 ±25.8	96.3 ±20.7	16.9 ±1.0	6.3 ±0.5	14.3 ±4.5
(b) Below adjacent "normal" intima	986.9 ±5.6	13.1 ±5.6	7.4 ±3.9	1.4 ±0.7	1.1 ±0.6	2.7 ±1.5

Mean values of three patients for each grade of atherosclerosis.

*Media histologically altered. Protein and lipid contents of elastin comparable to that of grade I plaque intima.

creases in cholesterol, particularly cholesteryl ester. Microscopic examination revealed that fragmentation of the elastic membranes extended deep into these media, frequently with deposition of stainable lipids on the degenerating medial elastica.

(b) AMINO ACID COMPOSITION OF AORTIC MEDIAL ELASTIN

The amino acid composition of elastin from the media of normal aortae as well as of medial elastin from normal appearing aortic areas adjacent to plaques was comparable to the amino acid composition of elastin from normal intimae. Likewise, elastin from the media below grade I plaques as well as elastin from the media below two of the grade II plaque samples had the amino acid composition of normal arterial elastin protein. On the other hand, elastin from the media below one of the grade II plaques as well as medial elastin below all grade III plaques showed increases in polar amino acids and decreases in cross-linking amino acids similar to those of intimal elastin from grade I plaques.

3. Lung elastin

Protein and lipid composition of lung elastin. As seen in Table VII, the protein content of elastin from two

normal human lungs ranged between 96.5 and 97.5%, whereas the lipid content of the lung elastin ranged between 2.5 and 3.4%. As with normal intimal or medial elastin of aorta, about 70% of the lipid contained in normal lung elastin was cholesterol with the major portion of the cholesterol being cholesteryl ester.

The amino acid composition of elastin protein from normal lungs appeared to be comparable to the amino acid composition of normal arterial elastin from human aorta.

DISCUSSION

The extracted arterial elastin was essentially free of glycoproteins, acid mucopolysaccharides, and collagen

TABLE VII
Protein and Lipid Composition of Elastin from Two Normal Lungs

Patient	Protein	Lipid	Ester cholesterol	Free cholesterol	Phospho-lipid	Triglyceride
<i>mg/g elastin</i>						
F. L.	974.7	25.3	14.9	4.0	4.2	2.2
S. W.	965.7	34.3	22.9	4.7	4.6	2.5

as indicated by the absence of neuraminic acid, uronic acid, hydroxylysine, and of significant amounts of hexosamine and hydroxyproline. A portion of the arterial lipids resisted alkali hydrolysis and remained associated with the extracted elastin protein. This lipid could not be separated from the elastin by ultracentrifugation in salt solutions of density 1.063 or 1.210. Repeated treatment of the arterial elastin with petroleum ether resulted only in the removal of 20% of this lipid fraction. On the other hand, when lipoproteins of hyperlipidemic serum or homogenates of fatty liver were treated with hot alkali in the same manner, all esterified lipids were hydrolyzed. Since the lipid associated with arterial elastin was not hydrolyzed by the base treatment and since petroleum ether proved to be rather ineffective in its removal of lipid from the elastin protein, it appears that the lipid moiety of arterial elastin is firmly bound to the protein moiety. The binding of the elastin lipids to the elastin protein also was supported by the observation that the elastin lipids migrated with the peptide moieties of elastase hydrolyzed elastin on polyacrylamide gel electrophoresis. It is unlikely that the presence of lipids in the extracted elastin was due to a transfer of extraelastin lipids during the preparation of elastin from the tissues. When serum lipids were added to intimal tissue, before the preparation of elastin, there was no change in the lipid content of the elastin as compared with elastin prepared from tissues without the addition of lipids. Furthermore, when an equal amount of normal and plaque intima was mixed before alkali extraction, the lipid content of the elastin from the mixture was intermediate between that of normal and plaque elastin. If a transfer of tissue lipids had occurred during the preparation procedures, one would expect a much higher lipid content of the mixed elastin since large amounts of plaque lipids were present in these tissue mixtures. These findings indicate that arterial elastin is a protein-lipid complex. It is of interest that elastin extracted from lung also revealed the presence of lipid.

The amino acid composition of normal intimal elastin was comparable to that reported by other authors for alkali extracted normal arterial elastin (26, 27). The amino acid composition of elastin prepared from atherosclerotic plaques was altered. With increasing severity of atherosclerosis, plaque elastin showed a progressive increase in polar amino acids and a progressive reduction in cross-linking amino acids. It is noteworthy that the lipid content of elastin from atherosclerotic plaques also increased progressively with increasing severity of atherosclerosis.

In preliminary studies the mode of binding of lipids to arterial elastin was tested by incubating defatted elastin protein with low density lipoproteins (LDL)

and very low density lipoproteins (VLDL) (9). These studies suggest that one of the mechanisms involved in the deposition of lipids in arterial elastin may be an interaction of the elastin protein with serum (or arterial) LDL and VLDL resulting in a transfer of lipids, especially of ester cholesterol, to the elastin. This uptake of lipids by the elastin appeared not to be due to a transfer of the intact lipoproteins. Incubations of defatted elastin with serum LDL and VLDL labeled in the protein moiety with ^{125}I indicated that the transfer of lipids from the lipoproteins occurred without binding of the protein moiety by the elastin. The prerequisite for the greater transfer of cholesteryl ester to plaque elastin than to normal elastin appeared to be the altered amino acid composition of plaque elastin protein.

The high content of cholesterol in elastin from atherosclerotic plaques contributed substantially to the total cholesterol content of plaque intima. In grade I and grade II plaques more than 30% of the total intimal cholesterol was found associated with plaque elastin. Previous studies (28) indicated that about half of the total intimal cholesterol contained in plaques is extractable as lipoprotein. It is possible that the major portion of the remaining intimal cholesterol is not extractable as a lipoprotein because it is bound to the insoluble elastin protein. In grade III plaques only about 20% of the total intimal cholesterol was associated with plaque elastin although elastin from grade III plaques had the highest cholesterol content. This decrease in the percentage of elastin-bound intimal cholesterol in grade III plaques was most likely due to a marked reduction of elastin protein as well as to an increase in intimal cholesterol which was not bound to elastin.

The amino acid and lipid composition of elastin was found to be changed only in those intimal and medial areas that showed microscopic evidence of atherosclerosis. Elastin from histologically normal intima adjacent to plaques had an amino acid and lipid composition similar to that of intimal elastin from normal aortae. Likewise, in the histologically normal media below plaques as well as below adjacent normal intima, the composition of elastin was not altered. However, the composition of elastin was abnormal in the histologically altered intima adjacent to grade III plaques as well as in the histologically altered media below grade III and below some of the grade II plaques. The changes in the amino acid and lipid composition of elastin in these intimal and medial areas were comparable to those of intimal elastin from grade I plaques.

It is of interest that the intima adjacent to grade III plaques contained almost as much cholesterol as did grade I plaques. And yet, the intima adjacent to grade III plaques did not show evidence of gross athero-

sclerosis, i.e., no raising of the intima with yellowish discoloration as in grade I plaques. An explanation for this lack of gross atherosclerosis may be that in these grossly normal appearing intimae about one-half (51%) of the total intimal cholesterol was contained in the intimal elastin and, therefore, no present in the cellular layers of the intima. On microscopic examination, the grossly normal intima adjacent to grade III plaques—and also the media below these plaques—revealed marked splitting and fragmentation of the arterial elastica, frequently with deposition of stainable lipids on the fragmented elastica. Foam cells only occasionally were present in these intimae. Radioautographic studies (9, 29) have shown that in severe atherosclerosis the labeled cholesterol is not deposited in the center of the lesion but accumulates most densely around the plaque, especially on the fragmented elastic membranes of the intimae on both sides of the plaque and in the media below the lesion. Apparently, in severe atherosclerosis, the atherosclerotic process spreads beyond the plaque into the adjacent relatively normal intima and subintima, involving the elastin of the elastic membranes before lipid accumulations are detectable in cells. The findings suggest that alterations in the protein and lipid composition of arterial elastin may be one of the earliest changes in atherosclerosis.

The alteration in the composition of elastin protein from atherosclerotic lesions may be due to two possible mechanisms, both of which may operate together.

1. The shift in the amino acid composition of plaque elastin may be attributable to a second protein which is closely associated with true elastin and which is equally resistant to hydrolysis by hot alkali as suggested by other workers (30–32). This second protein does not appear to be a lipoprotein (for reasons indicated above) but may be a glycoprotein. Glycoproteins have been found to be closely associated with arterial elastic fibers (33, 34).

2. The change in the amino acid composition of plaque elastin also may be due to the formation of new but defective elastin, replacing degenerated elastic tissue. The reduction in the cross-linking amino acids and the increase in lysine content in elastin of atherosclerotic plaques may be the result of an inhibition in the formation of cross-links in newly synthesized elastin as it is known to occur in lathyrism (35, 36), copper deficiency (37–39), and after treatment with penicillamine (40). As shown by Partridge, Elsdon, and Thomas (41) for the desmosines and by Franzblau, Faris, and Papaioannou (42) for lysinonorleucine, these cross-linking amino acids are composed of lysine molecules. The severe fragmentation of arterial elastic membranes seen in lathyrism (43–46) and copper deficiency (47, 48)

are believed to be due to the inhibition in the formation of cross-linking amino acids of newly formed elastin (49). It is possible that the splitting and fragmentation of arterial elastica in atherosclerosis also is related, at least in part, to such a metabolic defect.

Whatever the cause for elastica degeneration in atherosclerotic lesions may be, splitting and fragmentation of arterial elastic membranes is one of the most consistent findings in atherosclerosis and appears to be one of the earliest manifestations of the disease in humans (1–3, 9, 50–54) as well as in animals (4–7, 55–58). The results of the present study indicate that biochemical alterations of arterial elastin occur during the early development of the plaque. Such localized alterations in the composition of elastin of intimo-medial elastic membranes may be the basis of the initial elastica degeneration leading to one of the earliest, if not the first, focal lipid accumulation in the arterial intima. The diseased elastin of atherosclerotic lesions also may play an important role in the progression of atherosclerosis.

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