Enhancement of Cholesterol and Cholesteryl Ester Accumulation in Re-endothelialized Aorta

Domenick J. Falcone, BA, David P. Hajjar, PhD,
and C. Richard Minick, MD

The purpose of the experiments reported here was to determine chemically the character and quantity of lipid in re-endothelialized and de-endothelialized areas of rabbit aortas. The aortas of 22 rabbits, Groups I and II, were de-endothelialized with a balloon catheter, and the rabbits were maintained on a lipid-poor diet for 4 weeks. Thirteen rabbits, Group II, were then fed an egg-supplemented diet for 6 weeks. Nine rabbits, Group I, were continued on the lipid-poor diet for an additional 6 weeks. Control rabbits with uninjured aortas were fed a lipid-poor diet for 10 weeks (Group III) or an egg-supplemented diet for 6 weeks (Group IV). Nonesterified cholesterol and fatty acids, cholesteryl esters, triacylglycerols, and squalene were quantitated in re-endothelialized and de-endothelialized aorta by thin-layer chromatography and fluorometric analysis. The results indicate 1) that there was approximately three times as much nonesterified cholesterol and cholesteryl ester in re-endothelialized aorta of Groups I and II as compared with adjacent de-endothelialized aorta and 2) that in re-endothelialized aorta of Group II the amount of total cholesterol correlated with serum cholesterol concentration in contrast to adjacent de-endothelialized aorta, with no correlation over a range of nearly 900 mg/100 ml. These studies indicate that the presence of endothelium favors accumulation of aortic cholesteryl esters. The results suggest that arterial lipid accumulation is not simply a result of passive filtration but may result from metabolic differences in the re-endothelialized neointima. (Am J Pathol 1980, 99:81-104)

ATHEROSCLEROSIS is characterized by lipid accumulation in thickened fibromuscular intima. It has been suggested that arterial damage, particularly endothelial injury, is the primary event in both intimal thickening and lipid accumulation.[1-20] Central to this endothelial injury hypothesis are the widely accepted roles of endothelium as a thromboresistant surface and as a regulatory barrier for the transfer of macromolecules into the vessel wall.[21-28] It is posited that the absence of endothelium exposes the vessel wall to mitogenic factors, including those derived from platelets and low-density lipoproteins, resulting in replication of medial smooth muscle cells and formation of a neointima.[29-32] It is further postulated that the persistent absence of endothelium leads to continued in-

From the Department of Pathology, The New York Hospital–Cornell Medical Center, New York, New York.

Supported by Research Grants HL-01803, HL-18828, and HL-05851 from the National Heart, Lung and Blood Institute of the National Institutes of Health and a grant from The Cross Foundation. This report is a portion of the research which will be submitted as a thesis by the senior author to Cornell Graduate School of Medical Sciences in partial fulfillment of the requirements for a PhD in Biological Structure and Cell Biology.

Accepted for publication November 7, 1979.

Address reprint requests to C. Richard Minick, MD, Department of Pathology, The New York Hospital–Cornell Medical Center, 1300 York Avenue, New York, NY 10021.

0002-9440/80/0407-0081$01.00

crease in the influx of lipoprotein, causing lipid accumulation and atherosclerosis in this neointima.

In previous experiments we tested the hypothesis[16,17,20,33] that the absence of endothelium would lead to intimal thickening and lipid accumulation. The unexpected results of those experiments provided morphometric evidence that lipid accumulation and intimal thickness were significantly enhanced in aortas covered by regenerated endothelium as compared with adjacent areas lacking endothelium. Thus, results of those experiments were not consistent with the above hypothesis, since they indicated that reendothelialized intima, and not de-endothelialized intima, was especially prone to lipid accumulation and atherosclerosis. In experiments reported here we 1) characterized the aortic lipid chemically and compared the character and quantity of lipid in de-endothelialized, re-endothelialized, and uninjured aorta; 2) tested the relationship between lipid accumulation in de-endothelialized and re-endothelialized areas and serum cholesterol concentration; and 3) compared the quantity of lipid assessed morphometrically with that determined by chemical analysis.

Materials and Methods

Removal of Endothelium

Endothelium was removed by a balloon catheter from the aortas of 22 young New Zealand white female rabbits weighing from 2300 to 3300 g by the method of Baumgartner et al.[34] Briefly, a 4F thin-walled Fogarty embolectomy catheter (Edwards Laboratory, Santa Anna, Calif) was inserted into the right femoral artery of anesthetized rabbits and pushed into the proximal aorta. The balloon was inflated to a pressure of 450–500 mm Hg, and the inflated catheter was pulled through the aorta three times.

Experimental Groups

Forty rabbits were initially fed commercial rabbit ration relatively poor in lipid (Purina Rabbit Chow: total lipid approximately 2.0% by weight). Four weeks after de-endothelialization, 22 rabbits were randomly separated into Groups I and II (Text-figure 1). Nine rabbits (Group I) were continued on the lipid-poor diet for an additional 6 weeks until they were killed. Thirteen rabbits (Group II) were fed 100 g of an egg-supplemented diet containing 1 egg in 60 g of the commercial rabbit ration 5 days each week for 6 weeks and then killed. On weekends, Group II rabbits were fed the lipid-poor diet *ad libitum*. Two groups of unballooned rabbits served as controls. One group of 9 unballooned rabbits (Group III) was fed a lipid-poor diet for 10 weeks and then killed. An additional group of unballooned rabits (Group IV) was fed the lipid-poor diet for 4 weeks and then fed an egg-supplemented diet for an additional 6 weeks prior to killing.

Serum Cholesterol Concentrations

Rabbits of all experimental groups were bled prior to initiation of the experiment. The rabbits receiving the egg-supplemented diet were bled 3 weeks after the diet was initiated and on the day they were killed. The rabbits on the lipid-poor diet were bled at comparable intervals. In all instances, the rabbits were bled after a 12–18-hour fast. Serums

were stored at −20 C until assayed for total cholesterol by an autoanalyzer modification of the method of Levine and Zak.[35]

Autopsy Procedures

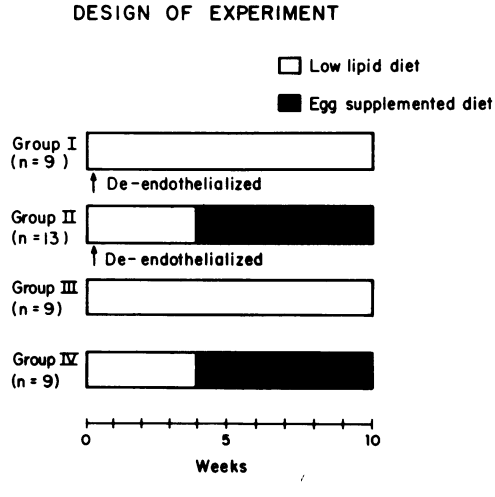
The rabbits were killed by an intravenous overdose of pentobarbital. In order to distinguish re-endothelialized and de-endothelialized areas of the aorta, 1 hour prior to killing 3 cc/kg of a 0.5% solution of the protein-binding azo dye Evans blue (w/v) in physiologic saline was injected into the rabbits. Areas of the aortic wall devoid of endothelium stained blue, while areas of aorta covered by endothelium restricted or excluded passage of the dye and remained unstained and appeared as nonblue islands or bands surrounded by de-endothelialized blue-staining areas (see Results).[20]

After the animals were killed, the aortas were dissected from adjacent tissues and maintained in cold saline at 4 C until frozen. Periadventitial debris and adherent blood was removed, and the adventitia was stripped. The artery was opened by a midventral incision extending from the arch to the iliac bifurcation. Representative tissue samples of the thoracic and abdominal segments were dissected and fixed in 10% neutral-buffered formalin. Tissue samples were divided into 2 portions each comprising blue areas and white and gray zones of nonblue areas. One portion of the tissue sample was embedded in paraffin and routinely processed for hematoxylin and eosin staining. The adjacent portion of each tissue sample was embedded in 7.5% gelatin, frozen in liquid nitrogen, and 3–5 μ frozen sections were prepared.[36] Frozen sections were stained with oil red O for lipid and counterstained with hematoxylin.

After the removal of tissue samples for microscopy, remaining portions of thoracic and abdominal segments were dissected into white and gray zones of nonblue areas and blue areas with the aid of a large field magnifier (Arthur H. Thomas Co, Philadelphia, Pa). Tissue from each zone and area of the thoracic and abdominal segments of a given rabbit was pooled, blotted to remove excess fluid, weighed, and placed in glass vials with Teflon- or foil-lined caps. Before storage at −70 C, the vials were flushed with nitrogen to prevent auto-oxidation of lipid.[37]

Aortic Lipid Analysis

All glassware was cleaned with chromic acid cleaning solution and thoroughly rinsed with distilled water. Only highly purified organic solvents were used (Mallinckrodt, St.



TEXT-FIGURE 1—Aortas of rabbits in Groups I and II were de-endothelialized with a balloon catheter at Day 0. Initially, both groups were fed lipid-poor diet. Group I was continued on this diet for 10 weeks. Four weeks after de-endothelialization, Group II was fed an egg-supplemented diet for 6 weeks. Rabbits with uninjured aortas in Groups III and IV were initially fed the lipid-poor diet. Group III was continued on this diet for 10 weeks. After 4 weeks Group IV was fed the egg-supplemented diet for 6 weeks.

Louis, Mo, Nanograde quality). Frozen samples of aortic tissue, weighing between 25 and 50 mg, were thawed at room temperature and minced at 4 C. Minced tissue was homogenized in a glass homogenizer/grinder in 2 ml of normal saline at 4 C. One milliliter of the homogenate was rehomogenized in 10 ml of cholorform–methanol (2:1, v/v) and the lipid was extracted according to Folch et al.[38] The lipid extract was then completely hydrogenated with the use of platinum oxide as a catalyst.[39] The extent of hydrogenation was confirmed by gas–liquid chromotography. Hydrogenation was necessary to avoid interference in fluorometric quantitation of lipids by unsaturated bonds.[39].

The hydrogenated extract and a standard containing a mixture of nonesterified cholesterol, cholesteryl esters, triacylglycerols, fatty acids, and squalene, at a concentration of 1 mg/ml, were then fractionated into the 5 lipid classes by thin-layer chromatography (TLC) with the use of a 2-solvent system as described by Hojnacki and Smith.[40] Briefly, the extract was concentrated under nitrogen and spotted on Whatman K6 silica gel analytical TLC plates (20 × 20 cm; 250 μ) activated as previously described.[39,40] Standards and samples were spotted 2 cm from the bottom of the TLC plate. The plate was removed from the TLC chamber and allowed to dry after Solvent 1 (diethyl ether/petroleum ether/glacial acetic acid [100/97/3, v/v/v]) had migrated to 9 cm above the bottom of the plate. The TLC plate was next placed in Solvent 2 (petroleum ether/diethyl ether [97/3, v/v]) in a second chamber, and the solvent was allowed to migrate 18 cm above the bottom of the plate.

We made the separated lipid classes visible by spraying the plate with 0.001% rhodamine 6G in 0.25 M potassium diphosphate and lipid spots quantitated by scanning fluorometry and compared with standards of known concentration. Peak areas from the fluorometric analyses for various concentration of samples and standards were determined by triangulation and expressed in units of square millimeters. The quantities of various lipid classes were expressed in micrograms lipid per milligram wet tissue weight. Quantity of lipid was not expressed in relation to the content of DNA since the result would have been misleading. We have shown in these experiments and previous experiments that the cellularity of aortic intima in de-endothelialized and re-endothelialized areas is significantly different (see Results and Table 1).

In order to test the precision of quantitation by scanning fluorometry, 10 microliters of the standard lipid mixture (1 mg/ml for each class) was applied at 2-cm intervals along the origin of a single TLC plate. The plate was processed as usual. Peak areas for 5 separate lipid classes were determined. The standard error was in all cases less than 4% of the mean.

Morphometric Analysis

Aortic cellularity was analyzed morphometrically in white zones of nonblue areas and adjacent blue areas of Group II animals. Cellularity was expressed as the number of cells per 1000 sq μ of intima. The number of nuclei were counted within a randomized intimal area of approximately 14,000 sq μ.

In order to test for a relationship between fatty change in the aortic wall and chemically defined lipid, aortic fatty change was assessed morphometrically in white zones of

Table 1—Intimal Cell Density of Blue and Nonblue Areas*

	Nonblue	Blue	Significance
Thoracic segment	2.76 ± .36	4.46 ± .56	$.005 < P < .01$
Abdominal segment	2.39 ± .28†	3.51 ± .28	$.002 < P < .005$

* White zones of nonblue areas.

† Cell density expressed as nuclei/1000 sq μ ± SEM.

nonblue areas of Group II rabbits. This was accomplished by employing, simultaneously, an ocular reticule with a net pattern and a linear micrometer. Micrometers were positioned so that lines were congruous. Boundaries of the area to be scanned were delineated with the use of a linear micrometer, and the degree of lipid change within the area was analyzed with the ocular reticule. All intersects were counted with the use of a 40× objective and 12.5× oculars (each square of the net micrometer was 144 sq μ). Each intersect falling on globular or granular lipid was counted as fatty change. Approximately 100 intersects were counted in the intima and underlying media of each white zone analyzed. One white zone was so analyzed in the abdominal and thoracic segment of each of 13 rabbits in Group II. Fatty change was then expressed as the percentage of intersects falling on lipid.

Statistical Analysis

Mean baseline serum cholesterol concentrations for Groups I and III and Groups II and IV were compared with the use of the Mann–Whitney nonparametric test, since variance ratio testing revealed significant differences between group variances.

During the experimental period, overall mean serum cholesterol concentrations for Groups I and III were compared using a *t* test. This *t* test could not be employed to test between the mean serum cholesterol concentration of Groups II and IV, since variance ratio testing demonstrated inequality of variances. Therefore, the Mann–Whitney nonparametric test was utilized.

In the thoracic and abdominal segments of each aorta, the quantity of each lipid class in the white and gray zones of nonblue areas and the blue areas of Groups I and II rabbits was compared with the use of a 2 × 2 × 3 (group × segment × zone) factorial analysis with repeated measures or observations on segments and zones. Since multifactorial analysis of variance requires equal replication, four animals were randomly excluded from Group II with the use of a random number table. After a significant overall analysis of variance, individual comparisons were effected by independent or paired *t* tests as indicated. Since this results in a series of *t* tests, our Type I error rate within the overall experiment is greater than 5%, but in each individual comparison the Type I error rate is 5%.[41–43] Since it was our prior intention to compare lipid quantities in the white and blue areas, the use of the *t* test is indicated.[42–44]

The quantity of each particular lipid class in the re-endothelialized white zones of nonblue areas of Group I and Group II was compared with the unballooned Groups III and IV with the use of a 4 × 2 (group × segment) factorial analysis with repeated measures on segments. In some cases the requirements of parametric analysis of variance were violated, therefore nonparametric Kruskal–Wallis analysis of variance was used. Since the Kruskal–Wallis analysis of variance is a single factor test, we were required to test for differences among Groups I–IV in thoracic and abdominal segments independently. In all cases, results of the parametric and nonparametric tests agreed where applicable. Only squalene demonstrated disparity. Nonparametric testing revealed differences between groups, while parametric testing did not. When the difference between variance of samples for squalene was reduced by log transformation of the data ($x^1 = \log [x + 1]$), the results of the parametric and nonparametric testing agreed. When a significant overall analysis of variance warranted further analysis, individual posteriori comparisons (comparisons made without prior intent) were effected by Duncan's multiple range test. Mean percentages of total cholesterol represented by nonesterified cholesterol and cholesteryl ester and the mean ratio of nonesterified and esterified cholesterol in the white zones of nonblue areas of injured aortas, Groups I and II, and uninjured aortas of Groups III and IV were also compared with the use of a 4 × 2 (group × segment) factorial analysis with repeated measures on segments. Following a significant analysis of variance, pair-wise comparisons were effected by Duncan's multiple range test.

We sought to define a relationship, if any, between total aortic cholesterol of blue and

nonblue areas of Group II rabbits and mean serum cholesterol concentration. Scatter diagrams suggested a linear relationship between the two variables. For each area and segment, simple linear regression equations were generated for 1) total aortic cholesterol, compared with mean serum cholesterol; 2) aortic cholesteryl ester, compared with mean serum cholesterol; and 3) aortic nonesterified cholesterol, compared with mean serum cholesterol concentrations. The significance of regression was tested by analysis of variance for each case. Testing between slopes and elevations (Y intercepts) was performed with the use of t tests.

Results

Serum Cholesterol Concentrations

Baseline serum cholesterol concentrations of Groups I–IV were as follows: Group I, 99.67 ± 17.61 mg/100 ml (mean ± SE); Group II, 86.50 ± 8.99 mg/100 ml; Group III, 49.00 ± 4.14 mg/100 ml; and Group IV, 44.56 ± 4.06 mg/100 ml. The mean baseline serum cholesterol of Groups I and III and Groups II and IV were significantly different ($P < .001$). During the experimental period, mean serum cholesterol concentrations of rabbits with de-endothelialized aortas that were fed the lipid-poor diet (Group I) ranged between 51 and 129 mg/100 ml, with an overall mean of 78.0 ± 3.2 mg/100 ml (mean ± SE). The mean serum cholesterol concentration of rabbits with uninjured aortas fed a lipid-poor diet (Group III) ranged between 28 and 66 mg/100 ml, with an overall mean of 56.0 ± 4.0 mg/100 ml. The overall mean serum cholesterol concentrations of Group I as compared with Group III was significantly different ($P < .05$). Diet-induced hypercholesterolemia was observed both in rabbits with de-endothelialized aortas, Group II, and in rabbits with uninjured aortas, Group IV, that were fed the egg-supplemented diet. Mean serum cholesterol concentrations of rabbits in Group II ranged between 127 and 1010 mg/100 ml, with an overall mean of 437.0 ± 66.5 mg/100 ml. Mean serum cholesterol concentrations of rabbits in Group IV ranged between 137 and 314 mg/100 ml, with an overall mean of 227.8 ± 22.3 mg/100 ml. Overall mean serum cholesterol concentrations for Groups II and IV were significantly different ($P < .05$). Thus, in both normocholesterolemic and hypercholesterolemic rabbits, balloon de-endothelialization of the aorta and subsequent reparative phenomena are associated with a significant increase in mean serum cholesterol concentration, as compared with normocholesterolemic and hypercholesterolemic unballooned control animals. These differences, although significant, may not be meaningful, since baseline serum cholesterol concentrations were also different.

Macroscopic Observations

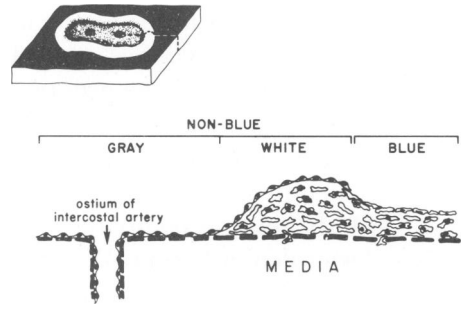
The pattern of re-endothelialization in the aorta after endothelial denudation has been previously described in detail by Minick et al.[20] The re-

sults of these experiments were similar. Briefly, following de-endothelialization there is fibromuscular intimal thickening, and endothelial cells regenerate simultaneously from the ostiums of branch vessels. When the animals of Groups I and II were given injections of Evans blue, the protein-binding dye stains the aortic wall lacking an endothelium blue. Areas of aortic wall covered by regenerated endothelium exclude the passage of the dye–protein complex, remain unstained, and appear nonblue. In these experiments, the aortic surface is characterized by blue, de-endothelialized areas that surround nonblue endothelial islands or bands. Each nonblue island or band surrounds the ostium of an aortic branch and has a complex structure composed of both a central gray zone without a thickened intima and an elevated peripheral white zone with thickened intima, both of which are covered by regenerated endothelium. White zones and central gray zones of nonblue areas will henceforth be referred to as white zones and gray zones.

When untreated animals, Group III, or animals fed an egg-supplemented diet, Group IV, were given injections of Evans blue, there was usually only little staining of the aorta in an inconsistent pattern. There were no grossly visible fatty lesions in the aortas of the rabbits of Groups III and IV.

Microscopic Observations

Microscopic features of the aortic wall following endothelial denudation and repair have been described previously (Text-figure 2).[20] The results of these experiments were similar. Briefly, in rabbits of Groups I and II, blue areas and white zones were characterized by fibromuscular intimal thickening and a normal underlying media. The intima in the white zone was thicker and covered by endothelium, compared with the adjacent intima of blue area, which lacked an endothelial cover. Gray zones were also covered by endothelium but lacked appreciable intimal thickening.



TEXT-FIGURE 2—Schematic representation of a cross-section of a nonblue re-endothelialized island and surrounding de-endothelialized blue area in thoracic segment of aorta. Each re-endothelialized nonblue island consists of a central gray zone exhibiting little intimal thickening and a more peripheral intensely white zone with thickened intima. The nonblue island is surrounded by a blue area characterized by thickened intima without an endothelial lining.

In the rabbits of Group II, fed egg-supplemented diets, pools of extracellular lipid and vacuolated, lipid-containing foam cells were prominent in the white zones of many animals. Similar fatty change was seen in adjacent blue areas, but it was less extensive. Oil-red-0–staining material was found in the intima and superficial media of blue areas and white zones in Group II rabbits. Although no attempt was made to quantitate and compare the degree of staining in blue and nonblue areas, the staining appeared to be most intense in the nonblue areas. In animals of Group I no evidence of fatty change was seen in sections stained with hematoxylin and eosin. No oil-red-0–staining material was seen in the aortic intima and media in these animals.

Morphometric Analysis

When the intimal cell density of Group II rabbits was assessed as nuclei per unit area, the blue areas were significantly more cellular than adjacent white zones in both thoracic and abdominal aortas (Table 1).

We could not demonstrate a significant relationship between fatty change assessed morphometrically in white zones of Group II rabbit aortas and the quantity of the major neutral lipid classes (see below). Neither aortic cholesteryl ester, triacylglycerol, nor the sum of these two lipid classes significantly correlated with fatty change estimated morphometrically. This was the case even when fatty change was assessed either in the intima and underlying media together or in the intima alone.

Tissue Lipid Analysis

By using multifactorial analysis of variance, the quantity of lipid in each class that accumulated in the white or re-endothelialized areas of thoracic and abdominal segments of injured aortas of Groups I and II was compared with that in the thoracic and abdominal segments of uninjured aortas of control Groups III and IV (see Tables 2 and 3). In this manner, we were able to assess the relative contribution of either diet or endothelial denudation and arterial repair, including endothelial regeneration on accumulation of these lipid classes. When the entire aorta was considered, all lipid classes measured including nonesterified cholesterol, cholesteryl esters, triacylglycerols, nonesterified fatty acids, and squalene differed significantly between groups.

Endothelial denudation and arterial repair (Group I) led to increased cholesteryl ester, nonesterified fatty acids, and squalene as compared with untreated controls (Group III) and increased cholesteryl ester and nonesterified fatty acids as compared with control Group IV. Diet-induced hypercholesterolemia (Group IV) led to increased nonesterified cholesterol,

Table 2—Lipid Content of Aorta

Lipid class	Group I—injured aorta			Group III—uninjured aorta	Significant differences ($P < 0.05$)
	Nonblue area				
	Gray zone	White zone	Blue area		
Thoracic segment					
Nonesterified cholesterol	.75 ± .18*	.80 ± .23	.69 ± .21	.33 ± .04	NS
Cholesteryl esters	.36 ± .11	.73 ± .23	.26 ± .08	.14 ± .06	white > blue white > uninjured
Nonesterified fatty acids	.81 ± .19	.81 ± .18	.78 ± .20	.36 ± .07	white > uninjured
Triacylglycerols	1.34 ± .65	1.39 ± .46	1.15 ± .40	1.21 ± .16	NS
Squalene	2.37 ± .79	2.02 ± .36	1.72 ± .66	.74 ± .19	white > uninjured
Abdominal segment					
Nonesterified cholesterol	.58 ± .25	.67 ± .16	.57 ± .30	.60 ± .15	NS
Cholesteryl esters	.60 ± .19	1.30 ± .32	.51 ± .19	.11 ± .05	white > blue white > uninjured
Nonesterified fatty acids	1.01 ± .44	.68 ± .17	1.02 ± .34	.45 ± .12	NS
Triacylglycerols	1.49 ± .49	2.00 ± .35	2.45 ± .49	2.06 ± .45	NS
Squalene	1.88 ± .63	1.71 ± .81	2.12 ± .79	.34 ± .08	white > uninjured

* Mean ± SE; μg lipid/mg wet weight.
NS = not significant.

Table 3—Lipid Content of Aorta

Lipid class	Group II—injured aorta			Group IV—uninjured aorta	Significant differences ($P < 0.05$)
	Nonblue area				
	Gray zone	White zone	Blue area		
Thoracic segment					
Nonesterified cholesterol	2.01 ± 1.01 *	2.60 ± .57	1.23 ± .30	.98 ± .16	white > blue white > uninjured
Cholesteryl esters	.97 ± .23	4.65 ± 1.28	1.63 ± .64	.33 ± .07	white > blue; gray white > uninjured
Nonesterified fatty acids	1.47 ± .67	1.74 ± .60	.74 ± .22	.33 ± .11	white > uninjured
Triacylglycerols	11.79 ± 6.86	9.45 ± 3.79	4.03 ± 1.64	2.81 ± .49	white > uninjured
Squalene	2.91 ± 1.58	2.05 ± .88	.77 ± .20	2.04 ± .43	NS
Abdominal segment					
Nonesterified cholesterol	2.13 ± .63	3.47 ± .68	1.41 ± .35	1.42 ± .22	white > blue white > uninjured
Cholesteryl esters	3.27 ± 1.02	6.21 ± 1.11	2.87 ± .61	.46 ± .10	white > blue; gray white > uninjured
Nonesterified fatty acids	.74 ± .16	.79 ± .12	.72 ± .26	.73 ± .19	NS
Triacylglycerols	11.36 ± 5.67	6.16 ± 2.25	8.04 ± 3.19	5.43 ± .65	NS
Squalene	1.53 ± .38	1.27 ± .34	1.60 ± .43	1.82 ± .18	NS

* Mean ± SE; μg lipid/mg wet weight.
NS = not significant.

triacylglycerol, and squalene in comparison with Group III and increased nonesterified cholesterol and triacylglycerol as compared with Group I. Arterial injury and repair, together with diet-induced hypercholesterolemia, Group II, led to an increase in all lipid classes as compared with Group III.

Aortas of rabbits of Group II had increased nonesterified cholesterol, triacylglycerols, cholesteryl esters, and nonesterified fatty acids as compared with either Group I or Group IV. In summary, it appears that feeding egg-supplemented diets particularly enhances aortic accumulation of triacylglycerols and nonesterified cholesterol. Endothelial denudation and arterial repair is associated with enhanced accumulation of nonesterified fatty acids and cholesteryl esters.

This interpretation is supported by the relative amount of cholesterol and cholesteryl ester in aortas of different experimental groups. As shown in Table 4, the relative proportion of total cholesterol represented by nonesterified cholesterol and cholesteryl ester was also different in animals with balloon-catheter–induced arterial injury as compared with uninjured normocholesterolemic and hypercholesterolemic rabbits. The percentages of total cholesterol represented by nonesterified cholesterol and cholesteryl ester were 75% and 25%, respectively, in the thoracic segment of uninjured aorta in normocholesterolemic Group III rabbits and 74% and 26% in hypercholesterolemic rabbits of Group IV. In the abdominal segment, relative percentages of cholesterol and cholesteryl ester in both Groups III and IV were similar to those in the thoracic segment. Following endothelial denudation and arterial repair, total aortic cholesterol increased and the relative proportion of nonesterified cholesterol and cholesteryl ester changed markedly. In the thoracic segment, the percentages of total cholesterol represented by free cholesterol and cholesteryl ester

Table 4—Ratio of Nonesterified and Esterified Cholesterol*

Aortic segment	Experimental groups			
	Group I	Group II	Group III	Group IV
Thoracic	1.64 ± .45† (51%/49%)‡	.99 ± .35 (42%/58%)	7.91 ± 3.44 (75%/25%)	3.91 ± .78 (74%/26%)
Abdominal	.60 ± .12 (36%/64%)	1.35 ± .93 (36%/64%)	14.63 ± 3.76 (85%/15%)	4.85 ± 1.23 (73%/27%)

* Overall mean ratio in white zones calculated from individual ratios of rabbits in Groups I–IV.

† Mean ± SE.

‡ Cholesterol/cholesteryl ester.

Comparison of ratios in thoracic and abdominal segments:
[Group III > Group IV] > Group I = Group II ($P < 0.05$).

was 51% and 49%, respectively, in injured aortas of normocholesterolemic Group I rabbits and 42% and 58% in hypercholesterolemic Group II rabbits. In the abdominal segment of aortas of Groups I and II the relative proportions of cholesterol and cholesteryl ester were similar to those in the thoracic segment. Thus, following diet-induced hypercholesterolemia, there was a marked increased of aortic cholesterol that primarily consisted of an increase of nonesterified cholesterol. However, the relative percentages of nonesterified cholesterol and cholesteryl ester did not change from the untreated controls. In contrast, following aortic injury of both normocholesterolemic and hypercholesterolemic rabbits, the increase was in large part cholesteryl ester, even though the total amount of cholesterol was greatly increased in hypercholesterolemic Group II animals.

The quantity of nonesterified cholesterol and fatty acids, cholesteryl ester, triacylglycerols, and squalene within zones and areas of thoracic and abdominal segments of aortas in Group I rabbits was compared with the quantity of these lipid classes in Group II rabbits (Table 5). When thoracic and abdominal segments were considered together, the amount of aortic cholesterol, cholesteryl ester, and triacylglycerol differed significantly between Group I and II rabbits. Further analysis of thoracic and abdominal segments demonstrated that nonesterified cholesterol in the thoracic white, abdominal white, gray, and blue areas of Group II rabbits was significantly greater than the nonesterified cholesterol in corresponding areas of Group I rabbits. In addition, quantities of cholesteryl ester, triacylglycerols, and nonesterified fatty acids of Group II were significantly greater in all zones and areas as compared with analogous zones and areas of Group I.

Within Groups I and II, significant zonal and area differences were detected for aortic cholesterol and cholesteryl ester (Tables 2 and 3). There were no significant differences between areas and zones for nonesterified fatty acids, triacylglycerols, and squalene. In thoracic segments of Group I rabbits, there was consistently more cholesteryl ester in the re-endothelialized white zones of thoracic segments as compared with adjacent deendothelialized blue areas. In the abdominal segment, the cholesteryl ester content of the white zones was greater than either adjacent gray zones or blue areas. There were no significant differences in nonesterified cholesterol in the corresponding zones and areas of Group I animals. In Group II animals, nonesterified cholesterol content of white zones in thoracic and abdominal segments was significantly greater than in the adjacent blue areas. Cholesteryl ester content in the white zone of both thoracic and abdominal segments was greater than in gray zones or blue areas

Table 5—Comparisons of Aortic Lipid Content of Groups I and II

	Nonblue areas								
	Gray zone			White zone			Blue areas		
	I	II	$P < .05$	I	II	$P < .05$	I	II	$P < .05$
Thoracic segment									
Nonesterified cholesterol	.75 ± .18*	2.01 ± 1.01	NS	.80 ± .23	2.60 ± .57	II > 1	.69 ± .21	1.23 ± .30	NS
Cholesteryl esters	.36 ± .11	.97 ± .23	II > 1	.73 ± .23	4.65 ± 1.28	II > 1	.26 ± .08	1.63 ± .64	II > 1
Nonesterified fatty acids	.81 ± .19	1.47 ± .67	NS	.81 ± .18	1.74 ± .60	NS	.78 ± .20	.74 ± .22	NS
Triacylglycerols	1.34 ± .65	11.79 ± 6.86	II > 1	1.39 ± .46	9.45 ± 3.79	II > 1	1.15 ± .40	4.03 ± 1.64	II > 1
Squalene	2.37 ± .79	2.91 ± 1.58	NS	2.02 ± .36	2.05 ± .88	NS	1.72 ± .60	.77 ± .20	NS
Abdominal segment									
Nonesterified cholesterol	.58 ± .25	2.13 ± .65	II > 1	.67 ± .16	3.47 ± .68	II > 1	.57 ± .30	1.41 ± .35	II > 1
Cholesteryl esters	.60 ± .19	3.27 ± 1.02	II > 1	1.30 ± .32	6.21 ± 1.11	II > 1	.51 ± .19	2.87 ± .61	II > 1
Nonesterified fatty acids	1.01 ± .44	.74 ± .16	NS	.68 ± .17	.79 ± .12	NS	1.02 ± .34	.72 ± .26	NS
Triacylglycerols	1.49 ± .49	11.36 ± 5.67	II > 1	2.00 ± .35	6.16 ± 2.25	II > 1	2.45 ± .49	8.04 ± 3.19	II > 1
Squalene	1.88 ± .63	1.53 ± .38	NS	1.71 ± .81	1.27 ± .34	NS	2.12 ± .79	1.60 ± .43	NS

* Mean ± SE; μg lipid/mg wet weight.
NS = not significant.

in Group II. In rabbits of both Groups I and II the quantity of cholesteryl ester in the white zones and in blue areas of abdominal segments was appreciably greater than in the corresponding zones and areas of the thoracic segments. However, these differences were not significant. In summary, de-endothelialization of the aorta and subsequent reparative phenomena in normocholesterolemic rabbits results in the preferential accumulation of cholesteryl ester but not nonesterified cholesterol within white zones of aortic wall covered by regenerated endothelium as compared with adjacent de-endothelialized aorta. De-endothelialization of the aorta in rabbits subsequently made hypercholesterolemic results in preferential accumulation of both nonesterified cholesterol and cholesteryl ester in re-endothelialized aorta as compared with adjacent de-endothelialized aortic wall.

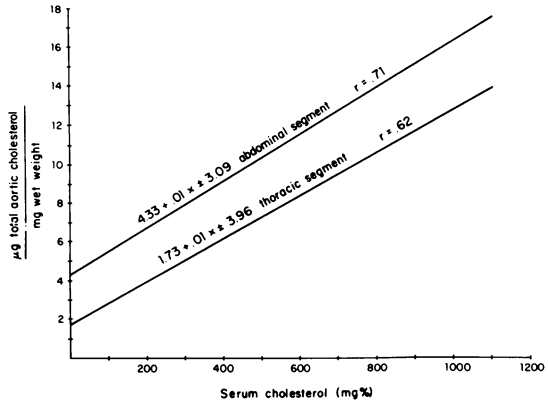
As indicated in Table 6, mean serum cholesterol concentration correlated significantly with total aortic cholesterol in white zones in both abdominal and thoracic segments of hypercholesterolemic Group II rabbits. Thus, the correlation between total cholesterol in the thoracic aorta was $r = 0.62$, $0.01 < P < 0.025$, and in the abdominal aorta, $r = 0.71$ $P < 0.005$. The coefficient of determination (r^2) for the thoracic and abdominal segments was 0.38 and 0.50, indicating that serum cholesterol concentration accounted for 38% and 50%, respectively, of the variation in total aortic cholesterol. The regression lines for the relationship between serum cholesterol concentration and total aortic cholesterol in thoracic and abdominal aortic segments are presented in Text-figure 3. The slopes of both lines are equal, and although the regression line for cholesterol in the abdominal aorta is above that for the thoracic aorta, indicating an increased propensity of the abdominal aorta to accumulate lipid at a given serum cholesterol concentration, we could not demonstrate a significant difference between elevations.

When total cholesterol was considered as nonesterified and esterified

Table 6—Correlation of Aortic Cholesterol With Serum Cholesterol Concentration in Group II Rabbits

	Aortic segment*	
	Thoracic	Abdominal
Total cholesterol	.62 $.01 < P < .025$	.71 $P < .005$
Nonesterified	.68 $.005 < P < .01$	.22 NS
Cholesteryl ester	.52 NS	.75 $P < .005$

* White zones of nonblue areas.



TEXT-FIGURE 3—In nonblue areas of both the thoracic and abdominal segments there was a significant positive correlation ($r = .71$, $P < .005$; $r = .62$, $.01 < P < .025$) between mean serum cholesterol concentration and total aortic cholesterol content of Group II rabbits. Although the simple linear regression line ($y = a + bx \pm$ standard error of regression) for the abdominal segment lies above that for the thoracic segment, elevations did not prove to be significantly different.

fractions, there was a significant correlation between serum cholesterol concentration and cholesteryl ester in the abdominal segment ($r = 0.75$, $P < .005$), but it was not possible to demonstrate a significant correlation between aortic cholesteryl ester and serum cholesterol concentration in the thoracic segment ($r = 0.52$, $P =$ NS). In the case of nonesterified cholesterol, there was a significant correlation with serum cholesterol concentration in the thoracic aorta ($r = 0.68$, $0.005 < P < 0.01$) but not in the abdominal segment.

In contrast to the findings in white zones, it was not possible to demonstrate a significant correlation between serum cholesterol concentration and total aortic cholesterol in blue areas of either thoracic or abdominal segments of the aorta. Thus, blue, or de-endothelialized, areas of the aortic wall accumulated approximately the same amount of cholesterol and cholesteryl ester over a wide range of serum cholesterol concentrations.

Discussion

Experiments reported here were designed to test the effect of arterial injury on arterial lipid accumulation and atherosclerosis. Specifically, these experiments sought to test the hypothesis that endothelial injury and desquamation will lead to increased accumulation of lipid and atherosclerosis. The results of these experiments do not support this hypothesis. Rather, our findings indicate the following: 1) In normocholesterolemic rabbits, there is significantly increased accumulation of cholesteryl ester in re-endothelialized areas of the injured aorta as compared with adjacent de-endothelialized areas. 2) In hypercholesterolemic rabbits, there is significantly increased accumulation of both cholesterol and cholesteryl ester in re-endothelialized areas of the injured aortic wall as compared with ad-

jacent de-endothelialized areas. 3) Accumulation of aortic total cholesterol correlates with serum cholesterol concentration in re-endothelialized areas but not in adjacent de-endothelialized areas. 4) Under the conditions of these experiments arterial injury and endothelial regeneration favor accumulation of cholesteryl ester and nonesterified fatty acids, while diet-induced hypercholesterolemia favors accumulation of nonesterified cholesterol and triacylglycerols. 5) The amount of intimal and medial lipid assessed morphometrically in re-endothelialized areas of previously injured aorta does not correlate with the quantity of neutral lipid assessed chemically.

The primary objective of these experiments was to compare the character and quantity of lipid in the wall of re-endothelialized white zones with those of adjacent de-endothelialized blue areas. In rabbits of Groups I and II there was approximately a threefold increase of cholesteryl ester in the white zones of the re-endothelialized areas of both the abdominal and thoracic segments of the injured aortas as compared with the adjacent de-endothelialized areas. In addition, the white zones of Group II rabbits contained approximately two times as much nonesterified cholesterol as adjacent blue areas.

The mechanism of this increased accumulation of nonesterified cholesterol and cholesteryl ester in re-endothelialized areas is unclear. Conceivably, this increased accumulation could result from increased endothelial transport. According to this hypothesis, endothelium enhances the transport of lipid or lipoprotein into the arterial wall. Thus, accumulation of lipid in the arterial wall would result from an active process and not passive filtration. In this regard, results of experiments of Vlodavsky et al and Fielding et al may be important.[45, 46] The findings of Vlodavsky et al[45] indicate that *in vitro* nonconfluent endothelium binds, internalizes, and degrades more low-density lipoprotein than confluent endothelium. The experiments of Fielding et al showed that vessels of perfused rats demonstrated nonsaturable uptake of chylomicron cholesteryl ester.[46] Assuming that internalization and degradation of lipids by cultured endothelial cells may be related to lipid transport, these results indicate that non-contact–inhibited endothelium near the edge of nonblue islands may transport more lipid than normal endothelium. Perhaps in hypercholesterolemic rabbits of Group II there is increased uptake of cholesteryl ester by endothelium, leading to damage of endothelium and loss of contact inhibiton. Such endothelial damage may then lead to increased internalization of low-density lipoprotein. In any event, it is difficult to believe that any of these mechanisms would lead to increased transport of lipoprotein as compared with adjacent de-endothelialized

areas. Since endothelium has been shown to act as a barrier to the influx of lipoprotein,[47] it is conceivable that endothelium may also act as a barrier for removal of lipid or lipoprotein. In the absence of endothelium there may be increased efflux of lipid or lipoprotein from the de-endothelialized areas. We know of no experiments where such luminal efflux of lipid has been shown to occur, but we cannot rule out a contribution by this mechanism.

Alternatively, intima covered by endothelium may have significant metabolic differences as compared with adjacent intima lacking an endothelial cover. Preliminary results of other experiments from our laboratory furnish morphometric and chemical evidence to indicate that there are differences in the quantity and type of glycosaminoglycans in re-endothelialized as compared with adjacent de-endothelialized areas.[17, 48] The results of these experiments may be important to our understanding of the role of endothelium in intimal lipid accumulation. Differences in the type and quantity of glycosaminoglycans indicate that there are important differences in the metabolism of connective tissue matrix proteins in re-endothelialized as compared with adjacent de-endothelialized intima. Synthesis of corneal glycosaminoglycans by corneal fibroblasts has been found to be dependent on the presence of corneal endothelium or epithelium in several species.[49–52] There may also be differences in the metabolism of other materials, eg, cholesterol and cholesteryl esters, that contribute to lipid accumulation in re-endothelialized areas. Sulfated glycosaminoglycans have been shown to inhibit a variety of lysosomal enzymes isolated from leukocytes,[53, 54] spleen,[55] and liver.[56] Since glycosaminoglycans are internalized by cultured mammalian cells,[57] in the *in vivo* condition the lysosomal breakdown of endocytosed lipoprotein and cholesteryl ester could be inhibited in areas containing increased quantities of glycosaminoglycan, which is incorporated as a lipoprotein–glycosaminoglycan complex or as free glycosaminoglycan.

Finally, there may be structural differences in re-endothelialized intima as compared with de-endothelialized intima that are important in the increased lipid accumulation in re-endothelialized areas. As noted above, preliminary results of our experiments indicate that there are differences in the quantity and type of glycosaminoglycans in re-endothelialized as compared with adjacent de-endothelialized areas. Glycosaminoglycans have been found to bind to low-density lipoproteins,[58] and glycosaminoglycan–lipoprotein complexes have been isolated from atherosclerotic arteries of humans and experimental animals.[59,60] Hence, even though the transport of lipoprotein into the de-endothelialized areas is as great or greater than re-endothelialized areas, increased lipid may accumulate in

the re-endothelialized areas as a result of increased quantities of glycosaminoglycans.

In re-endothelialized white zones of Group II rabbits, total aortic cholesterol was strongly associated with serum cholesterol concentration. In contrast, in adjacent de-endothelialized blue areas there was no correlation over a range of serum cholesterol concentration of approximately 900 mg/100 ml. These findings suggest that the accumulation of intimal lipid is not a simple matter of increased filtration of lipoprotein into the arterial wall and therefore not a direct function of serum cholesterol concentration. Rather, they suggest that there are important characteristics of the arterial intima that may determine whether or not accumulation of cholesterol and cholesteryl ester occur. When accumulation of nonesterified cholesterol and cholesteryl ester are considered separately, the results are more complex. Accumulation of nonesterified cholesterol in re-endothelialized white areas is significantly correlated with serum cholesterol concentration in the thoracic segment only. In contrast, serum cholesterol concentration correlated with accumulation of cholesteryl ester in the abdominal segment but not the thoracic segment. These differences appear to be highly significant and suggest that there may be important differences in the metabolism of cholesterol and cholesteryl ester in the abdominal and thoracic segments of the aorta following injury. Similar differences, if present in human aortas, may be important to our understanding of human atherosclerosis. Metabolic differences in the intima of thoracic and abdominal segments of human aortas may explain the well-known accentuation of atherosclerosis in the abdominal segment of human aortas. Preliminary experiments in our laboratory have indicated that there is less acid cholesteryl hydrolase activity in the abdominal aorta of rabbits as compared with corresponding thoracic segments. This may explain the increased deposition of esterified cholesterol in the abdominal aorta.[61]

There were significant differences in the character and quantity of lipid in re-endothelialized aortas of Groups I and II as compared with their respective controls, Groups III and IV. Re-endothelialized areas of injured aortas of normocholesterolemic rabbits of Group I contained significantly increased quantities of cholesteryl ester, nonesterified fatty acid, and squalene as compared with the uninjured aortas of untreated normocholesterolemic rabbits of Group III. Re-endothelialized areas of injured aortas of hypercholesterolemic rabbits of Group II contained significantly increased quantities of all five lipid classes analyzed as compared with the uninjured aortas of hypercholesterolemic rabbits of Group IV. Diet-induced hypercholesterolemia was associated with accumulation of nonesterified cholesterol, triacylglycerols, and squalene in the uninjured aortas

of Group IV rabbits. Conceivably, the increased quantities of lipid in the re-endothelialized areas of injured aortas of Group I and Group II rabbits could result from one or more of the following: 1) increased transport and/or binding of lipid or lipoprotein in the wall of the injured aorta as a consequence of the injury and subsequent reparative changes; 2) increased transport into the wall as a consequence of elevated serum cholesterol concentration, since the serum cholesterol levels in both groups of rabbits with injured aortas were significantly higher than their respective controls; and 3) altered metabolism of lipoprotein and lipid, in particular cholesterol and cholesteryl ester, by the wall of the injured aorta. Although we do not have sufficient information to determine which of these possibilities is of primary importance, some seem more likely than others.

In the instance of the rabbits with injured aortas that were fed lipid-poor diets, there was a selective increase of cholesteryl ester, non-esterified fatty acids, and squalene in the re-endothelialized areas of the aorta. It seems unlikely that this selective increase of these lipid classes could result from increased binding or transport of lipoproteins within the arterial wall. With regard to increased transport, preliminary experiments of Stemerman et al indicate that there are minute foci of increased permeability to horseradish peroxidase in re-endothelialized areas.[62] However, these findings would not appear to be pertinent, since there is no increase of any lipid class in the de-endothelialized blue areas of normocholesterolemic rabbits, even though these areas can be shown to be uniformly permeable to horseradish peroxidase. The increase in serum cholesterol concentrations of rabbits with injured aortas does not appear to explain the lipid accumulation in Group I rabbits, since the increases in serum cholesterol of Group I as compared with Group III during the experimental period were very modest. Moreover, diet-induced hypercholesterolemia alone (Group IV) appears to be associated with increases in cholesterol, triacylglycerols, and squalene, and not increased cholesteryl ester and nonesterified fatty acids, as seen in Group I. These findings suggest the possibility that there may be alterations of cholesterol and cholesteryl ester metabolism in the intima of the re-endothelialized areas of Group I animals. Alternatively, there may be a selective uptake of cholesteryl ester in the re-endothelialized areas of the injured aorta. The results of the experiments of Fielding et al suggest that there is nonsaturable uptake of chylomicron cholesteryl ester by blood vessels of the rat heart.[46] In this situation the increase in nonesterified fatty acids could be secondary to the increase in cholesteryl ester.

Previously injured aortas of hypercholesterolemic rabbits of Group II showed an increase of all lipid classes, ie, nonesterified cholesterol, choles-

teryl esters, triacylglycerols, nonesterified fatty acids, and squalene, as compared with untreated controls. This increase contrasts with that seen in Group I rabbits, which showed a selective increase of cholesteryl ester, nonesterified fatty acids, and squalene as compared with untreated control animals, Group III, and animals fed egg-supplemented diet, Group IV, which demonstrated an increase of nonesterified cholesterol, squalene, and triacylglycerols as compared with untreated controls. As might be expected, aortas of these Group II rabbits showed an increase of both the lipid classes that were associated with aortic injury and those associated with diet-induced hypercholesterolemia. However, the degree of change indicated a synergistic effect, since it is clearly greater than the additive effect of either treatment alone. Such a synergistic effect could result from accentuation of the mechanism(s) responsible for lipid accumulation following either treatment or could result from a new mechanism evoked by their combined effect.

There is surprisingly little information on the correlation between arterial lipid accumulation assessed by morphometric techniques and lipid accumulation analyzed chemically. Such information is particularly important when morphometric techniques are necessary to quantitate various materials present in segments of arterial tissue that are too small to analyze chemically and samples that are highly nonhomogeneous, eg, nonblue areas containing both white and gray zones as well as intima and media. In these experiments, we investigated the relationship between oil-red-O–stained neutral lipid in the aortic wall and the amount of neutral lipid assessed chemically ie, cholesteryl ester and triacylglycerols in the white zones of nonblue areas of thoracic and abdominal segments of the same rabbit. We were not able to demonstrate a significant relationship between aortic lipid accumulation assessed chemically and that assessed morphometrically, whether the intima alone or the intima and the media were analyzed morphometrically. Scatter diagrams indicate that morphometric assessment of lipid accumulation appeared to be insensitive to differences in the higher concentrations of aortic lipid as compared with chemical analysis. Thus, at relatively high concentrations morphometric analysis of oil red O stains may not be useful. In this regard, it is interesting that the basis for experiments reported here were studies in which lipid accumulation was quantitated morphometrically. Although the present experiments have provided new data that we could not obtain by morphometric analysis, results of the chemical analysis in these experiments have substantiated our previous findings in every respect.

In conclusion, the results of the experiments reported here indicate that arterial injury and repair enhance accumulation of lipid in the arterial

wall. The results provide quantitative chemical data to support our previous morphometric findings that neointima covered by regenerated endothelium is more prone to accumulate lipid, demonstrated in these experiments to be cholesterol and cholesteryl ester, than adjacent neointima lacking endothelium. Finally, our findings suggest that the presence of endothelium may evoke metabolic differences in the neointima that are important in intimal lipid accumulation.

References

1. Anitschkow NN: Experimental arteriosclerosis in animals, Arteriosclerosis: A Survey of the Problem. Edited by EV Cowdry. New York, Macmillan Publishing Co, 1933, pp 271–322
2. Duff GL: Experimental cholesterol arteriosclerosis and its relationship to human arteriosclerosis. Arch Pathol 1935, 20:81–123, 259–304
3. Taylor CB: The reaction of arteries to injury by physical agents, with a discussion of arterial repair and its relationship to atherosclerosis. Symposium on Atherosclerosis. Washington, DC, National Academy of Sciences, 1955, pp 74–90
4. Waters LL: The reaction of the artery wall to injury by chemicals or infection,[3] pp 91–98
5. Minick CR, Murphy GE, Campbell WG Jr: Experimental induction of atheroarteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet: I. Effect of repeated injections of horse serum in rabbits fed a dietary cholesterol supplement. J Exp Med 1966, 124:635–652
6. Minick CR, Murphy GE: Experimental induction of atheroarteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet: II. Effect of repeatedly injected foreign protein in rabbits fed a lipid-rich, cholesterol-poor diet. Am J Pathol 1973, 73:265–300
7. Hardin NJ, Minick CR, Murphy GE: Experimental induction of atheroarteriosclerosis by the synergy of allergic injury to arteries and lipid-rich diet. III. The role of earlier acquired fibromuscular intimal thickening in the pathogenesis of later developing atherosclerosis. Am J Pathol 1973, 73:301–326
8. Moore S: Thromboatherosclerosis in normolipemic rabbits: A result of continued endothelial damage. Lab Invest 1973, 29:478–487
9. Stemerman MB: Thrombogenesis of the rabbit arterial plaque: An electron microscopic study. Am J Pathol 1973, 73:7–26
10. Harker LA, Slichter SJ, Scott CR, Ross R: Homocystinemia: Vascular injury and arterial thrombosis. N Engl J Med 1974, 291:537–543
11. Day AJ, Bell FP, Moore S, Friedman R: Lipid composition and metabolism of thromboatherosclerotic lesions produced by continued endothelial damage in normal rabbits. Circ Res 1974, 34:467–476
12. Friedman RJ, Moore S, Singal DP: Repeated endothelial injury and induction of atherosclerosis in normolipemic rabbits by human serum. Lab Invest 1975, 31:404–415
13. Friedman RJ, Moore S, Singal DP, Gent M: Regression of injury-induced atheromatous lesions in rabbits. Arch Pathol Lab Med 1976, 100:189–195
14. Ross R, Harker LA: Hyperlipidemia and atherosclerosis: Chronic hyperlipidemia initiates and maintains lesions by endothelial cell desquamation and lipid accumulation. Science 1976, 193:1094–1100
15. Ross R, Glomset JA: The pathogenesis of atherosclerosis. N Engl J Med 1976, 295:369–377, 420–425

16. Minick CR, Stemerman MB, Insull W Jr: Effect of regenerated endothelium on lipid accumulation in the arterial wall. Proc Natl Acad Sci USA 1977, 74:1724–1728
17. Minick CR, Litrenta MM, Alonso DR, Silane MF, Stemerman MB: Further studies on the effect of regenerated endothelium on intimal lipid accumulation. Prog Biochem Pharmacol 1977, 13:115–122
18. Spaet TH, Rhee C, Geiger C: Delayed consequences of endothelial removal from rabbit aortae. Adv Exp Med Biol 1978, 102:165–173
19. Moore S, Ihnatowycz IO: Vessel injury and atherosclerosis. Adv Exp Med Biol 1978, 102:145–163
20. Minick CR, Stemerman MB, Insull W Jr: Role of endothelium and hypercholesterolemia in intimal thickening and lipid accumulation. Am J Pathol 1979, 95:131–158
21. Simionescu M, Simionescu N, Palade GE: Segmental differentiations of cell junctions in the vascular endothelium: Arteries and veins. J Cell Biol 1976, 68:705–723
22. Simionescu N, Simionescu M, Palade GE: Recent studies on vascular endothelium. Ann NY Acad Sci 1976, 275:64–75
23. Simionescu N, Simionescu M: The cardiovascular system, Histology. Edited by L Weiss and RO Greep, New York, McGraw-Hill Book Co, 1977, pp 373–431
24. Bell FP, Adamson IL, Schwartz CJ: Aortic endothelial permeability to albumin: Focal and regional patterns of uptake and transmural distribution of ^{131}I-albumin in the young pig. Exp Mol Pathol 1974, 20:57–68
25. Bell FP, Gallus AS, Schwartz CJ: Focal and regional patterns of uptake and the transmural distribution of ^{131}I-fibrinogen in the pig aorta *in vivo*. Exp Mol Pathol 1974, 20:281–292
26. Schwartz CJ, Gerrity RG, Lewis LJ: Arterial endothelial structure and function with particular reference to permeability, Atherosclerosis Reviews. Vol 3. Edited by RP Paoletti and AM Gotto Jr. New York, Raven Press, 1978, pp 109–124
27. Majno G, Joris I: Endothelium 1977: A review. Adv Exp Med Biol 1978, 104: 169–225, 481–526
28. Thorgeirsson G, Robertson AL: The vascular endothelium: Pathobiologic significance: A review. Am J Pathol 1978, 93:803–848
29. Ross R, Glomset J, Kariya B, Harker LA: A platelet-dependent serum factor that stimulates proliferation of arterial smooth muscle cells *in vitro*. Proc Natl Acad Sci USA 1974, 71:1207–1210
30. Antoniades HN, Scher CD, Stiles CD: Purification of human platelet-derived growth factor. Proc Natl Acad Sci USA 1979, 76:1809–1813
31. Fischer-Dzoga K, Fraser R, Wissler RW: Stimulation of proliferation in stationary primary cultures of monkey and rabbit aortic smooth muscle cells: I. Effects of lipoprotein fractions of hyperlipemic serum and lymph. Exp Mol Pathol 1976, 24:346–359
32. Chen RM, Getz GS, Fischer-Dzoga K, Wissler RW: The role of hyperlipidemic serum on the proliferation and necrosis of aortic medial cells *in vitro*. Exp Mol Pathol 1977, 26:359–374
33. Falcone DJ, Hajjar DP, Minick CR: Role of endothelium in intimal lipid accumulation (abstr). Fed Proc 1979, 38:1346
34. Baumgartner HR, Stemerman MB, Spaet TH: Adhesion of blood platelets to subendothelial surface: Distinct from adhesion to collagen. Experientia 1971, 27:283–285
35. Levine JB, Zak B: Automated determination of serum total cholesterol. Clin Chim Acta 1964, 10:381–384
36. Burkholder PM, Littell AH, Klein PG: Sectioning at room temperature of unfixed

tissues, frozen in a gelatin matrix, for immunological procedures. Stain Technol 1961, 36:89–91

37. Perkins EG (ed): Analysis of Lipids and Lipoproteins. Champaign, Ill, American Oil Chemists' Society, 1975
38. Folch J, Lees M, Sloane-Stanley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957, 226:497–509
39. Nicolosi RJ, Smith SC, Santerre RF: Simultaneous fluorometric analysis of five lipid classes on thin-layer chromatograms. J Chromatogr 1971, 60:111–117
40. Hojnacki JL, Smith SC: Separation of six lipid classes on one thin-layer chromatogram. J Chromatogr 1974, 90:365–367
41. Zar JH: Biostatistical Analysis. Englewood Cliffs, NJ, Prentice-Hall, Inc, 1974
42. Winer BJ: Statistical Principles in Experimental Design. New York, McGraw-Hill Book Co, 1962
43. Wike EL: Data Analysis. New York, Aldine-Atherton, 1971
44. Bruning JL, Kintz BL: Computational Handbook of Statistics. Glenview, Ill, Scott, Foresman and Co, 1977
45. Vlodavsky I, Fielding PE, Fielding CJ, Gospodarowicz D: Role of contact inhibition in the regulation of receptor-mediated uptake of low density lipoprotein in cultured vascular endothelial cells. Proc Natl Acad Sci USA 1978, 75:356–360
46. Fielding CJ: Metabolism of cholesterol-rich chylomicrons: Mechanism of binding and uptake of cholesteryl esters by the vascular bed of the perfused rat heart. J Clin Invest 1978, 62:141–151
47. Bratzler RL, Chisolm GM, Colton CK, Smith KA, Lees RS: The distribution of labeled low-density lipoproteins across the rabbit thoracic aorta *in vivo*. Atherosclerosis 1977, 28:289–307
48. Wight TN, Curwen KD, Homan WP, Minick CR: Effect of regenerated endothelium on glycosaminoglycan accumulation in the arterial wall (abstr). Fed Proc 1979, 39:1075
49. Anseth A: Influence of corneal epithelium on the incorporation of $^{35}SO_4$ into stromal glycosaminoglycans. Exp Eye Res 1971, 11:251–254
50. Cremer-Bartels G, Buddecke E: Factors regulating the keratan sulfate biosynthesis in bovine cornea. Exp Eye Res 1972, 14:171–172
51. Klintworth GK, Smith CF: A comparative study of extracellular sulfated glycosaminoglycans synthesized by rabbit corneal fibroblasts in organ and confluent cultures. Lab Invest 1976, 35:258–263
52. Smesler GK: Role of the epithelium in incorporation of sulphate in the corneal connective tissue, The Transparency of the Cornea. Edited by S Duke-Elder, ES Perkins. Oxford, Blackwell Scientific Publications, 1960, pp 125–130
53. Avila JL, Convit J: Physicochemical characteristics of the glycosaminoglycan-lysosomal enzyme interaction *in vitro*: A model of control and leucocytic lysosomal activity. Biochem J 1976, 160:129–136
54. Avila JL: The influence of the type of sulphate bond and degree of sulfation of glycosaminoglycans on their interaction with lysosomal enzymes. Biochem J 1978, 171:489–491
55. Robinson D, Stirling JL: N-acetyl-β-glucosaminidases in human spleen. Biochem J 1968, 107:321–327
56. Kint JA, Dacremont G, Carton D, Orye E, Hooft C: Mucopolysaccharidosis: Secondarily induced abnormal distribution of lysosomal isoenzymes. Science 1973, 181:352–354
57. Saito H, Uzman BG: Uptake of chondroitin sulfate by mammalian cells in culture: II. Kinetics of uptake and autoradiography. Exp Cell Res 1971, 66:990–96

58. Iverius PH: The interaction between human plasma lipoproteins and connective tissue glycosaminoglycans. J Biol Chem 1972, 247:2607–2613
59. Mawhinney TP, Augustyn JM, Fritz KE: Glycosaminoglycan-lipoprotein complexes from aortas of hypercholesterolemic rabbits: Part 1: Isolation and characterization. Atherosclerosis 1978, 31: 155–167
60. Srinivasan SR, Dolan P, Radhakrishnamurthy B, Berenson GS: Isolation of lipoprotein–acid mucopolysaccharide complexes from fatty streaks of human aortas. Atherosclerosis 1972, 16:95–104
61. Hajjar DP: Upublished observations, 1979
62. Stemerman MB, Mannarino AJ: Influence of hypercholesterolemia on the permeability of regenerating endothelium (abstr). Fed Proc 1979, 38:1456

Acknowledgments

We gratefully acknowledge the assistance of Carol Ibsen, Thomas Frenna, Theresia Laube, and Leslie Seiden. We also thank Dr. Martin Lesser, Biostatistics Laboratory, Sloan Kettering Institute, for assistance with the statistical analysis.