American Journal of Pathology, Vol. 147, No. 2, August 1995

Prominence of Coronary Arterial Wall Lipids in Human Heart Allografts

Implications for Pathogenesis of Allograft Arteriopathy

Bruce M. McManus,*† Kathleen J. Horley,* Janet E. Wilson,*† Gray T. Malcom,‡ Todd J. Kendall,† Rodney R. Miles,† Gayle L. Winters,§ Maria Rosa Costanzo,‖ Leslie L. Miller,¶ and Stanley J. Radio†

From the Cardiovascular Research Laboratory, Department of Pathology and Laboratory Medicine, St. Paul's Hospital, Vancouver, British Columbia, Canada; the Department of Pathology,‡ Louisiana State University, New Orleans, Louisiana; the Cardiovascular Registry,† Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska; the Department of Medicine‖ and Pathology,§ Loyola University Medical Center, Maywood, Illinois; and the Department of Cardiology,¶ St. Louis University, St. Louis, Missouri*

Transplant arteriopathy is a major late complication in human heart allograft recipients and the pathogenesis of such arteriopathy remains uncertain. The degree to which lipids and atheromata are involved in the arteriopathic lesions remains unsettled, and there is uncertainty regarding the significance of insudation or retention of lipids within the coronary artery walls of transplanted hearts. On current immunosuppressive regimens, most patients experience an increased serum total cholesterol and low-density lipoprotein cholesterol after transplant. Elevation of these blood lipids has an undetermined relationship to arteriopathy. We carried out morphological, morphometric, immunohistochemical, ultrastructural, and biochemical studies of particular coronary artery segments from 23 unselected explant or autopsy allografts and donor age-matched native coronary controls. Patients died of cardiac and non-cardiac reasons over a period of 4 to 1610 days after transplant. Atheromata were frequent, and diffuse intra- and extra-cellular accumulation of lipids in both intimal and medial walls was documented by oil red O positivity, immunohistochemical staining (muscle-specific α-actin), transmission and scanning electron microscopy, and biochemical analysis. Mean total cholesterol, esterified cholesterol, free cholesterol, and phospholipid content (μg/cm² intimal surface area) and concentration (μg/mg dry defatted weight) in arteriopathic coronaries were >10-fold higher than in comparable native coronary segments. Extent of lipids in the arterial walls was highly correlated with digitized percent luminal narrowing, mean daily and cumulative cyclosporin dose, and mean cumulative prednisone dose. Our data suggests strongly that lipid accumulation is an important early and persistent phenomenon in the development of transplant arteriopathy. (Am J Pathol 1995, 147:293–308)

The pathogenesis of transplant arteriopathy is unknown. The morphological characteristics of arteriopathy in transplanted solid organs have been described by numerous investigators over the past 25 years.[1–11] Despite documentation of foam cells and foci of extracellular lipid within the arterial walls of cardiac allografts, the degree to which lipids are involved and their pathogenetic role in the arterial lesions has not been fully characterized or understood. Early on, arteriopathic lesions in kidney allografts were found to be lipid-rich.[12] However, in recent discussions by some investigators arteriopathy was described as "lipid-poor," and an "inconstant" relationship between

Supported by NHLBI grant 5 RIO HL33778 and Heart and Stroke Foundation of British Columbia and Yukon grant G-94-MC-0256.

Accepted for publication May 5, 1995.

Address reprint requests to Bruce M. McManus, Department of Pathology and Laboratory Medicine, St. Paul's Hospital, University of British Columbia, 1081 Burrard St., Vancouver, BC Canada V6Z 1Y6.

Dr. Winters' present address: Department of Pathology, Brigham & Women's Hospital, Boston, MA. Dr. Costanzo's present address: Department of Medicine, Rush Presbyterian–St. Luke's Medical Center, Chicago, IL.

the arteriopathic process and vessel wall lipids has been suggested. Focus on the concept of a fibroproliferative lesion, particularly emanating from renal transplantation experience, has drawn attention away from the idea that lipids may play a significant role in the arteriopathic disease process.

Conventional cardiovascular risk factors, including hypercholesterolemia, have received considerable investigative attention as potential contributors to arteriopathy. After transplant, most patients experience an increase in serum total cholesterol and low-density lipoprotein (LDL) cholesterol levels when maintained on an immunosuppressive regimen which includes corticosteroids and cyclosporin.[12–16] Hyperlipidemia correlates with the development of angiographic arteriopathy in some series,[17,18] while other observers have found no such relationship.[1,19,20] Only rarely has direct histometric evidence of luminal narrowing by proliferative intimal lesions been correlated to lipid levels after transplant.[2] In multivariate analysis, the relationship between serum cholesterol and arteriopathic disease was stronger than any other potentially predictive factor, apart from post-transplant obesity.[2] The direct association of serum cholesterol with digitized luminal narrowing of allograft coronary arteries suggested an important role for lipids in development of arteriopathy.

Several additional hypotheses have been offered as explanations for transplant arteriopathy. The common theme of each hypothesis is arterial injury followed by a reaction of the arterial wall components to such injury. Immune-mediated injury, either by humoral or cellular mechanisms is virtually certain. The importance of humoral mechanisms is suggested by the presence of anti-endothelial antibodies in allograft recipients with arteriopathy.[21,22] Other investigators have proposed that transplant arteriopathy is primarily due to cellular mechanisms, whereby cytotoxic T lymphocytes cause "endothelialitis" as a manifestation of "vascular" rejection. Once present within the intima, T lymphocytes may promote transplant arteriopathy by the release of a variety of cytokines, growth factors, and inflammatory mediators.[23] Chronic immune reactivity of T lymphocytes to activated graft endothelial cells may lead to ongoing local release of cytokines, stimulation of smooth muscle cell proliferation, and matrix accumulation within the coronary arteries.[24] Another hypothesis centers on the role of viruses in induction of transplant arteriopathy. Studies of Marek's disease virus,[25] and observations indicating the potential importance of acid cholesteryl ester hydrolase in lipid overload of smooth muscle cells[26–27] have been bolstered by clinical studies of association by cytomegalovirus infection and arteriopathy.[28] As well, evidence has recently emerged linking cytomegalovirus to other proliferative lesions that follow arterial injury.[29]

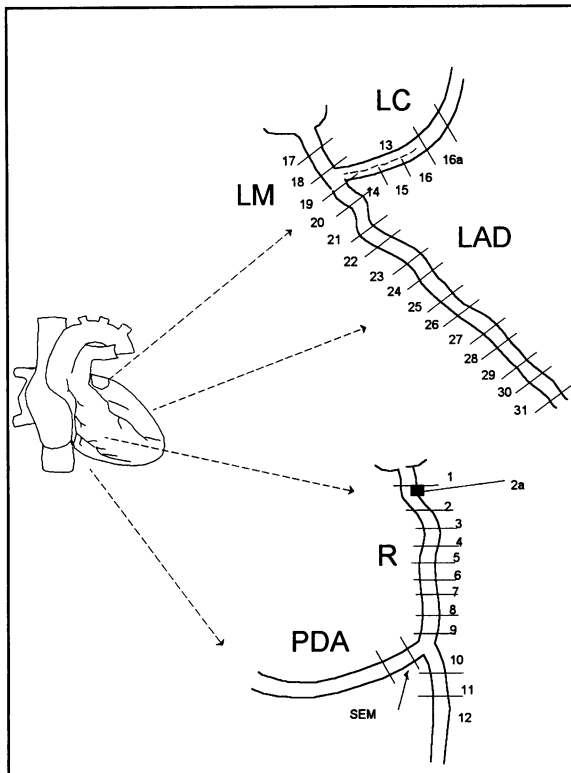
In light of the unresolved role that serum lipids play in transplant arteriopathy, combined with the continued uncertainty with respect to direct insudation or retention of lipids within coronary arteries of transplanted hearts, we have undertaken detailed morphological, morphometric, immunohistochemical, ultrastructural, and biochemical studies of selected epicardial coronary artery segments from human heart allografts. Importantly, the allografts are unselected and are derived from explant or autopsy, with cardiac (rejection- or nonrejection-related) or non-cardiac basis of transplant or death, representing a broad spectrum of implant duration times from 4 to 1610 days. We have attempted to examine the hypothesis that lipid storage within coronary arteries of transplanted hearts is a central process in the proliferative intimal thickening and medial degeneration of allograft arteriopathy.

Materials and Methods

Tissue Collection and Triage

Allografts were obtained as soon as possible after explant or autopsy, immersed and transported in RPMI 1640, after which they were rinsed in PBS, weighed, and photographed. The right (R) coronary artery was ligated with nylon thread as close as possible to its origin from the aorta. The vessel was then removed intact from the heart and rinsed in Hank's balanced salt solution (HBSS), cleaned of excess adventitial fat, and sectioned (Figure 1).[30] The left circumflex (LC) coronary artery was ligated as close as possible to the bifurcation of the left main coronary artery. The artery was opened longitudinally *in situ*, after which it was dissected from the heart, rinsed in HBSS, divided into halves longitudinally, and further sectioned (Figure 1). The left main (LM) and left anterior descending (LAD) coronary arteries were perfusion-fixed with 10% neutral buffered formaldehyde solution (formalin). The aortic root was cannulated with a stopper, the heart placed in a fixative bath and perfusion-fixed continuously at 100 mm Hg for 1 hour. After fixation, the LM and LAD were removed intact and sectioned subserially in a transverse fashion at 4 mm intervals (Figure 1). Sections were taken for histological study from other cardiac sites after overnight fixation.

Sections of epicardial coronary arteries from hearts archived in the Pathobiological Determinants of Atherosclerosis in Youth (PDAY) study were made avail-



SAMPLE	LENGTH	TISSUE USE	SPECIMEN PROCESSING
R 1	5 mm	Morphometrics	Formalin-Fixed
2*	15 mm	Lipid Analysis	Flash Frozen
2a*	1 mm x 2 mm	TEM	PAPG-Fixed
3*	5 mm	IHC, IF	OCT Frozen
4*	5 mm	PCR	Flash Frozen
5*	5 mm	IHC, Histo	Formalin-Fixed
6*	5 mm	ISH	Formalin-Fixed
7	5 mm	Morphometrics	Formalin-Fixed
8	15 mm	Archive	OCT Frozen
9	5 mm	Morphometrics	Formalin-Fixed
10	15 mm	Archive	Flash Frozen
11	5 mm	Morphometrics	Formalin-Fixed
12	Remaining	Lipid Analysis	Flash Frozen
12a R-PDA	10 mm	SEM	PAPG-Fixed
LC 13	30 mm	Archive	OCT Frozen
14	10 mm	PCR	Flash Frozen
15	10 mm	Lipid Analysis	Flash Frozen
16	10 mm	Archive	Flash Frozen
LC 16a	10 mm	SEM	PAPG-Fixed
LAD 17,18	5 mm	Morphometrics	½ Formalin-Fixed ½ OCT Frozen
19	5 mm	IHC, ISH, PCR	Formalin-Fixed
20,22,24, 26,28,30,32‡	5 mm	Morphometrics	Formalin-Fixed
21,23,25, 27,29,31‡	10 mm	Archive	½ Formalin-Fixed ½ OCT Frozen

*Section 2-6 (35mm) are opened
‡Sections 31 and 32 are taken if LAD is long enough

Figure 1. *Sectioning protocol for the epicardial arteries. Diagrammatic representation of specimen collection, processing, and use. Sections 2–6 (35 mm length) were opened longitudinally. LAD, left anterior descending coronary artery; LC, left circumflex coronary artery; LM, left main coronary artery; PDA, posterior descending artery; R, right coronary artery.*

able as donor age comparable, site-matched native artery controls. The coronary protocol for PDAY is comparable to that used in the conduct of this study.

Histological Examination

All formalin-fixed tissue sections were paraffin-embedded and 5-μm sections were stained with hematoxylin and eosin, Verhoeff's van Gieson elastin, and Movat's pentachrome. Each section was examined for the presence, extent, and distribution of intimal thickening, luminal narrowing, inflammatory infiltrates, disruption of the internal elastic lamina, lipid deposits in foam cells, and matrix accumulation. OCT-embedded frozen sections were cut and stained with oil red O, counterstained with hematoxylin and examined for the distribution, droplet size, and the amount of lipids.

Immunohistochemical Analysis

Selected sections prepared for oil red O staining were also stained immunohistochemically utilizing antibodies to muscle-specific α-actin (HHF-35, Enzo). Other paraffin-embedded sections were stained with antibodies to monocyte-macrophages (MAC 387, lysozyme), T cells (UCHL-1, CD2, OPD4-CD4, CD8), B cells (L 26), and NK cells (leu 7). All immunohistochemistry procedures have been described previously.[31,32]

Electron Microscopic Analysis

A section of the proximal R coronary artery wall (1 mm^3) was fixed overnight in phosphate buffered glutaraldehyde (pH 7.4) containing 15% saturated picric acid, and then post-fixed in osmium tetroxide for 30 minutes. The osmicated tissue was dehydrated in graded alcohols, incubated in two changes of propylene oxide, infiltrated with Araldite resin, and allowed to cure at 60°C overnight. Ultrathin sections were cut, placed on copper grids, and stained with uranyl acetate and lead citrate. The sections were viewed and photographed on a Phillips 201 transmission electron microscope.

Segments of the posterior descending coronary artery, formalin fixed, were sectioned transversely and mounted on pedestals of a Phillips Model 515 scanning electron microscope. The tissue was dehydrated through graded alcohols and Freon 113, the latter with critical point drying prior to viewing. Detailed examination of the intimal endothelial surface and the intimal and medial constituency was conducted.

Morphometric Analysis

Morphometric analysis of luminal narrowing was carried out on 23 formalin-fixed, Movat's pentachrome stained R (non-perfusion-fixed) and LAD (perfusion-fixed) coronary arterial segments utilizing a Video Image Analysis System computer program, version 2.11 (SCICOM Computer Consultants, Millbrae, CA), with digital input from a Kurta Corporation Series II mouse and drawing panel.[30] For each of the 23 allografts, an average of 4 transverse sections per R and 8 to 11 sections per LAD coronary arteries were digitized and the measurements were averaged for each vessel. Intimal, medial, and luminal areas were quantitated, with percent luminal narrowing (% LN) calculated using the following formula:

$$\% \text{ LN} = \frac{\text{Intimal area}}{\text{Intimal area} + \text{Luminal area}} \times 100. \quad (1)$$

Tissue Lipid Analysis

All biochemical lipid analyses were performed in the laboratory of Dr. Gray Malcom, LSU Medical Center and the lipid extractions are 100% complete.[33,34] The R coronary segments 2 (proximal R) and 12 (distal R), and LC segment 15 (proximal LC) were analyzed for lipid content (μg/cm^2 intimal surface area) and concentration (μg/mg dry defatted weight). The adventitia was stripped from each arterial segment and each segment was then lyophilized. Lipids were extracted from the lyophilized tissue by the method of Folch et al[35] after homogenization with chloroform-methanol. Phospholipid was determined by the method of Michelson[36] using phosphatidyl choline as a standard. An aliquot of the lipid sample was hydrolyzed with 2% KOH for determination of total cholesterol, and another, without base, for free cholesterol. Silyl ether derivatives of the sterols were prepared with trimethylsilyl reagent after the addition of a known mass of pure cholestane as a standard. Total and free cholesterol were quantitated by gas liquid chromatography. Esterified cholesterol were obtained by subtraction of free cholesterol from total cholesterol.

Statistical Analysis

All data were compiled on a 3090 IBM mainframe computer utilizing the VM/CMS 5.0 operating system and the SAS/FSP program, version 5. Descriptive statistics were prepared, and correlative studies, and univariate comparisons were carried out. All statistical evaluations were performed setting the Bonferroni probability of a type I (α) error at $P < 0.05$.

Results

Clinical Characteristics

The clinical features of the 23 patients whose coronary arteries were studied biochemically are presented in Table 1. Data on immunosuppressive therapy and rejection episodes for these patients are presented in Table 2.

The patients whose allografts were evaluated in this study included 13 with idiopathic dilated cardiomyopathy and 10 with either coronary atherosclerotic disease or coronary atherosclerotic disease plus myocardial consequences (ischemic heart disease). Implant durations ranged from as few as 4 days to as long as 1610 days with a mean of 544 days. Average age of recipients was 50 years (range 16 to 60 years), with 17 males and 6 females. The ultimate cause of death or explant in these recipients was rejection in 8, clinically visible transplant vascular disease in 2, sudden death in 4, heart failure in 1, post-transplant lymphoproliferative disease in 4, pneumonia in 1, Guillain-Barre disease in 1, and systemic infection in 1. The mean donor age was 30.1 years (with a range from 13 to 51 years), including 19 males and 4 females. The mean pre-transplant total serum cholesterol (mg/dl) was similar to post-transplant values (172 *versus* 172.5) while the serum triglycerides (mg/dl) rose from pre-transplant (148.8 to 161.8). These pre-transplant lipid values represent a mean of 3 pre-transplant measurements while the patient was on the waiting list. The post-transplant values reflect a mean derived from a minimum of 10 post-transplant measures.

Detailed information regarding mean blood levels of cyclosporin and daily and cumulative doses of cyclosporin and prednisone are presented in Table 2. The mean level of cyclosporin was 201 ± 62 mg/ml of whole blood (ranging from as low as 63 to as high as 330). The mean daily and cumulative doses of cyclosporin were 352 ± 126 mg and 140,357 ± 93,631 mg, respectively. The mean daily dose of prednisone was 32 ± 18 mg with a cumulative dose of 9,788 ± 6633 mg. Within the cyclosporin and prednisone

Table 1. *Clinical Features of 23 Allograft Recipients*

Patient no.	Primary diagnosis	Implant duration (days)	Age (yr)	Sex	Cause of death/ explant	Donor age (yr)	Donor sex	Total serum cholesterol (mg/dl) Pre-Tx	Total serum cholesterol (mg/dl) Post-Tx	Serum triglycerides (mg/dl) Pre-Tx	Serum triglycerides (mg/dl) Post-Tx
1	IDC	1432	27	F	HF	21	M	155	219	182	181
2	IHD	426	28	M	REJ	25	M	176	209	158	291
3	IDC	46	60	F	PTLD	44	F	227	151	107	168
4	IDC	55	56	M	PTLD	29	M	145	152	102	189
5	IHD	635	51	M	PTLD	13	M	156	249	49	181
6	IDC	611	16	M	REJ	47	M	114	109	128	119
7	IHD	180	61	M	PNEU	17	M	150	138	37	177
8	IDC	153	66	M	PTLD	36	M	226	113	191	122
9	CAD	147	65	M	CAD	35	M	186	NA	237	NA
10	CAD	1349	43	M	SD	51	M	211	114	189	224
11	IDC	411	28	M	CAD	45	F	92	NA	233	NA
12	IDC	4	44	F	REJ	33	F	129	130	152	119
13	CAD	63	60	M	GB	31	M	157	162	97	141
14	CAD	1145	57	M	SD	33	M	233	136	219	124
15	IHD	1190	57	F	REJ	22	M	175	246	172	249
16	CAD	1610	54	M	SD	47	M	226	260	92	78
17	IDC	1510	57	M	SD	35	M	145	161	31	32
18	IDC	323	63	M	REJ	32	M	178	227	108	172
19	IDC	300	50	M	REJ	18	M	181	154	409	179
20	IDC	210	45	M	REJ	20	M	129	163	76	110
21	IDC	450	49	F	INF	23	M	179	196	228	170
22	IDC	63	54	F	REJ	18	F	287	224	179	260
23	IHD	199	55	M	INF	17	M	99	109	46	112
Mean		544 (4–1610)	50 (16–66)	17M 6F		30 (13–51)	19M 4F	172 ± 47	173 ± 50	149 ± 86	162 ± 62

CAD, coronary artery disease; GB, Guillain-Barre disease; HF, heart failure; IDC, idiopathic dilated cardiomyopathy; IHD, ischemic heart disease; INF, infection; NA, not available; PNEU, pneumonia; PTLD, post-transplant lymphproliferative disorder; REJ, rejection; SD, sudden death; Tx = Transplant.

therapeutic regimens the daily doses varied widely. The number of total acute rejection episodes averaged 7 per patient for the period of study. For the purposes of this study, rejection episodes were defined by the "marker" positive biopsy. Of these episodes, only a small fraction were more than mild. Treated (treatable defined according to the Working Group classification as grade 2 or higher) nor untreated rejection episodes were correlated significantly (although positively) with arterial wall lipid levels regardless of the lipid moiety.

Relationships of digitized percent luminal narrowing of transversely sectioned epicardial coronary artery segments and either lipid content or lipid concentration determined biochemically, illustrate the generally strong prediction of luminal narrowing by arterial wall lipid fractions (r = 0.60 and 0.89 proximally and distally with correspondent P values <0.04 and <0.007 for total arterial cholesterol). Similarly, high correlations were found for other expressions of cholesterol content or concentration within the arterial walls, the consistently strongest relationship being between free cholesterol and luminal narrowing in the distal portion of the coronary arterial tree (content, r = 0.92 and concentration, r = 0.94). The phospholipid content was somewhat more variable in its relationship to luminal narrowing, but also tended in many segments to be highly related to luminal narrowing (distal phospholipid concentration relative to luminal narrowing, r = 0.88, $P < 0.009$).

Tissue Lipid Analysis

Mean lipid content (μg/cm^2 intimal surface area) levels of the proximal and distal R and proximal LC coronary artery segments including total cholesterol, free cholesterol, esterified cholesterol, and phospholipids from the allografts (Figure 2) were markedly elevated ($P < 0.05$) in comparison to the 90th percentile of donor age-comparable, site-matched coronary control tissue derived from the PDAY study (Figure 3). Likewise, mean lipid concentration (μg/mg dry defatted weight) of the proximal and distal R and proximal LC total cholesterol, free cholesterol, esterified cholesterol, and phospholipid were markedly elevated in the allografts (Figure 4). While total cholesterol content of proximal R coronary segments varied considerably between individual patients (79–6061 μg/cm^2), the total cholesterol was greater than the 90th percentile of PDAY control tissue in 19/23 (83%) allografts, and equal to the 42nd, 56th, 71st, and 79th percentile of

Table 2. *Immunosuppressive Therapy and Rejection Episodes of 23 Allograft Recipients*

Patient no.	Cyclosporin			Prednisone		No. of rejection episodes							
	Mean level (mg/ml)	Cumulative (mg)	Daily (mg/day)	Cumulative (mg/ml)	Daily (mg/day)	Grade							
						0	1A	1B	2	3A/B	4	TAc	Res*
1	157	193373	135	32178	23	19	3	1	1	2	0	7	3
2	192	143880	338	16367	38	14	4	1	0	0	0	5	0
3	294	21696	471	6143	134	2	0	1	0	2	0	3	0
4	246	14855	270	4990	91	3	0	1	0	0	0	1	0
5	212	167850	264	29565	47	14	7	0	2	4	0	13	1
6	232	149743	245	19405	32	4	0	6	0	5	1	12	6
7	195	39664	220	9610	53	4	4	7	0	3	0	14	0
8	239	62340	408	6044	40	13	1	0	0	0	0	1	0
9	110	112160	714	1455	31	8	0	1	0	1	0	2	0
10	68	NA	430	NA	NA	20	1	3	0	1	0	5	0
11	NA	365790	890	12330	30	14	0	9	4	0	0	13	0
12	NA	664	238	0	0	0	0	1	0	0	1	2	0
13	330	27410	370	1823	25	9	0	0	0	0	0	0	0
14	91	398645	348	4237	4	13	2	4	0	0	0	6	0
15	204	284170	238	18942	16	18	0	7	0	2	0	9	0
16	63	109510	68	7571	5	4	3	8	0	0	0	11	0
17	113	432180	286	5381	4	9	4	10	0	1	0	15	0
18	261	125585	455	4886	18	16	0	3	0	1	0	4	0
19	238	157523	523	5449	18	11	0	5	0	5	0	10	0
20	257	64150	332	4884	25	7	0	6	0	0	0	6	0
21	174	103525	233	17594	40	19	0	4	0	1	0	5	0
22	299	57556	341	3094	18	9	0	3	0	2	0	5	0
23	252	55585	274	3396	17	15	0	1	0	0	0	1	0
Mean ± SD	201±62	140357 ± 93631	352 ± 126	9788 ± 6633	32 ± 18	11	1.26	0.3	0.3	1.3	0.1	7	0.43

NA, not available; Res, resolving; TAc, total acute.
*Resolving is a category not currently used in the Working Formulation of the ISHLT, but commonly applied in the past to designate a lesser degree of rejection with healing following a biopsy specimen which is positive for acute rejection.

PDAY coronary artery tissue in the remaining allografts. Assessment as to whether differences in vessel wall lipid content or concentration are greater in the proximal LC or R as compared to the distal R coronary artery revealed only a statistical trend in this direction.

Morphometric Analysis

Mean luminal narrowing (percent cross-sectional area reduction) of non-perfusion-fixed R coronary arteries and perfusion-fixed LAD segments from the allografts ranged from 23.5 to 75.2% for the R coronary artery (mean, 55%) and from 10.2 to 76.6% for the LAD (mean, 44%).

Percent luminal narrowing of the LAD coronary arteries was strongly correlated with total cholesterol and cholesteryl ester content in the proximal R coronary and with total, free, and esterified cholesterol contents in the distal R coronary (Table 3). Arterial wall phospholipids, expressed both as content and concentration, were also strongly correlated with percent luminal narrowing in the LAD coronary artery (Table 3) (Figure 5). All arterial wall lipid moieties were weakly related with mean blood cholesterol (range: $r = 0.32$–0.38, $P < 0.07$–0.14) and triglyceride (range: $r = 0.11$–0.27, $P < 0.23$–0.62) levels. In addition, arterial wall lipids were correlated positively with mean daily and cumulative cyclosporin and prednisone doses.[37] Implant duration was not correlated with arterial wall lipid content or concentration (Table 4). This held true even when the implant duration was divided into 3 discrete time periods, early, middle, and late post-transplant, and comparisons were made for vessel wall lipids between these arbitrarily chosen intervals (Figure 6). However, for those patients with greater than one year implant duration, there was a trend toward higher arterial wall lipids. When arterial wall lipid values were analyzed with respect to cause of death (coronary artery disease, sudden death, and other), there were no significant differences among the groups.

Histological and Electron Microscopic Analysis

Excess extracellular lipid was demonstrated by histochemical staining of frozen intimal segments from 20 of 21 sets of coronary arteries studied in this manner. In lesions with <25% luminal narrowing, oil red O positivity was most prominent in the superficial (adluminal) zone where intimitis also began in a band-like, sub-endothelial pattern. Abundant extracellular lipid was present when a deeper, lipid-rich, atheromatous core had developed. The latter arteries included cholesterol

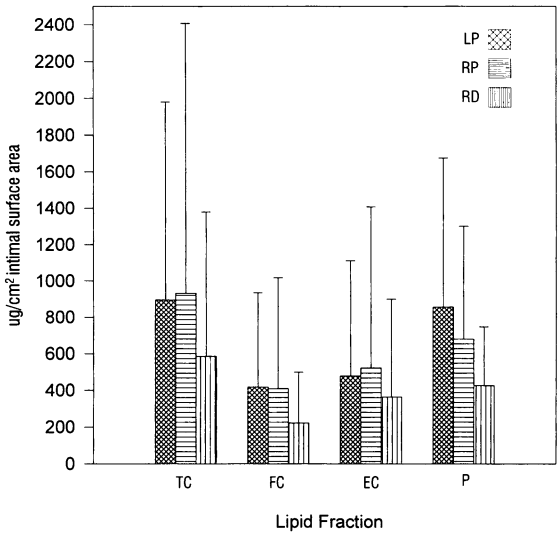


Figure 2. *Mean lipid content (μg/cm² intimal surface area) of proximal and distal right and proximal left circumflex coronary artery segments from 23 cardiac allografts. The lipid content is lower in the distal right coronary as compared with the proximal right or left circumflex. This contrasts to the lipid concentration (highest in the proximal left circumflex) and lower at any site in the right coronary artery (Figure 4). The latter difference may relate in part to greater relative dry defatted weight in the right coronary artery versus the circumflex. Standard deviations are indicated. EC, esterified cholesterol; FC, free cholesterol; LP, left proximal; P, phospholipids; RD, right distal; RP, right proximal; TC, total cholesterol.*

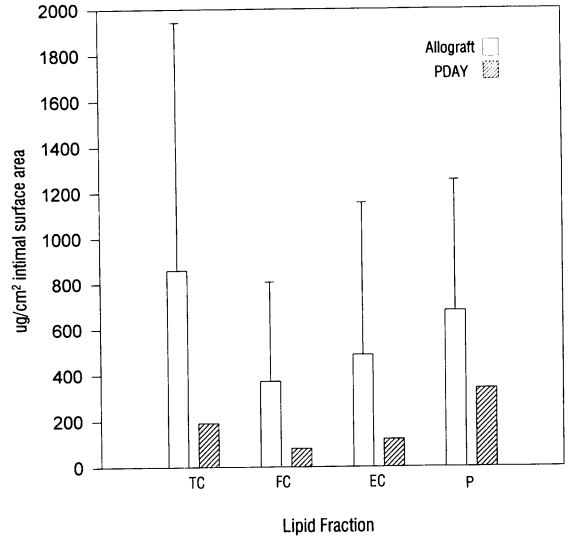


Figure 3. *Overall mean lipid content (μg/cm² intimal surface area) of epicardial coronary arteries from 23 cardiac allografts and 173 control native hearts (PDAY Study). Coronary segments from both study groups were triaged through comparable protocols including corresponding arterial sites. Standard deviations are indicated on allograft means only as the PDAY values are expressed as the mean at the 90th percentile and standard deviations were not included as part of the original analysis. EC, esterified cholesterol; FC, free cholesterol; P, phospholipids; TC, total cholesterol.*

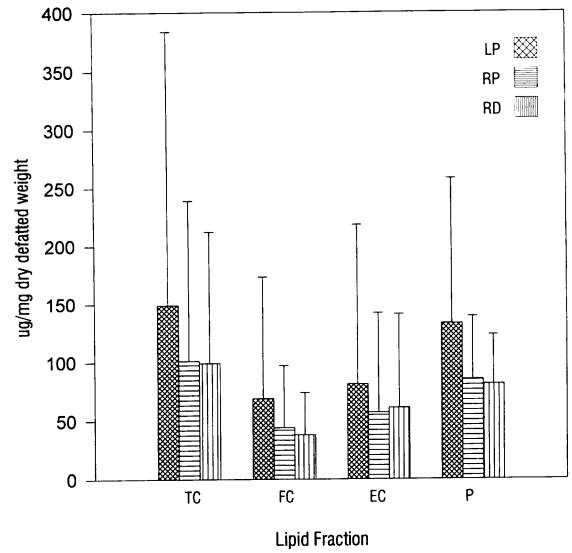


Figure 4. *Lipid concentration (μg/mg dry defatted weight) of proximal and distal right and proximal left circumflex coronary artery segments from 23 cardiac allografts. Standard deviations are indicated. EC, esterified cholesterol; FC, free cholesterol; LP, left proximal; P, phospholipids; RD, right distal; RP, right proximal; TC, total cholesterol.*

clefts, foam cells, and glycosaminoglycan-rich matrix, typically more circumferential than in native atherosclerotic disease. Lipid droplets were commonly associated with elastin fibers (internal elastic lamina) of the intima, especially in regions of disruption and reduplication. The internal elastic lamina was often focally interrupted, presumably by larger fenestrations,[38] and was at times completely destroyed in secondary coronary branches with moderate to severe disease. "Lipid transformation" of the adluminal component of the intima occurred very early post-transplant and before significant luminal narrowing had evolved (Figure 7, a and b). On dual staining of selected sections with oil red O for lipid and immunohistochemically (HHF-35) for smooth muscle cells, smooth muscle cells were shown to be lipid laden (Figure 7c).

Intra- and extracellular fat was generally less prominent histopathologically within the media than in the intima (Figure 7, d–f). However, in some arteries there was abundant intra- and extracellular medial lipid on standard and special histochemical stains (Figure 7, g–i) and on ultrastructural examination (Figure 8a). The medial lipid was associated with glycosaminoglycans on Movat's pentachrome stains, and in the most prominent cases was present in large extracellular pools or in contiguity with foam cell aggregates. It appears that *in situ* transformation of medial smooth muscle cells into foam cells occurs in absence of excessive disruption of the internal elastic lamina (Figure 7, g and h).

On transmission electron microscopy, intracellular intimal and medial lipid was most commonly present

Table 3. *Correlations between Percent Luminal Narrowing, as Measured in Perfusion-Fixed Left Anterior Descending Coronary Artery Segments, and Arterial Wall Lipid Contents and Concentrations in Proximal and Distal Right Coronary Artery Segments from the Correspondent Allografts*

	Lipid content (μg/cm² intimal surface area)				Lipid concentration (μg/mg dry defatted weight)			
	Proximal		Distal		Proximal		Distal	
Lipid fraction	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Total cholesterol	0.60	<0.04	0.89	<0.007	0.61	<0.03	0.87	<0.01
Free cholesterol	0.53	<0.08	0.92	<0.003	0.56	<0.05	0.94	<0.002
Esterified cholesterol	0.64	<0.03	0.84	<0.02	0.63	<0.03	0.80	<0.03
Phospholipid	0.60	<0.06	0.50	<0.25	0.45	<0.19	0.88	<0.009

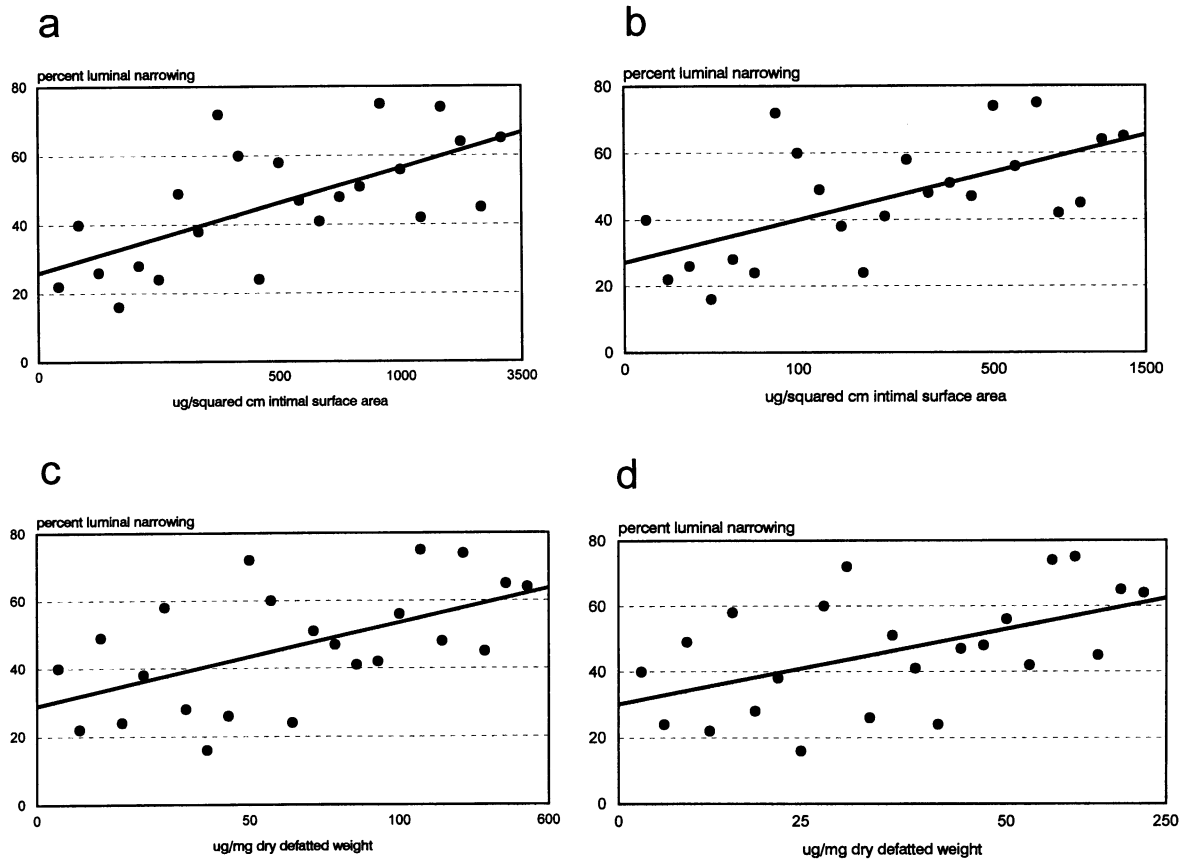


Figure 5. *Lipid content of quantitated total* (a) *and free cholesterol* (b) *as well as lipid concentration of quantitated total* (c) *and free cholesterol* (d) versus *luminal narrowing.*

within smooth muscle cells (Figure 8a). These smooth muscle cells were also in close association with glycosaminoglycan-rich matrix (Figure 8e). Macrophages and endothelial cells were also involved by lipid to the point of foam cell formation (Figure 8, b and f), but less often than smooth muscle cells. In deeper atheromatous cores, cholesterol cleft formation was observed (Figure 8c). Scanning electron microscopy revealed extracellular, spherical particles suggestive of lipid droplets or lipoprotein aggregates measuring about 1.5 to 4.5 μm in diameter, and being widely dispersed in the intima and media (Figure 8d). The aggregates are reminiscent of those observed in rabbit models of atherosclerosis.[39,40]

Discussion

Our studies of epicardial coronary arteries of human heart allografts have documented a striking deposition of both intracellular and extracellular lipids within the superficial and deep intima and media of mildly, moderately, and markedly narrowed arteries. Ultrastructural examination demonstrated that much of the

Table 4. *Correlation of Arterial Wall Lipid Values with Implant Duration*

Lipid fraction	Lipid content (μg/cm² intimal surface area)		Lipid concentration (μg/mg dry defatted weight)	
	r	*P*	*r*	*P*
Total cholesterol	0.1	0.65	0.19	0.37
Free cholesterol	0.11	0.6	0.22	0.31
Esterified cholesterol	0.09	0.69	0.17	0.43
Phospholipid	0.1	0.64	0.18	0.42

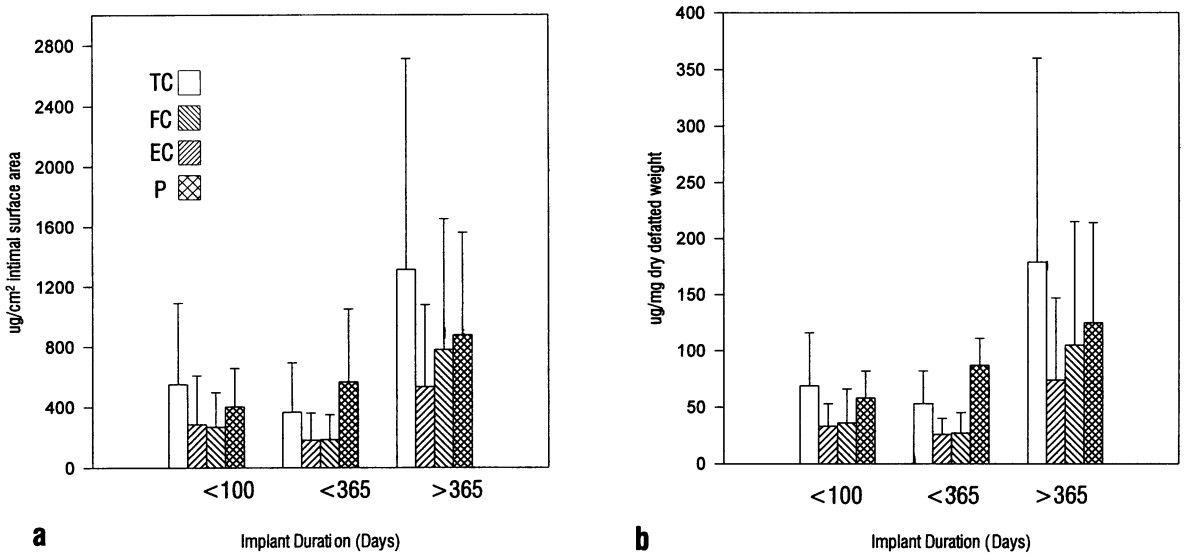


Figure 6. *Lipid fractions of coronary artery segments from 23 cardiac allografts displayed as three discrete post-transplant intervals, <100 days, <365 days, >365 days. As noted in the text, and in Table 4, no significant relationship between implant interval and arterial lipid levels was found. Standard deviations are indicated.* a. *Lipid content (μg/cm² intimal surface area),* b. *Lipid concentration (μg/mg dry defatted weight). EC, esterified cholesterol; FC, free cholesterol; P, phospholipids; TC, total cholesterol.*

intracellular intimal and medial lipid is within smooth muscle cells. Extracellular lipid deposition is especially prominent within deep atheromatous cores in the intima and was often associated with focally disrupted internal elastic laminae. Lipoprotein aggregates apparently occur within the intima and media of arteriopathic vessels. Content and concentration of lipids as determined by quantitative biochemistry are markedly elevated in the within the walls of the coronary arteries. Although pre-transplant and post-transplant serum lipid levels in these patients were not markedly elevated, the individual, mean, and typical coronary tissue levels of total, free, and esterified cholesterol, and of phospholipids were greater than the 90th percentile of donor age-comparable, site-matched PDAY coronary control tissue. In many coronary trees, the amount of lipid was 10 to 20 times more than normal native artery limits in donor age-comparable vessels. Thus, the lipid overload of allograft coronary arteries occurs in the face of only a modest blood-to-artery lipoprotein gradient, and much more rapidly than occurs in native atherosclerosis. Deposition of lipid within the arterial walls was prominent over a wide range of luminal narrowing by the arteriopathic process. Overall, a strong correlation between the amount of luminal narrowing and the content or concentration of lipids was demonstrated. Lipid deposition in the intima and media of coronary arteries in transplanted hearts appears to play an important early and ongoing role in transplant arteriopathy.

The trend toward more arterial wall lipids proximally appears to relate to the absolute mass of intima that is associated with a certain percent luminal narrowing or certain extent of intimal thickening in a proximal vessel *versus* a distal vessel. Thus, when lipid accumulates in the proximal vessels, be it the LC or the R, it is accumulating in a much larger mass of intima for the absolute intimal surface area of a proximal segment *versus* a distal segment. In addition, from our previous morphometric analyses,[30] we know that, by two independent quantitative assessments the intima is thicker proximally than distally in human arteriopathy. It is likely, because of the disproportion in mass

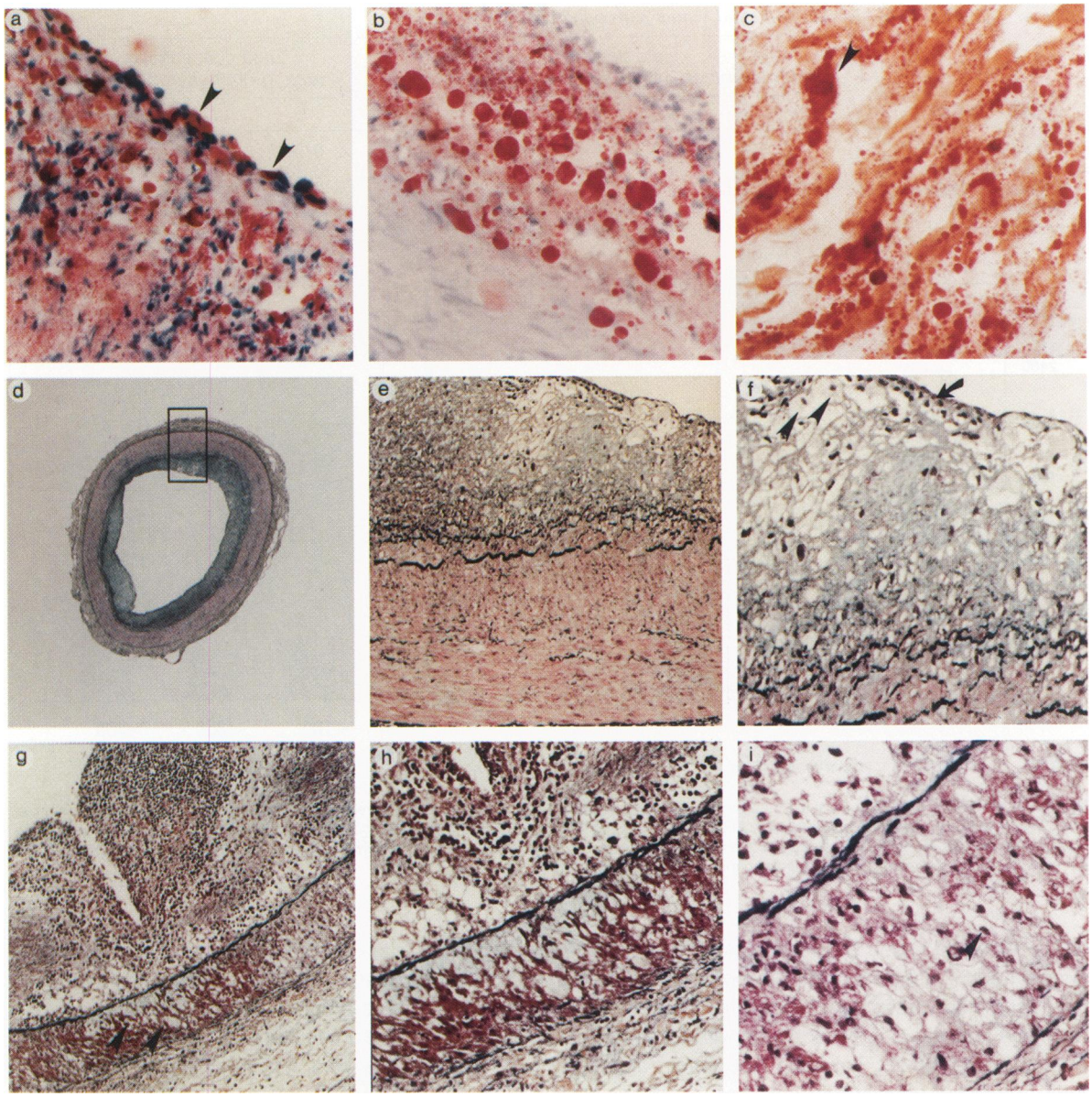


Figure 7. *Photomicrographs of oil red O stained lipid droplets and early and late lesions stained with Movat's pentachrome in allograft coronary arteries.* **a:** *Several fine lipid droplets are seen within the intima. In addition, numerous medium size lipid droplets are observed within endothelial cells at the luminal surface (arrowheads) (×100).* **b:** *Many large extracellular lipid droplets are observed within a mildly thickened intima (×100).* **c:** *Section of allograft coronary artery double-stained, histochemically with oil red O, and immunohistochemically with muscle specific α-actin antibody. Lipid droplets are extracellularly and intracellularly associated with smooth muscle cells (arrowhead) (×320).* **d:** *Early lesion in arteriopathic vessel exhibiting moderate intimal thickening rich in lipids and glycosaminoglycans (aqua color) (box) (×33).* **e:** *High power of a segment of allograft artery seen in* (**d**) *displaying prominent lipid deposits in close association with intimal glycosaminoglycan-rich matrix (aqua color) (×125).* **f:** *Lipid deposits are both intracellular in foam cells (arrowheads) and extracellular. A small subendothelial cellular infiltrate is observed (curved arrow) (×330).* **g:** *Late arteriopathic lesion is characterized by markedly inflamed intima with deep lipid deposits adjacent to the internal elastic lamina. The intima and media have a high concentration of glycosaminoglycans (arrowheads) in close proximity to lipid deposits (×33).* **h:** *On higher power, the medial lipid richness and glycosaminoglycan-rich matrix are emphasized, particularly adjacent to the internal elastic lamina (×66).* **i:** *The media has extensive foam cell transformation (arrowhead) with slight inflammation; both intracellular and extracellular lipid deposits are observed (×500).*

or volume of intima between equally thickened proximal and distal segments (required to produce a given amount of luminal narrowing proximally as opposed to distally), the proximal ratio (expressed as micrograms per centimeter squared intimal surface for the different lipid components) is greater than the distal. This observation is in contrast to the results in Figure 4 in which the proximal LC has a tendency toward

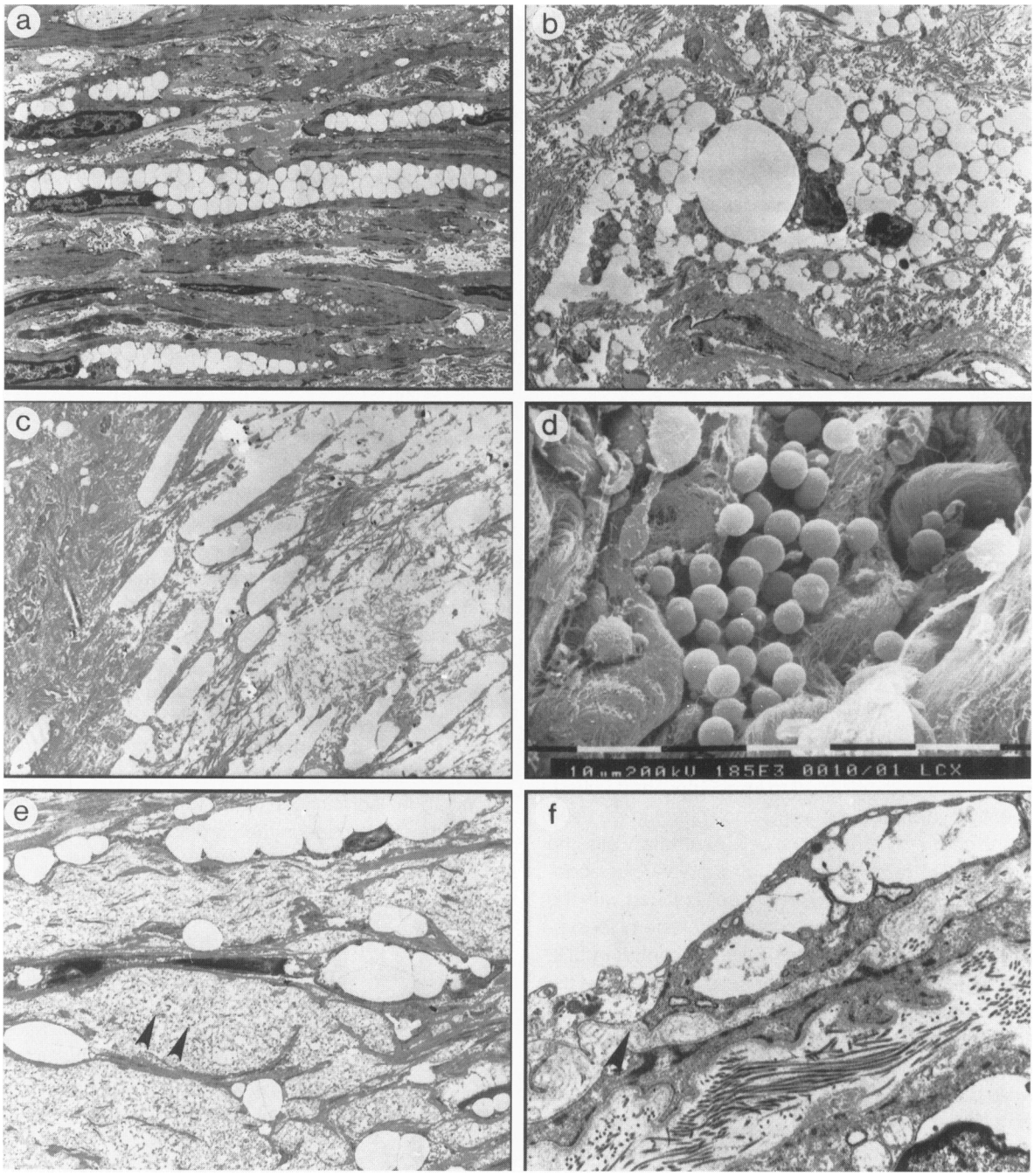


Figure 8. *Electron micrographs of arteriopathic coronary arteries (transmission:* **a–c, e, f;** *scanning:* **d**). **a:** *Numerous medial smooth muscle cells have prominent intracellular lipid droplets. Collagen fibers are present between the arrays of smooth muscle cells (×56000).* **b:** *A large cell resembling a monocyte-macrophage with engulfed lipid particles possibly surrounded by several large extracellular lipid droplets. Elastin and collagen fibers are observed at the bottom of the photomicrograph (×56000).* **c:** *Large deposits of extracellular lipid and "cholesterol cleft" formation are present deep within an allograft vessel (×54000).* **d:** *Scanning electron micrograph of medial tissue from transversely sectioned allograft coronary artery suggests probable extracellular lipid droplets or lipoprotein aggregates in proximity to smooth muscle cells. These structures may have accumulated on a primary basis extracellularly or from breakdown of foam cells. These aggregates (1.5–4.5 µm diameter) may represent complexes of lipoproteins and associated proteoglycans[74,75] (×1850).* **e:** *Extensive deposition of glycosaminoglycan-rich matrix (arrowheads) with foam cell transformation of smooth muscle cells depicted (×54000).* **f:** *Intracellular vacuoles, possibly representing lipid, are illustrated within an endothelial cell. Tight junctions are visible between endothelial cells and the basement membrane is depicted (arrowhead) (×55000).*

more (not significant) total cholesterol and phospholipid (as μg/mg dry defatted weight) than the proximal R or distal R.

The most important issue in this analysis relates to donor age-comparable arteries from control subjects (PDAY), wherein the precisely identical coronary location was used and one can evaluate the degree to which the transplant milieu has taken arteries of comparable age and accelerated the allo-atheromatous process. While comparisons to severely atherosclerotic arteries from non-transplant patients may be important, they are beyond the scope of this particular analysis and would not answer the question which we originally posed. Such information is widely available.[34]

In an attempt to understand how lipid might accumulate at such an accelerated rate in the arterial wall of human heart allografts, it is important to consider mechanisms by which lipid accumulation is proposed to occur within native atherosclerotic lesions. These "naturally occurring" mechanisms may provide a basis for understanding the aberrant lipid accumulation within arterial walls of human heart allografts. It is widely known that in the face of rising blood lipoprotein levels, increased deposition of lipids occurs in the arterial wall during atherosclerosis.[41,42] Cells normally acquire cholesterol for membrane synthesis primarily by receptor-mediated uptake of LDL which is internalized and delivered to lysosomes, and subsequently hydrolyzed releasing cholesterol to be used by the cell.[43–44] To reach the cells of arterial walls, the plasma LDL must pass through the vascular endothelium. *In vivo*, the endothelial cell is faced with the dual function of taking up plasma cholesterol for synthesis of its own membranes, and transporting LDL and other lipoproteins to other cells in the arterial tissue. It has been demonstrated that LDL is taken up and internalized through two parallel routes[45]; a relatively small amount of LDL is taken up by endocytosis, while most circulating LDL is transported across the endothelial cell by transcytosis via plasmalemmal vesicles. Disturbances in the capability of the endothelium to regulate transport of a physiological amount of LDL may lead to excessive accumulation of cholesterol within the arterial wall, much as in native atherosclerosis.[46–48] With increased levels of blood cholesterol, triglycerides, and lipoproteins in transplant patients,[2,49,50] transcytosis of LDL cholesterol and other molecules across arterial endothelium may be accelerated. In both normal and atherosclerotic arteries, LDL enters the intima in relatively large amounts.[51–53]

Virchow[54] proposed the concept that vessel injury initiates lipid imbibition and atherogenesis, and a number of investigators have contributed to the "response to injury" hypothesis of atherogenesis.[55–60] This hypothesis proposes that atherogenesis involves injury to the endothelium as the triggering event in the atherogenic process. Various investigators have shown that such injury leads to alterations in arterial permeability to lipid. Hajjar et al[60] have shown that lipids, especially cholesterol and cholesteryl esters, preferentially accumulate in re-endothelialized, as compared with de-endothelialized, areas of the aorta. This accumulation is due to two metabolic changes in the endothelial cells of re-endothelialized areas including a decrease in acid cholesteryl esterase activity with decreased cholesteryl ester hydrolysis, and an increase in acyl CoA:cholesterol acyltransferase activity with increased synthesis of cholesteryl esters. Davies et al[61] have demonstrated that mechanical wounding of endothelial cell cultures results in a significantly enhanced rate of pinocytosis per mg cell protein and supports the concept that pinocytosis in growing cells proceeds at a higher rate than in non-growing, quiescent cells. These experiments further support the possibility that enhanced transendothelial pinocytotic transport of lipoproteins by regenerating endothelium may have bearing on the deposition of lipids in areas of injured endothelium. In the case of transplant arteriopathy, endothelial injury not only may be important to the pathogenesis of the disease, but also may contribute to lipid accumulation. Endothelial damage in the cardiac allograft followed by de-endothelialization with subsequent re-endothelialization would alter lipid permeability and result in elevated transport of lipoprotein across the arterial wall thereby leading to lipid accumulation. An important question to consider regarding transplant arteriopathy is whether significant de-endothelialization with subsequent re-endothelization due to injury actually occurs. Focal denudation may occur repeatedly as waves of allo-immunological response occur to the graft. This issue has never been settled.

A third process which may explain rapid and excess lipid accumulation relates to differences in efflux of LDL, instead of increased lipid permeability. Arterial wall cells of the allograft with a decreased ability to remove LDL may achieve elevated LDL concentrations. It has been shown that LDL accumulates to a greater extent in lesion-susceptible than in lesion-resistant sites of the aorta of cholesterol-fed, normolipidemic rabbits.[62] It was demonstrated that the permeability to LDL did not increase in these lesion-susceptible sites as compared to lesion-resistant sites. Instead, the fractional rate of LDL efflux of the

arterial pool was lower in the lesion-prone sites than in lesion-resistant sites, resulting in LDL retention.[62]

Decreased LDL efflux could possibly be related to increased interactions of LDL with the extracellular matrix of the arterial wall. Such interactions may overshadow cellular processes designed to remove LDL. A similar mechanism could be responsible for elevated arterial lipid levels in human heart allografts, where large amounts of extracellular matrix accumulates. Proteoglycans in particular are thought to be of considerable significance in atheromatous processes. They modify lipids, cell adhesion and migration, and interact with growth factors which function to control cellular proliferation. As noted, a number of studies have demonstrated that LDL particles complex with proteoglycans via very specific basic amino acid residues,[68] and that these complexes stimulate uptake of lipid by macrophages, cholesterol ester synthesis, and conversion of macrophages into foam cells.[63–65] In addition to affecting intracellular processes, proteoglycan deposits within atherosclerotic plaques exhibit a propensity for lipid accumulation, and much of the lipoprotein in the arterial wall is believed to be complexed extracellularly to proteoglycan.[66] Our morphological investigation of transplanted arteries reflects the marked intra- and extracellular lipid accumulation in all stages of arteriopathic disease, and the role of proteoglycans in this process is most likely very significant (submitted for publication).

A fourth mechanism potentially responsible for lipid accumulation in transplant arteriopathy involves elevated lipid synthesis by cells within lesions. Cultured aortic cells from atherosclerotic lesions have an enhanced rate of lipid synthesis in comparison to non-atherosclerotic aortic cells,[67] which is further enhanced as the cells accumulate more lipid. Such a mechanism could be responsible for accumulation of lipid within arteriopathic lesions. Cytokines and growth factors are quite feasibly involved.

Finally, most allograft recipients are managed on immunosuppressive regimens involving cyclosporin, corticosteroids, and azathioprine to prevent graft rejection. Cyclosporin acts primarily on T helper lymphocytes, blocking interleukin-2-mediated proliferation.[68] Both cyclosporin and prednisone induce elevations in total cholesterol, LDL cholesterol, triglycerides, and apolipoprotein B100.[2,14,49,50,69,70] Cyclosporin may exert an increase in plasma LDL through the inhibition of enzymes in the bile acid pathway, reducing bile acid synthesis from cholesterol.[71] Lipoprotein (a) metabolism may also be affected by cyclosporin, given the relationship between LDL and lipoprotein (a). Lipoprotein (a) is an independent risk factor in coronary heart disease[72] and has been localized to arteriopathic lesions.[73] Cyclosporin itself is highly lipophilic; about 50% of a dose circulates in the plasma, of which 80% is bound to lipoproteins, especially LDL and HDL.[74] *In vitro* cyclosporin has a cytopathic effect on smooth muscle cell proliferation and promotes vacuolization[75,76]; however, *in vivo* it has a different effect. Cyclosporin treatment of de-endothelialized animals potentiates atheromatous lesion development.[77] The absence of endothelial injury of this interface by immune processes in allografts may contribute to potential for cyclosporin to participate in the arteriopathic process. In animals undergoing hyperalimentation[77] atheromatous lesions are also enhanced as compared with controls. In such animal models intimal thickening occurs early and is much more severe in cyclosporin-treated than in cyclosporin-untreated groups. Thus, cyclosporin accelerates the development of disease. While cyclosporin may augment arteriopathic lesions it is worth emphasizing that atheromatous arteriopathic lesions existed long before the first clinical use of the drug.

The data presented in this study are the first to document quantitatively the massive accumulation of lipids in arteries of human heart allografts. Clearly, this disease process has a major atheromatous component. The concurrent accumulation of lipids and proteoglycan-rich matrix may be causally related and the entire process deserves further examination.

Acknowledgments

The authors appreciate the technical assistance of Martin Cano with scanning electron microscopy and Patricia Best for transmission electron microscopy. Dr. Robert W. Wissler provided critical control samples from the PDAY study. Dr. Lorraine Verbugt contributed valuable statistical analyses. Sheldon L. Thieszen assisted with oil red O staining. Additionally, Dr. Dale G. Renlund graciously provided data on two allografts.

References

1. Billingham ME: Cardiac transplant atherosclerosis. Transplant Proc 1987, 19(Suppl 5):19–25
2. Winters GL, Kendall TJ, Radio SJ, Wilson JE, Costanzo-Nordin MR, Switzer BL, Remmenga JA, McManus BM: Posttransplant obesity and hyperlipidemia: major predictors of severity of coronary arteriopathy in failed human heart allografts. J Heart Transplant 1990, 9:364–371
3. Thomson JG: Production of severe atheroma in a transplanted human heart. Lancet 1969, 2:1088–1092

4. Bieber CP, Stinson EB, Shumway NE, Payne R, Kosek J: Cardiac transplantation in man. VII. Cardiac allograft pathology. Circulation 1970, 41:753–772
5. Kosek JC, Bieber C, Lower RR: Heart graft arteriosclerosis. Transplant Proc 1971, 3:512–514
6. Uys CJ, Rose AG: Pathologic findings in long-term cardiac transplants. Arch Pathol Lab Med 1984, 108: 112–116
7. Davies H, Al-Tikriti S: Coronary arterial pathology in the transplanted heart. Int J Pathol 1989, 25:99–118
8. Foerster A: Vascular rejection in cardiac transplantation: a morphological study of 25 human cardiac allografts. APMIS 1992, 100:367–376
9. Radio SJ, Kendall TJ, Malcom GT, Winters GL, McManus BM: Localization of intra- and extracellular lipid in coronary arterial walls from human heart allografts: histochemical and ultrastructural evidence. Lab Invest 1992, 66:21
10. Liu G, Butany J: Morphology of graft arteriosclerosis in cardiac transplant recipients. Hum Pathol 1992, 23: 768–773
11. Busch GJ, Galvanek EG, Reynolds ES: Human renal allografts: analysis of lesions in long-term survivors. Hum Pathol 1971, 2:253–298
12. Rudas L, Pflugfelder PW, McKenzie FN, Menkis AH, Novick RJ, Kostuk WJ: Serial evaluation of lipid profiles and risk factors for development of hyperlipidemia after cardiac transplantation. Am J Cardiol 1990, 66:1135–1138
13. Harris KPG, Russell GI, Parvin SP, Veitch PS, Walls J: Alterations in lipid and carbohydrate metabolism attributable to cyclosporin A in renal transplant recipients. Br Med J 1986, 292:16
14. Raine AEG, Carter R, Mann JI, Chapman JR, Morris PJ: Increased plasma LDL cholesterol after renal transplantation associated with cyclosporine immunosuppression. Transplant Proc 1987, 19:1820–1821
15. Ballantyne CM, Radovancevic B, Farmer JA, Frazier H, Chandler L, Payton-Ross C, Coranougher B, Jones PH, Young JB, Gotto AM: Hyperlipidemia after heart transplantation: report of a 6-year experience, with treatment recommendations. J Am Coll Cardiol 1992, 19:1315–1321
16. Tanaka H, Sukhova GK, Libby P: Interaction of the allogeneic state and hypercholesterolemia in arterial lesion formation in experimental cardiac allografts. Arterioscler Thromb 1994, 14:734–745
17. Gao SZ, Schroeder JS, Hunt S, Stinson EB: Retransplantation for severe accelerated coronary artery disease in cardiac transplant recipients. Am J Cardiol 1988, 62:876–881
18. Hess ML, Hastillo A, Mohanakumar T, Cowley MJ, Vetrovac G, Szentpetery S, Wolfgang TL, Lower RR: Accelerated atherosclerosis in cardiac transplantation: role of cytotoxic B-cell antibodies and hyperlipidemia. Circulation 1983, 68(Suppl II):II-94
19. Uretsky BF, Murali S, Reddy PS, Rabin B, Lee A, Griffith BP, Hardesty RL, Trento A, Bahnson HT: Development of coronary artery disease in cardiac transplantation patients receiving immunosuppressive therapy with cyclosporine and prednisone. Circulation 1987, 76:827–834
20. Gao SZ, Schroeder JS, Alderman EL: Clinical and laboratory correlates of accelerated coronary artery disease in the cardiac transplant patient. Circulation 1987, 76:56–61
21. Brasile L, Zerbe T, Rabin B, Clarke J, Abrams A, Cerilli J: Identification of the antibody to vascular endothelial cells in patients undergoing cardiac transplantation. Transplantation 1985, 40:672–675
22. Dunn MJ, Crisp SJ, Rose ML, Taylor PM, Yacoub MH: Anti-endothelial antibodies and coronary artery disease after cardiac transplantation. Lancet 1992, 339: 1566–1570
23. Hruban RH, Beschorner WF, Baumgartner WE, Augustine SM, Ren H, Reitz BA, Hutchins GM: Accelerated atherosclerosis in heart transplant recipients is associated with a T-lymphocyte-mediated endothelialitis. Am J Pathol 1990, 137:871–882
24. Salomon RN, Hughes CCW, Schoen FJ, Payne DP, Pober JS, Libby P: Human coronary transplantation-associated arteriosclerosis: evidence for a chronic immune reaction to activated graft endothelial cells. Am J Pathol 1991, 138:791–798
25. Fabricant CG, Fabricant J, Litrenta MM, Minick CR: Virus-induced atherosclerosis. J Exp Med 1978, 148: 335–340
26. Hajjar DP, Fabricant CG, Minick CR, Fabricant J: Virus-induced atherosclerosis: herpesvirus infection alters cholesterol metabolism and accumulation. Am J Pathol 1986, 122:62–70
27. Hajjar DP, Nicholson AC, Hajjar KA, Sando GN, Summers BD: Decreased messenger RNA translation in herpesvirus-infected arterial cells:effects on cholesteryl ester hydrolase. Proc Natl Acad Sci USA 1989, 86:3366–3370
28. Grattan MT, Moreno-Cabral CE, Starnes VA, Oyer PE, Stinson EB, Shumway NE: Cytomegalovirus infection is associated with cardiac allograft rejection and atherosclerosis. JAMA 1989, 261:3561–3566
29. Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE: Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 1994, 265:391–394
30. Lin H, Wilson JE, Kendall TJ, Radio SJ, Cornhill FJ, Herderick E, Winters GL, Costanzo MR, Porter T, Thieszen SL, McManus BM: Comparable proximal and distal severity of intimal thickening and size of epicardial coronary arteries in transplant arteriopathy of human cardiac allografts. J Heart Lung Transplant 1994, 13:824–833
31. Linder J, Cassling RS, Rogler WC, Wilson JE, Markin RS, Sears TD, McManus BM: Immunohistochemical characterization of lymphocytes in ventricular myocardium. Arch Pathol Lab Med 1985, 109:917–920
32. Chow LH, Ye YL, Linder J, McManus BM: Phenotypic

analysis of infiltrating cells in human myocarditis: an immunohistochemical study in paraffin-embedded tissue. Arch Pathol Lab Med 1989, 113:1357–1362
33. Malcom GT, Strong JP: The expression of results of lipid determinations in arterial tissues: mass per unit weight vs mass per unit area. Atherosclerosis 1981, 40:273–277
34. Malcom GT, Strong JP, Restrepo C: Atherosclerosis and lipid composition of the abdominal aorta. Lab Invest 1984, 50:79–86
35. Folch J, Ascoli I, Lees M, Meath JA, LeBaron FN: Preparation of lipid extracts from brain tissue. J Biol Chem 191;1951:833–841
36. Micholson OB: Photometric determinations of phosphorous as molbdovanadophosphoric acid. Anal Chem 1957, 29:60–69
37. McManus BM, Malcom G, Kendall TJ, Gulizia JM, Wilson JE, Winters G, Costanzo MR, Thieszen ST, Radio SR: Lipid overload and proteoglycan expression in chronic rejection of the human transplanted heart. Clin Transplant 1994, 8:336–340
38. Dunmore PJ, Song SH, Roach MR: A comparison of the size of fenestrations in the internal elastic lamina of young and old porcine aortas as seen with the scanning electron microscope. Can J Physiol Pharmacol 1990, 68:139–143
39. Guyton JR, Klemp KF: Early extracellular and cellular lipid deposits in aorta of cholesterol-fed rabbits. Am J Pathol 1992, 141:925–936
40. Berliner JA, Gerrity RG: Cellular events: pathology of atherogenesis. Molecular Genetics of Coronary Artery Disease. Edited by Lusis AJ, Rotter JI, Sparkes RS. Basel, Switzerland, Karger, 1992, pp 1–15
41. Nicoll A, Duffield R, Lewis B: Flux of plasma lipoproteins into human arterial intima: comparison between grossly normal and atheromatous intima. Atherosclerosis 1981, 39:229–242
42. Schonfeld G: Lipoproteins in atherogenesis. Artery 1979, 5:305–329
43. Brown MS, Kovanen SPT, Goldstein JL: Regulation of plasma cholesterol by lipoprotein receptors. Science 1981, 212:628–635
44. Goldstein JL, Brown MS: The low-density lipoprotein pathway and its relation to atherosclerosis. Annu Rev Biochem 1977, 46:897–930
45. Vasile E, Simionescu M, Simionescu N: Visualization of the binding, endocytosis, and transcytosis of low-density lipoprotein in the arterial endothelium in situ. J Cell Biol 1983, 96:1677–1689
46. Falcone DJ, Hajjar DP, Minick CR: Enhancement of cholesterol and cholesteryl ester accumulation in re-endothelialized aorta. Am J Pathol 1980, 99:81–104
47. Fielding PE, Vlodavsky D, Gospodarowicz D, Fielding CJ: Effect of contact inhibition on the regulation of cholesterol metabolism in cultured vascular endothelial cells. J Biol Chem 1979, 254:749–755
48. Minick CR, Stemerman MB, Insull W: Effect of regenerated endothelium on lipid accumulation in the arterial wall. Proc Natl Acad Sci USA 1977, 74:1724–1728
49. Rudas L, Pflugfelder PW, McKenzie FN, Menkis AH, Novick RJ, Kostuk WJ: Serial evaluation of lipid profiles and risk factors for development of hyperlipidemia after cardiac transplantation. Am J Cardiol 1990, 99:1135–1138
50. Hilbrands LB, Demacker PNM, Hoitsma AJ: Cyclosporin and serum lipids in renal transplant recipients. Lancet 1993, 341:765–766
51. Scott PJ, Hurley PJ: The distribution of radio-iodinated serum albumin and low density lipoprotein in tissues and the arterial wall. Atherosclerosis 1970, 11: 77–103
52. Sinapius D: Lipid deposition in the media of human coronary arteries. Atherosclerosis 1980, 37:87–96
53. Smith EB, Staples EM: Distribution of plasma proteins across the human aortic wall-barrier functions of endothelium and internal elastic lamina. Atherosclerosis 1980, 37:579–590
54. Virchow R: Phlogose und thrombose im gefabsystem. Gesammelte Abhandlungen zur Wissenschaftlichen Medicin. Frankfurt, Meidinger Sohn, 1856, p 458
55. Duguid JB: Pathogenesis of atherosclerosis. Lancet 1949, 2:925
56. French JE: Atherosclerosis in relation to the structure and function of the arterial intima, with special reference to the endothelium. Int Rev Exp Pathol 1966, 5:253–353
57. Ross R, Glomset JA: The pathogenesis of atherosclerosis. N Engl J Med 1976, 295:369–377:420–425
58. Ross R: The pathogenesis of atherosclerosis—a perspective for the 1990s. Nature 1993, 362:801–809
59. Ross R: The pathogenesis of atherosclerosis—an update. N Engl J Med 1986, 314:488–500
60. Hajjar DP, Falcone DJ, Fowler S, Minick CR: Endothelium modifies the altered metabolism of the injured aortic wall. Am J Pathol 1981, 102:28–39
61. Davies PF, Selden III SC, Schwartz SM: Enhanced rates of fluid pinocytosis during exponential growth and monolayer regeneration by cultured arterial endothelial cells. J Cell Physiol 1980, 102:119–127
62. Schwenke DC, Carew TE: Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. Arteriosclerosis 1989, 9:908–918
63. Camejo G, Olofsson S-O, Lopez F, Carlsson P, Bondjers G: Identification of Apo B-100 segments mediating the interaction of low density lipoproteins with arterial proteoglycans. Arteriosclerosis 1988, 8:368–377
64. Salisbury BFJ, Falcone DJ, Minick CR: Insoluble low-density lipoprotein-proteoglycan complexes enhance cholesteryl ester accumulation in macrophages. Am J Pathol 1985, 120:6–11
65. Vijayagopal P, Srinivasan SR, Radhakrishnamurthy B, Berenson GS: Lipoprotein-proteoglycan complexes

from atherosclerotic lesions promote cholesteryl ester accumulation in human monocytes/macrophages. Arterioscler Thromb 1992, 12:237–249
66. Camejo G: The interactions of lipids and lipoproteins with the intercellular matrix of arterial tissue: its possible role in atherogenesis. Adv Lipid Res 1982, 19: 1–53
67. Orevhov AN, Tertov VV, Smirnov VN: Lipids in cells of atherosclerotic and uninvolved human aorta. II. Lipid metabolism in primary culture. Exp Mol Pathol 1985, 195:43:187
68. Borel JF, Feurer C, Bubler HV, Staehelin H: Biological effects of cyclosporin A: a new anti-lymphocytic agent. Agent Actions 1976, 6:468–475
69. Harris KPG, Russell GI, Parvin SP, Veitch PS, Walls J: Metabolic effects of conversion from cyclosporine to azathioprine in renal transplant recipients. Proc EDTA-ERA 1985, 21:1010–1014
70. Segarra A, Chacon P, Vilardell M, Piera LL: Cyclosporin and serum lipids in renal transplant recipients. Lancet 1993, 341:766
71. Prince HMG, Meijer P, Hofstee B, Havekes LM, Kuipers F, Vonk RJ: Effects of cyclosporin A on LDL-receptor activity and bile cultures and in vivo in rat. Hepatology 1987, 7:1109 (abstr)
72. Sandkamp M, Funke H, Schulte H, Kohler E, Assmann G: Lipoprotein (a) is an independent risk factor for myocardial infarction at a young age. Clin Chem 1990, 36:20–23
73. Beisiegel U, Niendorf A, Wolf K, Reblin T, Rath M: Lipoprotein (a) in the arterial wall. Eur Heart J 1990, 11-(suppl E):174–183
74. Gurecki J, Warty V, Sanghvi A: The transport of cyclosporine in association with plasma lipoproteins in heart and liver transplant patients. Transplant Proc 1985, 1997;17:2002
75. Leszczynski D, Zhao Y, Yeagley TJ, Foegh ML: Direct and endothelial cell-mediated effect of cyclosporin A on the proliferation of rat smooth muscle cells in vitro. Am J Pathol 1993, 142:149–155
76. Ferns G, Reidy M, Ross R: Vascular effects of cyclosporine A in vivo and in vitro. Am J Pathol 1990, 137: 403–413
77. Emeson EE, Shen ML: Accelerated atherosclerosis in hyperlipidemic C57BL/6 mice treated with cyclosporin A. Am J Pathol 1993, 142:1906–1915