Hypercholesterolemia Increases Coronary Endothelial Dysfunction, Lipid Content, and Accelerated Atherosclerosis After Heart Transplantation

Louis P. Perrault, Florence Mahlberg, Christine Breugnot, Jean-Pierre Bidouard, Nicole Villeneuve, Jean-Paul Vilaine, Paul M. Vanhoutte

Abstract—Hyperlipidemia may increase endothelial damage and promote accelerated atherogenesis in graft coronary vasculopathy. To study the effects of hypercholesterolemia on coronary endothelial dysfunction, intimal hyperplasia, and lipid content, a porcine model of heterotopic heart transplantation, allowing nonacute rejection without immunosuppressive drugs, was used. A high cholesterol diet was fed to donor and recipient swine 1 month before and after transplantation. The endothelial function of coronary arteries of native and transplanted hearts from cholesterol-fed animals was studied in organ chambers 30 days after implantation and compared with endothelial function in arteries from animals fed a normal diet. The total serum cholesterol increased 3-fold in donors and recipients. Endothelium-dependent relaxations to serotonin, to the α2-adrenergic agonist UK14,304, and to the direct G-protein activator sodium fluoride were decreased significantly in allografted hearts compared with native hearts from both groups. relaxations to the calcium ionophore A23187 and bradykinin were decreased significantly in allografts from animals fed the high cholesterol diet. The prevalence of intimal hyperplasia was significantly increased in coronary arteries from hypercholesterolemic swine. There was a significant increase in the lipid content of allograft arteries of hypercholesterolemic recipients. Hypercholesterolemia causes a general coronary endothelial dysfunction, increases the prevalence of intimal hyperplasia, and augments the incorporation of lipids in the vascular wall after heart transplantation. Hyperlipidemia accelerates graft coronary atherosclerosis through its effects on the endothelium. (Arterioscler Thromb Vasc Biol. 2000;20:728-736.)

Key Words: endothelium • lipids • coronary arteries • transplantation • atherosclerosis

The normal endothelium contributes to the local regulation of vasomotor tone and the maintenance of a nonthrombogenic surface, acts as a selective barrier controlling permeability and transport, and regulates the adhesion and extravasation of neutrophils, monocytes, and lymphocytes and the proliferation of underlying vascular smooth muscle cells. These properties are due to the ability of endothelial cells to secrete endothelium-derived relaxing factors such as nitric oxide, endothelium-derived hyperpolarizing factor, and prostacyclin. Endothelial dysfunction is a fundamental initial step in the progression of atherosclerosis. Although heart transplantation remains the treatment of choice for medically unresponsive terminal heart disease and is associated with a 5-year survival of approximately 70%, coronary graft vasculopathy develops in a majority of transplant recipients and is the main cause of death beyond the first year after transplantation. Accelerated atherosclerosis is preceded by reduced dilatation of the coronary artery to endothelium-dependent agonists. This endothelial dysfunction is due to an immunologic injury directed at the endothelial cells and to other factors that cause endothelial activation and trigger a cascade of pathological events. Endothelial dysfunction is predictive of the development of graft coronary disease, which can be detected by intracoronary ultrasound, 1 year after graft implantation and of the occurrence of morbidity and death after transplantation.

In experimental animals and humans, hypercholesterolemia impairs endothelium-dependent relaxations both in the macrocirculation and microcirculation, which can occur in the absence of intimal thickening. In the early stage of the atherosclerotic process, endothelial dysfunction is limited to the pertussis toxin–sensitive Gi–protein–dependent pathway leading to nitric oxide formation. Oxidized LDLs induce, in vitro, a similar selective Gi–protein pathway leading to endothelial dysfunction and, at high concentrations, inhibit endothelium-dependent responses evoked by receptor-independent stimuli (A23187). Hypercholesterolemia increases production of the superoxide anion, which scavenges the endothelium-derived vasodilator, nitric oxide.

Hypercholesterolemia occurs in up to 80% of patients after transplantation, with significant increases in the ratio of total cholesterol to HDL cholesterol creating an atherogenic
miliu, and is incriminated in the development of graft atherosclerosis, early myocardial infarction, and death. Dietary and pharmacological control of dyslipidemia can revert the endothelial dysfunction and slow the progression of senile atherosclerosis, leading to an increased overall survival and freedom from cardiac ischemic events. Pravastatin reverses the early endothelial dysfunction and decreases the occurrence of graft coronary vasculopathy.

The purpose of the present study was to determine the effects of diet-induced hypercholesterolemia (1) on coronary endothelial dysfunction due to immune injury after transplantation, (2) on the development of intimal thickening in the vascular wall of coronary arteries, and (3) on the cholesterol content of coronary arteries from allografted hearts in a porcine model of heterotopic heart transplantation. The working hypothesis is that hypercholesterolemia is a compounding injurious factor to the endothelium of coronary arteries that is due to the immune injury from recipient cells and that it accelerates the development of vasculopathy, which is manifested by increased intimal hyperplasia and incorporation of lipids in the vascular wall.

Methods

Animals and Immunologic Studies
Forty-nine Large-White swine (EARL de Fresnelles, Boisemont, France) of either sex, aged 12 ± 0.8 weeks and weighing 20.2 ± 0.9 kg, were used. The experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85–23, revised 1985). All procedures used in the present study were approved by the local institutional committee on animal care. Preoperative blood samples were drawn for determination of blood type and the class I antigens of the Swine Lymphocytes Alloantigen (SLA) system by the microlymphocytotoxicity technique. Swine from the same litter were used to ensure a rate of recombination between the class I and class II regions of the major histocompatibility complex of less than 1% (Institut National de Recherches Agronomiques). The transplantations were performed between animals compatible for blood type and for the SLA class I antigen.

There was no statistically significant difference in sex distribution, age, or weight at the time of transplantation of donors and recipients in the normocholesterolemic group versus the high cholesterol group (data not shown).

Experimental Groups and Diets
Two different diet regimens were used: a standard piglet chow ad libitum (number 8, Pietrement) and a high cholesterol (2% cholesterol plus 10% lard) diet. To prevent excessive weight gain, the daily food intake was limited to an amount equal to 3% of body weight daily in swine fed the high cholesterol diet.

Six experimental groups were studied (Table 1). All animals were weighed weekly. Blood sampling was performed on day 1 of the diet, on the day of transplantation (4 weeks of the diet), and at euthanasia 30 days after graft implantation (8 weeks of the diet).

Regimens and Biochemistry Studies

Hematology Blood samples were processed for white and red blood cell counts, hemoglobin content, hematocrit, platelet number, and red blood cell indices (mean globular volume, mean globular hemoglobin content, and mean concentration of hemoglobin) by a Minos Vet analyzer (ABX).

Biochemistry Blood samples were processed with a Cobas 12 analyzer (Cobas-Mira, Roche) for liver function tests (lactate dehydrogenase, creatine kinase, glutamate oxalate transferase, glutamate pyruvate transferase, γ-glutamyltransferase, alkaline phosphatase, total bilirubin, albumin, and total protein content), renal function tests, and electrolytes (urea, creatinine, sodium, potassium, chloride, and calcium).

Serum Lipids Blood samples were processed for the following lipid profile with a Cobas 12 analyzer: total cholesterol, free cholesterol, triglycerides, phospholipids, and HDL cholesterol (HDL-C). LDL cholesterol (LDL-C) was calculated as the difference between total cholesterol and HDL-C.

Plasmatic Lipoprotein Distribution Studies Gel electrophoresis of the blood samples was performed for measurement of the HDL-C, LDL-C, and VLDL fractions of the cholesterol. Serum samples (40 mL) were left to coagulate at room temperature. Separation was performed on a polyacrylamide gel with a discontinuous gradient (Lipofilm SEBIA). Densitometric analysis of the lipid profiles was performed with a GS670 scanner (Bio-Rad). Dehydrated gels were stocked at room temperature by heating (warm air < 80°C).

Surgical Technique
Anesthesia and Cardiopulgia A 4:1 blood/crystalloid solution ratio was used to achieve cardioplegia of the donor heart. After systemic heparinization (3 mg/kg), a left retroperitoneal heterotopic transplant was performed with an end-to-side anastomosis between the donor ascending aorta and recipient abdominal aorta and between the donor main pulmonary artery and the recipient inferior vena cava. There was a statistically significant longer ischemic time in the normocholesterolemic group compared with the high cholesterol group (64 ± 6 versus 38 ± 3 minutes, respectively; P < 0.05; Table 2).

Postoperative Care
After standard ventilatory weaning, the animals were left to recover in temperature-controlled quarters. The preoperative diet was resumed, and water was fed ad libitum. No immunosuppressive drugs

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**TABLE 1. Experimental Groups**

<table>
<thead>
<tr>
<th>Type of Diet</th>
<th>Experimental Group</th>
<th>Duration of Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Control (n = 6)</td>
<td>8 wk</td>
</tr>
<tr>
<td>Standard native</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Standard allograft</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>High cholesterol (2% cholesterol + 10% lard)</td>
<td>Cholesterol control (no transplant) (n = 6)</td>
<td>8 wk</td>
</tr>
<tr>
<td>Cholesterol native</td>
<td>(n = 6)</td>
<td></td>
</tr>
<tr>
<td>Cholesterol allograft</td>
<td>(n = 6)</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2. Clinical Data: Standard Chow and High Cholesterol Transplant Groups**

<table>
<thead>
<tr>
<th></th>
<th>Standard Chow (n = 10)</th>
<th>High Cholesterol (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, wk</td>
<td>10 ± 0.4</td>
<td>12 ± 0.8</td>
</tr>
<tr>
<td>Sex ratio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Donor, male/female</td>
<td>8/2</td>
<td>3/3</td>
</tr>
<tr>
<td>Recipient, male/female</td>
<td>5/5</td>
<td>4/2</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>23.5 ± 1.4</td>
<td>20.2 ± 0.9</td>
</tr>
<tr>
<td>Ischemic time, min</td>
<td>64 ± 5</td>
<td>38 ± 3*</td>
</tr>
<tr>
<td>Contractility</td>
<td>(normal) 4/4</td>
<td>(normal) 4/4</td>
</tr>
<tr>
<td>Rejection grade</td>
<td>ISHLT 3B</td>
<td>ISHLT 3B</td>
</tr>
</tbody>
</table>

Age and weight values are mean ± SEM. *P < 0.05 vs standard chow.
were used. The recipients were euthanized 30 days after transplantation.

**Explantation Protocol and Experimental Groups**

In allografted hearts from normocholesterolemic and hypercholesterolemic swine, after pentobarbital anesthesia and ventilation, the allograft was excised rapidly. Native hearts were excised through a median sternotomy. Control hearts from nonoperated swine fed a 2% cholesterol+10% lard diet for 60 days and receiving the same sedation were also used. Hearts were excluded if the coronary arteries were thrombosed (n=4 allografted hearts 30 days after transplantation in the standard chow group). Experimental groups are summarized in Table 1.

**Vascular Reactivity**

The native, allografted, and control hearts were placed in a modified Krebs’ bicarbonate solution (composition in mmol/L: NaCl 118.3, KCl 4.7, MgSO4, 1.2, KH2PO4, 1.2, glucose 11.1, CaCl2, 2.5, NaHCO3, 25, and calcium EDTA 0.026; control solution). Oxygenation was ensured by using a 95% O2/5% CO2 gas mixture. The epicardial coronary arteries of the native and allografted hearts were dissected free from the epicardium, myocardium, and adventitial tissue and divided in rings (4 mm wide, 20 rings from the allograft and 20 rings from the native heart). Rings from the left anterior descending, left circumflex, and right coronary arteries were used randomly but were matched between native and allograft preparations in all experiments. The vascular reactivity of native, transplanted, and control coronary arteries was studied in organ chambers filled with control solution (20 mL) at 37°C. The rings were suspended between 2 metal stirrups, 1 of which was connected to an isometric force transducer. Data were collected with data acquisition software (IOS3, Emka Inc). All studies were performed in the presence of indomethacin (60 mmol/L). Responses to 5-hydroxytryptamine (5HT) creatinine contraction averaging 50% of the maximal contraction to KCl (30 mmol/L) at different levels of stretch, and then allowed to stabilize for 90 minutes. A maximal contraction was determined with potassium chloride (60 mmol/L). Rings were excluded if they failed to contract to potassium chloride (exclusion rate <5%).

After a wash and 30 minutes of stabilization, endothelium-dependent relaxations were studied in preparations contracted with prostaglandin E2 (range 2×10⁻⁸ to 2×10⁻⁷ mol/L) to achieve a contraction averaging 50% of the maximal contraction to KCl (60 mmol/L). Responses to 5-hydroxytryptamine (5HT) creatinine sulfate (serotonin, 10⁻¹⁰ to 10⁻⁷ mol/L, in the presence of 10⁻⁶ mol/L ketanserin, incubated 40 minutes before the addition of serotonin to block serotonin 5HT2 receptors), calcium ionophore A23187 (10⁻⁷ mol/L, to prevent the activation of β-adrenergic receptors).

Each preparation was stretched to the optimal point of its active length-tension curve (=4 g), as determined by measuring the contraction to potassium chloride (30 mmol/L) at different levels of stretch, and then allowed to stabilize for 90 minutes. A maximal contraction was determined with potassium chloride (60 mmol/L). Rings were excluded if they failed to contract to potassium chloride (exclusion rate <5%).

After a wash and 30 minutes of stabilization, endothelium-dependent relaxations were studied in preparations contracted with prostaglandin E2 (range 2×10⁻⁸ to 2×10⁻⁷ mol/L) to achieve a contraction averaging 50% of the maximal contraction to KCl (60 mmol/L). Responses to 5-hydroxytryptamine (5HT) creatinine sulfate (serotonin, 10⁻¹⁰ to 10⁻⁷ mol/L, in the presence of 10⁻⁶ mol/L ketanserin, incubated 40 minutes before the addition of serotonin to block serotonin 5HT2 receptors), calcium ionophore A23187 (10⁻⁷ mol/L to 10⁻⁵ mol/L), sodium fluoride (NaF, 0.5 to 9.5 mmol/L, in the presence of aluminum 5 mol/L creatinine chloride added 5 minutes before the addition of NaF), UK14,304 (10⁻⁵ to 10⁻³ mol/L, an α₁-adrenergic agonist), and bradykinin (10⁻¹⁰ to 10⁻⁵ mol/L) were compared between control, native, and allografted coronary rings 30 days after transplantation from swine fed either the standard chow or the lipid-rich regimen. No rings were exposed to more than one agonist in the course of the experiments. At the end of the experiments, endothelium-independent relaxations were studied with the use of 10⁻⁵ mol/L 3-morpholinosydnonimine (Sin-1), a nitric oxide donor.

**Histology**

**Myocardium**

Surgical myocardial biopsies were taken from the septum and right and left ventricular free walls at the time of explantation in all allografts and native hearts and fixed in formaldehyde (10%). Hematoxylin-eosin–safranin O staining was performed, and the biopsies were evaluated for rejection grade, extent of necrosis, and ischemic changes (Table 2).

**Coronary Artery Rings**

After each organ chamber experiment, coronary rings were fixed in 10% formaldehyde for 20 minutes at their optimal tension. All formalin-fixed tissue sections were embedded in paraffin, and 5-μm sections were stained with orcin. Each section was examined for the presence, extent, and distribution of intimal thickening, luminal narrowing, inflammatory infiltrates, and disruption of the internal elastic lamina by light microscopy. Grading of intimal thickening was performed by using a semiquantitative scale ranging from 0 to 4+. Intimal hyperplasia was considered to be present when there was abnormal smooth muscle cell accumulation within the intima (grades 1+ to 4+). All histological studies were read in a blinded fashion by an independent observer.

**Cholesterol and Phospholipid Determination in Coronary Arteries**

Cholesterol and phospholipids were determined in epicardial coronary arteries 30 days after transplantation. Segments from the left circumflex, left anterior descending, and right coronary arteries were excised from the native and from the transplanted hearts of 3 normocholesterolemic and 4 hypercholesterolemic animals. The adventitial fatty tissue was removed, and the dry weight was determined for each segment after an overnight desiccation at 40°C. Tissues were powdered in liquid nitrogen by using a Tenbroeck homogenizer and resuspended in 0.9% NaCl. The lipids were extracted according to the method of Bligh and Dyer. Free and total cholesterol mass determinations were conducted by gas liquid chromatography using cholesterol methyl ester as an internal standard. Samples were saponified by using the procedure of Klawe et al before total cholesterol assay. Phospholipid content was quantified by the method of Sokoloff and Rothblat.

**Drugs**

All solutions were prepared daily. Aluminum chloride, bradykinin, the calcium ionophore A23187, serotonin, indomethacin, ketanserin, propranolol, prostaglandin F₂α, NaF, and UK14,304 were purchased from Sigma Chemical Co. Sin-1 was synthesized at the Servier Research Institute.

**Statistical Analysis**

Relaxations and contractions are expressed as a percentage of the maximal contraction to prostaglandin F₂α, for each group and expressed as ±SEM; unless otherwise specified, n refers to the number of animals studied. ANOVA studies were performed to compare concentration-response curves with a Greenhouse-Geisser correction of sphericity (because of repeated measures). The Newman-Keuls test was used as the post hoc test. The Mantel-Haenszel χ² test was used for the comparison of the incidence of intimal hyperplasia between native and allografted coronary arteries at the time of explantation. A 1-way analysis with repeated measures was conducted to compare the cholesterol composition between the 3 coronary territories and to evaluate the effect of heart transplantation on coronary phospholipid content. The influence of diet and transplantation on the coronary cholesterol content was analyzed by a 2-way ANOVA. When differences were significant, a Newman-Keuls test was applied. All statistical evaluations were performed by setting the Bonferroni probability of a type I (β) error at P<0.05.

**Results**

**Hematology**

There were no statistically significant differences for white and red blood cell counts, hemoglobin, hematocrit, white blood cell count, platelet count, or red blood cell indices between baseline and end-of-diet values and no significant difference between animals fed the standard chow and those fed the high cholesterol diet (data not shown).

**Biochemistry**

There was a significant increase in serum creatinine in control animals and transplant recipients fed the cholesterol diet for 8 weeks (87±5 and 87±24 versus 144±20 and 144±28 mmol/L, respectively; P<0.001 versus day 1). There were no
statistically significant differences in all other biochemical values at the time of explantation between animals fed the standard chow versus the ones fed the high cholesterol diet (data not shown).

**Serum Lipids**
There were no statistically significant differences between baseline distributions in either group and no changes of distribution after diet feeding (data not shown). There were no statistically significant differences between baseline values (day 1 of the diet) between the swine fed the standard chow and those fed the cholesterol diet. There was a statistically significant increase in the total cholesterol and LDL-C in controls and transplant recipients fed the high cholesterol diet compared with those fed the standard chow. There were no statistically significant differences in the other measurements and in the ratio of HDL-C to total cholesterol. There were no significant changes in plasma lipids in standard chow–fed swine before and after transplantation (Figure 1). There was a progressive increase in the total cholesterol after 60 days of cholesterol feeding to 3-fold the baseline value (Figure 2). There was no effect of cholesterol feeding on the ischemic changes (subendocardial fibrosis) between the allografts of both groups and no evidence of transmural necrosis in either group. The myocardium from native hearts was normal in all instances.

**Vascular Reactivity Studies**
There were no statistically significant differences between native groups and allografts for contraction to potassium chloride and prostaglandin F₂₀ (please see Table I, published online at http://atvb.ahajournals.org/cgi/content/full/20/3/728/DC1). There were statistically lower contractions to KCl in the allografts compared with native arteries in both diet groups.

**Endothelium-Dependent Relaxations**

**Effect of Diet Per Se**
There was a statistically significant decrease of endothelium-dependent relaxations to serotonin, bradykinin, and the calcium ionophore A23187 in control cholesterol arteries compared with control standard arteries (Figures 4a, 5a, 6a). There were no statistically significant differences in endothelium-dependent relaxations to UK14,304 or NaF between the 2 groups (Figures 7a and 8a). There were no statistically significant differences in endothelium-dependent relaxations to serotonin, UK14,304, NaF, and bradykinin between native arteries from the standard versus the cholesterol group (Figures 4, 5b, 7, and 8).

**Effect of Transplantation**
There was a statistically significant decrease in endothelium-dependent relaxations to serotonin, UK14,304, and A23187 from allografted coronary arteries 30 days after transplantation compared with their respective native coronary arteries in the standard chow and cholesterol groups but no differences between the 2 groups. There was a significant decrease in endothelium-dependent relaxations to A23187 between allograft arteries from the cholesterol versus the native standard group (Figure 6b).

**Endothelium-Independent Relaxations**
There were no statistically significant differences in endothelium-independent relaxations to all agonists because all coronary
rings relaxed completely to the bolus of Sin-1 (data not shown).

Histology

Assessment of Intimal Hyperplasia

Effect of Diet per se
There were no statistically significant differences in the prevalence of subintimal thickening in coronary arteries from the controls fed cholesterol (without transplantation), arteries from the controls fed the standard chow, and arteries from native hearts of animals fed the standard chow and from native hearts of animals fed the cholesterol diet having undergone transplantation (Table 3). Normal swine carry intimal cushions that are preatherosclerotic lesions.

Effect of Transplantation
There was a statistically significant greater prevalence of subintimal thickening in coronary arteries from the allografts of animals fed the cholesterol diet compared with those of animals fed the standard chow (Table 3). There was no statistically significant difference in the severity of coronary intimal lesions in the allografts from animals fed the cholesterol-rich diet compared with those from the normcholesterolemic animals (Table 3).

Cholesterol Content of Coronary Arteries

Comparison of Lipid Composition Between Coronary Territories
The left anterior descending, the left circumflex, and the right coronary artery segments from native normcholesterolemic hearts (please see Figure I; Figures I to IV are published online at http://atvb.ahajournals.org/cgi/content/full/20/3/728/DC1) as well as coronary arteries from normcholesterolemic allografts and native and transplanted hearts from hypercholesterolemic animals (data not shown) had a similar composition in total, free, and esterified cholesterol. Further analyses were conducted independently of the coronary territory.
Effect of Diet per se

After 60 days of a cholesterol-rich diet, the total cholesterol content increased by 46% in coronary arteries from native hearts compared with arteries from hearts of normocholesterolemic animals (please see Figure III, published online) because of an increase in free cholesterol, whereas esterified cholesterol was similar in both groups.

Effect of Transplantation

The total and free cholesterol concentrations in coronary arteries 30 days after transplantation in normocholesterolemic swine were increased by 56% and 60%, respectively, compared with concentrations in native arteries. The esterified cholesterol remained unchanged (please see Figure II, published online), whereas the total phospholipid concentration increased by 53% after transplantation. The ratio of free cholesterol to phospholipids was maintained in both transplanted and native hearts (Table 4).

Effect of Diet and Transplantation

After 60 days of high cholesterol feeding (30 of which occurred after heart transplantation), coronary free cholesterol increased by an average of 50% compared with control values. Esterified cholesterol content remained unchanged. In allografted arteries from hypercholesterolemic animals, there was a greater accumulation of total cholesterol compared with that induced by hypercholesterolemia or by transplantation alone (please see Figure IV, published online). The effects of transplantation (Figure II, online) and hypercholesterolemia (Figure III, online) on coronary total and esterified cholesterol were additive in allografts from hypercholesterolemic animals compared with native arteries from normocholesterolemic animals, whereas the free cholesterol content remained similar (Figure IV, online).

Discussion

Hypercholesterolemia
The major findings of the present study are that hypercholesterolemia does not increase the extent of the selective early endothelial dysfunction due to rejection involving the G-protein–mediated pathway in this model but generalizes the dysfunction to involve relaxations to bradykinin and the calcium ionophore A23187 in allografts. Hypercholesterolemia affects only relaxations to the calcium ionophore in control and native coronary arteries. Hypercholesterolemia increases the prevalence but not the severity of coronary intimal thickening in the allografts from animals fed the high cholesterol diets compared with allografts from normocholesterolemic animals but has no effect on control or native hearts. There is a significant increase in the lipid content of native hearts from high cholesterol–fed swine compared with

Figure 6. Cumulative concentration-response curves to the calcium ionophore A23187 in rings of porcine coronary arteries with endothelium for the following groups: a, allograft standard (▲) and allograft cholesterol (●); b, native standard (●) and native cholesterol (▼); and c, control standard (▲) and control cholesterol (●). Responses are given as percentage of relaxation to the contraction induced by PGF2α. Results are presented as mean ± SEM. The asterisks indicate statistically significant differences between the 2 groups for the indicated concentration: *P < 0.05, **P < 0.01, and ***P < 0.001.

Figure 7. Cumulative concentration-response curves to UK14,304 in rings of porcine coronary arteries with endothelium for the following groups: a, allograft standard (▲) and allograft cholesterol (●); b, native standard (●) and native cholesterol (▼); and c, control standard (▲) and control cholesterol (●). Responses are given as percentage of relaxation to the contraction induced by PGF2α. Results are presented as mean ± SEM.
swine fed the standard chow. Transplantation, in the absence of hypercholesterolemia, causes a significant increase in cholesterol accumulation. Hypercholesterolemia and transplantation have additive effects on total and esterified cholesterol accumulation.

Vascular Reactivity

Effect of Diet
Hypercholesterolemia has been shown to cause impairment of relaxation to agonist-stimulating receptors coupled to endothelial pertussis toxin–sensitive G, proteins that are dysfunctional and have a reduced expression in human coronary arteries.19 Later, relaxations to bradykinin and ADP become impaired because of the dysfunction of other G proteins (eg, G, proteins).7 At the advanced stage of atherosclerosis, the response to A23187, reflecting the capacity of the final pathway to release nitric oxide, is reduced. In the present study, hypercholesterolemia did not increase the degree of impairment of endothelium-dependent relaxations to agonists coupled to G, protein or direct G-protein stimulation (eg, serotonin, a, adrenergic agonist UK14,304, and NaF), as evidenced by the absence of significant difference in relaxations to these agonists in the coronary arteries from control, native, and allograft arteries of hypercholesterolemic animals compared with animals fed the standard chow. This lack of effect of hypercholesterolemia on responses to receptor-mediated agonists that are mediated by G, proteins in control and native hearts may be related to the level and duration of hypercholesterolemia. Hypercholesterolemia generalized the endothelial dysfunction to bradykinin and the calcium ionophore A23187, agonists not using the G,–protein pathway (except in the native arteries for bradykinin). This effect of hypercholesterolemia on relaxation to bradykinin in the hypercholesterolemic group has not been observed before, whereas the effect on A23187 has been described with exposure to high levels of oxidized LDL.21 Induction of G,–protein dysfunction or an alteration of the endothelium–derived hyperpolarizing factor pathway could explain these observations.

Effect of Transplantation
Cellular rejection of the coronary endothelium after heart transplantation, without initial ischemia/reperfusion injury and in the absence of immunosuppressive drugs, in swine fed a standard chow causes coronary endothelial dysfunction without destruction of the endothelial cell lining.16 This impairment preferentially involves the pertussis toxin–sensitive G–protein–dependent pathway as described in human cardiac transplant recipients, in which vasodilatations to bradykinin are maintained, whereas there is paradoxical vasoconstriction to acetylcholine22 early after graft implantation. Damage sustained during cardiac preservation and reperfusion may cause endothelial dysfunction in the development of graft coronary vasculopathy.23 In the present model, no endothelial dysfunction is induced at the time of transplantation,15,16 which permits the evaluation of the effect of rejection and of hypercholesterolemia on the endothelial function and tone of the underlying vascular smooth muscle.

Effect of Diet Plus Transplantation
Rejection and high cholesterol feeding did not have additive effects in decreasing vasorelaxation within the time frame of the present study, probably because endothelial alterations induced by immune injury are already marked.16 The contribution of ischemia and reperfusion injury, which could trigger an early allogeneic reaction, cannot be assessed,24 but there was no histological difference in ischemic damage between the 2 groups of transplanted hearts.

Intimal Hyperplasia
Hypercholesterolemia and, specifically, elevated serum levels of oxidized LDL correlate with the development of graft

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TABLE 3. Incidence and Severity of Intimal Hyperplasia in Control, Native, and Allografted Hearts According to Different Diets

<table>
<thead>
<tr>
<th>Cholesterol</th>
<th>Rings With Hyperplasia/Total Rings, n/N (%)</th>
<th>Prevalence, %</th>
<th>1+</th>
<th>2+</th>
<th>3+</th>
<th>4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20/59 (37.2%)</td>
<td>45</td>
<td>35</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Native</td>
<td>19/57 (32.8%)</td>
<td>73.7</td>
<td>15.8</td>
<td>10.5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Allograft</td>
<td>38/61 (61.3%)*</td>
<td>44.7</td>
<td>36.8</td>
<td>13.2</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18/48 (37.5%)</td>
<td>72.2</td>
<td>27.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Native</td>
<td>53/146 (36.3%)</td>
<td>67.2</td>
<td>22.6</td>
<td>7.5</td>
<td>1.9</td>
<td></td>
</tr>
<tr>
<td>Allograft</td>
<td>46/98 (46.9%)†</td>
<td>37</td>
<td>52.2</td>
<td>10.9</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Severities of intimal hyperplasia were graded on a scale of 1+ to 4+, as described in Methods.

*P < 0.05 vs allograft standard and all others; †P < 0.05 vs control and native standard and control and native cholesterol.

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Figure 8. Cumulative concentration-response curves to NaF in rings of porcine coronary arteries with endothelium for the following groups: a, allograft standard (▲) and allograft cholesterol (●); b, native standard (●) and native cholesterol (○); and c, control standard (▲) and control cholesterol (●). Responses are given as percentage of relaxation to the contraction induced by PGF2α. Results are presented as mean ± SEM.
coronary vasculopathy, and low HDL levels are associated with subsequent sudden death and acute myocardial infarction in heart transplant recipients.25 Hypercholesterolemia increases endothelial production of superoxide anion destroying nitric oxide, promoting oxidation of LDL and lipid accumulation within the vessel wall26 and activating oxidant-sensitive transcription nuclear factors. This state of oxidative stress may be increased by low-grade subclinical injury from rejection and compounded by immunosuppressive drugs used in clinical heart transplantation, contributing to the development of graft coronary vasculopathy.27

The present study confirms that hypercholesterolemia increases the prevalence of early intimal lesions in the transplanted hearts; this has been documented 4 weeks after implantation in cholesterol-fed animals.28,29 Acceleration of the proliferative vascular damage by a factor of 3 to 4 is seen with cholesterol feeding, but frequency or severity of lesions is unchanged in chronically rejecting rat aortic allografts in the absence of hypertriglyceridemia30 and in apoE-deficient recipients of allotransplants.31 Cholesterol feeding has a limited effect on normal arteries, as shown in coronary arteries of control and native hypercholesterolemic animals.32 High cholesterol feeding simply accelerates the process without increasing the severity of lesions and may increase the proportion of fatty proliferative lesions in the early stages.

Coronary Artery Lipid Content

Hypercholesterolemia and heart transplantation independently increase the cholesterol content of swine coronary arteries. A 60% rise in free cholesterol is observed in rabbit native arteries after a 2-month cholesterol-rich diet with no accumulation of cholesteryl esters. In the absence of hypercholesterolemia, transplantation alone induces free cholesterol accumulation, which could be due to impairment of the barrier function of the endothelium and the secondary increase in vascular permeability observed during acute rejection in rat heart transplantation. Lipid overload of allograft coronary arteries occurs with only modest blood-to-artery lipoprotein gradients and more rapidly than in native atherosclerosis33 and could play an early and ongoing role in the development of transplant arteriopathy.34 Endothelial injury may initiate lipid deposition and atherogenesis by enhancing transendothelial pinocytic transport of lipoproteins by regenerated endothelium. Moreover, phospholipid content is also increased in coronary arteries from normocholesterolemic allografts, resulting in a free cholesterol-to-phospholipid cholesterol mass ratio of 0.2. The maintenance of this ratio corresponds to the initial adaptive response observed in vitro in cholesterol-loaded macrophages. Under these circumstances, phospholipid synthesis is induced when cellular free cholesterol increases to maintain cell viability and function and a ratio <0.4.32

An additive effect of hypercholesterolemia and transplantation was observed on total coronary cholesterol. The accumulation of esterified cholesterol in coronary arteries of hypercholesterolemic allografts was modest compared with the massive accumulation observed in advanced lesions. Nevertheless, the deposition of cholesteryl ester in hypercholesterolemic allografts is a hallmark of foam cell formation, which occurs through trapping of lipoproteins in the extracellular matrix or the activation of the cellular enzyme acyl coenzyme A cholesterol esterase participating in the maintenance of cellular cholesterol homeostasis by esterifying the free cholesterol released from the lyosomal hydrolysis of lipoprotein-derived cholesteryl esters. Stimulation of acyl coenzyme A cholesterol esterase transferase is directly related to the availability of excess cellular free cholesterol at a threshold that may be attained with hypercholesterolemic allografts but not with hypercholesterolemia or transplantation alone; this stimulation and its results may explain the absence of esterified cholesterol formation in the latter 2 situations.33 Transplantation alters the lipid composition of the arterial wall in the absence of hypercholesterolemia and immunosuppressive treatments secondary to an alteration of the barrier function of the endothelium. Hypercholesterolemia increases the accumulation of coronary cholesterol induced by transplantation. The accumulation of total cholesterol and cholesteryl esters in the coronaries from hypercholesterolemic allografts suggests an initiation of the foam cell formation process not assessed in the present study.

Lipid accumulation has been observed in transplanted human coronary arteries colocalized with proteoglycans and apolipoprotein deposits. In human pathological studies, increases in free esterified cholesterol and phospholipid wall content correlated with the degree of luminal stenosis. These increases did not differ among the 3 coronary territories. No link was observed between the lipid wall content, the duration of implantation, and the cause of death.33

The accumulation of lipids without endothelial inflammation or intimal thickening in the absence of rejection at biopsy suggests that lipid accumulation could be an early marker of endothelial injury that is due to an increased permeability of the endothelium secondary to immunologic injury.33 Focal denudation and functional alterations from repeated waves of alloimmune response could cause pathological endothelial activation, alteration of the permeability to lipids, and elevation of the transport of lipoprotein across the arterial wall leading to an accumulation of lipids.33 This may explain the greater accumulation of lipids in transplanted hearts com-

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<th>TABLE 4. Effect of Transplantation on Coronary Artery Lipid Content</th>
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<tr>
<td>Phospholipids, µg/mg dry wt</td>
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<td>Free cholesterol, µg/mg dry wt</td>
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<td>FC/PL. ratio</td>
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Values are mean±SEM (n=7). Coronary artery segments from native and transplanted normocholesterolemic hearts were analyzed for phospholipid (PL) and free cholesterol (FC) content as described in Methods. Values of P were obtained by a 2-way ANOVA without replication. NS indicates not significant.
pared with native hearts in the animals fed a standard diet and those fed a cholesterol-rich diet.

Lipid-lowering strategies achieving decreases in total and LDL cholesterol and increases in the HDL-to-LDL ratio significantly improve endothelial function, slow the progression of transplant coronary vascularopathy. Hypercholesterolemia exerts an adverse effect on endothelial function, accelerates intimal hyperplasia, and increases the accumulation of lipids in the vessel wall. Preservation of the release of protective endothelium-derived factors through minimization of immune injury and control of hyperlipidemia would favor maintenance of the homeostasis of the vascular wall through antithrombotic mechanisms and antiproliferative effects on smooth muscle cells.

References
Hypercholesterolemia Increases Coronary Endothelial Dysfunction, Lipid Content, and Accelerated Atherosclerosis After Heart Transplantation
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