Hyperlipidemia Accelerates Allograft Arteriosclerosis (Chronic Rejection) in the Rat

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Abstract The relevance of hyperlipidemia in allograft arteriosclerosis (chronic rejection) is controversial. Isolated hypercholesterolemia induced with cholesterol-cholic acid diet (CC-diet) or hypertriglyceridemia induced with glycerol-diet (G-diet) had no or only a protective effect on aortic allograft arteriosclerosis in the rat. Combined hyperlipidemia with both diets (CC+G-diet) enhanced allograft arteriosclerosis by doubling intimal thickness and cellularity (P<.05) but had no effect on host arteries. Compared with normolipidemic controls, the CC+G-diet increased the total serum cholesterol concentration 4.8-fold (P<.05). Levels of VLDL, and IDL increased 4.8- and 18.1-fold (P<.05), and their composition changed from triglyceride-rich to cholesterol-rich lipoproteins in an athrogenic direction. The CC+G-diet had no effect on the structure of inflammation in the vascular wall. Instead, significant lipid deposits were observed, and the expression of epidermal growth factor and insulin-like growth factor–1 was significantly elevated in the vascular wall. Thus, elevations in VLDL and IDL lipoprotein levels and their cholesterol content associate with the generation of allograft arteriosclerosis in rats. Deposition of lipids in the vascular wall seems to induce local synthesis of certain growth factors, which ultimately leads to the induction of smooth muscle cell replication. (Arterioscler Thromb. 1994;14:2032-2042.)

Key Words • hyperlipidemia • hypertriglyceridemia • hypercholesterolemia • chronic rejection • allograft arteriosclerosis • rat aortic transplant • lipoproteins

Chronic rejection is a leading cause of graft loss among long-term transplant recipients. The common manifestation of chronic rejection in all parenchymal organs is persistent perivascular and interstitial inflammation and fibrosis; thinning of vascular media with focal myocyte necrosis; occasional breaks in the internal elastic lamina; and a concentric, generalized allograft arteriosclerosis, ie, intimal thickening with proliferating smooth muscle cells (SMCs) underneath the endothelium. This disorder affects most of the intramyocardial branches of coronary arteries, the arteries and arterioles in the kidney, and the hilar and portal arteries of the liver.

The cause of chronic rejection is most likely multifactorial. Several risk factors have been identified. Histoincompatibility and frequency and intensity of acute rejection episodes are of prime importance. The half-life of a transplant bears a direct correlation to the number of mismatches, while no or only minor changes of chronic rejection are observed in syngeneic transplants in inbred animal models. Additional risk factors include hypertension, cytomegalovirus infection, very low or very high donor age, diabetes mellitus, and prolonged ischemia time.

Hypercholesterolemia and combined (mixed) hyperlipidemia are established risk factors for ordinary arteriosclerosis and coronary heart disease in humans. There also are reports showing that increases in triglyceride-carrying lipoprotein particles (chylomicra, VLDL) may carry differing degrees of vascular risk. Hyperlipidemia is also considered a risk factor for allograft arteriosclerosis, however, different opinions exist. In renal transplantation the increase in the relative risk of hypercholesterolemic patients is not particularly prominent, only 1.5-fold. Moreover, it is not known which of the lipid fractions are responsible for the observed effect.

Observations in experimental animals are equally controversial. Studies in rodent allograft models have demonstrated that feeding of the recipients with cholesterol-cholic acid-rich diet does not enhance intimal thickening in rat aortic allografts or in rat heart transplants, whereas Fellström et al were able to induce changes in rats. In the rabbit, where the cholesterol-rich diet induced arteriosclerotic alterations in cardiac allografts, similar changes were also found in recipients' own aorta. Lipoprotein subclasses were not monitored in any of these communications to establish a causal relationship to the observation.

We developed an animal model to investigate allograft vascular wall changes. Nonimmunosuppressed aortic allografts from DA to WF rat strain develop, after a spontaneously reversible initial acute rejection episode, vascular wall changes resembling those observed in human allografts in chronic rejection.

In this study we have manipulated the serum lipoproteins further by adding triglyceride-enhancing glycerol in the recipient diet and thereby induced isolated hypercholesterolemia, short-lasting isolated hypertriglyceridemia,
or combined hyperlipidemia in the recipient rat. We demonstrate that neither isolated hypercholesterolemia nor hypertriglyceridemia enhanced aortic allograft arteriosclerosis, but combined hyperlipidemia did.

Methods

Experimental Animals

Inbred WF (AG-B2, RTT) and DA (AG-B4, RTP) rat strains were used for transplantations. All animals were purchased from the Laboratory Animal Center, University of Helsinki, Helsinki, Finland. Rats weighing 250 to 350 g and of 2 to 4 months of age were used as donors and recipients. As a basic diet, pellets (Altromin No. 1314, Standard diet, Chr. Petersen A/S) were used, and tap water was given ad libitum.

Cholesterol-Cholic Acid- (CC-) Diet

The standard diet was supplemented with 4% cholesterol and 1% cholic acid (E. Merck) as follows. The pellets of the standard diet were moistened with tap water and thoroughly mixed with cholesterol and cholic acid into a homogeneous porridge. This diet was given to the experimental animals starting 1 week before transplantation until the rats were killed.

Glycerol- (G-) Diet

Hypertriglyceridemia was induced by 15% glycerol (Pro-labo) in drinking water. The diet was started 1 week before transplantation and continued until the animals were killed. 47

Preparative Separation of Lipoproteins

Blood samples were collected from killed rats, and serum was separated by low-speed centrifugation (2000 rpm, 30 minutes, 4°C). Lipoproteins were isolated by ultracentrifugation in a Beckman Optima TL ultracentrifuge using Beckman ½×2 polycarbonate centrifuge tubes and a TLA 100.3 rotor. For isolation of VLDL and IDL, tubes containing 0.8 mL of serum were overlaid with 1.0 mL of NaBr solution of a density of 1.0190 g/mL and ultracentrifuged (100 000 rpm, 3 hours, 20°C) using slow acceleration and deceleration (9/9, keypad number in the Beckman ultracentrifuge). After ultracentrifugation a thin, fairly yellowish layer containing VLDL and IDL was observed at the top of the tubes followed by a colorless section of 1 cm before a distinct yellowish layer containing LDL and HDL. The VLDL and IDL were removed from the top of the tubes by careful aspiration in a volume of 1 mL. The density of the infranatant was increased to 1.0900 g/mL by adding 0.12 mL of NaBr solution (d=1.535 g/mL) and was further used for LDL density gradient ultracentrifugation. To obtain LDL-, IDL-, and VLDL-free serum for HDL density gradient ultracentrifugation, the apoB-containing lipoproteins were removed by preparative ultracentrifugation. Serum (0.8 mL) in 1.2 mL of NaBr solution (d=1.535 g/mL) was overlaid with 1.1 mL of NaBr solution (d=1.0600 g/mL). After ultracentrifugation (100 000 rpm, 5 hours, 20°C), the supernatant layer containing VLDL, IDL, and LDL was removed by aspiration, and the infranatant was used for HDL density gradient ultracentrifugation. All density gradient ultracentrifugation studies were performed on fresh, unfrozen samples.

VLDL and IDL Density Gradient Ultracentrifugation

Density gradient ultracentrifugation was performed in a Beckman L8-70 ultracentrifuge with an SW-40TI swinging bucket rotor and Beckman Ultraclear ½×2 13-mL centrifuge tubes. The discontinuous gradient was prepared with NaBr solutions of distinct densities in the following order from the bottom to the top of the tube: 1 mL (d=1.0280 g/mL), 3 mL sample solution (1 mL VLDL+IDL in 2 mL NaBr solution, d=1.0100 g/mL), 3 mL (d=1.0060 g/mL), 3 mL (d=1.0030 g/mL), and 2.5 mL distilled water. All salt solutions contained 0.05% EDTA, and their densities were measured with a DMA 46 Digital Density Meter (Anton Paar). After ultracentrifugation (40 000 rpm, 55 minutes, 20°C), the centrifuge was allowed to stop with brake off. The bottoms of the tubes were punched, and three fractions in volumes of 5 mL, 5 mL, and 2.5 mL were obtained. The protein absorbance profiles in the tubes were monitored with an absorbance meter (Pharmacia), and the density gradient of the tubes was controlled with the DMA 46 density meter placed before the fraction collector. The first 5-mL fraction (d=1.0185 g/mL to 1.0060 g/mL) contained IDL; the second 5-mL fraction contained the dense VLDL1 (d=1.0060 g/mL to 1.0030 g/mL) and the 2.5-mL fraction (d=1.0030 g/mL to 0.9880 g/mL) contained the light VLDL2.

LDL Density Gradient Ultracentrifugation

The LDL subfractions LDL1, LDL2, LDL3, and LDL4 were separated by density gradient ultracentrifugation in a Beckman SW40 TI swinging bucket rotor and Beckman Ultraclear ½×2 tubes from the sample obtained from preparative ultracentrifugation in a Beckman Optima TL centrifuge. The discontinuous NaBr solution gradient was prepared by layering from the bottom to the top: 1.0 mL (d=1.1900 g/mL), 0.92 mL sample (the infranatant of a volume of 0.8 mL from VLDL+IDL separation in 0.12 mL NaBr, d=1.5350 g/mL), 2.0 mL (d=1.0600 g/mL), 2.0 mL (d=1.0550 g/mL), 2.0 mL (d=1.0460 g/mL), 0.5 mL (d=1.0910 g/mL). The tubes were centrifuged in a Beckman L8-70 ultracentrifuge (40 000 rpm, 24 hours, 23°C), and the rotor was allowed to stop without the brake being used. After ultracentrifugation the tubes were discharged from the top with the Beckman Recovery System and by infusing Maxidens solvent (Nyegaard & C. A/S) to the tubes. The protein absorbance profile and the density gradient of the tubes were monitored as described for VLDL+IDL. Four LDL subfractions — LDL1 (d=1.0190 g/mL to 1.0260 g/mL), LDL2 (d=1.0260 g/mL to 1.0340 g/mL), LDL3 (d=1.0340 g/mL to 1.0460 g/mL), and LDL4 (d=1.0460 g/mL to 1.0630 g/mL) — were collected in a volume of 2.0 mL each.

HDL Density Gradient Ultracentrifugation

The HDL density gradient ultracentrifugation method was based on the method previously described by Groot et al. 28 except that the apoB-containing lipoproteins first were removed from serum samples by a centrifugation in Beckman Optima TL centrifuge as described above. The density of the infranatant was increased by adding 0.5 g dry NaBr. One milliliter NaBr solution with a density of 1.3500 g/mL was pipetted to the bottom of Beckman Ultraclear ½×2 tubes, and the sample solvent was carefully layered above it. The discontinuous gradient was prepared by layering NaBr solutions d=1.2500 g/mL and d=1.2200 g/mL, 1.5 mL and 6.7 mL above the sample, respectively, and 2.0 mL of distilled water above the salt solutions. After ultracentrifugation in a Beckman 18-70 ultracentrifuge with an SW-40TI rotor (40 000 rpm, 18 hours, 20°C), the tubes were discharged from the top as described for the separation of LDL subfractions. Five 1.1-mL fractions corresponding to HDL1, HDL2, HDL3, HDL4, and HDL5 were collected.

Analytical Methods

Cholesterol, triglyceride, and phospholipid concentrations in serum and lipoprotein subclasses were measured by an enzymatic colorimetric method with a Cobas Mira analyzer (Hoffman-La Roche), using reagent kits (nos. 0715166 and 0722138, Hoffman-La Roche) for cholesterol and triglycerides.
Aortic Transplantations
A segment of the descending thoracic aorta (>3 cm long) was excised, perfused with phosphate-buffered saline (PBS), and used as a transplant. The rats were anesthetized with 240 mg/kg chloral hydrate IP, and 0.3 mg/kg buprenorphine hydrochloride SC (Temgesic; Reckitt & Colman) was used for postoperative pain relief. The graft was transplanted into a heterotopic position below renal arteries and above bifurcation in the abdominal cavity. Cranial suture line was made as close to renal arteries as technically possible to minimize the difference in diameter. End-to-end anastomosis was performed with 9-0 continuous nylon suture. The DA to WF strain combination was used for allografts. The grafts were removed at 1, 3, 5, or 6 months after transplantation and processed for histochemistry, frozen section immunohistochemistry, and biochemical determinations. Specimens for immunohistochemistry were immersed in Tissue-Tek (Miles, Inc) and snap frozen.

Histological Specimens and Staining
For evaluation of morphological changes, paraffin sections were stained with Mayer’s hematoxylin and eosin. Cross sections were prepared for quantitation of morphological changes in the graft.

Quantitation of Histology
The morphological changes were quantitated according to standard morphometric principles and expressed as point score units (psu), i.e., the mean number of points falling over a given anatomic area using straight cross-sectional lines and a 0.02-mm grid. The following variables were evaluated: the number of nuclei and the thickness of different layers of the graft, and expressed as point scores.

Immunohistochemistry
For immunohistochemistry, 3- to 4-mm-thick frozen sections were stained by immunoperoxidase technique using monoclonal anti-rat antibodies to leukocyte common antigen (LCA; Ox1; Sera Lab), to interleukin-2 receptor (IL-2 rec; a generous gift from Dr. J. Kupiec-Weglinski, Harvard Medical School), to MHC class II (Sera Lab), to interferon γ (DB1; Holland Biotechnology BV), to anti-smooth muscle α-actin (Bio-Makor), and to macrophages (OX42, MAS 370C; Sera Lab). The cryosections were fixed with chloroform for 30 minutes and then stained by the two-layer indirect immunoperoxidase technique. Briefly, the sections were incubated with appropriately diluted monoclonal antibody (usually 1:100), washed, and consecutively incubated with peroxidase-conjugated rabbit anti-mouse IgG and goat anti-rabbit IgG (Dako Immunoglobulins A/S), followed by treatment with a substrate solution containing chromogen 3-amino-9-ethyl-carbazole (AEC). Hydrogen peroxide was added to the AEC solution immediately before use. The samples were counterstained with Mayer’s hemalum solution and mounted.

Lipid Stainings
Unfixed frozen sections were stained with the oil-red-O method, which stains all lipids except those in solid state by the method described by Catalano and Lillie.

Growth Factor Measurements From Rat Aortas
A 1-cm piece of the recipient aortic allograft or of a normal aorta was removed and sliced. Aortas were first incubated for 30 minutes in an ice bath with 0.1 mol/L Na-phosphate buffer, pH 7.4, in 0.9% NaCl supplemented with 1% bovine serum albumin, 200 nmol/L Ca-ionophore (Sigma), and 0.1% NaN₃, and thereafter for another 30 minutes at +37°C in the same buffer. Exclusion of NaN₃ and/or Ca-ionophore from the incubation medium did not affect the growth factor determinations. Incubation was stopped by taking the aortas back to the ice bath. Liquids were collected from the incubation tubes for radioimmunoassay (RIA), and aortas were dried at +37°C overnight and weighed. Commercially available RIA kits (Amersham International PLC) for growth factors EGF, IGF, and PDGF-BB were employed. Normal nontransplanted aortas and syngeneic grafts were used as controls. The results were expressed as pg/mg dry wt of the graft.

Statistical Methods
Data are expressed as mean±SEM; in the case of nontransplanted aortas as mean±SD. If the data were not normally distributed, median and range also are given. The nonparametric Mann-Whitney U test (z corrected for ties) was used to evaluate the significances. Animals receiving different diets were compared with the control rats. Values of P<.05 were considered statistically significant.

Results
Serum Lipids and Lipoproteins
The serum lipid concentrations of nontransplanted WF rats in the control, CC-, G-, or CC+G-diet group after 3 weeks of feeding are presented in Table 1. The mean serum cholesterol concentration of animals receiving the CC- and CC+G-diets were 3.5 and 4.8 times higher, respectively, compared with the serum cholesterol concentration of control animals (P<.01 for both, Table 1). The animals receiving the G-diet did not differ from control animals with respect to the mean cholesterol level. Serum triglyceride levels of animals in the G- or CC+G-diet group tended to be higher than those
Dietary Effects on Triglyceride-Rich Lipoproteins (VLDL and IDL)

The individual protein absorbance curves of light VLDL particles (VLDL₁), more dense VLDL particles (VLDL₂), and IDL are presented in Fig 1. Animals on the CC- or CC+G-diet displayed more prominent VLDL₁ and IDL protein profiles than animals in the G-diet or control group. The mean VLDL₂ concentration of animals receiving the CC-diet was 2.7 times higher and that of animals receiving the CC+G-diet 4.8 times higher compared with the mean VLDL₂ concentration found in control animals (P < .05 for both; Table 3). Similarly, the IDL concentrations of animals on the CC- or CC+G-diet were 8.1 and 18.1 times higher than the IDL concentration in control animals (P < .05 for both; Table 3). The VLDL₁ concentrations were similar in all four diet groups. In addition, the IDL profiles of animals on the CC- or CC+G-diet had moved toward lower density, indicating lighter and more lipid-containing IDL particles (Fig 1). Animals on the CC- or CC+G-diet showed highly significant changes in the composition of VLDL₁, VLDL₂, and IDL. Animals on the CC-diet had 16%, 38%, and 42% higher content of cholesterol in VLDL₁, VLDL₂, and IDL, respectively, than control animals (P < .05 for all; Table 3). Similarly, animals on the combined diet (CC+G) had 34%, 52%, and 38% more cholesterol in VLDL₁, VLDL₂, and IDL particles, respectively, than control animals (P < .05 for all; Table 3). Animals receiving the combined diet (CC+G) and animals receiving the pure cholesterol diet (CC) differed from each other in two respects. First, the mean concentrations of VLDL₁ and IDL of animals on the CC+G-diet were 76% and 121% higher than the concentrations of these lipoproteins found in animals on the CC-diet (P < .01 for both). Second, animals on the CC+G-diet had 18% and 149% higher content of cholesterol in VLDL₁ and VLDL₂ particles, respectively, than animals receiving the CC-diet (P < .05 for both; Table 3). These findings indicate that the combined diet induced an enormous increase in the number of VLDL and IDL particles and turned them from cholesterol-poor to cholesterol-rich lipoproteins.

Low- and High-Density Lipoproteins

A very low concentration of LDL is typical in WF and DA rats. The individual protein absorbance curves in all four groups displayed almost plateau LDL profiles, and the measured LDL concentrations were extremely low in comparison with concentrations of VLDL, IDL, and HDL (Fig 1). None of the diets had any major effect on LDL. The HDL density distributions of the four groups were also similar (Fig 1).

Effect of Lipid Diet on Vascular Wall Changes

The vascular wall changes in allografts, with and without CC-, G₂, or CC+G-diet, are given in Fig 2. The histological changes in the CC-diet group followed the allograft controls for 3 months; thereafter, the diet was
Fig 1. Protein absorbance profiles of VLDL₁, VLDL₂, and IDL (left), of LDL subclasses (middle), and of HDL subclasses (right) obtained by density gradient ultracentrifugation. The individual curves show rat groups receiving either cholesterol-cholic acid– (CC–), glycerol- (G–), both CC+G–, or control-diet (n=3 to 5).
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<td>VLDL₁</td>
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<td>B</td>
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<td>C</td>
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<td>D</td>
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Values are given as mean±SEM. Chol indicates cholesterol; TG, triglycerides; PI, phospholipids; Prot, proteins, Group A, control (animals fed standard diet); B, cholesterol-cholic-acid diet; C, glycerol-diet; and D, both previous diets.

P values for differences in lipoprotein composition are given for cholesterol percentage only, as the other percent changes were mainly reciprocal to those in % cholesterol.

definitely not enhancing but rather protective for allograft arteriosclerosis, although the differences did not reach statistical significance. Adventitial inflammation increased from 6.4±1.0 to 11.8±6.0 psu, but media necrosis decreased from 1.0±0.2 to 1.5±0.6 psu, intimal cellularity decreased from 3.7±0.8 to 1.8±0.1 psu, and intimal thickness decreased from 2.9±0.4 to 1.8±0.2 psu.

Also, the G-diet was protective. It slightly reduced the inflammatory changes in the adventitia from 6.0±1.0 to 5.75±2.0 psu (P=NS), delayed the disappearance of nuclei in the media from 0.73±0.1 to 1.25±0.4 psu (P=NS), and delayed and reduced the appearance of nuclei in the intima from 0.85±0.4 to 2.24±0.3 psu (P<.01) and intimal thickness from 2.61±0.3 to 0.75±0.2 psu (P<.05).

However, when the recipient rat received the CC+G-diet, the result was entirely different: the combined diet still somewhat reduced the adventitial inflammation from 6.05±1.0 to 3.65±0.5 psu (P<.05) and had no effect on media necrosis but nearly doubled intimal nuclear contents from 2.24±0.3 to 4.22±0.2 psu (P<.05) and intimal thickness from 2.61±0.3 to 4.8±0.4 psu. However, when the experiment was continued for
longer than 3 months, the animals became ill, lost their appetite, and stopped drinking; they turned nonhyperlipidemic and had to be killed at <6 months.

**Lipid Stainings**

Frozen sections of allografts were stained with the oil-red-O method. Lipid droplets were demonstrable only very occasionally in the allograft vascular wall of hypercholesterolemic animals and never in hypertriglyceridemic or normolipidemic allograft recipients. Instead, in the CC+G-diet group, large amounts of lipid droplets were seen throughout the media and occasionally also in the intima (Fig 3).

**Effect of Hyperlipidemia on the Expression of Inflammatory Markers in the Aortic Allografts**

In nonimmunosuppressed rat aortic allografts, the acute rejection period occurs during the first month posttransplantation, whereafter the inflammatory response declines and the situation is stabilized. Immuno-enzyme peroxidase stainings were performed to investigate different inflammatory markers during chronic rejection, ie, 3 months after transplantation, when hyperlipidemic allografts were compared with normolipidemic controls. As seen in Table 4, IL-2 receptor expression was still slightly increased in diet-treated animals (CC and G) compared with control allografts. Although the
results were statistically significant, there were no biologically significant differences between the groups. The CC-diet seemed to slightly increase major histocompatibility complex II and macrophage (OX42) expression in the allograft, whereas there were no differences in a-actin and common leukocyte antigen in the vascular wall. a-Actin stain indicated preservation of SMCs in the median layer of the vascular wall in rats fed with combined cholesterol+cholic acid- and glycerol-diet (CC+G-diet). a indicates adventitia; m, media, and i, intima.

Effect of Hyperlipidemia on Growth Factor Expression of the Allograft Extract

RIA was performed to investigate the presence of different growth factors in allograft extracts after treatment for 3 to 6 months. Commercial kits for insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), and platelet-derived growth factor-β (PDGF-β) were used.

As seen in Table 5, the concentration of IGF-1 in the CC+G-fed animals was 3900±522 pg/dry wt compared with 1986±262 pg/dry wt of the controls (P = .008). The difference in EGF was even higher, 650±89 versus 221±34 (P = .003), but PDGF-β was under detection level, <200 pg. These results indicate activity of these growth factors in the development of allograft arteriosclerosis. There were no significant differences between controls and the CC- or G-diet groups.

Discussion

There is plenty of evidence on the role of hyperlipidemia in the development of atherosclerosis, which is one of the major reasons leading to cardiac transplantation via generation of coronary disease. The preoperative lipid levels may affect both cardiac allograft and patient survival. The use of immunosuppressive agents, particularly prednisolone and cyclosporine, may contribute to the development of hyperlipidemia and hypertension, with the main effect occurring during the first year after transplantation. Antihypertensive drugs such as thiazide diuretics or β-blocking agents may further influence lipid levels. Hence, hyperlipidemia is often considered an important risk factor when its effects on graft survival are investigated.

The relevance of hyperlipidemia in animal transplant models is a matter of controversy. Alonso et al were the first to show accelerated allograft arteriosclerosis in cholesterol-fed rabbits, a finding later confirmed by Cheng et al and Kuwahara et al. Fellström et al have reported similar results in rats, whereas we could not confirm these observations. While none of the studies gave detailed information on the composition of recipients' serum lipoproteins, all studies were designed to investigate the role of isolated hypercholesterolemia on allograft arteriosclerosis.

The effect of our three experimental diets on the serum lipoprotein patterns in nontransplanted rats was clearly evident after a 3-week diet. The CC-diet produced hypercholesterolemia with normal triglyceride levels and the G-diet produced a mild elevation in triglycerides with normal cholesterol levels, whereas the CC+G-diet produced hyperlipidemia combined with simultaneous elevation of cholesterol and triglyceride levels (Table 1). However, in animals with transplanted aortas these patterns were only partly maintained during prolonged dietary treatment of 3 months (Table 2).

After 3 months, elevated triglyceride levels in the CC- and CC+G-diet groups were not observed. However, the elevation in cholesterol observed in the CC+G-diet group had been further aggravated, most of the cholesterol being contained in the VLDL fraction. The slight elevations in triglyceride levels from the baseline (median 1.5 mmol/L) in the CC-diet (median 1.7 mmol/L) and CC+G-diet groups (median 2.3 mmol/L) in nontransplanted animals differed from the normal triglyceride levels in transplanted animals after a 3-week diet. The CC+G-fed rabbits, a finding later confirmed by Cheng et al and Kuwahara et al. Fellström et al have reported similar results in rats, whereas we could not confirm these observations. While none of the studies gave detailed information on the composition of recipients' serum lipoproteins, all studies were designed to investigate the role of isolated hypercholesterolemia on allograft arteriosclerosis.

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The increased susceptibility of rats receiving the CC+G-diet to arteriosclerotic changes while being re-
TABLE 4. Expression of Monoclonal Antibodies in the Vascular Wall of Rat Aortic Allografts at 3 Months

<table>
<thead>
<tr>
<th></th>
<th>IL-2r</th>
<th>Class II</th>
<th>IFN-γ</th>
<th>α-Actin</th>
<th>LCA</th>
<th>OX42</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allogeneic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control, n=5</td>
<td>A</td>
<td>0.2±0.2</td>
<td>2.1±0.4</td>
<td>0.3±0.2</td>
<td>0.4±0.2</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.0±0.0</td>
<td>0.8±0.3</td>
<td>0.2±0.2</td>
<td>0.8±0.3</td>
<td>0.5±0.3</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>0.0±0.0</td>
<td>1.3±0.5</td>
<td>0.1±0.1</td>
<td>2.4±0.2</td>
<td>0.9±0.3</td>
</tr>
<tr>
<td>G-diet, n=3</td>
<td>A</td>
<td>1.2±0.4</td>
<td>2.0±0.0</td>
<td>0.0±0.0</td>
<td>0.8±0.3</td>
<td>2.5±0.0</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.5±0.0*</td>
<td>1.0±0.0</td>
<td>0.3±0.3</td>
<td>2.0±0.0*</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>0.0±0.0</td>
<td>1.0±0.0</td>
<td>0.0±0.0</td>
<td>2.0±0.0</td>
<td>1.0±0.0</td>
</tr>
<tr>
<td>CC-diet, n=3</td>
<td>A</td>
<td>1.2±0.2*</td>
<td>2.8±0.2</td>
<td>0.7±0.3</td>
<td>0.0±0.0</td>
<td>2.7±0.3</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.5±0.3</td>
<td>2.0±0.0*</td>
<td>0.7±0.3</td>
<td>1.7±0.3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>0.0±0.0</td>
<td>1.5±0.3</td>
<td>0.0±0.0</td>
<td>3.0±0.0</td>
<td>1.5±0.3</td>
</tr>
<tr>
<td>G+CC-diet, n=3</td>
<td>A</td>
<td>0.8±0.3</td>
<td>2.1±0.1</td>
<td>0.7±0.3</td>
<td>0.2±0.2</td>
<td>2.0±0.2</td>
</tr>
<tr>
<td></td>
<td>M</td>
<td>0.3±0.1</td>
<td>0.9±0.1</td>
<td>0.7±0.3</td>
<td>0.7±0.3</td>
<td>0.6±0.2</td>
</tr>
<tr>
<td></td>
<td>I</td>
<td>0.0±0.0</td>
<td>1.1±0.3</td>
<td>0.0±0.0</td>
<td>2.3±0.3</td>
<td>0.9±0.3</td>
</tr>
</tbody>
</table>

IL-2r indicates interleukin-2 receptor; class II, MHC class II; IFN-γ, interferon gamma; α-actin, α-actin smooth muscle cells; LCA, leukocyte common antigen; OX42, macrophage marker MAS 370C; A, adventitia; M, media; and I, intima.

Arbitrary scale: 0 indicates no expression; 1, weak expression; 2, moderate expression; and 3, strong expression.

Groups: Control indicates standard diet; G-diet, glycerol diet; CC-diet, cholesterol and cholic acid diet; and CC+G-diet, both previous diets.

Lipid diet-fed animals are compared with standard diet controls. Significances by nonparametric Mann-Whitney U test, *P<.05.

TABLE 5. Presence of Growth Factors In the Vascular Wall of Allografts After 3 to 6 Months Feeding With Different Diets

<table>
<thead>
<tr>
<th></th>
<th>IGF-1</th>
<th>EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/dry wt</td>
<td>pg/dry wt</td>
</tr>
<tr>
<td>Allograft control, n=9</td>
<td>1866±262</td>
<td>221±34</td>
</tr>
<tr>
<td>Allograft G+CC-diet, n=5</td>
<td>3900±522*</td>
<td>650±89*</td>
</tr>
<tr>
<td>P=.008</td>
<td>P=.003</td>
<td></td>
</tr>
<tr>
<td>Allograft CC-diet, n=3-4</td>
<td>1153±187</td>
<td>127±5.0</td>
</tr>
<tr>
<td>P=.064</td>
<td>P=.052</td>
<td></td>
</tr>
<tr>
<td>Allograft G-diet, n=2</td>
<td>3550±850</td>
<td>224±55</td>
</tr>
<tr>
<td>P=.099</td>
<td>P=.81</td>
<td></td>
</tr>
</tbody>
</table>

IGF-1 indicates insulin-like growth factor 1 and EGF, epidermal growth factor.

Values are given at mean±SEM. Diet-fed animals are compared with standard diet-fed allografts.

P values by the Mann-Whitney U test, *P<.05 considered as significant.

sistant to the other two experimental diets is of interest. The differences observed in the serum lipoprotein pattern may at least partly explain this difference. Particularly the total and VLDL cholesterol concentrations were markedly increased in the CC+G-diet animals compared with the other two groups. Possibly, addition of glycerol to the cholesterol–cholic acid regimen may have improved the absorption of cholesterol from the intestine, resulting in more marked hypercholesterolemia. Most of the circulating cholesterol was contained in the VLDL fraction, particularly VLDL-2. Such cholesterol-enriched VLDL particles resemble the β-VLDL occurring in patients with type III hyperlipoproteinemia, which are regarded as highly atherogenic. We speculate that these particles contributed to the arteriosclerotic changes observed in the CC+G-diet group.

Rats with isolated hypertriglyceridemia and isolated hypercholesterolemia did not develop any more pronounced allograft arteriosclerosis than normolipidemic rats did. In fact, a slight protective effect could be observed. In contrast, when combined elevations in both serum cholesterol and triglyceride levels were produced, the result was remarkably different: the arteriosclerotic changes observed in the CC+G-diet group.

Intimal thickening and cellularity doubled, and total media necrosis developed. Intimal thickening is characterized by migration of SMCs into the intima and their proliferation. To proliferate, SMCs must change their phenotype from contractile to synthetic. This modulation is associated with increased binding and degradation of VLDL and increased LDL; in both cases, the binding appeared to be mediated via apolipoprotein B/E receptor. This reflects an increased need of cholesterol and other lipid components for membrane synthesis in the growing and rapidly proliferating synthetic SMCs. Lipid stainings demonstrated intensive accumu-
mulation of lipid droplets throughout the aortic media and, occasionally, in the intima. This was not seen in animals fed with other diets.

Growth factors may play an important role in the generation of allograft arteriosclerosis36-38 as well as in classic atherosclerosis.39,40 We observed significant elevations of IGF-1 and EGF in the aortic allografts of rats receiving the CC+G-diet compared with controls. Unexpectedly, we could not detect synthesis of PDGF-ββ. How do growth factors then act during chronic rejection? Cytokines are originally considered to be mediators implicated in immunity and inflammation, and growth factors are involved in the proliferation and chemotaxis of cells in organs and tissues.40 A damaged endothelial cell is capable of releasing cytokines like interferon-γ, IL-1, tumor necrosis factor-α (TNF-α), and of upregulating class II expression. These factors attract macrophages, and they start to produce PDGF-ββ and TGF-β, which in turn activate SMCs to proliferate and produce IGF-1, PDGF-ββ, TGF-β, and basic fibroblast growth factor (bFGF). PDGF-ββ is also secreted by platelets and endothelial cells.40 It is the most potent stimulator of SMC growth38 and also activates T lymphocytes.40 PDGF-ββ also acts as a chemotactic factor. TGF-β is produced by monocytes/macrophages, SMCs, endothelial cells, and activated T lymphocytes.40 It is secreted in vivo in inactive form, and the target specificity of the action may be determined by the ability of the cell to activate the latent complex.40 TGF-β induces SMC chemotaxis and growth and matrix formation, thus contributing fibroproliferative diseases,40 such as fibrosis seen in the kidney transplant.41 IGF-1 stimulates the migration of SMCs into the intima of the vessel, which is the key process during the generation of allograft arteriosclerosis.40 EGF is known to stimulate SMC growth, at least in cell culture.35

Inflammation markers detected by immunoperoxidase stainings did not differ among the experimental groups, thus indicating that hyperlipidemia did not alter immune response in the allografts.

Taken together, combined hyperlipidemia enhanced the development of allograft arteriosclerosis in the rat, and isolated hypercholesterolemia or hypertriglyceridemia did not. The mechanisms are still unclear, but the changes in the levels and composition of lipoproteins into a more atherogenic direction may be one of the contributing factors. Hyperlipidemia provides an excess of lipoproteins for membrane synthesis in the growing and proliferating synthetic SMCs. In addition, hyperlipidemia seems to stimulate growth factor synthesis in the allograft. These factors together may have enhanced demonstrated aortic allograft arteriosclerosis.

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References


Hyperlipidemia accelerates allograft arteriosclerosis (chronic rejection) in the rat.
A Räisänen-Sokolowski, M Tilly-Kiesi, J Ustinov, A Mennander, T Paavonen, M J Tikkanen and P Häyry

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