Temporal Relationships Between Circulating Levels of CC and CXC Chemokines and Developing Atherosclerosis in Apolipoprotein E*3 Leiden Mice

Nuala Murphy, K. Richard Bruckdorfer, David C. Grimsditch, Philip Overend, Martin Vidgeon-Hart, Pieter H.E. Groot, G. Martin Benson, Annette Graham

Objectives—CC and CXC chemokines are implicated in leukocyte recruitment during development of atherosclerotic lesions, suggesting circulating levels of chemokines may be useful serum markers of atherogenesis. Serum chemokine concentrations were measured in apolipoprotein (apo) E*3 Leiden mice and their nontransgenic littermates and related to the differing rates of atherogenesis in these animals.

Methods and Results—Mice were fed a high-fat, high-cholesterol (HFC/C) diet for 18 weeks. Circulating levels of JE/monocyte chemotactic protein-1 increased (P<0.05) after 2 to 4 weeks, coincident with development of diet-induced hypercholesterolemia, and remained elevated throughout the study. Circulating KC concentrations increased (P<0.05) after consumption of HFC/C diet; however, unlike JE, serum KC concentrations increased more rapidly in apoE*3 Leiden mice than their nontransgenic littermates. Heparic expression of JE and KC mRNA were detected by in situ hybridization in all mice fed HFC/C diet. Aortic expression of JE mRNA was seen only in apoE*3 Leiden mice within macrophage-rich atherosclerotic lesions. By contrast, no aortic expression of KC mRNA was detected by in situ hybridization.

Conclusions—Increases in serum chemokine concentrations did not reflect temporal aortic production of these molecules and proved less predictive than serum cholesterol of the markedly different extent of atheroma in apoE*3 Leiden and nontransgenic mice. (Arterioscler Thromb Vasc Biol. 2003;23:884-890.)

Key Words: chemokine ■ KC ■ JE ■ atherosclerosis ■ mice

Atherosclerosis is typified by a chronic inflammatory response involving the recruitment and activation of mononuclear leukocytes. In part, this process is controlled by the production of chemotactic cytokines (chemokines) by cells of the artery wall. Chemokines are members of a superfamily of small, secreted proteins (8 to 16 kDa) that mediate migration and activation of leukocytes and arterial cells by interacting with G-protein-coupled cell-surface receptors. Chemokines are divided into families according to the arrangement of the first 2 of 4 conserved cysteine residues, the 2 largest subfamilies being designated CC and CXC.

Members of the CXC (or α) family, particularly those that contain the Glu-Leu-Arg motif, principally induce the migration of neutrophils and not monocytes. Despite this apparent selectivity and the scarcity of neutrophils within atherosclerotic lesions, recent data implicate members of the CXC chemokine subfamily in atherogenesis. These include interleukin-8 (IL-8) and growth-related oncogene (GRO) chemokines that bind to CXCR-1/2 and CXCR-2 receptors, respectively. The murine ligands KC (GRO-α) and CXC chemokines are implicated in leukocyte recruitment during development of atherosclerotic lesions, suggesting circulating levels of chemokines may be useful serum markers of atherogenesis. Serum chemokine concentrations were measured in apolipoprotein (apo) E*3 Leiden mice and their nontransgenic littermates and related to the differing rates of atherogenesis in these animals.

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and macrophage inflammatory protein-2 (MIP-2; GRO-β/γ) bind to the murine CXCR-2 receptor homolog mIL-8RH. Increased expression of IL-8 has been shown in macrophages isolated from human atheromatous lesions, and in macrophages within macrophage-rich areas of human and murine atherosclerotic lesions. Macrophages in human and murine atherosclerotic lesions express CXCR-2 and mIL-8RH, respectively. Bone marrow transplantation studies demonstrated that the absence of leukocyte mIL-8RH resulted in fewer monocytes and reduced lesion size in the LDL receptor−/− model of atherosclerosis. Both IL-8 and GRO-α are capable of inducing monocyte adhesion in vitro, particularly under flow conditions; however, the effects of receiving mIL-8RH−/− bone marrow were more pronounced at later stages of lesion development in vivo, indicating a role in retention and expansion of intimal macrophages.

Circulating levels of chemokines are increased in several acute and chronic inflammatory and immune-related disorders, suggesting that their levels may reflect enhanced tissue expression and secretion of these molecules during disease. Little is known about circulating chemokine concentrations during the development of atherosclerotic lesions, but elevated plasma CC and CXC chemokines have been reported in patients with congestive heart failure, ischemia-reperfusion injury, or aortic aneurysms. Importantly, serum chemokine concentrations were associated with degree of disease severity.

This study examines the hypothesis that circulating levels of CC or CXC chemokines may prove useful serum markers of atheroma in transgenic mice expressing the human apoE*3 Leiden gene. These mice have impaired hepatic clearance of chylomicron and VLDL remnant lipoproteins and, when fed an atherogenic diet, develop marked hypercholesterolemia and accelerated atherosclerosis compared with their nontransgenic littermates. We measure circulating levels of CC (JE) and CXC (KC and MIP-2) chemokines in apoE*3 Leiden mice and their nontransgenic littermates consuming an atherogenic diet. We define the temporal relationships between increases in serum chemokines (JE and KC) and changes in serum lipids and compare hepatic chemokine expression and lipid accumulation with chemokine expression, macrophage content, and lesion development in the aortic root.

Methods

Please see the online data supplement, available at http://atvb.ahajournals.org.

Results

Serum Chemokines

The effects of feeding high-fat/high-cholesterol/cholate (HFC/C) diet on serum CC (JE) and CXC (KC and MIP-2) chemokine concentrations (Figure 1) were analyzed using terminal samples from mice culled at the time points indicated. At the start of the study, serum JE concentrations were higher in apoE*3 Leiden mice than in nontransgenic mice. Consumption of HFC/C diet caused rapid, and approximately equivalent, increases from baseline in serum JE in apoE*3 Leiden and nontransgenic mice. Serum JE concentrations were 3- to 5-fold higher at 4, 8, and 18 weeks in apoE*3 Leiden mice and 3- to 4-fold higher at 4, 12, and 18 weeks in nontransgenic mice consuming HFC/C diet compared with transgenic animals fed normal diet.

Serum KC levels were also higher at the start of the study in apoE*3 Leiden mice than in nontransgenic animals, but this increase was much more rapid in the transgenic animals (Figure 1B). Consumption of HFC/C diet increased serum KC significantly in apoE*3 Leiden and nontransgenic mice, at weeks 4, 8, and 18 in apoE*3 Leiden mice and at weeks 4 and 18 in nontransgenic mice fed HFC/C diet than in apoE*3 Leiden mice consuming normal diet.

Concentrations of the other mIL-8RH ligand, MIP-2, exhibited a more complex, almost biphasic profile and did not remain elevated throughout the study (Figure 1C). At the start of the study, serum MIP-2 levels were higher in apoE*3 Leiden mice consuming normal diet.
nontransgenic than in apoE*3 Leiden mice. Increases from baseline ($P<0.05$) in serum MIP-2 concentrations were observed at weeks 2, 4, 8, and 12 in apoE*3 Leiden mice fed HFC/C diet, but by week 18, MIP-2 levels fell below baseline values. At weeks 2, 4, and 18, levels of MIP-2 in apoE*3 Leiden and nontransgenic mice consuming HFC/C diet were significantly higher than those in the group of apoE*3 Leiden mice consuming normal diet. However, at no time were serum levels of MIP-2 higher in apoE*3 Leiden than in nontransgenic mice consuming the same diet.

**Lipids and Lipoproteins**

The effects of feeding HFC/C diet on serum cholesterol and triglyceride concentrations in apoE*3 Leiden and nontransgenic mice are shown in Figure 1 (see http://atvb.ahajournals.org). As expected,28 consumption of HFC/C diet for up to 18 weeks resulted in serum cholesterol levels that were higher (4- to 8-fold) in apoE*3 Leiden mice compared with nontransgenic mice ($P<0.00001$) and 10- to 18-fold higher than those in transgenic animals fed normal diet.

Serum triglyceride concentrations were higher throughout the study in both groups of apoE*3 Leiden mice than in nontransgenic mice. At all time points, serum triglyceride levels in nontransgenic animals were between 20% and 38% of the levels seen in apoE*3 Leiden mice consuming the same HFC/C diet. Serum triglyceride levels in the apoE*3 Leiden mice fed HFC/C diet were significantly lower (56%) at week 4 of the study than in apoE*3 Leiden mice fed normal diet.

Cholesterol lipoprotein profiles were analyzed at weeks 2, 4, 8, 12, and 18 using pooled serum samples from animals in the same experimental group. After 4 weeks of HFC/C diet, the lipoprotein profiles of the animals did not change substantially, and representative data from this time point are shown in Figure 1C (see http://atvb.ahajournals.org). As expected,28 HFC/C diet increased VLDL/LDL cholesterol in nontransgenic animals, with much larger increases in this fraction in the apoE*3 Leiden mice.

Livers of mice from both groups consuming HFC/C diet for 18 weeks contained more choleseryl ester but 3- to 4-fold lower triglyceride than those from apoE*3 Leiden mice consuming normal diet (Table). No significant differences in hepatic phospholipid or free cholesterol content were evident between the groups.

**Hepatic Chemokine Expression**

The earliest time points at which JE or KC mRNA could be detected by in situ hybridization were investigated using histological sections of liver and aorta from at least 4 animals per group. Representative sections are shown. Expression of KC or JE mRNA were not detected in hepatic sections from apoE*3 Leiden mice fed normal diet at any time (Figure 2A, see http://atvb.ahajournals.org).

Hepatic expression of both KC and JE mRNA were detected as early as week 2 in both apoE*3 Leiden and nontransgenic mice consuming HFC/C diet (Figures 2B, 2D, 2F, and 2H); more extensive expression of both chemokines were evident after 18 weeks on the same diet (Figures 2C, 2E, 2G, and 2I).

**Aortic Chemokine Expression, Macrophage Staining, and Lesion Development**

JE and KC mRNA expression patterns in the aortic root (Figure 2) were compared with serial sections stained for the presence of macrophages using the macrophage-specific antibody MOMA-2 (Figure 3). Lesion cross-sectional areas were 26- to 43-fold larger throughout the study in apoE*3 Leiden mice compared with nontransgenic mice fed HFC/C diet (Figure 3, see http://atvb.ahajournals.org).

After 4 weeks on atherogenic diet, early intimal lesions and MOMA-2-positive staining were evident in aortae from all (4 of 4) of the apoE*3 Leiden mice probed (Figures 3A and III). However, no expression of KC mRNA was found, and expression of JE mRNA was detected in aortic sections from only 1 of 4 apoE*3 Leiden mice (data not shown). Minimal evidence of macrophage staining, JE mRNA expression (data not shown), or lesion development could be detected in nontransgenic controls (Figures 3B and III).

After 8 weeks, larger intimal lesions had developed in apoE*3 Leiden mice, particularly in the aortic root (Figure 3, see http://atvb.ahajournals.org), and this coincided with the sites of JE mRNA expression (3 of 4) (Figure 2B); however, KC mRNA remained undetected (Figure 2C).

After 18 weeks on HFC/C diet, more advanced lesions were seen in apoE*3 Leiden mice, accompanied by extensive positive staining for macrophages within the core of the lesion in the aortic root (Figure 3C). Again, more extensive expression of JE (Figure 2D), but not KC mRNA, accompanied lesion development. No JE mRNA was detected when sections were incubated with sense probe (Figure 2E). Early fatty streak lesions were seen in nontransgenic mice at week 18 (Figure 3, see http://atvb.ahajournals.org), but no JE (Figure 3F) or KC mRNA expression was detected in these sections.

### Hepatic Cholesterol, Triglyceride and Phospholipid Content

<table>
<thead>
<tr>
<th>Group (Diet)</th>
<th>Cholesterol, mg/g wet weight</th>
<th>Triglyceride, mg/g wet weight</th>
<th>Phospholipid, mg/g wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free</td>
<td>Esterified</td>
<td>Esterified/Free</td>
</tr>
<tr>
<td>E*3 Leiden (HFC/C)</td>
<td>7.9±1.1</td>
<td>88.5±4.9*</td>
<td>11.7±1.5*</td>
</tr>
<tr>
<td>C57BL/6J (HFC/C)</td>
<td>6.6±0.8</td>
<td>92.5±14.1*</td>
<td>13.0±0.7*</td>
</tr>
<tr>
<td>E*3 Leiden (normal)</td>
<td>9.5±1.9</td>
<td>7.4±0.7</td>
<td>0.9±0.3</td>
</tr>
</tbody>
</table>

Values are mean±SE of at least 3 animals per group, fed either HFC/C or normal diet, for 18 weeks. The analyses were carried out as described in Methods on non-log-transformed data.

*Significantly different ($P<0.05$) from apoE*3 Leiden mice fed normal diet.
Correlations Between Serum Cholesterol and Chemokine Concentrations and Lesion Area

The value of serum JE and KC measurements in predicting the extent of murine atheroma were compared with serum cholesterol concentrations. Previously, it was established that total exposure to serum cholesterol, calculated as area under the curve (AUC) (see Methods), accurately predicted the extent of atheroma in apoE*3 Leiden mice. Here, serum cholesterol exposure again correlated closely with lesion area ($R = 0.93$) in apoE*3 Leiden mice but not their nontransgenic littermates. Lower positive correlations between lesion area and calculated AUC for serum JE ($R = 0.67$), KC ($R = 0.62$), and MIP-2 ($R = 0.62$) were seen in apoE*3 Leiden mice, but no such correlations were seen in nontransgenic animals consuming the same atherogenic diet. Significantly, highly positive correlations between serum cholesterol AUC and serum JE ($R = 0.86$ and 0.86), KC ($R = 0.89$ and 0.78), and

Figure 2. Photomicrographs of cross sections of aortic root from apoE*3 Leiden or nontransgenic mice (original magnification $\times 100$ unless otherwise stated). A, Section of aortic root from apoE*3 Leiden (Leiden) mouse fed normal diet for 18 weeks incubated with specific JE antisense (AS) probe. B and C, Sections of aortic root from apoE*3 Leiden mice consuming HFC/C diet for 8 weeks incubated with specific JE (B) or KC (C) antisense probes. D and E, Sections of aortic root from apoE*3 Leiden mice consuming HFC/C diet for 18 weeks incubated with JE antisense (D) or sense (S) (E) probes (D and E, original magnification $\times 40$). F, Section of aortic root from a nontransgenic animal consuming HFC/C diet for 18 weeks incubated with JE antisense probe. Arrowheads indicate positive expression of JE mRNA; no expression of KC mRNA was seen. L indicates lumen; VL, valve leaflet.

Figure 3. Photomicrographs of sections of aortic root stained for macrophages with MOMA-2 antibody (original magnification $\times 100$). Sections from apoE*3 Leiden (A) and nontransgenic (B) mice consuming HFC/C diet for 4 weeks. C, Aortic root sections from an apoE*3 Leiden mouse consuming HFC/C diet for 18 weeks showing (arrowheads) macrophages in the core of the lesion and in the adventitia. D, No specific staining was observed when sections were incubated with the isotype-matched control. L indicates lumen; VL, valve leaflet.
MIP-2 ($R=0.84$ and 0.82) were seen in apoE*3 Leiden and nontransgenic mice, respectively, consuming HFC/C diet.

**Discussion**

Atherosclerosis is a major cause of morbidity and mortality in Western societies. A serum marker reflecting clinically silent arterial pathogenesis would be a useful tool in assessing risk of developing coronary artery disease. We investigated the potential utility of systemic CC and CXC chemokines in this regard, because prototypical members of each of these chemokine subfamilies have been implicated in the recruitment or retention of monocyte-macrophages during atherogenesis. Consumption of an atherogenic diet induced sustained increases in serum CC and CXC chemokines, JE, and KC in both apoE*3 Leiden and nontransgenic mice. Serum levels of JE increased and remained approximately equivalent in both groups of animals, but serum KC increased much more rapidly in apoE*3 Leiden mice compared with their nontransgenic littermates (Figure 1B). We therefore investigated the temporal relationships between circulating chemokine concentrations, hepatic and aortic chemokine expression, lipid accumulation in the liver and serum, and development of atherosclerotic lesions in these animals.

ApoE*3 Leiden mice consuming an atherogenic diet develop atherosclerosis much more rapidly than nontransgenic mice fed the same diet (Figure 3, see http://atvb.ahajournals.org).27,28 In apoE*3 Leiden mice, lesion development seems directly related to the duration of hypercholesterolemia induced in these animals (above and Reference 28). Maximal increases in serum cholesterol concentrations were detected in both strains after 4 weeks on atherogenic diet (Figure 1A, see http://atvb.ahajournals.org), although serum cholesterol levels remained much lower in nontransgenic than in apoE*3 Leiden mice. However, despite the very differing rates of atherogenesis and degree of hypercholesterolemia induced by HFC/C diet, apoE*3 Leiden mice and their nontransgenic littermates had similar levels of hepatic lipids, particularly cholesteryl ester, after 18 weeks (Table).

Temporal increases in serum JE correlated with onset of hypercholesterolemia in both groups of animals consuming the atherogenic diet; however, the eventual extent of this rise was similar in both groups, suggesting that the absolute concentration of serum lipids was not a key determinant of serum JE levels. This contrasts with the reported effect of hypercholesterolemia on the expression of the JE/MCP-1 receptor, CCR-2, by circulating monocytes.30 Rather, serum levels of JE may be more closely associated with hepatic inflammation, possibly because of lipid accumulation. Indeed, hepatic expression of JE and KC mRNA were detected in both groups of mice after 2 weeks of consumption of the atherogenic HFC/C diet (Figure 2, see http://atvb.ahajournals.org), suggesting that the liver may be a primary source of serum chemokines in this study. Consumption of an atherogenic diet by C57BL mice has previously been shown to be associated with enhanced hepatic oxidative stress, activation of nuclear factor-kB, and increased expression of JE, KC, serum amyloid A, and heme-oxygenase mRNA.31,32 Indeed, circulating levels of serum amyloid A were increased and approximately equivalent in the 2 groups of animals consuming HFC/C diet in this study (data not shown).

The contribution of JE from developing aortic lesions to serum JE concentrations does not seem substantial, because serum JE levels in apoE*3 Leiden mice did not exceed those in the nontransgenic controls. Aortic expression of JE mRNA was also not seen until much later (8 weeks) in apoE*3 Leiden mice consuming HFC/C diet (Figure 2). Interestingly, positive macrophage staining could be detected within early lesions in both apoE*3 Leiden (4 weeks) and nontransgenic (4 weeks) mice (Figure 3) before detection of JE mRNA by in situ hybridization. Thus, apoE*3 Leiden mice seem to differ from apoE and LDL receptor–deficient models of atherogenesis, in which JE/MCP-1 expression seems to coincide directly with monocyte infiltration.29,33,34 This could be explained by the relative sensitivities of the techniques or probes used; alternatively, other chemokines may be involved in the very early stages of monocyte recruitment to the vessel wall in this murine model.

Systemic levels of ligands for mIL-8R, KC, and MIP-2 exhibited markedly differing profiles. The almost biphasic profile of serum MIP-2, seen in all the groups of animals, is intriguing, and the reasons underlying this finding are unclear. Because serum MIP-2 levels could not provide a reliable estimate of inflammatory status, they were not investigated additionally. By contrast, measurement of circulating levels of KC revealed differing inflammatory responses in the 3 groups of animals in this study. Maximal levels of serum KC were achieved by 4 weeks in apoE*3 Leiden mice consuming HFC/C diet and by 12 weeks in the nontransgenic controls and did not increase significantly in the apoE*3 Leiden mice fed normal diet. Previous reports of aortic CXC chemokine expression12–20 suggested intrasplenic expression of KC could be a factor contributing to the enhanced serum KC levels seen in apoE*3 Leiden mice. However, the apparent absence of KC mRNA within developing lesions in this study does not support this explanation. It seems more likely that the observed increases in serum KC are attributable to enhanced production of KC from the liver (above and References 31 and 32) and other tissues: For example, plasma IL-8 concentrations correlate directly with IL-8 expression by blood mononuclear cells,35–37 and patients with hypercholesterolaemia38 or congestive heart failure34 also exhibit enhanced IL-8 expression.

In conclusion, circulating levels of chemokines JE and KC are elevated and sustained during the chronic inflammatory response elicited by feeding an atherogenic diet to apoE*3 Leiden and nontransgenic mice. We propose that serum concentrations of these molecules may reflect their output from 1 or more sites of inflammation, assuming that clearance of these molecules from the circulation remains constant. Consumption of an atherogenic diet seems to trigger hepatic inflammation and chemokine expression, indicating that the liver may be an important source of the observed increases in systemic CC and CXC chemokine levels. Temporal increases in systemic JE and KC did not seem to reflect lesional expression of these molecules or, indeed, differentiate the markedly different extent of atheroma in apoE*3 Leiden mice compared with the nontransgenic controls.
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References
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Methods

Mice

Procedures involving animals were subject to internal review and UK Home Office regulations. Hemizygous transgenic mice expressing both human apoE*3 Leiden and apoC1 transgenes, were generated exactly as described previously. Non-transgenic littermates were used as controls. In the present study, female mice (60 transgenic and 40 non-transgenic) aged 8-10 weeks were allocated randomly to experimental groups on the basis of age and litter, and housed in groups of 4. Mice had free access to food and water.

Diet

Animals were fed normal rodent diet (RM1; Special Diet Services) before the experimental period. During the study (0-18 weeks) 40 apoE*3 Leiden mice and 40 of their non-transgenic littermates were fed high-fat/high-cholesterol/cholate (HFC/C) containing diet (Hope Farms); 20 apoE*3 Leiden mice were fed normal diet. HFC/C diet was formulated according to Nishina et al., and contained cocoa butter (15%), sodium cholate (0.5%), cholesterol (1%), sucrose (40.5%), cornstarch (10%), corn oil (1%), cellulose (4.7%), casein (20%), 50% w/v choline chloride (2%), methionine (0.2%) and a vitamin/mineral mixture (5.1%).

Tissue Preparation

After 2, 4, 8, 12, or 18 weeks of the study subgroups of mice were culled and hearts and livers were dissected out immediately, rinsed in 0.9% saline, immersed in OCT compound and snap-frozen in liquid N₂. Frozen tissues were stored at -70°C before sectioning.

Lipid and Lipoprotein Analysis

The mice were culled at least four hours after the start of the light phase, when food consumption is minimal, and blood samples taken from the aorta post mortem. Total serum cholesterol and triglyceride concentrations were measured enzymatically using commercially available kits (Boehringer Mannheim). An equal volume of serum from all the mice in each group was pooled and lipoproteins were separated by Superose 6B column chromatography, as described previously. Cholesterol concentration was determined in column eluate fractions.

Measurement of serum chemokine concentrations

JE, KC and MIP-2 concentrations were determined in diluted serum samples (1:1-1:4), using commercially available ELISA kits (R&D Systems). In preliminary experiments, identical serum chemokine concentrations were calculated for each of four serial dilutions, indicating that measurements were unaffected by the marked hyperlipidaemia evident in some samples.
Preparation of tissue sections

Serial sections (5µm) of the aortic root were cut as described previously, and used to assess macrophage content, chemokine expression and lesion cross-sectional area.

Immunohistochemical staining of tissue sections

Lesional macrophage content was determined by immunostaining with the antibody MOMA-2 (Serotech). Briefly, sections were air-dried, and fixed in acetone (-20°C). All subsequent incubations were carried out at room temperature. Endogenous peroxidase activity was quenched by incubating sections with H2O2 (0.3%) in methanol. Sections were washed with Tris-buffered saline/Tween (TBST; DAKO), and endogenous biotin blocked using an avidin/biotin blocking kit (Vector Labs). Sections were incubated overnight at 4°C with either anti-MOMA-2 (10µg/ml) or the equivalent concentration of the isotype control, rat IgG2a (Serotech). Sections were subsequently incubated with mouse preabsorbed goat anti-rat immunoglobulins (Biogenex), and then incubated with streptavidin conjugated peroxidase (Biogenex). Immunoreactivity was visualised by addition of dianaminobenzidine tetrahydrochloride substrate (Vector Labs). Sections were counterstained with Mayer’s haematoxylin (BDH), dehydrated through a graded series of alcohols, cleared in xylene and mounted in DPX.

Generation of riboprobes

JE/MCP-1 and KC cDNA was purchased from ATTC (Rockville, USA). As the plasmid containing the KC insert did not contain Sp6 or T7 promoter sites for generating sense and anti sense riboprobes, the insert was cut with restriction enzymes (Pst1) and cloned into PCRscript vector (Promega). Clones containing the insert were identified by restriction digest, and sequenced to check the orientation of the KC insert. Linearised plasmids were purified using the Wizard DNA clean-up kit (Promega).

In situ hybridization

Sections of aortic root and liver were used to investigate mRNA expression of chemokines, JE, and KC. Sections (5 µm) were fixed in fresh paraformaldehyde (4%) in DEPC treated PBS (pH 7.4), acetylated in acetic anhydride (0.25%) in triethanolamine (0.1 M)/NaCl (0.1 M) and dehydrated and delipidated through a graded series of alcohols and chloroform. Sense and antisense RNA probes were transcribed from linear templates, and labelled with [35S] UTP (Amersham Pharmacia) using Sp6 or T7 polymerase. Prior to ethanol precipitation of the probe, 1 µl of the reaction was run on a QuickPoint gel (Novex) to check for full-length transcripts. Probes were resuspended at 25,000 cpm/µl in hybridisation buffer (Denhardts solution (1x) containing 0.6M sodium chloride, 0.06M sodium citrate, deionised formamide (50%), dextran sulphate (10%, w/v), DTT (20 mM), polyadenylate (100 µg/ml), denatured salmon sperm DNA (100 µg/ml) and yeast tRNA (100 µg/ml)) and slides incubated overnight in a sealed humidified chamber at 55°C. After hybridisation, sections were
washed with sodium chloride (0.15M)/sodium citrate buffer (0.015M) (1 x SCC) at room temp, treated with RNaseA (20µg/ml), washed and then subjected to 3 high-stringency washes (0.5 x SSC) at 65°C (1x30 min) and room temp (2x10 min). Slides were dehydrated through a series of alcohols, air-dried and dipped in photographic emulsion (Amersham Pharmacia Biotech). Slides were exposed for 8 weeks at 4°C and developed using Kodak D19 (1:1 water), counterstained in toluidine blue, dehydrated through a graded series of alcohols and coverslipped using DPX as a mountant.

Analysis of hepatic lipid content

Hepatic lipids were extracted from liver homogenates, using a modified version of the Bligh and Dyer extraction method, where water was replaced by 0.2M KH$_2$PO$_4$, 2M KCl. Extraction efficiency was determined by incorporating radiolabelled cholesterol, cholesteryl ester, phosphatidylcholine and triolein into the extraction buffer. Lipids were dried under nitrogen, redissolved in chloroform and separated by t.l.c. (Whatman Linear K silica plate), using petroleum ether:diethyl ether:glacial acetic acid (90:30:1, by vol.) as the mobile phase. Lipids were identified by co-migration with authentic standards. Triglyceride, phospholipid, free and esterified cholesterol bands were extracted with hexane:propan-2-ol (3:2 by vol.), dried under nitrogen, resuspended in propan-2-ol, and assayed using commercially available kits (Boehringer).

Lesion Analysis

Sections were stained with Oil-Red-O, counterstained with Cole’s haematoxylin and used for quantitation of lesion development, as described previously. Lesion cross-sectional area was determined by morphometric evaluation of ten alternate sections of the aortic root, commencing where the three valve leaflets first appeared. Images were analysed using an Olympus BH-2 microscope and video-imaging system, exactly as described previously. All images were captured under identical lighting, microscope, camera and PC conditions. Atherosclerotic lesion areas were quantified by drawing around the lesions by hand using Optimas software. Absolute values for the cross-sectional area of the lesion were obtained by calibrating the software using an image of a micrometer slide taken at the same magnification.

Statistical Analysis

All the parameters described, except those indicated, were log-transformed in order to normalise the variances, and geometric means with 95% confidence intervals are quoted. Data for serum cholesterol, triglycerides, and chemokines were analysed using 2-way analysis of variance (ANOVA) because the mice were only sampled once when they were culled after either 2, 4, 8, 12 or 18 weeks. Comparisons were made between the apoE*3 Leiden mice fed HFC/C diet and either those fed normal diet, or the non-transgenic mice fed HFC/C diet, at each time point using a Dunnetts test. Differences between apoE*3 Leiden mice fed normal diet and non-transgenic mice were analysed post-hoc using the...
Bonferroni method. Pre-treatment data were transformed using the same method as the treatment data and then analysed using 1-way ANOVA. Comparisons were made as described above. For each timepoint in the study, the data for the available animals at that time was compared back to its baseline data using a repeated measures analysis of variance. From this analysis the within-animal variability was used to estimate the change from baseline for each group, along with a 95% confidence interval. Where the data was log-transformed, the change from baseline on the log scale was back-transformed to provide a ratio to baseline with confidence interval. The data for total lesion area were log-transformed and all sections analysed simultaneously using a split plot ANOVA. Comparisons of groups were made on the data from each week at the middle aortic section using the Bonferroni method. Differences were considered to be statistically significant if $p < 0.05$.

To investigate possible relationship(s) between lesion area, exposure of the arterial wall to increased concentrations of plasma cholesterol, and steady state levels of chemokines in the circulation, cholesterol and chemokine ‘exposures’ were calculated for each animal, as described previously. These were defined as the areas under the curve (AUC) in serum cholesterol or chemokine versus time plots. Analysis of serum chemokines was performed in terminal bleeds for each animal. To calculate cholesterol or chemokine exposure, we made best estimates of serum cholesterol or chemokine concentrations for the preceding week(s) for each animal. The AUC for each animal was calculated from the constructed plot of serum cholesterol or chemokine against period on diet, as described previously. Correlations were made using log-log plots of lesion area versus serum cholesterol AUC or serum chemokine AUC, and serum cholesterol AUC versus chemokine AUC.

References


Effect of diet and strain of mouse on serum cholesterol (A) and triglyceride (B) concentrations, and (C) lipoprotein profile concentrations (■, apoE*3 Leiden mice consuming HFC/C diet (n=8); ●, non-transgenic controls consuming HFC/C diet, (n=8); □, apoE*3 Leiden mice consuming normal diet, (n=4)). Values shown in (A) and (B) are geometric means±95% confidence intervals (a=significantly different ($p<0.05$) to apoE*3 Leiden mice consuming HFC/C diet, b= significantly different ($p\leq0.05$) to apoE*3 Leiden mice consuming normal diet).
Figure II

Photomicrographs of hepatic sections incubated with specific antisense (AS) probes to either KC or JE (original magnification x 250). Typical section (a) of liver from an apoE*3 Leiden mouse (Leiden) consuming normal diet for 18 weeks, labelled with the antisense probe for KC. Panels b), c), f) and g): sections of liver from non-transgenic (NTG) animals consuming HFC/C diet for 2 (b,f) or 18 (c,g) weeks, incubated with either KC (b, c) or JE (f, g) antisense probes, panels d),e),h), and i): hepatic sections from apoE*3 Leiden mice consuming HFC/C diet for 2 (d, h) or 18 (e, i) weeks, incubated with either KC (d, e) or JE (h, i) antisense probes. Arrowheads indicate positive clusters of chemokine expression.
Effect of feeding HFC/C diet for up to 18 weeks on the cross-sectional area of atherosclerotic lesions in the aortic roots of apoE*3 Leiden (n=8) and their non-transgenic littermates (n=8). Values shown are geometric means±95% confidence intervals (a=significantly different (p≤0.05) from non-transgenic mice consuming HFC/C diet).