Circulating levels of the chemokines JE and KC in female C3H apolipoprotein-E-deficient and C57BL apolipoprotein-E-deficient mice as potential markers of atherosclerosis development

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Abstract
We have investigated serum chemokines for their suitability as markers of atherosclerosis development in apoE (apolipoprotein E)-deficient (−/−) mice. Female C3H apoE−/− and C57BL apoE−/− mice were fed on either diet W (Western diet; 6 weeks) or normal rodent diet (12 weeks). Serum lipids (0, 6 and 12 weeks) and terminal chemokine levels were measured using commercially available assays, whereas the lesion area was determined using Oil-Red O-stained aortic sections. Serum lipids were higher in C3H apoE−/− mice for both diets throughout the study; however, lesions were significantly larger in C57BL apoE−/− mice fed on either diet. Chemokine levels were significantly lower in C3H apoE−/− mice fed on the normal diet, but no difference was observed between the two groups fed on diet W. We conclude that serum chemokine levels are potential markers for atherosclerosis susceptibility in C3H and C57BL apoE−/− mice fed on a normal rodent diet.

Introduction
Atherosclerosis is an inflammatory disease, which is the major cause of morbidity and mortality in the Western world due to complications such as myocardial infarction and cerebrovascular disease [1]. Atherosclerosis is initiated by the migration of monocytes into the arterial wall, where they are transformed into lipid-laden macrophages (foam cells) on exposure to oxidized low-density lipoprotein (oxLDL) [1]. The ‘fatty streaks’ that are formed may continue to develop into complex lesions with fibrous caps, in part because of chemokine release from the infiltrating leucocytes [1,2]. The result is an advanced plaque that is often unstable and highly prone to thrombosis [3].

Apolipoprotein E (apoE) mediates cellular uptake of atherogenic lipoproteins via the LDL receptor [4]. ApoE-deficient (apoE−/−) mice have reduced clearance of chylomicrons and VLDLs (very-low-density lipoproteins) from the plasma, resulting in hypercholesterolaemia [5,6]. Development of atherosclerotic lesions occurs even when these mice are fed on a normal diet, but is accelerated when fed on a Western diet containing cholesterol [7]. C3H/HeN apoE−/− (C3H apoE−/−) mice have been shown to have smaller lesions than C57BL/6J apoE−/− (C57BL apoE−/−) mice when fed on a high fat diet, despite C3H apoE−/− mice having significantly higher serum lipids [7].

Chemokines (chemoattractant cytokines), such as JE (murine homologue of the human CCR-2 chemokine monocyte chemoattractant protein-1) and KC (murine homologue of the human CXCR-2 chemokine Gro-α (growth-related oncogene α)), are involved in the inflammatory process of atherogenesis. JE has been reported to be located in the atherosclerotic lesions of the C57BL/6 129/Sv apoB-over-expressing mouse model [8] and in atherosclerotic lesions of apoE−/− Leiden mice [9]. However, KC was not detected in these lesions [9]. Despite this finding, there is some evidence that CXC chemokines such as KC may have a role in monocyte recruitment and migration in atherosclerosis [10].

The aim of this study was to investigate whether levels of serum chemokines could be used as potential markers of atherosclerosis in C3H and C57BL apoE−/− mice fed either on a Western or a normal diet.

Experimental
Procedures involving animals were subject to internal review and U.K. Home Office regulations. Female C3H/HeN (Charles River, Margate, Kent, U.K.) and C57BL/6J (Iffa Credo, L’Arbresle, France) mice were back-crossed on to the apoE−/− background and, at 10 weeks old, were fed on either a Western diet (diet W) for 6 weeks or a normal diet for 12 weeks in order to induce hypercholesterolaemia and atherosclerosis. Diet W was composed of cocoa butter (15%), cholesterol (0.25%), sucrose (40.5%), corn oil (1%) and 50% (w/v)
Effects of diet and mouse strain on serum cholesterol levels

Female C3H apoE−/− and C57BL apoE−/− mice were fed on diet W (n = 15 per group) for 6 weeks, or normal rodent diet (n = 10 per group) for 12 weeks. Serum cholesterol levels were measured for each individual animal at weeks 0, 6 and 12 (where appropriate) using a commercially available assay. Groups fed on diet W are shown by white bars (C3H apoE−/− mice) and black bars (C57BL apoE−/− mice); groups fed on a normal diet are shown by dotted bars (C3H apoE−/− mice) and grey bars (C57BL apoE−/− mice). Values shown are geometric means for each group ± confidence interval (bars labelled ‘a’, \( P < 0.0001 \) compared with C57BL apoE−/− mice consuming diet W; bars labelled ‘b’, \( P < 0.0001 \) compared with C57BL apoE−/− mice consuming a normal diet).

Choline chloride (2% solution; Hope Farms, Woerden, The Netherlands), whereas normal rodent diet (2014) included 14% protein and 3.5% fat (Harlan Teklad, Bicester, Oxon, U.K.). Serum was isolated from a blood sample at week 0 and a terminal blood sample after either 6 weeks (diet W) or 12 weeks (2014 diet), when the mice were killed by CO2 asphyxiation followed by exsanguination.

Atherosclerotic lesions were quantified using 10 (10 \( \mu m \)) serial sections of the aortic sinus with lipophilic Oil-Red O staining, as described previously [11].

Serum lipid levels were determined using a commercially available colorimetric enzymic assay at weeks 0, 6 and 12 (that for cholesterol was from WAKO, Alpha Labs, Richmond, VA, U.S.A.; that for triacylglycerols was from ABX Diagnostics, Shefford, Beds., U.K.), whereas serum chemokine levels were determined using commercially available colorimetric ELISAs (R&D Systems, Abingdon, Oxon, U.K.) after 6 or 12 weeks.

All responses were logarithmically transformed to normalize the variance, before statistical analysis by ANOVA was performed.

Results

Serum cholesterol levels (Figure 1) in the C3H apoE−/− mice were significantly higher (1.6-fold difference; \( P < 0.0001 \)) than those of C57BL apoE−/− mice at the beginning of the study. Cholesterol levels in C3H apoE−/− mice remained significantly higher than in C57BL apoE−/− mice whether they were fed on a normal diet or diet W (1.7- and 1.5-fold differences respectively; \( P < 0.0062 \)). Cholesterol levels were elevated in both strains fed on diet W, with a 3-fold increase in C3H apoE−/− mice and a 3.5-fold increase in C57BL apoE−/− mice compared with identical mice fed on a normal rodent diet (\( P < 0.0001 \)). Serum triacylglycerol levels were significantly higher (7.6-fold increase; \( P < 0.001 \)) for the C3H apoE−/− mice compared with C57BL apoE−/− mice at time zero (results not shown). Mice fed on diet W had significantly higher serum triacylglycerol levels (C3H apoE−/− mice, 0.69-fold increase; C57BL apoE−/− mice, 1.6-fold increase) compared with those fed on a normal diet. C3H apoE−/− mice had significantly higher triacylglycerol levels than C57BL apoE−/− mice when fed either on diet W (3.7-fold difference; \( P < 0.0001 \)) or on a normal diet (9-fold increase; \( P < 0.0001 \)).

Serum levels (Figure 2) of JE and KC were not significantly different between C3H apoE−/− and C57BL apoE−/− mice.
Effects of diet and mouse strain on atherosclerotic lesion development

Female C3H apoE<sup>−/−</sup> and C57BL apoE<sup>−/−</sup> mice were fed on either diet W (n = 15 per group) for 6 weeks or a normal rodent diet (n = 10 per group) for 12 weeks. The lesion area was determined for individual animals using 10 (10 µm) serial sections of the aortic sinus stained with lipophilic Oil-Red O. Values shown are geometric means for each group (±95% confidence interval). *P < 0.0001 compared with C57BL apoE<sup>−/−</sup> mice consuming diet W; †P < 0.0001 compared with C57BL apoE<sup>−/−</sup> mice consuming a normal diet.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet</th>
<th>Mean lesion area (µm&lt;sup&gt;2&lt;/sup&gt;)</th>
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<tbody>
<tr>
<td>C3H apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>W</td>
<td>3711 (1386–9674)*</td>
</tr>
<tr>
<td>C57BL apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>W</td>
<td>70 738 (45 592–109 720)</td>
</tr>
<tr>
<td>C3H apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Normal</td>
<td>391 (74–1284)†</td>
</tr>
<tr>
<td>C57BL apoE&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>normal</td>
<td>29 862 (17 564–50 772)</td>
</tr>
</tbody>
</table>

fed on a Western diet for 6 weeks. However, C3H apoE<sup>−/−</sup> mice fed on a normal diet for 12 weeks had significantly lower levels of JE (3.8-fold difference, P < 0.0001) and KC (2.6-fold difference, P < 0.0001) compared with the C57BL apoE<sup>−/−</sup> mice.

Atherosclerotic lesions (Table 1) in the aortic sinus were significantly smaller (P < 0.0001) in C3H apoE<sup>−/−</sup> mice compared with C57BL apoE<sup>−/−</sup> mice fed on a normal diet. Both groups of mice developed larger lesions when fed on a Western diet for 6 weeks; however, the lesions were still significantly smaller (P < 0.0001) in C3H apoE<sup>−/−</sup> compared with C57BL apoE<sup>−/−</sup> mice.

Table 1 | Effects of diet and mouse strain on atherosclerotic lesion development

Discussion
We have investigated whether circulating levels of serum chemokines JE and KC are markers for atherosclerosis using C3H and C57BL apoE<sup>−/−</sup> mice, which are known to be atherosclerosis-resistant and atherosclerosis-susceptible respectively. As may be anticipated, serum lipid levels are lower in both C3H and C57BL apoE<sup>−/−</sup> mice fed on a Western diet compared with those fed on a Western diet. However, irrespective of the diet, C3H apoE<sup>−/−</sup> mice had significantly higher serum lipid levels than C57BL apoE<sup>−/−</sup> mice. This is surprising, because C3H apoE<sup>−/−</sup> mice have significantly smaller atherosclerotic lesions compared with C57BL apoE<sup>−/−</sup> mice when fed on either a Western diet or a normal diet. It is known that C57BL apoE<sup>−/−</sup> mice are more susceptible than C3H apoE<sup>−/−</sup> mice to stimulation by oxLDL, resulting in increased expression of adhesion molecules on arterial endothelium [12]. Macrophages of C57BL/6J wild-type mice also accumulate cholesteryl ester in the cytoplasm more rapidly on feeding a 1% cholesterol diet than do those of C3H wild-type mice, and so they can more readily form foam cells [13].

Serum levels of JE and KC were not significantly different between C3H and C57BL apoE<sup>−/−</sup> mice fed on a Western diet despite a significant difference in lesion size between the two strains. This suggests that chemokines may be released from tissues other than the aorta and may indicate that liver inflammation induced on feeding diet W caused the large increase in serum chemokines [14,15].

Serum levels of JE and KC were significantly lower in the C3H apoE<sup>−/−</sup> mice fed on a normal diet compared with the C57BL apoE<sup>−/−</sup> mice. This suggests that serum chemokines are markers of early atherosclerosis, and may predict the development of lesions when there is no diet-induced inflammation.

In the present study, chemokines were measured only at the terminal time-point. Further studies are in progress to investigate the temporal relationships of serum chemokines in atherogenesis in these mouse models.

References

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