Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats

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The relative benefit of replacing saturated fatty acid with linoleic acids is still being debated because a linoleic acid-enriched diet increases oxidative and inflammatory stresses, although it is associated with a reduction in serum cholesterol levels. The present study was conducted to evaluate the effect of dietary supplementation of linoleic acid-rich (HL) fat, compared with a saturated fatty acid-rich (SF) fat on atherosclerotic lesion areas, serum and liver cholesterol levels, oxidative stress (urinary isoprostanes and serum malondialdehyde) and inflammatory stress (expression of aortic monocyte chemoattractant protein-1; MCP-1) in apo E-deficient mice. Male and female apo E-deficient mice (8 weeks old; seven to eight per group) were fed an AIN-76-based diet containing SF fat (50 g palm oil and 50 g lard/kg) or HL fat (100 g high-linoleic safflower-seed oil/kg) for 9 weeks. Compared with the SF diet, the HL diet lowered atherosclerosis (P<0·05). It reduced serum total cholesterol levels (P<0·05), increased HDL-cholesterol levels (P<0·05) and lowered liver esterified cholesterol levels (P<0·01). The HL diet-fed mice showed increased expression of MCP-1 mRNA (P<0·05), serum levels of malondialdehyde (P<0·05) and urinary excretion of 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2α (P<0·05). These results suggest that having biomarkers in vivo for oxidative stress and inflammatory status of endothelial cells does not necessarily indicate predisposition to an increased lesion area in the aortic root in apo E-deficient mice fed an HL or SF diet.

Apolipoprotein E-deficient mice: Linoleic acid: Monocyte chemoattractant protein-1: Saturated fatty acids

Endothelial dysfunction is a key variable of atherosclerosis where elevated serum cholesterol levels are associated with endothelial dysfunction (Vogel, 1999). Endothelial dysfunction is also associated with increased oxidative stress, an important promoter of inflammatory processes (Napoli et al. 2001). In man, saturated fatty acids increase the levels of LDL- and VLDL-cholesterol, and current recommendations include decreasing their intake as fatty acids increase the levels of LDL- and VLDL-cholesterol, diabetes and smoking, local factors including shear stress, genetic factors, and unknown factors determine the status of endothelial function (for a review, see Bonetti et al. 2003). The advantage of using animal models to examine the effect of dietary fats is that the level of serum cholesterol, the in vivo status of oxidative or inflammatory stresses and atherosclerosis can simultaneously be determined at the end of the study. Thus, we conducted the present study to investigate the effects of dietary enrichment of linoleic acid on serum and liver lipid levels, urinary excretion of isoprostanes as a biomarker of oxidative stress, expression of aortic monocyte chemoattractant protein-1 (MCP-1) as a biomarker of inflammatory status of endothelial cells (Bursill et al. 2004) and the extent of atherosclerosis in apo E-deficient mice.

Materials and methods

Animals and diets

ApoE-deficient mice purchased from Jackson Laboratory (Bar Harbor, ME, USA) in 1994 were used (Ni et al. 1998). Male and female apo E-deficient mice (8 weeks old) with an initial weight of 26·4 (se 0·6) g for male mice and 22·0 (se 0·6) g for female mice were divided into two groups, and were fed an AIN-76 diet

Abbreviations: 15-F2t-IsoP-M, 2,3-dinor-5,6-dihydro-8-iso-prostaglandin F2α; HL, linoleic acid-rich; 8-iso-PGF2α, 8-iso-prostaglandin F2α; MCP-1, monocyte chemoattractant protein-1; SF, saturated fatty acid-rich; TBARS, thiobarbituric acid reactive substances.

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Determination of 8-iso-prostaglandin F2α and notification no. 6 of the Government of Japan. Animal Experiments at the Faculty of Agriculture and the Graduate School of Veterinary Medicine, Kyushu University, Fukuoka, Japan, and law no. 105, 1987. The primer sequences of mouse MCP-1 were 5’-GAATTC-GGAGAGCATAGCCCTCGTAGATGG for the 3’-primer and 5’-GAATTCTACCAGTAGTCCGAGTCACAC for the 3’-primer. Also, the measurement of aortic β-actin mRNA levels was carried out as a housekeeping gene to confirm stable expression from these aortic samples. The primer sequences of mouse β-actin (Shimano et al. 1996) were 5’-GGATCCCGATGCTTGGTGAATGATCCCAATG for the 5’-primer and 5’-AAGGGCATACAGTCCGAGTCACAC for the 3’-primer. The amplification products obtained using the two primer pairs had 357 bp of MCP-1 or 254 bp of β-actin. The PCR amplification of MCP-1 as well as β-actin was carried out in 10 μl reaction mixtures composed of 1 μl cDNA solution, 2.5 units of Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA), 1 μl 10 × PCR buffer containing 2.5 mM-MgCl₂ (attached to the enzyme), 1-6 μM of each dNTP and 5 pmol each of the two oligonucleotide primers. The amplification conditions were as follows: 95°C, 2 min; 95°C, 1 min, 54°C, 30 s, 72°C, 1 min, for forty-two cycles; and finally 72°C, 5 min. The amplification products obtained using the two primer pairs had 357 bp of MCP-1 or 254 bp of β-actin. We cloned the PCR products into pGEM-T Easy (Promega, Madison, WI, USA) using a sequence vector to check the sequence with the 897

Linoleic acid-rich fats lower atherosclerosis

Table 1. Fatty acid composition of saturated fatty acid-rich fat (SF) and linoleic acid-rich fat (HL) (g/100 g total fatty acids) (Mean values with triplicate determination for each group)

<table>
<thead>
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<th>HL</th>
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<td>–</td>
</tr>
<tr>
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<td>3.4</td>
<td>7.0</td>
</tr>
<tr>
<td>16:1</td>
<td>2.2</td>
<td>–</td>
</tr>
<tr>
<td>18:0</td>
<td>9.2</td>
<td>3.6</td>
</tr>
<tr>
<td>18:1</td>
<td>4.3</td>
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</tr>
<tr>
<td>18:2</td>
<td>9.8</td>
<td>75.6</td>
</tr>
</tbody>
</table>
a DNA sequencer; Gene Rapid (Amersham Pharmacia Biosciences, Tokyo, Japan).

The aorta total RNA samples from these mice were subjected to RT-PCR as described earlier, but the cycle number was changed from forty-two to twenty cycles for MCP-1 and to sixteen cycles for β-actin. Southern-blotting hybridisation of the PCR products was carried out as described elsewhere (Wahl et al. 1979). The products on the nitrocellulose filter, Hybond NX (Amersham Pharmacia Biosciences, Tokyo, Japan) were hybridised to 32P-labelled probe of the cloned mouse MCP-1 or β-actin. The exposition intensities of these bands were then quantified by a Bio-imaging analyser FLA-5000 (Fuji Photo Film, Tokyo, Japan). We have confirmed that the concentrations (0.5, 1, 2 and 3 μl per 10μl reaction solution) of the cDNA from the aorta total RNA were linearly correlated with the radiation activities for MCP-1 (r 0.991; P<0.01).

Analyses of serum and liver lipids

Serum lipid levels were determined using commercially available kits (Cholesterol C test, Triglyceride G Test and Phospholipid B Test from Wako Pure Chemicals, Osaka, Japan and HDL-C2 from Daiichi Chemicals, Tokyo, Japan) and liver lipids were chemically determined as previously described (Tomoyori et al. 2004). The fatty acid composition of the liver and liver phosphatidylcholine was determined according to methods described previously (Carvajal et al. 2000).

Serum lipid peroxidation

Lipid peroxidation was quantified by measuring the serum concentration of thiobarbituric acid reactive substances (TBARS) determined to be malondialdehyde (MDA), a product of lipid peroxidation as previously described by Yagi (1976).

Serum nitrite plus nitrate

Serum nitrite plus nitrate (NO2 plus NO3) was determined as the final metabolites of NO as described previously (Ni et al. 2003).

Morphometric determination of atherosclerosis

Apo E-deficient mice were perfused with 50 ml PBS (pH 7.4) via a cannula inserted into the left ventricle, which allowed unrestricted efflux from an incision in the vena cava. After the aorta and its main branches were dissected from the aortic valve to the iliac bifurcation, perfusion of the heart was immediately continued with 50 ml of 10% (v/v) neutral formalin buffer solution (pH 7.4). The heart was removed and fixed in 10% (v/v) neutral formalin-buffered solution (Ni et al. 1998). To determine the cross-sectional lesion area, hearts containing aortic roots were processed for quantitative atherosclerosis assay as previously described (Tomoyori et al. 2004).

Statistics

The data were expressed as the means with their standard errors and were analysed by two-way ANOVA followed by a post hoc test (Bonferroni–Dunn method). Statistical analysis was carried out with Statcel (OMS, Saitama, Japan) and Excel 2000 (Microsoft, Redmond, WA, USA) and differences were considered to be statistically significant for P<0.05.

Results

There was a difference in the initial mean body weight between females (21.9 (SE 0.6) and 22.1 (SE 0.5) g for the SF and HL group, respectively) and males (26.4 (SE 0.7) and 26.4 (SE 0.5) g for the SF and HL group, respectively) (P<0.01). The final mean body weight for the females (32.0 (SE 1.4) and 33.1 (SE 1.5) g for SF and HL group, respectively) was smaller than that for males (41.9 (SE 1.0) and 43.1 (SE 0.8) g for SF and HL group, respectively) (P<0.01), but there was no significant dietary fat effect observed. The sex and type of dietary fat had no significant effect on the mean daily food intake: 4.2 (SE 0.1) and 4.2 (SE 0.1) g for SF- and LH-fed females, respectively; and 4.8 (SE 0.1) and 4.5 (SE 0.1) g for SF- and HL-fed males, respectively. There was also no significant difference due to sex and type of dietary fat on the relative liver weight (data not shown).

Table 2 shows the degree of atherosclerotic lesions as well as concentrations of the serum and liver lipids, serum NO2 plus NO3, serum TBARS and urinary isoprostanes in the apo E-deficient female and male mice fed a diet containing different fats. Two-way ANOVA revealed main effect of fats on the lesion size in the aortic root (P<0.01) and the serum LDL-cholesterol concentrations (P<0.01): the HL diet resulted in a more decreased lesion size and increased HDL-cholesterol concentrations than did the SF diet. There was no main effect of sex on these characteristics. A main effect of the fats was observed on the levels of serum total cholesterol (P<0.01), liver total cholesterol (P<0.01) and esterified cholesterol (P<0.01) and the serum TBARS (P<0.01). The mice fed the HL diet had a decreased concentration of serum total cholesterol and liver total or esterified cholesterol and increased concentration of serum TBARS compared with the SF-diet mice and these values were higher for males than for females. The atherosclerotic lesion area was significantly negatively correlated to the serum HDL-cholesterol level (r 0.13; P<0.05), but was not significantly related to the serum total cholesterol level (r 0.13; P>0.1). There was no main effect of fats on the concentrations of serum triacylglycerols and NO2 plus NO3. Table 2 also shows the urinary excretion of 8-iso-PGF2α or the β-oxidation product of 15-F2t-IsoP-M. Two-way ANOVA revealed a main effect of fats on the urinary levels of 15-F2t-IsoP-M (P<0.01), but not of 8-iso-PGF2α. Here, HL diet-fed mice showed increased excretion of the former isoprostane than compared with the SF diet-fed mice and females tended to excrete greater amounts of both isoprostanes. The atherosclerotic lesion area was not significantly related to 8-iso-PGF2α (r 0.11; P>0.1) or 15-F2t-IsoP-M (r 0.18; P>0.1) in the urine.

Fig. 1 shows MCP-1 mRNA expression over the whole aorta, where aortas that were unbroken from the aortic valve to the iliac bifurcation were used for RNA extraction. Main effects were found for fats on this expression: the HL diet-fed males exhibited increased expression of the mRNA compared with the SF diet-fed males; MCP-1 mRNA expression in females also tended to be lower.

Table 3 shows the fatty acid composition of the serum phosphatidylcholine. There was a prominent difference in the proportion of oleic, linoleic and arachidonic acids between the HL diet-fed and SF diet-fed mice, where a significant sex effect on their
proportions was seen, but only to a small extent. ANOVAs showed a significant diet effect on the proportion of saturated fatty acids (16:0 and 18:0), but the extent was smaller compared with the differences for the unsaturated fatty acids (.005). There were significant effects for fat (.05) (Bonferroni–Dunn test).

**Discussion**

The novel findings of the present study were the following: (1) atherosclerotic lesion area was greater in the apo E-deficient mice fed the SF diet than in those fed the HL diet, whereas the extent of biomarkers for oxidative stress (15-F2t-Isop-P-M) and inflammatory status (MCP-1 mRNA) in the arterial wall was greater in the latter group than in the former group; (2) the serum total cholesterol level was lower in the HL group than in the SF group, whereas the HDL-cholesterol level was higher in the former group than in the latter group. These results suggest that in apo E-deficient mice, the cholesterol traffic between the serum and arterial wall is the primary determinant for the atherosclerosis process.

In the present study, the HL diet-fed apo E-deficient mice had an increased proportion of linoleic and arachidonic acids in their serum phosphatidylcholine than did the SF diet-fed mice. It is probable that the elevation of these PUFA was associated with increased in serum TBARS and urinary isoprostanes, which are derived from arachidonic acid (Morrow et al. 1999). The results that the HL diet makes serum lipoproteins more susceptible to oxidation are in agreement with those of a previous in vitro study which found an increased rate in conjugate diene formation in LDL enriched with linoleic acid (Reaven et al. 1993), and an in vivo study that found increased urinary excretion of 8-iso-PGF2α in human subjects after consumption of a diet rich in linoleic acid compared with an oleic acid-rich diet (Turpeinen et al. 1998). Furthermore, the HL diet compared with the SF diet resulted in increased inflammatory stress as reflected in the increased expression of aortic MCP-1. It is probable that the elevation of MCP-1 mRNA was in part related to increased oxidative stress in mice of the HL group than in those of the SF group, because linoleic and arachidonic acids induce MCP-1 gene expression in cultured cells by activating the oxidative stress-responsive transcription factor NF-κB (Hennig et al. 1996; Lee et al. 2001).

Despite the above unfavourable effects of the HL diet, the present study found that compared with the apo E-deficient mice fed the SF diet, the HL diet-fed mice had decreased lesion sizes in the aortic root compared with those of the SF group, followed by a decreased serum cholesterol level and an increased level of the serum HDL compared with the SF group.
diet group. These favourable effects of the HL diet have also been observed in studies on the African green monkey (Rudel et al. 1995), LDL receptor-deficient human apo B-transgenic mice (Rudel et al. 1998), and LDL receptor-deficient mice (George et al. 2000). In these experiments, the SF diet contained palm oil (49 % palmitic acid and 37 % oleic acid; Rudel et al. 1995, 1998) or cocoa butter (43 % palmitic plus stearic acid and 35 % oleic acid; George et al. 2000) as the primary source of dietary fat. Therefore, the diets designated SF diets in the present and previous studies (Rudel et al. 1995, 1998; George et al. 2000) contained almost equal proportions of saturated fatty acids and MUFA. Hence, it may not be appropriate to term them SF diets. In addition, Calleja et al. (1999) reported that male apo E-deficient mice fed a chow-based diet containing 10 % sunflower-seed oil (56 % linoleic acid) had fewer lesions than did those fed 10 % palm oil. In contrast, Merkel et al. (2001) reported that there were no significant differences in the atherosclerotic lesion areas between HL diet-fed LDL receptor-deficient mice and SF diet-fed LDL receptor-deficient mice, and between HL diet-fed apo E-deficient mice and SF diet-fed apo E-deficient mice. In their experiments they (Merkel et al. 2001) used coconut oil (71 % saturated fatty acids and 19 % MUFA) as a primary source of dietary fats for the SF diet. For the same experiments with the LDL receptor-deficient mice, compared with the SF and HL diets, the MUFA-rich diet (oleic acid-enriched safflower-seed oil as primary fat; 71 % MUFA) significantly increased atherosclerosis in both sexes (Merkel et al. 2001). As described earlier, Rudel and colleagues (Rudel et al. 1995, 1998) used two animal models to compare the effect of various dietary fats on atherosclerosis. Animals fed an SF diet (palm oil as the primary fat source; 49 % palmitic acid and 37 % oleic acid) and a MUFA-rich diet (oleic acid-enriched sunflower-seed oil as the primary fat source; 71 % MUFA) developed equivalent numbers of atherosclerotic lesions and those fed the HL diet developed fewer. From these results, it is probable that dietary enrichment of linoleic acid is associated with a reduction in the extent of atherosclerotic lesion development in the atherosclerosis-susceptible mouse model. Furthermore, dietary fats rich in saturated fatty acids and MUFA rather than dietary fats enriched in saturated fatty acid alone appear to be more atherogenic than did linoleic acid-rich diets.

Although the present study was not designed to explore the mechanism whereby an HL diet positively prevents atherosclerosis susceptibility, the opposing results for the atherosclerotic lesion area and the MCP-1 mRNA expression between apo E-deficient mice fed the HL diet and SF diet suggest that dietary factors could be involved in influencing monocyte chemoattraction to arterial intima. Several animal studies found a role for the CC-chemokine receptor 2 in atherosclerosis (Boring et al. 1998; Guo et al. 2003). CC-chemokine receptor 2 is expressed on circulating monocytes, and this expression is upregulated in hypercholesterolemia and suppressed by elevated levels of HDL (Han et al. 1999). In the present study, the HL diet resulted in lower serum cholesterol and elevated HDL-cholesterol levels. Therefore, it remains to be determined if an HL diet is involved in lowering the recruitment of circulating monocytes into the arterial intima by reducing the expression of CC-chemokine receptor 2.

In summary, the present study found that an HL diet in vivo induces oxidative and inflammatory stresses as an adverse effect but improves serum lipoprotein cholesterol levels as a beneficial effect, and their net effect is an anti-atherogenic state in apo E-deficient mice. Therefore, having biomarkers in vivo for oxidative stress and inflammatory stress of endothelial cells does not necessarily show a predisposition toward atherosclerosis initiation or development in this animal model when fed different dietary fats.

Acknowledgements

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References


Table 3. Effect of dietary saturated fatty acid-rich fat (SF) and linoleic acid-rich fat (HL) on the fatty acid composition of serum phosphatidylcholine in female and male apolipoprotein E-deficient mice (g/100 g total fatty acids) (Mean values with their standard errors for seven to eight mice per sex for each group)

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<th>SE</th>
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<th>SE</th>
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a,b,cMean values within a row with unlike superscript letters were significantly different (P<0·05) (Bonferroni–Dunn test).
Linoleic acid-rich fats lower atherosclerosis


