Effect of Dietary Fish Oil on Coronary Artery and Aortic Atherosclerosis in African Green Monkeys

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Studies were carried out for 2.5 to 3 years in adult male African green monkeys (grivet subspecies) fed diets containing 22% of calories as lard or fish oil with 40% of calories as fat and 0.75 mg cholesterol/Kcal to determine if isocaloric substitution of menhaden fish oil for lard affects coronary artery atherosclerosis. The average total plasma cholesterol concentrations during the experimental period were significantly lower for the fish-oil group (231 ±37 mg/dl) compared to the lard group (360±44 mg/dl), but this difference did not become apparent until after 5 months of experimental diet consumption. High density lipoprotein cholesterol concentrations were 30% lower (p<0.01) for the fish-oil group also (57+5 vs. 82+6 mg/dl). Plasma triglyceride concentrations were low for both groups, but after about 5 months of diet consumption, they were higher for the animals fed fish oil (25±2 mg/dl) compared to their lard-fed counterparts (15±1 mg/dl). Coronary artery intimal area (in this case a measure of early atherosclerotic lesion size) was low in all animals but was significantly less (p<0.03) for the fish oil vs. lard groups (0.01 ±0.002 vs. 0.03±0.009 mm²). More atherosclerosis was found in other arteries, and a trend was seen of less atherosclerosis in the thoracic aorta and common carotid arteries of the fish-oil group. The size of lesions in the abdominal aorta was similar between diet groups, but microscopic examination of arteries of the lard group revealed relatively more cholesterol monohydrate crystals compared to the arteries of the fish-oil group. Chemical analysis showed that there was less esterified cholesterol (1.46±0.71 vs. 3.43±0.74 mg/g, p=0.04) and free cholesterol (3.7±2.15 vs. 7.05±1.68 mg/g, p=0.08) in the abdominal aortas taken from the animals fed fish oil. There was a significant correlation between low density lipoprotein (LDL) cholesteryl ester (CE) fatty acid ratio (i.e., saturated+monounsaturated/polyunsaturated species) and the amount of esterified (r=−0.59) and free (r=−0.63) cholesterol in the abdominal aortas. Compared to the lard group, animals fed fish oil had significantly lower LDL CE melting temperatures (26±1 vs. 38±1°C) and significantly smaller LDL particles (2.68±0.10 vs. 3.25±0.38 g/ml). Therefore, the potentially antiatherogenic effects of dietary fish oil include its ability to decrease the concentration, size, CE content, and CE melting temperature of plasma LDL.

Although deaths resulting from atherosclerotic heart disease have been declining in recent years, this disease is still the number one cause of death in the United States and most Westernized countries.1 The prevalence of coronary heart disease has stimulated interest in interventions that might retard atherosclerosis development and reduce the risk of coronary heart disease. Many studies have focused on dietary intervention because of the positive relationship between dietary cholesterol and fat intake and atherosclerotic heart disease.2 Recently, much interest has been generated by the observation that Greenland Eskimos appear to have a lower death rate from coronary heart disease than do Eskimos assimilated into Danish society.3 It has been suggested that the apparent lower coronary heart disease death rate may be related to the high consumption of cold-water marine products by the Greenland Eskimos compared to the more “Westernized,” saturated diets consumed in Danish society. The active ingredient in the marine products that is presumed to confer the cardioprotective effect is fish oil, which is an abundant source of ω-3 fatty acids.3 These epidemiological studies have motivated many to study the effect of ω-3 fatty acids on atherosclerosis development in animals so that environmental factors that influence lipoproteins and atherosclerosis development can be controlled more closely.4–14 These studies have yielded mixed results with increased11 and decreased4–6,8,10,12 atherosclerosis development in animals fed fish oil compared to control groups. To date, three studies have examined the effect of fish oil to decrease the development of coronary artery atherosclerosis.5,6,12 In two of these studies, balloon injury of the arteries was used to initiate atherosclerosis development. However,
steps leading to atherosclerosis progression with balloon injury may be different from those of diet-induced atherosclerosis alone.

The purpose of this study was to determine the effect of fish oil on the development of diet-induced coronary artery atherosclerosis in the African green monkey and to examine the relationships between plasma lipoproteins and atherosclerosis development. We chose the African green monkey because of its close phylogenetic relationship to humans and because it is a well-characterized animal model for atherosclerosis research.15–19 We have shown previously that diet-induced atherosclerotic lesions have a morphology, topography, and composition similar to those of humans20 perhaps due, in part, to the fact that the lesions develop in animals with levels of hypercholesterolemia typical of human beings at increased coronary heart disease risk. In addition, when fed atherogenic diets, African green monkeys have lipoprotein concentrations, distributions, and compositions similar to human beings at risk for coronary heart disease.15–19 We also have generated much information on lipoprotein metabolism in this species of nonhuman primates.21–26 Our results suggest that the isocaloric substitution of fish oil for lard in these animals may lessen the progression of coronary artery atherosclerosis and that some of the decrease in coronary artery disease is associated with a more favorable lipoprotein profile.

Methods

Animals

Twenty-four adult male African green grivets (Cercopithecus aethiops aethiops) were purchased from Primate Imports (Port Washington, NY) for this study. Animals were randomly assigned to one of two diet groups and fed the atherogenic diets for 2.5 to 3 years before necropsy. Each diet contained 42% of calories as fat with 0.76 mg cholesterol/Kcal. Half of the fat calories were derived from lard or menhaden oil and the other half were from egg yolk or egg-yolk replacement, a low cholesterol mixture that resembles egg yolk in composition. The egg-yolk replacement was substituted in the fish-oil diet to compensate for the additional cholesterol in fish oil (~9 mg/g) compared to lard (~1 mg/g). The lard diet fatty acid composition consisted of 38% saturated, 44% monounsaturated, and 13% ω-6 polyunsaturated, while the fish-oil diet contained 40% saturated, 34% monounsaturated, 10% ω-6 polyunsaturated, and 12% ω-3 polyunsaturated fatty acids. Approximately 5% of the fatty acids of each diet were unidentified. The diet compositions have been given in detail elsewhere.27 The diets were made in 10 kg batches, were stored at ~20°C until needed, and were allowed to thaw at 4°C before feeding. Animals were trained to eat 30-minute meals twice daily to minimize oxidation of the dietary fatty acids. Daily intake of ω-3 fatty acids was 3.8 g for the fish-oil group. During the course of the experiment, three animals in the fish-oil group died. One animal was diagnosed as having lymphoblastoma with plasmacytoid features (after 1.3 years on the diet), one had skeletal muscle necrosis suggestive of vitamin E deficiency (2 years on the diet), and one died from renal failure secondary to enteric disease (2 months on the diet). Two years after the start of the study, another animal in the fish-oil group developed macrocytic anemia, suggesting that the animal was vitamin E-deficient. The fish-oil group was then given a vitamin E supplement (100 IU/wk), which promptly alleviated the symptoms of vitamin E deficiency.

Plasma Lipoprotein Characterization

Blood samples were taken from animals after an overnight (18-hour) fast. Ketamine hydrochloride (10 mg/kg) was used to restrain each animal, and blood was then taken from the femoral vein into chilled tubes (4°C) containing 0.1% ethylenediaminetetraacetate (EDTA), 0.02% NaN3, 0.04% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (final concentrations) at pH 7.4. Low density lipoprotein (LDL) molecular weight was measured by using the agarose column method after isolation of the d<1.225 g/ml lipoproteins from plasma.28 Total plasma cholesterol (TPC) and high density lipoprotein (HDL) cholesterol and plasma triglyceride (TG) concentrations were determined with the Lipid Research Clinic methodology and an RA500 auto-analyzer as described previously.29,30 The apolipoprotein concentrations in plasma were measured by an enzyme-linked immunosorbent assay.31,32

Necropsy Methods

Because the animals of this study were utilized for liver perfusion studies, the necropsies were done over a 6-month period. The animals in each diet group were rank-ordered on the basis of total plasma cholesterol and were sacrificed for liver perfusion and atherosclerosis evaluation at weekly intervals according to their rank order, starting with the animals with the lowest TPC and alternating diet groups each week.

Before termination, all animals were fasted overnight. At sacrifice, animals were anesthetized with ketamine hydrochloride (25 mg/kg) and were weighed; a midline laparotomy was performed to expose the liver. The common bile duct and portal vein were cannulated and the hepatic artery was ligated. The liver was then flushed with perfusion medium, the inferior vena cava was ligated and severed, and the animal was exsanguinated. The thorax was then opened, the inferior vena cava was cannulated, and the liver was removed for further perfusion. The details of the liver perfusion and the results of those studies are presented elsewhere.30,33

The heart was removed and the inferior and superior vena cava, as well as the portal vein and artery, were ligated proximal to the heart. The heart was then perfusion-fixed with 10% neutral buffered formalin for 1 hour at 100 mm Hg. The aorta and carotid arteries were removed, cleaned of adventitia, and opened along the anterior midline. The arteries were placed flat on cardboard and were fixed by submersion in 10% neutral buffered formalin.

Morphometry Methods

Surface Evaluation Methods

After a minimum of 48 hours of fixation in 10% neutral buffered formalin, the aortas and carotid arteries were...
stained overnight in saturated Sudan IV stain for surface lipid determinations. Areas of flat red lesions were defined as fatty streak, areas of raised red lesions were defined as fatty plaque, and areas of raised white fibrous lesions were defined as fibrous plaque. The percentage surface involvement of each of these parameters was calculated by measuring the entire surface area of the abdominal aorta, thoracic aorta, right common carotid, and left common carotid arteries and then measuring the area of each lesion type on each artery. The percentage of surface area with atherosclerosis was defined as the sum of the percentage of each different lesion type. A Hipad digitizing tablet interfaced to an Apple II computer was used for all morphometric evaluations.

Sampling Methods

After surface measurements were determined, five equidistant, cross-sectional tissue blocks were taken from each thoracic and abdominal aorta for histological evaluation. The thoracic aorta was defined as that portion of the aorta between the aortic arch and the celiac artery. The abdominal aorta was defined as that portion between the celiac artery and the bifurcation of the aorta into right and left common iliac arteries.

Sampling of the carotid arteries was accomplished by trimming three equidistant, cross-sectional tissue blocks from each common carotid artery between the origin of the carotid artery from the brachiocephalic artery and the carotid artery bifurcation. An additional block was trimmed from each carotid artery bifurcation.

Hearts were opened following the flow of blood technique described by Hudson.34 Five cross-sectional serial tissue blocks of approximately 3 mm in length were trimmed perpendicular to the long axis of the artery beginning at the proximal extent of the left anterior descending coronary artery, left circumflex coronary artery, and the right coronary artery for histological evaluation.

The tissue blocks from each artery bed were dehydrated through increasing concentrations of ethanol and were embedded in paraffin. One 5 μm thick tissue slide stained with Verhoeff-van Gieson stain was obtained from each artery block.

Cholesterol Quantification of Abdominal Aorta

The remaining abdominal aorta, after sections were taken for histological analysis, was weighed and extracted by the method of Folch et al.35 Esterified and free cholesterol were separated by thin-layer chromatography and were quantified by the procedure of Rudel and Morris.36 The results were expressed as milligrams of free or esterified cholesterol per gram wet weight of aorta. Since these tissues were fixed and stained, the estimates of lipid represent only minimum estimates.

Morphometric Evaluation Methods

Morphometric evaluations of microscopic artery characteristics were made on each stained slide. Calibration factors were determined by measuring a dimensional marker of known length for each magnification used. Data were obtained from each slide for the following parameters: cross-sectional intimal area, maximum intimal thickness, maximum maximum intimal thickness, and percent lumen stenosis. These measurements are defined as follows:

**Cross-sectional Intimal Area.** Intimal area (i.e., lesion area) was determined by subtracting lumen area from area within the internal elastic lamina (IEL).

**Maximum Intimal Thickness.** Maximum intimal thickness was measured at the thickest lesion portion on each cross-sectional artery slide.

**Maximum Maximum Intimal Thickness.** Maximum maximum intimal thickness was determined by choosing the largest maximum intimal thickness from each artery bed evaluated.

**Percent Lumen Stenosis.** Percent lumen stenosis was defined as the intimal area divided by IEL area × 100 and was reflective of how much of the potential lumen area was occupied by the lesion.

**Grading of Cholesterol Monohydrate Crystals**

The relative amount of cholesterol monohydrate crystals in the five stained sections of the abdominal aorta was estimated by using a graded scale from 0 to 5, with 0 being no crystals and 5 being the maximum number of crystals. The scores from the five sections were averaged to give a mean score for each animal. Frozen sections of the abdominal aorta also were analyzed to confirm that the clefts in the stained tissue were due to the presence of cholesterol monohydrate crystals.

Statistical Analyses

The data are presented as means ± standard errors of the mean. Comparisons between dietary groups were made by using Student's t test. When data were not normally distributed, an In transformation was performed before using the t test. In one instance (whole plasma apolipoprotein [apo] B concentrations) the In transformation did not result in normally distributed values, so a Mann-Whitney test was performed to test for a group difference. Group differences for plasma lipid concentrations measured throughout the experiment were compared by repeated measures analysis of variance (ANOVA).

Results

TPC, HDL cholesterol, and TG concentrations were determined periodically throughout the entire study and are shown in Figure 1. In addition, the summary of several lipid and lipoprotein characteristics of these animals is given in Table 1. The chemical compositions of the lipoproteins after 8 months of diet treatment have been reported elsewhere.27 At baseline (time 0) the animals were consuming monkey chow and had TPC concentrations of approximately 125 mg/dl. When the atherogenic diets were consumed, TPC rose to between 300 and 400 mg/dl in both groups. TPC then decreased to ~230 mg/dl for the fish-oil group after 5 months and remained significantly lower than TPC for the lard group for the duration of the study (p < 0.04, repeated measures
The average TPC concentrations for the lard and fish-oil groups during the experimental period were $360 \pm 44 \text{ mg/dl}$ and $231 \pm 37 \text{ mg/dl}$, respectively (Table 1). HDL cholesterol concentrations increased from baseline (monkey chow) after initiation of the atherogenic diet for the lard group, and they decreased from baseline for the fish-oil group (Figure 1). Throughout the atherogenic diet period, animals fed the fish-oil diet had HDL cholesterol concentrations that were $\approx 30\%$ lower than those of the lard group ($57 \pm 5 \text{ vs. } 83 \pm 6 \text{ mg/dl}$, respectively; $p < 0.01$, repeated measures ANOVA). Plasma TG concentrations dropped for both groups when the animals were switched from monkey chow to the atherogenic diet. From 5 months to 29 months, the plasma TG concentrations for the group fed lard were significantly lower than those of the group fed fish oil ($p < 0.001$, repeated measure ANOVA). Average plasma TG concentrations during the entire experimental period were $15 \pm 1 \text{ mg/dl}$ for the lard group and $25 \pm 2$ for the fish-oil group. Note, however, that both groups had plasma TG concentrations much lower than those typical of human beings ($\approx 80 \text{ mg/dl}$). LDL cholesterol was estimated as the difference between total cholesterol and HDL cholesterol, since fasted African green monkeys have very low concentrations of very low density lipoproteins (VLDL) and intermediate density lipoproteins in plasma. This estimate of LDL cholesterol averaged $40\%$ lower ($p = 0.038$) for the fish-oil group over the experimental period. This difference is similar to that we found previously (34% lower LDL concentrations in the fish-oil group) using the agarose column method for LDL isolation for the same animals. Plasma apo A-I concentrations, but not apo B concentrations, were significantly lower ($p = 0.002$) for the fish-oil group compared to their lard counterparts. LDL molecular weight, a strong predictor of coronary artery atherosclerosis in African green monkeys, was significantly less ($p = 0.001$) for the fish-oil vs. the lard group.

The animals maintained their body weight on the atherogenic diets and there was no significant difference in mean body weight between the two diet groups (Figure 2). The mean body weights for the experimental period were $5.19 \pm 0.18 \text{ kg}$ for the lard group and $5.42 \pm 0.17 \text{ kg}$ for the fish-oil group. Thus, other than the temporary deficiency of vitamin E, the fish-oil diet maintained the growth and health of the animals over a 2.5- to 3-year period.

At the end of the experimental period, atherosclerosis was quantitated for the thoracic and abdominal aorta and the common carotid arteries (left and right averaged together). The percentages of total surface involvement and percentages of surface area involved with fatty streak are shown in Figure 3. The thoracic aorta of the lard group had 1.7 times as much total area involved with lesion as did that of the fish-oil group ($62.4 \pm 9.1 \%$ vs. $37.1 \pm 14.1 \%$ of surface, $p = 0.02$). The lard group also had 1.4 times as much of the thoracic aorta involved with fatty streak compared to the fish-oil group ($53.6 \pm 9.4 \%$ vs. $37.1 \pm 14.1 \%, p = 0.04$). The fish-oil group had no lesions in the thoracic aorta that were classified as plaque, but the lard group had small amounts of fatty plaque and...
Table 1. Lipid, Lipoprotein, and Apoprotein Characteristics of African Green Monkeys Fed Atherogenic Diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>TPC (mg/dl)</th>
<th>LDL-C (mg/dl)</th>
<th>HDL-C (mg/dl)</th>
<th>TG</th>
<th>Apo A-I</th>
<th>Apo B</th>
<th>LDL MW (g/μmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lard</td>
<td>360±44</td>
<td>270±47</td>
<td>83±6</td>
<td>15±1</td>
<td>267±18</td>
<td>125±12</td>
<td>3.25±0.38</td>
</tr>
<tr>
<td>(n=12)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil</td>
<td>231±37</td>
<td>172±39</td>
<td>57±5</td>
<td>25±2</td>
<td>181±12</td>
<td>112±13</td>
<td>2.68±0.10</td>
</tr>
<tr>
<td>(n=9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All values are the means±SEM. Values for TPC, LDL-C, and TG are the grand means for all observations during the atherogenic diet periods (see Figure 1). Values for apo A-I, apo B, and LDL MW are the means for two observations for each animal, one taken 8 months after the initiation of the atherogenic diets and one taken within 2 months of necropsy.

*Student's t test after natural log transformation. Not significant (NS) at p=0.05. †Student's t test. $NS at p=0.05 by Mann-Whitney test.

TPC=total plasma cholesterol, LDL-C=low density lipoprotein cholesterol, HDL-C=high density lipoprotein cholesterol, TG=triglycerides, MW=molecular weight, apo=apolipoprotein.

Figure 2. Body weights of African green monkeys during the experimental period of consuming an atherogenic diet containing either lard or fish oil. Each point represents the mean±SEM for 12 animals in the lard group and nine in the fish-oil group.

The results of the quantification of free and esterified cholesterol in abdominal aortas are shown in Figure 4. Each point represents the results from an individual animal, and the mean±SEM is also shown for each group. For reference, the amount of free and esterified cholesterol is shown for a control animal consuming monkey chow (open triangles). The lard group had significantly more esterified cholesterol in the abdominal aorta than did the fish-oil group (3.43±0.74 vs. 1.46±0.71 mg/g, p=0.04). Free cholesterol content was more variable than esterified cholesterol and on average was higher for the lard group than for the fish-oil group (7.05±1.68 vs. 3.70±2.15, p=0.08). Note that five of seven abdominal aortas analyzed in the fish-oil group...
addition, there was a strong correlation ($r=0.87$) between the histological score and the content of free cholesterol. The amount of atherosclerosis in the coronary arteries, thoracic aorta, and abdominal aorta. These data are shown in Table 2. Three measurements of abdominal aortic atherosclerosis were obtained at necropsy approximately 2 years later. The hepatic values were not statistically significant at $p=0.05$ perhaps because of variability in the data. There was no difference in the extent of atherosclerosis in the abdominal aorta for any of the three measurements.

We previously have shown that LDL molecular weight is highly correlated with the extent and severity of atherosclerosis in nonhuman primates fed atherogenic diets containing cholesterol and saturated fat.\cite{1,16,17,18} Atherogenic diets cause LDL cholesteryl ester (CE) composition to become more saturated, and the melting point of the LDL CE increases with increasing LDL size.\cite{37,38} We have previously shown that the melting temperature of LDL CE was $12^\circ C$ lower for animals in the fish oil vs. lard group ($26\pm 1$ vs. $38\pm 1^\circ C$, ref. 37). The relationship between several measurements of abdominal aortic atherosclerosis and plasma LDL was examined in this study (Figure 5). There was a significant correlation ($r=0.66$) between LDL size and the percentage of abdominal aorta involved with atherosclerotic lesions (top panel). Because the CE fatty acid ratio of LDL (i.e., the number of saturated+ monounsaturated CE species divided by the polyunsaturated species) increases with LDL size, we examined the relationship between this ratio and the amount of cholesterol in the abdominal aorta. There was a significant correlation between the LDL CE fatty acid ratio and the amount of esterified ($r=0.58$, Figure 5 middle) and free ($r=0.63$, Figure 5 bottom) cholesterol in the artery. The correlation of LDL CE melting temperature with esterified ($r=0.5$) and free ($r=0.43$) cholesterol in the abdominal aorta was also statistically significant but less than that of the CE fatty acid ratio (data not shown). Thus, the size, CE composition, and melting temperature of plasma LDL were significantly associated with the amount of abdominal aorta atherosclerosis measured by both morphometric and chemical analysis.

As noted previously, one animal in the fish-oil group (#464) had four times more free cholesterol in the abdominal aorta than did the others in that diet group. A closer examination of selected lipid, lipoprotein, and apoprotein measurements for #464 compared to the lard group and the animals in the fish-oil group was made (Table 3). The plasma values were from a single bleeding taken after 8 months of diet treatment; the hepatic values were obtained at necropsy approximately 2 years later. Plasma lipoprotein cholesterol concentrations and LDL molecular weight for #464 were more similar to values for the lard group than the fish-oil group. The peak melting temperature ($T_m$) for LDL CE for #464 was midway between the average value for the two diet groups. Hepatic CE accumulation for #464 was more similar to
that of the lard group than the fish-oil group. However, fatty acid analysis of plasma LDL and hepatic lipid fractions showed an enrichment of plasma LDL CE and phospholipid (PL) as well as hepatic TG and CE with ω-3 fatty acids for animal #464, indicating a more atherogenic fatty acid profile and more aortic atherosclerosis compared to other animals in the fish-oil group in spite of an equivalent enrichment of ω-3 fatty acids in plasma and hepatic lipids induced by dietary fish oil.

Discussion

The potentially beneficial effects of fish oil were first recognized when it was shown that Eskimos, whose consumption of fish and fish products was relatively high, had a lower incidence of coronary heart disease compared to a control Danish population. Other epidemiological studies have confirmed this initial finding, and it has been hypothesized that fish oil, an abundant source of ω-3 fatty acids, is the active agent in fish. Several studies have used diet-induced atherosclerosis to assess the effect of dietary fish oil on atherosclerosis progression in animal models. However, to date only three studies have focused on coronary artery atherosclerosis in animal models, and in two of those studies, balloon injury of the left anterior descending coronary arteries was used to initiate atherosclerosis. Using only diet-induced hyperlipidemia to induce atherosclerosis progression, we have found that isocaloric substitution of fish oil for lard was associated with less atherosclerosis in several arterial beds, including the coronary arteries. In addition, the decreased atherosclerosis in the fish-oil group was associated with a general lowering of plasma lipid and lipoprotein concentrations. These data suggest that the plasma lipid- and lipoprotein-lowering effect of fish oil in our animals resulted in a more beneficial atherosclerosis outcome. The characteristics of the atherosclerotic lesion also were affected by fish oil, as the amount of cholesterol monohydrate crystals in the abdominal aorta was significantly less compared to the lard group. Since there was a significant correlation between the amount of free and esterified cholesterol in the abdominal aorta and the CE fatty acid composition of LDL, the physical properties of the CE may be partially responsible for the decreased atherosclerosis and different lesion characteristics of the fish oil group.

Studies of the effect of fish oil on atherosclerosis development in animal models have had variable outcomes. Three studies have reported no effect of fish oil on atherosclerosis development, while decreased atherosclerosis was found in six studies when animals fed fish oil were compared to controls. Two studies have suggested more extensive atherosclerosis associated with fish-oil consumption. Part of the discrepancies among studies may be explained by differences in experimental design. A variety of animal models including the rat, rabbit, pig, rhesus monkey, and the Watanabe heritable hyperlipidemic (WHHL) rabbit were used in previous studies, and the length of atherosclerosis induction ranged from 2 weeks to 1 year. It also is interesting to note that only three of these studies used isocaloric replacement of fish oil for the control dietary fat, while all other studies used fish oil as a dietary supplement. Several distinguishing features of our study should be noted for comparison with the other studies. The present study had the longest duration of atherosclerosis induction (2.5 to 3 years) and the lowest average plasma lipid values (i.e., TPC=200 to 400 mg/dl vs. 500 to 1600 mg/dl for other studies). The lower TPC range is more relevant to human beings at increased risk for coronary heart disease. The fish oil in our study was isocalorically substituted for lard rather than given as a supplement, and the saturated fatty acid content of both diets was similar (38% in lard diet vs. 40% in fish-oil diet). As mentioned above, we quantified the development of atherosclerosis in the coronary arteries as well as other arterial beds. Finally, unlike some studies, we found that

### Table 2. Effects of Fish Oil on Aortic and Coronary Artery Atherosclerosis

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Intimal area (mm²)</th>
<th>Max. max. int. thickness (mm)</th>
<th>% lumen stenosis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coronary arteries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil</td>
<td>9</td>
<td>0.010±0.002</td>
<td>0.059±0.006</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Lard</td>
<td>12</td>
<td>0.034±0.009</td>
<td>0.103±0.019</td>
<td>3.7±0.7</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.026*</td>
<td>0.077*</td>
<td>0.011*</td>
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<tr>
<td><strong>Thoracic aorta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil</td>
<td>9</td>
<td>0.210±0.110</td>
<td>0.112±0.022</td>
<td>1.1±0.6</td>
</tr>
<tr>
<td>Lard</td>
<td>12</td>
<td>0.397±0.582</td>
<td>0.294±0.085</td>
<td>4.7±0.085</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>0.096*</td>
<td>0.074*</td>
<td>0.085*</td>
</tr>
<tr>
<td><strong>Abdominal aorta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fish oil</td>
<td>9</td>
<td>1.143±0.481</td>
<td>0.593±0.159</td>
<td>11.7±4.6</td>
</tr>
<tr>
<td>Lard</td>
<td>12</td>
<td>1.214±0.312</td>
<td>0.541±0.111</td>
<td>11.4±3.0</td>
</tr>
<tr>
<td>P value</td>
<td></td>
<td>NS*</td>
<td>NS*</td>
<td>NS*</td>
</tr>
</tbody>
</table>

Values are means±SEM.

*Student’s t test was performed after natural log transformation of the data. NS=not significant at p=0.05.

1Student’s t test.
Max. max. int. thickness=maximum maximum intimal thickness.
Figure 5. Relationships between low density lipoprotein (LDL) size and cholesteryl ester (CE) composition and abdominal aortic atherosclerosis for African green monkeys fed atherogenic diets containing lard or fish oil. Each point represents data from an individual animal. LDL CE fatty acid ratio is the ratio of saturated+monounsaturated to polyunsaturated cholesteryl ester species and was reported in a previous article.37 The line of best fit determined by linear regression analysis is shown for each graph.

decreased atherosclerosis development was associated with decreased concentrations of plasma lipids and lipoproteins. We wish to emphasize that TPC concentrations were not different between the two diet groups until 5 months after diet initiation and that greater differences in atherosclerosis development might have been apparent if the two groups of animals had expressed TPC differences 4 to 5 months earlier. A possible explanation for the delayed divergence of TPC between the groups is that a cumulative effect of the fish-oil diet occurred. If this were so, it might be a partial explanation for the studies that show a beneficial effect of long-term relatively low intakes of fish.

The potentially beneficial properties of fish oil are thought to be mediated through effects on platelet function and vascular reactivity as well as plasma lipids.39,40 This presumably explains the beneficial atherosclerosis outcome of the fish-oil group in the study of Weiner et al.5 in which plasma cholesterol concentrations were similar for both control and fish-oil supplemented groups. However, the balloon injury used to initiate atherosclerosis in that study involved endothelial denudation and presumably mural thrombosis as the major pathogenic event. Thus, the beneficial effect of fish oil in that study was apparently mediated through effects on platelet function and vascular reactivity and may represent a model for acute thrombotic events in advanced atherosclerotic lesions. In contrast, our study used only diet-induced hyperlipidemia to initiate atherosclerosis and, as a result, modeled the long-term effect of hyperlipidemia on arterial lipid deposition and atherosclerosis development. We are not aware of any evidence of a role for microthrombi in the pathogenesis of atherosclerosis in the African green monkey. In this study, the data from animal #464 appear particularly informative with regard to the role of thrombotic events in early atherosclerotic lesion development. This animal received the fish-oil diet, as confirmed by the content of ω-3 fatty acids in plasma and hepatic lipids (Table 3), but did not respond with a lowering of plasma lipids and lipoproteins typical of animals fed fish oil. Thus, platelet function and vascular reactivity presumably were altered by the fish oil in this animal and, according to the hypothesized anti-atherogenic properties of fish oil, this animal should have had less atherosclerosis than animals in the lard group. However, #464 had as much abdominal and thoracic aortic atherosclerosis (measured chemically and morphometrically) as animals in the lard group, suggesting that in our animal model, dietary fish oil must result in a lowering of plasma lipids and lipoproteins to affect a reduction in the amount of aortic atherosclerosis. The coronary artery intimal area for #464 was less than the average value for the lard group. Thus, there may be regional differences between the development of atherosclerosis and the association with lipoprotein properties. Taking these data together, we believe that fish oil may retard atherosclerosis development in animal models by its lipid-lowering properties and, in more advanced lesions, by its effect on platelet and vascular function. If this were true, then fish oil may be more beneficial with regard to atherosclerosis development than other polyunsaturated fats that only function to lower plasma lipid concentrations.

All the methods we have used to measure atherosclerosis demonstrated less lipid in the arteries from the animals fed fish oil, yet the intimal area measurements of sections of the abdominal aorta were not different. However, it was readily apparent in histologic sections that there was a greater connective tissue component in the
Table 3. Comparison of Plasma and Hepatic Lipid, Lipoprotein, and Apoprotein Variables in African Green Monkeys

<table>
<thead>
<tr>
<th></th>
<th>Diet group</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lard (n=12)</td>
<td>Fish oil (n=11)</td>
<td>#464 (Fish oil)</td>
</tr>
<tr>
<td><strong>Plasma variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPC (mg/dl)</td>
<td>372±55</td>
<td>249±32</td>
<td>517</td>
</tr>
<tr>
<td>LDL-C (mg/dl)</td>
<td>249±58</td>
<td>166±34</td>
<td>446</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>105±7</td>
<td>70±8</td>
<td>48</td>
</tr>
<tr>
<td>Apo B (mg/dl)</td>
<td>134±11</td>
<td>145±13</td>
<td>164</td>
</tr>
<tr>
<td>Apo A-I (mg/dl)</td>
<td>223±19</td>
<td>158±17</td>
<td>102</td>
</tr>
<tr>
<td>Tm (°C)</td>
<td>38±1</td>
<td>26±1</td>
<td>32</td>
</tr>
<tr>
<td>LDL MW (g/µmol)</td>
<td>3.43±0.11</td>
<td>2.91±0.12</td>
<td>3.58</td>
</tr>
<tr>
<td><strong>Hepatic variables</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE (mg/g)</td>
<td>340±16</td>
<td>450±29</td>
<td>577</td>
</tr>
<tr>
<td>CEFA (% ω-3)</td>
<td>175±20</td>
<td>123±20</td>
<td>243</td>
</tr>
<tr>
<td>TGFA (% ω-3)</td>
<td>18±7</td>
<td>10±15</td>
<td>159</td>
</tr>
<tr>
<td>LDL PL (% ω-3)</td>
<td>172±10</td>
<td>197±14</td>
<td>159</td>
</tr>
<tr>
<td>LDL CE (M/P)</td>
<td>25±15</td>
<td>292±28</td>
<td>472</td>
</tr>
<tr>
<td>LDL PL (% ω-3)</td>
<td>6.1±0.8 (n=7)</td>
<td>22.6±1.0 (n=7)</td>
<td>19.6</td>
</tr>
</tbody>
</table>

Values represent the means±SEM for the lard and fish-oil diet groups and the individual values for animal #464, which was assigned to the fish-oil group.

*Plasma variables were measured after 8 months of diet treatment and have been reported previously.27,37
†Hepatic variables were determined at necropsy at 2.5 to 3 years after initiation of the diet. Group means have been reported.30

Tm=peak melting temperature of LDL CE representing a liquid crystalline to liquid transition, M/P=molecules CE per LDL particle, CE=cholesteryl ester, PL=phospholipid, CEFA=cholesteryl ester fatty acid, TGFA=triglyceride fatty acid. See the legend to Table 1 for an explanation of the other abbreviations.

lesions in sections from monkeys fed fish oil. As is the case with human beings, atherosclerosis of African green monkeys develops first in the abdominal aorta. A possible explanation for part of the difference in microscopic appearance of the lesions would be that atherosclerosis was progressing at the same rate in the early months of the experiment when the TPC was the same between the groups of monkeys. There could have been some reduction in the amount of lipid in the lesions and replacement with connective tissue when plasma lipid levels fell in the fish-oil group by analogy with the course of events that occurs in regression studies. Since coronary artery atherosclerosis development lags behind that of the abdominal aorta, the fall in TPC prevented some of the development of coronary atherosclerosis in the monkeys fed fish oil.

We found that the physical properties of LDL, measured as CE melting temperature and CE fatty acid ratio, were significantly associated with the amount of atherosclerosis in our monkeys, measured both morphometrically and as cholesterol deposition in the abdominal aorta. The reason for this association is unclear but may be related to both uptake and efflux of CE by cells in the artery wall. In previous studies, large LDL were shown to result in greater cellular CE accumulation compared to cells incubated with equal molar concentrations of smaller LDL and the large LDL bound to cells with a higher affinity but a reduced capacity compared to smaller LDL.41,42 We have recently observed that plasma LDL from African green monkeys fed fish oil are smaller and contain fewer CE with lower melting temperatures compared to those from animals fed lard and also result in less cellular CE accumulation when incubated with fibroblasts at equal particle concentrations (W. Lentz, J. Parks, R. St. Clair, unpublished data). We have hypothesized that the physical state of the LDL core CE may influence the uptake of LDL by cells in the artery wall.44 This effect might be mediated by changes in the surface properties (i.e., apo B conformation) of LDL through surface-core interactions. Thus, LDL particles with a liquid crystalline CE core at body temperature might be taken up by cells more avidly than those LDL with liquid CE cores, with the result being greater cellular CE accumulation.

Cellular cholesterol efflux may also be affected by the physical state of CE. Cholesterol efflux from cells in culture is more rapid when the CE is in a liquid rather than liquid crystalline state.45 Accumulation of ω-3 CE in cells would lower the melting temperature of the CE droplet in a manner similar to that described for plasma LDL.37 A more rapid efflux of the liquid CE may also prevent the saturation of the cellular CE phase with free cholesterol.
and thus reduce the deposition of cholesterol monohydrate crystals in the atherosclerotic lesion. Cholesterol monohydrate crystals are more inert than CE and presumably are harder for the cell to remove.46-47,48 We noted greater numbers of sterol clefts, indicative of cholesterol monohydrate crystals, in the abdominal aortic lesions of the animals in the lard group compared to those in the fish-oil group, and the presence of excess free cholesterol in the artery was confirmed by chemical analysis. Therefore, the physical state of CE as well as the amount of CE appear to contribute to the development of atherosclerosis, as previously suggested by Small and Shipley.47

The response of HDL concentrations in human beings fed fish oil has been variable.49 However, we27 and others5,6,8,12 have consistently observed an HDL-lowering effect of fish oil in animal models. Since HDL concentrations are inversely associated with coronary heart diseases in human populations, dietary treatments that lower HDL concentrations do not override the beneficial effects of smaller LDL size, reduced LDL cholesterol concentrations, and lower LDL melting temperatures.

Acknowledgments

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Index Terms: coronary artery atherosclerosis • lipoproteins • plasma lipids • cholesteryl ester melting temperature • monkeys
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