Effect of Fish Oil on Atherogenesis in Watanabe Heritable Hyperlipidemic Rabbit

Alice H. Lichtenstein and Aram V. Chobanian

The effect of dietary supplementation with fish oil was investigated in the Watanabe heritable hyperlipidemic (WHHL) rabbit. Rabbits were fed Maxepa (1 g/kg body weight) from weaning for a 6-month period and were compared to controls. Fish oil supplements resulted in significant decreases in plasma triglyceride and cholesterol at 4 weeks after the start of the study, and these differences persisted over the 6-month period. No differences in the distribution of cholesterol or triglyceride among lipoprotein particles were found, nor were there differences in low density lipoprotein particle size. The decreases in plasma cholesterol and triglyceride were accompanied by significant increases in the percent of eicosapentaenoic and docosahexaenoic acids in both plasma and platelets. Fish oil supplementation resulted in a decrease in platelet count and response to the aggregating agents adenosine diphosphate, arachidonic acid, and collagen. Systolic blood pressure was significantly lower in the rabbits fed fish oil. At the end of the study period, aortic free and esterified cholesterol and intimal surface area covered with plaque were determined in the arch and in the descending and abdominal aorta. Significantly less cholesterol was found in the descending and abdominal aorta. These differences were mirrored by decreased activities of acid lipase and N-acetyl-β-glucosaminidase.

In conclusion, dietary fish oil resulted in a decrease in aortic lipid deposition in the WHHL rabbit, which may have been due to decreases in plasma triglyceride and cholesterol, platelet count and aggregability, and systolic blood pressure. (Arteriosclerosis 10:597–606, July/August)

Epidemiological evidence indicates that the consumption of diets rich in fish may have beneficial effects on plasma lipids, blood clotting, and overall cardiovascular health.1,2 Experimental studies in humans have provided evidence that diets supplemented with fish oil concentrates can confer some of these positive effects. There are a number of factors that have been attributed to the consumption of fish oil and which may possibly affect cardiovascular health. These factors include, but are not limited to, decreased plasma triglyceride,3,4 decreased platelet aggregation,5,6 decreased blood pressure,7,9 altered lipoprotein metabolism,8,10 and increased bleeding time.11,12 It is difficult to delineate which of these factor(s) may be important in the prevention of atherosclerosis because of the inherent variability of subjects in studies designed to investigate the area of fish oils and atherogenesis.

The Watanabe heritable hyperlipidemic (WHHL) rabbit exhibits a genetic deficiency of low density lipoprotein (LDL) receptors, which mimics the defect found in human homozygous familial hypercholesterolemia.13 This defect results in marked hypercholesterolemia and milder hypertriglyceridemia as well as the development of extensive atherosclerotic disease within the first year of life.14 This animal model of atherosclerosis is particularly appropriate for the current studies because the disease can be attributed to a genetic disorder seen in humans. It is also one of the few animal models of atherosclerosis that involves hypertriglyceridemia as well as hypercholesterolemia and a spontaneous atherosclerosis, which develops at a relatively young age.15

The present study investigated the effect of fish oil supplements on the progression of atherosclerosis in the WHHL rabbit. Fish oil feeding was started at weaning (2 months of age) and continued over a 6-month period. Two reports have been published on this topic. Rich et al.16 found no effect of fish oil supplements on plasma lipids, lipid hydroperoxides, or aortic atherosclerosis when assessed morphologically. Clubb et al.17 reported that dietary fish oil resulted in a reduction in plasma triglyceride and total, very low density lipoprotein (VLDL), LDL, and high density lipoprotein (HDL) cholesterol in female rabbits and VLDL cholesterol in male rabbits, as well as decreased platelet aggregation. However, no change in lesion progression was found when assessed morphologically. In contrast, our present results suggest a significant decrease in lesion development in the ascending and abdominal regions of the aorta when aortic atherosclerosis was assessed chemically by determining the cholesterol content of the aorta and biochemically by measuring the activity of lysosomal enzymes. Differences in surface area involvement of the vessel were not significantly different between the two groups of
rabbits and did not predict the changes seen in the vessel wall chemically or biochemically. This decrease may have been related to decreases in plasma lipids, blood pressure, and platelet aggregability.

Methods

Watanabe Heritable Hyperlipidemic Rabbits

Twenty WHHL rabbits (2 months old) weighing 1.71 ± 0.21 kg were obtained from the breeding colony maintained at the Boston University School of Medicine. This colony was established from homozygous WHHL rabbits kindly provided by Russell Ross of the University of Washington School of Medicine and Alan Fogelman of the University of California School of Medicine in Los Angeles, whose colonies originated from Dr. Yoshio Watanabe’s animals. There were five male and five female rabbits per group. These rabbits were derived from four litters, and each litter was divided equally between the control and fish oil-fed groups with balanced sex distribution. The rabbits used were at least a third-generation product of the original rabbits. The animals were housed individually and were given free access to water. Fish oil (1 g/kg body weight/day; Maxepa, generously provided by R.P. Scherer Corp., Troy, MI) was mixed with a pelleted diet (45 g/kg body weight/day, Agway ProLab High Fiber Rabbit Chow, Syracuse, NY). Analysis of the oil indicated that the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) contents were 26% and 21%, respectively, by weight, and there was, respectively, 2.2 mg of cholesterol and 1.4 mg of vitamin E per gram of oil. Control rabbits were treated identically except that the fish oil was omitted from the diet. Systolic blood pressure was monitored at monthly intervals by tail cuff plethysmography with a photoelectric cell detector developed in our laboratory.16 Pressures were recorded with an IITC Model 59 preamplifier and a Narcotrace 40 physiograph. Blood pressure recordings could not be made in two control animals due to the loss of tails soon after birth. The average blood pressure for each group for the final 3 months of the study was used for the statistical analyses.

Plasma Lipids

The rabbits were fasted overnight before blood sampling. Blood (approximately 2 ml) was removed from the central ear artery at monthly intervals. Total serum cholesterol, HDL cholesterol, and triglyceride concentrations were assayed with enzymatic techniques (Sigma Diagnostics, St. Louis, MO). Twice the amount of the recommended HDL precipitating reagent was used to ensure complete precipitation of the apolipoprotein (apo) B-containing lipoproteins. During the third month of the study, approximately 10 ml of blood was taken for plasma and platelet fatty acid analysis.

Fatty acid Analysis

Fatty acid methyl esters (FAME) were prepared from plasma, platelets, and homogenates of the ascending aorta and arch according to the method of LePage and Roy.18 The FAME were analyzed on a Hewlett-Packard 5890 gas-liquid chromatograph fitted with a 105 m fused silica capillary column (Quadrex Corp., New Haven, CT) with liquid phase RTX2330 (Restek Corp., Bellefonte, PA) and a flame ionization detector. Peak identification was validated by chromatography of mixtures of authenticated FAME.

Lipoprotein Analysis

Plasma lipoproteins were separated on 1% agarose gel by using a Corn ing electrophoresis system and were stained with fat red 7B to monitor lipid distribution. During the fourth month of the study, plasma from six randomly selected rabbits (three control, three fish oil-fed) was fractionated on a discontinuous density gradient according to a modification of the method described by Fisher et al.20 Briefly, a 3.0 ml sample of plasma was adjusted to a density of 1.21 g/ml with potassium bromide and was overlaid sequentially with potassium bromide solutions of density = 1.15 g/ml (1 ml), density = 1.063 g/ml (3 ml), density = 1.019 g/ml (3 ml), and normal saline (1 ml). The samples were centrifuged in a Beckman SW41 rotor at 35 000 rpm for 22 hours at 4°C. The contents of the ultracentrifuge tube were fractionated into 18 aliquots and were analyzed for cholesterol and triglyceride as described. The LDL subtraction distribution was determined by nondenaturing polyacrylamide-agarose gradient gel electrophoresis21 on plasma obtained just before sacrifice. Aliquots of whole plasma that had been stored at −70°C were run on 2% to 16% gels (Pharmacia, Piscataway, NJ) and were stained with Sudan black B. The migration patterns of the LDL were compared to those of human pooled plasma.

Platelet Aggregation

Platelet aggregation was assessed among 10 randomly selected rabbits from those not used for lipoprotein analysis (five control, five fish oil-fed) during the fourth month of the study by using a Model PAP-4 Platelet Aggregation Profiler (Bio/Data Cap, Hatboro, PA) fitted with a microvolume adaptor. Platelet-rich plasma and platelet-poor plasma were prepared from blood containing 0.011 M sodium citrate by centrifuging at 160 g for 10 minutes and 1000 g for 6 minutes, respectively. A total of 300 000 platelets were used per assay. The samples were analyzed within 3 hours of preparation. The aggregating agents evaluated were adenosine diphosphate (ADP) (50 mM, Chrono-Log, Havertown, PA); arachidonic acid (1.2 mM, Bio/Data); and collagen (20 mg/ml, Chrono-Log, Hatboro, PA). Percent aggregation at 1 minute and at maximum aggregation was analyzed. Platelet count and volume were determined by the clinical laboratory at University Hospital (Boston, MA).

Tissue Preparation

Rabbits were sacrificed after 6 months of fish oil supplementation with an overdose of intravenously administered sodium pentobarbital (100 mg/kg). The entire aorta from the aortic valve to the bifurcation was removed quickly and was placed in normal saline at 4°C. The heart, adrenals, kidney, and liver were removed for weight determinations. The aorta was opened along the mid-dorsal line to expose the intimal surface, and a
photograph was made. The aorta was divided into the ascending aorta and arch, the descending aorta, and the abdominal aorta by cutting 0.5 cm proximal to the first pair of intercostal arteries and 0.3 cm proximal to the celiac artery. The intimal-medial layer was dissected free of adventitia and was weighed and homogenized with a motor-driven glass-glass homogenizer at 0 to 2°C in 10 volumes of 0.25 M sucrose, 10 mM HEPES, pH 7.4. Aliquots were taken for protein, lipid, enzyme, and fatty acid determinations.

**Chemical Analysis**

The protein contents of the samples were determined by the method of Lowry et al. Lipids were extracted from aliquots of tissue homogenate according to the procedure of Folch et al. Free cholesterol (FC) and esterified cholesterol (CE) were separated by thin-layer chromatography as previously described, and the cholesterol content was determined according to the method of Rudel and Morris. The recovery of the FC and CE averaged 94% and 95%, respectively.

**Enzyme Assays**

Supernatants obtained by centrifuging aliquots of the descending aortic intima-medial homogenate at 10 000 rpm for 15 minutes at 4°C were used to determine enzyme activity. Lysosomal enzyme activity was assessed by measuring the activities of N-acetyl-β-glucosaminidase (NAGA) and acid lipase. NAGA and acid lipase activity were estimated by monitoring the hydrolysis of methylumbelliferyl-N-acetyl-β-D-glucosaminide and methylumbelliferyl palmitate, respectively.

**Morphologic Determinations**

Intimal surface area covered by plaque was quantitated by using the Optimas image processing and analysis system (BioScan, Edmonds, WA).

Statistical analyses were performed by using analysis of variance and Student's t tests for independent and paired samples. Data are reported as the means±standard deviations.

**Results**

The fish oil supplement appeared to be well tolerated by the WHHL rabbits. The rate of weight gains of the control and fish oil-fed rabbits were comparable. Final body weights did not differ significantly (2.88±0.42 kg and 2.99±0.28 kg for control and fish oil-fed rabbits, respectively). Plasma fatty acid patterns reflected dietary intake when assessed during the fourth month of the study (Table 1). Although the levels of all the major fatty acids evaluated differed significantly between the two groups of rabbits, the most striking alterations were the increases in circulating plasma levels of EPA and DHA in the fish oil-fed rabbits (13.0% EPA and 11.1% DHA) as compared to the control rabbits (0.2% EPA and 2.5% DHA). Significant decreases in the relative proportions of stearic, oleic, linoleic, and linolenic acid were also noted in the fish oil-fed rabbits as compared to controls.

**Table 1. Effect of Dietary Fish Oil Supplements on Plasma Fatty Acid Patterns In WHHL Rabbit**

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>Fish oil-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.3±0.1</td>
<td>0.5±0.1†</td>
</tr>
<tr>
<td>16:0</td>
<td>17.2±0.9</td>
<td>19.2±0.3†</td>
</tr>
<tr>
<td>18:0</td>
<td>11.0±0.4</td>
<td>9.2±0.6†</td>
</tr>
<tr>
<td>18:1 (w9)</td>
<td>24.3±2.2</td>
<td>11.2±0.6†</td>
</tr>
<tr>
<td>18:2 (w6)</td>
<td>36.2±2.8</td>
<td>25.3±1.7†</td>
</tr>
<tr>
<td>18:3 (w3)</td>
<td>3.2±0.4</td>
<td>2.5±0.2†</td>
</tr>
<tr>
<td>20:4 (w6)</td>
<td>2.2±0.4</td>
<td>2.5±0.1†</td>
</tr>
<tr>
<td>20:5 (w3)</td>
<td>0.2±0.1</td>
<td>13.0±1.3†</td>
</tr>
<tr>
<td>22:6 (w3)</td>
<td>2.5±1.8</td>
<td>11.1±1.2†‡</td>
</tr>
</tbody>
</table>

Values are given as percents and are the means±standard deviations.

There were 10 rabbits in both the control and the fish oil-fed groups.

A significant difference between control and fish oil-fed animals (t<0.01, t<0.001).

Plasma lipids were also affected by fish oil feeding in the WHHL rabbit. After 4 weeks on the fish oil-supplemented diet, plasma cholesterol levels decreased by 27% (p<0.01; Figure 1A). This reduction was maintained throughout the study period so that after 6 months on the diet, the average plasma cholesterol levels were 78% of the initial level. Plasma cholesterol levels in the control WHHL rabbits did not vary significantly throughout the study period and differed significantly from the fish oil-fed rabbits at all experimental time-points except 2 months. Plasma triglyceride levels in the fish oil-fed rabbits declined by 58% after 4 weeks on the diet (p<0.01). This reduction was maintained throughout the study period (Figure 1B). In contrast, plasma triglyceride levels in the control rabbits were relatively constant, with no significant changes during the 6-month period. HDL cholesterol levels averaged 7.7±2.6 mg/dl and 6.4±2.5 mg/dl, respectively, in control and fish oil-fed WHHL rabbits. There were no statistically significant changes in HDL cholesterol during the study period. When the data on plasma lipids were analyzed on the basis of sex, there were no significant differences between male and female rabbits within groups.

When plasma was electrophoresed on nondenaturing polyacrylamide-agarose gradient gels, no differences in LDL size were seen between the two groups of rabbits. It should be noted that LDL size or type in the WHHL rabbits corresponds to a position between LDL types 1 and 2. Similarly, no differences in the pattern of lipid staining on agarose gels were seen, either between groups of rabbits or over the time course of the study. Only one band was seen corresponding to LDL.

Depicted in Figure 2 are representative discontinuous density gradient distributions of plasma cholesterol and triglyceride derived from control (Figure 2A) and fish oil-fed (Figure 2B) WHHL rabbits. Virtually all of the cholesterol and triglyceride in both groups of rabbits was present in a band corresponding to the LDL.

At the beginning of the study, systolic blood pressure was about 100 mm Hg in both groups of rabbits (Table 2).
With advancing age, as we have previously reported, blood pressure rose in the WHHL rabbit. However, fish oil-feeding attenuated this trend so that during the latter half of the study, the supplemented group had a significantly lower systolic blood pressure than the control group ($p<0.05$).

The effects of dietary fish oil supplements on platelet count, volume, fatty acid composition, and aggregability were also assessed. Fish oil feeding resulted in a 24\% decrease in platelet count ($p<0.001$) over the 6-month period (Table 3). There was also a significant decrease in platelet volume in the fish oil-fed WHHL rabbits ($p<0.01$). Platelet fatty acid patterns reflected dietary intake (Table 4). There was a significant increase in platelet omega-3 fatty acid content in the fish oil-fed rabbits (12.3\% EPA and 5.5\% DHA) when compared to control rabbits (<0.1\% EPA and 3.3\% DHA). There was a concomitant decrease in the relative proportions of oleic, linoleic, and arachidonic acid within platelets in the fish oil-supplemented group.

The effects of ADP, arachidonic acid, and collagen were investigated with respect to their abilities to induce platelet aggregation. Shown in Figure 3 are data on maximum aggregation in response to these three platelet aggregating agents. In all cases, there was a decreased tendency of platelets from WHHL rabbits fed fish oil to aggregate, differences that reached statistical significance for ADP and collagen. Similar results were observed when aggregation was assessed at the 1-minute time-point. No evidence of spontaneous aggregation in either group was observed over a 5-minute period.

After 6 months of fish oil supplementation, the rabbits were sacrificed. There were no significant differences in liver, heart, adrenal, or kidney weights between the two
Table 4. Effect of Dietary Fish Oil Supplements on Platelet Fatty Acid Patterns in WHHL Rabbit

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Control</th>
<th>Fish oil-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>16:0</td>
<td>17.2±1.2</td>
<td>17.6±1.7</td>
</tr>
<tr>
<td>18:0</td>
<td>27.6±2.7</td>
<td>26.8±2.7</td>
</tr>
<tr>
<td>18:1 (w9)</td>
<td>10.4±3.2</td>
<td>7.5±1.1*</td>
</tr>
<tr>
<td>18:2 (w6)</td>
<td>29.0±1.3</td>
<td>20.0±1.8*</td>
</tr>
<tr>
<td>18:3 (w3)</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>20:4 (w6)</td>
<td>9.2±1.3</td>
<td>5.8±0.8*</td>
</tr>
<tr>
<td>20:5 (w3)</td>
<td>&lt;0.1</td>
<td>12.3±1.0*</td>
</tr>
<tr>
<td>22:6 (w3)</td>
<td>3.3±3.4</td>
<td>5.5±0.8*</td>
</tr>
</tbody>
</table>

Values are given in percents and are the means±standard deviations. There were 10 rabbits in both control and fish oil-fed groups.

A significant difference between control and fish oil-fed animals (*p<0.05, †p<0.01).

Table 5. Characteristics of WHHL Rabbits Fed Fish Oil-supplemented Diet for 6 Months

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Fish oil-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>1.27±0.27</td>
<td>1.39±0.31</td>
</tr>
<tr>
<td>Intima/medial weight</td>
<td>0.19±0.07</td>
<td>0.23±0.07</td>
</tr>
<tr>
<td>Arch</td>
<td>0.33±0.11</td>
<td>0.38±0.13</td>
</tr>
<tr>
<td>Descending</td>
<td>0.10±0.03</td>
<td>0.11±0.04</td>
</tr>
<tr>
<td>Abdominal</td>
<td>67.0±14.6</td>
<td>78.3±11.1</td>
</tr>
<tr>
<td>Liver</td>
<td>6.25±1.26</td>
<td>6.0±0.72</td>
</tr>
<tr>
<td>Heart</td>
<td>0.25±0.04</td>
<td>0.26±0.07</td>
</tr>
<tr>
<td>Adrenal</td>
<td>7.31±0.44</td>
<td>7.65±0.74</td>
</tr>
</tbody>
</table>

Values are given as grams and are the means±standard deviations. There were 10 rabbits in both the control and the fish oil-fed groups.

Table 6. Effect of Fish Oil Supplements on Percent of Intimal Surface Area Covered by Plaque in Aortic Segments of WHHL Rabbit

<table>
<thead>
<tr>
<th>Location</th>
<th>Control</th>
<th>Fish oil-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch</td>
<td>55.6±15.7</td>
<td>53.2±9.1</td>
</tr>
<tr>
<td>Descending</td>
<td>17.0±16.1</td>
<td>9.9±14.3</td>
</tr>
<tr>
<td>Abdominal</td>
<td>14.4±9.3</td>
<td>10.9±9.2</td>
</tr>
</tbody>
</table>

Values are given in percents and are the means±standard deviations. There were 10 rabbits in both the control and the fish oil-fed groups.

Table 7. Effect of Fish Oil Supplements on Free and Esterified Cholesterol Content in Aortic Segments of WHHL Rabbit

<table>
<thead>
<tr>
<th>Location</th>
<th>Lipid</th>
<th>Control</th>
<th>Fish oil-fed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arch</td>
<td>FC</td>
<td>8.5±3.1</td>
<td>7.1±2.9</td>
</tr>
<tr>
<td></td>
<td>CE</td>
<td>12.2±4.1</td>
<td>13.5±6.3</td>
</tr>
<tr>
<td>Descending</td>
<td>FC</td>
<td>4.1±1.6</td>
<td>2.2±1.4†</td>
</tr>
<tr>
<td></td>
<td>CE</td>
<td>5.8±3.2</td>
<td>3.1±2.8*</td>
</tr>
<tr>
<td>Abdominal</td>
<td>FC</td>
<td>7.7±4.4</td>
<td>4.3±2.5*</td>
</tr>
<tr>
<td></td>
<td>CE</td>
<td>6.4±3.9</td>
<td>4.1±4.6</td>
</tr>
</tbody>
</table>

Values are given as mg/g wet weight and are the means±standard deviations. There were 10 rabbits in both the control and the fish oil-fed groups.

A significant difference between control and fish oil-fed animals (*p<0.05, †p<0.01).

FC=free cholesterol, CE=cholesteryl ester.

The percent surface area covered by plaque is shown in Table 6. No differences in percent involvement of the ascending aorta and arch were seen. No statistically significant differences in surface area involvement were seen in the descending and abdominal regions of the aorta, although the intimal area covered by plaque tended to be less in the fish oil-fed rabbits.

The chemical composition of the aortic segments with respect to cholesterol is shown in Table 7. There were no significant differences in the FC or CE content of the ascending aorta and arch. However, in contrast to the morphologic data, dietary omega-3 fatty acid supplementation resulted in significantly less deposition of FC and CE in the descending region of the aorta. Additionally, both the FC and EC content of the abdominal aorta was decreased in the fish oil-fed animals, although only the concentration for FC reached statistical significance.
The chemical and enzymatic data, the percent of intimal surface area covered by plaque was not significantly different between the control and fish oil-fed rabbits. However, there tended to be less surface area involvement in those regions of the aorta which had lower concentrations of FC and CE, indicating that measures of surface area covered with plaque alone may not be as sensitive an assessment of degree of atherosclerosis as chemical or enzymatic measures.

Rich et al.16 has recently reported that supplementing the diet of WHHL rabbits for 1 year with fish oil had no effect on plasma lipids or percent surface area covered by atherosclerotic lesions. Clubb et al.17 subsequently reported that 6 months of fish oil feeding resulted in a significant decrease in plasma lipids (which was somewhat gender-specific), whereas the extent of surface area involvement of the lesions was unaffected. The additional variables assessed in the current study, lipid deposition in the aorta and activity of two aortic lysosomal enzymes, were not assessed in either of the two previous studies.

The lack of effect of fish oil supplements on altering the rate of lesion progression reported by Rich and coworkers may have been related to the absence of changes in plasma lipids also reported in their rabbits. The amounts of EPA fed to the WHHL rabbits was about half that fed in the other two studies. This was reflected in only a modest increase in plasma EPA (3.4%). The data of Clubb et al. are more comparable to that of our own. The duration of the two studies was the same (6 months) as was the amount of EPA fed to the rabbits (about 200 mg/kg/day) and the route of administration (mixed with chow). Both sets of WHHL rabbits showed a significant decrease in plasma cholesterol and triglyceride (restricted to females in the study of Clubb et al.), enrichment of platelet EPA, and decreased response of platelets to aggregating agents. The results of the two studies diverged with respect to the effect of fish oil on atherogenesis. On the basis of morphological data, Clubb et al. reported no effect of fish oil feeding on atherogenesis. On the basis of morphologic data, we likewise did not observe statistically significant differences in intimal surface area involvement; however, on the basis of chemical composition and enzymatic data of the vessel wall, we observed significantly less atherosclerosis in the aortas of the fish oil-fed rabbits. It is difficult to directly compare the data generated by the two methods used to assess the degree of lesion development. Quantitating the cholesterol content of an aorta provides a measure of excess material deposited in the vessel wall that might potentially narrow the vessel and decrease blood flow or interfere with the normal elasticity of the vessel. Data on lysosomal enzyme activity of the vessel wall has been shown to corroborate the chemical data. Surface area measures provide data on the proportion of the vessel that is lesional, hence the potentially thrombogenic or occlusive area of the vessel. Combining surface area data with measures of lesion thickness enhances the predictive value of the data, although interpretation is difficult due to marked heterogeneity within a lesion and the relatively few cross-sectional areas actually measured.
In the current study, lipid deposition was unaffected in the aortic arch when assessed after 6 months of fish oil supplementation. In the rabbit, atherosclerotic lesion formation usually progresses from the ascending aorta and arch and areas surrounding the bifurcations to the descending and abdominal aorta.29 Hence, the ascending aorta and arch are the first and most heavily affected areas under any circumstance and become lesioned when the animals are relatively young. Had the rate of lipid deposition in the ascending aorta and arch been increased by fish oil feeding, as suggested by the findings in the descending and abdominal aorta, shorter term studies would have been necessary to yield comparable data.

A number of other animal models of atherosclerosis have been used to investigate the effect of omega-3 fatty acids on disease progression. Weiner et al.30 reported that cod-liver oil supplements decreased the progression of coronary artery disease after balloon catheter injury in swine fed an atherogenetic diet (high cholesterol, high fat) for 8 months. Interestingly, the protective effect of cod-liver oil extended to vessels unaffected by the physical trauma and, unlike the present study, appeared unrelated to a reduction of plasma lipids. On the basis of decreased serum thrombomodulin and an altered pattern in platelet fatty acids, the authors concluded that the protective effect of the cod-liver oil may have been mediated through changes in prostaglandin metabolism. Davis et al.31 replaced coconut oil with Menhaden oil in a cholesterol-rich diet fed to rhesus monkeys for 12 months. They reported decreases in plasma cholesterol, percent aortic surface area covered by lesions, aortic FC and CE content, and aortic acid lipase, cholesterol esterase, and acylCoA:cholesteryl acyltransferase activities in fish oil versus coconut oil-fed animals. In contrast, Thiery and Seidell32 reported that Maxepa increased aortic surface involvement by atherosclerosis in New Zealand White rabbits fed a high cholesterol diet. Sanders33 has suggested that dramatic differences in the essential fatty acid or total fat content of the diets may have contributed to the findings. Additionally, only the rabbits fed fish oil were physically stressed by the route of administration (gavage).

The mechanisms by which fish oils may inhibit atherosclerosis could be related to several factors. As noted in the current study, significant decreases in blood pressure, plasma cholesterol, plasma triglyceride, and platelet aggregation were induced by fish oil supplements.

Elevated blood pressure appears to exacerbate atherosclerosis.34 We have recently reported that experimental hypertension markedly accelerates lesion development in the WHHL rabbit within a relatively short period of time (2 to 3 months).28 In the present study, supplementing normal rabbit chow with fish oil resulted in a significantly lower systolic blood pressure. Similar findings have been observed by others. Increasing dietary omega-3 fatty acids has reportedly decreased blood pressure in normal healthy volunteers,35 mild hypertensives,7,8,36 type II diabetics,37 patients with stable coronary heart disease,38 and hemodialysis patients.9,39-42 The data in normal subjects have been inconsistent.43,44,45 Whether the lower blood pressure in the fish oil-fed rabbits was responsible for the less severe aortic atherosclerosis is unknown, but it certainly could have been a contributing factor.

In our studies, a significant decrease in platelet count and volume was observed in the fish oil-fed WHHL rabbits. Observations of depressed platelet counts have been documented in humans on high fish diets and during fish oil administration. In patients with ischemic heart disease, Hays et al.46 reported a 15% decrease in platelet count after 5 weeks of fish oil supplements. Some studies have found a decreased platelet count in control subjects whose diets had been supplemented with fish or fish oil,35,43,47,48 but others have reported no change.8,49,50 Given the previously reported prolongation in platelet survival in subjects fed fish oil,46 the most probable cause of the decreased platelet count is reduced thrombopoiesis.

Dietary fish oil consistently reduces plasma triglyceride. In our studies, a pronounced drop in plasma triglyceride (58%) occurred within the first month and was maintained throughout the study period. The decrease in fasting plasma triglyceride has been attributed to a decreased synthetic rate of VLDL triglyceride49 and VLDL apo B.4 In the fed state, decreased plasma triglyceride has been attributed to an inhibition of chylomicron synthesis or secretion or to accelerated clearance due to decreased VLDL competition.40 Huff and Telford41 have recently presented kinetic data in miniature pigs, suggesting that dietary fish oil caused the secretion of a VLDL particle, which is preferentially converted to LDL. Decreases in plasma triglyceride of a similar magnitude to that observed in the present study have been reported for normolipidemic humans in both the fasting4,51-55 and postprandial4,56 state and in hyperlipidemics in the fed state.3,33 Decreases in VLDL triglyceride have also been frequently reported.3,9,23,54,56

The data on the effects of fish oil on plasma cholesterol concentrations have been less consistent. In the present study, fish oil supplements resulted in a clear and consistent decrease in plasma cholesterol. Since upregulation of the LDL receptor is unlikely to account for appreciable cholesterol clearance in the WHHL rabbit, the decrease in plasma cholesterol observed was probably due to either decreased de novo synthesis or increased nonreceptor-mediated clearance. Phillipson et al.3 has reported a decrease in plasma cholesterol in hypertriglyceridemic patients with Type IIB and Type V hyperlipoproteinemia after feeding of fish oil. In some studies of control subjects, fish oil feeding has reportedly decreased plasma cholesterol,51,52,55,56 but no change has been observed in others.36,49,53,54,57,58

Despite clear changes in plasma lipids, fish oil supplements had no perceptible effects on LDL size, electrophoretic mobility of the lipoprotein particles on agarose gels or on the distribution of cholesterol and triglyceride when the lipoproteins were fractionated on a discontinuous density gradient. Hence, we conclude that there probably was no shift in the distribution of cholesterol and triglyceride among the lipoprotein particles. Parks and Bullock57 studied the effect of substituting Menhaden oil for lard in the diet of African green grivet monkeys. In addition to a
significant decrease in plasma cholesterol, the fish oil-fed group had significantly smaller LDL particles.

Supplementing the diet with fish oil resulted in a dramatic increase in the relative proportion of plasma EPA and DHA and a concomitant decrease in the C-18 series of fatty acids. The responsiveness of plasma fatty acid levels to fish oil feeding has also been documented in humans. The apparent selective enrichment of EPA and DHA into platelets was of particular interest. The absolute increase in the percent of EPA (<0.1% to 12.3%) was strikingly higher than that of DHA (3.3% to 5.5%). This discrepancy may reflect selective incorporation of EPA compared to DHA into platelet membranes or retroconversion of the longer chain fatty acid into the shorter chain. The later hypothesis is supported by the work of Schacky and Weber who demonstrated that dietary supplementation with DHA resulted in elevated platelet levels of EPA. We also observed that with the enrichment of platelets with EPA and DHA, the relative proportion of arachidonic acid was almost half that in platelets isolated from control rabbits. EPA and DHA compete with arachidonic acid for metabolism by the cyclooxygenases and lipoxygenases; thromboxane A₂ and prostaglandin I₂ are produced at the expense of thromboxane A₅ and prostaglandin I₅. In contrast to thromboxane A₂, thromboxane A₅ has no platelet aggregating activity, and prostaglandin I₅ is an anti-aggregating substance. This scenario may have accounted for the decreased tendency of platelets isolated from the fish oil-fed WHHL rabbits to aggregate when challenged with collagen, ADP, or arachidonic acid.

Decreased platelet aggregation also has been reported by Brox and coworkers who fed cod-liver oil to both control subjects and patients with familial hypercholesterolemia. After 6 weeks of fish oil feeding, they found decreased collagen-induced platelet aggregation coupled with decreased platelet thrombin-induced thromboxane B₂ generation. The altered property of platelets observed in the present study may have either directly or indirectly contributed to the decrease in severity of aortic lesion development seen in the WHHL rabbit fed fish oil.

Similar proportions of palmitic, linoleic, and EPA were observed in plasma and platelets at the third month of the study. The platelets had approximately 2.5 times the amount of stearic acid, a lower proportion of linoleic acid, and virtually no detectable linolenic acid when compared to plasma. As expected, the relative proportion of arachidonic acid was higher in the isolated platelets than in plasma. When the two groups of rabbits were compared with respect to platelet fatty acid composition, the proportion of arachidonic acid was lower in the fish oil-fed rabbits relative to the control rabbits, the later group having increased proportions of EPA and DHA. Although the percent of EPA was nearly identical in plasma and platelets from both groups of rabbits, the proportion of DHA was approximately one half that in the platelets as observed in plasma in the fish oil-fed rabbits. Again, this may be indicative of either retroconversion of DHA to EPA or to selective enrichment of platelets.

The fatty acid patterns of the ascending aorta and arch more closely reflected the pattern of fatty acids seen in plasma rather than in platelets and suggested a direct uptake of the circulating lipoproteins. The relatively high levels of EPA and DHA in the aortic wall of rabbits fed fish oil also suggested that the majority of lesion formation occurred during the course of the study. Rosenfeld et al. also observed similarities in the fatty acid composition of the cholesteryl ester between plasma and the aorta of WHHL rabbits. Their previous work demonstrated that the early fatty streak lesion in the WHHL rabbit is composed of macrophage-derived foam cells.

The relevance of this study to persons with familial hypercholesterolemia is highly speculative. Fish oil feeding had a positive effect on plasma lipids, blood pressure, and platelet function. Taken with the decreased accumulation of cholesterol in the vessel wall, the evidence suggested that fish oil feeding may have retarded the rate of atherogenesis, but it clearly did not arrest it. The effect of fish oil feeding on platelet function in the WHHL rabbit was consistent between investigators. In contrast, the effect of fish oil feeding on plasma lipids appeared to be variable among the three studies thus far reported. Similar variability in response has been noted in hypercholesterolemic humans fed fish oil. Nevertheless, evidence from work with other animal species indicates that the beneficial effects of fish oil feeding with respect to atherogenesis may be independent of either factor. Although by no means an adequate treatment, in certain individuals, diets increased in fish or fish oil may exert some positive effects.

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References

Fish Oil, WHHL Rabbit, and Atherogenesis

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51. Saynor R, Verel D, Gillott T. The long-term effect of dietary supplementation with fish lipid concentrate on serum lipids, bleeding time, platelets and angina. Atherosclerosis 1984; 40:3–10

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A H Lichtenstein and A V Chobanian

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