Development of Atherosclerosis in Genetically Hyperlipidemic Rabbits during Chronic Fish-oil Ingestion

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The evidence for a reduction in cardiovascular mortality from fish oil is based on epidemiologic observations. To test whether fish-oil supplementation influences the development of atherosclerosis, we treated Watanabe heritable hyperlipidemic rabbits (WHHL), an inbred strain that spontaneously develops atherosclerosis, with 2.5 ml of MaxEPA fish-oil concentrate daily and compared them to a control group fed unsupplemented rabbit chow. Serial cholesterol and triglyceride levels were monitored as were plasma lipid hydroperoxides. The animals were given fish oil from the time of weaning until 1 year of age, when they were sacrificed and their aortas were compared for the extent of atherosclerosis. No significant differences in the cholesterol or triglyceride levels were noted between the two groups. Fatty acid hydroperoxide levels were also similar and were noted to increase from weaning (1.0±0.7/£M) to the time of sacrifice (1.8±1.5/£M, p<0.01). Fish oil had no influence on the extent of aortic atherosclerosis (25%±14% surface area for controls vs. 28%±19% for treated, p=NS), plaque thickness, or plaque volume after 1 year. We conclude that fish oil does not reduce the levels of serum cholesterol, lipid hydroperoxides, or aortic atherosclerosis in WHHL rabbits. The hypothesis that fish oil protects against atherosclerosis was not supported by this study.

breeders and were then mated with WHHL homozygous males. The subsequent offspring were either homozygous or heterozygous for the trait, a distinction made by obtaining a serum cholesterol level at the time of weaning (6 to 8 weeks of age). The cholesterol levels for the homozygous rabbits ranged between 669 and 1464 mg/dl and for the heterozygous rabbits between 72 and 306 mg/dl, thus allowing easy recognition of the homozygous group.

All rabbits were pasteurilla-free and maintained at 25°C in a temperature-controlled, laminar-flow room. No deaths from infection or other cause occurred in any of the study animals. The rabbits were fed a diet of standard rabbit chow (Purina #5123) and limited to 120 g per day. The rabbits were randomly assigned to control or treatment groups. The treated group had 13 male and two virgin female rabbits, and the control group, 11 males and two virgin females. The offspring from each litter were as evenly divided as possible to try to keep all potential genetic influences similar between both groups of study animals.

The treated rabbits were given 2.5 ml Maxepa fish-oil concentrate (generously supplied by the R.P. Scherer Corporation, Troy, MI) 6 days a week from the time of weaning until 1 year of age. This was accomplished by training the rabbits to remain stationary while the oil was slowly given via syringe into their posterior pharynx. This technique allowed the administration of a precise amount of fish oil to each rabbit without stress and prevented the possibility of oxidation of the fish oil if left standing in the rabbit food. Each rabbit consequently received 90 to 120 mg/kg/day of eicosapentaenoic acid (EPA), depending on the size of the rabbit. In addition, Maxepa contains 0.6% wt/wt cholesterol and one IU of vitamin E/ml, which resulted in the rabbits receiving approximately 15 mg/day cholesterol and 2.5 IU vitamin E/day. (This amount of vitamin E was previously shown not to affect hydroperoxide levels in the rabbit.)

**Biochemical Determinations**

Serum lipid levels were obtained by puncturing the middle ear artery of each rabbit at the time of weaning and at 4, 8, and 12 months of age. Total cholesterol and triglyceride levels were measured by standard enzymatic methods; the coefficient of variation for cholesterol was 3.2% and for triglyceride, 2.7%.

Lipid hydroperoxides were sampled at the time of blood sampling for lipid levels. Blood (5 ml) was collected into Vacutainer tubes (Becton Dickinson Labware) containing sodium citrate, and the plasma was separated by centrifugation for 15 minutes at 650 g at 4°C. Plasma was either kept briefly on ice for immediate assay or stored frozen at -15°C. The protein was partially removed from each plasma sample before assay by adding an equal volume of ethanol at 45°C and incubating it at 45°C for 20 minutes. The mixture was then chilled to -15°C for 20 minutes and centrifuged for 20 minutes at 650 g at -5°C. The supernatant was collected and assayed immediately for fatty acid hydroperoxide. Briefly, prostaglandin H synthase was injected into a reaction chamber containing 100 µM of arachidonic acid, 0.1 M Tris-HCL (pH 8.5), 1 mM phenol, 2.5 mM sodium cyanide, and 50 µl ethanol. Either standard hydroperoxide or the plasma sample was added to the assay in the ethanol immediately before the enzyme. The concentration of oxygen was monitored polarographically with an oxygen electrode and was recorded continuously; the reaction lag times were recorded and related to the pmol present in a 3-ml assay mixture. Samples were assayed in triplicate. The levels of nonesterified fatty acid hydroperoxide present in plasma samples were evaluated by reference to standard curves with 15-hydroperoxyarachidonate.

To ensure that the fish oil administered to the treated rabbits translated into an elevation in n-3 fatty acid circulating in the plasma, n-3 fatty acid levels were determined by random sampling of the rabbits by using gas chromatography. To 100 µl of plasma, 100 nmol butylated hydroxytoluene and 1 µmol methyl 11,14-eicosadienoate were added. Samples were extracted with 2 ml chloroform-methanol (2:1 vol/vol) and were transesterified in 1.5 ml 6% H2SO4 in methanol at 70°C for 4 hours. The methyl esters were extracted with hexane, and 500 nmol methyl tricosanoate was added as a further control to indicate the efficiency of the extraction of the internal standard. The solvent was evaporated under a nitrogen stream, and the residue was resuspended in 2 ml carbon disulfide for analysis. The gas chromatographic analysis was performed on a Packard Model 430 with a dropping needle injector and a 30 m Supelcowax 10 capillary column. Emission was isothermal at 230°C with the injector and detector at 260°C. Fatty acid methyl esters were identified by comparison of the retention times to those of standards. Quantities were determined by comparison of the peak area to that of the added methyl 11,14-eicosadienoate internal standard.

**Morphologic Determination of Extent of Atherosclerosis**

The rabbits were sacrificed at 12 months of age by an intravenous administration of anesthesia. An inflow cannula was inserted into the left carotid artery, and the proximal end was attached to a system designed to perfuse the arterial tree under controlled pressure. In brief, a head of pressure is maintained by a perfusion pump and monitored by a second catheter inserted into a femoral artery. The rate of flow from the pump can be regulated to maintain the desired pressure level. After fixation with 2.5% glutaraldehyde and Sorensen's buffer under a pressure of 100 mm Hg for 30 minutes, the entire aorta is excised, opened axially, pinned to a millimeter grid, and photographed. The preparation is then stained with Sudan IV and photographed again. For determination of the area covered by plaques, both the unstained and stained preparations were evaluated. Two standard regions of the aorta were utilized; the proximal descending thoracic aorta from the left subclavian artery to the celiac artery and the abdominal aorta from the celiac artery to the distal bifurcation. In each instance, photographic color transparencies were projected onto a digitizing plate coupled to a desk top computer. The outline of each lesion was traced, and the total surface area was determined. The quantitation system was programmed to provide the percent of total surface area covered by plaques.
To take into account possible differences in lesion thickness and cross-sectional area, complete transverse samples for histologic study were taken at 0.5, 2.5, 5, and 7.5 cm distal to the left subclavian artery. Paraffin-embedded sections of 7 µm were stained with hematoxylin and eosin and with the Gomori trichrome-aldehyde fuchsin stain for connective tissue. The sections were projected onto the digitizing plate by means of a microprojector, and the cross-sectional plaque areas were determined as percent of total artery wall area. Measurement of plaque thickness was also made at the point of maximal thickness at each level. An index of total lesion volume was then assigned to each specimen by establishing the product of the average of the maximal thickness at the thoracic levels and the percent of the surface area covered by plaques. The same was done for the abdominal segment utilizing the thickness at the 7.5-cm level.

### Statistical Analysis

The means and standard deviations for each of the measured variables were computed. Differences between the two groups were assessed with Student's t test for unpaired data. The relationships between variables were tested with the Pearson product moment correlation coefficient. Differences were significant if the p value was less than 0.05.

### Results

#### Serum Lipids

The total cholesterol and triglyceride levels of the two groups obtained at the time of weaning and at 4, 8, and 12 months of age were nearly identical at each age (Table 1). The significant downward trend of these lipids in the groups obtained at the time of weaning and at 4, 8, and 12 months of age has been previously described. The total cholesterol and triglyceride levels of the two groups were assessed with Student's t test for unpaired data. The relationships between variables were tested with the Pearson product moment correlation coefficient. Differences were significant if the p value was less than 0.05.

### Fatty Acid Hydroperoxides

The level of hydroperoxides was determined for the arterial plasma of New Zealand White rabbits (n=10) maintained on the standard diet and was 0.48±0.06 µM, similar to that reported for healthy human volunteers (0.5 µM). The mean value for fatty acid hydroperoxides in the WHHL rabbits on the standard diet (1.5±1.3 µM) was greater than that for the New Zealand White rabbits of similar age on the same diet (p<0.005) (see Table 1). The hydroperoxide levels for individual WHHL rabbits varied from month to month, ranging from 0 to greater than 2 µM during the course of the study. The average amount of lipid hydroperoxide observed after 4 months in the group of WHHL rabbits fed fish oil was similar to those rabbits fed the control diet. The values of hydroperoxide for individual WHHL rabbits receiving fish oil also fluctuated monthly over a wide range. The mean value of hydroperoxide for all WHHL rabbits increased with age from 1.0±0.7 µM to 1.8±1.5 µM (p<0.01).

To exclude the possibility that the WHHL rabbits failed to absorb the fish oil, the fatty acid contents of the total plasma from six WHHL rabbits were randomly determined before and after the period of dietary supplementation with fish oil. Before the animals received fish oil, no EPA was detected in their plasma. After two months of fish-oil supplementation, the level of EPA in the plasma reached 23.5±8 mg/100 ml, representing 3.4±1.6 molar percent of all plasma fatty acids. Similar concentrations of EPA have been reported in the plasma of Greenland Eskimos.

### Pathologic Analysis

In both groups of animals, lesions were most severe in the proximal aorta, particularly in the aortic arch. No grossly discernible differences could be detected in the overall distribution of the lesions between control animals and animals treated with fish oil. The histologic studies revealed intimal lesions consisting of accumulations of spherical foam cells and focal regions containing matrix fibers, both collagen and elastin. The lesions resembled those previously described for rabbits maintained on cholesterol-rich diets. There were no differences in the light microscopic appearance of the lesions in the two groups.

On the basis of extent of disease as determined from the percent luminal aortic surface area involved, no significant differences were evident (see Table 2 and Figure 1). For the WHHL rabbits, 25% of the surface area was involved (range, 9.6% to 64.3%), while the experimental group fed fish oil had 28% surface involvement (range, 11.0% to 80.6%). In the thoracic aortic region where lesions were most evident, 33% of the surface showed plaques (range, 4.9% to 82%) in the control group of WHHL rabbits fed fish oil had 28% surface involvement (range, 11.0% to 80.6%).
Table 2. Extent of Aortic Atherosclerosis in Two Groups at 1 Year of Age

<table>
<thead>
<tr>
<th></th>
<th>WHHL control</th>
<th>WHHL treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Surface area</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>25±14</td>
<td>28±19</td>
</tr>
<tr>
<td>Thoracic</td>
<td>33±24</td>
<td>33±22</td>
</tr>
<tr>
<td>Abdominal</td>
<td>17±9</td>
<td>21±16</td>
</tr>
<tr>
<td>Lesion thickness (mm)</td>
<td>0.14±0.08</td>
<td>0.18±0.12</td>
</tr>
<tr>
<td>Volume index (mm)</td>
<td>4.4±3.7</td>
<td>6.1±8.0</td>
</tr>
</tbody>
</table>

No statistically significant differences were observed between the groups for any of the measurements.

WHHL=Watanabe heritable hyperlipidemic rabbits.

Table 3. Percent Thoracic Aorta Surface Area Involved during Course of Study

<table>
<thead>
<tr>
<th>Number of animals</th>
<th>Control (%)</th>
<th>Treated (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=2</td>
<td>82</td>
<td>24</td>
<td>—</td>
</tr>
<tr>
<td>n=10</td>
<td>42</td>
<td>25</td>
<td>0.05</td>
</tr>
<tr>
<td>n=28</td>
<td>33</td>
<td>33</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS=not significant.

The distribution of aortic plaque luminal surface involvement, as percent of the total aortic surface area, is shown for the control Watanabe heritable hyperlipidemic (WHHL) rabbits and the WHHL rabbits given fish oil. No significant differences were apparent between the two groups. Marked variability in aortic involvement was noted in both groups.

Figure 1. The distribution of aortic plaque luminal surface involvement, as percent of the total aortic surface area, is shown for the control Watanabe heritable hyperlipidemic (WHHL) rabbits and the WHHL rabbits given fish oil. No significant differences were apparent between the two groups. Marked variability in aortic involvement was noted in both groups.

group, while in the fish-oil fed group, 33% of the surface was also involved (range, 12.1% to 93.0%). The less involved distal abdominal segment showed 17% involvement in the control animals (range, 5.5% to 36.5%) and 21% involvement in the group fed fish oil (range, 3.8% to 63.3%).

The average thickness of the lesions was similar for the two groups and was 0.14 mm (range, 0.03% to 0.27%) for the WHHL rabbits on normal chow and 0.18 mm (range, 0.03% to 0.43%) for the animals fed fish oil. The volume index for the descending aorta was 6.1 mm for the animals fed fish oil and 4.4 mm for the control group. In the thoracic aorta where the lesions were most prominent, the volume index was 7.2 mm for the WHHL rabbits without treatment and 9.5 mm for those fed fish oil. None of the measurements of aortic atherosclerosis were significantly different between the two groups.

Importance of Sample Size

From the analysis of the luminal surface involvement of plaque in the aortas done during the study, it was apparent that there was wide variability in the amount of atherosclerosis in both groups of animals (see Figure 1). This variability has been reported, but not addressed, in the published literature on the WHHL rabbit, although it is characteristic of the expression of atherosclerosis induced in other animals and of atherosclerosis in humans with familial homzygous hyperlipidemia.20 After two and 10 rabbits had been sacrificed, we noted a statistically significant effect of the fish oil (see Table 3) and reported a significant decrease in plasma hydroperoxide levels. However, because of the degree of variability in the aortic lesions in these rabbits, it was decided at that time to increase the number of rabbits for study to better test the hypothesis. When this was done, the perceived protective effects of the fish oil were lost.

Discussion

The evidence for a possibly beneficial influence of diets high in sea food on atherosclerosis in humans is primarily based on epidemiologic observations on the diets of populations and the incidence of cardiovascular events. The mechanism of this beneficial effect, like the mechanism of atherogenesis, remains speculative. The influence of omega-3 fatty acid on platelet function has been well studied22-23 and was not a focus of this project. Populations whose diets are high in fish oil and subjects on a Western diet supplemented with omega-3 fatty acids exhibit a prolongation in bleeding time and reduction in platelet aggregation. Recent studies indicate variable effects on neutrophil and macrophage function as well.7

The effect of fish oil on serum cholesterol levels is less consistent. Although omega-3 fatty acids may lower serum cholesterol, primarily by lowering very low density lipoprotein (VLDL) cholesterol, this occurs primarily in patients who have type II-B and type IV hyperlipidemias.24-25 Fish oil does not appear to be an effective cholesterol-lowering agent in the majority of people with elevated cholesterol and relatively normal triglycerides.26 Neither was it effective in lowering the cholesterol in the WHHL rabbit. Triglyceride levels can be reduced by dietary omega-3 fatty acids.
acids, apparently by suppressing triglyceride synthesis in the liver and reducing apolipoprotein B synthesis. We observed a modest but nonsignificant influence of fish oil on triglyceride levels in the WHHL rabbit, although VLDL lipoprotein metabolism in the WHHL rabbit differs from that in the human. 27

The influence of omega-3 fatty acids on lipid hydroperoxides is less well studied. Lipid hydroperoxides can damage vascular tissue and may be causal agents in the development of atherosclerosis. 28 It has been reported that patients with coronary heart disease have higher amounts of lipid hydroperoxides than do normal controls, 9 consistent with the concept that circulating hydroperoxides represent a chronic oxidant stress capable of either promoting atherogenesis directly or stimulating increases in intracellular hydroperoxides to reach atherogenic levels. The mean levels of lipid hydroperoxides in the WHHL rabbits estimated by the enzymatic assay were higher than those in New Zealand White rabbits, consistent with the possible participation of hydroperoxides in this atherogenic animal model. It was observed that the mean level of lipid hydroperoxide increased with the age of the rabbit, also consistent with the observation that the amount of atherosclerosis in these rabbits increases with age. 18 We were unable, however, to detect an influence of the ingestion of omega-3 fatty acids in reducing hydroperoxide levels. Although it remains possible that atherogenesis could be diminished by agents that reduce peroxide levels, it does not appear that omega-3 fatty acids are among them. The values of lipid hydroperoxide in an individual rabbit fluctuated considerably over the course of the study, suggesting that these levels are not in a steady state but rather in constant flux, perhaps in concert with the development of vascular lesions.

We found no influence of fish oil on the amount of atherosclerotic plaque observed in the aortas of the WHHL rabbit, either on surface involvement, distribution, plaque thickness, or plaque volume. The microscopic appearance of these plaques was similar to those observed in rabbits in whom atherosclerosis has been induced by high-fat diets. 17 Other studies looking at the influence of fish oil in animals have reported both a reduction 20-22 and an increase in atherosclerosis from fish-oil supplemented diets. 20,29 Discrepancies in the results of these studies might have several explanations. Although a dramatic model to study naturally occurring atherosclerosis, the WHHL rabbit may not be comparable to other models of atherosclerosis with respect to the influence of n-3 fatty acids on the disease process. A similar discrepancy has been reported on the effects of calcium blockers on atherogenesis in WHHL versus cholesterol-fed rabbits. 21,31 Species differences might also account for differences in the biologic effects of fish oil. Indeed, it has recently been reported that fish oil enhances monocyte adhesion and fatty streak formation in the rat. 30 Another important reason for the conflicting results of other studies, however, may relate to the number of animals studied. The observation of marked biologic variability in the amount of atherosclerosis in the rabbits that we studied is consistent with the disease process in all animal species. Thus, although the data from studies with small numbers of animals may turn out to be statistically significant, they may not necessarily be clinically meaningful. We, too, would have found a significant influence of fish oil on reducing atherosclerosis had we limited our study to 10 rabbits. The importance of sample size when reviewing and comparing other studies is underscored by our results.

We believe, however, that several aspects of this study relate to the human experience. The serum levels of omega-3 fatty acids attained in the rabbits receiving fish oil were similar to those observed in the Greenland Eskimo. 18 In addition, the main source of the dietary fatty acids in the study rabbits were derived from marine animals, also similar to the Eskimo population. Finally, by waiting for 12 months to assess the influence of the fish oil, we were able to evaluate the long-term effect of omega-3 fatty acids.

Our results suggest that the notion that fish oil protects against the development of atherosclerosis remains speculative. Fish oil might influence cardiovascular mortality by its antithrombotic effects on platelets, however, which would tend to reduce the risk of acute myocardial infarction, as recently supported by the results of the Physicians Health Study. 23 We did not test for the presence of acute myocardial infarction but rather for chronic atherosclerosis. Similarly, it is a paucity of acute myocardial infarction, rather than chronic atherosclerosis, that was observed in the Eskimo population. In this respect, diets high in omega-3 fatty acids may create an antithrombotic effect similar to that with the ingestion of the small amounts of aspirin. However, the uniform prescription of fish-oil capsules to adults with the expectation that it will either reduce their cholesterol or their development of atherosclerosis is not supported by this study.

References
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Index Terms: atherosclerosis • fish oil • omega-3 fatty acids • WHHL rabbits • lipid hydroperoxides
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