Prevention of Atherosclerosis in Sub-Human Primates by Chondroitin Sulfate A

By Lester M. Morrison, M.D., Katumi Murata, M.D., Ph.D., J. Joseph Quilligan, Jr., M.D., O. Arne Schjeide, Ph.D., and Leon Freeman, Ph.D.

ABSTRACT

Sixty-five squirrel monkeys (Saimiri sciurea) were studied for a year. During the last 9 months, spontaneously occurring atherosclerosis was accelerated by feeding a diet containing cholesterol and butter. Of six separate groups of animals, three groups were examined for the effects of acid mucopolysaccharides on the process of atherosclerosis. Monkeys treated with parenteral chondroitin sulfate A and fed the cholesterol-butter diet showed statistically significant reductions of serum total lipid levels and aorta lipid levels when compared to control groups on the cholesterol-butter diets. Macroscopic comparison of aortic atherosclerosis showed chondroitin sulfate A-treated animals to have the least atherosclerotic involvement. The acid mucopolysaccharide chondroitin sulfate A may be an effective preventive agent in the treatment of experimentally induced atherosclerosis.

ADDITIONAL KEY WORDS

inhibition of atherosclerotic plaques

acid mucopolysaccharide
squirrel monkeys

Despite a universal and critical need, the prevention and treatment of arteriosclerosis by any specific agent has not yet been demonstrated in the clinical practice of medicine.

Chondroitin sulfate A (CSA), an acid mucopolysaccharide, has been found in tissue culture studies in our laboratories to be a specific connective tissue-derived substance which possesses growth-stimulating and lipid-reducing properties,1-8 and is present at a level of 1.5 mg per L in normal human circulating plasma.4 The following preliminary report describes the results of treatment during a 9-month period with CSA and certain other agents in the inhibition of induced atherosclerosis in squirrel monkeys (Saimiri sciurea), a species which has been found by Middleton et al.5 and Portman and Andrus5 to exhibit a high incidence of naturally occurring arteriosclerosis with aortic sudanophilia which is similar in many respects to that in man.

Materials and Methods

The animals employed in this experiment were captured by professional animal trappers near Baranquilla, Colombia, South America, and were flown directly to the research compound.* During a 3-month screening period they were examined for parasites, pneumonia, tuberculosis and other diseases to which they are susceptible. Following preliminary trial housing arrangements, it was found that the monkeys did well when kept two to a cage. There was no sexual segregation since breeding or menstruation did not occur. Thirty-three male and 32 female 1- to 3-yr-old squirrel monkeys were selected at an initial body

*All animals in this colony were housed and cared for at the Research Primate Division, Asiatic Animal Imports, San Francisco, California, under the direct supervision of R. J. Veenstra, D.V.M. and E. Reid, D.V.M.
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Table 1
Grading of Atherosclerosis in Aortas of Monkeys

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No visible lesion.</td>
</tr>
<tr>
<td>0.5</td>
<td>Very slight; occasional or singular, nonelevated fatty streak.</td>
</tr>
<tr>
<td>1.0</td>
<td>Slight; yellowish and pale fatty streaks with stippled lipid elevations. These are very thin, superficial, lipid deposits appreciably raised above the intimal surface. The remainder of the intima is normal in appearance.</td>
</tr>
<tr>
<td>2.0</td>
<td>Mild; scattered lipid deposits with slight plaque formation.</td>
</tr>
<tr>
<td>3.0</td>
<td>Moderate; thicker lipid deposits with elevated fibrous plaques. Some specimens showed diffuse atheromatous lesions.</td>
</tr>
<tr>
<td>4.0</td>
<td>Marked atheromatous lesions with pronounced, diffuse lipid deposition.</td>
</tr>
</tbody>
</table>

No plaques with ulceration, necrosis, calcification or hemorrhage were observed in this 9 month experiment.

Weight of approximately 500 g and were divided into six groups which were treated as follows: Group I was fed a natural food stock diet (ground Purina Monkey Chow); groups II to VI inclusive were fed a diet consisting of 1.5% cholesterol, 20% butter and 78.5% ground Purina Monkey Chow. Group II consisted of 15 animals (8 males and 7 females); all other groups consisted of 5 males and 5 females each. Monkeys in groups III to VI inclusive received daily subcutaneous injections of the following: group III, saline solution; group IV, 10 mg of heparin; group V, 10 mg of CSA; and group VI, 10 mg of calf aorta extract.$ All injections were 1 ml amounts of either saline solution or test material in 1 ml saline solution. Food and water were provided ad libitum.

After an experimental period of 9 months all the surviving monkeys were sacrificed in a fasting state by withdrawal of blood directly from the heart under nembutal anesthesia. The serum was saved for lipid determinations. The aortas were removed and the surrounding adventitial tissue carefully stripped off. Macrosopic gradings of atherosclerosis (table 1) were carried out according to a slight modification of the method described previously.1,8 Segments of the aorta were preserved by freezing for lipid analyses. Total lipid of the serum and aorta was extracted by the method reported by Folch9 and weighed. At the time of sacrifice, specimens of aorta (approximately 5 X 10 mm in size) were taken from the same sites in each animal (the arch, thoracic and abdominal aortas) and were fixed in a 10% neutral formalin. Paraffin sections were prepared and stained with hematoxylin-eosin. Frozen sections were also prepared, stained for lipid with Sudan IV and counterstained with hematoxylin.

Results

Most animals in all groups gained weight during the course of the experiment. No statistical evidence for significant differences in body weight was observed among the various groups at the time of sacrifice (table 2). During the 9-month experimental period, the mortality was 0 to 20% for all groups except group VI, which received the calf aorta extract. In this group 60% (5 females and 1 male) died during the third and fourth month of the experiment. No gross pathological changes were found at the time of sacrifice in any animals in groups I through V in tissues other than the aortas. In group VI, changes were seen in vital organs, including the aorta, liver, kidneys, heart and lungs. Brains were not examined.

The average and range of the grading of atherosclerosis in each group are presented in table 2 and figure 1. In agreement with earlier findings,5,6 atherosclerotic lesions (although of minimal degree) were observed in the aortas of monkeys fed the unsupplemented stock diet (group I). The addition of cholesterol and butter to this diet resulted in a significant increase in the severity of lesions (groups II and III). The heparin group (IV) was comparable to groups II and III. The most severe lesions were observed among the survivors of group VI. The latter group consisted of only four animals, because the other six died earlier in the experiment with evidence suggesting an anaphylactic reaction. Possibly an anaphylactic reaction occurred to a smaller degree in the survivors.

As was recently pointed out by Hauss et al.10 this could be sufficiently stressful or traumatic.
TABLE 2
Comparison of Macroscopic Grading of Atherosclerosis and Serum Total Lipids

<table>
<thead>
<tr>
<th>Group</th>
<th>Survivors</th>
<th>Treatment</th>
<th>Macroscopic grading</th>
<th>Serum total lipids</th>
<th>Average body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td></td>
<td>Average Range</td>
<td>Mean ± SE</td>
<td>Initial Autopsy</td>
</tr>
<tr>
<td>I</td>
<td>8/8</td>
<td>Normal control</td>
<td>0.6 0-1.5</td>
<td>520 ± 29</td>
<td>505 661</td>
</tr>
<tr>
<td>II</td>
<td>11/14</td>
<td>Cholesterol-butter fed (CBF) control</td>
<td>2.0 0-4</td>
<td>596 ± 16</td>
<td>505 642</td>
</tr>
<tr>
<td>III</td>
<td>9/10</td>
<td>CBF + saline injections</td>
<td>2.0 0.5-3.5</td>
<td>553 ± 26</td>
<td>493 639</td>
</tr>
<tr>
<td>IV</td>
<td>8/10</td>
<td>CBF + heparin injections</td>
<td>2.0 1-3.5</td>
<td>552 ± 49</td>
<td>541 614</td>
</tr>
<tr>
<td>V</td>
<td>7/8</td>
<td>CBF + chondroitin sulfate A injections</td>
<td>0.4 0-1</td>
<td>466 ± 31</td>
<td>522 599</td>
</tr>
<tr>
<td>VI</td>
<td>4/10</td>
<td>CBF + calf aorta extract injections</td>
<td>3.5 3-4</td>
<td>494 ± 60</td>
<td>495 610</td>
</tr>
</tbody>
</table>

Significant differences in serum total lipids: II vs V ——— P < 0.01; III vs V ——— P < 0.05.

to accelerate the atherosclerotic process. Animals given CSA (group V), however, exhibited a striking reduction in the severity of atherosclerotic lesions compared to all other groups fed the cholesterol-butter diet and the lesions were indistinguishable in respect to the gross appearance of the aortas from animals fed the unsupplemented stock diet (group I).

Although a comparison of lipid deposits in aortas of all the groups indicated that there were no specifically affected portions, isolated fatty streaks or marked lipid deposits were more frequently observed at the aortic arch. Less frequent atheromatous changes were observed in the thoracic portion, where small lipid deposits surrounded the orifices of the thoracic arteries in some cases. Diffuse lipid deposits were intermediate in frequency in the abdominal aorta.

Only preliminary observations have been made on the histologic material. However, selected samples showed evidence of correlation with the different degrees of lipid infiltration already demonstrated in the gross materials. Microscopic changes at the early stage, in which the lipid deposits localize mostly in the intima, corresponded to grade 1 in the macroscopic differentiation.

In the more advanced stages, lipid infiltrated through the subintima into the medial portion of the aorta, but in some cases no elevation was observed macroscopically. In other cases, elevation of the intimal and subintimal layers was seen without lipid deposition.

With advance of the atherosclerotic process the number of foam or phagocytic cells containing lipids was increased. Lipid deposition extended into numerous intimal and subintimal cells and was also seen extracellularly. Some of the cells contained shrunken and pyknotic nuclei. Hyaline degeneration was detected in the medial layers inferior to the severe atheromatous lesions. No abnormal findings were seen in the other organs examined except in the group VI animals noted above.

As also shown in table 2, serum total lipid analyses obtained at sacrifice showed an average of 520 mg/dl in animals fed the unsupplemented stock ration and higher levels in animals of groups II, III and IV. The four survivors of group VI had a serum total lipid content slightly lower than that of animals in group I. The differences among the above groups, however, were not statistically significant. The CSA-treated animals (group V), however, showed significantly lower serum lipid than animals in groups II and III.

Table 3 shows the data on average total lipids (percent of dry weight) obtained by

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FIGURE 1

Arch portion of monkey aortas (magnification originally 250).

Upper left, normal monkey aorta in group I equivalent to macroscopic grade 0. Hematoxylin-eosin stain.

Upper right, lipid infiltration into thickened intima in group II equivalent to macroscopic grade 1. Hematoxylin-eosin stain.

Bottom left, superficial lipid deposits involving the intima in the early stage of atherosclerosis in group II equivalent to macroscopic grade 1. Sudan IV hematoxylin stain.

Bottom right, extensive lipid deposition in advanced atherosclerosis. In the intima there are several layered, phagocytosed cells with pyknotic nuclei. Lipids also infiltrate into subintima and media. Group II equivalent to macroscopic grade 4. Hematoxylin-eosin stain.

Extraction of aortic segments from each of the experimental groups. These data were surprisingly close to the amounts reported by Böttcher for total lipids in Stage II lesions from human atherosclerotic aortas. When comparisons were made, significantly lower amounts of lipids were observed in the CSA-treated animals in contrast to either group III or groups II and III combined. Although the number of animals studied is relatively small, significance was observed at the 0.05 level; a clear difference was discernible and was in agreement with observations of gross pathological changes.
TABLE 3
Comparison of Total Lipids of Monkey Aortas

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of aortas</th>
<th>Male</th>
<th>Female</th>
<th>% of total lipids mg of dry tissue</th>
<th>% of total lipids mg of dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>9.5 ± 1.5†</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>10</td>
<td>6</td>
<td>4</td>
<td>11.7 ± 1.3‡</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td>11.9 ± 0.3†</td>
<td>12.1 ± 0.3‡</td>
</tr>
<tr>
<td>IV</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>12.6 ± 3.3</td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>9.2 ± 0.5‡</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>17.0 ± 2.9</td>
<td></td>
</tr>
</tbody>
</table>

*Treatment for each group is listed in table 2.
†Mean and standard error.
‡Significant difference was found at 0.05 level of P value between group II and group V, and between the combined groups II + III and group V.
*Samples were determined in duplicate.

Discussion

Extensive studies of the inhibitory effects of the chondroitin sulfate acid mucopolysaccharides on experimental lipemia and atherosclerosis have been reported by Oshima and co-workers. These acid mucopolysaccharides are chondroitin sulfates derived from shark cartilage, presumably type C or D, and other sulfated polysaccharides from natural sources. As early as 1955, Kurita reported that administration of chondroitin sulfate C inhibited atherosclerosis and reduced plasma cholesterol levels in cholesterol-fed rabbits. Ohdoi found that sodium chondroitin sulfate when given orally at a level of 20 mg per kg of body weight per day inhibited the elevation of serum cholesterol, total lipids, beta-alpha lipoprotein ratio and atheromatous aortic lesions in cholesterol-fed cockerels. Murata observed that daily intravenous injections of 5 mg per kg of body weight of a chondroitin polysulfate prepared from shark cartilage significantly reduced the serum total lipid and serum cholesterol levels of cholesterol-fed rabbits and had an ameliorating effect on the severity of cholesterol-induced atherosclerosis. Oshima and associates have reported that the oral administration of 1.5 g of chondroitin sulfate daily to hypercholesteremic human subjects decreased serum total cholesterol by an average of 18% after 2 to 4 weeks; the decrease was maintained as long as the chondroitin sulfate was administered. Chondroitin sulfate at the above dosage to normal subjects, however, had no significant effect on serum cholesterol levels.

Present findings indicate that CSA inhibited increases in serum total lipids and atherosclerosis in squirrel monkeys fed a cholesterol and butter-containing diet. The use of CSA has not been previously reported for the prevention or treatment of atherosclerosis in humans or animals, although unpublished studies by one of us (L.M.M.) indicate that arterial extracts containing acid mucopolysaccharides, including CSA, were effective in ameliorating clinical symptoms associated with coronary, cerebral and peripheral arteriosclerosis in man. The CSA used in this experiment is comparatively pure and differs from other mucopolysaccharides in physical, chemical and biological properties. Further studies are indicated to determine the comparative effects of graded levels of CSA and other acid mucopolysaccharides following both oral and parenteral administration on the incidence and severity of atherosclerotic lesions in the aorta, coronary and cerebral arteries of squirrel monkeys, both under conditions of accelerated atherosclerosis induced by cholesterol feeding and upon the naturally occurring atherosclerosis on the stock diet. Studies are also indicated to determine the effects of such treatments on the reversal or regression of atherosclerotic lesions and the modus operandi of the observed effects.

Acknowledgment

Invaluable aid was rendered in this research by Stanford Gluck (deceased) of the Primate Research Division, Asiatic Animal Imports, San Francisco, R. J. Veenstra, D.V.M., E. Reid, D.V.M., Betty Dukes, B.S., Janet S. Brown, B. S., and Monica R. Stevens, B.S.

References


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