Atherogenesis during Low Level Hypercholesterolemia in the Nonhuman Primate

I. Fatty Streak Formation

Junichi Masuda and Russell Ross

Although a large body of data is available concerning atherogenesis in animals maintained at high levels of hypercholesterolemia, little data are available for animals maintained at lower levels of hypercholesterolemia for longer periods of time, closer to those observed in humans. The chronologic sequence of cellular events and interactions that occur during the formation of the lesions of atherosclerosis was investigated during relatively low level hypercholesterolemia (200 to 400 mg/dl) in a series of nonhuman primates (Macaca nemestrina). The arterial tree of each animal was examined by light microscopy and scanning and transmission electron microscopy. Immunohistochemical staining with monoclonal antibodies specific for smooth muscle cells, monocyte-macrophages, and T-lymphocytes was performed to analyze the cellular composition of the lesions. After 6 months of low level hypercholesterolemia, the surface of the aorta contained large numbers of adherent leukocytes, many of which were in the process of entering the artery. This resulted in irregularly shaped nodular elevations, or fatty streaks, preferentially located at branch sites and bifurcations. The fatty streaks consisted of intimal accumulations of numerous lipid-laden macrophages together with relatively small numbers of T-lymphocytes. With lesion progression, the thickness of the fatty streaks increased, and their surfaces became irregular and frequently showed disruptions of covering endothelial cells resulting in exposure of subendothelial macrophages. Platelet microthrombi were observed over some of the exposed macrophages at some branches or bifurcations in every animal studied. These observations made during the early phases of atherosclerosis lesion formation are virtually identical to those described in our previous reports in high level hypercholesterolemic nonhuman primates (600 to 1000 mg/dl) with the exception that the changes occurred more slowly in the lower levels of hypercholesterolemia. (Arteriosclerosis 10:164-177, March/April 1990)

Many studies have described the morphology of atherosclerosis lesion formation during very high levels of diet-induced hypercholesterolemia in various experimental animals, such as nonhuman primates,1-12 swine,13-15 rabbits,16-21 rats,22 and pigeons.23-24 The lesions that form in these animals are similar to those observed in familial homozygous hypercholesterolemia in humans, in whom very high levels of hypercholesterolemia occur endogenously.25 Very high levels of hypercholesterolemia were induced previously because of the time required to complete the experiments, the costs, and the assumption that the process of atherogenesis would be similar to that in humans. However, it has become clear that there are differences in the morphology and distribution of lesions among the different species of experimental animals as well as between animals and humans.26-30 Several studies demonstrated that the morphology and distribution of spontaneous and diet-induced atherosclerosis in nonhuman primates show striking similarities to those in humans.26-32 Nevertheless, there is little to no data available concerning the process of atherogenesis as it occurs in nonhuman primates kept at lower levels of hypercholesterolemia similar to those which occur in most humans. This study was designed to investigate the process of atherogenesis during low level hypercholesterolemia in nonhuman primates and to determine the similarities and differences in the lesions of atherosclerosis that form in these primates as compared to humans, who may be hypercholesterolemic for prolonged periods of time. It also permits us to determine the validity of the data obtained during high level hypercholesterolemia, which requires much less time for lesion induction, thus permitting access to data in a much shorter time period.

The chronologic sequence of cellular events that precede the formation of lesions with very high levels of hypercholesterolemia shows many similarities among nonhuman primates,1-3 swine,14-15 and fat-fed versus genetically hypercholesterolemic rabbits.16-17 In the current study, we examined the cellular interactions that take place during the early phases of lesion formation in
nonhuman primates with relatively low levels of hypercholesterolemia (200 to 400 mg/dl). We used light microscopy and scanning and transmission electron microscopy in these studies, and for immunohistochemical analysis we took advantage of a series of newly developed monoclonal antibodies that specifically recognize smooth muscle cells, monocyte-macrophages, and subsets of T-lymphocytes, respectively. The correlation of the immunohistochemistry with the light and electron microscopic studies provides an opportunity to analyze both the cellular interactions and the composition of the lesions that result from these interactions. In addition, these studies provide information that should be more applicable to understanding the cellular interactions that may occur in humans whose plasma cholesterol levels are closer to those observed in these studies. Furthermore, this approach allows us to ask whether the cellular events and lesions that form during relatively low level hypercholesterolemia are similar to or different from those we previously reported in short-term (13 months), very high level hypercholesterolemia. This article describes the cellular interactions that precede fatty streak formation in animals fed the diet for 6 months to 1 year. The accompanying article describes the cellular changes that occur during lesion progression and fibrous plaque formation in animals fed the same relatively low level cholesterol diet for periods up to 3.5 years.

Methods

Animals

Eight pigtail monkeys (Macaca nemestrina) between 3 and 5 years of age, with an average weight of 4 to 7 kg, were fed an atherogenic diet, and their plasma total cholesterol levels were maintained between 200 and 400 mg/dl. They were sacrificed after 6 months or 1 year on the diet. For scanning and transmission electron microscopic examination, two animals were sacrificed after 6 months on the diet and two after 1 year; for immunohistochemical examination, two animals were sacrificed after 6 months on the diet and two after 1 year. An additional four monkeys received normal monkey chow and served as controls (two for electron microscopy and two for immunohistochemistry). Procedures followed were in accordance with the Guide for Care and Use of Laboratory Animals, as issued by the U.S. Institute of Laboratory Resources.

Blood Samples

Each animal was bled periodically during the experimental period to monitor the changes in plasma lipid profiles of each animal. The values of plasma cholesterol (PC) and plasma triglyceride (PT) were measured every 2 or 3 months, and their lipoprotein fractions were determined every 6 months. These analyses were performed by the Northwest Lipid Research Clinic using established standard procedures.

Diet

The atherogenic diet mixture used in this study (Table 1) derived 42% of its calories from fat and was supplemented with cholesterol for a total concentration of 0.5 g of cholesterol for every 100 g of diet (0.5%). Since each animal responds differently to the atherogenic diet, PC and PT values were monitored every 2 or 3 months, and the cholesterol content of the diet was adjusted for each animal (0.1% to 0.5% of cholesterol content) to maintain PC levels between 200 and 400 mg/dl. The control animals were fed normal unsupplemented monkey chow.

Each monkey was housed in a single cage and fed daily ad libitum. Dietary intake was monitored, and bi-monthly determinations were made of body weight.

Animal Sacrifice and Perfusion Fixation for Electron Microscopy

Two different groups of animals were used for electron microscopic or for immunohistochemistry. The animals whose tissues were used for immunohistochemistry could not be perfusion-fixed because most of the antigens used as cell markers are denatured by glutaraldehyde. The animals whose arteries were examined by electron microscopy were anesthetized with ketamine (20 mg/kg) intramuscularly. Both femoral veins were exposed and cannulated for perfusion runoff. A cannula connected to a perfusion apparatus was inserted into the right carotid artery. Just before exsanguination, each animal was given an intravenous bolus injection of sodium pentobarbital intravenously, and the blood was flushed with lactated Ringer's solution at a flow rate of 400 ml/minute, which was required to maintain intrarterial pressure between 90 and 110 mm Hg. When the runoff was clear, 2 to 4 I of 2.5% glutaraldehyde in 0.1 M of phosphate buffer was pumped through at the same flow rate. After perfusion, the femoral veins and the cannulated carotid artery were clamped, and the arteries were fixed under pressure for an additional 30 minutes.

Tissue Preparation

After perfusion fixation, the entire aorta, iliac arteries, carotid arteries, and hearts were dissected from each animal. The arteries were cleaned of surrounding connective tissue, and the aorta and iliac arteries were cut into 18 segments as diagrammed in Figure 1. Each segment was further divided into two parts; one portion was a 2-mm section taken from the most representative area for light microscopic and transmission electron microscopic examination. The remaining larger portion was processed for scanning electron microscopy. All of the samples were immersion-fixed in glutaraldehyde overnight at 4°C. After fixation, each 2-mm section of artery was washed thoroughly in 0.1 M of phosphate buffer and was post-fixed in 0.1% osmium tetroxide (OsO4) buffered with 0.1 M of phosphate buffer for 3 hours at room temperature. The samples were then immersed in 50% ethanol for 15 minutes, followed by 1 hour in 70% ethanol containing 3% uranyl acetate. Dehydration was completed by using a graded series of increasing concentrations of ethanol followed by propylene oxide. Each sample was then embedded in Medcast (Ted Pella, Redding, CA). Three 1-μm sections were cut from each embedded ring and were stained with basic fuchsin-methylene blue.
Table 1. Atherogenic Diet Mixture and Control Diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Atherogenic diet</th>
<th>Control diet</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>g/100 g</td>
<td>Protein (g)</td>
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<tr>
<td>Casein, USP</td>
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<td>8.0</td>
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<td>Lactalbumin</td>
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<td>8.0</td>
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<tr>
<td>Wheat flour</td>
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<td>3.67</td>
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<td>Dextrin</td>
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<tr>
<td>Sucrose</td>
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<td></td>
</tr>
<tr>
<td>Applesauce</td>
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<td>0.018</td>
</tr>
<tr>
<td>Lard</td>
<td>12.0</td>
<td></td>
</tr>
<tr>
<td>Butter</td>
<td>3.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Beef tallow</td>
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<tr>
<td>Dried egg yolk</td>
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</tr>
<tr>
<td>Complete vitamin mixture (devold of vitamin D)</td>
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<td></td>
</tr>
<tr>
<td>Alphacel</td>
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<td></td>
</tr>
<tr>
<td>Hegsted salts mixture</td>
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<td>D$_3$ in corn oil</td>
<td>0.0625</td>
<td>(250 IU)</td>
</tr>
<tr>
<td></td>
<td>Total 100.0</td>
<td>20.85</td>
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<tr>
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<td>Wheat flour</td>
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<tr>
<td>Dextrin</td>
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<td>Applesauce</td>
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<td>Lard</td>
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<tr>
<td>Butter</td>
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<tr>
<td>Safflower oil</td>
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<td>Beef tallow</td>
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<td>Dried egg yolk (37)</td>
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<tr>
<td>Alphacel (1330)</td>
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<td>Hegsted salts mixture</td>
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<tr>
<td>D$_3$ in corn oil</td>
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<td>(250 IU)</td>
</tr>
<tr>
<td></td>
<td>Total 100.0</td>
<td>19.71</td>
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Atherogenic diet was equivalent to 0.300 mg cholesterol/cal diet; protein = 18% of calories; fat = 42% of calories; carbohydrates = 40% of calories. Atherogenic diet was supplemented with 0.36 g cholesterol/100 g diet to yield a final concentration of 0.5 g/100 g diet. Control diet was equivalent to 0.050 g cholesterol/cal diet; protein = 22% of calories; fat = 29% of calories; carbohydrates = 49% of calories.

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Slides were examined and photographed with a Zeiss photomicroscope III (Zeiss, Thornwood, NY). The areas of interest were selected, and corresponding thin sections (800 Å) were cut with an ultratome (LKB ultratome, Type 4) and placed on 400 mesh copper grids. The sections were counterstained with lead citrate and uranyl acetate and were examined with a JEOL 100B transmission electron microscope at 60 kV (JEOL-USA, Los Angeles, CA).

The larger arterial segments to be used for scanning electron microscopy were opened lengthwise and pinned on Teflon sheets. The tissue was washed in buffer and postfixed in 1% OsO$_4$ in 0.1 M phosphate buffer for 3 hours at room temperature. The tissue was then dehydrated through graded ethanol, critical-point-dried in CO$_2$ (Tousimis, Rockville, MD), and sputter-coated with a 300-Å layer of gold-palladium (Polaron, Warrington, PA). The specimens were examined with a JEOL 35C scanning electron microscope at 15 kV.

**Animal Sacrifice for Immunohistochemistry**

The animals were anesthetized with ketamine and sacrificed by an intravenous bolus injection of sodium pentobarbital. The entire aorta, iliac arteries, carotid ar-
teries, and heart were dissected from each animal. The arteries were cleaned of surrounding connective tissue on ice and opened lengthwise. After blood was removed from the surface by gentle rinsing with phosphate-buffered saline (PBS), the arterial tree was divided into 18 segments, as diagrammed in Figure 1. Two 2-mm sections were removed from each segment, and one section was embedded in OCT compound and frozen in liquid nitrogen. The other 2-mm section was immersion-fixed in methanol-Carnoy's fixative and subsequently embedded in paraffin.

**Monoclonal Antibodies**

Monoclonal antibodies, HHF35 and HAM56, were used as specific markers for smooth muscle cells and monocyte-macrophages, respectively, in both paraffin-embedded sections and frozen sections. Three monoclonal antibodies were applied to identify T-lymphocytes in frozen sections: MoAb 9.6, specific for CD2; OKT4a, for CD4; and G10.1, for CD8. Another antibody, 2H7, was used to look for B-lymphocytes in frozen sections. These monoclonal antibodies were raised against human anti-

gen, but they showed cross-reactivity against nonhuman primates sufficient for use in this study.

**Immunohistochemistry on Paraffin-embedded Tissue**

The immunoperoxidase avidin-biotin complex system with nickel chloride (NiCl2) color modification was employed on methanol-Carnoy's-fixed paraffin-embedded sections. Briefly, after 5-μm sections were de-paraffinized and rehydrated with PBS, the sections were pre-incubated with 3% normal horse serum for 10 minutes. Diluted primary antibodies (HAM56 1:2000 and HHF35 1:8000) were then applied to the sections and incubated for 30 minutes. With intervening washes in PBS, they were serially incubated with 1:200 dilutions in PBS of biotinylated horse, antimouse IgG (Vector Laboratories, Burlingame, CA) for 30 minutes; avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) at a 1:1 ratio, diluted 1:100 dilutions each in PBS for 30 minutes; 0.05% 3,3′-diaminobenzidine (DAB, Sigma Chemical, St. Louis, MO) in 200 ml 0.05M Tris buffer (pH 7.6) to which had been added 2 ml of 3% hydrogen peroxide and 1.0 ml of 8% NiCl2 solution for 10 minutes. Sections were counterstained with methylene blue, dehydrated in a graded series of alcohol concentrations, and then covered with coverslips.

**Immunohistochemistry of Frozen Sections**

Frozen sections approximately 6 μm in thickness were cut from fresh-frozen blocks of tissue and were mounted on glass slides. Since the fixation affects the sensitivity of immunostaining, we chose fixatives that demonstrated the strongest specific staining and reasonable background staining for each antibody. The following fixatives were used: ethyl ether for 9.6, OKT4a, and G10.1; acetone for 2H7; methanol for HAM56; and no fixation for HHF35. After appropriate fixation for 10 minutes and drying, the sections were immunostained by using the same procedures as described for the paraffin-embedded tissue with some modifications. For the frozen sections, we did not use NiCl2 discoloration; instead, a 1% solution of OsO4 was applied to the tissue sections after the DAB reaction to intensify the reaction product. The sections were counterstained with acid-fast red, dehydrated through a series of alcohol to xylenes, and covered with coverslips.

**Results**

**Control Animals**

Gross examination of the arterial segments from all four control animals demonstrated no visible abnormalities. Scanning electron microscopic observations revealed that the surfaces of the aortas of the control animals were flat and smooth, and were covered by a structurally intact endothelium with normal cell outlines similar to those described previously. There were occasional small focal areas that protruded into the lumen due to individual subendothelial foam cells beneath an intact endothelium. These foam cells were sporadically observed in focal areas in cross sections of arteries taken. 

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**Figure 1.** Schematic diagram for sampling of tissues. The aortic tree from each animal was divided into 18 segments, as shown. In animals studied by electron microscopy, each segment was further subdivided into two parts: a 2-mm section for light microscopy and transmission electron microscopy, and the remaining portion for scanning electron microscopy. The portion used for scanning electron microscopy was examined in its entirety either at very low or at increasing magnifications, so as to survey as much of the artery as possible. In animals studied by immunohistochemistry, two 2-mm sections were removed from each segment, and one section was embedded in OCT compound and frozen in liquid nitrogen. The other 2-mm section was immersion-fixed in methanol-Carnoy's fixative and subsequently embedded in paraffin.
from all four control animals. Their location had no apparent relation to anatomic level or to branches or bifurcations. The foam cells were lipid-laden macrophages, as determined by ultrastructural and immunohistochemical examination. The macrophages contained a ruffled cell surface, secondary lysosomes, and a characteristic distribution of nuclear chromatin and lacked a basement membrane. Staining with HAM56 also correlated with scattered positive monocyte-macrophages in the intima.

A few layers of intimal smooth muscle cells were found not only in cushions at branching sites, but also sporadically in non-branching areas similar to diffuse intimal thickenings in human arteries. The smooth muscle cells in these intimal thickenings rarely contained lipid droplets.

**Temporal Changes of Plasma Cholesterol and Triglyceride Levels**

Figure 2 shows the temporal changes in PC levels in the monkeys used in this study. After initiation of the atherogenic diet, PC levels progressively increased, and in most cases were maintained between 200 and 400 mg/dl, although some inevitable fluctuations and variations were observed. Some animals reached levels higher than we intended, probably due to variable intake of diet. This is probably one of the factors responsible for variations in the lesions described in this report. Temporal changes of PC levels in each animal are presented and discussed for each group.

Table 2 summarizes the temporal changes in the lipoprotein fractions of plasma cholesterol and triglycerides. The increase in plasma cholesterol was principally due to the increase in low density lipoprotein (LDL) cholesterol. Very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and high density lipoprotein 3 (HDL3) cholesterol also increased, whereas HDL2 cholesterol showed no significant changes. Values of plasma triglycerides and their lipoprotein fractions remained within the normal range.

**Types of Atherosclerotic Lesions in Animals on the Atherogenic Diet**

All eight hypercholserolemic animals contained lesions of atherosclerosis by gross examination and by light microscopic studies of paraffin-embedded and 1-μm plastic sections. The lesions are divisible into three major types.

**Fatty Streaks**

Fatty streaks were characterized by the presence of numerous foam cells that contained lipid vacuoles and round nuclei (Figures 3A, 3B, and 3C). The cells were usually round or oval, and their size varied due to the amount of lipid in their cytoplasm. The foam cells were macrophages as observed ultrastructurally and immunohistochemically. Spindle-shaped smooth muscle cells were also present in some of the fatty streaks, but were fewer in number and usually present beneath the layer of macrophages. When macrophages accumulated in multiple layers, lipid-laden smooth muscle cells were interspersed between the layers of macrophages (Figures 4A, 4B, and 4C). Such a lesion is interpreted to be a more advanced stage of a fatty streak.

**Fibrofatty Lesions (Transitional Lesions)**

The fibrofatty lesions consisted of approximately equal numbers of intimal lipid-laden macrophages and smooth muscle cells (Figures 5A, 5B, and 5C). The number of smooth muscle cells in the lesions was increased in comparison to those observed in the fatty streaks, and were present not only deep in the lesions, but in the surface layer near the lumen as well. They were usually present in regions with increased extracellular matrix, and sometimes appeared to form a layer of smooth muscle cells that might ultimately constitute a fibrous cap. However, the shoulder of this type of transitional lesion was generally composed of lipid-laden macrophages and closely resembled a fatty streak. In some cases, macrophages were located deep in the intima and were observed at sites occupied by necrotic cores later observed in fibrous plaques.

**Fibrous Plaques**

The fibrous plaques were characterized by a distinct, localized, fibrous-connective-tissue intimal thickening, consisting principally of smooth muscle cells surrounded by extracellular matrix (Figures 8A, 8B, and 8C in the accompanying article, page 183). An atheromatous core consisting of lipid-filled macrophages was usually present beneath the fibrous cap. However, lipid-filled macrophages did not form an atheromatous core in all of the fibrous plaques, and in some instances were scattered throughout the lesions, as indicated by immunostaining with HAM56 (Figures 12A, 12B, and 12C in the accompanying article, page 185). The fibrous plaque is described in greater detail in the accompanying article.
Table 2. Lipoprotein Fractions of Plasma Cholesterol and Triglyceride in Low Level Hypercholesterolemic Monkeys

<table>
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<tr>
<th>Time on diet</th>
<th>N</th>
<th>Total</th>
<th>VLDL</th>
<th>IDL</th>
<th>LDL</th>
<th>HDL₃</th>
<th>HDL₄</th>
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<tr>
<td>Cholesterol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pre</td>
<td>8</td>
<td>101.8±8.8</td>
<td>1.8±2.2</td>
<td>2.2±1.2</td>
<td>34.0±8.2</td>
<td>43.8±2.2</td>
<td>5.6±1.0</td>
</tr>
<tr>
<td>6 mo</td>
<td>8</td>
<td>317.8±110.9</td>
<td>13.5±21.1</td>
<td>46.6±55.8</td>
<td>176.8±65.6</td>
<td>47.5±22.8</td>
<td>16.5±3.2</td>
</tr>
<tr>
<td>1 yr</td>
<td>4</td>
<td>344.5±70.1</td>
<td>10.5±7.8</td>
<td>55.5±40.8</td>
<td>194.5±52.0</td>
<td>46.0±28.1</td>
<td>12.3±3.3</td>
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<td>Triglyceride</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>pre</td>
<td>8</td>
<td>32.2±18.5</td>
<td>6.4±7.5</td>
<td>0.0±0.0</td>
<td>3.6±4.4</td>
<td>11.2±3.5</td>
<td>1.0±0.9</td>
</tr>
<tr>
<td>6 mo</td>
<td>8</td>
<td>10.0±8.2</td>
<td>3.4±4.8</td>
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<tr>
<td>1 yr</td>
<td>4</td>
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<td>5.8±4.3</td>
<td>4.3±6.2</td>
<td>0.3±0.4</td>
</tr>
</tbody>
</table>

Values are given in mg/dl (means±SD).

Figure 3. Early stage of a fatty streak from the thoracic aorta of a monkey on the diet for 6 months. A. Hematoxylin and eosin (H&E) stain. B. Anti-macrophage antibody, HAM56. C. Anti-muscle antibody, HHF35. Avidin-biotin immunoperoxidase complex (ABC) method with nickel chloride modification in paraffin-embedded tissue sections counterstained with methyl green. One layer of round foam cells, HAM56-positive, is present beneath the endothelium. A few HHF35-positive cells (smooth muscle cells) are seen underneath the macrophages. ×120

Figure 4. Advanced stage of a fatty streak from the thoracic aorta of a monkey on the diet for 6 months. A. Hematoxylin and eosin (H&E) stain. B. Anti-macrophage antibody, HAM56. C. Anti-muscle antibody, HHF35. ABC method with nickel chloride modification in paraffin-embedded tissue sections counterstained with methyl green. HAM56-positive, lipid-filled macrophages accumulate in multiple layers, with some interspersed HHF35-positive smooth muscle cells. ×75

Cellular Changes and Lesions after 6 Months of Hypercholesterolemia

Cholesterol Levels

Four monkeys (Animals 1 to 4) were sacrificed after 6 months on the atherogenic diet. The temporal changes in PC observed in these four monkeys were individually quite different (Figure 2). The PC levels of Animals 1 and 3 gradually increased, whereas those of Animals 2 and 4 increased more rapidly and reached approximately 500 mg/dl at 4 months. They then decreased toward 400 mg/dl at the time of sacrifice as a result of decreasing the cholesterol content of their diet. Animals 2 and 4 were therefore exposed to more extensive hypercholesterolemia for a longer period of time than were Animals 1 and 3. As a consequence, the morphologic changes observed in these four monkeys were somewhat different and in each case appear to be related to the differences in their PC levels.

Types of Lesions and Distribution

Gross examination and low magnification scanning electron microscopic observations revealed that atherosclerotic lesions had a tendency to be localized around branch sites and bifurcations. In the aortic arch and
these lesions were present in the abdominal aorta and iliac arteries and were located at the branching sites. As noted above, Animals 2 and 4 had higher PC levels for a longer period of time. This factor is probably responsible for the differences in the lesions that were observed.

**Cellular Events**

Numerous leukocytes attached to endothelium covering irregularly shaped, nodular, elevated lesions were observed by scanning electron microscopy (Figure 6). Many of the adherent leukocytes were in the process of migrating on the surface of the endothelium, since they contained tail-like protrusions. Figure 7 is a composite scanning and transmission electron micrograph that demonstrates the entry of these leukocytes into the artery wall between endothelial junctions.

The irregularly shaped, nodular, elevated lesion that represents the early stage of fatty streak formation contained subendothelial monocyte-derived macrophages, many of which contained lipid and were foam cells, associated with an amorphous intimal insudate covered by thin, highly stretched endothelial cells (Figures 8A and 8B). Most of the foam cells were enlarged macrophages containing numerous lipid vacuoles. Immunohistochemistry with the monocyte-macrophage-specific monoclonal antibody, HAM56, confirmed this observation (Figure 3B). Relatively small numbers of lipid-free mononuclear cells were scattered among the foam cells. The morphology of most of these cells was consistent with that of lymphocytes (dense aggregated nuclear chromatin, a large nuclear/cytoplasmic ratio, and poorly developed intracytoplasmic organelles). Immunohistochemical staining of the corresponding lesions confirmed that T-lymphocytes were present in the fatty streaks (Figures 9A to 9D).

Lipid-laden smooth muscle cells were also observed in the fatty streaks but were usually located beneath the layer of macrophages (Figures 8B and 10). However, in the advanced stages of fatty streak formation that were observed in Animal 2, lipid-laden smooth muscle cells were interspersed between macrophages (Figure 10). The lipid droplets in the smooth muscle cells had somewhat different characteristics from those present in the

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**Figure 5.** Fibrofatty lesion (transitional lesion) from an iliac artery of a monkey on the diet for 6 months. A. Hematoxylin and eosin (H&E) stain. B. Anti-macrophage antibody, HAM56. C. Antimuscle antibody, HHF35. ABC method with nickel chloride modification of paraffin-embedded tissue sections counterstained with methyl green. HAM56-positive, macrophage-derived foam cells are scattered in the many smooth muscle cells, which appear to form a layer that may ultimately constitute a fibrous cap. x48

**Figure 6.** Scanning electron micrograph from a nonbranching region of the thoracic aorta in a monkey on the diet for 6 months. Many small irregularly shaped elevated lesions (fatty streaks) and scattered adherent leukocytes are seen on the intact endothelial surface. x260

In the thoracic aorta, the lesions were observed both in the areas near the branches as well as in non-branching areas (Figure 6). On the other hand, the lesions in the abdominal aorta and iliac arteries were often localized near branches, where the lesions had a V-shaped distribution involving the lateral leading edges and distal area of the flow divider.

Light microscopic examination of both paraffin- and plastic-embedded sections revealed that fatty streaks were the most prevalent lesions at 6 months and were present at most levels of the arterial tree in all four animals. Fatty streaks in Animals 1 and 3 appeared to be in the early stages of formation since they consisted of largely a single layer of lipid-filled macrophages beneath the endothelium (Figures 3A, 3B, and 3C). On the other hand, the lesions in Animals 2 and 4 displayed a histology consistent with an advanced stage of fatty streak formation; they consisted of multiple layers of lipid-filled macrophages together with small numbers of intervening smooth muscle cells (Figures 4A, 4B, and 4C).

Fibrofatty lesions were observed sporadically in all of the 6-month animals, whereas early-stage fibrous plaques were found only in Animals 2 and 4. Most of
macrophages. They were usually absent in the periphery of the smooth muscle cytoplasm and contained material not extracted by the ethanol dehydration process (Figure 10). The extracellular matrix between the smooth muscle cells was increased in amount and contained numerous small vesicular structures (Figure 11), similar to those previously described by Simionescu et al. as extracellular liposomes, together with newly formed elastin and collagen. Similar changes were also observed in the underlying media.

Although most of the surface area of the fatty streaks was covered by endothelium, small foci of disrupted endothelial cells that exposed large round foam cells, presumably macrophages, were observed. Such observations were made with increasing frequency in animals that had been hypercholesterolemic for longer periods of time, as described below.
Figure 9. Immunohistochemical staining of an early fatty streak, serial frozen sections from thoracic aorta after 6 months on the diet. 
ABC method with OsO4 intensification, counterstained with nuclear fast red. ×150

One Year of Hypercholesterolemia

Cholesterol Levels

Four animals (Animals 5 to 8) were sacrificed after 1 year on the atherogenic diet. Their PC levels were relatively well maintained between 200 and 400 mg/dl during the entire period (Figure 2). The PC levels of Animal 5 were consistently lower than those of the other animals, and never exceeded 280 mg/dl. The PC values of the other three animals reached 400 mg/dl, either by the time of sacrifice or within 2 months before sacrifice. Such differences in PC elevation are consistent with the extent and severity observed in the respective lesions of atherosclerosis.

Types of Lesions and Distribution

Gross and scanning electron microscopic observations of the surface of the aorta showed that the lesions were distributed largely at branches and bifurcations. Light microscopic examination of paraffin-embedded sections and of 1-μm sections revealed fatty streaks at almost every level of the arterial tree of all four animals. Fatty streaks were the predominant lesion in all of the animals on the diet for 1 year. In Animals 6 and 8, fibrofatty lesions and early stages of fibrous plaques were localized near branching sites of the thoracic and abdominal aorta, as well as the iliac arteries.

Cellular Events

Scanning electron microscopic examination revealed that the surfaces of the fatty streaks contained tremendous irregularities associated with marked disruption of the endothelial cells, resulting in exposure of the underlying lipid-laden macrophages (Figures 12A and 12B). Figure 12A shows disruption of the exceedingly thin endothelial cells that appeared to be stretched over large lipid-filled macrophages. The cytoplasm of the endothelial cells was dense, suggesting possible cell damage. In each of the monkeys, platelet microthrombi were frequently observed attached to the surface of the exposed macrophages (Figures 13A and 13B). Attachment and entry of leukocytes on the endothelial surface were also frequently seen on the surface of such fatty streaks (Figures 12A and 13A). Histologically, the intima of such advanced fatty streaks was thickened to a greater extent than was observed in the monkeys on the diet for 6 months and consisted of multiple layers of large lipid-
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Figure 10. A transmission electron micrograph of an advanced fatty streak, thoracic aorta at 6 months. This fatty streak contains multiple layers of subendothelial macrophage-derived foam cells with intervening lipid-laden smooth muscle cells. Note the difference in density of lipid droplets in the macrophages and the smooth muscle cells. They appear to be less extracted in the smooth muscle cells. x2200

filled macrophages together with small numbers of intervening lipid-laden smooth muscle cells. Lipid-free mononuclear cells, presumably lymphocytes, as well as some macrophages, were also intermingled in the lesions. Fibrofatty lesions and fibrous plaques were present at branching sites in Animals 6 and 8. When examined by scanning electron microscopy, the surface morphology of these lesions showed marked elevations of the endothelial surface; however, the irregularities of the endothelial surface were decreased compared with that of the fatty streaks. Cross sections of such regions revealed that a thin fibrous cap, consisting of a few layers of smooth muscle cells, had begun to form. In contrast, the shoulder regions of these lesions were highly irregular and contained disrupted endothelium associated with exposure of macrophages and numerous attached platelet thrombi.

Lymphocytes

All of the lesions that formed after 6 months and after 12 months on the diet contained T-lymphocytes. In general, they were distributed at loci similar to those containing macrophages (Figure 10). By using monoclonal antibodies able to discriminate between subsets of T-cells, the cells were found to be principally CD8-positive intermixed with relatively small numbers of CD4-positive cells. Few, if any, B cells were found anywhere within the lesions.

In general, most of the cells in each of the lesions examined appeared to be accounted for by the different antibodies used for immunohistochemical examination.

Discussion

Acute High Level Hypercholesterolemia Versus Chronic Low Level Hypercholesterolemia

One of the principal purposes of this study was to determine whether the cellular events and interactions, the cellular content of the lesions, and the types of lesions that form during relatively low level hypercholesterolemia are similar to, or different from, those that form during high level hypercholesterolemia. Since the principal relevancy of high level hypercholesterolemia to human disease appears to be associated with lesions that form in familial homozygous hypercholesterolemia, we were concerned that perhaps the cellular interactions and content of the lesions observed in high level hypercholesterolemia might not necessarily be relevant to the changes that occur in most humans. In most instances, humans have much lower plasma cholesterol levels, and it is assumed, although not proven, that most lesions form slowly and silently with time. Therefore we undertook these longer-term, relatively low level hypercholesterolemic studies in the nonhuman primate. They have clearly demonstrated that the cellular events that take place during the initiation, formation, and progression of fatty streaks are virtually identical to those observed during very high levels of hypercholesterolemia in nonhuman primates,1-2 swine,14-15 and rabbits.16-17

Cellular Events during Fatty Streak Formation

Adherence of Monocytes and Lymphocytes

The chronologic sequence of events that is observed during the early phase of fatty streak formation represents a special type of inflammatory response, it includes monocyte and lymphocyte adherence to endothelium and subendothelial penetration and migration of these leukocytes, resulting in fatty streaks that contain subendothelial lipid-laden macrophages intermingled with small numbers of non-lipid-filled macrophages and T-lymphocytes. Similar studies performed in swine,14-15 rabbits,16-17 rats,22 pigeons,23-24 and high level hypercholesterolemic nonhuman primates1-2 also showed an increase in the adherence of leukocytes to the endothelium and subendothelial migration followed by formation of a single layer of intimal macrophage-derived foam cells. The time required for this to occur is dependent upon the level of hypercholesterolemia. The observations in these studies are similar to recent observations showing leukocyte adherence in human coronary arteries in hearts removed for transplantation, suggesting that this is an ongoing process.40 Thus it seems probable that the same or highly similar cellular events occur during atherogenesis in humans.30

The molecular mechanisms of leukocyte adherence to endothelium and their migration between endothelial
cells into the subendothelial spaces are under intense investigation. Several recent studies have demonstrated that hypercholesterolemia induces chemotactic factor(s) for monocytes in lesion-prone areas of swine and pigeons. Berliner et al. showed that cultured endothelial cells produce a factor chemotactic for monocytes but not neutrophils, and that oxidized LDL and \( \beta \)-VLDL enhance this chemotactic activity. The nature of these factors has not yet been characterized. Other substances that can be produced by endothelial cells, smooth muscle cells, macrophages, and platelets, for example, platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-\( \beta \)) are also chemotactic for monocytes. Involvement of such factors in monocyte-endothelial adhesion and in subsequent subendothelial penetration of monocytes during atherogenesis needs further study, including cell-surface molecules such as the CD11/CD18 complex, the endothelial-leukocyte adhesion molecule-1 (ELAM-1), and the phorbol myristate acetate (PMA)-dependent monocyte receptor (Carlos et al., unpublished data). The capacity to control these events may permit the development of approaches to modify the inflammatory response that is clearly a part of the early phases of atherogenesis.

**Intimal Lipid Accumulation**

Simionescu et al. described extracellular cholesterol-rich liposomes in the arterial intima before the entrance of leukocytes into the intima in hyperlipidemic rabbits. They demonstrated by immunohistochemical and cytotoxic methods that apolipoprotein B and non-esterified cholesterol are present in these structures. We have observed similar extracellular liposome-like structures in the arterial intima of the monkeys after 6 months on the diet. Leukocyte adherence and fatty streak formation were also observed at this same time; however, we do not know whether these liposome-like structures occur before cell adhesion and entry or at the same time. If lipid permeation and deposition occur before adherence and entry of leukocytes, then these lipids or their breakdown products may themselves be responsible or may play a role in inducing arterial intimal cells to form chemoattractants for monocytes.

**Intimal Localization of Monocyte-Macrophages and T-Cells**

Both macrophages and T-lymphocytes can be demonstrated in the fatty streaks by immunohistochemistry with specific monoclonal antibodies. The majority of the T-lymphocytes were CD8-positive, suggesting that they may be either cytotoxic or suppressor T-cells. Smaller numbers of CD4-positive cells suggest that helper T-cells or inducer T-cells are also present. The lymphocytes were not observed to contain lipid droplets in their cytoplasm. Since both T-cells and macrophages are present during the early stages of fatty streak formation, it follows that they may form substances such as gamma-interferon and interleukins that may participate in some sort of inflammatory or immune response in the pathogenesis of the disease process. To demonstrate the presence of T-lymphocytes in nonhuman primates, it was necessary to use frozen sections because presently...
Figure 12. A. The surface of an advanced stage of fatty streak showing numerous irregularities associated with many disruptions of endothelial cells and exposure of macrophages. Attached leukocytes are also present. Thoracic aorta at 1 year. x750 B. A transmission electron micrograph demonstrating lipid-filled macrophages exposed to the lumen. Note the increased density of the cytoplasm of the endothelial cells showing disjunction. Thoracic aorta, 1 year. x7300

available monoclonal antibodies specific for T-cells and/or their subsets cannot recognize these cells in paraffin-embedded sections. These cells and their roles in the process of atherogenesis need to be further examined after additional reagents become available.

Smooth Muscle Accumulation

Smooth muscle cells are present in varying numbers in the fatty streaks. Although many of them migrate from the underlying media, some of them may be derived from pre-existing intimal smooth muscle cells, since scattered smooth muscle cells are present in the normal intima and are also found in larger numbers in intimal cushions that may occur at branch sites. These intimal smooth muscle cells may be one of the first targets of growth regulatory molecules released from macrophages and T-lymphocytes and of plasma proteins transported by the endothelial cells. Since adherence and subendothelial penetration of monocytes and lymphocytes frequently occur around branch sites, this may explain why pre-existing intimal smooth muscle cells are present in many fatty streaks and how they become involved in the further development of fibrous plaques.

Progression of the Fatty Streaks

As the fatty streaks progress, the intima increases in thickness and multiple layers form—layers consisting of lipid-filled macrophages together with small numbers of scattered lymphocytes alternating with lipid-laden smooth muscle cells and interspersed between the layers of macrophages. When viewed with the scanning elec-
tron microscope, the surface of the fatty streak has an irregular, nodular appearance with deep crevices between the nodules. Adherent leukocytes continue to be found on the endothelial cells covering the fatty streaks, which often contain focal areas of disruption of endothelial junctions with resultant exposure of the underlying lipid-filled macrophages to the circulation. These changes, especially disruption of the endothelial lining and exposure of macrophages, have been observed in hypercholesterolemic swine, rabbits, and nonhuman primates. Davies et al. found exactly the same events, including platelet adherence and microthrombi, in perfuse-fixed coronary arteries of human hearts removed for cardiac transplantation. The factors responsible for endothelial retraction and junction disassembly between the macrophages are unknown. Mechanical pressure and/or chemically mediated insult from the underlying macrophages could be responsible for this phenomenon. The stretched, amazingly thin endothelial cells, which sometimes show evidence of cell damage, suggest that endothelial insult does in fact occur. The role of these exposed macrophages and the microthrombi formed by the accumulated adherent platelets in inducing subsequent fibrinous plaque formation will be discussed in the following article. Platelet adherence and aggregation are phenomena commonly observed on exposed macrophages in all of the animals in this study. The factors responsible for the adherence of platelets on the macrophages are not understood; however, several possibilities have been proposed, including tissue factor recently observed in lesions associated with macrophages.

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