We investigated the influence of repeated pregnancy on diet-induced atherosclerosis in cynomolgus monkeys and sought to determine if circulating endogenous reproductive steroid levels were associated with the extent of coronary artery atherosclerosis. At necropsy, females which were pregnant one or more times were found to have coronary artery atherosclerosis which was one-fourth as extensive as that of intact females which had not been pregnant. Extent of coronary artery atherosclerosis correlated positively with mean total plasma cholesterol (Rho = 0.52, p < 0.01) and inversely with high density lipoprotein (HDL) cholesterol (Rho = -0.48, p < 0.01) concentrations; both decreased during pregnancy. Additionally, the extent of coronary artery atherosclerosis was found to have a strong inverse association (Rho = -0.66, p < 0.001) with an index (area-under-the-curve) of magnitude and duration of the pregnancy-induced elevation in plasma 17-B estradiol concentration. This association could not be explained by an interrelationship between estradiol area-under-the-curve and either plasma total or HDL cholesterol concentrations. There was no relationship between atherosclerosis extent and a similar index of plasma progesterone concentrations. These findings provide evidence for an inhibitory effect of endogenous estrogen on the progression of coronary artery atherosclerosis.

(Arteriosclerosis 7:378-384, July/August 1987)

It is well established that premenopausal white women are at low risk of coronary heart disease relative to white men of similar ages and, although direct evidence is lacking, it is widely believed that ovarian estrogen is responsible. However, it remains unproven whether coronary heart disease risk in women is influenced by conditions that influence endogenous estrogen levels. Studies of coronary heart disease risk in menopause, an estrogen deficiency state, and pregnancy, a hyperestrogenic state, have produced contradictory results.

With one exception, investigators addressing the relationship between pregnancy and atherosclerosis have examined only the association between number of pregnancies and incidence of coronary heart disease. The results are contradictory. In two studies it was found that there was an excess of pregnancies in women dying of coronary heart disease or in women with clinical evidence of coronary heart disease (angina pectoris or electrocardiographic changes suggestive of coronary heart disease). The results of three studies provided no evidence for an association between coronary heart disease risk and the number of pregnancies, and a fourth found no difference in the extent of coronary artery atherosclerosis as determined at autopsy in women dying while pregnant compared with nonpregnant women dying accidentally.

In a recent study by Beard et al., women whose first pregnancy was before the age of 25 were found to be at greater risk of coronary heart disease. The absolute numbers of pregnancies or births did not seem to be associated with coronary risk. These authors suggest that the observed association between age at first pregnancy and coronary risk may be explained by a confounding effect of psychosocial variables. Age at first pregnancy is highly correlated with level of education, and evidence exists that low socioeconomic status and low educational level are associated with increased risk of coronary heart disease. Psychosocial variables may also be confounding influences in those studies which found an association between the number of pregnancies and the risk of coronary heart disease. Socioeconomic status and level of education are examples of numerous variables that have the potential for confounding the results of epidemiologic studies of reproductive influences on coronary heart disease. In the absence of data collected prospectively, it remains unknown whether pregnancy influences the atherosclerotic process or risk of coronary heart disease in human beings.

For this reason we have studied the reproductive influences on atherosclerosis in a nonhuman primate model, the cynomolgus macaque. We have reported previously that female cynomolgus macaques, like premenopausal white women, are relatively protected against coronary artery atherosclerosis. Further, ovariectomy results in...
a loss of this "female protection," with an approximate doubling in extent of coronary artery atherosclerosis.\textsuperscript{27} We have also reported that the relative estrogen deficiency that often accompanies low social status in female monkeys is associated with a loss of "female protection."\textsuperscript{27}

In this report we describe the results of a study designed to determine the influence of repeated pregnancy on the extent of diet-induced atherosclerosis and to determine if the persistent hyperestrogenism of pregnancy is associated with inhibition of coronary artery atherosclerosis.

**Methods**

**Animals**

We studied 52 adult female cynomolgus macaques imported from Malaysia. For 30 months they were fed a diet designed to mimic the nutritional composition of the average North American diet (40% of calories as fat and 0.4 mg of cholesterol per Calorie).\textsuperscript{27} Twenty-nine females (Group 1) lived in social groups consisting of one male and four to six females. Twenty-seven were pregnant once or more (mean number of pregnancies = 2.4 ± 0.6). Twenty-three females in a second experimental group (Group 2) lived in similar social groups with vasectomized males and, thus, did not become pregnant. During the study, the social groups were housed separately in identical pens measuring 2.0 x 3.2 x 2.5 m. All procedures involving animals were conducted in compliance with state and federal laws, standards of the Department of Health and Human Services, and guidelines established by the institutional Animal Care and Use Committee.

**Risk Variables**

We studied several atherosclerosis risk variables. Total plasma cholesterol\textsuperscript{28} and plasma high density lipoprotein (HDL) cholesterol\textsuperscript{29} concentrations were determined at 7- to 15-day intervals during pregnancy and at 4- to 8-week intervals at other times.

Blood pressure\textsuperscript{30} was determined at 6-month intervals. Since pregnancy results in an altered carbohydrate metabolism, we determined the fasting blood glucose\textsuperscript{31} and insulin\textsuperscript{32} concentrations, and the glucose and insulin responses to an intravenous glucose challenge\textsuperscript{32} during each pregnancy. For each monkey, the values for plasma total and HDL cholesterol concentrations were plotted against time and an area-under-the-curve (AUC) (cm\textsuperscript{2}) was determined using a digitizer (Zeiss MOP III Image Analyzer, Thornwood, New York). These values were then compared to the overall means of plasma total and HDL cholesterol concentrations as predictors of atherosclerosis extent.

**Reproductive Steroids**

Since we were particularly interested in investigating the potential relationships between endogenous reproductive steroids and the extent of atherosclerosis in pregnant females, plasma 17-β estradiol and progesterone concentrations were determined at 7- to 15-day intervals during pregnancy and at 2- to 8-week intervals at other times.

For these determinations, plasma was separated by centrifugation and was frozen at -18° C until assayed. Progesterone and estradiol were extracted with diethyl ether and assayed without chromatography using triated steroids in a radioimmunoassay procedure. The progestosterone and estradiol assays utilized antisera which have been characterized previously for use in the macaque.\textsuperscript{33, 34}

We anticipated wide individual variation in the number of pregnancies, the duration of pregnancy, and the magnitude of gestational increases in plasma estradiol and progesterone concentrations. For this reason, plasma estradiol and progesterone were plotted against time and the AUC was determined using the digitizer. The AUCs were then used as indices of estradiol and progesterone "exposure" of each individual over the course of the experiment.

**Necropsy and Measurement of Atherosclerosis**

At the time of necropsy, the animals were anesthetized deeply with pentobarbital. The cardiovascular system was flushed with normal saline and was perfused with 10% neutral buffered formalin under a pressure of 100 mm Hg. After pressure fixation, five serial blocks were taken from each of the left circumflex, left anterior descending, and right coronary arteries. One section from each block was stained with Verhoeff-Van Geison stain. These sections were projected and the area occupied by intima and intimal lesion was measured with a digitizer. The extent of coronary artery atherosclerosis was expressed as the median intimal (plaque) area of the 15 sections of coronary artery. The carotid arteries and the aorta were opened longitudinally and were immersion-fixed in 10% buffered formalin. These segments were then stained with Sudan IV and isopropanol. For the thoracic and abdominal segments of the aorta (thoracic: 10 cm beginning at a point just distal to the aortic arch; abdominal: 10 cm beginning just distal to the branch of the celiac artery), gross evaluation by three investigators provided estimates of the total surface area affected with plaques. Next, five representative plaque cross sections were taken from each of the two aortic segments; plaque thickness (in millimeters) was measured in these microscopic slides by using the digitizer. The plaque volume was computed (in millimeters\textsuperscript{3}) as the total surface area covered with plaque multiplied by the mean intimal thickness of the plaque sections. At the carotid bifurcation, one standard cross section was taken for microscopic evaluation; in taking this section, bifurcation pads ("intimal cushions") were avoided. The lesion areas and thicknesses were measured (in millimeters\textsuperscript{2} and millimeters, respectively) with the digitizer. Five serial sections were taken from each common carotid and iliac-femoral artery and median intimal area (in millimeters\textsuperscript{2}) was determined for each by using the digitizer.

**Statistical Analysis**

Atherosclerosis extent data were not normally distributed. Therefore, the between-group comparisons were made using the Mann-Whitney U test\textsuperscript{35} and the associations between the risk variables and the extent of atherosclerosis were determined using Spearman's rank order correlation\textsuperscript{36} and Kendall partial rank correlation coeffi-
Results

Pregnancy Effects on Plasma Estradiol, Progesterone, Total Cholesterol, and HDL Cholesterol

As expected there was wide individual variation in the magnitude of gestational increases in plasma estradiol (200 to 1,000 pg/ml) and progesterone (2 to 20 ng/ml) concentrations. The characteristic changes in plasma sex steroid and lipid concentrations are represented in Figures 1 and 2. Figure 1 represents an individual (#841) with a relatively large gestational increase in plasma estradiol concentration. Figure 2 represents an individual (#546) with a relatively small gestational plasma estradiol increase. Both show similar biphasic gestational increases in plasma progesterone concentration and gradual, sustained decreases in both total plasma cholesterol and HDL cholesterol concentrations. Across all pregnancies, total plasma cholesterol levels decreased from a mean of 276 ± 67 mg/dl in the nonpregnant state to a mean of 239 ± 63 mg/dl during pregnancy and a mean of 199 ± 82 mg/dl at delivery. The plasma HDL cholesterol concentration decreased from a mean of 44 ± 13 mg/dl to a mean of 22 ± 5 mg/dl during pregnancy and a mean of 20 ± 8 mg/dl at delivery.

Effects of Pregnancy on Atherosclerosis, Total Plasma Cholesterol, and Plasma HDL Cholesterol

The differences between the two experimental groups in plasma lipids and the extent of coronary artery atherosclerosis are summarized in Table 1. Coronary artery atherosclerosis was one-fourth as extensive in Group 1 females (one or more pregnancies) (p<0.04, Mann-Whitney U test). The plasma total and HDL cholesterol concentrations are also summarized in Table 1. These values represent the mean of the means of all values for each animal regardless of the reproductive status at the time of sampling. The mean values calculated in this way correlated strongly with the AUCs determined for total plasma cholesterol (r=0.99) and HDL cholesterol (r=0.96). The mean values were arbitrarily chosen for use in subsequent analyses. Gestational decreases in total plasma cholesterol and HDL cholesterol resulted in lower overall means for both (Table 1). The fact that both were decreased resulted in there being no difference between the groups in the ratio of total plasma cholesterol to HDL cholesterol concentration (Table 1).

There were no differences between groups in the extent of atherosclerosis in carotid arteries, femoral arteries, or thoracic aorta. Group 1 females had less extensive atherosclerosis of the abdominal aorta (median plaque vol-
Table 1. Plasma Lipids and Coronary Artery Atherosclerosis in Pregnant and Nonpregnant Female Monkeys

<table>
<thead>
<tr>
<th>Monkey group</th>
<th>Coronary artery intimal area (mm²)</th>
<th>Total plasma cholesterol† (mg/dl)</th>
<th>Plasma HDL cholesterol† (mg/dl)</th>
<th>Plasma total/ HDL cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.009</td>
<td>260 ± 12</td>
<td>36 ± 2.0</td>
<td>7.2</td>
</tr>
<tr>
<td>(n = 27)†‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>0.036</td>
<td>312 ± 16</td>
<td>42 ± 2.8</td>
<td>7.4</td>
</tr>
<tr>
<td>(n = 23)§‡</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p&lt;</td>
<td>0.04</td>
<td>0.01</td>
<td>0.05</td>
<td>NS</td>
</tr>
</tbody>
</table>

*Mean ± SEM; these values represent the mean of all observations, regardless of reproductive status; compared using Student’s t test.
†Group 1 animals had one or more pregnancies.
‡Group 2 animals were not pregnant.
§Group 2 animals were not pregnant.

Blood Pressure, Carbohydrate Tolerance

A comparison of mean values for systolic and diastolic blood pressure determined at 6-month intervals revealed no differences between the two groups (Group 1: systolic blood pressure = 98 ± 10 mm Hg, diastolic blood pressure = 55 ± 7 mm Hg; Group 2: systolic blood pressure = 97 ± 14 mm Hg, diastolic blood pressure = 58 ± 10 mm Hg; p > 0.2, NS). These values represent the means of all recorded blood pressures (irrespective of reproductive status). Gestational influences on blood pressure were not assessed prospectively. However, a retrospective comparison of blood pressures recorded while animals were pregnant with those recorded when they were not pregnant revealed a lower systolic blood pressure (92 ± 16 mm Hg vs. 100 ± 11 mm Hg, p = 0.058, Student’s t test) associated with pregnancy. There was no difference in diastolic blood pressure (55 ± 14 mm Hg vs. 55 ± 7 mm Hg).

Gestational influences on carbohydrate tolerance in these monkeys have been reported previously.32 In brief, pregnancy resulted in a mean decrease of 17% in the plasma insulin response to a glucose intravenous glucose challenge (Rho = -0.48, p < 0.01), ratio of total plasma to HDL cholesterol concentration (Rho = -0.55, p < 0.01), ratio of total plasma to HDL cholesterol concentration (Rho = 0.39, p < 0.05), total plasma cholesterol concentration (Rho = -0.52, p < 0.01), ratio of total plasma to HDL cholesterol concentration (Rho = 0.55, p < 0.01), HDL cholesterol concentration (Rho = -0.48, p < 0.01), and the plasma insulin response to intravenous glucose challenge (Rho = -0.48, p < 0.01).

Risk Variable—Atherosclerosis Relationships

Among Group 1 females, estradiol AUC was the single variable that correlated most strongly with the coronary artery atherosclerosis extent (Rho = -0.66, p < 0.001). Other variables that correlated with the extent of coronary artery atherosclerosis were peak plasma estradiol concentration (an indicator of the magnitude of the gestational plasma estradiol increase) (Rho = -0.39, p < 0.05), total plasma cholesterol concentration (Rho = -0.52, p < 0.01), ratio of total plasma to HDL cholesterol concentration (Rho = -0.55, p < 0.01), HDL cholesterol concentration (Rho = -0.48, p < 0.01), and the plasma insulin response to intravenous glucose challenge (Rho = -0.48, p < 0.01).

Variables that did not correlate with the extent of coronary artery atherosclerosis were duration of pregnancy (total number of days), peak plasma progesterone concentration, plasma progesterone AUC, systolic blood pressure, diastolic blood pressure, and carbohydrate tolerance (fractional rate of glucose clearance).

Sex Hormone—Plasma Lipid—Atherosclerosis Interrelationships

Relationships between plasma lipid concentrations and atherosclerosis are summarized in Figures 4 and 5 with animals characterized as having high (greater than 90 cm²) or low (less than 90 cm²) estradiol AUC. Although extent of atherosclerosis was associated with total plasma cholesterol (Rho = 0.52, p < 0.01) (Figure 4) and plasma HDL cholesterol (Rho = -0.48, p < 0.01) (Figure 5) it is also apparent that with one exception, animals with high estradiol AUC were virtually free of coronary artery atherosclerosis regardless of total plasma cholesterol or HDL cholesterol concentration. The single exception was an individual with the highest mean total plasma cholesterol plasma estradiol concentrations of 407 pg/ml. Coronary artery atherosclerosis was much more extensive in these individuals (median = 0.049 mm², p < 0.01, Mann-Whitney U test) and was not different than atherosclerosis extent among Group 2 (nonpregnant) females (median = 0.036 mm², p > 0.2, Mann-Whitney U test).

Sex Hormone—Atherosclerosis Relationships

The relationship between endogenous plasma 17-β estradiol AUC and the extent of coronary artery atherosclerosis in Group 1 females is summarized in Figure 3. The coronary artery intimal area was inversely associated with plasma 17-β estradiol AUC (Rho = -0.66, p < 0.001). Females with AUCs greater than 90 cm² had mean peak plasma 17-β estradiol of 657 pg/ml and were pregnant an average of 12 of the 30 months of the experiment. With one exception, these females were virtually unaffected with coronary artery atherosclerosis (median = 0.001 mm²). In contrast, females with AUCs less than 90 cm² were pregnant an average of only 8 months and had mean peak
Figure 4. Relation between total plasma cholesterol concentration and extent of coronary artery atherosclerosis (intimal area) with monkeys classified as having high (>90 cm²) or low (<90 cm²) area-under-the-curve (AUC) of plasma 17-β estradiol concentration.

(365 mg/dl) and the lowest mean plasma HDL cholesterol (16 mg/dl) concentrations, and, therefore, by far the highest total plasma cholesterol/HDL cholesterol ratio (23.1); this value was more than three times greater than the group mean (7.2) and nearly double the next highest value (12.0).

There was no association between estradiol AUC and either total plasma cholesterol (Rho = -0.26, NS) or plasma HDL cholesterol (Rho = -0.32, NS). This further indicates that the association between estradiol AUC and the extent of coronary artery atherosclerosis cannot be accounted for by an interrelationship with total plasma cholesterol or HDL cholesterol concentrations. The Kendall partial rank correlation was used to eliminate statistically the effects of variation in total plasma cholesterol and HDL cholesterol concentrations on the relationship between estradiol AUC and extent of coronary artery atherosclerosis. The result is a negligible influence on the strength of this association (Tau = -0.47, p<0.002) by either total plasma cholesterol (Tau [partial] = -0.43, p<0.007) and HDL cholesterol (Tau [partial] = -0.42, p = 0.007).

Figure 5. Relation between plasma HDL cholesterol concentration and extent of coronary artery atherosclerosis (intimal area) with monkeys classified as having high (>90 cm²) or low (<90 cm²) area-under-the-curve (AUC) of plasma 17-β estradiol concentration.

Discussion

As we have reported previously, ovariectomy, which results in a persistent estrogen deficiency, also results in an approximate doubling of the extent of coronary artery atherosclerosis. In contrast, the results of the present study indicate that the effect of repeated pregnancy, a persistent hyperestrogenic state, is a reduction in the extent of coronary artery atherosclerosis to one-fourth that of intact, nonpregnant female monkeys. These findings are suggestive of an inhibitory effect of endogenous estrogen on the development of atherosclerosis. Stronger evidence for the existence of such a relationship is found in the observation that plasma 17-β estradiol concentration AUC, an index of magnitude and duration of the gestational elevation in plasma estradiol concentration, was inversely correlated with the extent of coronary artery atherosclerosis and was the single variable that correlated most strongly with the extent of coronary artery atherosclerosis. Indices of only magnitude (peak plasma estradiol concentration) or duration (number of days pregnant) correlated weakly or not at all.

Also, although pregnancy resulted in decreased total plasma cholesterol and HDL cholesterol concentrations, the relationship between estradiol AUC and the extent of atherosclerosis appears to be independent of pregnancy-associated changes in plasma lipid concentrations. The ratio of total plasma cholesterol to HDL cholesterol was, in this experiment and in other experiments involving monkeys and human beings, a stronger predictor of the extent of coronary artery atherosclerosis or coronary heart disease risk than either measure alone. In this experiment, there was no difference between the two treatment groups in the ratio of total plasma cholesterol to HDL cholesterol. Further there was no association between estradiol AUC and either total plasma cholesterol or HDL cholesterol concentrations. Also, as shown in Figures 4 and 5, there seems to be no relationship between either total plasma cholesterol or HDL cholesterol concentrations and the extent of coronary artery atherosclerosis among animals in the upper half of the distribution of estradiol AUC. Finally, the Kendall partial rank correlation, used to eliminate statistically the effects of variation in total plasma cholesterol and HDL cholesterol concentrations on the relationship between estradiol AUC and extent of coronary artery atherosclerosis, indicated a negligible influence of either.

It is important to note that the current study did not include lipoprotein fractionation or an assessment of compositional heterogeneity within lipoprotein fractions. A more complete evaluation of lipoprotein distribution and composition may have accounted for the unexplained variation in atherosclerosis extent associated with pregnancy. Yet, the evidence described here for an independent influence of endogenous estrogen on the atherosclerotic process perhaps is not surprising in light of a previous study of gender differences in the extent of atherosclerosis which also addressed gender differences in the distribution and chemical composition of the major lipoprotein classes. Multi-variate analyses of these data revealed that variation in plasma lipoprotein concentration or composition accounted statistically for no more than one-half of the observed variation in the extent of coronary artery atherosclerosis.
Furthermore, despite numerous studies of human populations, it remains uncertain whether "female protection" can be explained entirely by gender differences in plasma lipoprotein patterns or other known risk variables. It also remains uncertain whether estrogen treatment of postmenopausal women influences the atherosclerotic process or coronary heart disease risk. This has been the subject of a large number of prospective epidemiologic studies. Although the weight of the evidence seems to favor the existence of a beneficial effect of estrogen treatment, there is one exception. However, in that study it was acknowledged that the increased coronary heart disease risk associated with postmenopausal estrogen use was statistically significant only among cigarette smokers. Also, this study included an adjustment for variation in high density lipoprotein cholesterol. This seems unwarranted in the light of evidence that beneficial influences on plasma HDL may play a role in a protective effect of estrogen treatment. Thus, the evidence published from prospective studies seems to favor heavily a beneficial effect of postmenopausal estrogen use on coronary risk, at least in nonsmokers.

Since it is uncertain whether gender differences in coronary heart disease risk or a protective effect of endogenous estrogen can be explained by variation in known risk factors, it remains possible that they may be mediated, at least in part, either through risk factors that have not yet been described or through a direct influence on cellular or molecular events occurring in the arterial intima. Estrogen and progesterone receptors have been found in arterial endothelial or smooth muscle cells of several mammalian species. A recent study has shown that treatment of ovariectomized baboons with 17-estradiol results in a redistribution of arterial intracellular estrogen receptors from the cytoplasmic fraction to the nuclear fraction and an increase in the cytoplasmic concentration of progesterone receptors. These findings imply a role for sex steroids in the regulation of arterial cell function. Other animal studies have shown that estrogen treatment results in reductions in lipoprotein-induced arterial smooth muscle cell proliferation, inhibition of the myointimal proliferation associated with mechanical endothelial injury, reduced arterial cholesterol ester influx and hydrolysis, inhibition of platelet aggregation, decreased collagen and elastin production, increased collagen and elastin degradation, and increased prostacyclin production by arterial smooth muscle cells. These studies provide further evidence that vascular estrogen receptors are physiologically functional and that elevations in circulating levels or potencies of endogenous or exogenous estrogen may inhibit atherogenesis by inhibiting foam cell formation, platelet aggregation, smooth muscle cell proliferation, and the accumulation of collagen and elastin.

The findings of this study indicate that repeated pregnancy is associated with a marked reduction in the extent of diet-induced coronary artery atherosclerosis and provides evidence that endogenous estrogen has an inhibitory effect on the atherosclerotic process. It is important to note that the pregnancy-associated inhibition of atherosclerosis was limited to the coronary arteries and abdominal aorta. In contrast, ovariectomy resulted in the exacerbation of coronary, carotid, and femoral artery atherosclerosis, but not aortic atherosclerosis. These findings may have relevance to the observation that gender differences in the extent of atherosclerosis in humans are limited to the coronary arteries, and perhaps the femoral arteries, and seems to provide evidence for the existence of regional differences in the susceptibility to reproductive influences on atherosclerosis. It seems reasonable to speculate that regional differences in arterial steroid receptor content or distribution may be involved.

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