Estrogen and Progesterone Replacement Therapy Reduces Low Density Lipoprotein Accumulation in the Coronary Arteries of Surgically Postmenopausal Cynomolgus Monkeys

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Abstract

The effect of estrogen and progesterone replacement therapy on the initiating events in atherogenesis was studied in surgically postmenopausal cynomolgus monkeys. Monkeys were ovariectomized and divided randomly into two groups, one receiving 17β-estradiol and cyclic progesterone treatment ($n = 9$) and ovariectomized controls receiving no hormone replacement therapy ($n = 8$). The monkeys were fed a moderately atherogenic diet for 18 wk to accelerate the early pathogenic processes but not to be of sufficient duration to produce grossly visible atherosclerotic lesions. Sex hormone replacement therapy decreased the accumulation of LDL and products of LDL degradation in the coronary arteries by > 70% while having no significant effect on plasma lipid, lipoprotein, or apoprotein concentrations. Arterial intimal lesions were small with no difference between groups. The reduction in arterial LDL metabolism occurred very early in the pathogenesis of atherosclerosis and was independent of indices of endothelial cell injury, such as enhanced endothelial cell turnover or leukocyte adhesion to the endothelium. Results of this study suggest that one mechanism by which sex hormone treatment inhibits the initiation of atherosclerosis is a direct effect at the level of the arterial wall by suppressing the uptake and/or degradation of LDL. (*J. Clin. Invest.* 1991. 88:1995–2002.) Key words: atherosclerosis • sex hormones • lipoproteins • menopause • endothelium

Introduction

Although coronary heart disease is more prevalent in men than women, it remains the leading cause of death in women in Western societies (1). Both natural and surgical menopause are associated with accelerated development of coronary artery atherosclerosis and the clinical expression of coronary heart disease, and among postmenopausal women, estrogen replacement therapy has been shown to decrease the risk of coronary

heart disease (2, 3). Taken together, these observations suggest a protective effect of estrogen on the pathogenesis of coronary artery atherosclerosis and the occurrence of coronary heart disease.

The Lipid Research Clinics Program Follow-up Study (3) found that the cardiovascular mortality in estrogen users was about one-third of that of nonusers. After statistical adjustment for risk factors (blood pressure, smoking, and age), Bush et al. (3) determined that about half of the protective effect of estrogen use was accounted for by estrogen-induced increases in high density lipoprotein cholesterol (HDLC)[1] and decreases in low density lipoprotein (LDL) cholesterol concentrations. Thus, half of the protective effect of estrogen use must be mediated by mechanisms other than its effects on plasma LDL and HDL cholesterol concentrations.

The purpose of this study was to investigate the influence of sex hormone therapy on early atherogenic processes in cynomolgus monkeys. In another animal model of atherogenesis, the hypercholesterolemic rabbit, increases in the arterial concentration of undegraded LDL and arterial rates of LDL degradation appear to be early events in the pathogenesis of atherosclerosis (4); these aspects of the interaction of LDL with the arterial wall are exaggerated once atherosclerotic lesions are present (5, 6). Thus, to study the influence of sex hormones on atherogenesis, this study was designed to assess metabolic effects (accumulation of LDL and its degradation within the artery) and cellular events (intimal proliferation, leukocyte adhesion to the arterial wall, and endothelial cell turnover) that are believed to occur before the formation of grossly detectable atherosclerotic lesions.

The cynomolgus monkey was chosen for this study because females of this species have a menstrual cycle similar to that of human females (7–9), and female cynomolgus monkeys, like human females, develop less coronary artery atherosclerosis than their male counterparts (10, 11). This effect has been shown to be influenced by the estrogenic state of the monkey (10–12). Females that are ovariectomized have estradiol concentrations similar to males and more extensive atherosclerosis than intact females, while pregnancy and the resultant increase in plasma estradiol concentrations is associated with decreased atherosclerosis (12). Also, we have shown recently that hormone replacement therapy results in a decreased extent of coronary artery atherosclerosis in surgically postmenopausal monkeys (13). We report here that sex hormone replacement mark-

1. *Abbreviations used in this paper:* ANCOVA, analysis of covariance; ANOVA, analysis of variance; FCR, fractional catabolic rate; HDLC, high density lipoprotein cholesterol; LAD, left anterior descending; LCX, left circumflex; TC, tyramine cellobiose; TPC, total plasma cholesterol.

edly suppresses the accumulation of ^{125}I-tyramine cellobiose (TC)-LDL in the coronary arteries, as determined by the arterial content of ^{125}I-TC which reflects undegraded LDL and products of LDL degradation, without affecting other indices of atherogenesis including endothelial cell proliferation and leukocyte adhesion to the endothelium.

Methods

Animal study. 17 adult female cynomolgus monkeys (*Macaca fascicularis*) of Indonesian origin were obtained from Charles River Research Primates (Port Washington, NY). The animals averaged 7.0±0.5 yr of age (mean±SEM) as estimated by dentition. After a 3-mo quarantine period during which the animals were fed Monkey Chow (Ralston Purina Co., St. Louis, MO), they were randomized into two groups. Two animals, one from each group, entered the protocol each month. The experimental period was 18 wk during which the monkeys consumed a moderately atherogenic diet containing 40% of calories as fat and 0.25 mg cholesterol per kilocalorie (13). Based on past experience, the 18-wk period of cholesterol feeding was selected to accelerate the early pathogenic processes in the development of atherosclerosis, but not to be of sufficient duration to produce grossly visible atherosclerotic lesions. 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. Surgical procedures (ovariectomy and arterial catheterization) were performed while the animals were anesthetized with ketamine hydrochloride (10 mg/kg i.m.) and butorphanol tartrate (0.05 mg/kg i.m.), whereas minor procedures (blood sampling and implant manipulations) were performed while the animals were sedated with ketamine hydrochloride (15 mg/kg i.m.).

Hormone manipulations. The experimental period began 3 wk after ovariectomy. Estrogen and progesterone replacement therapy was accomplished by means of silastic implants (Dow Corning Corp., Midland, MI) that were placed subcutaneously between scapulae (13). In the treatment group, physiologic sex hormone concentrations were maintained using two implants, one containing 17β-estradiol and one containing progesterone. Estrogen implants remained in place continuously while progesterone implants were inserted for the last 2 wk out of each 4 wk to approximate discrete follicular and luteal phases as well as the combined estrogen-progestin therapy commonly prescribed for postmenopausal women. Ovariectomized controls, not receiving hormone replacement therapy, received empty implants inserted on a similar schedule. Plasma progesterone and estradiol concentrations were assayed at weeks 4, 12, and 16 by radioimmunoassay utilizing antisera which have been characterized for use in monkeys (14, 15). These samples were taken at the end of the 2 wk with progesterone implants.

Plasma lipids and lipoproteins. Blood samples were collected into EDTA (1 mg/ml final concentration) after the animals were fasted overnight. Determinations were made of total plasma cholesterol (TPC), triglycerides, HDLC, and HDL subfractions 2 and 3 (HDL$_2$ and HDL$_3$) at baseline (before ovariectomy, experimental diet, and hormone manipulations) and 2, 4, 6, 10, 12, and 18 wk. LDL cholesterol was estimated from total plasma cholesterol and HDLC concentrations (16). TPC and triglycerides were measured by automated enzymatic procedures. The cholesterol procedure is based on the method of Allain et al. (17), while the triglyceride procedure is based on the method of Fossati and Prencipe (18). Plasma HDLC was determined by the heparin-manganese precipitation procedure as described in the Manual of Laboratory Operations of the Lipid Research Clinics Program (19). The differential dextran sulfate precipitation method of Gidez et al. (20) was used to determine HDL$_2$ and HDL$_3$ cholesterol concentrations. Analyses for TPC, triglyceride, and HDLC are in full standardization with the CDC-NHLBI Lipid Standardization Program. LDL molecular weight (21, 22), and apoproteins (apo) A1 (23) and B (24) were determined once at baseline, and at weeks 8 and 14.

Isolation and labeling of LDL. To isolate autologous LDL, blood samples (20 ml) were collected at weeks 14 and 15 after the animals were fasted overnight. The LDL was isolated by combined ultracentrifugation and agarose column chromatography as described previously (25, 26). The only modification of this method was that 0.02% sodium azide was mixed with the blood for bacteriostasis and was removed by dialysis before injection into the monkeys. Approximately 8–12 mg of LDL protein, as measured by the method of Lowry (27), were isolated from each animal.

One aliquot (6.4–9.6 mg) of each LDL preparation was coupled to ^{125}I-tyramine cellobiose (TC), as described previously (28, 29). A second aliquot (1.6–2.4 mg) of each LDL preparation was labeled with ^{131}I using the iodine monochloride method (30) as described previously (25). Specific activities of the ^{125}I-TC-LDL and ^{131}I-LDL averaged 251±66 and 67±18 cpm/ng protein, respectively (mean±SEM) for 17 preparations. For ^{125}I-TC-LDL and ^{131}I-LDL, TCA-soluble radioactivities (10% final concentration) averaged 10±4% and 4±1.5% and radioactivities extractable in chloroform/methanol (31) averaged 4±0.5% and 8±3%, respectively. Just before injection, the two aliquots of each LDL preparation were combined and sterilized by filtration (0.45 μm). LDLs were used within 1 wk of labeling.

In vivo studies. After 18 wk of consuming the atherogenic diet, radiolabeled LDLs were injected into each animal and the animal was necropsied 24 h later. To facilitate the studies with labeled LDLs, indwelling femoral venous and arterial catheters were inserted. Each monkey was then fitted with a nylon mesh jacket and attached to a flexible metal tether (Alice King Chatham Medical Arts, Los Angeles, CA) in its cage (32). The labeled LDL (5.60±1.29 × 10^7 cpm ^{131}I and 1.33±0.33 × 10^9 cpm ^{125}I) were injected through the venous catheter 1 d after inserting the catheters. Subsequent blood samples were collected in EDTA (0.1% final concentration) from the arterial catheter at 5, 15, 30, and 60 min and 3, 7, 13, and 24 h after injection to determine the plasma decay of labeled LDL.

To label replicating endothelial cells, we injected the animals intravenously with ^{3}H-thymidine at a dose of 0.5 mCi/kg body weight per injection at three time points (17, 9, and 1 h before necropsy) (33).

Necropsy and measurement of atherosclerosis. The animals were necropsied immediately after the last blood sample was collected for the plasma decay of LDL. The animals were anesthetized with sodium pentobarbital (60 mg/kg body weight, i.v.), and were perfused with lactated Ringer's solution through catheters introduced into the left ventricle. An incision was made in the vena cava through which blood containing labeled LDL and perfusate were removed. When the vena caval effluent became colorless, the perfusate was changed to a modified Karnovsky's fixative (29). After perfusing for 5 min, the vena cava was clamped and pressure was maintained at 100 mmHg for an additional 15 min to provide adequate fixation for electron microscopy and radiolabeled LDL.

The heart was removed and the left anterior descending (LAD) and left circumflex (LCX) coronary arteries were dissected free. Adventitia was removed and the arteries were fixed in modified Karnovsky's fixative for 24 h. Segments were taken as shown in Fig. 1 for histologic and electron microscopic study and for studies of the interaction of radiolabeled LDL with the artery.

One section (5 μm) was taken from each paraffin block (segment *A*, Fig. 1) and stained with Verhoeff-van Gieson (34). The sections were projected and intimal thickness was measured at 4-mm intervals with a digitizer (35). The extent of coronary artery atherosclerosis was expressed as the mean intimal thickness (millimeters).

Segments (segment *B*, Fig. 1) were also prepared for scanning electron microscopy as described previously (33). 200 fields (0.006 mm^2 each) were examined at a magnification of 1,250 using a Philips 501 scanning electron microscope. Endothelial cell turnover rates were expressed as number of labeled nuclei per square millimeter of endothelial surface observed. Although the radiolabeled LDL contributed to cellular radiographic grains, this label was easily distinguished due to its more uniform cytoplasmic distribution as opposed to the nuclear location of ^{3}H-thymidine. The number of adherent leukocytes (identified
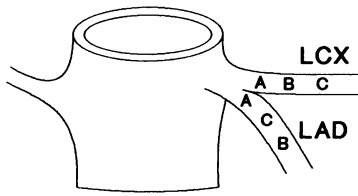
Figure 1. Segments from the left circumflex (*LCX*) and left anterior descending (*LAD*) coronary arteries were evaluated. Segment *A* was prepared for light microscopy, segment *B* for scanning electron microscopy, and segment *C* for the interaction of LDL with the arterial wall.

as cells between 8 and 15 μm in diameter) was also determined in segment *B* and results are reported as number of cells per square mm.

Finally, the interaction of LDL with the coronary arteries was evaluated (segment *C*, Fig. 1). [125]I and ^{131}I radioactivity in arterial tissues and plasma were determined in a well-type gamma counter (model 5500B; Beckman Instruments, Inc., Fullerton, CA). Both total ^{125}I and ^{131}I radioactivity in plasma and the fraction of plasma radioactivity that was protein bound (as determined by TCA precipitable radioactivity, 10% final concentration) were determined. Arterial samples, LAD and LCX, averaged ($\pm$SEM) 12.2$\pm$1.6 mg and 12.1$\pm$2.0 mg, respectively, with no difference between groups. Radioactivities in all samples were corrected for overlap of the energy spectra of the two isotopes, for background radioactivity and for isotopic decay. Arterial samples were counted for $\sim$ 40 min for each isotope during which an average of 169,739$\pm$52,754 gross counts of [125]I and 6,585$\pm$793 gross counts of [131]I accumulated, giving a 2 Sigma counting error of < 0.5% for [125]I and < 3.0% for [131]I. Background was counted until a minimum of 10,000 counts accumulated resulting in a 2 Sigma counting error of < 2%. The coefficient of variation on net counts per minute for [125]I present in arterial samples was 0.9$\pm$0.1%. For approximately one-fourth of the arterial samples, no net [131]I radioactivity could be detected. For the remaining samples, the coefficient of variation on net counts per minute for the [131]I present was 11.8$\pm$3.0%. Because the ^{131}I present in arterial samples was too low to be determined accurately, arterial ^{131}I data will not be presented.

Analysis of LDL studies. Clearance of LDL from plasma was determined from the decay of protein-bound radioactivity in the plasma during the 24 h from injection of labeled LDL until death. Data for the plasma decay of protein-bound radioactivity of each monkey were fitted with biexponential equations using a nonlinear regression algorithm (BMDP Statistical Software, Los Angeles, CA). The fractional catabolic rate (FCR) of LDL was determined from the rate constants and coefficients defined by the biexponential fit to the radioactive decay in the plasma (36). Similarly, the fraction of the dose irreversibly degraded at the time of death was calculated from the decay of ^{125}I-TC-LDL in plasma (4, 29, 37).

At the end of the experiment, coronary arteries were fixed in a modified (half-strength) Karnovsky's fixative (29). Fixation in this manner preserves both products of ^{125}I-TC-LDL degradation trapped in lysosomes and undegraded ^{125}I-TC-LDL that has either not yet been taken up by arterial cells or is within cells but has not yet been degraded (28, 29). Thus, for tissues fixed in this manner, radioactivity from ^{125}I-TC represents both undegraded LDL and products of arterial LDL degradation. The arterial ^{125}I-TC radioactivity (counts per minute per gram) was normalized by the average protein-bound plasma ^{125}I-TC radioactivity during the metabolic experiment to express the arterial ^{125}I-TC radioactivity in a form (nanoliters per gram per hour) which is independent of the plasma LDL concentration and the dose of LDL that was injected. The average plasma ^{125}I-TC radioactivity ([cpm/nl]$\ast$h) was determined from the area under the plasma decay curve (38). If a sufficient amount of ^{131}I radioactivity had accumulated in the arteries, this would have allowed us to determine the amount of undegraded LDL present and then calculate rates of LDL degradation after correcting for the amount of arterial ^{125}I-TC representing undegraded LDL. Because there was not enough ^{131}I radioactivity to obtain an

accurate estimate of undegraded LDL, we report here only data for ^{125}I-TC, which represents the sum of undegraded LDL and LDL degradation products.

Statistical analysis. Data for arterial morphology were analyzed after square root transformation while lipid, lipoprotein, and apoprotein concentrations and arterial ^{125}I-TC accumulation were analyzed after logarithmic transformation to reduce skewness and equalize group variances. Variables measured during the baseline period (body weight, lipids, lipoproteins, and apoproteins) that significantly predicted the outcome measures were identified by multiple regression and used in group comparisons as covariates to reduce the variability in outcome measures by accounting for variability in pretreatment factors. Statistical comparisons between treatment groups were made by analysis of variance (ANOVA) and covariance (ANCOVA). However, results of analyses were similar whether or not pretreatment variables were included. Analyses were performed with the BMDP statistical package (BMDP Statistical Software, Los Angeles, CA). Means presented in the text are reported with the SEM. Comparisons resulting in $P \leq 0.05$ were considered significantly different.

Results

Reproductive steroids. Ovariectomized controls without hormone replacement had undetectable levels of plasma estradiol (< 20 pg/ml) and very low concentrations of plasma progesterone (0.29$\pm$0.03 ng/ml). In contrast, animals receiving estrogen and progesterone replacement therapy had physiologic concentrations of both 17β-estradiol (124$\pm$29 pg/ml) and progesterone (5.4$\pm$0.3 ng/ml; $P < 0.001$ for both estradiol and progesterone concentrations, control vs. hormone replacement).

Plasma lipoproteins. Baseline plasma lipid, lipoprotein, and apoprotein measures were compared between groups to determine whether there were differences before treatment (Table I). Although there were small (nonsignificant) differences between groups in several of the variables at baseline, by random chance apo A1 was significantly different ($P < 0.05$). To account for variability in pretreatment values, we performed all statistical analyses using baseline variables as covariates. As shown in Table I, the atherogenic diet produced a moderate hypercholesterolemia in both groups. Hormone therapy did not produce a significant change in plasma lipid and lipoprotein concentrations except for LDL molecular weight ($P < 0.05$). Due to the possibility that LDL of different molecular weights would be metabolized at a different rate than the animal's own LDL, autologous LDL was used for the studies of the interaction of LDL with the artery.

Plasma decay kinetics. Fractional catabolic rates of LDL, determined from the plasma decay curves for ^{125}I-TC-LDL and ^{131}I-LDL, were not significantly different (0.054$\pm$0.005 vs. 0.063$\pm$0.006 pools h^{-1}, ^{125}I-TC-LDL vs. ^{131}I-LDL, $P = 0.27$), indicating that type of radiolabel did not influence the rate of plasma clearance (see Fig. 2 for representative decay curve). Fractional catabolic rates were not affected by hormone treatment (0.052$\pm$0.008 vs. 0.056$\pm$0.008 pools h^{-1}, ^{125}I-TC-LDL, control vs. hormone treated, $P = 0.71$). Consistent with that observation, monkeys in both groups degraded similar fractions of the injected dose (0.56$\pm$0.04 vs. 0.58$\pm$0.04, ^{125}I-TC-LDL, control vs. hormone treated, $P = 0.70$).

Arterial accumulation of ^{125}I-TC. Preliminary analyses revealed that when treatment effects on the LAD and LCX were considered together in a repeated measures analysis, no significant differences between arteries were found ($P > 0.62$), thus, subsequent analyses were done using the mean of these two

Table I. Effect of Hormone Treatment on Plasma Lipids, Lipoproteins, Apoproteins, and Body Weight

	Control (n = 8)		Hormone replacement (n = 9)	
	Baseline	Treatment	Baseline	Treatment
Total plasma cholesterol (TPC) (*mg/dl*)	127±7	383±30	138±6	341±30
HDL cholesterol (HDLC) (*mg/dl*)	61±4	52±5	71±6	71±11
HDL$_2$ subfraction (*mg/dl*)	36±3	25±3	46±5	46±9
HDL$_3$ subfraction (*mg/dl*)	25±2	29±3	25±2	27±3
TPC:HDLC	2.2±0.2	9.8±1.5	2.0±0.2	8.2±2.0
LDL cholesterol (TPC-HDLC) (*mg/dl*)	66±7	331±33	67±3	270±32
Triglyceride (*mg/dl*)	38±4	28±5	46±6	43±7
Apoprotein A1 (*mg/dl*)	166±9*	149±13	240±28	210±28
Apoprotein B (*mg/dl*)	99±16	175±12	89±8	160±17
LDL molecular weight (*g/μmol*)	3.23±0.12	3.35±0.03*	3.28±0.11	3.21±0.07
Body weight (*kg*)	3.54±0.24	3.38±0.20	3.64±0.16	3.51±0.16

Values are means±SEM. Baseline values were determined before cholesterol feeding, ovariectomy, and hormone manipulations. Statistical analyses were performed after logarithmic transformation. Treatment values were determined at multiple times during the experimental protocol; for controls (atherogenic diet with ovariectomy alone) or hormone replacement (ovariectomy with estrogen and progesterone replacement). Statistical analyses were performed after logarithmic transformation with baseline variables as covariates. * $P < 0.05$ for control vs. hormone replacement.

arteries. Multiple regression analysis revealed that no pretreatment (baseline) characteristics were significant predictors of LDL accumulation; therefore effects of hormone replacement were analyzed using ANOVA. The principal finding of these studies was that the combined accumulation of products of LDL degradation and undegraded LDL in the arterial tissue (^{125}I-TC accumulation) was more than threefold greater in animals not receiving hormone replacement (Table II).

Histologic and ultrastructural analysis. The effects of hormone therapy on intimal thickness, endothelial cell turnover, and the number of adherent leukocytes on the arterial wall were examined. Preliminary analyses revealed that when treatment effects on the LAD and LCX were considered together in a repeated measures analysis, no significant differences between arteries were found (all *P* values > 0.52). Thus, all subsequent analyses were done using the combined mean of these two arteries.

Multiple regression was used to determine which pretreatment (baseline) characteristics of the animals were significant predictors of outcome measures. Pretreatment values of HDL$_2$
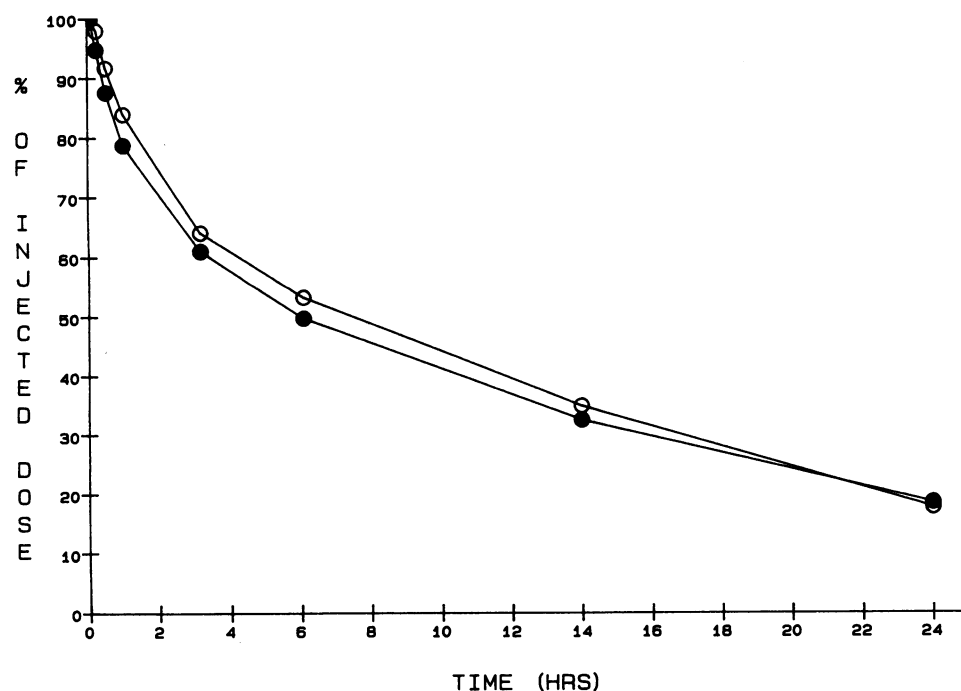

Figure 2. Representative plasma decay curve for ^{125}I-TC-LDL (*solid circles*) and ^{131}I-LDL (*open circles*). Monkey 2530 was injected with 5.34 × 10^8 cpm ^{125}I-TC-LDL and 6.16 × 10^7 cpm ^{131}I-LDL. Data were fitted with biexponential equations using a nonlinear regression algorithm. Fractional catabolic rates (FCR) were determined as described in Methods. FCRs for ^{125}I-TC-LDL and ^{131}I-LDL were 0.079 and 0.077 pools/h, respectively.

TIME (HRS)

Table II. Effect of Hormone Treatment on Accumulation of ^{125}I-TC and Morphologic Characteristics of Coronary Arteries

	Control ($n = 8$)	Hormone replacement ($n = 9$)	P
Accumulation of ^{125}I-TC			
$(nl/g/h)$*	5954±2612	1753±479	0.04
Intimal thickness (mm^2)‡	0.006±0.002	0.007±0.003	0.60
Endothelial cell turnover			
rate $(cells/mm^2)$‡	0.47±0.23	0.79±0.46	0.69
Adherent cells $(cells/mm^2)$*	2.9±0.7	2.5±0.5	0.80

Values are means±SEM.
* Statistical analyses were performed by ANOVA after transformation.
‡ Statistical analyses were performed by ANCOVA after transformation.

accounted for 29% of the variance in mean intimal thickness and pretreatment values of plasma triglyceride concentrations, HDL_2 concentrations, and the TPC:HDLC ratio together accounted for 53% of the variance in endothelial cell turnover rates. No pretreatment variables significantly predicted the number of adherent leukocytes. The effects of treatment on endothelial cell turnover and adherent leukocytes were then analyzed by ANCOVA, using significant baseline predictors as covariates.

Hormone therapy over this 18-wk period did not have a significant effect on intimal thickness, endothelial cell turnover rate, or the number of adherent leukocytes (Table II). The intimal lesions produced by the moderate hypercholesterolemia of 4 mo duration were very small and did not differ in thickness between groups. A photomicrograph of a typical coronary artery is shown in Fig. 3. Although most of the intima appeared normal, small focal accumulations of foam cells and small areas of diffuse intimal thickening were observed in some histologic sections. The endothelial cell turnover rates and the number of adherent cells were also small, suggesting limited

amounts of endothelial injury. Thus, the changes produced with this degree and duration of hypercholesterolemia were mild with no difference between groups in any of the morphologic measurements examined.

Discussion

This study was designed to investigate the effects of estrogen and cyclic progesterone replacement therapy on atherogenesis in surgically postmenopausal monkeys. Events which may play a role in initiating atherogenesis, such as the uptake and accumulation of LDL by the arterial wall, endothelial cell damage, and adherence of leukocytes to the endothelium were evaluated. Because we were interested in the early events in the pathogenesis of atherosclerosis, monkeys were studied after a short period of moderate hypercholesterolemia and, therefore, before the appearance of grossly visible atherosclerotic lesions (Fig. 3). This is particularly important for studies of LDL interaction with the arterial wall because rates of LDL degradation are markedly enhanced when atherosclerotic lesions are present (5, 6).

Effects of hormone therapy on atherogenesis may be mediated, at least in part, by plasma lipoprotein concentrations and composition. The effects of sex hormones on plasma lipoprotein distribution have been studied extensively. Premenopausal women (2, 3) and monkeys (10, 12) typically have lower plasma LDL cholesterol concentrations and higher HDLC concentrations than males of similar age. The difference in HDLC is principally in the HDL_2 subfraction, the fraction most strongly associated (negatively) with coronary artery atherosclerosis (2, 3). In the present study, the lack of a significant effect of hormone therapy on plasma lipids and lipoproteins, despite trends in the expected directions (Table I), may relate to the short period of study, the limited number of animals available for study, or the potency and route of administration of the hormones.

The route of administration has been shown to affect changes in plasma lipids (see reference 2 for review). In general, whereas both oral and parenteral treatments increase circulat-
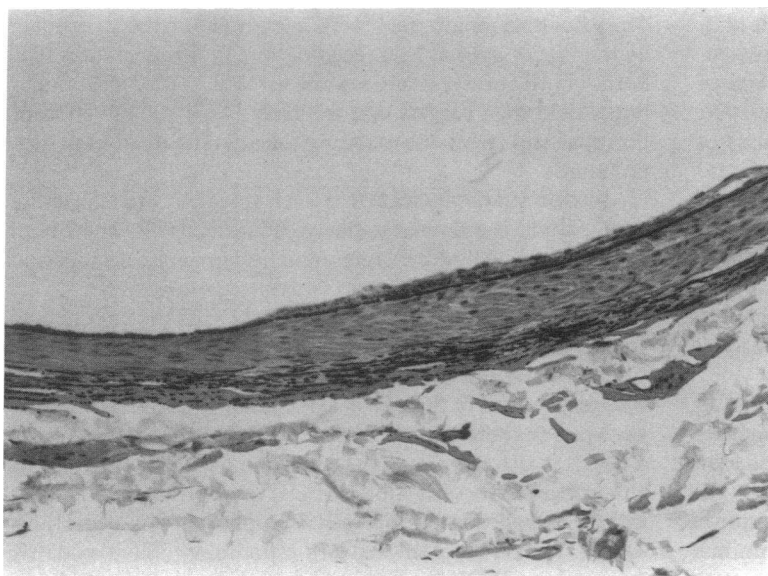

Figure 3. Photomicrograph of a portion of a typical Verhoef van Gieson–stained section of a coronary artery after 18 wk of a moderately atherogenic diet. The majority of the intimal surface appeared normal histologically except for a small intimal thickening shown here which involved ~ 10% of the intimal surface. Magnification, 200.

ing estradiol concentrations to a similar degree, in the study of Basdevant et al. (39) only oral treatment reduced LDL cholesterol, and in the study of Fahraeus et al. (40) only oral treatment increased HDLC concentrations. It is possible that treatments that also improve lipoprotein patterns may be even more effective in inhibiting development of atherosclerosis. However, other studies in rabbits (41) and monkeys (13) have found inhibitory effects of estrogen on atherosclerotic lesion development despite no effect on plasma lipoproteins. Using a hormone replacement method identical to the one described here, Adams et al. (13) found that after 30 mo, animals treated with either estrogen alone or estrogen plus progesterone had significantly less diet-induced coronary artery atherosclerosis than ovariectomized controls. Furthermore, the antiatherogenic effects of hormone replacement were independent of changes in plasma cholesterol, lipoprotein cholesterol, apo A1 and B concentrations, HDL subfraction heterogeneity and LDL molecular weight (13). This suggests that estrogen with or without added progesterone may have a protective effect on the development of atherosclerosis that is independent of its effects on plasma lipoproteins and that in the present study, the addition of progesterone may not have affected atherogenesis.

Differences in LDL molecular size may have important pathophysiologic implications because LDL size and particle heterogeneity are important predictors of coronary artery atherosclerosis and coronary heart disease in nonhuman primates (42). Parks et al. (43) found that treatment with the oral contraceptive Demulen (ethinyl estradiol and ethynodiol diacetate) decreased the LDL molecular weight significantly. However, in the study by Adams et al. (13), effects of hormone replacement therapy on LDL molecular weight were small and transitory. Because the study by Adams et al. (13) was similar in design to the present study, except for a longer experimental period, this suggests that any effects of hormone replacement therapy on LDL molecular weight may be insignificant over longer periods of time. However, we cannot discount the possibility that the interaction of LDL with the arterial wall may be influenced by subtle differences in LDL particle composition and heterogeneity induced by hormone replacement. Hormone treatment may also influence the expression of LDL receptors in the arterial wall. An estrogen-induced increase in LDL receptors has been seen in rat liver (44–46) and in swine granulosa cells (47). Eriksson (48) has reported that in human beings, a pharmacologic dose of ethinyl estradiol increased the fractional clearance rate of LDL, presumably secondary to upregulation of LDL receptors. In our study, where physiologic concentrations of estradiol and progesterone were coadministered, there was no effect on the clearance of LDL from plasma, suggesting that at these concentrations there is no enhancement of LDL receptor activity. This suggests that in this study, the effects of sex hormones on the accumulation and/or degradation of LDL by the arterial wall is probably not secondary to alterations in LDL receptor function, but rather suggests an independent effect of estrogen and/or progesterone on the artery.

Because of the brief duration of the atherogenic stimulus, there was no grossly visible atherosclerosis and intimal changes in the coronary artery (intimal thickness, number of adherent leukocytes, or endothelial cell turnover rate) were minimal (Table II). As shown in the photomicrograph (Fig. 3), the amount of microscopically visible changes in the intima were minimal with no differences between groups (Table II). Clarkson et al.

(33) found an endothelial cell turnover rate of ~ 0.6 cells/mm^2 in the coronary arteries of control monkeys fed a nonatherogenic diet, whereas monkeys fed an atherogenic diet for 35 wk exhibited about a sixfold higher turnover rate. The values obtained in the present study are comparable to the control values reported in the earlier study (33), suggesting that the 18 wk of moderate hypercholesterolemia in the current experiment did not damage endothelial cells enough to stimulate cell proliferation. The number of adherent leukocytes, generally 80% monocytes (49), was also low and comparable to that found by Clarkson et al. (33) for the coronary arteries of control monkeys. Thus, the effect of hormone replacement on LDL accumulation and/or degradation occurred before any differences in these other indices of endothelial cell dysfunction.

Hough and Zilversmit (41) investigated the effects of estrogen treatment (17-estradiol cypionate) of cholesterol-fed rabbits on net arterial influx and hydrolysis of plasma cholesteryl ester entering the artery. Despite no significant effect on plasma cholesterol concentrations or lipoprotein patterns, estrogen treatment decreased lesion development significantly. In each group, net cholesteryl ester influx was positively correlated with the extent of atherosclerosis while the percentage of newly entering cholesteryl ester hydrolyzed by the artery was independent of the extent of atherosclerosis. When normalized by aortic cholesterol, net influx of plasma cholesteryl ester did not differ between estrogen-treated and untreated rabbits. In contrast, the percentage of newly entered cholesteryl ester hydrolyzed by the artery was suppressed by estrogen treatment; this effect was evident whether or not differences in aortic cholesterol were considered.

In the present study we had intended to determine directly the arterial rate of degradation of LDL protein. Unfortunately, due to the insufficient ^{131}I radioactivity in the artery of some animals, we were not able to obtain reliable estimates of the portion of the arterial ^{125}I-TC accumulation representing undegraded LDL with which to correct the total arterial ^{125}I-TC accumulation for all animals. However, in the monkeys for which the coefficient of variation of the net arterial ^{131}I radioactivity was 10% or less, $\sim 60\%$ of the ^{125}I-TC accumulation represented products of arterial LDL degradation; there was no significant difference between treated and untreated animals. Thus, our data for arterial ^{125}I-TC accumulation reflect primarily the rate of arterial LDL degradation. Our observation that hormone treatment decreased the arterial ^{125}I-TC accumulation would then suggest that the most likely site of action of hormone treatment was to reduce the arterial rate of LDL degradation.

In vitro studies indicate that after uptake by cells the entire LDL particle is delivered to the lysosome as a unit, where both protein and cholesteryl ester moieties undergo degradation (50). Therefore, both the report that estrogen decreases hydrolysis of cholesterol ester by rabbit artery (41) and the present data for ^{125}I-TC accumulation support the conclusion that estrogen treatment inhibits the catabolism of LDL by the arterial wall. The mechanism of this effect is unclear. Estrogens could influence one or more of the intracellular steps in the processing of LDL. It is also possible that sex hormone deficiency promotes the intraarterial conversion of LDL to a form which is more rapidly degraded by arterial cells. In support of this idea, Huber et al. (51) have reported that 17β-estradiol inhibited oxidative conversion of LDL to a form which stimulated cholesteryl ester

accumulation in macrophages. Alternatively, it may be that subtle differences in LDL composition induced by hormone deficiency increase degradation by the artery. Further studies will be needed to sort out these possible explanations for the decrease in arterial LDL accumulation associated with estrogen/progesterone replacement.

In summary, sex hormone replacement therapy decreased the ^{125}I-TC accumulation by coronary arteries by > 70% while having no significant effect on plasma lipids, coronary artery endothelial cell turnover, or leukocyte adhesion. Our data suggest that suppression of the accumulation and/or degradation of LDL may be an important mechanism by which natural or surgical menopause exacerbate atherosclerosis. This study further suggests that changes in LDL metabolism by the arterial wall can occur very early in the pathogenesis of atherosclerosis and independent of changes in endothelial cell function associated with endothelial cell injury, such as enhanced turnover rate or leukocyte adhesion.

Acknowledgments

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