Variable Expression of the Estrogen Receptor in Normal and Atherosclerotic Coronary Arteries of Premenopausal Women

Douglas W. Losordo, MD; Marianne Kearney, BA; Elizabeth A. Kim, BA; Jaclynn Jekanowski, BA; Jeffrey M. Isner, MD

Background The relative absence of coronary atherosclerosis in premenopausal women has been established. Estrogen is presumed to play a role in the protection of coronary arteries from atherosclerosis, and part of this protective effect appears to be mediated by amelioration of serum lipids. However, all of the atheroprotective effect of estrogen is not explained by alteration of serum lipids. In this study, we attempt to identify evidence of estrogen receptors in coronary artery specimens of female patients and in human vascular smooth muscle cells.

Methods and Results Postmortem coronary artery specimens were obtained from premenopausal (n = 18) and postmenopausal (n = 22) women who died with significant coronary artery disease (n = 19) and from noncardiac causes with normal coronary arteries (n = 21). Sections were examined for evidence of estrogen receptor expression using a monoclonal antibody stain. Radioligand binding assays for estrogen receptors were performed on human vascular smooth muscle cells in culture, and gel retardation assays were performed to confirm the presence of functional estrogen receptors. Estrogen receptor expression was identified by immunostaining in a total of 21 coronary arteries, with the majority of normal arteries (15 positive of 21 total, P = .0117) demonstrating evidence of estrogen receptor expression. Conversely, a minority (6 of 19, P = NS) of atherosclerotic arteries were positive for estrogen receptor expression. Furthermore, the relation between estrogen receptor expression and absence of coronary atherosclerosis was most evident in premenopausal subjects, with 10 of 12 normal arteries in this group demonstrating evidence of estrogen receptors, whereas only 1 of 6 atherosclerotic coronary arteries was positive (P = .0062). Radioligand binding assays confirmed the presence of estrogen receptors at significant concentrations in intact human vascular smooth muscle cells. Gel retardation assays also documented the presence of functional estrogen receptors in extracts from human vascular smooth muscle cells.

Conclusions This investigation provides evidence of estrogen receptors in smooth muscle cells from human coronary arteries. The demonstrated relation between the presence of the receptors and the absence of atherosclerosis in premenopausal women suggests that these receptors may play a functional role in coronary atheroprotection. (Circulation. 1994;89:1501-1510.)

Key Words • atherosclerosis • receptors, estrogen

The relative protection from coronary atherosclerosis conferred upon premenopausal women is well established. Heberden commented on the sex difference in the incidence of angina pectoris in 1803. Others have documented the lower incidence of coronary atherosclerosis in premenopausal women in epidemiological and autopsy studies. The increased incidence of atherosclerosis in women who undergo premature menopause also has been well described. Finally, there is increasing evidence that treatment with replacement estrogen after menopause will reduce cardiovascular mortality.

Animal studies have provided further evidence of the effects of estrogen on the vascular system. Estrogen treatment prevented collagen and elastin accumulation in the aortic wall in normotensive rats and thickening of the aorta in hypertensive rats and similarly suppressed atherosclerosis in primate coronary arteries. Alternatively, progesterone administered alone resulted in an increase in fatty streak formation in castrated baboons, whereas animals receiving estradiol and progesterone together had the fewest lesions. Thus, in an intact animal preparation, there have been multiple studies clearly suggesting an effect of sex steroids on vascular biology and specifically suggesting an antiproliferative effect of estrogen. These studies lend experimental support to the hypothesis, brought forward by epidemiological studies, that female sex hormones are protective against coronary atherosclerosis. What these studies do not address, however, is the mechanism whereby the presence of estrogen is translated into an effect on the biology of the arterial wall.

Endogenous and exogenous estrogens have been observed to alter the levels of serum lipids and lipid metabolism in humans. In experimental animals fed an atherogenic diet, administration of estrogen inhibited or reversed atheroma formation and was associated with reversion of lipid levels toward normal. Thus, well-established experimental animal data have demonstrated a clear atheroprotective effect of estrogen. These studies further demonstrate that estrogen administration results in a more normal lipid profile in animals fed a high cholesterol diet. Furthermore, these studies are corroborated by human evidence demonstrating a favorable alteration in lipid levels in the presence of endogenous or exogenously administered...
estrogen as well as changes in lipid metabolism, which would explain these alterations in lipoprotein profiles. The experimental animal and human data provide a partial explanation for the salutary effect of estrogen on the incidence of coronary atherosclerosis. The changes in serum lipids noted in human patients, however, fail to fully account for the discrepancy in the incidence of coronary disease between men and premenopausal women. A direct effect of estrogen on the arterial wall is suggested by animal and human experimental data. Vasodilation in response to estrogen administration was first noted in the rabbit ear artery and later in human umbilical and primate coronary arteries. Gender differences in the contractile response of the aorta in rats have been shown, as has estrogen-dependent sexual dimorphism of rat vascular smooth muscle cells. While this experimental evidence provides further suggestion of a direct estrogen effect on vascular tissue, neither a systemic effect nor a non–receptor-mediated effect of estrogen is excluded by these studies.

Examination of vascular smooth muscle cells in culture, however, has provided further data suggesting a direct estrogen effect on vascular tissue. Nichols et al demonstrated decreased protein synthesis in rat vascular smooth muscle cells in culture when exposed to estrogen. Isolating vascular tissue from the systemic effects of sex steroid administration in this study, therefore, provided evidence of a direct effect of estrogen on cells of the arterial wall.

Epidemiological and experimental evidence point to a significant effect of estrogen on vascular biology and suggest the possibility of a direct estrogen effect on cells of the arterial wall. Based on these previous experimental data, a direct action of estrogen on human vascular tissue appears to be a plausible explanation for the apparent atheroprotective effect of estrogen in human patients.

While protection from coronary atherosclerosis is enjoyed by most premenopausal women, a minority of premenopausal women are afflicted with significant coronary disease. If the atheroprotective effect of estrogen is partially mediated by direct action on the arterial wall, it is possible that the occurrence of coronary atherosclerosis in female patients may be precipitated by one of two events: (1) the loss of circulating estrogen or (2) absolute or relative lack of estrogen receptor expression by target cells in the arterial wall. The first of these occurs when female patients reach menopause and is clearly associated with an increase in the incidence of coronary disease. Premature atherosclerosis in female patients, however, may be mediated by a failure of target cells in the vessel wall to adequately express estrogen receptors, therefore abrogating the possibility of atheroprotection by circulating estrogen. This latter possibility, however, has not been investigated.

This study, therefore, was designed to test the hypothesis that premature atherosclerosis in women is associated with decreased local expression of estrogen receptors in the vessel wall. To test this hypothesis, we examined coronary artery specimens from female patients to determine if there was evidence of estrogen receptor expression. Furthermore, by examining samples from both normal and atherosclerotic arteries, this study sought to identify if a relation existed between estrogen receptor expression and the presence or absence of coronary artery atherosclerosis.

**Methods**

**Coronary Artery Specimens**

The autopsy records of St Elizabeth's Hospital were reviewed to identify patients who had undergone cardiac postmortem examination and who met criteria for one of the following four categories: (1) premenopausal women without coronary disease, (2) premenopausal women with severe coronary atherosclerosis, (3) postmenopausal women with normal coronary arteries, or (4) postmenopausal women with severe coronary atherosclerosis.

For the purposes of this study, severe atherosclerosis was defined as a luminal cross-sectional area narrowing of >75% measured histologically. We specifically attempted to identify the maximum number of premenopausal women with atherosclerosis, since testing our hypothesis would rely to a large extent on examination of specimens from this group. It was anticipated that coronary artery specimens from this group would be the least numerous and that the remaining categories would then be matched in size.

**Estrogen Receptor Immunohistochemical Staining**

Although assays of fresh-frozen tissue remain the standard for estrogen receptor analysis in breast carcinoma, prior studies have demonstrated specific identification of estrogen receptor protein by monoclonal antibodies applied to formalin-fixed, paraffin-embedded tissue. In this investigation, we applied a method developed by Hiort et al, which was verified previously against the results of the standard biochemical assay for estrogen receptors. This technique uses a commercially available antibody preparation (Abbott Laboratories). A total of 12 sections from each specimen were examined immunohistochemically.

Slides were deparaffinized in xylene and ethanol and rinsed in phosphate-buffered saline (PBS). Sections then were transferred to a solution of Ficin (Sigma Chemicals) for 15 minutes. After rinsing in tap water, the slides then were immersed in PBS and transferred to incubation dishes. Sections were covered with two or three drops of DNase solution (5 mg DNase [Sigma Chemicals] per milliliter of 0.05 M Tris, pH 7.4, with 1.2 MgSO4). After 15 minutes, sections were rinsed in PBS, and the blocking agent (normal goat serum) from the manufacturer's kit was added for 15 minutes. After draining the blocking agent, the primary antibody was applied, and the slides were incubated at 4°C overnight. Sections then were rinsed in PBS, and bridging antibody was applied for 30 minutes at room temperature. Endogenous peroxidase activity was blocked with the application of solution consisting of 80 mL methyl alcohol plus 20 mL 3% H2O2. After 30 minutes, slides were rinsed with tap water, after which PBS and a solution of peroxidase-antiperoxidase were added for 1 hour. The chromogen solution consisted of diaminobenzidine (DAB), cobalt chloride (CoCl2), and hydrogen peroxide.
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50 µm

50 µm
(H₂O₂), according to the method of Hiort et al. After application of the chromogen solution to the sections, tissues were observed under the microscope during development and were compared with the reaction seen in positive control breast tissue. The reaction was terminated when the positive control tissue demonstrated color development and while the negative control remained colorless. Positive controls, provided by breast carcinoma tissue, and negative controls, in which estrogen receptor antibody was omitted, were performed with each staining run. All immunohistochemistry was analyzed without the investigator’s knowledge of the subject’s age or menopausal status.

**Histopathology**

Adjacent sections of the coronary artery specimens examined for estrogen receptor expression in this study were stained with antibody to vascular smooth muscle α-actin (HHF 35, Enzo Diagnostics) and with trichrome elastic stain. The smooth muscle α-actin stain was used to determine the presence and location of smooth muscle cells within the arterial wall and plaque. The trichrome elastic stain tissue was used to identify the internal and elastic laminae and thus provides the anatomic landmarks in each coronary specimen necessary to document the presence and extent of atherosclerosis. Histological morphometry was performed, directly measuring the cross-sectional area of the residual lumen and the area subtended by the internal elastic lamina. Cross-sectional area narrowing was calculated as 100 minus (lumen area divided by area bounded by internal elastic lamina).

**Human Vascular Smooth Muscle Cell Culture**

Human vascular smooth muscle cells (hVSMC) were obtained from internal mammary arteries excised at the time of coronary bypass surgery. Internal mammary arteries are typical of arterial conduits for bypass to the left anterior descending coronary artery. After the artery is dissected from the chest wall, it typically needs to be trimmed of any redundant artery at the time of the anastomosis. These segments of arteries have been routinely supplied to us by the Division of Cardiothoracic Surgery. Immediately after the artery was ligated and trimmed, it was placed in ice-cold, serum-free, phenol red-free culture medium (M199, Gibco) supplemented with penicillin (100 µg/mL) and streptomycin (100 µg/mL) and transported to the cardiovascular cell culture facility within 30 minutes. Phenol red–free M199 was used because phenol red has been shown to exert a weak estrogen-like effect in cell culture. Samples then were washed with fresh medium, after which the vessels were opened longitudinally and the luminal side was gently scraped with a scalpel blade to dislodge endothelial cells. The specimen then was turned over, and the adventitia was carefully removed by gently scraping with a scalpel blade. Because poor adherence of atherosclerotic tissue to culture substrate has been a limitation of successful culture of hVSMC, artery specimens were planted on plastic culture dishes that had been precoated with human fibronectin. All specimens were placed into fibronectin-coated, six-well plastic culture dishes and cut into 1- to 2-mm fragments. Each fragment was immersed in a drop of culture medium supplemented with 15% dextran-coated, charcoal-stripped fetal bovine serum (FBS) and placed in a humidified incubator (5% carbon dioxide/95% air) at 37°C. Serum was dextran charcoal stripped to remove any steroid hormone from the serum. Fragments were allowed to attach to the surface of the culture well during the next 12 to 18 hours, after which an additional 1 mL of serum-supplemented culture medium was added. Medium is exchanged every 2 days after the onset of cell outgrowth. After the cells reached confluence, the tissue fragments were removed and the cells were enzymatically dispersed (0.25% trypsin, 1 mMol/L EDTA), pooled, counted, and subcultured into 24-well plates at a density of 5000 cells per cm².

The identity of cells as vascular smooth muscle was confirmed in each explant by staining with a monoclonal antibody to α-actin, by the absence of staining with factor VIII–related antigen, and by the tendency of the cells to grow in a characteristic hill-and-valley pattern. Cells in passage 2 were used for all of the studies outlined herein.

**Estrogen Receptor Radioligand Binding Assay**

Estrogen receptor assay was performed using a radioligand binding assay, which was developed for use in evaluating the estrogen receptor status of breast carcinoma and has been adapted for use in cell culture. Human vascular smooth muscle cells growing in 24-well plates were exposed to (2,4,6,7-3H)estradiol (New England Nuclear) in concentrations from 1 to 40 nmol/L, alone or in the presence of 200-fold excess unlabeled 17β-estradiol. The cells were allowed to incubate at 37°C for 30 minutes. The cell culture plates then were placed on ice, the medium was aspirated, and cells were washed five times in ice-cold calcium and magnesium–free PBS. The bound, labeled estradiol was then solubilized with 70% EtOH, and total radioactivity was determined by liquid scintillation counting. All assays were performed in duplicate.

Calculation of receptor number was performed according to the method of Scatchard. Specific binding of radiolabeled ligand was determined by subtraction of bound radioactivity in the wells in which excess unlabeled steroid was present from the bound radioactivity in the wells incubated without unlabelled hormone. Receptor numbers were calculated per 10⁶ cells.

**Gel Shift Assay**

Gel shift assays are widely used in the study of DNA binding proteins, including receptors. The assay is based on the fact that complexes of DNA and protein will migrate more slowly through a polyacrylamide gel than uncomplexed DNA. We used the gel shift assay to identify the presence of estrogen receptor protein in the extract from hVSMC. In the presence of estrogen, the estrogen receptor should form a complex with the estrogen response element. By labeling the response element with ³²P, the complexes can be identified by their altered electrophoretic mobility.

**Whole-Cell Extract Preparation**

Confluent 100-mm plates of cultured hVSMC were incubated in 10% charcoal-stripped FBS in phenol red–free M199 for 48 hours, after which some of the cells were exposed to estradiol (10⁻⁷ mol/L) for 1 hour. Cells were then lysed by freezing at −80°C in 0.1 mL of buffer containing 20 mMol/L Tris-HCl, 2 mMol/L DTT, 0.4 mol/L KCl, and 20% glycerol. Cells were thawed over ice and centrifuged for 20 minutes at 4°C. The supernatant was used as the crude whole-cell extract.

**Estrogen Response Element**

Previously published sequences for the estrogen response element (ERE) were synthesized commercially and dimerized at room temperature to form a double-stranded oligonucleotide probe.

**5'-End Labeling**

The 37-bp probe containing the ERE was end-labeled with ³²P in a reaction catalyzed by T4 polynucleotide kinase, according to the manufacturer’s guidelines (Promega).

**Gel Shift Assay**

Whole-cell extracts from hVSMC that had been incubated in the presence or absence of estradiol were incubated with binding buffer for 10 minutes (final buffer concentrations, 1 mMol/L MgCl₂, 0.5 mMol/L EDTA, 0.5 mMol/L DTT, 50 mMol/L NaCl, 10 mMol/L Tris-HCl, 0.05 mg/mL poly[dI-dC]). The labeled oligonucleotide was then added, and the mixture...
was incubated at room temperature for 20 minutes. Reactions were terminated by adding a gel-loading buffer containing 250 mmol/L Tris-HCl, 40% glycerol, 0.2% bromphenol blue, and 0.2% xylene cyanol.

Controls included addition of excess unlabeled oligonucleotide probe, exclusion of estradiol, exclusion of cell extract, and exclusion of labeled oligonucleotide.

Reactions were run on a 5% nondenaturing acrylamide gel. The gel was prerun for 30 minutes at 20 mA at 4°C and then run at 30 mA at 4°C until the bromphenol dye was approximately halfway down the gel. The gel then was vacuum dried and exposed to autoradiography film overnight at −80°C.

**Immunocytochemistry**

To confirm the presence of estrogen receptor (ER) protein in cultured hVSMC, a second antibody to human ER was used for immunostaining. This antibody, designated ER-21 (generously provided by Geoffrey Greene, University of Chicago) is a rabbit polyclonal antibody directed at the N-terminus of the ER.

Cells first were fixed in 4% paraformaldehyde for 7 minutes, then rinsed in TBS (Tris-buffered saline) for 10 minutes. Endogenous peroxidase activity was blocked and nonspecific staining decreased with 1% hydrogen peroxide, 20% normal goat serum, and 1% bovine serum albumin in TBS for 20 minutes. Primary antibody then was added to the slides at a final concentration of 2 μg/mL in 1% goat serum, 0.1% gelatin, and 0.5% Tween X-100. The negative control slides were treated with rabbit IgG. Both solutions were diluted in TBS. After incubating for 40 hours at 4°C, slides were washed three times in TBS for 5 minutes each wash. The secondary antibody (Vector Rabbit Elite Kit, Vector Laboratories), biotinylated anti-rabbit IgG, then was applied for 90 minutes. Slides then were washed in TBS for 5 minutes each, after which the avidin-biotin complex reagent was applied for 90 minutes. Slides then were washed three times in TBS for 5 minutes each wash. The sections then were incubated with diaminobenzidine (0.05% in TBS) for 10 minutes and then rinsed for 5 minutes in distilled water. The slides were coverslipped with aqueous media and examined. After confirming the absence of staining of the negative control cells, coverslips were removed, and these slides were lightly counterstained with hematoxylin and recovered slips.

**Statistical Analysis**

Numerical data are presented as mean±SEM. Paired groups of data were compared using the Student's t test. Contingency table analysis was used to analyze differences within subgroups of premenopausal and postmenopausal subjects.

**Results**

The age range of the study cohort is shown in Table 1. There were 18 premenopausal women (age, 31.6±1.9 years; range, 19 to 43) and 22 postmenopausal women (age, 71.8±2.3 years; range, 52 to 90) included in this investigation. The coronary arteries of these subjects were classified as normal in 21 cases and atherosclerotic (mean cross-sectional area narrowing, 86.8±5.6%; range, 77% to 98.8%) in 19 cases, based on morphometric examination of light microscopic sections (Table 1). The ages of subjects with normal or atherosclerotic arteries in each menopausal category were not statistically different (Table 1). Contingency table analysis revealed no statistically significant association between menopausal status and the presence or absence of coronary atherosclerosis in the group of patients included in this analysis (Table 1).

**Immunohistochemical Staining**

Positive control tissue, consisting of a breast carcinoma previously shown by radioligand binding assays to express high levels of estrogen receptor, was used in all staining runs.

Fig 1 shows a coronary artery specimen from a premenopausal woman with severe atherosclerosis. Staining with monoclonal antibody to the estrogen receptor was negative. The elastic trichrome stain identifies the anatomic boundaries of the artery, demonstrating severe atherosclerotic narrowing of the native arterial lumen by atherosclerotic plaque.

Fig 1 also shows a severely atherosclerotic artery from a postmenopausal woman stained positively for estrogen receptors. Cells in the medial layer demonstrate blue-black staining consistent with estrogen receptor expression.

Fig 2 shows a coronary artery specimen from a premenopausal woman who died from noncardiac causes. Estrogen receptor expression in this artery is indicated by blue staining of individual cells within the arterial wall. The accompanying elastic trichrome stain of an adjacent tissue section demonstrates that the coronary artery in this case is widely patent. An adjacent section stained with antibody to smooth muscle α-actin stain is also shown and demonstrates the presence of smooth muscle cells both in the intima and the media. The location of the α-actin–positive cells in the media corresponds to the location of the estrogen receptor–positive cells seen above.

Estrogen receptor expression in the coronary arteries of all subjects studied is shown in Table 2. Of the 21 normal arteries studied, 15 (71.4%) of these showed evidence of estrogen receptor expression; in contrast, among 19 atherosclerotic arteries, 13 (68.4%) showed no evidence of estrogen receptor expression when assayed immunohistochemically. Contingency table analysis revealed that differences between these groups were statistically significant (P=.0117).

The association between the presence of estrogen receptor expression and absence of coronary atherosclerosis was highly significant when the results of staining of arteries of premenopausal women were analyzed separately. In 12 normal arteries, estrogen receptor expression was shown immunohistochemically in 10 (83.3%), whereas in 5 of 6 atherosclerotic arteries, no evidence of estrogen receptor expression was observed. The differences between these groups were highly statistically significant, with P=.0062 (Table 2).

**Table 1**. Distribution and Ages of Patients Whose Coronary Arteries Were Included in the Study According to Menopausal Status at the Time of Death and the Presence or Absence of Coronary Atherosclerosis

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Age, y (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Premenopausal</td>
<td>18</td>
<td>31.6±1.9</td>
</tr>
<tr>
<td>Normal</td>
<td>12</td>
<td>31.2±2.1</td>
</tr>
<tr>
<td>Atherosclerotic</td>
<td>6</td>
<td>32.6±4.3</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>22</td>
<td>71.8±2.3</td>
</tr>
<tr>
<td>Normal</td>
<td>9</td>
<td>75.1±5.0</td>
</tr>
<tr>
<td>Atherosclerotic</td>
<td>13</td>
<td>69.4±1.7</td>
</tr>
</tbody>
</table>
When the results of the immunohistochemical staining for estrogen receptor expression in the coronary arteries of postmenopausal subjects were analyzed separately, the impact of estrogen receptor expression was no longer evident (Table 2). In nine normal coronary arteries of postmenopausal female patients, estrogen receptor expression was observed in five (55.6%) and was absent in four (44.4%). Among the atherosclerotic
arteries, 4 of 12 (33.3%) arteries showed evidence of estrogen receptor expression. The differences between these groups did not achieve statistical significance. Thus, in the postmenopausal subjects studied, evidence of estrogen receptor expression was not significantly associated with protection from coronary atherosclerosis, presumably because levels of the ligand (estrogen) are absent or insufficient.

**Radioligand Binding Assays**

Studies using the radioligand binding assay in vascular smooth muscle cell culture were performed on hVSMC outgrowth from normal human arteries. Assay for the estrogen receptor in this system was performed by adapting a technique previously established for receptor assay in breast carcinoma tissue to the assay of estrogen receptor in hVSMC.38,39 As shown in Fig 3, characteristic saturation plots are generated from these studies. The binding of receptors is saturable in the concentration range studied, similar to results obtained of estrogen receptor binding in vascular smooth muscle cell culture of other species.38 The binding curve on the bottom of Fig 3 indicates specific binding of the radiolabeled ligand to estrogen receptors in hVSMC. The binding curve demonstrated here could not be caused by nonspecific interaction of labeled steroid because this is controlled for by the series of experiments performed in the presence of 200-fold excess unlabeled estradiol. From these same data, the precise quantity of estrogen receptors is calculated to equal $1.16 \times 10^{-13}$ mol/L per $10^6$ hVSMC. These data also represent a high-affinity estrogen receptor, as is indicated by the calculated Kd of $8 \times 10^{-9}$. These data represent the first verification of high-affinity estrogen receptors in human vascular smooth muscle tissue.

**Gel Retardation Assay**

Further evidence of the presence of functional estrogen receptors in hVSMC derived from normal human arteries is provided by the gel shift assays, an example of which is shown in Fig 4. In the presence of estradiol, whole-cell extract from hVSMC, and a labeled oligonucleotide probe containing the ERE, a complex is formed between the estrogen receptor and the ERE. This complex is identified by the band in the first lane of the gel, which results from the retarded migration of the labeled ERE through the gel when it binds to the receptor protein present in the cell extract. All negative controls show no evidence of this mobility shift. Thus, both the ligand binding assays in intact cells and the assay for functional receptors in cell extracts confirm the presence of estrogen receptors in hVSMC.

**Immunocytochemistry**

Staining of cultured hVSMC with the ER-21 antibody to human estrogen receptors is depicted in Fig 5.

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**TABLE 2. Results of Immunostaining for Estrogen Receptors in Coronary Arteries: Distribution of Coronary Arteries Examined According to Presence or Absence of Atherosclerosis and Immunohistological Evidence of Estrogen Receptors**

<table>
<thead>
<tr>
<th>Estrogen receptors</th>
<th>Normal</th>
<th>Atherosclerotic</th>
<th>Normal</th>
<th>Atherosclerotic</th>
<th>Normal</th>
<th>Atherosclerotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>15</td>
<td>6</td>
<td>10</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Negative</td>
<td>6</td>
<td>13</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>8</td>
</tr>
</tbody>
</table>

*P = .0117*

*Significant association between estrogen receptor expression and absence of coronary atherosclerosis.

†No statistical relation between estrogen receptor expression and the presence or absence of coronary atherosclerosis.

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**Fig 3. Radioligand binding assay: Binding curves generated by incubation of cultured human vascular smooth muscle cell with $^3$H-estradiol. Top, Specifically bound labeled estradiol (calculated by subtracting binding that occurred in the presence of unlabeled 200-fold excess estradiol from $^3$H-estradiol binding in cells incubated with only labeled hormone) is plotted against the concentration of labeled estradiol. The equation generating the rectangular hyperbola is $Y=A \times X/(B+X)$, where $A=8\text{max}$ and $B=K_d$. Bottom, Scatchard plot of $^3$H-estradiol binding in human vascular smooth muscle cells.**
of these women, obviating any potentially protective effect of the receptor alone.

Two additional independent techniques were used to identify the expression of estrogen receptors in human vascular smooth muscle cells. The radioligand binding assays, performed on intact cells, and the gel retardation assays, performed on extracts from smooth muscle cells, both confirmed the presence of functional estrogen receptors.

In sum, these studies present data confirming the expression of estrogen receptors by human vascular smooth muscle cells and suggest an association between diminished receptor expression and the occurrence of premature coronary atherosclerosis in premenopausal women.

There were several confounding issues raised by the initial immunohistochemistry. Although the evidence of positive staining of arterial tissue was consistently observed, the staining pattern was not always typical of that seen in other tissues studied by this technique, particularly breast cancer. This may be due in part to the well-known difficulty in detecting the estrogen receptor immunohistochemically in specimens stored in formalin and may be compounded by the fact that the majority of the specimens used in this study had been stored for many years. The pattern of staining seen in some specimens raised questions as to the precise location of estrogen receptors in coronary arteries.

In addition, this immunohistochemical method is qualitative, making a precise quantification of receptor expression impossible. The positive control tissue used in all cases was a sample of breast carcinoma, which had been independently shown to be expressing estrogen receptors at high levels. This raised the possibility that tissues defined as "estrogen receptor negative" by this technique may have been expressing estrogen receptors at some level lower than that of the control tissue and therefore were arbitrarily defined as negative. Furthermore, the possibility of a threshold level of estrogen receptor expression necessary for atheroprotection could legitimately be raised by these findings.

To respond to these concerns and to provide clear evidence of estrogen receptors in human vascular tissue, the next phase of this investigation was performed with the goal of confirming estrogen receptor expression in human vascular tissue. A method of quantifying estrogen receptor expression in hVSMC in culture was developed for this purpose. We could not rely on autopsy tissue because functional estrogen receptor assays require fresh or frozen tissue. Radioligand binding assays and gel shift assays confirmed estrogen receptor expression in hVSMC. These findings are compatible with the interpretation of the immunohistochemical data demonstrating diminished rather than absent estrogen receptor expression in atherosclerotic arteries.

Estrogen, as is the case with all steroids, acts by binding to a specific receptor. Establishing a direct, genomically mediated mechanism of estrogen action on the vessel wall therefore required demonstration of specific estrogen receptor in the target tissue. Estrogen receptors have been demonstrated in canine peripheral and coronary arteries, in cultured rat aortic vascular smooth muscle cells, and in human vascular endothelial cells. The present study confirms the existence of these receptors in hVSMC, rendering a direct

![Figure 4](http://circ.ahajournals.org/). Gel retardation assay. Band in left lane corresponds to complex of estrogen receptor and labeled estrogen response element. Controls included exclusion of labeled oligonucleotide probe, exclusion of estradiol (rendering receptor inactive), addition of excess unlabeled oligonucleotide (which competes for binding to the estrogen receptor preventing binding of labeled probe), and exclusion of cell extract. All controls are appropriately negative, indicated by the absence of the band seen in lane 1. E<sub>2</sub> indicates estradiol; LABEL, labeled oligonucleotide probe; WCE, whole-cell extract; and XS UN-LAB, excess unlabeled oligonucleotide probe.

Positively stained cells are shown with negative control cells lightly counterstained with hematoxylin (they would otherwise be undetectable by light microscopy). Thus, using a secondary antibody to human estrogen receptors, clear antigenic evidence of receptor expression by hVSMC is present.

**Discussion**

In this study, we have demonstrated evidence for estrogen receptor expression in the smooth muscle cells of human coronary arteries. Immunohistochemical staining for the estrogen receptor demonstrated positively stained cells both above and below the internal elastic lamina that were associated with cells also staining positively for human smooth muscle a-actin.

Furthermore, immunohistochemical staining in premenopausal and postmenopausal women indicates differential estrogen receptor expression in normal versus atherosclerotic arteries. Specifically, atherosclerotic arteries of premenopausal women demonstrated distinctly diminished expression of estrogen receptors compared with normal arteries of premenopausal women. A strong association between diminished immunohistochemical evidence of estrogen receptors in the presence of atherosclerosis in these premenopausal women is thus demonstrated.

While immunological evidence for estrogen receptor expression is variable among the postmenopausal arteries we studied, no statistically significant relation was observed between the presence or absence of atherosclerosis and estrogen receptor expression in the postmenopausal population. The absence of any such relation could be explained by the estrogen-deficient state...
action of estrogen on arterial biology possible. While the mechanism(s) of a direct genomic effect of estrogen remain to be elucidated, this investigation provides evidence that such an effect of estrogen on vascular smooth muscle cell biology is possible and may account for the “non-lipid-mediated” atheroprotective effect of estrogen.

Acknowledgments
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