Hormone therapy has been associated with reduced risk of coronary heart disease events in observational studies of healthy postmenopausal women (1). Although favorable effects of estrogen therapy on lipoproteins may account for much of this apparent benefit, direct vascular effects may also be of importance, including increased nitric oxide bioactivity, enhanced fibrinolysis and reduction in levels of soluble cell adhesion molecules (2-4). However, levels of C-reactive protein (CRP) are increased with hormone therapy in healthy postmenopausal women suggesting that estrogen may initiate or aggravate inflammation (5-8) that in some women could contribute to the progression and clinical expression of atherosclerosis.

The Heart and Estrogen/progestin Replacement Study (HERS) is the first randomized, double-blind, placebo-controlled clinical trial testing the potential cardiovascular benefit of hormone therapy to be completed in postmenopausal women (9). This secondary prevention trial randomized 2,763 postmenopausal women with coronary artery disease (CAD) to conjugated equine estrogens (CEE) 0.625 mg combined with medroxyprogesterone acetate (MPA) 2.5 mg daily, or placebo daily, in addition to conventional management. After an average follow-up of 4.1 years, there were no significant differences between hormone therapy and placebo treatment groups in the primary outcomes of nonfatal myocardial infarction (MI) and coronary heart disease death. However, there was a statistically significant time-trend of more coronary heart disease events—especially MI—in the hormone therapy group than there was in the placebo group during the first year after randomization, but there were fewer events in years 4 and 5 of treatment. In a preliminary report of 2,245 postmenopausal women in the Nurses’ Health Study cohort with CAD, a two-fold increased risk of MI or coronary
heart disease death was observed within the first year after initiation of hormone therapy in women with prior MI compared with nonusers of hormones (10). Almost 30 years ago a similar observation was made in the Coronary Drug Project of men with previous MI; those men randomized to CEE therapy had a significantly increased MI event rate early after randomization, resulting in early termination of the CEE 5 mg treatment arm of the clinical trial (11).

Pathological studies have shown that MI is commonly associated with rupture of the fibrous caps of atherosclerotic plaques, exposing the thrombogenic lipid-rich contents of the plaque to blood (12–14). Inflammatory cells and activated smooth muscle cells commonly associated with atheromatous plaques secrete matrix metalloproteinases (MMP) (15). After activation by plasmin and other enzymes, MMPs may digest the matrix proteins of the fibrous cap, thus weakening the cap and predisposing it to rupture with thrombus formation (16). In this regard, increased expression of enzymatically active MMPs has been detected in human atherosclerotic plaques (17–19). Further, increased levels of MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in serum have been reported in CAD patients with unstable angina or acute MI (20).

Because hormone therapy has been associated with increased levels of CRP in healthy postmenopausal women, which could indicate activation of inflammatory cells, this study was designed to assess the effect of hormone therapy on markers of inflammation, including MMP expression, in postmenopausal women with CAD who were on appropriate medical management.

**METHODS**

Ten women (average age 66 years; range 59 to 76 years) with angiographically documented CAD (>70% stenosis of at least 1 coronary artery at the time of diagnostic cardiac catheterization) and left ventricular ejection fractions ≥40% were enrolled in this study. All patients were Canadian Cardiovascular Society functional class I or II and remained on medical management (including aspirin [9], statin lipid-lowering drugs [7], calcium channel blocking agent [5], beta-adrenergic blocking agents [5], angiotensin-converting enzyme inhibitors [3] and chronic nitrates [1]) during the entire study. Two women were treated with oral hypoglycemic drugs for adult-onset diabetes mellitus. In this double-blind study, patients were randomized to CEE 0.625 mg daily (combined with MPA 2.5 mg daily in five women with uterus intact) or identical placebo(s) daily, each for one month, with one month off of investigational therapy before crossover to the alternate therapy. This protocol was approved by the Institutional Review Board of the National Heart, Lung and Blood Institute, and informed written consent was obtained from all study participants.

**Laboratory assays.** Blood samples for laboratory assays were obtained between 8 and 9 AM after overnight fasting and approximately 1 h after taking morning medications and study drugs. Samples were immediately coded so that investigators performing laboratory assays were blinded to subject identity and study sequence. Estrone and 17β-estradiol levels were measured in serum by radioimmunoassay in order to determine compliance with study drugs. Total cholesterol and glycerol-blanket triglycerides in the serum were quantified by automated enzymatic techniques. Serum high-density lipoprotein cholesterol was quantified after dextran sulfate precipitation of other lipoproteins. Serum low-density lipoprotein cholesterol levels were directly quantified by an immunoabsorption method. The following markers of inflammation were measured in serum in batches with duplicate samples: CRP (two site chemiluminescent enzyme immunometric assay, sensitivity: 0.01 mg/dL; Immulite, DPC, Los Angeles, California), interleukin-6 (IL-6; ELISA; R&D Systems, Minneapolis, Minnesota) and the cell adhesion molecules E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) (ELISA; R&D Systems, Inc., Minneapolis, Minnesota). Plasminogen activator inhibitor-1 (PAI-1) antigen levels in plasma were determined by sandwich ELISA assay (Biopool, Ventura, California).

Total MMP-9 (gelatinase B, proenzyme and active enzyme bound to inhibitor) was measured in serum with duplicate samples by sandwich ELISA (Quantikine, R&D Systems, Minneapolis, Minnesota). Plasma samples were collected in EDTA tubes (necessary for PAI-1 determination) and, thus, could not be assayed for MMP because of interference by EDTA with the assay. Additionally, MMP-2 and MMP-9 were analyzed by zymography, with determination of the digestion of gelatin in polyacrylamide gels by serum samples, as previously described (21). Serum aliquots (0.5 μL for MMP-2 and 1.0 μL for MMP-9) were added to 10 μL loading buffer and loaded on 10% polyacrylamide gels (Novex) containing 0.1% gelatin. After electrophoresis, gels were incubated in 0.05 mol/L Tris-HCl, pH 7.5, containing 0.2 mol/L NaCl, 5 mmol/L CaCl₂ and 2.5% Triton X-100, for 30 to 60 min at room temperature and subsequently incubated for 16 h at 37°C in the same buffer without Triton X-100. The gels were then stained with Coomassie blue (0.25% Coomassie blue/45.4%...
methanol/9.2% glacial acetic acid) and destained (75% ethanol/25% glacial acetic acid). Clear zones against the blue background indicated the presence of proteolytic activity. Renaturation of MMP-2 and MMP-9 with Triton X-100 allows both latent and active forms to be detected. The zymography gels were scanned by a ScanMaker 5 (Microtek International, Inc., Hsinchu, Taiwan) using Adobe Photoshop (Adobe Systems Inc., Mountain View, California). Bands on the scan were analyzed by the Macintosh version of Image, and the relative densitometric areas were calculated by integrating the area under each peak. Areas are expressed as arbitrary units.

**Statistical analysis.** Data are expressed as means ± standard deviation. After testing for normality, Student paired t test (E-selectin, ICAM-1, VCAM-1, MMP-9, PAI-1) or Wilcoxon signed rank test (CRP, IL-6) was used to compare values before and after each therapy. Pearson correlation coefficient analysis was used to assess associations between measured parameters. p values <0.05 were accepted as statistically significant.

**RESULTS**

Compliance with study medications was excellent, as manifested by increases in serum estrone and 17β-estradiol levels in all women during the hormone treatment phase of the study (Table 1). Hormone replacement therapy lowered levels of total cholesterol and low-density lipoprotein cholesterol by approximately 10% and increased levels of high-density lipoprotein cholesterol and apolipoprotein (A-1) each by approximately 10%. Triglycerides were unchanged with hormone therapy compared with placebo values.

Assay values for markers of inflammation are provided in Table 2. C-reactive protein and IL-6 values in patients on placebo were highly correlated (r = 0.928, p < 0.001). However, there was no correlation between levels of MMP-9 and CRP (r = 0.135, p = 0.710) or IL-6 (r = 0.207, p = 0.566) on placebo. Hormone therapy did not significantly increase levels of CRP and IL-6 in serum but significantly lowered PAI-1 levels in plasma compared with placebo values. Hormone therapy significantly reduced levels of the cell adhesion molecules E-selectin, ICAM-1 and VCAM-1 (Fig. 1), with similar magnitude of reduction from placebo values in women on CEE alone compared with women on combination hormone therapy (E-estradiol 20% vs. 13%, ICAM-1 7% vs. 7%; VCAM-1 8% vs. 9%).

Hormone therapy significantly increased serum MMP-9 compared with placebo (Table 2), with higher levels of MMP-9 in 9 of 10 women compared with respective placebo values (Fig. 2). Increases in MMP-9 levels on hormone therapy appeared to be similar in women on CEE alone compared with women on combination hormone therapy. There was no correlation between the placebo MMP-9 levels and the increase in MMP-9 on hormone therapy relative to placebo (r = 0.492, p = 0.149). The only patient (D) who had lower levels of MMP-9 on hormone therapy had increases in CRP (0.77 to 1.03 mg/dL) and IL-6 (2.67 to 3.45 pg/mL) compared with placebo values. For the group, there was no correlation between the increases in CRP and in MMP-9 on hormone therapy relative to placebo (r = 0.150, p = 0.680) or the increases

**Table 1. Effects of Hormone Therapy on Hormone and Lipid Values**

<table>
<thead>
<tr>
<th>Lipid Values</th>
<th>Placebo</th>
<th>Hormone Therapy</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone (pg/mL)</td>
<td>22 ± 17</td>
<td>98 ± 59</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>15 ± 8</td>
<td>50 ± 24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lipids (mg/dL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>220 ± 38</td>
<td>202 ± 52</td>
<td>0.337</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>133 ± 32</td>
<td>121 ± 50</td>
<td>0.364</td>
</tr>
<tr>
<td>Apolipoprotein B</td>
<td>121 ± 24</td>
<td>119 ± 39</td>
<td>0.863</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>41 ± 6</td>
<td>46 ± 13</td>
<td>0.097</td>
</tr>
<tr>
<td>Apolipoprotein A-I</td>
<td>124 ± 16</td>
<td>136 ± 14</td>
<td>0.010</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>249 ± 275</td>
<td>215 ± 146</td>
<td>0.622</td>
</tr>
</tbody>
</table>

HDL = high-density lipoprotein; LDL = low-density lipoprotein.

**Table 2. Effects of Hormone Therapy on Markers of Inflammation and MMP-9 Expression in Serum**

<table>
<thead>
<tr>
<th>Markers of inflammation</th>
<th>Placebo</th>
<th>Hormone Therapy</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-reactive protein (mg/dL)</td>
<td>0.61 ± 0.50</td>
<td>0.88 ± 1.13</td>
<td>0.358</td>
</tr>
<tr>
<td>Interleukin-6 (pg/mL)</td>
<td>3.04 ± 1.47</td>
<td>4.33 ± 4.78</td>
<td>0.283</td>
</tr>
<tr>
<td>E-selectin (ng/mL)</td>
<td>56.3 ± 20.6</td>
<td>46.9 ± 18.3</td>
<td>0.006</td>
</tr>
<tr>
<td>ICAM-1 (ng/mL)</td>
<td>304 ± 78</td>
<td>282 ± 74</td>
<td>0.013</td>
</tr>
<tr>
<td>VCAM-1 (ng/mL)</td>
<td>657 ± 214</td>
<td>605 ± 218</td>
<td>0.010</td>
</tr>
<tr>
<td>MMP-9 (ng/mL)</td>
<td>501 ± 285</td>
<td>648 ± 349</td>
<td>0.020</td>
</tr>
<tr>
<td>Gelatinolytic activity</td>
<td>1,250 ± 644</td>
<td>1,517 ± 748</td>
<td>0.036</td>
</tr>
<tr>
<td>(densitometric U)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI-1 (ng/mL)</td>
<td>42.3 ± 26.8</td>
<td>28.5 ± 21.0</td>
<td>0.030</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation.

ICAM-1 = intercellular adhesion molecule-1; MMP-9 = matrix metalloproteinase-9; PAI-1 = plasminogen activator inhibitor-1; VCAM-1 = vascular cell adhesion molecule-1.
in IL-6 and in MMP-9 on hormone therapy relative to placebo \((r = -0.246, p = 0.494)\).

By zymography, proteolysis of gelatin by 92 kDa MMP-9 was greater in serum samples from the hormone therapy phase relative to placebo \((1,517 \pm 748 \text{ vs. } 1,250 \pm 644 \text{ densitometric U, } p = 0.036)\), with greater gelatinolysis in 8 of 10 women on hormone therapy than on placebo (Fig. 3), including eight of nine women who had higher serum MMP-9 levels, as measured by ELISA on hormone therapy compared with placebo values (Fig. 2).

By zymography, proteolysis of gelatin by the 72 kDa MMP-2 was similar on placebo \((956 \pm 228 \text{ densitometric U})\) and hormone therapy \((880 \pm 245 \text{ densitometric U})\), and no patient had higher MMP-2 levels on hormone therapy relative to placebo values.

**DISCUSSION**

We have previously reported that hormone replacement therapy significantly reduces serum levels of cell adhesion molecules in healthy postmenopausal women who were not taking other medications at the time of their study (4). We found in this study that hormone replacement therapy significantly reduced levels of the three cell adhesion molecules—E-selectin, ICAM-1, VCAM-1—measured in our study relative to placebo values in women with CAD, despite continued medical management with drugs previously shown to reduce cardiovascular risk (aspirin, statin lipid-lowering therapy, angiotensin-converting enzyme inhibitors). Serum concentrations of cell adhesion molecules have been reported to be higher for patients with CAD than in healthy subjects (22). Although the biological function in serum remains unclear, the clinical relevance of cell adhesion molecules has been suggested by several observational studies. Thus, E-selectin, ICAM-1 and VCAM-1 have been demonstrated in human atherosclerotic arteries by immunohistochemistry (23, 24). In the Atherosclerosis Risk in Communities (ARIC) study (25), higher serum levels of E-selectin and ICAM-1 were found in patients with CAD and carotid artery atherosclerosis than in healthy control subjects; E-selectin levels correlated positively with the carotid artery thickness measured by ultrasound in this study. Rohde et al. (26) reported significant correlations between ICAM-1 and VCAM-1 levels and carotid artery intimal–medial thickness in 92 men and women referred to their echocardiography laboratory. Men in the Physician’s Health Study (27) with the highest quartile of ICAM-1 levels were found to be at greater cardiovascular risk than men in the lowest quartile. Our findings are consistent with the observation of Caulin-Glaser and coworkers (28) who found higher serum levels of E-selectin, ICAM-1 and VCAM-1 in postmenopausal women with CAD not on hormone therapy than postmenopausal women with CAD on hormone therapy at the time of cardiac catheterization.

Hormone therapy and MMP. In this study hormone therapy increased serum levels of MMP-9 in women with CAD, as determined by monoclonal antibody immunoassay and by zymography. By these independent assays, the majority of women had higher MMP-9 levels on hormone therapy than on placebo, whether on CEE alone or on CEE combined with MPA. This effect of hormone therapy was seen despite continuation of medications shown to reduce..
cardiovascular risk for patients with CAD. Levels of CRP and IL-6, nonspecific markers of inflammation associated with atherosclerosis and increased cardiovascular risk (29–33), were slightly increased on hormone therapy, consistent with recent reports performed in healthy postmenopausal women (5–8) suggesting that estrogen may have initiated or aggravated inflammation in our study population with CAD. Increased vascular inflammation could increase synthesis of MMP-9 by inflammatory cells or activated smooth muscle cells within the vessel wall. However, there was no correlation between the relative increases in CRP or in IL-6 and in MMP-9 to support a proinflammatory effect of estrogen as a stimulus for increased MMP-9 expression.

**Mechanism of increased MMP-9 expression.** The increase in MMP-9 during hormone therapy is compatible with direct enhancement of enzyme synthesis by estrogen. Although classical estrogen response elements have not been identified in the promoter regions of MMPs, several MMPs, including MMP-9, have consensus sequences for activator protein-1 (AP-1) sites (34–37). Estrogen bound to its receptor (especially ERα [38]) activates transcription of target genes under control of AP-1 response elements by binding to the Jun-Fos heterodimer of AP-1, facilitating activation of the AP-1 response element with enhanced transcription (38–40). Of interest, MMP-2, which does not have an AP-1 site in its promoter region, was not altered by estrogen treatment in our study as measured by zymography. In this regard our findings differ from the report of Wingrove et al. (41) in which human vascular smooth muscle cells in culture were found to increase the synthesis of MMP-2 at physiological concentrations of 17β-estradiol.

**Study limitations.** A limitation of this study is that the increase in serum levels of MMP-9 in our study participants on hormone therapy does not prove increased enzymatic MMP activity on matrix proteins within the atherosclerotic plaque. Indeed, the MMP-9 measured by zymography in our study was in the 92 kDa proenzyme form and likely bound to inhibitors (TIMP-1, α2-macroglobulin). However, Galis et al. (17) have shown that MMPs in human atherosclerotic plaques have gelatinase activity, as assessed by in situ zymography, which suggests that local synthesis of MMPs within the plaque may exceed the local synthesis of inhibitor proteins.

**Potential for metalloproteinase activation.** Hormone therapy in postmenopausal women may also provide a mechanism for activation of MMPs in the vessel wall. We have previously shown that hormone therapy reduces plasma levels of PAI-1, a principal inhibitor of plasminogen activators (3). This results in increased plasmin activity, as evidenced by proportionate increases in serum levels of D-dimer, the breakdown product of cross-linked fibrin enzymatically digested by plasmin. Plasmin also activates MMPs by converting the inactive zymogen form of the enzyme to the active proteolytic form (42–44). Similar to the effect of hormone therapy previously shown in healthy postmenopausal women (40), plasma levels of PAI-1 were reduced in women with CAD in this study. Accordingly, the combination of increased expression of MMP-9 and the potential for increased plasmin-mediated activation of MMP-9 in women with vulnerable plaques could result in the digestion of matrix proteins that comprise the fibrous cap, with plaque rupture and thrombosis.

**Acknowledgments**

The authors wish to thank Londa Hathaway, RN, and Rita Mincemoyer, RN, for their assistance in the conduct of the clinical trial; Rene Costello, MT, for his excellent technical assistance and Toni Julia for typing the manuscript.

**Reprint requests and correspondence:** Dr. Richard O. Cannon, III, National Institutes of Health, Building 10, Room 7B15, 10 Center Drive MSC-1650, Bethesda, Maryland 20892-1650. E-mail: cannonr@nih.gov.

**REFERENCES**