Raloxifene-Mediated Increase in Matrix Metalloproteinase-1 Production by Activated Monocytes

Jeanette A. Ardans, Arnon Blum, Paul R. Mangan, Shlomo Wientroub, Richard O. Cannon III, Larry M. Wahl

Abstract—Matrix metalloproteinases (MMPs), proteolytic enzymes produced by monocytes, may contribute to atherosclerotic arterial wall remodeling and to plaque rupture. Because estrogen influences the synthesis of MMPs, we examined the effect of raloxifene, a selective estrogen receptor modulator, on monocyte MMP production. Human primary blood monocytes treated with raloxifene (10 μmol/L) in the presence of lipopolysaccharide (LPS) or tumor necrosis factor-α and granulocyte-macrophage colony–stimulating factor induced a 2- to 3-fold increase in MMP-1 production by monocytes. The enhancement of MMP-1 production by raloxifene in LPS-activated monocytes occurred through a cyclooxygenase-2– and prostaglandin E2–independent mechanism. Additionally, compared with monocytes acquired during the placebo phase, peripheral blood monocytes from 5 of 6 healthy postmenopausal women treated with raloxifene (60 mg daily for 1 month) in a clinical trial produced significantly higher levels of MMP-1 when the monocytes were activated with LPS. Furthermore, serum obtained during the raloxifene phase from 4 of these subjects, when added to control monocytes, significantly enhanced LPS-induced MMP-1 production compared with that from serum obtained during the placebo phase. In summary, raloxifene increases the production of MMP-1 in activated monocytes; this effect may be favorable in atherosclerotic arterial wall remodeling but unfavorable for plaque stability.

Key Words: atherosclerosis ■ matrix metalloproteinases ■ monocytes ■ plaque ■ raloxifene

Monocytes/macrophages, prominent cells in inflammatory sites that are associated with connective tissue turnover, are abundant in atherosclerotic plaques. Monocytes/macrophages at these sites colocalize with matrix metalloproteinases (MMPs), proteolytic enzymes produced by monocytes/macrophages that are capable of degrading all extracellular matrix components. MMPs may contribute to beneficial atherosclerotic arterial wall remodeling as well as to the degradation of the connective tissue framework in the plaque, thus leading to rupture and subsequent ischemic events.

Raloxifene, a selective estrogen receptor modulator that is currently under investigation as an alternative to hormone replacement therapy, may be useful in the prevention of coronary artery disease in postmenopausal women and in reducing the risk of breast cancer without increasing the risk of endometrial cancer. We have recently reported that oral estrogen replacement therapy increases levels of serum MMP-9 when administered to postmenopausal women with coronary artery disease. Moreover, estrogen has been reported to increase MMP-2 production by smooth muscle cells. Because raloxifene shares some biological properties of estrogen, we examined how raloxifene might influence the production of MMPs by monocytes.

Methods

Purification of Human Monocytes

Human peripheral blood monocytes were obtained from normal female volunteers by leukapheresis, as previously described, and from healthy postmenopausal women participating in a clinical trial (approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute) testing the effects of raloxifene versus placebo on risk factors for atherosclerosis. Informed written consent was obtained from all subjects.

Culture Conditions

Purified monocytes were cultured in serum-free, phenol red–free DMEM (BioWhittaker) supplemented with 2 mmol/L L-glutamine (Mediatech) and 10 μg/mL gentamicin sulfate (BioWhittaker). Purified monocytes were plated at 5×10⁶/mL DMEM in 12-well plates or at 20×10⁶/4 mL DMEM in 60-mm plates (Falcon, Becton Dickinson) and adhered for 30 minutes at 37°C before reagents were added.

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added. Raloxifene (Eli Lily) was added to cultures for 4 hours at 37°C before stimulation with lipopolysaccharide (LPS [Escherichia coli 055:B5], 200 ng/mL, Difco) or tumor necrosis factor (TNF-α (50 ng/mL, PeproTech) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 50 ng/mL, PeproTech). Culture supernatants were then collected 24 or 36 to 48 hours after LPS stimulation for cyclooxygenase (COX)-2 and MMP-1/MMP-9 analysis, respectively, and 36 to 48 hours after cytokine stimulation for MMP-1 analysis. Each experiment was repeated a minimum of 3 times with different female donors.

### Laboratory Assays
Proteins in the conditioned medium from 3 independent experiments were analyzed by Western blot for MMP-1 or tissue inhibitor of MMP (TIMP)-1 with peptide-specific antibodies (generously provided by Dr Henning Birkedal-Hansen, National Institute of Dental and Craniofacial Research, Bethesda, Md) or by zymography for MMP-9 detection, as previously described. Additionally, as previously detailed, COX-2 membrane protein was measured from 3 independent experiments by Western blot using an antibody against COX-2 (Cayman Chemical). Aliquots from these experiments were analyzed for prostaglandin E2 (PGE2) production by ELISA (Neogen Corp) according to the manufacturer’s suggestions.

### Raloxifene Study in Postmenopausal Women
In the present double-blind study, 6 subjects were randomized to raloxifene (60 mg) or placebo daily for 1 month, with 1 month off therapy before crossover to the alternate therapy. Peripheral blood mononuclear cells (PBMCs) and serum were isolated from each subject 1 hour after ingestion of either raloxifene or placebo at the end of each treatment period. PBMCs were plated at an equivalent of 2 × 10^6 monocytes/mL. DMEM, stimulated with 200 ng/mL LPS, and incubated for 36 to 48 hours at 37°C. Supernatants were collected, frozen, and analyzed for MMP-1 production by ELISA (Oncogene Research Products) according to the manufacturer’s suggestions. Subject sera, collected at the same time as the PBMCs and frozen until later use, were added to control monocytes obtained from a normal female volunteer at an equivalent of 5 × 10^5 monocytes/mL, serum in polypropylene tubes and then incubated for 18 hours at 37°C. Cultures were washed 3 times with serum-free, phenol red–free DMEM, plated in 12-well plates (in 1 mL DMEM), adhered at 37°C. Cultures were washed 3 times with serum-free, phenol red–free DMEM, plated in 12-well plates (in 1 mL DMEM), adhered for 30 minutes at 37°C, and stimulated with LPS. Supernatants were collected 36 to 48 hours after stimulation and analyzed in duplicate by ELISA for MMP-1 production.

### Results

#### Raloxifene Enhances MMP-1 Production by Activated Monocytes
Although raloxifene at concentrations of 0.1 to 1 μmol/L in the presence or absence of LPS had no effect on MMP-1 or MMP-9 production by monocytes, raloxifene at 10 μmol/L induced a 2- to 3-fold increase in MMP-1 production by LPS-activated monocytes, whereas MMP-9 was unaffected (Figure 1A). Raloxifene had no effect on TIMP-1 production in the presence or absence of LPS (Figure 1A). Additionally, raloxifene (0.1 to 1 μmol/L) in the presence or absence of TNF-α and GM-CSF had no effect on MMP-1 production; however, 10 μmol/L raloxifene in the presence of TNF-α and GM-CSF induced a 2- to 3-fold increase in MMP-1 (Figure 2).

#### Regulation of Raloxifene-Induced MMP-1 Production by LPS-Activated Monocytes Is COX-2 and PGE2 Independent
Because COX-2 can mediate the release of PGE2, which regulates MMP production, we evaluated the effect of raloxifene on COX-2 and PGE2 production. Raloxifene alone or in combination with LPS had no effect on COX-2 production (Figure 1B) or PGE2 levels (Figure 1C).

### Discussion
The findings in the present study demonstrate that monocytes exposed to raloxifene in vitro or in vivo produce significantly...
Numerous factors regulate MMP production, including hormones, growth factors, and cytokines\textsuperscript{12}; therefore, we evaluated the effects of raloxifene on MMP production. Our data demonstrate that the interaction of raloxifene with monocytes in vitro is not sufficient to induce MMP-1 or MMP-9 production; however, 10 \textmu mol/L raloxifene in the presence of LPS significantly enhances monocyte MMP-1 production by 2- to 3-fold, whereas MMP-9 is not affected (Figure 1A). Interestingly, although LPS has been used as a classic monocyte stimulant, we also show that 10 \textmu mol/L raloxifene in the presence of TNF-\alpha and GM-CSF, a specific cytokine combination that has previously been shown to induce MMP-1 by monocytes,\textsuperscript{8} likewise enhances MMP-1 production (Figure 2). Compared with LPS, the cytokines may be more site specific and pathophysiologically relevant in vivo factors because TNF-\alpha has been isolated from human atherosclerotic plaques\textsuperscript{2} and because in vitro interactions between monocytes and endothelial cells result in the production of GM-CSF.\textsuperscript{13} Finally, although estrogen (60 mg daily) would never be used therapeutically, when 10 \textmu mol/L 17\beta-estradiol was added to monocytes in the presence of LPS, a 2- to 3-fold induction of MMP-1, similar to that of raloxifene, was observed (data not shown). This observation may provide insights into the mechanism of raloxifene-induced MMP-1 production by activated monocytes.

Similar to results obtained in our in vitro culture model, we observed that compared with LPS-activated PBMCs obtained during the placebo phase, PBMCs obtained from 5 of 6 postmenopausal women who were treated with raloxifene (60 mg daily) produced significantly more MMP-1 when the PBMCs were activated with LPS (Figure 3A). In addition, monocytes obtained from a normal female donor that were exposed to the serum from raloxifene-treated women produced significantly more MMP-1, after exposure to 4 of the 6 sera, when activated than did monocytes treated with serum acquired during the placebo phase (Figure 3B). The concentration of raloxifene required to stimulate MMP-1 production in monocytes from healthy female donors was substantially higher than the peak plasma concentrations reported in healthy volunteers on raloxifene therapy.\textsuperscript{14,15} However, if PBMCs sequester raloxifene or if albumin and other plasma proteins bind raloxifene in vivo, then the plasma levels reported in healthy volunteers taking raloxifene may have significantly underestimated the quantity of bioavailable raloxifene in plasma.\textsuperscript{15} Our data obtained from the postmenopausal women, although limited in number in this initial study, corroborate the in vitro findings and warrant further study.

MMP enzymatic activity is regulated, in part, by TIMPs; thus, the ratio of MMP-1 to TIMP-1 production indicates the relative enzymatic activity of MMP-1. We show that raloxifene had no effect on TIMP-1 production in the presence or absence of LPS (Figure 1A); therefore, the net effect of MMP-1 to TIMP-1 production favors MMP-1 activity and connective tissue degradation.

The production of MMPs is regulated by signaling mechanisms that involve prostaglandin-dependent and -independent mechanisms.\textsuperscript{8,10,11} To evaluate whether raloxifene mediated its effect through a prostaglandin-dependent mechanism, we analyzed COX-2 and PGE\textsubscript{2} production. The findings in the present study demonstrate that the induction of.

Figure 3. PBMCs isolated from healthy postmenopausal women during the raloxifene and placebo phases of the clinical trial were stimulated with LPS, and supernatants were collected 48 hours later. Supernatants were analyzed in duplicate by ELISA for MMP-1 production (A). Serum collected at the same time as the PBMCs, and frozen until later use, was added to monocytes obtained from a normal female volunteer. Cultures were then washed with serum-free DMEM and stimulated with LPS, and supernatants were collected 48 hours later. Supernatants were analyzed in duplicate by ELISA for MMP-1 production (B).

higher levels of MMP-1 when activated than do control monocytes or monocytes from placebo-treated postmenopausal women. We also show that the induction of MMP-1 by raloxifene in LPS-activated monocytes is mediated through a PGE\textsubscript{2}-independent and COX-2–independent mechanism.

The prominence of monocytes/macrophages in atherosclerotic plaques may be pathologically significant in the progression of this disease. Human atherosclerotic plaques prone to rupture are distinguished from stable plaques by the presence of monocyte/macrophage infiltrates in the fibrous cap and shoulder regions of the plaque.\textsuperscript{1} Monocytes/macrophages produce MMPs, a family of proteolytic enzymes that contribute to connective tissue remodeling in normal and inflammatory sites. MMP-1, an interstitial collagenase that degrades collagen I, II, III, VII, VIII, and X, has been isolated in human atherosclerotic plaques\textsuperscript{2} and not in nonatherosclerotic vascular tissues.\textsuperscript{2,3} Recent studies have demonstrated that macrophages and MMP-1 are colocalized in areas of increased collagenolysis within rupture-prone plaques but not in stable plaques.\textsuperscript{2} Cellular and signaling mechanisms that regulate plaque stability remain unclear; however, monocytes/macrophages and MMPs may influence plaque rupture. On the other hand, MMPs may also contribute to favorable arterial remodeling (compensatory enlargement) in early atherosclerosis and to the removal of excess matrix protein in the arterial wall, thus improving compliance.
MMP-1 by raloxifene in LPS-activated monocytes is COX-2 independent (Figure 1B) and PGE$_2$ independent (Figure 1C). Future studies to elucidate the potential mechanism by which raloxifene may increase MMP-1 production in activated monocytes will focus on the effect of raloxifene on the binding of specific transcription factors to the MMP-1 promoter.

In summary, monocytes exposed to raloxifene in vitro or in vivo showed a 2- to 3-fold increase in MMP-1 production when activated. Raloxifene-induced MMP-1 production could be beneficial for atherosclerotic arterial wall remodeling but harmful for the stability of vulnerable plaques.

References
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