Medroxyprogesterone Acetate Enhances Monocyte-Endothelial Interaction Under Flow Conditions by Stimulating the Expression of Cell Adhesion Molecules

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Context: Monocyte adhesion to endothelial cells is an important initial event in atherosclerosis and is partially mediated by adhesion molecule expression on the cell surface. Although estrogens inhibit atherosclerosis development, effects of coadministered progestogen remain controversial.

Objective: We examined the effects of progestogen on cytokine-stimulated human umbilical venous endothelial cell (HUVEC) expression of adhesion molecules.

Design: In HUVECs, adhesion molecule mRNA levels were measured by real-time PCR. Protein expression was quantified by immunocytochemistry and ELISAs. To mimic the monocyte adherence to endothelial cells, we used a flow chamber system to assess progestogen effects on U937 monocytoid cell adherence to HUVEC monolayers. We also examined the suppression effects of adhesion molecules with small interference RNAs.

Results: mRNA levels of adhesion molecules in HUVECs treated with medroxyprogesterone acetate (MPA) or 17β-estradiol + MPA were 1.7- to 2.5-fold higher than those in the control. MPA increased the protein expression of E-selectin, P-selectin, and intercellular adhesion molecule-1 compared with that for the control (83.0 ± 0.7, 34.8 ± 1.2, and 5.4 ± 0.0 ng/mL, respectively), whereas other progestogens or 17β-estradiol additive to progestogens did not significantly change expression. MPA significantly increased U937 monocytoid cell adherence compared with the control (56.0 ± 1.5 vs 46.5 ± 3.5 adherent cells per 10 fields) but did not increase adherence to HUVECs with knocked down intercellular adhesion molecule-1.

Conclusions: MPA increases cell adhesion molecule expression on HUVECs, causing increased numbers of monocytes to adhere to HUVECs. These MPA effects may be a risk factor for atherosclerosis on endothelial cells in postmenopausal women receiving hormone replacement therapy. (J Clin Endocrinol Metab 99: 2188–2197, 2014)

Although the incidence of coronary heart disease (CHD) is lower in premenopausal women than in men, the incidence in postmenopausal women exceeds that in age-matched men with similar risk profiles (1–3). Indeed, estrogen replacement therapy in postmenopausal women exerts a protective effect on the reduction of CHD risk by 50% (4, 5). The Women’s Health Initiative (WHI), the largest randomized prospective controlled trial, was conducted to test the beneficial effect of continuous combined hormone therapy with conjugated equine estrogen (CEE) + medroxyprogesterone acetate (MPA) as a prog-

Abbreviations: AR, androgen receptor; CEE, conjugated equine estrogen; CHD, coronary heart disease; Dex, dexamethasone; DG, dydrogesterone; DHT, dihydrotestosterone; DNG, dienogest; E2, 17β-estradiol; EIA, enzyme immunoassay; ER, estrogen receptor; FBS, fetal bovine serum; GR, glucocorticoid receptor; HBSS, Hank’s balanced salt solution; HF, hydroxyflutamide; HRT, hormone replacement therapy; HUVEC, human umbilical venous endothelial cell; ICAM-1, intercellular adhesion molecule-1; LNG, levonorgestrel; LPS, lipopolysaccharide; MPA, medroxyprogesterone acetate; NETA, norethisterone acetate; P4, natural progesterone; PR, progesterone receptor; RU486, mifepristone; siRNA, small interfering RNA; VCAM-1, vascular cell adhesion molecule-1; WHI, Women’s Health Initiative.
estrogen. However, it was discontinued in 2002 owing to an observed increased risk in coronary events, stroke, and venous thromboembolism as well as breast cancer (6). The other arm of WHI, involved in testing CEE alone for women with prior hysterectomy, was also discontinued owing to an absence of cardioprotective effects, although it reported no observed, significant increase in the incidence of coronary events or breast cancer (7). Although CEE + MPA had been used in a majority of large-scale trials until termination of the WHI, subsequent trials have tested the risk and benefit of estrogen-progestogen therapy with progestogens other than MPA, ie, norethisterone acetate (NETA), levonorgestrel (LNG), dydrogesterone (DG), dienogest (DNG), and natural progesterone (P4). Fournier et al (8) showed in a prospective cohort study of French women that increased the risk of breast cancer was not associated with estrogen + P4 or DG, whereas estrogen alone and estrogen + other types of progestogens, including MPA, were associated with a significant increase in the risk. It is noteworthy that CHD risk according to progestogen type has not yet been demonstrated in large-scale trials. Furthermore, few basic studies have investigated the vascular functions associated with various types of progestogens (9–14).

Monocyte adhesion to endothelial cells is an important event at the onset of atherosclerosis. Adhesion involves the multistep process of rolling of the circulating cells, firm adhesion, and transendothelial cell migration. Such intercellular adhesion requires specific receptor-ligand interactions between adhesion receptors on the monocyte. These correspond with endothelial cell adhesion molecules such as E-selectin, P-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (15–18). Various proteins and chemical compounds such as inflammatory cytokines have been known to regulate the expression of these adhesion molecules (19). Among these compounds, estrogens have been shown to decrease the expression of cell adhesion molecules in endothelial cells (20). Several studies have shown that certain types of progestogen modify the expression of adhesion molecules on human endothelial cells and diversely influence hormone replacement therapy (HRT) with respect to the risk of CHD (9, 11–14). We have previously shown that some types of progestogens increase the expression of cell adhesion molecules in human umbilical vein endothelial cells (HUVECs) (11).

In the present study, we examined the effect of synthetic progestogens, including MPA and P4, which are used for HRT, on the expression of cell adhesion molecules on HUVECs. Elevated monocyte adherence by MPA was observed using a well-defined parallel plate flow chamber under flow conditions that mimic the actual adherence of monocytes to endothelial cells.

Materials and Methods

Steroids

P4, MPA, NETA, LNG, dihydrotestosterone (DHT), mifepristone (RU486), and hydroxyflutamide (HF) were purchased from Sigma-Aldrich. DG was purchased from Tront Research Chemicals Inc. DNG was kindly gifted by Mochida Pharmaceutical Corp. 17β-estradiol (E2) and dexamethasone (Dex) were purchased from Nacalai Tesque, Inc.

Cell cultures and treatments

This protocol has been approved by the Kyoto Prefectural University of Medicine Institutional Review Board. Female infant umbilical cords were obtained from patients who had no endocrine complications and had undergone normal delivery; the samples were transported to the laboratory in PBS without calcium and magnesium. HUVECs were separated using the method described by Jaffe et al (21). The cell suspension was plated in M199 medium (without phenol red) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corp), l-glutamine (2 mM), thymidine (2.4 mg/mL), heparin sodium (10 IU/mL), endothelial cell growth factor (10 ng/mL; Biomedical Technologies Inc), penicillin (100 IU/mL), and streptomycin (100 µg/mL). The cells were cultured at 37°C under a humidified atmosphere of 5% CO2-95% air in 75-cm2 tissue culture flasks until cells reached preconfluence. Cell purity was confirmed by the appearance of typical cobblestone morphology and the presence of von Willebrand factor antigen. Cells were used between passages 1 and 3. The cells were harvested by trypsinization using 0.02% EDTA solution containing 0.05% trypsin (Invitrogen Corp), plated onto six- or 96-well plates and coverslips that had been coated with 0.1% gelatin, and cultured until cells reached preconfluence. The medium was exchanged to MEM (without phenol red) containing 4% charcoal-treated FBS, penicillin (100 IU/mL), streptomycin (100 µg/mL), and the cells were preincubated for 12 hours. The cells were incubated for 24 hours with incubation medium containing recombinant human IL-1β (40 U/mL; Genzyme Corp) and steroids. Each steroid was dissolved in ethanol to a final concentration of 0.1% per well.

Real-time PCR analysis

Total RNA was extracted from the HUVECs using TRIzol Reagent (Invitrogen Corp) according to the manufacturer’s instructions. RNA (1 µg) from each sample was reverse transcribed to cDNA and amplified using ReverTra Ace quantitative PCR reverse transcriptase master mix (TOYOBO). Gene expression levels were analyzed using real-time PCR. The forward and reverse primers for E-selectin, P-selectin, ICAM-1, and VCAM-1 are listed in Supplemental Table 1, published on The Endocrine Society’s Journals Online web site at http://jcem.endojournals.org. The cycle time values obtained were used to quantify the relative expression of the genes of interest. The cycle time values were first normalized to the internal control gene (GAPDH), and then the fold changes of target genes in all the groups were calculated and represented as relative expression values.
Enzyme immunoassay (EIA)

The 96-well plates with HUVEC monolayers were washed twice with PBS containing 1% bovine serum albumin and 0.05% sodium azide (wash buffer) and fixed by addition of 4% paraformaldehyde in PBS for 15 minutes at room temperature. For creating a standard curve, each 96-well plate was coated with diluted Capture antibody (R&D Systems), and standards were added according to the manufacturer’s instructions. After the wells were washed twice with wash buffer, mouse antihuman E-selectin monoclonal antibody (1.0 μg/mL), mouse antihuman P-selectin monoclonal antibody (1.0 μg/mL), mouse antihuman ICAM-1 monoclonal antibody (1.0 μg/mL), mouse antihuman VCAM-1 monoclonal antibody (1.0 μg/mL) (R&D Systems) were added to each plate and incubated for 30 minutes at 37°C. The wells were washed twice with wash buffer, followed by addition of streptavidin-horseradish peroxidase complex (Dako) and incubation for 30 minutes at 37°C. After two washes with wash buffer, o-phenylenediamine dihydrochloride in citric-acid phosphate buffer (pH 5.0) was added and incubated for 30 minutes at 37°C. The wells were washed twice with wash buffer, followed by addition of streptavidin-horseradish peroxidase complex (Dako) and incubation for 30 minutes at 37°C. After two washes with wash buffer, o-phenylenediamine dihydrochloride in citric-acid phosphate buffer (pH 5.0) was added and reacted for 5 minutes at room temperature. The reaction was stopped by the addition of 1 M sulfuric acid. Absorbance was measured at 490 nm with an ELISA reader (model 550; Bio-Rad Laboratories).

Immunocytochemistry

After HUVECs grown on glass slides were treated with 4% paraformaldehyde, the slides were incubated first with 0.1 M glycine in PBS and then with 0.1% Triton X-100 in PBS. The slides were washed, incubated with 5% swine serum in PBS for 1 hour, diluted 1:200 with either antibody against human E-selectin (Santa Cruz) or human ICAM-1 (Santa Cruz Biotechnology Inc), and incubated with biotinylated antimouse IgG antibody. The slides were incubated with the standard labeled streptavidin-antibody biotin kit (Dako), washed in carbonate buffer, developed in 3,3’-diaminobenzidine hydrochloride solution, and counterstained with Mayer’s hematoxylin.

Cell adhesion assay

U937 monocytoid cells were obtained from Health Science Research Resources Bank and grown in RPMI 1640 medium (Life Technologies, Inc) containing 10% FBS. Confluent HUVECs were exposed to IL-1β (40 U/mL) for 24 hours in the presence or absence of other treatments. The adhesion assays were performed by the addition of U937 cells to each monolayer with agitation at 21°C. Nonadhering cells were removed by a gentle wash, and monolayers were fixed with 4% paraformaldehyde. The number of adherent cells was blindly determined by counting 10 different fields, using an ocular grid and a ×20 magnifying lens.

Flow chamber system

We assessed the effect of shear stress on the adherence of U937 monocytoid cells to HUVEC monolayers induced by IL-1β. To produce well-defined shear stress, we used a flow chamber system previously described by Gerszten et al (22), with modification (23). Briefly, the chamber consisted of a glass slide with a confluent HUVEC monolayer that was attached to a polycarbonate base. These two flat surfaces were held approximately 270 μm apart by a SILASTIC brand rubber gasket (Dow Corning). Flow across the monolayer was controlled with a syringe pump (Harvard Apparatus). Shear stress was calculated by the momentum balance for a Newtonian fluid. The viscosity of water at 37°C was used as an approximation of the viscosity of Hanks’ balanced salt solution (HBSS) (P = .007). The wall shear stress along the HUVEC monolayers was equal to T = 3 μg/mL, where T was the wall shear stress, μ was the coefficient of viscosity, a was the half-channel height, and b was the channel width. The shear rate was given by T/μ. Endothelial monolayers on the cover glass were exposed to IL-1β (40 U/mL for 4 h at 37°C in a humidified atmosphere with 5% CO2, and washed once with HBSS. Subsequently, a U937 monocytoid cell suspension (10 000 cells/mL) in HBSS was perfused through the flow chamber. Experiments were videotaped using a color camera mounted on an inverted microscope. Adherent cells were defined as cells that remained stationary for a period of 30 seconds or longer. The number of adherent U937 monocytoid cells was counted 5 minutes after the perfusion of cells.

Small interfering RNA (siRNA) transfection

For the siRNA experiment, HUVECs, at greater than 85% confluence, were transiently transfected with scrambled or validated siRNA for ICAM-1 (Ambion) using Lipofectamine LTX (Invitrogen) according to the manufacturer’s instructions. RNA was extracted 48 hours after transfection, and cDNA was synthesized according to the methods described above. The suppression of ICAM-1 was then confirmed by real-time PCR and EIA.

Statistical analysis

All data are expressed as mean ± SEM for three separate experiments. Differences in progestogen stimulation were analyzed with one-factor ANOVA followed by the Bonferroni-Dunn test for multiple comparisons. Differences in siRNA experiments and suppression experiments were analyzed with two-way ANOVA and the post hoc Tukey test. P < .05 was considered significant.

Results

MPA but not other progestogens increased mRNA expression of adhesion molecules in HUVECs

We first performed quantitative real-time PCR to examine how MPA affects cell adhesion molecules in HUVECs. Our preliminary study revealed that MPA resulted in a dose-dependent stimulation of adhesion molecule expression in the range between 10 and 100 nM (Supplemental Figure 1). In consideration of the clinical settings, we fixed the concentrations of the progestogens at 100 nM. Our findings indicate that MPA and E2 + MPA treatment resulted in 1.4- to 1.6-fold increases in mRNA levels for E-selectin in HUVECs compared with the control (P < .01, Figure 1A). The expression of P-selectin in HUVECs treated with MPA or E2 + MPA was approximately 1.7-fold higher than the control (P < .01, Figure 1B). The ICAM-1 level was also significantly higher than the con-
MPA but not other progestogens increased protein levels of adhesion molecules in HUVECs

We next demonstrated the expression of cell adhesion molecules at the protein level in HUVECs treated with several progestogens by EIA. Our findings revealed that MPA increased the expression of E-selectin, P-selectin, and ICAM-1 compared with the control (83.0 ± 0.7 vs 72.3 ± 0.1 ng/mL, 34.8 ± 1.2 vs 31.0 ± 0.1 ng/mL, and 5.4 ± 0.0 vs 4.9 ± 0.1 ng/mL, respectively) (P < .05, Figure 2, A–C). The expression of E-selectin and ICAM-1 in HUVECs was

Figure 1. MPA stimulation of the mRNA expression of adhesion molecules in HUVECs. Steroid-deprived and serum-starved HUVECs were treated for 24 hours in the presence of various progestogens (100 nM P4, 100 nM DG, 100 nM MPA, 100 nM DNG, 100 nM LNG, or 100 nM NETA) with or without 1 nM E2. The cells were then stimulated by IL-1β (40 U/mL) for 4 hours. Relative mRNA expression of E-selectin (A), P-selectin (B), ICAM-1 (C), and VCAM-1 (D) was measured by real-time PCR, as described in Materials and Methods. Data are expressed as mean ± SEM from three experiments with triplicate assays. Ct, cycle time. **, P < .01 vs vehicle only.

Figure 2. MPA stimulation of the protein expression of adhesion molecules in HUVECs. HUVECs were treated in the presence of various progestogens with or without E2, as described in Figure 1. E-selectin (A), P-selectin (B), ICAM-1 (C), and VCAM-1 (D) protein expression levels were quantified by EIA, as described in Materials and Methods. Data are expressed as mean ± SEM from four experiments. *, P < .05 vs vehicle only; **, P < .01 vs vehicle only.
significantly higher than that for the control (76.3 ± 1.9 vs 72.3 ± 0.1 ng/mL and 5.1 ± 0.0 vs 4.9 ± 0.1 ng/mL, respectively) when HUVECs were treated with E2 + MPA (P < .05, Figure 2, A and C). In contrast, other progestogens did not increase the protein levels of cell adhesion molecules (Figure 2, A–D). Additionally, increased expression of E-selectin and ICAM-1 on the surfaces of HUVECs after MPA exposure was evident from the higher intensity of immunocytochemistry staining (Figure 3, A and B).

MPA but not other progestogens increased the number of monocytoid cells adhered to HUVECs

We next performed cell adhesion assays to verify whether increased expression of cell adhesion molecules actually exerted an effect on the adherence of U937 monocytoid cells to HUVECs. During MPA treatment, the number of U937 monocytoid cells adhered to HUVECs significantly increased compared with the control (127.3 ± 2.1 vs 117.5 ± 3.5 adherent cells per 10 fields, P < .05, Figure 4A), but this effect was not observed with other progestogens or those combined with E2 (Figure 4A). Mimicking the actual adherence of monocytes to endothelial cells, we used the flow chamber system to clarify the effects of progestogens on cell numbers under flow conditions. During MPA treatment, the adherence of U937 monocytoid cells significantly increased compared with the control (56.0 ± 1.5 vs 46.5 ± 3.5 adherent cells per 10 fields, P < .05, Figure 4, B and C). In contrast, E2, P4, E2 + P4, E2 + DG, and E2 + DNG significantly reduced the number of adherent monocytoid cells to HUVECs (P < .05, Figure 4B).

Is MPA action mediated by the progesterone receptor (PR), glucocorticoid receptor (GR), and/or androgen receptor (AR)?

Unlike P4 and other synthetic progestogens tested, MPA also has an affinity for both GR and AR in addition
Figure 5. Effects of the PR and GR antagonist and/or AR antagonist during MPA treatment and effects of the GR and/or AR agonists on the expression of endothelial adhesion molecules and adhesive interactions of U937 monocyteid cells on HUVECs. HUVECs were treated in the presence of 100 nM P4 or 100 nM MPA with or without 1 μM RU486 and/or 1 μM HF, as described in Figure 1. A, Relative mRNA expression of adhesion molecules on HUVECs. Data are expressed as mean ± SEM from three experiments with triplicate assays. *, P < .05 and **, P < .01 vs corresponding treatment groups with vehicle; #, P < .05. B, Protein expression levels of adhesion molecules were quantified by EIA, as described in Materials and Methods. Data are expressed as mean ± SEM from four experiments. *, P < .05 and **, P < .01 vs corresponding treatment groups with vehicle; #, P < .05. HUVECs were treated in the presence of 100 nM MPA, 100 nM Dex, and/or 100 nM DHT, as described in Figure 1. C, The numbers of adherent U937 monocyteid cells on HUVEC monolayers during flow conditions. Adherent cells were counted after washing, as described in Materials and Methods. The total numbers of adherent cells in 10 randomly selected microscopic fields are presented in the figure.
to PR. We therefore tested whether the elevated expression of adhesion molecules and the increased adherence of U937 on HUVECs during MPA treatment could be suppressed by the PR/GR antagonist RU486 or the AR antagonist HF. We confirmed the expression of PR, GR, and AR on HUVECs (Supplemental Figure 2). Real-time PCR analysis and EIA revealed that RU486 partially attenuated MPA stimulation, resulting in increased adhesion molecule expression (P < .05). Conversely, HF tended to produce limited augmentation of MPA stimulation. In contrast, neither suppression nor stimulation was observed during P4 treatment (Figure 5, A and B). Similarly, in the flow chamber system, RU486 partially attenuated MPA stimulation of the adherence of U937 monocytoid cells to HUVECs, whereas HF did not alter MPA stimulation. Additionally, neither RU486 nor HF affected P4 suppression of the adherence of monocytoid cells (Figure 5C).

In addition to suppression experiments, we next tested whether the ability of MPA to induce cell adhesion molecules and to stimulate cell adhesion could be mimicked by GR and AR agonists. We used Dex as a GR agonist and DHT as a potent AR agonist. Real-time PCR analysis revealed that MPA, Dex, DHT, and Dex + DHT all significantly increased mRNA levels of all adhesion molecules tested (P < .05, Figure 5D). With EIA, the protein levels of all adhesion molecules tested also significantly increased when the HUVECs were treated with MPA and Dex + DHT (P < .05, Figure 5E). In the flow chamber system, however, only MPA but not Dex and/or DHT significantly increased cell adhesion, compared with the control (Figure 5F).

### Reduction of the expression of ICAM-1 prevents the increase of adhered monocytoid cells to HUVECs

To confirm that the increase in ICAM-1 expression induced by MPA leads to the greater adherence of monocytoid cells to HUVECs, we evaluated the effects of steroids tested in HUVECs treated with siRNA for ICAM-1. The results of this analysis confirmed suppression of ICAM-1 in treated HUVECs (Figure 6, A and B), whereas the flow chamber system revealed that MPA treatment attenuated U937 monocytoid cell adherence to the siRNA-transfected HUVECs as compared with the control (P < .01) (Figure 6C).

**Discussion**

The results of this present study demonstrate that MPA, unlike other progestogens, stimulates mRNA and protein expression of cell adhesion molecules on HUVECs. Such molecules, ie, E-selectin, P-selectin, ICAM-1, and VCAM-1, have been known to directly mediate the adherence between monocytes and endothelial cells (15). Monocytes adhered to endothelial cells migrate to the intima of vascular walls and participate in the uptake of oxidative low-density lipoprotein-cholesterol in atherogenesis. Therefore, the long-term exposure of cell adhesion molecules on vascular endothelial cells in postmenopausal women receiving HRT may lead to increased risk of CHD. Previous studies in vitro have clarified the mechanisms by which estrogen exerts beneficial effects on the expression of endothelial cell adhesion molecules (20, 24, 25). Caulin-Glaser et al (20) revealed that E2 strongly inhibits IL-1-mediated membrane E-selectin and VCAM-1 induction and that this inhibition is abrogated by an E2 antagonist, ICI 164 384, demonstrating a specific estrogen receptor (ER)-mediated induction of adhesion molecules.

It has also been reported that E2 significantly attenuates the effects of TNF-α on ERK1/2 activity and adhesion molecule expression in HUVECs (24). Wang et al (23) suggested that nitric oxide synthesis increases through the ERβ-mediated pathway that suppresses apoptosis and nuclear factor-κB activity in endothelial cells that down-regulates cell adhesion molecule expression on endothelial cells via the ERβ/nitric oxide/nuclear factor-κB pathway. Although favorable effects of estrogens on the endothelial expression of cell adhesion molecules have been reported, the functions of progestogens have remained controversial. One previous clinical trial demonstrated that the administration of MPA combined with CEE decreased the plasma levels of human endothelial cell adhesion molecules through a reduction of estrogen-induced C-reactive protein, which enhances the expression of adhesion molecules (26). Because various factors contribute to the expression of cell adhesion molecules and other proatherosclerotic compounds in vivo, it was not clear whether MPA directly increased the expression of cell adhesion molecules. The results of this study, however, demonstrate that MPA (E2 + MPA) directly increases the expression of cell...
adhesion molecules in HUVECs. The different effects of MPA for the daily doses of 2.5 mg and 5.0 mg on the cardiovascular parameters might be attributed, in part, to the dose-dependent stimulation of MPA in the range between 10 and 100 nM.

To our knowledge, the results of this study also demonstrate for the first time that pretreatment of HUVECs with MPA, regardless of the presence or absence of E2, enhanced adhesion of U937 monocytoid cells to HUVECs, although similar enhancement was not observed with other progestogens. Moreover, E2, P4, E2 + P4, E2 + DG, and E2 + DNG pretreatment conversely diminished adhesion, which is not detected by the conventional cell adhesion assay. The parallel plate flow chamber system was initially designed to investigate monocyte-endothelial interactions under physiological conditions. Because laminar flow causes monocytes to roll on endothelial cells, the flow condition more closely mimics physiological conditions than rotated or static models (22, 23, 27). Therefore, the assay under flow conditions is shown to be more sensitive than the cell adhesion assay under conditions of agitation. Epidemiological and experimental studies indicate several atheroprotective effects of endogenous estrogen, which intervenes from progression of atherosclerosis and vascular inflammation (24, 28, 29). The results of previous animal studies showing that MPA attenuates beneficial effects of estrogen on endothelial function (30, 31) are also consistent with the results of our flow chamber system analysis.

To further examine the mechanism of enhanced adhesion with MPA in relation with the enhanced expression of cell adhesion molecules, we used HUVECs with knocked-down ICAM-1. As expected, MPA did not stimulate the adhesion of monocytoid cells, suggesting that enhancement of adhesion with MPA treatment is mediated by increased cell adhesion molecules on HUVECs.

Several studies have also suggested that MPA inhibits the protein expression of VCAM-1 induced by bacterial lipopolysaccharide (LPS) in HUVECs (26, 32). IL-1β, which was used in the present study, is known to be induced by the LPS/toll-like receptor 4 signal transduction pathway. The expression of proinflammatory cytokines, including IL-1 and TNF-α, is regulated by the myeloid differentiation primary response gene 88-dependent pathway after stimulation of the LPS/toll-like receptor 4 pathway, which also stimulates the myeloid differentiation primary response gene 88-independent pathway during inflammation (33). Therefore, there are some differences between the signal transduction mediated by bacterial LPS and that mediated by IL-1. In the present study, we used IL-1β, which is activated downstream in the multistep in-

**Figure 6.** The differences in the numbers of adherent U937 monocytoid cells on HUVEC monolayers for treatment with ICAM-1 siRNA or control siRNA. A, Relative mRNA expression of ICAM-1 on HUVECs that were transiently transfected for 24 hours or 48 hours with scrambled (control) or ICAM-1 siRNA and treated for 24 hours with or without 1 nM E2 and/or 100 nM MPA. Data have been expressed as mean ± SEM from four experiments. B, HUVECs, transfected with scrambled (control) or ICAM-1 siRNA, were treated for 24 hours with or without 1 nM E2 and/or 100 nM MPA. U937 monocytoid cells at 10 000/mL were perfused over HUVEC monolayers and stimulated by IL-1β (40 U/ml) for 4 hours, and adherent cells were counted 5 minutes after perfusion of cells, as described in Materials and Methods. The total numbers of adherent cells in 10 randomly selected microscopic fields are shown. Data are expressed as mean ± SEM from three experiments. C, cycle time; *, P < .05; **, P < .01.
flamatory response, to minimize the influence of other inflammatory mediators.

Synthetic progestogens can be classified into three major subtypes: 17α-hydroxyprogesterone derivatives (ie, MPA and DG), 19-nortestosterone derivatives (ie, NETA, LNG, and DNG), and 17α-spirospironolactone derivatives (ie, drosiprenone). Individual synthetic progestogens bind with different affinities to various steroid receptors, such as the PR, ER, AR, GR, and mineralocorticoid receptor, thereby exerting unique and complex actions on target tissues (34). MPA is characterized by its ability to cross-bind to both GR and AR, whereas NETA and LNG cross-bind to AR. Of the progestogens tested, only MPA cross-binds to GR. We have demonstrated here that the PR/GR antagonist contributed to attenuation of MPA stimulation of the expression of adhesion molecules and the adhesion of monocytoid cells. Conversely, the AR antagonist tended to augment MPA stimulation, possibly due to the antiinflammatory effects of androgens. In contrast, treatments with natural P4, which specifically binds to PR, did not produce either suppression or stimulation. Furthermore, concomitant addition of Dex and/or DHT enhanced the expression of cell adhesion molecules yet failed to stimulate monocytoid cell adhesion to the same extent as MPA in our flow chamber system.

These results suggest that the effects of MPA on adhesion molecules are induced in part by GR and that MPA possesses some additional functions related to the adherence of monocytoid cells to endothelial cells in addition to cross-reactivity with steroid receptors. Neither MPA nor any of its metabolites occur naturally in humans, and absorbed MPA is not converted to P4. There is accumulated evidence suggesting that P4 exerts favorable actions on the cardiovascular system and the brain, whereas MPA exerts adverse effects on these tissues (35, 36). However, in vitro studies have limitations that prevent investigation of the actual effects of progestogens on the cardiovascular system. Further studies are needed to elucidate the mechanism by which MPA stimulates cell adhesion.

After discontinuation of the WHI, recent clinical trials have started to design protocols using other type of progestogens. To date, however, only a limited number of large-scale trials have been reported. A prospective cohort study in French women revealed no increased risk of breast cancer associated with estrogen + P4 or DG, whereas the risk increased with estrogen alone and estrogen + other type of progestogens, including MPA (8). A national cohort study of Danish women by Løkkegaard et al (9) reported an increased risk of myocardial infarction with continuous combined estrogen-progestogen therapy but not with the cyclic regimen, although they did not show associations with the progestogen type. In smaller-scale trials, increased risk of venous thromboembolism risk has been reported in conjunction with oral ingestion of estrogen and nonpregnane progestogens (eg, noregestrel acetate) but not micronized P4 or progestogen (eg, MPA, DG, and chlormadinone acetate) (37). Continuous combined HRT containing 0.5 mg E2 and 2.5 mg DG has been shown to be effective in alleviating vasomotor symptoms and improving the quality of life (38). Compared with the use of other HRTs, the use of E2 + DG for a period of several months to a few years has not been found to be associated with a higher risk of cardiovascular events (39). Continuous use of a combination of 1 mg E2 valerate and 2 mg DNG has been shown to reduce moderate to severe hot flushes (40). In addition to P4, both DG and DNG seem to be promising synthetic progestogen alternatives to MPA, as supported by our in vitro results, but larger clinical trials would be needed to evaluate the effects of each progestogen on the cardiovascular system.

In conclusion, we have shown that MPA increases the expression of cell adhesion molecules on HUVECs in vitro, causing increased adherence of U937 monocytoid cells to HUVECs. These findings suggest that MPA administered for HRT may be a risk factor for atherosclerosis in postmenopausal women. Further clinical trials are required to investigate a synthetic progestogen for HRT as an alternative to MPA.

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References

5. Grady D, Rubin SM, Peititi DB, et al. Hormone therapy to prevent
40. Endrikat J, Graesser T, Mellinger U, Ertan K, Holz C. A multicenter, prospective, randomized, double-blind, placebo-controlled study to investigate the efficacy of a continuous-combined hormone therapy preparation containing 1 mg estradiol valerate/2 mg dienogest on hot flushes in postmenopausal women. Maturitas. 2007;58:201–207.