Androgen Exposure Increases Human Monocyte Adhesion to Vascular Endothelium and Endothelial Cell Expression of Vascular Cell Adhesion Molecule-1

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Background—Male sex is an independent risk factor for coronary artery disease. Owing to the importance of monocyte adhesion to endothelial cells in the development of atherosclerosis, we hypothesized that androgens might promote this process. We therefore studied the effects of the nonaromatizable androgen dihydrotestosterone (DHT) on human monocyte adhesion to human endothelial cells and on endothelial cell–surface expression of adhesion molecules.

Methods and Results—Human umbilical vein endothelial cells (HUVECs) were grown to confluence in media supplemented with postmenopausal female serum, then exposed for 48 hours to either DHT (40 and 400 nmol/L), with or without the androgen receptor blocker hydroxyflutamide (HF) (4 \( \mu \text{mol/L} \)); HF alone; or vehicle control (ethanol 0.1%). Human monocytes obtained by elutriation were incubated for 1 hour with the HUVECs at 37°C, and adhesion was measured by light microscopy. Compared with vehicle control, monocyte adhesion was increased in the androgen-treated HUVECs in a dose-dependent manner (116 ± 6% and 128 ± 3% for DHT 40 and 400 nmol/L respectively; \( P < 0.001 \)). HF blocked this increase (\( P \geq 0.3 \) compared with control). Surface expression of endothelial cell adhesion molecules was measured by ELISA and revealed an increased expression of vascular cell adhesion molecule-1 (VCAM-1) in the DHT-treated HUVECs (125 ± 5% versus 100 ± 4% in controls; \( P = 0.002 \)), an effect also antagonized by HF (\( P \geq 0.3 \) compared with controls). Furthermore, the DHT-related increase in adhesion was completely blocked by coincubation with anti–VCAM-1 antibody. Comparable results were obtained in arterial endothelial cells and in endothelium stimulated with the cytokine tumor necrosis factor-\( \alpha \).

Conclusions—Androgen exposure is associated with increased human monocyte adhesion to endothelial cells, a proatherogenic effect mediated at least in part by an increased endothelial cell–surface expression of VCAM-1.

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Key Words: hormones ■ atherosclerosis ■ cell adhesion molecules

There is an important sex difference in coronary heart disease risk, with an earlier onset of disease in males and excess mortality in men throughout adult life.\(^1\)\(^,\)\(^2\) Although there is a growing abundance of clinical and basic science data supporting a favorable effect of estrogen and perhaps progesterone on female cardiovascular risk and atherogenic processes,\(^3\)\(^–\)\(^6\) there are comparatively few data on the possible proatherogenic effects of androgens. Recent animal studies in chicks\(^7\) and cynomolgus monkeys\(^8\) support this possibility, documenting increased plaque formation associated with testosterone treatment. Few mechanistic studies, however, have examined the vascular effects of androgens in humans, although we have recently demonstrated an association between androgen deprivation and enhanced endothelial function in older men consistent with a deleterious effect of androgens on vascular reactivity.\(^9\)

Monocyte adhesion to endothelial cells is an important early event in atherogenesis, controlled in part by expression of adhesion molecules on the endothelial cell surface.\(^10\)\(^,\)\(^11\) We therefore aimed to explore the effects of the potent nonaromatizable androgen dihydrotestosterone (DHT) on monocyte–endothelial cell adhesion and on the expression of endothelial cell adhesion molecules using primary human cells.

Methods

Materials

Phenol red–free variable amino acid RPMI cell culture medium was obtained from Life Technology. The sex-steroid hormone used was DHT (Sigma Chemical Co) because it cannot be aromatized to estrogenic metabolites. Hydroxyflutamide (HF), a nonsteroidal androgen-receptor antagonist, was a gift from Dr David Handelsman, Department of Andrology, Royal Prince Alfred Hospital, Sydney, Australia. Interleukin-1\( \beta \) (IL-1\( \beta \)) was obtained from Genzyme Corp.

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Tumor necrosis factor-α (TNF-α) was obtained from Boehringer Mannheim. Mouse anti-human monoclonal antibodies against vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin were obtained from Becton-Dickinson, and isotype mouse IgG1 and IgG2 not directed against endothelial cell antigens were obtained from ICN Immunobiologics. Sheep anti-mouse antibody–horseradish peroxidase conjugate was obtained from Amersham International.

**Endothelial Cell Culture**

Human umbilical vein endothelial cells (HUVECs) were harvested enzymatically from male infant umbilical cords under sterile conditions as described by Minter et al.12 and established as primary cell cultures in phenol red–free M199 (Gibco Life Sciences) containing l-glutamine 2 mmol/L (ICN Biomedicals), 0.5% endothelial cell growth promoter (Starr Produkte AG), penicillin 100 U/mL, streptomycin 0.1 mg/mL, and 20% filtered human serum from healthy postmenopausal female volunteers not taking hormone replacement therapy. Human umbilical arterial endothelial cells (HUAECs) were harvested and cultured by the same methods. Commercially bottled media had been filtered at 0.1 mmol/L, and powdered medium was reconstituted with endotoxin-free water and filtered at 0.2 mmol/L. Endothelial cell monolayers (passages 2 to 4) were propagated on gelatin-coated flasks in phenol red–free medium, then trypsinized and replated onto gelatin-coated 24-mm-diameter tissue-culture wells or 96-well plates for monocyte adhesion and cell adhesion molecule expression studies, respectively. Wells were gelatin coated with 1 mL/5 cm². Hemaccel (Behringwerke AG) diluted 1:250 in media had been filtered at 0.1 mmol/L, and powdered medium was reconstituted with endotoxin-free water and filtered at 0.2 mmol/L. Cells were eluted at between 16 and 17 mL/min. Lymphoprep-derived mononuclear cell fraction was loaded at 9 mL/min into an elutriation chamber (Beckman Instruments, Inc). The elutriation M/E centrifuge equipped with a JE-6B elutriation rotor and a 4.2-mL sample cup (Beckman Instruments, Inc) was obtained from Amersham International.

**Isolation of Human Monocytes**

White blood cells (Red Cross Blood Bank) were obtained from the peripheral blood of healthy human volunteers, and monocytes were removed within 24 hours of collection by density gradient separation of the white cells on Lymphoprep (Nycomed Pharma) followed by counterflow centrifugation elutriation at 20°C, as previously described by our group.13,14 by use of a Beckman J2–21 M/E centrifuge equipped with a JE-6B elutriation rotor and a 4.2-mL elutriation chamber (Beckman Instruments, Inc). The elutriation buffer was HBSS without calcium or magnesium (Sigma) supplemented with EDTA (0.1 g/L) and 1% heat-inactivated human serum. The system and tubing were rinsed with 250 mL each of 70% ethanol, endotoxin-free water, 6% hydrogen peroxide, endotoxin-free water, and elutriation buffer in that order before the Lymphoprep-derived mononuclear cell fraction was loaded at 9 mL/min into the elutriation rotor chamber (200 rpm at 20°C). Flow rate was increased by 1 mL/min increments every 10 minutes, and monocytes were typically eluted at between 16 and 17 mL/min. Collected fractions were examined by a Cytospin system (Shandon and Wright’s stain (DiffQuik, Laboratory-Aids)). Monocyte purity of >90% and viability of >95% by Trypan blue exclusion were confirmed on light microscopy, and the monocytes were resuspended in RPMI containing 2% human serum and used immediately for adhesion studies.

**Monocyte–Endothelial Cell Adhesion Assay**

Endothelial cells were propagated for ≥1 passage in phenol red–free RPMI supplemented with 20% human postmenopausal female serum from healthy donors. This serum was used because of the low baseline levels of sex-steroid hormones (estradiol <75 pmol/L, progesterone <0.8 mmol/L, and testosterone <2 nmol/L). Confluent endothelial monolayers were established in 24-mm-diameter wells before incubation for 48 hours with the following treatments: (1) control wells treated with 0.1% ethanol (used to dissolve the sex-steroid hormones); (2) DHT 40 nmol/L; (3) DHT 400 nmol/L (the normal range of testosterone in human male serum is 4 to 40 nmol/L); (4) DHT 40 nmol/L and HF 400 nmol/L; (5) DHT 400 nmol/L and HF 4 μmol/L; and (6) HF 4 μmol/L. Each treatment group was divided after 24 hours of hormone treatment into basal and stimulated states, the latter receiving IL-1β (50 U/mL) or TNF-α (500 U/mL) for the final 24-hour period. Separate adhesion experiments were performed 5 times for control and DHT-treatment groups and 3 times for HF exposure. Each experiment used at least triplicate wells for each condition. An additional series of experiments investigated the interaction of DHT and IL-1β in terms of stimulating cell adhesion and involved sequential and/or coinuculation conditions, as described in the Results section.

The adhesion assay involved the addition of 1.5 × 10⁶ monocytes per milliliter of RPMI/2% human serum to the endothelial monolayer and incubation for 1 hour at 37°C under 5% CO2 in air. After 1 hour, nonadherent cells were removed by gentle washing with a 1000-μL automatic pipette (Gilton), and the 1-mL suspension was stored on ice until the cell concentration was determined with a Neubauer hemocytometer (Weber Scientific). The initial suspensions and the suspension from each well were counted 4 times by an observer blinded to the treatment conditions. The percentage of adherent monocytes was calculated by comparison with the initial concentration. This method has been shown to have a low intraobserver error, with a coefficient of variation of <5% and maximal basal adhesion after 1 hour of incubation.13 Basal monocyte–endothelial cell adhesion in these experiments was 25±5%, increasing with IL-1β stimulation to 55±6% (P<0.01).

**Endothelial Cell Adhesion Molecule Expression**

The cell-surface expression of adhesion molecules on the endothelial cell monolayers exposed to different treatments was assessed by an ELISA technique. Confluent cell monolayers were established in 96-well plates and, as was done for the adhesion studies, exposed for 48 hours to the control or hormone treatments as outlined above, with or without IL-1β stimulation (50 U/mL) for the last 24 hours of treatment. Wells were then washed twice with HBSS; monoclonal antibodies to ICAM-1, VCAM-1, E-selectin, and isotype mouse IgG (0.1 μg in 100 μL of HBSS with 10% heat-inactivated human serum) were added and left for 30 minutes. The layers were washed 3 times with HBSS and 0.05% Tween 20 before a 30-minute incubation with sheep anti-mouse antibody–horseradish peroxidase conjugate (1:500 in 100 μL of HBSS with 10% heat-inactivated human serum and 0.05% Tween 20). After an additional 4 washes, 150 μL of ABTS substrate (Kirkegaard and Perry Laboratories) was added to each well and allowed to develop for 15 minutes. Results were expressed as units of optical density measured at 414 nm with an ELISA plate reader (Titertek Multiscan, Flow Laboratories).

**Adhesion Assay With Neutralizing Antibodies to Cell Adhesion Molecules**

Endothelial cells grown to confluence and exposed to different hormone conditions (as described above) were incubated with human monoclonal antibodies (2 μg/mL) to either ICAM-1, VCAM-1, E-selectin, and isotype mouse IgG (0.1 μg in 100 μL of HBSS with 10% heat-inactivated human serum) were added and left for 30 minutes. The layers were washed 3 times with HBSS and 0.05% Tween 20 before a 30-minute incubation with sheep anti-mouse antibody–horseradish peroxidase conjugate (1:500 in 100 μL of HBSS with 10% heat-inactivated human serum and 0.05% Tween 20). After an additional 4 washes, 150 μL of ABTS substrate (Kirkegaard and Perry Laboratories) was added to each well and allowed to develop for 15 minutes. Results were expressed as units of optical density measured at 414 nm with an ELISA plate reader (Titertek Multiscan, Flow Laboratories).

**Statistical Analysis**

All descriptive data are expressed as mean±SEM, and the data were analyzed on SPSS for Windows 6.0. Because each experiment involved both endothelial cells and monocytes from different donors, results for the adhesion assays and ELISAs for cell adhesion molecule expression were expressed as a percentage of the control condition within each experiment. Groups were compared by 1-way ANOVA.
ANOVA followed by the Student-Newman-Keuls test for multiple comparisons and independent-samples t tests for comparisons between groups. Statistical significance was inferred at a 2-sided value of $P$, 0.05.

Results
Monocyte-Endothelial Cell Adhesion and Expression of Endothelial Cell Adhesion Molecules
Androgen exposure was associated with an increase in monocyte adhesion to IL-1β-stimulated endothelial cell monolayers (Figure 1). This was dose related (116±6% and 128±3% for DHT 40 nmol/L and 400 nmol/L, respectively, compared with control; $P<0.001$ by ANOVA and $P<0.01$ for each DHT concentration compared with control) (Figure 2) and was not observed in basal state (non–IL-1β–stimulated) endothelium (data not shown). This androgen-mediated increase in adhesion was abolished by cotreatment with the androgen-receptor antagonist HF (95±8% and 105±4% for DHT 40 nmol/L-HF 400 nmol/L and DHT 400 nmol/L-HF 4 μmol/L, respectively; $P>0.3$ compared with control). HF alone did not affect adhesion (94±6% compared with controls; $P=0.4$). These results suggest an androgen-mediated amplification of monocyte–endothelial cell adhesion that is at least partially receptor dependent.

The effect of androgen treatment on endothelial cell–surface adhesion molecule expression was then studied with an ELISA technique. Treatment of endothelial cell monolayers for 24 hours with IL-1β increased cell-surface expression of all 3 cell adhesion molecules: ICAM-1 (optical density, 0.55±0.02 versus 0.98±0.02; $P<0.001$), VCAM-1 (optical density, 0.22±0.01 versus 0.48±0.03; $P<0.001$), and E-selectin (optical density, 0.12±0.01 versus 0.30±0.02; $P<0.02$). In the stimulated state, there was an increase in endothelial surface expression of VCAM-1 with DHT compared with control wells (DHT 40 nmol/L 125±5% and DHT 400 nmol/L 123±7% versus control 100±4%; $P<0.01$ for each comparison) (Figure 3). Furthermore, this increase in...
VCAM-1 expression was reduced to control levels in HUVECs treated with both DHT and HF (97±6% compared with control; *P=0.7). HF alone did not alter VCAM-1 expression (105±7% versus control; *P=0.5). There was no association between treatment condition and surface expression of either ICAM-1 or E-selectin (data not shown). In the basal state (without IL-1β), surface expression of VCAM-1, ICAM-1, and E-selectin did not change significantly between treatment conditions, similar to the results of the adhesion studies described above. In all experiments, there was no significant binding of isotype IgG to endothelial cells in either the basal or stimulated state.

These results were derived from endothelial cells coincubated with IL-1β during the final 24 hours of treatment exposure. To further explore the interaction between DHT and IL-1β, similar experiments were performed with sequential exposures in which (1) DHT was washed off the endothelial cells completely after 48 hours, followed by a subsequent 24-hour exposure to IL-1β, or (2) IL-1β was added to the media for 24 hours (and then washed off) before a 48-hour exposure to DHT (that is, the reverse of experiment 1 above). In both cases, the IL-1β– and DHT-exposed HUVECs demonstrated increased adhesion and increased VCAM-1 expression compared with DHT-vehicle (ethanol 0.1%)-treated controls similarly exposed to IL-1β. In experiment 1, DHT exposure before IL-1β increased adhesion (DHT 400 nmol/L 131±4% versus 100±2% for controls; *P=0.03) and VCAM-1 expression (DHT 400 nmol/L 110±3% versus 100±2% for controls; *P=0.03). In experiment 2, IL-1β exposure before DHT treatment also increased adhesion (DHT 400 nmol/L 124±3% versus 100±1% for controls; *P=0.03) and VCAM-1 expression (DHT 400 nmol/L 131±6% versus 100±7% for controls; *P=0.01). In each case, HF antagonized the effect of DHT (data not shown).

To demonstrate whether these effects would also be observed with arterial endothelial cells, additional experiments were performed with HUAECs. DHT-treated HUAECs were similarly associated with an increase in monocyte adhesion after IL-1β stimulation (DHT 400 nmol/L 131±7% versus 100±7% for controls; *P<0.03), and this effect could be blocked by coincubation with HF (DHT 400 nmol/L-HF 88±4%; *P=0.1 compared with control). Furthermore, as was seen in the equivalent HUVEC experiments, this androgen-mediated increase in monocyte–endothelial cell adhesion was associated with an increase in VCAM-1 expression (DHT 400 nmol/L 116±5% versus 100±1% for controls; *P=0.04), which was reduced by coadministration of HF (107±3%; *P=0.1 compared with control).

To demonstrate that this effect was not confined to endothelium costimulated with DHT and IL-1β, experiments were performed with another cell-adhesion–promoting cytokine, TNF-α. DHT-treated HUVECs stimulated with TNF-α also showed increased monocyte adhesion (DHT 400 nmol/L 130±1% versus 100±2% for controls; *P=0.002) and VCAM-1 expression (DHT 400 nmol/L 114±2% versus 100±2% for controls; *P=0.02). As seen with IL-1β stimulation, the androgen-mediated increase in adhesion and VCAM-1 expression observed in TNF-α–stimulated endothelium could be antagonized by HF (100±5% and 103±8% for adhesion and VCAM-1 expression, respectively; *P>0.5 compared with control). E-selectin and ICAM-1 expression were not significantly different between conditions (102±6% and 101±4% for E-selectin and ICAM-1, respectively, after androgen exposure; *P>0.5 compared with control).

### Neutralizing Antibodies to Cell Adhesion Molecules

The ELISA results showing increased surface expression of VCAM-1 on HUVECs treated with androgen were confirmed by repeat adhesion studies involving a 60-minute incubation of the endothelial monolayers with antibodies to VCAM-1, ICAM-1, and E-selectin before the addition of human monocytes. In these experiments, VCAM-1 antibody effectively eliminated the androgen-related increase in monocyte–endothelial cell adhesion (DHT 400 nmol/L 91±6% compared with control 97±5%; *P=0.4) (Figure 4). ICAM-1 antibody reduced overall monocyte adhesion compared with baseline but did not change the relative increase seen between control and DHT treatment (DHT 400 nmol/L 113±8% compared with control 75±4%; *P=0.002); results were similar for E-selectin (DHT 400 nmol/L 118±6% compared with control 93±8%; *P=0.02). As expected, incubation with all 3 cell adhesion molecule antibodies greatly reduced overall adhesion and eliminated any difference between control and androgen-treated wells (DHT 400 nmol/L 74±4% compared with control 75±3%; *P=0.9).

### Discussion

The effect of androgens on different aspects of atherogenesis has received little attention despite the marked male predisposition to occlusive vascular disease.1,2 In the present study, we have demonstrated that androgen exposure leads to a dose-related and receptor-mediated increase in human monocyte adhesion to endothelial cells, a key early event in atherosclerosis. This effect is mediated at least in part by an
androgen receptor–dependent increase in endothelial cell expression of the important adhesion molecule VCAM-1.

A proatherogenic effect of androgens is supported by recent work in experimental animals. For example, Adams et al. documented an approximate doubling of coronary artery plaque size in female postmenopausal cynomolgus monkeys treated with testosterone and a cholesterol-enriched diet, and Hutchison et al. documented arterial endothelial dysfunction in hypercholesterolemic rabbits that were administered androgens. Similar data are not available in humans.

The incidence of coronary deaths in men aged 35 to 64 years exceeds that in age-matched females by up to 500%. Within each gender, however, there has been no consistent association between androgen levels and cardiovascular event rates. Although most cross-sectional data suggest either no effect or an inverse correlation between serum testosterone levels and cardiovascular event rates in men, prospective studies have not shown a significant correlation. Therefore, although small physiological variations in androgen levels within genders may not correlate clearly with patterns of atherosclerosis, it is likely that the large difference in androgen levels and receptors between genders is a significant contributor to the sex difference in cardiovascular risk. In the body, testosterone is partially metabolized by aromatases to estrogen and by 5α-reductase to DHT. Testosterone may therefore not be a clear marker of androgen action owing to its estrogenic metabolites. In the present study, the androgen DHT was used to assess the effect of androgens on monocyte–endothelial cell adhesion for several reasons: it is nonaromatizable, thus avoiding confounding estrogenic effects, and it is one of the most potent androgens in androgen-sensitive tissues, binding to the cytoplasmic androgen receptor 2 to 10 times more avidly than testosterone.

In the present study, DHT-treated endothelial cells showed an increased surface expression of VCAM-1, which suggests increased production and/or recruitment of VCAM-1 to the endothelial cell surface. In concordance with the functional adhesion data, this androgen-mediated increase in surface expression of VCAM-1 was abrogated by HF, which indicates that these processes are mediated at least in part via androgen receptors. Interestingly, 17β-estradiol, the potent estrogen, decreases endothelial cell adhesion molecule expression and monocyte adhesion, also via its sex-steroid receptor. The intracellular events accounting for such changes require further study; however, receptor-activated stimulation of gene transcription is a likely mechanism. Of note, the endothelial cells treated with androgen in the current experiments were coincubated with whole human serum. This suggests that androgen-mediated monocyte–endothelial cell adhesion is a physiologically relevant event, even in the presence of lipoproteins such as HDL, which have been shown to be protective against cell adhesion molecule expression.

Our experiments involved an in vitro model of HUVECs and monocytes obtained from peripheral blood by elutriation, which may differ from the in vivo situation. Endothelial cells in vivo do not usually express high levels of ICAM-1, VCAM-1, or E-selectin, whereas the HUVECs used in our experiments did express these adhesion molecules, presumably owing to the tissue-culture environment. This may be similar to the situation seen in atherosclerosis in which cytokines such as IL-1β and TNF-α are present and endothelial expression of adhesion molecules is stimulated. In this context, the androgen-related increase in cell adhesion observed after stimulation with IL-1β or TNF-α, as well as the similarity of findings with both venous and arterial endothelial cells in the present experiments, is consistent with potentially important effects in vivo at arterial sites prone to atherosclerosis.

The interaction between androgen and cytokine required to promote monocyte–endothelial cell adhesion did not require coinubcation with DHT and IL-1β and was observed regardless of the order of DHT or IL-1β stimulation. Although the exact intracellular mechanism of this interaction is not known, these data suggest that the sequential or simultaneous presence of androgen and cytokine might promote monocyte adhesion in the in vivo situation.

In the present study, we observed an androgen-related increase in endothelial cell expression of the adhesion molecule VCAM-1. Recent reports have confirmed an important role for VCAM-1 in atherogenesis. Studies have demonstrated a significant upregulation of VCAM-1 in plaques found in diet-induced atherosclerosis in animal models and in human atherosclerotic lesions. In contrast to other adhesion molecules, VCAM-1 in isolation is able to mediate leukocyte adhesion via integrin interaction, whereas E-selectin and ICAM-1 can mediate only part of the complex adhesion process. In addition, VCAM-1 expression precedes and is correlated with the degree of macrophage accumulation in human plaques. In our experiments, IL-1β-induced adhesion of monocytes to endothelium was only reduced by ~30% in the presence of anti–VCAM-1, anti–ICAM-1, and anti–E-selectin antibodies, which suggests either incomplete blockade of these adhesion molecules or the coexistence of other important proadhesion factors not blocked in our study. These might include surface-associated chemokines such as monocyte chemoattractant protein, growth-regulated protein, and monocyte colony stimulating factor, as well as other, as-yet-unidentified adhesion molecules.

In summary, DHT increases human monocyte adhesion to vascular endothelium, at least in part through an androgen receptor–mediated effect on endothelial expression of VCAM-1. This androgen-mediated increase in monocyte adhesion may be an important mechanism in the greater susceptibility of men to the development of premature atherosclerosis.

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