Androgen Receptor Expression Is Greater in Macrophages From Male Than From Female Donors
A Sex Difference With Implications for Atherogenesis

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Background—Male sex is an independent risk factor for the extent and severity of atherosclerosis. The influence of androgens on foam cell formation, a key event in atherogenesis, has not yet been investigated.

Methods and Results—Primary human monocytes were allowed to differentiate into macrophages. RNA was then extracted from healthy male-donor (n=8) and premenopausal female-donor (n=8) macrophages, and message for the androgen receptor (AR) was examined by RT-PCR. There was a significantly higher level of AR mRNA in macrophages isolated from men than in those from women (0.64±0.06 versus 0.15±0.02 amol/μg total RNA; P<0.001). AR mRNA levels were similar in macrophages from postmenopausal and premenopausal women (P=0.16). The functional consequence of this sex difference was then explored. Lipid-loading studies were performed on male (n=9) macrophages treated with the androgen dihydrotestosterone (DHT) and/or the AR antagonist hydroxyflutamide. These showed that DHT caused a dose-dependent and receptor-mediated increase in macrophage cholesteryl ester content (109±10%, 117±3%, and 120±4% for 4, 40, and 400 nmol/L DHT, respectively, as a percentage of control, P=0.002; 95±8% for DHT with hydroxyflutamide, P=0.58 versus controls). By contrast, there was no significant effect of androgen on lipid loading in female-donor macrophages (P>0.2 versus controls).

Conclusions—Sex differences in androgen-mediated macrophage lipid loading may contribute to the greater prevalence and severity of atherosclerosis in men. (Circulation. 2000;101:224-226.)

Key Words: atherosclerosis■cells■hormones

Men have an earlier onset and higher incidence of atherosclerosis than women.1 The potential protective effects of estrogens on vascular structure and function have been studied extensively. Much less work, however, has addressed the possible effects of androgens on atherogenesis. In animal models, androgen treatment may increase plaque formation in the aorta and coronary vessels.2,3 Furthermore, in humans, we have recently demonstrated that androgens enhance monocyte adhesion to endothelial cells4 and are associated with impaired vascular reactivity.5

Foam cell formation is a key early event in atherosclerosis and is largely due to the uptake of modified lipoproteins, principally LDL, by peripheral blood monocyte-derived macrophages (MDMs) in the arterial wall.6,7 The possibility that androgen receptors (ARs) and androgen-related intracellular pathways may be involved in the process of lipid loading and foam cell formation in atherosclerosis has not been explored previously. We therefore investigated whether the AR is expressed in primary human MDMs and whether there is a sex difference in its expression and have examined the biological effects of androgen stimulation on cholesteryl ester (CE) formation/lipid loading in these cells.

Methods

Isolation of Human Monocytes
White cell concentrates (Red Cross Blood Bank) were obtained from the peripheral blood of individual healthy men (20 to 58 years old) and premenopausal (18 to 45 years old) and postmenopausal (45, 55, and 72 years old) women. The postmenopausal women all had had no menstruation for >3 years and had follicle-stimulating hormone levels >40 U/L. Monocytes were removed by density gradient separation and counterflow centrifugation elutriation.8 Monocyte purity was >90% and viability >95% (trypan blue) in all experiments.

Culture and Analysis of Human MDMs
Monocytes isolated by elutriation were resuspended in phenol red–free RPMI (Life Technology) and allowed to adhere for ≥1.5 hours at 37°C under 5% CO2 in air. Thereafter, phenol red–free RPMI containing 10% postmenopausal female human serum (17β-estradiol <75 pmol/L, progesterone <0.8 nmol/L, and testosterone <2 nmol/L), penicillin G (50 U/mL), and streptomycin (50 µg/mL)
was added. Changes of medium occurred every 2 to 3 days, and hormone treatments (dihydrotestosterone, DHT, Sigma, at 4 to 400 nmol/L with or without the AR antagonist hydroxyflutamide, HF, 400 nmol/L) were added from days 3 through 10 with each change of medium. In the control conditions, no DHT or HF was added. Serum testosterone levels in healthy men range from $\approx 4$ to 40 nmol/L. Lipid loading was achieved on days 8 through 10 during a 48-hour incubation with 50 $\mu$g/mL of acetylated LDL in medium containing 10% (vol/vol) lipoprotein-deficient human serum (d$\approx 1.25$). Cell viability for each treatment condition was 90% to 95%. LDL was isolated from plasma from healthy, normolipidemic subjects, and macrophage cell extracts were analyzed by high-performance liquid chromatography according to previously described methods.9

RNA Extraction and Measurement of AR mRNA Levels
After 7 to 8 days of culture in RPMI supplemented with postmenopausal serum, male- and female-donor MDMs were assessed for AR message.

Total RNA was extracted from each male- and female-donor experiment (1.5$\times$10$^6$ macrophages per well), and RNA was quantified with the SYBR Green II assay (Molecular Probes).10 Androgen and $\beta$-actin (control) primers were designed from the published sequence of the human genes11,12: AR (sense), 5'-AGATGGGCTGACTTCCAGAAG-3'; AR (antisense), 5'-ATGCTGTGATCATGCTCTGGA-3'. Competition-based quantitative RT-PCR was performed with an AR competitor template as described elsewhere.12

Statistical Analysis
AR mRNA values are expressed as mean±SEM. Statistical significance was analyzed with Fisher's exact test. Lipid-loading results are also expressed as mean±SEM of at least triplicate wells per experiment per condition for each male and female donor. Results for each condition in each donor were calculated as a percentage of the control values (100%). Statistical significance by an independent-samples t test, Fischer's exact test where n=6, or a 1-way ANOVA for trend, as appropriate, was inferred at a 2-sided value of $P<0.05$.

Results

AR Expression in MDMs
Male-donor MDMs (n=8) expressed levels of AR mRNA $\approx 4$ times greater than those isolated from premenopausal female-donor cells (n=8) (0.64±0.06 versus 0.15±0.02 amol/µg total RNA; $P<0.001$). This was evident on semiquantitative RT-PCR (Figure 1A), in which representative male samples showed distinct AR bands (545 bp), whereas the female samples showed less visible bands under the same conditions, and was confirmed by quantitative competition-based RT-PCR. AR mRNA levels were also low in cells from 3 postmenopausal females (0.18, 0.24, and 0.26 amol/µg RNA, respectively, $P<0.001$, versus male levels, $P=0.16$ versus premenopausal female levels) (Figure 1B).

Effects of Androgen Exposure on Macrophage Lipid Loading
The functional effects of this sex difference in AR expression were then explored. Androgen exposure was associated with a dose-dependent increase in intracellular CE content in male-donor MDMs (n=9) (CE as a percentage of control, 109±10%, 117±3%, and 120±4% for DHT 4, 40, and 400 nmol/L, respectively; $P=0.002$ by ANOVA; Figure 2). This androgen-associated increase in CE accumulation was abrogated by coincubation with the nonsteroidal AR antagonist HF (CE content 95±8% for DHT 40 nmol/L and HF 400 nmol/L versus controls, $P=0.58$). HF 400 nmol/L alone did not alter CE content significantly (110±7%, $P=0.1$). Free cholesterol (FC) content in male-donor macrophages was not altered significantly by androgen exposure ($P=0.40$). Absolute lipid-loading values in macrophages from 1 male donor (but comparable in nature to the others) were as follows: for control cells, CE 62±4 nmol/mg cell protein, FC 139±16 nmol/mg; and for DHT 40 nmol/L–treated cells, CE 78±2 nmol/mg cell protein, FC 132±11 nmol/mg cell protein.

In contrast to the male-donor cells, CE content in female-donor macrophages (n=4) did not increase with androgen exposure (CE 86±11% and 75±15% for DHT 40 nmol/L and
400 nmol/L, respectively, \( P > 0.2 \) versus controls). Similarly, FC accumulation was unchanged by androgen treatment.

**Discussion**

Although the male predisposition to atherosclerosis is well documented, there are few studies assessing the effects of androgens on atherogenic processes. In this study, we have demonstrated a marked sex difference in the AR mRNA status of mature MDMs from healthy human subjects. Because MDMs play a key role in the early development of atherosclerosis, with macrophage lipid uptake leading to foam cell and fatty streak formation, we postulated that a greater expression of AR in these cells might contribute to the male predisposition to atherosclerosis. Indeed, our data indicate that the androgen dihydrotestosterone caused a dose-related and receptor-mediated increase in human macrophage lipid accumulation in male- but not female-donor cells. This is probably due to higher levels of AR in the male MDMs; however, it is possible that androgens may also influence lipoprotein metabolism via other pathways.

Sex differences have previously been documented in the levels of AR message found in macrophage-like synoviocytes, and in rat aortic smooth muscle cells, androgen-receptor protein levels are higher in male than in female animals. In these experimental studies, however, no functional correlates of these observations were investigated. In this first study of AR expression in human MDMs, the observed sex difference in AR message was associated with enhanced lipid loading by male- but not female-donor cells, consistent with a proatherogenic effect.

Dihydrotestosterone was used in these experiments because it is a potent, nonaromatizable physiological androgen that is specific for the AR. This avoids the potentially confounding effects of estrogenic metabolites that arise from the aromatization of testosterone, noting that estrogens may cause it is a potent, nonaromatizable physiological androgen and estrogen receptors are present in human synoviocytes, and in rat aortic smooth muscle cells, androgen regulation of thromboxane A2 receptors in rat aortic smooth muscle cells. Therefore, it is likely that androgens may also influence lipoprotein metabolism via other pathways.

(although additional pathways of modified LDL uptake also occur in vivo).

In summary, the androgen dihydrotestosterone increases CE accumulation in MDMs from male but not female subjects. Because this effect is receptor-mediated, the most likely reason for its sex specificity relates to the significantly higher levels of AR mRNA found in male- than in female-donor macrophages. These data are consistent with a significant effect of androgen exposure on foam cell formation in men.

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