Testosterone inhibits tumor necrosis factor-α-induced vascular cell adhesion molecule-1 expression in human aortic endothelial cells

Haruhiko Hatakeyama*, Makoto Nishizawa, Atsushi Nakagawa, Shigeru Nakano, Toshikazu Kigoshi, Kenzo Uchida

Department of Internal Medicine, Division of Endocrinology, Kanazawa Medical University, Ishikawa 920-0293, Japan

Received 22 August 2002; revised 11 September 2002; accepted 11 September 2002

First published online 27 September 2002

Edited by Jacques Hanoune

Abstract **We investigated the effect of testosterone (T) on tumor necrosis factor-α (TNF-α)-induced expression of vascular cell adhesion molecule-1 (VCAM-1) in human aortic endothelial cells. Incubation of these cells with T resulted in a dose-dependent reduction in the expression, with this reduction completely abolished by a selective androgen receptor blocker. Electrophoretic mobility shift assay demonstrated that T inhibited TNF-α-induced activation of the transcriptional nuclear factor-κB, which is critical for the inducible expression of VCAM-1, probably through the suppression of the nuclear translocation. Our results may suggest an inhibitory effect of T on atherogenesis, providing a novel insight into the consideration of the pathogenesis of atherosclerosis.**

Key words: Testosterone; Vascular cell adhesion molecule-1; Atherosclerosis; Nuclear factor-κB

1. Introduction

The issue of testosterone (T) replacement in older men is just beginning to be addressed. There is agreement that T levels decline progressively with advancing age, and that many of the physiological changes that occur with aging are similar to those associated with androgen deficiency in young men. Some studies have reported that T administration to older men with low or low normal T levels improves various physiological indices [1,2]. However, it is premature to make a general recommendation about T replacement in these men, because the risks of long-term T administration, particularly the risk of cardiovascular disease, are unknown.

T, the main circulating androgen, can be converted into 5α-dihydrotestosterone (5αDHT) by the action of the enzyme 5α-reductase (5α-R) in the target tissues such as the prostate before binding to androgen receptor (AR). T is also the substrate for the enzyme aromatase which irreversibly transforms the androgen into estrogen (estradiol). There are two 5α-R isozymes in humans. The type 1 5α-R (5α-R1) displays a low affinity for T, while the type 2 isozyme (5α-R2) shows a high affinity for the androgen and is generally considered the main isoform in the target tissues [3–5].

One of the initial events in atherogenesis is the activation of endothelial cells. The activation by atherogenic stimuli such as cytokines and oxidative free radicals results in the enhanced expression of cell surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 and E-selectin, which in turn facilitates the attachment of blood leukocytes to endothelial surfaces [6,7]. Recent evidence indicates that the promoter region of genes for adhesion molecules contains nuclear factor-κB (NF-κB), a transcriptional activator protein, binding sites which are essential for the expression of these genes [8]. Tumor necrosis factor-α (TNF-α), the principal atherogenic cytokine, mediates its cellular responses via activation of the NF-κB [9].

There is a widespread perception that T supplementation increases the risk of atherosclerotic heart disease. However, the available data do not support this premise, and the mechanisms by which T may promote atherogenesis have not been determined [10]. Since leukocyte recruitment into the vascular wall following adhesion to endothelial cells is a crucial step in the pathogenesis of atherosclerosis, it appeared promising to investigate whether T, a presumed atherogenic substance, might modulate endothelial adhesion molecule expression. In the present study, we examined the effect of T on TNF-α-induced VCAM-1 expression and also on the activation of NF-κB in cultured human aortic endothelial cells (HAEC).

2. Materials and methods

2.1. Cell culture

HAEC were cultured according to the supplier's instruction (Clonetics, Walkersville, MD, USA). Cells at passage 4–6 were used for the experiments.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR assay was performed as described previously [11]. AR primers were 5′-CCAGGAGACCTGCCTGATCTG-3′ and 5′-CCTCCTGTAGTTTCAGATTACC-3′ [12]. 5α-R1 primers were 5′-AGCAGATACTTGAGCCA-3′ and 5′-CCAAAATAGTTGGCTGC-3′ [3]. 5α-R2 primers were 5′-ACATTACTTCCACAGGACATTT-3′ and 5′-AGGAAATTGGCTCCAGA-3′ [13]. P450 aromatase primers were 5′-GAATATTGGAAGGATGCACAGACT-3′ and 5′-GGGTAAAGATCATTTCCAGCATGT-3′ [14].

2.3. Assay of 5α-R activity

5α-R activity was measured by a radiometric conversion assay as

*Correspondence author. Fax: (81)-76-286 6927.
E-mail address: hataha@kanazawa-med.ac.jp (H. Hatakeyama).

Abbreviations: VCAM-1, vascular cell adhesion molecule-1; HAEC, human aortic endothelial cells; NF-κB, nuclear factor-κB; 5α-R, 5α-reductase; T, testosterone; 5αDHT, 5α-dihydrotestosterone; AR, androgen receptor; TNF-α, tumor necrosis factor-α; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay

0014-5793/02/$22.00
PII: S0014-5793(02)03440-3

previously described [15]. In brief, confluent HAEC were incubated in phenol red-free and serum-free medium containing 100 nM [1,2,6,7-^{3}H]T (Amersham Pharmacia Biotech, Buckinghamshire, UK) for 2 h at 37°C, after which steroids were extracted with ethyl ether and were resolved by thin layer chromatography. Blanks were run for each assay, using culture dishes without cells. Radioactivities corresponding to T and 5αDHT were determined. 5α-R activity was calculated as counts per minute (cpm) for 5αDHT/(cpm for T+cpm for 5αDHT)×100.

2.4. Whole cell binding assay

AR binding assay was performed as previously described [15]. In brief, confluent HAEC were incubated in phenol red-free and serum-free medium with 2% bovine serum albumin containing 0–16 nM [1,2,6,7-^{3}H]T or [1,2,4,5,6,7-^{3}H]5αDHT (Amersham Pharmacia Biotech) for 2 h at 37°C in the absence or presence of 500 nM of unlabeled steroids to determine the total and non-specific binding, respectively. At the end of the incubation, the cells were washed four times with serum-free medium, solubilized in 0.1 N NaOH. The radioactivity of the content was counted with a liquid scintillation counter. The remaining content was used for the determination of protein by a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA).

2.5. Northern blot analysis

Confluent cells were incubated with test compounds or vehicle (0.1% ethanol) for 24 h in phenol red-free and serum-free medium, and thereafter were stimulated with 20 ng/ml TNF-α (Sigma, St. Louis, MO, USA) for 4 h. Northern blot analysis was performed as previously described [16] with the use of total RNAs (20 μg) isolated from HAEC and human VCAM-1 cDNAs. The cDNA probe was originally obtained by RT-PCR with HAEC total RNA. The sequence was verified by the dideoxy chain termination method.

2.6. Nuclear extraction and electrophoretic mobility shift assay (EMSA)

HAEC were treated with test compounds and/or TNF-α, as described above. The nuclear extracts were prepared by the method of Schreiber et al. [17]. EMSA for NF-κB was performed as described [18]. The sequence of double-stranded oligonucleotide used to determine the DNA binding abilities of NF-κB was derived from the human VCAM-1 gene promoter [19]. The NF-κB consensus sequence is shown in bold face: 5′-CTGCCCT**GGGTTTCCCC**TTGAA**GGGATTTCCC**TCCGCC-3′.

2.7. Statistical analysis

All values are expressed as mean ± S.D. Significance of differences between group means was assessed by Student's unpaired *t*-test or one-way ANOVA followed by Tukey–Kramer multiple comparison test. *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

First, we examined the expression of the AR gene in HAEC. Using the RT-PCR method, amplified products with the expected size corresponding to the transcripts were detected (Fig. 1). Cloning and sequence analysis of the PCR products demonstrated that these bands had the known sequence of the human AR mRNA (data not shown). The transcript of the 5α-R1 gene was obtained from HAEC after 35 cycles of PCR were performed. We failed to detect the products of 5α-R2 and aromatase mRNAs in HAEC, even after 35 cycles of PCR.

Next, 5α-R activity was measured by a radiometric conversion assay. Under these incubation conditions, there was no significant 5α-R activity in these cells (10.2 ± 2.0%) compared with blank assays (8.6 ± 2.1%) ($n = 6$ in each group, $P = 0.21$).

HAEC were tested for their ability to bind to [^{3}H]T or [^{3}H]5αDHT. The specific binding of both ligands was saturable (Fig. 2). Scatchard analyses of the binding data revealed a single class of binding sites for T with an apparent K_d of

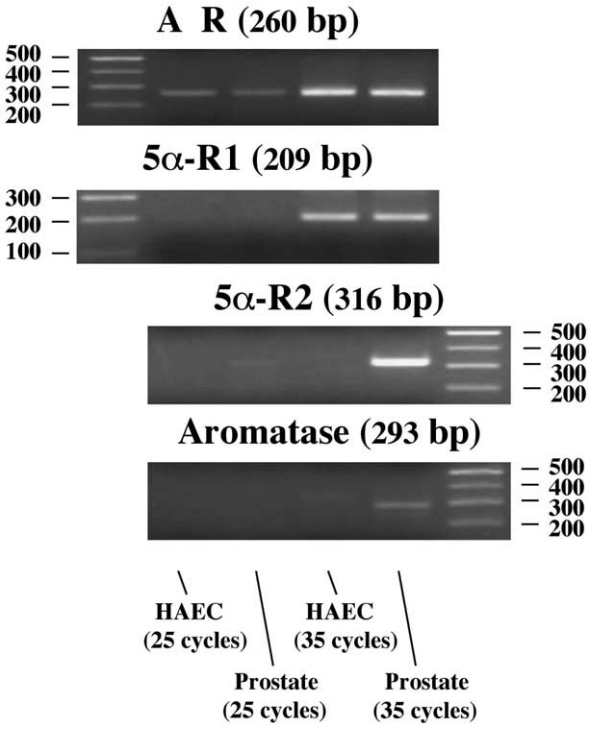

Fig. 1. Detection of AR, 5α-R1, 5α-R2 and aromatase mRNAs in HAEC by RT-PCR. Total RNAs (1 μg) were amplified by RT-PCR as described. The prostate total RNA (Sigma, St. Louis, MO, USA) was used as a positive control. Each experiment was repeated three times, and the data shown are representative of three independent results.

2.01 ± 0.42 nM and an apparent B_{max} of 44.8 ± 8.5 fmol/mg protein, and for 5αDHT with a K_d of 1.92 ± 0.55 nM and a B_{max} of 51.8 ± 10.5 fmol/mg protein.

To examine the effect of androgens on TNF-α-induced VCAM-1 mRNA accumulation, Northern blot analysis was performed. Since T and 5αDHT had similar B_{max} and K_d values and no significant 5α-R activity was observed in HAEC, we tested the effect of T in the following experiments. As shown in Fig. 3, TNF-α increased the VCAM-1 mRNA level. Treatment with T inhibited the VCAM-1 expression in a concentration-dependent manner, with a maximal decrease (49 ± 16%) at 1 μM. The inhibition of VCAM-1 expression was completely blocked by the concomitant administration of a steroidal AR blocker, cyproterone acetate (CPA).

To determine whether T regulates VCAM-1 expression by inhibiting NF-κB activation, we performed EMSA with nuclear extracts and cytoplasmic extracts from HAEC. EMSA with the oligonucleotide probe containing NF-κB binding sites in the human VCAM-1 gene promoter showed that TNF-α enhanced two of the gel-retarded bands (Fig. 4A, lanes 1 and 3). Specificity of the complexes was demonstrated with antibodies against NF-κB subunit Rel A (p65) and p50, which supershifted the complexes, and by competition with an excess of unlabeled oligonucleotide (Fig. 4A, lanes 6–9). T markedly decreased the intensity of the shifted bands produced by nuclear extracts treated with TNF-α, and the decrease of NF-κB activation was partially abolished by the presence of AR blocker (Fig. 4A, lanes 3–5). Cytoplasmic extracts from the same cells showed little difference (Fig.

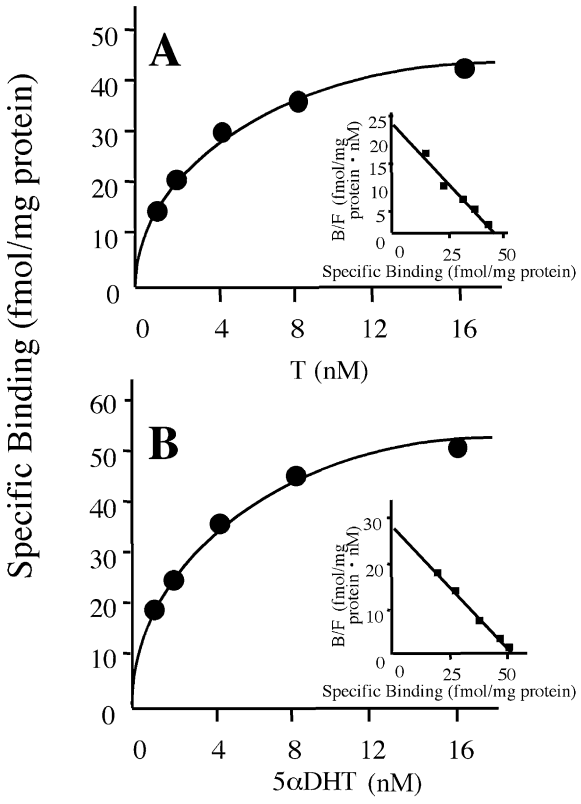

Fig. 2. Saturation curves and Scatchard analyses of [^{3}H]T (A) and [^{3}H]5αDHT (B) binding to HAEC. Each point represents the mean of triplicate assays. Non-specific binding was less than 10% of total binding in all assays.

4B, lanes 3–5). T alone did not exhibit any significant differences in NF-κB activation (Fig. 4A, lanes 1 and 2).

4. Discussion

Leukocyte adhesion to the endothelium via adhesion molecules is one of the earliest events in atherosclerosis. It has been

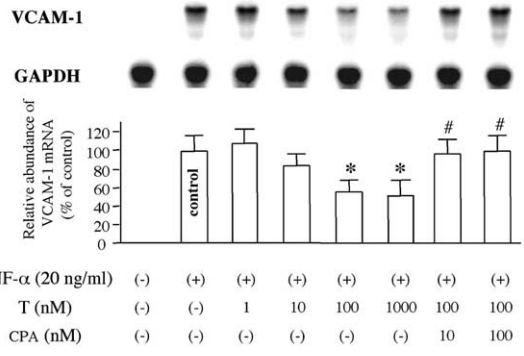

Fig. 3. Effect of T on TNF-α-induced VCAM-1 expression in HAEC. Confluent cells were incubated with test compounds or vehicle (0.1% ethanol) for 24 h in phenol red-free and serum-free medium, and thereafter were stimulated with 20 ng/ml TNF-α for 4 h. Experiments were repeated three times with equal results. The individual result was obtained by the calculation of the intensity of the VCAM-1 band divided by that of the GAPDH band, and related to the mean of control group. $^*P < 0.05$ compared with control; $^\#P < 0.05$ compared with cells cultured with 20 ng/ml TNF-α and 100 nM of T in the absence of CPA, a steroidal antiandrogen.

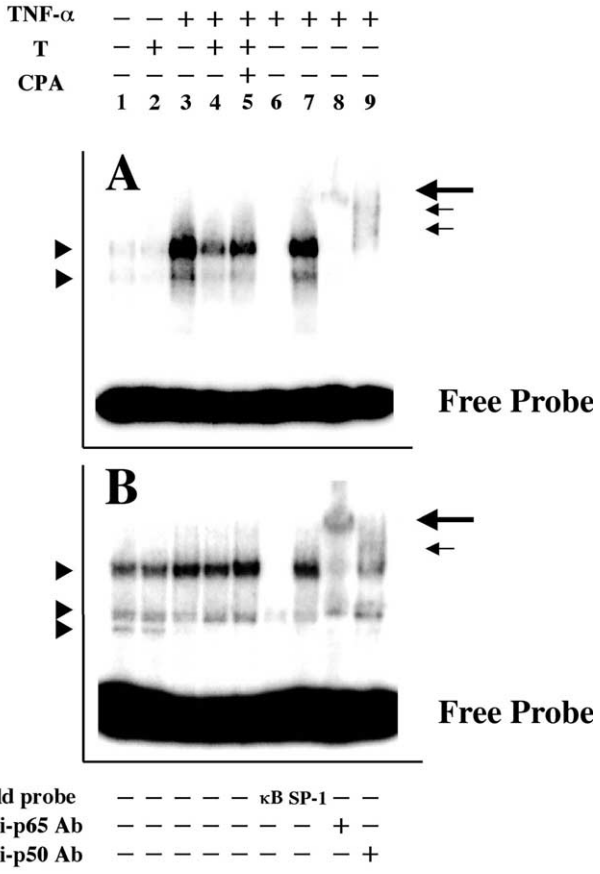

Fig. 4. Effect of T on DNA binding activity of NF-κB. HAEC were treated as described in Fig. 3. EMSA with use of the oligonucleotide probe specific for the NF-κB binding of human VCAM-1 gene promoter was performed on nuclear extracts (A) and cytoplasmic extracts (B) with no reagents (lanes 1–5), 50-fold excess of unlabeled NF-κB oligonucleotide (lane 6), 50-fold excess of unlabeled stimulatory protein-1 (SP-1) oligonucleotide (lane 7), anti-p65 antibody (anti-p65 Ab, lane 8) and anti-p50 antibody (anti-p50 Ab, lane 9). Arrowheads indicate NF-κB binding to DNA. Large and small arrows indicate supershifted bands in lanes 8 and 9, respectively. Data shown are representative of three separate experiments.

suggested that VCAM-1 plays a very important role in the recruitment of leukocytes into the vascular wall [20,21]. Recent evidence indicates that the promoter region of the VCAM-1 gene contains NF-κB binding sites which are essential for the expression of the gene [9]. NF-κB, a transcriptional activator factor, is present in the cytoplasm of unstimulated cells in an inactive form, complexed to the inhibitory protein IκB. Cellular activation by cytokines such as TNF-α induces phosphorylation and degradation of IκB, with subsequent liberation and translocation of NF-κB to the nucleus [8]. NF-κB then binds to specific DNA sequences in the 5′-flanking region of VCAM-1 gene [22].

In the present study, we demonstrated that T inhibits TNF-α-induced VCAM-1 expression and NF-κB activation through an interaction with AR in HAEC. Since EMSA with cytoplasmic extracts showed little difference in the NF-κB binding, and T alone did not exert significant effects, the mechanism by which T inhibits TNF-α-induced VCAM-1 expression is probably mediated by inhibition of the TNF-α-induced NF-κB nuclear translocation. Because there is no evidence that androgen response elements exist on the

VCAM-1 gene promoter, it is unlikely that T-bound AR directly interacts with the 5′-flanking region of the VCAM-1 gene. Simoncini et al. demonstrated that 17β-estradiol decreased VCAM-1 gene expression through the inhibition of the NF-κB nuclear translocation, with the inhibition associated with decreased IκB kinase activity [23].

Our results provide evidence for the presence of AR by RT-PCR and receptor binding methods. In the binding study, T and 5αDHT had similar B_{max} and K_d values. Wilson et al. demonstrated that AR has essentially identical capabilities to bind T and 5αDHT in rat testis, epididymis and prostate [24]. McCrohon et al. reported that 5αDHT increased human monocyte adhesion to vascular endothelium, at least in part through an AR-mediated effect on endothelial expression of VCAM-1 [25]. In many androgen target tissues, it has been suggested that 5αDHT is the active form of androgen and T is reduced to 5αDHT by the enzyme 5α-R before binding to AR. However, abundant physiologic and genetic data demonstrate that T and 5αDHT are not biologically equivalent. Conformational changes that occur as a result of the binding of different ligands form the structural basis for the recruitment of different cofactors to nuclear receptors. These changes may contribute to the diversity of androgen action. Powers and Florini found that T, not 5αDHT, stimulates mitotic activity in a myoblast culture system and this result suggests a possibility that T affects muscles by direct interaction with the AR [26]. This is supported by the finding that 5α-R activity in muscle tissue was lower than that in accessory sex organs [27]. Although we detected the small amount of mRNA of 5α-R1 which displays a low affinity for T, no significant 5α-R activity was observed in the present study.

Clinical manifestations of atherosclerosis, such as coronary heart disease, occur at a higher frequency in the western world. It has been postulated that male sex along with other factors accelerates the progression rate of the atherosclerotic process. However, available data do not support the role of androgens in atherogenesis. Alexandersen et al. have suggested an anti-atherogenic effect of T in animal studies [28]. Further studies of the possible benefits of androgens in the prevention of atherosclerosis are needed. Nevertheless, the present findings provide novel insight into the consideration of androgen replacement therapy and understanding of the pathogenesis of atherosclerosis.

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