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# The Effect of Estrogen and Androgen on Androgen Receptors and mRNA Levels in Uterine Leiomyoma, Myometrium and Endometrium of Human Subjects

**Jiro Fujimoto,* Miki Nishigaki, Masashi Hori, Satoshi Ichigo, Toshiya Itoh and Teruhiko Tamaya**

*Department of Obstetrics and Gynecology, Gifu University School of Medicine, 40-Tsukasa-Machi, Gifu, Gifu 500, Japan*

**This study was designed to show the effect of estrogen and androgen on the level of testosterone and dihydrotestosterone specific binding sites (TBS and DHTBS, respectively) and to clarify the implication of androgen receptor mRNA expression to TBS and DHTBS in human uterine tissues. Estrogen mainly induces the increase of TBS and androgen receptor mRNA in uterine endometrium and leiomyoma. TBS increased by estrogen are downregulated when testosterone is given along with estrogen, while androgen receptor mRNA increased by estrogen was not significantly altered by testosterone with estrogen in endometrium and leiomyoma. These results suggest that the androgen receptor mRNA determined might encode TBS and that testosterone may stimulate the metabolic rate of TBS, or inhibit the translation rate of androgen receptor mRNA to TBS. Furthermore, the biological character of leiomyoma is considered to be an endometrial type.**

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## INTRODUCTION

Human uterus is changed morphologically and biochemically during the menstrual cycle. Cellular proliferation of endometrium (EM), myometrium (MM) and leiomyoma (LM) in the uterus is stimulated by estrogen (E). This proliferation is inhibited by progesterone (P) after ovulation. On the other hand, cellular differentiation of these tissues is stimulated by P. Additionally, testosterone (T) is secreted from the ovaries and adrenals in reproductive women. The serum level of T is 10 times higher than that of estradiol-17$\beta$ [1, 2]. In addition, androgen demonstrates the biological effect on the uterus [3]. Therefore, the biological effects of T on these uterine tissues (EM, MM or LM) can be implicated from the aspect of expressions of androgen receptor (AR) and its mRNA.

It is well known that the effect of T is different from that of its 5$\alpha$-reductase metabolites, dihydrotestosterone (DHT) [4]. In brief, T is related to cellular differentiation and DHT to cellular proliferation. Namely, T stimulates embryonic Wolffian-duct development, sperm generation, muscle growth and sexual behavior, while DHT stimulates the virilism (growth) of external genital tracts and sexual extragenital development [5, 6]. It has been proposed that different binding sites for T and DHT are present in various tissues of rabbits [7].

This study was designed to show the effects of estrogen and androgen on the level of T and DHT binding sites and the expression of AR mRNA in the tissues described above and to clarify the implication of AR mRNA expression to T and DHT binding sites in human tissues.

## EXPERIMENTAL

### *Chemicals*

[1,2,6,7-$^3$H]-testosterone ([$^3$H]-T, 98 Ci/mmol) and dihydro[1,2,4,5,6,7-$^3$H]-testosterone ([$^3$H]-DHT, 125.9 Ci/mmol) were purchased from New England Nuclear Corporation, Boston, MA). Estradiol-17$\beta$, diethystilbestrol (DES, 3,4-bis[*p*-hydrooxyphenyl]-3-hexene), testosterone(T) and dihydrotestosterone

*Correspondence to J. Fujimoto.
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(DHT) were from Sigma Chemical Co. (St Louis, MO). All other reagents were of experimental grade.

*Preparation of samples for the assay of steroid binding sites*

Agreements for the experiments below were obtained from the patients and the Research Committee for human subjects, Gifu University School of Medicine. Twenty-five patients, ranging from 34 to 52 years of age, were selected from those who underwent hysterectomy for uterine leiomyoma at the Department of Obstetrics and Gynecology, Gifu University School of Medicine, between November 1992 and August 1993. None of the patients had received any therapy except for the sex steroid administration indicated. The patients were given an intramuscular injection of 10 mg estradiol dipropionate (Ed) or 10 mg Ed plus 100 mg testosterone cypionate (Tc) 1 week before hysterectomy. The tissues obtained immediately after hysterectomy were snap-frozen in liquid nitrogen. All subsequent steps were carried out at 4°C, except when indicated.

Tissues (400 mg) were homogenized in a Polytron homogenizer (Kinematica, Luzern, Switzerland) in 2 ml TEG buffer (10 mM Tris–HCl, 1.5 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, pH 7.4). The amount of DNA was determined in the 100 $\mu$l aliquot of this suspension before the first centrifugation by the method of Burton [8].

The suspension was centrifuged at 800 ***g*** for 10 min and the supernatant was centrifuged at 160,500 ***g*** for 60 min. This supernatant, designated as the cytosol fraction, was treated with the same volume of 0.3% dextran-coated charcoal (DCC) for 15 min to remove all free steroids.

The 800 ***g*** pellet was resuspended in 0.5 ml KCl buffer (10 mM Tris–HCl, 1.5 mM EDTA, 10% glycerol, 0.6 M KCl, pH 8.5) for 60 min with vigorous mixing every 15 min and centrifuged at 90,300 ***g*** for 30 min. This supernatant, designated as the nuclear KCl extract fraction, was treated with 0.3% DCC for 15 min.

The 90,300 ***g*** pellet was homogenized in 1 ml TEG buffer and designated as nuclear KCl insoluble fraction.

Each soluble fraction was diluted with an adequate volume of TEG buffer at a concentration of at least 1 mg protein/ml to avoid the interference of various proteins of each sample. Protein levels in samples were measured by the method of Bradford [9].

*Testosterone binding sites (TBS) and dihydrotestosterone binding sites (DHTBS) assay*

Each fraction (500 $\mu$l) was incubated with 0.2–5.0 nM [$^3$H]-T or [$^3$H]-DHT and a 200-fold molar excess of DHT or T, respectively, and those with or without a 200-fold molar excess of T for TBS assay or of DHT for DHTBS assay, respectively, at 20°C for 90 min. The bound form of the soluble fractions (cytosol and nuclear KCl extract fractions) was determined by 0.3% DCC adsorption method, and that of the insoluble fraction (nuclear KCl insoluble fraction) by washing and pelleting [7]. Scatchard plot analysis [10] was carried out.

*Isolation of RNA and reverse transcription (RT)*

Total RNAs from tissues were isolated by acid guanidium thiocyanate–phenol–chloroform extraction method [11]. Total RNA (3 $\mu$g) was reverse-transcribed with Moloney Murine leukemia virus reverse transcriptase (MMLV-RTase, 200 units, Gibco BRL, Gatherburg, MD) in 20 mM Tris–HCl, pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/ml BSA, 10 mM DTT and 0.5 mM deoxynucleotides using random hexamer (50 ng Gibco BRL, Gatherburg, MD) at 37°C for 60 min. RT reaction was heated at 94°C for 5 min to inactivate MMLV-RTase.

*Amplification of AR mRNA by polymerase chain reactions (PCR)*

PCR for AR mRNA was carried out, with reverse-transcribed cDNAs from total RNAs of tissues as templates and 0.1 $\mu$M specific primers (AR-S: [1652-1671, Exon 2] 5′-ACTTTCCACCCCAGAAGACC-3′, AR-A: [1936-1955, Exon 4] 5′-TCAGTGGGGCTGGTGGTGCT-3′) for the coding sequences of cDNA of human AR [12] in 10 mM KCl, 20 mM Tri–HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.15 mM deoxynucleotide phosphates, Vent DNA polymerase (2 units/100 $\mu$l reaction, New England Biolab, Beverley, MA) using an IWAKI thermal sequencer TSR-300 (Iwaki Glass, Tokyo, Japan). Thirty-five cycles of PCR for AR mRNA, consisting of 1 min at 94°C for denaturation, 1 min at 55°C for annealing and 1 min at 72°C for extension, were performed. Twenty-six cycles of PCR for glyceraldehyde-3-phosphate dehydrogenese (GAPDH, a house keeping gene) mRNA, as an internal marker to normalize AR mRNA expression levels, with specific primers: GAPDH-S:[71-96, Exon 2] 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, GAPDH-A:[1030-1053, Exon 8] 5′-CATGTGGGCCATGAGGTCCACCAC-3′] [13], were performed under the same conditions as PCR for AR mRNA.

*Analysis of quantities of AR mRNA (Southern blot for PCR products)*

PCR products were applied to 1.2% agarose gel and electrophoresis was performed at 50–100 VC. PCR products were capillary-transferred to Immobilon transfer membrane (Millpore Corp. Bedford, MA) for 16 h. The membrane was dried at 80°C for 30 min and was UV-irradiated to fix PCR products tightly. PCR products on the membrane were prehybridized in 1 M NaCl, 50 mM Tris–HCl, pH 7.6 and 1% SDS at 65°C for 1 h and were hybridized in the same solution with

the biotinylated random-primed probes, synthesized from entire cDNA of AR with Plex Luminesent Blotting Kits for labelling (Millipore Corp., Bedford, MA), at 65°C for 16 h. Specific bands with the biotinylated probes were detected with Plex Luminesent Kits (Millipore Corp., Bedford, MA) and the membrane was exposed to X-ray films at room temperature for 10 min. The intensity of luminesence required to express AR mRNA level was standardized with that of GAPDH mRNA, a house keeping gene.

Statistical analysis was performed with Student's $t$-test; differences were considered significant when $P$ was less than 0.05.

## RESULTS

### *The expression of TBS and DHTBS in EM, MM and LM*

Saturation and Scatchard plot analyses were performed in cytosol, nuclear KCl extract and nuclear KCl insoluble fractions of the samples. Representative analyses are shown in Fig. 1. The levels of AR expression in uterine tissues were evaluated by summing up the maximal binding sites ($B_{max}$) of TBS or DHTBS in the cytosol fraction, in the nuclear KCl soluble fraction and in the nuclear KCl insoluble fraction, after normalizing each level with the DNA amount in individual samples.

In TBS, the mean dissociation constant ($K_d$) and $B_{max}$ in EM, MM and LM are shown in Table 1 and Fig. 2(A). The administration of Ed or Ed plus Tc did not affect the $K_d$. TBS levels in EM and LM were significantly ($P < 0.05$) increased by Ed and these increases were significantly ($P < 0.05$) inhibited by Ed plus Tc [Fig. 2(A)]. These steroids did not affect the TBS level in MM.

In DHTBS, the mean $K_d$ and $B_{max}$ in EM, MM and LM are shown in Table 1 and Fig. 2(B). The administration of Ed or Ed plus Tc did not affect the $K_d$, shown in Table 1 and Fig. 2(B). DHTBS levels were not significantly modulated by Ed or Ed plus Tc [Fig. 2(B)].

### *The expression of AR mRNA in EM, MM and LM*

Amplified AR DNA fragment from AR mRNA in EM, MM and LM was analyzed by Southern blotting (Fig. 3). The level of GAPDH mRNA expression as an internal marker was almost uniform in all given cases. The level of AR mRNA expression was standardized by that of GAPDH mRNA. The mean level of AR mRNA without treatment was assigned as 100 arbitrary units/GAPDH mRNA (AU/GAPDH).

The administration of Ed increased the AR mRNA in EM and LM ($P < 0.05$), but not in MM (Fig. 4). The level of the AR mRNA in EM and LM increased by Ed was not significantly modified by Tc in each uterine tissue.

## DISCUSSION

Androgen is possibly implicated in the biological function of the female reproductive tract. In fact, androgen demonstrates biological effects on the uterus, with the cooperative effects of estrogen [3]. The uterus consists of the endometrium, the myometrium and occasionally myomas which are derived from the myometrium. The cellular growth and functional differentiation of those tissues are regulated by sex

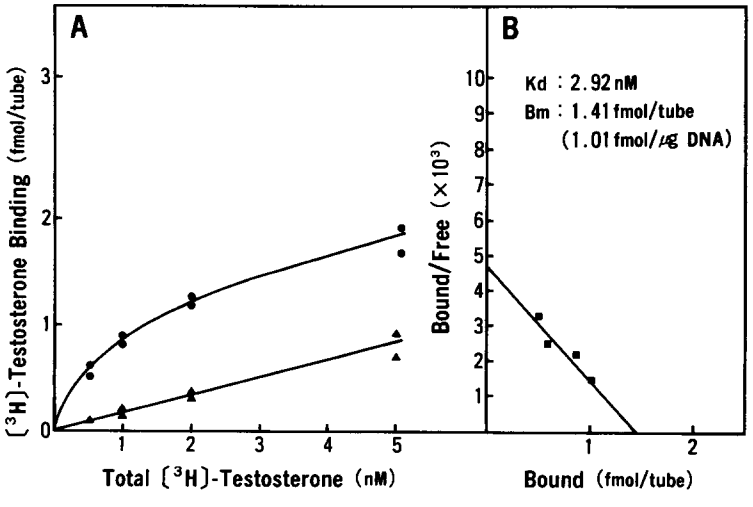


**Fig. 1. Saturation and Scatchard plot analyses for testosterone binding sites (TBS) in endometrial cytosol as a representative analysis. Cytosol fraction (500 μl) of uterine endometrium was incubated with 0.2–5.0 nM [³H]-T and a 200-fold molar excess of DHT, and those with or without a 200-fold molar excess of T at 20°C for 90 min. Radioactivity of the bound form was determined by DCC adsorption method. (A) Total binding in cytosol is indicated as [³H]-T binding plus a 200-fold molar excess of DHT (●). Non-specific binding in cytosol is indicated as [³H]-T plus a 200-fold molar excess of DHT and T (▲). (B) Scatchard plot analysis was carried out as for (A).**

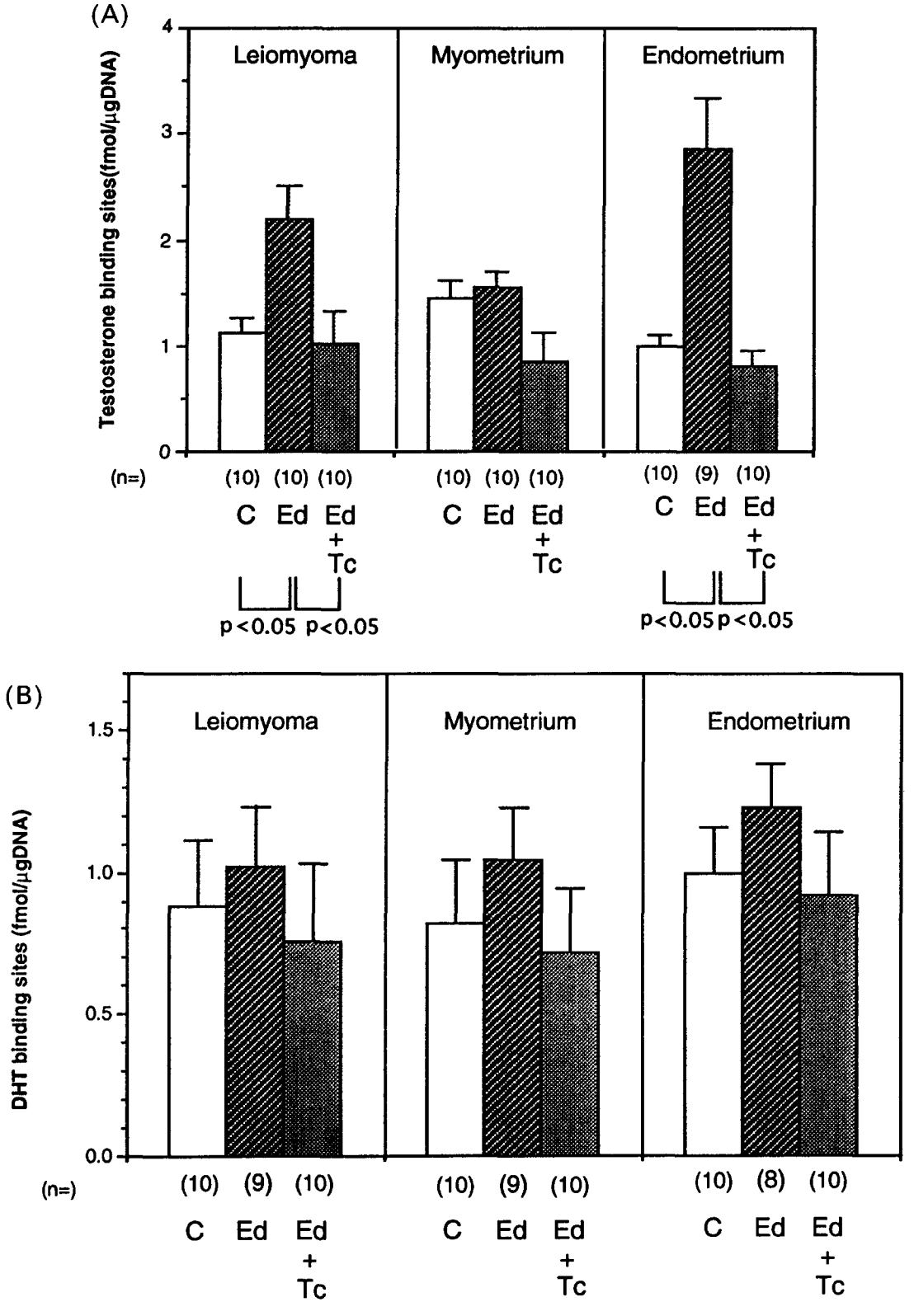


Fig. 2. TBS (A) and DHTBS (B) levels in uterine endometrium (EM), myometrium (MM) and leiomyoma (LM). The cytosol, nuclear KCl extract and nuclear non-KCl extract fractions (500 $\mu$l) in each uterine tissue were incubated at 20°C for 90 min with [$^3$H]-T or [$^3$H]-DHT and a 200-fold molar excess of DHT or T, respectively, and those with or without a 200-fold molar excess of T for TBS assay (A) or DHT for DHTBS assay (B). Radioactivity of the bound form was determined. Scatchard plot analysis was carried out. Cellular expression levels of steroid receptors were evaluated by summing up the maximal TBS (or DHTBS) of each fraction. Data are the mean $\pm$ SD of 8–10 determinations for uterine tissue. The value of $P < 0.05$ means of statistic significance difference. Ed and Tc treatments were described in Table 1. All abbreviations are the same as those described for Table 1.

*Table 1. Dissociation constants of testosterone and dihydrotestosterone binding sites in leiomyomata, myometria and endometria*

| Sample | | TBA Control | TBA Ed | TBA Ed + Tc | DHTBS Control | DHTBS Ed | DHTBS Ed + Tc |
|---|---|---|---|---|---|---|---|
| EM | | | | | | | |
| [$n$ = ] | | [10] | [9] | [10] | [10] | [8] | [10] |
| $K_d$: | Mean ± SD | 2.94 | 2.92 | 2.47 | 2.69 | 2.33 | 2.25 |
| (nM) | | 0.48 | 0.52 | 0.17 | 0.64 | 0.43 | 0.53 |
| MM | | | | | | | |
| [$n$ = ] | | [10] | [10] | [10] | [10] | [9] | [10] |
| $K_d$ | Mean ± SD | 2.37 | 2.38 | 2.54 | 2.86 | 2.66 | 2.21 |
| (nM) | | 0.49 | 0.67 | 0.82 | 0.52 | 0.34 | 0.93 |
| LM | | | | | | | |
| [$n$ = ] | | [10] | [10] | [10] | [10] | [9] | [10] |
| $K_d$ | Mean ± SD | 2.74 | 2.62 | 2.28 | 2.82 | 2.87 | 2.23 |
| (nM) | | 0.45 | 0.34 | 0.23 | 0.72 | 0.23 | 0.88 |

The cytosol, nuclear KCl extract and nuclear non-KCl extract fractions in EM, MM, LM were incubated with 0.2–5.0 nM [$^3$H]-Tor DHT plus a 200-fold molar excess of DHT or T, respectively, and those with or without a 200-fold molar excess T for TB, or DHT for DHTB at 20°C for 90 min. Radioactivity of the bound form was determined and Scatchard plot analysis was carried out. The mean (±SD) dissociation constants ($K_d$) of TBS and DHTBS were determined from the 3 fractions of individual samples.

EM, endometria; MM, myometria; LM, leiomyomata; Ed, estradiol dipropionate; 10 mg intramuscular injection; Tc, testosterone cypionate, 100 mg intramuscular injection.

steroids. The endometrium and myoma demonstrate marked change under the effect of estrogen. It is reasonable to evaluate the biological effects of sex steroids on these tissues comparatively.

It is well known that androgens, testosterone, dihydrotestosterone and the other metabolites demonstrate individually different biological effects [4]. It has been considered that the biological difference between T and DHT is caused by an individual translation-mode of androgen-induced proteins after T or DHT binding to the AR [14], or by an individual affinity of T or DHT to the single AR [15]. However, it is plausible that there are various aberrant forms of androgen receptor for demonstrating different biological effects. It has been proposed that different binding sites for T and DHT are present in a rabbit model [7]. T and DHT demonstrate different biological effects, differention and proliferation of cells, respectively, as mentioned previously. Therefore, it is worthwhile evaluating androgen effects on the target tissues from the regulation of TBS and DHTBS levels.

In male dogs, androgen plus estrogen leads to a higher incidence of prostate hypertrophy than androgen alone does [16]. This suggests that estrogen induces androgen receptor and exerts a synergistic effect on the growth of the prostate under DHTBS activity, while DHT contributes to cell proliferation in male target tissues. However, in female target tissues with marked proliferation such as endometrium and myoma, the evidence of the induction of TBS is different from that in the male targets, as has been demonstrated in this study.

Estrogen induces the proliferation of the uterus [17], under the induction of the various proteins. Among them, the receptors for sex steroids, estrogen [18], progestin [19] and androgen are induced by estrogen. In the present study, estrogen markedly increased the TBS level especially in EM and LM, but minimally induced the increasing change of the DHTBS level. There is, therefore, tissue-specific induction of androgen receptors.

In general, the effect of estrogen on the growth in EM is morphologically distinct in comparison with that in MM [20] whilst that in LM seems to be of the endometrial type for the growth. Therefore, estrogen-inducible TBS is demonstrated in the proliferative tissues such as EM and LM, while T itself exerts a differential effect on the cells [5, 6]. TBS changed by E and A seems to contribute to a sort of cell differentiation in EM. LM derived from MM has a similar character to that of EM in response to estrogen and androgen. TBS in LM are considered to be for a sort of cell differentiation. T can downregulate TBS in EM and LM as shown in the present study. Therefore, in female target tissue with remarkable proliferation such as EM and LM, T appears to be more important for the biology than DHT, under the estrogen inducible response of TBS as presented in this study. Similarly, progesterone demonstrates cell differentiation [20] under the downregulation of progesterone receptor [21].

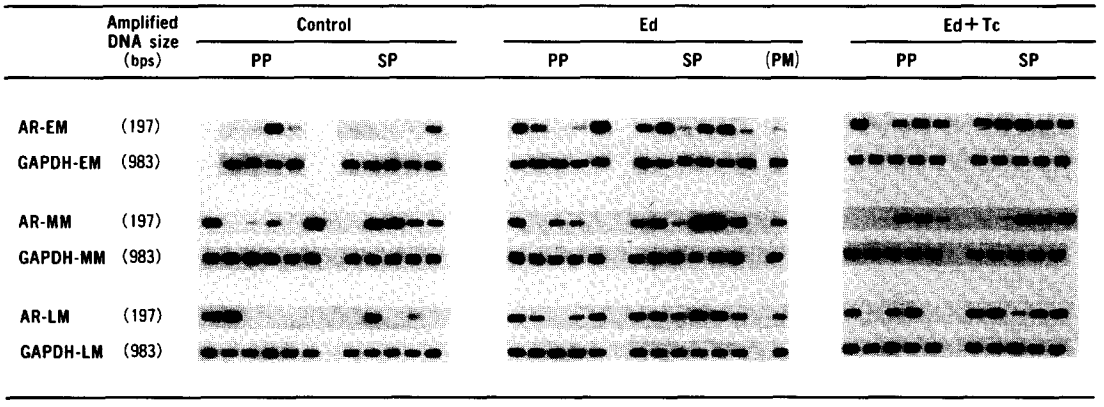


Fig. 3. Southern blot analysis of reverse transcription-polymerase chain reaction (RT-PCR) products from total RNA of EM, MM and LM. Total RNAs were subjected to RT with a random hexamer (50 ng). AR and GAPDH cDNAs subjected to RT were amplified in 35 cycles and 26 cycles of PCR, respectively. PCR products were hybridized with deoxyoligoprobes synthesized from the sequences of AR and GAPDH cDNAs between specific primers. AR, androgen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. All abbreviations are the same as those described for Table 1.

The treatment of E or E plus T does not modulate the $K_d$ of TBS and DHTBS, suggesting no alteration of the quality of sex steroid binding sites in human uterine tissues. Therefore, the intensity of E and T actions might depend upon the quantity of binding sites in a target organ, except for post-receptor modulation. E and E plus T induced the expression of AR mRNA in EM and LM, where TBS were increased by E, and this increase was reduced by T as a down-regulation. However, AR mRNA expression was not decreased by T. E or E plus T may slightly modulate the quality of DHTBS in each uterine tissue. These results suggest that AR mRNA might encode TBS in uterus, and that T might stimulate the metabolic rate of TBS, or inhibit the translation rate of AR mRNA to TBS in EM and LM.

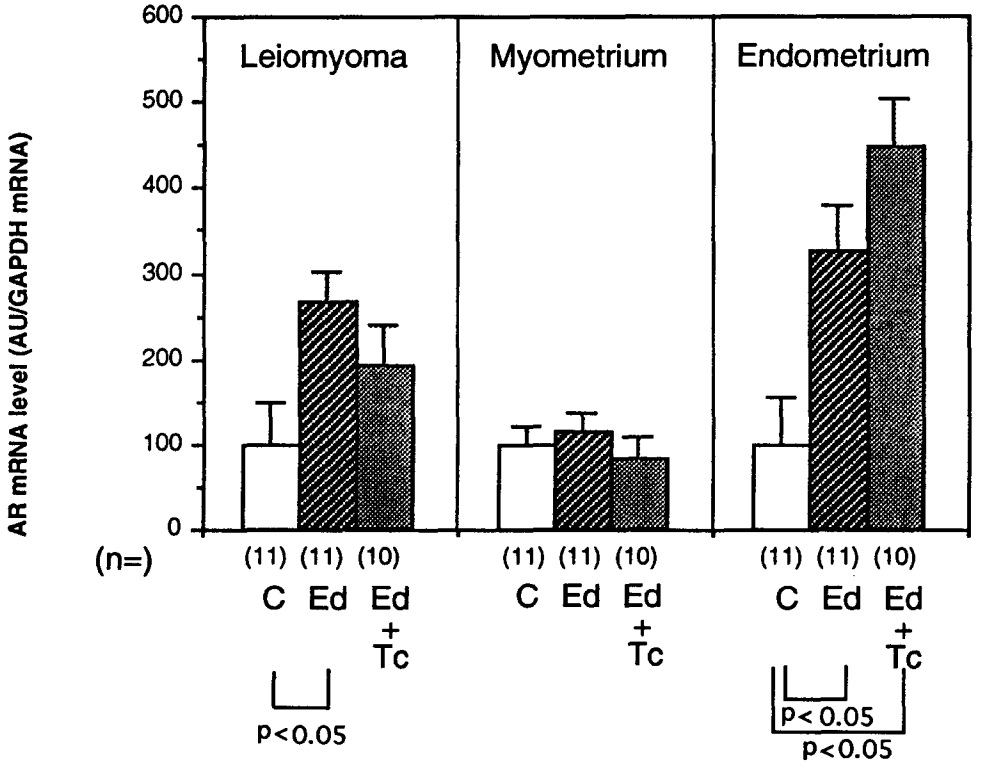


Fig. 4. AR mRNA expression levels standardized by the level of GAPDH mRNA expression in EM, MM and LM under Ed or Ed plus Tc administration. The signal intensity in Southern blot (Fig. 3) was analyzed with a densitometer and standardized by the signal intensity of GAPDH PCR products in uterine endometria. Data are the mean ± SD of four to six determinations. AR mRNA expression levels without treatment were assigned with 100 AU/GAPDH mRNA. All abbreviations are the same as those described for Table 1.

Finally, the different biological effects of T and DHT might be present on the uterus and myoma at the detection of TBS and DHTBS, while T predominantly demonstrates biological effects such as cell differentiation on the tissues with marked cell proliferation induced by estrogen via estrogen-inducible TBS.

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