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Divergent Effects of Retinoic Acids on the Expression of ERα and 17β-Hydroxysteroid Dehydrogenase Type 2 in Endometrial Carcinoma Cells (RL 95-2)

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The effects of E2 are dependent on ERs and local E2 concentration in target cells. Modulation of intracellular E2 concentration involves the action of 17β-hydroxysteroid dehydrogenase (17HSD) type 2, the enzyme converting E2 to estrone. In the present study, the influence of RAs on the growth of endometrial cancer cell line RL 95-2 as well as the expression of ERs and 17HSD type 2 have been investigated. It was found that RAs repress the growth of RL 95-2 cells, which express all subtypes of RXR and RAR, as examined by RT-PCR. Also, quantitative RT-PCR analysis showed that both ERα and ERβ are present in RL 95-2 cells, and Western blot assay further revealed that ERα expression was decreased by *all trans*-RA treatment. In contrast, RAs induced 17HSD type 2 mRNA expression in a dose- and time-dependent fashion. This stimulatory effect was also detected at the level of *in vivo* oxidative 17HSD activity in cultured cells. On the other hand, the abundance of 17HSD type 2 mRNA was not altered by RAs in cultured normal epithelial cells isolated from human early- and late-secretory endometrium. The data indicate that RAs have an inhibitory effect on the growth of RL 95-2 cells and a cross-talk with the estrogen pathway in estrogen-responsive endometrial cancer cells. (*J Clin Endocrinol Metab* 87: 640–649, 2002)

ESTROGENS PRIMARILY PRODUCED in the ovary are essential sex steroid hormones for female development and reproduction. A key biological effect of estrogens is to modulate the growth and function of the reproductive tissues (such as the endometrium) and a number of peripheral tissues (for example, the breast). However, a large body of epidemiological evidence has suggested that estrogens, mainly 17β-estradiol (17β-E2), are involved in certain neoplastic process in the breast and the endometrium (1–4).

Estrogens have two main molecular forms: estrone (E_1) and E2. Both molecules can bind to ERs, which mediate the cellular effect of estrogens. Because E_1 displays only approximately 1% of the biological potency of E2 (5), the physiologically significant molecule of estrogens is therefore E2. The action of estrogen is dependent on the expression level of ERs and intracellular E2 concentration in target cells. Current opinion supports the idea that intracellular E2 concentration is not only determined by endocrine production but also largely affected by local biosynthesis and metabolism in target cells (6).

E_1 and E2 can be converted to each other by 17β-hydroxysteroid dehydrogenases (17HSDs), an enzyme family catalyzing the interconversion between low-activity sex steroids (17-ketosteroids) and more potent forms (17β-hydroxysteroids). To date, at least eight 17HSD isoenzymes have been identified from humans. The various 17HSDs are encoded by individual genes, seeming to be different from each other in amino acid component, subcellular localization, tissue distribution, substrate specificity, catalytic property, and cofactor preference (reviewed in Ref. 7). The highly estrogen-specific 17HSD type 1 prefers converting E_1 to E2, thus acting as an E2-synthesizing enzyme. In contrast, 17HSD type 2 can use either estrogens or androgens as substrates, and it primarily catalyzes the oxidative reaction of E2 to E_1, or T to androstenedione (8). It has been suggested that among 17HSDs characterized so far, the type 2 enzyme plays the most substantial role in inactivating E2 in classical and nonclassical steroid hormone target tissues (9). Coordinate expressions of 17HSD type 1 and type 2 in some estrogen target cells modulate intracellular E2 concentration, and hence the action of estrogens (10).

As the main target cells of estrogen action, human endometrial epithelial cells express both 17HSD type 1 and type 2 (8, 9, 11, 12). However, the expression level of the type 2 enzyme is considerably higher than that of the type 1 enzyme (8, 12). Correspondingly, the dominant 17HSD activity in the endometrium is the oxidation of E2 to E_1 (13). Thus the type 2 enzyme is the main 17HSD protein responsible for regulating the local E2 level in the endometrium. The abundance of this enzyme in the endometrium is fluctuated concurrently with the blood progesterone level, being highest during mid- to late-secretory phase in the cycle. Thus, it is speculated that 17HSD type 2 expression is subjected to regulation of progesterone (12). So far, however, neither progesterone nor other factors have been shown to directly regulate 17HSD type 2 expression. Information about the regulatory factors controlling its endometrial expression will significantly assist an inside understanding of estrogen action on the endometrium, both normal cyclic growth and neoplastic development.

RAs, the biological derivatives of vitamin A, have been

Abbreviations: AMV, Avian myeloblastosis virus; *at*-RA, *all trans*-RA; 9*cis*-RA, 9*cis*-retinioc acid; DCC-FCS, FCS treated with dextran-coated charcoal; DES, diethylstilbestrol; E_1, estrone; FD, DMEM/F12; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 17HSD, 17β-hydroxysteroid dehydrogenase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetraziolum bromide.

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demonstrated to inhibit the cell proliferation of estrogen-sensitive breast cancer cells (14, 15). Recent studies also revealed that RAs interact with the estrogen biosynthesis pathway by stimulating 17HSD type 1 expression in steroidogenic as well as estrogen target cells (16–18). Thus, it is very interesting to understand whether RAs play a role in regulating estrogen action in endometrial carcinoma cells. In this study, we have investigated the effect of RAs on the growth of endometrial carcinoma cell line RL 95-2. This estrogen-responsive epithelial cell line, derived from human endometrium (19), is absent from 17HSD type 1 but has considerable expression of 17HSD type 2 (8). We also studied the existence of ERα and ERβ in this cell line, using various methods, as well as the influence of RAs on the expression of ERα, ERβ, and 17HSD type 2. In addition, we examined the expression of RXRs and RARs in RL 95-2 cells, using RT-PCR analysis.

Materials and Methods

Chemicals

[2,4,6,7-^{3}H]E2 (75 Ci/mmol) and [2,4,6,7-^{3}H]E_1 (102 Ci/mmol) were from Amersham Pharmacia Biotech (Little Chalfont, UK). [α-^{32}P]deoxycycidine triphosphate (3000 Ci/mmol) was obtained from Yahui Biomedical Engineering Inc. (Beijing, China). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetraziolum bromide (MTT), diethylstilbestrol (DES), *al trans*-RA (*at*-RA), trypsin, and FCS were the products of Sigma (St. Louis, MO). Unlabeled E2 and E_1 were purchased from Steroids (Wilton, MD). Type IA collagenase and other supplies for cell culture were from Life Technologies, Inc. (Grand Island, NY). Avian myeloblastosis virus (AMV) reverse transcriptase, *Taq* DNA polymerase, ribonuclease inhibitor, and restriction enzymes were purchased from Promega Corp. (Madison, WI). 9*cis*-retinioc acid (9*cis*-RA) was a gift from F. Hoffman-LaRoche Inc. (Basel, Switzerland). The plasmids pBluescript-ERα and pBluescript-ERβ, containing the cDNA fragment of human ERα (nt 1210–1648) and ERβ (nt 1495–1974), respectively, were kindly provided by Dr. Yi-ming Mu (Department of Endocrinology, General Hospital of People's Liberation Army, Beijing, China).

Culture and treatment of RL 95-2 cells

Human endometrial carcinoma cell line RL 95-2 was obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM/F12 (1:1, FD) containing 10 mM HEPES, 5 μg/ml insulin, and 10% FCS at 37 C in humidified 5% CO_2 incubator. Before each experiment, the cells were allowed to grow to 70–80% confluence, in the dishes, before being subjected to various stimuli present in the media, containing 5% FCS treated with dextran-coated charcoal (DCC-FCS), for the indicated times. Control samples were treated similarly but without stimuli. After stimulation, total RNA was isolated from the cultured cells.

Primary culture of human endometrial epithelial cells

Human endometrial tissues of early secretory phase (n = 3) and late secretory phase (n = 3) were obtained from normal cycling women who accepted hysterectomy, in Haidian Hospital (Beijing, China), because of uterine myoma. The project was approved by the local ethics committee, and informed consent was provided by the patients. After an operation, the tissues were immediately collected from the uterus and transported to the laboratory. The epithelial cells were isolated and cultured according to a method described elsewhere (20, 21). In brief, the tissues were minced into small pieces, which were then subjected to digestion of 0.05% type IA collagenase-0.02% deoxyribonuclease I, in serum-free FD, at 37 C, by shaking for 1.5–2 h. Subsequently, the digestion mixture was filtrated through two stainless steel sieves (180 μm and 38 μm). To separate the epithelial cells from the stromal cells, the component of digestion mixture passed through a 180-μm sieve but captured by 38-μm sieve was fractionated by BSA gradient gravity sedimentation. The epithelial cell clumps were collected at the bottom of the fractionation tube whereas the stromal cells remained in suspension. Further purification of epithelial cells was performed by differential attachment. After culturing the cells for 3–4 h, stromal cells got attached to the plates, whereas the epithelial cell clumps stayed in suspension. Thus, the epithelial cell clumps were harvested from suspension and digested by 0.1% trypsin-0.02% EDTA until only small clumps were present. The final purification was achieved by allowing the stromal cells to attach to the plates for 30 min in the incubator. A sufficient amount of medium (serum-free FD) containing isolated epithelial cells was transferred to new dishes to obtain 70–80% confluence after plating. An immunocytochemical study showed that 90–94% cells are stained by the antibody against cytokeratin (result not shown). After attachment, the cells were treated with RAs, and total RNA was purified for Northern blot analysis.

Measurement of cell growth

RL 95-2 cells were applied to 96-well plates in a density of 2000 cells/well and incubated for 48 h for attachment. To eliminate the estrogens existing in culture media, the cells were cultured in phenol red-free DMEM (Life Technologies, Inc.) containing 5% DCC-FCS and various stimuli for the indicated time. To detect the cell number, the media were replaced by 0.5 mg/ml MTT in PBS (200 μl/well), and the cells were incubated for 4 h under cell culture condition. After that, the MTT was removed and 100 μl DMSO was applied to each well, followed by shaking the plates for 5 min. The concentration of the solvable dye was measured at a wavelength of 595 nm and a reference wavelength of 690 nm.

Isolation of total RNA

Total RNA from cultured RL 95-2 cells and normal endometrial epithelial cells was isolated using TRIzol reagent (Life Technologies, Inc.) according to the manufacturer's instructions.

RT-PCR analysis of RXRs, RARs, and ERs

The primers used for RT-PCR analysis are summarized in Table 1. To perform RT reaction, 1 μg total RNA isolated from RL 95-2 cells was included in AMV reverse transcriptase reaction buffer containing 0.25 mM deoxynucleotide triphosphates and 1 μM antisense primer. The reaction started with denaturing at 65 C for 5 min and was continued by adding 10 U AMV reverse transcriptase and 20 U ribonuclease inhibitor to the reaction. The total 20-μl mixture was incubated at 37 C for 1 h, and the reaction was terminated by heating the sample at 95 C for 5 min. Then, the tubes were placed in ice until next application. For PCR amplification, 2 μl of the RT product was included in 20 μl mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM deoxynucleotide triphosphates, 1 μM sense primer, and 5 U *Taq* DNA polymerase. The reaction was carried out by denaturing at 94 C for 20 sec, annealing at 56 C for 10 sec, and extension at 72 C for 10 sec (30 cycles in total). At the end of the last cycle, an additional extension at 72 C for 2 min was conducted.

Southern blot analysis

PCR products of ERα and ERβ were separated on a 2% agarose gel stained with ethidium bromide. After electrophoresis, the DNA was denatured by soaking the gel in 1.5 M NaCl/0.5 N NaOH solution for 45 min. Consequently, the gel was incubated in the neutralization solution for 30 min. Then, the DNA was transferred to a Hybond nylon membrane (Amersham International, Buckinghamshire, UK) by pressure blotting. The membrane was probed by a ^{32}P-labeled 0.44-kb ERα cDNA fragment (obtained by digestion of pBluescript-ERα with *Bam*HI and *Eco*RI) and then a 0.48-kb ERβ cDNA fragment (produced by digestion of pBluescript-ERβ with *Bam*HI and *Hin*dIII), respectively. The probes were labeled using Prime-a-gene Labeling System (Promega Corp.).

Quantitative RT-PCR analysis of ERα and ERβ

Quantification of the mRNA of ERα and ERβ in RL 95-2 cells was performed by competitive RT-PCR, based on a method described by Saghizadeh *et al.* (22). In this method, a quantitative cRNA competitor

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TABLE 1. Summary of oligonucleotide primers used for RT-PCR

Gene	Primer sequence (5′→3′)	Positions (nt)	Product size (bp)
ERα	Sense: GGAGACATGAGAGCTGC	1210–1226	439
	Antisense: CCAGCAGCATGTCGAAG	1632–1648	
ERβ	Sense: TCTTGTTCTGGACAGGG	1495–1511	480
	Antisense: TTACTGTCCTCTGCCGG	1958–1974	
RARα	Sense: CTGCCCGACACCTGGGGGC	123–141	160
	Antisense: GTGTGGTAGGGGTCGGTGG	264–282	
RARβ	Sense: TTCATGATTCGGGGCTGGGAAAAA	322–345	154
	Antisense: CGTTCCCTCTAGTACAAAC	457–475	
RARγ	Sense: GACTCTTTGCGGCTGGTGC	434–452	162
	Antisense: GGGTTCCTCTACCGGAGAG	577–595	
RXRα	Sense: ATGGACACCAAACATTTCCTGC	76–97	268
	Antisense: GGGACCCGAAGTCGTGAC	325–343	
RXRβ	Sense: ATGTCTTGGGCCGCTCGCC	118–136	133
	Antisense: GCTGCCGGGACCGACCTAG	232–250	
RXRγ	Sense: GAGGCTCCCCTGGCCACAC	74–92	129
	Antisense: GGTGCCTGAGACTCACGTC	184–202	

specific to target mRNA is added to the RT reaction in a series of concentrations, together with a certain amount of total RNA. The cRNA competitor produces a cDNA that competes the native cDNA in PCR amplification. The copy number of target mRNA in the sample is calculated according to the point of equivalence, where cRNA-derived and native mRNA-derived cDNAs are equally produced.

To prepare specific competitors, an 88-bp fragment was deleted from ERα cDNA by digestion of pBluescript-ERα with *Bgl*II and *Nco*I, whereas a 140-bp fragment was eliminated from ERβ cDNA by treatment of pBluescript-ERβ with nuclease Bal 31 (TaKaRa Biochem, Dalian, China). The deletion constructs, which contain T3 promoter in the pBluescript backbone, were employed to generate the mutant cRNA of ERα or ERβ by *in vitro* transcription, using T3 RNA polymerase and Ribomax Transcription Kit (Promega Corp.). The copy number of resultant cRNA was calculated from the absorbance at 260 nm and the molecular weight. To perform quantitative RT-PCR, various amounts of mutant cRNA were added to an RT reaction together with 1 μg total RNA. PCR amplification was carried out for 30 cycles under the condition described above. The RT-PCR products were resolved on 2% agarose gel stained with ethidium bromide, and the gel photographs were analyzed using Personal Densitometer SI (Molecular Dynamics, Inc., Sunnyvale, CA). Plots of the ratio of mRNA-derived and cRNA-derived products were linear, and the point of equivalence was determined by linear regression analysis. The copy number of ERα or ERβ transcripts per microgram of total RNA is expressed as mean ± SD from the results of three independent experiments.

Nuclear protein extraction and Western blot analysis

Nuclear extracts were prepared from vehicle- and *at*-RA-treated RL 95-2 cells by the method employed previously (17, 23). Fifty micrograms of nuclear extract was loaded onto 7.5% SDS-PAGE gel and separated by electrophoresis, followed by transfer of proteins to nitrocellulose membrane (Life Technologies, Inc.) using a pressure blotting system. The membranes were blocked in TBS buffer [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.05% Tween-20] containing 5% milk, at room temperature for 1 h, and then incubated with 5 μg/ml antibody against ERα or ERβ (Affinity BioReagents, Inc., Golden, CO) at 4 C overnight. The blots were washed three times with TBS and incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at room temperature for 1 h. ER proteins were visualized using ECL Western blotting detection reagents (Amersham International plc, Little Chalfont, UK). To determine protein molecular size, a standard molecular weight marker from Bio-Rad Laboratories, Inc. (Richmond, CA) was run in the adjacent lane to ERα or ERβ. The autoradiographic signals were measured by using Molecular Dynamics, Inc. Personal Densitometer SI.

Northern blot analysis of 17HSD type 2 mRNA

The 17HSD type 2 cDNA fragment (nt 115-1244) was obtained by RT-PCR using total RNA isolated from RL 95-2 cells as template. The fragment was constructed into pBluescript SK ± vector and confirmed by sequencing. Northern analyses were performed according to a method described previously (18). A 1.0-kb 17HSD type 2 cDNA fragment resulting from *Sac*I digestion was used as the probe, which was ^{32}P-labeled by Nick Translation System (Promega Corp.). Membranes were also hybridized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA-probe for the quantification of samples loaded. Autoradiographic signals were quantified by densitometry analysis using Molecular Dynamics, Inc. Personal Densitometer SI.

Oxidative and reductive 17HSD activity measurements

A total of 5.0×10^5 RL 95-2 cells/well were applied to 6-well plates, and the cells were allowed to attach to the plates for 48 h. They were then cultured in media containing 5% DCC-FCS and 100 nM RAs for 24 h. For measuring oxidative 17HSD activity, the media were removed from the wells, and 2 ml of serum-free FD containing 100 nM unlabeled E2 and 2.5×10^6 cpm of [2,4,6,7-^{3}H]E2 were added. For the detection of reductive 17HSD activity, 100 nM unlabeled E_1 and 2.5×10^6 cpm of [2,4,6,7-^{3}H]E_1 were included in the medium. The cells were incubated for 1 h at 37 C under cell culture condition, and the consequent steps followed the method previously described (18).

Statistics

All experiments were done at least two times, and statistical significance was determined by ANOVA or *t* test, where appropriate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Results

The proliferation of RL 95-2 cells was inhibited by RAs

To examine the effect of RAs on cell proliferation, RL 95-2 cells were cultured for 48 h with 1.0–1000 nM *at*-RA or 9*cis*-RA, whereas the vehicle-treated cells were employed as control. As shown in Fig. 1A, the growth of RL 95-2 cells was inhibited by RAs in a dose-dependent manner, compared with vehicle. Furthermore, the cells were cultured for 7 d in the presence of E2 and RAs. The data presented in Fig. 1B show that exposure to 100 nM E2 slightly increased the growth of RL 95-2 cells ($P = 0.036$). Again, either *at*-RA or 9*cis*-RA, in the concentration of 100 nM, significantly inhibited the cell proliferation. However, when the cells were treated with either *at*-RA or 9*cis*-RA alone, or in combination with E2, the extents of the antigrowth effect were observed at similar levels.

RL 95-2 cells were also cultured for 7 d in serum-free medium containing 100 nM E2, 10 ng/ml EGF, and 0.1%

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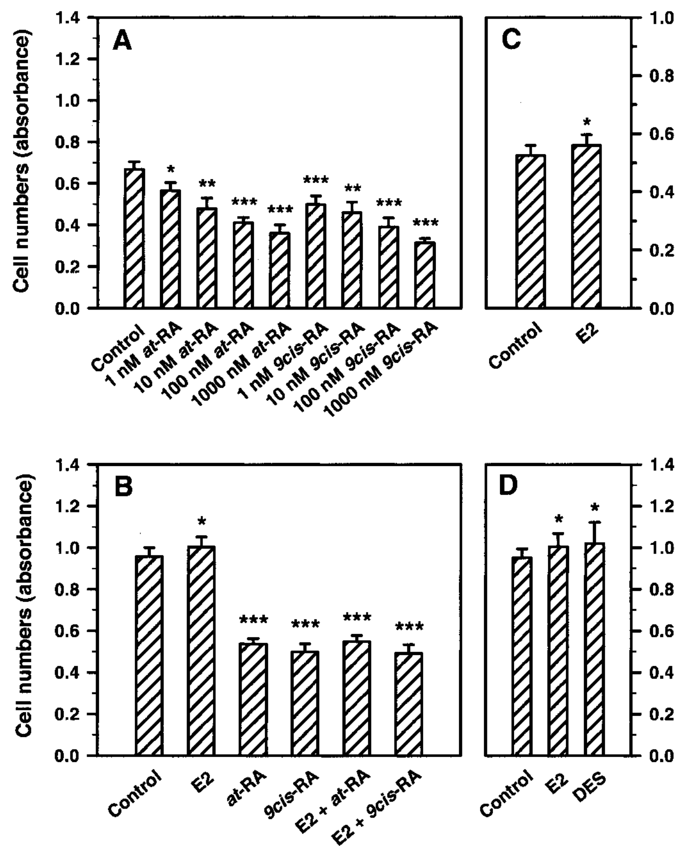

FIG. 1. Effect of RAs on the growth of RL 95-2 cells. A, Cells were cultured for 48 h in the presence of various doses of *at*-RA or 9*cis*-RA, ranging from 1.0–1000 nM. The result represents the mean ± SD of three independent experiments, with duplicate samples in each. B, Cells were cultured for 7 d in the presence of 100 nM E2, 100 nM *at*-RA or 9*cis*-RA, and 100 nM E2 plus 100 nM *at*-RA or 9*cis*-RA, respectively. The result represents the mean ± SD of 6 independent experiments, with 12 parallel samples in each. C and D, Cells were cultured for 7 d in (C) serum-free media containing 0.1% BSA, 10 ng/ml EGF, and 100 nM E2; whereas the control cells were cultured without E2, and (D) in the presence of 10 nM DES or 100 nM E2. The results represent the mean ± SD of 4 independent experiments, with quadruplet samples in each.

BSA. Although the cells grew more slowly than in the presence of DCC-FCS, treatment with E2 resulted in an increase of 6.6% ($P = 0.029$) in cell number (Fig. 1C). On the other hand, when the cells were cultured for 7 d in the medium containing 5% DCC-FCS and 10 nM DES (Fig. 1D), the growth of cells was increased by 7.2% ($P = 0.033$), whereas treatment of cells with 100 nM E2 at the same time caused an increase of cell number of 5.3% ($P = 0.023$) in the presence of 5% DCC-FCS.

Profile of RXRs and RARs in RL 95-2 cells

To explore whether RXRs and RARs are present in RL 95-2 cells, RT-PCR analyses were carried out using the primer set specific to each subtype of RXR and RAR. All members of RXR and RAR, including RXRα, RXRβ, RXRγ, RARα, RARβ, and RARγ, could be detected when total RNA isolated from RL 95-2 cells was used as template (Fig. 2).

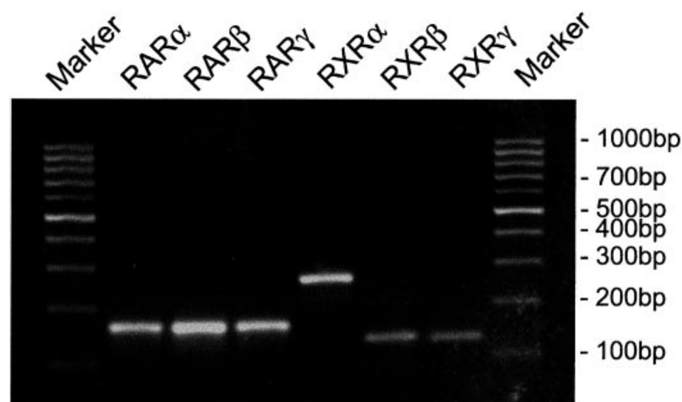

FIG. 2. RT-PCR analysis of RXRs and RARs in RL 95-2 cells. Gel photograph shows the result of RT-PCR amplification, which generated the cDNA fragment of RARα (160 bp), RARβ (154 bp), RARγ (162 bp), RXRα (268 bp), RXRβ (133 bp), and RXRγ (129 bp), using total RNA isolated from RL 95-2 cells as template. Two independent experiments were done, with the same results, and the representative data are shown.

Existence of ERα and ERβ mRNAs in RL 95-2 cells

The ERα has been shown to be present in RL 95-2 cells, whereas it is not known whether the ERβ is expressed. RT-PCR analysis, using total RNA isolated from RL 95-2 cells as template, demonstrated a 439-bp fragment of ERα, as expected (Fig. 3A, *right panel*). Of additional interest was the appearance of ERβ in a corresponding size of 480 bp. To further confirm the authenticity of ERα and ERβ expression in RL 95-2 cells, Southern blot analyses of RT-PCR products were carried out. When the ^{32}P-labeled ERα cDNA fragment was used as a probe, a strong signal, representing a 439-bp cDNA fragment, was observed merely in the lane in which the RT-PCR product of ERα was applied (Fig. 3A, *middle panel*). When the membrane was probed by the ^{32}P-labeled ERβ cDNA fragment, signals were displayed only in the lane loaded with the RT-PCR product of ERβ (Fig. 3A, *left panel*). The major band displayed is 480 bp, identical with the expected size of ERβ fragment.

To identify the amount of ERα and ERβ transcripts in RL 95-2 cells, quantitative RT-PCR assays were conducted. A total of 5.0×10^5 to 7.5×10^6 copies of cRNA competitor for ERα and 2.5×10^5 to 5.0×10^6 copies of cRNA competitor for ERβ were added to each RT-PCR, in which 1 μg total RNA were included. As shown in Fig. 3, B and C, the cRNA competitors for ERα and ERβ resulted in 351- and 340-bp cDNA fragments during amplification, respectively, compared with the 439-bp and 480-bp cDNA fragments generated by the native transcripts of ERα and ERβ. Furthermore, the amount of native mRNA-derived cDNA products of ERα and ERβ decreased linearly, in parallel with the increase of cRNA competitors added (plot not shown). Linear regression analyses revealed that the copy numbers of ERα and ERβ transcripts per microgram total RNA in RL 95-2 cells are $24.0 \pm 1.4 \times 10^6$ and $13.6 \pm 0.6 \times 10^6$, respectively.

Inhibitory effect of at-*RA on the protein level of ERα in RL 95-2 cells*

It has been previously reported that 9*cis*-RA reduced the expression of ERα in estrogen-responsive breast cancer T47D

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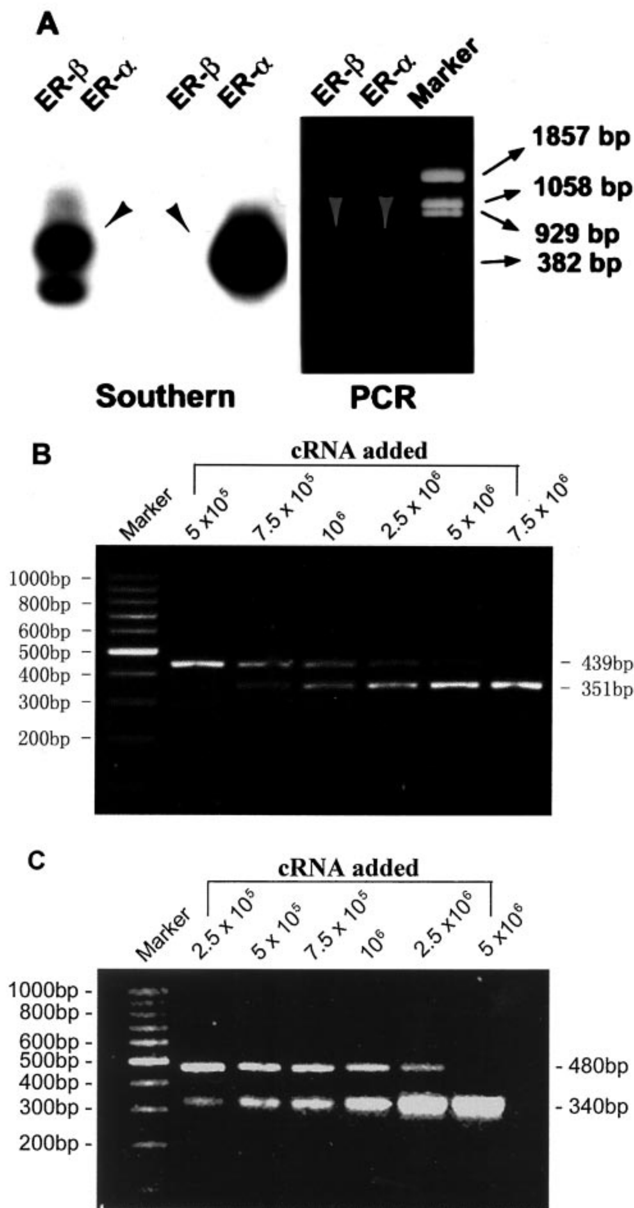

FIG. 3. Expression of ERα and ERβ in RL 95-2 cells. Each experiment was independently conducted, three times, with identical results, and the representative figures are shown. A, Gel photograph shows the result of RT-PCR analysis using total RNA isolated from RL 95-2 cells as template. Positions of ERα (439 bp) and ERβ (480 bp) fragments are indicated by *white arrow* (*right panel*). Southern blot analysis of RT-PCR products further identifies ERα and ERβ signals, which are pointed out by *black arrow* (*left and middle panels*). B and C, Quantitative RT-PCR analyses of ERα and ERβ. The cRNA competitor (5×10^5–7.5×10^6 copies for ERα and 2.5×10^5–5×10^6 copies for ERβ) was added to each reaction containing 1 μg total RNA. The native ERα and ERβ mRNA resulted in 439- and 480-bp fragments, respectively, whereas their cRNA competitors generated 351- and 340-bp fragments, correspondingly.

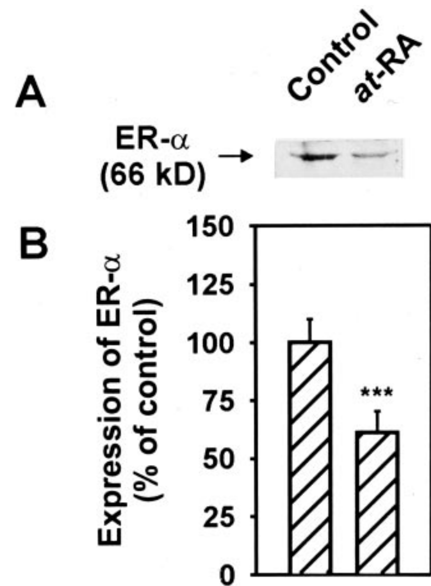

FIG. 4. Effect of *at*-RA on ERα protein expression in RL 95-2 cells. Fifty micrograms of nuclear proteins, after vehicle treatment (lane 1) and stimulation by 100 nM *at*-RA for 24 h (lane 2), was subjected to SDS-PAGE for Western blot analysis. A, The figure depicts the film after exposure for 1 min with the immunoblotting membranes, and the ERα signals are indicated by the *arrow*. B, *Scale bars* indicate mean ± range resulting from densitometry analysis of ERα signals of two independent experiments. The expression level of the control sample is defined as 100%.

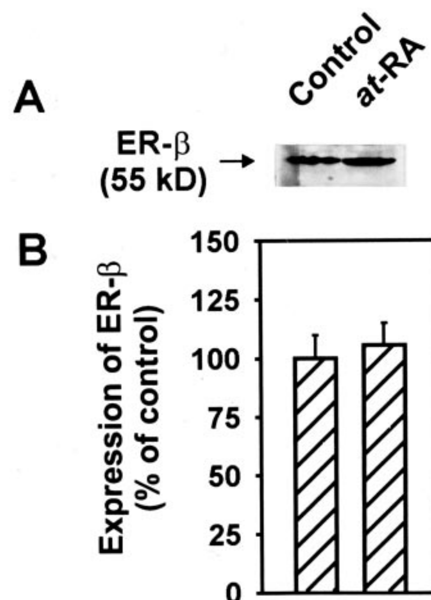

FIG. 5. Effect of *at*-RA on ERβ protein expression in RL 95-2 cells. The details of the experiment were described in the legend for Fig. 4A. The picture depicts the film after exposure for 1 min with the immunoblotting membranes, and the ERβ signals are indicated by the *arrow*. B, *Scale bars* indicate mean ± range obtained from the results of densitometry analysis of ERα signals of two independent experiments.

cells (14). This led us to examine whether RAs have a similar effect on RL 95-2 cells. Thus, nuclear proteins were extracted from RL 95-2 cells, which had been treated with 100 nM *at*-RA for 24 h. Western blot analysis showed that the level of ERα protein was decreased to approximately 60% after stimulation (Fig. 4). On the other hand, the amount of ERβ protein remained unchanged after treatment (Fig. 5).

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The expression of 17HSD type 2 mRNA and oxidative 17HSD activity were increased by RAs in RL 95-2 cells

To understand the possible role of RAs in regulating 17HSD type 2 expression in RL 95-2 cells, Northern blot analyses were performed after the cells had been stimulated by RAs in various doses, ranging from 1.0–1000 nM, for 6–48 h. Increased expression of 17HSD type 2 mRNA (1.5 kb) could be detected after stimulation for 24 h with 1.0 nM 9*cis*-RA, and the induction further increased up to 1000 nM in a dose-dependent manner. A similar effect was also observed with the treatment of *at*-RA but to a lesser extent (Fig. 6). Also, after an induction for 6 h with 100 nM *at*-RA or 9*cis*-RA, increases in 17HSD type 2 expression were detected, and the stimulation seemed to be time-dependent up to 48 h, as detected (Fig. 7).

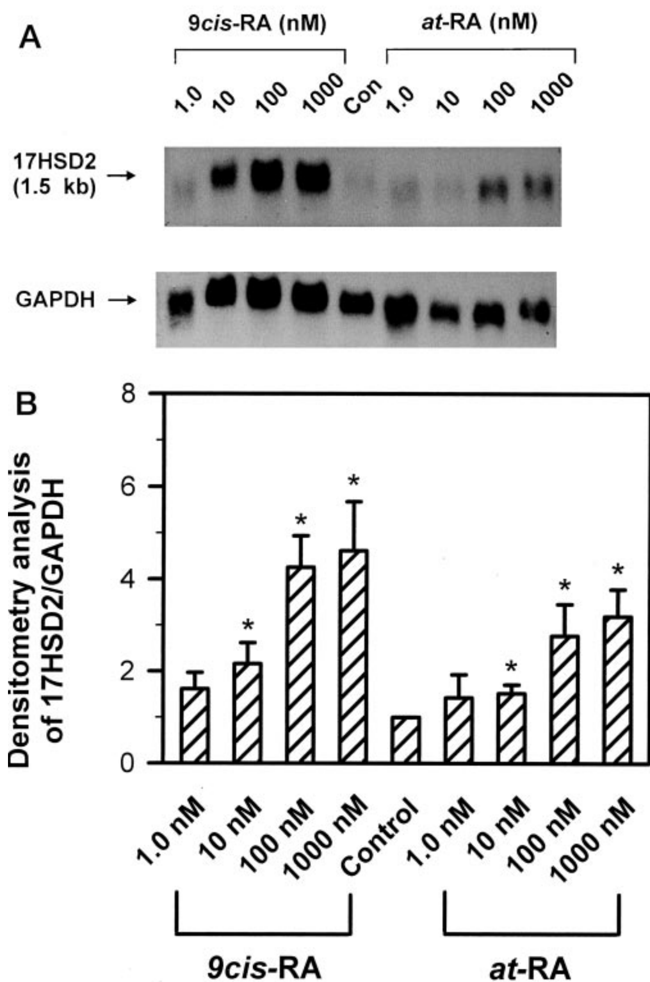

FIG. 6. Dose-dependent stimulation of 17HSD type 2 mRNA expression by RAs in RL 95-2 cells. A, Fifteen micrograms of total RNA was loaded into each lane after vehicle treatment and stimulation with 1.0–1000 nM *at*-RA (*right panel*) or 9*cis*-RA (*left panel*) for 24 h, followed by electrophoresis and subsequent Northern blot analysis. In the *upper panel*, the position of the 1.5-kb 17HSD type 2 mRNA is indicated by the *arrow*. In the *lower panel*, hybridization with a reference probe, GAPDH cDNA, is demonstrated. B, *Scale bars* indicate mean ± SD resulting from densitometry analysis of hybridization signals (17HSD type 2/GAPDH) of three independent experiments. The expression level of the control sample is defined as 1.0.

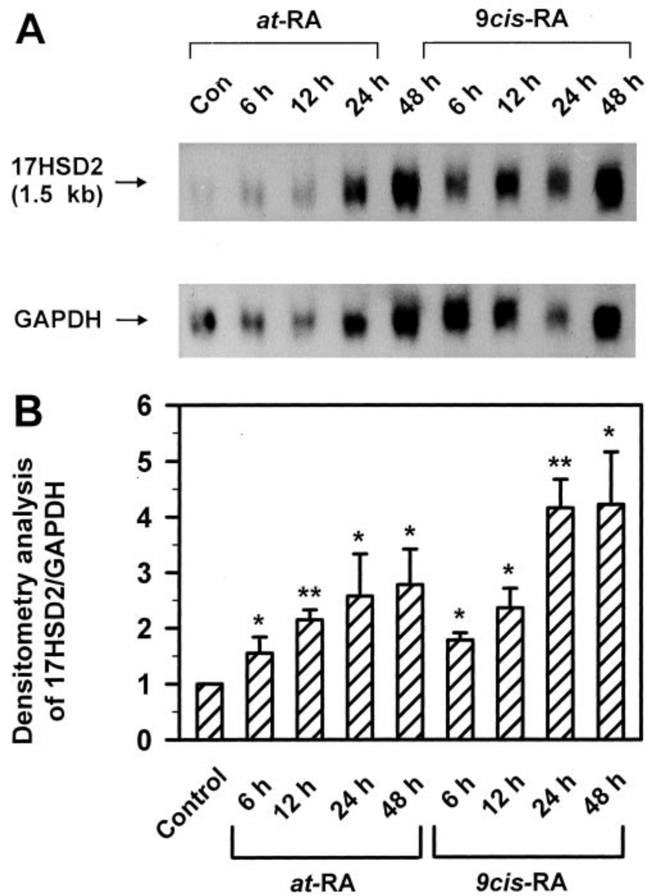

FIG. 7. Time-dependent stimulation of 17HSD type 2 mRNA expression by RAs in RL 95-2 cells. A, Fifteen micrograms of total RNA was loaded into each lane after vehicle treatment and stimulation with 100 nM *at*-RA (*left panel*) or 9*cis*-RA (*right panel*) for 6, 12, 24, and 48 h, followed by electrophoresis and subsequent Northern blot analysis. In the *upper panel*, the position of the 1.5-kb 17HSD type 2 mRNA is indicated by the *arrow*. In the *lower panel*, hybridization with a reference probe, GAPDH cDNA, is demonstrated. B, *Scale bars* show mean ± SD resulting from densitometry analysis of hybridization signals (17HSD type 2/GAPDH) of three independent experiments. The expression level of the control sample is defined as 1.0.

The influence of RAs was also investigated at the enzyme activity level. As shown in Fig. 8, the primary estrogenic 17HSD activity in RL 95-2 cells is oxidation. After treatment of the cells with 100 nM *at*-RA or 9*cis*-RA for 24 h, the *in vivo* oxidative 17HSD activity was increased to over 2.5-fold. In addition, the reductive 17HSD activity was also enhanced to a similar extent.

The expression of 17HSD type 2 mRNA was not altered by RAs in normal human endometrial epithelial cells

The effect of RAs on the expression of 17HSD type 2 mRNA was also studied in primary cultured epithelial cells obtained from normal human endometrium at early and later secretory stages. Northern analysis did not reveal the stimulatory effect of RAs on the expression of 17HSD type 2 mRNA after treatment of the cells with 100 nM *at*-RA or 9*cis*-RA for 24 h (Fig. 9).

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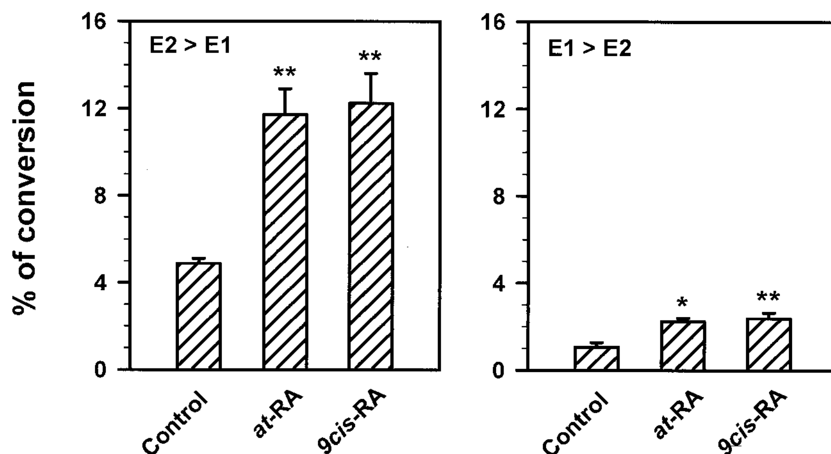

FIG. 8. Effect of RAs on 17HSD activities in cultured RL 95-2 cells. After stimulation with RAs, RL 95-2 cells were incubated in 2 ml serum free-FD containing substrates E_1 or E2 (200 pmol) for 1 h. 17HSD activity is expressed as a percentage of the substrate converted. The results are given as the mean ± SD of three independent assays, and duplicate samples were analyzed in each. A, Oxidative 17HSD activity of the cells after treatment with 100 nM *at*-RA or 9*cis*-RA for 24 h. B, Reductive 17HSD activity of the cells after stimulation with 100 nM *at*-RA or 9*cis*-RA for 24 h.

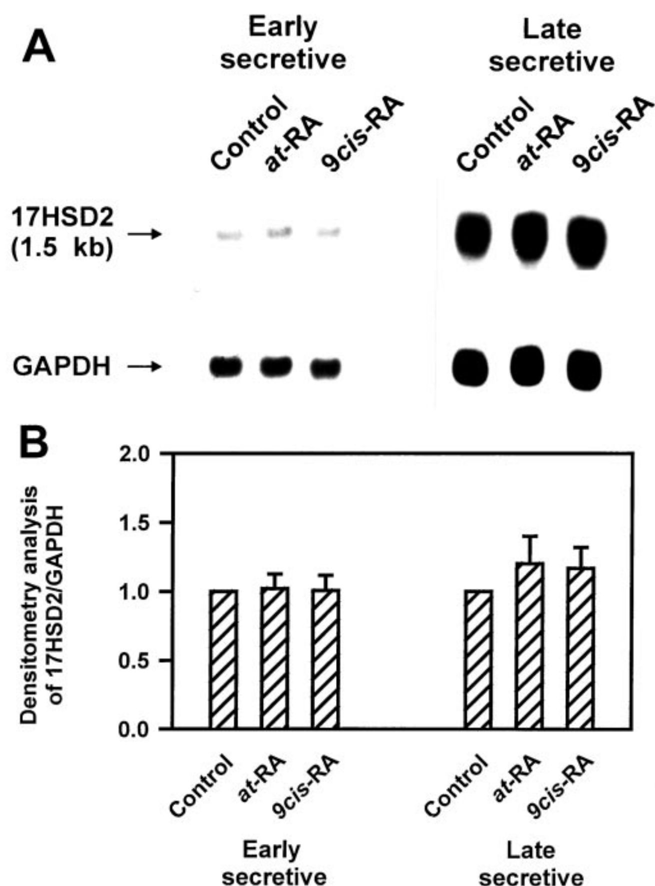

FIG. 9. Influence of RAs on 17HSD type 2 mRNA expression in primary cultured normal human endometrial epithelial cells. A, For Northern blot analysis, 15 μg total RNA was loaded into each lane after vehicle treatment and stimulation with 100 nM *at*-RA or 9*cis*-RA for 24 h. Three specimens of early and late secretive phases were individually analyzed, with similar results, and the representative figures are shown. In the *upper panel*, the position of the 1.5-kb 17HSD type 2 mRNA is indicated by the *arrow*. In the *lower panel*, hybridization with a reference probe, GAPDH cDNA is demonstrated. Please notice that the intensity of hybridization signals given by early secretive samples are not comparable with those provided by late secretive samples, because the experiments were separately performed. B, *Scale bars* demonstrate mean ± SD resulting from densitometry analysis of hybridization signals (17HSD type 2/GAPDH) of three independent experiments. The expression level of the control sample is defined as 1.0.

Discussion

Although the influence of retinoids on endometrial cancer cells has been recently studied (24–26), it remains unknown whether the cell growth is affected by retinoids. In the present study, we demonstrated that RAs inhibit the proliferation of estrogen-responsive endometrial cancer RL 95-2 cells (Fig. 1A). Together with the previous observation that RAs down-regulated the growth of estrogen-inducible breast cancer cells (14, 15), it is suggestive that RAs may have a general inhibitory effect on the proliferation of certain estrogen target cells. In our experiments, however, E2 only slightly promoted the growth of RL 95-2 cells (Fig. 1B), which is not a typical action of E2 on its target cells. At this point, it is difficult to explore whether the inhibition of the cell growth involves the antiestrogen effect of RAs. The poor response of RL 95-2 cells to E2 might be attributable to the abundant expression of 17HSD type 2, which greatly metabolizes E2 to E_1 (8), or the presence of sulfotransferase, which intensively converts estrogens to their sulfate forms (27). On the other hand, however, DES, a potent nonsteroid estrogen, also promoted the proliferation of RL 95-2 cells, with a slight increase in cell number, although the cells have certain copies of ERα and ERβ transcripts. Therefore, studies in sequence and function of ERα and ERβ or the pathways of their action in RL 95-2 cells remain to be further investigated.

Accumulating evidence suggests that RAs play a role in regulating the function of the endometrium (28, 29). Also, RAs have been reported to affect the expression of a number of genes in the endometrium, such as matrix metalloproteinases and IL-6 (30, 31). Although the profile of retinoid receptors of epithelial cells has been elucidated (32–34), the effect of RAs on the proliferation of normal epithelial cells is still unknown. Usually, the epithelial cells isolated from the endometrium contain 5–10% stromal cells, which are able to grow faster than epithelial cells, even if in the serum-free media. This makes it technically difficult to perform the growth experiments of epithelial cells because the change of cell number would not reflect merely the proliferation status of epithelial cells.

Endometrial and breast epithelial cells are the major targets of estrogen action. Epidemiological evidence revealed that estrogens also play a role in endometrial neoplastic

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development (2–4). Estrogens exert their cellular effects through ERs, which belong to the nuclear receptor superfamily. Recently, a new ER, termed ERβ, has been identified (35, 36). Hence, the previously found ER is now designated as ERα. These two protein molecules share some identities as well as differences, such as chromosomal localization, amino acid component, tissue distribution, and ligand affinities (37–41), suggesting that they may have different roles in various tissues. Both ERα and ERβ are expressed in human endometrial epithelial cells (42). Identically, our study demonstrated that not only ERα, but also ERβ, is present in RL 95-2 cells (Fig. 2), which originated from endometrial epithelial cells (19). It needs to be pointed out that an additional fragment, which appears shorter than the ERβ fragment, was detected by Southern analysis of RT-PCR products (Fig. 2A). Sequence analysis revealed that this fragment, which has some homology with the ERβ fragment, results from the unspecific PCR amplification (data not shown).

RAs play an important role in development, cell differentiation, and cell proliferation (43). They have been investigated in several systems as potential agents of chemoprevention and treatment of human cancer. Relevant studies showed that RAs inhibit the proliferation of estrogen-sensitive breast cancer cells, and RARα plays a role in the process (14, 15). The retinoid-induced growth inhibition of breast cancer cells coincided with down-regulation of ERα, indicating a possible interaction between proliferation inhibition of RAs and estrogen action (15). Recently, it has been demonstrated that transcription of RARα is activated by estrogen (44, 45). Therefore, decrease in ERα expression may, in turn, decline the expression of RARα. In this study, we show that protein expression of ERα was inhibited by *at*-RA in endometrial cancer cells (Fig. 4), another cell type responsive to estrogen action. Although ERβ is present in RL 95-2 cells as well, its protein expression is not affected by *at*-RA (Fig. 5), indicating that ERα and ERβ may play different roles in the same cells. In addition to RL 95-2, at least two other endometrial cancer cell lines, KLE and Ishikawa, express ERα (46, 47). The influence of RAs on their ERα expression needs to be examined for a further understanding of the cross-talk between the retinoid and estrogen pathways in endometrial cancer cells.

Apart from the down-regulation of ERα, decrease in intracellular E2 concentration may diminish estrogen action. Indeed, RAs stimulated the expression of 17HSD type 2 in RL 95-2 cells (Figs. 6 and 7), which may, in turn, decline the intracellular E2 level and, therefore, the action of estrogens. This observation was also confirmed at the level of the oxidative estrogenic 17HSD activity (Fig. 8). To date, the expression of 17HSDs has been studied in three endometrial cancer lines, including RL 95-2, HEC-1-A, and HEC-1-B (8). None of them were found to produce 17HSD type 1, and only RL 95-2 was shown to significantly express 17HSD type 2. At this point, the presence of 17HSD type 1 and type 2, as well as the effect of RAs on their expression, should be examined in other endometrial cancer cell lines.

It is well known that the cellular effects of RAs are mediated by RXRs and RARs. Usually RXRs and RARs form heterodimers, which interact with RA response elements in target genes and consequently activate the gene transcription (48). 9*cis*-RA is a ligand for both RXRs and RARs, but the affinity to RXRs is 40-fold higher than that to RARs. On the other hand, *at*-RA is primarily a ligand for RARs and only activates RXRs at high concentration (49). Therefore, 9*cis*-RA is usually a more potent activator of retinoid. Identically, 9*cis*-RA seems to be a more potent stimulator for the expression 17HSD type 2 mRNA (Figs. 6 and 7). A similar phenomenon was also found in our previous study, in which 9*cis*-RA more actively induces 17HSD type 1 expression in choriocarcinoma cells (18).

The oxidative 17HSD activity in human endometrium exhibits a cyclic fluctuation during the menstrual cycle, being highest in the midsecretory phase, and the activity is induced by progesterone (13, 50). Accordingly, 17HSD type 2 mRNA expression was detected only in the secretory (but not in the proliferative) phase of human endometrium (9, 12, 34). These data strongly indicate that progesterone regulates the endometrial expression of the enzyme. On the other hand, RA decreases the expression of PR in human breast cancer cells and, as a result, diminished the cell response to progestin (51, 52). In RL 95-2 cells, PR is not expressed; and therefore, RAs may bypass PR and stimulate the expression of 17HSD type 2.

It was suggested in a recent report by Ito *et al.* that retinoids may regulate 17HSD type 2 expression in endometrial epithelial cells during the secretory phase (34). In the present study, however, RAs were not observed to affect 17HSD type 2 expression in cultured normal epithelial cells from secretory endometrium (Fig. 9). Of course, the system of *in vitro* cultured endometrial epithelial cells lacks paracrine factor(s), which might be needed for the action of RAs. Even so, the effect of RAs on normal endometrial epithelial cells would be apparently different from that on RL 95-2 cells, in terms of 17HSD type 2 expression.

There have been several reports about the retinoid receptors in human endometrium, showing the mRNA expression of RARα, RARβ, RARγ, and RXRα using Northern analysis as well as immunoreactivity of RARα, RARβ, RXRα, and RXRγ in epithelial cells throughout the cycle (32–34). Meanwhile, RXRβ was not found in endometrial epithelial cells by immunostaining (34), whereas the data from Northern analysis is not available so far. Regarding the profile of retinoid receptors in RL 95-2 cells, RT-PCR analysis detected the existence of all subtypes of RXR and RAR (Fig. 2). Thus, absence of RXRβ in normal endometrial epithelial cells, if it is true, may give one explanation of the differential effects of RAs between RL 95-2 and cultured normal endometrial epithelial cells. Also, there exists a possibility that a certain transcription factor(s), which is involved in mediating the effects of RAs, becomes available or activated in cancer cells and, therefore, induces the expression of 17HSD type 2. An additional consideration is that in secretory phase endometrial samples, stimulated by progesterone, the expression of 17HSD type 2 has been induced and, therefore, that no further effect of RAs could be observed. A shortcoming of the present study is that the proliferative phase endometrial samples were not investigated. Nevertheless, an interesting question can be raised: Is the stimulatory effect of RAs on 17HSD type 2 expression generally selective to certain endometrial cancer cells? More investigation with other suit-

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able endometrial carcinoma cell lines (and primarily, cultured endometrial cancer cells from different patients) should be carried out to answer this question.

In brief, the results of the present investigation demonstrate that the proliferation of RL 95-2 cells is repressed by RAs. It was also found that both ERα and ERβ are present in RL 95-2 cells, but only ERα is subjected to down-regulation by *at*-RA. On the other hand, RAs stimulate the expression of 17HSD type 2, which functions as an E2-inactivating enzyme. Hence, the dual effects of RAs may diminish the action of estrogen. Another finding is that RAs do not affect 17HSD type 2 expression in normal endometrial epithelial cells, raising a questionable as to whether the stimulatory effect of RAs on 17HSD type 2 expression is true in certain endometrial cancer tissues.

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