High-Level Expression of the Retinoic Acid Receptor β Gene in Normal Cells of the Uterine Cervix Is Regulated by the Retinoic Acid Receptor α and Is Abnormally Down-Regulated in Cervical Carcinoma Cells

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ABSTRACT

Retinoic acid (RA) is essential for regulation of epithelial cell differentiation. The intracellular effects of RA are mediated by RA-binding nuclear receptors, including the RA receptors (RARs) α, β, and γ. The ligand-activated receptors induce the transcription of target genes by binding to RA-responsive elements in the promoter regions. One target gene is the RARβ gene, which encodes a potential tumor suppressor. Loss of RA inducibility of RARβ gene expression is assumed to play a role in the development of several types of human carcinomas, including carcinomas of the uterine cervix. We have analyzed RARβ gene expression in normal cervical cells and in cervical carcinoma cell lines. The results show that the RARβ mRNA levels are high and RA inducible in the primary keratinocytes, whereas they are low and not inducible or only slightly inducible by RA in all of the cervical carcinoma cell lines analyzed. The basal and the RA-induced RARβ mRNA levels tend to increase with senescence of the normal cells. Fusion of primary ectocervical keratinocytes with HeLa cervical carcinoma cells revealed that the characteristics of RARβ gene expression of the normal cells are dominant over that of the tumor cells. Using synthetic retinoids with receptor-preferential agonist activities and a RARα-specific antagonist, we show that RARα is the major endogenous RAR subtype for induction of RA-dependent RARβ gene expression. Taken together, our results indicate that abnormal down-regulation of RARβ gene expression may be an important step in the multifactorial process of cervical carcinogenesis.

INTRODUCTION

Vitamin A and its biologically active metabolites, the retinoids, are required for normal growth and development of vertebrates and are key regulators of epithelial cell differentiation (1, 2). These natural retinoids, as well as their structurally related synthetic analogues, have diverse biological properties that may potentially contribute to their antitumor effect. They inhibit cell proliferation and angiogenesis, and they induce cell differentiation and apoptosis, or programmed cell death (3, 4). Retinoids are currently used in prevention and therapy of various preneoplastic and neoplastic diseases (5, 6). The effects of RA3 and related natural and synthetic compounds are mediated through two classes of nuclear receptors, the RARs and the RXRs. Both receptor classes contain three members (α, β, and γ), each encoded by a distinct gene (7–9). They belong to the steroid-thyroid hormone receptor superfamily of ligand-inducible transcription factors (10, 11). Retinoid receptors activate transcription in a ligand-dependent manner by binding to RAREs located in the promoter region of target genes. Additionally, there is evidence that the ligand-free receptors can inhibit gene transcription (12). The ligand all-trans RA binds and activates RARs but not RXRs, whereas 9-cis RA is a bifunctional ligand that binds and activates both RARs and RXRs (13, 14). In DNA binding and transcriptional activation by ligand, retinoid receptors function as heterodimers of RXR and RAR or as RXR homodimers. RXR/RAR heterodimers bind to response elements that generally consist of two direct repeat half-sites of the consensus sequence AGGTCA spaced by 2 or 5 bp (DR2 and DR5 elements). In contrast, RXR homodimers bind and transactivate target genes containing DR1 response elements.

One of the target genes of retinoid receptors is the gene encoding the RARβ receptor (15). In its promoter region, a DR5 RA-responsive element named βRARE was identified that mediates RA-induced RARβ gene expression in many different cell types (16–18). The RARβ gene is expressed primarily in normal epithelial tissue, where its expression is up-regulated in response to RA treatment. Alterations in the structure and expression of the RARβ gene are associated with human carcinogenesis. In one case of a human hepatocellular carcinoma, disruption of the RARβ gene by integrated hepatitis B virus DNA was found (19). Reduced or undetectable RARβ mRNA levels have been demonstrated in many malignant epithelial cell lines derived from carcinomas of the lung (20–23), epithelial ovarian cancer (24), squamous carcinomas of the oral pharynx (25), and breast cancer (26). Compared with normal tissue, a decrease in RARβ mRNA levels was also observed in squamous carcinoma of the head and neck region (27) and in premalignant oral lesions (28). All of these findings indicate that the specific loss of RARβ expression may be an important event in tumorigenesis. This hypothesis is supported by the observation that transfection of human epidermoid lung cancer cells with a RARβ expression plasmid results in decreased tumorigenicity in nude mice (29).

Alterations in the RA-regulated RARβ gene expression might also contribute to the development of malignant tumors of the uterine cervix, as has been suggested by the analysis of somatic cell hybrids between HeLa cervical carcinoma cells and normal human cells. It has been shown previously that RA treatment strongly induces RARβ gene expression in the nontumorigenic HeLa × normal cell hybrids, but not in the parental HeLa cells or in tumorigenic hybrid segregants (30, 31).

In the present study, we have examined whether RARβ gene expression is generally altered in cervical carcinoma cell lines compared with the normal human cervical epithelium. RARβ mRNA levels were determined in RA-treated and untreated primary cervical cells and in a variety of cervical carcinoma cell lines. Furthermore, we analyzed RARβ expression levels in senescing human cervical keratinocytes and in freshly generated hybrids between HeLa and normal ectocervical epithelial cells. By using synthetic retinoids with receptor-preferential agonist and antagonist activity, we have analyzed synthetic retinoids with receptor-preferential agonist and antagonist activity, we have analyzed synthetic retinoids with receptor-preferential agonist and antagonist activity, we have analyzed
RA-dependent regulation of RARβ gene expression in HeLa × fibroblast hybrid cells and in cultured ectocervical keratinocytes.

MATERIALS AND METHODS

Retinoids. The natural and synthetic retinoids at-RA, 9-cis RA, Ro 40-6055 (RARα-agonist), Ro 19-0645 (RARβ-agonist), Ro 48-2249 (RARβ-agonist), Ro 44-4753 (RARγ-agonist), Ro 47-2077 (RARγ-agonist), Ro 25-7386 (RXR-agonist), and Ro 41-5253 (RARα-antagonist) were synthesized at F. Hoffmann-La Roche, Ltd. (Basel, Switzerland and Nutley, NJ). The profiles of the retinoids are summarized in Table 1. Retinoids were solubilized in DMSO as 10 mM stock solutions and kept at −17°C under liquid nitrogen. Fresh dilutions were made in DMSO for each experiment.

Cell Lines, Primary Cells, and Culture Conditions. The human cervical carcinoma cell lines C4-I, SiHa, CaSki, HT3, MS751, and C33A were obtained from the American Type Culture Collection (Rockville, MD). The hybrid cell lines ESH100 P6 (HeLa × normal foreskin keratinocyte; nontumorigenic) and 444 (HeLa × normal fibroblast; nontumorigenic) were kindly provided by Dr. Eric Stanbridge (University of California, Irvine; Ref. 32). Cells of all cervical carcinoma cell lines, as well as primary human fibroblasts and the hybrid cells, were maintained in DMEM (pH 7.2) supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells of the cell line W12, established from an HPV16-positive CIN lesion (33), were kindly provided by Dr. Margaret Stanley (Cambridge University, Cambridge, England), and primary keratinocytes from penile foreskin tissue were provided by Dr. Matthias Dürrst (Heidelberg, Germany). W12 cells, as well as primary keratinocytes, were cultivated in a 3:1 mixture of DMEM and F12 medium enriched with adenine (18.2 μg/ml), insulin (5 μg/ml), cholera toxin (10−10 M), epidermal growth factor (10 ng/ml), hydrocortisone (0.4 μg/ml), glutamine (2 mM), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FCS (DMEM/F12; Ref. 34).

Endocervical and ectocervical cultures were derived from cervical biopsies obtained from hysterectomy specimens to be free of cervical intraepithelial neoplasia by histological examination. Ectocervical biopsies were taken from an area that was distant from the squamocolumnar junction, and endocervical tissue was selected from an area high in the cervical canal distant from the squamocolumnar junction. Specimens were placed in cold DMEM immediately after surgical removal. After two rinses in fresh medium, most of the stroma was removed and used for culturing primary fibroblasts. The epithelial tissue and the remaining underlining stroma were washed twice in calcium and magnesium-free PBS (pH 7.0), minced into smaller fragments, and placed with the epithelial side down in 6-cm Petri dishes with trypsin (0.25% w/v in PBS). After 30 min, the epithelial cells were carefully scraped into fresh medium, collected by centrifugation, and plated on tissue culture dishes with a feeder layer of lethally irradiated 3T3 mouse fibroblasts (2 × 10⁶ fibroblasts/cm²). Cells were grown in the enriched DMEM/F12 medium.

Four h before retinoid treatment, the normal culture medium was replaced by medium containing 10% charcoal-stripped serum. Cells were exposed to different concentrations of retinoids for 24 or 48 h. The final concentration of the solvent DMSO was always 0.1% (v/v). Control cultures received medium with 0.1% (v/v) DMSO alone.

Somatic Cell Fusion. HeLa universal fuser cells (D98OR) were kindly provided by Dr. Eric Stanbridge. These cells exhibit both a dominant (ouabain resistance) and a recessive (hypoxanthine phosphoribosyltransferase-negative) selection marker and were maintained in DMEM containing 2 × 10−4 M ouabain and 10−4 M 6-thioguanin. For cell fusion, 10⁶ primary ectocervical keratinocytes and 10⁶ HeLa universal fuser cells were mixed. The next day, cell fusion was achieved by adding 1 ml of prewarmed 50% polyethylene glycol 1500 (Boehringer Mannheim) and incubation for 1 min. The cells were washed with PBS and incubated overnight with fresh medium. The next day, the cells were replated at low density (1:2, 1:4, and 1:6 split ratios) and incubated in DMEM containing ouabain and hypoxanthine-aminoimidiner-thymidine for selection of ouabain-resistant/hypoxanthine phosphoribosyltransferase-positive cell hybrid clones. Individual clones were isolated after 4 weeks and subcultured continuously for more than 25 population doublings, at which stage they were considered immortal.

DNA and RNA Analysis. Total high molecular weight DNA was isolated from cells as described previously (35). DNAs were digested with TaqI according to the manufacturer’s instructions, electrophoretically separated on 1% agarose gels, transferred to Hybond-N membranes (Amersham Buchler, Braunschweig, Germany), and attached by UV exposure plus baking. The DNA probes were labeled with 32P by random priming (Stratagene, Heidelberg, Germany). Hybridization was done at 68°C in 0.75 M NaCl/1% SDS solution, 0.1 mg/ml tRNA, and 1% SDS. Filters were washed in 2X SSC and 0.1% SDS at 68°C and exposed to Reflection X-ray films (Dupont NEN, Germany) at −70°C using intensifier screens.

Total RNA was isolated from cells by the acidic guanidinium isothiocyanate procedure (36). For Northern blot analysis, RNAs were denatured and fractionated on 1% agarose gels using MOPS buffer (20 mM 3-(N-morpholino)-propanesulfonic acid, 5 mM sodium acetate, and 1 mM EDTA). RNAs were transferred to Hybond-N membranes using 10X SSC buffer and attached by UV exposure plus baking. Probe labeling, hybridization, and washing were performed as described for the Southern blots.

Recombinant Plasmids. Reporter plasmid D1 contains an approximately 5.2-kb HindIII-BamHI fragment of the RARβ2 transcription-control region inserted directly upstream of the luciferase reporter gene (16). It was kindly provided by Dr. Anne Dejean (Institut Pasteur, Paris, France). Plasmid pAc-Gal contains the 4.3-kb EcoRI-AluI fragment of the human β-actin promoter linked to the Escherichia coli β-galactosidase gene (30).

Transient Transfection and Luciferase Reporter Analysis. Transfection of cells was performed by calcium phosphate precipitation according to Chen and Okayama (37). One day before transfection, 1.6 × 10⁶ cells per 6-cm Petri dish were seeded, and 4 h before transfection, the normal culture medium was replaced by DMEM with 10% charcoal-stripped FCS. The DNAs of the promoter-luciferase reporter plasmid (3 μg) and the pAc-Gal plasmid (1 μg) for internal standardization were mixed and adjusted to the total amount of 6.5 μg DNA with pBlueScript. Cells of four dishes in parallel were incubated with the calcium phosphate/DNA mixture for 16–18 h at 37°C and 3% CO₂ and were then washed twice with medium. Two of the four dishes received fresh medium containing different concentrations of retinoids, and the remaining two dishes received medium with DMSO solvent alone. Incubation was carried out for 24 h at 37°C and 3% CO₂. Cells were harvested, and luciferase activity was measured. β-galactosidase activity of cotransfected pAc-Gal was determined to account for variations in transfection efficiency.

Isolation and DNA Sequence Analysis of cDNA Clones. A HeLa cDNA library in phage ANM1149 (kindly provided by H. Sommer, Max-Planck-Institut für Züchtungsforschung, Cologne, Germany) was used to isolate clones containing RARα-cDNA inserts. Phage DNA was cleaved with EcoRI, and the

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<th>Activation EC₅₀ (tm)⁷</th>
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Table 1 Retinoid receptor profiles of the pan- and RAR-preferential agonists used in this study

⁶ IC₅₀, retinoid concentration at which labeled all-trans RA binding is inhibited by 50%.

⁷ EC₅₀, retinoid concentration at which 50% of the maximal induction of gene expression is observed.
cDNA inserts were subcloned into pBluescript SK+ plasmid vector. DNA sequencing was performed by the dideoxy chain termination method using the primer walking approach with 11 primers to sequence both strands of the inserts. Automated sequencing was carried out on a model 373A automated sequencer (Perkin-Elmer, Weiterstadt, Germany) using the ABI-Prism dye-terminator cycle sequencing kit (Perkin-Elmer) according to the manufacturer’s instructions. A total of 0.15 μg of template DNA was used per reaction.

RESULTS

Our previous analysis of HeLa and HeLa hybrid cells had revealed RA-mediated induction of RARβ gene expression in the nontumorigenic HeLa hybrid cells but not in the parental HeLa cervical carcinoma cells or in tumorigenic segregants (30, 31). These results indicated that loss of RA responsiveness of the RARβ gene may play a role in cervical carcinogenesis. For comparison of normal and tumor cells, we have examined in this study the basal and RA-dependent mRNA levels of RARβ in cultured primary keratinocytes of the human uterine cervix and in cervical carcinoma-derived cell lines.

RARβ Expression in Epithelial Cells of the Normal Uterine Cervix. Two morphologically distinct types of epithelium are present in the uterine cervix, a nonkeratinizing stratified squamous epithelium covering the ectocervix and a simple columnar epithelium lining the cervical canal. For our analysis of RARβ gene expression, keratinocytes from the ecto- and endocervical parts of the biopsies were cultivated separately.

High basal levels of RARβ mRNA were detected in both ecto- and endocervical keratinocytes, and RA treatment resulted in a further elevation (Fig. 1). Similar results were obtained with the cultured cervical keratinocytes from biopsies of 10 additional donors (data not shown). To distinguish between the endo- and ectocervical cells, CK13 was used as a marker. The CK13 protein is present in the stratified epithelium of the ectocervix but absent from the simple epithelium of the endocervix (38). Hybridization of the RNA filters with a CK13-specific DNA probe produced strong signals in the RNAs isolated from the ectocervical cell cultures, but produced weak signals in the RNAs from the endocervical cell cultures (data not shown). Thus, the cultured endocervical cells were not contaminated or were only slightly contaminated by ectocervical cells. Taken together, the Northern blot data show that the RARβ gene is expressed at a high basal and RA-inducible mRNA level in primary ecto- and endocervical epithelial cells.

The cultured primary cervical keratinocytes senesce after approximately six passages (which correspond to about 10 population doublings). We examined whether the RARβ gene expression is altered in senescing cervical keratinocytes. With increasing passage number, the basal mRNA levels of the RARβ gene were found to be up-regulated, whereas expression of RARα remained unchanged (Fig. 2). RA treatment resulted in elevated RARβ mRNA levels also in the senescing cells. These results show that senescence of cervical epithelial cells is correlated with an up-regulation of RARβ gene expression. Primary cervical fibroblasts exhibited only low basal levels of RARβ transcripts, which were strongly induced by RA treatment (Fig. 2).

RARβ Gene Expression in Cervical Carcinoma Cell Lines. In addition to HeLa cells (30), the RARβ mRNA levels were analyzed by Northern blot hybridization in untreated and RA-treated cells of the cervical carcinoma cell lines CaSki, C4-I, C33A, HT-3, ME180, MS751, SiHa, and SW756. Fig. 3 shows the results for the cell lines CaSki, MS751, and ME180. In all of the carcinoma cell lines tested, the basal RARβ mRNA levels were very low, and they were not inducible or only slightly inducible by RA treatment. Because the established cell lines are grown in DMEM, whereas the primary keratinocytes are cultivated in enriched DMEM/F12 in the presence of feeder cells, we examined whether the cell culture conditions might influence RARβ gene expression. Therefore, the RARβ gene expression in these cell lines was compared with that in primary cervical keratinocytes. These results show that the RARβ gene is expressed at a high basal and RA-inducible mRNA level in primary cervical keratinocytes, but not in the cervical carcinoma cell lines.
Thus, the low and RA-unresponsive RARβ gene expression levels are caused by the cell culture conditions. Taken together, the results show that the basal expression level and the RA responsiveness of the RARβ gene are severely reduced in cervical carcinoma cell lines compared with normal cervical epithelial cells.

A low basal and uninducible RARβ mRNA level was also observed in the W12 cell line (Fig. 4), which was established from a CIN 1 lesion (33). Furthermore, in primary keratinocytes from human penile foreskin, basal RARβ expression was very low and remained nearly unaffected by RA treatment (Fig. 4). The latter result indicates that regulation of RARβ gene expression is different in keratinocytes derived from different epithelia. In contrast to RARβ, the genes encoding the RARα and RARγ subtypes were expressed at constitutively high mRNA levels in the carcinoma cell lines and in the primary cells. The basal expression of both receptor genes remained unaffected by RA treatment (the results for RARα are shown in Fig. 4).

RARβ Gene Expression in HeLa × Ectocervical Keratinocyte Hybrid Cells. The nontumorigenic HeLa × foreskin keratinocyte hybrid cells of line ESH100 P6 show a RARβ gene expression pattern comparable with that of primary cervical keratinocytes (i.e., high basal level and induction by RA; see Fig. 1). Thus, we examined whether strong basal expression and RA inducibility of the RARβ gene are also features of hybrids between HeLa and primary cervical epithelial cells. Tumorigenic HeLa universal fuser cells (D980R) were fused with normal ectocervical keratinocytes. Cell hybrids were selected in medium containing hypoxanthine-aminopterin-thymidine and ouabain, and four hybrid clones (CGH1, CGH2, CGH3, and CGH5) were analyzed further. Formation of stable hybrids was confirmed by Southern blot hybridization of TaqI-digested cellular DNAs with a human repetitive DNA probe. Characteristic fragments of both parental cell types were present in the four clones (data not shown), thus demonstrating their hybrid nature.

The HeLa × ectocervical cell hybrids were tested for basal expression and RA regulation of the RARβ gene (Fig. 5). In contrast to HeLa, but similar to the normal parental cells, the four hybrid clones showed high basal RARβ mRNA levels, which could be enhanced further by RA treatment. These results indicate that the characteristics of RARβ gene expression of the primary keratinocytes are preserved in the HeLa × ectocervical epithelial cell hybrids.

Identification of the Endogenous RAR Subtype Responsible for Ligand Induction of RARβ Gene Expression in 444 Cells. The genes encoding the RARα and RARγ subtypes are constitutively expressed in the 444 hybrid cells (30) and in the primary cervical cells (see above). To determine the endogenous RAR subtype responsible for the ligand-dependent induction of RARβ gene expression, synthetic retinoids with receptor-preferential agonist or antagonist activities were used. 444 cells were treated with different concentrations of either one of the pan-agonists (at-RA or 9-cis RA) or one of the agonists preferentially activating RARα (Ro 40-6055), RARβ (Ro 19-0645 and Ro 48-2249), RARγ (Ro 44-4753 and Ro 47-2077), or RXR (Ro 25-7386; Refs. 3 and 39). In addition, the cells were treated with at-RA or one of the agonists in combination with the RARβ-selective antagonist Ro 41-5253 (40). The results of the Northern blot analyses are shown in Fig. 6. A and B. The two natural RAs strongly induced RARβ gene expression. Efficient induction of RARβ mRNA levels was also obtained with the RARα-agonist. In contrast, almost no induction was observed with the RARγ-agonist. The RARβ-agonist Ro 19-0645 also stimulated RARβ gene expression, albeit weaker than the RARα-agonist. However, with the RARβ-agonist Ro 48-2249, which has a lower cross-reactive binding affinity for RARα
48-2249 is due to the fact that both RAR(3-agonists also bind to and approximately passage 4 (CxK)l and of four stable, freshly generated hybrid clones (CGH excess, respectively, inhibited RA-mediated or RARa-agonist-mediated induction of RAR@. The filter was hybridized with the RAR(3-probe and rehybridized with GAPDH.

Inhibition of RAR@ Selective Antagonist. It seems unlikely that this blockade is due to a binding of the RAR@-selective antagonist to RARf3, because the antagonist has an almost 70-fold lower binding affinity for RARf3 than for RARα (EC50 = 4700 nM versus EC50 = 70 nM). Rather, the activation of RAR@ by Ro 19-0645 is probably blocked by the RAR@-antagonist.

To examine whether the RAR@-specific induction of RAR@ mRNA levels is caused by transcriptional activation, transient transfection analyses were performed in 444 cells using the luciferase reporter plasmid D1. This plasmid contains an approximately 5.2-kb fragment of the RARf3 transcription control region (16). After transfection, the cells were treated with either RA or one of the RAR agonists in the absence or presence of the RARα-agonist. As shown in Fig. 7, retinoid-mediated induction of luciferase activity was blocked by the antagonist with full suppression requiring a 1000-fold excess of antagonist over agonist. These results indicate that activation of transcription initiation of the RARf3 gene by RARα is the primary cause for the increase of mRNA steady-state levels. Taken together, the results of the Northern analyses and the transient transfection experiments identify RARα as the principal endogenous RAR subtype regulating ligand induction of RARf3 gene expression in 444 cells.

Primary Structure of the RARα Coding Region in HeLa Cells. The identification of RARα as the major ligand-dependent inducer of RARβ in 444 cells raised the question of whether the RA unresponsiveness of RARβ in HeLa cells might be due to inactivating mutations in the protein-coding part of the RARα gene. Several RARα-specific cDNA clones were isolated from a HeLa cDNA library, and the inserts of three of them were completely sequenced. No mutations could be detected, indicating that the RARα gene is intact in HeLa cells.

Influence of RARα on RARβ Gene Expression in Primary Keratinocytes. We next examined whether RARα also plays a role for RARβ gene expression in normal cervical keratinocytes. Treatment of keratinocyte cultures with the RARα-specific antagonist resulted in a strong reduction of basal RARβ mRNA levels (Fig. 8, Lanes 1–3), which was a dose-dependent effect (Fig. 8, Lanes 5–8). The down-modulation was due to an unspecific toxic effect, because the transcript levels of RARα and of the cellular housekeeping gene GAPDH were not altered. Moreover, the reduced RARβ mRNA levels could be reinduced to the normal basal level by subsequent treatment with RA or with the RARα-agonist, respectively (Fig. 8, Lanes 10–12). These results indicate that RARα is a major regulator of RARβ gene expression in normal epithelial cells of the ectocervix.

The data further suggest that the high basal RARβ mRNA levels may be caused by the residual RA present in the serum, which could be sufficient for activation of RARα and transcription of the RARβ gene. This assumption is supported by the finding that keratinocytes maintained in medium with normal serum showed elevated RARβ mRNA levels compared with cells shifted for 28 h to medium with charcoal-stripped serum (Fig. 8, compare Lanes 4 and 1). More strikingly, cultivation of keratinocytes in medium with stripped serum for 28 and 52 h resulted in a time-dependent strong reduction of the RARβ transcript levels (Fig. 8, compare Lanes 5 and 9), which were reinducible by RA treatment (data not shown).

DISCUSSION

In this study, we show that differences in the expression and retinoid regulation of the RARβ gene exist between cultured primary epithelial cells of the human uterine cervix and cervical carcinoma cell lines. In the normal cells, the basal RARβ mRNA levels are high and can be induced further by RA treatment (Figs. 1 and 2). In contrast, the basal RARβ mRNA levels are low and not inducible or only slightly inducible by RA in the cervical carcinoma cells (Figs. 3 and 4). Loss of high and RA-inducible RARβ gene expression seems to be a general feature of cervical carcinoma cell lines because no exception has been found thus far. These results support the assumption that defects in RARβ gene expression resulting in low and uninducible mRNA levels may play a role in the multifactorial development of cervical cancer. Abnormally low expression levels and RA uninducibility of the RARβ gene have also been found in several other human cancer types, in particular in lung and breast cancer (see "Introduction"). It has been shown that RARβ is the essential receptor mediating the growth-inhibitory effect of RA in breast cancer cells (41). The tumorigenicity of RARβ-nonexpressing lung cancer cells in mice could be reduced by introduction of a RARβ gene expression plasmid (29). Regarding cervical cancer cells, it was shown for HeLa cells that the cell proliferation in vitro is efficiently suppressed by transient overexpression and ligand activation of RARβ (42). Taken together, the data indicate that RARβ is a negative regulator of cell growth and tumorigenicity in certain cell types, including epithelial cells of the uterine cervix, breast, and lung. Consequently, defects in RARβ gene expression may be one pathway leading to the escape of such cells from growth regulation.

Using synthetic retinoids with receptor-preferential agonist activities and a RARα-specific antagonist, we show in this study that the
The primary cervical epithelial cells show high basal RARβ mRNA levels that could be efficiently reduced by the RARα-specific antagonist. Cultivation of the primary cells in medium with charcoal-stripped serum resulted in a decrease of the basal RARβ mRNA levels (Fig. 8) that could then be reinduced by RA or the RARα-agonist. These data allow us to conclude that the basal RARβ mRNA level in the normal cervical epithelial cells is actually an induced one. The low residual RA concentration in the untreated serum seems to be sufficiently high to trigger a constant RARα-mediated induction of RARβ gene expression in the normal cervical epithelial cells. In contrast, the normal cervical fibroblasts show a very low basal RARβ mRNA level. Apparently, they need higher RA concentrations for RARβ induction than the cervical keratinocytes. The reason for this difference in ligand responsiveness of RARβ gene expression between the two cell types is not known.

Analysis of the nontumorigenic HeLa × fibroblast hybrid cell line
444 and the tumorigenic segregants gave the first indication that loss of RARβ gene expression may be involved in cervical carcinogenesis (30, 31). In addition, in a pair of HeLa × foreskin keratinocyte hybrids, ligand inducibility of RARβ gene expression as observed in the nontumorigenic line was lost in the tumorigenic segregant (30). In the present study, freshly established hybrids between HeLa cells and primary ectocervical epithelial cells show a RARβ expression profile comparable to that of the normal parental cells (i.e., high basal level and RA inducibility; Fig. 5). The hybrid clones are considered immortal because they have now been expanded for more than 25 population doublings without showing any signs of senescence. The combination of immortality with “normal” RARβ gene expression and ligand regulation suggests that these hybrid cells may be helpful in identifying the genes that participate in the ligand-dependent regulation of RARβ gene expression.

With serial passage of the cultured human keratinocytes, the expression of the RARβ gene becomes elevated, whereas expression of RARα remains unchanged (Fig. 2). The high levels of RARβ mRNA in senescent keratinocytes could be superinduced by treatment with 10^{-6} M RA. A selective up-regulation of RARβ mRNA levels was also demonstrated recently in late-passage mammary epithelial cells (26) and, under conditions of RA induction, in aging human dermal fibroblasts that have a reduced proliferative capacity (43). These findings suggest that RARβ might play a role in normal cellular senescence, a condition that ultimately decreases cell proliferation. Thus, loss of RARβ expression and function could be involved in the process of immortalization. This is in line with the uninducible and almost undetectable RARβ mRNA level of the immortal but nontumorigenic W12 cells (Fig. 4). However, the HeLa × ectocervical epithelial cell hybrids clearly show that an immortalized phenotype and a high RA-inducible RARβ expression level are not mutually exclusive.

Because RARα obviously plays a central role in RA-dependent regulation of RARβ gene expression, the loss of RA inducibility in the tumor cells might be explained by alterations of RARα structure, function, or expression. However, the RARα mRNA levels in the cervical carcinoma cell lines were similar to those in the primary cells. Therefore, we examined the possibility of inactivating mutations by sequencing cDNA clones of the endogenous RARα gene of HeLa cells; no mutations could be detected, indicating that the endogenous RARα in HeLa cells is intact. It remains to be determined whether the RARα gene is also unaltered in the other cervical carcinoma cell lines.

When the structure and the expression of the RARα gene are normal in the carcinoma cells, then the question is why RARα is nevertheless unable to induce RARβ transcription. To activate transcription, the RARE-bound RARα receptor must transmit the activation signal to the basal transcription complex. Recent studies have revealed the existence of several different coactivators or transcriptional intermediary factors that interact with RARs and other nuclear receptors and mediate the ligand-dependent activation (44–46). With normal RARα gene structure and expression, it is tempting to assume that loss or inactivation of one of these mediator proteins leads to the loss of RA inducibility of the RARβ gene in the carcinoma cells. Alternatively, the aberrant synthesis or activity of an inhibitor may cause the effect. We have shown in a previous study that full RA-dependent induction of the RARβ promoter in 444 cells requires several upstream cis-elements, in addition to the promoter-proximal RARE, which together seem to constitute a higher-order RA-responsive domain (31). Thus,
activation of RARβ gene transcription by RARα probably involves a plethora of protein factors. Identifying which factors are critical for interaction with RARα in the ligand-dependent activation of RARβ transcription in the normal epithelial cells and which are inactivated in the carcinoma cells will be a fascinating task for future analysis.

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References

High-Level Expression of the Retinoic Acid Receptor β Gene in Normal Cells of the Uterine Cervix Is Regulated by the Retinoic Acid Receptor α and Is Abnormally Down-Regulated in Cervical Carcinoma Cells

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