Expression and Up-Regulation of Retinoic Acid Receptor-β Is Associated with Retinoid Sensitivity and Colony Formation in Esophageal Cancer Cell Lines

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ABSTRACT

Retinoids exhibit chemotherapeutic and chemopreventive activities, possibly due to their ability to modulate cell growth, differentiation, and apoptosis. These effects are thought to be mediated by nuclear retinoid acid (RA) receptors (RARs) and retinoid X receptors, each of which includes three subtypes (α, β, and γ) that act as transcription factors. To determine whether RARs play a role in mediating the effects of RA on human esophageal cancer (HEC) cells, we analyzed the effects of RA on: (a) the growth, differentiation, and apoptosis in seven HEC cell lines; (b) receptor expression; (c) receptor modulation by RA; and (d) expression of receptors in 20 surgical HEC specimens. RA inhibited the growth of five of seven cell lines and also the constitutive expression of the squamous differentiation markers cytokeratin 1 and transglutaminase 1 in all cell lines. The growth inhibition by RA was due to the induction of apoptosis in the five cell lines. All seven cell lines expressed RAR-α and RAR-γ, and four cell lines showed some changes by RA, but not associated with apoptosis. In contrast, RAR-β was expressed in five of seven cell lines and up-regulated by RA in these five cell lines, which were associated with apoptosis. Two cell lines that failed to express RAR-β showed no growth inhibition or apoptosis and no RAR-β inducibility. Interestingly, only two of the two cell lines were able to form colonies in soft agar. RAR-α, RAR-β, and RAR-γ mRNAs were expressed in all 20 adjacent normal esophageal tissues. The expression of RAR-α and RAR-γ remains positive in HEC specimens, but RAR-β expression was detected in only 6 of 20 HEC specimens. These data suggest that the expression of RAR-β is associated with response of HEC cells to RA and that the loss of RAR-β expression may be associated with HEC development.

INTRODUCTION

HEC is characterized by an advanced pathological stage at presentation and associated with poor prognosis. The overall 5-year survival rate of esophageal cancer patients is still under 10% (1–5). Although esophageal carcinoma is less frequent in the United States than in Asian countries, ~12,300 new cases will still be diagnosed in 1998, and 11,900 patients will die of their disease in the United States (6). Recently, epidemiological studies have reported that the incidence of esophageal adenocarcinoma is increasing faster than nearly any other cancer in the United States (1–5). The reasons for this increase are not well understood. Therefore, early identification and new approaches to prevention and treatment for esophageal carcinoma are urgently needed. One possible approach may be to use retinoids chemoprevention because of their action in the upper aerodigestive tract and potential to reduce the incidence of and mortality from esophageal carcinoma (7–10).

Retinoids, a group of structural and functional analogues of vitamin A, are established modifiers of epithelial cell growth and differentiation in vitro and in vivo. Various retinoids have been shown to suppress or reverse epithelial carcinogenesis and prevent the development of invasive cancer in many animal models, including those cancers of the skin, lung, oral cavity, and esophagus (10–19). Vitamin A deficiency induces hyperkeratotic change in esophageal mucosa in experimental animals (11, 19). A clinical trial using N-4-(ethoxycarbonylphenyl) retinamide, a synthetic retinoid, demonstrated that cancer incidence among the treatment group with severe esophageal dysplasia was reduced by 43.2% compared with the placebo group (9, 19). The chemopreventive effects of this retinoid in a high-risk population indicated that N-4-(ethoxycarbonylphenyl) retinamide is effective in the prevention of esophageal cancer (9). However, the results of two more recent trials in Linxian, China, in which a combination of nutrients and vitamin A was given, were inconclusive (20–21). Therefore, additional studies of retinoid signal transduction pathway are necessary to understand RA action in HEC.

Retinoids are known to exert their biological effects by binding to specific nuclear retinoid receptors, which belong to a steroid/thyroid hormone-receptor superfamily (22–27). The nuclear retinoid receptors are divided into RARs and retinoid X receptors, both composed of three subtypes (α, β, and γ). Like other members of this family, the nuclear retinoid receptors are ligand-activated, DNA-binding, trans-acting, transcription-modulating proteins. RARs and retinoid X receptors form either homodimers or heterodimers that bind to a specific DNA sequence, the RA response element, in the promoter regions of genes to modify the expression of target genes. Several retinoid-regulated genes carrying these response elements in their regulatory regions have been identified. Each subtype of nuclear retinoid receptors is thought to regulate the expression of distinct genes because the subtypes exhibit specific patterns of expression during embryonal development and different distributions in adult tissues (22–27).

Our previous studies, together with others, have demonstrated that altered expression of these receptors is associated with malignant transformation in human epithelium cells (27–41). In this study, we explored the expression of RARs and their modulation by RA in HEC cells.

MATERIALS AND METHODS

Cell Lines and Surgical Specimens. The HEC cell lines TE-1, TE-2, TE-3, TE-7, TE-8, TE-12, and TE-13 were obtained from the First Department of Pathology, Hiroshima University School of Medicine (Hiroshima, Japan). TE-1, TE-3, and TE-7 are from well-differentiated HEC, TE-8 and TE-12 are from moderately differentiated HEC, and TE-2 and TE-13 are from poorly differentiated HEC.

Tissue samples taken from 20 esophageal cancer patients were used in this study and also obtained from the above-mentioned institute. These samples were routinely fixed in 10% buffered formalin and embedded in paraffin. All of the specimens were cut into 4-μm sections using procedures aimed at minimizing RNase contamination.
Cell Culture and Treatment. The cell lines were plated in tissue culture dishes and grown in DMEM with 10% FBS at 37°C in a humidified atmosphere of 95% air and 5% CO₂. To examine the effects of retinoids, the cells were plated in regular medium for 24 h. The medium was then replaced with either control medium (containing 0.01% DMSO) or ATRA dissolved in DMSO (stock solution of 10 mM) diluted into the medium before each experiment. The medium was completely replaced with a fresh one every 72 h.

At the end of the experiment, the cells were fixed with 10% trichloroacetic acid, stained with 0.4% sulforhodamine B in 1% acetic acid, and then the optical densities were determined using an automated spectrophotometric plate reader at a single wavelength of 490 or 530 nm. Viability was tested by exclusion of trypan blue (0.1%), and the percentage of growth inhibition was calculated by the equation: % growth inhibition = (1 - ODt/ODc) × 100, where ODt and ODc are the optical densities in treated and control cultures, respectively.

DNA Extraction and Gel Electrophoretic Analysis of DNA Fragmentation. Soluble DNA was extracted as described (42). Briefly, after a 1-, 3-, or 5-day treatment with 1 μM ATRA, the cells floating in medium were collected by centrifugation, and the cells that remained attached to the dish on day 5 were detached by scraping. The cells were centrifuged into a pellet and resuspended in Tri-EctDA buffer (pH 8.0). The plasma membrane of the cell was lysed in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100 (TX-100) on ice for 15 min. The lysate was centrifuged at 12,000 × g for 15 min to separate soluble (fragmented) from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 μg/ml) at 37°C for 1 h, followed by treatment with proteinase K (100 μg/ml) in 0.5% SDS, at 50°C for 2 h. The residual material was extracted with phenol/chloroform, precipitated in ethanol, electrophoresed on a 1.8% agarose gel, and stained with ethidium bromide.

Residual material was extracted with phenol/chloroform, precipitated in ethanol, and then the optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 490 or 530 nm. The percentage of growth inhibition was calculated from two independent triplicate studies using the equation: % con- 

RT-PCR. For RT-PCR analysis, 5 μg of total cellular RNA, purified from monolayer cultures of HEC cells by Tri-reagent (MRC), were incubated to digest potential contamination of genomic DNA with RNase-free DNase at 37°C for 30 min and then purified by phenol/chloroform extraction, precipitated in isopropanol, and washed in ethanol. The cleaned RNA was subjected to cDNA synthesis in a reverse transcription mixture (2.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-Cl (pH 8.4), 10 mM DTT, 1 mM dNTP, 200 units of SuperScript II reverse transcriptase, and 2.5 μM random hexamers; Life Technologies, Inc., Gaithersburg, MD) at 42°C for 50 min. Template RNA was then destroyed by RNase H treatment. PCR was then performed on the cDNA product in each tube using different primer sets. Primers for RAR-α were (sense) 5’-GTCTTTCGTGACACTCAACAG-3’ and (antisense) 5’-GCCCTGGATCCGTCTCACA-3’; these primer sets amplified a 333-bp PCR product. Primers for RAR-β were (sense) 5’-CGTGGATTGCTCTGACTGACT-3’ and (antisense) 5’-CATGTGAGGCTTGTGGTCGTC-3’; these primers amplified a 220-bp PCR product. Primers for RAR-γ were (sense) 5’-AAATCCAGCATTCCGGGCGG-3’ and (antisense) 5’-GGGTTCCTCGAGGGGAG-3’. These primer sets amplified a 122-bp PCR product. Primers for β-actin were (sense) 5’-ATCGCCACCCACCTCTCTCAATAGAGTCG-3’ and (antisense) 5’- CGTCATACCTGGTGTGATCCACATCTGC-3’; these primers amplified an 838-bp PCR product. The PCR reaction was performed in 1.5 mM MgCl₂, 50 mM KCl, 20 mM Tris-Cl (pH 8.4), 1 mM dNTP, 1 unit of Taq DNA polymerase, and 0.2 μM primers in a thermal cycler (Peltier thermal cycler, PTC-200; MJ Research Inc. Watertown, MA). The reaction was initiated with a 3-min incubation at 95°C, followed by 35 amplification cycles (95°C for 30 s, 58°C for 1 min, and 72°C for 1 min) and a final extension step (72°C for 10 min and then storage at 4°C). Equal volumes of the PCR product from each sample were subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and photographed.

In Situ Hybridization. A previously described method of nonradioactive in situ hybridization was used (28, 43). The quality and specificity of the digoxigenin-labeled antisense and sense riboprobes were determined using Northern blotting, and the specificity of the binding of antisense riboprobes was verified using negative control sections (28, 43).
Statistical Analysis. The McNemar test was performed to determine the association between adjacent normal tissues and tumors. \( P \) was generated by Statistics version 3.0a (StatSoft, Tulsa, OK).

RESULTS

Growth Inhibition of TE Cells by ATRA Is Due to Induction of Apoptosis. To test the effect of ATRA on the growth of esophageal cancer cells, the TE cells in monolayer culture were treated with 0.1, 1, or 10 \( \mu M \) ATRA for 1, 3, or 5 days. Maximal effects were reached when TE cells were treated with 1 or 10 \( \mu M \) ATRA for 5 days (Fig. 1). The treatment resulted in growth inhibition of five cell lines at different grade, whereas TE-1 and TE-8 cells were resistant to ATRA even at 10 \( \mu M \) (Fig. 1). To determine the possible mechanism of growth inhibition by ATRA, we performed DNA fragmentation and TUNEL assays. DNA fragmentation analysis demonstrated that a 5-day treatment with 1 \( \mu M \) ATRA induced apoptosis in the five sensitive cell lines (Fig. 2). One- or 3-day treatment had no or only minimal effect on most cell lines except for TE-3 and TE-7, which showed some apoptosis on day 3. TUNEL assay also showed ATRA-induced apoptosis in these five cell lines, and computer analysis of the data revealed that TE cells except TE-1 and TE-8 accumulated in sub-G1 phase of cell cycles, followed by 1 \( \mu M \) ATRA treatment for 5 days.

Expression of RAR mRNA by Northern Blot and RT-PCR. The expression of RARs was determined in HEC cell lines by using Northern analysis of total cellular RNA (Fig. 3). All seven HEC cell lines expressed RAR-\( \alpha \) and RAR-\( \gamma \) when grown in monolayer cultures in medium supplemented with 10% FBS, but RAR-\( \beta \) was expressed only in five of the seven lines. Furthermore, the two cell lines TE-1 and TE-8 that were negative for RAR-\( \beta \) by Northern

![DNA ladder formation of TE cells induced by ATRA from two independent experiments. TE cells were treated with DMSO as control or 1 \( \mu M \) ATRA for 5 days. Both floating and adherent cells were collected, and soluble DNA from each cell fraction was extracted and electrophoresed on a 1.8% agarose gel. The gels were stained with ethidium bromide and photographed.](image)

![Northern blotting analysis of expression of RARs and their modulation by ATRA in HEC cells. HEC cells were grown in monolayer cultures with or without 1 \( \mu M \) ATRA for 5 days, total cellular RNA was extracted and subjected to Northern blot analysis. The experiments were then repeated. The photographs of the Northern blots underwent quantitative analysis by IS-1000 Digital-Imaging System (Alpha Innotech Corp., San Leandro, CA), and the results were plotted on the bottom of the figure.](image)

![RT-PCR analysis of RAR expression in HEC cells. RT-PCR was performed on total cellular RNA from the same preparation as for Northern blotting. RNA (5 \( \mu g \)) from control and treated cells were used for cDNA synthesis, and one-tenth of the newly synthesized cDNAs underwent PCR amplification for 35 cycles by different primer sets, specific for RARs or \( \beta \)-actin. PCR products were then subjected to electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, and photographed. This experiment was repeated twice.](image)

![Northern blot analysis of squamous cell differentiation markers in HEC cell lines. HEC cells were grown on monolayer cultures in DMEM with 10% FBS supplemented with or without 1 \( \mu M \) ATRA for 5 days, total cellular RNA was extracted, and the level of mRNAs for the cytokeratin K1 and transglutaminase I were determined by Northern blotting from duplicate experiments.](image)
blotting were also negative by much more sensitive RT-PCR (Fig. 4). RAR expression was analyzed in these cell lines after treating them with 1 μM ATRA for 5 days when marked effects on growth were observed (Fig. 1). Expression of RAR-α and RAR-γ show some changes in TE-2, TE-3, TE-8, and TE-12, respectively after addition of ATRA to these cells. The increase in RAR-α occurred in cell line TE-8, which is resistant to growth inhibition; therefore, RAR-α increase may not be involved in apoptosis. RAR-γ increase in TE-2, which was responsive to ATRA, but decreased in TE-3, which is very sensitive to RA; thus, the changes in RAR-γ were not consistent and may not be related to apoptosis induced by ATRA. In contrast, RAR-β expression was up-regulated by ATRA in five of seven cell lines. RAR-β was not detected in TE-1 and TE-8 cell lines after ATRA treatment even when RT-PCR was used (Fig. 3 and Fig. 4). The five cell lines with the up-regulation of RAR-β by ATRA treatment also showed apoptosis (Fig. 2). In contrast, the two cell lines without the up-regulation of RAR-β expression by ATRA failed to show any apoptosis by ATRA (Fig. 2).

Fig. 6. Localization of RAR mRNAs in HEC surgical specimens by in situ hybridization. A, formalin-fixed and paraffin-embedded sections from esophageal carcinoma and its adjacent normal tissues were hybridized with RAR-β antisense probe, and both showed positive staining in cytoplasm. B, consecutive sections of formalin-fixed and paraffin-embedded HEC and its adjacent normal tissues were hybridized with RAR-α, RAR-β, or RAR-γ antisense by using digoxigenin-labeled cRNA probes, which results in purple to blue staining of the positive signal in the cytoplasm, and with RAR-β sense probe for staining control.
**Colonial Formation Assay.** The ability of cells to form colonies in agarose is indicative of anchorage independence and is used as an *in vitro* criterion of transformation. We, therefore, used it to examine whether the cells could form colonies in soft agar and attempted to find out its correlation with RAR-β expression. The results show that the five cell lines, which expressed RAR-β failed to form any colonies in soft agar when 2000 cells were seeded for 60-mm dish, whereas the two cell lines, which expressed no RAR-β did form colonies (140 colonies were seen in TE-1 and 15 colonies were seen in TE-8). Treatment with ATRA (1 μM) failed to inhibit colony formation in TE-1 and TE-8 cells in soft agar. Two hundred sixty-three colonies were formed in ATRA treated TE-1 cells and 15 colonies were formed in ATRA-treated TE-8 cells.

**Suppression of Squamous Cell Differentiation by ATRA.** The expression of the squamous differentiation markers cytokeratin K1 and keratinocyte transglutaminase I was determined in the seven cell lines using Northern blotting. The results demonstrated that TE-2, TE-7, and TE-12 expressed both markers, TE-3 and TE-8 expressed only cytokeratin K1, and TE-13 expressed only transglutaminase I; in contrast, TE-1 expressed neither of the two markers (Fig. 5). ATRA treatment suppressed both markers in all cell lines that expressed them constitutively (Fig. 5). However, there seems to be no correlation between the expression of these two differentiation markers and ATRA sensitivity or expression of RAR-β.

**Expression of RARs in Surgical Specimens of HEC.** We analyzed the expression of RARs in tissue specimens from esophageal cancer patients, which contained both HEC and adjacent normal tissues. All 20 adjacent normal tissues expressed mRNAs for the three RARs, as detected by *in situ* hybridization (Fig. 6). RAR-α and RAR-γ mRNAs were also detected in HEC. In contrast, RAR-β mRNAs was detected in only 6 of the 20 HEC specimens. The difference in RAR-β expression between tumor and adjacent normal tissues was statistically significant (*P* < 0.005). Semiquantitatively analytic data are also shown in Table 1. The results indicate that the loss of RAR-β expression might be associated with HEC development.

**DISCUSSION**

This is the first detailed and systematic study of RA effects and its nuclear receptors on cultured HEC *in vitro* and on receptor expression *in vivo*. The *in vitro* data of the present study revealed the effect of retinoid on the growth, differentiation, and apoptosis of HEC cell lines, as well as the relationship between its biological effect and the expression of its nuclear receptor RAR-β. Growth inhibition due to induction of apoptosis of five of seven HEC cell lines with ATRA seems to be associated with RAR-β expression and its up-regulation. TE-1 and TE-8 cells failed to express RAR-β and also did not respond to RA-induced growth inhibitory effects. Our *in vivo* data demonstrated for the first time that RAR-α and RAR-γ was expressed in esophageal carcinoma specimens from patients, whereas RAR-β expression was lost in 14 of 20 cases. Two recent clinical trials of 13-*cis* RA in the patients with advanced esophageal cancer were unsuccessful (44, 45). We propose that these failures may, at least in part, be due to the loss of RAR-β expression in these cancers.

It is well established that retinoids can modulate epithelial cell growth, differentiation, and apoptosis *in vitro* and *in vivo* (8, 9, 12–19, 26, 27, 33, 42, 46). Retinoids can prevent abnormal squamous differentiation of epithelial cells in nonkeratinizing tissues physiologically (46, 47). Retinoids can also reverse squamous metaplasia, which develops during vitamin A deficiency. In this study, ATRA inhibited the expression of transglutaminase I and cytokeratin K1 in HEC, but these effects did not correspond to growth inhibition of HEC cells. Similar results were reported for other squamous cell carcinomas (33, 47–49). Studies with experimental animals and clinical trials have demonstrated that retinoids suppress carcinogenesis in a variety of epithelial tissues including skin, trachea, lung, and oral mucosa (8, 9, 12–19, 26). It was suggested that retinoids might restore premalignant and malignant lesions to the normal nonkeratinizing phenotype and also restore responsiveness to normal growth-control mechanisms, thereby suppressing carcinogenesis and preventing squamous cell carcinoma development.

Studies with different cancer cell lines have demonstrated that retinoids can suppress cell proliferation in monolayer cultures (33), inhibit the formation of squamous cancer cell colonies in soft agar (48), and decrease the growth of multicellular spheroids (49). A recent study limited to two HEC cell lines demonstrated that 4-HPR treatment inhibited cell growth by suppressing epidermal growth factor receptor signaling (50). The TE series of cell lines exhibited different sensitivities to RA in monolayer culture, and the growth inhibition was due to the induction of apoptosis shown by DNA fragmentation and TUNEL assays. Although the expression of RAR-α and RAR-γ showed some changes after the treatment with 1 μM ATRA, there seemed to be no association with cell survival, and only the expression and up-regulation of RAR-β by ATRA was associated with RA-induced apoptosis of TE cells. This may imply that RAR-β plays a role in esophageal cancer response to RA treatment.

Studies by others and by us have clearly demonstrated that altered expression of retinoid receptors is associated with malignant transformation in various human cells (27–41). Altered expression of RAR-β is a common event in different types of tumors, including head and neck, lung, breast, and pancreas (27–41), although lost expression of RAR-α or RAR-γ has been reported also (41). For instance, chromosomal translocation inactivates RAR-α during malignant transformation in human promyelocytic leukemia (34). Our present study is the first report about loss of RAR-β expression in esophageal carcinoma. The sensitivity of TE cells to RA seems to be correlated not only with the expression but also with up-regulation of RAR-β. Lacking RAR-β expression or up-regulation, TE-1 and TE-8 cells failed to respond to RA and formed colonies in soft agar. This indicates that RAR-β may possess tumor suppressor function, as has been proposed and indicated by previous studies with other cell types (37–40, 51–54). We have also observed that head and neck squamous cancer cell line 1483 *in vitro* expressed RAR-β in monolayer cultures, but lost the expression in nude-mouse tumors. Previous studies showed that many lung cancer cell lines that did not express RAR-β were also resistant to growth inhibition by RA (37, 39). The selective suppression of RAR-β expression may be related to the process of malignant transformation in epithelial cells (30, 31, 35–40, 52, 54). Support for this contention was provided by the report that lung carcinoma cells expressing a transfected RAR-β exhibited decreased tumorigenicity in nude mice (38) and the observation that transgenic mice expressing antisense RAR-β developed lung cancer (54).

In the present study, analysis of surgical specimens from esophageal cancer patients further confirmed that expression of RAR-β is frequently lost in HEC, although the expression of RAR-α and RAR-γ was decreased in some cases by semiquantitative comparison with adjacent normal epithelium of the esophagus. This finding is in agreement with the studies in head and neck, lung, and breast cancers, and also other types of cancers (27–41). Previously, we found RAR-β expression was selectively suppressed in head and neck cancer and, in the early stages of carcinogenesis, in the oral cavity (28, 29). In non-small cell lung cancers, several groups (30, 36–39) have reported

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4 Unpublished data.

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the abnormalities in the expression of RAR-β. It is also true in breast cancers (31, 40, 51, 52). Altogether, the selective suppression of RAR-β expression may be related to the process of malignant transformation in epithelial cells.

The mechanism underlying the lack of expression of the RAR-β gene remains unknown. Deletion of chromosome 3p may exist in some HEC tumors (55), but no homozygous deletions or rearrangements of the gene were found by Southern blotting in the DNA isolated from head and neck squamous cancer cell lines that do not express the RAR-β mRNA (35). A study using HeLa cells suggested that the loss of RAR-β expression may not be caused by mutations in the RA response element or other proximal regulatory elements of the RAR-β promotor (56). In addition, the alteration of nuclear retinoid receptor coactivators or corepressors for the RAR-β gene may also account for the loss of expression (57–60). Finally, expression of RAR-β may depend on the cellular levels of retinoids because this receptor is inducible by retinoids in different human malignant cell lines. Loss of RAR-β expression in premalignant oral lesions correlated with a low cellular level of RA concentration, which indicates that there is some defect in retinoid uptake or metabolism (61). This conclusion is also supported by the finding that RAR-β expression is selectively reduced in several organs during vitamin A deficiency and is enhanced by RA, as demonstrated in studies with rats (62, 63). All of the above findings suggest that multiple mechanisms may be involved in the loss and reduction of RAR-β expression.

Cancer is a genetic disease. Genetic alterations in HEC are frequently exhibited in chromosomes 3p, 5q, 9p, 9q, 14q, 17q, and/or 18q (64, 65). p53 and p16 mutation and overexpression of p53 and p16 are frequently exhibited in chromosomes 3p, 5q, 9p, 9q, 13q, 17p, 17q, and 18q. Moreover, there is a selective reduction of RA response elements in squamous-cell carcinoma of the head and neck. N. Engl. J. Med., 323: 795–801, 1990.


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