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Differential Effects of Retinoic Acid on Growth and Apoptosis in Human Colon Cancer Cell Lines Associated with the Induction of Retinoic Acid Receptor β

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ABSTRACT. Retinoids are well known as potential chemopreventive and chemotherapeutic agents against a variety of human cancers. Here, we report that retinoic acid (RA) induced differential growth inhibition in human colon cancer cell lines: while DLD-1, HT-29, and WiDr were relatively resistant, HCT-15 and Colo201 were relatively sensitive. All-*trans*-retinoic acid caused morphological and biochemical changes such as membrane shrinkage, chromatin condensation, and DNA cleavage, which are typical features of cells undergoing apoptosis in sensitive cell lines. Although retinoic acid receptor (RAR)α, β, γ and retinoid X receptor α were expressed in all cell lines examined, a significant induction of RARβ by all-*trans*-RA was observed only in sensitive cell lines, suggesting important roles of RARβ in RA sensitivity. When a vector containing the *RAR*β gene was introduced into a relatively resistant cell line, DLD-1, the cells acquired RA sensitivity. Further, we found that the RARβ transfectants of DLD-1 expressed an enhanced level of c-Myc and Bax proteins, which may result in the increased susceptibility of the cells to all-*trans*-RA-induced apoptosis. In summary, our data demonstrated that RA induced growth inhibition and apoptosis in human colon cancer cells and that the induction of RARβ may mediate the retinoid action. BIOCHEM PHARMACOL **59**;5:485–496, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. all-*trans*-retinoic acid; apoptosis; RARβ; colon cancer

Retinoids, vitamin A, and its natural and synthetic derivatives regulate a wide array of biological processes including cellular proliferation, differentiation, and development [1]. They have been recognized as promising agents for chemoprevention and chemotherapy for a variety of human cancers, including epithelial cancer and promyelocytic leukemia [1–3]. The biological and pharmacological effects of retinoids are mainly mediated through two classes of nuclear receptors, RAR† and RXR, which belong to the steroid/thyroid hormone receptor superfamily [4, 5]. Multiple isoforms of retinoid receptors, i.e. RARα, β, and γ and RXRα, β, and γ, are expressed in a unique pattern during development and differentiation. Among the receptors, RARβ is primarily expressed in epithelial cells, suggesting that RARβ may be involved in maintaining normal homeostasis of epithelia. Consistent with that notion, the lack of or abnormal expression of RARβ has been reported in several human tumors including lung carcinoma, squamous cell carcinoma of the oral pharynx, and breast carcinoma [6–9].

Colorectal cancer has been one of the most common malignancies in Western society, but its incidence is increasing in East Asian countries, perhaps due to the adaptation of Western lifestyles, including diet. In colonic epithelium, a dynamic balance between cell production at the base and cell death at the surface of the colonic crypts is precisely regulated to maintain the cellular homeostasis of the tissue [10, 11]. Since apoptosis is a part of the final differentiation step for colonic enterocytes, a progressive inhibition of apoptosis may cause a cancerous transformation that further progresses to malignancy in the colon [12, 13]. In fact, apoptosis has been related to the survival rate in colorectal adenoma and carcinoma patients [14]. Therefore, the factors/agents that control the balance between cellular proliferation and death in colon epithelium are of considerable importance. Thus far, growth factors such as transforming growth factor-β, cytokines such as interferon γ, and chemotherapeutic drugs/differentiation-inducing agents such as 5-fluorouracil, sodium butyrate, and nonste-

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† *Abbreviations:* RAR, retinoic acid receptor; RXR, retinoid X receptor; RA, retinoic acid; RARE, retinoic acid response element; PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; RT-PCR, reverse transcriptase–polymerase chain reaction; CAT, chloramphenicol acetyltransferase; and β-gal, β-galactosidase.

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roidal anti-inflammatory drugs have been reported to induce apoptosis in human colon cancer cells [15–18]. Epidemiological studies have shown that vitamin A deficiency increased the incidence of colon cancer and that an adequate amount of vitamin A supplement reduced the risk of the cancer [19]. However, the effects of retinoids on the growth of human colon cancer cells have not been extensively examined [20–23]. In general, the protective/therapeutic potential of retinoids against human cancer has been considered to be primarily due to the inhibition of cellular proliferation and the stimulation or restoration of the differentiation potential of their target cells. Recent studies, however, have demonstrated that retinoids induced apoptosis in certain types of normal and cancer cells which partially contribute to the observed biological/pharmacological effects of retinoids [9, 24, 25]. Therefore, we undertook this investigation to examine the effects of RA on the growth of human colon cancer cells and to determine whether or not RA induces apoptosis in these cells.

Here, we show that all-*trans*-RA/9-*cis*-RA inhibited the cellular growth of human colon cancer cells. Further, we provide evidence that all-*trans*-RA induced apoptosis in the cells that were accompanied by morphological changes such as plasma membrane blebbing and DNA fragmentation. Importantly, the inhibitory effects of retinoids on cell growth are correlated with the induction of RARβ in response to all-*trans*-RA in human colon cancer cell lines. When RARβ was overexpressed in a retinoid-insensitive cell line, DLD-1, the cells acquired RA sensitivity. Further, we showed that the overexpression of RARβ was associated with the increased expression of c-Myc and Bax. In summary, our data demonstrated that RA induced growth inhibition and apoptosis in human colon cancer cell lines and that the induction of RARβ may mediate the retinoid action.

MATERIALS AND METHODS

Cell Lines and Culture

The human colon cancer cell lines DLD-1 (ATCC CCL 221), HT-29 (ATCC HTB 38), HCT-15 (ATCC CCL 225), Colo201 (ATCC CCL 224), Colo205 (ATCC CCL 222), and WiDr (ATCC CCL 218) were obtained from the American Type Culture Collection and maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) except for Colo201 cells, which were grown in RPMI containing 20% FBS. For RA treatment, the medium was changed to MEM containing 1% charcoal-stripped FBS after cells were attached to culture dishes.

Growth Inhibition Assays

Cellular proliferation experiments were performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays using the Cell titer[96] (Boehringer Mannheim) according to the manufacturer's instructions. Cells were seeded at an initial density of 3000 cells per well in 96-well plates. The media containing fresh retinoid was added every 2 days for the first 5 days. The number of viable cells was determined at day 9 by measuring their capacity to convert a tetrazolium salt into a blue formazan product.

Total RNA Preparation and RT–PCR

Total RNA was prepared using the Qiagen RNeasy kit (Qiagen) following the manufacturer's instructions. Single-stranded DNA was synthesized from RNA in a reaction mixture containing 100 ng random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (GIBCO BRL). The PCR reaction mixture contained 10 pmol of each forward and reverse primer, 2 units of Taq DNA polymerase (Perkin Elmer), and cDNA templates. Amplification was carried out for 20 to 30 cycles depending on target genes in a thermal cycler. Each cycle consisted of a 1-min denaturation at 94°, 1-min annealing at 54°, and a 1-min extension at 72°. The sequences of primers used for analysis were as follows: RARα (forward: 5′-CAAGTG CATCATTAAGACTG-3′, reverse: 5′-GGCTGCTCC AGGTCCTGGCG-3′), RARβ (forward: 5′-TGGAGTT TGCTAAACGTCTG-3′, reverse: 5′-GTAGCTTATCT ACTTTTGTC-3′), RARγ (forward: 5′-TGGAGATGG ATGACACCGAG-3′, reverse: 5′-GGTGGGGACCAG GCTGCGAG-3′), RXRα (forward: 5′-CGGGATCCT CCTGGCCACCG-3′, reverse: 5′-TCCAGGCATTTG AGCCCGAT-3′), and β-actin (5′-CGTGGGCCGCCC TAGGCACCA-3′, reverse: 5′-TTGGCTTAGGGTTCA GGGGGG-3′). All the genes were analyzed under the conditions in which PCR products were exponentially amplified.

PI Staining for Fluorescence Microscopy and Flow Cytometry

To examine nuclear morphology by fluorescence microscopy, cells were collected, washed once, and resuspended in PBS. The cell suspension was placed on coverslips precoated with aminopropyltriethoxysilane and incubated for 5 min at room temperature to allow cells to attach. The coverslips with adhered cells were fixed with ice-cold 80% methanol for 30 min, followed by 3 washes with PBS. Cells were stained for 5 min with PI (50 μg/mL in PBS) and then washed 3 times with PBS before mounting onto glass slides with 50% glycerol in PBS. The nuclear morphology of cells was examined with an UV fluorescence microscope and photographed. Alternatively, to assess subdiploid DNA content, total cells were collected and fixed in 70% ethanol in PBS at −20°. Cells were then washed and stained with 50 μg/mL PI in the presence of 100 μg/mL RNase A for 30 min at 37° in the dark. DNA content was analyzed by a FACStar Plus flow cytometer (Becton Dickinson). Apoptotic cells with subdiploid DNA staining were found in the 'sub-G_0/G_1' peak and the percentage of such cells was calculated.

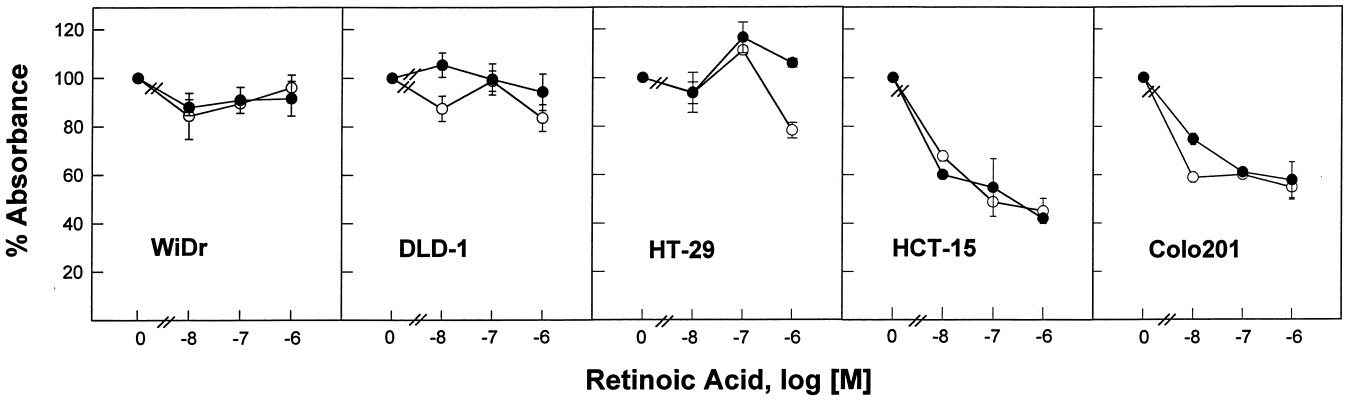


FIG. 1. Effects of retinoic acid on the growth of human colon cancer cell lines. A total of 3000 cells per well was seeded in 96-well culture plates and treated with various concentrations of all-*trans*-RA (closed circles) and 9-*cis*-RA (open circles) for 9 days. Details are described in Materials and Methods. Results were normalized and expressed as means ± SE ($N = 3$) of the control. Data represent results derived from a single experiment, which was repeated at least 3 times with similar results.

Analysis of Oligonucleosomal DNA Fragments

Cells were collected by centrifugation and resuspended in TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). An equal volume of lysis buffer containing 20 mM EDTA, 0.05% (v/v) Triton X-100, and 10 mM Tris–HCl (pH 8.0) was added and incubated on ice for 30 min. The lysates were then centrifuged for 15 min at 4° at 13,000 *g* to separate cell debris containing intact chromatin (pellet) from DNA fragments (supernatant). The fragmented DNA in supernatant was precipitated overnight at −20° with ethanol. DNA was resuspended in TE buffer and subsequently treated with RNase A and proteinase K. The obtained samples were electrophoresed for 4 hr at 70 mV on 1.8% agarose gels. The separated DNA was stained with ethidium bromide and visualized by UV light.

Assessment of DNA Fragmentation by TUNEL Assay

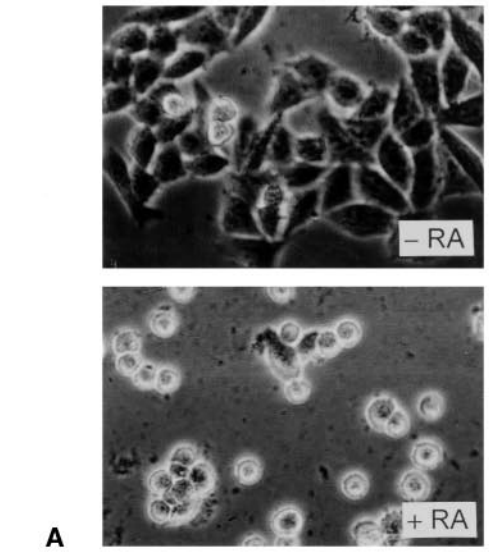
The TUNEL assay was carried out essentially according to the methods of Liu *et al.* [9]. Briefly, cells were harvested and fixed with 1% formaldehyde in PBS for 15 min on ice. After washing with PBS, cells were resuspended in 70% ice-cold ethanol and immediately transferred to a freezer until use. Following fixation, cells were incubated in a solution containing 0.2 M sodium cacodylate (pH 7.0), 2.5 mM $CoCl_2$, 25 mM Tris–HCl, 0.25 mg/mL BSA, terminal deoxynucleotidyl transferase (Oncor Inc.), and 2 nmol of biotin-16-dUTP (Boehringer Mannheim) for 30 min at 37°. The cells were then washed with PBS and incubated in a staining solution containing 40 μg/mL fluoresceinated avidin, 4 × saline–sodium citrate buffer (1 × SSC: 0.15 M NaCl, 0.015 M Na-citrate), 0.1% Triton X-100, and 5% non-fat dried milk for 30 min at room temperature in the dark. Following staining, cells were resuspended in a solution of formaldehyde/PBS (1/5, v/v), and fluorescence was measured by the FACStar Plus flow cytometer.

Transient and Stable Transfection Assay

The reporter gene containing two copies of βRARE (→ →) was used in the experiments [9]. Colon cancer cells (2×10^5 cells/well) were seeded in a 6-well culture plate. Reporter plasmid (1 μg) and β-gal expression vector (0.5 μg) were used for transfection using Lipofectin® (GIBCO BRL) according to the manufacturer's instructions. After 24 hr of retinoid treatment, CAT activity was determined and normalized for transfection efficiency by the corresponding β-gal activity. For stable transfection, pRC/cytomegalovirus-RARβ [9] recombinant plasmid was stably transfected into DLD-1 using Lipofectin®. Positive clones were selected using G418 (400 μg/mL) (GIBCO BRL).

Flow Cytometry for Detection of c-Myc, Bax, and Bcl-2 Proteins

For staining of c-Myc, cells were fixed with ice-cold methanol/acetone (1:1, v/v) overnight at −20° and washed with buffer containing 2% BSA, 5% glycerol, and 0.2% Tween 20 in PBS. Cells were then incubated with 4 μL mouse anti-c-Myc antibody (Santa Cruz Biotech) for 30 min at 4°. For staining of Bcl-2 and Bax, cells were fixed with 2% paraformaldehyde in PBS overnight at 4° and washed with buffer containing 2% BSA in PBS. Cells were then resuspended in 0.1% Triton X-100 for 15 min at room temperature and washed, and then incubated with 1 μL each of hamster anti-Bcl-2 antibody (PharMingen) or rabbit anti-Bax antibody (Oncogene Research Product) for 30 min at 4°. After the cells were stained with primary antibody, they were washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (IgG) (c-Myc), anti-hamster IgG (Bcl-2), or anti-rabbit IgG (Bax) for 30 min at 4°. Irrelevant antibody (normal mouse IgG) was run in parallel as negative controls for c-Myc and Bcl-2 staining. After staining, cells were washed once and resuspended in PBS. Stained cells were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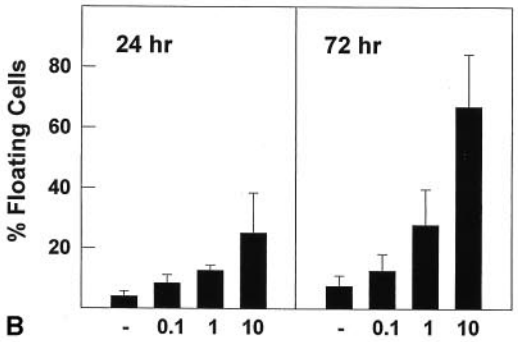
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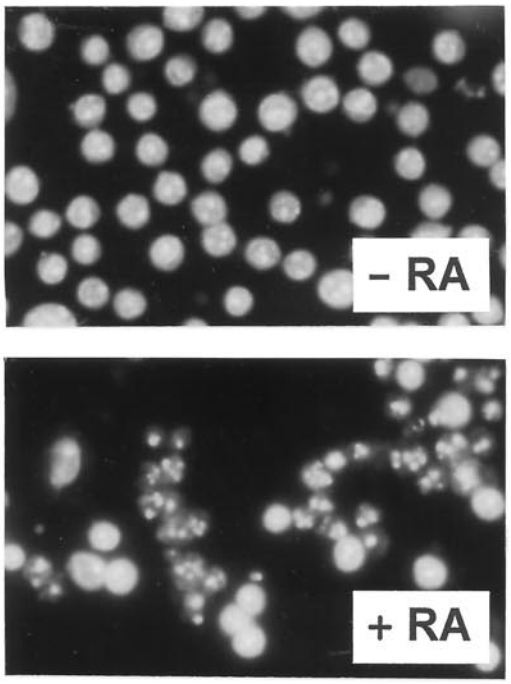
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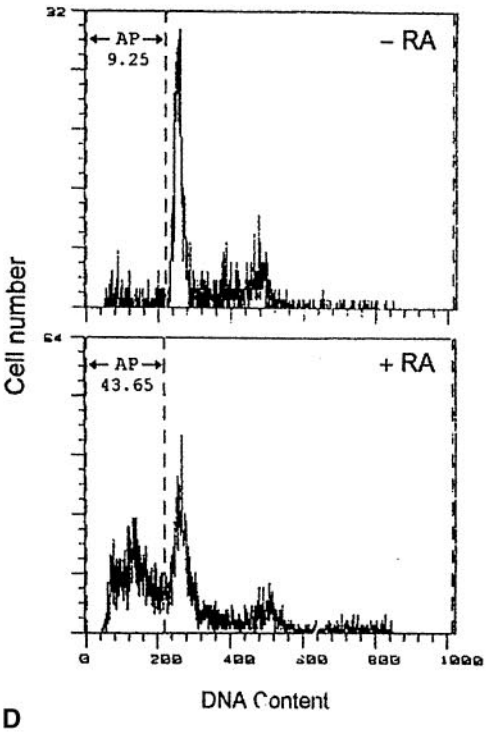
← AP→
9.25
– RA
← AP→
43.65
+ RA
Cell number
DNA Content

D

acquired in the FACStar Plus flow cytometer. Data were presented on histograms plotted as fluorescence intensity against cell number and analyzed using a PC lysis software program (Beckton Dickinson).

RESULTS

Retinoic Acid-Induced Growth Inhibition and Apoptosis in Colon Cancer Cells

We tested whether all-*trans*-RA and 9-*cis*-RA suppressed growth of human colon cancer cell lines using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The cell growth of DLD-1, WiDr, and HT-29 was not significantly retarded in the presence of RA, showing that the cells were relatively resistant to RA treatment (Fig. 1). HCT-15 and Colo201 were relatively sensitive to RA-induced growth inhibition in that the growth was inhibited at a 10-nM concentration of all-*trans*-RA (Fig. 1). All-*trans*-RA and 9-*cis*-RA were almost equally effective in repressing the growth of colon cancer cells. Overall, the colon cancer cell lines used in this study were relatively resistant to all-*trans*-RA compared to other types of human cancer cell lines such as MCF-7 (data not shown). We observed considerable inhibitory effects only when medium containing a low concentration of serum was used.

The cell growth inhibition induced by all-*trans*-RA was characterized in a sensitive colon cancer cell line, HCT-15. We observed that when HCT-15 cells were treated with all-*trans*-RA, they became shrunken and balled up, eventually becoming detached and floating into medium, which is a typical phenomenon observed in cells undergoing apoptosis (Fig. 2A). While cell numbers were reduced by all-*trans*-RA treatment, the percentage of floating cells increased dose and time dependently (Fig. 2B). Chromatin condensation, a typical morphological change in apoptosis, was visualized by PI staining. When all-*trans*-RA-treated HCT-15 cells were stained with PI, a high proportion of cells was found to have condensed chromatin which was highly fluorescent (Fig. 2C). Cellular DNA content analysis by flow cytometry showed the typical profile of cells undergoing apoptosis (Fig. 2D). A distinct, well-quantifiable region below G_1 flow cytometry profiles represented a subpopulation of apoptotic cells in which chromatin condensation and DNA degradation reduced the stainability of PI. The percentage of apoptotic cells after treatment with all-*trans*-RA (10 μM) for 3 days was about 45%. All-*trans*-RA induced a typical DNA laddering pattern in the sensitive cells in both agarose gel electrophoresis and

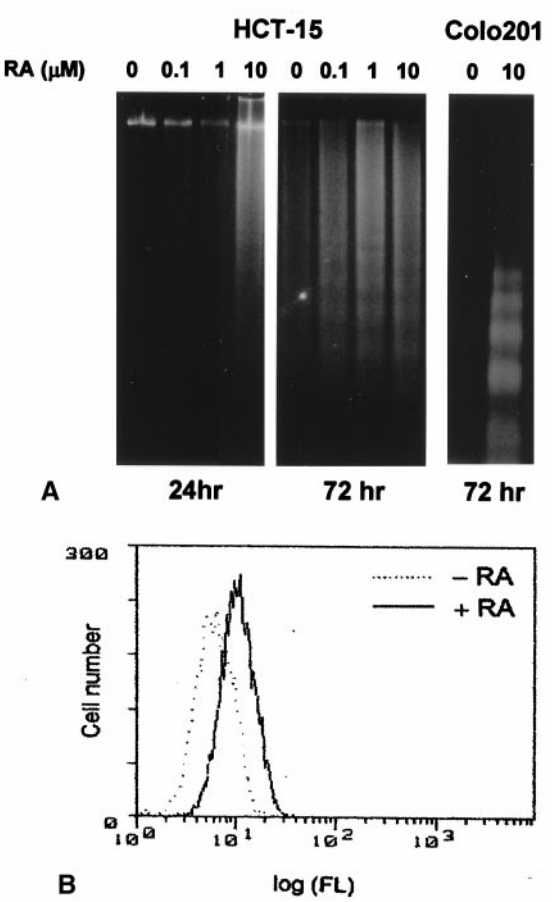

FIG. 3. DNA fragmentation in HCT-15 cells induced by all-*trans*-RA. (A) Agarose gel electrophoresis. HCT-15 or Colo201 cells were treated with or without various concentrations of all-*trans*-RA for different time periods. Fragmented DNA was extracted, subjected to agarose gel electrophoresis, and stained with ethidium bromide. (B) TUNEL assay. HCT-15 cells were cultured in the presence or absence of 10 μM all-*trans*-RA for 72 hr. Cells were then fixed, labeled with biotin-16-dUTP by the terminal deoxynucleotidyl transferase and stained with avidin–fluorescein isothiocyanate. The stained cells were analyzed with a FACStar Plus flow cytometer. FL = fluorescence.

FIG. 2. Morphological changes induced by all-*trans*-RA in HCT-15 cells. HCT-15 cells were cultured with or without various concentrations of all-*trans*-RA as described in Materials and Methods. (A) Phase contrast microscopy. After 48 hr of incubation in the presence or absence of 10 μM all-*trans*-RA, cells were photographed by a phase-contrast microscope. (B) Measurement of floating cells. After incubation for the indicated time periods with various concentrations of all-*trans*-RA, floating cells and attached cells were counted using a hematocytometer. Total cell numbers without all-*trans*-RA treatment (control) at 24 and 72 hr were 1.1×10^6 and 2.38×10^6, respectively. The bars represent the percentage of floating cell numbers out of the total cell numbers. Data represent the means of 3 independent experiments. Error bars indicate standard deviations. (C) PI staining. HCT-15 cells were cultured in the presence or absence of 10 μM all-*trans*-RA for 72 hr and allowed to adhere to aminopropyltriethoxysilane-coated coverslips. The coverslips were then fixed and stained with propidium iodide. Stained cells were examined by a fluorescence microscope and photographed. (D) Determination of apoptosis in all-*trans*-RA-treated HCT-15 cells by DNA content analysis of PI-labeled cells. HCT-15 cells were cultured in the presence or absence of 10 μM all-*trans*-RA for 72 hr and stained with PI as described in Materials and Methods. DNA fluorescence histograms of PI-stained cells were obtained by flow cytometry. Apoptotic cells with subdiploid DNA staining (Ap) were shown and the percentage of such cells was indicated.

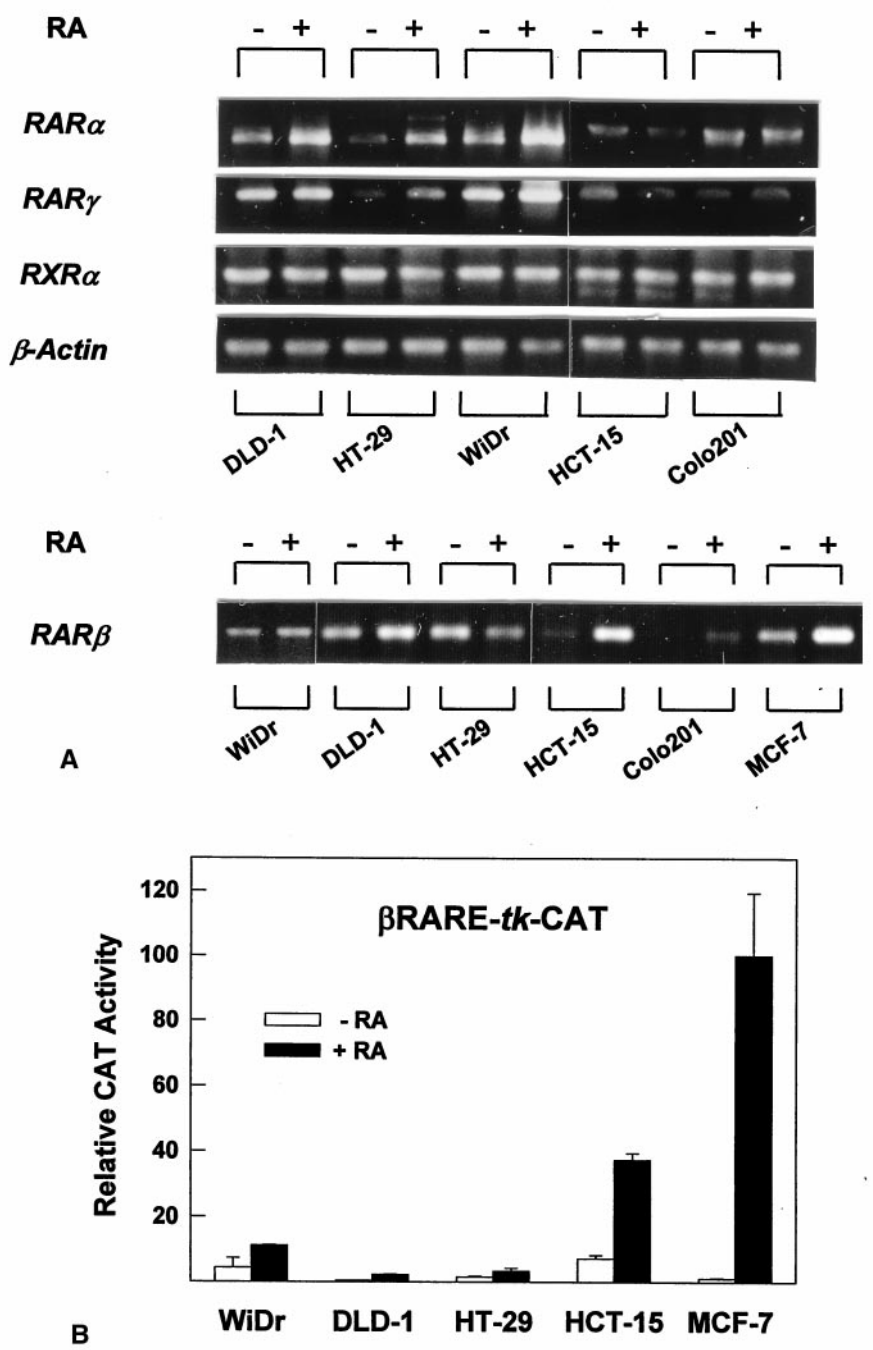

FIG. 4. Expression of retinoid receptors in human colon cancer cell lines. (A) Expression of RARα, RARβ, RARγ, and RXRα. The expression of RARα, RARβ, RARγ, and RXRα was determined by RT–PCR with primers that are specific for each receptor subtype. The expression of β-actin was monitored as a control. To determine the effects of all-*trans*-RA, cells were treated with 1 μM all-*trans*-RA for 48 hr before RNA preparation. (B) Activity of the βRARE reporter gene in colon cancer cell lines. The βRARE-*tk*-CAT together with the β-gal expression vector was transiently transfected into the indicated cells as described in Materials and Methods. Transfected cells were treated with or without 1 μM all-*trans*-RA for 24 hr and CAT activity was measured. CAT activity normalized by β-gal activity was indicated as relative CAT activity. Data represent the means of 3 independent experiments. Error bars indicate standard deviations.

TUNEL assays (Fig. 3). A weak degree of DNA fragmentation was also observed in the relatively resistant colon cancer cells only when cells were treated with 10 μM all-*trans*-RA (data not shown). The results indicated that the cytotoxicity induced by retinoic acids was determined by the susceptibility of the colon cancer cells to undergo apoptosis.

Expression of RARα, RARβ, RARγ, and RXRα in Colon Cancer Cells

The expression of the retinoid receptors in human colon cancer cell lines was examined by RT–PCR using receptor subtype-specific primers (Fig. 4A). RARα and RARγ were

expressed at different levels in all the colon cancer cell lines tested. However, the degree of baseline expression did not correlate with their individual sensitivity to all-*trans*-RA. An increase in RARα gene expression after all-*trans*-RA treatment (1 μM) was observed in most of the cell lines tested. Induction of RARγ was also observed in DLD-1, HT-29, and WiDr. In contrast, RXRα gene expression was not inducible in all the cell lines tested. RARβ transcripts were detected at variable levels in the colon cancer cells, but remarkable induction was only seen in HCT-15 and Colo201 (Fig. 4A). Induction of RARβ in a retinoid-sensitive human breast cancer cell line, MCF-7, was shown for comparison [9]. Induction of RAR activity was also observed in transient transfection experiments using a reporter gene encoding βRARE, a retinoic acid response element found in the promoter of the RARβ gene, i.e. βRARE-*tk*(thymidine kinase)-CAT. As shown in Fig. 4B, all-*trans*-RA induced reporter gene activity significantly in HCT-15, it being comparable to reporter gene activity in MCF-7. However, reporter gene activity was not significant in other colon cancer cell lines in the presence or absence of all-*trans*-RA (Fig. 4B). When RARβ expression plasmid was transiently co-transfected with the βRARE-*tk*-CAT into DLD-1 cells, the reporter gene activity was further enhanced (data not shown), suggesting that endogenous RARs may not be sufficient to deduce retinoid sensitivity in colon cancer cells.

Overexpression of RARβ Gene in DLD-1-Acquired Retinoid Sensitivity

To further study the role of RARβ induction in RA sensitivity in colon cancer cells, we established cell lines that overexpress the RARβ using stable transfection of a vector containing full-coding sequences of the RARβ. A total of 13 clones were selected by G418 screening, and two clones that express a high level of RARβ transcripts were employed for further studies (Fig. 5A). The fold induction measured by the βRARE-*tk*-CAT reporter gene in DLD-1-β11 and DLD-1-β13 was significantly greater than that of wild-type (wt) (Fig. 5B). The effects of all-*trans*-RA on the growth of DLD-1-β11 and DLD-1-β13 were analyzed. The growth of the colon cancer cells in the presence or absence of all-*trans*-RA was measured by counting viable cells after trypan blue staining. Although the growth rate was diminished after 40 hr of all-*trans* RA treatment, DLD-1-wt proliferated exponentially in the presence of all-*trans*-RA (Fig. 6A). In contrast, the growth of DLD-1-β11 was apparently arrested and that of DLD-1-β13 was decreased time dependently in the presence of all-*trans*-RA. To determine whether or not apoptosis was induced by RA treatment in DLD-1-β13, DNA fragmentation was analyzed. As shown in Fig. 6B, DNA fragmentation was observed as early as 12 hr after all-*trans*-RA treatment in DLD-1-β13. The percentage of apoptotic cells measured by PI staining increased time dependently in DLD-1-β11 and DLD-1-β13, while no significant increase was observed in

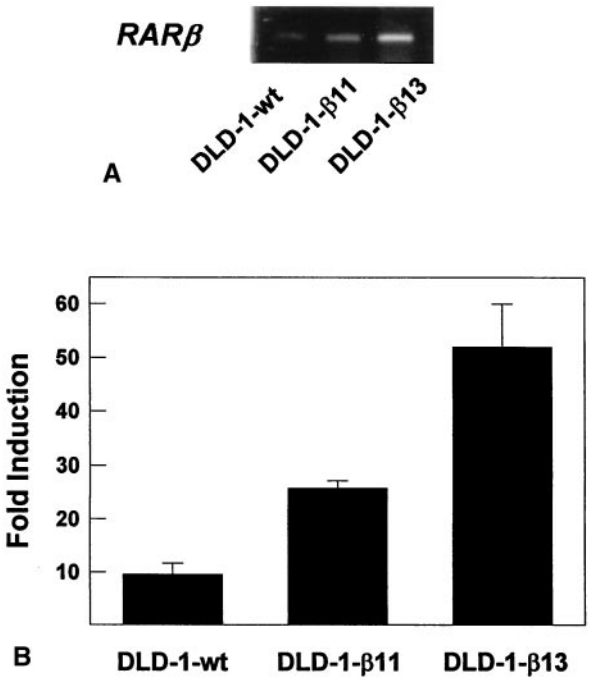

FIG. 5. Expression of RARβ in RARβ-transduced DLD-1 cells. (A) Expression of RARβ in the DLD-1-β11 and DLD-1-β13. PCR was performed using cDNA template synthesized from total RNA extracted from the RARβ transfectants as described in Materials and Methods. (B) The βRARE reporter gene activity in colon cancer cell lines. The βRARE-*tk*-CAT, together with the β-gal expression vector, was transiently transfected into the indicated cells as described in Materials and Methods. Transfected cells were treated with or without 1 μM all-*trans*-RA for 24 hr and CAT activity was measured. The fold induction was calculated from the CAT activity normalized by corresponding β-gal activity. Data represent the means of 3 independent experiments. Error bars indicate standard deviations.

DLD-1-wt after all-*trans*-RA treatment. A representative data profile is shown in Fig. 6C. In the absence of all-*trans*-RA, we also observed an increase in the apoptotic cell subpopulation in DLD-1-β11 and DLD-1-β13, although less so than in the presence of all-*trans*-RA (data not shown).

Analysis of the Expression of c-Myc, Bax, and Bcl-2 Proteins in RARβ Transfectants

A number of cellular proteins either enhance or inhibit apoptosis through a variety of pathways, and the susceptibility of cells to apoptosis is most likely regulated by the expression of such protein factors. We analyzed the expression of c-*myc*, *bax*, and *bcl-2* gene products that are known as positive or negative regulators of apoptosis in DLD-1-wt, DLD-1-β11, and DLD-1-β13. As shown in Fig. 7, the expression of c-Myc was significantly increased ($P < 0.05$, paired *t*-test) in DLD-1-β11 and DLD-1-β13. While the expression of Bcl-2 did not differ significantly in DLD-1-wt, DLD-1-β11, and DLD-1-β13, the expression of Bax was higher in DLD-1-β11 and DLD-1-β13 than in DLD-1-wt.

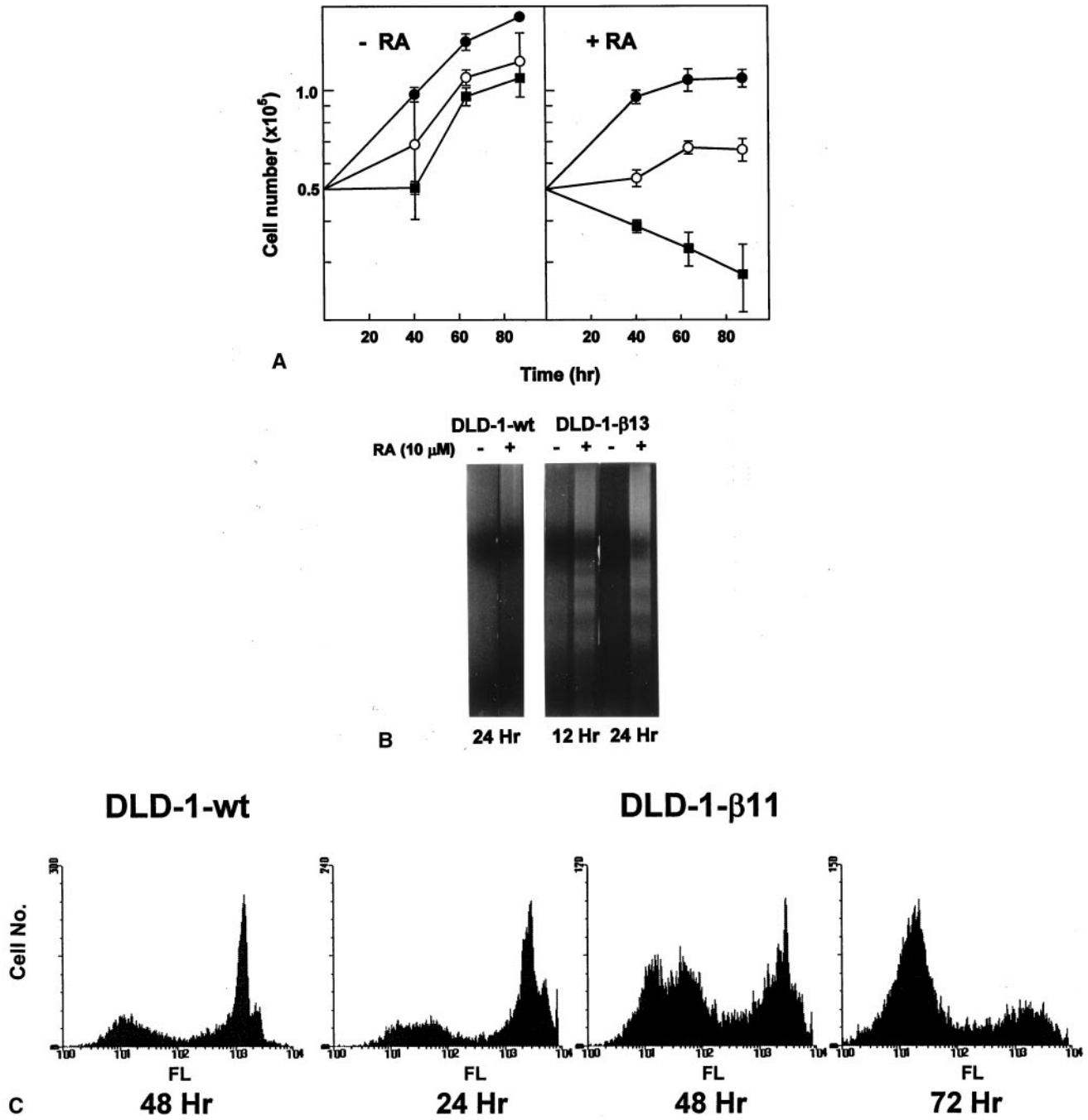

FIG. 6. Effects of retinoids on the growth of the RARβ-transduced DLD-1 cells. (A) Growth inhibition assay. After the DLD-1-wt (open circles), DLD-1-β11 (closed circles), and DLD-1-β13 (closed squares) cells were incubated for the indicated time periods with 10 μM all-*trans*-RA, viable cells were counted using a hematocytometer after trypan blue staining. Data represent the means of 3 independent experiments. Error bars indicate standard deviations. (B) DNA fragmentation. DLD-1-wt and DLD-1-β13 cells were treated with or without 10 μM all-*trans*-RA for the indicated time periods. Fragmented DNA was extracted, subjected to agarose gel electrophoresis, and stained with ethidium bromide. (C) PI staining. DLD-1-wt and DLD-1-β11 cells were cultured in the presence or absence of 10 μM all-*trans*-RA for 72 hr and stained with PI as described in Materials and Methods. DNA fluorescence histograms of PI-stained cells were obtained by flow cytometry. FL = fluorescence.

DISCUSSION

As apoptosis is a normal part of the terminal differentiation program in colonic epithelial cells, a progressive inhibition of apoptosis may cause cancerous transformation which further progresses to malignancy in the colon [12, 13]. Therefore, the factors that control the balance between cellular proliferation and apoptosis are of considerable importance in colonic epithelium. One of the potential compounds that control the apoptotic process could be retinoids; however, their effects on the development and

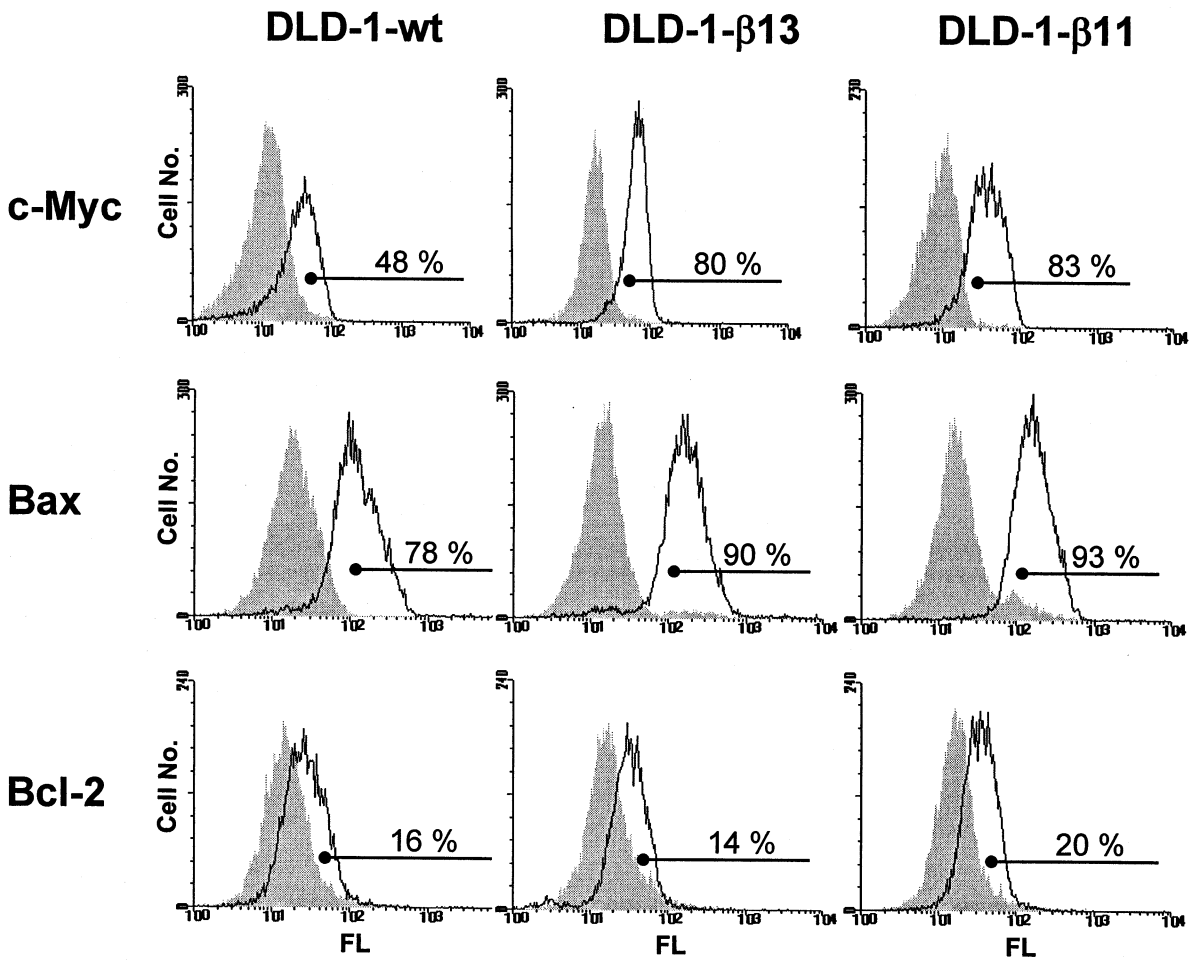

FIG. 7. Expression of c-Myc, Bcl-2, and Bax proteins in the RARβ-transduced DLD-1 cells. c-Myc, Bcl-2, and Bax proteins were detected by flow cytometry using specific antibodies as described in Materials and Methods. Similar results were obtained from at least 3 independent experiments.

growth of colon cancer have not been extensively examined. In this study, we investigated the effects of all-*trans*-RA and 9-*cis*-RA on growth and apoptosis in 5 human colon cancer cell lines. The sensitivity of individual cell lines to the effects of RA were diverse: DLD-1, HT-29, and WiDr were relatively resistant, while HCT-15 and Colo201 were relatively sensitive (Fig. 1). Our results were in agreement with others in that retinoids do not control the growth of all colon cancer cells in the same manner. Previous studies reported that retinoids repressed cell growth of Caco-2 [26], but did not significantly modulate that of HT-29 [22] and LoVo [23]. Further, enhancement of cell growth in MC-26 by retinoids has been observed [23]. Our results, together with others, may suggest that the responses of colon cancer cells to retinoids are determined by intracellular machinery such as nuclear retinoid receptors, retinoid-metabolizing enzymes, and co-activators/co-repressors that distribute cell type specifically. In the colon cancer cell line HCT-15, we demonstrated that all-*trans*-RA treatment caused typical features of apoptosis such as membrane shrinkage, chromatin condensation, and DNA fragmentation (Figs. 2 and 3). Our data indicated that the growth inhibitory effects obtained by all-*trans*-RA treatment were due, at least in part, to the induction of apoptosis in the cells.

A significant induction of RARβ was observed only in RA-sensitive cell lines, suggesting that induction of RARβ expression may partially explain the differential sensitivity to RA in colon cancer cells. Relevant to our observation, the role of RARβ in the maintenance of intestinal epithelial cell differentiation has been implicated [26]. Similar to colon cancer cells, abnormalities in the expression level and function of RARβ have been observed in other types of human cancers. A number of lung cancer cell lines have failed to express RARβ [6, 8]. The induction of RARβ was observed only in estrogen receptor (ER)-positive human breast cancer cells that were sensitive to RA, but not in ER-negative cell lines that were resistant to RA-induced growth inhibition [9, 27]. Importantly, a significant association between the induction of RARβ and the clinical responses of premalignant oral lesions to all-*trans*-RA has been reported [28]. Together, these results indicated that RARβ plays a role in the maintenance of epithelium and that abnormal expression and function of the gene may contribute to tumorigenesis.

Our data suggested that autoregulation of RARβ plays an

important role in the response of colon cancer cells to all-*trans*-RA. Colon cancer cell lines in which RARβ was not induced by all-*trans*-RA treatment may express inhibiting factors, such as activating protein-1 (AP-1), that could antagonize transactivation of the βRARE present in the promoter region of RARβ. RA-resistant cells may have decreased levels of co-activators and/or increased levels of co-repressors that are specifically associated with the transcription of RARβ. A recent report on the amplification and overexpression of AIB1, a steroid receptor co-activator-1 family member, in human breast and ovarian cancer cells may be relevant to the abnormal transcriptional regulation of the RARβ gene in certain types of cancer cells [29]. Alternatively, there is a possibility that mutations in the proximal regulatory region of the RARβ gene promoter may exist in the cells. Indeed, hypermethylation of normally unmethylated CpG islands in the promoter region of RARβ in DLD-1 has been reported as a mechanism of poor expression of the gene [30]. As RARβ was proposed to be responsible for a significant part of the antiproliferative action of RA, identification of factors that inhibit activation of the RARβ promoter may provide a better understanding of the growth control and transformation process of colon epithelia. Recently, Ferrari *et al.* [31] showed that RARγ acted as a repressor of RARβ transcription in the absence of RA and that RA treatment reduced the expression of RARγ, whereas it induced RARβ expression in neuroblastoma cells. We observed a moderate induction of RARγ upon all-*trans*-RA treatment in DLD-1, HT-29, and WiDr, but a reduced expression of the gene by all-*trans*-RA in HCT-15 (Fig. 4A), suggesting a possible association of RARγ in the transcriptional regulation of RARβ in colon cancer cells.

A fundamental question that remains unanswered is how all-*trans*-RA and RARβ trigger apoptosis in colon cancer cells. Our observation raises the possibility that RARβ regulates specific genes that are important for apoptosis. The target genes could be various oncogenes such as *bcl-2*, *p53*, *bax*, *ras*, c-*myc* and others, the expression of which largely determines the susceptibility of tumor cells to undergo apoptosis. Among these, c-Myc and Bcl-2/Bax family proteins have been implicated in colorectal tumorigenesis [13, 32–36]. Frameshift mutations in the bax gene were found in hereditary nonpolyposis colorectal carcinoma [37] and the Bcl-x(L) to Bax ratio was suggested as a factor that determines chemosensitivity of colon cancer cells to 5-fluoruracil [38]. c-Myc Functions to arrest the cell cycle and/or induces apoptosis [39, 40]. Consistently, the overexpression of c-Myc has been reported to confer susceptibility on tumor necrosis factor α- or chemotherapy-induced apoptosis [41, 42]. Interestingly, we found that overexpression of RARβ was accompanied by an increase in c-Myc and Bax protein expression (Fig. 7), suggesting that the proteins in DLD-1-β11 and DLD-1-β13 may play roles in sensitization of the cells to all-*trans*-RA-induced apoptosis. In fact, we found that DLD-1-β11 and DLD-1-β13 were more sensitive than DLD-1-wt to other apoptotic stimuli such as serum deprivation, sodium butyrate, and indomethacin treatment.* Given the ability of deregulated c-Myc in the induction of apoptosis, we speculated that apoptosis induced by c-Myc and RARβ might share some common mechanisms. Molecules such as Bax, p53, ornithine decarboxylase, and dihydrofolate reductase have been identified as targets of c-Myc [43], and the caspase 3-like proteases Fas/Fas Ligand have been shown to function in c-Myc-induced apoptosis signaling [44]. Therefore, the question as to whether such steps are shared by the apoptosis induced by all-*trans*-RA in colon cancer cells is of interest for future investigation.

In summary, our data demonstrated that RA induced apoptosis in human colon cancer cells and that the induction of RARβ may mediate the retinoid action. However, much work remains to be done before we can fully understand the mechanism by which RARβ participates in the normal regulation of divergent cellular fates of proliferation, differentiation and apoptosis in colon epithelia. Clinical studies which examine the expression of RARβ and its responsiveness to retinoids in conjunction with a survey of apoptosis regulators and growth factors may provide new prognostic information about human colon cancer and may identify potential new targets for cancer therapy.

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References

1. Gudas L, Sporn MB, and Roberts AB, Cellular biology and biochemistry of the retinoids. In: *The Retinoids*. 2nd ed. (Eds. Sporn MB, Roberts AB and Goodman DS), pp. 443–520. Raven Press, New York, 1994.
2. Moon RC and Mehta RG, Chemoprevention of mammary cancer by retinoids. *Basic Life Sci* **52:** 213–224, 1990.
3. Lippman SM, Kessler JF and Meyksens FL, Retinoids as preventive and therapeutic anticancer agents. *Cancer Treat Rep* **71:** 493–515, 1987.
4. Pfahl M, Apfel R, Bendik I, Fanjul A, Graupner G, Lee M-O, LaVista N, Lu X-P, Piedrafita J, Ortiz MA, Salbert G and Zhang X-K, Nuclear retinoid receptors and their mechanism of action. In: *Vitamins and Hormones: Steroids* (Ed Litwack G) Vol. 49, pp. 327–381. Academic Press, San Diego, 1994.
5. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM, The nuclear receptor superfamily: The second decade. *Cell* **83:** 835–839, 1995.
6. Geber JF, Moghal N, Frangioni JV, Sugarbaker DJ and Neel BG, High frequency of retinoic acid receptor β abnormalities in human lung cancer. *Oncogene* **6:** 1859–1868, 1991.
7. Hu L, Crowe L, Rheinwald JG, Chambon P and Gudas L, Abnormal expression of retinoic acid receptors and keratin 19 by human oral and epidermal squamous cell carcinoma cell lines. *Cancer Res* **51:** 3972–3981, 1991.
8. Zhang X-K, Liu Y, Lee M-O and Pfahl M, A specific defect in

the retinoic acid response associated with human lung cancer cell lines. *Cancer Res* **54:** 5663–5669, 1994.
9. Liu Y, Lee M-O, Wang H-G, Li Y, Hashimoto Y, Klaus M, Reed JC and Zhang X-K, Retinoic acid receptor mediates the growth-inhibitory effect of retinoic acid by promoting apoptosis in human breast cancer cells. *Mol Cell Biol* **16:** 1138–1149, 1996.
10. Gavrieli Y, Sherman Y and Ben-Sasson SA, Identification of cell death *in situ* via specific labeling of nuclear DNA fragmentation *J Cell Biol* **119:** 493–501, 1992.
11. Shaw P, Bovey R, Tardy S, Sahli R, Sordat B and Costa J, Induction of apoptosis by wild-type p53 in a human colon tumor-derived cell line. *Proc Natl Acad Sci USA* **89:** 4495–4499, 1992.
12. Bedi A, Pasricha PJ, Akhtar AJ, Barber JP, Bedi GC, Giardiello FM, Zehnbauer BA, Hamilton SR, and Jones RJ, Inhibition of apoptosis during development of colorectal cancer. *Cancer Res* **55:** 1811–1816, 1995.
13. Kikuchi Y, Dinjens WN and Bosman FT, Proliferation and apoptosis in proliferative lesions of the colon and rectum. *Virchows Arch* **431:** 111–117, 1997.
14. Baretton GB, Diebold J and Christoforis G, Apoptosis and immunohistochemical bcl-2 expression in colorectal adenomas and carcinomas. Aspects of carcinogenesis and prognostic significance. *Cancer* **77:** 255–264, 1996.
15. Hague A, Manning AM, Hanlon KA, Huschtscha LI, Hart D and Paraskeva C, Sodium butyrate induces apoptosis in human colonic tumor cell lines in a p53-independent pathway: Implications for the possible role of dietary fiber in the prevention of large-bowel cancer. *Int J Cancer* **55:** 498–505, 1993.
16. Shchepotin IB, Soldatenkov V, Bruas RR, Nauta RJ, Shabahang M and Evans SR, Apoptosis of human primary and metastatic colon adenocarcinoma cell lines *in vitro* induced by 5-fluorouracil, verapamil, and hyperthermia. *Anticancer Res* **14:** 1027–1031, 1994.
17. Wang CY, Eshleman JR, Willson JK and Markowitz S, Both transforming growth factor-β and substrate release are inducers of apoptosis in a human colon adenoma cell line. *Cancer Res* **55:** 5101–5105, 1995.
18. Elder DJ, Hague A, Hicks DJ and Paraskeva C, Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: Enhanced apoptosis in carcinoma and *in vitro*-transformed adenoma relative to adenoma cell lines. *Cancer Res* **56:** 2273–2276, 1996.
19. Willett WC and Hunter DJ, Vitamin A and cancers of the breast, large bowel and prostate: Epidermiologic evidence. *Nutr Rev* **52:** S53–S59, 1994.
20. Stopera SA and Bird RP, Effects of all-*trans* retinoic acid as a potential chemopreventive agent on the formation of azoxymethane-induced aberrant crypt foci: differential expression of c-*myc* and c-*fos* mRNA and protein. *Int J Cancer* **53:** 798–804, 1993.
21. Kawamori T, Tanaka T, Hirose Y, Satoh K, Hara A, Torihara M, Tamai Y, Yamahara J and Mori H, Suppression of azoxymethane-induced colonic aberrant crypt foci by dietary exposure to a novel synthesized retinoidal butenolide, 5-hydroxy-4-(2-phenyl-(*E*)ethenyl-2(5*H*)-furanone, in rats. *Cancer Res* **92:** 159–165, 1995.
22. Kane KF, Langman MJ and Williams GR, Antiproliferative responses of two human colon cancer cell lines to vitamin D_3 are differentially modified by 9-*cis* retinoic acid. *Cancer Res* **56:** 623–632, 1996.
23. Stewart LV and Thomas ML, Retinoids differentially regulate the proliferation of colon cancer cell lines. *Exp Cell Res* **233:** 321–329, 1997.
24. Bruel G, Benoit G, De Nay D, Brown S and Lanotte M, Distinct apoptotic responses in maturation sensitive and resistant t (15;17) acute promyelocytic leukemia N84 cells. 9-*cis* retinoic acid induces apoptosis independent of maturation and Bcl-2 expression. *Leukemia* **9:** 1173–1184, 1995.
25. Piedrafita J and Pfahl M, Retinoid-induced apoptosis and Sp1 cleavage occur independently of transcription and require caspase activation. *Mol Cell Biol* **17:** 6348–6358, 1997.
26. McCormack SA, Viar MJ, Tague L and Johnson LR, Altered distribution of the nuclear receptor RAR beta accompanies proliferation and differentiation changes caused by retinoic acid in Caco-2 cells. *In Vitro Cell Dev Biol Anim* **32:** 53–61, 1996.
27. van der Burg B, van der Leede BM, Kwakkenbos-Isbrucker L, Salverda S, de laat SW and van der Saag PT, Retinoic acid resistance of estradiol-independent breast cancer cells coincides with diminished retinoic acid receptor function. *Mol Cell Endocrinol* **91:** 149–157, 1993.
28. Lotan R, Xu XC, Lippman SM, Ro JY, Lee JS, Lee JJ and Hong WK, Suppression of retinoic acid receptor-β in premalignant oral lesions and its up-regulation by isotretinoin. *N Eng J Med* **332:** 1405–1410, 1995.
29. Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner JJ, Guan XY, Sauter G, Kallioniemi OP, Trent JM and Meltzer PS, AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* **277:** 965–968, 1997.
30. Cote S and Momparler RL, Activation of the retinoic acid receptor β gene by 5-aza-2′-deoxycytidine in human DLD-1 colon carcinoma cells. *Anticancer Drugs* **8:** 56–61, 1997.
31. Ferrari N, Pfahl M and Levi G, Retinoic acid receptor γ1 ($RAR\gamma_1$) levels control $RAR\beta_2$ expression in SK-N-BE2(c) neuroblastoma cells and regulate a differentiation-apoptosis switch. *Mol Cell Biol* **18:** 6482–6492, 1998.
32. Hague A, Hicks MD, Chapman M and Paraskeva C, Bcl-2 expression in human colorectal adenomas and carcinomas. *Oncogene* **9:** 3367–3370, 1994.
33. Langlois NE, Lamb J, Eremin O and Heys SD, Apoptosis in colorectal carcinoma occurring in patients aged 45 years and under: Relationship to prognosis, mitosis, and immunohistochemical demonstration of p53, c-myc and bcl-2 protein products. *J Pathol* **182:** 392–397, 1997.
34. Kawasaki Y, Monden T, Morimoto H, Murotani M, Miyoshi Y, Kobayashi T, Shimano T and Mori T, Immunohistochemical study of p53 expression in microwave-fixed, paraffin-embedded sections of colorectal carcinoma and adenoma. *Am J Clin Pathol* **97:** 244–249, 1992.
35. Krajewska M, Moss SF, Krajewska S, Song K, Holt PR and Reed JC, Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. *Cancer Res* **56:** 2422–2427, 1996.
36. Tsujitani S, Shirai H, Tatebe S, Sugamura K, Ohfuji S, Gomyo Y, Maeta M, Ito H and Kaibara N, Apoptotic cell death and its relationship to carcinogenesis in colorectal carcinoma. *Cancer* **77**(Suppl 8): 1711–1716, 1996.
37. Sakao Y, Noro M, Sekin S, Nozue M, Hirohashi S, Itoh T and Noguchi M, Microsatellite instability and frameshift mutations in the Bax gene in hereditary nonpolyposis colorectal carcinoma. *Jpn J Cancer Res* **89:** 1020–1027, 1998.
38. Nita ME, Nagawa H, Tominaga O, Tsuno N, Fujii S, Sasaki S, Fu CG, Takenoue T, Tsuruo T and Muto T, 5-Fluorouracil induces apoptosis in human colon cancer cell lines with modulation of Bcl-2 family proteins. *Br J Cancer* **78:** 9986–9992, 1998.
39. Marcu KB, Bossone SA and Patel AJ, *myc* function and regulation. *Annu Rev Biochem* **61:** 809–860, 1992.
40. Shi Y, Glynn JM, Guilbert LJ, Cotter TG, Bissonnette RP and Green DR, Role for c-*myc* in activation-induced apoptotic cell death in T cell hybridomas. *Science* **257:** 212–214, 1992.

41. Klefstrom J, Vastrik I, Saksela E, Valle J, Eilers M and Alitalo K, c-*myc* induces cellular susceptibility to the cytotoxic action of TNF-α. *EMBO J* **13:** 5442–5450, 1994.
42. Dong J, Natio M and Tsuruo T, c-Myc plays a role in cellular susceptibility to death receptor-mediated and chemotherapy-induced apoptosis in human monocytic leukemia U937 cells. *Oncogene* **15:** 639–647, 1997.
43. Lewis BC, Shim H, Li Q, Wu CS, Lee LA, Maity A and Dang CV, Identification of putative c-*myc*-responsive genes: Characterization of *rcl*, a novel growth-related gene. *Mol Cell Biol* **17:** 4967–4978, 1997.
44. Hueber AO, Zornig M, Lyon D, Suda T, Nagata S and Evan GI, Requirement for the CD95 receptor-ligand pathway in c-*myc*-induced apoptosis. *Science* **278:** 1305–1309, 1997.