All trans-retinoic acid induces apoptosis via p38 and caspase pathways inmetaplastic Barrett’s cells

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Hormi-Carver K, Feagins LA, Spechler SJ, Souza RF. All trans-retinoic acid induces apoptosis via p38 and caspase pathways inmetaplastic Barrett’s cells. Am J Physiol Gastrointest Liver Physiol 292: G18–G27, 2007. First published August 24, 2006; doi:10.1152/ajpgi.00237.2006.—Retinoids such as all-trans-retinoic acid (ATRA) have been used as chemopreventive agents for a number of premalignant conditions. To explore a potential role for retinoids as chemopreventive agents for Barrett’s esophagus, we studied ATRA’s effects on apoptosis in a nonneoplastic, telomerase-immortalized,metaplastic Barrett’s cell line. We treated the Barrett’s cells withATRA in the presence and absence of inhibitors to p53 (pSRZ-siRNA-p53), p38 (SB-203580 and p38 siRNA), and the caspase cascade (z-Val-Ala-Asp-fluoromethyl ketone). We determined the effects of ATRA and the various inhibitors on apoptosis using cell morphology, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling staining, cleaved caspase-3 immunofluorescence, and Annexin V staining. We also determined how ATRA in the presence and absence of the inhibitors affected apoptosis following low-dose UV-B irradiation. ATRA induced apoptosis and increased the expression of p53 protein in a dose-dependent fashion. The apoptotic effect of ATRA was abolished by treatment with inhibitors of both p38 and caspase, but not by p53 interfering RNA (RNAi). Inhibition of p38 also prevented expression of cleaved caspase-3, suggesting that ATRA activates p38 upstream of the caspase cascade. We found that ATRA sensitized immortalized Barrett’s cells to apoptosis induced by low-dose UV-B irradiation via a similar mechanism. ATRA induces apoptosis in Barrett’s epithelial cells and sensitizes them to apoptosis induced by UV-B irradiation via activation of p38 and the caspase cascade, but not through p53. This study elucidates molecular pathways whereby retinoid treatment might prevent carcinogenesis in Barrett’s metaplasia and suggests a potential role for the use of safer retinoids for chemoprevention in Barrett’s esophagus.

Barrett’s esophagus; retinoids; caspase cascade; p53

THE INCIDENCE OF ESOPHAGEAL adenocarcinoma, one of the mostdeadly gastrointestinal malignancies, has increased more than sixfold over the past 30 years in the United States (18). The major risk factors for this tumor are gastroesophageal reflux disease and Barrett’s esophagus, the condition in which reflux-damaged, esophageal squamous epithelium is replaced by ametaplastic, intestinal-type epithelium. Esophageal adenocarcinoma develops in patients with Barrett’s esophagus at therate of ~0.5% per year (20). Clearly, an agent that could halt the progression from metaplasia to malignancy in Barrett’s esophagus would be highly desirable, but there is no agent with proven chemopreventive efficacy for this condition. 

There are data to suggest that retinoids, which are derived from vitamin A, might have chemopreventive effects inBarrett’s esophagus. Retinoids are known to play an important role in promoting differentiation and/or apoptosis in both normal and tumor cells (2, 4, 5, 13). Epidemiological studies suggest that deficiency of β-carotene, a vitamin A provitamin, increases the risk of esophageal adenocarcinoma (6, 12). A number of naturally occurring retinoids, including all-trans-retinoic acid (ATRA), 9-cis retinoic acid, and 13-cis retinoic acid have been used with variable success as chemopreventive and chemotherapeutic agents (2). However, systemic use of these natural retinoids has been limited by their toxicities at therapeutic doses. As a result, less toxic synthetic retinoids are being evaluated in vitro and in vivo as potential therapeutic agents (17, 21, 25).

The mechanisms underlying the chemopreventive and chemotherapy effects of the retinoids are not completelyunderstood (9). It is known that retinoic acid binds to retinoic acid receptor and to retinoid X receptor in the nucleus, forming receptor homo- and heterodimers that trigger the transcription of genes promoting cell growth arrest and differentiation (1). Natural and synthetic retinoids also have been shown to induce apoptosis independent of nuclear retinoid receptors by bothp53-dependent and p53-independent mechanisms in human non-small-cell lung carcinoma cells and in primary cultures of orbital fibroblasts from patients with Graves’ ophthalmopathy (16, 23, 24). Retinoid-induced apoptosis has been shown to be mediated by activation of the p38 mitogen-activated protein kinase (MAPK) and by the expression of caspases in normal human epidermal keratinocytes, human leukemia cells, spontaneously immortalized human keratinocytes (HaCaT) cells, and ovarian carcinoma cells (3, 9, 19, 26).

13-Cis retinoic acid was tried as a chemopreventive agent for Barrett’s esophagus in a phase II clinical trial, but the trial was stopped because the agent induced esophageal ulcerations (8). There have been no subsequent trials of retinoids for chemoprevention in Barrett’s esophagus, perhaps because offears that the agents are caustic to the esophagus. With the

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development of the less toxic synthetic derivatives of vitamin A, retinoids remain a promising class of chemopreventive agents. We used ATRA, which has been used successfully to treat promyelocytic leukemia, to explore a potential role for retinoids as chemopreventive agents for patients with Barrett’s esophagus. We exposed a novel, nonneoplastic, telomerase-immortalized, metaplastic Barrett’s epithelial cell line to ATRA and sought to determine its effects on apoptosis and the molecular mechanisms underlying this effect.

**MATERIALS AND METHODS**

**Cell lines.** A telomerase-immortalized, nonneoplastic Barrett’s epithelial cell line derived from biopsies taken from a patient with benign Barrett’s esophagus was cultured in keratinocyte basal media, KBM-2, (Cambrex Biologicals, Walkersville, MD) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 0.4 μg/ml hydrocortisone, 20 ng/ml recombinant epidermal growth factor, 20 μg/ml adenine, 5 μg/ml transferrin, 5 μg/ml insulin (all from Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin (Invitrogen, Grand Island, NY), 0.1 nM cholera toxin (Calbiochem, San Diego, CA), and 70 μg/ml bovine pituitary extract (Hammond Cell Technology, Windsor, CA) (10, 14). Before ATRA treatment or UV irradiation, subconfluent Barrett’s cells were placed in KBM-2 media prepared as above, but without serum, epidermal growth factor, and insulin overnight at 37°C and 5% CO2. Cellular morphology was documented using the Metamorph imaging system (Universal Imaging, Downingtown, PA).

**UV-B irradiation.** Genomic damage caused by UV-B irradiation is a well-established inducer of apoptosis and p53 expression (7). We used UV-B irradiation to assess whether our telomerase-immortalized Barrett’s cells maintain the ability to undergo apoptosis and to express functional p53. UV-B irradiation was carried out with a homemade box built with four ultraviolet-B bulbs (280–320 nm wavelength, Philips Lighting, Peabody, MA). The applied UV-B dose was measured with a radiometer (IL-1400A Radiometer, International/Photometer). Equally seeded wells of Barrett’s cells at 70% confluence were irradiated with 50, 100, 200, and 400 J/m2 of UV-B. In preliminary experiments, we determined that UV-B irradiation in doses greater than 50 J/m2 had significant growth inhibitory effects on our Barrett’s cells. On the basis of these preliminary findings, we selected to investigate further the effects of UV-B irradiation within this dosage range.

**ATRA.** Equally seeded wells of Barrett’s cells at 70% confluence were exposed to all-trans-retinoic acid (ATRA) at concentrations ranging from 0–5 μM (Sigma, St. Louis, MO). In preliminary experiments, we determined that concentrations of ATRA less than 1 μM had no significant effect on growth of our Barrett’s cells. On the basis of these preliminary studies, we investigated further the effects of 1 and 5 μM ATRA.

**Treatment time points for ATRA, UV-B irradiation, or both.** In all experiments combining both ATRA treatment and UV-B irradiation, equally seeded wells of Barrett’s cells at 70% confluence were first exposed to media containing either 1 or 5 μM ATRA for 24 h, after which the ATRA-containing medium was removed and the cells were then irradiated with either 50 or 100 J/m2 of UV-B. Fresh medium without ATRA was then added for an additional 24 h. The cells were then harvested for analysis after a total of 48 h. We maintained the same experimental procedure for cells treated only with ATRA or only irradiated with UV-B so that all treatment groups underwent two media changes before the cells being harvested for analysis after a total of 48 h. For ATRA treatment alone, equally seeded wells of Barrett’s cells at 70% confluence were exposed to media containing either 1 or 5 μM ATRA for 24 h, after which the ATRA-containing medium was removed and replaced with medium without ATRA for an additional 24 h. The cells were then harvested for analysis after a
Barrett’s cells at 70% confluence underwent a media change to fresh non-ATRA-containing medium for 24 h, after which the medium was harvested after a total of 48 h. For UV-B irradiation alone, equally seeded wells of Barrett’s cells at 70% confluence underwent a media change to fresh non-ATRA-containing medium for 24 h, after which the medium was harvested by trypsinization, stained with Annexin V-FITC and 50 μg/ml of propidium iodide (PI), then immediately analyzed by flow cytometry (FACScaliber, Becton Dickinson, Franklin Lakes, NJ). To distinguish between cells in early stages of apoptosis and necrotic or late-stage apoptotic cells, staining with Annexin V-FITC was combined with PI staining. Cells in the early stage of apoptosis were negative for PI but stained with Annexin V-FITC, whereas dead cells and late-stage apoptotic cells stained for both PI and Annexin V-FITC. Experiments were performed in triplicate, and a total of 10,000 cells were analyzed in each individual experiment. We considered apoptotic cells as those cells staining positively with Annexin V-FITC but not with PI (early-stage apoptosis). The number of cells staining for Annexin V-FITC was expressed as a percentage of the total number of cells (10,000) analyzed in each individual experiment. For the TUNEL and cleaved caspase 3 immunofluorescence assays, cells were fixed with 4% paraformaldehyde for 15 min. The sections were washed, and incubated overnight at 4°C in a appropriate antibody incubation for 1 h in 0.1 M PBS, 2% BSA, and 0.3% Triton X-100, washed, and incubated overnight at 4°C in a appropriate antibody dilution followed by a 1:100 dilution of goat anti-mouse, or rabbit anti-goat IgG FITC-conjugated secondary antibody (Sigma). Specificity was determined by omitting either the primary or secondary antibody from the incubation.

**Detection of apoptosis.** Apoptosis rates were assessed quantitatively by use of Annexin V (BD Biosciences, San Diego, CA) and qualitatively by immunofluorescence with terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL; Roche, Indianapolis, IN) per the manufacturer’s instructions. Briefly, cells were harvested by trypsinization, stained with Annexin V-FITC and 50 μg/ml of propidium iodide (PI), then immediately analyzed by flow cytometry (FACScaliber, Becton Dickinson, Franklin Lakes, NJ). To distinguish between cells in early stages of apoptosis and necrotic or late-stage apoptotic cells, staining with Annexin V-FITC was combined with PI staining. Cells in the early stage of apoptosis were negative for PI but stained with Annexin V-FITC, whereas dead cells and late-stage apoptotic cells stained for both PI and Annexin V-FITC. Experiments were performed in triplicate, and a total of 10,000 cells were analyzed in each individual experiment. We considered apoptotic cells as those cells staining positively with Annexin V-FITC but not with PI (early-stage apoptosis). The number of cells staining for Annexin V-FITC was expressed as a percentage of the total number of cells (10,000) analyzed in each individual experiment. For the TUNEL and cleaved caspase 3 immunofluorescence assays, cells were fixed with 4% paraformaldehyde for 15 min. The sections were washed, and incubated overnight at 4°C in a appropriate antibody dilution followed by a 1:100 dilution of goat anti-mouse, or rabbit anti-goat IgG FITC-conjugated secondary antibody (Sigma). Specificity was determined by omitting either the primary or secondary antibody from the incubation.

**Western blotting.** The cells were harvested by scraping into extraction buffer that contained 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 7.4), and protease inhibitors. Equal amounts of protein were separated by SDS-PAGE; protein concentrations were determined by using the BCA-200 protein assay kit (Pierce, Rockford, IL). After separation and transfer to nitrocellulose membrane, membranes were then incubated with 1:1,000 dilutions of mouse monoclonal anti-human p53 (Cell Signaling, Beverly, MA) or rabbit polyclonal antibodies against cleaved caspase-3 or the phosphorylated form of p38 and total p38 (Cell Signaling). Horseradish peroxidase secondary antibody (Cell Signaling) was used at 1:2,000, and chemiluminescence was determined by using the ECL detection system (Pierce, Rockford, IL). At least three independent studies were performed. Membranes were then stripped by using Restore stripping buffer (Pierce) and were reprobed with β-actin (Sigma) or with total p38 (Cell Signaling) to confirm equal loading. Relative band intensities were determined by densitometry using the MultiAnalyst software package (Bio-Rad, Hercules, CA).

**Caspase and p38 MAPK inhibitors.** The caspase pathway was blocked by using the pan-caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone (z-VAD-fmk, Alexis, Carlsbad, CA) dissolved in DMSO and used at 60 μmol/l. The p38 pathway was blocked by using SB-203580 (Calbiochem, San Diego, CA) as previously described and using siGenome SMART selection-designed small interfering RNA (siRNA) duplexes against p38 (MAPK14, Dharmacon, RNA technologies, Lafayette, CO) (22). Equally seeded wells of Barrett’s cells were transiently transfected with 120 nM siRNA using LipofectAMINE PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. As controls, cells were transfected
with double-stranded RNA oligonucleotides designed against laminin A/C (synthesized at the RNA Oligonucleotide Synthesis Core facility, University of Texas Southwestern Medical Center) or treated with LipofectAMINE PLUS reagent alone. Cells were pretreated with z-VAD or SB-203580 for 1 h and then treated with ATRA alone or in combination with UV-B. Forty-eight hours after transfection with p38 siRNA, cells were treated with ATRA alone or in combination with UV-B.

*p53 RNAi infection.* Barrett’s cells were infected with viral medium containing either pSRZ-siRNA-p53 or the empty vector pSRZ (generous gift of Dr. John Minna, University of Texas Southwestern Medical Center) after addition of 4 mg/ml of Polybrene (Sigma). Forty-eight hours after the infection, cells were treated with ATRA alone or in combination with UV-B.

*p53 Protein expression by flow cytometry.* Barrett’s cells infected with viral media containing either pSRZ-siRNA-p53 or the empty vector pSRZ were harvested by trypsinization and stained with a 1:100 dilution of p53 antibody (Cell Signaling) in PBS with 2% BSA at 4°C for 30 min. Cells were then washed in PBS, and FITC-conjugated secondary antibody diluted 1:50 in 2% BSA was added at 4°C for 45 min. Cells were washed again in PBS and detected by flow cytometry.

Statistical analyses. The data were collected from at least three independent experiments. Quantitative data are expressed as means ± SE. Statistical analysis was performed by using ANOVA and the Student-Newman-Keuls multiple-comparison test with the Instat for Windows statistical software package (GraphPad Software, San Diego, CA). *P* values <0.05 were considered significant for all analyses.

RESULTS

UV-B irradiation induces apoptosis and p53 protein expression in a dose-dependent fashion in Barrett’s epithelial cells. Barrett’s epithelial cells were irradiated with UV-B at doses of 50, 100, 200, and 400 J/m², and apoptosis was assessed by morphology, TUNEL, cleaved caspase 3, and Annexin V. We found no difference in morphology and only a few cells stained for TUNEL and cleaved caspase 3 in both irradiated and nonirradiated Barrett’s cells at either 50 or 100 J/m² compared with untreated controls (Fig. 1). In contrast, cells irradiated with either 200 or 400 J/m² were small and shrunken, a morphology that suggests an apoptotic phenotype and demonstrated marked staining for TUNEL and cleaved caspase 3 (Fig. 1). Annexin V staining also showed a dose-dependent increase in apoptosis with UV-B irradiation (Fig. 2A). These findings show that UV-B irradiation at 200 and 400 J/m², but not at 50 or 100 J/m², induces apoptosis in metaplastic Barrett’s cells.

UV-B irradiation is known to induce both apoptosis and p53 protein expression. Therefore, we used Western blotting to
evaluate p53 protein expression in response to UV-B irradiation. Compared with the nonirradiated controls, p53 expression increased in a dose-dependent manner following UV-B irradiation (Fig. 2B). These findings demonstrate that UV-B irradiation causes metaplastic Barrett’s cells to undergo apoptosis and to increase p53 expression in a dose-dependent fashion.

**ATRA induces apoptosis and p53 protein expression in a dose-dependent fashion in Barrett’s epithelial cells.** Barrett's epithelial cells were cultured in the presence of 1 or 5 μM ATRA, and apoptosis was assessed by morphology, TUNEL, cleaved caspase 3, and Annexin V. Following either dose of ATRA, the Barrett’s cells exhibited substantial morphological abnormalities including the protrusion of elongated spikes from the cell surface and loss of cell-cell contact (Fig. 3). TUNEL and cleaved caspase 3 staining demonstrated few apoptotic cells in the control, but many in the ATRA-treated groups (Fig. 3). Annexin staining confirmed that ATRA treatment caused a dose-dependent increase in apoptosis (Fig. 2C).

Fig. 4. ATRA sensitized Barrett’s cells to the apoptotic effects of UV-B irradiation. A: results of 24 h of ATRA treatment before low-dose UV-B irradiation on apoptosis as determined by Annexin V staining. The histograms depict the mean ± SE of at least 3 individual experiments. *P < 0.05 compared with controls; +P < 0.05 compared with ATRA alone. B: representative Western blot demonstrating an increase in p53 protein expression following low-dose UV-B irradiation in cells pretreated with 24 h of ATRA; β-actin served as a loading control.

Fig. 5. ATRA alone or in combination with UV-B activated the caspase cascade. Representative Western blots demonstrating an increase in the active, cleaved form of caspase-3 protein following treatment with ATRA alone for 24 h (A) and in combination with UV-B (B). C: UV-B alone did not increase cleaved caspase-3 expression. Hela is used as positive control for cleaved caspase-3; β-actin was used as a loading control; C, control Barrett’s cells.
Western blotting showed a dose-dependent increase in p53 protein expression following ATRA treatment (Fig. 2D).

ATRA sensitizes Barrett’s cells to the apoptotic effects of UV-B irradiation. Like our Barrett’s cells, human keratinocytes have been shown to resist the apoptotic effects of low-dose UV-B irradiation (<200 J/m²) (7). After treatment with ATRA, however, even low-dose UV-B irradiation induces apoptosis in human keratinocytes (15). Noting those data, we treated our Barrett’s cells with either 1 or 5 μM ATRA followed by irradiation with either 50 or 100 J/m² of UV-B (doses that did not induce apoptosis in our untreated Barrett’s cells; see Figs. 1 and 2A). Annexin V staining demonstrated a marked increase in p53 protein expression following low-dose UV-B irradiation in cells treated with ATRA alone (Fig. 4B). These data show that treatment with ATRA sensitizes Barrett’s cells to apoptosis induced by irradiation with 100 J/m² of UV-B.

ATRA induces apoptosis via activation of the caspase cascade. We next sought to determine whether the caspase cascade is involved in apoptosis induced by ATRA alone or in combination with UV-B. We performed Western blotting to analyze the expression of cleaved caspase-3 in Barrett’s cells after treatment with ATRA 1 or 5 μM alone or in combination with UV-B; Barrett’s cells treated with 50 and 100 J/m² of UV-B alone served as a negative control. Similar to our findings using the immunofluorescence assay for cleaved caspase-3, Western blotting demonstrated the presence of cleaved caspase-3 protein in cells treated with ATRA alone (Fig. 5A) and in combination with UV-B (Fig. 5B). Low-dose UV-B irradiation alone did not induce cleaved caspase-3 protein expression (Fig. 5C). These data suggest that the caspase cascade is activated in apoptosis induced by ATRA alone and in combination with low-dose UV-B.

Inhibition of p53 does not prevent ATRA-induced apoptosis. We next sought to determine whether apoptosis induced by ATRA alone or in combination with UV-B is dependent on p53. We exposed metastatic Barrett’s cells to virus-containing
medium produced from 293T cells transfected with either the viral vector pSRZ-siRNA-p53 or an empty vector pSRZ as a control. To determine the efficiency of p53 inhibition by viral infection with pSRZ-siRNA-p53, Western blotting for p53 was performed in the presence and absence of ATRA. We found complete absence of p53 expression in metaplastic Barrett’s cells following infection with pSRZ-siRNA-p53 at baseline and after treatment with 1 or 5 μM ATRA (Fig. 6A). These findings were confirmed by flow cytometry analysis for p53 expression (Fig. 6B).

Following infection with pSRZ-siRNA-p53, Barrett’s cells were again exposed to 24 h of 1 and 5 μM ATRA alone or in combination with 100 J/m² UV-B; apoptosis was assessed by cell morphology and Annexin V. Untransfected cells and cells transfected with the empty vector pSRZ served as controls. Infection with p53 siRNA did not alter basal rates of apoptosis or rates of apoptosis following irradiation with 100 J/m² as determined by Annexin V staining (Fig. 7). Moreover, there was no effect of p53 inhibition by siRNA on rates of apoptosis induced by 1 μM ATRA alone or in combination with 100 J/m² UV-B by cell morphology (data not shown) or by Annexin V (Fig. 7). These data suggest that apoptosis induced by ATRA alone or in combination with low-dose UV-B is independent of p53.

**ATRA activation of the p38 MAPK pathway induces apoptosis.** A variety of proapoptotic stimuli, including the synthetic retinoid CD437, have been shown to exert their apoptotic effects via activation of p38 MAPK (3, 9, 26). Therefore, we determined whether ATRA induced apoptosis in metaplastic Barrett’s cells via activation of p38. We performed Western blotting for active, phosphorylated p38 in cells treated with ATRA alone or in combination with UV-B. For controls we used untreated cells and cells exposed to low-dose UV-B alone. We found that ATRA alone or in combination with UV-B markedly increased levels of phosphorylated p38 relative to nontreated controls (Fig. 8, A and B). We also found an increase in p38 levels in Barrett’s cells irradiated with 50 or 100 J/m² compared with nonirradiated controls (Fig. 8C).

**We then treated the cells with the p38 inhibitor SB-203580 and determined the rates of apoptosis following treatment with**

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**Fig. 7.** Inhibition of p53 did not prevent ATRA-induced apoptosis. Results of UV-B irradiation, ATRA treatment, or both and p53 RNAi on apoptosis as determined by Annexin V staining. Note that inhibition of p53 does not block apoptosis induced by 24 h of ATRA treatment alone or in combination with UV-B. The histograms depict the mean ± SD of at least 2 individual experiments. (*P < 0.05 compared with controls.)

**Fig. 8.** Western blots for p38 phosphorylation. p38 Phosphorylation increased with ATRA alone, ATRA in combination with UV-B, and UV-B alone. A: p38 phosphorylation increased in Barrett’s cells treated with 1 or 5 μM ATRA for 24 h. B: p38 phosphorylation increased in cells pretreated with 24 h of ATRA in combination with 50 or 100 J/m² UV-B irradiation. C: irradiation with 50 or 100 J/m² UV-B alone increases phosphorylation of p38. Total p38 and β-actin were used as loading controls.
found following transfection of double-stranded laminin A/C RNA or following treatment with LipofectAMINE PLUS reagent alone (data not shown). We found that p38 siRNA had no effect on basal rates of apoptosis but significantly decreased apoptosis induced by 1 μM ATRA alone or in combination with UV-B compared with untreated controls (Fig. 9B); transfection with double-stranded laminin A/C RNA oligonucleotides or treatment with LipofectAMINE PLUS reagent alone had no effect on apoptosis at baseline or following treatment with ATRA alone or in combination with UV-B (data not shown). These data suggest that p38 activation mediates apoptosis induced by ATRA alone or in combination with low-dose UV-B.

**Activation of p38 MAPK occurs upstream of caspase activation in ATRA-mediated apoptosis.** Activation of p38 can occur either upstream or downstream of caspase activation during apoptosis (3, 11). To determine where caspase activation occurs following treatment with ATRA alone or in combination with UV-B, metaplastic Barrett’s cells were treated with either the caspase inhibitor Z-VAD-fmk or the p38 inhibitor SB-203580, and the effects on p38 activation and cleaved caspase-3 expression were determined by Western blotting. We found that Z-VAD-fmk did not inhibit p38 phosphorylation following treatment with ATRA alone or in combination with UV-B (Fig. 10A). However, treatment with SB-203580 eliminated expression of cleaved caspase-3 induced by both ATRA alone and in combination with low-dose UV-B (Fig. 10B). These data suggest that p38 MAPK activation occurs upstream of the caspase cascade following treatment with ATRA alone or in combination with low-dose UV-B in metaplastic Barrett’s cells.

**Fig. 9. Inhibition of p38 prevented ATRA-induced apoptosis. A:** Western blots for p38 following p38 RNAi infection. Note that p38 protein expression is inhibited following transfection with p38 RNAi at baseline and after treatment with either 1 or 5 μM ATRA for 24 h. **B:** results of UV-B irradiation, ATRA treatment, and p38 siRNA on apoptosis as determined by Annexin V staining. Note that inhibition of p38 blocks apoptosis induced by 24 h of ATRA treatment alone and in combination with UV-B. The histograms depict the mean ± SD of at least 2 individual experiments. (*P < 0.05 compared with controls.)

1 or 5 μM ATRA alone or in combination with low-dose UV-B. The effect of SB-203580 on inhibiting apoptosis induced by 1 μM or 5 μM ATRA alone or in combination with low-dose UV-B was apparent by cell morphology (data not shown). Using Annexin V, we determined the effect of SB-203580 on the rate of apoptosis in cells treated with 1 μM or 5 μM ATRA alone or in combination with low-dose UV-B. We found that SB-203580 significantly decreased apoptosis induced by 1 μM or 5 μM ATRA alone or in combination with UV-B compared with untreated controls (data not shown). There was no effect of SB-203580 on basal rates of apoptosis or rates of apoptosis following treatment with 50 or 100 J/m² UV-B (data not shown).

We next confirmed our findings by transfecting the cells with p38 siRNA oligonucleotides and determining the rates of apoptosis following treatment with 1 μM ATRA alone or in combination with low-dose UV-B using Annexin V. To determine the efficiency of p38 siRNA on inhibiting p38 expression, Western blotting was performed in the presence and absence of ATRA. We found a marked decrease in p38 expression in metaplastic Barrett’s cells following transfection with the siRNA at baseline and after treatment with 1 or 5 μM ATRA (Fig. 9A). No decrease in ATRA-induced p38 expression was
DISCUSSION

The rapidly increasing incidence of esophageal adenocarcinoma has led to an interest in identifying chemopreventive agents to prevent this deadly tumor. Since the majority of these tumors arise from Barrett’s esophagus, agents that halt the neoplastic progression of the metaplastic, intestinal-type epithelium would be desirable. Since epidemiological data suggest that deficiency of β-carotene, a vitamin A provitamin, increases the risk of esophageal adenocarcinoma, we sought to determine whether retinoids, which are derivatives of vitamin A, would have a role as chemopreventive agents for patients with Barrett’s esophagus (6, 12). We selected to study the effects of the naturally occurring retinoid ATRA, a representative agent of this class of compounds, because it has been used successfully to treat patients with promyelocytic leukemia (2). We found that ATRA induces apoptosis in a dose-dependent fashion in a novel, nonneoplastic, telomerase-immortalized, metaplastic Barrett’s cell line. To our knowledge, this is the first study demonstrating that ATRA induces apoptosis in the metaplastic cells of Barrett’s esophagus.

Retinoids have been shown to induce apoptosis by both p53-dependent and p53-independent mechanisms in human non-small-cell lung carcinoma cells and in primary cultures of orbital fibroblasts from patients with Graves’ ophthalmopathy (16, 23, 24). Using UV-B irradiation, we found that our telomerase-immortalized Barrett’s cells maintain the ability to undergo apoptosis and to express functional p53. To determine whether ATRA-induced apoptosis is p53-dependent in Barrett’s metaplastic cells, we infected the cells with a retroviral vector containing p53 interfering RNA (RNAi). We found no difference in the level of apoptosis between cells infected with the p53 RNAi and empty-vector control cells after ATRA treatment, suggesting that p53 is not necessary for ATRA-mediated apoptosis in metaplastic Barrett’s cells.

A number of proapoptotic agents have been shown to exert their effects via activation of the p38 MAPK (3, 9, 26). Therefore, we explored whether p38 mediated ATRA-induced apoptosis in our metaplastic Barrett’s cells. Cells were treated with the p38 inhibitor SB-203580 or with p38 siRNA before treatment with ATRA. We found that inhibition of p38 blocked apoptosis, suggesting that the p38 MAPK pathway mediates ATRA-induced apoptosis in Barrett’s metaplastic cells. During the mediation of apoptosis, activation of p38 can either precede or follow activation of the caspase cascade. For example, the activation of p38 and UV-B-induced apoptosis were blocked by treatment with the pan-caspase inhibitor Z-VAD-fmk in HaCaT cells, indicating that p38 activation occurred downstream of caspase activation (3). In Jurkat T cells, treatment with Z-VAD-fmk also inhibited p38 activation during receptor-mediated apoptosis (11). However, p38 activation was not blocked by Z-VAD-fmk in Jurkat T cells during chemically induced apoptosis, suggesting that activation of p38 occurred upstream of the caspase cascade (11). These observations show that, although different stimuli ultimately may activate the same components of a proapoptotic pathway, the sequence in which those components are activated proceeds in a stimulus-and cell type-specific manner. In our Barrett’s cells, therefore, we studied both the effects of the pan-caspase inhibitor Z-VAD-fmk on p38 activation and the effects of SB-203580 on cleaved caspase-3 expression. We found that Z-VAD-fmk did not inhibit p38 phosphorylation following exposure to ATRA, whereas treatment with SB-203580 eliminated ATRA-induced expression of cleaved caspase-3. These data suggest that, during ATRA-induced apoptosis in Barrett’s metaplastic cells, p38 MAPK activation occurs upstream of the caspase cascade.

Treatment with ATRA has been shown to sensitize human keratinocytes to apoptosis following low-dose UV-B irradiation (15). Therefore, we treated our Barrett’s cells with ATRA before exposing them to low doses of UV-B irradiation that, without ATRA, did not affect the basal rate of apoptosis. We found that, following irradiation with 100 J/m² of UV-B, both the 1 μM and 5 μM doses of ATRA produced significantly more apoptosis than that seen in cells treated with either dose of ATRA alone. Moreover, we found that the mechanism underlying apoptosis induced by the combination of ATRA and UV-B was similar to that of ATRA alone, involving activation of the p38 MAPK upstream of the caspase cascade and independent of p53.

In conclusion, we have shown that ATRA induces apoptosis and increases the expression of p53 protein in a dose-dependent fashion in a nonneoplastic, telomerase-immortalized, metaplastic Barrett’s cell line. The apoptotic effect of ATRA is abolished by treatment with inhibitors of both p38 and caspase, but not by p53 RNAi. Moreover, we have found that ATRA activates the p38 MAPK upstream of the caspase cascade and that treatment with ATRA sensitizes Barrett’s cells to UV-B-induced apoptosis via a similar mechanism. To our knowledge, this is the first study demonstrating that ATRA induces apoptosis in the metaplastic cells of Barrett’s esophagus, and we have elucidated molecular pathways whereby retinoid treatment might prevent carcinogenesis in this condition. Although the known toxicity of ATRA may preclude its use as a chemopreventive agent, our findings suggest a potential role for the use of safer retinoids for chemoprevention in Barrett’s esophagus.

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REFERENCES