Retinol Inhibits the Growth of All-Trans-Retinoic Acid–Sensitive and All-Trans-Retinoic Acid–Resistant Colon Cancer Cells through a Retinoic Acid Receptor–Independent Mechanism

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Abstract
Retinol (vitamin A) is thought to exert its effects through the actions of its metabolite, all-trans-retinoic acid (ATRA), on gene transcription mediated by retinoic acid receptors (RAR) and retinoic acid response elements (RARE). However, retinoic acid resistance limits the chemotherapeutic potential of ATRA. We examined the ability of retinol to inhibit the growth of ATRA-sensitive (HCT-15) and ATRA-resistant (HCT-116, SW620, and WiDR) human colon cancer cell lines. Retinol inhibited cell growth in a dose-responsive manner. Retinol was not metabolized to ATRA or any bioactive retinoid in two of the cell lines examined. HCT-116 and WiDR cells converted a small amount of retinol to ATRA; however, this amount of ATRA was unable to inhibit cell growth. To show that retinol was not inducing RARE-mediated transcription, each cell line was transfected with pRARE-chloramphenicol acetyltransferase (CAT) and treated with ATRA and retinol. Although treatment with ATRA increased CAT activity 5-fold in ATRA-sensitive cells, retinol treatment did not increase CAT activity in any cell line examined. To show that growth inhibition due to retinol was ATRA, RAR, and RARE independent, a pan-RAR antagonist was used to block RAR signaling. Retinol-induced growth inhibition was not alleviated by the RAR antagonist in any cell line, but the antagonist alleviated ATRA-induced growth inhibition of HCT-15 cells. Retinol did not induce apoptosis, differentiation or necrosis, but affected cell cycle progression. Our data show that retinol acts through a novel, RAR-independent mechanism to inhibit colon cancer cell growth. (Cancer Res 2005; 65(21): 9923-33)

Introduction
Colorectal cancer is currently the third leading cause of death due to cancer in the United States. Retinoids, a group of compounds consisting of vitamin A (retinol), its natural metabolites, and several synthetic compounds, have been shown to act as cancer chemopreventive agents (for reviews, see refs. 1–3). In rats, retinol, 9-cis-retinoic acid, and 4-(hydroxyphenyl)retinamide can inhibit the formation of carcinoogenic-induced aberrant crypt foci, a precursor to colon cancer, as well as colon tumors (4–7). Retinyl palmitate was recently shown to inhibit high-fat diet–induced aberrant crypt foci (7). Additionally, several in vitro studies indicate that retinoids have potent antiproliferative effects on colon cancer cell lines and may hold potential for both chemoprevention and chemotherapy of colon cancer.

In almost all of the above studies, the retinoid examined has been an isomer of retinoic acid or a synthetic retinoid such as 4-(hydroxyphenyl)retinamide. Although these compounds are effective at inhibiting all-trans-retinoic acid (ATRA)–sensitive cell growth, the use of exogenous ATRA to study the effects of vitamin A assumes that all of the biological phenomena attributed to retinol are due to ATRA. The diet contains very little ATRA (8). Rather, the diet contains vitamin A in two forms: (1) previtamin A carotenoids and (2) preformed vitamin A as retinol and retinyl esters. Retinyl esters are cleaved within the intestinal lumen to yield retinol. Therefore, human colonocytes are exposed primarily to retinol, the focus of this study. Within most cells, retinol is either esterified for storage or metabolized to ATRA. ATRA exerts its effects on cell growth and differentiation by binding to retinoic acid receptors (RAR) located in cell nuclei. RARs heterodimerize with retinoid X receptors (RXR) and bind to retinoic acid response elements (RARE) located in the regulatory regions of retinoid-responsive genes. When ATRA binds to the RAR member of the RAR/RXR heterodimer, gene transcription via RARE is induced (for review, see ref. 2).

Retinoic acid resistance is believed to be due to a defect in RARα, RARβ, or RARγ induction in response to ATRA (for review, see refs. 2, 9–11). Retinoic acid resistance occurs when tumors or tumor-derived cell lines cease to growth inhibit or differentiate in response to treatment with ATRA. Retinoic acid resistance is a common phenomenon and appears to arise spontaneously in numerous types of cancer and tumor-derived cell lines. The defective receptor varies with cell line but RARβ expression is frequently lost.

The objective of the present study was to determine if retinol could inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cell lines in vitro. Because the ATRA-resistant cell lines lack one or more RARs, their use allowed us to determine the effects of retinol on cell growth, exclusive of the effects of ATRA. Our data show that retinol itself inhibits the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells through an ATRA- and RAR-independent mechanism.

Materials and Methods

Cell culture. Three human colorectal adenoma cell lines, HCT-15, SW620, and WiDR, and one human colon carcinoma cell line, HCT-116, were obtained from the American Type Culture Collection (Manassas, VA) and grown as recommended. HCT-15 cells were grown in MEM, HCT-116 in McCoy’s medium, and SW620 and WiDR cells in DMEM in a humidified
atmosphere at 37°C with 5% CO2. All medium was supplemented with 10% FCS and antibiotics (1,000 units/mL penicillin and 1,000 μg/mL streptomycin). The experiment was repeated with each cell line grown in either supplemented DMEM or McCoy’s medium. Medium type did not affect cell growth. Cells were seeded in 12-well culture dishes at a density of 1 × 10^4 cells per well. The following day, the medium was removed and replaced with medium containing 0, 0.1, 1, or 10 μM ATRA or all-trans-retinol (Sigma, St. Louis, MO). All retinoids were prepared as 10 mmol/L stocks in 100% ethanol. All treatments, including control, received equal volumes of ethanol vehicle and all retinoid manipulations were performed in the dark. All treatments were done in duplicate. Cells were harvested using trypsin and counted via hemocytometer every 24 hours for 4 days.

Retinoid extraction and high-performance liquid chromatography analysis. To examine retinol metabolism, cells were seeded in 60 mm dishes at the following densities to yield 60% to 80% confluence and maximum high-performance liquid chromatography (HPLC) detection sensitivity at the time of harvest: 5 × 10^5 cells/dish for 24 hours, 2.5 × 10^5 cells/dish for 48 hours, 1 × 10^5 cells/dish for 72 hours, and 5 × 10^5 cells/dish for 96 hours. Twenty-four hours after plating, cells were treated with 0, 1, and 10 μM retinol for 24, 48, 72, or 96 hours. Sixteen hours before harvest, the culture medium was removed and replaced with medium containing 5% FCS and 50 nmol/L [3H]retinol (specific activity = 52.5 Ci/mmol). Cells and medium were harvested 2, 4, 8, and 16 hours after the addition of label as described previously (12). A control of labeling medium without cells was also incubated for 16 hours. F9 murine teratocarcinoma cells, treated with 1 μM ATRA for 48 hours and incubated with 50 nmol/L [3H]retinol for 16 hours, were used as a positive control for 4-oxotretinoin production (13). Retinoids were extracted and separated using a Waters Millennium HPLC system as described previously (14).

Cell transfection and chloramphenicol acetyltransferase assays. To examine the possibility that an undetected metabolite of retinol was activating RAR/RXR-mediated transcription, all cell lines were transiently transfected with pRARE-chloramphenicol acetyltransferase (CAT; generously provided by Dr. Dianne Soprano). Cells were seeded onto 12-well plates at a density of 1.75 × 10^5 cells/well and incubated overnight in FCS-supplemented medium. The following day, cells were transfected using LipofectAMINE 2000 (Promega, Madison, WI) according to the protocol of the manufacturer with 1 μg of pRARE-CAT and 0.5 μg of pSV-β-gal. Twenty-four hours later, the transfection medium was removed and the cells were treated with fresh medium containing 0, 1, and 10 μM ATRA or retinol. The cells were harvested after treatment for 24 or 48 hours and assayed for β-galactosidase (β-Galactosidase Enzyme Assay System; Promega) and CAT (CAT Enzyme Assay System; Promega) activity as per instructions of the manufacturer. CAT activity was corrected for transfection efficiency using the β-galactosidase activity.

Reptinoic acid receptor antagonist assays. To determine if retinol was inhibiting cell growth via RAR, the pan-RAR-agonist, AGN 193109 was used to block RAR function. The RAR pan-agonist was synthesized by Allergan, Inc. (Irvine, CA). Cell lines were plated at a density of 1 × 10^5 in 12-well plates and allowed to attach overnight. The following day, HCT-15 cells were treated with 0 and 1 μM ATRA or retinol with and without 10 μM/AGN 193109. HCT-116, SW620, and WIDR cells were treated with 0 and 1 μM retinol with and without 10 μM/AGN 193109. Control cells received an equal volume of DMSO and ethanol vehicle. Cells were harvested after treatment for 48 hours (HCT-15) or 96 hours (HCT-116, SW620, and WIDR). All treatments were done in duplicate. The pharmacologic, 10 μM/L, concentrations of ATRA and retinol were not examined because 100 μM/AGN 193109 was toxic to the cells.

Detection of apoptosis. Nuclear staining via D,3-diaminobenino-2-phenylindole (DAPI) and flow cytometry analysis, described below, were used to determine if cell growth inhibition was due to apoptosis. For DAPI staining, cells were plated at 1 × 10^5 cells per well in 12-well plates before treatment with 0, 1, and 10 μM retinol. Cells incubated for 4 hours at 37°C with 4 μg/mL camptothecin served as the positive control for apoptosis. Both adherent and floating cells were harvested every 24 hours for 4 days. The cells were centrifuged and washed with PBS to remove all traces of media. Cells were then incubated with 2 μg/mL DAPI for 10 minutes at 37°C before counting at 400× magnification with an Olympus upright fluorescence microscope. To obtain cell counts, at least three different locations on each slide were used. Two hundred cells were counted at each location yielding a minimum of 600 cells counted per slide. Cells with segmented nuclei were scored as apoptotic.

Cellular differentiation. Alkaline phosphatase activity was used to determine if retinol was inhibiting cell growth by inducing cellular differentiation. All cell lines were plated on 60 mm dishes at a density of 5 × 10^5 cells per plate. Twenty-four hours later, cells were treated with 0, 1, and 10 μM retinol or 2 mmol/L sodium butyrate (positive control) for 96 hours. Alkaline phosphatase activity was determined as described previously (15). Alkaline phosphatase activity was measured by the conversion of p-nitrophenyl phosphate (19.8 mmol/L) to p-nitrophenol by 0.1 mL of cell lysate in 100 mmol/L glycine buffer, containing 1 mmol/L MgCl2 (pH 10). Alkaline phosphatase enzyme activity was corrected for lysate protein content and expressed as percentage positive control.

Necrosis assays. Trypan blue exclusion assays were used to measure cell death. Briefly, an aliquot of the cells harvested for the growth curve assays was pelleted by centrifugation, resuspended in 0.5 mL HBSS, and incubated with an equal volume of 0.4% trypan blue solution (Sigma-Aldrich, St. Louis, MO) for 5 minutes at room temperature before counting with a hemocytometer. Blue cells were scored as necrotic.

Flow cytometry analysis. To determine if growth inhibition was due to cell cycle arrest and to confirm the absence of apoptosis through lack of a sub-G1 peak, cells were seeded on 60 mm dishes at a density of 3 × 10^5 (HCT15 and SW620) or 2 × 10^5 (HCT-116 and WIDR) cells per dish to provide 50% to 60% confluence at the time of harvest. To synchronize, cells were plated in serum-free medium for 24 hours and treated with 1 (HCT-15) or 3 μg/mL (HCT-116, SW620, WIDR) aphidicolin for an additional 24 hours. The following day, the cells were washed with PBS and treated with fresh FCS-supplemented media containing 0, 1, and 10 μM/L retinol. Cells were harvested, fixed in 70% ethanol overnight, and stained with 40 μg/mL propidium iodide as described previously (16). At least 10,000 cells were analyzed per sample using a FACScalibur machine (Becton Dickinson, San Jose, CA). DNA content was determined using ModFit software version 3.0 (Verity Software House, Inc., Topsham, ME).

Results

Growth of all-trans-retinoic acid–resistant colon cancer cells is inhibited by retinol. The ability of retinol to inhibit cell growth was examined in three ATRA-resistant human colon cancer cell lines HCT-116 (17), SW620 (18), and WIDR (11). An ATRA-sensitive cell line, HCT-15, was chosen to serve as a positive control for the inhibitory effects of ATRA on colon cancer cell growth (19). Serum concentrations of retinol range from 0.5 to 2 μmol/L (20). Therefore, 0.1 μmol/L was selected to represent a subphysiologic, and 1 μmol/L a physiologic, concentration of retinol. The highest level, 10 μmol/L retinol, was used as a pharmacologic, but potentially therapeutically relevant, concentration. There is very little ATRA (4-14 nmol/L) in the serum (21, 22). ATRA levels were chosen to match the concentrations of retinol used and to reflect ATRA levels commonly found in the literature (9–11, 23).

After 96 hours of treatment, the growth of HCT-15 cells was inhibited by ATRA (Fig. 1A) as expected. In addition, HCT-15 cell growth was also inhibited by retinol in a dose-responsive manner. Cells treated with 10 μmol/L retinol exhibited the largest degree of inhibition to 36.7 ± 7.8% of control. HCT-116 cell growth was inhibited slightly by 0.1 and 1 μmol/L retinol but this decrease was not significant when compared with the same concentrations of ATRA (Fig. 1B). However, HCT-116 cell growth was significantly inhibited by 10 μmol/L retinol (37.5 ± 9.2% of control) when compared with 10 μmol/L ATRA (74.3 ± 4.7% of control), SW620 and WIDR cell growth was significantly inhibited by treatment with 0.1 and 1 μmol/L retinol for 96 hours when compared with ATRA.
Retinol inhibits the growth of ATRA-resistant human colon cancer cells. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDR (D) cells were seeded and treated with 0, 0.1, 1, or 10 μmol/L ATRA or retinol. All treatments were performed in duplicate. Cells were counted via hemocytometer daily for 4 days. Columns, mean for three experiments; bars, SE. Left, percentage growth inhibition exhibited by human colon cancer cell lines after 96 hours of treatment with increasing amounts of ATRA or retinol. Statistical analysis was done using t tests comparing ATRA to retinol for each concentration. *Significantly different from ATRA, P < 0.05. Right, growth rates of HCT-15 (A), HCT-116 (B), SW620 (C), and WiDR (D) cells grown for 4 days with increasing amounts of retinol.
(Fig. 1C and D), indicating that physiologic levels of retinol can inhibit the growth of ATRA-resistant cells. At 10 μmol/L concentrations, there was no significant difference in the ability of ATRA and retinol to inhibit SW620 and WiDR cell growth. The highest concentration of ATRA, 10 μmol/L, inhibited cell growth slightly in all cell lines examined (Fig. 1). These data show that retinol can inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells.

Retinol is not metabolized to bioactive compounds. To determine if retinol was metabolized to a bioactive compound,
The amount or [3H]ATRA synthesized from [3H]retinol by WiDR slightly by 0.1 or 1.2. As can be seen in Fig. 1B retinol for 96 hours and included in the experiment shown in Fig. 2. However, both control and retinol-treated cells metabolized [3H]retinol to [3H]ATRA. The time point displayed in Fig. 2 (48 hours of retinol treatment followed 8 hours of incubation with 50 nmol/L [3H]ATRA) showed the largest concentration of 50 nmol/L [3H]ATRA synthesis by HCT-116 and WiDR cells of any time point examined. When corrected for cell number, HCT-116 cells treated with the vehicle control synthesized 0.15 nmol/L [3H]ATRA/million cells from 50 nmol/L [3H]retinol. Cells treated with 10 μmol/L retinol synthesized 0.55 nmol/L [3H]ATRA/million cells from 50 nmol/L [3H]retinol. Because the metabolism of 50 nmol/L [3H]retinol reflects the metabolism of 10 μmol/L retinol (12, 14, 24), we can assume that if HCT-116 cells were treated with 10 μmol/L retinol, 0.11 μmol/L of ATRA would be produced per million cells. There were 5.4 × 10^5 cells on a duplicate plate of HCT-116 cells treated with 10 μmol/L retinol for 96 hours and included in the experiment shown in Fig. 2. As can be seen in Fig. 1B, HCT-116 cell growth is inhibited only slightly by 0.1 or 1 μmol/L ATRA when compared with control. The amount of [3H]ATRA synthesized from [3H]retinol by WiDR cells was even less than that synthesized by the HCT-116 cell line. Therefore, the small amount of ATRA produced by these cell lines when treated with 10 μmol/L retinol cannot be responsible for the decrease in cell number that occurs when these cells are treated with 10 μmol/L retinol.

Retinol does not induce retinoic acid response element-chloramphenicol acetyltransferase reporter gene expression. To confirm that retinol was not metabolized to a bioactive compound that could transactivate RARE-mediated gene transcription, each colon cancer cell line was transiently transfected with pRARE-CAT and treated with ATRA or retinol. The pRARE-CAT construct contains only the nucleotides corresponding to the RARE found in the regulatory region of the RARβ2 gene linked to a CAT promoter.

Figure 3A shows that treatment of ATRA-sensitive HCT-15 cells with both 1 and 10 μmol/L ATRA for 24 or 48 hours resulted in an increase in CAT activity to 4.98 ± 0.39-fold over control at 48 hours for cells treated with 10 μmol/L ATRA. Because HCT-15 cells were ATRA sensitive, we were surprised to find that treatment of HCT-15 cells with retinol did not increase CAT activity to >1.80 ± 0.06-fold over control at 48 hours for cells treated with 1 μmol/L retinol (Fig. 3A). However, this lack of CAT activity reflects the metabolism data (Fig. 2A-D), showing that HCT-15 cells do not metabolize retinol to ATRA.

Neither ATRA nor retinol increased CAT activity >1.7-fold over control in any of the three ATRA-resistant colon cancer cell lines (Fig. 3B-D). The lack of CAT activity in cells treated with ATRA confirms the inability of these cells to respond to ATRA via RAR/RARE-mediated mechanisms as described previously (11, 17, 18). Although the HCT-116 cells converted a small amount of [3H]retinol to [3H]ATRA, these cells lack RAR (17). The absence of an increase in CAT activity in response to retinol treatment in the HCT-116 and WiDR cell lines shows that the small amount of ATRA produced by these cells does not induce RAR/RARE-mediated gene transcription. SW620 cells did not metabolize [3H]retinol to [3H]ATRA (Fig. 2) and the lack of CAT activity in SW620 cells when treated with retinol both confirms the metabolism data and shows that an RAR-activating metabolite of retinol is either not present or is incapable of activating RAR/RARE-mediated gene transcription. In summary, the inability of retinol to increase CAT activity in any of the cell lines examined, including ATRA-sensitive HCT-15 cells, shows that retinol is not inducing retinoic acid–mediated gene transcription, confirming our metabolism data, and indicating that retinol may be acting exclusive of the RAR to inhibit colon cancer cell growth.

Retinol is not acting through retinoic acid receptors to inhibit cell growth. To confirm that the growth inhibition exhibited by cells treated with retinol was not mediated by the retinoic acid/RAR/RARE retinoid signaling mechanism, all cell lines were treated with a RAR pan-antagonist, AGN 193109. This antagonist, when added at 10 times the concentration of agonist, blocks the ability of agonist to bind to RAR (25). HCT-15 cells treated with 1 μmol/L ATRA and 10 μmol/L AGN 193109 served as a positive control for the ability of AGN 193109 to block RAR-mediated cell growth inhibition. Because 1 μmol/L ATRA does not inhibit HCT-116, SW620, or WiDR cell growth, we did not test the effects of the combined treatment of AGN 193109 and ATRA in these cell lines. As shown in Fig. 4A, AGN 193109 blocked ATRA-induced growth inhibition in HCT-15 cells, as expected. However, AGN 193109 did not block growth inhibition due to retinol treatment in any of the four cell lines examined, including the ATRA-sensitive HCT-15 cell line (Fig. 4). The inability of AGN 193109 to block retinol-induced growth inhibition confirms the results of the metabolism and RARE reporter experiments, which also indicate that retinol is not acting via retinoic acid/RAR/RARE signaling mechanisms.
to affect cell growth even in the ATRA-sensitive, HCT-15 cell line. Unlike the ATRA-resistant cell lines, HCT-15 cells contain all of the cellular machinery required for induction of ATRA/RAR/RARE-mediated gene transcription and growth inhibition (11). As shown in Fig. 4A, ATRA is acting via this mechanism to inhibit the growth of HCT-15 cells. In contrast, retinol is acting via a novel, receptor-independent mechanism to inhibit the growth of both ATRA-resistant and, surprisingly, ATRA-sensitive human colon cancer cell lines.

Figure 3. Retinol does not induce RARE-CAT reporter gene expression. HCT-15 (A), HCT-116 (B), SW620 (C), and WGR (D) cells were transiently transfected with 1 µg pRARE-CAT and 0.5 µg pSV-β-gal using LipofectAMINE 2000. Twenty-four hours following transfection, cells were treated with 0, 1, and 10 µmol/L ATRA or retinol. Cells were harvested 24 and 48 hours after treatment and CAT and β-galactosidase assays were done. CAT activity was normalized for transfection efficiency using the β-galactosidase activity. The CAT activity in control cells treated with the ethanol vehicle was set equal to one and all other values are expressed as fold induction. Columns, mean of three separate experiments; bars, SE.

Retinol does not induce apoptosis, differentiation, or necrosis in all-trans-retinoic acid–resistant colon cancer cells.

To determine the mode by which retinol inhibits the growth of colon cancer cells, apoptosis was examined by nuclear staining using DAPI (Fig. 5, left) and fluorescence-activated cell sorting (FACS) analysis of DNA content (Fig. 6). The percentage of DAPI-stained cells exhibiting segmented nuclei was <10% in all cell lines at all time points and treatments examined. FACS analysis failed to detect a sub-G1 peak in any of the cell lines when treated with
retinol, confirming the absence of apoptosis (Fig. 6). In addition, in all cell lines, <4% apoptosis was detected with TUNEL assay and no apoptosis was detected by poly(ADP)-ribose polymerase (PARP) cleavage or DNA laddering (data not shown). Therefore, retinol does not inhibit colon cancer cell growth by inducing apoptosis.

Alkaline phosphatase assays were performed to determine if retinol was inhibiting colon cancer cell growth by inducing cellular differentiation. Retinol does not induce alkaline phosphatase activity in HCT-116, SW620, or WiDr cells (Fig. 5, right). Retinol increased alkaline phosphatase activity slightly in HCT-15 cells (Fig. 5A). In contrast, treatment with sodium butyrate resulted in a large increase in alkaline phosphatase activity in each cell line. These data indicate that retinol is not inhibiting cell growth by inducing cellular differentiation in the three ATRA-resistant cell lines. A small increase in alkaline phosphatase activity in HCT-15 cells treated with retinol may indicate that cellular differentiation accounts for part of the retinol-induced decrease in growth.

To ensure that retinol was not inducing necrosis, trypan blue dye exclusion assays were performed on the adherent cells used for the growth curve experiments described in Fig. 1. The percentage of cells that stained with trypan blue dye varied between 0.1% and 7% and no consistent pattern was exhibited under any treatment condition at any time point (data not shown). Therefore, necrosis is not responsible for the growth inhibition exhibited by colon cancer cells treated with retinol.

Retinol affects cell cycle progression. Treatment with 10 μmol/L retinol increased the percentage of cells in G0-G1 while decreasing the percentage of cells in S phase in the HCT-15, SW620, and WiDr cell lines (Fig. 6A, C, and D). Treatment with retinol decreased the percentage of HCT-15 cells in G2-M, slightly increased the percentage of HCT-116 and SW620 cells in G2-M, and notably increased the percentage of WiDr cells in G2-M. In contrast, the percentage of HCT-116 cells in G0-G1 was not affected by retinol (Fig. 6B), but retinol decreased the percentage of HCT-116 cells in S phase. As can be seen in Fig. 1D, control HCT-116 cells continued to divide in a linear manner, whereas HCT-116 cells treated with 10 μmol/L retinol ceased to divide between 24 and 48 hours of treatment. This result, when considered in light of the absence of apoptosis, differentiation, and necrosis in the HCT-116 cell line despite strong growth inhibition by retinol, may indicate that retinol acts to slow the overall rate of cell division and increase the generation time of this cell line.

**Discussion**

This study shows that retinol inhibits the growth of both ATRA-sensitive and ATRA-resistant human colon cancer cell lines. We provide three lines of evidence that retinol is acting independent of the established ATRA/RAR/RARE retinoid signaling pathway. The first line of evidence indicates that retinol is not metabolized to bioactive compounds, such as ATRA, in two of the four cell lines examined. The remaining two cell lines synthesized only small amounts of ATRA from retinol. Second, we show that retinol does not activate RARE-mediated gene transcription. Finally, we present evidence that a RAR antagonist blocks the ability of ATRA to inhibit the growth of ATRA-sensitive HCT-15 cells, as expected, but does not block the ability of retinol to inhibit the growth of any cell line examined. The most surprising outcome of this study is that retinol is not acting through a RAR-dependent pathway in ATRA-sensitive HCT-15 cells. Therefore, even in the presence of functioning RAR, retinol does not inhibit cell growth by the actions of its metabolite ATRA, because this metabolite is not present in ATRA-sensitive HCT-15 cells (Fig. 2).
The ability of retinol to inhibit colon cancer cell growth is particularly interesting given that colon cancer cell lines produce little or no ATRA (Fig. 2). This finding is supported in a recent study by Jette et al. (26) that used Northern blot analysis to show that colon cancer cell lines, including HCT-116, lack retinol dehydrogenases 5 and L, and therefore the ability to synthesize ATRA. The metabolism of retinol by colon cancer cells was not examined in the study by Jette et al. (26). In contrast, our data shows that the HCT-116 cell line is capable of synthesizing very small amounts of ATRA from retinol (Fig. 2). This discrepancy is perhaps due to the ability of HPLC to detect extremely small amounts of [3H]retinoids compared with the relative lack of sensitivity of Northern blot analysis.

4-Oxoretinol and anhydroretinol are two naturally occurring retinoids capable of inhibiting cell growth. 4-Oxoretinol acts via RARs (13), much like ATRA, whereas anhydroretinol acts via a receptor-independent cytosolic mechanism to inhibit cell growth.

Figure 5. Retinol does not induce apoptosis or cellular differentiation in ATRA-resistant human colon cancer cell lines. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDR (D) cells were plated and treated with 0, 1, or 10 μmol/L retinol as described in Materials and Methods. To measure apoptosis (left), floating and adherent cells were harvested every 24 hours, centrifuged, and washed with PBS to remove all traces of media. Cells were stained with 2 μg/mL DAPI for 10 minutes in 37°C before observation. To measure cellular differentiation (right), cells were plated as described and treated with 0, 1, or 10 μmol/L retinol or 2 mmol/L sodium butyrate for 96 hours. Alkaline phosphatase activity was determined as described (15) by the conversion of p-nitrophenyl phosphate to p-nitrophenol at 410 nm. Alkaline phosphatase enzyme activity is expressed as percentage positive control. Columns, mean for three independent experiments; bars, SE.
Neither compound was formed from retinol by any of the cell lines we examined (Fig. 2). We cannot eliminate the possibility that an unknown bioactive metabolite of retinol was formed that existed only briefly or was not detected by our HPLC protocol. However, the inability of retinol to induce CAT activity in cells transfected with a pRARE-CAT construct (Fig. 3) as well as the inability of a pan-RAR antagonist to block the effects of retinol on cell growth (Fig. 4) support the metabolism data.

We chose to use the pan-RAR antagonist, AGN 193109, to block RARs because this compound exhibits a high affinity for RAR (25).

Figure 6. Retinol alters cell cycle progression but does not induce apoptosis. HCT-15 (A), HCT-116 (B), SW620 (C), and WiDR (D) cells were synchronized with serum starvation for a total of 48 hours and the addition of aphidicolin during the last 24 hours of serum starvation. Following synchronization, fresh medium containing 10% FCS and 0 (left) or 10 μmol/L retinol (right) was added for 24 hours before fixation and staining with propidium iodide. At least 10,000 cells were analyzed per sample using a FACSCalibur machine. Absence of a sub–G₀-G₁ indicates that apoptosis was not induced by retinol treatment. Dark gray, G₀-G₁; and G₂-M hatched, S phase. One representative experiment of two is shown.
Although a genetic approach would have been more specific, the dominant-negative RAR construct available is activated by retinol (29), making it inappropriate for this study. Therefore, we included a positive control, showing that AGN 193109 blocks ATRA-induced growth inhibition in the HCT-15, ATRA-sensitive cell line (Fig. 3A), to indicate that AGN 193109 is functioning to block RAR-mediated growth inhibition.

Retinoids have been previously shown to inhibit cancer cell growth by increasing cellular differentiation, inducing apoptosis, or causing cell cycle arrest. With respect to colon cancer, retinoids tend to induce tumor apoptosis both in vitro (11, 30, 31) and in vivo (4, 32, 33). The cell lines examined in this study showed no apoptosis in response to retinoid treatment (Figs. 5, left, and 6). In contrast, retinol induced G0-G1 arrest in three of the cell lines examined (Fig. 6). Although retinol failed to increase the percentage of cells in G0-G1 in the HCT-116 cell line, growth inhibition in these cells could be due to an overall increase in generation time because retinol does decrease cell growth (Fig. 1B). The differing responses between the HCT-116 cell line and the other three may reflect the heterogeneity of these cell lines, tumor stage (carcinoma versus adenoma), and presence or absence of various proteins in each cell line, e.g., adenomatous polyposis coli or p53.

Retinoids tend to induce cell cycle arrest by blocking the G1-S phase transition (for review, see ref. 1). Unfortunately, the effect of retinoids on cell cycle regulatory proteins seems to be cell type specific (1). For example, in carcinogen-exposed immortalized human bronchial epithelial cells, ATRA-induced G1 arrest is associated with decreased cyclin D1 protein levels due to ubiquitin-mediated degradation of cyclin D1 (34, 35). In contrast, ATRA-induced G1 arrest in MCF-7 breast cancer cells is associated with decreased pRB phosphorylation, whereas cyclin D1, p21WAF1/CIP1, cdk4, and cdk6 activity either does not change or with decreased pRB phosphorylation, whereas cyclin D1, p21WAF1/CIP1, cdk4, and cdk6 activity either does not change or decreases slightly, depending on the study (36–38).

This study shows that retinol is acting independent of the RAR to inhibit colon cancer cell growth. Previously, retinoids have been shown to exert their receptor-independent effects via interactions with protein kinase Cα (39), F-actin (40), c-Raf kinase (28), regulating mitochondrial membrane potential (41), generating reactive oxygen species (42), increasing intracellular ceramide levels (43), activating c-Jun NH2-terminal kinase (44), inducing ubiquitin-dependent proteolysis (34, 35), and affecting mitogen-activated protein kinase (45, 46), phosphatidylinositol 3-kinase (PI3K)/Akt (47), and epidermal growth factor receptor signaling (48). The Hammerling lab has shown that the retinoid family, retinol, and ATRA can bind protein kinase Cα and affect its redox activation (39). In contrast to our present study, they speculate that retinol antagonizes anhydroretinol and increases cell survival by binding to c-Raf and augmenting its response to reactive oxygen species generated during UV irradiation; however, the link between cell growth and c-Raf activation was not directly examined (28).

Because anhydroretinol induces apoptosis, we do not expect retinol to affect cell growth by interacting with c-Raf or any of the other pathways listed above that induce apoptosis.

In conclusion, this study shows that retinol acts through a novel mechanism to inhibit the growth of both ATRA-sensitive and ATRA-resistant colon cancer cells by affecting cell cycle progression. Resistance to ATRA is a common phenomenon and limits the use of retinoic acid derivatives as chemotherapy. We speculate that retinol, or a derivative of it, may prove an effective therapy to treat colorectal cancer.

References


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Retinol Inhibits the Growth of All-Trans-Retinoic Acid–Sensitive and All-Trans-Retinoic Acid–Resistant Colon Cancer Cells through a Retinoic Acid Receptor–Independent Mechanism

Eun Young Park, Alice Dillard, Elizabeth A. Williams, et al.


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