Differential Regulation of Apoptosis in Normal *versus* Transformed Mammary Epithelium by Lutein and Retinoic Acid[1]

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Abstract

We examined the effects of all-*trans* retinoic acid (ATRA) and lutein (a nonprovitamin A carotenoid), on apoptosis and chemosensitivity in primary normal human mammary epithelial cells, SV40 transformed mammary cells, and MCF-7 human mammary carcinoma cells. ATRA and lutein selectively induced apoptosis in transformed but not normal human mammary cells. In addition, both compounds protected normal cells, but not transformed cells, from apoptosis induced by the chemotherapy agents etoposide and cisplatin. Furthermore, lutein and ATRA selectively increased the ratio of Bcl-xL:Bax protein expression in normal cells but not transformed mammary cells, suggesting a possible mechanism for selective modulation of apoptosis. The differential effects of lutein and ATRA on apoptotic pathways in normal *versus* transformed mammary epithelial cells may have important implications for chemoprevention and therapy.

Introduction

There have been a number of investigations demonstrating the chemoprotective effects of selected retinoids and carotenoids. This has been demonstrated in animal tumor models as well as in epidemiological studies in humans (1–4). The molecular mechanisms responsible for these protective effects are unclear. However, one mechanism suggested for effects of retinoids on carcinogenesis involves the ability of these compounds to induce apoptosis or programmed cell death in epithelial tumor cells (5, 6). All-*trans* retinoic acid has been shown to induce significant levels of apoptosis in breast, ovarian, and squamous carcinoma cells and can sensitize tumor cells to chemotherapy-induced apoptosis (5–8). Retinoids given at the high concentrations necessary to achieve this effect, however, are toxic (5, 6). There have been fewer studies investigating the effects of retinoids and carotenoids on normal breast epithelium. This is

of considerable importance, because the therapeutic potential of these compounds depends on their selectivity in normal *versus* transformed cells.

Of the 600 known carotenoids, only 10% function as vitamin A precursors in mammals (9). Therefore, understanding the role of nonprovitamin A carotenoids in cancer chemoprevention is important. Although β-carotene and the nonprovitamin A carotenoid canthaxanthin have been shown to inhibit mammary carcinogenesis in mouse and rat models (1–4), the molecular mechanisms of their chemopreventive action remain unknown. Thus far, the suggested mechanisms of anticancer action of carotenoids include singlet oxygen quenching, immunoenhancement, protection against cellular mutagenesis, and up-regulation of specific connexins (gap junction proteins; Refs. 10–12). Thus, there is growing evidence suggesting that the chemopreventive properties of carotenoids are independent of the antioxidant activity of these compounds (13).

Lutein is a nonprovitamin A carotenoid found in broccoli and spinach. Lutein has chemopreventive activity in mouse models of murine breast and colon cancers (14, 15). It has been suggested that lutein, as well as another carotenoid, zeaxanthin, can account for part of the decreased breast cancer risk for women on high vegetable and fiber diets (2–4). Dietary lutein was associated with decreased breast cancer risk and estrogen receptor-positive status in premenopausal disease (16).

We examined the effects of both a retinoid, ATRA,[3] and lutein on modulation of apoptotic pathways in normal human mammary epithelial cells as well as similar cells transformed with SV40. The latter cells have inactivated *p53* and *pRB* proteins (17). We also examined the effects of ATRA and lutein on MCF-7 cells. These cells are a fully transformed human mammary carcinoma cell line with wild-type *p53* and high levels of Bcl-2 (18). In this study, we demonstrate the differential effects of these compounds on apoptosis and chemosensitivity in normal *versus* transformed mammary cells. In addition, we examined the effects of these compounds on expression of members of the Bcl-2 protein family. We report that ATRA and lutein have differential effects on the apoptotic threshold of normal *versus* transformed mammary cells and are associated with changes in expression of *bcl-2* family members. These experiments have important implications for understanding the role of ATRA and lutein in chemoprevention and for developing strategies to increase the therapeutic index of cancer treatments.

Materials and Methods

Reagents. ATRA and lutein were purchased from Sigma Chemical Co. (St. Louis, MO) and Kemin Industries (Des

[3] The abbreviations used are: ATRA, all-*trans* retinoic acid; THF, tetrahydrofuran; MSU-1, Michigan State University-1 medium; SRF, serum replacement factor; PI, propidium iodide; CDDP, cisplatin.
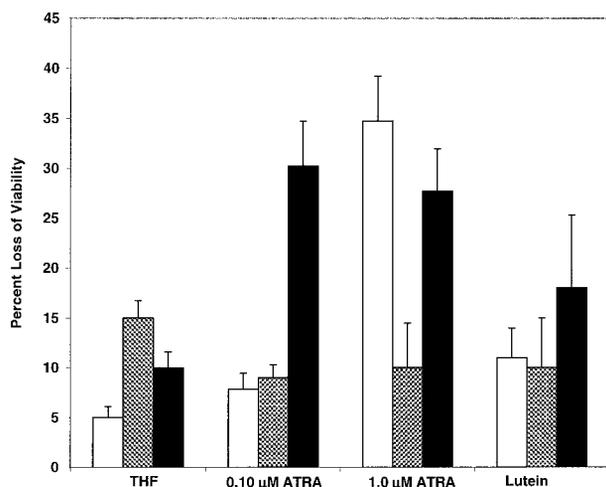
Fig. 1. Effects of ATRA and lutein on viability of normal and transformed mammary cells. Cell viability was measured on day 4 for each cell type. The graph shows the percentage of viable cells in ATRA- or lutein-treated samples with respect to the THF control for a given cell type. ATRA (1.0 μM) significantly decreased the viability of normal mammary cells with respect to the THF control (35% decrease; $n = 6$; $P = 0.005$). Both ATRA concentrations caused a significant (30%) decrease in viability of MCF-7 tumor cells ($n = 6$, $P = 0.001$ for 0.10 μM; and $n = 3$, $P = 0.004$ for 1.0 μM). Lutein induced a significant decrease (17%) in viability of MCF-7 tumor cells ($n = 6$, $P = 0.002$). □, normal cells; ▨, SV40-transformed cells; ■, MCF-7 tumor cells. *Bars*, SD.

Table 1 Effects of lutein and ATRA on apoptosis in normal *versus* transformed human mammary epithelial cells

The normal cells, SV40-transformed cells, and MCF-7 tumor cells were grown in the presence of ATRA or lutein for 4 days. Apoptosis was quantitated after staining cells with PI. For each cell type, the values for the percentage of PI-positive cells should be compared with the corresponding value in the control (THF) sample. No significant increase in PI staining was observed in normal mammary cells treated with ATRA or lutein. In SV40-transformed cells, only ATRA (1.0 μM) significantly increased staining ($n = 2$; $P = 0.028$). In MCF-7 tumor cells, PI staining was significantly increased by ATRA ($n = 3$; $P = 0.038$ for both concentrations) and lutein ($n = 3$; $P = 0.016$) relative to that in the control (THF).

	% of cells stained with PI ($\pm$SD)		
Sample	Normal mammary cells	SV40-transformed cells	MCF-7 breast cancer cells
THF	3.90 (1.00)	13.00 (2.00)	5.20 (1.10)
0.10 μM ATRA	4.80 (0.50)	26.50 (4.50)	16.10 (2.90)
1.0 μM ATRA	4.40 (0.30)	21.50 (0.50)	16.00 (3.00)
Lutein	5.30 (1.00)	19.50 (7.50)	13.60 (1.05)

Moines, IA), respectively. Each compound was dissolved in THF (99.9%; Aldrich Chemicals, Milwaukee, WI; Ref. 15), which was stored under N_2 gas and sealed to prevent peroxide formation. Crystalline ATRA and lutein were stored in the dark at $-20°$C. Lutein was used at a final concentration of 7 μM, based on levels reported in sera of human subjects (19, 20). ATRA was used at final concentrations of 0.10 and 1.0 μM. Stock solutions of ATRA and lutein (500-fold in THF) were prepared for each experiment such that the final THF concentration in lutein/ATRA-treated samples was 0.20%. Control samples were treated with THF alone (0.20%).

Cell Culture. Normal human mammary epithelial cells were derived from reduction mammoplasties as described (21). Normal mammary cells were cultured in MSU-1 medium + 5% fetal bovine serum (21). For experiments, cells were switched to MSU-1 medium without serum but including SRFs. These factors include: human recombinant epidermal growth factor (0.5 ng/ml), β-estradiol (10^{-8} M), insulin (5 μg/ml), hydrocortisone (0.50 μg/ml), thyroid hormone (T3 at 2×10^{-8} M), and human transferrin (5 μg/ml). SV40 transformed cells are counterparts of normal cells transfected with SV40 DNA and are grown in MSU-1-SRF medium + cholera toxin (1 ng/ml; Sigma) and bovine pituitary extract (0.40%; Pel Freez, AR). MCF-7 tumor cells were cultured in DMEM + 5%, fetal bovine serum, and 10 μg/ml insulin.

For experiments, the three cell types were seeded at 2×10^4/well in 24-well plates, respectively, in MSU-1-SRF media. Each cell type was treated with THF, lutein, or ATRA for 4 days. Medium with and without compounds was replaced on day 2. Cell viability was measured on days 2 and 4 by trypan blue exclusion/Coulter counter. Both methods gave similar results. For chemotherapy experiments, cells were pretreated with ATRA or lutein for 3 days, followed by treatment (24 h) with and without the drugs in the presence of fresh THF, lutein, or ATRA.

Measurement of Apoptosis. Apoptosis was assessed by monitoring nuclear morphology after staining cells with PI, as described (22). The Cell Death ELISA was performed as described (23).

Western Blotting. After 4 days of treatment with and without THF, lutein, or ATRA, total lysates were prepared from attached and detached cells of each cell type. The Bradford assay was used to quantitate total protein within each sample. For normal cells, 20 μg of total protein of each sample were loaded per lane. For tumor cells, 50 μg of total protein from each sample were loaded per lane. Selected blots were reprobed with an actin antibody to ensure equal loading of lanes.

Western blot analysis was done as described (23), with antibodies to human Bcl-2, Bcl-xL, or Bax. The mouse monoclonal Bcl-2 antibody and Rabbit polyclonal antibodies against human-Bcl-x were from Dako Corp. (Carpinteria, CA) and Transduction Laboratories (Lexington, KY), respectively. The mouse monoclonal antibody against human Bax and the goat antimouse/antirabbit antibodies conjugated to horseradish peroxidase were from PharMingen (San Diego, CA) and Ameresco Corp. (Solon, OH), respectively. A chemiluminescence kit (Amersham, Arlington Heights, IL) was used to visualize protein bands. In each experiment, three X-ray film exposures (10 s to 2 min) of the same blot were scanned to calculate expression levels of Bcl-xL, Bcl-2, or Bax proteins.

Statistical Analysis. Significance of the effects of ATRA or lutein in the three cell types were analyzed by the students unpaired t test using a confidence level of 95% ($P = 0.05$). Results of t tests are included in the figure legends.

RESULTS

Effects of ATRA and Lutein on Growth and Apoptosis in Normal and Transformed Human Mammary Epithelial Cells. Studies have demonstrated that ATRA can induce apoptosis in transformed mammary cells (5–7). To determine whether this is also the case for the nonprovitamin A carotenoid, lutein, and to compare the effects of these compounds on normal *versus* transformed cells, we investigated the effects of ATRA and lutein on growth and apoptosis in normal human mammary epithelial cells and similar cells transformed with SV40. We compared these effects to a fully transformed human mammary carcinoma cell line, MCF-7.

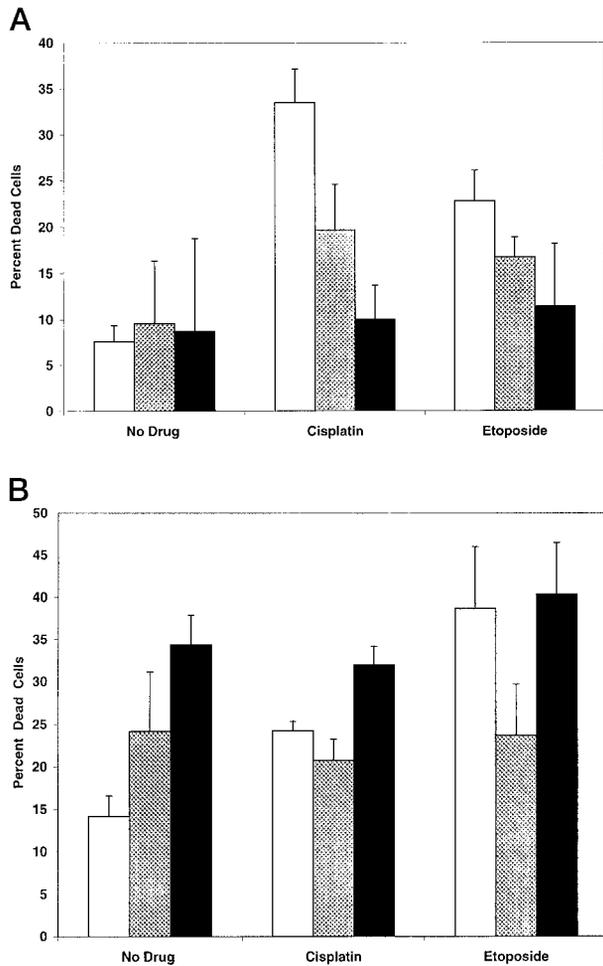ATRA reportedly binds serum proteins and has different

Fig. 2. *A,* ATRA and lutein protect normal mammary cells from chemotherapy-induced cell death. Normal mammary cells were pretreated with THF, ATRA, or lutein for 3 days. The cells were then treated for an additional 24 h with CDDP (50 μM) or etoposide (100 μg/ml) in the presence of fresh THF, ATRA, or lutein, and the percentage of viability was determined. In the presence of the control solvent THF, etoposide and CDDP each induced significant cell death [($n = 5$, $P = 0.001$) for each drug]. In the presence of lutein, etoposide and CDDP did not induce significant death in normal cells (lutein + etoposide *versus* lutein alone: $n = 5$, $P = 0.460$) and (lutein + CDDP *versus* lutein alone: $n = 5$, $P = 0.500$). ATRA partially protected normal cells from apoptosis induced by etoposide ($n = 4$, $P = 0.030$ for ATRA + etoposide *versus* ATRA alone) and CDDP ($n = 4$, $P = 0.002$ for ATRA + CDDP *versus* ATRA alone). *Bars,* SD. *B,* ATRA and lutein do not alter chemosensitivity in MCF-7 breast cancer cells. MCF-7 tumor cells were treated with THF, ATRA, or lutein in the presence or absence of etoposide or CDDP as described in *A.* Each agent induced a significant increase in dead cell number in the presence of THF ($n = 3$, $P = 0.005$ for etoposide; and $n = 3$, $P = 0.003$ for CDDP). In the absence of drugs, lutein alone caused a significant increase in cell death ($n = 5$, $P = 0.001$). ATRA also induced significant cell death in MCF-7 cells ($n = 5$, $P = 0.017$). Neither compound significantly affected the sensitivity of MCF-7 tumor cells to CDDP or etoposide. *Bars,* SD. *A* and *B:* $\square$, THF; ▨, 0.10 μM ATRA; ■, lutein.

effects when used *in vitro* in serum-containing *versus* serum-free media (24). Thus, all experiments on MCF-7 tumor cells were performed in the MSU-SRF used for culture of normal cells and SV40 transformed cells, thereby facilitating comparisons between the three cell lines. However, similar results were obtained in serum-containing medium (data not shown).

The three cell lines were cultured in MSU-SRF medium, and viability was measured on day 4. For each cell type, the

Table 2   Cell Death ELISA: Effects of lutein and ATRA on normal human mammary cells treated with/without CDDP and etoposide

Normal human mammary cells were pretreated with and without lutein, ATRA, or THF for 3 days, followed by a 24-h exposure to CDDP or etoposide. Cytoplasmic extracts from 3×10^3 total cells (attached + detached) per sample were isolated for the ELISA. The ELISA data represent the no. of units of cytoplasmic DNA-histone per sample. CDDP and etoposide each induced significant apoptosis in THF ($n = 2$; $P = 0.043$ for CDDP and $P = 0.050$ for etoposide). In the presence of ATRA, neither drug caused significant apoptosis ($n = 2$; $P = 0.273$ for ATRA + CDDP *versus* ATRA, and $P = 0.300$ for ATRA + etoposide *versus* ATRA alone). Lutein also blocked drug-induced death ($n = 2$; $P = 0.375$ for lutein + CDDP *versus* lutein, and $P = 0.138$ for lutein + etoposide *versus* lutein alone).

	Mean number of units of cytoplasmic DNA-histone[a] ($\pm$SD)		
Sample	No drug	CDDP	Etoposide
THF	1.00 (0.12)	3.50 (0.75)	4.40 (1.11)
0.10 μM ATRA	0.85 (0.17)	1.17 (0.25)	1.10 (0.19)
Lutein	0.75 (0.15)	0.95 (0.20)	2.25 (0.87)

[a] 1.0 unit DNA-histone at 405 nm = the amount of horseradish peroxidase activity conjugated to anti-DNA-histone antibodies, specifically bound to a cytoplasmic extract from 3×10^3 HL-60 cells treated with camptothecin (2 μg/ml for 4 h).

percentage of loss of viability in THF-, lutein-, or ATRA-treated samples is shown (Fig. 1). Lutein and ATRA (0.10 μM) did not significantly affect viability of normal or SV40-transformed mammary cells. However, ATRA (1.0 μM) significantly decreased viability of normal mammary cells. Fig. 1 also shows that ATRA (0.10 and 1.0 μM) and lutein significantly decreased the viability of MCF-7 tumor cells.

To determine whether lutein and ATRA decreased viability in MCF-7 tumor cells by induction of apoptosis, we performed PI staining to quantitate apoptotic nuclei. Table 1 demonstrates the lack of induction of apoptosis by either ATRA or lutein in normal mammary epithelial cells. Thus, ATRA (1.0 μM) decreased viability (Fig. 1) without inducing apoptosis (Table 1) in normal cells. These data are consistent with a report showing that ATRA induced apoptosis in transformed human mammary cells but not in normal human mammary epithelial cells (25). In SV40-transformed cells, only ATRA (1.0 μM) induced significant apoptosis. In contrast, both concentrations of ATRA induced a significant 3-fold increase in PI staining of MCF-7 tumor cells relative to the control (THF) sample. Lutein also induced a significant (2.60-fold) increase in apoptosis in MCF-7 tumor cells. The MCF-7 cell data represent an underestimation of apoptosis induction because it was measured in attached cells, and there were substantial numbers of detached cells, >90% of which were nonviable. The data in Table 1 and Fig. 1 suggest that ATRA and lutein inhibit the viability of MCF-7 tumor cells by inducing apoptosis. However, neither compound induced apoptosis in normal mammary epithelial cells.

Differential Effects of ATRA and Lutein on Chemotherapy-induced Apoptosis in Normal and Transformed Human Mammary Epithelial Cells. The above experiments demonstrated that both ATRA and lutein show selective induction of apoptosis in transformed cells compared with normal human mammary epithelial cells. Because a variety of chemotherapeutic agents induce apoptosis, we examined the ability of ATRA (0.10 μM) and lutein to alter the apoptotic threshold in normal and transformed human mammary epithelial cells. The higher concentration of ATRA (1.0 μM) was not used because it significantly inhibited the viability of normal mammary cells and MCF-7 tumor cells (Fig. 1).
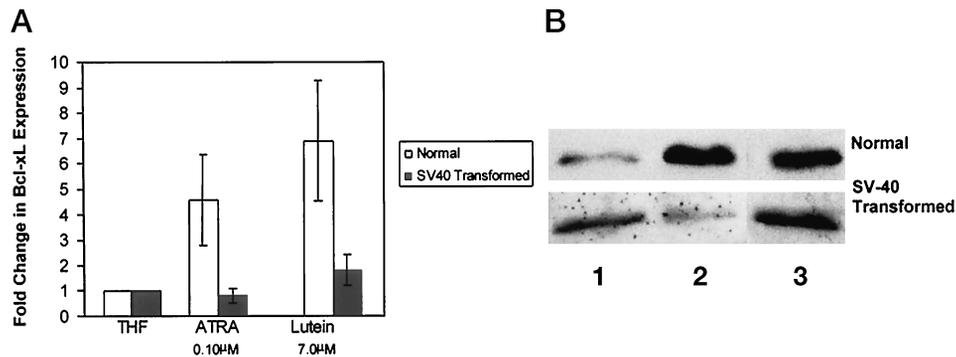
Fig. 3. *A,* effects of ATRA and lutein and on Bcl-xL expression in normal and SV40-transformed cells. The graph shows Bcl-xL protein expression in normal and SV40-transformed cells after treatment with ATRA or lutein for 4 days. In normal cells, the fold changes in Bcl-xL expression induced by ATRA ($n = 3$, $P = 0.025$) are shown relative to Bcl-xL levels in the control (THF) sample. Lutein significantly induced Bcl-xL protein expression in normal cells ($n = 3$, $P = 0.012$). In SV40-transformed cells neither compound significantly affected Bcl-xL expression. *Bars,* SD. *B,* Bcl-xL expression assessed by Western blot. ATRA and lutein each induced Bcl-xL expression in normal mammary cells (*top panel, Lanes 2* and *3*) relative to the THF control (*Lane 1*). The *bottom panel* shows that Bcl-xL expression was not significantly affected by ATRA or lutein in SV40-transformed cells (*Lanes 2* and *3 versus Lane 1*).

Fig. 2*A* shows the effects of ATRA (0.10 μM) and lutein on the viability of normal mammary cells treated with the chemotherapeutic agents, etoposide or CDDP. In normal mammary cells, etoposide and CDDP induced a 3.0- and 4.50-fold increase in dead cell numbers, respectively. However, the induction of drug-induced apoptosis in normal mammary cells was completely blocked by lutein. Similarly, ATRA (0.10 μM) decreased the sensitivity of normal cells to each drug by 2-fold.

Fig. 2*B* shows that MCF-7 tumor cells underwent significant CDDP-and etoposide-induced cell death, which was not significantly affected by lutein/ATRA. In the absence of drug, ATRA and lutein each caused a significant (1.70- and 2.60-fold) increase in dead cell numbers, respectively, in MCF-7 tumor cells with respect to the control (THF). This result is consistent with the decreased viability and increased apoptosis induced by ATRA and lutein in MCF-7 tumor cells (Fig. 1 and Table 1).

To determine whether the chemoprotective effects of lutein and ATRA in normal cells were attributable to the modulation of drug-induced apoptosis, we performed the Cell Death ELISA, which measures DNA degradation in apoptotic cells (Table 2). After 24 h of exposure, both drugs induced significant apoptosis in normal mammary cells (3–5 fold). Table 2 shows that lutein and ATRA fully blocked CDDP and etoposide-induced apoptosis in normal cells. Fig. 2*A* also suggested that lutein effectively protected normal cells from apoptosis induced by both drugs. Thus, both by cell viability and the Cell Death ELISA assay, lutein and ATRA blocked chemotherapy-induced death in normal but not transformed cells. In MCF-7 tumor cells, the Cell Death ELISA showed that both drugs induced a 2-fold increase in apoptosis, which was not significantly affected by ATRA or lutein (data not shown). This result is consistent with the data shown in Fig. 2*B*.

Effects of ATRA and Lutein on Expression of Bcl-xL, Bcl-2, and Bax Proteins in Normal and Transformed Human Mammary Epithelial Cells. The experiments described above demonstrated that ATRA and lutein have differential effects on the induction and modulation of apoptosis in normal *versus* transformed human mammary epithelial cells, suggesting that the apoptotic threshold may be differentially regulated by these compounds in these cell types. The *bcl-2* family of genes has been demonstrated to play a key role in regulating the apoptotic threshold in many cell types. This threshold is modulated by the

ratio of inhibitors of apoptosis such as Bcl-xL and Bcl-2 relative to inducers of apoptosis such as Bax (26, 27).

To determine whether the effects of ATRA and lutein on apoptotic pathways were associated with modulation of the *bcl-2* family of genes, we examined the effects of these compounds on Bcl-2, Bcl-xL, and Bax expression. Normal, SV40 transformed, and MCF-7 carcinoma cells were exposed to ATRA or lutein, and protein expression was assessed by Western blotting. The average fold change in expression of each protein induced by a given compound was calculated relative to its expression in the corresponding control (THF) sample. This was done for each cell type, and the results were graphed in Figs. 3–5. These figures also include representative Western blots showing the effects of lutein and ATRA on the expression of Bcl-xL, Bcl-2, and Bax in each of the three cell types.

Fig. 3*A* shows that ATRA and lutein induced a 5- and 7-fold increase, respectively, in Bcl-xL expression in normal mammary cells relative to the control (THF). Fig. 3*B* shows the Western blot (*Top panel, Lanes 2* and *3 versus Lane 1*). In contrast to normal mammary cells, ATRA and lutein did not significantly alter Bcl-xL expression in SV40 transformed cells (Fig. 3*B, bottom panel*). Data for the MCF-7 carcinoma cells are not shown because these cells (early passage) lack Bcl-xL expression.

Fig. 4 shows the effects of ATRA and lutein on Bcl-2 expression in the three cell types. In normal cells, ATRA induced a significant 3.50-fold increase in Bcl-2 expression relative to the control (THF; Fig. 4*A*). Lutein did not induce a statistically significant increase in Bcl-2 expression in normal cells. The corresponding Western blot shows this data (Fig. 4*B, top panel: Lanes 2* and *3 versus Lane 1*). Neither compound significantly affected Bcl-2 expression in the SV40-transformed cells (Fig. 4*B, middle panel*) or in MCF-7 carcinoma cells (Fig. 4, *A* and *B, bottom panel*). Similarly, expression of the proapoptotic protein Bax was not significantly affected by lutein or ATRA in any of the three cell types (Fig. 5).

In summary, ATRA and lutein have differential effects on the expression of Bcl-2 family members in normal *versus* transformed human mammary epithelial cells. Lutein selectively increased the ratio of Bcl-xL:Bax in normal cells but not transformed mammary cells. Similarly, ATRA selectively increased the ratio of Bcl-xL + Bcl-2:Bax in normal mammary cells only. These data suggest a possible mechanism for the
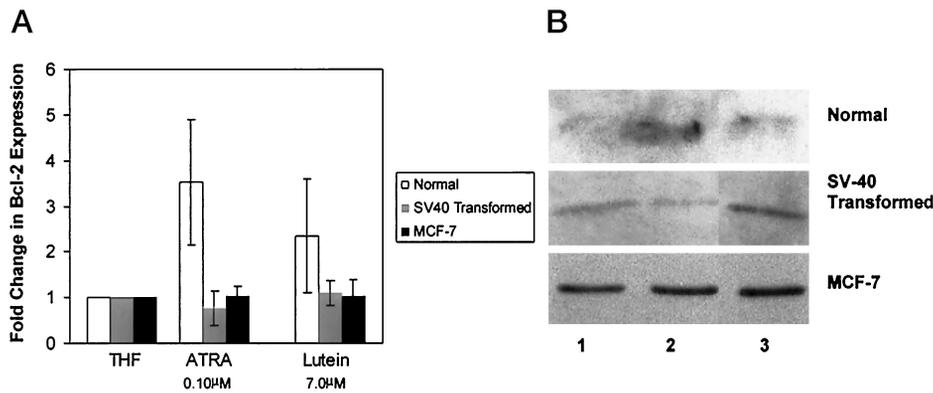
Fig. 4.   *A,* effects of ATRA and lutein on Bcl-2 expression in normal and transformed mammary cells. The graph shows the fold changes in Bcl-2 expression in normal, SV40-transformed cells, and MCF-7 tumor cells, after treatment with ATRA or lutein for 4 days. Bcl-2 expression was significantly induced by ATRA in normal cells ($n = 3$, $P = 0.033$) relative to the control (THF). In SV40-transformed cells and MCF-7 tumor cells, no significant changes in Bcl-2 expression were induced by ATRA. Lutein did not significantly alter Bcl-2 expression in any of the cell types. *Bars,* SD. *B,* Bcl-2 expression assessed by Western blot. The *top panel* shows that ATRA significantly increased Bcl-2 expression in normal mammary cells relative to the THF control (*Lane 2 versus Lane 1*). Bcl-2 expression was not significantly affected by ATRA in SV40-transformed cells (*middle panel*) or in MCF-7 tumor cells (*bottom panel*). Lutein did not significantly affect Bcl-2 expression in any of the cell types (*Lane 3 versus Lane 1*).

Fig. 5.   *A,* effects of ATRA and lutein on Bax expression in normal cells, SV40-transformed cells, and MCF-7 cells. The graph shows fold changes in Bax expression in normal mammary cells, SV40-transformed cells, or MCF-7 tumor cells after treatment with ATRA or lutein for 4 days. Neither compound induced significant changes in Bax expression in any of the three cell types. *Bars,* SD. *B,* Bax expression assessed by Western blot. *Lanes 2* and *3* show that Bax expression was unchanged by ATRA (*Lane 2*) or lutein (*Lane 3*) in normal cells (*top panel*), SV40 transformed cells (*middle panel*), and MCF-7 tumor cells (*bottom panel*), relative to their respective THF controls (*Lane 1* of each panel).
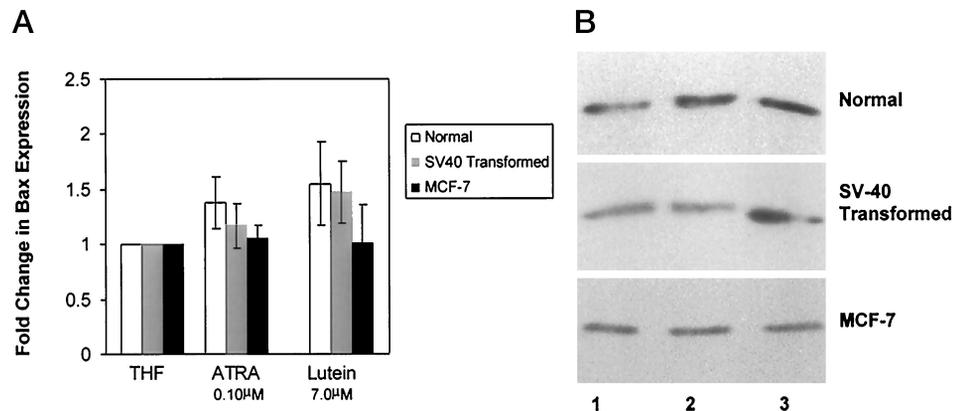
ability of lutein and ATRA to protect normal cells, but not MCF-7 tumor cells, from apoptosis induced by etoposide and cisplatin.

Discussion

In this study, we demonstrate that ATRA and lutein selectively induce apoptosis in transformed cells compared with normal human mammary cells. The differential effects of these compounds on apoptosis and chemosensitivity in normal *versus* transformed mammary cells may in part be related to the differential effects of these compounds on the expression of Bcl-xL, Bcl-2, and Bax expression in these cells.

This study is the first to demonstrate that lutein, a non-provitamin A carotenoid, has significant and markedly different effects on apoptosis in normal mammary cells compared with transformed counterparts. Because lutein is a nonprovitamin A carotenoid, its mechanism of action is likely independent of the retinoic acid receptor family. Whether lutein signals via a specific receptor is as yet unknown. However, both compounds selectively increased the ratio of Bcl-xL:Bax expression in normal mammary cells and protected these cells from apoptosis induced by etoposide and CDDP. Thus, these data suggest that lutein and ATRA are similar in their ability to modulate the

apoptotic threshold. Although the experiments were performed in serum-free medium to obviate the effects of serum-binding proteins, similar differential effects of ATRA and lutein on *bcl-2* family expression and apoptosis were observed in normal mammary cells *versus* MCF-7 tumor cells in serum-containing medium (data not shown). Thus, the differential effects of ATRA and lutein on the apoptotic threshold of normal *versus* tumor mammary cells occur in the presence of serum and albumin that may be found *in vivo.*

The results demonstrating that lutein and ATRA raise the apoptotic threshold in normal mammary cells but induce apoptosis in mammary carcinoma cells are consistent with reports showing that the carotenoids β-carotene and canthaxanthin are selectively toxic in malignant tumor lines but not in normal keratinocytes (28). Whether these and other carotenoids have differential effects on apoptosis pathways in normal *versus* transformed cells remains to be determined. Interestingly, a number of other chemopreventive agents, such as aspirin (29), sulindac (30), and the retinoid *N*-4-hydroxyphenylretinamide (31), are thought to exert their action through selective induction of apoptosis in transformed cells. Our data suggest that the chemopreventive effects of ATRA and lutein in mammary carcinogenesis may be attributable to similar mechanisms.

The molecular mechanisms responsible for the differential effects of ATRA and lutein on Bcl-2 family members in normal *versus* transformed human mammary cells are unknown. However, elevated Bcl-2 expression has been observed in differentiating hematopoietic, neural, and epithelial tissues (32). Interestingly, ATRA-induced differentiation of neuroblastoma cells was accompanied by marked induction of Bcl-2 and drug resistance (33). We found that ATRA and lutein selectively increased the ratio of Bcl-xL:Bax expression in normal mammary cells and protected these cells from apoptosis induced by etoposide and CDDP. However, in MCF-7 tumor cells, ATRA and lutein induced significant apoptosis without altering the ratio of Bcl-2:Bax expression, suggesting that these compounds can induce apoptosis, independent of regulation of expression of *bcl-2* family members (Table 1; Figs. 2*B*, 4, and 5). Candidate genes known to be regulated by ATRA include *p53*, *p21*, *c-myc*, and transforming growth factor β (34). Therefore, the effects of ATRA and lutein on the expression of these genes in normal *versus* transformed human mammary epithelial cells need to be investigated.

A variety of mammary carcinoma cells show increased expression of either Bcl-2 or Bcl-xL compared with nontransformed mammary cells (35–37). We have suggested that the expression of these inhibitors of apoptosis may be required for mammary transformation, because up to 80% of breast carcinomas overexpress one or the other of these gene products. Thus, these genes may be constitutively expressed in transformed mammary cells but regulated by retinoids and carotenoids in nontransformed mammary cells. Although these studies do not directly establish a causal relationship between modulation of Bcl-2 family members and apoptotic thresholds, other studies demonstrating chemoresistance by overexpression of Bcl-2 (or Bcl-xL) by transfection are consistent with this mechanism (38, 39).

In summary, these studies demonstrate the selective effects of the nonprovitamin A carotenoid lutein and ATRA on apoptosis pathways in normal *versus* transformed human mammary epithelial cells. The chemopreventive properties of these compounds may relate to their differential effects on apoptosis pathways in normal *versus* transformed mammary cells. Furthermore, the selective protection of normal mammary cells compared with transformed cells by lutein suggests that clinical trials investigating the effect of this nontoxic compound on chemotherapeutic efficacy and toxicity may be warranted.

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