Differential Regulation of Apoptosis in Normal versus Transformed Mammary Epithelium by Lutein and Retinoic Acid

Venil N. Sumantran, Rong Zhang, David S. Lee, and Max S. Wicha

Department of Internal Medicine and Comprehensive Cancer Center, Cancer and Geriatrics Center, University of Michigan, Ann Arbor, Michigan 48109-0942

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, 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
or ATRA. With ATRA or lutein for 3 days, followed by treatment (24 h) 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.

Normal mammary epithelial cells were cultured in MSU-1 medium (21). Normal mammary cells were derived from reduction mammoplasties as described (21). Normal mammary epithelial cells and similar cells transformed with SV40 were grown in MSU-1-SRF medium (21). SV40-transformed cells, and MCF-7 breast cancer cells were cultured in DMEM (Sigma) and bovine pituitary extract (0.40%; Pel Freez, AR). For experiments, cells were switched to MSU-1-SRF medium without serum but including SRFs. These factors include: human recombinant epidermal growth factor (0.5 ng/ml), human-Bcl-xL, Bcl-2, or Bax proteins.

Significance of the effects of ATRA or lutein in the three cell types were analyzed by the students 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 effects on the plasma proteins binding and on the overall biological activity of the compound. ATRA reportedly binds serum proteins and has different effects on the plasma proteins binding and on the overall biological activity of the compound. The effects of ATRA and lutein on growth and apoptosis were assessed in normal and 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).

**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. 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 anti-bcl-2 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/anti-rabbit 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 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.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Normal mammary cells</th>
<th>SV40-transformed cells</th>
<th>MCF-7 breast cancer cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>3.90 (1.00)</td>
<td>13.00 (2.00)</td>
<td>5.20 (1.10)</td>
</tr>
<tr>
<td>0.10 μM ATRA</td>
<td>4.80 (0.50)</td>
<td>26.50 (4.50)</td>
<td>16.10 (2.90)</td>
</tr>
<tr>
<td>1.0 μM ATRA</td>
<td>4.40 (0.30)</td>
<td>21.50 (0.50)</td>
<td>16.00 (3.00)</td>
</tr>
<tr>
<td>Lutein</td>
<td>5.30 (1.00)</td>
<td>19.50 (7.50)</td>
<td>13.60 (1.05)</td>
</tr>
</tbody>
</table>

**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.
Bar 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 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 quantify 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 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).

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>No drug</th>
<th>CDDP</th>
<th>Etoposide</th>
</tr>
</thead>
<tbody>
<tr>
<td>THF</td>
<td>1.00 (0.12)</td>
<td>3.50 (0.75)</td>
<td>4.40 (1.11)</td>
</tr>
<tr>
<td>0.10 μM ATRA</td>
<td>0.85 (0.17)</td>
<td>1.17 (0.25)</td>
<td>1.10 (0.19)</td>
</tr>
<tr>
<td>Lutein</td>
<td>0.75 (0.15)</td>
<td>0.95 (0.20)</td>
<td>2.25 (0.87)</td>
</tr>
</tbody>
</table>

*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 × 10^6 HL-60 cells treated with camptothecin (2 μg/ml for 4 h).*
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. 3A 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). In contrast to normal mammary cells, ATRA and lutein did not significantly alter Bcl-xL expression in SV40 transformed cells (Fig. 3B, 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 3.5-fold increase in Bcl-2 expression relative to the control (THF; Fig. 4A). Lutein did not induce a statistically significant increase in Bcl-2 expression in normal cells. The corresponding Western blot shows this data (Fig. 4B, top panel: Lanes 2 and 3 versus Lane 1). Neither compound significantly affected Bcl-2 expression in the SV40-transformed cells (Fig. 4B, 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
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. 2B, 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.

Acknowledgments

We gratefully acknowledge the gifts of SV40 transformed type II cells provided by Dr. C. C. Chang from Michigan State University (MSU). We also thank Dr. James Trosko (MSU) for helpful insight on the normal and SV40-transformed human mammary cells. We thank Dr. Adam Drenowski for support for Rong Zhang. We appreciate Dr. Cheryl Rock's help with project design and information on the properties of retinoids and carotenoids. Dr. David Beidler helped us design the chemotherapy experiments.

References


Downloaded from cebp.aacrjournals.org on April 9, 2017. © 2000 American Association for Cancer Research.


Differential Regulation of Apoptosis in Normal versus Transformed Mammary Epithelium by Lutein and Retinoic Acid

Venil N. Sumantran, Rong Zhang, David S. Lee, et al.


Updated version  Access the most recent version of this article at: http://cebp.aacrjournals.org/content/9/3/257

Cited articles  This article cites 37 articles, 19 of which you can access for free at: http://cebp.aacrjournals.org/content/9/3/257.full.html#ref-list-1

Citing articles  This article has been cited by 10 HighWire-hosted articles. Access the articles at: /content/9/3/257.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.