All *trans*-retinoic acid acts synergistically with hydroxytamoxifen and transforming-growth factor β to stimulate apoptosis in MCF-7 breast cancer cells

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Abstract

The anti-estrogen 4-hydroxytamoxifen (TAM) and vitamin A-related compounds, the retinoids, in combination act synergistically to inhibit growth of breast cancer cells *in vitro* and *in vivo*. To clarify the mechanism of this synergism, the effect of TAM and all *trans*-retinoic acid (AT) on proliferation of MCF-7 breast cancer cells was studied *in vitro*. TAM and AT acted synergistically to cause a time-dependent and dose-dependent inhibition of MCF-7 cell growth. In a temporally related manner, TAM+AT acted synergistically to downregulate Bcl-2 mRNA and Bcl-2 protein expression, and to stimulate apoptosis. TAM and AT each blocked cell cycle progression throughout 7 days of treatment but without any synergistic or additive effect on this process, indicating a selective synergism for apoptosis.

The negative growth factor-transforming growth factor β (TGF β) is secreted by these cells and was studied as a potential mediator of the synergistic effects of TAM+AT

on apoptosis. TAM+AT acted synergistically to induce a fivefold increase in TGF β 1 secretion over 72 h. TGF β 1 alone had no apoptotic effects on these cells; however, TGF β 1 in combination with AT acted synergistically to inhibit growth, to downregulate Bcl-2 mRNA and Bcl-2 protein expression, and to stimulate apoptosis of these cells in a manner comparable with that noted for TAM+AT. The synergism of both TAM+AT and TGF β 1+AT for apoptosis was suppressed by estradiol. Co-incubation of TAM+AT with anti-TGF β antibody did not block downregulation of Bcl-2 protein expression or stimulation of apoptosis. The synergistic effects of TAM+AT on apoptosis therefore occur independently of TGF β , although TGF β may interact with AT in a novel manner to provide another important anti-proliferative mechanism for breast cancer cells.

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Introduction

The anti-estrogen tamoxifen (TAM) and vitamin A-related compounds, the retinoids, in combination act synergistically to inhibit growth of breast cancer cells. TAM acts additively/synergistically with all *trans*-retinoic acid (AT) (Wetherall & Taylor 1986, Fontana 1987, Koga & Sutherland 1991) or 4-hydroxyphenylretinide (fenretinide (Coradini *et al.* 1997)) to inhibit growth of estrogen receptor (ER)-positive breast cancer cells *in vitro*. *In vivo*, TAM acts synergistically with either 9-*cis*-retinoic acid or AT (Anzano *et al.* 1994) to reduce tumor number and tumor burden, and acts synergistically with fenretinide to prevent development, prevent recurrence following excision, and reduce the incidence and number of nitrosomethylurea-induced tumors in rats (Ratko *et al.* 1989). Clinically, TAM plays a major role in the prevention and treatment of breast cancer (Early Breast Cancer Trialists' Collaborative Group 1992, Fisher *et al.* 1998),

encouraging the development of means for further enhancing its activity.

The mechanism of the synergistic action of anti-estrogens and retinoids in combination on breast cancer cells is not known; however, there are important similarities in the actions of these agents. Both retinoids and anti-estrogens block cell cycle progression at G₀G₁, and both agents reduce the hyperphosphorylated form of the retinoblastoma protein (pRb), indicating an important convergence point for their action (Wilcken *et al.* 1996). Both retinoids and anti-estrogens downregulate Bcl-2 gene and protein expression (Wang & Phang 1995, Zhang *et al.* 1999) and stimulate apoptosis in breast cancer cells (Perry *et al.* 1995, Chen *et al.* 1996, Liu *et al.* 1996, Toma *et al.* 1997). These similarities raise the possibility that synergism involves shared anti-proliferative pathways. In addition, there is evidence to suggest that enhancement may be mediated through interaction of one or both of these agents with endogenous growth-inhibitory

substances. The negative growth factor, transforming-growth factor β (TGF β), is secreted by these cells, and in turn can act in an autocrine manner on breast cancer cells to inhibit growth (Danforth & Sgagias 1996, Perry *et al.* 1995). Importantly, it has been shown that exogenous TGF β can interact with AT to inhibit growth of breast cancer cells as well as other cell types. Valette & Botanch (1990) demonstrated that AT (1 μ M) and TGF β (10 pM), in combination, acted synergistically to inhibit MCF-7 cell growth in a time- and dose-dependent manner over 6 days in culture. Others have shown that AT in combination with TGF β acts additively/synergistically to inhibit growth of colon cancer cells (Hoosein *et al.* 1988), glioblastoma cells (Helseth *et al.* 1988), and epidermal keratinocytes (Tong *et al.* 1990). It is well known that TAM acts on breast cancer cells to stimulate secretion of TGF β (Knabbe *et al.* 1987, Perry *et al.* 1995). These findings raise the intriguing possibility that TGF β may interact with AT to mediate the synergism of AT with TAM. To clarify the mechanism of synergy between TAM and retinoids, the anti-proliferative effects of TAM and AT on MCF-7 human breast cancer cells were studied *in vitro*. These studies indicate that AT acts synergistically with both TAM and TGF β to stimulate apoptosis in these cells; however, these two processes appear to act independently.

Materials and Methods

Chemicals

AT, TAM, 17 β -estradiol, leupeptin, and aprotinin were purchased from Sigma Corporation, St Louis, MO, USA. Monoclonal antibody to Bcl-2 protein was obtained from Calbiochem, San Diego, CA, USA. Recombinant human TGF β 1 (rhTGF β), anti-TGF β neutralizing antibody (no. AB-100-NA), and ELISA for detection of TGF β 1 and TGF2 were obtained from R & D Systems, Minneapolis, MN, USA.

Cell line and cell culture

MCF-7 human breast cancer cells were obtained from the Michigan Cancer Foundation, Detroit, MI, USA. Cell cultures were maintained in Costar flasks with Improved Minimum Essential Medium (IMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS; Biofluids Inc., Rockville, MD, USA), glutamine (2 mM), and gentamicin (40 mg/l). The FCS, unless indicated, did not undergo processing to remove endogenous steroids, in agreement with previous studies (Marth *et al.* 1984, Fontana 1987, Koga & Sutherland 1991, Butler & Fontana 1992, Koli *et al.* 1997, Ko *et al.* 1998, Wu *et al.* 1998). For studies evaluating estradiol, cells were plated in phenol red-free IMEM supplemented with heat-inactivated FCS stripped

of endogenous steroids by charcoal. After 48 h the medium was replaced with fresh medium containing added substances as indicated in the figure legends. All steroids for experimental studies were diluted from 1000-fold stock concentrates. Ethanol carrier was added to control media; final ethanol content of media was <0.1%. For cell growth studies evaluating TGF β , rhTGF β 1 (no. 240-B; R & D Systems, Minneapolis, MN, USA) was used. For cell counts, cells were harvested in dilute trypsin-EDTA and counted in isotone in a Coulter counter (Coulter Electronics, Hialeah, FL, USA). The doubling time for cell growth was calculated from the slope of a regression line fitted to the time course for each treatment group (Hayflick 1973). The additive or synergistic interaction of test substances on cell growth in combination was determined from the following formula (Berenbaum 1981, Koga & Sutherland 1991): $(Ac/Ae) + (Bc/Be) = 1$, where Ae and Be are doses of A and B alone that produce some specified growth-inhibitory effect, and Ac and Bc are their doses in combination that also have this same inhibitory effect. Values <1 indicate synergism, values =1 indicate additive effects, and values >1 indicate antagonism.

Quantitative analysis of apoptosis

Quantitative detection of cytoplasmic histone-associated DNA fragments Cells (1×10^5) from control or treatment groups were lysed, centrifuged at 20 000 g, and the supernatant assayed for DNA fragments by the Cell Death ELISA (Roche Diagnostics Corp., Indianapolis, IN, USA) according to the manufacturer's instructions.

Quantitative determination of DNA strand breaks

Detection and quantitation of apoptosis was determined by a two-color staining method which labels DNA breaks and total DNA. This was performed with the APO-BRDU kit (Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Briefly, a monolayer of cells was harvested in PBS/0.04% EDTA, fixed in 1% para-formaldehyde (4 °C for 15 min), followed by 70% ethanol (–20 °C overnight). Cells were washed and recovered in FACS buffer (PBS/0.5% bovine serum albumin/0.02% sodium azide). Cells were then labeled with brominated deoxyuridine triphosphates (Br-dUTP) for 60 min at 37 °C, stained with FITC (30 min at room temperature), and labeled with propidium iodide/RNAase solution. Flow cytometry of apoptotic cells and cell cycle analysis were performed using a Becton-Dickinson FACSCAN equipped with 488 nm argon laser, and the Cell Quest computer program (Becton-Dickinson Corp., San Jose, CA, USA).

Immunoblotting

Cell pellets from control or treatment groups were lysed in 2 volumes lysis buffer (Tris, 10 mM, pH 7.4; NaCl,

150 mM; EDTA, 5 mM; 1% Triton-X100, leupeptin and aprotinin, 0.5 μ g/ml each and 1 mM phenylmethylsulfonyl fluoride (Keane *et al.* 1996)). Cell lysates were centrifuged at 10 000 *g*, and 30 μ g total protein fractionated on 16% Tris/glycine/SDS gels (Invitrogen Corp., Carlsbad, CA, USA). Proteins were electrophoretically transferred to nitrocellulose, blocked with 5% non-fat dry milk, probed with anti-Bcl-2 monoclonal antibody (1–2 μ g/ml PBS/0.05% Tween) washed, complexed with secondary horseradish peroxidase antibody and developed with ECL detection reagents (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The membranes were stripped with PBS/0.1% Tween-20, reblocked and re-probed with anti- β actin antibody (1:2000 dilution) as above. X-ray films were examined by densitometry and images quantitated using an Adobe Photoshop imager (Adobe Systems Inc., San Jose, CA, USA) and IP Labgel (Scanalytics Inc., Fairfax, VA, USA). The following densitometric ratio was calculated for each image: Densitometric ratio = (treatment/actin)/(control/actin).

RNA extraction, cDNA synthesis, and quantitative RT-PCR

Cell monolayers of control or treatment groups were harvested in Hanks' balanced salt solution with a cell scraper, and total RNA was extracted from cell pellets with Trizol reagent (Invitrogen Corp.) according to the manufacturer's instructions. Total RNA was quantitated by spectrophotometry and stored at -70°C until assay.

cDNA synthesis was performed on total RNA with the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen Corp.) according to the manufacturer's instructions. Three hundred nanograms of total RNA and 150 ng/ml of random hexamers were used for each synthesis. Each experiment included a no-reverse transcriptase control. Final cDNA preparations were treated with RNase H (37°C for 30 min) and stored at -20°C until assay.

Quantitative RT-PCR was carried out on the ABI Prism 7700 Sequence Detector Thermal Cycler (Applied Biosystems, Foster City, CA, USA). Bcl-2 expression was detected using a predeveloped primer and probe set (Assays-on-Demand; Applied Biosystems). The probe contained a 6-carboxyfluorescein (FAM) reporter dye at the 5' end and 6-carboxytetramethylrhodamine (TAMRA) quencher dye at the 3' end. Each reaction mixture consisted of 25 μ l TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2.5 μ l Assays-on-Demand gene expression assay mix, and 22.5 μ l H_2O containing 300 ng cDNA equivalents. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as endogenous standard. PCR reactions were performed in 96-well optical reaction plates. Cycling conditions were $95^{\circ}\text{C} \times 10$ min for denaturation, followed by 40 cycles of amplification at 15 s at 95°C and 1 min at 60°C . The threshold cycle was measured for each reaction.

Quantitation of RNA expression was determined by the method of Jeyaseelan *et al.* (2001), which calculates the ratio between the expression of Bcl-2 to the expression of GAPDH in the calibrator and in the probe. Untreated MCF-7 cells served as the calibrator. A calibration curve was constructed over an input range of cDNA from 3 to 3000 ng. The amplification of the cDNA was linear over this range.

TGF β studies

Isometric TGF β 1 and TGF β 2 concentrations of the cell monolayer media were determined by their respective ELISAs (R & D Systems, Minneapolis, MN, USA). Briefly, MCF-7 cells were plated in IMEM supplemented with glutamine (2 mM), and 5% FCS stripped of endogenous steroids with dextran-coated charcoal. After 48 h, the monolayers were washed twice with warm PBS and changed to serum-free IMEM supplemented with transferrin (2 μ g/ml) to which test substances were added (Arteaga *et al.* 1993). After the incubation times indicated in the Figures, the medium was collected, leupeptin (1 μ g/ml) and aprotinin (1 μ g/ml) added, and samples either assayed immediately or stored at -70°C . At the time of assay, samples were acid activated to pH 1.5 with 1.0 M HCl, neutralized with NaOH/HEPES to pH 7.4, and assayed for TGF β 1 and TGF β by the ELISA specific for the respective isomeric TGF β 2 according to the manufacturer's instructions. There was no cross-reactivity between ELISAs for the alternate isomeric forms. All TGF β concentrations were expressed as pg/ 10^6 cells of the feeder monolayer.

TGF β neutralization studies were conducted using an anti-TGF β antibody (no. AB-100-NA; R & D Systems, Milwaukee, WI, USA). This antibody completely neutralizes the activity of TGF β 1, TGF β 2, and TGF β 3 isomeric forms, as determined by the manufacturer. A concentration (20 μ g/ml) was selected which completely neutralized 300 pg/ml active exogenous rhTGF β 1 in IMEM supplemented with 5% unstripped FCS (growth inhibition over 7 days: control/normal rabbit IgG/TGF β 1, $28.9 \pm 3.5\%$; anti-TGF β antibody/TGF β 1, $0.3 \pm 2.4\%$; $P < 0.01$). Cell monolayers were incubated with media containing test substances and anti-TGF β antibody (20 μ g/ml) or a control normal IgG antibody, and Bcl-2 protein expression or apoptosis measured as indicated in the figure legends.

Statistical methods

Statistical comparisons were analyzed by Kruskal–Wallis non-parametric comparisons of the means or Student's *t*-test using StatView statistical software (SAS Institute Inc., Cary, NC, USA). Data points represent the means $\pm$ S.E.M. for three to four experiments unless stated otherwise.

Figure 1 Inhibition of MCF-7 cell growth by TAM and AT. A monolayer of cells was incubated with vehicle or TAM (100 nM) and/or AT (1 μM) and cell numbers determined at the indicated time points. Cell growth is expressed as the percent inhibition from control. **P*<0.05 vs TAM or AT at day 5 or day 7.

Results

Growth inhibition of MCF-7 cells by TAM and AT

AT and TAM in combination acted synergistically in a time-dependent manner to inhibit MCF-7 cell growth (Fig. 1). The synergy was dose dependent: at TAM=100 nM, synergism was seen at AT=10⁻⁶ M to 10⁻⁷ M (*P*<0.05). At TAM=10 nM, synergistic effects were noted at AT=10⁻⁶ M to 10⁻⁸ M (*P*<0.05), and at AT=10⁻⁸ M, synergism was noted at TAM=0.10 nM to 10.0 nM (*P*<0.05). Doubling times were calculated from the time courses using TAM=100 nM and AT=1 μM, and were as follows: control, 1.75 ± 0.1 days; TAM, 2.86 ± 0.4; AT, 2.64 ± 0.5; TAM+AT, 4.7 ± 0.5). Synergy was confirmed by the method of Berenbaum (1981), which revealed a ratio of 0.02 at concentrations for each agent which provided for growth inhibition of approximately 50.0%.

Effect of TAM and AT on apoptosis, cell cycle progression, and cell differentiation

To determine if the synergistic inhibition of growth was due to the stimulation of apoptosis, the effect of AT+TAM on DNA fragmentation was studied at 4 days and 7 days of treatment. As shown in Fig. 2, at 7 days of treatment, TAM and AT acted synergistically to stimulate apoptosis three- to fourfold over control cells. This was confirmed by an alternative method which measured Br-dUTP-labeled DNA strand breaks. The spontaneous apoptotic rate in untreated cells by this method was approximately 1–2%. At 7 days of treatment, TAM and AT in combination acted synergistically to stimulate apoptosis (Table 1), in agreement with the findings by the DNA fragmentation assay.

Figure 2 Stimulation of apoptosis by TAM and AT. Cells were incubated with vehicle (CONT) or TAM (100 nM) and/or AT (1 μM) and apoptosis determined by DNA fragmentation ELISA on day 4 or day 7 as described in Materials and Methods. Values are expressed relative to control which is set to 1 at day 4 and day 7. **P*<0.01 vs CONT, AT, TAM, day 7.

To further define regulation of apoptosis by TAM+AT, the effects of these agents on expression of the anti-apoptotic protein Bcl-2 was studied by immunoblot. This showed that, at 4 days, TAM+AT acted synergistically to downregulate Bcl-2 protein expression, and these effects persisted through 7 days of treatment (Fig. 3). When the effects on Bcl-2 mRNA at 72 h was studied by quantitative RT-PCR, the combination of TAM+AT acted synergistically to downregulate Bcl-2 mRNA levels (Fig. 4). No cDNA synthesis was noted in the reaction which lacked reverse transcriptase, and cDNA synthesis was linear over an mRNA input range of 3–3000 ng.

Table 1 Stimulation of apoptosis by TAM and AT. A monolayer of cells was incubated with vehicle or TAM (100 nM) and/or AT (1 μM) and percentage of total cells undergoing apoptosis determined by the APO-BRDU method on day 4 or day 7 as described in Materials and Methods

	Incidence of apoptosis (%)
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Treatment group	
Day 4	
Control	1.3 ± 0.3
TAM	1.7 ± 0.7
AT	3.0 ± 2.0
TAM/AT	2.7 ± 1.7
Day 7	
Control	2.5 ± 1.0
TAM	29.4 ± 8.9
AT	24.8 ± 7.6
TAM/AT	69.1 ± 9.5*

**P*<0.05 vs control, TAM, AT at day 7.

Figure 4 Modulation of Bcl-2 mRNA expression by TAM, AT, or TGFβ. Cells were treated with vehicle (CONT) or AT (1 μM), TAM (100 nM) and/or rhTGFβ1 (TGF; 5 ng/ml) for 72 h, and Bcl-2 mRNA levels determined by quantitative RT-PCR as described in Materials and Methods. **P*<0.05 vs CONT, AT, TAM; #*P*<0.05 vs CONT, AT, TGFβ.

To determine the specificity of the synergistic effect of TAM+AT for apoptosis, regulation of cell cycle progression and cell differentiation was studied. Treatment with TAM (100 nM) alone or AT (1 μM) alone each blocked cell cycle progression at G₀G₁, delaying entry into S phase (Fig. 5). At this dose there was no additive or synergistic effect on cell cycle progression however, at either 4 days and 7 days of treatment. When the effect of TAM+AT on cellular epithelial membrane antigen (EMA) expression, an indicator of epithelial differentiation, was studied by immunoblot, no synergistic effect of the combination TAM+AT was seen on EMA expression at either 4 or 7 days of treatment (data not shown).

Modulation of TGFβ by AT/TAM

TGFβ is an autocrine regulator of cell growth in MCF-7 cells, and its secretion is stimulated by TAM (Perry *et al.* 1995, Chen *et al.* 1996, Knabbe *et al.* 2000). Regulation of TGFβ secretion by TAM+AT was studied using specific ELISAs to quantitate medium TGFβ content. Untreated cells secreted both TGFβ1 and TGFβ2 isomeric forms,

Figure 3 Modulation of Bcl-2 protein expression by TAM, AT, or rhTGFβ1. Cells were treated with vehicle (CONT) or TAM (100 nM) and/or AT (1 μM) and/or rhTGFβ1 (5 ng/ml), and Bcl-2 protein or actin protein expression of cell lysates determined by immunoblot analysis at day 4 (top panel) and day 7 (bottom panel). (A) Protein expression for each treatment group was quantitated and is expressed in the histogram relative to control as described in Materials and Methods. (B) A representative immunoblot example for the respective histogram. (Top panel) **P*<0.01 vs CONT. (Bottom panel) #*P*<0.05 vs CONT; +*P*<0.05 vs CONT, AT, TAM; ^*P*<0.05 vs CONT, TGFβ.

Figure 5 Regulation of cell cycle phase by TAM and AT. Cells were incubated with vehicle (Cont) or TAM (100 nM) and/or AT (1 μM) and percent of cells in G₀G₁ or S phase determined at day 4 or day 7 by flow cytometry.

with a slight predominance of TGFβ1 (62.8%; (Table 2)). The media of these studies were acid-activated prior to the TGFβ assay, and thus the TGFβ content which was measured was the total quantity secreted by these cells. TAM alone caused a significant increase in TGFβ1 secretion, and in combination TAM+AT had a marked synergistic effect on TGFβ1 secretion, increasing media levels five-fold over 72 h (Table 2), with a smaller but significant stimulation of TGFβ2 secretion.

Interaction of AT and TGFβ to regulate growth and apoptosis of MCF-7 cells

The enhanced secretion of TGFβ by TAM+AT over 72 h preceded the synergistic inhibition of growth at 5–7 days

Table 2 Stimulation of TGFβ1 and TGFβ2 secretion by TAM and AT. Cells were incubated with vehicle or TAM (100 nM) and/or AT (1 μM) and the media content of acid-activated TGFβ1 or TGFβ2 determined by ELISA at 72 h as described in Materials and Methods. The concentrations of TGFβ1 and TGFβ2 are expressed as pg/10⁶ cells per 72 h of the feeder monolayer

	Media content
TGFβ1	
Control	101.1 ± 17.4
AT	124.7 ± 16.2
TAM	232.6 ± 20.6 [#]
TAM+AT	493.8 ± 58.4 [*]
TGFβ2	
Control	59.6 ± 10.9
AT	65.3 ± 12.2
TAM	105.4 ± 19.1
TAM+AT	127.7 ± 20.9 ⁺

^{*}*P*<0.01 vs control, AT, or TAM; [#]*P*<0.01 vs control for TGFβ1; ⁺*P*<0.05 vs control for TGFβ2.

Figure 6 Effect of TGFβ1 and AT on MCF-7 cell growth. A monolayer of cells was incubated with vehicle or rhTGFβ1 (TGF; 5 ng/ml) and/or AT (1 μM) and cell numbers determined at the indicated time points. Cell growth is expressed as a percent of inhibition from control. ^{*}*P*<0.01 vs TGFβ or AT, days 5, 6, 7.

by TAM+AT, and TGFβ1 has been shown to interact with AT to inhibit cell growth (Valette & Botanch 1990). When AT was studied in combination with TGFβ1, a synergistic inhibition of growth was observed which was time-dependent (Fig. 6) and dose-dependent for both AT (in the range of 10^{−6} M to 10^{−8} M; *P*<0.01) and for TGFβ1 (in the range of 0.05 to 5.0 ng/ml; *P*<0.01). When the effects of TGFβ on Bcl-2 expression and apoptosis was studied, TGFβ+AT was found to act synergistically to downregulate Bcl-2 mRNA expression at 72 h (Fig. 4), and Bcl-2 protein expression at 4 days and 7 days (Fig. 3). This was associated with a time-dependent synergistic stimulation of apoptosis by AT+TGFβ at 7 days of treatment (Fig. 7).

Figure 7 Regulation of apoptosis by TGFβ1 and AT. Cells were incubated with vehicle (CONT) or AT or rhTGFβ1 (TGF) as described in Fig. 6, and apoptosis measured on day 7 by DNA fragmentation ELISA. ^{*}*P*<0.05 vs CONT, AT or TGFβ, day 7.

These studies of AT, TAM, and TGF β were conducted in media supplemented with unstripped calf serum. To clarify the effects of estradiol, the synergy of AT+TAM and AT+TGF was studied in charcoal-stripped calf serum supplemented with estradiol at concentrations ranging from 0.05 to 5.0 nM. Estradiol at 0.05 and 0.5 nM did not suppress the synergistic effect of AT+TAM on cell growth ($P < 0.01$); however, the synergistic effect of AT+TGF β

on cell growth was suppressed at all concentrations of estradiol, and a concentration of 0.5 nM estradiol suppressed the synergistic stimulation of apoptosis for both AT+TAM and AT+TGF (Fig. 8).

To determine if TGF β mediates TAM+AT synergism, cells were co-incubated with TAM+AT and neutralizing anti-TGF β antibody, and Bcl-2 protein expression were studied at 4 days and 7 days of treatment. TAM+AT downregulated Bcl-2 protein expression, confirming the action of these two agents; however, this was not reversed at either of the two time points by anti-TGF β antibody (Fig. 9). When the effect of the antibody on TAM+AT stimulation of apoptosis at 7 days was studied, no reversal of the apoptotic process was seen, consistent with the effects on Bcl-2 protein expression (Fig. 9).

Discussion

The ability of TAM and AT to act synergistically in combination to inhibit growth of breast cancer cells represents an important means for enhancing the anti-proliferative effects of these two agents. The present report has demonstrated that the synergism of TAM+AT for growth inhibition was due to the selective stimulation of apoptosis. This is supported by the finding that TAM+AT acted synergistically to downregulate Bcl-2 mRNA and Bcl-2 protein expression, and to stimulate DNA fragmentation in a temporally related manner, without any additive or synergistic effect on cell cycle progression or cell differentiation. Downregulation of Bcl-2 is causally related to apoptosis in MCF-7 cells (Perillo *et al.* 2000), and the downregulation of mRNA levels is consistent with synergism occurring at the transcriptional level. These studies were conducted in media containing unstripped calf serum, and TAM and AT each have anti-estrogenic properties, suggesting the possible involvement of estrogen in these effects. 17 β -Estradiol upregulates Bcl-2 transcription in MCF-7 cells, and binding of the ER to two *dis*-acting elements in the Bcl-2 coding region can result in an additive or synergistic stimulation of Bcl-2 gene expression (Perillo *et al.* 2000). Estradiol suppressed the synergistic stimulation of apoptosis by

Figure 8 Effect of estradiol on cell growth and apoptosis by TAM, AT, and TGF β 1. Cells were incubated in phenol red-free media supplemented with charcoal-stripped calf serum. (top panel) Medium was supplemented with TAM (100 nM) and/or AT (1 μ M) and the indicated concentrations of 17 β -estradiol, and cell counts were determined at day 7 (* $P < 0.05$ vs respective AT, TAM). (middle panel) Medium was supplemented with AT (1 μ M) and/or rhTGF β 1 (TGF; 5 ng/ml) and the indicated concentrations of 17 β -estradiol, and cell counts were determined at day 7. (bottom panel) Medium was supplemented with TAM (100 nM) and/or AT (1 μ M) and/or rhTGF β 1 (TGF; 5 ng/ml) and estradiol (0.5 nM) and apoptosis measured at day 7 by DNA fragmentation ELISA as described in Materials and Methods. * $P < 0.05$ vs control.

Figure 9 Effect of anti-TGF β antibody on TAM+AT downregulation of Bcl-2 protein and stimulation of apoptosis. Cells were incubated with vehicle (CONT) or TAM (100 nM)+AT (1 μ M) and either anti-TGF β antibody (AB) or normal IgG antibody (NL). (Top panel) Bcl-2 protein expression was determined by immunoblot analysis on day 4 and day 7. * P <0.01 vs CONT-NL, CONT-AB, day 4; # P <0.01 vs CONT-NL, CONT-AB, day 7. (Bottom panel) Apoptosis was measured on day 7 by DNA fragmentation ELISA, and values expressed as described in Fig. 2. * P <0.01 vs CONT-NL, CONT-AB.

TAM+AT, suggesting a potential role of the ER in regulating these effects.

The finding that Bcl-2 plays an important role in the synergism of TAM+AT suggests a new convergence point for the action of these agents. Evidence has been presented by others that there are convergence points for retinoids and anti-estrogens in both the cell cycle pathway and the apoptotic pathway. Wilcken *et al.* (1996) studied the effects of AT and the anti-estrogen ICI 164,348 on human breast cancer cells (T47D) and found that both agents inhibited cell cycle progression in the G₁ phase. They found, however, that both AT and the anti-estrogen reduced the amount of hyperphosphorylated pRb within 48 h, indicating that pRb was a point of convergence for the mechanism of action of AT and anti-estrogen. The

present report expands these findings by indicating a second point of convergence for TAM and AT in the apoptotic pathway at the level of Bcl-2 mRNA and Bcl-2 protein. The Bcl-2 oncoprotein inhibits both cell cycle progression (Mazel *et al.* 1996) and apoptosis (Korsmeyer 1992), and can be modulated by exogenous substances (Wang & Phang 1996). Bcl-2 is closely linked to pRb, with overexpression of Bcl-2 blocking pRb dephosphorylation (Wang *et al.* 1996). The finding that TAM and AT downregulated Bcl-2 provides a potential mechanism for reinforcing pRb dephosphorylation by these agents, further promoting inhibition of cell cycle progression. In addition, modulation of Bcl-2 would indicate a convergence point for TAM and AT upstream to that of pRb.

The possibility that TAM+AT were acting in an indirect manner to stimulate apoptosis through regulation of TGF β secretion was studied. TAM+AT acted synergistically to stimulate TGF β secretion. TGF β 1 was the principle isomer secreted by these cells, and was the isomer whose secretion was most enhanced by TAM+AT. AT was found to act synergistically with TGF β 1 to inhibit growth, downregulate Bcl-2 mRNA and protein expression, and stimulate DNA fragmentation. This was temporally equivalent to that for TAM+AT, had a comparable dose-response relationship for AT, and involved comparable downregulation of Bcl-2 protein and mRNA expression and stimulation of apoptosis. The synergism of TGF β 1+AT on apoptosis was also suppressed by estradiol. Together, these findings suggested that TGF β might be mediating the synergistic action of TAM+AT on apoptosis through its interaction with AT. Co-incubation of TAM+AT with anti-TGF β antibody, however, did not block TAM+AT downregulation of Bcl-2 protein or block stimulation of apoptosis, indicating that the synergism of TAM+AT occurs independently of TGF β . The neutralizing antibody was shown to completely neutralize the concentration of active TGF β in the media, and the antibody was present throughout the 7 days of incubation, providing sustained neutralizing capacity. The possibility that incomplete neutralization was because the antibody could not gain access to all of the TGF β , such as if some were sequestered between the cells and the culture dish as proposed by Tobin *et al.* (2001) for other studies, thus seems unlikely. Studies from this laboratory have shown that approximately 75% of TGF β secreted by these MCF-7 cells is in the latent form and 25% in the active form (Danforth & Sgagias 1996). The degree to which the latent form might interfere with neutralization of the endogenous active form by the neutralizing antibody is not known. This possibility notwithstanding, the findings indicate that two parallel anti-proliferative processes, TAM+AT and TGF β 1+AT, are in effect and act without apparent cross-over. The mechanism of synergism for each remains to be defined; however, Bcl-2 transcription will be an important focal point for future studies.

In conclusion, TAM+AT and TGF β +AT represent two important means for enhancing growth inhibition of breast cancer cells. AT may allow increased efficacy of TAM, a widely used agent in the prevention and management of breast cancer. Both AT and TGF β are naturally occurring agents, TGF β is secreted by a variety of cell types including both epithelial and stromal breast cancer cells, and plasma levels of both substances have been well documented in breast cancer patients. The synergism between AT and TGF β 1 represents an important anti-proliferative mechanism with the potential to inhibit breast cancer cell growth at the primary, local/regional, or distant sites.

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