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QUARTERLY

Can transforming growth factor-β_1 and retinoids modify the activity of estradiol and antiestrogens in MCF-7 breast cancer cells?

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Retinoic acid and transforming growth factor-β (TGF-β) affect differentiation, proliferation and carcinogenesis of epithelial cells. The effect of both compounds on the proliferation of cells of the hormone sensitive human breast cancer cell line (ER+) MCF-7 was assessed in the presence of estradiol and tamoxifen. The assay was based on [^{3}H]thymidine incorporation and the proliferative activity of PCNA- and Ki 67-positive cells. The apoptotic index and expression of the Bcl-2 and p53 antigens in MCF-7 cells were also determined. Exogenous TGF-β_1 added to the cell culture showed antiproliferative activity within the concentration range of 0.003–30 ng/ml. Irrespective of TGF-β_1 concentrations, a marked reduction in the stimulatory action of estradiol (10^{-9} and 10^{-8} M) was observed whereas in combination with tamoxifen (10^{-7} and 10^{-6} M) only 30 ng/ml TGF-β_1 caused a statistically significant reduction to aproximately 30% of the proliferative cells. In further experiments we examined the effect of exposure of breast cancer cells to retinoids in combination with TGF-β_1. The incorporation of [^{3}H]thymidine into MCF-7 cells was inhibited to 52 ± 19% (control =100%) by 3 ng/ml TGF-β_1, and this dose was used throughout. It was found that addition of TGF-β_1 and isotretinoin to the culture did not decrease proliferation, while TGF-β_1 and tretinoin at low concentrations (3×10^{-8} and 3×10^{-7} M) reduced the percentage of proliferating cells by aproximately 30% (67±8% and 67±5%, $P < 0.05$ compared to values in the tretinoin group). Both retinoids also led to a statistically significant decrease in the stimulatory effect of 10^{-9} M estradiol, attenuated by TGF-β_1. In addition, the retinoids in combination with TGF-β_1 and tamoxifen

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Abbreviations: E_2, 17-β-estradiol; ER, estrogen receptor; PCNA, proliferating cell nuclear antigen; TAM, tamoxifen; TGF-β_1, transforming growth factor-β_1.

(10^{-6} M) caused a further reduction in the percentage of proliferating cells. Immunocytochemical analysis showed that all the examined compounds gave a statistically significant reduction in the percentage of cells with a positive reaction to PCNA and Ki 67 antigen. TGF-β_1, isotretinoin and tretinoin added to the culture resulted in the lowest percentage of PCNA positive cells. However, the lowest fraction of Ki 67 positive cells was observed after addition of isotretinoin. The obtained results also confirm the fact that the well-known regulatory proteins Bcl-2 and p53 play an important role in the regulation of apoptosis in the MCF-7 cell line, with lowered Bcl-2 expression accompanying easier apoptotic induction. The majority of the examined compounds act *via* the p53 pathway although some bypass this important proapoptotic factor.

Retinoic acid controls cell differentiation, proliferation and carcinogenesis. The mechanism of its action is based mainly on the activation of RAR and RXR receptors (Chambon, 1995) and on the action of CRBP I and CRABP II proteins, which are involved in retinoid autoregulation (Smith *et al.,* 1991; Durand *et al.,* 1992). The RAR and RXR receptors share sequence homology with other members of the steroid receptor superfamily, such as estrogen or vitamin A receptors. Retinoids complexed to their receptor can activate or repress transcription from retinoic acid response elements in the promoters of retinoid-sensitive genes. It is known that RARs and RXRs can form homodimers or heterodimers and RXRs heterodimerize with multiple members of the steroid receptor superfamily. This heterodimerization plays an important role in the regulation of the nuclear receptor-dependent signaling pathways.

The mechanism of retinoid-induced cell death is not yet well understood. Apoptosis can be induced in a number of human cancer cell lines, including breast cancer cells (Sheikh *et al.,* 1995). The mechanisms likely to stimulate apoptosis include p21 induction (Shao *et al.,* 1995; Liu *et al.,* 1996), AP-1 complex induction (Schadendorf *et al.,* 1996), suppression of Bcl-2 expression and/or TGF-β induction (Roberts & Sporn, 1992), and IGF-3 induction (Gucev *et al.,* 1996). The expression of RAR and ER (estrogen receptors) is highly correlated in breast cancer, and the growth of many ER(+) breast cancer cell lines is arrested by retinoic acid. Moreover, retinoic acid can also induce apoptosis in breast cancer cells (Nagy *et al.,* 1995). It was shown that in the ER(+) MCF-7 cell line retinoid acid decreases the Bcl-2 level (Gross *et al.,* 1999) and down-regulates cyclin D1 and CDK2 protein levels and kinase activity (Teixeira & Pratt, 1997). On the other hand, expression of cyclin D1 sensitizes ER(+) breast cancer cells to retinoic acid-induced mitochondrial death pathway through Bax activity, cytochrom *c* release and caspase-9 cleavage (Niu *et al.,* 2001). The effect of retinoic acid in the cell is regulated by peptide growth factors, with the major role played by the TGF-β family. Practically all cells have functional receptors for these peptides, which explains their unique regulatory role both in physiological and pathological processes (Sporn & Roberts, 1991).

Mammals have three known TGF-β isoforms, defined as TGF-β-1, -2 and -3 (Barnard *et al.,* 1990; Sporn & Roberts, 1992). In *in vitro* conditions, the major role of the TGF-β cytokines is to inhibit the growth of epithelial cells and thus they can strongly affect the growth of many neoplasms of epithelial origin (Reiss & Barcellos-Hoff, 1997). In carcinogenesis, they play a dual role depending on the response of neoplastic cells to these polypeptides. Transformable cells are sensitive to TGF-β-dependent arrest of the cell cycle. In the early phase of carcinogenesis TGF-β play a suppressive function and are able to affect the reaction of antiestrogens and retinoids. However, during the neoplastic process, when the population of transformable cells consists of TGF-β-resistant mutated

cells, an environment rich in bioactive TGF-β selectively promotes the expression of these cells (Reiss, 1999). It has been shown that this can be related to specific genetic changes (Markowitz *et al.,* 1995) and defects of receptors for TGF-β (Birchenall-Roberts *et al.,* 1995; Kim *et al.,* 1997).

As summarized above, the TGF-β family modulates the action of retinoids. Since neoplasm treatment is still a problem, new ways of potential treatment in oncological therapeutic schemes are badly needed. The aim of the study was to investigate whether exogenous TGF-β_1 and retinoids can affect the response of the human breast cancer MCF-7 cell-line to estradiol and tamoxifen. Effective neoplastic therapy involves also the apoptosis pathway induction in cancer cells and thus it appears important to search for pro- and antiapoptotic factors regulating this process.

MATERIALS AND METHODS

Materials. 13-*cis* retinoic acid (Isotretinoin), all-*trans* retinoic acid (Tretinoin), tamoxifen (citrate salt tamoxifen), 17-β-estradiol (1,3,5[10]-estratriene-3,17-β-diol) and transforming growth factor-β_1 (TGF-β_1) were obtained from Sigma (St. Louis, MO, U.S.A). The following antibodies: bcl-2 oncoprotein: monoclonal mouse antibody (clone 124), chromosomal translocation t (14,18); p53 protein: monoclonal mouse antibody (clone DO-7); PCNA – proliferating cell nuclear antigen: monoclonal mouse antibody (clone PC 10) and Ki 67: monoclonal mouse antibody (clone Ki 67) were obtained from Dako (Glostrup, Denmark).

Preparation of chemicals. Isotretinoin and tretinoin were diluted in ethyl alcohol and then in the culture medium, to final concentrations of $3 \times 10^{-8} - 3 \times 10^{-3}$ M. Tamoxifen and 17-β-estradiol were added to the culture at a concentration of 10^{-7} or 10^{-6} M and 10^{-9} or 10^{-8} M, respectively. Transforming growth factor-β_1 was diluted in the culture medium to final concentrations of 0.0003–30 ng/ml. In the later phases of the experiment its concentration was 3 ng/ml.

Culture of cell line MCF-7. The study was carried out on the hormone sensitive cell line (ER+) MCF-7 of human breast cancer (American Type Culture Collection, Rockville, MD) in DMEM medium (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% FBS (Sigma, St. Louis, MO, U.S.A.), 50 μg/ml streptomycin, and 100 U/ml penicillin in 75 cm^2, plastic flasks (Nunc, Roskilde, Denmark), at 37°C, in a humid incubator with 5% CO_2/95% air. The cell line was passaged once a week. The cells for the experiment were obtained from passages 3–7 and inoculated in 24-well plates (Nunc, Roskilde, Denmark) at 5×10^4 cells/well and grown to 85% confluence in Dulbecco's modified Eagle's medium (DME/F12, Sigma, St. Louis, MO, U.S.A.) supplemented as above. During the experiments, cells were detached with 0.05% trypsin/0.02% EDTA (Sigma, St. Louis, MO, U.S.A.).

Experiments were conducted in plates in DME/F12 Ham (Sigma, St. Louis, MO, U.S.A.), supplemented with a synthetic substitute of CPSR-1 serum (Sigma, St. Louis, MO, U.S.A.). Incubation of the MCF-7 cells with the examined substances was performed for 24 h.

[^{3}H]thymidine incorporation. Cell proliferation in the culture was assessed based on incorporation of [^{3}H]thymidine (Amersham, U.K., specific activity 925 GBq/mmol), after incubation of the cell culture in the medium with or without the examined substances. Two hours prior to the termination of the experiment, [^{3}H]thymidine was added to the culture at 18.8 KBq/well. After 2–3 washings of the culture with cold phosphate buffer, trypsinisation and precipitation (3 washings with 10% trichloroacetic acid), the precipitate was flooded with Instagel scintillation fluid (Packard, Groningen, The Netherlands). Radioactivity was expressed in dpm per well.

Immunocytochemical examinations. Immunocytochemical examinations were carried out in chambers for histochemical exami-

nations (Lab-tek 4 well chamber slide, Nunc, Naperville, IL, U.S.A.). Cell material was fixed with cytofix (Cytofix, Merck, Darmstadt, Germany). A 2-step streptavidin-biotin LSAB kit + HRP kit (with horse-radish peroxidase) was used for detection. The antigen-antibody reaction was visualized with the chromogen DAB (diaminobenzidine). The results were presented as the percentage of immuno-positive cells in the culture (at 10^3 cells/sample).

Determination of apoptotic index. Determination of cell viability and analysis of apoptotic and necrotic cells were based on a 72-h culture. Staining was performed with the method of Wright-Giemsa, using a Fisher Leuko Stat kit. MCF-7 cells, cultured in 6-well plates (Nunc, 5×10^4 cells/well), were stained with 10 mM acridine orange and 10 nM ethidium bromide following apoptosis induction. After removal of the medium the cells were detached with 0.05% trypsin and 0.02% EDTA for 1 min, and rinsed. Cell suspension (250 μl) was mixed with 10 μl of acridine-ethidinum mixture, and 200 cells/sample were examined in a fluorescence microscope (Nikon) for live cells with normal nuclei, live cells with apoptotic nuclei, necrotic cells with normal nuclei, and necrotic cells with apoptotic nuclei.

Statistical analysis. In all the experiments, mean values ± standard deviation (S.D.) for 4 measurements of each parameter were calculated. The Mann-Whitney test was used to perform statistical analysis.

RESULTS

The effect of exposure of breast cancer MCF-7 cells to TGF-β_1 and TGF-β_1 in combination with 17-β-estradiol or with tamoxifen on the incorporation of [^{3}H]thymidine

In the present study exogenous TGF-β_1 at 0.003–30 ng/ml showed antiproliferative activity. Simultaneous addition of TGF-β_1 and estradiol caused a statistically significant reduction in the percentage of proliferating cells. For the increasing concentrations of TGF-β_1, 3 and 30 ng/ml, and estradiol at 10^{-8} M, it was 112.2 ± 6.5% and 84.6 ± 6.8% compared to the 123.5 ± 6.4% in the 10^{-8} M estradiol group ($P < 0.05$), whereas for 0.3, 3 and 30 ng/ml TGF-β_1 and 10^{-9} M estradiol it was 112.3 ± 9.7%, 106.1 ± 6.5% and 40.7 ± 5.4%, compared to the 189.2 ± 62.6% in the 10^{-9} M estradiol group ($P < 0.05$) (Fig. 1). Moreover, only 30 ng/ml TGF-β_1 in combination with tamoxifen (10^{-7} or 10^{-6} M) caused a statistically significant reduction in the percentage of proliferating cells to 38.2 ± 6.7% and 33.6 ± 6.9%, respectively, compared to the 86.8 ± 28.8% in 10^{-7} M tamoxifen group and 51.1 ± 4.0% in 10^{-6} M tamoxifen group ($P < 0.05$) (Fig. 2).

The effect of exposure of breast cancer MCF-7 cells to 13-*cis* retinoic acid (isotretinoin) and all-*trans* retinoic acid (tretinoin) in combination with TGF-β_1, 17-β-estradiol or tamoxifen on the incorporation of [^{3}H]thymidine

The incorporation of [^{3}H]thymidine into MCF-7 cells was inhibited by 3 ng/ml TGF-β_1 to 51.5 ± 18.8% (control = 100%), and this was the dose used in our experiment with retinoids.

Simultaneous addition of TGF-β_1 and isotretinoin did not decrease proliferation, while TGF-β_1 and tretinoin at low concentrations (3×10^{-8} and 3×10^{-7} M) reduced the proliferating cells by approximately 30% (66.7 ± 8.4% and 67.1 ± 5.5%, respectively, $P < 0.05$), compared to the values in the tretinoin group (Figs. 3, 5). The stimulatory effect of 10^{-9} M estradiol, attenuated by TGF-β_1, was statistically significantly decreased by 3×10^{-4} or 3×10^{-3} M isotretinoin (41.9 ± 7.2% and 3.0 ± 0.1%, $P < 0.05$), and 3×10^{-6} or 3×10^{-3} M tretinoin (80.8 ± 9.4% and 1.3 ± 0.4%, $P < 0.05$), (Figs. 3, 5).

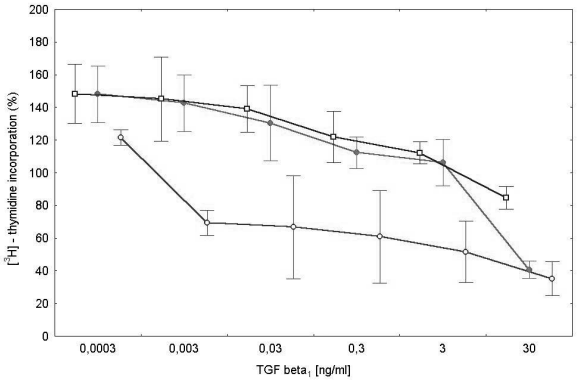

Figure 1. Influence of TGF-β_1 and TGF-β_1 combined with estradiol (E_2) on [^{3}H]thymidine incorporation into MCF-7 breast cancer cells.

Exposure time 24 h. Symbols: (○) TGF-β_1 and no other additions; (●) TGF-β_1 + E_2 (10^{-9} M); (□) TGF-β_1 + E_2 (10^{-8} M).

During 24 h incubation of MCF-7 cells in the presence of TGF-β_1 and at a pharmacological concentration of tamoxifen (10^{-6} M), the percentage of proliferating cells was increased (78.8 ± 7.7%). This percentage was reduced in a statistically significant manner when at the same time retinoids were added to the culture. The concentrations of retinoids which decreased the proliferation of cells in the

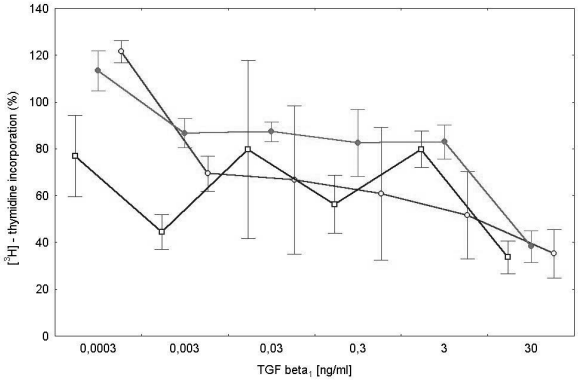

Figure 2. Influence of TGF-β_1 and TGF-β_1 combined with tamoxifen (TAM) on [^{3}H]thymidine incorporation into MCF-7 breast cancer cells.

Exposure time 24 h. Symbols: (○) TGF-β_1 and no other additions; (●) TGF-β_1 + TAM (10^{-7} M); (□) TGF-β_1 + TAM (10^{-6} M).

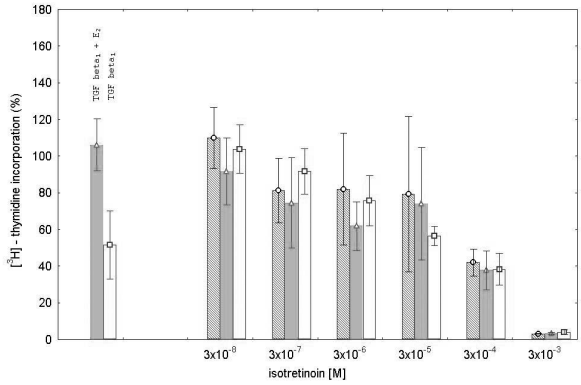

Figure 3. Influence of isotretinoin, isotretinoin combined with TGF-β_1, and isotretinoin combined with TGF-β_1 and estradiol on [^{3}H]thymidine incorporation into MCF-7 breast cancer cells.

Exposure time 24 h. Symbols: open bars, isotretinoin and no other additions; filled bars, isotretinoin + TGF-β_1 (3 ng/ml); hatched bars, isotretinoin + TGF-β_1 (3 ng/ml) + E_2 (10^{-9} M).

presence of TGF-β_1 and estradiol were also effective with TGF-β_1 and tamoxifen, although the decrease in case of combination of TGF-β_1 with tamoxifen was lower. 3×10^{-8} M

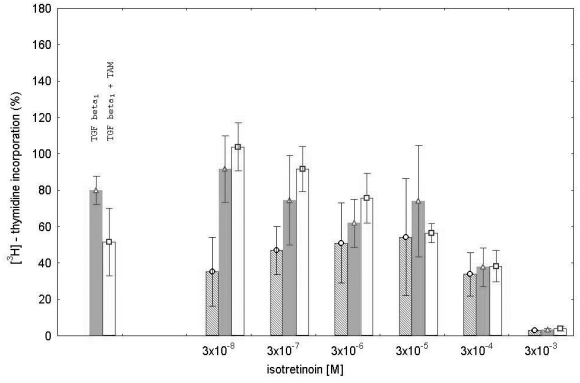

Figure 4. Influence of isotretinoin, isotretinoin combined with TGF-β_1, and isotretinoin combined with TGF-β_1 and tamoxifen on [^{3}H]thymidine incorporation into MCF-7 breast cancer cells.

Exposure time 24 h. Symbols: open bars, isotretinoin and no other additions; filled bars, isotretinoin + TGF-β_1 (3 ng/ml); hatched bars, isotretinoin + TGF-β_1 (3 ng/ml) + TAM (10^{-6} M).

isotretinoin was the lowest concentration showing an inhibitory effect (35.2 ± 18.8%, $P < 0.05$), (Figs. 4, 6).

Determination of the expression of PCNA, Ki 67, Bcl-2 and p53 antigens in MCF-7 cells

Following a 24-h culture of MCF-7 cells in the presence of TGF-β_1 or isotretinoin, the percentage of PCNA-positive cells was the lowest, compared to control (35.7 ± 3.7% and 38.7 ± 3.2%). Combination of the examined compounds with TGF-β_1 did not cause further reduction.

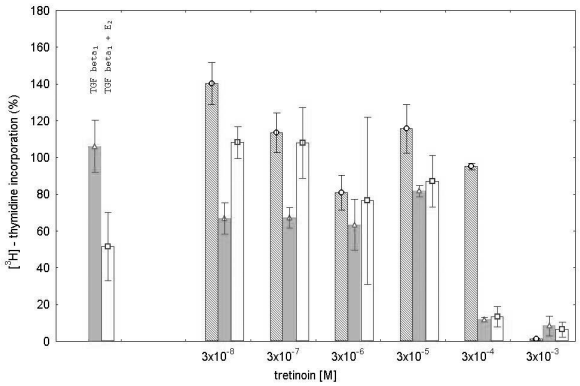

Figure 5. Influence of tretinoin, tretinoin combined with TGF-β_1, and tretinoin combined with TGF-β_1 and estradiol on [^{3}H]thymidine incorporation into MCF-7 breast cancer cells.

Exposure time 24 h. Symbols: open bars, tretinoin and no other additions; filled bars, tretinoin + TGF-β_1 (3 ng/ml); hatched bars, tretinoin + TGF-β_1 (3 ng/ml) + E_2 (10^{-9} M).

The exposure of the culture to isotretinoin resulted in a reduction in the percentage of Ki 67-positive cells to 38.7 ± 2.9%, i.e., the lowest value (Table 1).

The incubation of the MCF-7 cells in the presence of any of the examined compounds alone or in combinations caused a distinct and statistically significant decrease in the percentage of Bcl-2 positive cells (Table 1).

The percentage of p53 positive cells was the highest when both isotretinoin and TGF-β_1 were simultaneously added to the culture (Table 1).

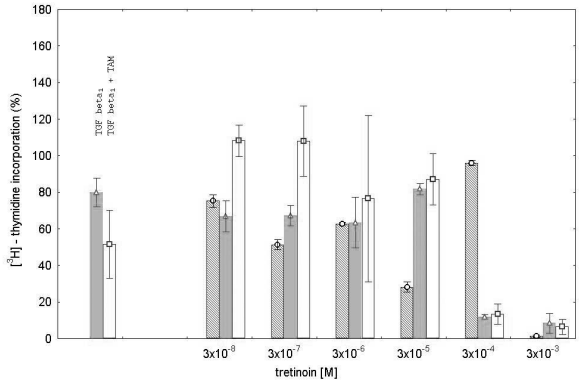

Figure 6. Influence of tretinoin, tretinoin combined with TGF-β_1, and tretinoin combined with TGF-β_1 and tamoxifen on [^{3}H]thymidine incorporation into MCF-7 breast cancer cells.

Exposure time 24 h. Symbols: open bars, tretinoin and no other additions; filled bars, tretinoin + TGF-β_1 (3 ng/ml); hatched bars, tretinoin + TGF-β_1 (3 ng/ml) + TAM (10^{-6} M).

Determination of the apoptotic index

The highest percentage of apoptotic cells was obtained in media containing isotretinoin and a combination of isotretinoin with TGF-β_1 (35.9 ± 4.5% and 33.0 ± 2.3%). Only 12.0 ± 4.9% were found to be apoptotic after culture supplementation with TGF-β_1 alone (Table 2).

DISCUSSION

The TGF-β cytokines affect a number of cell functions, including proliferation, differentiation, migration and adhesion (Massaque *et al.,* 1992), *via* type I and II receptors (TGF-βRI and TGF-βRII), which belong to a large family of receptor serine/threonine kinases (Attisan *et al.,* 1990). TGF-β inhibits pro-

liferation of epithelial cells, including neoplastic ones (Kalkhoven *et al.,* 1995), although this effect is sometimes dominated by promotion of oncogenes and induction of carcinogenes (Pierce *et al.,* 1995). In hormone sensitive breast neoplasms growth factors and steroid hormones are released and co-operate in complex regulation of cell functions. The action of TGF-β in cell culture depends on the type of cells, general conditions of the culture and the presence of other polypeptide growth factors (Desruisseau *et al.,* 1996; Hietanen *et al.,* 1998; Cupp *et al.,* 1999; Hishikawa *et al.,* 1999). The MCF-7 cells produce the receptor proteins TGF-βI and TGF-βII (Jakowlew *et al.,* 1997), and hence exogenous TGF-β_1 can inhibit proliferation of these cells in a dose-dependent manner (Lafon *et al.,* 1995) ten times more efficiently than TGF-β_2 (Arric *et al.,* 1990). It was shown that exogenous TGF-β_1 at 0.1–1.0 ng/ml inhibited proliferation of epithelial neoplasms of the vagina (Hietanen *et al.,* 1998). When added to a culture of human prostatic cancer cells, it inhibited proliferation in 40% (Desruisseau *et al.,* 1996). Although early studies of Karey *et al.* (1988) did not confirm a significant effect of exogenous TGF-β_1 at $\leq 20.0\ \mu$g/ml on MCF-7 cell culture (Karey & Sirbascu, 1988), we found an inhibitory effect of this cytokine on the incorporation of [^{3}H]thymidine and proliferation of the examined cell line.

Table 1. Percentage of PCNA, Ki 67, Bcl-2 and p53 positive MCF-7 breast carcinoma cells

Group	PCNA	Ki 67	Bcl-2	p53
Control	84.5 ± 7.1	98.3 ± 2.1	25.0 ± 5.0	27.2 ± 1.7
TGF-β_1	35.7 ± 3.7 $P < 0.0001$	67.5 ± 1.7 $P < 0.0001$	6.7 ± 1.8 $P < 0.0005$	40.5 ± 2.3 $P < 0.0001$
Isotretinoin	38.7 ± 3.2 $P < 0.0001$	38.7 ± 2.9 $P < 0.0001$	6.1 ± 1.2 $P < 0.0004$	40.2 ± 2.5 $P < 0.0001$
Isotretinoin + TGF-β_1	49.0 ± 0.8 $P < 0.0001$	64.2 ± 3.7 $P < 0.0003$	16.0 ± 2.1 $P < 0.02$	50.5 ± 3.1 $P < 0.0001$
Tretinoin	44.2 ± 3.2 $P < 0.0001$	49.7 ± 2.1 $P < 0.0001$	10.3 ± 3.0 $P < 0.001$	28.1 ± 3.1 n.s.
Tretinoin + TGF-β_1	57.1 ± 1.2 $P < 0.004$	61.0 ± 1.0 $P < 0.0001$	9.7 ± 2.1 $P < 0.001$	37.6 ± 2.2 $P < 0.0005$
Tamoxifen	49.5 ± 1.5 $P < 0.0001$	50.0 ± 2.2 $P < 0.0003$	5.5 ± 0.8 $P < 0.0001$	30.1 ± 1.1 n.s.
Tamoxifen + TGF-β_1	58.2 ± 1.7 $P < 0.004$	66.3 ± 3.1 $P < 0.0001$	5.9 ± 0.7 $P < 0.0003$	41.2 ± 2.0 $P < 0.0001$

3×10^{-5} M isotretinoin, 3×10^{-5} M tretinoin, TGF-β_1 (3 ng/ml) and 10^{-5} M tamoxifen; exposure time 24 h. Data presented as mean values ± S.D. (n = 4); statistically significant differences relative to the control group; n.s., statistically not significant.

Sun *et al.* (1994) reported that a low level of expression of TGF-βRI or TGF-βRII may limit the response of neoplastic cells to exogenous TGF-β (Sun *et al.,* 1994), and in the case of TGF-β_1, this depends on a decrease in type II receptor expression (Liu *et al.,* 2000). According to Liu *et al.* (2000) the sensitivity of MCF-7 cells to exogenous TGF-β_1 may also depend on the number of passages.

It has been proven that the antiproliferative action of TGF-β_1 does not block the activity of estrogen receptors (Perry *et al.,* 1995; Stoica *et al.,* 1997), but inhibits the proliferation of

MCF-7 cells through inhibition of the cell cycle in phase G_1. This is due to a decrease in the activity of Cdk2 kinase without Cdk protein modification and cyclin expression, and is correlated with an increase in the accumulation of p21WAF in the cell nucleus (Mazars *et al.*, 1995).

and that their chemopreventive action *in vitro* occurs *via* local activation of TGF-β. The antiestrogens and retinoids (all-*trans* retinoic acid) can reverse (*via* a TGF-β mediated pathway) the repressing effect of estrogen on *AIB1* gene expression in the cell line MCF-7. This may be of significance in cancer progression (Lau-

Table 2. Influence of isotretinoin, tretinoin and TGF-β_1 on apoptosis in MCF-7 breast carcinoma cells.

	Control	TGF-β_1	Isotretinoin	Isotretinoin + TGF-β_1	Tretinoin	Tretinoin + TGF-β_1	Tamoxifen	Tamoxifen + TGF-β_1
Viable cells (%)	93.2 ±1.5	80.7 ±4.3 $P <$ 0.002	56.8 ±2.0 $P <$ 0.0001	8.2 ± 3.7 $P <$ 0.0001	72.5 ±2.3 $P <$ 0.0003	70.5 ±2.7 $P <$ 0.0003	68.5 ± 6.2 $P <$ 0.0001	67.7 ± 4.3 $P <$ 0.0001
Apoptotic cells (%)	1.8 ±0.8	12.0 ±4.9 $P <$ 0.007	35.9 ±4.5 $P <$ 0.0001	33.0 ± 2.3 $P <$ 0.0001	16.7 ±2.0 $P <$ 0.0001	20.2 ±2.2 $P <$ 0.0001	30.7 ± 6.1 $P <$ 0.0001	31.5 ± 2.5 $P <$ 0.0001
Necrotic cells (%)	4.9 ±1.9	8.5 ± 2.3 n.s.	8.5 ± 2.3 n.s.	8.7 ± 2.3 n.s.	12.5 ±4.4 $P <$ 0.02	10.7 ±1.5 $P <$ 0.02	2.0 ± 0.8 n.s.	2.5 ± 0.5 n.s.

100% = viable (%) + apoptotic (%) + apoptotic/necrotic (%) + necrotic (%) cells. 3 x 10^{-5} M isotretinoin, 3 × 10^{-5} M tretinoin, TGF-β_1 (3 ng/ml) and 10^{-5} M tamoxifen; exposure time 72 h. Data presented as mean values ± S.D. (n = 4); statistically significant differences relative to the control group; n.s., statistically not significant.

We observed a significant decrease in the stimulatory effect of estradiol in the presence of TGF-β_1, although the findings of Stewart *et al.* (1992) suggested that exogenous TGF-β added to the culture only slightly reduced the estradiol-induced growth of cell line MCF-7. While tamoxifen used in a pharmacological dose effectively inhibited MCF-7-cell proliferation (Perry *et al.,* 1995), it had no significant effect in the presence of TGF-β_1 at or below 3 ng/ml.

Retinoids and SERM's (selective estrogen receptor modulators) are the most widely used factors in chemoprevention and treatment of neoplastic diseases. It has been shown that retinoids, like tamoxifen, increase the production and activation of TGF-β in cell cultures (Knabbe *et al.,* 1987; Glick *et al.,* 1989; Colletta *et al.,* 1990; Koli *et al.*, 1997), including breast cancer cell lines,

ritsen *et al.,* 2002). *In vivo* studies, however, demonstrated no significant effect of tamoxifen and 9-*cis* retinoic acid on the expression of TGF-β in mammary ductal epithelium or periductal stroma (Zujewski *et al.,* 2001), although Beenken *et al.* (2002) suggested that the TGF cytokines take part in the protective effect of 13-*cis* retinoic acid in dysplastic oral leukoplakia. Our previously published findings confirmed the inhibitory effect of the examined retinoids *in vitro* on the proliferation of breast cancer MCF-7 cells in a dose-dependent manner (Czeczuga- Semeniuk *et al.,* 2001). The cytokines (IFN-α, INF-γ, TNF-α, TGF-β, INF-β) used in experimental cultures of various transformed cell lines differ in their antiproliferative action (Frey *et al.,* 1991; Bollag *et al.*, 1992; Coradini *et al.,* 1997). In the presence of retinoids, TGF-β and TNF-α inhibit MCF-7 cell proliferation most effectively, causing a

synergistic effect (Bollag *et al.,* 1992). In our study, it was shown that TGF-β_1 with low concentrations of retinoid were the most effective combination and exogenous TGF-β_1 only increased the antiproliferative effect of low tretinoin concentrations, which may play a role in the regulation of MCF-7 cell growth. In the study of Bollag *et al.* (1992), statistically significant inhibition of proliferation was observed after simultaneous application of TGF-β_1 at 3 ng/ml and tretinoin at 3×10^{-6} M (MTT method). When evaluating the staining of PCNA- and Ki 67-positive cells, we found that the combination of TGF-β_1 with the examined compounds did not reduce the percentage of positive cells in the respective groups.

Estrogens can control cell proliferation through an effect on TGF-β receptor expression (Massaque *et al.,* 1992). Estradiol stimulates growth of hormone sensitive breast cancers mainly through the reduction in TGF-β_2 and TGF-β_3 mRNA expression, but does not affect TGF-β_1 mRNA expression (Arric *et al.,* 1990; Jeng *et al.,* 1993). Addition of 17-β-estradiol to the culture containing exogenous TGF-β_1 and retinoids had a significant effect on inhibition of proliferation of breast cancer MCF-7 cells mainly at high concentrations of retinoids. Since retinoids, unlike estradiol, increase the expression of TGF-β_1 and TGF-β_2 (Cupp *et al.,* 1999), their addition to the culture may promote the action of exogenous TGF-β_1.

Although tamoxifen increased the expression of TGF-β receptors in the MCF-7 cell line (Koli *et al.,* 1997) and secretion of the respective proteins (Knabbe *et al.,* 1987), and when added to the culture medium caused a fourfold increase in TGF-β_1 activity (Chen *et al.*, 1996), we observed its inhibitory effect in the presence of TGF-β_1 and low concentrations of isotretinoin and threefold higher concentration of tretinoin. Retinoic acid can also induce secretion and activation of TGF-β_1 or TGF-β_2 by epithelial cells (Glick *et al.,* 1989; Danielpour, 1996). It seems interesting that in the presence of 3×10^{-4} M tretinoin, both estradiol and tamoxifen increased proliferation to 95%.

Apoptosis can be induced by a number of factors, and its regulation in neoplastic cells is still not fully elucidated. It has been shown based on the MCF-7 cell model that retinoids can induce programmed cell death (Toma *et al.,* 1997; Mangiarotti *et al.,* 1998; Czeczuga-Semeniuk *et al.,* 2001; Niu *et al.*, 2001). TGF-β behaves in a similar way, but the reaction is partly mediated by CTGF (Hishikawa *et al.,* 1999). The effect of tamoxifen on this process may occur through the secretion of active TGF-β (Chen *et al.,* 1996). We observed the largest number of cells with morphological features typical of apoptosis, i.e., condensation and fragmentation of chromatin and cytoplasm vacuolization, after application of isotretinoin alone or in combination with exogenous TGF-β_1. However, in case of the combination of isotretinoin with TGF-β_1, the over twofold increase in the percentage of Bcl-2-positive cells is difficult to explain, since of all cell proteins Bcl-2 can most strongly inhibit apoptosis induced by numerous physiological and pathological factors (Boise *et al.,* 1993). The lack of a significant increase in p53 expression in MCF-7 cells in the presence of tretinoin and tamoxifen may suggest that p53 does not take part in the apoptotic pathway induced by these factors.

TGF-β_1 affects diverse cellular processes and its action can be modified by hormones and other growth factors involved in proliferation, differentiation and apoptosis. Also, a crosstalk between epithelial cells and stromal fibroblasts of the mammary gland should be considered. Our findings are difficult to interpret, but they seem to suggest that the examined compounds used in combination may be more effective in the treatment of breast cancer than monotherapy. The increased efficacy may be due to retinoic acid ability to modulate angiogenesis in the tumor microenvironment through downregulation of TGF-β_1 secretion (Liss *et al.,* 2002) and indi-

cate that the loss of cell response to retinoids and TGF-β may play a role in cancer progression (Hietanen *et al.* 1998).

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