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CELL PROLIFERATION IN DIMETHYLBENZ(A)ANTHRACENE(DMBA)-INDUCED RAT MAMMARY CARCINOMA TREATED WITH ANTIESTROGEN TOREMIFENE

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Cell proliferation during antiestrogen toremifene treatment was studied using the DMBA-induced rat mammary carcinoma model. The volume corrected mitotic index (M/V INDEX) and the S-phase fraction (SPF) determined by flow cytometry (FCM) were used as proliferation markers. Two series of rats (A and B) treated with two dose levels of toremifene were used. The two series of tumors appeared to have different growth properties. In series A the tumors were rapidly growing with high proliferation rate. In this series, toremifene (3 mg/kg for 4 weeks) reduced significantly the mean MV/INDEX, but the slight reduction of the mean SPF was not significant. In series B the tumors grew slowly and had low levels of proliferation markers. One-third of the tumors were spontaneously stable in the untreated group. Higher dose of toremifene was used in this series (12 mg/kg for 4 weeks), and the number of regressing or stable tumors was 58% compared with 31% in series A. Taking into consideration the high number of spontaneously stable tumors in series B, it may be concluded that about one-third of the tumors regressed or remained stable due to toremifene treatment in both series. The reduction of the M/V INDEX was significant only when the regressing treated tumors were compared with the growing controls. The reduction of the SPF was not significant. We think that the M/V INDEX is a more appropriate method to measure cell proliferation than is the SPF in this tumor model, where the tumors are heterogenous and, e.g., spontaneous apoptosis is known to be frequent.

The major growth stimulatory mechanisms of estrogens in their target organs include recruitment of dormant cells to the proliferation cycle, thus increasing the growth fraction, shortening the overall cell cycle time mainly by reducing the average length of the G1 phase and decreasing the cell loss factor (cell death) (1). Antiestrogens, the competitive inhibitors of estrogens, are now used for treatment of all stages of breast cancer (2–4). Tamoxifen, which has been best characterized in this context, decreases the growth fraction and lengthens the G0/G1 phase (5–7) and probably increases the cell death rate in vitro (8, 9). The reduction of the SPF and accumulation of the cells in the G0 and G1 phases have not, however, been demonstrated in vivo (10, 11).

Toremifene is a triphenylethylene compound, a non-steroidal antiestrogen with characteristics close to those of tamoxifen (12, 13). Toremifene has already shown its therapeutic potential in the treatment of advanced human breast cancer (14), with responses similar to tamoxifen, and these two compounds are now considered to be cross-resistant and equally effective in equivalent doses (15, 16). The advantage of toremifene compared with tamoxifen is that toremifene has been better tolerated in higher doses than tamoxifen both in preclinical and clinical studies on breast cancer (17). In addition, toremifene has shown antitumor effect against estrogen receptor negative tumors,

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Table 1

The number of regressing and growing DMBA-induced mammary tumors in untreated control rats and in toremifene treated rats with two different dose levels

	Series A		Series B	
	CONTR	TOR 3 mg/kg	CONTR	TOR 12 mg/kg
Number of rats	5	5	5*	8
Tumors at the start of the treatment	7	7	15	26
Total number of tumors during the experiment	16	16	22	31
Tumors at the end of the treatment	16	14	22	26
Complete regression (CR) during the treatment	0 (0%)	2 (13%)	0 (0%)	5 (16%)
Regressing and stable tumors (including CR)	1 (6%)	5 (31%)	6 (27%)	18 (58%)
New tumors during the treatment	9 (56%)	9 (56%)	7 (32%)	5 (16%)
Growing tumors at the end of treatment (including new tumours)	15 (94%)	11 (69%)	16 (73%)	13 (42%)

*2 rats of the initial 7 were excluded (see text).
CONTR, untreated control rats; TOR, toremifene treated rats

such as human desmoid tumors and experimental murine uterine sarcoma (13, 18). The possibility to use toremifene in high doses may expand the therapeutic potential beyond the classical competitive inhibition of estrongens, and result in stronger antitumor effect (13, 19, 20).

The mechanisms of the antitumor action of antiestrogens are only partially understood (21). There has been some controversy about whether antiestrogens are capable of inducing cell death or only cytostasis (22). Wärri et al. (23) recently showed how toremifene was able to induce apoptosis in MCF-7 human breast cancer cells in vitro, and in vivo when grown in nude mice. In dimethylbenz(a)anthracene(DMBA)-induced rate mammary carcinoma model, toremifene reduces the mitotic activity and modifies the apoptosis, which spontaneously is great in this tumor model (24). Toremifene reduces the SPF in MCF-7 cells in vitro (25), but the effect of toremifene on cell cycle parameters has not been studied in vivo.

We studied the effects of toremifene on cell proliferation with two dose levels using the established DMBA-induced rat mammary carcinoma model (26). In tumors regressing after treatment, the volume corrected mitotic index M/V INDEX (27) and the SPF, were lower, but the grade of the reduction seemed to be dependent on the pre-existing level of proliferation in tumors which are known to be heterogeneous (28).

Material and Methods

Animals and toremifene treatment. Two series of female Sprague-Dawley rats were used in the present study. Mammary carcinomas were induced in 50-day-old rats with 12 mg of dimethylbenz(a)anthracene according to Huggins & Yang (26). In series A, after 46 days from the tumor induction, 5 rats received toremifene 3 mg/kg (low dose) by gastric intubation for 29 days, while 5 rats served as controls and received toremifene vehicle only for the same time. In series B, after 74 days from the tumor induction, 8 rats received toremifene 12 mg/kg (high dose) for 25 days, with 7 control rats. Of the controls in series B, two rats had to be excluded from the final evaluation since one rat did not develop any tumors and one died during the experiment. The animals received standard chow and water under conventional laboratory conditions. The width and the length of the tumors were recorded by weekly palpation, and the volume was calculated according to formula (13):

$$\text{Tumor volume} = \pi \times (\text{width})^2 \times \text{length}/12$$

The tumor growth rate (cm^3/day) was estimated by dividing the difference between the tumor volume at the end of the experiment and at the start of the experiment by the number of days of treatment.

Tumor sampling. After 4 weeks of toremifene treatment, the animals were killed by CO_2 and all palpable

Table 2

The mean tumor burden, tumor volume and growth rate of DMBA-induced rat mammary tumors at the end of the 4 weeks' treatment in untreated animals and in animals treated with two different doses of toremifene

	Series A			Series B		
	CONTR	TOR 3 mg/kg	p*	CONTR	TOR 12 mg/kg	p*
Mean tumor volume at the start of the treatment (cm^3)	0.17	0.07	NS	0.12	0.16	NS
Mean tumor volume at the end of the treatment (cm^3)	1.22	0.49	0.032	0.49	0.24	NS
Mean total tumor volume/rat at the start of the treatment (cm^3)	0.56	0.22	NS	0.55	0.61	NS
Mean total tumor volume/rat at the end of the treatment (cm^3)	3.77	1.41	NS	2.15	0.92	< 0.050
Tumor growth rat (cm^3/day)	0.035	0.013	0.050	0.015	0.003	0.004

*Wilcoxon rank sum test.
CONTR, untreated control rats; TOR, toremifene treated rats

tumors were dissected and fixed with 10% formalin for preparation of histological specimens. The number of tumors at the end of the experiment in series A was 16 in the control group (5 rats) and 14 in the toremifene group (5 rats) (Table 1). In series B, the number of the tumors at the end of the experiment was 22 in the control group (5 rats) and 26 in the toremifene group (8 rats) (Table 1).

Histology. Five-micron sections were cut from the formalin-fixed and paraffin-embedded blocks and stained with haematoxylin and eosin. At histology, special attention was paid to the presence or abscence of necrotic and atrophic areas. The number of tumor samples evaluable for histology and flow cytometry in series A was 16 from the control group and 13 from the toremifene group, and 20 and 24 in series B respectively. The non-evaluable tumors were those which had completely regressed during treatment and those for which the sample was unrepresentative.

M/V INDEX. The volume corrected mitotic index (M/V INDEX) was measured from the sections with $\times 40$ objective according to Haapasalo et al. (27):

$$\text{M/V INDEX} = k(\Sigma MI)/(\Sigma Vv)$$

where ΣMI is the sum of mitotic figures in the counted fields, ΣVv the sum of the volume proportions of neoplastic epithelium, k a constant for the area of the field ($100/\pi r^2$), and r the radius of the microscope field (mm). The M/V INDEX gives the absolute number of mitotic figures/mm^2 of tumor epithelium. A total of 10 to 30 fields selected by systematic randomisation (30–100% of the area in the section) were counted and the area fraction (%) of the epithelium was estimated subjectively. The radius of the microscope field was 0.242 mm.

Flow cytometry (FCM). Fifty μm sections were cut from the paraffiin blocks of the mammary tumors for DNA FCM, and the sections were deparaffinized as described earlier (29). FCM was done with a FACScan flow cytometer (Becton-Dickinson Immunocytometry System, Mountain View, CA, USA). DNA was stained using propidium iodide (Sigma, St Louis, USA) immediately before FCM (30). A 488 nm argon laser line at 600 mW was used for fluorescence excitation. Before analysis, all samples were filtered through polyester silk gauze (ESTAL MONO, type 110 HD, pore size 50 μm, Switzerland). The SPF was analyzed using the rectangular method (31). The height of the rectangle was measured as the mean of a segment about 10 to 15 channels long at the lowest part of the curve over the SPF region, which was usually in the middle or near the G2/M peak.

Statistics. The significance of differences between the results obtained in the treatment groups were calculated using the Wilcoxon rank sum test, and the correlation analyses were performed using Spearmans' correlation coefficient.

Results

Growth pattern of tumors. The tumors of two series of rats appeared to have different growth properties. In series

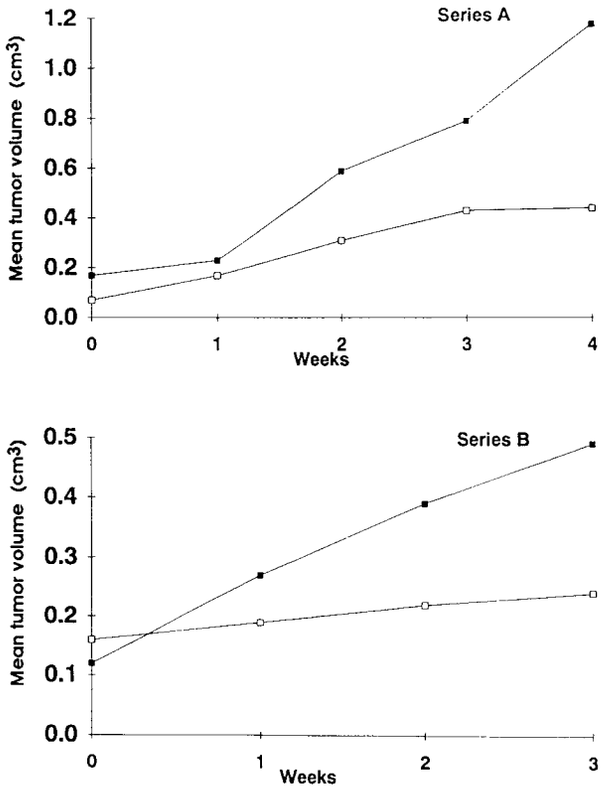

Figure. Growth pattern of DMBA-induced rat mammary carcinoma treated with two different doses of toremifene. The mean tumor volume in each week: ■ control tumors. □ toremifene treated tumors. series A = 3 mg/kg; series B = 12 mg/kg.

A, the rats had few tumors (1–2 tumors/animal) which were large and grew rapidly. In series B, the rats had more tumors (3–4 tumors/animal) but the tumors were small and slowly growing (Table 1 and 2).

Response to treatment. The gross response to toremifene treatment was seen as regression of the growth curve of the treated tumors (Figure). Toremifene did not reduce the total average number of the tumors in the animals (Table 1), but the tumors were smaller in the treated animals than in the untreated ones (Table 2). In series A, 31% of the treated tumors responded to treatment and in series B 58% of the treated tumors regressed or remained stable during treatment (Table 1). In the control group of series B, 27% of the tumors remained constant in size or even slightly regressed. The growth rate of the treated tumors was significantly lower than the growth rate of the untreated tumors in both series (Table 2). In series B, toremifene inhibited the formation of new tumors but not so in series A (Table 1).

Histology. The tumors were composed of hyperplastic cuboidal epithelium in nests, within which glandular ducts could be recognized, surrounded by various amounts of intervening fibrous stroma. Mast cells and lymphocytic infiltration were prominent in the stroma. The dominant tissue components were epithelium, stroma, cells with abundant clear cytoplasm (lipid cells), glandular luminae and necrosis. The effect of toremifene was seen as focal reduction of the cell layers around the lumina (atrophy) with simultaneous formation of numerous luminal openings in these areas, which were located near the stromal walls in the periphery of the epithelial nests. Necrosis was seen only in small areas of some tumors, in equal amounts in the treated and untreated tumors. In series B, in which the tumors were older, the tumors from both groups contained fibrosis and the toremifene-treated tumors could not easily be distinguished from the untreated ones. More tumors were atrophic in toremifene-treated rats, but the number of necrotic tumors was within 95% confidence limit, both in the control and the treated groups (4 and 3 respectively). Pyknotic nuclei interpreted as apoptotic cells were numerous in both series, both in untreated and treated tumors.

Table 3

The M/V INDEX and the SPF in DMBA-induced rat mammary tumors treated with two different doses (Series A and B) of toremifene for 4 weeks

	Growing tumors	Regressing and stable tumors	All tumors
Series A			
M/V INDEX			
CONTROL	14.9 (n = 15)	20.3 (n = 1)	15.2 (n = 16)
TOR 3 mg/kg	8.1 (n = 10)	4.9 (n = 3)	7.4 (n = 13)
SPF			
CONTROL	5.6 (n = 15)	5.2 (n = 1)	5.2 (n = 16)
TOR 3 mg/kg	4.9 (n = 10)	2.8 (n = 3)	4.4 (n = 13)
Series B			
M/V INDEX			
CONTROL	4.6 (n = 15)	0.9 (n = 5)	3.7 (n = 20)
TOR 12 mg/kg	3.6 (n = 12)	2.9 (n = 12)	3.2 (n = 24)
SPF			
CONTROL	3.3 (n = 15)	1.8 (n = 5)	3.0 (n = 20)
TOR 12 mg/kg	3.0 (n = 12)	2.3 (n = 12)	2.7 (n = 24)

M/V INDEX, volume corrected mitotic index; SPF, S-phase fraction; CONTROL, untreated control tumors; TOR, toremifene treated tumors. For statistics, see text

M/V INDEX and SPF. The M/V INDEX and the SPF were evaluable in all 16 control tumors and in 13/16 toremifene-treated tumors in series A, and in 20/22 control tumors in 24/31 toremifene-treated tumors in series B. Both the untreated and toremifene-treated tumors were diploid. The overall levels of the SPF and the M/V INDEX were different in the two series: the tumors in series A had a high M/V INDEX and those in series B had a low M/V INDEX (Table 3). The SPF was low in each series, and even lower in series B (Table 3).

The reduction in the proliferation rate during toremifene treatment was evident when comparing the proliferation

markers in the subgroups of the growing control tumors with the regressing or stable treated tumors (Table 3): the M/V INDEX in series A: 14.9 vs. 4.9, $p < 0.05$; the SPF in series A: 5.6 vs. 2.8, $p = 0.05$; the M/V INDEX in series B: 4.6 vs. 2.9, $p = 0.05$; the SPF in series B: 3.3 vs. 2.3, $p = NS$. In series B, both proliferation markers were lower in the group of spontaneously stable untreated tumors than in the regressing tumors in the treated group (Table 3). The only regressing tumor in series A, control group, appeared to have a surprisingly high M/V INDEX, 20.3. The SPF of this tumor was, however, moderate, 5.2. In series B, there was a good positive correlation with the M/V INDEX and the SPF both in the treated ($r = 0.59$, $p < 0.01$) and untreated tumors ($r = 0.69$, $p < 0.01$), but not in series A ($r = 0.29$, $p = NS$ for controls, and $r = 0.20$, $p = NS$ for toremifene treated tumors).

Discussion

The aim of the present study was to examine the effects of antiestrogen toremifene on proliferation in the DMBA-tumor model, with two proliferation markers, the volume corrected M/V INDEX and the SPF. Two different doses of toremifene were used with the hope that inhibition of cell proliferation would be more effective with the higher dose. The lower dose level, 3 mg/kg/day corresponds to the dose of 60 mg/day used in the clinical situation for treatment of human breast cancer (14). The higher dose, 12 mg/kg, was chosen in conformity with some clinical studies, in which toremifene has been used in four times higher doses than the conventional ones and yet been well tolerated (18). The level of 1 to 2 mg/kg is the minimum level at which toremifene is able to cause tumor regression in the DMBA-tumor (13, 20).

The two rat series used in the present study appeared to have different proliferative capacity: Series A with fast growing, large tumors with high proliferation marker levels, and series B with slowly growing, small tumors with low proliferation marker levels. At the time of the carcinogen exposure the rats were at the age of 50 days. At the start of toremifene treatment the rats in series B were older than those in series A (155 vs. 96 days). The proliferation of the DMBA-tumors is likely to decline with time even without any treatment, measured by SPF from fine-needle aspirates. In four weeks' time, the SPF may decline from 12.8 to 7.4 (data not shown).

The overall inhibition of the growth of the already established tumors was similar at the two dose levels (Figure). The higher dose of toremifene was more effective in inhibiting the new tumor formation than the lower dose. The proportion of regressive or stable tumors was 31% in the rats treated with the lower dose of toremifene (series A) and 58% in those treated with the higher dose (series B). It has to be noted that in the series treated with the higher dose, 27% of the tumors in the untreated group remained spontaneously stable, thus indicating an overall response of approximately 30% in this series. Kangas et al. (13) and diSalle et al. (20) have reported similar responses with high doses of toremifene on the DMBA-tumor model, although they failed to show a stronger inhibition of new tumor formation with higher doses. The growth of carcinogen-induced mammary tumors is variable, and spontaneous stabilization and even spontaneous regression of the tumors may occur (28). Hormone sensitivity of the DMBA-tumor model has been a consistent feature, but in contrast to human breast cancer, DMBA-tumors are dependent on prolactin (32). Ovariectomy and LHRH analogues have been the most potent tumor inhibitors (33). Because of the distinctly different growth patterns seen in the two rate series in our material, the comparison of the treatment response with each dose level has to be interpreted cautiously.

The reduction of the SPF in breast cancer cells has been demonstrated to occur during antiestrogen treatment in vitro, but this phenomenon has not been shown in vivo (11). Most studies on this subject have been done on heterotransplated tumors of human breast cancer cell lines grown in nude mice. We observed, that toremifene reduced both the SPF and the volume corrected M/V INDEX when comparing the subgroups of actually regressing tumors in the treated rats with the growing tumors in the untreated animals. If the whole tumor population, in which both the spontaneously stabilized untreated and non-responding treated tumors are included, is taken into the difference analysis, a misleading conclusion that the SPF is not decreasing may be reached. Suprisingly, in series B, the spontaneously stabilized tumors in the untreated rats had lower proliferative activity than the regressing toremifene treated tumors measured by both proliferation markers (Table 3). We know from our previous studies that apoptosis in this tumor model is spontaneously great, and there is evidence, that toremifene may modify apoptosis (24), and the regression during toremifene treatment may in part be due to apoptosis (23). In series B, the mitotic activity in the untreated spontaneously stabilized tumors was very low, and tumors may have been regressed because of spontaneous apoptosis. In series A, one tumor in the untreated control group was regressing and still had a very high mitotic activity (Table 3). This tumor was re-examined and the possible role of necrosis or apoptosis was considered. The tumor was large and appeared to have only modest amount of apoptotic cells and no necrosis. The explanation for the controversial proliferation markers may be the tumor volume, because in a large tumor there may be areas with different proliferation capacity.

The correlation with the M/V INDEX and the SPF was expected to be better. Only in series B was the correlations significantly positive. To insure accurate assesment of mitotic counts, it is essential that the tissue sample is fixed

properly and early after sampling, and that the sampling is done adequately at the microscopic level (34). Counting mitoses is highly reproducible if the morphological criteria for a mitosis are strictly followed (34). The most important disadvantages of flow cytometry are the need to disrupt tissues so that spatial resolution of tissue subpopulations is lost, and the inclusion of admixed non-neoplastic cells in samples (35). One confusing factor affecting the SPF may be that in contrast to M/V INDEX, the whole cell population in the tumor is measured, including stromal and lymphatic cells which are abundant in DMBA-tumors (36). Cells entering apoptosis may also be included in the SPF, because the phase from which the cells are entering apoptosis is not known. Other proliferation markers, such as PCNA and BrdUrd incorporation, have been shown to increase before apoptosis in prostatic epithelium after castration (37). Hence, in tissues with abundant spontaneous apoptosis the SPF may not be the optimal proliferation marker. In the DMBA-tumor model we feel that the M/V INDEX is a more appropriate measure.

In the DMBA-tumor model, toremifene seems to dominantly interfere with the cell turnover by reducing mitotic activity, but probably also by modifying the spontaneous apoptosis, depending on the prevailing mitotic and apoptotic rate.

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