Tamoxifen Enhances Cell Death in Implanted MCF7 Breast Cancer by Inhibiting Endothelium Growth

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Abstract

Magnetic resonance imaging at high spatial resolution and histochemical staining were applied to monitor the influence of tamoxifen versus estrogen on the growth, endothelial density, and extent of necrosis in tumors of MCF7 human breast cancer cells implanted in nude mice. Concomitantly with tamoxifen growth arrest, a highly significant decrease, by more than 2-fold, in the endothelial density of viable tumor regions had occurred, together with a significant increase in the extent of necrosis. The results suggest that the antiestrogenic activity of tamoxifen in breast cancer, which results in enhanced necrosis and tumor regression, is due to the inhibition of angiogenesis and of endothelial growth, thus reducing vascularization and impairing tumor perfusion.

Introduction

The antiestrogenic activity of tamoxifen [Z-1-p-(β-dimethylaminoethoxy)phenyl-1,1-diphenyl but-1-ene] is being used extensively in hormonal therapy of breast cancer, at all stages of the disease (1). Currently it is being assessed as a preventive agent for breast cancer (2). Despite extensive research and clinical experience with this drug, its exact mode of action in inducing tumor regression is still not clear (3, 4). Tamoxifen activity against breast cancer was originally attributed to its ability to bind competitively to estrogen receptors and inhibit estrogen induced mitogenic activity, but other mechanisms of action, not related to the presence of estrogen receptors, have also been reported (1, 4). Recently, experiments with a bacterial polysaccharide and tamoxifen in MCF7 tumors showed that tamoxifen exhibits anti-angiogenesis properties (5). An estrogen independent angiostatic activity of tamoxifen and other antiestrogens was demonstrated by Gagliardi and Collins (6) in the chick chorioallantoic membrane. On the basis of in vivo MRI studies of MCF7 tumors implanted in athymic mice, we have proposed a mechanism for the anticancer action of tamoxifen which attributed the critical activity of the drug to its capacity to inhibit angiogenesis and impair neovascularization (7, 8). To test this hypothesis we investigated in this tumor model the distribution and density of the endothelial cells that comprise the tumor blood vessels and capillaries by immunohistochemistry in parallel to monitoring tumor growth and necrosis by MRI.

Materials and Methods

Animals and Tumors. Female C57H-sc athymic mice, 6 weeks old, were obtained from the Weizmann Institute Animal Services. MCF7 human breast cancer cells, cultured routinely as described previously (8), were inoculated s.c. (10⁶ cells/mouse) in the right flank of the mice. Before the cell inoculation a pellet of 17β-estradiol (0.72 mg/pellet, 45 days release time; Innovative Research, Toledo, OH) was implanted underneath the skin of the back. Tamoxifen treatment was initiated when the tumors reached a size of ~1 cm³, by removing the estrogen pellet and implanting a tamoxifen pellet (5 mg/pellet, released over 60 days with blood levels of 3–4 ng/ml; Innovative Research). In control tumors a new estrogen pellet was implanted after 45 days. Another control experiment of removing the estrogen pellet and implanting a placebo pellet (Innovative Research) was also conducted. The size of these placebo treated tumors was measured by caliper assuming a hemiellipsoidoid volume (4π/3) × (length/2) × (width/2) × (thickness/2).

MRL. MR images were recorded with a 4.7-T spectrometer (Biospec 4.7/30; Bruker) using a 7.5-cm Bruker radiofrequency probe. Multislice, spin echo T2 weighted images were recorded with an echo time of 68 ms and a repetition time of 2400 ms at a spatial resolution of 1 x 0.2 x 0.2 mm. Tumor volume was determined with an experimental error of less than 5% from the tumor area measured with the standard Bruker program for image analysis, the slice thickness, and the slice to slice distance. Average size values are presented as mean ± SEM.

Histology. Tumors from estrogen and tamoxifen treated mice were removed after cervical dislocation. The histological plane corresponding to the central transverse section of the final MR images was established by a single cut through the tumor. Following fixation in 4% formaldehyde solution, paraffin embedded histological slices (~4 μm thick) were stained with hematoxylin-eosin and modified trichrome method. Endothelial cells were specifically stained with GSL-1. The GSL-1 lectin binds specifically to α-galactosyl residues and marks the vascular endothelium in mice (10). Sections (4 μm) were deparaffinized and rehydrated. The endogenous peroxidase was inactivated with 3% H₂O₂ and washed in TBS, pH 7.6, followed by preincubation in nonimmune goat serum for 30 min at room temperature. The sections were then incubated for 60 min with biotinylated GSL-1 (Vector Laboratories, Burlingame, CA) at a concentration of 0.1 mg/ml, washed with TBS, and treated (30 min) with avidin-biotin-peroxidase complex (Vector Laboratories) and washed again with TBS. The peroxidase was activated by incubation for 15 min in 0.1 M acetate buffer (pH 5.2) containing 3% H₂O₂ and 3% 3-aminoethylcarbazole. Finally, the slices were washed in distilled water and tap water, counterstained with hematoxylin, dehydrated, and coverslipped with permount. Image analysis of sections stained for endothelial cells with GSL-1 was performed with a GALAI CUE-2 system (11). The analysis was performed on regions containing exclusively viable cancer cells depicted by the hematoxylin stain. In the tamoxifen treated tumors these regions did not develop necrosis, although they were exposed to the effects of the drug. In each GSL-1 stained section of control and of tamoxifen treated tumors, three to five regions (0.27 mm² each) in exclusively viable regions were selected randomly. The area of stained endothelial cells was determined with an experimental error of less than 5%, by measuring the area above a threshold intensity of stain in each
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The tumors showed a significant (26 ± 5%) reduction in the mean tumor size (Table 1). Four tamoxifen treated tumors continued to grow at a rate similar to that of the placebo treated tumors increasing in size by 31 ± 8% after 1 week and 51 ± 13% after 2 weeks of treatment. These tumors were considered nonresponders and were analyzed separately from the remaining 17 tumors that showed a clear response.

In addition to the accurate size determination, the MR images provided information on the distribution and extent of viable tumor cells and necrotic areas. As was shown previously (7, 9), regions of viable tumor cells appeared gray in the T2 weighted images while necrotic regions appeared brighter due to their high water content and long T2 (Figs. 1 and 2). Initially all the tumors exhibited mostly viable tumor tissue (Fig. 2) and a small fraction of necrosis (<10%). In the estrogen treated tumors

<table>
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<th>Table 1</th>
<th>Changes in size and percentage of necrosis (A) and in percentage of endothelial cell density (B) in control and tamoxifen treated tumors</th>
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<td>Control tumors (n = 5)</td>
<td>Size (cm³)</td>
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<td>% of necrosis</td>
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<td>Tamoxifen responding tumors (n = 17)</td>
<td>Size (cm³)</td>
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<td>% of necrosis</td>
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|         | B                                                                                                                                |
|         | Size (cm³) | % of area of endothelial cells |
| Control tumors (n = 10) | 0.75 ± 0.13 | 10.2 ± 2.1 |
| Tamoxifen responding tumors (n = 16) | 0.70 ± 0.10 | 4.4 ± 0.5⁺ | (P < 0.0001) |

*Mean ± SEM.
⁺A statistically significant change relative to control.
*After 2 weeks, n = 14.
A 14 tumors examined after 2 weeks of treatment and 2 tumors after 1 week of treatment.

Results

Mice inoculated with MCF7 cells developed tumors which were about 1 cm³ in size within 8 weeks. The tumors were then monitored according to the following protocols: (a) Five estrogen treated control tumors with an average size of 0.78 ± 0.11 cm³ were monitored by MRI three times within 2 weeks (0, 1, and 2 weeks); (b) 21 tumors grown in the presence of supplemented estrogen, with an average size of 1.05 ± 0.11 cm³, were monitored by MRI and immediately after the first monitoring the estrogen pellet was replaced with a pellet of tamoxifen. The treated mice were reexamined by MRI after 1 week (n = 21) and 2 weeks (n = 18) of treatment, and all were then sectioned for histological studies (3 tumors were sectioned for histology after 1 week); (c) 8 estrogen treated control mice with tumors with an average size of 0.75 ± 0.13 cm³ were monitored once by MRI and were sectioned for histological studies; (d). The growth of 10 tumors with an average size of 0.73 ± 0.18 cm³ was monitored by caliper immediately before and then several times after the replacement of the estrogen pellet by a placebo pellet.

The control tumors monitored by MRI continued to grow in the presence of estrogen and the average increase in their size was 56 ± 6% after 1 week and 114 ± 15% after 2 weeks (tumor sizes are summarized in Table 1). The placebo treated tumors also continued to grow by 21 ± 11 and 53 ± 17% after 1 and 2 weeks, respectively, despite the lowering of estrogen level to normal endogenous values. The growth of most tumors treated with tamoxifen (17 of 21 tumors) was halted within the first week of treatment and in fact a small decrease in size of 10 ± 4.5% was already observed. After 2 weeks of treatment the tumors showed a significant (26 ± 5%) reduction in the mean tumor size (Table 1). Four tamoxifen treated tumors continued to grow at a rate similar to that of the placebo treated tumors increasing in size by 31 ± 8% after 1 week and 51 ± 13% after 2 weeks of treatment. These tumors were considered nonresponders and were analyzed separately from the remaining 17 tumors that showed a clear response.

In addition to the accurate size determination, the MR images provided information on the distribution and extent of viable tumor cells and necrotic areas. As was shown previously (7, 9), regions of viable tumor cells appeared gray in the T2 weighted images while necrotic regions appeared brighter due to their high water content and long T2 (Figs. 1 and 2). Initially all the tumors exhibited mostly viable tumor tissue (Fig. 2) and a small fraction of necrosis (<10%). In the estrogen treated tumors
the mean fraction of necrosis in a central tumor slice did not change significantly (Table 1) although analysis of the change in the necrotic fraction of each tumor revealed in these fast growing tumors an average increase of 140 ± 83% (percentage of necrosis increased in 3 tumors and decreased in 2 tumors) after 2 weeks. In the tamoxifen treated tumors, despite the reduction in their size, a significant increase in the mean fraction of the necrotic area was observed after 2 weeks (Table 1). Analysis of the change in the fraction of necrosis in each tumor revealed an average increase of 264 ± 124% in 9 tumors and the same or a reduction in the necrotic fraction in 5 and 3 tumors, respectively. After 2 weeks of treatment the average increase in the necrotic fraction reached 464 ± 165% (n = 10) while in the remaining tumors a decrease (n = 3) or the same fraction (n = 1) was observed. In the 4 nonresponding tumors that continued to grow in the presence of tamoxifen necrosis was also significantly enhanced from 5.0 ± 2.0 to 15.2 ± 2.5% after 2 weeks of treatment.

Inspection of the tumor periphery, before sectioning for histological studies, clearly revealed that the endothelium growth and capillary sprouting exhibited an outside-inside pattern in all cases. A net of large blood vessels surrounding the peripheral areas of the tumor and the microcapillaries extended from the periphery into the center of the tumor were observed.

To determine whether the tamoxifen enhanced necrosis was associated with a quantitative reduction in tumor vascularity we measured the density of the endothelial cells that compose the microcapillaries, in regions of viable tumor cells close to the tumor periphery. A specific biotinylated lectin that interacts with endothelial cells (GSL-I) enabled us to stain these cells selectively (Fig. 3). The density of endothelial cells (percentage of area occupied by endothelial cells) was determined by image analysis of the stained areas as described in “Materials and Methods.” The mean percentage of endothelial cell area in the viable regions of estrogen treatment, control tumors was 10.2 ± 2.1% (33 regions in 8 tumors) and in the viable regions of tumors treated with tamoxifen it was 4.4 ± 0.5% (57 regions in 14 tumors after 2 weeks of treatment and in 2 tumors after 1 week of treatment). The mean endothelial density in nonresponding tumors after 2 weeks of tamoxifen treatment was 7.9 ± 1.4% (n = 7). The reduced endothelial cell density as a result of tamoxifen treatment (Table 1) was highly significant (P < 0.0001) and strongly supports our hypothesis that tamoxifen inhibits endothelial cell proliferation.

It is important to note that the tamoxifen treated tumors and the control tumors were initially matched in size. Moreover, the reduction in endothelial density and the increase in necrosis as a result of tamoxifen treatment occurred despite the lack of growth of these tumors.

Discussion

MCF7 human breast cancer tumors can develop and grow in athymic mice only in the presence of 17ß-estradiol (12). Replacing estrogen with tamoxifen rapidly inhibits the growth of these tumors and induces growth arrest and regression (12–15). These studies as well as in vitro investigations of breast cancer cell cultures indicated that tamoxifen is a cytostatic rather than a cytotoxic agent. Recently studies on the effect of estrogen ablation on MCF7 tumors (16) and the effects of a new antiestrogen, toremifene, on MCF7 and ZR-75-1 tumors (17) indicated cessation of cell proliferation and activation of programmed cell death which preceded reduction in tumor volume. As seen in our previous in vivo NMR studies on MCF7 tumors (7–9), the results presented here have shown that tamoxifen induces tumor cell death and the appearance of central necrotic regions within 1–2 weeks of treatment. Histological inspection of these tumors also indicated the presence of necrosis in the tamoxifen treated tumors without revealing apoptotic cells. However, we cannot rule out the possibility that programmed cell death had occurred in the treated tumors prior to our observations.

It is reasonable to assume that if tamoxifen induced cell death or programmed cell death by directly affecting the tumor cells, this induction would also be observed in vitro in cultured tumor cells. Studies of the same MCF7 cell variant in culture, using phenol red free medium that contained charcoal stripped fetal calf serum indicated a slow down of the logarithmic growth rate of about 60% in the presence of tamoxifen (2 μM) relative to 17ß-estradiol (3 nm), but no cell death (8). This result, which confirms similar experiments in other laboratories (see, e.g., Ref. 15), also favors a mechanism that does not attribute a direct killing effect to tamoxifen.

Tumor growth ceased during tamoxifen treatment and a significant regression occurred after 2 weeks of treatment, yet in most of these tumors (in 53% of the tumors after 1 week and in 75% of the tumors after 2 weeks of treatment) the fraction of central necrosis increased considerably. In general, during tumor growth necrosis would evolve in nonvascularized regions which are distant from the main blood vessels at the tumor periphery, as observed in rapidly growing MCF7 tumors (this work and Ref. 7). The central tumor region is more dependent on perfusion by the microcapillaries than the periphery. Furthermore, in the tamoxifen treated tumors a highly significant reduction (P < 0.0001) of more than 50% in the endothelial density...
had occurred in viable tumor regions. These two events, reduction in endothelial cell density and the concomitant increase of necrosis, lend support to our hypothesis that tamoxifen, by inhibiting angiogenesis and endothelium growth, impairs delivery of nutrients and oxygen, thereby leading to cell death.

Regression due to tamoxifen treatment was observed in 17 of 21 tumors while all the 10 placebo treated tumors continued to grow. These results clearly demonstrate that tumor regression was induced by tamoxifen and did not result from the withdrawal of the supplementing estrogen. The endogenous estrogen in the placebo treated mice appeared to be enough to maintain continuous growth of these tumors, although the rate of growth was about 2-fold slower than that of the estrogen treated tumors. In 4 tamoxifen treated tumors, which continued to grow at a rate similar to that of the placebo treated tumors, the fraction of necrosis increased after 2 weeks of treatment by approximately 3-fold but the small reduction in endothelial cell density was insignificant ($P < 0.35$). This observation may suggest that tamoxifen growth arrest of tumor cells is not necessarily synchronous with its inhibition of endothelial cell growth and induction of necrosis.

Angiogenesis and neovascularization are fundamental steps in tumor growth and metastasis (18). Neovascularization is particularly critical in rapidly growing tumors such as the MCF7 implanted tumors. The dynamics of neovascularization are affected by the gradual compression and disruption of capillaries and by the continual formation of new capillaries induced by tumor secreted angiogenic factors (19). The production of these factors as well as the other growth factors and growth inhibitory factors, which work by autocrine and paracrine mechanisms, has been shown to be regulated in breast cancer by estrogen (20). Thus we propose that the reduction in endothelium density caused by tamoxifen resulted from its antiangiogenic activity, inhibiting production and secretion of angiogenic stimulatory factors.

Further support to an estrogen receptor mediated tamoxifen inhibition of angiogenesis was provided in the report of Tanaka et al. (5) on the effects of bacterial polysaccharide and tamoxifen on MCF7 tumors. In this report it was shown that the number of capillary lumens in the capillary nets surrounding the tumor masses of MCF7 estrogen receptor positive tumors was markedly reduced (~50%) by treatment with tamoxifen, while in MCF7 tumors of estrogen receptor negative and tamoxifen resistant variant cells (U-2 line) the number of capillary lumens stayed the same in control and tamoxifen treated tumors. Preliminary studies in our laboratory of estrogen independent MDA MB 231 human breast tumors implanted in the same athymic mice as the MCF7 tumors showed that tamoxifen had no effect on the rate of growth and extent of necrosis of these tumors ($n = 4$). This observation also suggests a receptor mediated antiestrogen effect of tamoxifen. On the other hand Gagliardi and Collins (6) showed recently that tamoxifen as well as other antiestrogenic drugs inhibit angiogenesis in the chick egg chorioallantoic membrane (6). This inhibition was not removed in the presence of high estrogen (6). It was therefore suggested that the angiostatic activity of the antiestrogens was exerted via mechanisms other than their inhibition of estrogen action. In this system, unlike observations in breast carcinomas which are estrogen receptor positive, tamoxifen could have acted by a direct membrane mediated mechanism (4).

In summary, the reduction in the density of endothelial cells and the increase in the fraction of necrosis following tamoxifen arrest and regression of MCF7 tumors support the hypothesis that tamoxifen inhibition of angiogenesis and consequently of neovascularization is the critical activity which causes remission of estrogen receptor positive breast cancer. This hypothesis is also relevant for understanding the role of tamoxifen in breast cancer prevention since it predicts that tamoxifen will inhibit tumor entrance into the angiogenic phase thereby delaying tumor progression.

Acknowledgments

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References

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