Growth Inhibition in Response to Estrogen Withdrawal and Tamoxifen Therapy of Human Breast Cancer Xenografts Evaluated by in Vivo $^{31}$P Magnetic Resonance Spectroscopy, Creatine Kinase Activity, and Apoptotic Index

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ABSTRACT

Estrogen withdrawal versus tamoxifen (TAM) treatment was compared in two human breast cancer xenografts, the estrogen-dependent ZR75-1 and its estrogen-independent subline ZR75/LCC-3. The following parameters were determined: tumor growth, NTP:P, by $^{31}$P magnetic resonance spectroscopy, apoptotic index, and creatine kinase (CK) activity. Tumors of each line were grown in ovariectomized nude mice during stimulation from a s.c. 17$\beta$-estradiol pellet. At a tumor size of approximately 350 mm$^3$, the pellet was removed from one-half of the animals. The remaining one-half served as controls. In parallel experiments, injections of TAM were initiated instead of estrogen withdrawal. Estrogen withdrawal as well as TAM induced growth inhibition of ZR75-1 tumors, whereas ZR75/LCC-3 was resistant to both types of therapy. Growth inhibition of ZR75-1 by estrogen withdrawal, but not by TAM, was accompanied by an 80% increase of the NTP:P ratio ($P < 0.01$) and a significantly decreased cytosolic CK activity ($P < 0.001$). No significant change in pH was observed. These changes seemed not to be related to changes in apoptotic index. None of the described changes occurred in ZR75/LCC-3. The present data indicate: (a) ZR75-1 and ZR75/LCC-3 xenografts respond differently to estrogen withdrawal and TAM with regard to growth inhibition, $^{31}$P magnetic resonance spectroscopy, and CK activity; (b) estrogen withdrawal, but not TAM, induces a decrease in the CK activity of estrogen-dependent tumor tissue, and (c) increased apoptosis did not explain the growth inhibition and the increase in NTP:P, induced by estrogen withdrawal. The results indicate other growth inhibitory mechanisms of TAM in addition to competitive inhibition of the estrogen receptor.

INTRODUCTION

The presence of estrogen receptors and the hormone-dependent growth of a significant portion of human breast cancers is the rationale for endocrine therapy of this disease. The most commonly used drug in endocrine therapy of breast cancer is the nonsteroidal antiestrogen TAM, but also estrogen withdrawal (ovarian ablation) is clinically used in adjuvant (1) as well as metastatic (2) treatment of breast cancer. In fact, the demonstrated effect of adjuvant chemotherapy in high-risk premenopausal estrogen receptor-positive breast cancer patients may be due to a concomitant chemotherapy-induced ovarian dysfunction (3).

Noninvasive $^{31}$P MRS studies of energy metabolism in breast cancer cells (4, 5) and tumor transplants (6–9) before and after hormonal manipulation have been performed to address the possible role of this method in clinical response evaluation and to further elucidate the biochemical mechanisms involved in the response to endocrine manipulation in breast cancer (10). We have recently demonstrated a relationship between estrogen withdrawal and an increase in NTP:P, as measured by $^{31}$P MRS of estrogen-dependent human breast cancer xenografts (11). Morphological studies of hormone-dependent breast (12, 13) and prostate (14) tumors after anti-hormonal therapy have demonstrated a significantly increased amount of apoptotic cells compared with untreated tissue (15). The inhibitory effect of TAM through the estrogen receptor was previously assumed to be caused by competitive inhibition, but recent data indicate that other mechanisms of action are also involved in TAM-induced growth inhibition (16, 17). Thus, the effect of TAM as compared to estrogen withdrawal remains controversial.

On this background, we used the estrogen-dependent/TAM-sensitive human breast cancer line ZR75-1 and its estrogen-independent/TAM-resistant subline ZR75-1/LCC-3 to investigate: (a) whether the previously observed increase in NTP:P, in xenografts growth inhibited after estrogen withdrawal also occurred after effective TAM treatment; (b) if the increase in NTP:P, was related to regulation of pH and CK activity; and (c) if the observed phenomenon could be explained by an increased amount of cells undergoing programmed cell death (apoptosis).

MATERIALS AND METHODS

Cell Lines. Stock cultures of the estrogen-dependent and receptor-positive human breast cancer cell line ZR75-1 were maintained in Eagle’s MEM with phenol red and Earle’s salt containing 5% fetal bovine serum, while the estrogen-independent and receptor-negative ZR75-1/LCC-3 was routinely carried in Iscove’s MEM without phenol red and supplemented with 5% charcoal-stripped FCS. ZR75-1/LCC-3 was isolated by selection for estrogen independence in vivo (11). Serum was stripped of endogenous steroids by treatment with dextran-coated charcoal and sulfatase as described by Clarke et al. (18). Cells were harvested at 80% confluency, using a cell scraper, and 2 $\times$ 10$^6$ cells were inoculated into each flank of 8–10-week-old ovariectomized NMRI nu/nu athymic nude mice (BOMMICE, Ry, Denmark). The mice carrying ZR75-1 tumors received an E$_2$ pellet (60-day release, 0.72 mg; Innovative Research, Toledo, Ohio) s.c. at the time of inoculation. ZR75-1/LCC-3 was maintained in ovariectomized mice without E$_2$ pellets to avoid selection of estrogen-dependent cells. Proliferating tumors were excised, cut into approximately 1-mm$^3$ pieces, and serially transplanted to a minimum of 20 ovariectomized mice for each experiment.

$^{31}$P MRS. Tumors were allowed to grow under continuous estradiol stimulation until they reached a size of $\approx$10 mm in diameter. Estrogen withdrawal or TAM therapy (0.1 mg i.p. daily; Ref. 19) was initiated on day 0. $^{31}$P MR spectra were obtained from each tumor on days 1, 2, 6, and 14. Immediately before MRS, the mice were anesthetized briefly (4–6 min) with propanidide i.p. (Sombrevin, 500 mg/kg; Gideon Richter, Budapest, Hungary) and placed in a flexible PVC tube with the tumor protruding through a hole concentric with the radiofrequency coil. To provide exact fixation, the tail of the animal was taped to the tube. In this setup, the breathing was not compromised, and furthermore, breathing movements were not transmitted to the tumor. Thus, during the spectral recording, the mice were restrained but unanesthetized. A 14-mm outside diameter, two-turn surface coil was located over the tumor in a special probe. A Vivospec spectrometer (Otsuka Electronics, Inc.) with a wide-bore 4.7 T magnet (Magnex, Inc.) was used. Sixty-four scans were...
acquired with a 10-s repetition time. The applied pulse width was 16 μs, corresponding to a flip angle of approximately 70°; these parameters ensured an acceptable signal-to-noise ratio and no signal saturation. The peak ratios were calculated by manual integration of each peak. pH was calculated from the distance between the P, and the PCr peak (20, 21).

**Growth Curves.** Mean tumor growth curves were constructed according to a transformed Gompertz function, as described elsewhere (22), using two orthogonal tumor diameters. The growth curves included only measurements from tumors that could be followed during the full observation period of 23–26 days. Tumor size was normalized to the size on day 0.

**Tissue Preparation.** As in the MRS experiments, at least 20 tumors of each tumor line were allowed to grow with an implanted estradiol pellet until they reached a size of ≥10 mm in diameter (350 mm3). On a day defined as day 0, the mice were separated into two groups. In one of these groups, the estradiol pellet was removed by surgical incision; in the others, the tumors were allowed to grow during continuous estradiol stimulation. Three tumors from each of the resulting four groups (ZR75-1 ± E2 and ZR75-1/LCC-3 ± E2) were excised from the animals on days 2, 6, and 14. One-half of each tumor was fixed in 4% buffered formaldehyde and embedded in paraffin. The other one-half was placed on ice and cut into small pieces before homogenization. Tumor size was normalized to the size on day 0.

Estrogen Withdrawal. Estrogen withdrawal induced a significant increase in NTP:P, to approximately 180% of the initial value in the estrogen-dependent tumor ZR75-1 (Fig. 2) but no change in NTP:P, of ZR75/LCC-3 (Fig. 3) during the 14-day observation period. In ZR75-1 tumors, estrogen withdrawal induced a statistically significant growth inhibition as shown in Fig. 2. The growth of ZR75/LCC-3 tumors was unaffected by estrogen withdrawal (Fig. 3). The mean pH for ZR75-1 was 6.97 ± 0.17 and 6.95 ± 0.14 for ZR75/LCC-3. pH in both tumor lines did not change following estrogen withdrawal (data not shown).

Estrogen withdrawal induced a significant increase in the cytosolic CK activity in ZR75-1 (P = 0.002) compared to estrogen-supplemented tumors, whereas no changes in CK activity were seen in

**RESULTS**

A total of 72 tumors were included (35 ZR75-1 and 37 ZR75/LCC-3). A representative 31P MRS spectrum of ZR75-1 is shown in Fig. 1. In both tumor lines, no significant differences in growth were found when comparing the pretherapeutic growth in tumors allocated to TAM treatment and estrogen withdrawal.

Estrogen Withdrawal. Estrogen withdrawal induced a significant increase in NTP:P, to approximately 180% of the initial value in the estrogen-dependent tumor ZR75-1 (Fig. 2) but no change in NTP:P, of ZR75/LCC-3 (Fig. 3) during the 14-day observation period. In ZR75-1 tumors, estrogen withdrawal induced a statistically significant growth inhibition as shown in Fig. 2. The growth of ZR75/LCC-3 tumors was unaffected by estrogen withdrawal (Fig. 3). The mean pH for ZR75-1 was 6.97 ± 0.17 and 6.95 ± 0.14 for ZR75/LCC-3. pH in both tumor lines did not change following estrogen withdrawal (data not shown).
ways or other mechanisms of action. Estrogen ablation is in concert with other published results of antiestrogen receptor. It could be mediated by one of the above-mentioned alternative pathways or other mechanisms of action.

**DISCUSSION**

In the estrogen-dependent ZR75-1 xenografts, significant changes in NTP:Pᵢ and CK activity were induced by estrogen withdrawal, whereas no such effects were observed following TAM therapy, although both types of treatment induced statistically significant growth inhibition (Fig. 2). The present MRS data concerning estrogen withdrawal from estrogen-dependent and -independent human breast tumors (Figs. 2 and 3) are consistent with similar observations in nitrosomethylurea-induced rat mammary tumors (6, 7). In contrast, we were not able to confirm the increase in NTP:Pᵢ after TAM therapy of estrogen-dependent human xenografts demonstrated by Furman et al. (8, 9) and in induced rat mammary tumors by Baluch et al. (30). The tamoxifen-induced NTP:Pᵢ increase in the xenograft studies (8, 9) was found following concomitant estrogen withdrawal and initiation of tamoxifen therapy and was, thus, probably caused by the estrogen withdrawal and not by the TAM therapy. In the present study, TAM therapy during continuous estrogen stimulation induced significant growth inhibition of estrogen-dependent tumors but no change in NTP:Pᵢ(Fig. 2). The use of ovariectomized mice was an essential part of our experimental design to fully disclose the effect of estrogen withdrawal and of TAM therapy. Transplantation of estrogen-dependent breast cancers into intact female mice without estrogen supplementation would lead to reduced or no take of the xenografts (31). Furthermore, the endogenous estrogen production in estrogen-supplemented intact mice is not sufficient to sustain the tumor growth rate after E₂ pellet withdrawal, as recently demonstrated by others (32). On the other hand, an intact endogenous estrogen production would potentially conceal the full effect of E₂ pellet withdrawal.

Lactate dehydrogenase (33) and glucose-6-phosphate dehydrogenase (34) have been shown to be regulated by estrogen, but also TAM regulates certain metabolic enzymes such as phosphofructokinase and α-glycerolphosphate dehydrogenase (35) and a decrease in glycolytic rate after initiation of tamoxifen perfusion has been found in MCF-7 cells in vitro (5). Creatine kinase is responsible for the exchange of high-energy phosphates between ATP and PCr. The demonstrated decrease in cytosolic CK activity in estrogen-depleted ZR75-1 xenografts (Table 1) does not, however, necessarily change the intracellular concentration of the substrate or the product of the reaction, and fluctuations in enzyme activity may be without metabolic consequences. Still, upon metabolic transitions, the decreased activity will affect the rate by which the cells are able to adjust to a new equilibrium. The possible role of CK activity in growth regulation is supported by Walker and Kaye (36), who found increased concentration of mRNA for CK in the rat uterus shortly after estrogen injections and a concomitant weight increase of the uterus. Others have found increased CK activity in different tumor types compared with their tissue of origin (37, 38), and recent studies have demonstrated a marked growth-inhibitory effect on different tumor types of dietary...
The relationship between NTP:P_i and CK activity is complex. The increased NTP:P_i after estrogen withdrawal in estrogen-dependent tumors could be caused by a decreased energy demand for tumor growth, but previous data have indicated that a similar phenomenon observed by 31P MRS is probably due to stromal factors such as changes in tumor vascularization or increased tumor content of connective tissue (8, 9). This hypothesis is supported by data from 31P MRS studies of other treatment modalities, e.g., radiotherapy (41, 42). Our previous data on estrogen withdrawal in four human breast cancer xenografts indicate that the observed NTP:P_i increase after estrogen withdrawal in estrogen-dependent tumors is due to a decrease in P_i concentration (11), and the constancy of the PCr:NTP ratio in the present study further supports the assumption that the observed increase in NTP:P_i has no direct relation to regulation of energy metabolism. Mean tumor pH was close to neutral in both xenografts, as found previously in other tumor lines (20). The relative contribution of intracellular and extracellular pH to this value is unsettled, but it is generally agreed that pH measured by MRS represents intracellular and extracellular pH to this value is unsettled, but it is generally agreed that pH measured by MRS represents intracellular pH due to the small relative size of the extracellular space and short relaxation time of extracellular P_i. In tumors, the intracellular space is maintained neutral to alkaline, whereas the extracellular space is more acidic (20, 21).

We were unable to demonstrate an increased amount of apoptotic cells in estrogen-depleted or tamoxifen-treated ZR75-1 tissue (Table 2). This finding is in contrast to the study of MCF-7 tumors by Kyprianou et al. (12) and MXT mouse mammary tumors by Szende et al. (13), who found a significantly increased frequency of apoptosis after estrogen withdrawal. In both of these studies, only morphological criteria without specific labeling of fragmented DNA was used (43, 44). The method used for quantification of apoptosis is probably crucial for the outcome. This problem is demonstrated by Wärr et al. (45), who found an increased number of apoptoses in MCF-7 cells grown in vitro after estrogen withdrawal and/or tamoxifen therapy compared to estrogen-stimulated cells when morphological time-lapse video microscopy was used for identification of apoptotic cells. In contrast, they found no difference in DNA fragmentation between treatment groups when the DNA degradation in MCF-7 cells was analyzed by gel electrophoresis. As stated, the reason for this divergence could be that DNA fragmentation is only present in a short period of the total apoptotic process, but it should be kept in mind that identification of apoptotic cells from morphology only implies the risk of misclassification, since necrotic cells can be difficult to distinguish from apoptoses. This would lead to an overestimation of the number of apoptotic cells. In the same study (45), ZR75-1 xenografts were analyzed by electron microscopy 10 days after initiation of tamoxifen treatment. Several apoptotic cells were found, but quantification or statistical comparison was not reported.

Whether estrogen withdrawal and tamoxifen therapy are cytostatic or cytotoxic remains controversial (30, 46). As described by Kyprianou et al. (12), estrogen withdrawal from the MCF-7 line induced significant tumor regression (indicating a cytotoxic effect) and a concomitantly increased percentage of cells undergoing apoptosis. Other MCF-7 variants seem to have lost their cytotoxic response to estrogen withdrawal, since the maximum response to E_2 withdrawal was static growth, i.e., without regression (46). Thus, an inhibitory but noncytotoxic effect of estrogen withdrawal and/or tamoxifen therapy would lead to an overestimation of the number of apoptotic cells. In the same study (45), ZR75-1 xenografts were analyzed by electron microscopy 10 days after initiation of tamoxifen treatment. Several apoptotic cells were found, but quantification or statistical comparison was not reported.

Table 2 Median (Ai) for the two xenografts after estrogen withdrawal and TAM therapy

<table>
<thead>
<tr>
<th></th>
<th>ZR75-1</th>
<th>ZR75/LCC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>+E_2</td>
<td>0.12 (0.06-0.18)</td>
<td>0.51 (0.08-0.7)</td>
</tr>
<tr>
<td></td>
<td>n = 10</td>
<td>n = 7</td>
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<tr>
<td>-E_2</td>
<td>0.18 (0.09-0.25)</td>
<td>0.21 (0.16-0.41)</td>
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<td></td>
<td>n = 9</td>
<td>n = 8</td>
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<tr>
<td>-TAM</td>
<td>0.08 (0.06-0.16)</td>
<td>0.10 (0.08-0.22)</td>
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<td></td>
<td>n = 9</td>
<td>n = 9</td>
</tr>
<tr>
<td>+TAM</td>
<td>0.1 (0.08-0.22)</td>
<td>0.2 (0.12-0.34)</td>
</tr>
<tr>
<td></td>
<td>n = 9</td>
<td>n = 9</td>
</tr>
</tbody>
</table>

a Numbers in parentheses, SD.

*Numbers in parentheses, ranges.

The numbers in parentheses are the ranges of the median values for each group.

Table 1 Cytosolic and mitochondrial CK activity (units/g) at 37°C in ZR75-1 and ZR75/LCC-3 xenografts after estrogen withdrawal and TAM therapy

<table>
<thead>
<tr>
<th></th>
<th>Cytosolic CK activity* (µmol/min × g wet weight)</th>
<th>Mitochondrial CK activity* (µmol/min × g wet weight)</th>
<th>Total CK activity* (µmol/min × g wet weight)</th>
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<tbody>
<tr>
<td></td>
<td>+E_2</td>
<td>-E_2</td>
<td>+E_2</td>
</tr>
<tr>
<td>ZR75-1</td>
<td>12.78</td>
<td>8.31 b</td>
<td>4.92</td>
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<td></td>
<td>(2.70)</td>
<td>(2.87)</td>
<td>(1.02)</td>
</tr>
<tr>
<td>ZR75/LCC-3</td>
<td>18.96</td>
<td>20.20</td>
<td>3.04</td>
</tr>
<tr>
<td></td>
<td>(5.25)</td>
<td>(14.5)</td>
<td>(1.23)</td>
</tr>
<tr>
<td>ZR75-1</td>
<td>8.97</td>
<td>8.43</td>
<td>4.86</td>
</tr>
<tr>
<td></td>
<td>(2.37)</td>
<td>(2.59)</td>
<td>(1.03)</td>
</tr>
<tr>
<td>ZR75/LCC-3</td>
<td>18.42</td>
<td>16.79</td>
<td>3.94</td>
</tr>
<tr>
<td></td>
<td>(5.32)</td>
<td>(2.30)</td>
<td>(1.60)</td>
</tr>
</tbody>
</table>

a Numbers in parentheses, SD.

b P < 0.01.
ACKNOWLEDGMENTS

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REFERENCES

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