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Characterization of tamoxifen stimulated MCF-7 tumor variants grown in athymic mice

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Abstract

The non-steroidal antiestrogen tamoxifen (TAM) is successfully used to treat all stages of breast cancer in both pre- and postmenopausal women. Unfortunately, most women treated with TAM eventually develop resistant tumor recurrences which require intervention with a second-line endocrine therapy, or cytotoxic chemotherapy if the recurrence is completely endocrine insensitive. There is evidence that some recurrences may in fact be TAM stimulated. MCF-7 human breast cancer cells grown as solid tumors in athymic mice chronically treated with TAM reproducibly develop a TAM stimulated phenotype (Osborne *et al.*, Eur J Cancer Clin Oncol 23: 1189–1196, 1987; Gottardis and Jordan, Cancer Res 48: 5183–5187, 1988; Osborne *et al.*, J Natl Cancer Inst 83: 1477–1482, 1991; Wolf *et al.*, J Natl Cancer Inst 85: 806–812, 1993). Tumors of this type may provide a useful model for a subset of therapeutic failures in the clinic. Therefore, we have extensively studied this model in an attempt to define the mechanism or mechanisms leading to TAM stimulated growth. In this paper we describe the characteristics of 4 TAM stimulated MCF-7 tumor variants. All of these tumors are growth stimulated by TAM, but vary in their response to estradiol (E_2) treatment, and grow poorly in placebo treated hosts. All tumor variants express estrogen receptor (ER) RNA and protein, which at the RNA level appear to be down regulated by TAM, and to a greater extent by E_2. All tumors also express epidermal growth factor receptor (EGFR) RNA, which is down regulated by TAM, and further down regulated by E_2. However, among the tumor variants analyzed, ER and EGFR levels appear to be inversely related. Further, despite the expression of ER by all 4 TAM stimulated tumor variants, E_2 induction of progesterone receptor expression is very weak or entirely absent.

Introduction

Breast cancer is the most common form of cancer among Western women, and is second only to lung cancer as a cause of death. Treatment of breast cancer by endocrine manipulation was first reported by Beatson [1] nearly a century ago. In 1900 Boyd [2] performed what may be regarded as the first clinical trial of endocrine treatment for breast cancer. He found that out of 30 premenopausal breast cancer patients, 11 had a favorable response to oophorectomy. The discovery of diethylstilbestrol (DES) [3, 4] and its paradoxical antitumor effect at high doses [5] made endocrine therapy available to postmenopausal women with advanced breast cancer as well. As with oophorectomy, roughly one third of pa-

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tients treated with DES responded to therapy [6]. However, no method was available to predict which patients would respond.

The discovery of the estrogen receptor (ER) by Jensen and Jacobson [7], and their suggestion that hormone responsive tumors might contain this protein whereas nonresponsive tumors would not, finally provided a means to distinguish potential responders from nonresponders to endocrine therapy. Later, it was suggested that even better prediction of response could be made by also assaying for the estrogen-inducible progesterone receptor (PR), which would indicate the presence of a functional ER [8]. By selecting patients with ER and PR positive tumors, the response rate to endocrine therapy can be increased to nearly 80% [9–11].

Numerous endocrine interventions have been used for the treatment of advanced breast cancer, all of which gave similar response rates, albeit with side effects of varying severity. One of the agents used was the triphenylethylene antiestrogen tamoxifen (TAM). Response rates to TAM treatment were similar to other endocrine agents, but with a much milder profile of associated side effects. Initially TAM was applied as a palliative agent for the treatment of advanced disease in elderly patients thought to be too infirm to undergo more aggressive treatments [12]. During the past 20 years, the application of TAM has expanded from being a palliative treatment to the principal endocrine therapy that produces a survival advantage for stage I and II breast cancer [13].

TAM is generally believed to act as a tumoristatic and not tumoricidal agent. It is therefore not surprising that longer durations of TAM treatment are more effective at controlling tumor growth, as has been demonstrated repeatedly both in the laboratory [14] and the clinic [13]. However, the cytostatic nature of TAM treatment means that residual tumor cells remain in the patient's body, growth arrested but not eradicated. Consequently, most TAM treated patients eventually experience disease recurrence. Some recurrences require treatment with cytotoxic chemotherapy. Others will have second-line responses to alternative forms of endocrine therapy, indicating that the resistance to TAM is specific, but that the estrogen response machinery is still functional. There is some evidence that a subset of recurrences may actually be stimulated to grow by TAM. Anecdotal reports of patients experiencing objective response after TAM withdrawal [15–17], as well as one small trial which reported withdrawal responses in 10% of patients with advanced breast cancer [18], suggest that TAM stimulated tumor growth may be a clinically relevant problem.

In a previous report we described the reproducible development of tamoxifen (TAM) stimulated MCF-7 tumor variants [19], which occurred in athymic mice bearing MCF-7 tumors chronically treated with either TAM or the nonisomerizable analog fixed-ring TAM. The work in this paper is an extension of that work, and also builds upon studies reported by Gottardis and Jordan [20] and others [21] on the development of TAM stimulated MCF-7 tumor variants. It had been suggested that TAM stimulated tumor growth might arise because tumors acquire the ability to eliminate TAM and its antiestrogenic metabolites selectively [22, 23] or convert them to compounds with reduced antiestrogenicity and elevated estrogenicity [22–24]. Our previous report demonstrated that in our TAM stimulated tumor model, neither of these mechanisms occurred, and that additional investigations were necessary to elucidate the mechanism or mechanisms leading to TAM stimulated growth [19]. In this paper, we further characterize the original MCF-7 TAM tumor as well as three new TAM stimulated MCF-7 variants which are maintained as serially transplantable tumors in our laboratory.

Materials and methods

Athymic mice and tumor transplants

MCF-7 and MCF-7 TAM breast tumors were maintained as serially passaged solid tumors in ovariectomized athymic nude mice (Harlan Sprague Dawley, Madison, WI) treated with either estradiol (E_2) or TAM capsules. Serial passaging procedures and capsule preparation have previously been described in detail [25–27]. Briefly, tumors were routinely passaged by removing a >1.0 cm diameter tu-

mor from an animal, trimming away all fat, skin, and necrotic tissue, and mincing the remaining viable tissue into approximately 1 mm^3 pieces in a bath of cold Ca/Mg-free heavy balanced salt solution. Tumor pieces were then implanted with a 13 gauge trocar into the thoracic mammary fat pads (1/side) of 4 to 5 week-old athymic mice. At the time of tumor transplantation, all animals were also implanted subcutaneously with a Silastic capsule containing either E_2, TAM or no drug (placebo control). Tumor measurements were performed weekly using calipers, and tumor cross sectional areas were calculated using the formula:

$$(\text{long axis}/2) \times (\text{short axis}/2) \times \pi$$

Data are reported as mean tumor area for each treatment group; error bars show standard error of the mean.

For tumor harvests, animals were killed by cervical dislocation, tumors were removed and cleared of all skin, fat, and obvious necrosis, and snap frozen in liquid nitrogen. Frozen tumor specimens were stored at –80° C until use.

RNA isolation and northern blot analysis

Total RNA was prepared from tumors by the method of Chomczynski and Sacchi [28]. Tumors were pulverized in liquid nitrogen and the resulting powder was homogenized in GITC solution (4 M guanidinium isothiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M β-mercaptoethanol) in a Dounce pestle homogenizer. Tumor homogenates were extracted with acidified phenol and total RNA was recovered by isopropanol precipitation from the aqueous phase. Poly A+ selected RNA was prepared by a modification of the protocol of Badley et al [29]. Total RNA (600–1000 μg) was diluted in 5 ml RNA isolation buffer (0.5 M NaCl, 0.2 M Tris-HCl pH 7.5, 1.5 mM $MgCl_2$, 2% SDS) and added to a pellet of ~75 mg oligo dT cellulose (Boeringher Mannheim, Indianapolis, IN). The resulting slurry was incubated at room temperature on a tube rotator for 2 h. Oligo dT cellulose pellets were washed 4 times with 0.5 M NaCl, 10 mM Tris-HCl, pH 7.5, and poly A+ RNA was eluted with 10 mM Tris-HCl, pH 7.5. A two step process for purifying poly A+ RNA was necessary since the large amount of tissue debris prevented efficient isolation directly from tumor homogenates. RNA was electrophoresed on 1% agarose, 7% formaldehyde gels and transferred to Hybond-N (Amersham, Arlington Heights, IL) using a Milliblot vacuum transfer apparatus (Millipore, Bedford, MA). Blots were UV fixed in a Stratalinker illuminator (Stratagene, La Jolla, CA) and prehybridized at 42° C in buffer containing 25 mM Na_3PO_4, pH 6.5, 5 × SSC, 5 × Denhardt's solution, 50 μg/ml denatured salmon sperm DNA, 50% formamide, and 10% dextran sulfate, and hybridized for 16–24 hr in the same buffer containing 1–2 × 10^6 dpm/ml of radiolabeled probe. cDNA probes were ^{32}P double labeled by random primer extension using nucleotides ^{32}P-α-dCTP and ^{32}P-α-dATP (3000 mCi/mmol, DuPont NEN, Hoffman Estates, IL). Membranes were washed 3 times for 1 h each with 2 × SSC containing 0.2% SDS at room temperature and 10–15 min at 65° C with 0.1 × SSC containing 0.2% SDS. Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY) at –70° C. For quantitation of RNA signal intensity, blots were also exposed to a phosphorimager screen and band intensity was quantitated by signal integration using a Molecular Dynamics phosphorimage analysis system (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

Whole cell extracts were prepared by pulverizing tumors in liquid nitrogen and homogenizing the tumor powder in 10 volumes of buffer containing 10 mM Tris-HCl pH 7.4, 0.6 M NaCl, 1.5 mM EDTA, and 10 mM β-mercaptoethanol. Homogenates were incubated on ice for 20 min and then centrifuged for 30 min at 100,000 × g. An aliquot of the resulting whole cell extract was immediately mixed with 2 × SDS sample buffer (final concentration 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2.3% SDS, 100 mM β-mercaptoethanol), boiled for 3 to 5 min, and frozen at –70° C until use. Another aliquot was imme-

diately snap frozen in liquid nitrogen for determination of protein concentration.

Protein concentrations were determined using the Biorad assay reagent (Biorad, Richmond, CA). Whole cell extracts (150 μg total protein) in SDS sample buffer were boiled for 2 min immediately prior to loading on denaturing polyacrylamide gels (10% acrylamide, 19:1 acrylamide:bisacrylamide). One lane on each gel was loaded with a molecular weight mixture (Biorad, Richmond, CA) containing a range of marker proteins from 14.4 to 97.4 kDa. Gels were run at 75 V per gel for 1 h. Proteins were then transferred to nitrocellulose using a Multiphor II semi-dry electroblotting transfer apparatus (Pharmacia LKB, Piscataway, NJ), at 34 mA per blot for 2–3 h. Nitrocellulose was stained with Ponceau S stain (20 mg/ml Ponceau S, 300 mg/ml trichloroacetic acid, 300 mg/ml sulfosalycilic acid) in order to visualize molecular weight markers and check for even protein loading. Blots were blocked overnight in 1 × PBST (20 mM K_2HPO_4 pH 7.5, 150 mM NaCl, 0.1% Tween-20) containing 50% calf serum, which also removed the Ponceau S stain. The following day, blots were incubated with primary antibody (H222 for ER, KD68 for PR, Abbot Labs, North Chicago, IL) for 2 h in 1 × PBST containing 10% calf serum. Blots were washed with PBST and incubated with secondary antibody (goat anti-rat IgG conjugated to alkaline phosphatase, HyClone, Logan, UT) for 2 h in 1 × PBST plus 10% calf serum. Blots were again washed with PBST and then incubated with substrate solution containing 0.33 mg/ml nitro blue tetrazolium and 0.17 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Promega) in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, and 25 μM $ZnCl_2$. When sufficient color intensity was reached, development was stopped by washing blots with 7% acetic acid. Blots were rinsed with distilled water, dried, and photographed.

Results

Generation and growth characterization of TAM stimulated tumors

MCF-7 tumors growing in athymic mice chronically

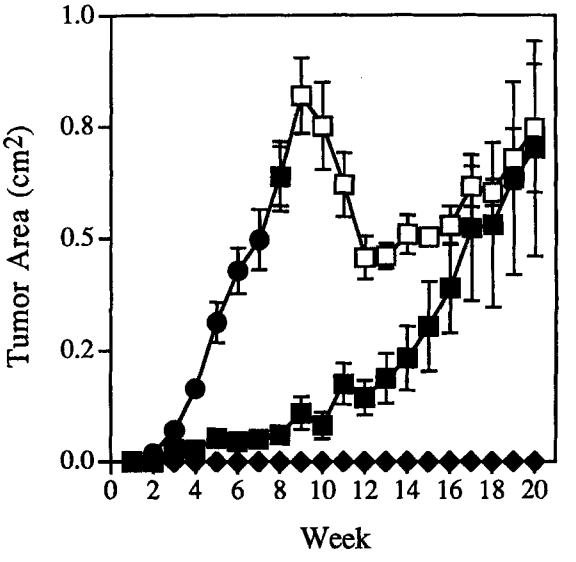

Fig. 1. Generation of new TAM stimulated tumors. Pieces of an MCF-7 tumor (passage 4) were implanted into the mammary fatpads of 18 ovariectomized athymic mice and treated with E_2 (●), TAM (■), or placebo (◆) capsules (6 mice per group). At week 8, animals treated with E_2 were switched to TAM (□). Tumor take rates were 12 of 12 sites for animals initially treated with E_2, 7 of 12 sites for TAM animals, and 0 of 12 for placebo animals.

treated with TAM reproducibly develop a TAM stimulated phenotype [19–22]. In order to carry out these studies we decided to generate new TAM stimulated tumors to compare to the previously established MCF-7 TAM tumor [20] which, at the time these experiments were carried out, had been maintained in our laboratory for 5 years and serially passaged through 21 generations of athymic mice.

In order to establish new TAM stimulated variants, we carried out an experiment similar to the one in which the MCF-7 TAM tumor was generated [20]. MCF-7 cells (10^7 cells per site) were inoculated into the mammary fatpads of E_2 treated ovariectomized athymic mice. E_2 stimulated tumor growth in all inoculated mice (data not shown). When the average tumor area reached ~0.7 cm^2, a tumor was harvested and serially transplanted into E_2 treated athymic mice, as described in Materials and methods. This process was repeated 2 more times, and then pieces of the passage 4 MCF-7 tumor were implanted into 18 4–5 week old ovariectomized athymic mice which were treated with either E_2, TAM, or placebo capsules (6 mice per group). Tumor growth was observed almost immediately in E_2

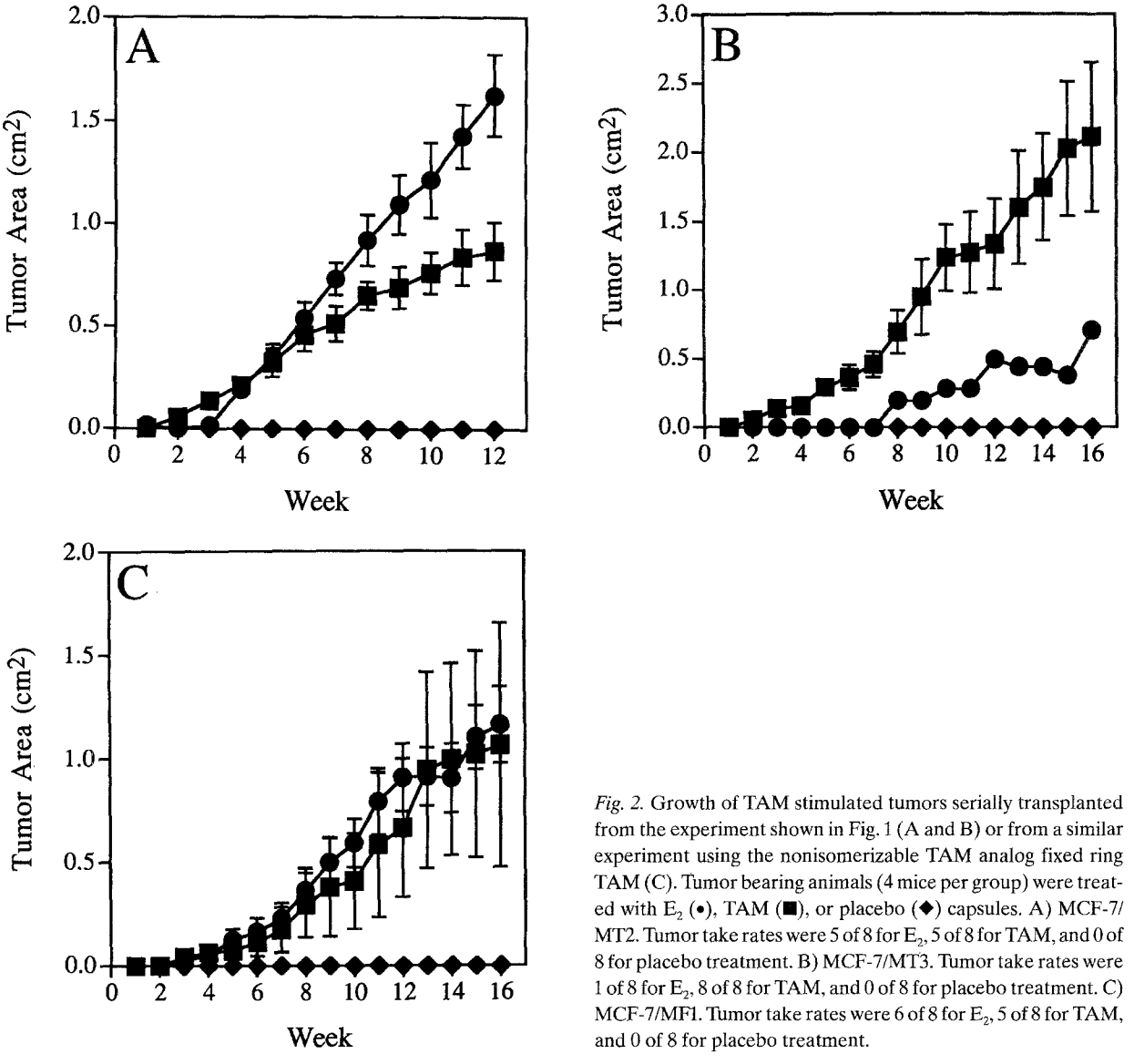

Fig. 2. Growth of TAM stimulated tumors serially transplanted from the experiment shown in Fig. 1 (A and B) or from a similar experiment using the nonisomerizable TAM analog fixed ring TAM (C). Tumor bearing animals (4 mice per group) were treated with E_2 (•), TAM (■), or placebo (◆) capsules. A) MCF-7/MT2. Tumor take rates were 5 of 8 for E_2, 5 of 8 for TAM, and 0 of 8 for placebo treatment. B) MCF-7/MT3. Tumor take rates were 1 of 8 for E_2, 8 of 8 for TAM, and 0 of 8 for placebo treatment. C) MCF-7/MF1. Tumor take rates were 6 of 8 for E_2, 5 of 8 for TAM, and 0 of 8 for placebo treatment.

treated animals (tumors grew in 12 of 12 sites), but virtually no growth was observed initially in TAM or placebo treated mice (Fig. 1). After 8 weeks, capsules were removed from the E_2 treated group and replaced with TAM capsules. Tumors treated in this fashion regressed partially, but we began to observe tumor regrowth after about 8 to 10 weeks of TAM treatment. In mice treated with TAM from the time of tumor implantation, tumor growth was delayed compared to animals treated initially with E_2, but by week 12 TAM stimulated tumor growth was observed in 8 of 12 sites (Fig. 1). No tumor growth was observed in placebo treated animals during the entire course of the experiment.

In order to determine whether these tumors were TAM stimulated versus endocrine independent, serial transplantation experiments were performed. An animal from the group initially treated with E_2 and then switched to TAM (Fig. 1) was sacrificed at week 22 (14 weeks exposure to TAM), and one of the tumors was transplanted into a new generation of ovariectomized recipient mice treated with either E_2, TAM, or placebo. This tumor is referred to as MCF-7/MT2. A similar experiment was initiated using a mouse from the continuous TAM treat-

ment group at week 20 (Fig. 1). This tumor is referred to as MCF-7/MT3. We also carried out a serial transplant experiment with a tumor generated by growth in a mouse initially treated with E_2 for 8 weeks and then switched to the non-isomerizable TAM analog fixed-ring TAM for 11 weeks (data not shown). This tumor is referred to as MCF-7/MF1. Recent work from our laboratory [19] has demonstrated that the *in vitro* and *in vivo* activities of TAM and fixed-ring TAM are indistinguishable. Therefore the MCF-7/MF1 tumor is now maintained in our laboratory with TAM rather than the less readily available fixed-ring analog.

Figure 2A shows the growth characteristics of the tumor designated MCF-7/MT2, which was derived from an MCF-7 bearing animal initially treated with E_2 and then switched to TAM. MCF-7/MT2 tumors have growth characteristics similar to those initially reported by Gottardis and Jordan [20] for the TAM dependent MCF-7 TAM tumor. Both E_2 and TAM stimulated the growth of this tumor, but no tumor growth was observed in animals treated with placebo capsules. Figure 2B shows the growth of tumor MCF-7/MT3, a TAM dependent tumor derived from an MCF-7 bearing athymic mouse treated with TAM from the time of tumor implantation. MCF-7/MT3 tumors grew rapidly in TAM treated hosts, but only one tumor grew out of 8 sites implanted in 4 E_2 treated animals, and no growth was observed in animals bearing placebo capsules. This growth pattern is similar to that observed for the late passage MCF-7 TAM, which is stimulated to grow by TAM, but does not grow well when implanted into E_2 treated hosts, and is in fact induced to regress upon E_2 administration [30, 31]. Figure 2C shows the growth of tumor MCF-7/MF1, which has growth characteristics very similar to the MCF-7/MT2 tumor.

In order to generate sufficient tumor material to perform the experiments described below, we implanted 12 animals with each tumor variant, and treated them with the 'normal' growth stimulatory ligand for that variant. Mice bearing MCF-7 wild type tumors (passage 6) were treated with E_2, while MCF-7 TAM (passage 22), MCF-7/MT2, MCF-7/MF1, and MCF-7/MT3 (each at passage 2) tumors were implanted into TAM treated mice. When the

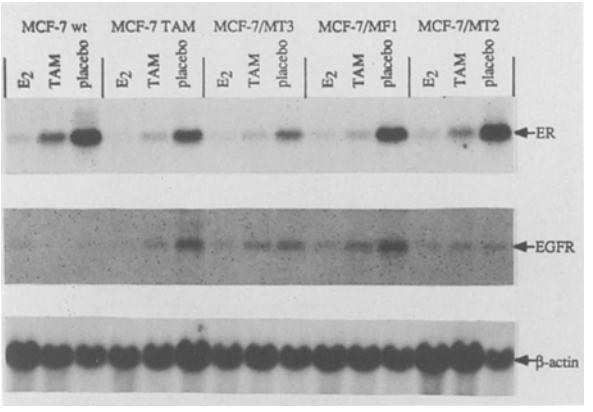

Fig. 3. Northern blot analysis of poly A+ RNA isolated from MCF-7 wild type and TAM stimulated variant tumors. ER RNA is shown in the upper panel, EGFR RNA in the middle panel, and β-actin RNA, probed as a loading control, in the bottom panel.

mean tumor cross sectional area for a tumor type reached approximately 0.5 cm^2, animals bearing that tumor were randomized into 3 groups of 4 animals each. One group continued to be treated as it had initially, and capsules were removed from the other two groups and replaced so that for each tumor there were 3 treatment groups: E_2, TAM, and placebo. This approach was followed for experimental consistency, since tumor growth rates in placebo treated animals vary widely for each tumor, wild type tumors do not grow initially in TAM treated hosts, and likewise, MCF-7 TAM and MCF-7/MT3 tumors do not grow well in E_2 treated hosts. Thus it would have been inappropriate to use all three treatments with each tumor from the time of implantation. Animals were sacrificed and tumors were harvested 3 to 4 weeks after the treatment switch, before MCF-7 TAM and MCF-7/MT3 tumors in E_2 treated animals could regress completely.

Northern blot analysis of tumor RNA

Poly A+ RNA was prepared, electrophoresed, and transferred to a nylon membrane as described in Materials and methods. The top panel of Fig. 3 shows tumor RNA probed for ER. Figure 4A shows relative levels of ER expression in each sample af-

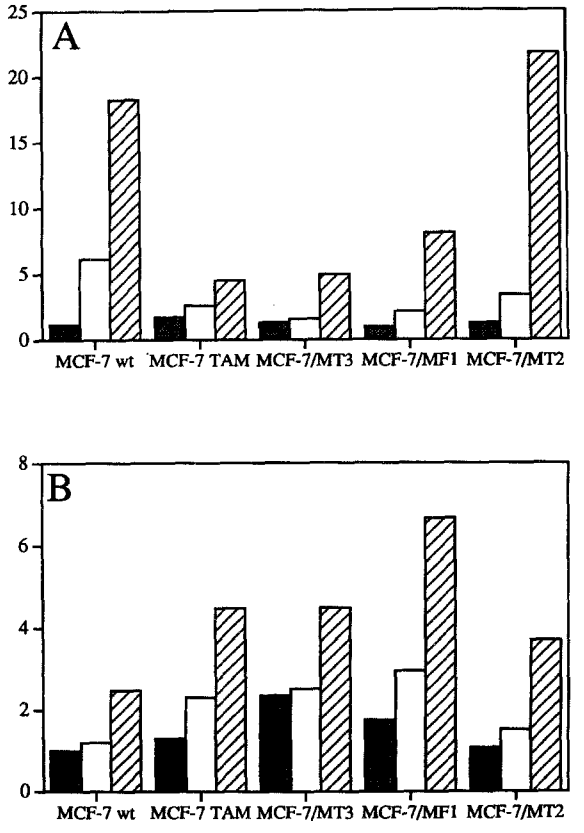

Fig. 4. Quantitation of A) ER and B) EGFR RNAs. Blots shown in Fig. 3 were quantitated by phosphorimage analysis and ER and EGFR levels were normalized to β-actin levels. The lowest ratio was arbitrarily set to 1.0 and all other levels expressed as multiples of that value. Stippled bars: E_2; white bars: TAM; cross-hatched bars: placebo treated tumors.

ter normalization to β-actin levels (Fig. 3, bottom panel). The same pattern of ER mRNA regulation occurred in all tumors examined, although the relative abundance varied from one tumor variant to another. ER RNA levels were highest in tumors from placebo treated mice and lowest in animals from E_2 treated mice. Thus, whether an MCF-7 derived tumor was growth stimulated or inhibited by TAM, both E_2 and TAM downregulated ER RNA, but TAM always did so to a lesser extent than E_2.

The middle panel of Fig. 3 shows the same blot as described above, stripped and reprobed for epidermal growth factor receptor (EGFR) RNA. Figure 4B shows relative levels of EGFR expression in each sample after normalization to β-actin levels (Fig. 3, bottom panel). Control of EGFR expression follows a pattern similar to that for ER, with levels for a given tumor variant being highest in the placebo treated lanes and lowest in the E_2 treated lanes. It is interesting to note that the two tumor variants (MCF-7 wt and MCF-7/MT2) which express the highest levels of ER RNA in the placebo treated animals express the lowest EGFR levels.

A blot was also probed for PR RNA, but even with high specific activity probes (see Materials and methods) and exposure times in excess of 3 weeks, only a very weak signal was detectable, and only in the E_2 treated wild type MCF-7 tumor (data not shown).

Western blot analysis of tumor ER and PR

Previous work from our laboratory has shown that PR protein could be detected in MCF-7 samples by ligand binding or immunological assay even though little or no PR RNA was detectable by northern blot analysis (Fritsch, Jeng, Pink, and Jordan, unpublished observations). We therefore decided to do western blot analyses of ER and PR expressed by the MCF-7 wild type and variant tumors used in these experiments.

Whole cell extracts containing 150 mg total protein were size fractionated on 10% polyacrylamide gels and then transferred to nitrocellulose and probed with either H222 anti-ER or KD68 anti-PR antibody. The blot probed with anti-ER is shown in Fig. 5A. All tumors expressed ER protein of the expected 65 kDa size, but regulation did not exactly parallel that for ER RNA. ER protein levels were always lowest in the E_2 treated lane for a given tumor, but for the MCF-7 wild type, MCF-7 TAM, and MCF-7/MF-1 tumors, ER protein levels from TAM treated animals appeared to be upregulated compared to placebo treated animals. No conclusions can be drawn for the MCF-7/MT3 tumor since there was insufficient tumor from the E2 treated MCF-7/MT3 group to prepare whole cell extracts. The lower band in both Figs. 5A and 5B is mouse IgG, which cross reacts with the goat anti-rat IgG second antibody. Differences in IgG intensity are due to differing amounts of blood contaminating the tumor tissue.

A western blot probed for PR is shown in Fig. 5B.

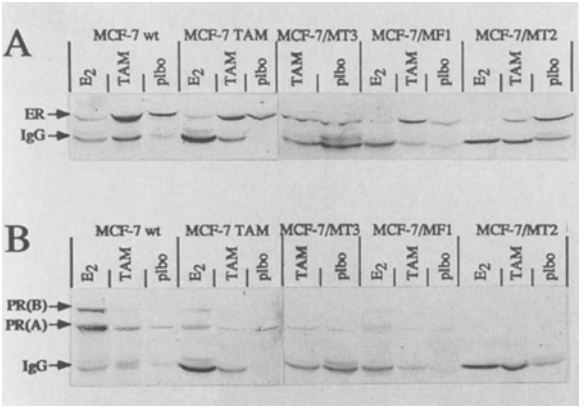

Fig. 5. Western blot analysis of ER and PR protein expression by MCF-7 tumor variants. A) Blot probed with anti-ER antibody H222. The upper band is ER, and the lower band is mouse IgG, which cross reacts with the secondary antibody. B) Blot probed with anti-PR antibody KD68. The upper band is the ~120 kDa PR form B, the middle band is the ~99 kDa PR form A, and the lower band is mouse IgG, which cross reacts with the secondary antibody.

Both the ~99 kDa A form and ~120 kDa B form of the PR are apparent in the whole cell extract from E_2 treated MCF-7 wild type tumor. Both bands are also present in the TAM treated wild type tumor but were induced to a much weaker extent. In the lanes containing whole cell extracts from MCF-7 TAM tumors, E_2 induced PR expression is also apparent, but to a much lower extent than seen in the wild type tumor. In the remaining MCF-7 TAM lanes, and extracts from the other three tumors, no PR form B is detectable, little if any of the A form can be seen, and no regulation of expression is apparent. As with the ER blot (Fig. 5A) there is no E_2 treated lane for the MCF-7/MT3 tumor since there was insufficient material to prepare a whole cell extract.

Discussion

Previous reports from our laboratory and others have focused on describing the development of TAM stimulated MCF-7 breast tumor growth [19–22], and addressing the hypothesis that this form of treatment failure arises because tumors acquire the ability to eliminate TAM and its metabolites from the cell [19, 22, 23], and/or convert them to compounds with reduced antiestrogenic or heightened estrogenic activity [19, 22–24]. Although an attractive hypothesis, we have previously demonstrated that neither mechanism can be responsible for the TAM stimulated growth of MCF-7 tumor variants maintained in our laboratory [19].

In this paper we further characterize 4 TAM stimulated MCF-7 tumor variants which we have developed by exposing pieces of an MCF-7 solid tumor to chronic TAM administration in ovariectomized athymic mice. As shown in Fig. 1, TAM stimulated growth can develop either from a fairly large tumor mass which was initially grown up under E_2 treatment and switched to TAM, or from small tumor pieces implanted into mice treated with TAM from the beginning. Upon serial transplantation, TAM stimulated tumor variants grew as rapidly in TAM treated mice as the original wild type MCF-7 tumor grew in E_2 treated animals (compare Fig. 2, A-C, to the E_2 treated growth of MCF-7 tumors in Fig. 1). Two of the TAM stimulated variants (MCF-7/MT2, Fig. 2A, and MCF-7/MF1, Fig. 2C) were also stimulated to grow by E_2. However, the MCF-7/MT3 tumor (Fig. 2B) did not grow well in E_2 treated hosts. Unlike the MCF-7 TAM tumor, which required several years of TAM treatment to become refractory to E_2 stimulation [30, 31], MCF-7/MT3 tumors were E_2 intolerant from the time of first serial transplantation. The 'late passage' nature of MCF-7/MT3 tumors may reflect the more stringent conditions under which these tumors were developed, since the pool of cells from which a TAM stimulated tumor could develop was much smaller for MCF-7/MT3 than for MCF-7/MT2 or MCF-7/MF1.

Despite differences in growth responses to TAM and E_2, all four TAM stimulated tumor variants show similar patterns of regulation for ER and EGFR RNA (Figs. 3 and 4). However, placebo treated ER RNA levels in 3 of the 4 TAM stimulated tumor variants were much lower than those for the wild type MCF-7 tumor. The only exception was the MCF-7/MT2 tumor, which, as described in the accompanying paper [32], is the only TAM stimulated tumor that expresses a mutant ER. In all 4 TAM stimulated tumor variants, TAM treatment appeared to down regulate ER message more nearly to E_2 treated levels than was the case in the wild

type tumor. This difference was not reflected in ER protein levels, however. ER protein levels in TAM treated MCF-7, MCF-7 TAM, and MCF-7/MF1 tumors were actually higher than those in placebo treated controls. ER levels appeared to be roughly equal in MCF-7/MT3 tumors, but in the TAM treated MCF-7/MT2 tumor, the ER protein level was much lower than in the placebo treated tumor. There have been reports that TAM treatment can artifactually increase apparent ER concentrations as determined by immunoassay because the anti-ER antibody H222 binds with higher affinity to a TAM occupied receptor [33, 34]. However, that cannot be the reason for the results shown in Fig. 5A. Since western blots involve the immunological detection of denatured proteins run on SDS acrylamide gels, TAM binding could not artifactually increase the antigenicity of ERs taken from TAM treated tumors in this case.

Perhaps TAM stabilizes the ER protein whereas E_2 does not, so that both ligands can induce down regulation of ER at the RNA level, but an increase in protein turnover is only accomplished by an E_2 occupied ER. Although consistent with our data, this hypothesis is not supported by recent findings that show E_2 increases the half-life of the ER [35] when compared to the unoccupied receptor. An alternate possibility is that an E_2 occupied ER might be bound much more tightly to nuclear matrix components, and our whole cell extract preparation procedure was not effective enough to extract the entire ER population from the nuclei of E_2 treated tumor cells. Interestingly, the only tumor in which the ER protein level is lower in the TAM treated sample than in the placebo sample is the MCF-7/MT2, which expresses a mutant ER. This suggests that whatever post translational regulation E_2 causes in a wild type receptor is mimicked by TAM in the mutant ER containing tyrosine instead of aspartate at position 351.

EGFR RNA regulation does not vary qualitatively among TAM stimulated and TAM inhibited tumors. However, EGFR RNA levels appear to be somewhat higher in TAM stimulated tumors than in the MCF-7 wild type, and particularly in the 3 tumors (MCF-7 TAM, MCF-7/MT3, and MCF-7/MF1) in which no mutant ER was detected [32]. It may be the TGF-α/EGFR pathway is of greater importance to the TAM stimulated phenotype in the absence of other factors, such as a mutant ER.

Both ER and EGFR RNA regulation appear to be qualitatively unchanged between TAM stimulated and TAM inhibited tumors. Although ER protein and RNA regulation does not appear identical, E_2 and TAM have consistent effects on ER expression in all tumors expressing wild type ER, regardless of whether they are inhibited or stimulated by TAM. However, both ER and EGFR are under negative regulatory control by the ER in MCF-7 cells. In contrast, it appears that during the conversion from TAM inhibited to TAM stimulated growth, positive regulation of PR protein expression by ER ligands is strongly curtailed if not lost entirely. This is contrary to what one might expect, i.e., that PR in a TAM stimulated tumor would be strongly induced by both TAM and E_2 instead of just E_2, but this is not the case. This suggests that the transcriptional control mechanisms regulating the induction of growth and the induction of other E_2 responsive genes such as PR are different. It may be that TAM stimulated tumor cells have begun expressing transcription factors characteristic of a cell type, such as liver or endometrium, which responds to TAM as a stronger agonist than does a breast cell (reviewed in [36]). In the process, expression of transcription factors which control PR expression in TAM inhibited wild type MCF-7 cells may have been lost. Obviously, before this hypothesis can be fully tested, the proteins making up ER associated transcription complexes in various estrogen responsive cell types must be identified and characterized.

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