

Prevention of solely estrogen-induced mammary tumors in female ACI rats by tamoxifen: evidence for estrogen receptor mediation

S A Li, S J Weroha, O Tawfik¹ and J J Li

Hormonal Carcinogenesis Laboratory, Division of Etiology and Prevention of Hormonal Cancers, Kansas Cancer Institute and the Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160-7417, USA

¹Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160-7417, USA (Requests for offprints should be addressed to S A Li; Email: sli@kumc.edu)

Abstract

There is increasing evidence that both endogenous and exogenously ingested estrogens play a primary role in sporadic breast cancer causation. To establish further that solely estrogen-induced mammary oncogenesis in female ACI rats is an estrogen receptor (ER α)-driven process, we show for the first time that concomitant treatment with the antiestrogen, tamoxifen citrate (TAMc), completely prevents the induction of 17 β -estradiol (E₂)-induced mammary gland tumors (MGTs). This finding is also supported by the reduced mammary gland (MG) lobulo-alveolar development and proliferative activity observed in TAMc+E₂-treated animals compared with MGs from animals treated with E₂ alone. These data also correlated with a marked decrease in the number of MG cells expressing ER α and progesterone receptor (PR) in immunostained MG tissue sections from TAMc+E₂-treated animals. Additionally, a marked decline in the level

of expression of ER α 47, 56 and 66 kDa forms, and PR-A and PR-B was seen in TAMc+E₂-treated MGs, compared with MGs treated solely with E₂. Thus, both ER α and PR MG profiles in TAMc+E₂-treated rats essentially revert to their respective receptor profiles seen in untreated control and TAMc-alone-treated rats. The presence of 56 and 54 kDa isoforms in chronically E₂-treated MGs and in MGTs respectively may contribute to fostering the enhanced E₂-dependent growth response of both precursor and frank MGT epithelial cells. These findings are consistent with an ER α /PR-mediated MG cell proliferation, a prerequisite for generating the high frequency of chromosomal instability seen in E₂-induced ductal carcinomas *in situ* and primary MGTs in female ACI rats reported by us previously.

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Introduction

Sporadic breast cancer (BC) accounts for 90–95% of all BC cases among women (King *et al.* 1993, Li CI *et al.* 2000). While it is believed that the etiology of human BC is multi-factorial, there is increasing evidence that both endogenous naturally occurring estrogens and exogenously ingested estrogenic agents play a crucial role in sporadic BC causation (Colditz *et al.* 1995, Toniolo 1997). Numerous epidemiological studies have shown that early menarche, late age at menopause, late age at first full-term pregnancy, nulliparity, absence of lactation, and post-menopausal estrogen replacement therapy are associated with increased BC risk (Pike *et al.* 1993, Colditz *et al.* 1995, Henderson & Feigelson 2000, Mahavni & Sood 2001). Even dietary and lifestyle factors, such as fat and alcohol intake, result in elevated circulating estrogen levels (Howe *et al.* 1991, Wu *et al.* 1999). Recent findings from the Breast Cancer Prevention Trials (Fisher *et al.* 1998) in which tamoxifen treatment showed a dramatic decline in

BC risk (44–55%) in women considered at increased risk for the disease provided compelling evidence for a primary role for estrogens in BC etiology. While synthetic chemical carcinogen-induced mammary tumor models have dominated experimental BC research in the past 50 years, solely estrogen-induced mammary murine models have been generally neglected, despite the fact the latter have been known for a longer time (Noble *et al.* 1940, Geschickter & Byrnes 1942, Shay *et al.* 1949, Huggins *et al.* 1959, Bots & Willighagen 1975). Numerous early studies have shown that many rat strains are highly susceptible to estrogen-induced mammary tumors, including Noble, ACI and Long-Evans (60–100%), and to a lesser extent, August, Wistar-Wag and Sprague-Dawley (36–42%) (Nelson 1944, Dunning *et al.* 1953, Cutts & Noble 1964, Blankenstein *et al.* 1977). Recently, we have shown that there are distinctive critical differences between synthetic chemical carcinogen- and environmental carcinogen-induced breast tumors, both ductal carcinoma *in situ* (DCIS) and primary tumors, and solely

estrogen-induced mammary neoplasms (Li SA *et al.* 2000, Li *et al.* 2002). These differences include early genomic destabilization (aneuploidy) and *c-myc* amplification, both features commonly found in human BCs. To further establish that mammary gland (MG) estrogen-induced oncogenesis in female ACI rats is an estrogen receptor (ER α)-driven process, we now demonstrate, for the first time, that concomitant treatment with tamoxifen completely prevents the induction of these mammary tumors by estrogen. Moreover, characterization of ER α and progesterone receptors (PR) during mammary tumor induction further supports our contention for a crucial role of estrogens in the oncogenic process of the ACI rat MG.

Materials and Methods

Animals and treatment

Intact female ACI rats, 6 weeks of age (Harlan SD, Indianapolis, IN, USA), were housed in facilities certified by the American Association for the Accreditation of Laboratory Animal Care. The rats were acclimated for at least 1 week prior to treatment, and then randomly distributed into various control and treatment groups. They were maintained on a 12 h light:12 h darkness cycle, and freely fed Ralston-Purina Teklad Rodent Diet 8604 (Teklad, Madison, WI, USA) and tap water. The animal studies were carried out in adherence to the guidelines established in the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Resources, NIH 1985). The female ACI rats were divided into five groups, each group contained 6–12 animals: group 1 remained untreated and served as control; groups 2 and 3 received a single 20 mg pellet of 17 β -estradiol (E $_2$), containing either 2 or 3 mg E $_2$, plus 18 or 17 mg cholesterol respectively; group 4 received a single 40 mg pellet of tamoxifen citrate (TAMc); and group 5 received a 40 mg pellet of TAMc, and 1 week later, a 20 mg pellet of E $_2$ containing 3 mg E $_2$. Hormone pellets, prepared without binder by Hormone Pellet Press (Leawood, KS, USA), were implanted subpannicularly in the shoulder region as described previously (Li *et al.* 2002). After 6 months of treatment, all the rats were killed by decapitation, subjected to macroscopic examination, and the presence of mammary gland tumors (MGTs), both number and location, was recorded. The abdominal-inguinal MGs and the MGTs, freed of connective and necrotic tissue respectively, were quickly removed. Portions of these tissues were immediately frozen in liquid nitrogen and stored at -80°C for future analysis, while others were fixed in 10% buffered formalin, followed by a rapid paraffin-embedding process. Uterine tissue from ACI female rats (ER α and PR), and dorsal and ventral prostates from male Sprague-Dawley (SD) rats (ER β) were also harvested and processed in the same manner as the MG samples as positive controls for Western and Northern blot analyses.

Histological and immunohistochemical analysis

For histopathological evaluation, MG sections (6 μm) were prepared from paraffin-embedded blocks and stained with hematoxylin and eosin. For immunohistochemical analysis, sections were dewaxed and treated with 3% H $_2$ O $_2$ for 15 min to block endogenous peroxidases. Antigens were retrieved (Dako Target Retrieval Solution; Dako, Carpinteria, CA, USA) by heating in a water bath set at 97°C for 40 min for ER α , 15 min for PR staining, and 10 min for proliferating cell nuclear antigen (PCNA) staining. After blocking with 6% of the appropriate serum in 1% BSA, the primary antibodies, rabbit polyclonal ER α MC20 and PR C19, and PCNA mouse monoclonal PC10 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were applied to the sections overnight at 4°C . Tissues were also incubated in the presence of their respective blocking peptides to determine the specificity of the immunoreactions. Slides were counterstained with hematoxylin, dehydrated in alcohol, and mounted in Permount medium (1:1 Permount:xylene) before being examined under the microscope.

RIA

Blood was collected during decapitation. Serum samples were collected after centrifugation and stored at -20°C until needed. E $_2$ circulating levels were determined by RIA, as previously described by us (Li *et al.* 1994), using an ultra-sensitive E $_2$ kit from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA).

Western blot analysis

For Western blot analysis of ER α , ER β , PR, PCNA and actin, MG, MGT and uterine cytosolic fractions from 6–12 female ACI rats/group were used, as well as the prostates of six intact male SD rats. The tissue samples, harvested from individual rats from each group, were homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, NY, USA) in a lysate buffer containing: 50 mM Tris-HCl pH 7.4, 0.2 M NaCl, 2 mM EDTA, 0.5% NP-40, 50 mM NaF, 0.5 mM Na $_3$ VO $_4$, 20 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 10 $\mu\text{g}/\text{ml}$ aprotinin, 10 $\mu\text{g}/\text{ml}$ leupeptin and 1 mM dithiothreitol, and centrifuged at 12 500 *g* for 20 min at 4°C . The tissue lysates were collected and centrifuged again as before. The supernatant fractions were collected and their protein content determined with BCA reagents (Pierce, Rockford, IL, USA). Protein aliquots (20 μg) were electrofractionated on 4–20% gradient Tris-glycine SDS-PAGE under reducing conditions. Thereafter, proteins were transferred onto nitrocellulose membranes, blocked with non-fat milk, and probed with primary antibodies followed by secondary antibodies conjugated with horseradish peroxidase. The immunoblots were visualized by chemiluminescence using

ECL Western blot detection reagents (Amersham Life Science, Arlington Heights, IL, USA). The intensity of each signal was quantified by densitometric scanning (Molecular Dynamics, Sunnyvale, CA, USA). Equal loading was monitored by hybridization of the same membranes with an anti- β -actin antibody. The primary antibodies and their respective blocking peptides for ER α , PR and PCNA were the same used for the immunohistochemical analysis. For the detection of ER β , three different antibodies were tested: L-20 and H-150 of human origin and corresponding to the carboxy- and amino-termini respectively, and Y-19 of mouse origin and corresponding to the amino-terminus. For β -actin, a mouse monoclonal C2 primary antibody was used. All the antibodies were obtained from Santa Cruz Biotechnology.

Northern blot analysis

Total RNA was extracted as previously described (Chomczynski & Sacchi 1987) with some modifications. Briefly, tissue samples were homogenized for 60 s in 5 ml 4 M guanidinium isothiocyanate using a Polytron set at maximum speed, followed by phenol/chloroform extraction. Total RNA was precipitated with isopropanol and dissolved in ribonuclease-free water. The RNA concentration was determined by absorbance at 260 nm. The RNA was separated by electrophoresis in a denaturing formaldehyde agarose gel, and blotted onto Hybond nylon membranes (Amersham Life Science). The RNA was crosslinked to the membranes by irradiation for 1 min under u.v. light, and baked for 3 h at 65 °C. A random priming probe kit from Promega (Madison, WI, USA) was used to label a 700 bp cDNA synthesized from a linearized human ER α cDNA, and a 56 bp oligonucleotide probe specific for the rat ER β and glyceraldehyde 3-phosphate dehydrogenase (GADPH). The blots were hybridized for 16–18 h at 65 °C with 32 P-labeled ER α and ER β cDNA probes. After washing twice with $2 \times$ SSC/0.1% SDS, the membranes were exposed for 18–72 h at -70 °C to Kodak XAR-5 film (Eastman Kodak, Rochester, NY, USA) with intensifying screens. Control for equal loading and blotting of RNA was performed by stripping blots in boiling $0.05 \times$ SSC/0.1% SDS and rehybridizing with antisense riboprobes generated from cDNA clones for GADPH.

Results

Serum E₂ levels, body and tissue weights

Employing a single 20 mg pellet containing either 2 or 3 mg E₂, serum E₂ concentrations were assessed at monthly intervals. Over a 6 month treatment, the serum E₂ values ranged from 52 to 89 pg/ml and from 112 to 145 pg/ml after a 2 or 3 mg dose of E₂ respectively (Fig.

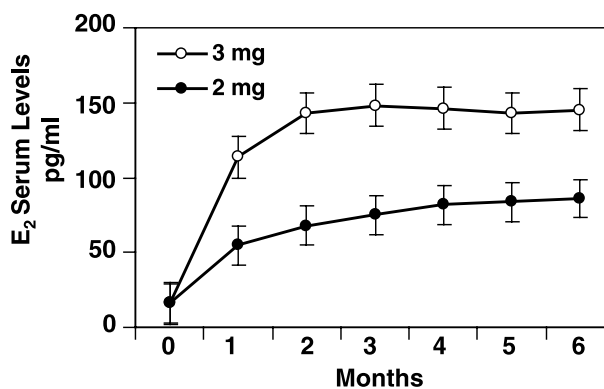


Figure 1 Serum E₂ levels determined at monthly intervals in intact female ACI rats treated with either 2 or 3 mg E₂. The data represent the means \pm S.E.M. of three or four individual samples measured in duplicate.

1). The E₂ serum levels plateau rapidly after 2 months of either E₂ treatment (Fig. 1; Table 1). At the higher dose of E₂, a 100% MGT incidence was attained (Table 2), in the absence of either body weight loss or mortality (Table 1). A 100% MGT incidence was also found at the lower E₂ dose but the MGTs were slightly smaller and less numerous (data not shown). Concomitant treatment with TAMc did not significantly alter the E₂ serum levels compared with the group receiving E₂ treatment alone (Table 1). Relative to untreated control animals, female rats receiving E₂ alone exhibited a significant increase in MG and uterine wet weights, while a smaller, although significant, rise in MG wet weight was observed after the combined TAMc+E₂ treatment (Table 1).

Prevention of solely E₂-induced mammary oncogenesis by TAMc

Female rats treated with 3 mg E₂ for 6 months yielded a 100% MGT incidence, and an average of 15.6 ± 1.6 (S.E.M.) MGTs/rat (Table 2). In contrast, no MGTs were detected in either control untreated or TAMc-treated rats. Importantly, after histological examination of MG serial sections, animals receiving TAMc+E₂ did not exhibit evidence of MGT formation after 6 months of continuous treatment (Table 2).

MG and MGT morphology

MG tissue sections from the control untreated group revealed a limited number of lobular units which were composed of small terminal ducts surrounded by occasional ductules or acini (Fig. 2A). Terminal ducts and acini were lined by two cell layers. The inner layer was composed of luminal cuboidal epithelial cells, while the outer layer consisted of myoepithelial cells. MG sections from TAMc-treated animals (Fig. 2B) were almost identical to those of the control untreated group (Fig. 2A), with a similar

Table 1 Body weight (BW), MG and uterus weights, and serum E₂ levels of ACI rats treated with E₂, and/or TAMc alone or in combination for 6 months. Values are the means ± S.E.M.

Treatment	No. of rats	BW (g)	MG wet weight (g/100 BW)	Uterine wet weight (g/100 BW)	E ₂ serum levels (pg/ml)
Untreated	6	198 ± 2	1.03 ± 0.03	0.24 ± 0.01	20 ± 3
TAMc 40 mg	8	187 ± 3	0.91 ± 0.07	0.14 ± 0.01 ¹	18 ± 2
E ₂ 3 mg	12	175 ± 8	1.88 ± 0.15 ¹	0.41 ± 0.02 ¹	140 ± 7 ¹
TAMc 40 mg+E ₂ 3 mg	8	186 ± 5	1.47 ± 0.08 ¹	0.22 ± 0.05	145 ± 8 ¹

¹Indicates significant difference ($P \leq 0.05$) compared with untreated group (Student's *t*-test).

Table 2 Prevention of E₂-induced MGTs in female ACI rats by TAMc. ACI rats were treated with E₂, and/or TAMc alone or in combination for 6 months. Values are the means ± S.E.M.

Treatment	No. of rats	No. of rats with tumors	Percent of rats with tumors	Tumor multiplicity/rat
Untreated	6	0	0	0
TAMc 40 mg	8	0	0	0
E ₂ 3 mg	12	12	100	15.6 ± 1.6
TAMc 40 mg+E ₂ 3 mg	8	0	0	0

number of lobular units and cellular composition. As anticipated, after E₂ treatment (Fig. 2C) a series of morphological changes became evident including enlargement of the lobular units, formation of new units, and marked epithelial hyperplasia with nuclear enlargement and prominence of nucleoli. TAMc+E₂ treatment (Fig. 2D) resulted in a significant decrease in the proliferative activity of the MG as reflected by the reduced number of lobular units, and the smaller size of the cellular and nuclear components, compared with MG sections of E₂-treated female ACI rats (Fig. 2C). Multiple malignant neoplastic foci were noted after 6 months of E₂ treatment (Fig. 2E). These malignant foci exhibited histological features of malignant transformation such as nuclear enlargement, pleomorphism, hyperchromasia and prominent nucleoli. Foci with early atypical dysplastic changes that proceed a frankly malignant process were also evident.

Immunohistochemical analysis of ER and PR in MG and MGT samples

In control untreated MG tissue samples, ER α (Fig. 2F) and PR (Fig. 2K) were expressed only in 5–10% of the terminal ducts and lobules. Staining for these hormone receptors appeared extremely heterogeneous. In MGs from TAMc-treated animals, the pattern of ER α expression (Fig. 2G) was very similar to that observed in the control untreated group (Fig. 2F). A modest increase in PR expression (Fig. 2L) was observed in this treatment group. After 6 months of E₂ treatment, ER α (Fig. 2H) and PR (Fig. 2M) were upregulated in 70–95% of the MG cells. TAMc+E₂ treatment (Fig. 2I), however, resulted in

a significant reduction of ER α expression in MG cells to levels approaching those observed in the control untreated MGs (Fig. 2F). A slight increase in PR expression was observed after treatment with TAMc+E₂ (Fig. 2N), when compared with control untreated animals (Fig. 2K); however, these levels were markedly lower from those observed after E₂ treatment alone (Fig. 2M). In all MGTs examined, a consistent high expression of ER α (Fig. 2J) and PR (Fig. 2O) was observed.

Expression of MG and MGT ER α and β isoforms

Western blot analysis established the presence of ER α in female ACI rat MGs and MGTs. MGs from untreated rats revealed a single ER α 56 kDa isoform (Fig. 3, upper panel). After 6 months of E₂ treatment, the appearance of a 47 kDa variant and a moderate expression of a 66 kDa ER α form, probably the full length ER α , were observed. While the primary E₂-induced MGTs exhibited modest expression of both the 56 and the 66 kDa ER α forms, a 54 kDa ER α isoform, present in control untreated uterus, was also detected. It was evident that the 54 kDa isoform was the predominant form in MGTs. Strikingly, when compared with E₂-treatment alone, both the MG 56 kDa and the 47 kDa ER α isoforms were markedly reduced (>5.0-fold) after TAMc+E₂ treatment. TAMc treatment alone depressed the expression of the MG 56 kDa ER α variant (~2.0-fold) compared with the untreated control group. Uteri from the untreated control group exhibited high levels of ER α expression of both the 66 and the 54 kDa isoforms. ER α expression in Western blots was completely abolished in the presence of its respective

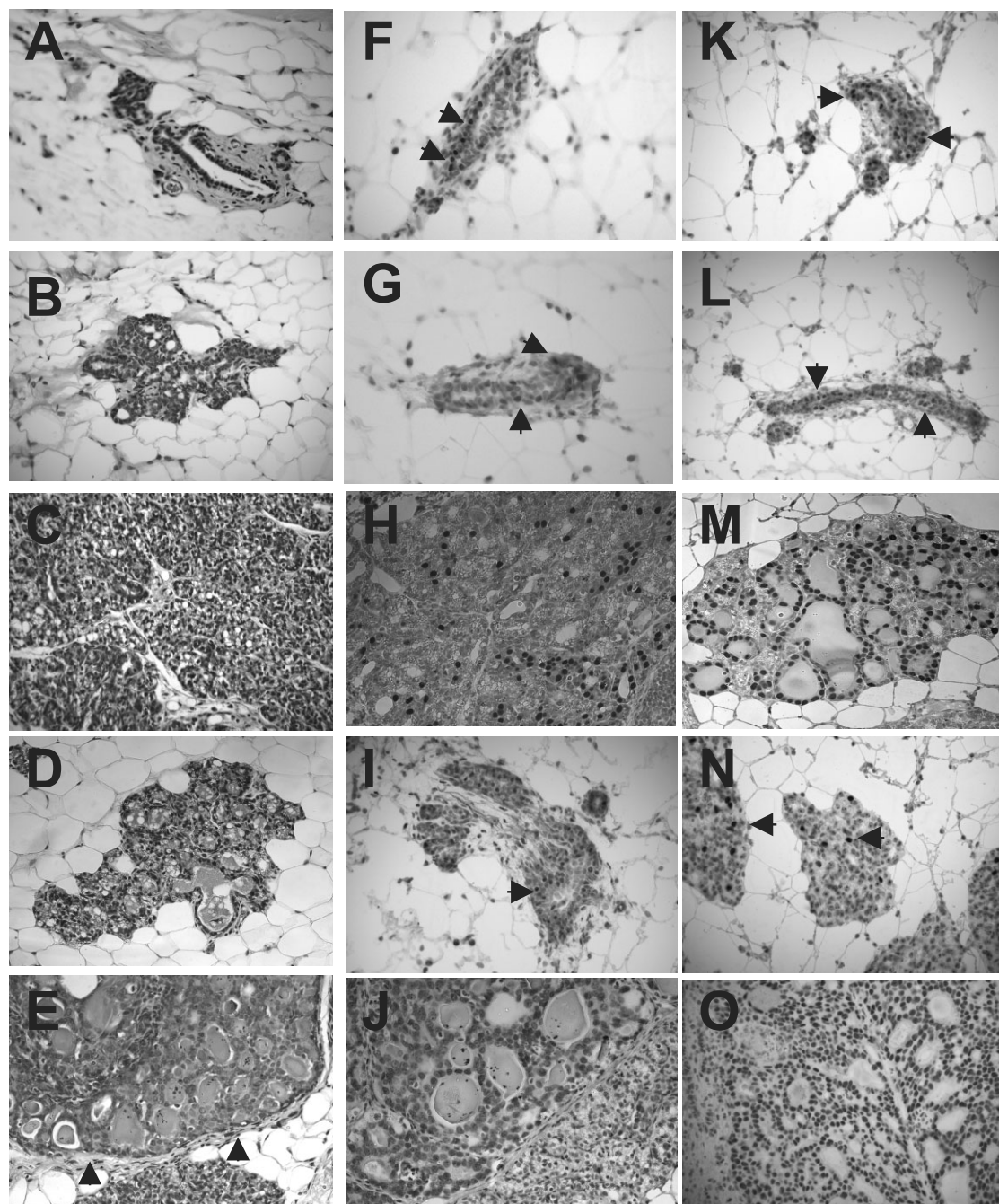


Figure 2 Consecutive sections of MG tissue samples, after 6 months of treatment, were stained separately with hematoxylin and eosin (A–E) or antibodies to ER α (F–J) and PR (K–O). (A) Normal MG section from an untreated rat exhibiting modest alveolar development and adipocytes; (B) TAMc-treated rat MGs showing modest ductal and alveolar development; (C) E₂-treated rat MG depicting an increase in proliferative activity compared with that of untreated controls (A) and TAMc-treated (B) rats; (D) TAMc+E₂-treated MG exhibiting moderate alveolar development; (E) DCIS after 6 months of E₂ treatment (arrows) nested within surrounding hyperplastic MG cells – the MGT cells showed a marked variation in size and shape, and mitotic figures; (F and K) normal MG showing nuclear (arrows) ER α (F) and PR (K) staining in ductal epithelial cells; (G and L) TAMc-treated MG sections with few cells (arrows) exhibiting weak to moderate ER α (G) and PR (L) nuclear staining; (H and M) E₂-treated MG with hyperplastic cells intensely stained for nuclear ER α (H) and PR (M); (I and N) TAMc+E₂-treated MG sections showing few cells (arrows) with ER α (I) or PR (N) nuclear staining; (J and O) DCIS with numerous cells intensely stained for nuclear ER α (J) and PR (O). Magnifications $\times 400$.

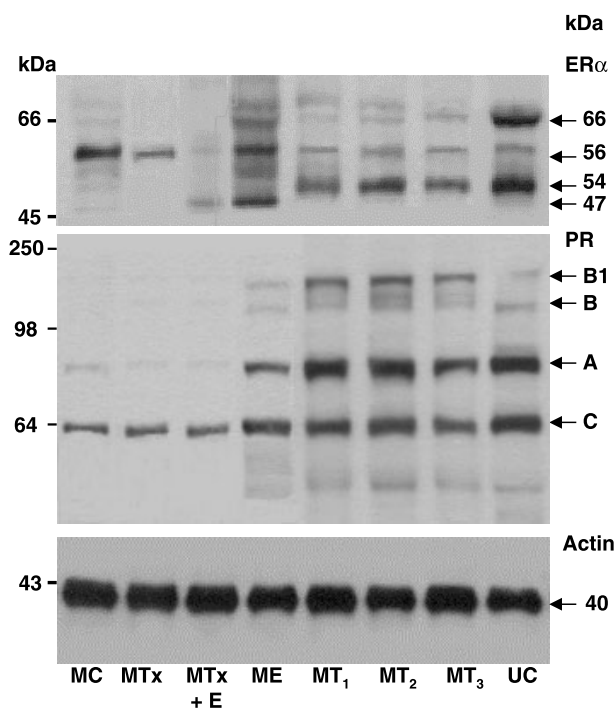


Figure 3 Representative ER α and PR expression in MG and MGT samples. Western blot was performed on 20 μ g protein aliquots after 6 months of treatment. MC, untreated, TAMc-treated (MTx), TAMc+E₂-treated (MTx+E), E₂-treated (ME), three individual E₂-induced MGT (MT₁ to MT₃), and untreated uterus (UC). The primary antibodies used were ER α MC20, β -actin C10 and PR C19. The relative amount of protein was determined by densitometry. The figure is representative of three independent experiments.

blocking peptide. No positive signals for ER β were observed in any of the MG or MGT samples tested with three different ER β antibodies, specific for ER β of either mouse or human origin. However, dorsal and ventral rat prostate samples, used as positive controls, showed an intense \sim 50 kDa band that was neutralized in the presence of the proper blocking peptides (data not shown).

Expression of MG and MGT PR forms

MGs from both the untreated control and the TAMc-treated groups exhibited essentially the same level of expression of PR-C (Fig. 3 middle panel). Upon treatment with E₂ for 6 months, both PR-A and PR-B were induced, albeit PR-B exhibited only weak expression. PR-A in E₂-induced primary MGTs was \sim 6.0- to \sim 10.0-fold higher than that observed in untreated control MG samples. TAMc+E₂ treatment yielded PR expression profiles similar to those seen in either untreated control or solely TAMc-treated MGs. PR-A was extremely low in all of these groups. In MGTs, a prominent PR-B1 form was consistently detected, which was much less evident in

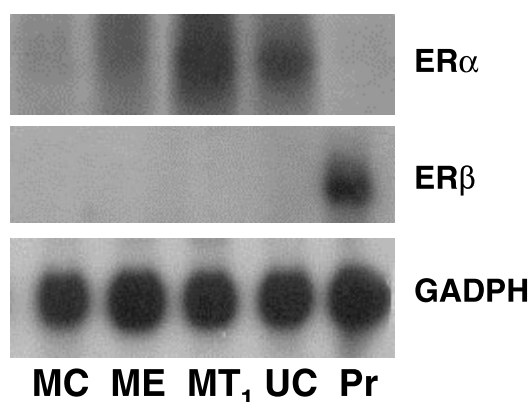


Figure 4 Representative Northern blot analysis of ER α and ER β during E₂-induced MG carcinogenesis. Total RNA was prepared from ACI rat MGs from untreated controls (MC), after 6 month of E₂ treatment (ME), and mammary tumors (MT), uterine tissue samples from untreated controls (UC) and SD rat prostate (Pr). Equal loading (10 μ g) was assessed by GADPH. This figure is representative of three independent experiments.

MGs from the solely E₂-treated group. Untreated control uteri showed predominant expression of the PR-A and PR-C forms, but only a weak expression of PR-B.

ER α and ER β transcript expression in MG and MGTs

Northern blot analysis revealed an increase in ER α mRNA expression after 6 months of E₂ treatment compared with untreated control MGs (Fig. 4). A further rise in ER α transcript expression was observed in E₂-induced primary MGTs. In contrast, ER β mRNA expression was not detected in any of the MG, MGT or uterine tissues examined. Rat prostate samples, however, resulted in marked ER β mRNA expression, thus serving as a positive control.

Cell proliferation

A comparison of the MG proliferative activity in response to the various treatments was determined by the level of PCNA expression employing Western blot analysis. When compared with the control untreated group, after 6 months of E₂ treatment, a 3.5-fold increase in MG PCNA expression was detected (Fig. 5). In contrast, the groups treated with TAMc alone (1.4-fold) or in combination with E₂ (1.2-fold) showed levels of expression similar to those observed in the untreated control group (1.0-fold). Moreover, solely E₂-induced primary MGTs showed a 5.8- to 6.0-fold increase in PCNA expression. Similar changes were observed in MG tissue sections from all the treatment groups examined after PCNA immunohistochemical analysis (data not shown).

Discussion

The female ACI rat is particularly sensitive to E₂-induced MG oncogenesis. Employing doses of 2 and 3 mg E₂,

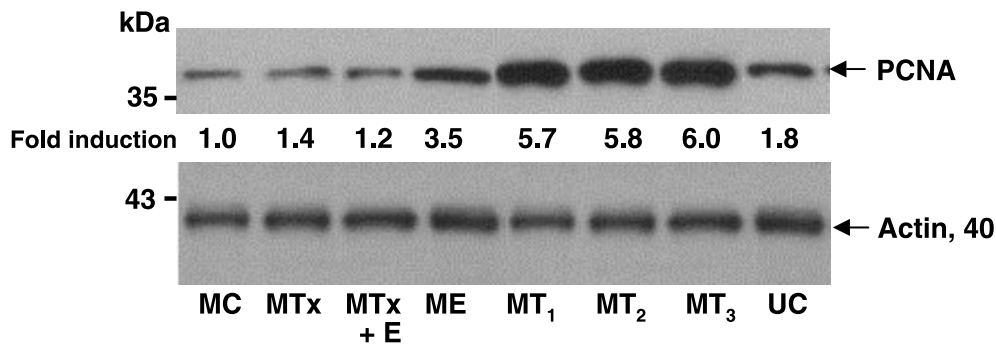


Figure 5 PCNA expression in MG and MGT samples. Western blot analysis was performed after 6 months of treatment on 20 µg protein aliquots. MC, untreated, TAMc-treated (MTx), TAMc+E₂-treated (MTx+E), E₂-treated (ME), three individual primary E₂-induced MGT (MT₁ to MT₃) and untreated uterus (UC). The primary antibodies used were PCNA (PC10) and β-actin (C10). The relative PCNA levels were determined by densitometry. The figure is representative of three independent experiments.

100% MGT incidence was obtained, which resulted in E₂ circulating levels between 50 and 89, and 112 and 145 pg/ml respectively. These E₂ concentrations are either within or approach physiological serum concentrations (17–88 pg/ml) previously reported in normal untreated cycling SD rats (Butcher *et al.* 1974). At doses of 3 mg, the serum E₂ levels were 22–60% lower than those previously reported to obtain 100% MGT incidence (Shull *et al.* 1997). Although very low levels of circulating E₂ are sufficient to induce a high incidence of MGTs in female ACI rats, the minimal MG oncogenic dose of E₂ has yet to be established.

Results presented herein, for the first time, demonstrate that concomitant TAMc treatment completely prevented the E₂ induction of MGT in female ACI rats, supporting the supposition that E₂-induced MG oncogenesis, in this animal model, is an ERα-mediated process. Most interestingly, this conclusion is supported by the decreased levels of expression of ERα (47, 56 and 66 kDa forms) and PR (A and B) in MGs treated with TAMc+E₂, compared with MGs treated solely with E₂. These data also correlated with a marked decrease in the number of MG cells expressing ERα and PR in immunostained MG tissue sections from TAMc+E₂-treated animals. In relation to TAMc treatment, these findings are consistent with a substantial reduction in BC risk reported when this antiestrogen was administered to women at high risk to develop the disease (Fisher *et al.* 1998). No ERβ expression was detected in either untreated or E₂-treated MGs and E₂-induced MGTs. These observations are consistent with the lack of ERβ expression in MG and MGTs induced by N-methyl-N-nitrosourea (NMU) in SD rats (Thordarson *et al.* 2001).

Although it remains to be established, the presence of 56 and 54 kDa isoforms in MGs and MGTs respectively, after chronic treatment with E₂, suggests that these isoforms may contribute to fostering the growth advantage seen in frank MGT epithelial cells, by differentially

affecting signaling responses. Moreover, the 54 kDa ERα isoform, consistently the prominent isoform present in E₂-induced primary MGTs, was also the major ERα isoform present in the uterus. A study comparing the ERα content in MGs of different rat strains, including the female ACI rat, at a given physiological state (18–20 days of lactation), has shown that the levels of MG ERα are very similar, 56–75 fmol/mg protein (Wiehle & Wittliff 1983). Although a single, 66–67 kDa, ERα native form has been reported in MGs of female untreated SD rats (Saji *et al.* 2000), estrogen/androgen-treated MGs and -induced MGTs in male Noble rats exhibit multiple ERα isoforms (Liao *et al.* 1998), comparable in many respects with the ERα isoforms reported herein for similar MG tissues of female ACI rats.

There is considerable evidence that both estrogen and progesterone positively affect MG gene expression and subsequent cell proliferation (Shyamala *et al.* 2000, Hofseth *et al.* 1999, Fata *et al.* 2001, Ginger *et al.* 2001, Raafat *et al.* 2001). Morphologically, progesterone treatment alone promotes a marked increase in the number of tertiary branching of primary and secondary breast tissue networks (Atwood *et al.* 2000). Consequently, the substantial rise in PR-A, and to a lesser extent PR-B in MGs and MGTs shown herein, after E₂ treatment, is consistent with the increased MG ductal branching and lobular-alveolar development reported in transgenic PR-A and PR-B mice (Shyamala *et al.* 2000), and during MGT cell proliferation (Liao *et al.* 1998). Taken together, these findings are supportive of an ERα/PR-driven cell proliferation in MGs of intact female ACI rats.

Common characteristics between estrogen- and synthetic chemical carcinogen (dimethylbenzanthracene and NMU)-induced MGTs include their histological features and estrogen dependence (Dao 1962, Russo & Russo 1996, Thordarson *et al.* 2001). Moreover, MGTs induced by synthetic chemical carcinogens can be prevented by antiestrogens (Jordan 1976, Gottardis & Jordan 1987).

However, crucial differences exist in MGT causation since both DCIS and primary MGTs induced by estrogens are highly aneuploid and exhibit a high frequency of *c-myc* amplification (Li SA *et al.* 2000, Li *et al.* 2002), whereas DCIS and MGTs induced by synthetic chemical carcinogens are largely diploid and exhibit high frequencies of *H-ras* activation (Zhang *et al.* 1991, Li *et al.* 2002). It is evident that the DCIS and MGTs induced by estrogen alone more closely resemble human DCIS and invasive ductal BCs, since the latter are also highly aneuploid (Alanen *et al.* 1997, Arnerlov *et al.* 2001), and show a high frequency of *c-myc* amplification (Escot *et al.* 1986, Watson *et al.* 1993).

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