ABSTRACT

The aim of this study was to investigate the effects of estradiol and tamoxifen (TAM) on the growth of human endometrial carcinomas in athymic mice. Tissues from primary tumors were implanted into estradiol-treated mice. In passage 2, animals were treated with (a) placebo, (b) estradiol, (c) estradiol plus TAM, and (d) TAM alone. The size of the tumors was measured weekly. Estrogen receptors (ER) were determined with the dextran-coated charcoal method and/or ER enzyme-linked immunosassay. Progesterone receptors were measured with the dextran-coated charcoal technique. Of 16 primary tumors, 2 grew in the athymic mice and were studied further. Tumor EL was positive for ER (145 fmoI/mg protein) and progesterone receptors (993 fmoI/mg protein). Tumor EL in passage 2 was not significantly stimulated by estradiol, but was stimulated by a combination of estradiol and TAM. Treatments (estradiol, estradiol plus TAM, or TAM) all increased tumor growth in passage 3. Tumor BR and a metastasis BR-MET were ER and progesterone receptor negative, applying dextran-coated charcoal, ER enzyme-linked immunoassay, and immunocytochemistry. The BR and BR-MET cells contain the complete ER gene but do not express any measurable amounts of ER mRNA as quantitated by Northern blot analysis, using a complete ER complementary DNA probe. In all animal passages the growth rate was significantly higher in estradiol-treated mice compared with the control. TAM alone had some growth stimulatory effect, but much smaller than that observed in the estradiol group. TAM inhibited estradiol-stimulated growth. These results suggest that estradiol and possibly TAM are capable of stimulating tumor growth in the athymic mice independently from ER, potentially through a host-mediated mechanism.

INTRODUCTION

The triphenylethylen TAM has been widely accepted as an adjuvant agent for the treatment of breast cancer. Recent reports demonstrate that the long-term (>5 years) administration of adjuvant TAM is superior in terms of prolonged disease-free survival than the 1- to 2-year treatment regimens (1–3). These clinical data and also experimental observations suggest that TAM acts as a cytostatic rather than cytotoxic agent (4, 5). Indefinite adjuvant TAM treatment for stage II and stage I breast cancer patients has now been recommended. TAM is generally regarded as a safe drug, but the concept of long-term therapy has raised further interest in studying the potential side effects. One concern is that TAM might have estrogenic potential in some target tissues. This could be a desired effect on bone for the prevention of osteoporosis (6, 7), but might be detrimental in the uterus, if TAM acts as a mitogen.

Anecdotal case reports suggest an association between the administration of TAM and the development of endometrial carcinoma (8), and a recent large clinical trial of adjuvant tamoxifen therapy has shown an increased incidence of endometrial carcinoma in the tamoxifen-treated arm with long-term therapy (9). It should be noted though that this study shows an effective decrease in the appearance of second primary breast cancers (9). Although laboratory data that demonstrate that TAM can inhibit mammary tumors are available (4, 5), data addressing the question of TAM-stimulated endometrial tumor growth are limited. Satyaswaroop et al. (10) performed a successful heterotransplantation of hormone-responsive human endometrial adenocarcinomata in athymic mice. The growth of two of these tumors was stimulated by estradiol implants, but also by TAM treatment (11, 12). Interestingly, bitransplantation of a TAM-stimulated endometrial tumor on one side of an athymic mouse with an estrogen-sensitive breast tumor (MCF-7) on the other side, results in the control of breast tumor growth by TAM but the stimulation of endometrial tumor growth (13).

The initial aim of this study was to investigate the influence of estradiol and TAM on the growth of primary human endometrial carcinoma transplanted into athymic mice. We have found, however, that an ER-negative endometrial tumor can grow more rapidly by treating the athymic mice with estradiol.

MATERIALS AND METHODS

Materials

Balb/c-nu nu ovariectomized mice (4–5 weeks old) were obtained from Sprague-Dawley, Madison, WI, and kept in a pathogen-free environment. Estradiol (1.7 mg, 8-week sustained release) and TAM (5 mg, 4-week sustained release) pellets were purchased from Innovative Research of America, Rockville, MD, [3H]R5020 (specific activity, 86.7 Ci/mmol) and [3H]estradiol (specific activity, 101.0 Ci/mmol) from NEN, Boston, MA. The ER-EIA kit for the measurement of ER and the ER-ICA kit for immunocytochemical localization of the ER were obtained from Abbott Laboratories, Chicago, IL.

An affinity-purified goat anti-rat IgG antibody was purchased from Jackson Immuno Research, West Grove, PA, and was used as a replacement for the original Abbott bridging antibody. The plasmid HEO, which contains a complete ER CDNA and was used for Northern blot analysis of tumor RNA was kindly provided by Professor Pierre Chambon, Strasbourg, France.

Experimental Design

Tissue samples from primary human endometrial adenocarcinomata were obtained from the operating room under sterile conditions. The specimens were kept in Hanks’ buffered saline solution for transport and preparation. The tumor was cut into pieces of 1 to 2 mm in diameter and implanted bilaterally into the anterior axillary region of 4–6 athymic mice. At the same time, estradiol pellets were implanted s.c. into the backs of all animals. If tumors grew up to a transplantable size of approximately 1 x 1 cm and tumor incidence in the passage was at least 50%, the largest tumor was used for transplantation into the next passage. For further passages tumors were always taken from estradiol-treated mice. In the second and consecutive passages groups of 6 animals were assigned to different treatment regimens. In most experiments four groups were compared. (a) Control (placebo); (b) estradiol; (c) estradiol plus TAM; and (d) TAM alone. When necessary estradiol pellets were replaced every 8 weeks and TAM pellets every 4 days.

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The abbreviations used are: TAM, tamoxifen; ER, estrogen receptor; PR, progesterone receptor; ER-ICA, estrogen receptor immunocytochemistry; ER-EIA, estrogen receptor enzyme-linked immunoassay; cDNA, complementary DNA; DCC, dextran-coated charcoal.
ENHANCED GROWTH OF ER-NEGATIVE ENDOMETRIAL CARCINOMAS BY ESTRADIOL

Steroid Receptor Measurements

Estrogen Receptors. Tumors were homogenized in 10 mM Tris/HCl, pH 7.3, 1.5 mM EDTA, 5 mM sodium molybdate, 0.4 mM KCl, 0.01% monomethylglycine buffer for ER-EIA or 10 mM Tris/HCl, pH 7.4, 1 mM EDTA, 5 mM sodium molybdate, 10 mM sodium glyceraldehyde, 0.01% monomethylglycine buffer for DCC with a Polytron homogenizer (Brinkman Instruments). ER determination of the primary tumor specimens and of tumors grown in animals from the placebo treatment arm were performed by applying the DCC method. Aliquots (100 µl) of cytosols were incubated with 6 concentrations of [3H]estradiol (0.1 nM to 3 nM) with and without the presence of 200-fold excess of unlabeled diethylstilbestrol. After an 18-h incubation period, unbound labeled ligand was removed with DCC. Binding capacity and affinity were determined by Scatchard analysis. Tumors grown in the mice were assayed for ER by using the ER-EIA kit, following the original protocol except for using the high salt nuclear extraction buffer for homogenization. It has been shown previously that the addition of 0.4 mM KCl does not affect the measurement of ER with the ER-EIA (14).

Frozen sections were taken from the tumors for immunocytochemical localization of the ER. The ER-ICA kit from Abbott Laboratories was applied according to the original protocol. The ER monoclonal antibodies raised against ER from MCF-7 breast tumors is known to interact with human uterine ER (15), but the original bridging antibody was replaced by an affinity-purified goat anti-rat IgG antibody in order to avoid cross-reactivity with mouse IgG, present in the tumors when grown in the mice. The concentration of this second antibody was 100 µg/ml with an incubation time of 2 h. The ER-ICA assay was performed with both the original and the substitute bridging antibody with 4 primary breast tumors to compare the sensitivity of the techniques.

Progesterone Receptors. PR in all tumors was determined with the same DCC technique that has been applied for ER measurements. [3H]R5020 and R5020 were used as labeled and unlabeled ligand, respectively.

RNA and DNA Analysis. Genomic DNA was purified and analyzed by dot blotting and Southern blotting as described elsewhere (16). The DNA was transferred to Hybond N as per the manufacturer’s recommendations.

RNA purification and Northern blotting were performed as described previously (17). The RNA was also transferred to Hybond N by using the manufacturer's instructions.

All blots were analyzed by using a 3P-labeled nick-translated EcoRI insert from the plasmid HEO which was graciously supplied by Dr. Pierre Chambon (18). HEO contains the 1.8-kilobase pair long open reading frame of the human ER cDNA (19).

RESULTS

Tumor Take

Of 16 implanted primary human endometrial adenocarcinomata, 2 grew in the first animal passage and could be studied further in consecutive passages. Table 1 shows a comparison of these tumors and lists the patients' characteristics. Tumor EL represents the typical slow-growing endometrial adenocarcinoma and tumor BR represents a very aggressive adenosquamous carcinoma; 21 weeks after tumor BR was obtained, an abdominal metastasis was removed from the same patient which grew in mice as well, and was named BR-MET.

Histology

Tumor EL was described as a well-differentiated adenosquamous carcinoma, grade I, according to the International Federation of Gynecology and Obstetrics classification (20). The tumor maintained its morphological appearance and grade throughout all animal passages (Fig. 1A).

Primary tumor BR had been diagnosed as moderately differentiated adenosquamous carcinoma with International Federation of Gynecology and Obstetrics grade II. Again the histopathological evaluation of tumors grown in mice showed that the morphology stayed the same, except for an increased frequency of necrosis.

Tumor BR-MET. The abdominal metastasis from patient BR was diagnosed as grade III adenocarcinoma in most tumors grown in the mice, as grade II in some cases. There were more mitotic figures and necrotic areas than in the primary tumor. Macroscopic examination and serial sections of liver and lungs of the animals bearing tumors BR and BR-MET provided no evidence for metastatic spread.

Hormone Receptor Measurements

Tumor EL. The primary tumor specimen obtained from surgery had an ER concentration of 145 fmol/mg and a PR concentration of 993 fmol/mg protein. Tumors grown in the athymic mice remained ER positive, though at a lower level. ER and PR concentrations in passage 3 are shown in Table 2.

Tumor BR. Ligand-binding assays performed with the DCC method showed that both ER and PR were positive in the primary tumor obtained from surgery. The same technique applied for tumors from the placebo-treatment groups gave very low levels of specific binding (2.65 fmol/mg) protein. PR was undetectable in tumor BR. The ER result was confirmed by ER-EIA in all animal passages [3.45 ± 1.1 (SE) fmol/mg] protein.

Immunocytochemistry

Immunocytochemical staining with a modified ER-ICA protocol showed specific nuclear staining in the case of tumor EL (Fig. 2, A and B) and no specific staining of any tumor cells in tumor BR (Fig. 2, C and D). Positive immunoperoxidase reaction of the hormone-sensitive endometrial carcinoma EnCa 101 (11), which served as a positive control, is demonstrated in Fig. 2, E and F. Several primary breast cancer specimens were included in this experiment in order to demonstrate the sensi-

| Table 1 Characteristics of tumors EL and BR and clinical data of the patients |
|-----------------|------|---------------|---|
|                | EL   | BR            |
| Age            | 87 yr| 63 yr         |
| Estrogen replacement | No   | Premarin (0.3 mg), Provera (10 mg) last 10 days of each cycle |
| Tumor           | Adenocarcinoma, invasive, well differentiated | Adenosquamous carcinoma moderately differentiated |
| Grade           | FIGO* I | FIGO II–III |
| Growth          | Slow | Fast          |
| ER              | Positive | Positive |
| PR              | Positive | Negative |
| Course          | No recurrence | Metastatic spread, death 6 months after diagnosis |

* FIGO, International Federation of Gynecology and Obstetrics.
ENHANCED GROWTH OF ER-NEGATIVE ENDOMETRIAL CARCINOMAS BY ESTRADIOL

Table 2 ER and PR concentrations of tumor EL in passage 3 for the different treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ER (fmol/mg)</th>
<th>PR (fmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>46.0 ± 7.0</td>
<td>15.4 ± 10.9</td>
</tr>
<tr>
<td>Estradiol</td>
<td>11.9 ± 2.5</td>
<td>53.6 ± 9.4</td>
</tr>
<tr>
<td>Estradiol + TAM</td>
<td>43.9 ± 9.6</td>
<td>39.6 ± 5.9</td>
</tr>
<tr>
<td>TAM</td>
<td>43.9 ± 9.6</td>
<td>53.6 ± 9.4</td>
</tr>
</tbody>
</table>

* Mean ± SEM.
* ND, not determined.

Tumor Growth

The 1.7-mg estradiol cholesterol pellets have previously been shown to stimulate the growth of MCF-7 breast cancer cells (ER positive) implanted into athymic mice (13, 21, 22). Tumors do not grow without the sustained release pellets. Periodic sampling of blood from athymic mice with estradiol pellets (n = 4) showed high circulating levels of estradiol (determined by radioimmunoassay as previously described (23)): 5141 ± 432 (1 week), 3130 ± 272 (2 weeks), 3113 ± 436 (4 weeks), 1738 ± 137 (8 weeks), mean ± SEM pg/ml serum. TAM pellets (5 mg) have previously been shown to inhibit the growth of MCF-7 tumors enhanced by 1.7-mg estradiol pellets (13, 21, 22). Circulating levels of TAM could be detected (<20 ng/ml), but not accurately quantitated by high-performance liquid chromatography (24). Nevertheless, TAM pellets inhibit the uptake of [3H]estradiol into uterus, vagina, and MCF-7 tumors in ovariectomized athymic mice.5

Growth of the ER-positive endometrial carcinoma EL in passage 2 is illustrated in Fig. 5. This tumor was stimulated to growth more by a combination of estradiol and TAM, than by estradiol alone. Tumor incidence in this tumor was low (50%). In passage 3, no significant differences in tumor growth could be observed between the different treatment groups. After 12 weeks, the tumor areas (cm² ± SEM) were: control, 0.78 ± 0.11 (n = 4); estradiol, 1.45 ± 0.25 (n = 40); estradiol plus TAM, 1.32 ± 0.22 (n = 5); TAM, 1.16 ± 0.25 (n = 6). Nevertheless, in groups treated with estradiol with or without TAM, the tumors were larger than controls.

The growth characteristics of the ER-negative tumor BR are shown in Fig. 6. In animal passage 3 there was only minimal growth in the control group. Estradiol was capable of stimulating growth significantly over control. The addition of TAM in the estradiol plus TAM group decreased the growth to the level of TAM alone. It is worthwhile to note that in the control group only 4 tumors occurred from 12 implantation sites. The TAM-only group also had a low tumor incidence of 6 (12), while in the estradiol and estradiol plus TAM group 10 and 11 tumors grew to a measurable size, respectively. Growth curves in the animal passages 2 and 4 showed similar characteristics, although there was an increase in the growth rates in the estradiol and control group with serial passages (data not shown).

A comparison between the growth of the primary tumor BR...
Fig. 2. Immunocytochemical staining for ER of frozen sections from tumors grown in mice (A–F) and primary breast cancer tissue (G and H). The Abbott ER-ICA kit was applied by replacing the original bridging antibody with an affinity-purified goat anti-rat IgG antibody (for details see “Materials and Methods”). Hematoxylin was used as a nuclear counterstain. ER-positive tumor EL was incubated with the primary antibody H222 (A), and with a control antibody (B), tumor BR with the primary antibody (C), and with a control antibody (D), the ER-positive endometrial carcinoma EnCa 101 with the primary antibody (E), and a control antibody (F) and a borderline-positive breast tumor (ER: 14 fmol/mg) with the primary antibody (G) and a control antibody (H).
ENHANCED GROWTH OF ER-NEGATIVE ENDOMETRIAL CARCINOMAS BY ESTRADIOL

0.1 0.2 0.4 0.8 1.0 2.0 3.0 ug DNA

T47D
MCF-7
T47D
BR
BR-MET

Fig. 3. DNA dot blot analysis of total DNA from T47D, MCF-7, BR, and BR-MET cells. DNA was denatured with 0.1 N NaOH at 68°C for 30 min, neutralized with HCl, and spotted onto Hybond N (prewetted with 20x standard saline citrate [150 mM NaCl and 15 mM sodium citrate, pH 7]), using a BRL Hybri-dot manifold. The DNA was fixed to the membrane by UV irradiation for 5 min, prehybridized overnight at 42°C with shaking, and then hybridized with the 32P-labeled HEO-insert probe. After washing the blot was exposed to Kodak X-OMAT AR film for 36 h, using Quanta III intensifying screens.

In an experiment with different treatment groups (passage 2) estradiol enhanced BR-MET growth about 2-fold over the control group. TAM had no effect. Tumor incidence and growth rate of the control group were considerably higher than the ones seen in tumor BR (Fig. 8).

DISCUSSION

Growth response of the ER-positive tumor EL to hormonal treatment in passage 2 is in agreement with result from Satyaswaroop's ER-positive tumor EnCa 101 (12, 13). Maximal growth was observed with both estradiol and TAM pellets, suggesting an additive estrogenic effect of both substances. Our results suggest that increased growth of endometrial carcinoma in athymic mice as a result of TAM treatment is not uncommon.

In passage 3 the exact growth pattern seen in passage 2 was

and the metastasis BR-MET in estradiol-treated mice, is illustrated in Fig. 7. The metastasis had a 6-fold increase of the growth rate compared to the primary tumor under the same experimental conditions.
not be reproduced, although the treatment increased tumor growth and tumor EL remained ER positive. Lower ER concentrations in the estrogen treatment group and higher PR concentrations in the estradiol plus TAM and TAM treatment groups (Table 2) suggested a functional estrogen receptor. PR could not be determined in the estradiol group because of inadequate amounts of tumor.

It is important to appreciate that TAM may not only affect the tumor directly, either through the ER or possibly non-ER-related binding sites (25), but also indirectly via the host. TAM exhibits estrogen-like actions in the mouse uterus and vagina in short-term (3-day) assays, although continuous therapy tends to produce a partial estrogen-like effect on the uterus (26–28). Studies using the sustained release preparations of estradiol (1.7 mg) and TAM (5 mg) demonstrated that TAM can inhibit estradiol-stimulated growth of MCF-7 breast tumors (ER positive) implanted in athymic mice (21, 22). However, long-term therapy (up to 6 months) with TAM can result in the selection of MCF-7 tumor cells that grow in response to either TAM or estradiol (22). The reasons for the unusual target site-specific effects of tamoxifen are at present unknown, but the fact that tumor heterotransplants are supported in a mouse host that exhibits estrogen-like effect toward the drug should not be dismissed.

Growth stimulation by estradiol in the case of the ER-negative tumor BR is in disagreement with the model of estrogen action and most experiments involving ER-negative tumors and cell lines. The transplantable ER-negative endometrial carcinoma EnCa V obtained by Satyaswaroop et al. (11) showed no response to estradiol treatment, but its growth characteristics differ widely from our ER-negative tumor BR. EnCa V grows to an average tumor area of approximately 2 cm² after only 3 weeks, while BR grows up to an average of 0.2 cm² without hormone supplement after a period of 9 weeks.

Since this is the first report of a growth stimulation of an ER-negative tumor from an estrogen target tissue by estradiol in athymic mice, it was particularly important to prove the absence of estrogen receptors in tumor BR. Ligand-binding assays and enzyme-linked immunosassays were uniformly negative. An explanation for the growth stimulation of tumor BR by estradiol could be that scattered ER-positive cells in a predominantly ER-negative tumor react to an estradiol stimulus by secreting growth factors and subsequently stimulating the majority of ER-negative cells in a paracrine fashion. Sparse ER-positive cells might not be detectable by means of a biochemical assay because of a dilution effect. To rule out this possibility, an immunocytochemical technique was applied, but this did not detect any ER-positive cells. Finally, the cells do not express any mRNA that codes for ER, even though they contain equal amounts of apparently complete ER genes as compared with the cell lines MCF-7 and T47D. It is implausible that these cells are expressing any functional ER in the absence of any mRNA coding for the ER.

We believe that the findings that estradiol enhances tumor growth could be explained by three hypotheses: (a) estradiol stimulates the tumor directly through a mechanism independent from ER. This would be in contrast to the current model of estrogen action and would be the only observation of this kind which leads us to discard this concept. Nevertheless, a hypothesis based upon the release of inhibitors (estracolyones) by estradiol from the cell surface of target tissues has been proposed (29) which might be consistent with our present observations; (b) the growth-stimulatory effect of estradiol might be mediated through ER present in this tumor below the sensitivity of our assays. It seems unlikely, however, that the very low amounts of ER found are capable of mediating such a pronounced effect; (c) estradiol stimulates growth of this tumor indirectly by modulating the host. Estrogen-stimulated prolactin release from the pituitary gland might cause permissive effects for the initiation of growth by other hormones, e.g., somatomedins or thyroxine (by an as yet unknown mechanism).

Similarly, estromedins (30) or growth factors from mouse tissues or organs might cause growth of the ER-negative tumor by an endocrine or paracrine mechanism (31). Another possible mechanism would be a further immunosuppression of the partially immunodeficient athymic mice. It is known that athymic mice have normal or even increased activities of natural killer cells (32) and that estradiol is a strong inhibitor of natural killer cell activity in normal mice (33, 34). This effect might only become relevant by influencing tumor growth in the relatively slow-growing tumors, since the difference between the control and estradiol-treated groups was less obvious in the faster-growing tumor BR-MET compared to BR and not observed in the very fast-growing ER-negative endometrial tumor EnCa V (4). Slow-growing tumors of the larynx have recently been shown to grow more rapidly with estrogen therapy (35). The authors suggested that ER, that has been reported to be present in some of the tumor types (though not determined in their study), may allow estrogen to stimulate tumor growth. It is possible that their finding is consistent with ours and that estrogen is enhancing tumor growth through a host-mediated mechanism.

ACKNOWLEDGMENTS

We want to thank Dave Whitford for performing steroid receptor assays on the primary endometrial tumors.

REFERENCES

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Enhanced Growth of an Estrogen Receptor-negative Endometrial Adenocarcinoma by Estradiol in Athymic Mice


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