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Photoaffinity Labeling of the Progesterone Receptor from Human Endometrial Carcinoma[1]

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ABSTRACT

A nude mouse model for the growth of human endometrial carcinoma and hormonal modulation of the progesterone receptor (PR) was established previously. This study describes the effect of 17β-estradiol and tamoxifen (TAM) on growth rate and PR concentration in a hormonally responsive human endometrial tumor (EnCa 101) grown in this experimental system and presents the first characterization of human endometrial carcinoma PR. EnCa 101 was transplanted subcutaneously into ovariectomized, BALB/c, nu/nu athymic mice and grown under 17β-estradiol-stimulated, TAM-stimulated, and control conditions. Both 17β-estradiol and TAM increased the growth rate of EnCa 101 in nude mice, and a parallel increase in the cytosol PR concentration was observed, from 130 ± 55 (SD) fmol/mg protein to 1,311 ± 598 fmol/mg protein and 710 ± 310 fmol/mg protein, respectively. PR was partially purified by phosphocellulose and DEAE cellulose chromatography, and the DEAE eluate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and photoaffinity labeling with [17α-*methyl*-^{3}H]promegestone ([^{3}H]R5020). Two PR-negative tumors (EnCa K and EnCa V) were also examined in parallel. Coomassie blue staining of gels revealed that the protein patterns of all of the partially purified preparations from EnCa 101, EnCa K, and EnCa V were essentially identical. In contrast, photolabeling and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of EnCa 101 grown in the presence of 17β-estradiol or TAM revealed incorporation of [^{3}H]R5020 into proteins of molecular weight ~116,000 and 85,000. Labeled proteins of molecular weight 66,000, 45,000, and 35,000 were also observed. In each case, the labeling was competable with excess non-radioactive R5020. No incorporation of [^{3}H]R5020 was observed in EnCa 101 grown in the absence of estrogen, nor was any observed in EnCa K or EnCa V.

INTRODUCTION

It is well established that steroid responses within target tissues are elicited through the mediation of steroid-specific, high-affinity, intracellular binding proteins (receptors). Numerous studies have shown the presence of, and changes in, the levels of the ER[3] and PR within normal and neoplastic endometrium (1-5). We have developed an experimental system for studying the biology of human endometrial carcinoma (6), in which malignant tumors of human endometrium are grown and serially passaged in castrated nude mice. We have shown that the morphological and biochemical characteristics of the original tumor remain essentially unchanged (7). The characteristics of a 17β-estradiol-sensitive (EnCa X) tumor and a 17β-estradiol-insensitive (EnCa V) tumor have been published previously (6). One of the most important features of the nude mouse model is the possibility of comparing a tumor under various conditions of hormonal treatment with the same tumor grown in control conditions. This provides an in-built control for every experiment and highlights the suitability of this system for the study of hormone-dependent parameters in human endometrial carcinoma. One such parameter is PR, which we have shown to be increased by 17β-estradiol or TAM in the 17β-estradiol-sensitive EnCa X tumor grown in the nude mouse (6, 8). We now describe the growth characteristics and response to 17β-estradiol and TAM of another well-differentiated endometrial carcinoma (EnCa 101) and the characerization of PR from this tumor by photoaffinity labeling.

MATERIALS AND METHODS

Chemicals. [1,2,6,7-^{3}H]Progesterone (80-110 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), and [^{3}H]R5020 (87 Ci/mmol) and unlabeled promegestone were obtained from New England Nuclear (Boston, MA). Radioactive products were used for receptor measurements or photoaffinity labeling after verification of radiochemical purity. Electrophoresis supplies were obtained from Bio-Rad (NY), and molecular weight standards for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad and Pharmacia (Piscataway, NJ). DEAE cellulose (DE52) and phosphocellulose were Whatman products (Fisher Scientific, Philadelphia, PA). Control, 17β-estradiol, and TAM pellets were obtained from Innovative Research of America (Rockville, MD) and were designed to maintain the stated levels in blood for 60 days.

Animals. Ovariectomized female athymic BALB/c nu/nu nude mice were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained in isolated barrier facilities.

Tumors. Endometrial carcinomas EnCa 101, EnCa K, and EnCa V were derived from primary human endometrial tumors grown in nude mice. The EnCa 101 tumor is histologically well differentiated and is ER positive. The EnCa K and EnCa V tumors are ER negative, and their growth rates are unaffected by 17β-estradiol. The EnCa K tumor is histologically moderately differentiated, and the EnCa V tumor is poorly differentiated.

Tumor Transplantation. Tumor tissue (100 mg) was transplanted subcutaneously in the infrascapular region of 4- to 6-week-old ovariectomized female athymic BALB/c nu/nu nude mice. Animals bearing EnCa 101 tumors received control, 17β-estradiol (20 pg/ml), or TAM (20 ng/ml) pellets. Animals bearing EnCa K or EnCa V tumors received control or 17β-estradiol pellets (200 pg/ml), but only tumors from 17β-estradiol-exposed animals were used for photolabeling. Pellets were implanted

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[3] The abbreviations used are: ER, estrogen receptor; PR, progesterone receptor; TAM, tamoxifen; SDS, sodium dodecyl sulfate; EnCa 101, well-differentiated, estradiol receptor-positive, transplantable human endometrial carcinoma; EnCa K, moderately differentiated, estradiol receptor-negative, transplantable human endometrial carcinoma; EnCa V, poorly differentiated, estradiol receptor-negative, transplantable human endometrial carcinoma; [^{3}H]R5020, [17α-*methyl*-^{3}H]promegestone; PEMTG buffer, 50 mM potassium phosphate:10 mM EGTA:10 mM Na_2MoO_4:12 mM thioglycerol:10% glycerol, v/v, pH 7.0.

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subcutaneously in the flank contralateral to the tumor. The growth rate of tumors was followed at weekly intervals by determination of tumor size with vernier calipers. When the geometric mean diameter of tumors reached 1 cm, animals were killed by cervical dislocation, and tumors were excised and weighed. Tumors were pulverized and stored in liquid N_2 for cytosolic PR determination and photolabeling with [^{3}H]R5020.

Measurement of Cytosol PR Concentration. PR concentrations were measured in 105,000 × *g* supernatants of tumor homogenates in Tris:EDTA:dithiothreitol buffer (20 mM Tris-HCl:3 mM EDTA:1 mM dithiothreitol:0.01% sodium azide, pH 7.8, 4°) according to the procedure described previously (6, 9). Receptor concentrations were calculated by Scatchard analysis (10) of specific binding data. Protein concentrations were determined by the method of Bradford (11).

Photoaffinity Labeling with [^{3}H]R5020. Tumor tissue was homogenized in PEMTG buffer at 0°, and cytosol was prepared. Cytosol was stirred with phosphocellulose (3:1, v/v, 60 min) in the same buffer, and the drop-through fraction was collected and chromatographed on DEAE-cellulose (1.5 g/g tissue). After extensive washing with PEMTG buffer, elution was effected with 0.15 M KCl in PEMTG. Receptor-containing fractions were pooled and incubated in the dark with [^{3}H]R5020 (30 nM, 2 h, 0°) ± a 100-fold excess of unlabeled R5020 in the presence of cortisol (1 μM, to prevent [^{3}H]R5020 binding to glucocorticoid receptors). Free and bound R5020 were separated by dextran-coated charcoal, and samples were frozen (−80°) for irradiation (4 min, 5 cm from a mercury vapor lamp; Hanovia 200 W; Ace Glass, Vineland, NJ). Irradiated samples were frozen (−80°) and analyzed by SDS-polyacrylamide gel electrophoresis within 24 h.

SDS-Polyacrylamide Gel Electrophoresis. 7.5% acrylamide gels were prepared and run according to the method of Laemmli (12). Samples were heated (2 min, 90°) in a solution (1:4, v/v) containing glycerol (10%, v/v), 2-mercaptoethanol (5%, v/v), and SDS (3%, w/v) in Tris buffer with bromophenol blue as tracking dye and layered onto the gel. Standard proteins were included in every run. After electrophoresis, gels were fixed and stained with Coomassie G250 (13) and then sliced into segments (2 mm) and dissolved in H_2O_2 (0.5 ml, 50°, 6 h) for the determination of radioactivity in a Beckman LS6800 liquid scintillation counter.

RESULTS

The growth rate of the well-differentiated EnCa 101 tumor in nude mice was increased in the presence of 17β-estradiol or TAM (Chart 1), and the response to both agents paralleled that

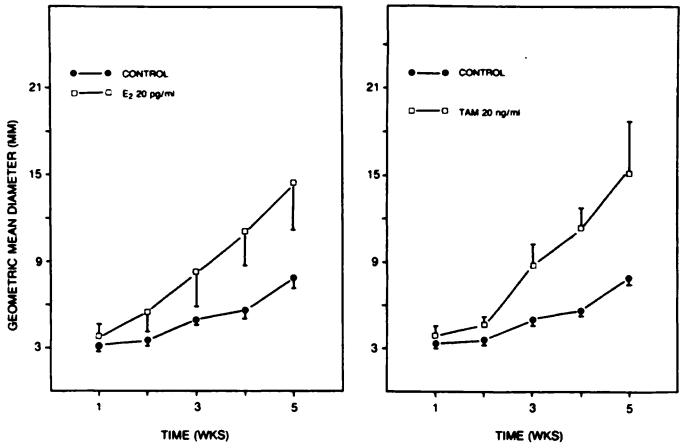

Chart 1. Effect of 17β-estradiol and TAM on EnCa 101 tumor growth in ovariectomized nude mice. Animals bearing EnCa 101 tumors received control, 17β-estradiol (20 pg/ml), or TAM (20 ng/ml) pellets. The growth rate of tumors was followed at weekly intervals by determination of tumor size with vernier calipers. When the geometric mean diameter of tumors reached around 1 cm, animals were sacrificed and tumors were excised. Results are expressed as the mean; *bars*, SD (n = 4). E_2, 17β-estradiol.

Table 1

Effect of 17β-estradiol and TAM on EnCa 101 tumor weight and PR concentrations

	n	Tumor wt. (g)	Cytosol PR (fmol/mg protein)
EnCa 101 control	4	0.25 ± 0.05[a]	130 ± 55
+17β-estradiol (20 pg/ml)	4	1.70 ± 1.00	1,311 ± 598
+TAM (20 ng/ml)	4	1.91 ± 0.41	710 ± 310

[a] Mean ± SD.

Table 2

Biological characteristics and PR concentrations of tumors chosen for photolabeling

Tumor	Histological Grade	Growth with 17β-estradiol	Passage (no.)	Cytosol PR fmol/mg protein		
				Control	17β-estradiol	TAM
EnCa 101[a]	I	+	23	130	1,660	1,180
EnCa K	II	−	10	<10	<10	ND[b]
EnCa V	III	−	7	<10	<10	ND

[a] Single tumors were chosen from the 17β-estradiol and TAM-stimulated experimental groups described in Table 1. Pooled tumors from the control group were used for photolabeling.
[b] ND, not done.

described previously for the 17β-estradiol-sensitive, well-differentiated EnCa X endometrial carcinoma (6, 8). The increased growth rate was reflected in an increase in the tumor weight from 0.25 ± 0.05 (SD) g (control) to 1.70 ± 1.00 g in the presence of 17β-estradiol and 1.91 ± 0.41 g in the presence of TAM and was accompanied by an increase in the cytosol PR concentrations in 17β-estradiol and TAM-treated tumors (Table 1).

PR from these human endometrial carcinomas grown in nude mice was characterized by photoaffinity labeling with [^{3}H]R5020. Tumors were selected from individual EnCa 101-bearing animals grown under 17β-estradiol-stimulated (20 pg/ml), TAM-stimulated (20 ng/ml), and control conditions. Two PR-negative tumors were also included as controls: EnCa K, an ER-, PR-negative, moderately differentiated endometrial carcinoma, the growth rate and PR concentration of which are unaffected by 17β-estradiol (data not shown), and EnCa V, a poorly differentiated, 17β-estradiol-insensitive tumor the characteristics of which have been published previously (6). The properties of the tumors chosen for photolabeling are summarized in Table 2.

The irradiation protocol was designed to yield maximal covalent attachment of [^{3}H]R5020 with minimal light or heat induced damage to PR. Although the efficiency of photolabeling was generally low (2–5%), blank experiments revealed that preparations irradiated in the absence of steroid retained over 85% of the [^{3}H]progesterone binding capacity present in unirradiated controls (data not shown). Tumor cytosols were partially purified by chromatography with phosphocellulose and DEAE cellulose. In PR-containing tumors this yielded preparations purified around 10-fold with respect to the cytosol. Preparations were partially purified before photolabeling, since [^{3}H]R5020 was found to bind (noncompetably) to a cytosolic protein of a molecular weight around 68,000. This protein did not co-chromatograph with PR on DEAE cellulose and was thereby eliminated.

Coomassie blue staining of SDS-polyacrylamide gels revealed that the protein patterns of the partially purified preparations from EnCa 101 (control, 17β-estradiol, and TAM stimulated), EnCa K, and EnCa V were essentially identical. In contrast, photolabeling of EnCa 101 grown in the presence of 17β-estra-

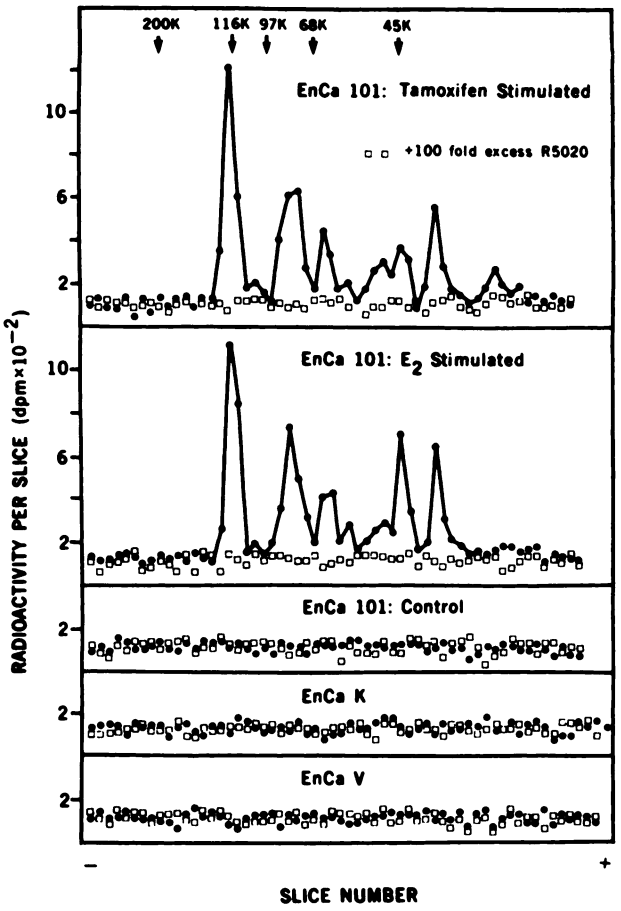

Chart 2. *In vitro* photoaffinity labeling of human endometrial carcinoma. Tumor cytosols were partially purified and labeled *in vitro* (2 h, 0°) with 30 nM [^{3}H]R5020 and cortisol (1 μM) alone (●) or together with a 100-fold excess of unlabeled R5020 (□). Hormone-receptor complexes were irradiated for 4 min using a mercury vapor lamp, and irradiated samples were analyzed by SDS-polyacrylamide gel electrophoresis. Lanes (containing 200 μg total protein) were sliced and counted, and the molecular weights of radioactive peaks were determined by their migration rate relative to that of stained molecular weight standards. Experimental details are described in "Materials and Methods." E_2, 17β-estradiol.

diol or TAM showed covalent labeling of [^{3}H]R5020 to proteins of molecular weight ~116,000, 85,000, 66,000, 45,000 and 35,000 (Chart 2). In each case, the labeling was competable with excess non-radioactive R5020. No incorporation of [^{3}H]R5020 was observed in EnCa 101 control, EnCa K, or EnCa V (Chart 2).

DISCUSSION

The nude mouse model of human endometrial carcinoma was developed in this laboratory, and the growth, histological features, and steroid receptor concentrations of a well-differentiated (EnCa X) and a poorly differentiated (EnCa V) tumor have been documented extensively in this system (6–8). This report presents certain features of another well-differentiated tumor (EnCa 101) growing in the nude mouse model. This tumor shows differential growth in the presence of 17β-estradiol or TAM and a concomitant increase in the cytosol PR concentration in response to both agents. TAM behaves as an estrogen agonist in this regard, as was shown previously for EnCa X (8). Nevertheless, we have observed that the cytosolic PR concentrations are generally lower in TAM-treated than in 17β-estradiol-treated tumors, despite the 1000-fold difference in dose.

The fact that PR concentrations can be modulated by 17β-estradiol or TAM in this system highlighted its suitability for the study of PR regulation in human endometrial carcinoma. To this end, tumor cytosol proteins were partially purified and irradiated in the presence of [^{3}H]R5020; covalent attachment of [^{3}H]R5020 by photolabeling was used as a probe for the receptor in a complex mixture of proteins. It is noteworthy that PR-negative tumors (EnCa K and EnCa V) showed no photolabeling. Similarly, EnCa 101 grown under control conditions and containing low PR also showed no labeling. However, when EnCa 101 was grown in the presence of 17β-estradiol or TAM and photolabeled, a number of radioactive peaks were observed on SDS-polyacrylamide gel electrophoresis. The number and intensity of the peaks observed were virtually identical in 17β-estradiol- and TAM-treated tumors, and a major peak was revealed corresponding to a protein of molecular weight ~116,000; secondary peaks of molecular weights ~85,000, 45,000, and 35,000 and a minor peak at M_r 66,000 were also revealed. The molecular weight of human endometrial PR has not been described previously, but there are several reports on the molecular weight of human uterine PR: 110,000 (14); 108,000 and 43,000 (15); 42,000 (16); and 42,000 and 27,000 (17). The peaks observed at M_r ~116,000 and 45,000 in the present study are reasonably consistent with these data; furthermore, the peaks at M_r ~116,000 and 85,000 are also consistent with the molecular weight reported for human breast cancer cell (T47D) PR (18).

The large number of specifically labeled peaks obtained in this study was unexpected, and the reason is presently unknown. However, other data (not shown) on the photolabeling of PR in a separate experiment reveal labeling only at M_r 116,000 and 85,000. This suggests that PR may have been broken down under the conditions of this experiment, possibly by proteolytic attack. Proteolysis has been proposed as the explanation for the lack of consistency in the molecular weight reported for human uterine PR (16). Furthermore, it has been reported that PR is susceptible to leupeptin-sensitive protease(s) in the human uterus (19) and in human breast cancer cytosols (20). In other mammalian as well as non-mammalian systems, PR has been shown to be sensitive to protease attack. Chick oviduct PR has been the most extensively studied, and it is susceptible to breakdown from subunits of M_r 117,000 and 79,000 to fragments of M_r 43,000 and 23,000 (21, 22). A recent report also supports protease involvement in the rabbit and suggests that rabbit uterine PR is composed of a single subunit of M_r 110,000 (23). In the present study, there is an indication that all of the bands specifically labeled by [^{3}H]R5020 are PR associated, as they are only observed in EnCa 101 grown in the presence of 17β-estradiol or TAM and are completely absent in the same tumor grown under control conditions and in PR-negative tumors (EnCa K or EnCa V). Therefore, the involvement of proteolysis of PR in the generation of the multiple peaks observed appears likely, and this possibility is presently under investigation.

In summary, 17β-estradiol and TAM promote an increase in the growth rate and cytosolic PR concentration in a well-differentiated human endometrial carcinoma (EnCa 101) grown in nude mice. EnCa 101 grown under control conditions, as well as two PR-negative tumors of different histological grades, contained no or low PR and failed to show photolabeling by the

synthetic progestin [^{3}H]R5020. However, EnCa 101 grown in the presence of 17β-estradiol or TAM showed an increase in the cytosolic PR concentration, and this was mirrored by the appearance of photoaffinity-labeled proteins of molecular weight ~116,000, 85,000, 66,000, 45,000, and 35,000. This is the first characterization of the PR from human endometrial carcinoma, and these studies establish the suitability of the nude mouse model as an easily manipulable and well-controlled system for the study of PR.

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