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Failure of Progestins to Induce Estradiol Dehydrogenase Activity in Endometrial Carcinoma, *in Vitro*[1]

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ABSTRACT

Estradiol dehydrogenase (E_2DH) is a well-known progesterone-dependent enzyme in human endometrium, and its induction has been proposed as a means to test hormonal sensitivity of endometrial carcinoma. While administration of progestins to some patients with endometrial carcinoma resulted in increased endometrial E_2DH activity, efforts to induce this enzyme, *in vitro*, in these tumors have been unsuccessful. The reasons for such failure were investigated in the present study. Progesterone receptor (PR) concentrations and E_2DH activities were simultaneously measured in proliferative and malignant endometria under organ culture conditions. Cytoplasmic PR concentrations were determined by Scatchard plot analysis of [^{3}H]progesterone binding in fresh samples and in tissue explants incubated in nutrient medium at 37° in a humidified 5% CO_2 atmosphere for various periods of time. Parallel incubations of explants with and without 500 ng medroxyprogesterone acetate per ml were carried out for monitoring E_2DH induction. In proliferative endometrium, the progesterone-specific binding sites remained stable during the culture periods, and the E_2DH activities were stimulated severalfold by medroxyprogesterone acetate. In contrast, the PR concentrations in carcinoma explants were undetectable after a 24-hr period, and this was associated with a lack of increase in E_2DH activity. These findings provide evidence that progestin-induced endometrial E_2DH activity is a receptor-mediated phenomenon. In addition, these results demonstrate clearly that the ineffectiveness of progestin to induce E_2DH in endometrial cancer specimens, *in vitro*, is related to the instability of PR under culture conditions. It is suggested that any experiment designed to follow effects of steroids on target tissues must take into account the stability of steroid receptors under *in vitro* conditions.

INTRODUCTION

Activity of the enzyme E_2DH[3] is increased severalfold in the human endometrium during the luteal phase of the menstrual cycle (15, 20). Results from both *in vitro* and *in vivo* studies have shown that progesterone regulates the activity of E_2DH in the human endometrium (8, 9, 21). Administration of progestins to patients with endometrial adenocarcinoma also results in the enhancement of E_2DH activity in some of these tumors (5, 14). Extension of these studies to cultured explants of endometrial carcinoma was attempted to develop a simple *in vitro* test for predicting responsiveness of endometrial tumors to progestin therapy. While in normal proliferative endometrium there was a consistent induction of E_2DH activity with progestin, neoplastic tissue maintained under identical conditions persistently failed to respond to this agent. Since all cancer specimens used in this study initially contained PR, it was reasoned that the lack of response of this tissue to added progestin may be due to (*a*) the instability of PR under culture conditions or (*b*) a defect in the receptor mechanism in the tumor explants, *e.g.*, translocation of PR to the nucleus, interaction of the steroid:receptor complex with the chromosomal machinery, etc. The first possibility was tested by measuring PR concentrations in cultured explants of both normal and neoplastic tissue at various time intervals. Simultaneous measurements of E_2DH activity were also carried out in explants of these tissues cultured in parallel for 2 days in the absence or presence of the progestin, MPA. Our results indicate that the inability of progestin to induce E_2DH activity in endometrial carcinoma is due to the instability of PR in these tissue explants. In addition, the close correlation between the maintenance of PR concentrations and response to progestin, *in vitro*, provides additional evidence for receptor mediation in progestin induction of E_2DH activity in human endometrium.

MATERIALS AND METHODS

Reagents. The radioactive steroids, [1,2,6,7-^{3}H]progesterone (90 Ci/mmol), 17β[1,2-^{3}H]estradiol (50 Ci/mmol), and [4-^{14}C]estrone (50 mCi/mmol), were purchased from New England Nuclear and used for PR measurements and enzyme assays after verification of radiochemical purity. Crystalline steroids, estrone, estradiol, and progesterone were obtained from Steraloids, Wilton, N. H., and NAD^+ was from Sigma Chemical Co.; MPA was a gift from The Upjohn Company, Kalamazoo, Mich.

Tissue. Endometrial carcinoma specimens were obtained from postmenopausal women undergoing dilatation and curettage or hysterectomy. Endometria were also obtained from premenopausal women undergoing dilatation and curettage or curettage for various disorders. Only those tissues that were histologically determined to be from proliferative stage of the menstrual cycle were included in these studies.

Organ Culture. Endometrial curettings were transported to the laboratory in sterile, ice-cold Ham's F-10 medium (Flow Laboratories) containing 10% fetal calf serum (Flow), 1% antibiotic-antimicotic mixture (Grand Island Biological Co.), insulin (10 μg/ml) (Eli Lilly and Co.), 17β-estradiol (2.5 ng/ml), and D-glucose (final concentration, 5 mg/ml). All preparative procedures for culture studies were carried out asceptically in sterile hoods. The endometrial curettings were washed free of blood clots, cut into 1-cu mm pieces, and placed over filters on stainless steel grids in 60- x 15-mm culture dishes (Falcon Plastics). The filter was kept moist and in contact with about 3 ml of culture

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[3] The abbreviations used are: E_2DH, estradiol dehydrogenase; PR, progesterone receptor; MPA, medroxyprogesterone acetate; TED buffer, 20 mM Tris-HCl (pH 7.8) containing 3 mM EDTA, 1 mM dithiothreitol, and 0.01% sodium azide.

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medium. The cultures were incubated for 2, 24, and 48 hr at 37° in a humidified 95% air:5% CO_2 atmosphere in a Wedco CO_2 incubator when stability of PR was determined. When E_2DH induction was followed, parallel cultures of explants were maintained for 2 days with or without the addition of MPA (500 ng/ml) to the culture medium.

Assay of Cytosol PR in Human Endometrium. The PRs were measured in the cytosol preparations of fresh endometrial tissue and in cultured explants according to the procedure of Bayard *et al.* (1) with the following modifications: (*a*) the receptor concentrations were estimated by Scatchard analysis of specific binding data (17) instead of single-point assays; and (*b*) the removal of free and loosely bound [^{3}H]progesterone was effected by a 5-min instead of a 30-min incubation of the reaction mixture at 0° with dextran-coated charcoal (0.05% dextran and 0.5% charcoal in TED buffer).

All procedures were carried out at 0°. The fresh endometrial tissue and cultured explants were washed twice in 10 ml TED buffer and homogenized in 20 volumes of the same buffer using a motor-driven glass-glass homogenizer with 15-sec bursts at 1000 rpm 3 to 5 times, each separated by a 30-sec interval. The receptor assay was carried out in cytosol fractions of these homogenates after a 105,000 × *g* centrifugation for 1 hr. For determination of total bound [^{3}H]progesterone, aliquots (200 μl) of cytosol were incubated with increasing concentrations of [^{3}H]progesterone (0.5 to 5 nM) and 100-fold excess concentrations of cortisol for 3 hr. Identical samples containing 100 times excess unlabeled progesterone were used for determination of nonspecifically bound [^{3}H]progesterone. Free and loosely bound radioactive ligand were removed from the cytosol preparations by the addition of 100 μl ice-cold TED buffer and 300 μl chilled TED buffer containing Dextran-Coated Charcoal followed by a 5-min incubation at 0°. After centrifugation at 2000 rpm for 5 min, an aliquot of the supernatant was counted for radioactivity using 4 ml Dimiscint (National Diagnostics, Inc., Somerville, N. J.) in minivials in a Beckman Model 7500 liquid scintillation spectrometer with an automatic quench correction feature and counting efficiencies of 48% for tritium.

Specific binding was obtained by subtracting nonspecific binding from total binding at each concentration of the ^{3}H-steroid. Results were subjected to Scatchard plot analysis providing the concentration of receptor sites (fmol/mg cytosol protein) and the apparent K_d. Protein concentration was determined in an aliquot of cytosol preparation by the Coomassie dye-binding method (3) using bovine serum albumin as standard.

Enzyme Assays. E_2DH activity was measured according to the method of Tseng and Gurpide (20). The 800 × *g* supernatant (1 mg protein concentration per ml) was incubated with excess substrates 17β-[^{3}H]estradiol (20 μM) and NAD^+ (1.4 μM) in 0.05 M Tris, pH 8.0, at 37°, and the rate of formation of the product was monitored at 1, 3, 6, and 9 min. Aliquots (0.1 ml) taken at these intervals were mixed immediately with 2 ml methanol containing 1000 cpm [^{14}C]estrone, 500 g estrone, and 500 g 17β-estradiol. Estradiol and estrone were separated by thin-layer chromatography using the solvent system, chloroform:ethyl acetate (4:1). The products were extracted, and their radioactivity was determined using the Beckman Model 7500 liquid scintillation spectrometer with counting efficiencies of 48% for tritium and 71% for ^{14}C. The enzyme activity is expressed as nmol product formed per mg protein per hr and calculated as reported previously (16). Protein concentration in the assay mixture was determined by the Coomassie dye method (3).

RESULTS

In agreement with results published previously (20, 22), explants of proliferative endometrium responded consistently to progestin, *in vitro*, with more than a 2-fold increase in the activity of E_2DH over control explants. In contrast, the activity of E_2DH in fragments of endometrial carcinoma, cultured under identical conditions, was not inducible with progestin (Table 1). Parallel receptor measurements in these cancer tissues indicated the presence of PR in freshly obtained cancer specimen (results not presented).

Scatchard plot analysis of specific [^{3}H]progesterone binding of cytosol fractions of normal and neoplastic endometrium further indicated that the K_d for PR in both of these tissues were essentially similar, ranging from 1 to 5 nM (Chart 1).

Since sensitivity to progestin, *in vitro*, may require the continued presence of PR during the culture period, we examined the stability of PR in cytosol fractions of both proliferative endometrium and carcinoma tissue at various time intervals. The E_2DH activity was simultaneously assayed in another portion of these explants maintained for 2 days with or without MPA. In proliferative explants, a considerable portion of the [^{3}H]progesterone-specific binding sites was maintained for 24 hr under culture conditions without any significant change in K_d. In addition, the receptor stability correlated with the ability of MPA to induce E_2DH activity in parallel cultures of these explants. Since the E_2DH activity in the explants was determined after a 48-hr culture period, PR concentration was also determined in fragments of one proliferative endometrium maintained for the same length of time. Comparison of PR concentrations of fresh tissue with that maintained in culture for 48 hr shows that [^{3}H]progesterone binding sites of normal endometrium are remarkably stable (Chart 2). On the other hand, PR in endometrial carcinoma was very unstable. The [^{3}H]progesterone binding sites were undetectable in carcinoma explants cultured for 24 hr (Chart 2). About 60 to 75% of the original binding sites were degraded at the end of 2 hr at 37°. This instability of PR in carcinoma explants is reflected by the

Table 1

Induction of E_2DH by progestin in normal and neoplastic endometrium in vitro

Explants of proliferative endometrium and endometrial carcinoma were cultured in nutrient medium for 2 days at 37° in a humidified 5% CO_2:air atmosphere in the absence (control) and presence (MPA) of 500 ng MPA per ml. The E_2DH activities were assayed in 800 × g supernatants of homogenates of these explants.

	Estrone formed (nmol)/mg protein/hr	
Endometrium	Control	MPA
Proliferative (n = 8)	2.4 ± 0.9[a]	5.3 ± 2.1
Carcinoma (n = 9)	2.7 ± 1.8	2.9 ± 2.1

[a] Mean ± S.D.

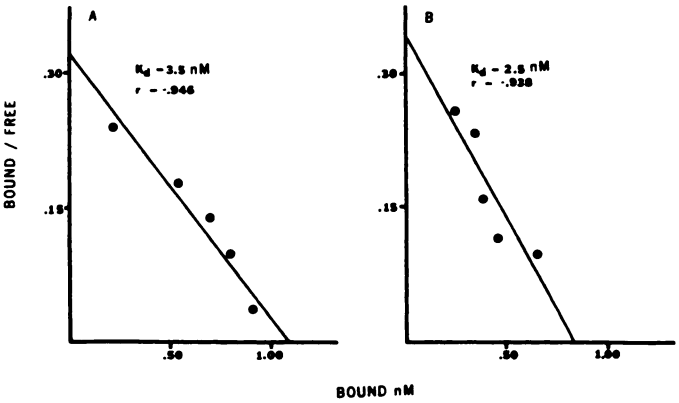

Chart 1. Representative Scatchard plots of specific [^{3}H]progesterone binding in cytosol fractions of endometrial carcinoma (*A*) and proliferative endometrium (*B*).

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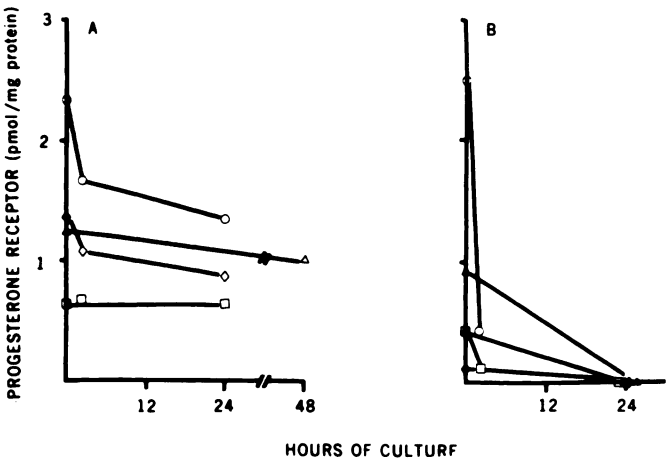

Chart 2. Stability of cytosol PR in proliferative endometrium (*A*) and endometrial carcinoma (*B*) under culture conditions. *Symbols*, separate tissue samples.

noninducibility of E_2DH by progestin, *in vitro*, in parallel cultures.

DISCUSSION

Since the original studies of Kelley and Baker (7), it is generally accepted that about 30 to 35% of patients with metastatic carcinoma of the endometrium will respond to progestin therapy. However, methods for predicting accurately which patients will respond to treatment with progestin are lacking. Several investigators have proposed the use of PR status as an indicator of progesterone sensitivity of endometrial tumors (2, 4, 6, 10, 11). It has also been suggested that any test which reflects the presence of a functional PR would be a better indicator of progestin reponsiveness than a mere demonstration of PR in tumors (12). Indeed, following the demonstration of induction of E_2DH activity by progestin in normal endometrium, Pollow *et al.* (14) showed that *in vivo* administration of progestin to patients with endometrial carcinoma resulted in increased activity of this enzyme in some tumors. These findings were later confirmed by other investigators (5). Attempts to reproduce these results in cultured explants of endometrial carcinoma, *in vitro*, were unsuccessful, and it was surmised that this may be related to the problem of viability of cancer tissue under culture conditions (16). However, the present results on the *in vitro* stability of PR in normal and neoplastic endometrium indicate that sensitivity to progestin is intimately related to the continued presence of PR in cultured tissues. The receptors for progesterone in proliferative endometrium were remarkably stable for 1 to 2 days at 37°, and the continued presence of PR was reflected by the simultaneous stimulation of E_2DH activities in tissue explants. The PR concentrations of adenocarcinoma explants, on the other hand, were quite unstable. About 60 to 75% of the original binding sites were lost within 2 hr of culture at 37°. As indicated above, since the continued maintenance of PR within the tissue is essential for eliciting progesterone effects, it is not surprising that these explants were nonresponsive in regard to E_2DH induction. There was no detectable change in the K_d between proliferative endometrium and cancer specimen, as estimated from Scatchard plot analysis of [^{3}H]progesterone binding data.

The reasons for the instability of PR in cancer tissue are unclear. Release of proteolytic activity by various tumors has been reported. Secretion of considerable amounts of plasminogen activator activity by endometrial carcinoma specimens has also been shown (19). We have also observed that, while carcinoma explants elaborate high plasminogen activator activity concentrations, the culture fluids of normal endometrium contain negligible amounts of this enzyme.[4] Notides *et al.* (13) showed that human uterus contains a serine protease which accounted for the multiple molecular species of estradiol receptors. Most of this activity, however, was localized to the myometrium. Fluctuations in protease activity as a function of the menstrual cycle were, unfortunately, not followed by these investigators. The remarkable stability of PR in cultured proliferative endometrium, noted in the present study, suggests that either the uterine protease activity is specific to estrogen receptor or that there is negligible protease activity during the proliferative phase of the menstrual cycle.

Although the role of progesterone in the induction of E_2DH activity in the human endometrium has been accepted widely, receptor mediation in this process has not been established. The existence of a correlation between PR levels and E_2DH activity in the human endometrium during the menstrual cycle and the response of endometrial carcinoma to progestin administration, as measured by increased E_2DH activity in these tissues, were reported (18). The present findings on the close relationship between PR stability and induction of E_2DH activity by progestin, *in vitro*, provide additional support for the receptor mechanism in this system. Unequivocal establishment of PR mediation in this phenomenon, however, would require the demonstration of a temporal sequence, namely, cytosol PR → nuclear PR → induction of E_2DH, in progestin-exposed endometrium.

In conclusion, we have identified the dramatic decrease in PR concentration in explants of endometrial carcinoma as the reason underlying the repeated failure to induce E_2DH activity *in vitro*. Identification of agents which may prevent receptor degradation within carcinoma tissue and their addition to these cultures may be expected to render these tissues responsive to progestin. The intimate relationship between PR stability under culture condition and induction of E_2DH in these explants has provided further evidence for the receptor mediation in progestin effect. Finally, it is suggested that any experiment designed to follow the effects of steroids on target tissues must take into account the stability of steroid receptors under *in vitro* conditions.

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