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# Estrogen Receptor Expression in Serially Cultivated Rat Endometrial Cells: Stimulation by Forskolin and Cholera Toxin

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Serially propagated with 3T3 feeder layer support, epithelial cells derived from normal rat endometrium expressed estrogen receptor activity. Specific binding of 17- $\beta$ -estradiol was in the range of 30-60 fmol/mg of protein and was of high affinity ( $K_d = 0.3$  nM). A survey of cell lines derived from several other normal epithelia showed that rat vaginal and human cervical cultures also had high-affinity estrogen receptors (6-13 fmol/mg of protein), while rat epidermal and esophageal cells had no detectable activity. In the endometrial cultures, receptor levels were elevated nearly two- to fourfold by cholera toxin or forskolin in the medium. This effect was detectable after 4 hr but not 1 hr of treatment and did not occur in the presence of cycloheximide. We conclude that serially cultivated rat endometrial cells retain hormonal properties expressed in vivo while exhibiting some keratinocyte character. These cells may provide a useful model for study of receptor modulation.

Recent improvements in serial cultivation of keratinocytes (Rheinwald, 1980) have led to considerable progress in understanding their differentiation program (Green, 1980). With 3T3 feeder layer support, this cell type can be propagated from stratified squamous epithelia of a variety of species. From the rat, continuous epithelial cell lines are readily derived not only from epidermis, esophagus, and vagina but also nonkeratinized and even glandular epithelia (Heimann and Rice, 1983a; Phillips and Rice, 1983). These cells all show substantial keratinocyte character in culture—evidence for convergent reprogramming of their differentiated state.

A recurring problem in the study of many epithelia is their minimal expression in culture of distinctive character in vivo (Wigley, 1975), which could be explained by the sort of reprogramming observed (Phillips and Rice, 1983). However, intrinsic differences in regulation of keratinocyte properties (Heimann and Rice, 1983a; Phillips and Rice, 1983) and response to toxic agents (Heimann and Rice, 1983b) are evident in the cultured rat epithelial cells, showing that the convergence is incomplete. Moreover, that such convergent cells from rabbit epidermis and esophagus upon transfer from culture to a suitable environment (the flank of athymic mouse) are capable of reexpressing keratin profiles synthesized in vivo (Doran et al., 1980) emphasizes the potentially malleable nature of the phenotypic switch. Thus scrutiny of the differentiated state of cultured epithelial cells may help clarify the degree of reprogramming the cells undergo and suggest responsible mechanisms to explore.

The high-affinity steroid-binding receptor proteins mediating hormone action have been useful markers in

assessing differentiated function in primary cultures and tumor cell lines derived from sex-steroid-responsive tissues. The finding in cultured cells of female genital tract origin that estrogen receptors are expressed offers, in addition, an opportunity to examine modulation of receptor level by agents known to affect keratinocyte physiology. Agents raising intracellular cyclic AMP have not been reported previously to alter sex steroid receptor levels.

## MATERIALS AND METHODS

### Cell culture

Epithelial cells were cultivated with support from feeder layers of lethally irradiated 3T3 cells according to standard methods (Rheinwald, 1980) in Dulbecco-Vogt Eagle's medium supplemented with fetal bovine serum (5%), hydrocortisone (0.4  $\mu$ g/ml), epidermal growth factor (10 ng/ml), adenine (25  $\mu$ g/ml), biotin (0.1  $\mu$ g/ml), and cholera toxin (9 ng/ml) except as specified. Human ex-cervical epithelial cultures were initiated as tissue explants, freed of fibroblasts as necessary by vigorous rinsing with 0.5 mM EDTA in isotonic saline (Sun and Green, 1976), and used in the second subcultivation. Epithelial cell lines previously derived by serial cultivation from normal tissues of Sprague-Dawley rats (Heimann and Rice, 1983a; Phillips and Rice, 1983) were

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employed between passages 8–16 (endometrial) or 6–10 (vaginal, epidermal, pituitary, mammary, esophageal).

### Estrogen receptor content

Newly confluent cultures were rinsed three times with serum-free medium, harvested, and disrupted by sonication in 10mM Tris HCl (pH 7.4)-2 mM dithioerythritol containing  $\text{Na}_2\text{MoO}_4$  (10mM) for receptor stabilization (Anderson et al., 1980). The extract (typically of four 100-mm cultures in a final volume of 4 ml) was adjusted to 0.4 M in KCl, stirred at 0°C for 20 min, and centrifuged at 100,000g for 45 min. The supernatant fraction was divided into 0.2-ml aliquots and incubated 1 hr at 30°C (Watson et al., 1977) with various concentrations in duplicate or triplicate of [ $^3\text{H}$ ]-17 $\beta$ -estradiol (106 Ci/mmol; New England Nuclear, Boston, MA) with or without a 100-fold excess of unlabeled estradiol. For analyzing effects of KCl extraction or addition of forskolin or cholera toxin to the medium, assays contained 1–2 nM [ $^3\text{H}$ ]-estradiol. Bound and free steroid were separated at 0–2°C using 80 mg of hydroxyapatite HTP (BioRad Laboratories, Richmond, CA) per sample, a method insensitive to ionic strength and protein concentration (Pavlik and Coulson, 1976). The washed pellets were eluted twice with ethanol and scintillation counted at 33–38% efficiency. Receptor content was normalized to supernatant protein content measured using Coomassie G-250 dye (Bradford, 1976). Results were analyzed by the method of Scatchard (1949). DNA content per cell was measured with diphenylamine (Burton, 1956). Forskolin was purchased from Calbiochem (La Jolla, CA), cholera toxin from Schwartz/Mann (Spring Valley, NY), and unlabeled steroids from the Sigma Chem. Co. (St. Louis, MO).

### RESULTS

Serially cultivated cells derived from normal rat and human female genital tract epithelia expressed substantial estrogen binding activity at nM levels of estrogen. Figure 1 presents the results of several representative experiments, and Table 1 summarizes these and other data. Of the several cell types surveyed, rat endometrial cultures had the highest receptor content, four- to sixfold higher than rat vaginal and human cervical cultures. The binding showed the usual specificity for steroid ligands seen for estrogen receptor. When endometrial cell extracts were incubated with 2 nM [ $^3\text{H}$ ]-17 $\beta$ -estradiol and a 200-fold excess of other steroids, diethylstilbestrol was as effective as unlabeled 17 $\beta$ -estradiol in inhibiting binding, while hydrocortisone, progesterone, and testosterone were less than 5% as effective (Rice et al., 1984). With each epithelial cell type, the binding was of high affinity, with measured  $K_d$  approximately 0.3 nM (Table 1). The Scatchard plots (Fig. 1c) were linear over the ranges illustrated. However, in the rat vaginal and hu-

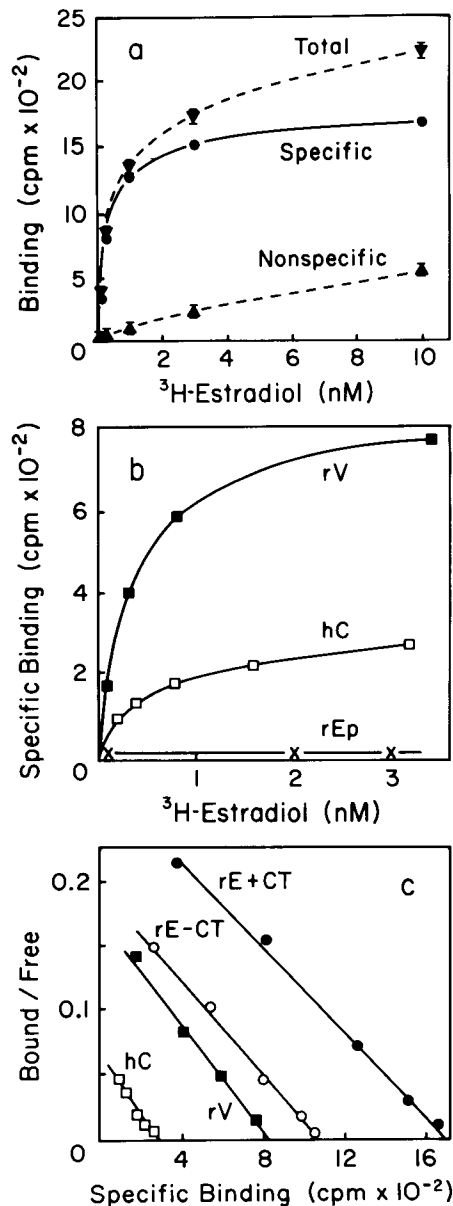


Fig. 1. Concentration dependence of [ $^3\text{H}$ ]-17 $\beta$ -estradiol binding in epithelial cell extracts. Panel a shows total ( $\blacktriangledown$ ) and nonspecific ( $\blacktriangle$ ) binding (without and with 100-fold excess of unlabeled estradiol, respectively) obtained with endometrial cell extracts. Error bars indicate standard deviations of triplicate determinations. Specific (total minus nonspecific) binding is plotted for rat endometrial ( $\bullet$ , rE), vaginal ( $\blacksquare$ , rV), and epidermal ( $\times$ , rEp) and human cervical cells ( $\square$ , hC) in panels a and b, where protein concentrations were 1.7, 6.1, 2.6, and 1.25 mg/ml, respectively. Scatchard plots of the data (same symbols) are given in panel c, including endometrial cells (1.7 mg protein per ml of extract) grown without cholera toxin (CT) supplementation of the medium ( $\circ$ ).

TABLE 1. Estrogen receptors in cultured epithelial cells<sup>1</sup>

Tissue origin	Content (fmol/mg prot)	$K_d$ (nM)
Rat endometrium (5)	46 ± 16	0.34 ± 0.08
Rat vagina (2)	7.5 ± 1.4	0.26 ± 0.06
Rat epidermis (2)	0	—
Human exocervix (2)	11 ± 2.3	0.37 ± 0.12

<sup>1</sup>Mean ± standard deviation (No. of independent experiments in parenthesis).

man cervical cultures, a small amount of low-affinity binding activity was also evident at 10–20 nM concentrations of estradiol. In contrast to these three epithelial cell types, rat epidermal (Fig. 1b), esophageal, and mammary cells were devoid of high-affinity binding activity. In repeated experiments, rat pituitary cells had a barely detectable activity (~1 fmol/mg prot.) that was too low to characterize adequately.

Initial experiments with the various epithelial cells showed better growth in medium supplemented with untreated serum instead of serum depleted of steroids by charcoal extraction. Since treatment of the endometrial cells for a day in medium with extracted serum had little effect on receptor content, they were grown subsequently using the untreated serum. Under these conditions, only 35–40% of the total measured receptor was extractable in low salt buffer. This result was a consequence of both the lower specific activity ( $62 \pm 5\%$ ) and the lower yield of protein ( $59 \pm 9\%$ ) obtained in the low-salt extracts compared to those in parallel adjusted to 0.4 M in KCl. A similar finding was made in one experiment with rat vaginal cells.

Effects of growth factors in the medium were examined on expression of estrogen receptor activity in the rat endometrial cells. In preliminary experiments, the cells were inoculated and grown for 3 days in medium supplemented with epidermal growth factor, hydrocortisone, and cholera toxin as usual. One factor was then removed, and the cells were assayed 4 days later as they reached confluence. Omission of epidermal growth factor or hydrocortisone from the medium had little effect, but the cultures from which cholera toxin was removed exhibited typically only 50–55% as much binding activity. In confirmation, subsequent experiments showed that, although similar in growth rate, cultures treated with cholera toxin starting from inoculation had nearly twice the receptor activity of those grown without the

toxin (Fig. 2). This result evidently was due to an increase in receptor number, since the measured affinity for estradiol was not significantly altered (Fig. 1c).

Compared to control cultures without cholera toxin in the medium, cells treated with this agent for 4 hr exhibited considerably higher receptor levels, nearly as high as those exposed for a day or more (Fig. 2a). Treatment for 1 hr, however, had no significant effect (not shown). In contrast to cholera toxin, which is known to raise cyclic AMP after a lag period, reaching maximal levels in an hour in some cells (Fishman, 1980), forskolin activates adenyl cyclase within minutes (Seamon and Daly, 1981). Treatment with this diterpene (100  $\mu$ M) for 1 hr elicited no response, but by 4 hr the measured receptor level was three- to fourfold that in parallel unexposed cultures (Fig. 2a). Receptor content in cultures treated with 10  $\mu$ M forskolin was approximately twice that in untreated cultures, consistent with the concentration dependence of the compound in other systems (Seamon and Daly, 1981). Concomitant treatment of the cells with cycloheximide, an effective inhibitor of keratinocyte protein synthesis at 10  $\mu$ g/ml (Rice and Green, 1978), prevented the response both to forskolin and to cholera toxin but produced little alteration in receptor level in cultures without these two agents (Fig. 2b).

## DISCUSSION

In the present work, serially cultivated epithelial cells derived from normal rat endometrium exhibited substantial levels of estrogen receptor. Although these cells exhibit some keratinocyte character (Phillips and Rice, 1983), clearly within their repertoire in vivo upon vitamin A deprivation (Wolbach and Howe, 1925) or certain other pathological conditions, retention of this hormonal property is not excluded, suggesting the same may be true during squamous metaplasia. Based on measured values of 60 pg of protein and 11 pg of DNA per endometrial cell, the corresponding receptor levels are calculated as nearly 2,000 binding sites per cell and 250 fmol of receptor per mg DNA under standard growth conditions, somewhat lower than values obtained with an endometrial cell line from a prepubescent Wistar-Furth rat exhibiting 6,800 sites per cell (Sonnenshein et al., 1974). The values obtained for the cervical cells are at the low end of the range reported for cervical tissue in vivo, while the ratio of receptor levels in the cultured rat endometrial and vaginal cells is similar to that reported for human endometrial and cervical tissue (Sanborn et al., 1975).

Retention of receptor expression in the cultured female lower genital tract epithelial cells is in marked contrast to their virtual absence in the cultured rat mammary and pituitary cells, although some uncertainty exists for the latter regarding the actual cell types of origin within the glands. Loss of receptor activity cannot be due to adoption of keratinocyte character per se, but probably reflects the degree of divergence from the normal differentiated state elicited by cultivation. These observations emphasize the importance of appropriate culture conditions for expression of hormonal receptors and responses. The continued expression of other sex steroid receptors under the present culture conditions would be of interest in a variety of cell types.

The effect of agents elevating intracellular cyclic AMP levels to increase receptor activity in the endometrial

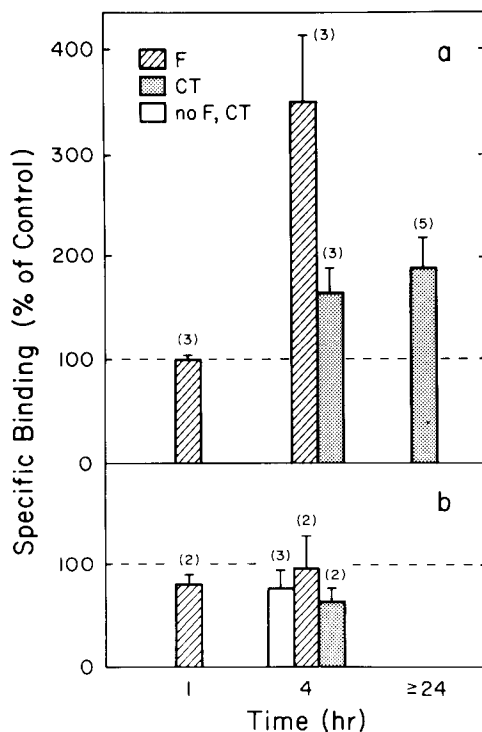


Fig. 2. Effect of forskolin (F, 100  $\mu$ g/ml) and cholera toxin (CT, 9 ng/ml) on estrogen receptor levels. Newly confluent endometrial cell cultures were treated in the absence (a) or presence (b) of cycloheximide (10  $\mu$ g/ml). Results are normalized to 100% for untreated cultures measured in parallel in each experiment (dashed line). The number of independent determinations, each an average of duplicate samples, is given in parentheses.

cells raises the possibility such a phenomenon is important in initiating or maintaining responsiveness *in vivo*. Binding activity of steroid hormone receptors is known to be modulated by phosphorylation, recently shown directly with the estrogen receptor (Auricchio et al., 1984). The present findings are compatible with cyclic AMP stimulation of protein kinase activity to produce the observed result, but suggest protein synthesis may be important as well. Further exploration of specific protein expression influenced by cyclic AMP levels may help elucidate the mechanism of receptor elevation. Chemical agents such as forskolin that elevate intracellular cyclic AMP levels can have a spectrum of effects on the hormonal program of responsive cells corresponding to physiological processes (Ranta et al., 1984). The degree to which the cultured rat endometrial cell program is altered remains to be evaluated. However, the present phenomenon appears to be an element in the induction by cholera toxin of decidualization in hamster uterus, hypothesized to be mediated by an observed increase in estrogen receptor level that normally occurs through physiological processes (Alleva et al., 1984).

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