Estradiol/Progesterone Interaction in Normal and Pathologic Breast Cells

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INTRODUCTION

The possibility that an unopposed estrogen effect mainly due to an insufficiency of progesterone secretion during the luteal phase might be involved in the promotion of human breast cancer remains a challenging hypothesis.[1-4] However, it will take many years before epidemiologists can collect enough objective data to verify this hypothesis and then implement it in the prevention of breast cancer.

In the meantime it remains necessary to collect basic and experimental information on the interaction between estradiol and progesterone at the level of breast cells. Indeed, since such information is already available regarding the human endometrium, the paucity of studies on human breast tissue is appalling.

This review is an attempt to collect the information that has been obtained thus far on this topic.

SYNERGISTIC ACTION OF ESTRADIOL PLUS PROGESTERONE

In most target cells of the female genital tract, cell differentiation involves the successive and synergistic action of estradiol and progesterone. It is now well established that estrogen priming increases progesterone receptor titers in the target cell. Estrogenic induction of progesterone receptor synthesis would seem to provide a basis for the priming effect of estrogen in the preparation of target tissue for subsequent progesterone response.[5,6] The augmentation of progesterone receptor synthesis by estrogen action involves the stimulation of RNA and protein synthesis, and this response appears to be regulated by the estrogen receptor system.[6,7] Progesterone receptor—at least in the uterus—is controlled by its own physiological ligand.[7] An important decrease in progesterone receptor is observed when progesterone is administered to guinea pigs whose concentration of estrogen-stimulated receptor is at its highest level. Progesterone probably exerts its effect through an enhancement of

receptor inactivation rate. In other words, there seems to be an autolimitation of progesterone action. These different sequences in receptor synthesis and inactivation first primed by estradiol have been essentially observed in the uterus of cycling animals and also in women.[8] In the breast, the presence of progesterone receptors has been demonstrated in human mammary tumors, and it has been proposed that these receptors, whose synthesis is controlled by estrogen in the uterus, might serve as markers of estrogen action in breast cancer.[9] Thus the presence of progesterone receptor in a cancerous breast tumor would indicate that the entire sequence involving estrogen binding to cytosolic receptor, movement of the receptor complex into the nucleus, and stimulation of a specific end product can be achieved in the tumor cell. This would rule out the existence of a defect beyond the binding step.[9] At the present time this sequence of receptor synthesis throughout the menstrual cycle has not been demonstrated in normal breast.

However, it is well known that estradiol and progesterone act synergistically in the breast as well as in the endometrium.[2] Estradiol is the hormone initially responsible for the differentiation and development of the ductal epithelium, increasing mitotic activity of the cylindrical cells of the internal layer of the ducts.[10] Progesterone acts synergistically with estrogens on the distal part of the duct, favoring differentiation into acini. This conclusion is based upon many experimental observations.[11] Generally, in those species in which the cycle consists mainly of a follicular phase, the mammary gland primarily contains a ductal system. Well-developed alveoli, on the other hand, are found for the most part only in species that have a definite luteal phase.

In 1941, Lyons and McGinty[12] demonstrated that the administration of estrone to immature male rabbits produced good ductal growth with almost negligible alveolar formation. When animals were treated with the same dose of estrone plus increasing doses of progesterone, the high doses of progesterone counteracted mammary duct ectasia induced by estrogen alone and permitted instead an extensive alveolar proliferation. In other words the existence of a harmonious ovarian cyclic function seems to ensure a perfect mammary development. In particular, the presence every month of a corpus luteum that secretes a sufficient quantity of progesterone for a normal duration of time permits a coherent organization of the ductal system and the adjacent connective tissue.[13,14]

Indirect information on the possible cyclic changes in the content and the intracellular distribution of estradiol receptors (ER) and progesterone receptors (PR) was obtained from studies of fibroadenomas removed in young women at different times of the menstrual cycle.[15] This breast tumor was chosen as material since it offers a relatively homogeneous epithelial concentration resembling that of the normal breast from an anatomic and hormonal point of view.[15] Only fibroadenomas with high epithelial cell density and scarce or absent fibrosis were studied. In these tumors cyclic changes in the distribution of cytosolic and nuclear receptors of estradiol (ER_c and ER_n) and progesterone (PR_c and PR_n are very similar to those noted in the human endometrium.[8,16,17] These results suggest that receptors in both tissues are under the control of cyclic ovarian secretion. Indeed, during the follicular phase (FIG. 1) the increase in ovarian secretion seems to be responsible for a progressive increase in cytosolic and nuclear ER since estradiol stimulates synthesis of its own receptor.[18,19] During the menstrual cycle, the higher level of ER in the nucleus as compared to that in the cytosol may be explained by the increase in plasma estradiol and the translocation of the E_2-receptor complex from the cytosol to the nucleus.[20] During the luteal phase, the decrease in ER_c and ER_n probably reflects the action of progesterone secreted by the corpus luteum since progesterone is known to inhibit ER synthesis.[7,21,22] Moreover, the variations in PR_c and PR_n levels observed in fibroadenomas throughout the menstrual cycle (FIG. 2) give valuable information on the

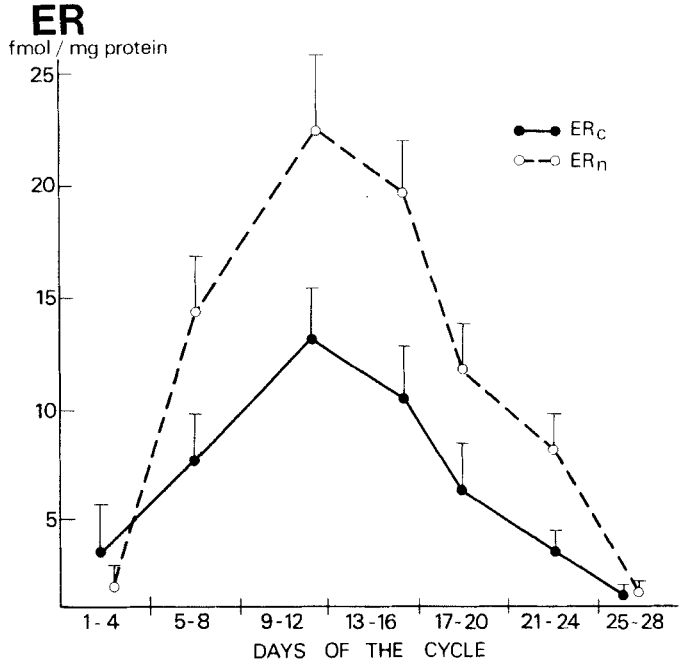

FIGURE 1. Variation in ER_c (●——●) and ER_n (○- - ○) levels in 34 fibroadenomas during different phases of the menstrual cycle. Each point represents the mean ± SEM (n = 4 or more). (From Kuttenn *et al.*[15] Reprinted by permission from the *Journal of Clinical Endocrinology and Metabolism.*)

hormone dependency of progesterone receptors. The estrogen dependency of PR[5,23] is reflected by the high level of progesterone at the end of the follicular phase. The translocation of PR from the cytosol to the nucleus at the beginning of the luteal phase seems to be mainly due to the occurrence of progesterone secretion.[24] The decrease in progesterone receptor levels during the luteal phase probably reflects the inhibition by progesterone of its own receptor.[21] The moderate increase in PR_c noted at the end of the luteal phase may be explained either by the normal decrease in plasma progesterone at the end of the luteal phase, or by the frequent luteal insufficiency noted in these patients.[2,3] Milgrom *et al.*[21] observed similar cyclic variations in PR in rat endometrium.

In addition, it is interesting to observe the variations in concentration and distribution of cytolosic and nuclear receptors of estradiol and progesterone in fibroadenomas removed from women treated with progestagens or estrogen-progestagen combinations (FIG. 3). When estrogen-progestagen combinations are well balanced, they induce a cell distribution of PR similar to that observed during a normal luteal phase. The correction of luteal insufficiency in patients with fibroadenomas induces a progesterone receptor translocation into the nucleus, which is more important than what is noted in fibroadenomas removed during the luteal phase.[15]

ANTIESTROGEN EFFECT OF PROGESTERONE IN BREAST CELLS

Experimentally there are many indirect data to suggest an antagonism between estradiol and progesterone at the level of breast tissue. Many investigations have shown that the mammary gland of different species reacts differently to estrogen when the hormone is administered in physiological or supraphysiological doses or in combination with progesterone or not.[2,4]

High doses of estrogen administered for a long time to castrated female rats induce proliferation and dilation of the lobules in the glandular tissue with formation of cysts and overgrowth of the epithelium.[14] In addition, estrogen provokes an increase of circumcanalicular and intralobular connective tissue.

The successive sequence of mammary alterations following the administration of estradiol to female rats[25] includes the proliferation of tubular system secretion, the dilatation of ducts, and the formation of cysts and fibrosis. These changes observed under the effect of supraphysiological doses of estrogen seem to be comparable to those of human fibrocystic disease.[3,14] In contrast, when estradiol is administered in combination with progesterone, complete and proper development of the mammary

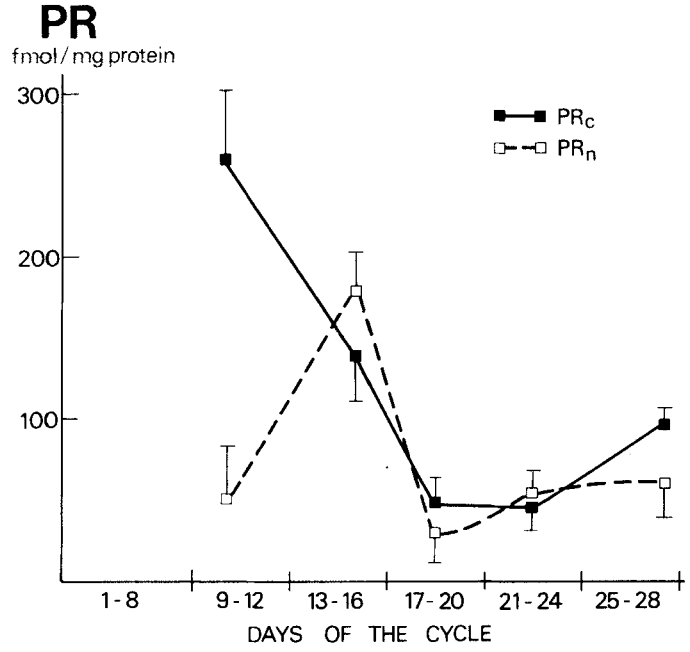

FIGURE 2. Variations in PR_c (■——■) and PR_n (□----□) levels in 26 fibroadenomas during different phases of the menstrual cycle. Each *point* represents the mean ± SEM (n = 4 or more). (From Kuttenn *et al.*[15] Reprinted by permission from the *Journal of Clinical Endocrinology and Metabolism.*)

gland is observed when the ratio between estrogen and progesterone is adequate.[12] Cowie *et al.*[20] found that a combination of estrogen and progesterone in castrated goats resulted in a uniform development and secretion when the dose of estrogen remained small (0.25 mg/day). An increase of the estrogen dose to 1 mg/day produced cysts and epithelial proliferation.

Biochemically, the antiestrogen activity of progesterone is well documented. The mechanism by which progesterone and progestins exert their antiestrogenic action in women includes a reduction of estrogen secretion in systemic circulation, inactivation of estradiol by metabolism at the target tissue, and a lowering of levels of estrogen receptors in these tissues.[27]

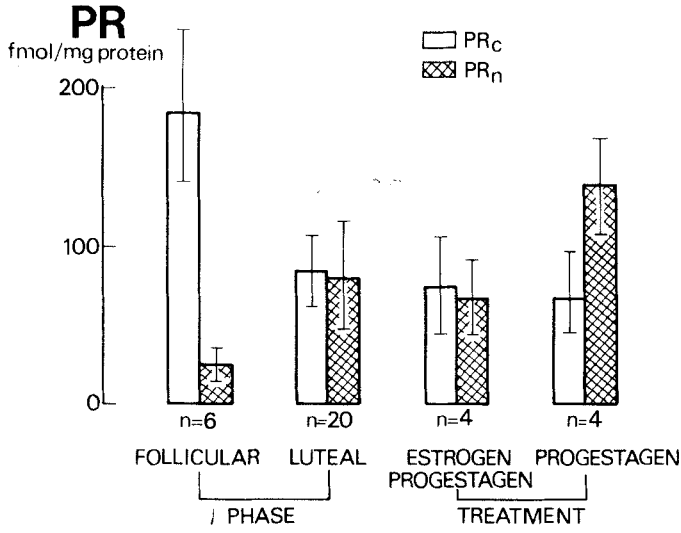

FIGURE 3. Parallel study of PR levels (mean ± SEM) in the cytosol (□) and nuclear fractions (⊠) of fibroadenomas of women treated with a combination of estrogen-progestagen or progestagen alone compared with PR levels during the follicular and luteal phases of the menstrual cycle. (From Kuttenn *et al.*[15] Reprinted by permission from the *Journal of Clinical Endocrinology and Metabolism.*)

EFFECTS ON BLOOD LEVELS OF ESTROGENS

Progestins in pharmacologic doses may lower the circulating levels of estrogens by suppressing gonadotropins and ovarian function. In particular, androstane derivatives are strong antigonadotropic agents. In a large investigation of women with benign breast disease, we were able to confirm that lynestrenol administered from day 10 to day 25 of the menstrual cycle not only suppressed the ovulation peak of LH, but also resulted in estradiol levels less than or equal to 50 pg/ml.[3,28]

EFFECTS ON ESTROGEN RECEPTOR LEVELS

Another mechanism by which progestins can be antiestrogenic is their ability to lower estrogen receptor levels in the endometrium.[27,29] How this effect is mediated has not been biochemically elucidated. It has been suggested that the decrease in intracellular concentrations of estradiol, brought about by the increase in 17β-hydroxysteroid dehydrogenase (see further), may be responsible for the decline in estradiol-binding sites. Experiments on rats have shown, however, that progestins affect estrogen receptor levels, even though they do not influence the activity of the enzyme.[27] Progestins do not alter estradiol affinity for binding to its receptor, and it is likely that their effect involves a reduction of the estrogen receptor synthesis. In the hamster uterus, progestins decrease the levels of nuclear estrogen receptors through processes inhibited by actinomycin or cycloheximide.[5]

Uncertainties concerning the heterogeneity of the estradiol receptor complicate the definition of the problem. In particular Clark *et al.*[30] found two distinct types of cytosol receptors: one (type I) is translocated by estrogens into the nucleus, the other (type II) is not. These authors also described the presence of two types of nuclear receptors. According to recent data of Clark *et al.*,[30] progesterone blocks the estrogen stimulation of nuclear type II sites and inhibits uterine growth.

In human tissue there is no direct proof of progesterone antiestrogenic activity's being mediated by action on the receptor. Only indirect information is available. First, in fibroadenomas removed at different times in the menstrual cycle, as reported above,[15] there was a continuous decrease in the level of both ER_c and ER_n throughout the luteal phase, proven by increased plasma progesterone levels (FIG. 1). In addition, when patients with fibroadenomas are treated for 30 days before surgery with progestins (10 mg of lynestrenol daily with percutaneous progesterone,[3,28] their fibroadenomas do not contain any trace of estradiol receptor (unpublished data).

EFFECT ON BREAST CELL MULTIPLICATION

Most of the data on the effects of progesterone and progestins on estrogen-dependent cell multiplication have been reported by Rochefort and coworkers.[31] These authors studied *in vitro* the effect of the progestin R 5020 on the growth of human breast cancer cell line T 45D and R 25. While R 5020 has no effect on cell growth when tested alone, it significantly inhibits the growth of cells in the presence of estradiol. This effect is more clear after 10–12 days of progestin treatment and is dose-dependent. Progesterone is less effective than R 5020 because of its rapid metabolism in the cells. These results emphasize the antiestrogenic activity of progesterone and progestins in cultured breast cancer cells since these steroids prevent the stimulation of cell growth by estradiol. When we used the technique of primary culture of human normal breast cells (see further) in our laboratory, preliminary results showed that estradiol stimulates and that the progestin R 5020 slows down the incorporation of [^{3}H]thymidine into DNA of cells in culture (in preparation). These results are an additional proof of the antagonistic effect of estradiol and progestin on mammary cell proliferation.

ESTROGEN METABOLISM AT TARGET LEVEL: THE IMPORTANCE OF 17β-HYDROXYSTEROID DEHYDROGENASE

Current concepts emphasize the regulation of receptor levels as the primary means of intracellular control of hormonal activity. The high affinity of estradiol for its receptor leads to the idea that the receptor level is the main factor in determining the amount of the available hormone bound under physiologic conditions. However, other considerations suggest that hormone-metabolizing enzymes in the cell may interfere with the capture of the hormone by the receptor.[27] The main enzyme involved in the antiestrogenic activity is the progesterone-dependent 17β-hydroxysteroid dehydrogenase.[16,17] The activity of the NAD-dependent 17β-hydroxysteroid dehydrogenase (E_2DH) plays an important antiestrogen role indeed since it converts a potent estrogen—estradiol (E_2)—into a less active one: estrone (E_1). Indeed, the cytosolic estrogen receptor has a lower affinity for E_1 and the complex dissociates more rapidly.[30] Previous studies have demonstrated that E_2DH activity was present in human endometrium and other tissues.[16,28,32] An important investigation was therefore carried out in our laboratory in order to study this enzyme activity in breast cells. Two sorts of investigation were performed: (1) with fibroadenomas and (2) with normal breast cells in culture.

Fibroadenomas

This benign tumor was chosen for receptor studies because it offers a relatively homogeneous epithelial concentration that is very close to that of normal breast tissue. Breast fibroadenomas were surgically removed from 54 patients.[30] Surgery was performed in 28 of these patients during the follicular phase and in 18 others during the luteal phase. Eight other patients were under hormonal treatment: Five received progestin therapy (10 mg lynestrenol/day for 10 days) and three were treated with progesterone percutaneously applied to the breast (50 mg/day in an alcoholic gel allowing 10-15% local absorption of the total dose of progesterone).[28]

When E_2DH activity was studied according to the day of the menstrual cycle (FIG. 4), it was observed that during the follicular phase the enzyme activity was low and did not significantly vary. After ovulation, E_2DH activity progressively increased to reach its maximal level at the end of the luteal phase. This delayed increase in E_2DH activity during the luteal phase might be due to the inadequate corpus luteum with low progesterone secretion in most patients with fibroadenomas.[2,32] Under such conditions, E_2DH stimulation might occur more slowly and later than during a normal luteal phase. However, the variations in E_2DH activity observed at different phases of the menstrual cycle in the patients studied are in agreement with *in vitro* results obtained by different investigators studying human and monkey endometrium.[16,17,34–36]

It is interesting to note that the increase in E_2DH activity seen during the luteal phase varies according to the level of epithelial cellularity of the fibroadenoma studied (FIG. 5). E_2DH activity was relatively high in tumors with high epithelial cellularity, but was as low as during the follicular phase in tumors with significant fibrosis and no epithelial cells. These results may be correlated with studies on E_2 and progesterone receptors in fibroadenomas.[2,3,15] Progesterone receptors disappear rapidly during the course of the disease, and since E_2DH seems to be dependent on the presence and

efficiency of the progesterone receptor, it is tempting to consider E_2DH activity as a particularly fine index of cellular differentiation, at least in benign breast lesions. It would therefore be interesting to test such an enzyme activity in breast cancer in order to determine whether it is a more sensitive marker of hormone dependency than is the progesterone receptor.

The marked increase in E_2DH activity noted in fibroadenomas in patients treated with either oral progestin or topically applied progesterone is yet further proof that this enzyme is progesterone-dependent (FIG. 6). It also provides an additional basis for the treatment of benign breast disease with locally or systemically administered progesterone or progestins. In addition to the antiestrogenic effect of progesterone and

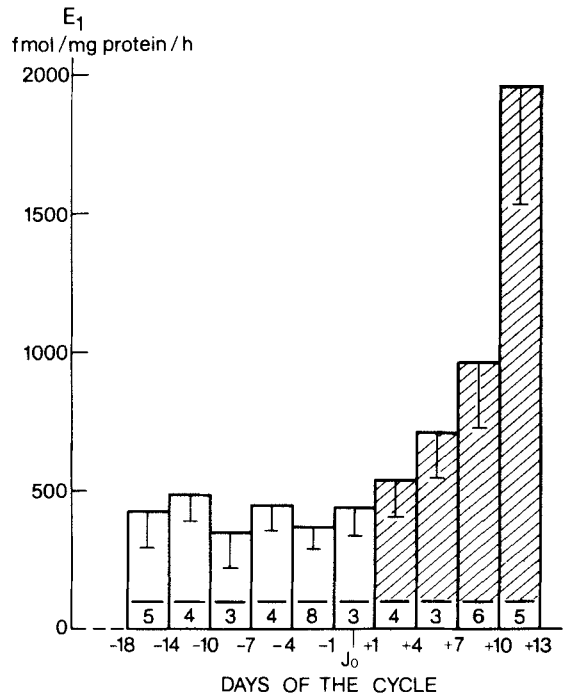

FIGURE 4. E_2DH activity (mean ± SEM) at different times during the menstrual cycle. E_2DH activity (mean ± SEM) □, follicular phase; ▨, luteal phase. J_0 designates the day of ovulation, according to basal body temperature measurement. (From Fournier *et al.*[32] Reprinted by permission from the *Journal of Clinical Endocrinology and Metabolism.*)

progestin due to the decrease in ER synthesis, the progesterone-dependent E_2DH activity efficiently opposes the elevated concentration of E_2 inside the breast tissue, particularly in the case of insufficient production of progesterone by corpus luteum.[2,33]

These results confirm our previous hypothesis on the hormonal background and buttress our proposition for treatment of benign breast disease with progesterone and progestins as a putative and potentially preventive action against the cocarcinogenic role of estrogen in the development of breast cancer.[3,14,15]

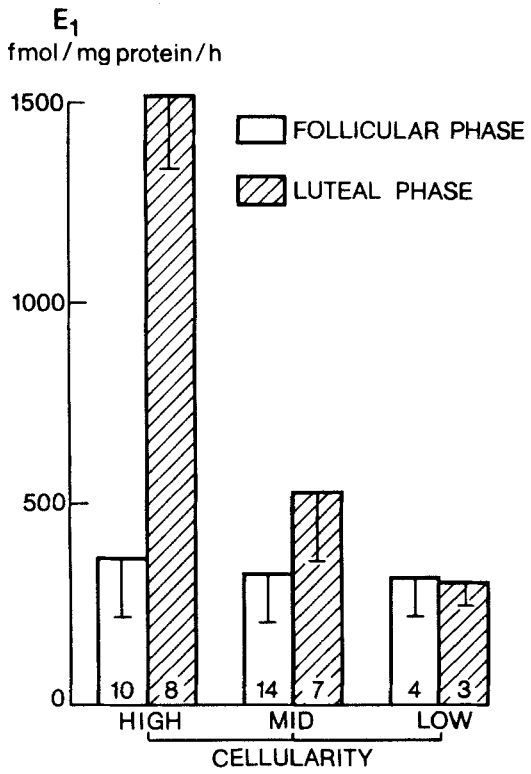

FIGURE 5. Variations in E_2DH activity (mean ± SEM) between the follicular phase (□) and the luteal phase (▨) of the menstrual cycle in breast fibroadenomas, according to the density of epithelial cellularity. (From Fournier *et al.*[32] Reprinted by permission from the *Journal of Clinical Endocrinology and Metabolism.*)

Primary Cultures of Normal Breast Cells

Primary cultures of normal breast cells prepared from surgical specimens taken during reduction mammoplasty were used to study the activity of the enzyme 17β-hydroxysteroid dehydrogenase (E_2DH). This study was performed on both epithelial and stromal cells separated after collagenase digestion of the tissue on a Percoll gradient and then cultured as monolayers in Ham's F10 medium supplemented differently for epithelial cells or fibroblasts.[36] Epithelial cells and fibroblasts have completely different morphologic patterns and growth rates. Epithelial cells are small, round and slightly tear-shaped, with a voluminous nucleus which is round and very refringent (FIG. 7A). Fibroblasts are characteristically spindle-shaped with a small nucleus and filaments extending in several directions (FIG. 7B).

The kinetics of estrone (E_1) formation after incubation of epithelial cells with [^{3}H]estradiol for periods of time ranging from 15 to 40 minutes show that the reaction

was linear for the first 30 minutes (FIG. 8). In fibroblast culture, E_1 formation was much slower and 24 hr incubation was necessary to obtain the same amount of E_1 that was obtained after 1-hr incubation with epithelial cells (FIG. 8).

When estradiol was added to the culture medium, it has no effect on E_2DH activity on either kind of cell. The affinity and capacity of E_2DH were greater in epithelial cells with apparent $K_m = 0.6 \pm 0.1$ μM and $V_{max} = 250$ to 360 pmols/μg DNA/hr, while they were 10 ± 1 μM and 50 to 70 pmol/μg DNA/hr, respectively, in fibroblast cultures (FIG. 9).

Moreover, the E_2DH activity was 2 to 5 times higher in epithelial cells cultured in the presence of the progestin medroxyprogesterone acetate (MPA), whereas it remained unchanged in fibroblasts cultured under the same conditions (FIG. 8). This increase in E_2DH activity was dose-dependent from 10^{-10} to 10^{-7} M MPA(FIG. 10) and was inhibited by both actinomycin D and cycloheximide.

This system of differential breast cell cultures appears to be a fruitful tool for the study of the hormone-dependence of normal breast growth and differentiation. Because of the presence of E_2DH, epithelial cells are more apt to undergo and moderate estradiol action. Moreover, epithelial cells are a possible site of progesterone modulation of E_2DH activity. Therefore E_2DH could be a good marker for both epithelial cells and their hormone-dependence.

Epithelial cells indeed seem to be very sensitive to estradiol and capable of modulating its action because the presence of E_2DH. They are also the site of a possible modulation of E_2DH activity by progesterone. Inhibition of progestin E_2DH stimu-

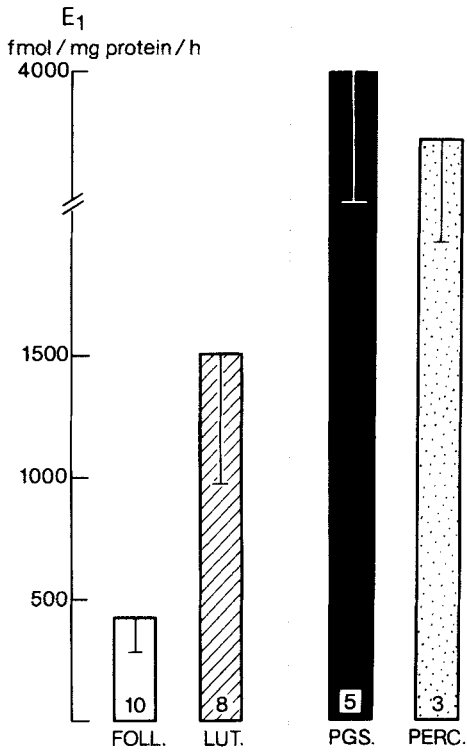

FIGURE 6. E_2DH activity in high epithelial cellularity fibroadenomas surgically removed during the follicular (FOLL; □) or luteal phase (LUT; ▨) of the menstrual cycle, and under progestagen therapy [lynestrenol *per os* (PGS; ■) or percutaneous progesterone topically applied to the breast (PERC; ⊡)]. (From Fournier *et al.*[32] Reprinted by permission from the *Journal of Clinical Endocrinology and Metabolism.*)

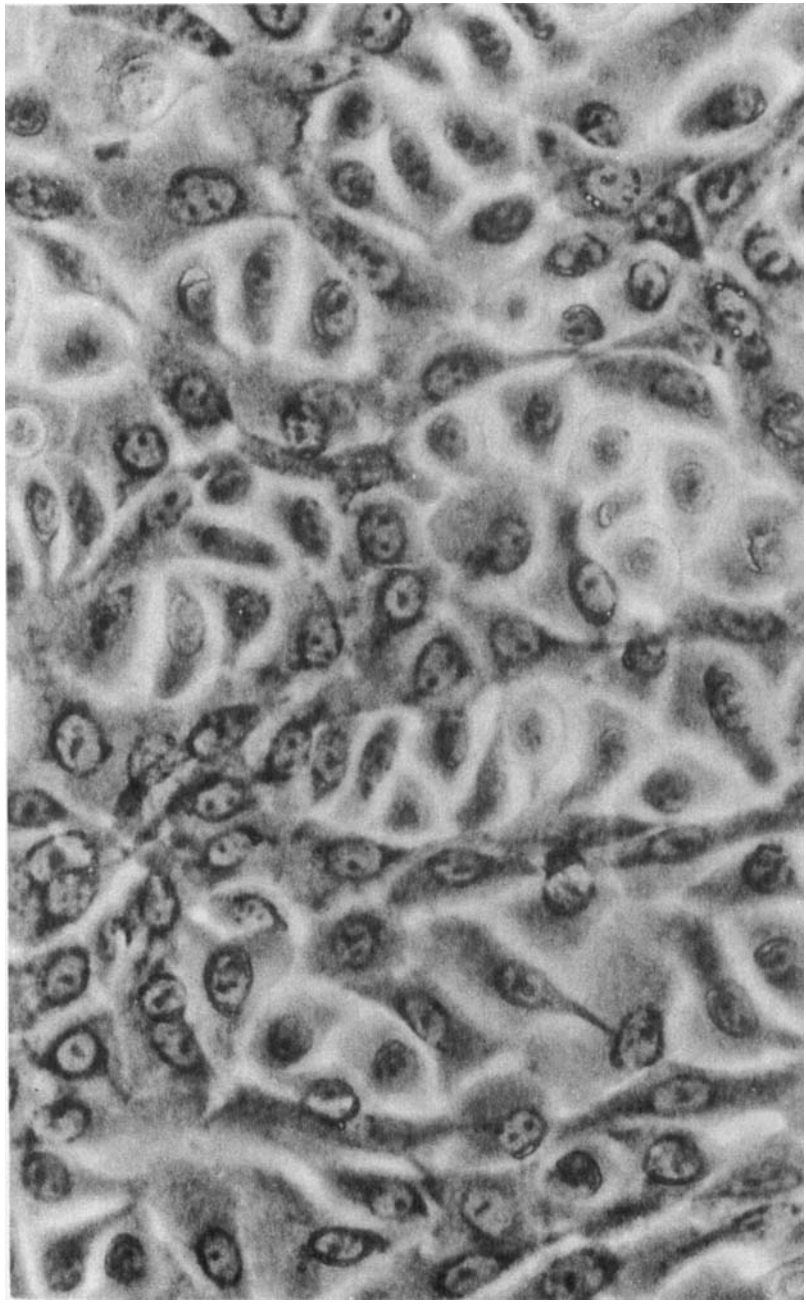

FIGURE 7A. Morphologic aspect of epithelial cells from the normal human breast under phase-contrast microscopy after 16 days of culture. Magnification $\times 100$. (From Prudhomme *et al.*[37] Reprinted by permission from *Endocrinology.*)

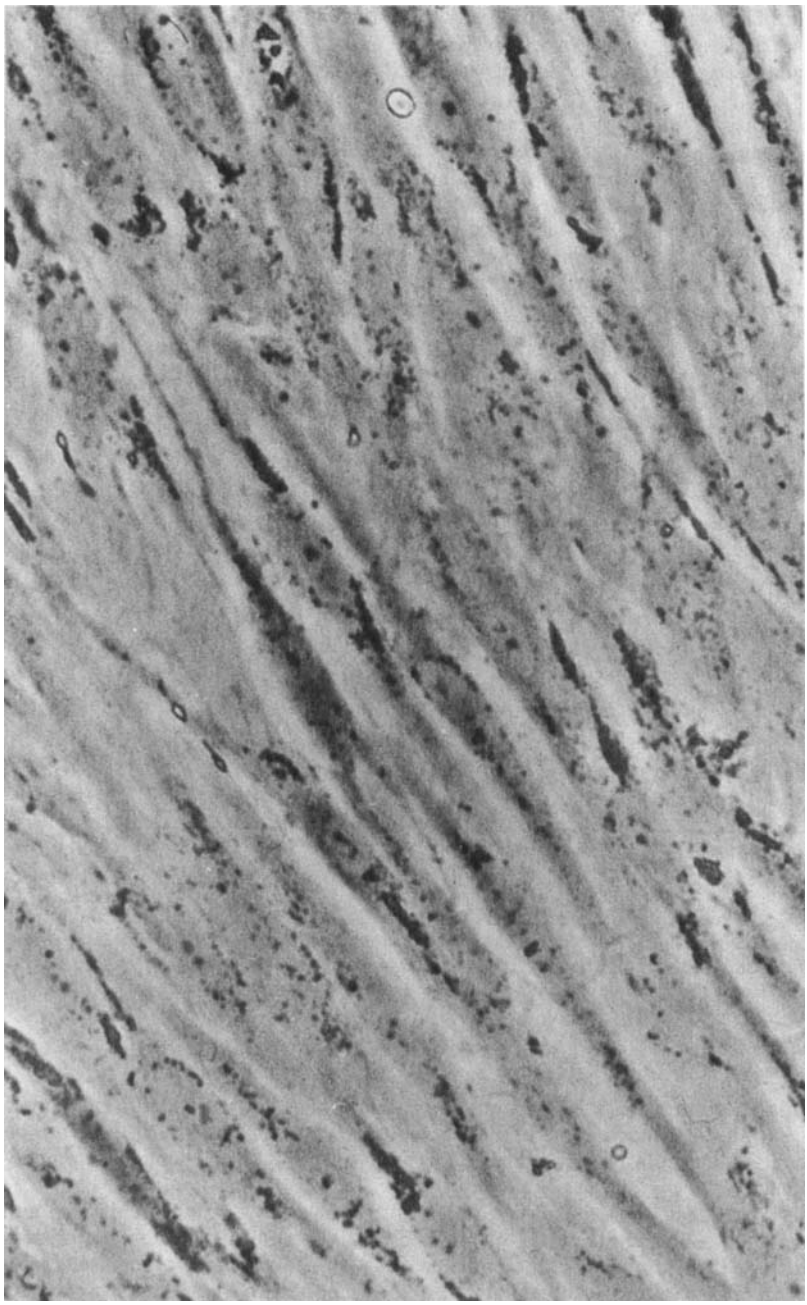

FIGURE 7B. Morphologic aspect of stromal cells under phase-contrast microscopy after 8 days of culture. Magnification ×100. (From Prudhomme *et al.*[37] Reprinted by permission from *Endocrinology*.)

lation, by both cycloheximide and actinomycin D, in epithelial cells indicates that this stimulation requires active protein synthesis and implies, at least partially, an action at the level of transcription. *In vivo,* E_2DH may be involved in the regulation of estradiol action and mammary growth, once ovulation and progesterone secretion occur.

Therefore, in the epithelial components of the breast, as in the endometrium, progesterone controls estradiol action by at least two mechanisms, including the inhibition of estradiol receptor synthesis and the stimulation of E_2DH.

Since E_2DH is progesterone-dependent, its activity in estradiol and progesterone target tissues indicates that the progesterone receptor is present and functional. In contrast to E_2DH, which is measurable even with rather small quantities of cells, the progesterone receptor requires large quantities of cells in order to be studied. The progesterone receptor is known to be E_2-dependent. The fact that the presence of

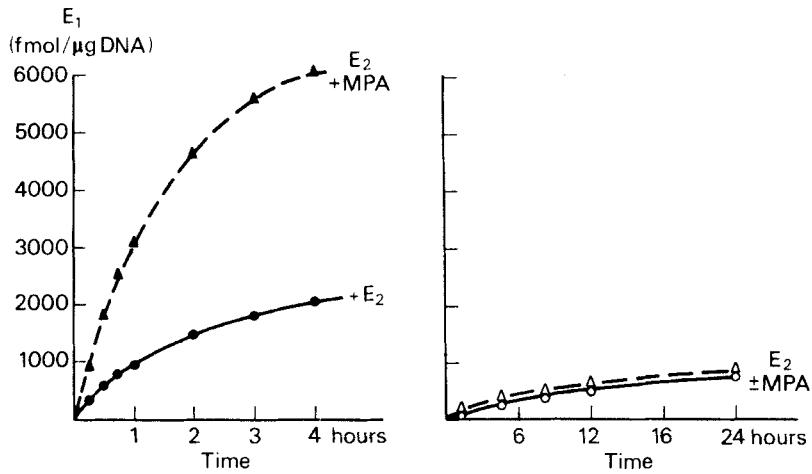

FIGURE 8. Time course of E_1 formation after incubation of $[^3H]E_2$ (incubation medium 5 ml, final concentration = $2nM$) in normal human breast epithelial cells (*left*) or fibroblasts (*right*) cultured in the presence of either $E_2(10^{-8}M)$ alone (no difference from control) or E_2 + MPA ($10^{-8}M$). (From Prudhomme *et al.*[37] Reprinted by permission from *Endocrinology.)*

estradiol is necessary for MPA to stimulate E_2DH in this study is an additional proof of the estradiol-dependence of the progesterone receptor. E_2DH could therefore be considered a particularly good marker for both epithelial cells and their hormone-dependency.

These data once more raise the problem of the respective roles of epithelial and connective tissue in the mammary gland: Is connective tissue only a support or does it participate in the hormonal environment of the epithelial structures themselves? In any event, this system of differential cell culture appears to be a productive model for the study of normal breast receptivity to hormones and hormonal action on breast growth.

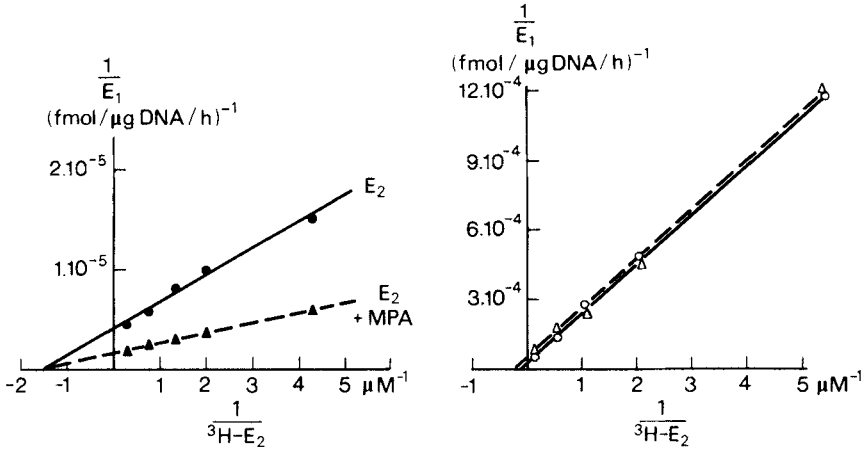

FIGURE 9. Lineweaver-Burk representation of E_2DH activity after incubation of normal human breast epithelial cells (*left*) or fibroblasts (*right*) with different concentration of $[^3H]E_2$. Cells were cultured in the presence of either $E_2(10^{-8}M)$ alone (no difference from control) or $E_2 \pm$ MPA ($10^{-8}M$). (From Prudhomme *et al.*[37] Reprinted by permission from *Endocrinology.*)

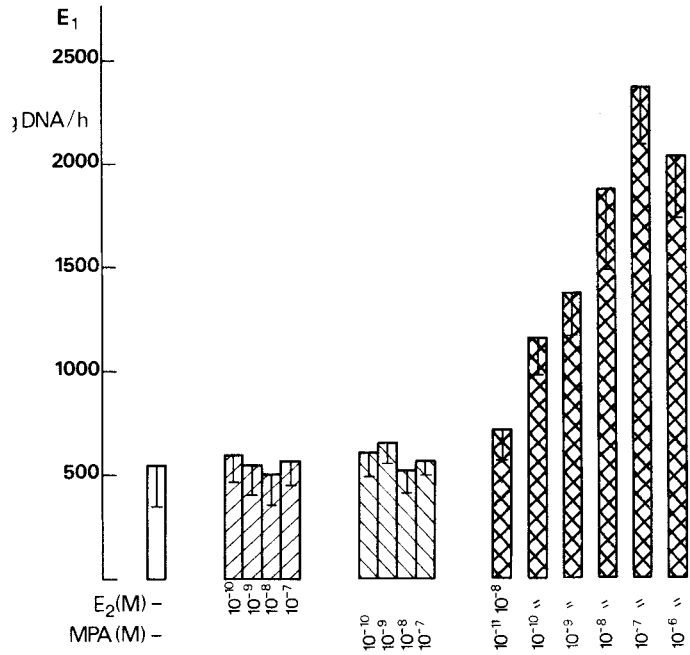

FIGURE 10. E_2DH activity in normal human breast epithelial cells cultured in the absence or the presence of different concentrations of E_2 (no difference from control) and/or MPA. (From Prudhomme *et al.*[37] Reprinted by permission from *Endocrinology.*)

SUMMARY

In most target cells of the female genital tract, adequate cell differentiation is obtained via the successive and synergistic actions of estradiol (E_2) and progesterone (P). This mainly due to the fact that progesterone receptor (PR) synthesis involves the prior action of estradiol through its receptor (ER). In normal breast, E_2 stimulates the growth of the ductal system whereas lobular development depends on progesterone secretion. In other words E_2 + P, when secreted in an adequate balance, permit the complete and proper development of the mammary gland. On the other hand progesterone may also have an antagonistic action against E_2. The antiestrogen activity of progesterone is mediated through a decrease in the replenishment of E_2 receptor and the synthesis of 17β-hydroxysteroid dehydrogenase, which leads to an accelerated metabolism of E_2 to E_1 in the target organ itself. These biochemical events, which have been well documented in the endometrium, have also been shown in cultures of normal breast epithelial cells as well as in differentiated fibroadenomas with high cellular density. In addition, data from the literature show that E_2 added to human breast cells increases cell multiplication by means, eventually, of the synthesis of growth factors. Progesterone and progestins have a reverse effect. Data from our laboratory indicate that in normal cultured cells E_2 and progestins are also antagonists with regard to cell multiplication. From these different data, it is postulated that in human beings, long periods of a luteal-phase defect leading to an unopposed estrogen effect might be a promoter of carcinogenesis in the breast.

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