



# Progesterone effect on cell growth, ultrastructural aspect and estradiol receptors of normal human breast epithelial (HBE) cells in culture

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## Abstract

The stimulating effect of estradiol (E2) on breast cell growth is well documented. However, the actions of progesterone (P) and its derivatives remain controversial. Additional information is therefore necessary. On a culture system of normal human breast epithelial (HBE) cells, we observed an inhibitory effect on cell growth of a long-term P treatment (7 days) in the presence or absence of E2, using two methods: a daily cell count providing a histometric growth index, and [<sup>3</sup>H]-thymidine incorporation during the exponential phase of cell growth. A scanning electron microscopy study confirmed these results. Cells exhibited a proliferative appearance after E2 treatment, and returned to a quiescent appearance when P was added to E2. In both studies, P proved to be as efficient as the synthetic progestin R5020. Moreover, the immunocytochemical study of E2 receptors indicated that E2 increases its own receptor level whereas P and R5020 have the opposite effect, thus limiting the stimulatory effect of E2 on cell growth. In the HBE cell culture system and in long-term treatment, P and R5020 appear predominantly to inhibit cell growth, both in the presence and absence of E2. © 2000 Elsevier Science Ltd. All rights reserved.

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## 1. Introduction

It is unanimously accepted that estradiol (E2) stimulates breast cell multiplication; as a consequence, E2 may increase the risk of errors at the time of cell replication and act as a “promoter” of breast carcinogenesis. In contrast, the role of progesterone (P) and its derivatives remains debated. An antimitotic effect of P and progestins has been observed in animal models [1,2], breast cancer cell lines [3–6] and also normal breast tissue and cultured cells [7–12]. However, some authors have suggested that P might have a mitogenic

effect during the menstrual cycle [13,14]. Norsteroid progestins have been shown to stimulate cell growth in breast cancer cell line artificial models [15,16] and progestin administration has been accused of increasing the risk of breast cancer [17,18]. Considering these discrepancies, any new data concerning the action of P and its derivatives would be welcome. Indeed, it is important to have a clear definition of the normal hormonal balance required for growth and differentiation of the human breast tissue and to determine the pathophysiological function of each hormone, so as to establish the most appropriate therapeutic and possibly preventive use. This information would be the basis for choosing the right estrogen/progestogen balance in contraceptive pills, hormone replacement therapy of menopause, and to develop strategies for preventing breast cancer.

We have previously observed that synthetic proges-

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tins have an antiestrogen effect on normal human breast epithelial (HBE) cells in culture, routinely obtained from surgical reductive mammoplasty samples [19]. Medroxyprogesterone acetate (MPA) and promegestone (R5020) reduce the oxydative ( $E2 \rightarrow E1$ )  $17\beta$ -hydroxysteroid dehydrogenase enzyme activity [9,19] and ER immunostaining [20].

The purpose of this paper was to determine the role of P on HBE cell multiplication and ultrastructural appearance studied in scanning electron microscopy (SEM). P regulation of the E2 receptor (ER) was also studied. The results indicate that P controls the mitogenic effect of E2, as shown by the reduced HBE cell growth in the presence of P, and also the quiescent appearance of the cells in SEM. This effect may be a consequence of the lower ER level after P treatment. The addition of P alone did not induce any proliferative effect.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Ham F10 without phenol red, Hank's balanced salt solution (HBSS) and trypsin were obtained from Life Technologies (Cergy-Pontoise, France). Human serum was provided by the Centre National de Transfusion Sanguine (Les Ulis, France). Collagenase and epidermal growth factor (EGF) were purchased from Boehringer Mannheim Biochemicals (Meylan, France). Diaminobenzidine in tablets, hyaluronidase, cholera toxin, transferrin, bovine pancreas crystalline, insulin, triiodo-L-thyronine (T3), cortisol (F), estradiol (E2) and progesterone (P) from Sigma (St. Louis, MO). Promegestone (R5020) and  $[6\text{-}^3\text{H}]$  thymidine (S.A. 15 Ci/mmol) were purchased from New England Nuclear Corporation (Dupont de Nemours, Paris, France). Human estradiol receptor (ER) antibody H222spy were generously provided by G.L. Greene (Chicago II). Goat anti-rat immunoglobulin (IgG) and monoclonal rat peroxidase–antiperoxidase (PAP) complex were obtained from Jackson ImmunoResearch Laboratory (Immunotech, Marseille, France).

### 2.2. Tissue collection

Specimens of normal human breast tissue were obtained from women (aged 15–27 years) who underwent reduction mammoplasty. These patients had no history of benign breast disease, and in all patients pathological study of the tissue revealed only normal breast tissue.

### 2.3. Enzymatic digestion

The breast tissue enzymatic digestion procedure has been previously described [19]. Briefly, the tissue was enzymatically digested with collagenase (0.15%) and hyaluronidase (0.05%) in Ham F10 added with serum, and then filtered consecutively through 500-, 300- and 150- $\mu\text{m}$  sieves in order to retain any indigested tissue. Cell material retained on a final 60- $\mu\text{m}$  sieve was used for epithelial cell culture.

### 2.4. Culture procedure

The cells were pelleted, distributed into T25 plastic flasks, and maintained at 37°C in a humidified atmosphere with 5%  $\text{CO}_2$ . The basal culture medium consisted of Ham F10 without phenol red, containing  $\text{NaHCO}_3$  (0.24%), kanamycin (1 mg/ml), F (5 ng/ml), T3 (6.5 ng/ml), cholera toxin (10 ng/ml), transferrin (5  $\mu\text{g}/\text{ml}$ ), insulin (0.12 U/ml), EGF (10 ng/ml) and 5% compatible human serum.

In case of accidental contamination of epithelial cell cultures by fibroblasts, a brief washing with trypsin (0.25%, 1 ml) was used to eliminate the fibroblasts, which are more rapidly detached from the surface of the plastic flask than epithelial cells.

### 2.5. Steroid treatments

In order to study the effect of P on cell growth and compare it with the effect of R5020, after 6–8 days of primary culture, cells were replated at  $2 \times 10^5$  cells/T25 flasks. After a 24-h attachment and equilibration, steroids were added: E2 ( $10^{-8}$  M)  $\pm$  P ( $10^{-8}$ – $10^{-6}$  M) or  $\pm$  R5020 ( $10^{-8}$ – $10^{-6}$  M) for 3–7 days. All the steroids were added in ethanol at a concentration never exceeding 0.1%. Because of its rapid metabolism in cultured HBE cells, P was added twice a day. All the media were changed every other day.

### 2.6. Cell growth study

#### 2.6.1. Histometry

Cell growth was studied by counting cells daily by a manual histometric method, using the eyepiece of a microscope equipped with a micrometric 19-nm square grid as reported previously [9,21]. The lattice of the grid forms points at the intersections of the perpendicular lines. The points of intersections coinciding with epithelial cells are counted. Counting is carried out on seven different areas of each T25 flask covering a total of 247 points. Every day, points falling on cells are counted, thus providing an histometric cell growth index (HGI).

### 2.6.2. [ $^3$ H]-thymidine incorporation

Cells in exponential growth phase were treated with steroids for 3 days and [ $^3$ H]-thymidine (1  $\mu$ l/ml) was added for the last 3 h. The cells were, then, washed three times with buffer (100 mM NaCl, 10 mM EDTA and 10 mM Tris pH 7.0) and harvested. After 15 min centrifugation at 400  $\times$ g, the pellet was resuspended in 1 ml buffer and sonicated (four times, 5 s). Radioactivity was counted on 500  $\mu$ l of the suspension with 10 ml of Picofluor (Packard) in a Tricarb 300C spectrometer. The other 500  $\mu$ l were used for DNA assay.

### 2.7. DNA determination

DNA was assayed by the fluorimetric method of Brunk et al. [22] using calf thymus DNA as standard.

### 2.8. ER study by immunocytochemistry

Effects of E2 and P on ER were studied using immunocytochemistry as already described [20]. In brief, the cells were harvested and cytospun. The slides were fixed with 3.7% formaldehyde at room temperature, followed by methanol and acetone at  $-20^{\circ}\text{C}$ ; after washing in phosphate buffer saline (PBS), they were successively incubated with rat monoclonal anti-ER antibodies, a bridging goat anti-rat IgG antibody and, finally, with rat-PAP. Staining was revealed with diaminobenzidine and cells were counterstained with light green. The number of positive cells was evaluated. A staining intensity scale was established: strong (+++ or ++++), moderate (++) , weak (+). Intra-assay variations were less than 10%. Inter-assay variation did not exceed 15%.

### 2.9. SEM

Cells were grown on plastic dishes. After 7 days of steroid treatment, cells were washed with PBS and small circles (about 5 mm diameter) were cut up from the plastic surface of the petri dishes. Cells were then fixed with glutaraldehyde (2.5%) overnight at  $4^{\circ}\text{C}$ , and dehydrated with alcohol before critical point drying. The samples were coated with gold and examined with a JEOL electron microscope.

### 2.10. Data analysis

The influence of the various hormone culture conditions on parameters of cell multiplication (HGI,  $^3\text{H}$ -thy) and the percentage of ER immunostained cells were compared within the series of cultures established using cells from the same patient. Each measurement was carried out on parallel triplicate flasks or slides, and the results were expressed as the mean  $\pm$  SD.

## 3. Results

### 3.1. Growth study by histometry: dose-dependent inhibitory effect of P on cell growth

*In the absence of E2* (Fig. 1, left). When increasing concentrations of P ( $10^{-8}$ – $10^{-6}$  M) were added to the culture medium of the HBE cells, a dose-dependent inhibitory effect was observed on HBE cell growth. Inhibition became apparent at  $10^{-8}$  M, and was strongest at  $10^{-6}$  M. When the effects of P and R5020 were compared at the highest concentration studied ( $10^{-6}$  M), the inhibitory effect of P was of the same order of magnitude as that observed with R5020.

*In the presence of E2* (Fig. 1, right). When increasing concentrations of P were added to E2 ( $10^{-8}$  M), P was also observed to dose-dependently inhibit HBE cell growth, albeit to a lesser extent than in the absence of E2.

### 3.2. Comparison of the inhibitory effects of P and R5020 on [ $^3$ H]-thymidine incorporation in the cells

A 3-day treatment with E2 ( $10^{-8}$  M) led to an approximately 30% increase in [ $^3$ H]-thymidine incorporation comparatively to the control cells (Fig. 2).

The addition of increasing concentrations ( $10^{-8}$ – $10^{-6}$  M) of P or R5020 to E2, dose-dependently reduced the E2-induced incorporation of [ $^3$ H]-thymidine with a maximum effect at  $10^{-6}$  M. Inhibition was equivalent whether R5020 or P was used (Fig. 2).

In the absence of E2, P also dose-dependently reduced [ $^3$ H]-thymidine incorporation (data not shown).

### 3.3. ER immunocytochemical study: effects of E2 and P alone or in combination

An immunocytochemical assay of ER was performed on human breast epithelial cells, treated for 3

Table 1  
Variation in ER immunostaining in HBE cells in culture according to steroid treatments<sup>a</sup>

ER immunostaining	Positive cells (%)	Intensity of staining
Control	47 $\pm$ 3	+++
E2	59 $\pm$ 4	++++
P	39 $\pm$ 3	+
E2 + P	50 $\pm$ 3	++

<sup>a</sup> HBE cells were cultured for 8 days (1) in the absence of steroids: control cells, or (2) in the presence of E2 ( $10^{-8}$  M), or (3) E2 ( $10^{-8}$  M) + P ( $10^{-7}$  M), or (4) P ( $10^{-7}$  M) alone.

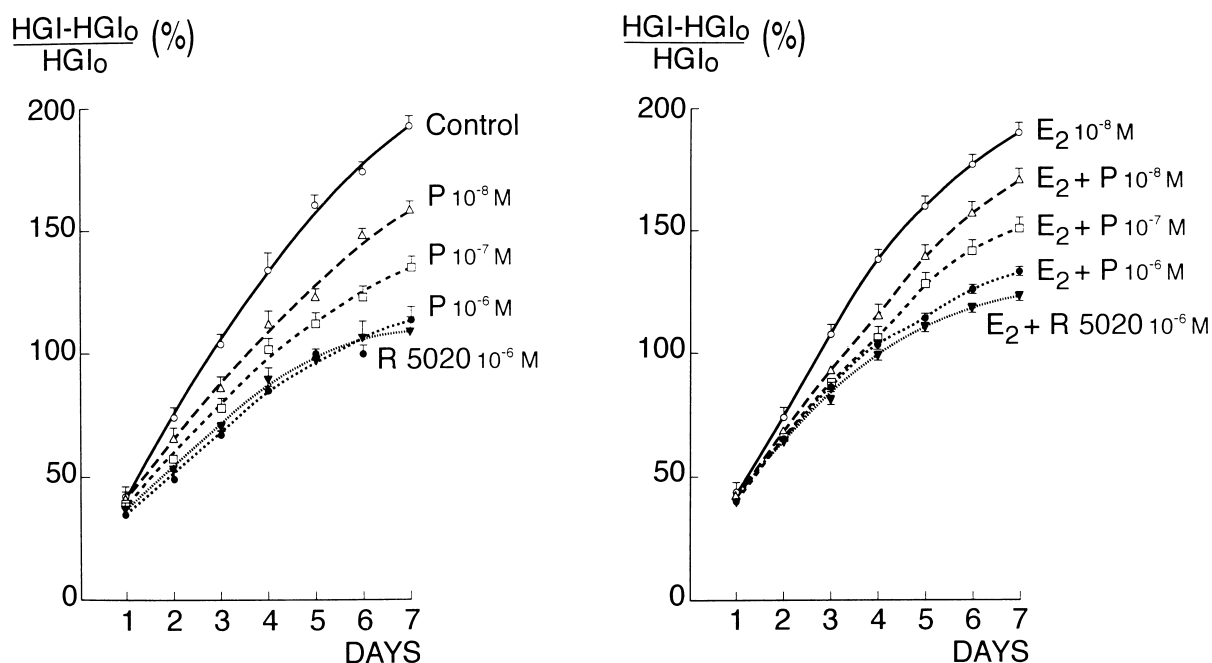


Fig. 1. The effects of estradiol (E2), progesterone (P) and the progestin promegestone (R5020) on HBE cell growth in culture. HBE cells grown to confluency in primary cultures were plated at a density of  $5 \times 10^5$  cells per T25 flask. Twenty-four hours later (day 0), P or R5020 was added either alone or combined with E2 at the indicated concentrations. The study of cell growth was based on the daily determination of the HGI (see Materials and methods) and the results expressed as percentage of increase in HGI as compared to its value on day 0 (HGI<sub>0</sub>). Left panel: no progestin (control), P ( $10^{-8}$ – $10^{-6}$  M), R5020 ( $10^{-6}$  M). Right panel: same culture and hormone conditions, but with  $10^{-8}$  M E2.

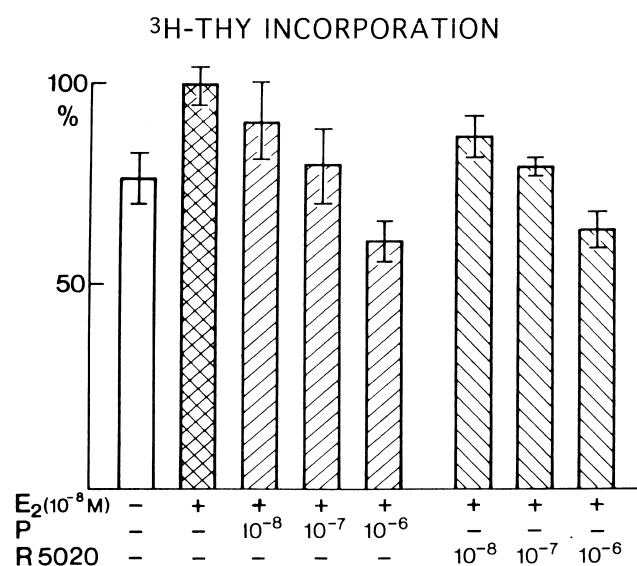


Fig. 2. The effects of estradiol (E2), progesterone (P) and the progestin promegestone (R5020) on [ $^3$ H]-thymidine ( $^3$ H-thy) incorporation in HBE cells in culture. Cells in exponential growth phase were treated with steroids:  $\pm$ E2, alone or combined with P or R5020 at the concentrations indicated, for 3 days and [ $^3$ H]-thymidine added for the last 3 h. Cells were then harvested, sonicated, [ $^3$ H]-thy counted, and DNA assayed. Results express cell incorporation of [ $^3$ H]-thymidine in each hormonal condition, compared with maximum incorporation obtained with E2 ( $10^{-8}$  M) alone, taken as the reference, i.e., 100%.

days with E2 ( $10^{-8}$  M), or P ( $10^{-7}$  M), or a combination of the two.

Results are presented on Fig. 3 and in Table 1. Nuclear staining was observed under each hormonal condition. The percentage of positive cells and the intensity of staining varied depending on the type of hormonal treatment. The percentage of ER<sup>+</sup> cells was greater and the intensity of staining was higher in E2-treated cells than in control cells (Fig. 3(a) and (c)).

When P was added to E2 (Fig. 3(d)), the percentage of ER<sup>+</sup> cells as well as the intensity of staining were lower, similar to the results observed in untreated cells. When P was added alone (data not shown), staining intensity and percentage of ER<sup>+</sup> cells were even lower than in control cells.

#### 3.4. Scanning electron microscopic study: effect of E2 $\pm$ P

Control cells cultured in the basal medium without the addition of any hormone were polygonal and flattened, with extending filaments (Fig. 4(a)). At higher magnification, microvilli were scarce (Fig. 4(b)).

After 8 days of E2 treatment, the cells lost their flattened appearance and become heterogeneous, with small round cells protruding from the lining sheet (Fig. 5(a)). At higher magnification, a marked increase in the number and density of microvilli was observed compared to the surface of control cells (Fig. 5(b)).

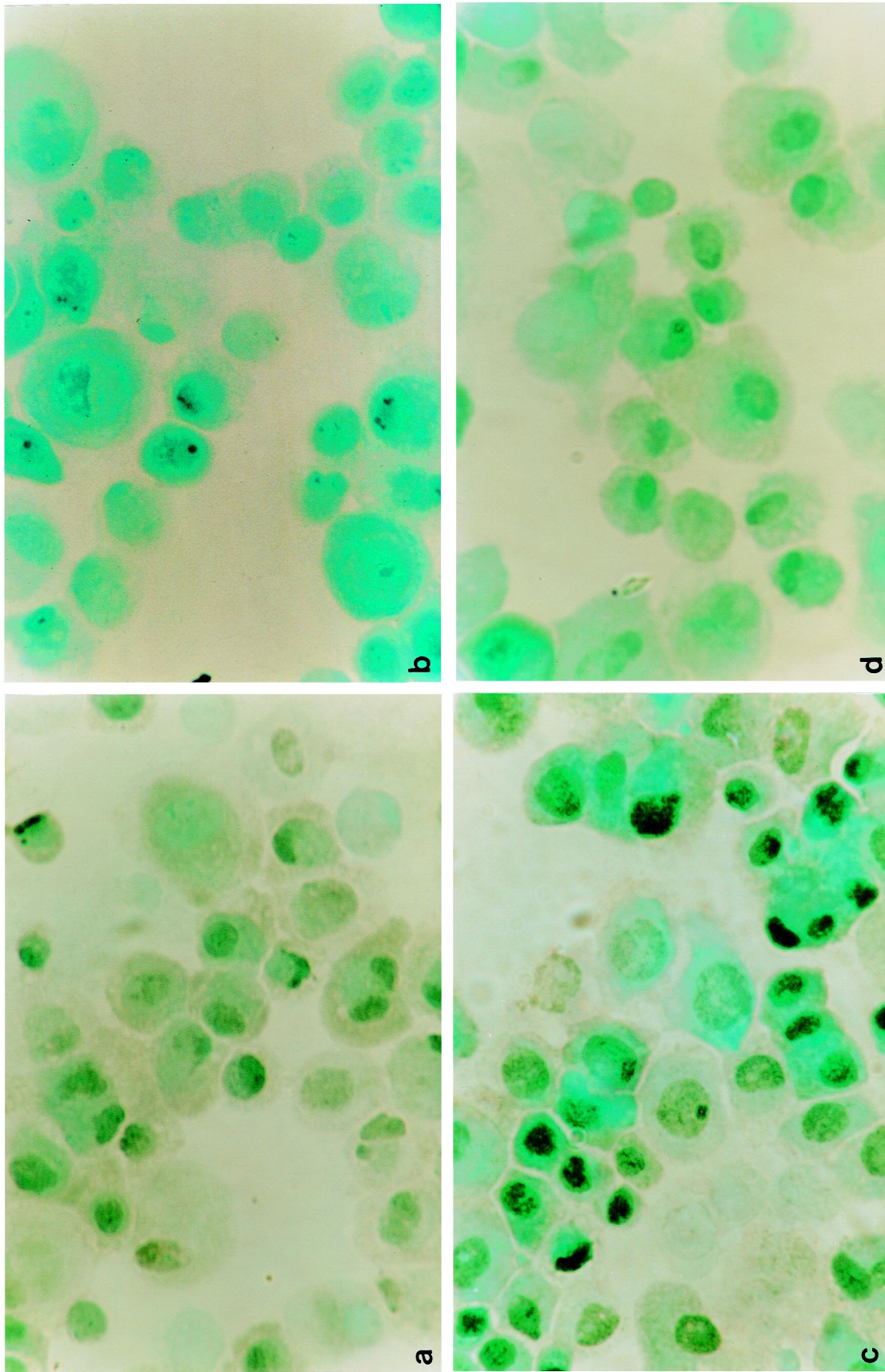


Fig. 3. Stimulatory effect of estradiol (E2) and inhibitory effect of progesterone (P), on estradiol receptor (ER) immunostaining in normal HBE cells. HBE cells were cultured for 8 days: (a) in the absence of steroids, (c) in the presence of E2 ( $10^{-8}$  M), or (d) E2 ( $10^{-8}$  M) + P ( $10^{-7}$  M). The cells were cytospun and immunocytochemical study of ER was carried out using the monoclonal anti-ER antibody H222 Spγ. (b) negative control: the primary antibody was replaced by normal IgG. Counterstaining: light green. Magnification, 400 $\times$ .



When P was added to E2, the cells resumed their flattened appearance (Fig. 6(a)) and the number of microvilli, which was high in the presence of E2 alone, markedly decreased (Fig. 6(b)). When the cells were treated with P alone, most were polygonal and stretched out, and microvilli were very scarce. Cells treated with R5020 ( $10^{-7}$  M) with or without E2, showed a quiescent appearance similar to that observed with P (data not shown).

#### 4. Discussion

The antimitotic and therefore protective effect of P and progestins has been inferred from experimental animal studies, showing that P prevents or decreases the occurrence of carcinogen- and estrogen-induced mammary tumors [23–26].

Most hormone-dependence studies of the human breast have been carried out on cancer cell lines. Using

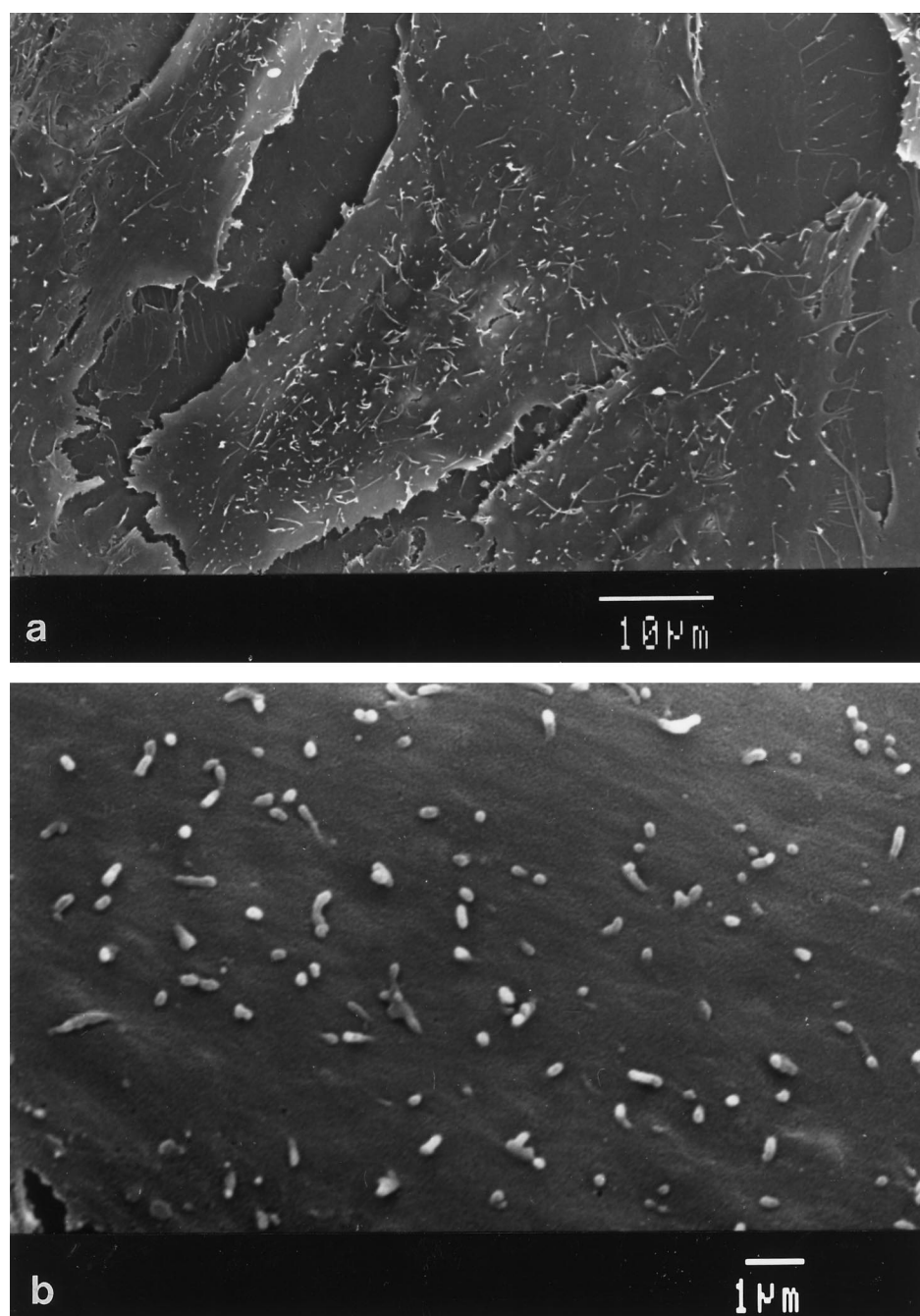


Fig. 4. HBE cells cultured in basal medium. SEM analysis: (a) control cells are polygonal and flattened (1500 $\times$ ), (b) scarce and short microvilli are observed on the cell surface (7500 $\times$ ).

such models, Vignon et al. [5] and Horwitz et al. [6] have shown that the progestin R5020 clearly inhibits the growth of estrogen-dependent T47D cells.

Studies have also been performed on the normal human breast in vivo, using breast biopsies obtained at various times of the menstrual cycle. Vogel et al. [7] observed numerous mitoses of the duct epithelium during the follicular phase, suggesting proliferative activity. In contrast, they observed very few mitoses

during the luteal phase, and considered it to be a more quiescent phase. In normal breast in vitro, Welsch et al. [27] have shown, using explants from normal or adenomatous breast tissue, that P inhibited the [ $^3\text{H}$ ]-thymidine incorporation induced by E2. Longman and Buehring [10] showed that P or progestins, when added alone, did not stimulate cell growth in explants of normal mammary tissue. When added to ethinyl-estradiol, which had the greatest stimulatory effect,

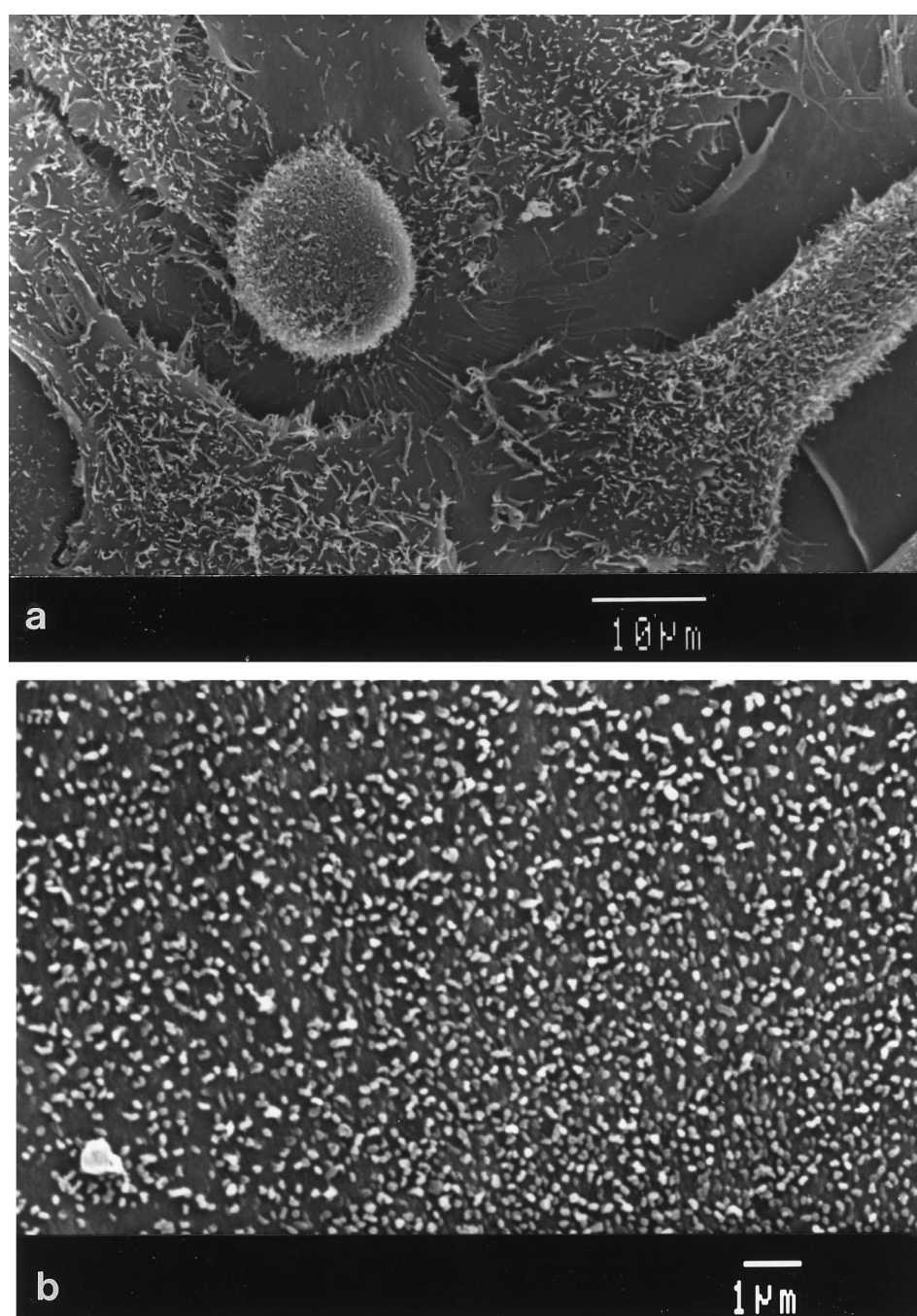


Fig. 5. HBE cells cultured in medium supplemented with E2 ( $10^{-8}$  M) for 8 days SEM analysis: (a) small round cells protrude from the lining sheet (1500 $\times$ ), (b) numerous and short microvilli cover the surface of the cells (7500 $\times$ ).

progestins slowed down cell growth. In our laboratory, we have previously described the inhibitory effect of the progestin R5020 on the growth of normal human breast epithelial cells in culture, whether or not treated by E2 [8,9,28].

However, the antimitotic effect of P has been disputed by some authors. Ferguson and Anderson [13] claimed to have observed the greatest number of mitoses in the epithelial cells from normal breast tissue obtained on days 24–25 of the cycle in women undergoing surgery for benign breast disease, and concluded

that progesterone could have a mitogenic effect. In a further study of patients taking oral contraceptives, Anderson et al. [29] observed a correlation between the number of mitoses in breast tissue and the estrogen potency of the contraceptive (low, medium or high), but no correlation with the progestogen content. Potten et al. [30] observed the highest rate of mitoses on day 21 of the cycle, which is too early in the luteal phase to be attributed to the cumulative effect of secreted P. Rather, high rate of mitoses probably reflects the cumulative effect of E2 since the beginning of the

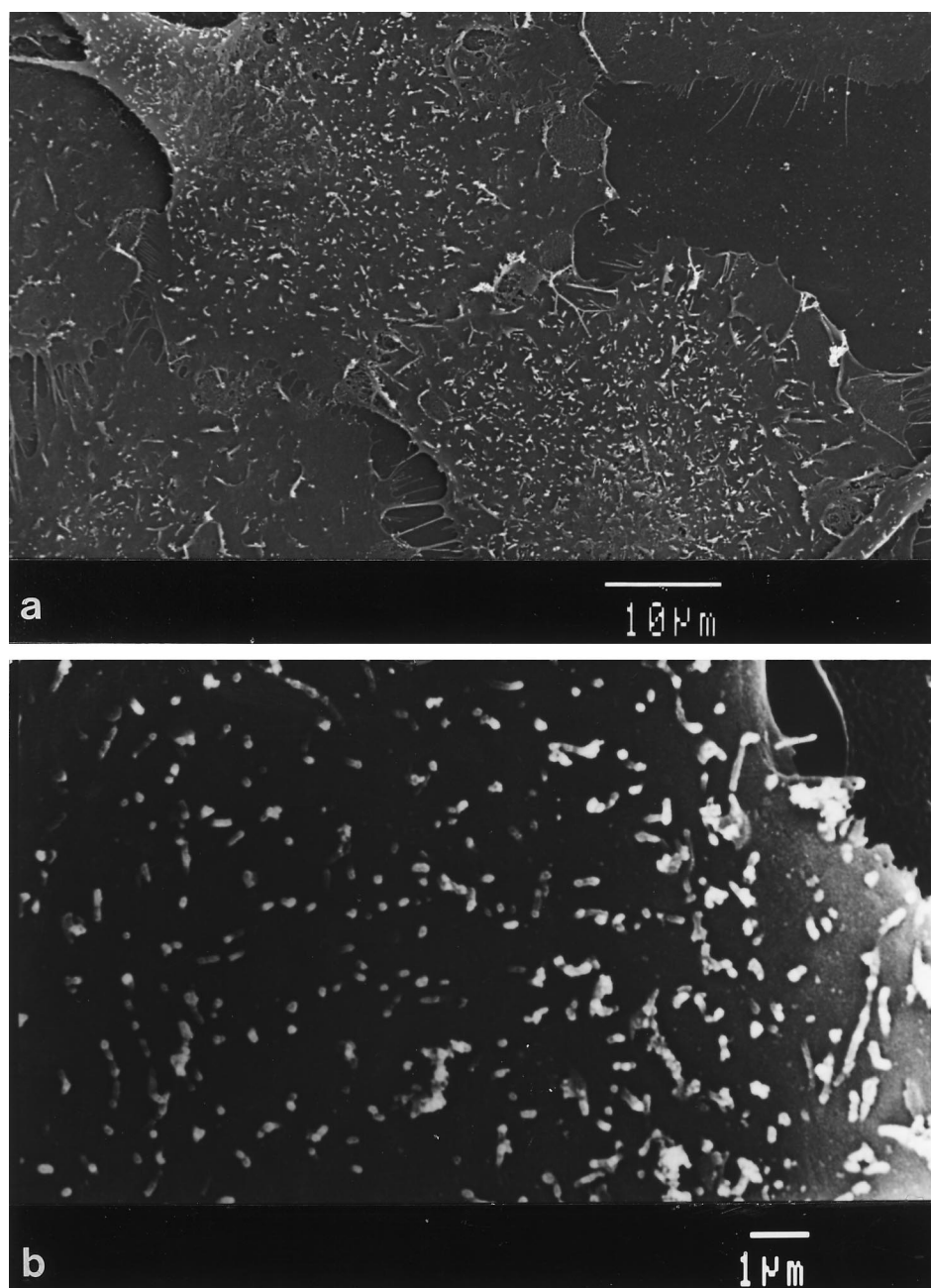


Fig. 6. HBE cells cultured in medium supplemented with P  $\pm$  E2 for 8 days. SEM analysis. After E2 ( $10^{-8}$  M) + P ( $10^{-7}$  M) treatment: (a) cells were homogeneously flat, without any round cells (1500 $\times$ ), (b) far fewer microvilli (7500 $\times$ ) were observed than in the presence of E2 alone.



cycle. In an article published in 1983, Pike et al. [17] suggested that oral contraceptives containing the highest doses of progestogen increased the risk of breast cancer. However, the Swyer test used to evaluate the progestogen potency of these pills has been criticised as non-specific. In particular, the pills that he claimed to contain the highest progestogen content were, in fact, the highest in estrogen. The most recent and controversial article is by Bergkvist et al. [18], who evaluated the breast cancer risk in post-menopausal women receiving estrogen replacement therapy. Whereas the global risk was 1.1, the authors found a relative risk of 4.4 when a progestogen was combined with estrogen in the treatment. However, this evaluation was based on only 10 patients. The group of Jordan [15,16] also concluded that norsteroid progestins had proliferative effect, however, the study had been carried out in an artificial model of transfected mammary cancer MCF-7 cells. A detailed epidemiological review examining the questionable relationship between progestin exposure in contraceptives and hormone replacement therapies, and the breast cancer risk failed to find evidence of an association between progestins and breast cancer [31,32].

To provide more information concerning the effect of P in the normal human breast, we have developed a culture system of separated normal breast epithelial and stromal cells. These cells maintain their hormone dependence in primary and even secondary culture [8,9,19,20] and constitute an irreplaceable tool for the study of normal cell hormone dependence. The effect of P was studied on epithelial cells and compared to the effect of the progestin R5020. However, since P is extensively metabolised in culture, we overcome the problem by adding P twice a day in the culture medium.

The effect of progesterone on cell growth was studied over 7 days using a histometric method providing a growth index, as previously described [9]. In addition, [ $^3\text{H}$ ]-thymidine incorporation into the cells was assayed during the exponential growth phase of the secondary culture, i.e. on day 3. The ultrastructural appearance of the cells, studied by SEM, provided another approach to evaluate the proliferative or quiescent state of the culture. In parallel, an immunocytochemical study of ER was performed to determine whether the ER regulation is involved in P action.

Growth of the HBE cells was inhibited in a dose-dependent manner by the addition of P. However, as previously observed with R5020 [9], P was more effective in inhibiting cell growth, when given alone, than in the presence of E2, suggesting that the proliferative effect of E2 predominates over a presumed priming effect of estrogen on progesterone receptor levels [3]. The inhibitory effect of P and R5020 seemed to be

quite similar. The [ $^3\text{H}$ ]-thymidine incorporation assay confirmed these results.

Ultrastructural characteristics of normal human breast epithelial cells examined by SEM indicated that, in basal culture medium, cells showed low proliferative activity and appeared essentially quiescent. The alteration of cell shape and surface observed after the addition of  $\text{E2} \pm \text{P}$  or  $\pm \text{R5020}$  confirmed the hormone-dependence of HBE cells in culture: the addition of E2 led to an abundance of round cells which exhibited dense microvilli, strongly suggesting the emergence of a population of actively dividing cells. In contrast, when P or the progestin R5020 was added to E2, striking differences were observed: the emergence of proliferating cells were inhibited, cells were flattened as in control cultures and the number of microvilli was reduced. These changes were more pronounced in the absence of E2, in agreement with our observations on cell growth.

When ER was studied by immunocytochemistry, a hormonal regulation of nuclear ER immunostaining was observed in HBE cells: E2 increased and P decreased ER staining. By stimulating ER in normal HBE cells, E2 tends to amplify its own action, whereas P, by decreasing ER level, tends to limit E2 action. This decrease of E2 receptor level is one of the mechanisms by which P controls the stimulatory effect of E2 on cell growth.

Despite the contradictory results published concerning the effect of P or progestins on cell growth [5,6,9–15,17,18,20,27,28,30,33], it appears from our study that in this culture system of normal human breast epithelial cells, E2 stimulates cell growth whereas P and progestins inhibit it, in both the presence and absence of E2.

Some stimulatory effect observed with P or progestins could be explained by a primary transient effect on the cell cycle, as recently described by Musgrove et al. [34], who have shown in T47D that the progestin medroxyprogesterone acetate (MPA) slows cell growth. However, with a biphasic effect, it causes a transient acceleration of the cell cycles that have already started, then blocks cells in the G0/G1 phase and prevents them from entering further cycles [35]. More recently, Groshong et al. [36] observed the same biphasic effect of P and R5020 on T47D-YB cancer cell growth with a final G1 phase arrest.

Indeed, P is implicated in end-bud differentiation into acini and it can be postulated that a stimulatory action of P on the acinar cells could be a prerequisite for its action on cell differentiation. Nevertheless, all these stimulatory actions of P constitute short-term proliferative effects, the long term action of P or progestins being mainly antiproliferative. Further studies are now necessary to indicate whether, like progestins in breast cancer cells, P exerts a biphasic effect on nor-

mal breast epithelial cells, with a transient acceleration of the cell cycles already started.

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