Effect of Estradiol on the Ultrastructure of the MCF7 Human Breast Cancer Cells in Culture¹

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ABSTRACT

We have analyzed the effect of estradiol and of two classes of antiestrogens on the morphology of the MCF7 human breast cancer cell line by scanning and transmission electron microscopy. Estradiol progressively increased the number and the length of microvilli at the cell surface. The density of the microvilli network increased between 2 and 11 days of estrogen treatment, while the cells became more globular and less tightly attached to the surface of the dish.

Estradiol also progressively transformed cells into secretory cells containing, at Day 2, large, clear mitochondria and, at Day 4, rough endoplasmic reticulum and Golgi complexes. At Day 6, secretory granules (diameter, 0.2 μm), which mainly contained glycoproteins, were first observed in the cytoplasm. By Day 8, they were concentrated at the cell membrane and being liberated into the medium. Larger granules (diameter, 0.8 μm), which probably contained lipids, were observed later (Day 11). Cell cultures in 10% fetal calf serum not treated by charcoal contained secretory granules.

The modifications were induced by physiological concentrations of estradiol but not 5α-dihydrotestosterone. Progesterone (10 nm for 8 days) completely inhibited the effect of estradiol on the microvilli and secretory activity. Tamoxifen or hydroxytamoxifen did not induce secretory activity but did alter the cell morphology compared to control cells. The effects of estradiol were observed in other estrogen receptor-positive breast cancer cell lines (ZR 75-1, T 47 D) but not in an estrogen receptor-negative cell line (BT 20).

This morphological evidence that estrogens modify the cell surface of breast cancer cells in culture and transform them into "secretory cells" complements evidence that they induce the release of a glycoprotein with a molecular weight of ≅50,000 into the culture medium (Cell, 20: 352-362, 1980). (The molecular weight was found first to be 46,000. It seems to be closer to 52,000 in a 10% polyacrylamide gel and by using the NEN-labeled proteins as molecular weight markers.)

INTRODUCTION

The mechanism by which estrogens stimulate the growth of breast cancer cells is still unknown (14) even though human breast cancer cell lines are now available to investigate in vitro the direct effect of estrogen on cell proliferation and differentiation.

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The MCF7, estrogen-responsive human breast cancer cell line (25), which was established from a pleural effusion, has been studied extensively. These cells have some biochemical markers, such as steroid hormone receptors (5), which indicate that they have retained a certain differentiated state. The stimulation of their proliferation by estrogens remains controversial (18, 24), even though it is accepted that their growth is specifically inhibited by antiestrogens (9, 17). In contrast, the induction by estrogen of specific proteins, such as the progesterone receptor (13), plasminogen activator (6), and other nonidentified proteins (8, 29), is now well documented.

We have shown previously that 17β-estradiol specifically induced a glycoprotein with a molecular weight of ≅50,000 which is released into the extracellular medium (29). This suggests that it could be a secreted protein; however, the specific shedding of a membrane protein has not been excluded. Therefore, using electron microscopy, we examined whether, under similar conditions, estrogens were able to directly modify the surface of MCF7 cells and/or their general secretory machinery.

We also tested the effect of both synthetic (TAM³) and natural (progesterone) antiestrogens because the mechanism by which they prevent the growth of some human breast cancers is not clearly defined (17, 26). More precisely, our purpose was to ascertain (a) whether TAM which inhibits MCF7 cell growth alters the morphology of the cells and (b) whether progesterone has a direct antiestrogenic activity on breast cancer cells in vitro (26).

MATERIALS AND METHODS

Materials. All steroids (99% pure) were donated by the Roussel-Uclaf Research Center, Romainville, France. Ovine prolactin (NIH-PS-12) was obtained from the Endocrinology Study Section, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Md. TAM and 4-hydroxytamoxifen were a gift from ICI-England.

All culture media were from Flow Laboratories, Inc. FCS was obtained through Seromed-France.

Cell culture. MCF7 and ZR 75-1 cells, donated by Dr. M. Lippman, are cultured routinely in Dulbecco’s modified Eagle’s medium containing 10% FCS, bovine insulin (0.6 μg/ml), and penicillin/streptomycin (25 IU/25 μg/ml) at 37° in a humidified atmosphere with 5% CO₂. BT 20 cells which were obtained through the Mason Research Institute and T 47 D cells which were donated by Dr. I. Keydar (Tel Aviv, Israel) were maintained in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% FCS, bovine insulin (0.6 μg/ml), and antibiotics.

Hormone Treatment. Before hormone treatment, the MCF7 cells were withdrawn from estrogens by culture for 4 days in the above

³ The abbreviations used are: TAM, tamoxifen; FCS, fetal calf serum; DCC, dextran-coated charcoal; TEM, transmission electron microscopy; SEM, scanning electron microscopy.
medium containing 10% DCC-treated FCS, which was prepared as described (28). The concentration of free estrogen in the DCC-treated FCS was shown by radioimmunooassay to be less than 5 pg/ml. The MCF7 cells, at passages 102 to 148, were then plated in T-25 flasks (TEM) or on glass coverslips (SEM) at cell densities which ensured that they reached near cell confluency at the end of each hormone treatment.

Cells maintained in medium containing 10% DCC-treated FCS and no added hormone are referred to as control cells. The steroid hormones (estradiol, diethylstilbestrol, 5a-dihydrotestosterone, or dexamethasone) were tested routinely at a final concentration of 10 nm for 8 days in a 10% DCC-treated FCS medium, and the medium was changed every 2 days. The synthetic antiestrogens TAM and its metabolite 4-hydroxytamoxifen were tested at 1 and 0.1 μM, respectively. Steroids and drugs were added in ethanol solution so that the final concentration of ethanol was <0.1%. For experiments with estradiol, several concentrations and lengths of treatment were tested as indicated in the results. The cells grown on coverslips were fixed directly for TEM. The cells grown in T-25 flasks were trypsinized and prepared for TEM.

**TEM.** The cell pellet was fixed with glutaraldehyde (1.5%) for 2 hr and then with osmium tetroxide (1%) for 1 hr in phosphate buffer at pH 7.3; it was then embedded in araldite or Epon. The sections (Reichert ultramicrotome) were stained according to the method of Reynolds (22). The Thiéry histochemical reaction was used to stain the glycoproteins in granules (27). The grids were examined with a Philips EM 301 microscope at 80 or 60 kV.

**SEM.** The cells grown on coverslips were fixed with glutaraldehyde (1%) for 45 min and then with osmium tetroxide (1%) for 1 hr in phosphate buffer at pH 7.3. The coverslips were rinsed in phosphate buffer and dehydrated through a graded series of alcohol and isooamy acetate before critical point drying. The samples were mounted and shadowed with gold; they were then examined and photographed in a JEOl Model JSM-35 scanning electron microscope at 25 kV.

### RESULTS

We have compared the cell surface (by SEM) and the ultrastructure (by TEM) of MCF7 cells grown with or without 10 nm estradiol as described in "Materials and Methods."

**Effect of Estradiol on the Cell Surface (SEM).** The surface of control cells (DCC-treated FCS) observed by SEM was smooth with short and rare microvilli (0.1 to 0.2 μm in diameter) and infrequent blebs (Fig. 1a). The cells were firmly attached to the glass coverslips as also visualized by light microscopy, from their degree of spreading. In the estradiol-treated cells, an increase in the number and length of microvilli was noticed as early as 24 hr after treatment. After 2 days of treatment (Fig. 1b), the cells were uniformly covered with microvilli. By 8 days (Fig. 1c), the cell surface was covered with a dense network of long microvilli (Fig. 1c, inset) and with numerous blebs of different sizes. Smaller particles (0.4 to 0.8 μm in diameter) were concentrated in large bunches. After 8 days, the microvillous network did not change further, but larger granules (2 to 8 μm in diameter) were seen at the cell surface. In addition, phase-contrast light microscopy indicated that the cells became more rounded and less attached to the dish.

For cells maintained in 10% untreated FCS, which contains endogenous steroids, the density of the surface microvilli was less than in the estradiol-treated cells though significantly greater than in the withdrawn control cells (not shown). A different increase in microvilli was observed 8 days after estradiol (10 nM) treatment with 2 other estrogen receptor-positive human breast cancer cell lines, ZR 75-1 (10) and T47D (15), but there was no increase with an estrogen receptor-negative human breast cancer cell line, BT20 (not shown).

**Effect of Estradiol on Secretion (TEM).** Cells cultured in 10% untreated FCS had a high metabolic activity as visualized by TEM (Fig. 2a). Their mitochondria were large and clear with thin cristae. Electron-dense granules (0.2 μm in diameter) were seen associated with bundles of microfilaments, indicating secretory activity. When the cells were cultured in a DCC-treated FCS medium for 5 to 15 days (control cells), most of the cells were quiescent with small, dark mitochondria and no Golgi complexes, rough endoplasmic reticulum, or secretory granules. In 30 to 50% of the nuclei, the chromatin was condensed, and the nucleolus was small (Fig. 2b). No marked differences were found between cells withdrawn for 5 and 15 days, which suggest that 5 days of culture in DCC-treated serum is sufficient to remove endogenous hormones.

After estradiol treatment, the cells progressed through 3 periods of activity. (a) From Days 1 to 4, most of the cells showed an intense metabolic activity with larger (×2.5), clearer mitochondria characterized by thin and numerous cristae (Fig. 2c). The nuclei of all the cells contained completely dispersed chromatin and one to 3 large nucleoli which were centrally located (Fig. 2c). (b) From Days 4 to 8, 50% of the cells contained cisternae of rough endoplasmic reticulum associated with numerous ribosomes. Each cell contained one or more Golgi complexes with distended sacculles and vesicles (Fig. 3a). In some cells, electron-dense bodies of 0.2-μm diameter at different stages of maturation (Fig. 3b) were observed close to the Golgi. These features suggested the beginning of a secretory process. (c) After Day 8, while some cytoplasmic organelles decreased in number (Golgi complex, endoplasmic reticulum) or in size (mitochondria), numerous electron-dense granules of 0.2- and 1-μm diameter were seen close to the cell surface (Fig. 2d). Some of them were detaching from the membrane, suggesting an exocytosis process (Fig. 3c). These granules were more numerous than in the 10% FCS-treated cells. The small granules (0.2 μm) consisted of electron-dense cores surrounded by a loose membrane. After extrusion, the cores remained intact but had lost their membranes. Positive histochemical periodic acid-Schiff staining, according to the method of Thiery (27), showed that the matrix of the dense core consisted mainly of glycoprotein (Fig. 3d). The bigger granules (1 μm), which do not have a membrane and probably contain lipids, appeared from Day 8 onwards, and their number and volume increased up to Day 11 (Fig. 2d).

It is possible, but not proven, that the 0.2-μm granules observed by TEM and the 0.4-μm granules visualized by SEM are identical. They both appeared at the cell membrane at the same time, and the apparent difference in granule size could be due to an overestimation because of the gold-shadowing process in SEM.

The epithelial character of the cells, estradiol treated or untreated, was verified by the presence of tight junctions and desmosomes. In no case could we observe any viral or virallike particles in the MCF7 cells.

**Hormone Specificity.** The hormone specificity was tested only on the MCF7 cells. The maximum effect of estradiol on the number of secretory granules per cell and on the number of secretory cells was obtained at physiological concentrations of estradiol (1 to 10 nM). A similar increase in microvilli and secretory granules was obtained after treatment with the syn-
Striated estrogen diethylstilbestrol (10 nM) (not shown). At 1 μM concentration, estradiol altered the structure of the mitochondria and provoked heterogeneous vacuoles in the cytoplasm and nuclear pyknosis, suggesting a toxic effect of the steroid.

We then looked at the morphological effect of the 2 antiestrogenic compounds described in "Materials and Methods." Progesterone alone was inactive at 10 nM concentration. The cell surface (examined by SEM) was regular and flat. The microvilli were rare and always shorter (Fig. 4a) than in estradiol-treated cells (Fig. 4d). No secretory granules were found in the cytoplasm which was characterized by the scarcity of organelles examined by TEM (not shown); however, at the same low concentration, progesterone was able to prevent the effect of estradiol (10 nM). The frequency and length of microvilli (Fig. 4c) and the number of secretory granules (Fig. 5a) were decreased markedly compared to those found in estradiol-treated cells (Figs. 2d and 4d). The effects of TAM and of 4-hydroxytamoxifen, its high-affinity metabolite, were then evaluated, as described in "Materials and Methods," under conditions that decrease cell growth.

Both drugs were unable to induce microvilli at the cell surface, which remained flat and smooth with infrequent blebs (Fig. 4b). In addition, the cells were modified as compared to control cells. Primary lysosomes and autophagic vacuoles of secretory-like material were seen in most cells (Fig. 5b), and heterogenous bodies containing pseudomyelinated figures (Fig. 5b, insert) were present in some of the cells. The majority of the nuclei contained dispersed chromat. Some nuclei, however, were pyknotic and contained clumps of retracted and condensed chromatin.

We have also tested the effect of other hormones on the cell morphology in an attempt to assess the specificity of the effects observed with estrogens. 5α-Dihydrotestosterone did not produce any secretory activity in cells when analyzed by TEM. By contrast, the glucocorticoid dexamethasone increased the frequency of both microvilli and secretory granules, although to a lesser degree than did estradiol. Ovine prolactin (50 ng/ml) and glucocorticoids, but not 5α-dihydrotestosterone, were also able to induce secretory granules. However, the patterns of labeled proteins secreted after estradiol (29), dexamethasone (29), or prolactin treatment are different. Thus, as with cell proliferation which can be stimulated by several hormones or growth factors, the secretory activity of the cells can also be modulated by different, but not all, types of hormones which probably act via their own receptors. These estrogen-stimulated responses are therefore less specific as estrogen-induced proteins observed in the same cells (29).

That progesterone abolishes the effects of estradiol on the cell surface and secretory activity of MCF7 cells indicates that this steroid is an estrogen antagonist which acts directly on the breast cancer cells.

The respective effects of estradiol and progesterone on the secretory activity of these cells were rather unexpected. In normal mammary glands (21), as well as in normal endometrium (2), estradiol is mitogenic on epithelial cells while progesterone stimulates secretion. We show here that, in breast cancer cells, estradiol stimulates secretory activity and that progesterone inhibits the stimulation. The significance of this stimulation of the secretory activity by estradiol in these cells is unknown. By analogy with normal mouse mammary glands, in which the synthesis and secretion of lactose synthetase and casein are stimulated by estradiol in organ cultures, it could reflect an increased differentiation of the epithelial mammary cells (3, 4). However, there is no or very little milk protein synthesis in MCF7 cells (19), which indicates that the estradiol effect observed may be associated with the tumorigenic nature of these cells. The mitogenic effect of estradiol on MCF7 cells in culture (17) has been questioned (24). However, we have observed recently this effect (7) and propose that there may be a close relationship between the stimulation by estradiol of protein secretion and of cell proliferation. Optimal effects were obtained after more than 6 days of continuous exposure of cells to estradiol in the absence of progesterone. These conditions of "unopposed" estrogen stimulation are not found normally in females during the menstrual stimulation but only at puberty and premenopausal stages (16).

The other effect which estradiol had on the morphology of the cells was to increase markedly the number and length of their microvilli. In rat pituitary cells, estradiol has been shown to increase rapidly the length and number of surface microvilli (1) and to stimulate the production of prolactin. In contrast, in hepatoma tumor cells, glucocorticoids decrease both the frequency of microvilli and the production of plasminogen activator (11), although they do induce tyrosine aminotransferase synthesis. Microvilli have been described previously on the cell surface of various mammary glands (12) and on MCF7 cells grown in normal medium on collagen sponge (23), but no attempt was made to define which regulatory factor or hormone is responsible for their presence. It is not known whether or not these modifications of the cell membrane are indicative of increased differentiation (1), transformation (20, 30), or prolif-

* F. Vignon, unpublished observations.
eration of the cells. The effect on the microvilli was accompa-
nied by cell detachment (Fig. 1c). Similar modifications have
been induced by testosterone in androgen-dependent mouse
mammary tumor cells (30).

The effects of estradiol on the cell surface morphology and
secretory activity agree with biochemical evidence that estrad-
oi increases the amount of total proteins labeled by $^{35}$S-
methionine and released into the culture medium 2- to 3-fold
and the amount of a specific and major glycoprotein with a
molecular weight of $\approx 50,000$ 10- to 15-fold (29). However, a
lag was observed between the time at which increased $^{35}$S-
methionine incorporation by the protein with a molecular weight
of $\approx 50,000$ was detected (2 days) and that at which increased
exocytosis of secretory granules could be seen by TEM (8
days). Because estradiol induced drastic changes in the
plasma membrane more rapidly, work is now in progress to
determine whether the estradiol-induced glycoprotein with a
molecular weight of $\approx 50,000$ is secreted by exocytosis or is a
membrane constituent which is shed into the medium in the
presence of estrogen.

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Fig. 1. Effect of estradiol (10 nM) analyzed by SEM. a, control cells grown in DCC-treated FCS for 8 days. Few, short microvilli can be seen on the surface of the flattened cells. b, cells treated with estradiol for 2 days. Note the increase in number and length of microvilli. c, cells treated with estradiol for 8 days. Numerous blebs and microvilli can be seen, and the cells have become more rounded. ——, 1 μm. Inset, high magnification of microvilli. × 20,000.
Fig. 2. Effect of 10% FCS and estradiol, analyzed by TEM. 
a. cells cultured in Dulbecco's modified Eagle's medium with 10% FCS. CH, chromatin; MI, mitochondria large and clear; arrows, secretory granules; and MF, microfilaments. 
b. control cells grown in DCC-treated FCS for 8 days. CH, chromatin; MI, mitochondria small and dark. 
c. cells treated with estradiol for 2 days. DCH, dispersed chromatin; NU, large nucleoli; MI, mitochondria large and clear; and G, Golgi saccules distended. 
d. cells treated with estradiol for 8 days. Clusters of numerous secretory granules 0.2 μm in diameter (small arrow). Large, clear granules 1 μm in diameter (large arrow). 
\( DCH \), dispersed chromatin; bar, 1 μm.

Fig. 3. Secretory processes in cells treated with estradiol (10 nm) for 8 days. 
a. distended saccules of Golgi complexes close to smooth endoplasmic reticulum (SER) and nucleus (N). 
b. distended saccules of Golgi complex with vesicles, secretory granules at different stages of maturation (arrows), and rough endoplasmic reticulum (RER). 
c. extrusion of secretory granules at the plasma membrane level. 
d. Thiéry histochemical glycoprotein reaction (28) on secretory granule. bar, 0.2 μm.
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Fig. 4. Hormone specificity analyzed by SEM after 8 days of hormone treatment (see 'Materials and Methods'). a, progesterone. Occasional short microvilli are visible on flattened cells. b, 4-hydroxytamoxifen. Rare microvilli are seen on flattened cells. c, progesterone plus estradiol. A few microvilli are seen on flattened cells. d, estradiol. Numerous microvilli are seen on rounded cells. The direct antiestrogenic effect of progesterone is evidenced by the striking difference between c and d. bar, 1 μm.

Fig. 5. Effect of antiestrogens analyzed by TEM after 8 days of treatment (same conditions as in Fig. 4). a, progesterone plus estradiol. No secretory granules are seen, and chromatin (CH) is more condensed, compared to Fig. 2d. M, mitochondria. b, 4-hydroxytamoxifen. No secretory granules are visible. V, autophagic vacuoles; LY, lysosomes visible in the cytoplasm close to the nucleus; DCH, dispersed chromatin. Inset, pseudomyelinated autophagic vacuoles. bar, 1 μm. X 50,000.
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