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ARTICLES

Estrogen-Stimulation of Postconfluent Cell Accumulation and Foci Formation of Human MCF-7 Breast Cancer Cells

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Abstract Foci, nodules of cellular overgrowth, that appear after confluence are an in vitro characteristic of malignant transformation. A well-studied in vitro model of estrogen-dependent tumors is the MCF-7 cell line, derived from a pleural metastasis of a human breast adenocarcinoma. We report that cultivation of MCF-7 cells, using routine methods, results in extensive estrogen-stimulated postconfluent cell accumulation characterized by discrete three-dimensional arrays. Side view Nomarski optical sections revealed these to be principally multicellular foci with occasional domes and pseudoacinar vacuoles. This effect on MCF-7 cell growth occurs in media containing fetal bovine serum but not with calf serum or charcoal-dextran-treated fetal bovine serum unless supplemented with estrogens. Foci formation starts 5–6 days after confluence, and the number of foci generated is a function of the concentration of added estrogens. Foci formation is suppressed by the antiestrogens Tamoxifen and LY 156758. Addition of progesterone, testosterone, or dexamethasone had little or no effect, while various estrogens (ethinyl estradiol, diethylstilbestrol, and moxestrol) induced foci development. Clones derived from single cells of the initial MCF-7 population revealed a wide variance in estrogen-induced foci formation, demonstrating heterogeneity of this tumor cell line. The postconfluent cell growth of the estrogen receptor-deficient cell line, MDA-MB-231, contrasted with MCF-7 by developing an extensive multilayer morphology devoid of discrete structures. The tumorigenic potential of the MCF-7 cells used in our experiments was confirmed by their estrogen-dependent growth in immunosuppressed male BDF1 mice.

These data suggest an estrogen receptor-based mechanism for the development of multicellular foci during postconfluent growth of MCF-7 cells. After confluence, foci, in contrast to the quiescent surrounding monolayer, retain proliferating cells. Focus formation, therefore, reflects the heterogeneous responsiveness of these cells to estrogens and should provide a model permitting in vitro comparisons between the progenitor cells of multicellular foci and the monolayer population.

Key words: estrogen dependent, tumor cell heterogeneity, postconfluent growth, antiestrogen

The MCF-7 cell line was isolated from a pleural metastasis of human breast adenocarcinoma and typically forms a continuous cell monolayer at confluence, when grown on plastic substrates [1]. This differs markedly from the histopathology of the pleural exudate source where clusters of cellular overgrowth and pseudoacini were readily apparent [2]. These features of the original isolate were duplicated by growing MCF-7 cells on either collagen-coated cellulose sponges [3] or in artificial capillary networks [4]. In the former study, large multicellular clusters with occasional luminal structures were the dominant morphological motif. In contrast to other studies using hydrophilic plastic substrates, Pourreau-Schneider et al. [5] described cluster and dome formation when MCF-7 cells grew as dense, confluent cultures. In addition to these morphological markers, postconfluent and preconfluent cultures differ in their physiological responses. For example, postconfluent cultures of MCF-7 cells, when compared to preconfluent cultures, are responsive to insulin [6] and estrogen [7]. In agreement with the studies of Pour-

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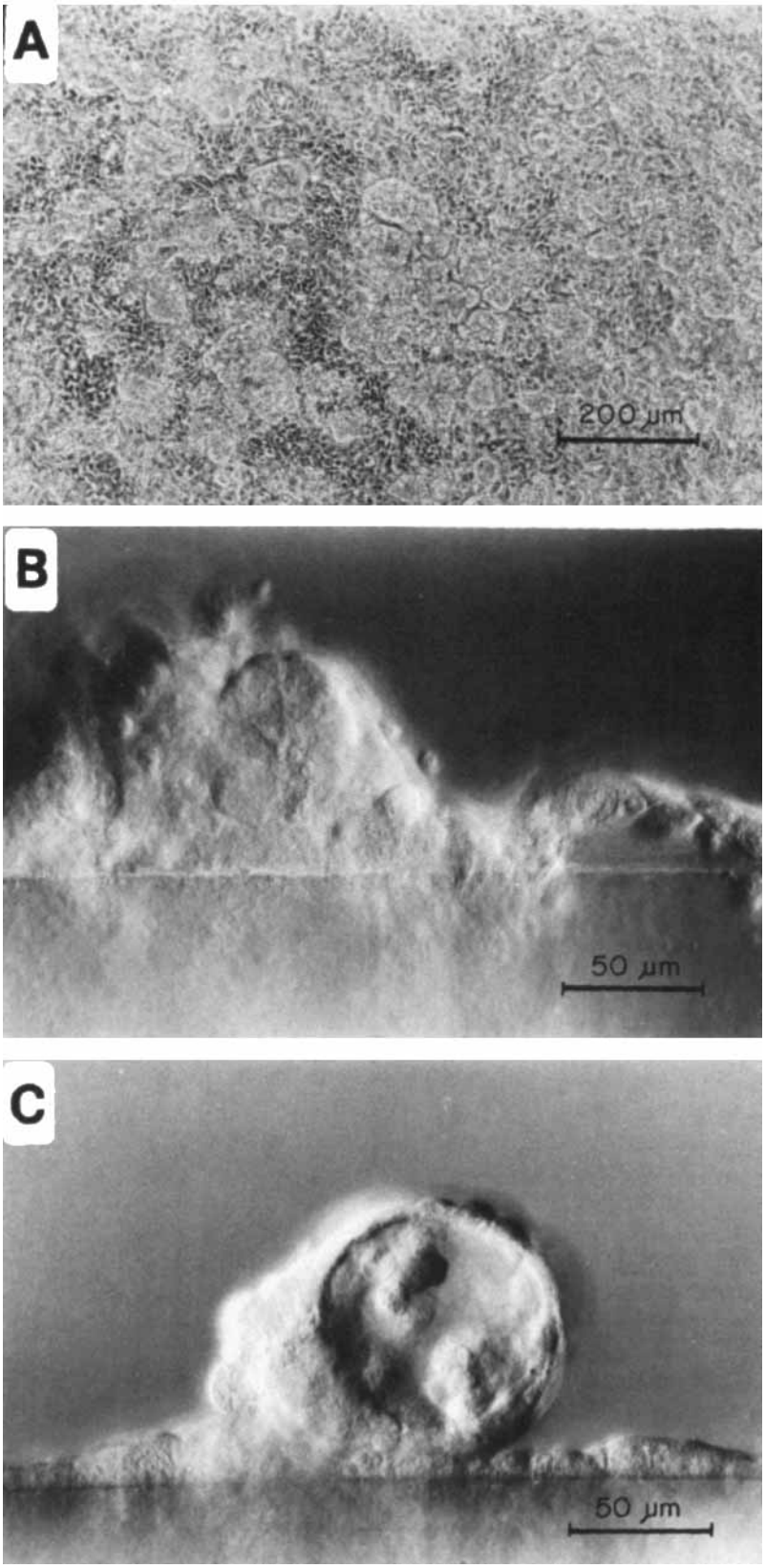

Figure 1.

reau-Schneider et al. [5], Gierthy and Lincoln [8] observed that MCF-7 cells exhibit postconfluent cell accumulation and development of multicellular aggregates (foci) following prolonged culture on a plastic substrate in 5% fetal bovine serum-supplemented medium.

Interestingly, the postconfluent cell accumulation and focus formation in MCF-7 cells are inhibited by 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), while preconfluent cell growth and viability are unaffected [8]. Previous studies have shown TCDD to be a potent antiestrogen as demonstrated by its reversible suppression of estrogen enhancement of tissue plasminogen activator (t-PA) activity in MCF-7 cells [9] and by its reduction of estrogen-induced uterotropic activity in rodents [10,11].

The association of the antiestrogen, TCDD, as an inhibitor of both postconfluent focus formation [8] and estrogen-regulated t-PA activity in MCF-7 cells [9] led to the present study to provide insight into this relationship. We demonstrate and characterize the estrogen stimulation of postconfluent cell accumulation and focus development in regard to agonist specificity, estrogen receptor involvement, growth kinetics, morphology, and cellular heterogeneity.

MATERIALS AND METHODS

Chemicals

Ethynyl estradiol, 17 β-estradiol (E_2), Hoechst dye, and dexamethasone were from Sigma Biochemicals, St. Louis, MO. The following compounds were obtained from Steraloids, Inc., Wilton, NH: progesterone, testosterone, moxesterol, and diethylstilbestrol. Analytical grade dimethyl sulfoxide (DMSO) was purchased from the Aldrich Chemical Company, Milwaukee, WI. Tamoxifen citrate was purchased from ICI America, Inc., Wilmington, DE, and Keoxifen (LY156758) was a gift of Dr. L.J. Black, Lilly Co., Indianapolis, IN. Cyclosporine A was purchased from Sandoz, Inc., East Hanover, NJ, and male BDF_1 mice were obtained from the Jackson Laboratory, Bar Harbor, ME.

Cell Lines

A strain of the human metastatic mammary adenocarcinoma cell line MCF-7 was obtained from Dr. Alberto C. Baldi, Institute of Experimental Biology and Medicine, Buenos Aires, Argentina. Stock cultures were maintained in plastic tissue culture flasks (Costar) using medium (DF_5) consisting of Dulbecco's modified Eagle's medium (DMEM) with penicillin (100 U/ml) and streptomycin (100 μg/ml) supplemented with 5% fetal bovine serum (FBS), 10 ng/ml insulin, L-glutamine (2 mM), and nonessential amino acids. The complete medium was filter sterilized using 500 ml capacity 0.2 μm pore-size plastic nalgene filter units from Nalgene, Rochester, NY.

Focus Development

Stock MCF-7 cells were suspended in DF_5 after treatment with trypsin (0.25%), seeded into 24-well plastic tissue culture plates (2 cm^2/well) at a density of 10^5 cells/well in 1 ml of medium, and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The cultures were refed at 24 h and every 4–5 days thereafter with 2 ml of DF_5 or medium supplemented with calf serum (CS) with various concentrations of experimental compounds in DMSO ($\leq 0.1\%$ final concentration) or DMSO alone. After 14 days or at indicated times in the kinetic studies, the cultures were fixed with formalin in phosphate-buffered saline (PBS) and stained with Giemsa (for microscopic evaluation) and 1% Rhodamine B (for foci counting). The latter was done using a New Brunswick automated colony counter modified to magnify the image of the microscopic multicellular foci. The foci retained the red Rhodamine B stain to a greater extent than did surrounding monolayer cells, affording an appropriate contrast for discrimination and enumeration. All experimental protocols used phenol red-free medium.

DNA Assay

Cell number per well was derived from fluorometric assay of total DNA of each culture using Hoechst dye. Medium was removed from the 24-well plates at indicated times, and the cultures were stored in a −20°C freezer. Cultures were examined to ensure cell adhesion to the well surface. For DNA analysis, frozen cultures

Fig. 1. Morphology of long-term MCF-7 cultures. The cells were seeded and maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. **A:** Overview of 14 day cultures that exhibit foci (phase-contrast). **B:** Nomarski-optics side view of cultures incubated for 14 days as in A. Note the prominent multilayered focus on left as distinguished from the dome composed of a detached monolayer on right of this panel. **C** shows a typical pseudoacinus, which is a less frequent feature of the 14 day control cultures.

were thawed, 1 ml Hoechst stain (20 μg/ml) in PBS, pH 7.0, was added, and the cultures were disrupted for 3–5 s with a probe sonicator. The suspension was transferred to a 1 ml aliquot of Hoechst stain and fluorescence determined in an Aminco-Bowman spectrofluorometer at 370 nm excitation and 450 nm emission. The cell number was calculated from the linear portion of a DNA standard curve constructed from a dilution series of MCF-7 cells that were trypsin treated and enumerated with a Coulter particle counter model ZM before processing.

Side View Microscopy

MCF-7 cells were grown in 24-well plates containing Formvar-coated coverslips as described by Roth et al. [12]. The Formvar with associated cells was removed from the coverslip after 14 days of incubation, folded to form a cell-lined edge, and wet mounted for side viewing of the viable cells by high-resolution optical sectioning using Nomarski differential interference-contrast microscopy.

MCF-7 Clone Establishment

Stock cultures were suspended as single cells by trypsin digestion, diluted, and seeded at a concentration of 1 cell/0.2 ml DF_5/well in 96-well plates. After overnight incubation to allow for attachment, the wells, which contained a single cell by microscopic examination, were marked and incubated to allow clonal expansion. At confluence, the clones were harvested by trypinization and expanded to 2 cm^2 wells of 24-well plates and then to 25 cm^2 plastic flasks. They were then tested for E_2 responsive focus development.

Cell Proliferation Assay

Proliferating cells were identified in cultures by immunodetection of 5-bromo-2′-deoxyuridine (BrdU) incorporation into newly synthesized DNA using an Amersham cell proliferation assay (Amersham RPN20). After various treatments, cultures were exposed to BrdU and incubated for 2 h followed by fixation and sequential incubation with mouse anti-BrdU, peroxidase conjugated anti-mouse Ig, and diaminobenzidine (DAB) substrate with iron-nickel intensifiers. Cells incorporating BrdU exhibited black nuclei indicative of the peroxidase-DAB reaction product.

In Vivo Tumor Growth

MCF-7 cells (2.5×10^6 solidified in fibrin clots) were transplanted and grown as xenografts under the kidney capsule of male BDF_1 mice immunosuppressed by daily subcutaneous treatment with cyclosporine A (60 mg/kg) as previously described [13]. The viability of the MCF-7 cells following solidification of the fibrin clots was insured by trypan blue exclusion. Estrogen support was provided by subcutaneous placement of Silastic E_2 implants at the time of tumor implantation, resulting in a serum E_2 concentration of 100 pg/ml. Estrogen dependency was determined as tumor growth only in the presence of these implants.

RESULTS

Foci Morphology

Our previous studies [8] demonstrated that postconfluent cultures of MCF-7 cells grown in FBS supplemented medium had accumulations of cellular aggregates superimposed on a monolayer background (Fig. 1A). These aggregates were designated foci, a polarity-independent, three-dimensional solid array of cells. However, other structures, such as domes formed by a single-cell layer, could be mistaken for aggregates when viewed by standard phase microscopy. To distinguish between these possibilities, cells were grown on a flexible plastic substrate that was then folded to form a cell-lined edge. The folded edge was viewed from the side with Nomarski differential interference optics [12]. The distinction between a focus and a dome is clearly seen in Figure 1B; the focus is a relatively solid collection of cells in a morula-like structure, while the dome comprises the space covered by a monolayer. The foci first appeared after about 9 days and increased in size over the next 5 days. A small fraction of the aggregates developed into pseudoacini, characterized by a cellular stem and sphere with a sharply delineated central vacuole (Fig. 1C).

Effect of Serum Source on Foci Development

We initially evaluated the effect that different serum sources, i.e., estrogen-rich versus estrogen-poor, might have on focus formation. Cultures were seeded in DF_5 and, after an overnight incubation, refed with the indicated concentrations of FBS, charcoal-dextran-treated FBS, or CS. The data are presented in Figure 2. After 10 to 14 days of incubation, foci were seen in cul-

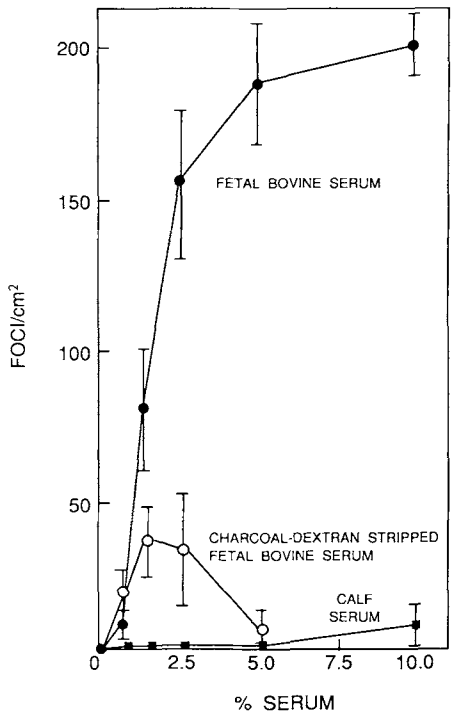

Fig. 2. The effect of serum type on foci development in MCF-7 cell cultures. Cells were seeded in DF_5, which after 24 h was replaced with medium supplemented with fetal bovine serum (●), charcoal-dextran-stripped fetal bovine serum (○), or calf serum (■) at the indicated concentration. The cultures were maintained on the media and refed as described in Materials and Methods for 14 days, fixed, and stained and the foci counted. The values are the average of four replicates ± S.E.M.

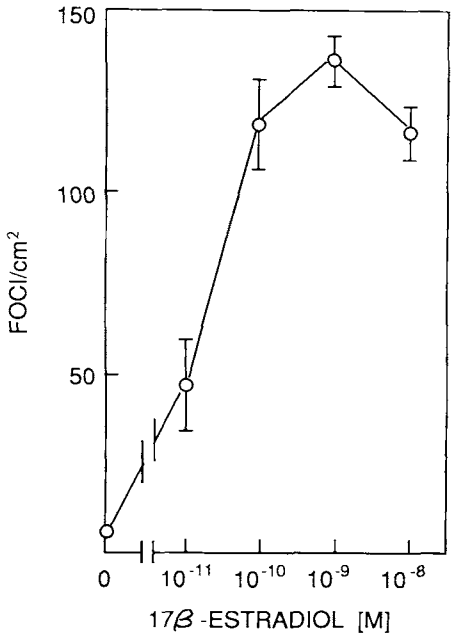

Fig. 3. The effect of E_2 concentration on the frequency of foci in MCF-7 cell cultures. MCF-7 cells were seeded and maintained in 5% calf serum supplemented medium with the indicated concentration of E_2 for 14 days. The cultures were then fixed and stained and the foci counted as described in Materials and Methods. The values are the average of four replicates ± S.E.M.

tures grown in FBS concentrations exceeding 1%. There were no foci formed in cultures grown in CS and relatively few in cultures grown in charcoal-dextran-treated FBS. These serum sources differed markedly in their E_2 content; FBS had an E_2 concentration of 30 pg/ml (0.11×10^{-9} M), while both CS and the charcoal-dextran-treated FBS had concentrations < 5 pg/ml, a level below the detectable limit of the direct radioimmunoassay using Coat-A-Count kits obtained from Diagnostic Products (Los Angeles, CA).

Effect of 17 β-Estradiol on Foci Development

The finding that focus formation was not supported in CS permitted evaluation of E_2 as a factor in this process. MCF-7 cells were cultured in DMEM supplemented with 5% CS (DC_5) alone or supplemented with E_2 in the range 10^{-11} M to 10^{-8} M, and foci formation was assessed after 14 days of culture. The results are shown in Figure 3 where a concentration-responsive increase in the number of foci was seen. These data indicate that E_2 enhances foci development in a dose-dependent manner over a physiologic concentration range of the hormone.

Dependence of Foci Development on Estrogenic Activity and Functional Estrogen Receptors

Hormone specificity was examined by testing estrogens other than E_2 and nonestrogenic steroid hormones for activity in promoting foci formation. The results are presented as EC_{50} values (Table I). Ethinyl E_2 and E_2 were nearly equivalent, while moxestrol and the nonsteroidal agonist diethylstilbestrol had the same EC_{50} value of 1×10^{-9} M. In contrast, neither testosterone nor dexamethasone showed any promotion of foci formation at concentrations up to 1×10^{-5} M, while progesterone showed limited stimulation at the nonphysiological levels of 1×10^{-6} to 1×10^{-5} M. Among the tested agonists, only estrogens appeared effective in increasing the number of foci after 14 days of incubation when MCF-7 cells were grown in DC_5.

To assess the role of estrogen receptors in foci formation, Tamoxifen and LY156758, both an-

TABLE I. Effect of Estrogenic Agonists, Nonestrogenic Steroids, and Antiestrogens on Focus Development in MCF-7 Cultures*

Compounds	EC_{50}[a]	IC_{50}[b]
A. Estrogenic agonists		
17β-Estradiol	5×10^{-11} M	—
Ethinyl estradiol	10^{-10} M	—
Moxestrol	10^{-9} M	—
Diethylstilbestrol	10^{-9} M	—
B. Nonestrogenic steroids		
Progesterone	10^{-6} to 10^{-5} M	—
Testosterone	$>10^{-5}$ M	—
Dexamethasone	$>10^{-5}$ M	—
C. Antiestrogens		
Tamoxifen citrate	—	10^{-6} M
LY156758	—	10^{-8} M

*MCF-7 cultures were exposed to various concentrations of estrogenic agonists, nonestrogenic steroids, or antiestrogens in the presence of 1×10^{-8} M 17 β-estradiol for 14 days, after which they were fixed and stained and the foci counted.
[a]EC_{50} is the effective concentration required to achieve 50% of the maximal stimulation of the number of foci.
[b]IC_{50} is the effective concentration required to inhibit 50% of the foci accumulated in the presence of E_2, 1×10^{-8} M.

tagonists of E_2 binding to unoccupied receptors, were added to MCF-7 cells grown in DC_5 with 1×10^{-8} M E_2 and the number of foci determined after 14 days of culture. The results are presented as IC_{50}, the inhibitor concentration required for 50% inhibition under these conditions (Table I). The approximate IC_{50} values were 1×10^{-6} M for Tamoxifen and 1×10^{-8} M for LY156758. The stimulation of foci by 1×10^{-8} M E_2 and the inhibition of that stimulation by 1×10^{-7} M LY156758 are shown in Figure 4 for 14 day cultures. The inhibition by Tamoxifen and LY156758 supports the concept that E_2 stimulation of foci formation involves an estrogen receptor-based mechanism.

Another approach to the estrogen dependency of foci formation was the comparison under identical conditions of MCF-7 cells with MDA-MB-231, an estrogen-independent cell line lacking significant estrogen receptors. At 14 days of culture, MDA-MB-231 cultures, examined by standard light microscopy, showed diffuse, generalized overgrowth devoid of the discrete aggregates seen with estrogen-exposed MCF-7 cells. These differences were more apparent when cultures were observed from the side using Nomarski optics (Fig. 5). In the absence of E_2, MCF-7 cultures appeared as monolayers devoid of any overgrowths (Fig. 5A). With cultures grown in E_2, a series of multicellular aggregates, like those

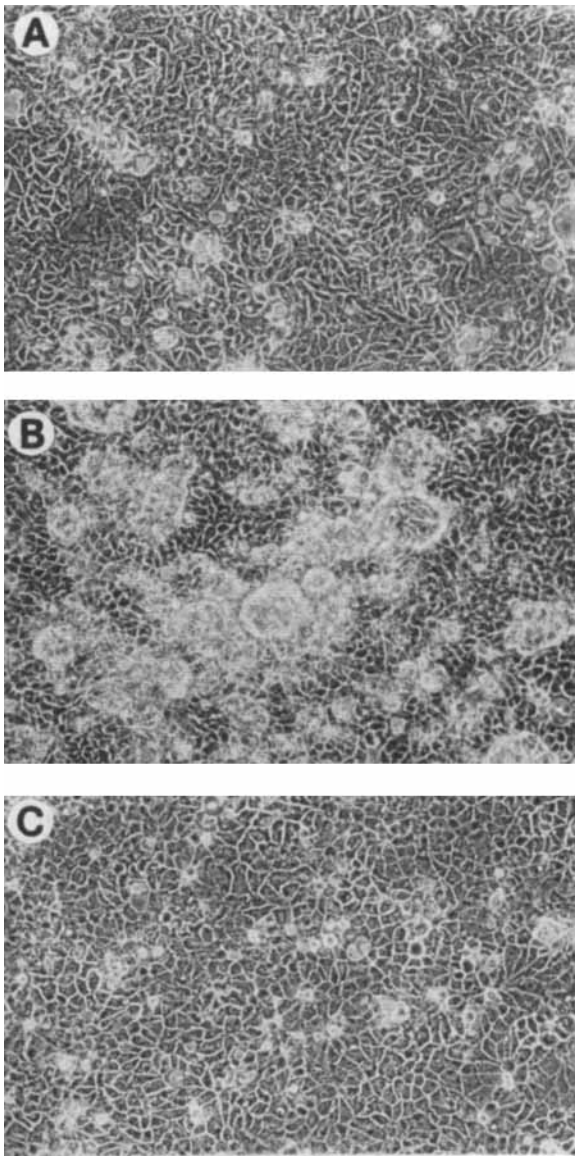

Fig. 4. Inhibition of foci development by the antiestrogen LY156758 in E_2-treated MCF-7 cultures. MCF-7 cells were cultured untreated (**A**), with 1×10^{-8} M E_2 (**B**) or with 1×10^{-8} M E_2 and 1×10^{-7} M LY156758 for 14 days in DC_5 with medium replacement every 4–5 days as described. Note the absence of foci in panel A (untreated), foci development in panel B (E_2-treated), and the inhibition of these foci in panel **C** (E_2 + LY156758).

depicted in Figure 1B, were seen (Fig. 5B). Again, there was a clustering of cells above the plane of the monolayer. Representative side views of MDA-MB-231, cultured under the same conditions as the MCF-7 cells, are shown in Figure 5C,D. There was no difference in the overall patterns regardless of the additions of hormone. In all cases, an irregular diffuse overgrowth of 2–3 cell layers was seen, indicating the lack of monolayer formation and, instead, a generalized

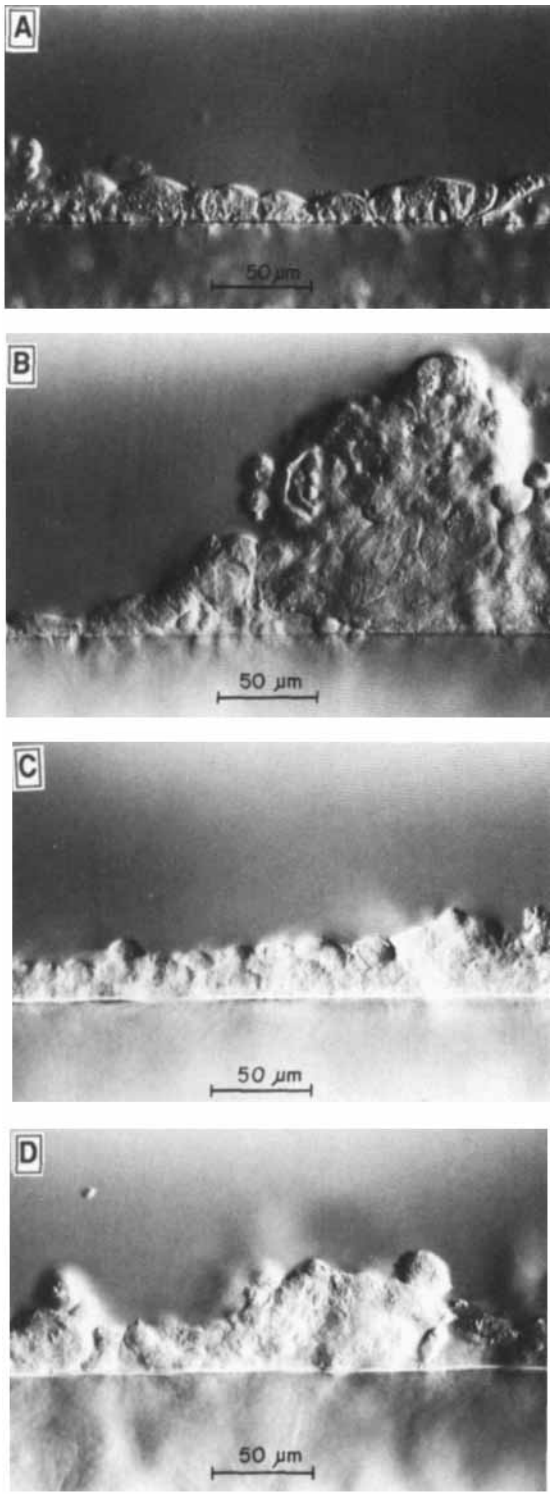

Fig. 5. Postconfluent development of MCF-7 (**A,B**) and MDA-MB-237 (**C,D**) cells. Nomarski side views (as in Fig. 1B,C) of control (A,C) and 10^{-9} M 17β-estradiol (B,D)-treated cultures. All cultures were maintained for 14 days in DC_5. Note the induction of foci over a monolayer by E_2 treatment (A,B) in the MCF-7 cultures. Similar treatment of MDA-MB-231 fails to alter their nonstructured multilayered morphology.

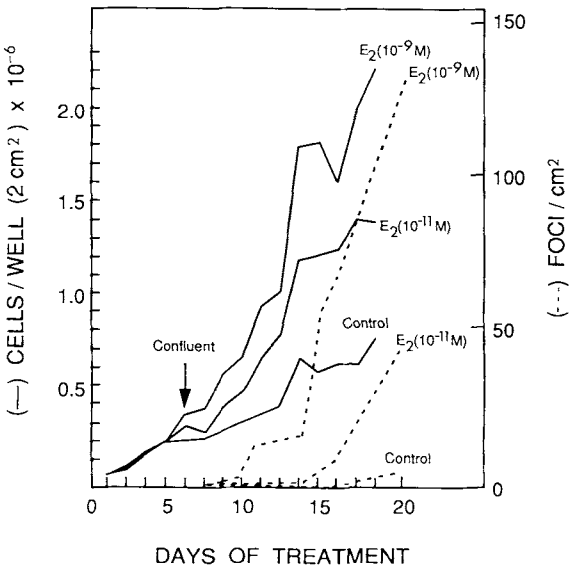

Fig. 6. Effect of 17β-estradiol on the time course of cell accumulation and foci development in MCF-7 cells. MCF-7 cells were seeded and maintained in DC_5 supplemented with the indicated E_2 concentrations as described in Materials and Methods. Daily cell counts were performed on trypsin-suspended cultures (solid line), and from days 7 to 20, foci were enumerated in fixed and stained cultures (dotted lines). Confluence, defined as the first appearance of a contiguous monolayer, was reached between 5 and 6 days after seeding (arrow). The values are the average of four replicates, S.E.M. ≤ 10% for each point.

cellular overgrowth. There was nothing comparable to the morphology seen in Figure 4B, corroborating the absence of well-marked, discrete structures seen in top views of MDA-MB-231 grown in the presence or absence of estrogens.

Temporal Aspects of 17β-Estradiol Enhancement of Postconfluent Cell Accumulation and Foci Development

Few data are available on postconfluent cell proliferation despite the attention directed to the influence of estrogens on the exponential growth of MCF-7 cells. We studied the effect of E_2 on MCF-7 cell accumulation from 1 to 20 days postseeding by exposing preconfluent, 24 h cultures to DC_5 alone or supplemented with 1×10^{-11} M or 1×10^{-9} M E_2. Samples were collected daily for cell number determination. As seen in Figure 6, E_2 induced a relatively modest dose-dependent increase in preconfluent cell number until day 6, at which time cultures were near or at confluence as determined by visual observation. At this point, a small, but E_2-concentration-related, increase in cell number was apparent.

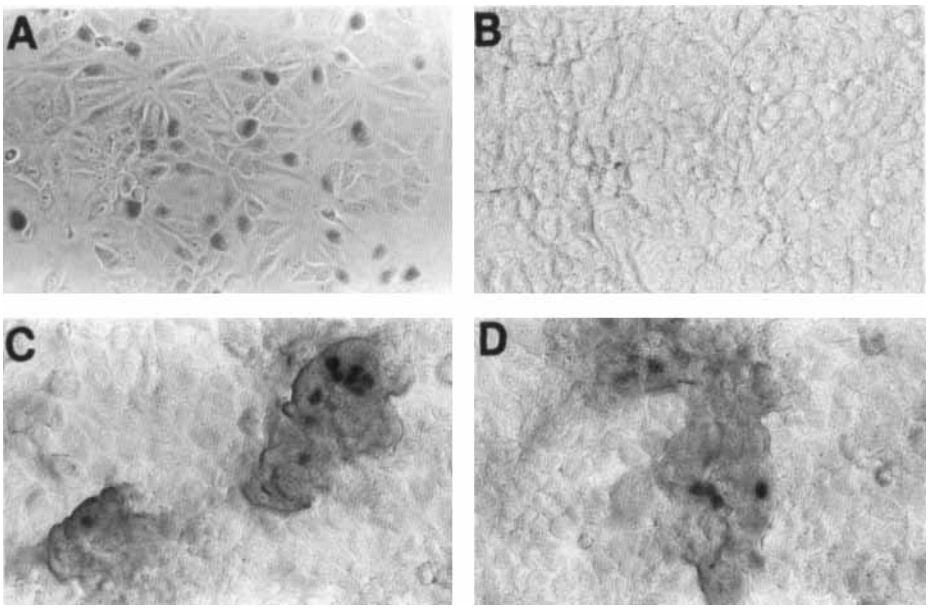

Fig. 7. Restriction of cell proliferation to foci in 1×10^{-9} M E_2-treated 14 day MCF-7 cultures. Incorporation of 5-bromo 2'-deoxyuridine, shown by black nuclei and indicative of DNA synthesis, was determined for MCF-7 cultures under various conditions. The random staining patterns typical of preconfluent proliferation is seen in nontreated 4 day cultures (**A:** phase-contrast) and the absence of staining is apparent in the non-E_2-treated, high-density 14 day monolayer (**B**). Labeled nuclei are associated only with the foci of the 10^{-9} M E_2-treated cultures at 14 days (**C,D**) 200×.

After confluence was attained, there was a persistent increase in cell number in all cultures consistent with an attenuation of density-dependent inhibition of cell proliferation. However, the striking feature was that cultures treated with E_2 exhibited a very large, concentration-related increase in cell numbers during this time compared to nonestrogenized controls.

To determine the relation of foci formation to this E_2-induced cell accumulation, replicate cultures were collected daily, fixed, and stained and the foci counted. The results, shown in Figure 6, demonstrate that they appear in the estrogenized cultures only after 9–10 days in the presence of 1×10^{-9} M E_2 and after 15 days in the presence of 1×10^{-11} M E_2. Very few, if any, foci appear in cultures devoid of added hormone for at least up to 30 days, which was the study duration. These data indicate that the sensitivity of foci formation to E_2 is similar to that of cell accumulation and that foci development begins well after confluence has been attained.

The proliferation status of cells was examined to determine if replication was generalized or restricted to foci in E_2-treated MCF-7 cultures at day 14. The results of this study, using BrdU incorporation and anti BrdU antibody with a peroxidase detection system, are shown in Figure 7. The random staining pattern of cultures approaching confluence at day 4 is shown in Figure 7A. It is clear that the population of cells forming the monolayer in the control cultures at day 14 (Fig. 7B) is devoid of label indicative of DNA synthesis as are the monolayer cells surrounding the foci in 1×10^{-9} M E_2-treated cultures (Fig. 7C,D). In contrast, BrdU-labeled nuclei are prominently displayed in the foci of these E_2-treated cultures.

Heterogeneity of MCF-7 Cells for Estrogen-Stimulated Foci Development

Experiments were done to characterize the estrogen sensitivity of cells composing the E_2-induced foci to determine if they consisted of a discrete, stable population of cells. Foci from 14 day cultures treated with 1×10^{-9} M E_2 were dislodged from the culture surface by a hard shake, and this foci-rich population was disaggregated to single cells by trypsin treatment as determined by visual observation, reseeded, and cultured in the presence or absence of 1×10^{-9} M E_2. After 14 days, the E_2-treated cultures had both foci comparable in number to those formed by the stock MCF-7 cells and cells arranged in a monolayer, without evidence of enrichment of focus-forming progenetor cells (data not shown).

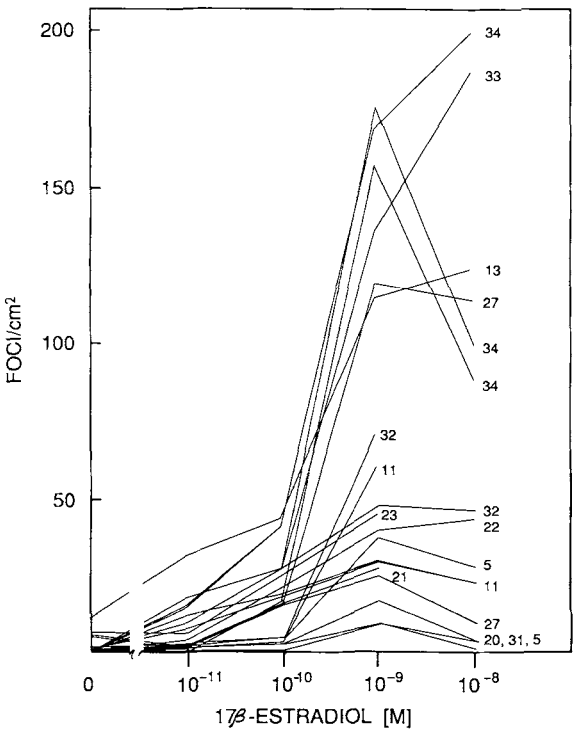

Fig. 8. Variation of MCF-7 clones to E_2-stimulated focus development. MCF-7 clones were developed and tested for E_2 sensitivity for focus development at 14 days as described. The clones, identified by number, exhibited a wide range of sensitivity to E_2, indicative of the heterogeneity of this cell line.

Clones established from stock MCF-7 cultures were tested for sensitivity to E_2 with respect to foci formation. Cloned cells were seeded and cultured in the presence of various concentrations of E_2 for 14 days. The results shown in Figure 8 demonstrate heterogeneity in the ability of the MCF-7 clones to develop foci in response to E_2. These data substantiate that heterogeneity of MCF-7 cell populations [14] also extends to hormonal promotion of postconfluent foci.

Confirmation of MCF-7 Estrogen Dependence and MDA-MB-231 Estrogen Independence

MCF-7 cells may lose their estrogen requirement for in vivo tumorigenicity depending on conditions of in vitro culture. Therefore, it was necessary to validate that the MCF-7 cells used in this study responded in vivo in mice to estrogen by induction of tumors. Measurements of the tumor diameter were taken after 2 and 3 weeks to assess tumor growth, after which time the mice were sacrificed. The results shown in Table II verify that the MCF-7 cells used in these in vitro studies formed estrogen-dependent tumors in vivo. The in vivo estrogen independence of the MDA-MB-231 cell line was also tested in this experiment. In contrast to MCF-7, the results indicate that implants of MDA-MB-231 cells grew equally with or without added E_2—a finding compatible with the in vitro results (Fig. 5) indicating estrogen independence.

TABLE II. Effects of Estrogen on Growth of MCF-7 and MDA-MB-231 Tumor Xenografts*

Treatment	Tumor size[a] MCF-7	MDA-MB-231
Control		
Day 0	2.13 ± 0.13	2.03 ± 0.12
Day 14	2.08 ± 0.08	2.64 ± 0.13
Day 21	1.67 ± 0.08	2.99 ± 0.15
17β-Estradiol implanted		
Day 0	2.26 ± 0.13	2.43 ± 0.09
Day 14	2.95 ± 0.13[b]	2.90 ± 0.07
Day 21	3.30 ± 0.08[b]	3.22 ± 0.08

*MCF-7 or MDA-MB-231 cells (2.5×10^6 solidified in fibrin clots) were implanted under the renal capsule of male BDF_1 mice immunosuppressed with cyclosporin A. Mice were estrogenized by s.c. placement of Silastic E_2 implants at the time of tumor implantation. Tumor diameter measurements were taken 2 and 3 weeks after implantation.

[a]Mean diameter in mm, average of three to six replicates ± SE.

[b]Significantly different from controls, $P = 0.05$ by the Wilcoxon Two Sample Rank Sum Test.

DISCUSSION

In this study, we demonstrated that, in MCF-7 cultures, the formation of multicellular clusters (foci) is an estrogen-stimulated postconfluent process, probably involving an estrogen receptor-based mechanism. Foci are seen as a piling up and overlapping of cells on a monolayer background. In contrast to the general overgrowth of estrogen-nonresponsive MDA-MB-231 cells, foci appeared as morula-like structures in estrogen-treated MCF-7 cultures, with some ultimately presenting as luminal pseudoacini. Both structures are reminiscent of the original malignant exudate from which the cell line was isolated.

MCF-7 cells are transformed, yet are a heterogeneous population, such that some cells exhibit the malignant phenotype by forming foci in response to estrogen, while others maintain a monolayer morphology characteristic of non-transformed cells in vitro. This heterogeneity is apparently an epigenetic phenomenon as there

was no enrichment of focus-forming cells in the progeny of foci-derived parental cells. A wide variance in sensitivity to E_2 in the formation of foci was seen with clones derived from stock MCF-7 cultures, comparable to the results reported for other determinants by Butler et al. [14]. Analysis of the kinetics of foci production identify three distinct cellular accumulation phases: preconfluent, postconfluent but prefocal, and postconfluent with foci. This separation presents an opportunity to study estrogen-regulated processes as they relate to each of these distinct phases.

At least three possible mechanisms for foci formation are apparent from the data presented here. These putative mechanisms could act independently or in overlapping sequence and not all are based on E_2-stimulated postconfluent cell proliferation. First, it is possible that postconfluent proliferation is stimulated by E_2 randomly, resulting in increasing cell density with time. At a particular density, compression from surrounding cells may cause cellular detachment from the plastic substratum in specific areas while intercellular adhesion is maintained. Ultimately, the detached cells are forced by the encroachment of neighboring cells into accumulations seen as foci on a monolayer background. Alternatively, random postconfluent proliferation may result in cellular detachment from the substratum as well as from adjacent cells resulting in sloughing into the medium. This would lead to an apparently static monolayer that is actually an approximation of a steady state of cell proliferation and cell loss. In this case, added E_2 may not affect cell proliferation, but instead cause local enhancement of intercellular adhesion and, based on the heterogeneity of the population, subsequent focal accumulation of these adherent cells rather than their loss to the medium. This invokes an E_2 modulation other than increased proliferation and does not conflict with an epigenetic process indicated by our study. A third possibility is that foci are the progeny of a discrete population of cells with increased E_2 sensitivity for cell proliferation. Here, cells are stimulated to divide with equal probability until confluence was reached. At this time, the progeny of E_2-sensitive cells would have a selective advantage for proliferation and continue to divide, forming aggregations of cells on a monolayer of the nonproliferating neighboring cells. We have seen that BrdU incorporation (reflective of DNA synthesis) occurs in focus-forming cells of 14 day cultures and not in the surrounding monolayer. However, this is a static endpoint and is not informative as to mechanism.

In regard to these possibilities, it is conceivable that structural alterations dependent on estrogen treatment of MCF-7 cells underlie the development of foci and the more structured pseudoacini. A key factor in this development may be decreased substratum adherence of a subpopulation of estrogen-responsive MCF-7 cells, permitting principally upward and lateral migration of the focus-forming cells. Insight into the changes in cell shape and adhesiveness was gained by examining the rearrangement of cytoskeletal and adhesion structures after estrogenization [15]. In this study, the MCF-7 cells were arranged in clusters with irregular gland-like features. The most prominent estrogen-induced change was the appearance of cytoplasmic protrusions rich in F-actin, especially in the cells at the cluster's periphery. Actin-containing ruffles were also common along the dorsal membranes of these cells, while a profuse keratin filament network was arranged in a perinuclear distribution. Conversely, there were no remarkable hormone-induced changes in vimentin or microtubule distribution, but there was a significant reduction in vinculin at the protrusions' tips, indicative of loss of adhesion plaques. These results support a hypothesis for cellular dehiscence based on decreased vinculin expression in estrogen-treated MCF-7 cells.

Increased number and length of microvilli on MCF-7 cells were directly attributed by Vic et al. [16] to estradiol addition. This was accompanied by an overall conversion to "secretory cells" exemplified by alterations in rough endoplasmic reticulum, Golgi complexes, and secretory granules. The cells appeared rounded and partly detached from the plastic substrate. A decreased adherence to plastic after estrogen addition was also observed by others [17] and further supports an estrogen-mediated decrease in cell-stratum interaction resulting in the cell accumulation and foci development.

Marked changes in the MCF-7 cell morphology after estrogen addition, including preeminent microvilli and formation of cytoplasmic pseudopods, reflect increased cellular secretory activity, including release of proteases. This may be related to foci induction by estrogen since hormone-stimulated production of cathepsin D [18] and tissue plasminogen activator [19] pro-

vide proteases capable of extracellular proteolysis, a possible means for degrading extracellular matrix. Selective loss of adhesion plaques and modification of extracellular matrix in the vicinity of the progenitor subpopulation may remove constraints for upward and lateral cellular migration or proliferation and the subsequent cell accumulation resulting in foci formation as opposed to the diffuse overgrowth observed with MDA-MB-231 cells, which exhibit profuse constitutive protease production.

In the case of the more structured pseudoacini, these processes may be combined with vectorial differences, such as the retention of junctional complexes in outer epithelial cells rather than inner ones as seen by Russo et al. [20], resulting in their discrete luminal architecture. By growing MCF-7 cells in medium supplemented with 10% fetal bovine serum on collagen-coated sponges, Russo et al. [3] found the same multicellular architecture as seen in the original exudate. These structures resemble those described in the present study, but the authors did not define the role of estrogens in this pleomorphic development. In monolayers, the cells were connected by tight junctions, intermediate junctions, and desmosomes, with abundant microvilli on their free surfaces [1]. In the hollow clusters attached to collagen-coated cellulose sponges, microvilli and junctional complexes were prominent on the cells of the outer surface [20]. The association of microvilli on the outer layer of cells, rather than inner, was seen in these ductule-like structures, so the authors concluded they were pseudoacini, rather than the true acini seen in normal mammary glands.

In summary, there is a marked estrogen-mediated increase in cell numbers and foci formation of MCF-7 cells during the postconfluent growth phase. The effect is restricted to estrogen agonists and proportional to hormone concentration. Inhibition by the antagonists Tamoxifen and LY156758 suggest an estrogen-receptor-based mechanism. The formed foci have a distinct circumscribed structure similar to the histopathology of the original pleural exudate from which the MCF-7 cell line was derived and in stark contrast to the diffuse multilayers of MDA-MB-231 cells. The present finding that focus formation in postconfluent MCF-7 cell cultures is dependent on estrogen agonists provides an alternative method of examining the relative contributions of estrogen-regulated factors to this process.

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