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Estrogen Receptor Status and Estradiol Sensitivity of MCF-7 Cells in Exponential Growth Phase

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Abstract—*Proliferative patterns of MCF-7 human breast cancer cells have been reported to influence their estrogen receptor (ER) contents. However, the experimental conditions under which these variations in ER contents were described differed from those commonly used for maintaining exponential growth. We, therefore, investigated whether or not MCF-7 receptor status also fluctuated under normal growth conditions. MCF-7 cells were cultured up to 4 days in 96-multiwell dishes. On each day, cell number was spectrophotometrically assessed after fixation and coloration of the cells with hematoxylin; corresponding ER content was measured by the Abbott enzyme immunoassay in KCl extracts. At the three plating densities tested (5, 10 and 20 × 10^3 cells/ml), an obvious parallel was found between the cell number and the ER content suggesting an unchanged receptor status throughout the culture period. Regression analysis confirmed this impression. Additional fractionation by SDS–PAGE of total MCF-7 proteins extracted at various times of the culture (up to 7 days in 35 mm Petri dishes) gave identical patterns suggesting that ER synthesis is regulated as the majority of proteins. Growth experiments indicated that this situation conferred a constant estrogenic sensitivity to the cells: 24 h exposure to 10^{-8} M estradiol on either the 1st, 2nd, 3rd or 4th day after plating resulted in the same increase in cell number. All these data indicated that ER contents of MCF-7 cells were maintained at a constant level under exponential growth which resulted in a constant estrogenic sensitivity.*

INTRODUCTION

AMONG models developed for the study of the hormone sensitivity of human breast cancers, the MCF-7 mammary tumor cell line has proven especially useful since its growth is modulated by a variety of steroid and peptide hormones. In this regard, the influence of estrogens has been extremely well documented [1]. Thus, physiological concentrations of estradiol-17β (E_2) (range = 10^{-11} to 10^{-8} M) are known to induce the synthesis and secretion of several peptides including growth factors which in turn produce a trophic effect by an autocrine regulation process [2]. At high concentrations ($\geq 10^{-5}$ M), E_2 produces a strong growth inhibition paralleling the clinical observation of a therapeutic effect of pharmacological doses of estrogens [1].

Several reports demonstrated the direct participation of estrogen receptor (ER) in the estrogenic responses observed at low E_2 concentrations [1]. Other reports revealed that proliferative patterns of the cells influenced the ER levels: the exponential growth phase was shown to be associated with the highest receptor concentration, the confluence to a period of progressive decline of concentration [3]. Moreover, evidence was reported that slowly proliferative cells contained higher amounts of ER than fast proliferative ones [4]. Finally, cell synchronization experiments suggested that ER synthesis occur throughout the whole cell cycle with a decrease during the S phase and a predominance in G1 and G2 [4].

Reported conditions to assess these variations in ER contents differ from those used for maintaining an exponential growth. We, therefore, decided to investigate whether or not receptor concentration also fluctuated under such conditions. Our data clearly indicated that ER remained at a constant level along a whole exponential phase. The present paper describes these investigations and, moreover, shows that this property confers to the cells a constant sensitivity to the trophic action of E_2.

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MATERIALS AND METHODS

Chemicals

E_2 was purchased from Sigma, St Louis, MO; hematoxylin from Chroma, Stuttgart, F.R.G. Protein standards for molecular weight calibration were from Pharmacia, Sweden. All reagents were of analytical grade.

Culture materials

Earle's based minimal essential medium (MEM), Hanks balanced salt solution (HBSS), fetal calf serum and L-glutamine were purchased from Gibco (Glasgow, U.K.); penicillin, streptomycin from Difco (Detroit, MI) and gentamicin from Schering (Kenilworth, NJ). Closed T-25 and 96-multiwell dishes were from Falcon (Becton Dickinson).

Cell growth evaluation

MCF-7 cells were maintained at 37°C in monolayer culture in closed T-25 dishes in MEM supplemented with L-glutamine (0.6 mg/ml), gentamycine (40 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% inactivated fetal calf serum (inactivation: 1 h at 56°C).

Cell growth was assessed by a spectrophotometric method recently developed in our laboratory [5]. This method which uses the high efficiency of the 96-multiwell tissue culture dishes gives an accurate evaluation of the cellular content of the monolayer without any cell harvest. Briefly, MCF-7 cells were removed from a monolayer (trypsin 0.05%, EDTA 0.025%) and plated in 96-multiwell dishes at either 5, 10 or 20 × 10^3 cells/ml. They were cultured at 37°C in a humidified 95% air, 5% CO_2 atmosphere for a maximum of 5 days, a period known to maintain an exponential growth. At the time of spectrophotometric measurement, the medium was removed and the monolayer successively washed with running tap water, fixed with 90% ethanol and colored with hematoxylin. The intensity of the coloration which is a measure of the cell number was then determined at $\lambda_{540\,nm}$ with a multiscan spectrophotometer (Flow Laboratories Inc.).

E_2 growth stimulation was assessed according to this procedure with serum depleted of endogenous steroids (dextran-coated charcoal treatment: 0.5% charcoal, 0.005% dextran in 1.5 ml medium/ml serum). On either the 1st, 2nd, 3rd or 4th day after plating, half of the cells were exposed for 24 h to E_2 at a final concentration of 10^{-8} M; the other half to the medium (control). After hormonal stimulation, culture was pursued until spectrophotometric measurements.

Estrogen receptor assay

In two independent experiments, MCF-7 cells were plated at 5, 10 and 10 × 10^3 cells/ml in 96-multiwell dishes to be cultured up to 4 days. ER content of the cells was measured along the experiment according to the following procedure. On each day, a dish was taken and frozen overnight at −80°C after removal of the growth medium. Fifty microliters of a buffered 0.5 M KCl solution was then added to each well of the dish (buffer: 10 mM potassium phosphate pH 7.4 containing 1.5 mM EDTA and 1 mM monothioglycerol). After 1 h of exposure to this solution, a time period sufficient for the total extraction of steroid receptors [6], ER concentration was measured in an aliquot of 100 μl by the Abbott enzyme immunoassay (ER-EIA); this aliquot was taken from pooled extracts (400 μl; 8 wells). The high sensitivity as well as the ability of ER-EIA to measure receptors saturated by serum estrogens, justified the selection of this assay.

Protein fractionation by SDS–PAGE

MCF-7 cells were plated at 50 × 10^3 cells/ml and cultured up to 7 days in 35 mm Petri dishes. On either the 1st, 3rd, 5th or 7th day, cells were harvested with a rubber policeman, washed in HBSS and stored at −20°C in a buffered SDS-mercaptoethanol solution (~ 2.5 × 10^3 cells in 20 μl) until sodium dodecyl sulfate electrophoresis (SDS–PAGE) [7]. Electrophoresis of cell lysates (3 min at 100°C) was conducted at 50 mA for ~ 3.5 h; standard proteins of known molecular weight were run in parallel. Gels were finally treated for staining and coloration by Coomassie Brilliant Blue.

RESULTS

Figure 1 gives the ER contents of MCF-7 cells in exponential growth phase (two independent experiments; full and dotted lines). The left panels of the figure describe the growth curves (semi-logarithmic plotting of the data produced straight lines confirming the exponential growth; doubling time ~ 20 h); the right panels, the corresponding ER concentrations. At the three plating densities tested (5, 10, 20 × 10^3 cells/ml) an obvious parallel was found between the cell number (assessed by spectrophotometric measurement) and the receptor levels suggesting a significant correlation between both parameters; linear regression analysis of pooled data confirmed this impression (Fig. 2; $r = 0.92$; $p<0.001$). The mean ER concentration of ~ 5 fmol/40 × 10^3 cells estimated from this statistical analysis corresponded to a value previously established by incubating MCF-7 cells with [^{3}H]E_2 (whole cell assay [8]) which confirms the total recovery of the receptors under our extraction procedure.

The linear relationship between the cell number and the receptor concentration is indicative of an unchanged ER status throughout the culture period.

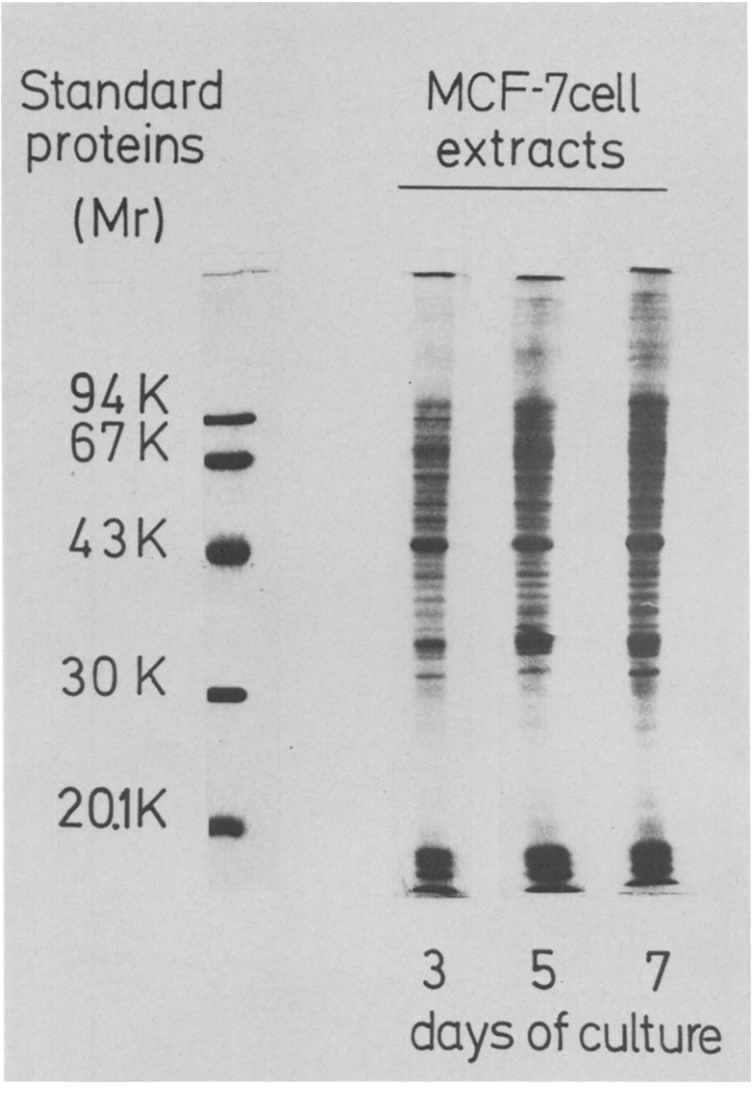

Fig. 3. Electrophoretic patterns of proteins from MCF-7 cells in exponential growth phase (from 3rd to 7th day of culture; intensity of the pattern from the 1st day of culture was too low to be reproduced). Proteins were separated on SDS slab gels and revealed by Coomassie Brillant Blue staining.

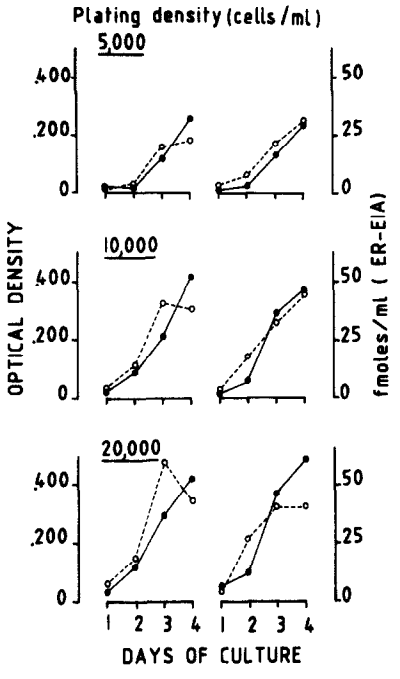

Fig. 1. MCF-7 cells in exponential growth phase. Relationship between growth and ER content of the cells in two independent experiments (full and dotted lines). In each experiment, cells were plated at 5, 10 *and* 20 × 10^3 *cells/*ml; *growth was assessed spectrophotometrically and receptor measured in KCl extracts by the Abbott enzyme immunoassay.*

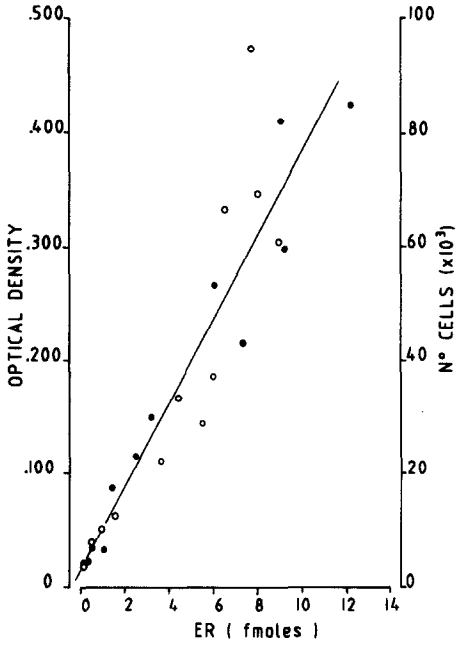

Fig. 2. MCF-7 cells in exponential growth phase. Correlation of cell number and corresponding ER content (pooled data from Fig. 1; *linear correlation* y = 0.013 + 0.037 x, r = 0.92). *Cell number was estimated from the optical densities by reference to reported calibration data* [5].

Fractionation of the total cell proteins by SDS–PAGE gave identical patterns along the whole culture (Fig. 3) suggesting that ER synthesis is

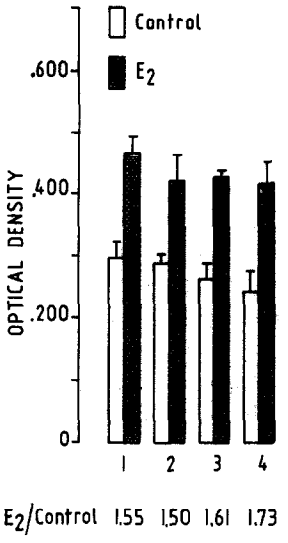

Fig. 4. Influence of an estradiol pulse on MCF-7 cell growth. Growth was estimated spectrophotometrically after 5 *days of culture (plating density* = 5000 *cells/*ml). *Treated cells were exposed to* 10^{-8} M *estradiol for* 24 h *on either the* 1*st,* 2*nd,* 3*rd or* 4*th day after plating.*

regulated as the majority of the proteins. A constant sensitivity to E_2 seemed a logical consequence of this observation. Growth experiments were designed to verify this statement.

Throughout the 5-day culture period, MCF-7 cells were submitted at various times to a unic estrogenic pulse expecting to produce a same growth increase along the whole experiment (24 h exposure to 10^{-8} M E_2 on either the 1st, 2nd, 3rd or 4th day after plating). A spectrophotometric evaluation of the cell number gave the expected result: the same increase in color intensity was observed after each exposure to E_2 (Fig. 4). To avoid any misleading data which could result from a difference in cell adherence properties under E_2 stimulation, growth was also assessed with 35 mm Petri dishes by measuring the DNA content [9] of harvested cells. Remarkably, the same growth stimulation was also found along the whole culture in this control experiment (ratio E_2/C = 1.40–1.43).

DISCUSSION

Our data indicate that ER are maintained at a constant level under exponential growth phase of MCF-7 cells, which results in a constant sensitivity of this cell line to estrogens. This property is reminiscent of a situation previously described *in vivo* in the MXT mouse mammary tumor model [10]. Injection of a physiological dose of E_2 to castrated mice bearing this hormone-sensitive tumor increased its thymidine labeling index. Remarkably, the labeling increase as well as the ER content of the tumor remained constant up to 6 weeks after its implantation. It seems, therefore, that both *in vitro* and *in vivo*, estrogen sensitivity of mammary tumor cells do not change during cell replication in the

absence of exogenous stimulus or treatment. This may explain why, in human breast cancers, ER status does not markedly fluctuate along the natural course of the disease [11].

ER levels are influenced by a variety of factors which consequently modulate the estrogen sensitivity of the cells [12–14]. According to the present data, for plating densities producing similar growth rates, one can assume identical receptor concentrations. Hence, under exponential growth, cell density would not influence the molecular mechanisms controlling the E_2 sensitivity; this conclusion would also hold for antiestrogens which are also acting through ER. Whether or not these mechanisms are modified under non-exponential growth conditions still remains to be established.

The extent of estrogenic growth stimulation varies among reported studies. Increases measured here after 24 h in the presence of E_2 (~ 40–70%) are not extremely low in regard to those we usually found after 120 h [14], indicating that a significant growth stimulation does not require a permanent presence of the hormone. Experiments are required to establish the appropriate E_2 concentration as well as the administration schedule to produce an optimal stimulation. In this regard, on ZR-75-1 breast cancer cells, it has been shown that the growth increase is dependent of the E_2 concentration in a non-linear fashion as predicted by the low of mass action which governs the interaction between the hormone and its receptor [15]. Moreover, several E_2 pulses led to a total cell number which could be predicted by summarizing the effects of all the individual pulses [15] which is consistent with the concept that ER measures the overall time of exposure to the hormone in a linear fashion.

Although our studies are of a fundamental nature they are not devoid of therapeutic relevance. Research aimed at achieving the best synergism between cytotoxic agents and hormones is being pursued in several laboratories and clinical centers [16]. The results reported here may be helpful in the design of experimental protocols for estrogenic recruitment of breast cancer cells. Indeed, they suggest that tumor growth stimulation (recruitment) may be obtained with an equivalent potency independently of the time of E_2 administration to tumor cells in the exponential growth phase.

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