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Cell Cycle Analysis of Estrogen Stimulated Growth of the Human Breast Cancer Cell Line, MCF-7

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Abstract—*The estrogen receptor positive human breast cancer cell line, MCF-7, can be growth inhibited by high concentrations of newborn calf serum (NCS). MCF-7 cells grown with high concentration of NCS can be growth stimulated by* 10^{-8} M *estradiol, and the growth stimulation seems to involve the abolishment of the effect of inhibitory activity in serum. Flow cytometry has been used to determine cell cycle parameters such as distribution of cells in the different phases of the cell cycle and growth fraction. The cell cycle analysis revealed that addition of* 10% *NCS to cultures grown with* 0.5% *fetal calf serum (FCS) increased the doubling time by elongating the G1 transit time. Estradiol stimulation occurred through a shortening of the G1 transit time. An effect on growth fraction was observed neither during growth inhibition with high NCS concentration nor during growth stimulation with estradiol. Cells which are growth stimulated by estradiol have an activated estrogen receptor mechanism as indicated by the presence of filled nuclear estrogen receptors and high level of progesterone receptors. We suppose that a possible mechanism for this estrogen stimulation could be induction of synthesis of growth factors which annul the effect of the inhibitory activity present in NCS.*

INTRODUCTION

THERE HAS been much controversy as to whether or not the human breast cancer cell line MCF-7 can be growth stimulated by estradiol. In some studies [1–9] varying degrees of growth stimulation by estradiol have been demonstrated while others have reported no effect of estradiol on the *in vitro* growth of MCF-7 cells [9–13]. Our own studies have shown no direct effect of estradiol on growth of MCF-7 cells in tissue culture although estrogen receptors were translocated to the nucleus and induced progesterone receptor synthesis [13].

Since estrogens are required for the growth of MCF-7 cells in athymic mice [11, 14] the lack of a direct mitogenic effect of estradiol on MCF-7 cells *in vitro* may be explained by an indirect mechanism of action [11]. Three mechanisms for indirect action of estrogens have been suggested. Firstly, estrogens may induce the synthesis of growth factors *in vivo* which are responsible for tumor growth as shown by Sirbasku who has found that estrogens induce the synthesis of growth factors in rodent uterus, kidney, and liver [15]. Secondly, estrogens may work in cooperation with a growth factor which is present in the serum and, thirdly, estrogens may annul the effect of a growth inhibitory activity present in the serum [8, 11, 16].

An indirect action of estradiol in growth stimulation of MCF-7 cells involving cooperation with factors present in serum could easily explain why – only in some laboratories – MCF-7 cells can be growth stimulated by estradiol. The importance of serum factors for estradiol stimulation has been described in several papers during the last years, and the general picture is that high concentration of estrogen deprived or estrogen poor serum is the best growth condition for stimulation by estradiol [6–8, 11, 16]. Under these growth conditions estrogens seem to neutralize the action of inhibitory activity in serum [8, 16].

Sutherland et al. [17] have reviewed the papers on the effect of estrogens on cell proliferation and cell cycle kinetics. They discuss a number of *in vivo* studies on estrogen stimulated growth, but only one study includes cell cycle kinetic data on estrogen stimulation of growth of a human breast cancer cell line *in vitro* [2]. By use of growth curves, ^{3}H-thymidine labelling indices and pulse-label experiments Weichelbaum *et al.* have found that 10^{-9} M estradiol enhanced the rate of cell proliferation of MCF-7 cells by shortening the overall cell cycle time. The proportion of cells in *S*-phase was increased and they suggest that the shortening of the mean cycle time may be due to a reduction in the G1 phase of the cell cycle [2]. A similar observation

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of an increase in the S-phase fraction has recently been described for the human breast cancer cell line T47D grown in presence of 10^{-9}M estradiol [18]. In this study we have used flow cytometry to estimate the growth fraction and the distribution of MCF-7 cells in the different phases of the cell cycle in cultures which are growth inhibited by newborn calf serum and cultures with 10% newborn calf serum which are growth stimulated by 10^{-8} M estradiol. The involvement of estrogen receptors in the estrogen stimulated growth is discussed.

MATERIALS AND METHODS

Cell cultures

MCF-7 cells were kindly supplied from the Human Cell Culture Bank, Mason Research Institute, Rockville, MD, U.S.A. The cells were propagated in plastic T flasks (Nunc, Denmark) in growth medium composed of Dulbecco's MEM + Ham's F 12 (1 : 1) supplemented with glutamine 2 mM, insulin 6 ng/ml and 0.5% heat inactivated fetal calf serum (FCS).

Growth curves

For the experiments the cells were plated at a cell density of 1.25×10^5 per T-25 flask in growth medium. After 6 hr the cultures were divided into four groups. Group 1 continued in normal growth medium with 0.5% FCS, group 2 received medium with 0.5% FCS + 10% NCS, group 3 received medium with 10% NCS, and group 4 received medium with 10% NCS + 10^{-8} M estradiol. Medium was renewed each day from day 2. Three cultures from each group were trypsinized and the cells counted in a Bürker–Türck chamber at day 3, 4, 5, 6, 7, and 10. Estimates of doubling times were based on the cell count from day 3 to 6.

Determination of growth fraction

5-Bromodeoxyuridine, BUdR (final concentration 0.02 mM) was added to four T-25 flasks in each group at day 3. In each group nuclei from cells in two T-25 flasks were prepared for flow cytometry (as described below) after the duration of one doubling time and after the duration of two doubling times. The GO fraction is estimated as the proportion of cells which contain a G1 amount of DNA without incorporated BUdR after the duration of two doubling times. The GO fraction estimated after the duration of one doubling time is 2–12% higher than the GO fraction estimated after two doubling times indicating that the cultures contain a small fraction of cells with an extended cell cycle time. The growth fraction was calculated as 100% minus the GO fraction.

Flow cytometry

Nuclei for flow cytometry were prepared as described previously [19]. For conventional flow cytometric cell cycle analysis the nuclei were stained with propidium iodide (50 μg/ml, Sigma) and RNase (0.1 mg/ml, Sigma Type 1A) for at least 30 min. The samples for determination of growth fraction were stained with mithramycin (20 μg/ml, Mithracin®, Pfizer) and $MgCl_2$ (25 mM) [20]. Stained chicken and trout erythrocyte nuclei were used as internal DNA reference. Further details of the cell cycle analysis are described in [19]. The flow cytometer used was a Becton Dickinson FACS IV Cell Sorter with a Spectra Physics 5W argon laser (488 nm, 400 mW for propidium iodide, 457 nm, 100 mW for mithramycin). The C.V. of the G1 peak was 2.5–3.1 with propidium iodide and 3.7–5.3 with mithramycin.

Statistics

The number of nuclei analyzed in each sample was greater than 20,000. The phase fractions of the DNA histograms were estimated by fitting the observed distributions of fluorescence (deconvolution) by maximum likelihood as described earlier [19].

Receptor determinations

Near confluent cultures grown for 1 week in the respective media were harvested and cytosol and nuclear extract prepared as described in [13]. Free estrogen and free progesterone receptor were determined by the dextran charcoal technique [21]. Filled nuclear estrogen receptors were determined by the hydroxylapatite assay described by Garola and McGuire [22] with the small modification that the nuclear extract obtained after the ultracentrifugation was diluted three times to avoid salt effects on the binding of hormone to receptor and on the binding of receptor and receptorcomplex to hydroxylapatite.

RESULTS

In our laboratory the human breast cancer cell line MCF-7 is routinely propagated in medium with 0.5% FCS. Under these culture conditions no growth stimulation can be obtained by addition of estradiol [13]. If MCF-7 cells are transferred to medium with 10% NCS or NCS is added to the cultures with 0.5% FCS, the doubling time is increased from 24 hr to 48 hr or 36 hr (Fig. 1). Cells grown with 10% NCS + 10^{-8} M estradiol have a doubling time of 22 hr (Fig. 1) but the growth rate of the estrogen stimulated culture does not exceed the growth rate in the control cultures with 0.5% FCS. It should be noticed that the cultures with rapid growth rate achieve a much higher cell density than the cultures with 10% NCS.

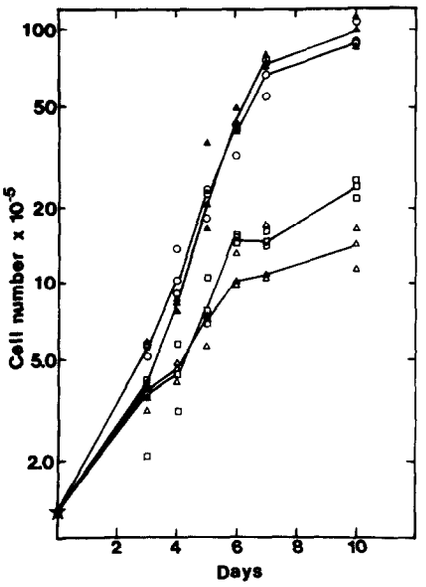

Fig. 1. Growth curves for cultures grown with 0.5% *FCS* (○), 10% *NCS* (△), 10% *NCS* + 0.5% *FCS* (□) *and* 10% *NCS* + 10^{-8} M E_2 (▲). *The lines are drawn between median cell number of three flasks. The cell number at day* 0 (+) *is the number of cells seeded per flask.*

A cell cycle analysis of the growth of cultures in the presence of 0.5% FCS, 10% NCS, 0.5% FCS + 10% NCS and 10% NCS + 10^{-8} M estradiol has been performed. Samples for flow cytometry were prepared at day 3, 5, 7 and 10 of the experiment. Representative DNA histograms from day 3 are shown in Fig. 2. The percentage of cells in the different phases of the cell cycle has been estimated and Fig. 3 shows the result. It may be seen that the distribution of cells in the different phases of the cell cycle in the cultures which are growth stimulated by estradiol is very similar to the distribution of cells in the control cultures with 0.5% FCS. The cultures with 0.5% FCS + 10% NCS and 10% NCS alone have more cells in the G1 phase and a lower percentage of cells in the *S* phase than control cultures and cultures with 10% NCS + 10^{-8} M estradiol.

Differences in the distribution of cells in the different phases of the cell cycle can be due to changes in the transit times, the growth fraction or a selective cell loss. The cell loss in these experiments is negligible as judged by phase contrast microscopy, assuming that dead cells do not escape visual detection within 1 day. We have determined the growth fraction by use of the bromodeoxyuridine – mithramycin technique described earlier [19]. We found that during exponential growth (day 3–6) the growth fraction in cultures with 10% NCS and 10% NCS + 0.5% FCS is 92% and 91% respectively, whereas the growth fraction in cul-

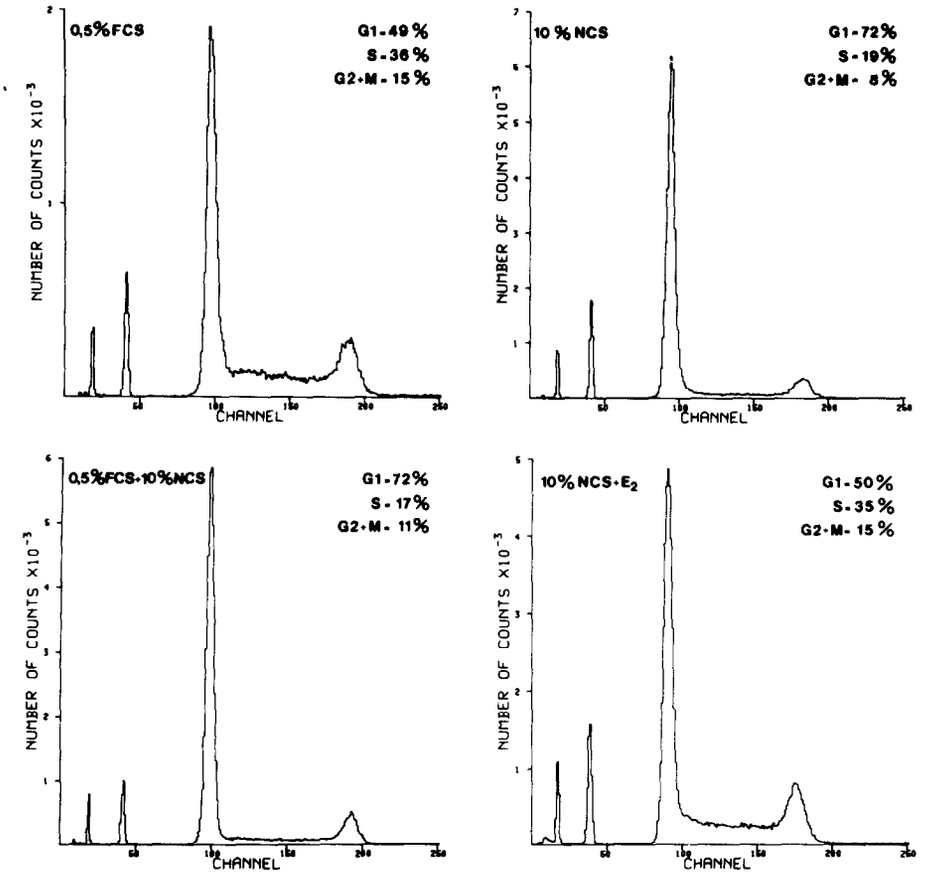

Fig. 2. DNA histograms from cells harvested at day 3. *The DNA was stained with propidium iodide and measured by flow cytometry with chicken and trout erythrocyte nuclei as internal reference.*

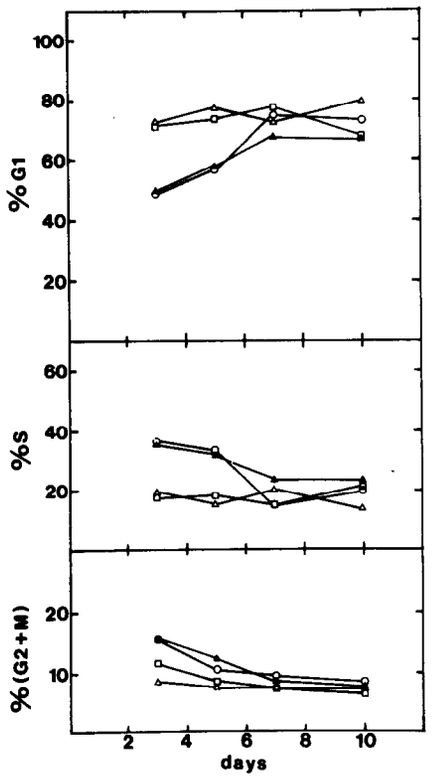

Fig. 3. Cell cycle parameters of cultures grown with 0.5% *FCS* (○) 10% *NCS* (△), 10% *NCS* + 0.5% *FCS* (□) *and* 10% *NCS* + 10^{-8} M E_2 (▲). *The proportion of cells in the different phases of the cell cycle is calculated from DNA histograms as presented in Fig.* 2.

Table 1. *Estrogen and progesterone receptor content in MCF-7 cells grown with* 0.5% *FCS*, 10% *NCS and* 10% *NCS* + 10^{-8} M *estradiol*

	Cytosol		Nuclear extract
Culture medium	Free ER*	Free PgR*	Filled ER*
0.5% FCS	348 ± 152	44 ± 17	~10 †
10% NCS	213 ± 5	<10	<10
10% NCS + 10^{-8} M E_2	<10	849 ± 308	290 ± 36

* Fmol per mg protein ± S.D.
† Close to the detection limit
Near confluent cultures of MCF-7 cells grown for 1 week with 0.5% FCS, 10% NCS or 10% NCS + 10^{-8} M E_2 were harvested and cytosol and nuclear extract were prepared as described in Materials and Methods. Free estrogen and free progesterone receptors were determined by the dextran charcoal technique [20], and filled nuclear estrogen receptors determined by the hydroxylapatite technique [21]. The numbers in the table are the average of three receptor determinations ± S.D. The amount of filled nuclear estrogen receptors in cells grown with 0.5% FCS is indicated as being close to the detection limit of 10 fmol/mg protein since two experiments gave values below the detection limit and one experiment gave 38 fmol/mg protein.

tures with 0.5% FCS or 10% NCS + 10^{-8} M estradiol is 98%.

We have determined the estrogen and progesterone receptor content and distribution in MCF-7 cells grown in presence of 0.5% FCS, 10% NCS and 10% NCS + 10^{-8} M estradiol, Table 1. The majority of the estrogen receptors in cultures grown with 0.5% FCS is found as free receptors in the cytosol, however a small amount of filled nuclear receptors are also determined. The progesterone receptor content is low but significantly above the detection limit of 10 fmol per mg protein. Cells grown with 10% NCS contain free estrogen receptors, no free progesterone receptors and no filled nuclear estrogen receptors whereas cells propagated with 10% NCS + 10^{-8} M estradiol have no free estrogen receptors, a high amount of free progesterone receptors and filled nuclear estrogen receptors.

DISCUSSION

We have previously shown that MCF-7 cells adapted to growth in FCS from 5 to 0.05% cannot be growth stimulated by estradiol [13]. However, when the MCF-7 cells are grown in high concentration of estrogen-poor serum, estradiol can stimulate the growth considerably [7, 8, 16]. The growth stimulation is an annullation of the effect of inhibitory activity in serum [8, 16]. In this paper we present a cell cycle analysis of the effect of the inhibitory activity in NCS as well as the effect of estradiol on cultures grown with NCS. An inhibitory effect of NCS in the presence of 0.5% FCS excludes that the inhibition is due to lack of growth factors in NCS and demonstrates the presence of inhibitory activity in NCS. The slightly higher growth rate obtained by the addition of 0.5% FCS to 10% NCS may be ascribed to the presence of small amounts of estrogens in FCS as indicated by the presence of free progesterone receptors and filled nuclear estrogen receptors in cultures grown with 0.5% FCS (Table 1). The growth stimulation by estradiol is demonstrated by the decrease in doubling time from about 48 hr for the cultures grown with 10% NCS to about 22 hr in cultures grown with 10% NCS + 10^{-8} M estradiol. Since the growth rate in the estrogen stimulated cultures does not exceed the growth rate in cultures grown with low FCS concentration, these results support our earlier observation that estradiol stimulation occurs through an annullation of the effect of inhibitory activity in serum [16, 23].

The cell cycle analysis showed that addition of 10% NCS to cultures grown at 0.5% FCS increased the proportion of cells in the G1 phase of the cell cycle. A similar increase in the G1 fraction is observed when MCF-7 cells are treated with the

antiestrogen tamoxifen (10^{-6} M), and this increase was found to be due to a decrease in the growth fraction [19]. The present work revealed only a small difference in the growth fraction between the rapidly growing cultures and the cultures which were growth inhibited by NCS. This small difference in growth fraction cannot alone account for the great difference in doubling time. We therefore assume that high NCS concentration reduces the growth rate by increasing the transit time in the G1 phase of the cell cycle rather than by decreasing the growth fraction. Correspondingly, the estradiol stimulation occurs through a decrease of the G1 transit time. It is a general observation that cultures with a long generation time have a high G1 phase fraction, and Gross *et al.* [24] have recently shown that insulin stimulation of growth of MCF-7 cells is accompanied by a decreased G1 transit time.

The measurements of estrogen and progesterone receptor content and the distribution of estrogen receptors within the cells grown with 10% NCS and 10% NCS + estradiol indicate that cells grown on 10% NCS proliferate without an activated estrogen receptor mechanism since all estrogen receptors are determined as free receptors in the cytosol and no progesterone receptors can be determined. Addition of estradiol results in the presence of only filled nuclear estrogen receptors and induction of progesterone receptor, synthesis, demonstrating that cells under these growth conditions proliferate with an activated estrogen receptor mechanism. The receptor determinations on cells grown with 0.5% FCS indicate that a fully activated estrogen receptor mechanism may not be a prerequisite for rapid growth since these cells grow rapidly with a minimal activation of the estrogen receptor mechanism. However, cells which are growth inhibited by high concentration of NCS may require an activated estrogen receptor mechanism to achieve rapid growth since we have previously found that only estrogen receptor positive human breast cancer cell lines grown with 10% NCS can be growth stimulated by estradiol [16]. By use of a tamoxifen resistant subline of MCF-7 cells, the AL-1 cell line, we have further indicated that only cells with a functional receptor mechanism can be growth stimulated [16].

The present cell cycle analysis has revealed that the growth stimulation occurs through a shortening of the transit time in the G1 phase of the cell cycle, and we assume that the functional estrogen receptor mechanism results in production of growth factors which annul the effect of the inhibitory activity in NCS. Furthermore, estrogen stimulated cells grow to a much higher cell density (Fig. 1) indicating that the estradiol stimulation also trigger the cells to grow to an increased total cell number. The increase in total cell number may be due to lack of density inhibition in the estrogen stimulated cells since cells grown with 10% NCS tend to grow in monolayer whereas cells grown with 10% NCS + estradiol pile up upon one another and grow in multilayers.

The growth of MCF-7 cells in athymic mice requires estrogen stimulation. The shortening of the cell cycle time as well as the increased ability to grow without density inhibition may be essential for the tumor growth. Whether estrogen dependent growth of human breast cancer is due to both a shorter cell cycle time and escape from density inhibition remains to be elucidated.

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