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# Relationship between Proliferative Activity and Cellular Hormono-dependence in the MCF-7 Breast Cancer Cell Line*

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Research on kinetic and hormonal features of breast cancer has led to the development of indices which either reflect accurately the prognosis (incorporation of tritium labelled thymidine) or predict the response to hormonal treatment (presence and concentration of estrogen and progesterone receptors). However, the relationship between cellular proliferation and tumour hormono-dependence has been little studied so far. We describe this relationship in the hormone-dependent MCF-7 cell line cultured in monolayers in MEM + 10 % FCS or MEM + 10 % FCS (s). We have found that: 1) cellular proliferation and estrogen or progesterone receptor concentration were mutually dependent, the greatest estradiol binding capacity was obtained in cells in which mitotic activity had been slowed down ($G_0/G_1$) by the antiestrogenic action of hydroxytamoxifen added to the culture; 2) the presence of estradiol in the culture medium induced marked changes in the synthesis and catabolism of estrogen and progesterone receptors; and 3) both receptors acted as functional proteins whose intracellular concentrations varied depending on the phases of the mitotic cycle.

Key words: Cellular proliferation, Hormono-dependence, MCF-7 cell line.

In recent years, breast cancer biology has been the subject of comprehensive studies resulting in the use of the tumour's kinetic features and hormono-dependence characteristics either to establish the prognosis or to predict the response to treatment (4, 16, 19, 20, 30). The $^3$H-thymidine labelling index (TLI), determined *in vitro* on tumour samples, has been found to correlate significantly with tumour be-

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havior (20, 21, 29). Together with axillary status, TLI is also a major predictive factor of clinical course, although no mutual dependence between these two indicators has been found (20). Also, estrogen receptor (ER) phenotype appears to be a predictor of clinical course. Despite claims to the contrary (1, 5), our findings as well as those of other groups indicate that overall, patients with ER (+) breast cancer have longer survival times and disease free intervals than patients whose tumours are ER (−) (1, 5, 10, 13, 18).

The value of receptor phenotype as a prognostic factor appears to be related to the rate of tumour cell proliferation (20, 23, 26). Undifferentiated tumours with rapid growth kinetics (high TLI index) are more frequently ER (−) than slowly proliferating, well-differentiated tumours (22). Thus the evidence to date supports the assumption that the parameters used to define a tumour's cellular kinetics and hormone dependence should allow us to characterize it in biological terms. The experiments described in this paper were intended to investigate if *in vitro* ER and progesterone receptor (PgR) levels in cultured breast cancer cells are related to cellular growth rate and cell cycle phase.

## Materials and Methods

*Chemicals.* — The following chemicals were used in all experiments: a) 2,4,6,7-$^3$H-17-β-estradiol (Amersham) with a specific activity between 85 and 110 Ci/mmol and a radioactive concentration of 1 mCi/ml; b) 17-α-methyl-$^3$H-promegestone (New England Nuclear), with a specific activity between 77 and 101 Ci/mmol and a radioactive concentration of 1 mCi/ml; c) 17,β-estradiol (Sigma); d) 17,α-methyl-promegestone (Russel-UCLA); e) hydroxytamoxifen (OH-TAM) (ICI-Farma); f) methyl-$^3$H-thymidine ($^3$H-TdR) (Amersham).

*MCF-7 cell line.* — The experimental model used (ER(+) breast cancer cell line) was the MCF-7 cell line, established by SOULE *et al.* (27) from a menopausal patient with metastatic ductal infiltrating carcinoma. In addition to the presence of ER, which enables cells to express metabolic and structural changes in response to estrogens (2, 17), the MCF-7 cell line is positive for PgR as well as a number of other hormone receptors (12).

The MCF-7 cells were maintained in monolayer cultures (Costar T 25 flasks) in minimum essential medium with Earle's salts (MEM) supplemented with 10 % heat inactivated fetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin and 40 µg/ml gentamycin). All cultures were maintained in a humidified atmosphere of 95 % air and 5 % $CO_2$ at 37 °C. In some experiments, MEM was supplemented with heat inactivated FCS treated with charcoal dextran to remove endogenous estrogen ($FCS_{(s)}$). Cell population growth was assessed by DNA determination using the BURTON's method (3).

*Cell synchronisation procedure.* — A cellular suspension in MEM+FCS (1.5 ml aliquots containing 2-3 × $10^4$ cells/ml) was seeded on Petri dishes (32 mm in diametre). After allowing 24 h for cells to attach and begin exponential growth, the medium was supplemented with 0.5 ml of MEM + FCS and 4 × $10^{-7}$ M OH-TAM was added. This was followed by incubation at 37 °C for 72 h, a period constituting the initial phase of treatment in all experiments performed in the present study.

*Incorporation of tritiated thymidine.*— Tritiated thymidine incorporation was studied in cells released from the antiestrogenic action of OH-TAM at different times (0, 6, 12, 18, 24 and 30 h) after rescuing with MEM + FCS or MEM + $FCS_{(s)}$. The nutrient medium was discard-

ed and the cells were incubated with a solution of $^3$HTdR (1 μCi/ml) in MEM for 1 h, followed by washing in saline. The cellular monolayer was then treated with 5 % trichloracetic acid (TCA) for 30 min at 4 °C. Washing in saline and treatment with TCA were repeated twice, after which the monolayer was washed in absolute ethanol to remove DNA-unbound-$^3$HTdR and fix the cells to the support layer. Finally, the cells were treated with 1 N NaOH for 45 min at 37 °C to solubilize the residual cellular material. An aliquot of this solution, neutralized with 1 N ClH, was analysed in a scintillation counter (Beckman LS2800) with 4 ml Normascint scintillation fluid (Scharlau). Each experiment was performed in quadruplicate.

*Hormone receptor assays ($E_2$ and Pg in intact cells).*— After removing the OH-TAM, intracellular levels of estradiol and progesterone receptors were determined at the same intervals as mentioned above in intact cells (24) rescued with MEM + FCS or MEM + $FCS_{(s)}$. After discarded the nutrient medium, ER or PgR levels were determined (6, 24, 31) by adding five concentrations of labelled hormones in MEM to each dish: $5 \times 10^{-9}$ to $5 \times 10^{-10}$ M. Nonspecific binding was determined in a parallel experiment involving the addition to the labelled hormone solution of a 500-fold excess of cold hormone. Each concentration was assayed in quintuplicate. After incubation for 1 h at 37 °C the radioactive solution was discarded and the cells were washed with cold saline. To extract incorporated hormones, cells were treated with 2 ml absolute ethanol for 20 min at room temperature. An aliquot of the resulting solution was counted in a Beckman LS-2800 scintillation counter with 4 ml Normascint (Scharlau). The data were Scatchard transformed and these values were used to calculate maximum binding ($B_{max}$: fmol/μg DNA) and the association constant (M/l).

## Results and Discussion

*Incorporation of tritiated thymidine.*— The uptake of $^3$HTdR by MCF-7 cells treated for 72 h with OH-TAM and subsequently rescued by replacing the nutrient medium is plotted against time in figure 1. Once released from the antiestrogenic action of OH-TAM, changeover of the medium was sufficient to trigger the growth of cells arrested in $G_0/G_1$ (28). Maximum $^3$H-TdR was observed within 18-24 h after replacing the medium, indicating that the synchronised cell fraction was entering the period of DNA synthesis. Minimum incorporation resulted 30 h post-rescue, coinciding with the $G_2$/M phase of the mitotic cycle.

*Quantification of estrogen and progesterone receptors on synchronised MCF-7 cells.*— Estrogen and progesterone recep-

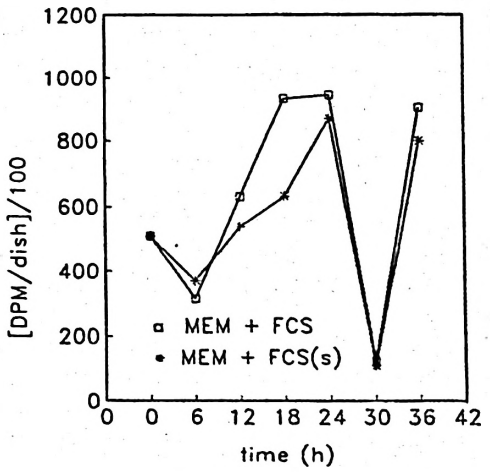


Fig. 1. *$^3$H-Thymidine incorporation in partially synchronised MCF-7 cells.*
Cells were maintained for 72 h in medium containing $10^{-7}$ M OH-TAM and then rescued with MEM supplemented either with 10 % FCS or 10 % $FCS_{(s)}$. At indicated time points cells were incubated with $^3$H-TdR for one hour.

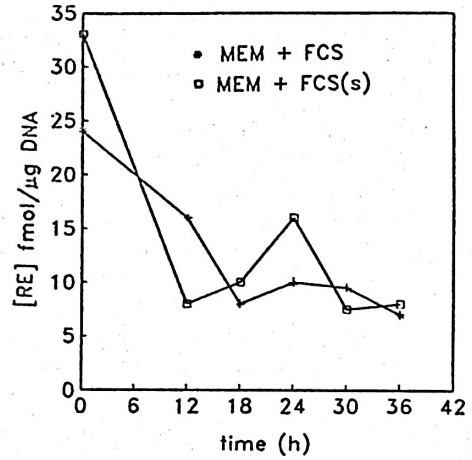


Fig. 2. *Estrogen receptor concentration in partially synchronised MCF-7 cells.*
Cells were maintained for 72 h in medium containing $10^{-7}$ M OH-TAM and then rescued with MEM supplemented either with 10 % FCS or 10 % $FCS_{(s)}$. At indicated time points cells were assayed in quadruplicate dishes for both total and non specific binding using five concentrations of tritiated estradiol and 500-fold excess of cold competitor. The concentration of specific ER binding sites was determined by Scatchard analysis.

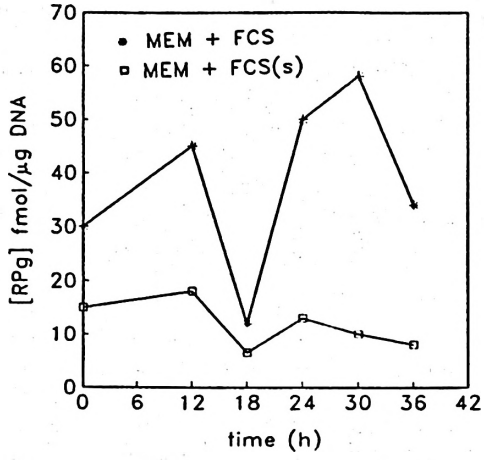


Fig. 3. *Progesterone receptor concentration in partially synchronised MCF-7 cells.*
Cells were maintained for 72 h in medium containing $10^{-7}$ M OH-TAM and then rescued with MEM supplemented either with 10 % FCS or 10 % $FCS_{(s)}$. At indicated time points cells were assayed in quadruplicate dishes for both total and non specific binding using five concentrations of tritiated promegestone and 500-fold excess of cold competitor. The concentration of specific PgR binding sites was determined by Scatchard analysis.

tor levels were measured after medium changeover in accordance with the following sequence: a) Most cultured cells in $G_0/G_1$ (0 h). b) Synchronised cell population in $G_1$ (6-12 h). c) Cells start DNA synthesis (18 h). d) Cells near the end of DNA synthesis (24 h). e) Cells in transition from $G_2$ to M (30 h). f) New mitotic cycle begins (36 h).

Figures 2 and 3 show the specific binding capacity of steroid hormones to MCF-7 cells from the start of the recruitment experiment, 72 hours after OH-TAM treatment. Intracellular concentrations of estradiol and progesterone receptors clearly fluctuated throughout the mitotic cycle.

After synchronisation with $10^{-7}$ M OH-TAM in whole FCS for 72 h, estradiol receptor concentration was highest in cell cultures at the moment of rescue, i.e. when most cells were in phase $G_0/G_1$ (fig. 2), characterised by slow growth kinetics. At this time the cultures can be assumed to contain a large proportion of resting cells. Upon renewing the medium, the cells entered the mitotic cycle, and ER levels fell rapidly to minimum values within approximately 18 h after rescue in our experiments. One implication of these findings is that the beginning of division in resting cells requires the degradation of the receptor molecule. When cells reached the DNA synthesis phase (18-

24 h after medium changeover), receptor levels rose slightly. Finally, between 30 and 36 h after rescue, maximum binding capacity once again fell.

In experiments with $FCS_{(s)}$ (fig. 2), the curve of ER concentration versus time, although similar in shape to that obtained in cultures with whole FCS, showed a higher peak 24 h postrescue. After ER consumption in the course of the metabolic processes carried out in preparation for the synthesis phase, intracellular levels of ER increased notably, reaching maximum values at the end of S phase. During $G_2$/M, receptor levels declined. The fundamental difference between the two experiments was treatment of FCS with charcoal dextran in the second to remove endogenous estrogen. Hence the different shape of the two curves could be attributed to the presence or absence of hormone in the culture medium.

The intracellular level of ER is thought to be the factor which regulates the cell's sensitivity to this hormone (15). Our findings offer an explanation, on a molecular basis, for the proliferative response of OH-TAM treated MCF-7 cells to medium changeover. After 72 hours of culture in MEM + FCS with $10^{-7}$ M OH-TAM, the cells presented maximum levels of ER, possibly as a result of a change in the rate of receptor degradation. Peak amounts of ER per cell were recorded when OH-TAM was removed from the culture medium, while estradiol in the FCS or added to the nutrient medium (32) led to hyperactivity as defined by the increase in the growing fraction together with a drop in cellular doubling time (DT) of the population. In clinical terms this may explain some of the recent findings in ECOG (25) and NSABP groups (8), which suggest that in breast cancer patients, tamoxifen treatment withdrawal is associated with a rise in relapse rates.

Moreover, estrogen receptor is known to be synthesised and degraded extremely rapidly; in the presence of estradiol, receptor degradation is accelerated (7). The prevalence of degradation over synthesis in the first phase of our experiments may explain the initial drop of the curve. During DNA replication however, the relationship between the rates of synthesis and degradation must at some point become inverted, so that the net result is a considerable rise in the intracellular level of ER.

The changes in intracellular levels of PgR, both in FCS and $FCS_{(s)}$ cultured cells (fig. 3), followed similar patterns. Upon entering the mitotic cycle, both cultures showed increases in PgR levels, suggesting that PgR is synthesised during the period immediately following release from the antiestrogenic effects of OH-TAM. Progesterone receptor is likely to fulfil its metabolic role during the period which precedes the DNA synthesis phase, as levels of this receptor fell dramatically between 12 and 18 h and again between 30 and 36 h postrescue. In contrast, PgR synthesis predominates in the S phase of the mitotic cycle. PgR synthesis in cultures with estrogen depleted FCS, although showing an overall pattern similar to that seen in whole FCS cultures, was less intense, owing most likely to the lack of estradiol in the medium. This result is unsurprising given that PgR is one of the products of estrogen activity (9, 11).

Our findings have the following implications: 1) cellular proliferation and estrogen or progesterone receptor concentration were mutually dependent, the greatest estradiol binding capacity was obtained in cells in which mitotic activity had been slowed down ($G_0/G_1$) by the antiestrogenic action of hydroxitamoxifen added to the culture; 2) the presence of estradiol in the culture medium induced marked changes in the synthesis and catabolism of estrogen and progesterone receptors; and 3) both receptors acted as functional proteins whose intracellular concentrations varied depending on the phases of the mitotic cycle.

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## Resumen

Se analiza la relación entre proliferación celular y hormonodependencia, utilizando como modelo la línea celular de cáncer de mama humano MCF-7 cultivada en monocapa en MEM + 10 % FCS o MEM + 10 % $FCS_{(s)}$ + antibióticos a 37 °C en atmósfera saturada de humedad con 5 % de $CO_2$. Los resultados demuestran: 1) La proliferación celular y la concentración de receptores de estrógenos y de progesterona son mutuamente dependientes, la mayor capacidad de enlace para estradiol se encuentra en células cuya actividad mitótica se retarda (fase $G_0/G_1$) por la acción antiestrogénica del hidroxitamoxifeno adicionado al medio de cultivo. 2) La presencia o ausencia de estradiol en el medio de cultivo induce fuertes cambios en la síntesis y catabolismo dr los receptores de estrógenos y de progesterona. 3) Ambos receptores actúan como proteínas funcionales y sus concentraciones intracelulares varían dependiendo de las necesidades metabólicas de las células.

Palabras clave: Proliferación celular, Hormonodependencia, Línea celular MCF-7.

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