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INTERACTION OF ESTRADIOL AND HIGH DENSITY LIPOPROTEINS ON PROLIFERATION OF THE HUMAN BREAST CANCER CELL LINE MCF-7 ADAPTED TO GROW IN SERUM FREE CONDITIONS

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Summary : The responsivness of the human mammary carcinoma cell line MCF-7 to estradiol and tamoxifen treatment has been studied in different culture conditions. Cells from exponentially growing cultures were compared with cells in their initial cycles after replating from confluent cultures ("confluent-log" cells). It has been observed that estradiol stimulation of tritiated thymidine incorporation decreases with cell density and that "confluent-log" cells are estrogen unresponsive for a period of four cell cycles in serum-free medium conditions. On the other hand, growth of cells replated from exponentially growing, as well as from confluent cultures, can be inhibited by tamoxifen or a combined treatment with tamoxifen and the progestin levonorgestrel. This growth inhibitory effect can be rescued by estradiol when cells are replated from exponentially growing cultures. The growth inhibitory effect cannot be rescued by estradiol alone (10^{-10} to 10^{-8}M) when cells are replated from confluent cultures. In this condition, the addition of steroid depleted serum is necessary to reverse the state of estradiol unresponsiveness. Serum can be replaced by high density lipoproteins but not by low density lipoproteins or lipoprotein deficient serum. The present data show that estradiol and HDL interact in the control of MCF-7 cell proliferation.

The MCF-7 human mammary carcinoma cell line (1) is a model system for the study of the regulation of breast cancer cell growth by estrogens and antiestrogens. These cells contain high levels of estrogen receptor, are estrogen-responsive and their growth is inhibited by antiestrogens such as tamoxifen (2-5). We have recently reported the adaptation of this cell line to grow in serum free medium on an extracellular matrix produced by bovine corneal endothelial cells (6). In these conditions, cells are still hormone-responsive. We demonstrate in the present report that culture age influences the state of estrogen sensitivity but not the growth inhibitory effect induced by tamoxifen or by a combined treatment with tamoxifen and the progestin levonorgestrel. The

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reversibility of this growth inhibitory effect by estradiol can be clearly demonstrated on cells replated from exponentially growing cultures. In contrast this growth inhibitory effect is not reversed by estradiol on cells replated from confluent cultures and studied in their initial cycles after replating ; estradiol activity is recovered after the addition of steroid-depleted serum in a dose-dependant manner between 1 and 10 %. We also show that the serum can be replaced by high density lipoproteins (HDL), but neither by low density lipoproteins (LDL) fraction nor by the lipoprotein deficient serum (LPDS).

MATERIAL AND METHODS

Adaptation of the MCF-7 cell line to grow in serum free conditons on extracellular matrix has been previously reported (6). Cells were grown in RPMI 1640 (Gibco) supplemented with 2 mM glutamine, 1 µM insulin (Novo Laboratories) and 0.1 µM transferrin (Sigma) ; the culture medium was changed every other day. Cells were harvested with 0.05 % trypsin - 0.02 % EDTA (w/v) in phosphate buffered saline (PBS) from exponentially growing cultures or from confluent T 75 flasks. The tritiated thymidine (^{3}H-thymidine) incorporation studies was performed on cells plated in 35 mm Petri dishes (Nunclon, Roskilde, Denmark ; 8.55 sq cm surface area). Cells were treated with estradiol, tamoxifen (ICI Pharmaceuticals) and levonorgestrel (13-ethyl-17 hydroxy-18,19-dinor-pregn-4-en-20-yn-3-one ; Wyeth-Byla) diluted in ethanol at a final concentration of 0.1 % in the culture medium . At the times indicated in the text, they were pulsed with ^{3}H-thymidine (28 Ci/mmole ; Amersham, Bucks, England) 1 µCi/ml for 1 h ; the plates were then rinced with PBS and cells harvested with 1 ml 0.05 % trypsin -0.02 % EDTA in PBS for counting in a cell counter (Coulter Electronics, Inc.) or precipitated with 1 ml 10 % trichloracetic acid on 0.45 µm Millipore filters for radioactivity counting (Packard Liquid Scintillation Counter). The preparations of charcoal-treated calf serum, calf and human HDL, LDL or LPDS have been previously described (6,7).

RESULTS AND DISCUSSION

As shown in Fig. 1, a 24 h estradiol stimulation of ^{3}H-thymidine incorporation into trichloracetic acid-precipitable material of MCF-7 cells regularly passed from exponentially growing culture was dependant upon cell density. When cells reached confluency, a state of estrogen recalcitrance was clearly apparent. These observations could have been expected since it is known that, as cultures reach confluency, the percentage of G_o-G_1 cells increases (8). However, when MCF-7 cells which had reached confluency were replated at low density (10^4 cells /sq cm), it was observed that these "confluent-log" cells were still unresponsive to estradiol stimulation (10^{-11} to 10^{-8}M) for four cell cycles (Fig. 1). On the other hand tamoxifen (5 x 10^{-7}M) was able to decrease thymidine

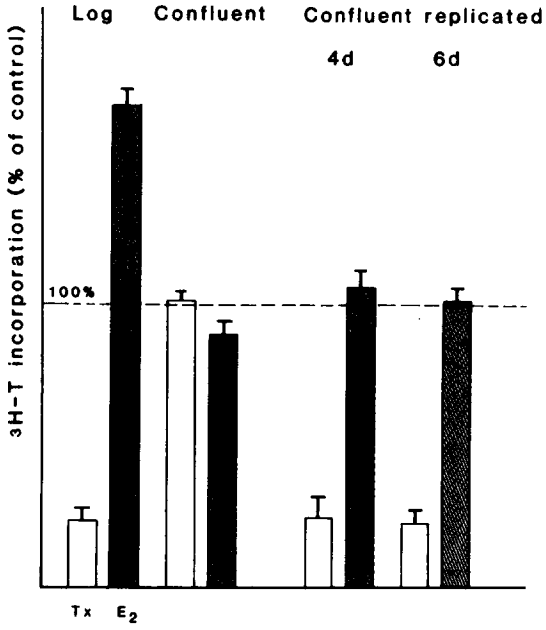

Fig. 1.

Effect of estradiol (10^{-8}M) and tamoxifen (5 x 10^{-7}M) on (^{3}H)-thymidine incorporation into tricholoacetic acid insoluble material of MCF-7 cells. Cells from exponentially growing cultures were studied at low (log) or high (confluent) densities. Cells from confluent cultures were studied after plating at low density (confluent-log). Estradiol or tamoxifen were added in ethanol (0.05 %). Control cultures received ethanol alone. Twenty four hour later, (^{3}H)-thymidine (1 µCi/ml of medium) was added to each dish for 1 h before cell harvesting. Data are expressed as mean ± sd.

incorporation (Fig. 1) and slow down cell multiplication (not shown) of log as well as "confluent-log" growing cells after 4 days treatment. As reported by other authors (8-10), the antiestrogen blocked cell cycle progression in G_1 ; the cell synchronisation was obtained even more completely by a combined treatment with tamoxifen and the progestin levonorgestrel (10^{-8}M) (11). In these conditions cells stopped growing and the incorporation of ^{3}H-thymidine decreased to a plateau, 25 % of the incorporation in untreated cells (11). The serum-free conditions enabled us to study the respective effect of estradiol and serum on the growth rescue of these tamoxifen-levonorgestrel treated cells. To prevent the effects of unspecific binding by serum proteins, the concentration of tamoxifen was increased to 10^{-6}M when serum was added to the culture medium. In exponentially growing cells, the cell cycle block could be reversed by incubation with 10^{-8}M estradiol, resulting in a synchronized cohort of cells progres-

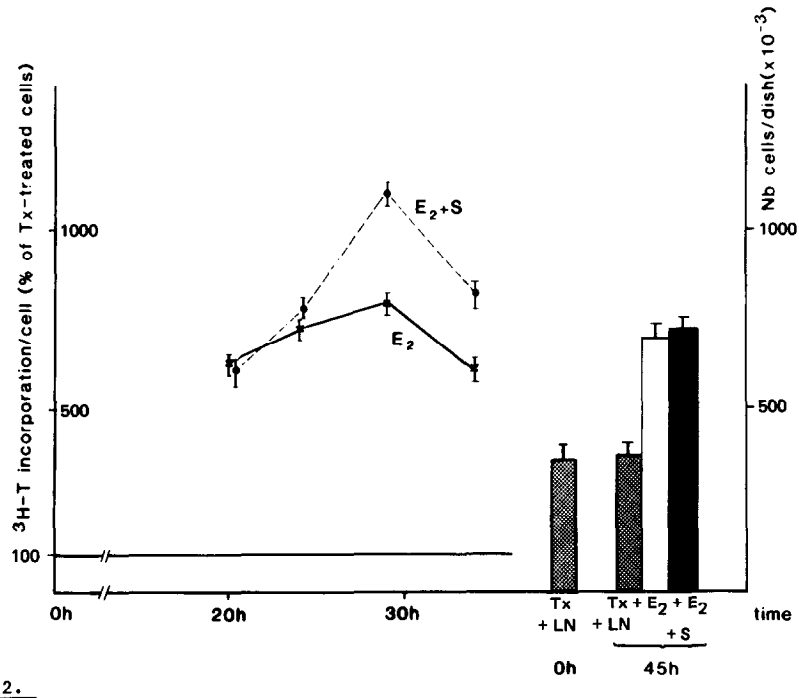

Fig. 2.

Effect of estradiol on (^{3}H)-thymidine incorporation and cell counts of exponentially growing MCF-7 cells treated with tamoxifen and levonorgestrel for 4 days in serum free conditions on extracellular matrix. Exponentially growing cells were plated in 35 mm Petri dishes at low densities (5 x 10^4 cells/dish). Twenty four hours later, the medium was changed and 5 x 10^{-7}M tamoxifen and 10^{-8}M levonorgestel were added in ethanol (0.05 %). Four days later, estradiol (10^{-8}M in 0.05 % ethanol) $\pm$ 4 % steroid-depleted calf serum was added and (^{3}H)-thymidine incorporation and cell counts were measured at times indicated.

sing through the S phase, with a peak of ^{3}H-thymidine incorporation measured 28-30 h, and a doubled cell count measured 45h after estradiol addition (Fig. 2). The addition of 4 % steroid-depleted calf serum resulted in a significant increase of ^{3}H-thymidine incorporation after 29h, but did not change the general figure of this parameter as a function of time. In contrast (fig. 3), "confluent-log" cells were not rescued by estradiol alone (10^{-11} to 10^{-8}M) during the time period of the study (45 h). A progressive increase of ^{3}H-thymidine incorporation was noted with a significant increase in cell number after 86h, which resulted eventually in a late growth rescue. The addition of 4 % steroid-depleted calf serum gave variable results. Some of these batches resulted in a significant increase of ^{3}H-thymidine incorporation after 29 h, and in cell number after 86 h ; in this last condition, cell growth became arrested

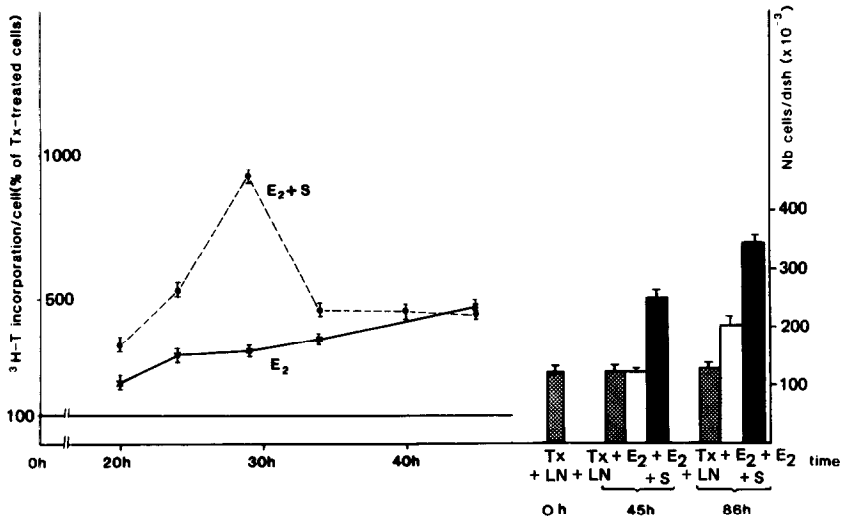

Fig. 3.

Effect of estradiol and steroid-depleted calf serum on (^{3}H)-thymidine incorporation and cell counts of "confluent-log" MCF-7 cells treated for 4 days with tamoxifen and levonorgestrel. Conditions are as described in the legend of Fig. 2 except that "confluent-log" cells were plated from confluent cultures.

again after one new cell generation. Other batches were inefficient. This variability may have been due to the incomplete removal of estrogens during the dextran-coated charcoal treatment (12). In all cases however, the simultaneous addition of steroid-depleted serum and estradiol (10^{-8}M) demonstrated a permissive effect of serum over the estradiol activity (Fig. 4) in a dose dependant

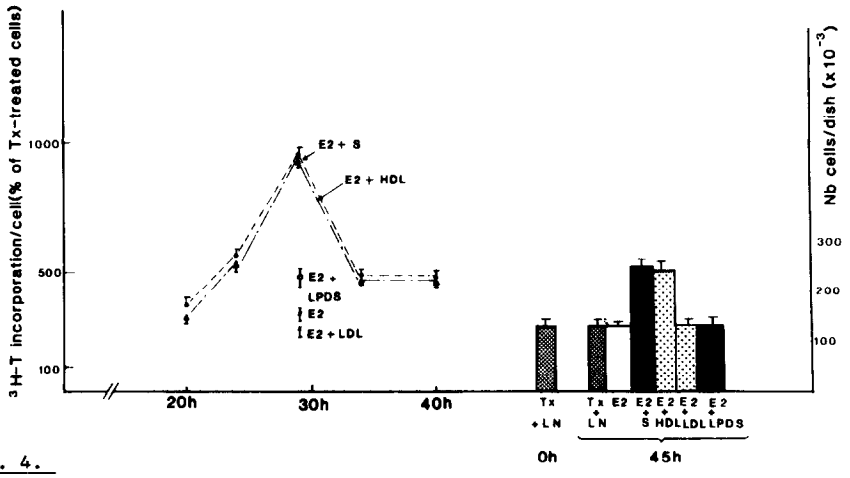

Fig. 4.

Effect of estradiol and HDL on tritiated-thymidine incorporation and cell counts of "confluent-log" MCF-7 cells treated for 4 days with tamoxifen and levonorgestrel. Conditions are as described in the legend of Fig. 2 and 3 except that estradiol (10^{-8}M) was added in absence or presence of serum (4 %), human HDL (500 µg protein/ml), human LDL (200 µg protein/ml) or calf LPDS (4%).

manner between 1 and 10 % (not shown). In the presence of estradiol and serum, cell growth would continue to reach cell confluency.

In an attempt to define the serum component(s) able to rescue the cell growth and to enable a permissive effect to the estradiol action, factors shown to influence the growth of breast cancer cells (13-16) were tested. Insulin and transferrin (components of the serum free medium), prolactin (1 µg/ml), Dexamethasone (10^{-8}M), L-triiodothyronine (10^{-8}M), EGF (10 ng/ml), FGF (10 ng/ml), alone or in combination, were ineffective. On the other hand calf and human HDL (10-900 µg protein/ml) were able to rescue cell growth and enable a permissive effect to the estradiol action (Fig. 4 and 5). In the same experiments human LDL were not active (Fig. 5) and appeared to be partially toxic to the cells ; calf LPDS was very partially active (Fig.5). Human LPDS was found also to be somehow toxic, in agreement with a recent report by other authors (17).

We, therefore conclude that the estrogen responsiveness of the thymidine incorporation in these cells is dependant upon the culture age at the time of plating. The characterization, in certain conditions, of a temporary state of

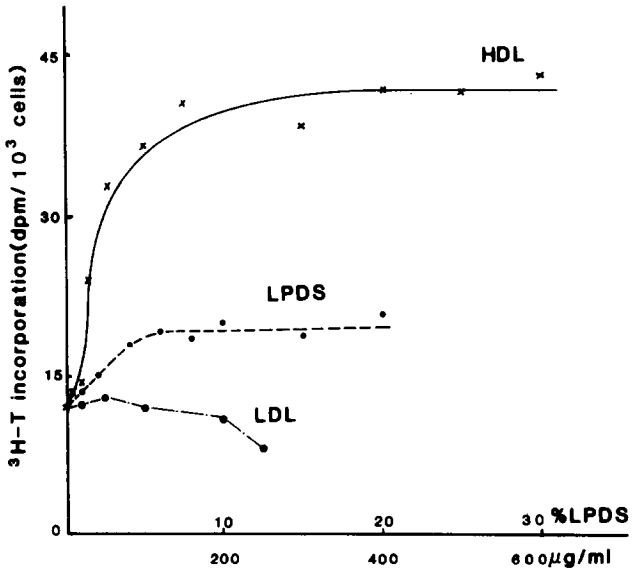

Fig. 5.
Effect of increasing concentrations of human HDL, human LDL or calf LPDS in presence of estradiol (10^{-8}M) on (^{3}H)-thymidine incorporation of MCF-7 cells replated from confluent cultures and treated with tamoxifen and levonorgestrel for 4 days in serum free conditions on extracellular matrix.

estradiol unresponsiveness confirm our previous report (6) and underlines the importance of defining such parameters in this type of study. The activity of tamoxifen, on the contrary is not similarly influenced by cell culture conditions. The present study also confirms the presence in serum of (a) "factor(s)" which can influence expression of the growth response to estradiol (18) and enable the growth rescue of tamoxifen-levonorgestrel inhibited cells; such "factor(s)" is (are) apparently part of the high density fraction of the lipoproteins. These data are in agreement with recent reports which have described the mitogenic action of HDL for different cell types such as granulosa cells, corneal endothelial, vascular endothelial, vascular smooth muscle cells and human tumor cells (7,19,20). They present for the first time indications of an interaction between estradiol and HDL in the control of cell proliferation of the human breast cancer line MCF-7. The molecular mechanisms of this interaction remain now a matter of inquiry.

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