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PULSATILE VERSUS CONTINUOUS ESTRADIOL EXPOSURE IN INDUCING PROLIFERATION OF CULTURED ZR-75.1 HUMAN BREAST CANCER CELLS

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Summary—Estradiol induces proliferation of ZR-75.1 human breast cancer cells cultured as a monolayer using minimum essential medium supplemented with 10% charcoal treated fetal bovine serum. Comparing continuous and pulsatile estradiol treatment we could not observe any amplification or limitation of growth effects when we corrected for the different time of exposure. We could perfectly predict cell number by summarizing growth effects of all estradiol pulses and therefore conclude that estrogen receptor is a "sensor" that measures the time of estradiol exposure in a linear manner whereas the estradiol concentration is recognized in a non-linear fashion as predicted by the law of mass action which governs steroid–receptor interaction.

INTRODUCTION

Approximately 35% of mammary carcinomas are responsive to endocrine manipulation such as castration, adrenalectomy, or administration of antiestrogens. Proliferation of these breast cancer cells is directly influenced by estrogens, the action of which is mediated by the estrogen receptor [1–4]. Estrogen target tissues have been widely studied using cell culture or animal models [5–10]. Human breast cancer ZR-75.1 cells are a good tool for investigating hormone action since they respond reproducibly to estrogen manipulation by changes in cell number [11]. Growth stimulation was observed in serum free culture media [12] or media containing charcoal treated serum [11, 13]. Estradiol effects on breast cancer cells are dose dependent and 1–10 pM estradiol significantly increases the cell number. About 1 nM estradiol is sufficient to attain maximal growth stimulation [11, 14]. In all these experiments estradiol concentrations remained constant and therefore growth effects were observed under steadily state hormonal conditions. On the other hand, in laboratory animals administration of steroids is often performed in a manner that no stable hormone levels are obtained. Direct comparison of experiments under such different hormonal conditions are therefore doubtful unless we know that the fluctuation of the hormone level itself has no additional effect on the hormone action. For some receptors an interesting rate theory has been proposed in which it is suggested that hormone effect is a function not of receptor occupation alone, but of the formation rate of the hormone–receptor-complex [15]. Luteinizing-hormone-releasing hormone for example stimulates the hypophysis only when it is secreted in a pulsatile manner. Continuous administration, however, decreases gonadotropin release [16]. The purpose of this investigation was to determine whether estrogen induced proliferation is not only dependent on estradiol concentrations, but may also be altered by fluctuation in estradiol concentrations. Such a dependence of fluctuation could alter our current view on hormonal therapy, e.g. in breast cancer, or on the interpretation of experiments in laboratory animals.

EXPERIMENTAL

Material

Estradiol-17β was obtained from Sigma (Sigma Chemical Co., St Louis, MO) and a 1 mM solution in ethanol was prepared. Minimum essential medium (MEM), L-glutamine, and non-essential amino acids were from Eurobio, Paris, France. Fetal bovine serum (FBS) was obtained from Seromed (Seromed GmbH, Munich, F.R.G.). Penicillin and streptomycin were obtained from Serva, Heidelberg, F.R.G., and amphotericin B from Arcana, Austria. Culture flasks and plates were purchased from Falcon (Becton Dickinson and Co., Oxnard, CA) or Costar (Cambridge, MA).

Cell culture

Human breast cancer cell line ZR-75.1 originated from ascitic fluid and is well characterized as an estrogen sensitive cell line [17]. These cells were generously supplied by Dr R. J. B. King, Imperial Cancer Research Fund, London. Cells were cultured in MEM supplemented with charcoal treated FBS (10% FBS-CT), glutamine (2 mM), non-essential amino acids (1% v/v), streptomycin (10 μg/ml), penicillin (10 U/ml), amphotericin B (5 μg/ml). Charcoal treatment of FBS as well as the maintenance of a stock culture was performed as described recently [13].

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Estrogen induced growth

Cells from stock flasks were harvested using trypsin (0.05%)–EDTA (0.02%) in saline and then seeded in Nunc 24 well tissue culture plates (Nunc 14 64 85, Roskilde, Dk) as 1 ml suspensions ($1–1.5 \times 10^5$ cells/ml) in MEM 10% FBS-CT. The cells were allowed to attach over night, after which the medium was changed and the appropriate amount of estradiol added. For each experiment a dose response relation with cells treated continuously with 0, 0.01, 0.1, and 1 nM estradiol was performed with 8 wells per group. After 9–12 days of treatment cells were harvested using trypsin–EDTA in saline and enumerated using an electronic particle counter (Coulter Electronics Ltd, Dunstable, U.K.) as described [13]. Simultaneously with these experiments cells were treated with estradiol (0.01–1 nM) in a pulsatile manner. The length of the estradiol pulses varied between 8 h and 6 days. Each exposure to estradiol was followed by two fluid changes with estrogen free medium. Culture dishes with continuously administered estradiol were rinsed in the same manner with medium containing estradiol in the respective concentration. Estradiol induced growth for each concentration was defined as the difference in cell number between the continuously treated (n_1 to n_3) and the control groups (n_0). If we assume as null hypothesis that estradiol fluctuations have no additional effect on proliferation we shall be able to calculate the number of cells in our experimental model by the following formula:

$$\text{Number of cells} = n_0 + \sum_{i=0}^{i=3} (n_i - n_0) \times t_i/T$$

t_0 = time of incubation with 0 nM estradiol
t_1 = time of incubation with 0.01 nM estradiol
t_2 = time of incubation with 0.1 nM estradiol
t_3 = time of incubation with 1 nM estradiol

n_0 = cell number after continuous exposure to 0 nM estradiol
n_1 = cell number after continuous exposure to 0.01 nM estradiol
n_2 = cell number after continuous exposure to 0.1 nM estradiol
n_3 = cell number after continuous exposure to 1 nM estradiol

T means total duration of culture until enumeration. The principle of this formula is the calculation of estradiol induced proliferation under pulsatile estradiol by summarizing the effect of each pulse and adding it to the estradiol independent growth. The effect of each pulse was derived from the estradiol induced growth per time unit. This was obtained from experiments where cells were continuously exposed to estradiol. A daily 8 h pulse of 1 nM estradiol followed by 16 h estradiol free medium should therefore reduce the estradiol induced growth to 1/3 of the effect of 1 nM estradiol administered continuously.

Statistical evaluation

Multiple comparisons were performed by the Kruskal–Wallis [18] one way analysis of variance by ranks or the Dunnet-t-test [19]. Two treatments were compared by the Wilcoxon–Mann–Whitney test. The Wilcoxon matched pairs signed rank test, Pearson correlation coefficient, and linear regression by least square method were used to analyze the relationship between calculated and observed numbers of cells with different estradiol treatments [20].

RESULTS

Estradiol increased the number of ZR-75.1 cells in each experiment in a dose related manner (Figs 1–3). 0.01 nM was always sufficient to provoke significant growth stimulation ($P < 0.01$). In Figs 2 and 3 1 nM estradiol was the most effective concentration while Fig. 1 shows the same growth rate under 0.1 and 1 nM estradiol. This difference may be due to biological variations which are frequently observed in *in vitro* systems. In addition to the estradiol induced proliferation, cell growth was also observed in unsupplemented medium. After an incubation period of 9–12 days cell number in the control group on average was nearly double the original. By decreasing 4 times for 24 h the estradiol concentration from 1 to 0.01 nM (Fig. 1A) or to zero (Fig. 1B) [unsupplemented medium], during a total incubation period of 11 days the cell number was significantly ($P < 0.01$ for both) reduced compared to continuous 1 nM estradiol exposure. Although the mean estradiol concentration was about the same (0.68 nM for A vs 0.64 nM for B), the number of cells was higher in group A than in group B ($P < 0.01$). Calculated values were in good agreement with the observed number of cells (A: −2%; B: −3%). Daily 8 h pulses of 1 nM estradiol over a 0.01 nM estradiol continuous exposure (exp. C, Fig. 2) led to a lower amount of cells than daily 16 h pulses over a 0.01 nM estradiol continuous exposure [exp. D, Fig. 2] ($P < 0.01$). In addition, C and D were statistically distinguishable from the group treated continuously with 0.01 nM estradiol ($P < 0.05$ and $P < 0.01$ respectively). Daily 8 h pulses of 0.01 nM (exp. E) or 1 nM estradiol (exp. F) led to low growth stimulation in good agreement with the calculated number of cells (deviation −4% and +5%). Results of exp. E and exp. F were statistically distinguishable ($P < 0.01$). In the experiments shown in Fig. 3 the calculated and observed number of cells did not agree as well as in the two previous experiments, with deviations from −24% to +13%. With the exception of experiment I (4 days 1 nM estradiol, then 4 days unsupplemented medium and finally 4 days 1 nM), estradiol concentration was changed only once. Interestingly the formula used overestimated the number of cells in all cases where the higher concentration of estradiol was applied in the first half of the incubation period (exp. G: −21%; exp. H: −12%; exp. L: −13%;

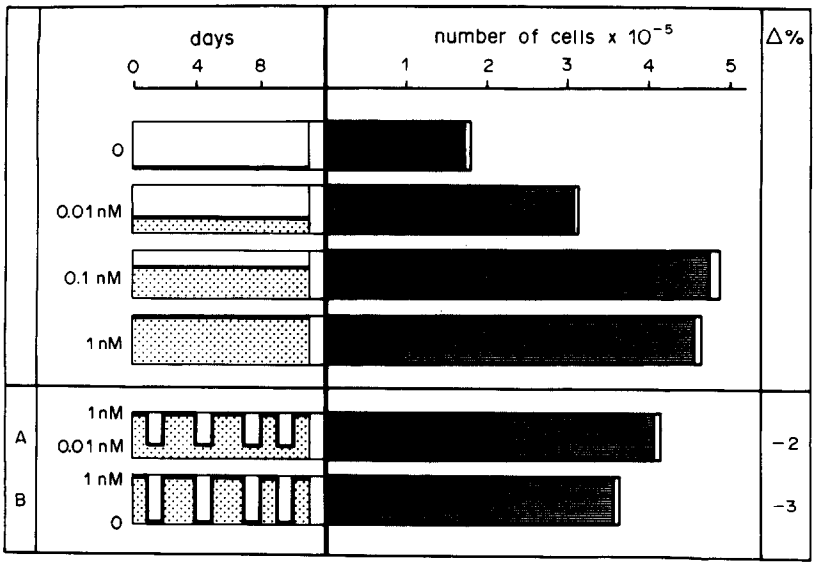

Fig. 1. Growth effects of continuous and pulsatile estradiol treatment on ZR-75.1 cells cultured in MEM 10% FBS-CT. The bars on the left show schematically the manner of incubation. The dotted area represents duration and concentration of estradiol in the medium. The first 4 groups were treated continuously with estradiol (0–1 nM). In experiment A and B cells were treated with 1 nM estradiol for 5 intervals and with 0.01 nM or unsupplemented medium for the four intervening intervals of 24 h. Striped bars on the right represent growth effects and are expressed as the mean cell number of 8 wells counted (+SE) after 11 days of treatment. Percentage of difference between estimated and observed cell number was calculated according to the formula: $\Delta\% = 100 \times (1 - \text{calculated number of cells/observed number of cells})$. The estimation of growth effects was performed as described in Experimental. Constants necessary for calculation, i.e. n_0 and the number of cells for the different estradiol concentrations (n_1 to n_3) were taken from the 4 groups treated with constant estradiol concentrations.

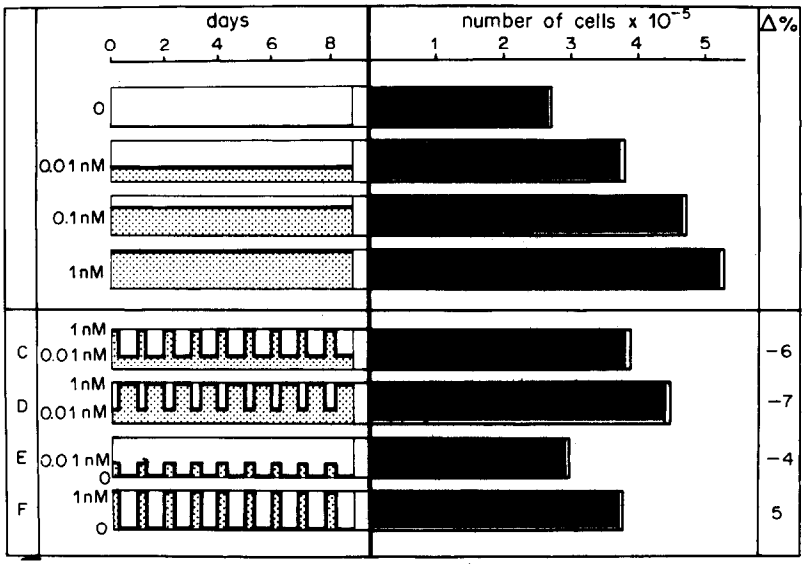

Fig. 2. Proliferation of ZR-75.1 cells under continuous and pulsatile estradiol. Cells for these experiments were cultured for 9 days in the manner indicated by the bars on the left. In experiments C and D cells were treated with 1 nM estradiol for periods of 8 and 16 h respectively after which the medium was changed to 0.01 nM estradiol for 16 and 8 h respectively. This procedure was repeated every day over the full incubation period. Experimental groups E and F were treated daily for 8 h with 0.01 and 1 nM estradiol respectively. Thereafter medium was changed to unsupplemented MEM 10% FBS-CT. For other details see Fig. 1.

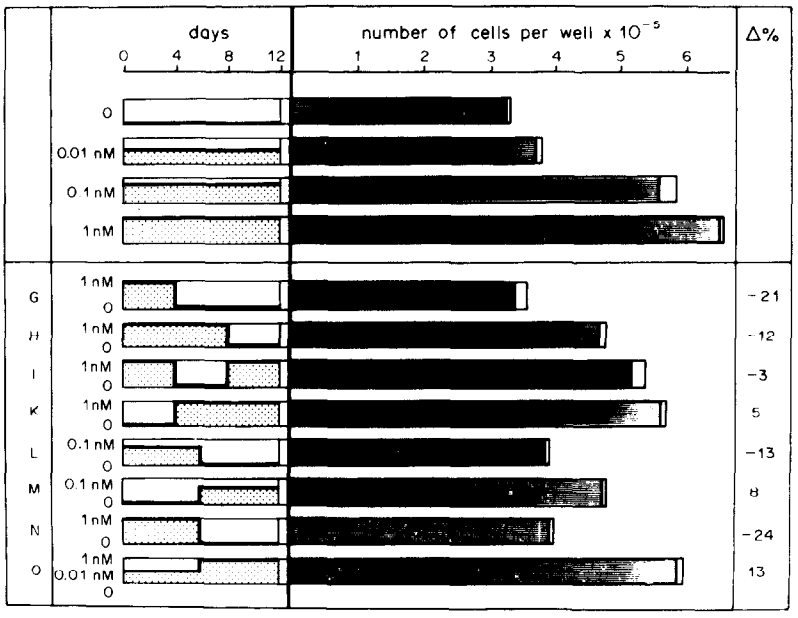

Fig. 3. Growth effects of continuous and pulsatile estradiol administration on ZR-75.1 cells. For these experiments cells were cultured for 12 days. For experiment G 1 nM estradiol was administered only the first 4 days whereas for the remaining 8 days the medium was unsupplemented. During the first 8 days cells were treated with 1 nM estradiol in experiment H. In experiment I cells were treated with 1 nM estradiol from day 0–4 and day 8–12. The growth effect of an 8 day exposure to 1 nM estradiol beginning at day 4 was documented in experiment K. In experiment L and M cells were treated with 0.1 nM estradiol during the first and second half of incubation respectively. For experiment N 1 nM estradiol was administered for the first 6 days, and finally experiment 0 served to demonstrate the effect of a low dose of estradiol (0.01 nM) during the first half of the incubation period followed by a higher dose (1 nM) during the remaining 6 days. For further explanation see Fig. 1.

exp. N: −24%). On the other hand, more cells than calculated were found if the higher estradiol concentrations were administered in the second half of the incubation period (exp. K: +5%; exp. M: +8%; exp. 0: +13%). For the experiments H, I, and K we expected the same cell number but this was not the case as the number of cells differed depending on the period of predominant estradiol exposure. A balanced estradiol administration (i.e. the same time of exposure in the first and second half of the culture period) led to a number of cells between the two other experiments (−3%). In exp. L and M we also expected the same number of cells, but we found a statistically significantly higher proliferation in exp. M where cells were incubated the last 6 days with 0.1 nM estradiol ($P < 0.01$). The number of cells calculated and observed in groups where estradiol concentration was changed at least twice correlated highly significantly ($P < 0.001$) [Fig. 4] and showed a linear function with a slope of about 45°. Median deviation of calculated and observed number of cells was −3% and statistically not significant. All experimental groups with "asymmetric" estradiol treatment deviated more or less from the calculated line (Fig. 4), depending in which half of the incubation period the higher estradiol concentration was applied.

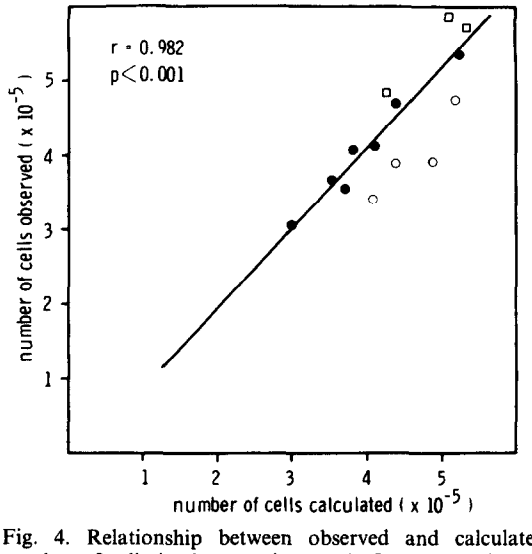

Fig. 4. Relationship between observed and calculated number of cells in the experiments A–O. ● Experiments with more than one change in estradiol concentration (A, B, C, D, E, F, and I). □ Experiments with predominant estradiol treatment in the second half of incubation period (K, M, and O). ○ Experiments with predominant estradiol treatment in the first half of the incubation period (G, H, L, and N). The straight line was constructed by linear regression using the 7 experiments with more than one change in estradiol concentration.

DISCUSSION

Earlier studies have demonstrated that the estrogen receptor is an estradiol "sensor" which works between 1 pM and 1 nM estradiol only. In this range, estradiol concentration is measured by its receptor in a non linear scale because of the intrinsic affinity of the receptor ($K_d = 10^{-10}$–10^{-9} M) and because the steroid receptor interaction underlies the law of mass action. Therefore doubling the estradiol concentration does not lead to a 2-fold growth induction. Our data strongly suggest that duration of estradiol treatment, however, is "measured" linearly. We could not observe any amplification or diminution of growth effects induced by fluctuating estradiol concentrations. In all cases where hormone concentration was changed at least twice, the recovered number of cells was perfectly predicted by summarizing the effects of all estradiol pulses. The good agreement of experimental and estimated values allows us to conclude:

(1) As reported earlier ZR-75.1 cells show estrogen independent growth [13].
(2) Estradiol induces proliferation of ZR-75.1 cells which is dependent on the concentration in a non linear fashion and on the overall duration of treatment in a linear fashion.

The estrogen receptor mechanism rapidly recognizes any changes in ligand concentration exerting an unchanged response. In our system we were unable to observe neither tachyphylaxis nor sensitization by repeated pulses of estradiol. Therefore changes in estrogen receptor content which may occur as a consequence of our treatment schemes do not significantly affect cell response. The estrogen receptor mechanism is not disturbed by pulsatile estradiol administration since the different rhythm tested did not lead to a deviation between calculated and experimental results. Discrepancies between calculated and observed data were only seen in the experiments shown in Fig. 3, where estradiol concentration was changed only once. This may be explained by the growth characteristic of cells cultured as monolayer. After seeding, cells initially undergo a lag-phase with slow proliferation and relative resistance to stimulatory as well as inhibitory agents. Cells growing in the logarithmic phase are highly sensitive to substances affecting proliferation. The formula used to predict the cell number does not account for this difference in estradiol sensitivity but averages the effect during the whole incubation period. Therefore, predominant estradiol administration in the lag phase results in a decreased growth stimulation compared to a calculated one. On the other hand, prevalent estradiol exposure in the log phase should lead to a proliferation superior to the predicted one. In all these cases our model led to the expected discrepancies between calculated and observed number of cells (Fig. 4). We never observed the so called "estrogen-rescue" phenomenon in our experiments which should lead to an underestimation of proliferation in estrogen-"hungry" cells. Estradiol application in exp. K and M (Fig. 3) both resulted in a lower growth rate ($P < 0.01$) than was observed under continuous estradiol exposure.

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