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THE ROLE OF ESTROGENS ON THE PROLIFERATION OF HUMAN BREAST TUMOR CELLS (MCF-7)

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Summary—The cloned human breast tumor cell line C_7MCF7-173 behaved as an estrogen-dependent tumor in the nude mice. In contrast, E_2 added to serum-less media did not increase the multiplication rate of these cells over the values obtained in the control cultures. Media supplemented with charcoal-dextran stripped (CD) human female serum (FHS) resulted in inhibition of cell proliferation in a concentration-dependent pattern (40% = 20% > 10% > 5% > 2.5%). E_2 addition to all but the 2.5% CDFHS significantly increased the proliferation rate of these cells. The E_2 concentration required to attain maximal proliferation rate increased as the serum concentration of the medium increased (e.g. 3×10^{-11} M for 10% CDFHS, 3×10^{-10} M for 40% CDFHS). E_2 concentrations higher than the one needed to achieve maximal proliferation rate resulted in decreased cell yields (shut-off mechanism). Similar effects were obtained with synthetic and other natural estrogens. CD fetal bovine serum (FBS) also inhibited the proliferation of C_7MCF7-173 cells; however, at similar concentration the inhibitory effect of CDFHS was more potent than the one obtained with CDFBS. The addition of "growth factors" (insulin, Epidermal Growth Factor and transferrin) and non-estrogenic steroids to 10% CDFHS failed to overcome the inhibitory effect of this serum. These results suggest that: (1) human and fetal bovine sera contain a specific inhibitor of the proliferation of E_2-sensitive cells (estrocolyones), and (2) E_2 promotes cell proliferation by neutralizing this inhibitor.

INTRODUCTION

The mechanism of estradiol-17β (E_2) action on cell proliferation is poorly understood [1–5]. Researchers in this field are currently following three distinct working hypothesis: (i) the direct-positive hypothesis, according to which E_2 by itself triggers the multiplication of its target cells [3, 4, 6–8]; (ii) the indirect-positive hypothesis, by which E_2 triggers the synthesis and/or release of (a) a growth factor (estromedin) [2, 9] that in turn causes proliferation of the E-sensitive cells; or (b) a facilitating factor (plasminogen activator) [10] that would ease the invasion of the surrounding space by these cells. And, (iii) the indirect negative hypothesis [11] by which (a) E_2 blocks the synthesis and/or release of a specific inhibitor of E_2-sensitive cells secreted by an intermediary organ [12], and/or (b) plasma estrogens may neutralize the action of a putative inhibitor [13, 14]. Compatible with all three working hypotheses, E_2 would limit its own proliferative response by directly inducing the synthesis of an intracellular inhibitor that shuts off the constitutive capacity for proliferation of these target cells [15–19].

We explored this fundamental issue of control of cell multiplication under the direct and the indirect positive hypotheses; the data collected were not entirely compatible with either hypothesis [5, 11, 20, 21]. We therefore explored and subsequently adopted the indirect negative hypothesis based on what we considered increasingly compelling evidence in its favor [11, 22–26]. This paper describes experiments using C_7MCF7-173 human breast tumor cells, and provides evidence for the role of a blood-borne specific inhibitor of cell multiplication on the proliferative response to estrogens.

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EXPERIMENTAL

Cell lines

We obtained MCF7 cells passage 173 from Dr C. McGrath of the Michigan Cancer Foundation, Detroit, MI [27, 28]. The data in this article were obtained using the C_7MCF7-173 clone; however, the uncloned population in culture provided comparable results. Cells were routinely grown in 10% FBS supplemented media in an atmosphere of 5% CO_2/95% air and 100% humidity at 37°C.

KLE cells passage 34, derived from a human endometrial carcinoma, have estrophilins and develop into tumors in nude mice regardless of the E_2 supplementation to these animals [29]. They were generously supplied to us by Dr G. Richardson, Massachusetts General Hospital, Boston, MA.

Cell proliferation experiments in culture

Approximately 10^4 cells/well were plated into Costar 3512 Multiplates in 1% FBS in Dulbecco modified Eagle's media (DME). They were allowed to attach for 24 h and then the seeding medium was replaced by an experimental one. Cells were harvested in triplicate during the exponential phase with a lysing solution [10% Zapoglobin (Coulter Electronics, Hialeah, FL) in 0.5% Triton X-100, 2 mM $MgCl_2$ 15 mM

NaCl 5 mM Phosphate buffer pH 7.4] and counted on a Coulter Counter Apparatus, Model Zf (Coulter Electronics, Hialeah, FL). The results were expressed as cell numbers per well (mean ± SD). Cell multiplication curves were plotted on a logarithmic scale in the ordinate against time in days in the abscisa. The mean generation time (t_D) was used as a measure of the proliferation rate. t_D is the time interval in which an exponentially growing culture doubles its cell number. t_D is calculated from the equation:

$$\alpha = \frac{1}{t} \times \ln \frac{C_t}{C_o}$$

where C_o is the initial cell number, C_t is the cell number at time $= t$ and α is the instantaneous cell proliferation rate constant. t_D is the value ln Co/Ct would take when $C_t = 2\,C_o$. The t_D is expressed in time units, i.e. hours. The slopes of the different growth curves were calculated by fitting the experimental data to a straight line by regression analysis of the pairs ln cell numbers/time [5]. Cell yield measurements instead of proliferation rates were performed when many variables were compared in the same experiment. In this instance, cells were harvested simultaneously in the late exponential phase (11–14 days after seeding).

Experimental media

To test the effect of E_2 on C_7MCF7-173 cells in serum-less medium we used the combination of DME plus Ham's F12 media (1:1) supplemented with Epidermal Growth Factor [EGF] (final concentration, 100 ng/ml) and Transferrin [T] (final concentration, 25 μg/ml) purchased from Collaborative Research Inc., Lexington, MA (Lot No. 83–213 and 83–283, respectively) and Insulin [I] (final concentration 100 ng/ml) [Lot 615–07J–256] graciously donated by Eli Lilly Co., Indianapolis, IN. This serum-free medium [30] was changed every 48 h. To test the effect of human sera we used: (a) sera from healthy cycling women drawn at days-01 and -14 of their menstrual cycle (FHS), and (b) sera from adult healthy men and post-menopausal women. Sera were then filtered through a 0.45 μm pore size Nalgene filter unit and stored in a freezer at −20°C. All sera were heat-inactivated at 56°C for 30 min, unless otherwise indicated in the text. To obtain plasma, blood was drawn into a collecting device containing anticoagulant citrate phosphate dextrose solution, U.S.P. (Fenwal Labs, Division of Travenol Labs Inc., Deerfield, IL 60015). The citrated plasma was defibrinogenated by heat-inactivation (56°C for 30 min), centrifuged, sterilized by filtration, and stored at −20°C.

Removal of sex steroids from serum was accomplished by charcoal–dextran (CD) adsorption (0.5% charcoal–0.05% dextran T70). The extraction was carried out at 37.5°C for 3 h. To monitor the extraction efficiency, comparable volumes of the different sera were equilibrated for 16 h at room temperature with [^{3}H] E_2 or [^{3}H]testosterone at concentrations of 10^{-9} M and 10^{-8} M, respectively, prior to charcoal extraction; 99% of the label was removed by this treatment.

Estrophilin concentrations in C_7MCF7-173 cells

Cells grown in roller bottles were harvested and processed as described elsewhere to determine the presence and characteristics of the estrophilins in these cells [31, 32].

Steroids

E_2 and testosterone were purchased from Calbiochem, San Diego CA; 5α-dihydrotesterone (DHT), progesterone, hydrocortisone, and diethylstilbestrol (DES) were purchased from Sigma Co., St Louis, MO. Moxestrol (R_{2858}) was generously supplied by Dr J. P. Raynaud, Roussell-UCLAF, Romainville, France.

E_2 Concentrations in the different experimental sera

E_2 concentration was determined by RIA using the method described by Goodman [33]. The standard curve ranged from 2 to 20 pg; 50% displacement occurred at 7–8 pg. The water blanks ranged from 1 to 1.5 pg and were substracted from the values obtained with serum samples of similar volume. We used Niswender's antibody 244.

Animal experiments

5×10^6 C_7MCF7-173 cells were inoculated subcutaneously in the interscapular region of nude (nu/nu) mice from a Balb/c background (Charles River Breeding Labs, Wilmington, MA). Mice were checked twice weekly throughout the experiment (8 weeks). Mice were separated in 4 groups: (1) ovariectomized (2) intact (3) ovariectomized implanted with a silastic tube (1 cm in length, 0.025 in i.d., 0.047 in o.d.) filled with E_2–cholesterol (1:10); and (4) ovariectomized mice implanted with a similar size silastic tube filled with E_2 only. The tubes were implanted subcutaneously. The tumor incidence rates in the different experimental groups were subjected to the chi-square test (both in 2×2 and $2 \times k = 4$ configurations).

At the end of these experiments mice were anesthetized with ether, tumors were then excised and fixed in Bouin's fixative to obtain histologic slides for light microscopy. Portions of the uterus of these mice were also processed to establish a correlation between the endometrial status and the tumor histopathology.

RESULTS

E-dependent C_7MCF7-173 cell proliferation in nude mice

Table 1 compares the tumor incidence among the different experimental groups. The stringent estrogen dependence of C_7MCF7-173 cells regarding cell pro-

liferation is evidenced by the complete lack of tumor takes in the ovariectomized group, and the high take (almost 100%) in the E_2-treated ovariectomized ones ($P < 0.001$). The E_2 plasma levels present in the tumor bearing animals were within the physiological range in humans, and slightly higher than the values normally found in mice.

The histology of the C_7MCF7-173 tumors was similar to that described for the uncloned MCF7 tumors [28, 34]. Cells were arranged in cords and in groups; they were hypertrophied and abundant mitotic figures could be seen. A comparable degree of hypertrophy and hyperplasia could be seen in the endometrial lining of the uterus in these mice.

C_7MCF7-173 as genuine E-target cells

C_7MCF7-173 cells contain estrophilins whose subcellular distribution and physicochemical properties are similar to those in the uncloned MCF7 cell line [34]. The sedimentation profile of the intracellular estrophilin revealed a 4.0S estrogen binder at high salt (500 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$). The estrophilin-E_2 dissociation constant at equilibrium was between 1 and 5×10^{-10} M (not shown).

Effect of E_2 over the multiplication of C_7MCF7-173 cells grown in medium supplemented with female human sera

Figure 1 compares the effect of E_2 on the yield of C_7MCF7-173 cells grown in medium supplemented with 10% day-01 FHS treated as follows: (a) "fresh" (non heat-inactivated); (b) heat-inactivated; (c) CD "fresh"; and (d) CD heat-inactivated. CD treatment of both "fresh" and heat-inactivated sera resulted in almost complete inhibition of cell proliferation. Addition of E_2 increased the cell yield to values close to those found with "fresh" or heat-inactivated sera supplemented with E_2. Similar results were obtained with day-14 FHS. Since both the "fresh" and heat-inactivated CDFHS were quantitatively similar in their inhibitory efficiency, all the experiments described below were done with heat-inactivated

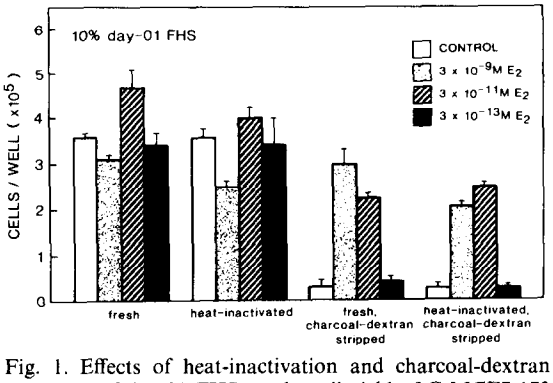

Fig. 1. Effects of heat-inactivation and charcoal-dextran treatment of day-01 FHS on the cell yield of C_7MCF7-173 cells maintained in media supplemented with 10% FHS and different E_2 concentrations. Values in this and subsequent figures represent the mean ± SD of 3 determinations.

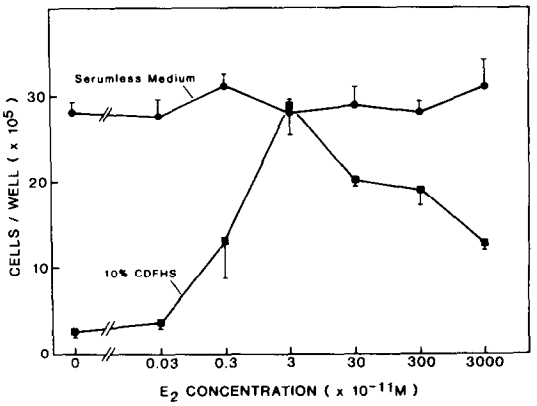

Fig. 2. Effect of E_2 on the cell yield of C_7MCF7-173 cells maintained in (a) 10% day-01 CDFHS; and (b) chemically defined medium (DME/F12 plus insulin, Epidermal Growth Factor and transferrin).

CDFHS. No significant variability in the inhibitory potency of the CDFHS from over 40 donors studied has been detected so far.

Effect of E_2 over the multiplication of C_7MCF7-173 cells grown in serum-less medium

Figure 2 compares the effect of a wide range of E_2 concentrations on the proliferation of C_7MCF7-173 cells in (a) serum-less medium and (b) 10% day-01 CDFHS. Cells grown in defined medium supplemented with transferrin, EGF and insulin achieved similar yields regardless of the concentration of E_2 (3×10^{-11} M to 3×10^{-8} M) in the medium. These results confirm those reported by Butler *et al.* [10]. In contrast, the cells in 10% day-01 CDFHS were strongly inhibited. E_2 increased their yield; the maximal yield was achieved at 3×10^{-11} M E_2. It is worth noting that E_2 concentrations from 3×10^{-10} M to 3×10^{-8} M resulted in a 25–50% decrease of the cell yield obtained with 3×10^{-11} M E_2.

Effect of CDFHS concentration on the proliferation of C_7MCF7-173 cells

The effect of decreasing levels of day-01 CDFHS over the cell yield, both with and without E_2 was investigated (Fig. 3). The results show that: (a) there was an inverse relationship between the concentration of serum and cell yield, i.e. the less serum, the more cells; (b) the maximal stimulation in E-treated cultures was basically similar between 2.5 and 20% serum concentrations; and (c) the difference between E_2-stimulated and control decreased and finally disappeared as the concentration of serum in the culture medium decreased. Similar results were obtained with day-14 CDFHS (not shown).

In 10% CDFHS supplemented medium cells maintained in the absence of E_2 did not seem to multiply, i.e. the cell number was maintained constant throughout the experiment [$t_D = 377.0$ h, correlation coefficient (r) = 0.99]. The cultures treated with E_2

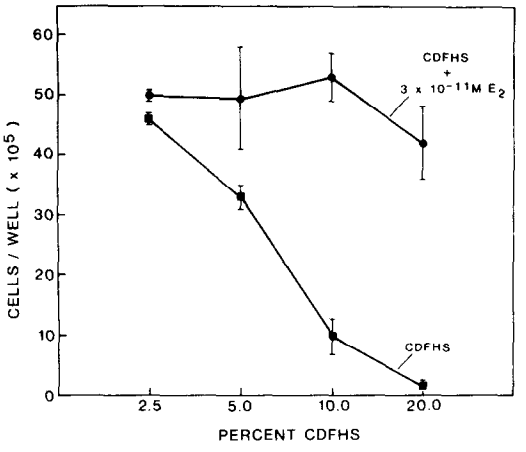

Fig. 3. Effect of different concentrations of CDFHS on the cell yield of C_7MCF7-173 cells maintained in media with or without 3×10^{-11} M E_2.

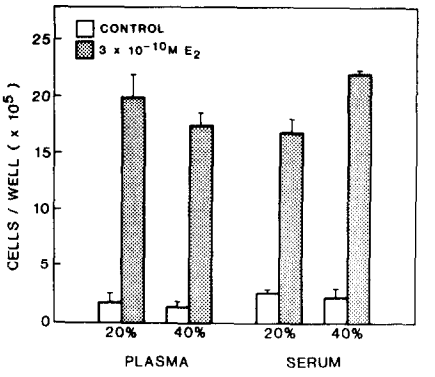

Fig. 4. Comparison between the inhibitory effect of different concentrations of CDFHS and CDFH plasma drawn from the same donor on the proliferation of C_7MCF7-173 cells in media containing no E_2 or 3×10^{-10} M E_2.

multiplied exponentially with a t_D of 39.6 h ($r = 0.99$), similar to the one found in cells growing in 10% heat-inactivated FHS ($t_D = 38.8$ h, $r = 0.99$) [not shown].

Effect of male and postmenopausal female human sera and human plasma

CD male and post-menopausal FHS supplemented to DME prevented cell proliferation in a fashion similar to that seen with day-01 and day-14 CDFHS (not shown); E_2 reversed this inhibitory effect. Figure 4 compares the effect of human CD plasma and CD serum, and shows that the inhibitory effect is comparable in both fluids.

Specificity of the inhibitory effect of serum upon E-sensitive cell proliferation

KLE human endometrium carcinoma cells grow as an autonomous tumor in nude mice [29]. These cells proliferated at similar rates in medium supplemented with 10% CDFHS or CDFBS, regardless of the presence of estrogens in the media (Fig. 5).

Effect of estrogens, other steroids and growth factors upon the multiplication of C_7MCF7-173 cells in medium supplemented with CDFHS

Estrogens (E_2, R2858, DES) significantly increase the cell yield over the control (Fig. 6). E_1 and E_3 also increased the cell yield (not shown). Androgens, progestagens and glucocorticoids failed to increase the cell yield over the control values showing that the inhibitory effect is reversed specifically by estrogens. In addition, growth factors (IET) did not reverse the inhibitory effect of 10% CDFHS [14].

Effect of serum concentration on the E_2 dose needed to obtain maximal cell yield

A comparison of the E_2 dose–response curves obtained in cultures supplemented with 5, 10, 20 and 40% day-01 CDFHS was made (Fig. 7). The E_2 concentration needed to obtain maximal yield increased as the serum concentration in the medium increased.

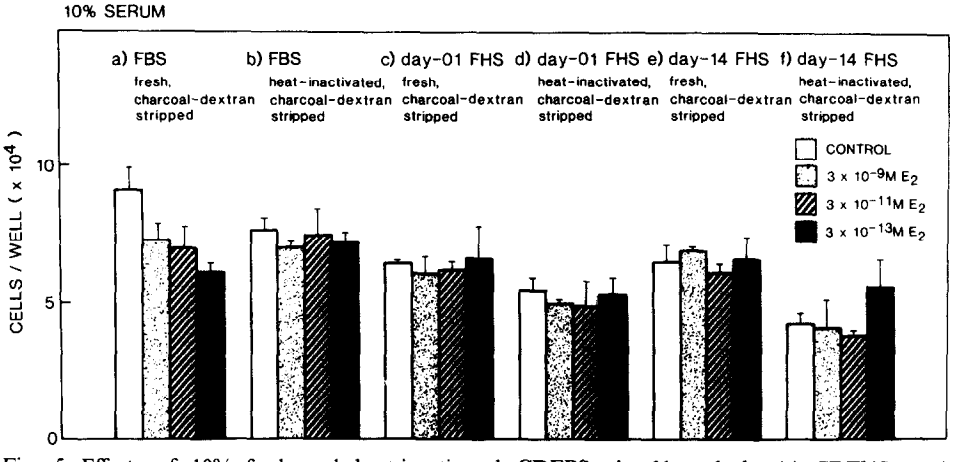

Fig. 5. Effects of 10% fresh and heat-inactivated CDFBS, day-01 and day-14 CDFHS-supplemented media on the proliferation of KLE cells maintained in media supplemented with different E_2 concentrations.

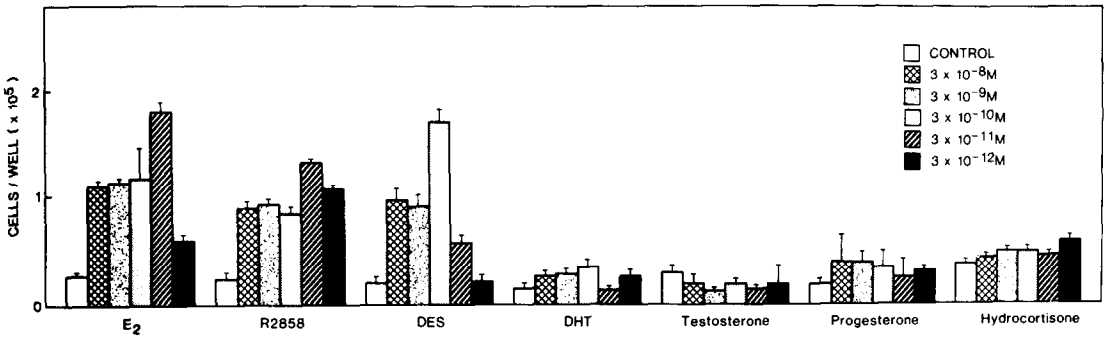

Fig. 6. Effect of several estrogens and steroid hormones on the proliferation of C_7MCF7-173 cells maintained in media supplemented with 10% day-01 CDFHS.

Effect of CDFBS over the cell yield of C_7MCF7-173 cells

C_7MCF7-173 cells were grown in medium supplemented with (a) CDFHS, and (b) CDFBS. The maximal cell yield was achieved with 3×10^{-11} M E_2. However, the cell yield in the unstimulated cultures varied inversely with the serum concentration. In addition, at similar serum concentrations CDFHS was more inhibitory than CDFBS (Fig. 8). We compared the proliferation rate of C_7MCF7-173 cells in medium supplemented with 10% CDFBS with and without addition of 3×10^{-11} M E_2 [14]. The cultures in medium not supplemented with E_2 were stationary for the first 7 days ($t_D = 131.6$ h; $r = 0.98$); the slope of the proliferation curve changed abruptly thereafter ($t_D = 53.3$ h; $r = 0.99$). The cultures in medium supplemented with E_2 multiplied at a rate much higher than the unstimulated cultures ($t_D = 50.9$ h; $r = 0.99$).

DISCUSSION

The experimental data presented provide important information about how E_2 operates upon the proliferation of its target cells. C_7MCF7-173 cells, as well as the uncloned MCF7 cells, proliferate maximally in medium supplemented with 10% FHS. Charcoal–dextran adsorption results in a marked reduction of the ability of this serum to sustain cell proliferation (Fig. 1 and ref. 14). Although CD treatment may remove many low molecular weight components from the sera, maximal proliferation rates were attained by resupplying only E_2 (Figs 1 and 2). In contrast, E_2-sensitive cells proliferated at comparable rates in serum-less medium regardless of the presence of E_2. The lack of a positive proliferative effect by E_2 could be explained by postulating that (a) E_2 *per se* does not induce cell proliferation [5, 9–11, 26, 35]; and (b) growth factors (insulin, EGF and transferrin) in the serum-free medium are as effective as E_2 in inducing cell proliferation. However, the latter interpretation does not concur with the known specificity of E_2 in animals. In addition, these cells do not proliferate in oophorectomized nude mice (Table 1); these animals have, however, normal levels of EGF, transferrin, and insulin. Moreover, Butler *et al.* [10] obtained similar results to the ones reported in Fig. 1 using media supplemented with synthetic components but lacking hormones and growth factors. Under these conditions, the cells proliferated as fast as in 10% FBS. Recently, Darbre *et al.* [36] reported a proliferative effect by E_2 on

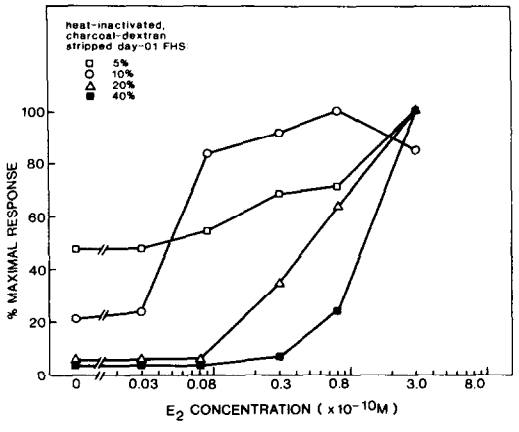

Fig. 7. Dose–response curve on the effect of E_2 on the cell yield of C_7MCF7-173 cells maintained in media supplemented with different concentrations of day-01 CDFHS.

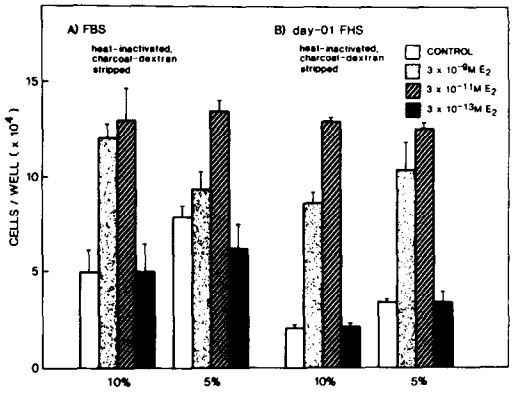

Fig. 8. Comparison between the inhibitory effect of CDFBS and day-01 CDFHS on the proliferation of C_7MCF7-173 cells maintained in media containing different E_2 concentrations.

Table 1. Effect of estradiol on the tumor incidence of C_7MCF7-173 cells inoculated into nude mice

Treatment	Plasma E_2 concentration range (pg/ml)	Number of animals with tumors	Total number of animals
Ovariectomized	12.6–25.4	0	12
Ovariectomized + E_2-cholesterol implant	57.7–66.3	15*	16
Ovariectomized + E_2-only implant	79.2–100.1	11*	16
Intact cycling females	24.4–40.9	4	12

*Statistically significant when compared to ovariectomized mice only ($P < 0.001$).

ZR75-1 cells growing in serum-free medium. Both the time course and the dose–response of this effect differed from the ones reported by the same authors using CD bovine serum-supplemented medium [6]. These differences may indicate that these two sets of results may be due to two different mechanisms.

Cells grown in 10% CDFHS plus E_2 at 3×10^{-11} M achieve maximal proliferation rate (Fig. 2). Higher concentrations of E_2 resulted in cell yields significantly higher than those in control cultures, but lower than those achieved with 3×10^{-11} M E_2. We interpret the effect of hyperphysiological E_2 concentrations as the "in culture" counterpart of the well known biphasic E_2 effect in the live animal [14–19].

It is noteworthy that E_2 supplemented to 10% CDFHS resulted in a significant increase in the proliferation rate of C_7MCF7-173 cells while the same amount of E_2 supplemented to serumless medium had no effect. To reconcile this paradox we explored the role of CDFHS concentration on the expression of E_2 sensitivity for cell proliferation. Figures 3, 7, 8 and data presented before [14] show that: (a) there was an inverse relationship between the concentration of CD serum and the cell yield; (b) the maximal cell yield in E_2-supplemented cultures was basically similar at all serum concentrations; and (c) the difference between E_2-stimulated and control cultures decreased and finally disappeared as the concentration of CD serum in the culture medium decreased. These results are compatible with the notion that CDFHS contains an inhibitor of the proliferation of estrogen-sensitive cells. We are calling this inhibitor estrocolyone (from the Greek κωλεω, to inhibit); this inhibitor is being diluted when the serum concentration in the medium is lowered. This inhibitory effect is abolished by E_2. The E_2 concentration needed to reverse this inhibition increases as the serum concentration increases (Fig. 7).

The inhibitory effect present in human serum and plasma (Fig. 4) seems to be highly specific for genuine E_2-sensitive cells. Estrophilin-positive human endometrial tumor cells that behave autonomously in nude mice multiplied maximally in CDFHS supplemented media, regardless of the E_2 concentration (Fig. 5). Moreover, of the steroids tested so far, only natural and synthetic estrogens reversed the inhibitory properties of CDFHS (Fig. 6). CDFBS inhibited the proliferation of C_7MCF7-173 cells, indicating that the inhibitor was operative in all the species studied. However, at similar doses CDFHS was more potent than CDFBS (Fig. 8) suggesting that the human and the bovine estrocolyones are not identical. We have already shown that the inhibitory effect of CDFBS decayed with time in culture, and finally disappeared [14]. We interpret this "spontaneous" loss of inhibitory potency as degradation of the estrocolyones.

We explored the effect of "growth factors" (IET) on cell proliferation in medium supplemented with variable concentrations of CDFHS [14]. These results complement those in Fig. 2 because they show that: (a) E_2 supplementation does not increase cell yield when added to diluted concentrations of CDFHS; (b) the addition of IET does not neutralize the inhibitory effect of 10% CDFHS; and finally, (c) the sole addition of E_2 is sufficient to neutralize the inhibitory effect of 10% CDFHS on C_7MCF7-173 cells, pointing towards the specificity of the inhibitory effect of human serum. This interpretation is compatible with what happens in the ovariectomized animal; here, the nutrients and putative "growth factors" are present, and while some cells multiply, the E_2-sensitive ones do not. Only when E_2 is administered to the ovariectomized animal do the latter cells proliferate (Table 1).

The data presented do not reveal the mechanism whereby E_2 abolishes the inhibitory effect of CDFHS. In this regard, we have considered two possibilities: (a) E_2 would act on the target cells rendering them refractory to the serum-borne estrocolyones; and (b) E_2 would cancel the effects of the serum-borne estrocolyones by direct interaction with them, i.e. changing their conformation so they would not be recognized by the target cells. We are presently exploring this latter model. Figure 9 schematically represents our interpretation of the data gathered so far explaining the control of the proliferation of E_2-sensitive cells: in the animal, plasma E are distributed among the plasma E-binders (SHBG, albumin, etc.) and the estrocolyones. Only the free estrocolyones are recognized by the E_2-sensitive cells, which will then be prevented from multiplying. E_2 bound to the inhibitor would render it biologically inactive (Fig. 9A). In culture conditions, heat-inactivation destroys SHBG, and thus, more E become available for binding to the inhibitor; E_2-sensitive cells proliferate maximally in heat-inactivated serum supple-

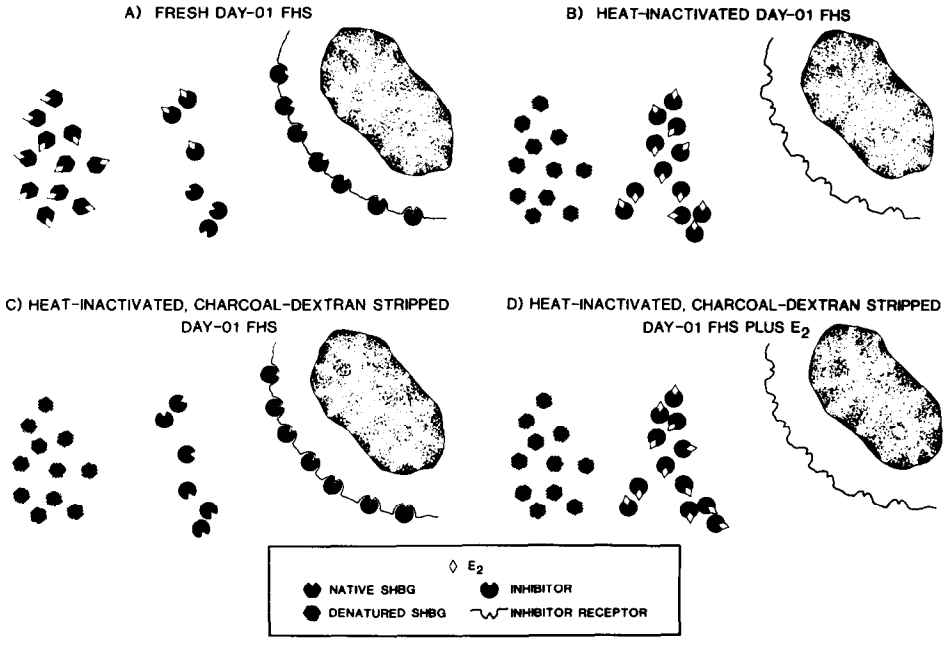

Fig. 9. Model for the mechanism of inhibition of cell proliferation by serum. The interaction between the inhibitor and the E_2-sensitive cells is modulated by the availability of E_2. SHBG trapping of E_2 is eliminated by heat-inactivation at 56°C for 30 min.

mented medium (Fig. 9B). Charcoal-dextran stripping removes E_2, and the free estrocolyones prevent the cells from multiplying (Fig. 9C). Finally, the addition of E_2 to CD serum-supplemented medium results in E_2 binding to the estrocolyones, and cell proliferation ensues (Fig. 9D).

An additional significant implication provided by our work is represented by the possibility to study cell cycle events using a physiologically synchronized population of cells that are neither starved (as those synchronized by serum deprivation) nor intoxicated (by the use of drugs that affect one or more steps within the cell cycle). Cells kept for 12 days in 10% or higher concentrations of CDFHS can be induced to enter the cycle simply by the addition of 3×10^{-11} M E_2.

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