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# TISSUE CULTURE CONDITIONS DETERMINE THE EFFECTS OF ESTROGEN AND GROWTH FACTORS ON THE ANCHORAGE INDEPENDENT GROWTH OF HUMAN BREAST CANCER CELL LINES

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**Summary**—We determined the effects of epidermal growth factor, insulin-like-growth-factor-1 and estradiol on the anchorage independent growth of the estrogen receptor positive human breast cancer cell lines MCF7 and T-47D. In serum free conditions growth factors but not estrogen induced a dose dependent stimulation of growth in both cell lines. The ability of estrogen to induce colony formation of early passage MCF7 cells (< 100) was strictly correlated to the concentration of sulfatase and charcoal treated calf serum (CCS) with a maximal effect at a concentration of 5% CCS and 10 nM estradiol. CCS alone had no stimulatory effect on the anchorage independent growth of early passage MCF7 cells, but increased colony formation in late passage (> 1000) MCF7 and T-47D cells. The growth of late passage MCF7 cells was inhibited by antiestrogen. Thus, the presence of serum components is necessary for the effect of estrogen but not for the effects of growth factors on the anchorage independent growth of estrogen receptor positive human breast cancer cell lines; after a prolonged period of tissue culture serum components switch their function from indirectly modulating estrogen effects to directly stimulating growth in the absence of estrogen.

## INTRODUCTION

Many different tumor cells have been shown to produce and to release growth regulatory factors that are widely believed to influence the growth and behavior of these cells (autocrine growth control [1]). A mitogenic effect on anchorage dependent growth of breast cancer cell lines has been shown for epidermal growth factor (EGF), insulin-like-growth-factor-1 (IGF-1) and basic fibroblast growth factor (basic FGF) [2–4].

It has been known for about 100 years that many breast cancers in both humans and experimental animals are estrogen dependent, but only within the last twenty years estrogen responsive cell lines have been described [5, 6]. The estrogen effect on estrogen receptor positive human breast cancer cells *in vitro* depends on tissue culture conditions [2, 7–10]. It has been shown that estradiol has no effect on cell proliferation in serum free or growth factor deprived media [2, 7–10]. To explain these data it has been proposed that estrogen antagonizes a growth inhibitory effect caused by a serum factor [8]. Other studies have provided evidence for the presence of a serum factor, as a necessary condition for the effect of estradiol [2, 7, 9, 10]. Insulin has been proposed as one candidate for mediating estrogen effects, based on the finding that in growth factor depleted serum, estradiol potentiates the stimulatory effect of insulin on cell proliferation [7].

While most of the studies mentioned above have been done in anchorage dependent proliferation assays, less is known about the interaction of estrogen and growth factors in anchorage independent growth assays. Because the ability to grow in soft agar is one of the best indicators of a cell's tumorigenic capacity [1], we set out to determine if estradiol, IGF-1, EGF, alone or in combination, stimulated the cloning of the estrogen receptor positive human breast cancer cell lines MCF7 and T-47D in soft agar

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supplemented with sulfatase and charcoal treated calf serum (CCS) compared with serum free conditions.

## EXPERIMENTAL

### Cell culture

MCF7 cells were obtained from the Michigan Cancer Foundation (Detroit, MI). T-47D and ZR75-1 cells were acquired from the American Type Culture Collection (Rockville, MD). All cell lines were cultured in phenol red free IMEM (improved minimal essential medium, Gibco Laboratories, Chagrin Falls, OH) supplemented with 10% fetal bovine serum (FBS, Gibco) and routinely tested for mycoplasma contamination. MCF7 cells were used in early (< 100) and late passage (> 1000).

### Growth factors

EGF (derived from mouse submaxillary gland, tissue culture grade) was obtained from Bethesda Research Laboratories (BRL, Gaithersburg, MD). Recombinant IGF-1 and basic FGF were acquired from Amgen Biologicals (Thousand Oaks, CA).

### Anchorage independent growth assays

In 35 mm tissue culture dishes (Costar, Cambridge, MA) a bottom layer of 1.0 ml IMEM containing 0.6% Bacto Agar (Difco, Detroit, MI) was prepared. When the bottom layer was solidified, the indicator cells were added in triplicates in a 0.8 ml top layer containing the sample in 0.4% agar. Plates were incubated 9–14 days at 37°C in 5% $CO_2$. Agars were supplemented with a concentration of 5% sulfatase and CCS (5%), which was prepared as described previously [11]. Serum free agars were provided with transferrin (2 mg/l, Sigma, St Louis, MO), bovine serum albumin (BSA) (200 mg/l, crystallized, Sigma) and fibronectin (1 mg/l, Collaborative Research, Bedford, MA). For experiments done in 5% CCS cells were plated 5–7 days before the experiment in IMEM containing 5% CCS. For serum free agars, cells were washed at least once in medium containing transferrin, BSA and fibronectin in the same concentration as the agar. 10,000–20,000 Cells were seeded per dish. Cell colonies larger than 60 $\mu$m were counted using a Bausch and Lomb stem cell colony counter. Cells were always passaged one or two days before the experiment.

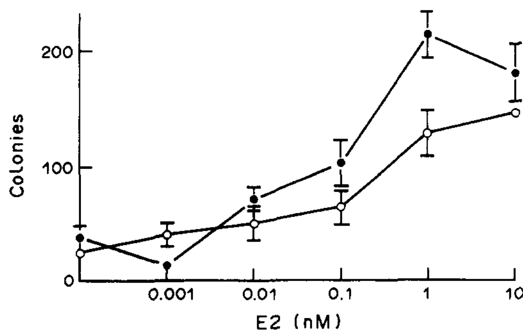


Fig. 1. Effect of increasing concentrations of estradiol on the anchorage independent growth of early (< 100) passage MCF7 cells in medium supplemented with 5% sulfatase and CCS; in the absence (○) and in the presence (●) of IGF-1 (100 ng/ml). Cells were plated in 5% CCS 1 week before the experiment as described in Experimental. Each experiment was repeated twice.

### Statistical analysis

Each experiment was repeated twice and statistical analysis was done by using a Student's $t$-test. Differences were considered statistically significant, if $P = 0.01$.

## RESULTS

### *Influence of estradiol, IGF-1, EGF and basic FGF on the growth of MCF7 and T-47D cells in agar supplemented with 5% sulfatase and CCS*

The anchorage independent growth of early passage MCF7 cells (< 100) was stimulated by estradiol in a dose dependent way with a maximal effect at a concentration of 1 nM (Fig. 1). IGF-1 but not EGF potentiated this effect about 1.5–2 fold (Fig. 1). The estrogen stimulated growth of ZR-75-1 cells was also potentiated by IGF-1 and EGF in 10% FBS (data not shown). Growth factors added alone (Table 1) or in combination (not shown) did not increase cloning. The anchorage independent growth of late passage MCF7 cells (> 1000) was already maximally stimulated by 5% CCS (Fig. 2). Estrogen did not further stimulate the cloning (Fig. 2). The growth of late passage MCF-7 in 5% CCS was inhibited by hydroxytamoxifen (Fig. 2). The growth rate of T-47D cells (late

Table 1. Stimulation of anchorage independent growth of estrogen receptor positive human breast cancer cell lines in the presence (+) and in the absence (−) of CCS

| | MCF7 early passage | | MCF7 late passage | | T-47D | |
|---|---|---|---|---|---|---|
| | +CCS | −CCS | +CCS | −CCS | +CCS | −CCS |
| E2 | + | − | − | − | + | − |
| EGF | − | − | − | − | + | + |
| IGF-1 | − | + | − | + | − | + |
| b-FGF | − | − | − | − | − | − |

For details see Experimental. E2 = estradiol, b-FGF = basic FGF.

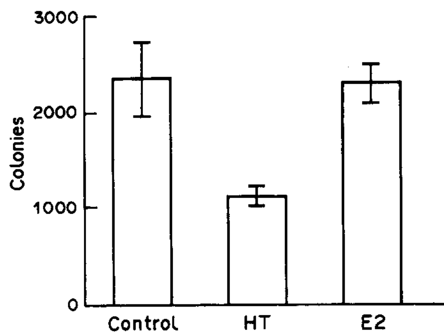


Fig. 2. Effect of estradiol (E2, 10 nM) and hydroxy-tamoxifen (HT, 100 nM) on the anchorage independent growth of late passage (> 1000) MCF7 cells in medium supplemented with 5% CCS. For details see legend to Fig. 1.

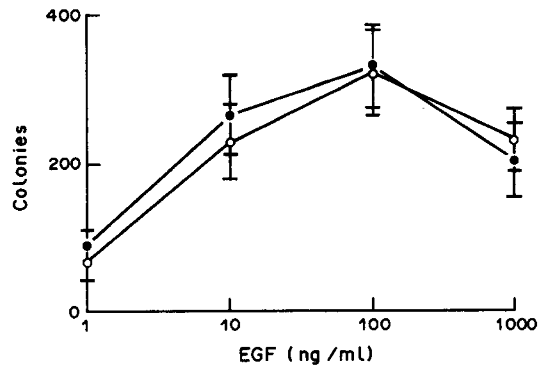


Fig. 4. Effect of increasing concentrations of EGF on the anchorage independent growth of T-47D cells in serum free medium; in the absence (○) and in the presence (●) of estradiol (10 nM) as described in Fig. 3.

passage) was stimulated by estradiol or EGF (Table 1). IGF-1 was ineffective, and there was no synergism with estradiol (not shown). Basic FGF had no effect (Table 1).

*Influence of estradiol, IGF-1, EGF and basic FGF on the growth of MCF7 and T-47D cells in serum free agar*

The anchorage independent growth of MCF7 cells in serum free conditions was increased by IGF-1 but not by EGF in a dose dependent manner (Fig. 3, Table 1), independent of the number of cell passages (data not shown). The anchorage independent growth of T-47D cells was increased by EGF or IGF-1 in a dose dependent way (Fig. 4, Table 1). There was no synergism of the two growth factors (not shown). In both cell lines estradiol alone or together with growth factors had no effect (Figs 3 and 4). Basic FGF was ineffective (Table 1).

*Influence of the concentration of CCS on the effect of estradiol*

10 nM estradiol, which was maximally effective in agar supplemented with 5% CCS, was added to early passage MCF7 cells together with concentrations of CCS ranging from 0–5%. The growth stimulation by estradiol was directly correlated with the concentration of CCS [Fig. 5(A)]. The maximal effect was reached at a concentration of 5% [Fig. 5(A)]. CCS alone neither inhibited nor stimulated colony formation in early passage MCF7 cells [Fig. 5(A)]. In late passage MCF7 cells [Fig. 5(A)] and T-47D cells [Fig. 5(B)]CCS alone increased the anchorage independent growth in a dose dependent way. This effect was further increased by

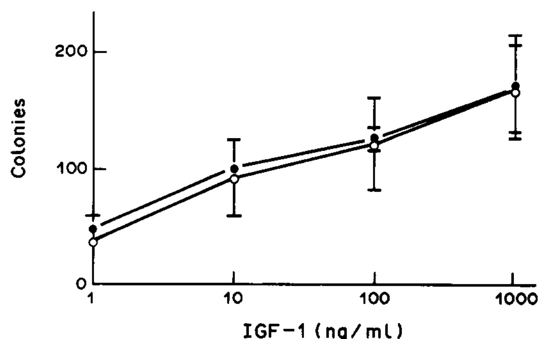


Fig. 3. Effect of increasing concentrations of IGF-1 on the anchorage independent growth of MCF7 cells in serum free medium supplemented with transferrin, BSA and fibronectin as described in Experimental; in the absence (○) and in the presence (●) of estradiol (10 nM).

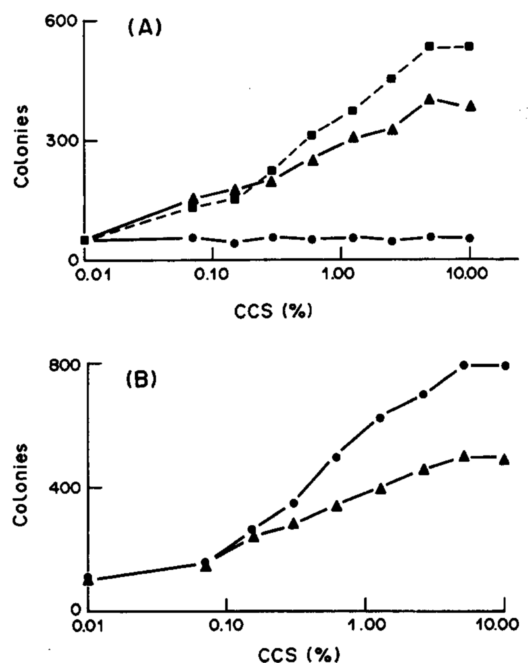


Fig. 5. (A) Effect of increasing concentrations of CCS on the anchorage independent growth of early (●——●) and late (■---■) passage MCF7 cells. Media were supplemented with transferrin, BSA and fibronectin; (▲——▲) = early passage MCF7 cells in the presence of estradiol (10 nM). (B) Effect of increasing concentrations of CCS on the anchorage independent growth of T-47D cells in the absence (▲) and presence (●) of estradiol (10 nM).

estradiol in T-47D cells [Fig. 5(B)] but not in late passage MCF7 cells (data not shown).

## DISCUSSION

We found that the effect of estradiol on the anchorage independent growth of estrogen receptor positive human breast cancer cell lines depended on the presence of CCS. We observed that IGF-1 stimulated the anchorage independent growth of both MCF7 and T-47D cells in serum free conditions, whereas EGF induced increased colony formation only in T-47D cells. The effect of estrogen in the presence of CCS was potentiated by IGF-1, which, if given alone had no effect. Similar results were obtained with insulin and estradiol (data not shown). These findings are consistent with reports of synergism between insulin and estradiol on the anchorage dependent growth of MCF7 cells in serum depleted of growth factors [7] and the observation that estrogen increases the amount of mRNA of the IGF-1 receptor [12]. It has also been reported that progesterone amplifies the stimulatory effect of insulin on the anchorage dependent growth of T-47D cells [13].

It has been postulated that serum factors are necessary mediators of an estrogen effect on human breast cancer cells [2, 9, 10]. According to our results these factors cannot be insulin, IGF-1 or EGF alone or in combination. The finding of a synergistic effect of estrogen and IGF-1 in the presence of serum (Fig. 1) indicates the possible existence of unknown serum proteins modulating the estrogen effect together with IGF-1. Other studies indicate that estrogen overcomes the effects of a growth inhibitor in serum [8, 14]. Some authors have found stimulatory as well as inhibitory factors in serum [15]. The hypothesis that estrogen overcomes a growth inhibitory effect of serum is not supported by our data in this system. In that case, increasing serum concentrations would induce an inhibition of growth compared to serum free conditions. This was not observed.

It has been shown that EGF stimulates the anchorage dependent proliferation of MCF-7 cells [2–4]. We found an EGF induced growth stimulation in T-47D but not MCF7 cells. One reason for the different results might be that in anchorage independent conditions the number of EGF receptors in MCF-7 cells, which is 20 × lower than in T-47D cells [3], is not sufficient to mediate induction of colony formation.

Our results are consistent with results obtained from experiments in anchorage dependent growth conditions in that serum free conditions are optimal for studying effects of growth factors without the disturbing effects of serum proteins [2]. This has also been shown for the effects of transforming growth factors (TGFs) on the anchorage independent growth of NRK-fibroblasts [1]. Because the ability to grow in soft agar is one of the best indicators of a cell's tumorigenic capacity [1], the finding that growth factors increase the rate of colony formation of breast cancer cell lines in serum free conditions emphasizes their potential importance as stimulators of tumor growth. The stimulation of anchorage independent growth in serum free conditions was optimal in medium supplemented with transferrin, fibronectin and BSA. The spontaneous cloning, which gave a background of 25–100 colonies, was probably due to an effect of the supplements. This has also been shown for cell proliferation in anchorage dependent conditions [2].

It has been shown that estrogen receptor positive human breast cancer cells become estrogen independent after prolonged tissue culture in anchorage dependent conditions in the absence of estrogen [16]. These cells still have estrogen receptors, and their growth is inhibited by antiestrogens [16]. We have demonstrated that this is also true for anchorage independent conditions. Our late passage MCF-7 cells grew in soft agar in the absence of estrogen and their growth was still inhibited by antiestrogen. It is intriguing to speculate that after prolonged tissue culture other factors in serum or secreted by the cells can replace estrogen. It has been shown that human breast cancer cells, whose growth *in vitro* is independent of estrogen but still inhibited by antiestrogen, form tumors in nude mice in the absence of estrogen [17].

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