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Estrogen Inhibits the Growth of MCF-7 Cell Variants Resistant to Transforming Growth Factor-beta

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Human breast cancer MCF-7 cells containing estrogen receptor are killed by transforming growth factor-beta (TGF-β). We isolated variants of MCF-7 highly resistant to TGF-β. Variants ES-1 and ES-4 were cloned, and the growth of ES-1 and ES-4 was found to be inhibited by estradiol, whereas estradiol stimulated the growth of the parental MCF-7 cells. ES-1 cells contained about 2-fold higher level of estradiol receptor than MCF-7 cells. Addition of estradiol to the culture medium for MCF-7 and the variant changed the expression of several secreted proteins. The repertoire of secreted proteins was markedly altered in the variant. Polypeptides of molecular weight 52,000 (52 K), 65 K and 160 K were increased about 10- to 50-fold in both estradiol-treated MCF-7 and ES-1 cells. Polypeptide of 130 K was decreased in estradiol-treated ES-1 cells while this polypeptide was increased about 4-fold in estradiol-treated MCF-7, as compared with untreated MCF-7. Polypeptide of 100 K was specifically secreted in ES-1 whether or not estradiol was present, but there appeared to be no significant amount of the 100 K protein in MCF-7. The estradiol-hypersensitive phenotype is discussed in relation to its aberrant expression of secreting proteins.

Key words: MCF-7 cell variants — TGF-β-resistance — Estrogen effect

The human breast cancer MCF-7 cell line has been widely used to study hormone action in culture. MCF-7 cells contain estrogen receptor (ER)[1] and the cell growth is stimulated by estrogen.[2] Addition of estradiol (E_2) to the culture medium of MCF-7 cells induced estrogen-regulated proteins as well as pS2RNA[3,4]: Synthesis of pS2RNA of about 600 nucleotides is specifically regulated by estrogen in MCF-7 cells. To analyze the action of steroids, somatic cell variants altered in the response to steroids should be blocked at various levels. Variants resistant to an anti-steroidal drug, tamoxifen, have been isolated from MCF-7[5,6] and the effects of E_2 or anti-estrogens on these variants have been studied.[4] A study on the isolation of estrogen-inhibited variants of MCF-7 has been reported.[7] In our laboratory, we have also studied clones from MCF-7 with defective ER activity.[8]

Growth of MCF-7 has been found to be inhibited by a transforming growth factor-beta (TGF-β),[9] an autocrine growth factor of tumor cells.[10] Knabbe *et al.*[11] have recently reported that estrogen or antiestrogen regulates the secreted amount of TGF-β in MCF-7 cells, and the secreted TGF-β in the medium is supposed to affect the cell growth of MCF-7. During studies on the effect of TGF-β on the cell growth of MCF-7 and its variants with altered estrogen responses, we noticed that ER-positive breast cancer cell lines such as MCF-7 were highly sensitive to the growth-inhibitory action of TGF-β, but estrogen-nonresponsive cell lines were not. We have isolated variants resistant to the cytocidal action of TGF-β from MCF-7 cells, and to characterize the variants, we first examined their responses to estrogens in this study.

Materials and Methods

Cell Lines and Cell Culture Human breast cancer MCF-7 cell line[12] and its ER-defective variant, U-2, have been described previously.[8,13] A tamoxifen-resistant variant, R-27, derived from MCF-7[5] was donated to us by Dr. H. Nawata (Kyushu University School of Medicine, Fukuoka). The cells are

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grown in RPMI 1640 medium supplemented with sodium bicarbonate (2 g/liter) and 10% (v/v) untreated fetal bovine serum (FBS) which had been treated with dextran-coated charcoal to eliminate serum estrogens.[8] To examine the effect of various doses of estrogen on cell growth, exponentially growing cells (3×10^4 per dish) were plated in 35-mm dishes and were incubated at 37° overnight. Then they were exposed to estradiol and incubation was continued. Every 2 days the medium was changed and viable cells were counted by trypan blue dye exclusion at the indicated time.

Chemicals and Isotopes [^{3}H]E_2 (93.0 Ci/mmol) and nonradioactive E_2 were purchased from New England Nuclear (Boston, Mass.) and Sigma Chemical Co. (St. Louis, Mo.) respectively. All chemicals were dissolved in ethanol. TGF-β was kindly donated to us by Dr. R. K. Assoian (NCI, Bethesda, Md.).

Cell Growth Conditions To examine the effect of E_2 on cell growth, exponentially growing cells (3×10^4/dish) were plated in 35-mm dishes and were incubated at 37° overnight. Then, they were exposed to $10^{-8}M$ E_2 and incubation was continued. Every 2 days the medium was changed and viable cells were counted by trypan blue dye exclusion at the indicated time.

Assay of Cytosolic ER by Scatchard Analysis To determine the level of ER in cytosol, Scatchard analyses were performed.[8] Cells grown in medium containing charcoal-treated serum were washed 3 times with PBS and harvested with a rubber policeman. The cells were pelleted by centrifugation at 800g for 5 min, and suspended in TED buffer (10 mM Tris, 1.5mM EDTA, 0.5mM dithiothreitol pH 7.4) for ER assay. After homogenization of the cells in a glass Dounce homogenizer until more than 90% had been lysed (about 50 strokes), the homogenate was centrifuged at 800g for 5 min and the supernatant was centrifuged at 105,000g for 60 min. To measure ER by Scatchard analysis, 200 μl of the supernatant was incubated with various doses of [^{3}H]E_2 in the presence or absence of 200-fold excess of unlabeled E_2 in a total volume of 500 μl for 15 hr at 4°. Unbound E_2 was then removed by incubation with 250 μl of 3% dextran-coated charcoal in TED buffer for 30 min at 4° and by centrifugation at 4,000g for 10 min. Radioactivity of the supernatant (300 μl) was counted as described above.

Cell Survival Assay by Colony Formation The sensitivity of cells to E_2 was measured in terms of plating efficiency. One thousand exponentially growing cells were plated in each of a series of 60-mm plastic dishes. Next day, 5 μl of E_2 at various doses was added to the culture media; 5 μl of ethanol was added to control dishes. After 2 weeks of incubation in a CO_2 incubator, colonies were stained with 2.5% Giemsa and counted as described previously.[13]

Isolation of Estrogen Growth-inhibitory Variants from MCF-7 Cells Exponentially growing MCF-7 cells at 1×10^6 per 100-mm dish in MEM and 10% FBS were cultured at 37° with 10 ng/ml TGF-β, a dose high enough to show cytotoxicity. In each dish, two to three colonies appeared after two weeks of incubation. Incubation was continued for one to two weeks in the absence of TGF-β. Colonies on each of five dishes were then independently isolated and cloned. During the treatment with TGF-β, the medium was replaced with fresh medium containing TGF-β every 4 to 5 days. Two repurified clones, ES-1 and ES-4, were used in this study.

Polyacrylamide Gel Electrophoresis (PAGE) of Proteins Secreted from [^{35}S]Methionine-labeled Cells MCF-7 and ES-1 cells were plated in 100-mm dishes in the presence of 10% charcoal-treated serum and $10^{-8}M$ E_2 or ethanol. Media were changed every two days, and after 4 days of growth, cells were labeled with [^{35}S]methionine after the method of Westley and Rochefort.[3] Cells were labeled in 3 ml of methionine-free minimal essential medium (MEM) containing 10 μCi per ml of [^{35}S]methionine without or with $10^{-8}M$ E_2 for 5 hr, and then media were centrifuged at 9,000g for 5 min and the supernatant was mixed with an equal volume of buffer containing 5% β-mercaptoethanol, 2% SDS, 10% glycerol and 0.125M Tris-HCl, pH 6.8. The samples were heated at 100° for 5 min and the amount containing 40,000 dpm was analyzed on 1 mm thick 10% acrylamide slab gel with a 4.5% stacking gel. The gels were dried and processed for fluorography using Kodak XAR film as described previously.[14]

Results

Effect of E_2 on Growth Curves of Estrogen Growth Inhibitory Variants Two variants, ES-1 and ES-4, were selected as clones resistant to the cytocidal action of TGF-β (Table I) (see also "Materials and Methods"). As can be seen in Table I, ER-positive MCF-7 is sensitive to TGF-β, but an ER-defective clone U-2[8] and a tamoxifen-resistant clone, R-27[5] derived from MCF-7, were resistant to TGF-β. EC_{50}, the dose required to inhibit the cell survival by 50% of control, was estimated to be 0.03 ng/ml for MCF-7 cells, which is comparable to the value given by Roberts *et al.*[9] These data suggest that there might be a close correlation between cellular sensitivity to TGF-β and ER level. We thus examined whether ES-1 and ES-4 showed altered sensi-

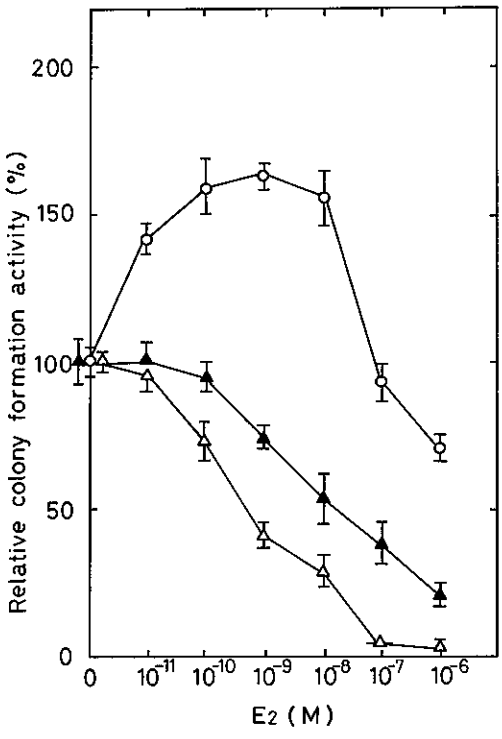

Table I. ER Level and Cellular Sensitivity to TGF-β of MCF-7 and Its Variants

Cell lines	ER level[a]	EC_{50}[b] (ng/ml)	References
MCF-7	58 fmol/mg protein	0.03	this study
R-27	similar to MCF-7	>20	(ref. 5)
U-2	not detectable	>20	(ref. 8)
ES-1	112 fmol/mg protein	>20	this study
ES-4	not tested	>20	this study

a) *K*d values of ER for MCF-7 and ES-1 were deduced from Fig. 4.
b) EC_{50} of TGF-β was obtained from dose-response curves to TGF-β of various cell lines when colony formation assay was done, and each value is the average of duplicate dishes.

Fig. 1. Effect of various doses of E_2 on cell survival of MCF-7, ES-1 and ES-4. Exponentially growing cells (10^3 per 60 mm dish) were plated and the next day the cells were further incubated without or with various doses of E_2 for 10 days. Colonies were scored from three plates for each dose of E_2 and each point shows the mean $\pm$ SE. The colony number corresponding to 100% in the absence of E_2 is 87 (MCF-7), 238 (ES-1) and 240 (ES-4). MCF-7 (○), ES-1 (△) and ES-4 (▲).

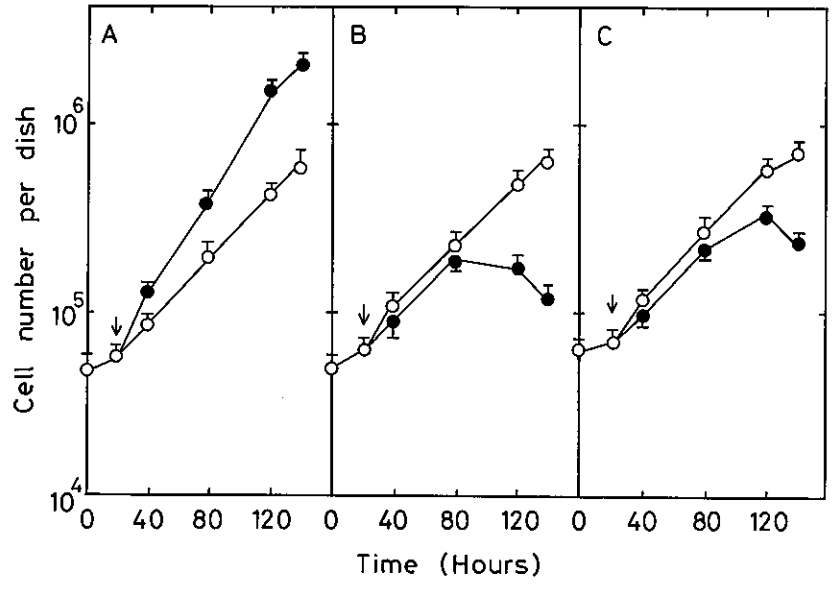

Fig. 2. Effect of E_2 on the growth of MCF-7 (A), ES-1 (B) and ES-4 (C). Exponentially growing cells (3×10^4/dish) were plated and the next day as indicated by the arrow the cells were further incubated without (○) or with (●) $10^{-8}M$ E_2. At the indicated time, the viable number of the cells was scored; each point is the average of triplicate trials. Bars, SE.

tivity to E_2. As shown in Fig. 1, colony formation of MCF-7 cells was enhanced 1.5- to 1.7-fold over the control when 10^{-11} to $10^{-8}M$ E_2 was added to the culture medium. By contrast, cell survival of ES-1 and ES-4 cells decreased as a function of E_2 dose. E_2 at 10^{-8} to $10^{-6}M$ decreased the cell survival of ES-1 or ES-4 to less than 50% of the control in the absence of E_2, whereas 10^{-7} to $10^{-6}M$ E_2 only slightly affected the cell survival of MCF-7 cells (Fig. 1). There appeared to be differential sensitivity to the cytocidal action of E_2 between MCF-7 and ES-1 or ES-4. ES-1 was rather more sensitive to the inhibitory action of E_2 than ES-4.

The growth of MCF-7 is dependent on exogenously added E_2.[7] We compared the effect of E_2 on the growth behavior of ES-1 or ES-4 with that of MCF-7. Figure 2 shows growth curves of MCF-7, ES-1 and ES-4 in the absence or presence of $10^{-8}M$ E_2. Addition of E_2 to culture medium of MCF-7 cells increased their growth rates as expected, and stimulation was significant at $P<0.01$. By contrast, $10^{-8}M$ E_2 failed to stimulate the growth rates of ES-1 and ES-4, and instead their cell growth rates declined gradually from about 60 hr after addition of $10^{-8}M$ E_2 (Fig. 2). E_2 was stimulatory to the growth of MCF-7, but inhibitory to the growth of ES-1 or ES-4.

Microscopic observation of MCF-7 and ES-1 cells cultured in the absence or presence of $10^{-8}M$ E_2 for 4 days at 37° was carried out (Fig. 3). ES-1 cells looked smaller and rounded, with less cytoplasm than MCF-7 in the absence of E_2. E_2 appeared to increase the

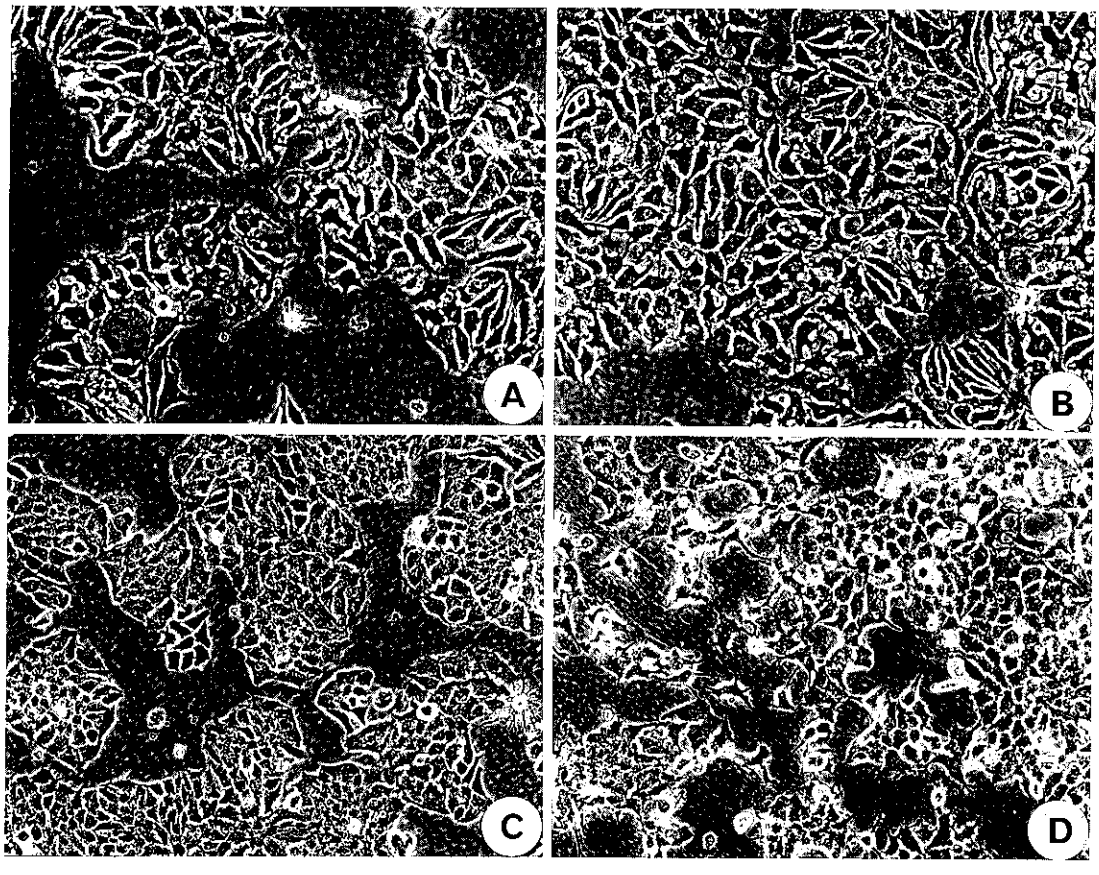

Fig. 3. Changes in morphology of MCF-7 (A, B) and ES-1 (C, D) grown in the absence (A, C) or presence (B, D) of $10^{-8}M$ E_2 for 4 days. Cells grown in monolayer culture on plastic dishes were analyzed by phase-contrast microscopy (Diaphot-TMD; Nikon Co., Tokyo). ×200.

cell number of MCF-7, and the cell morphology was similar in the absence and presence of E_2. ES-1 in the absence of E_2 often showed piling-up of the cells to form islands as shown in Fig 3, and the cells were flat, with many spike-like protuberances in the presence of E_2. ES-1 and ES-4 showed a chromosome number ranging from 45 to 96 with a modal number of 71, like MCF-7.

Comparison of Cellular Levels of ER in MCF-7 and ES-1 Since the cell population of ES-1 was sensitive to growth inhibition by exogenously added E_2, we considered that ES-1 and ES-4 cells might have a modified capacity to take up E_2. The amounts of E_2 incorporated into ES-1 or ES-4 cells were about 2-fold greater than in MCF-7 (M. Mawatari, data not shown). We compared the cytosolic ER levels of MCF-7 and ES-1 cells as shown in Fig. 4A. From a Scatchard analysis for the cytosol fraction (Fig. 4B), the specific activity of the ER was estimated to be 58 fmol/mg protein with a *K*d of $4.3\times10^{-10}M$ in MCF-7 cells, and the cytosolic fraction of ES-1 cells showed about 2-fold greater receptor number than MCF-7 cells: ES-1 had a specific activity of 112 fmol/mg protein with a *K*d of $3.1\times10^{-10}M$ ER. MCF-7 cells also contain progesterone receptor (PR), and addition of E_2 to the culture medium enhances the cytosol PR level in MCF-7.[5,8] From the saturation binding kinetics of a radioactive analog of progesterone, [^{3}H]R5020, with the cytosol fraction, MCF-7 and ES-1 cells had 106 and 110 fmol/mg protein of PR, and the level of PR was increased more than 5-fold in both MCF-7 and ES-1 cells by incubation with $10^{-8}M$ E_2 (M. Mawatari and Y. Hagino, unpublished data).

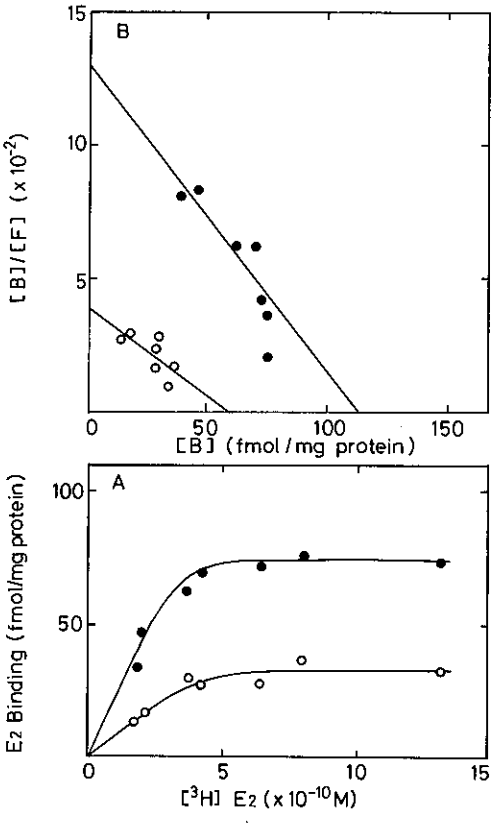

Fig. 4. Binding of [^{3}H]E_2 to ER in the cytosol fraction of MCF-7 and ES-1 cells and Scatchard analysis. Cytoplasmic fractions of MCF-7 (○) and ES-1 (●) were incubated with various doses of [^{3}H]E_2 in the absence or presence of unlabeled E_2. Unbound E_2 was removed by dextran-coated charcoal, and radioactivity in the supernatant fraction was counted. The saturation binding kinetics is shown in (A) and a Scatchard analysis in (B). [B]=concentration of [^{3}H]E_2 bound to proteins; [F]=concentration of free tracer.

Effect of Estrogen on Secretory Proteins Estrogen induces expression of various proteins with molecular weights of 52,000 (52 K) and 160 K in human breast cancer cells.[3] Cellular responses to estrogens can be assayed by analyzing SDS-PAGE patterns of the secretory proteins. SDS-PAGE patterns of proteins secreted into the medium from cells which had been prelabeled with [^{35}S]methionine were compared between MCF-7 and ES-1 cultured in the absence and presence of E_2. In good agreement with previous reports,[3,4] MCF-7 cells secreted the 52 K (band e), the 65 K (band d) and the 160 K (band a) proteins at much higher rates in the presence of E_2 than in its absence (Fig. 5): densitometric analysis showed about 10- to 50-fold increase of these three proteins in E_2-treated MCF-7 cells. The secretion of the three different proteins of 160 K, 65 K and 52 K from ES-1 cells was enhanced by exogenously added E_2 to essentially the same extent as in the case of MCF-7 cells. However, expression of two other proteins of 100 K (band c) and 130 K (band b) was found to be altered in ES-1 cells (Fig. 5). Protein of 100 K was con-

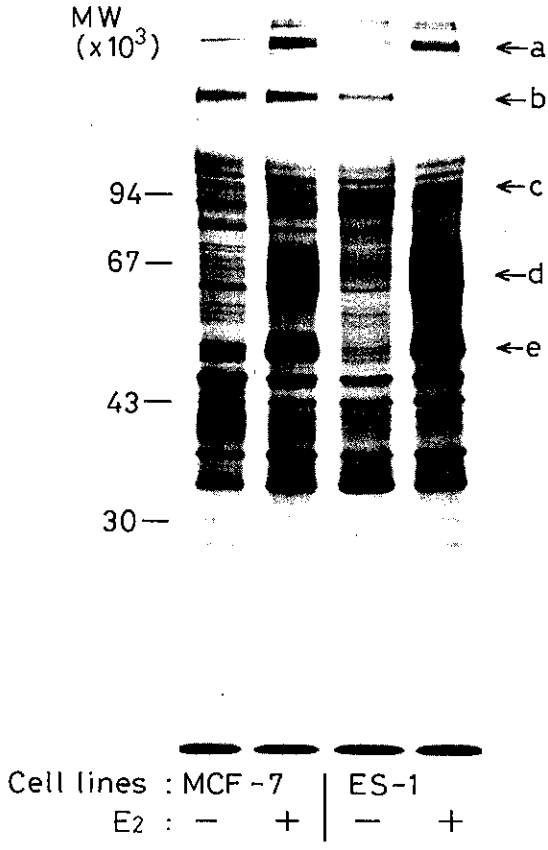

Fig. 5. Comparison of proteins secreted from MCF-7 and ES-1 cultured in the absence or presence of E_2. MCF-7 and ES-1 were cultured in the presence or absence of $10^{-8}M$ E_2 for 4 days. Then the cells were labeled for 5 hr with [^{35}S]methionine, and the medium was recovered and subjected to SDS-PAGE, followed by the fluorographic procedure. Molecular weight is based on the results for standard marker proteins.

stitutively secreted from ES-1 cells in much higher amounts than from MCF-7 cells, whether or not E_2 was added. E_2 inhibited the secretion of 130 K protein in ES-1, but not in MCF-7: this protein was increased about 4-fold in E_2-treated MCF-7 cells as compared with untreated cells. MCF-7 and ES-1 cells showed highly reproducible PAGE patterns of secretory proteins, as shown in Fig. 5, when independent assays were performed. In ER-defective U-2 cells, no secretion of 52 K protein appeared whether or not E_2 was present (Y. Hagino *et al.*, unpublished data).

Discussion

Roberts *et al.*[9] have reported bifunctional regulation of TGF-β to inhibit the growth of some cell lines and ennhance anchorage-independent growth of other cell lines. MCF-7 is one of the human cell lines sensitive to the cytocidal action of TGF-β. Our present study suggests that the cellular sensitivity of mammary carcinoma cell lines to TGF-β may be closely correlated with the cellular response to E_2: ER-positive MCF-7 cells are sensitive to TGF-β, whereas ER-defective or other clones with altered response to E_2 are not. Selection of TGF-β-resistant clones from MCF-7 provides a novel type of mutant of mammary carcinoma cell line with altered responses to E_2. However, the cellular level of ER in ES-1 is only about 2-fold higher than that of MCF-7 cells: ES-1 contains a 2-fold greater number of ER than MCF-7 while the affinity of ER is not changed significantly between MCF-7 and ES-1 (see Fig. 4). Transfer of E_2 or an E_2-induced message into the nucleus appears to occur actively in ES-1 or ES-4 as well as in MCF-7. In fact, the secretion of 52 K and 160 K proteins is enhanced by exogenously added E_2 in both MCF-7 and ES-1 cell lines. Bronzert *et al.*[7] have isolated estrogen-inhibited variants of MCF-7 cells after exposing the cells to high specific activity of 16-α-[^{125}I]iodoestradiol. Their variants secrete proteins similar to those of the parental MCF-7 cells when E_2 is present. Their variants show very similar growth behavior to ES-1 and ES-4, but our variant also shows secretion of some other specific proteins in the presence of E_2 (see Fig. 5). Our present clones thus appear to be different from their variants. On the other hand, TGF-β-resistant mutants have also been recently isolated from a mink lung epithelial cell line.[15] Although there appears to be no difference in the TGF-β receptor activity between the mutant and the parent clone, secretion of 48 K and 73 K proteins is stimulated in the parental cells, but not in the resistant clone. Since the cellular responses of their lung cell lines to E_2 are not yet determined, comparison of the cellular properties of their clones with those of our clones is not yet feasible.

One could argue whether E_2-induced cytotoxicity of ES-1 or ES-4 cells is mediated

either through E_2 *per se* or through any intermediate regulated by E_2. MCF-7 cells produce a variety of polypeptides with growth-promoting potential: a 52 K protein,[4] TGF-β, TGF-α and IGF-I-related growth factors.[16-19] Knabbe *et al.*[11] have reported that MCF-7 secretes the active form of TGF-β, and that secretion of TGF-β is induced significantly by treatment of MCF-7 with antiestrogens. They propose that TGF-β is the autocrine and paracrine growth factor to regulate growth of MCF-7 cells in the presence of E_2 or antiestrogens. We could not observe significant amounts of TGF-β secreted into the culture medium of ES-1 cells without or with E_2 (Y. Kuratomi and Y. Hagino, unpublished data) when assayed in terms of colony-forming ability of normal rat kidney (NRK) cells in soft agar.[20,21] At present, it remains to be studied why TGF-β-resistant clones of MCF-7 show increased sensitivities to E_2. We have just succeeded in the isolation of several clones derived spontaneously from ES-1 which could grow in the presence of $10^{-8}M$ E_2 (K. Matsui, unpublished data). With these clones, we can now examine whether cellular acquisition of TGF-β-resistant phenotype is closely linked with the altered sensitivity of ES-1 (or ES-4) to E_2.

Our present study suggests that ES-1 (or ES-4) and MCF-7 cells carry both common and different pathways for E_2-mediated gene expression. Secretion of 160 K, 65 K and 52 K proteins was similarly enhanced by exogenously added E_2 in ES-1 and MCF-7. Although the level of 100 K protein secreted into the culture medium of MCF-7 is only slight, if any, the 100 K protein is secreted from ES-1 cells at much higher rates even in the absence of E_2. The secretion of the 130 K protein is inhibited by E_2 in ES-1 cells, but not in MCF-7 cells. These data suggest a specific gene regulation in ES-1 cells which is different from that of the parental cells. Other unknown proteins altered in their levels in ES-1 cells might be involved in the E_2-induced cytotoxicity. Alternatively, expression of any protein which is required for cell growth may be specifically abolished in the variant in the presence of E_2.

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