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Evidence of an EGF/TGF-α–Independent Pathway for Estrogen-Regulated Cell Proliferation

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Abstract To elucidate the relationship between epidermal growth factor (EGF)/transforming growth factor (TGF-α) and estradiol-17β (E) in cell proliferation, we examined their effects on the breast cancer cell line, CAMA-1. While E was able to consistently induce cell proliferation under a variety of experimental conditions, EGF/TGF-α was without effect. Despite the presence of the receptor (EGFR) gene, mature EGFR protein and mRNA were not detected by radioreceptor assay, ^{35}S Met-labelling, and the Intron Differential RNA/PCR method under conditions in which cells remain responsive to E. Furthermore, TGF-α is not an autocrine factor in CAMA-1 cells. We demonstrated unequivocally that EGF/TGF-α interaction with EGFR is not an obligatory event in mediating estrogen-stimulated cell proliferation.

Key words: breast cancer cell line, CAMA-1, Intron Differential RNA/PCR, gene expression, EGF receptor

It has been known for decades that breast cancer is under the influence of a number of hormones [1–4]. In particular, estrogen stimulation and antiestrogen inhibition of tumor growth have been extensively studied in relation to the treatment of this disease with endocrine manipulation or with adjuvant therapy of antiestrogens. In addition, numerous studies have focused on estrogen action on cell proliferation in monolayer breast cancer cells. We have used CAMA-1 cell line to elucidate the mode of estrogen action in cell proliferation [2,5–9]. Previous studies have shown that CAMA-1 cells contained estrogen receptor and that estrogen was able to induce a sevenfold increase of progesterone receptor, modulate the level of membrane acyltransferase activity, increase thymidine uptake and cell number of CAMA-1 cells, promote the traverse of G1 phase cells to S phase by shortening the G1 phase duration without significantly altering the cell cycle time, and increase the proportion of S phase cell population.

Several growth factors (GFs) have been identified in breast cancers [10–12] and their expression in cancer cells in vitro may have important roles as autocrine and/or paracrine regulators of cell proliferation. While the involvement of steroid hormones and GFs in promoting or inhibiting cell proliferation in breast cancer cells has been demonstrated in many studies [1–10], the interrelationship of their actions, if any, is poorly understood. Recently, it was postulated that estrogen-induced cell proliferation might be mediated by GFs such as EGF/TGF-α [13,14], which might act as "second messengers"; however, thus far, there is no definitive proof of this hypothesis. In addition, conflicting results were presented in a recent report that estrogen-induced cell proliferation in MCF-7 was not blocked by antibodies against EGFR [15]. We deemed it worthwhile to examine the relationship of estrogen and EGF/TGF-α in cell proliferation of CAMA-1 cells. Our results, obtained by using several techniques in determining EGFR, show that CAMA-1 cells do not contain the EGFR protein nor do they express the mRNA under the culture conditions in which estrogen can elicit a mitogenic response in CAMA-1. Furthermore, EGF does not affect cell proliferation in the presence or absence of estrogen. These results present strong evidence that estrogen action on cell proliferation in this breast cancer cell line is not mediated via EGF/TGF-α and EGFR pathway.

MATERIALS AND METHODS

Materials

Antibodies to EGFR extracellular domain and α-^{32}P and ^{35}S were purchased from Amersham,

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antibodies to intracellular domain were obtained from ICN Pharmaceuticals (Irvine, CA), nocodazole, methyl [5-(2-thienyl carbonyl)-1 H-benzimidazol-2-yl] carbamate, was a product of Sigma Chemical Co. (St. Louis, MO), fetal bovine serum was purchased from HyClone Laboratories, Inc. (Logan, UT), mouse EGF was obtained from Collaborative Research (Bedford, MA), Percoll was purchased from Pharmacia LKB Biotech, Inc. (Piscataway, NJ), and ^{3}H-thymidine was made by New England Nuclear (Boston, MA). Antibodies immobilized on protein A beads were prepared by incubating EGFR antibodies at 4°C for 2 h with protein A Sepharose (RepliGen, Cambridge, MA). Oligonucleotides (Primer 1: 5′-CGCTGCTGGCTGCGCTCTG; Primer 2: 5′-AGCCACCTCCTGGATGGTC; Probe: 5′-TCAGCCTCCAGAGGATGTTC) were synthesized with an Applied Biosystem 391 DNA Synthesizer.

Methods

Cell culture. Cell culture and cell synchronization of CAMA-1 cells to the mitotic phase by nocodazole have been previously described [9]. Cancer cells in log phase growth were trypsinized, washed, and plated in 12-well cluster plates (Costar). Cells in medium lacking serum, phenol red, growth factor, estrogen were added to wells containing appropriate combinations of the above [9]. For each treatment at each time point triplicate wells were trypsinized and cell counts were estimated using a Coulter counter.

Incorporation of ^{3}H-thymidine. Sufficient CAMA-1 cells were plated on 450 cm^2 culture surface in estrogen-free medium (including 10% dextran-charcoal stripped fetal bovine serum (DCFBS), without phenol red) to achieve 70–80% confluence after 5 days. Fresh medium was then exchanged for old and cells were incubated for another 20 h before addition of nocodazole to a final concentration of 50 ng/ml. After 16 h cells, predominantly synchronized in M phase, were shaken off and applied to a discontinuous gradient of Percoll containing nocodazole; centrifugation was for 20 min at 400*g*. Cells at the density interfaces 1.04/1.05 and 1.05/1.06 were pooled, washed, and plated at 50,000 cells per well in 24-well cluster plates in the presence of 10^{-8} M E_2, 10 ng/ml EGF, or vehicle control. Medium included 1% DCFBS and 10% serum substitute [9]. At indicated times after plating, triplicate wells were pulse labeled with 0.5 uCi ^{3}H-thymidine for 1 h. After washing, cells were digested with 200 μl of 1 N NaOH at room temperature for half an hour, and the digest was neutralized with an approximately equal volume of 1 M HCl. Aliquots of the cell digest were used for determination of thymidine incorporation which serves as a quantitation of the new S phase population. Radioactivity was determined with a Beckman Liquid Scintillation System LS 2800 and counts were corrected for cell number.

Biosynthesis of EGFR protein. Cells (10^5) were seeded into 6-well plates in media supplemented with 10% FBS. On the next day, monolayers were washed with PBS and then incubated in 2% Met and 2% FBS-DMEM containing 50 uCi/ml of ^{35}S Met at 37°C for 16 h. At the end of incubation, cells were scraped off, washed, and lysed. Cell lysates were subjected to immunoprecipitation with protein A Sepharose-linked EGFR monoclonal antibodies at 4°C for 2 h. Beads were washed and boiled, and the bound radioactivities were eluted with SDS-PAGE sample buffer, separated on a 7.5% SDS-PAGE and visualized by fluorography.

Radioreceptor assay for EGFR. Cells were seeded into 12-well plates in regular medium. After 2–3 days, near confluent monolayer was washed with PBS and then incubated at 4°C or 22°C with ^{125}I-EGF (4 ng/ml) in the presence or absence of radioinert EGF (1000 ng/ml) for various periods. ^{125}I-EGF binding to EGFR was conducted according to procedure previously published [16]. At the end of incubation, the medium was removed, and cells were washed and solubilized in 0.5 N NaOH and neutralized with 1N HCl. Cell bound EGF radioactivity was determined in a Beckman gamma counter 4000.

Detection of mRNA by ID RNA/PCR. Total RNAs were isolated from cultured cells by a modification of the quanidine thiocyanate method of Chirgwin et al. [17]. Total RNAs (10 μg) were primed with 40 pmol of down-stream primers (Primer 2) and reversely transcribed into cDNAs by 200 units of M-MLV reverse transcriptase (BRL) in a total 50 μl volume, 37°C, 1 h. An aliquot (10 μl) of the resulting mixture was denatured by incubating at 95°C for 10 min and added to a 100 μl PCR mix with 2.5 units of Taq polymerase according to conditions previously established for ID RNA/PCR method [12]. The design of the primers and probe is illustrated in Figure 1. The expected molecular weight of cDNA PCR product derived from these primers (see Materials) is 221 bp, as

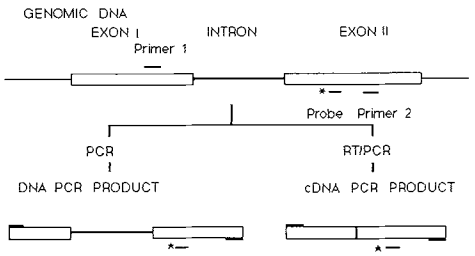

Fig. 1. A schematic representation of the ID RNA/PCR method. For detail, see text.

detected by Southern analysis. Twenty-five to 50 cycles of denaturing (94°C, 30 sec), annealing (45°C, 1 min), and extension (72°C, 30 sec), with an additional one minute incubation at 72°C after the completion of the last cycle, were performed on a Twin-Block Thermocycler (Ericomp Inc., San Diego, CA). The same amount of total RNAs (2 μg) was used in the PCR amplification to serve as a negative control.

Determination of genomic DNA. The 1.84 kb EcoR1 fragment from pHER-A64-1 (ATCC 57484), which includes coding sequences for 2 cystein-rich domains and the transmembrane domain of EGFR [18], was used to prepare radiolabeled EGFR probe with α-^{32}P dATP by a random-priming procedure (Amersham). High molecular weight genomic DNA (10 μg), isolated as described [19], was digested with excess EcoR1, electrophoretically fractionated on 1% agarose, capillarily transferred to nylon membrane, and hybridized [20]. Washing was carried out for 30 min at room temperature in 5× SSC-0.1% SDS, 30 min at 60°C in 2× SSC-0.1% SDS, and the filter was exposed to X-ray film for 5 days at −70°C. λ Phage DNA, digested with Hind III, was used as a size marker.

RESULTS

EGF/TGF-α Effect on Cell Proliferation

Since cell proliferation in CAMA-1 was totally arrested in the absence of serum, we first investigated the effect of mouse EGF on cell growth under a suboptimal level of serum in an attempt to minimize estrogen and EGF/TGF-α present there. As shown in Figure 2A, CAMA-1 cells grew better in 10% FBS than in 1% FBS or steroid-stripped serum prepared by treatment with dextran-coated charcoal (DCFBS). In 1% DCFBS, these cells were able to show a low response to estrogen (0.1 nM) stimulation, as has been previously demonstrated [9]. However, estrogen in 1% DCFBS was unable to restore cell growth to the level of 1% FBS, indicating that certain biomolecules other than estrogen have been removed by dextran-charcoal treatment. The addition of EGF (0.5 or 5.0 ng/ml) to culture with 1% FBS did not result in cell growth different from control cells without added EGF during a 7-day period. Under these culture conditions, we demonstrated that several gynecologic cancer cell lines were responsive to EGF, in a dose-dependent manner, in stimulating thymidine uptake and cell proliferation (Leung, unpublished data). We were concerned that the lack of response to EGF might be contributed by cells cultured in a low serum condition. Further testing of EGF on CAMA-1 cells under 10% dialysed serum again failed to show any stimulatory effect (Fig. 2B). Under this condition, TGF-α at 5 ng/ml was also ineffective. However, in a parallel experiment conducted simultaneously, observable stimulation by estradiol-17β at 10 nM was demonstrated in 10% dialysed (Fig. 2B) or DCFBS (Fig. 2C).

The lack of response of CAMA-1 cells to EGF was surprising in view of the fact that they differed from MCF-7 cells which not only responded to estrogen but also to EGF [3,11,13,21]. To rule out a possible experimental artifact attributed to residual estrogenic effect which might mask EGF action in these cells we undertook more vigorously controlled culture conditions. Since regular MEM medium contains phenol red which can act as a weak estrogen, CAMA-1 cells were cultured for three days in phenol red-free MEM (-pMEM) and 10% DCFBS to deplete endogenous source of estrogen. On the fourth day, they were incubated for 16 h with nocodazole, according to previously established procedure [9], to arrest cells at mitotic phase. This partially synchronized population was then enriched by centrifugation on a discontinuous Percoll gradient, and mitotic cells settled at 1.04/1.05 and 1.05/1.06 density interface. These cells were plated in -pMEM supplemented with 1% DCFBS and a 10% serum substitute according to previously described procedure [9]. The effects of estradiol (10 nM) and EGF (10 ng/ml) were tested on this synchronized mitotic phase cell population. As shown in Figure 3, estrogen was able to enhance these cells to incorporate ^{3}H-thymidine, a measure of the new S-phase population, while EGF was without significant effect. Results from the above experiments showed that CAMA-1 cells did not respond to EGF in vitro.

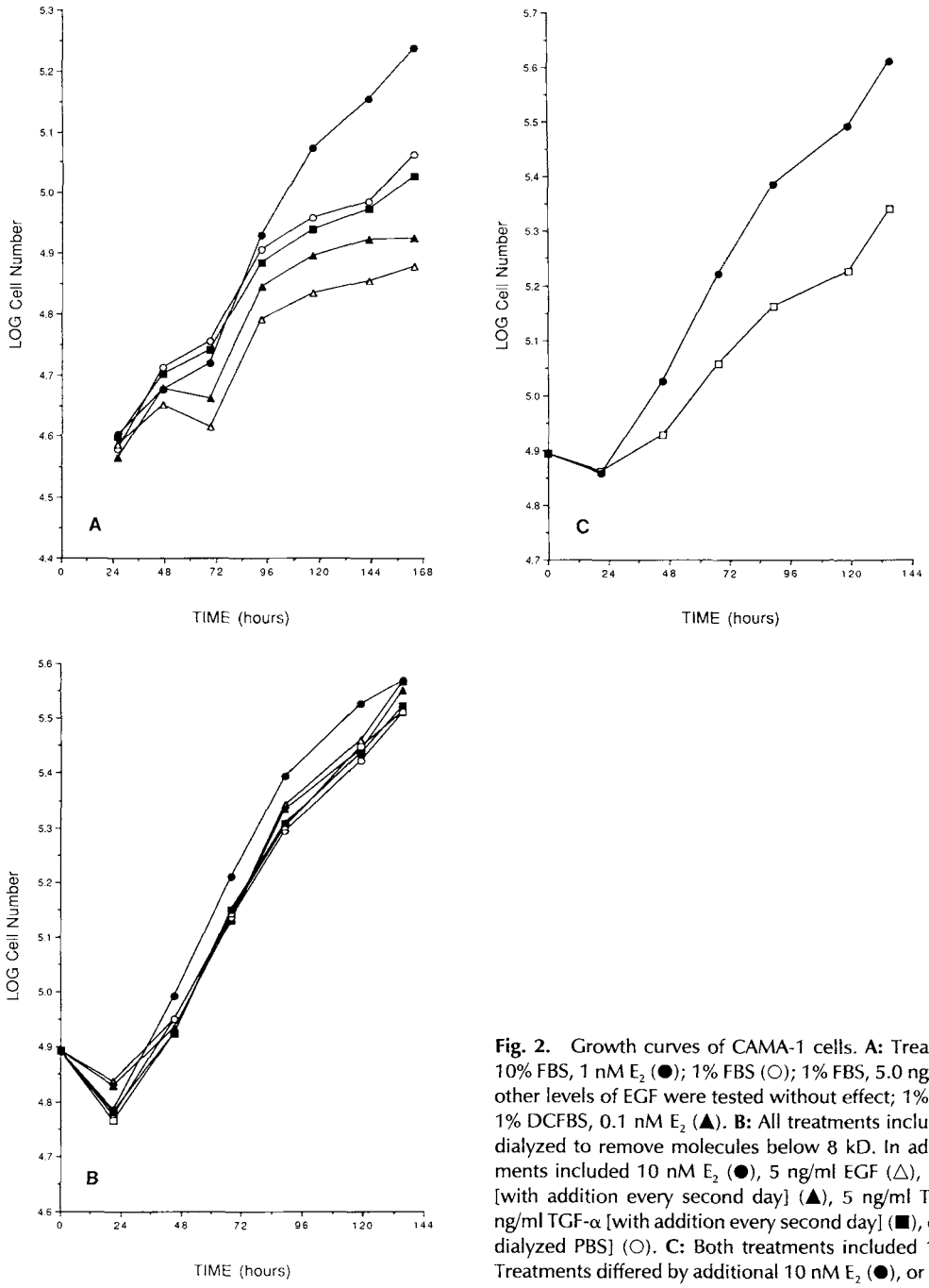

Fig. 2. Growth curves of CAMA-1 cells. **A:** Treatments were 10% FBS, 1 nM E_2 (●); 1% FBS (○); 1% FBS, 5.0 ng/ml EGF (■); other levels of EGF were tested without effect; 1% DCFBS (△); 1% DCFBS, 0.1 nM E_2 (▲). **B:** All treatments include 10% FBS, dialyzed to remove molecules below 8 kD. In addition, treatments included 10 nM E_2 (●), 5 ng/ml EGF (△), 5 ng/ml EGF [with addition every second day] (▲), 5 ng/ml TGF-α (□), 5 ng/ml TGF-α [with addition every second day] (■), control [10% dialyzed PBS] (○). **C:** Both treatments included 10% DCFBS. Treatments differed by additional 10 nM E_2 (●), or no E_2 (□).

Determination of EGFR Protein

The lack of a biological response to EGF/TGF-α in an estrogen-responsive cell line is of particular interest because it can serve as a good model to differentiate EGF/TGF-α–induced cell proliferation from estrogen-induced cell proliferation. Since EGF mediates its biological activity by binding to its cognate membrane receptor, we then explored the binding of ^{125}I-EGF to the cell surface by a radio-receptor assay [16]. Consistent with its lack of biological activity, we found that CAMA-1 cells did not have any specific ^{125}I-EGF binding on the cell surface (Fig. 5). In contrast, OVCAR-3 and CAOV-3 ovarian cancer cells [22] that responded to EGF growth modulation (data not shown) showed specific binding in the same assay. The lack of EGFR was further demonstrated in a pulse experiment by incubating CAMA-1 cells with ^{35}S-Met at 37°C for 16 h. EGFR from cell lysate was isolated by immuno-

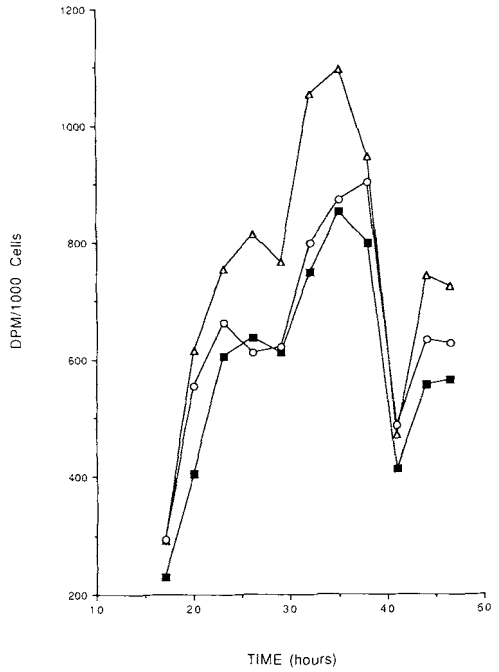

Fig. 3. Pulsed incorporation of ^{3}H-TdR into synchronized CAMA-1 cells. 10^{-8}M E_2 (△), 10 ng/ml EGF (○) and vehicle control (■).

precipitation with monoclonal antibodies to EGFR [23] and the precipitates were analyzed on SDS-PAGE. Figure 4 shows that EGFR was present in OVCAR-3 cells as a 170 kD protein. In contrast, cell lysates of CAMA-1 cells precipitated with antibodies against either the intracellular- or the extracellular-domain of EGFR did not show this band.

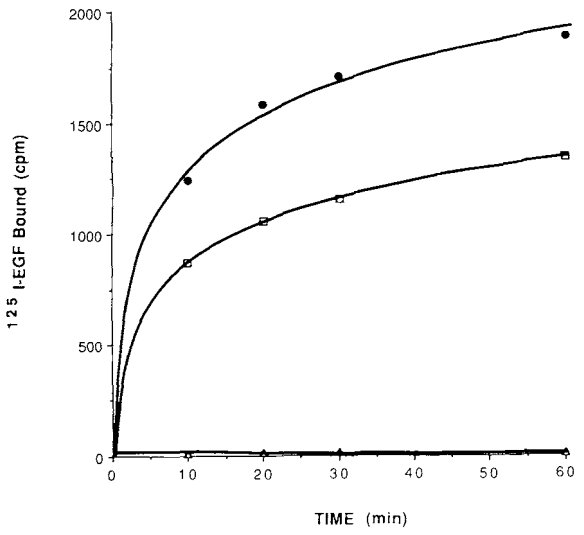

Fig. 4. Determination of EGFR by radioreceptor assay. Figure shows specific binding of CAOV-3 (●), OVCAR-3 (□), and CAMA-1 (△).

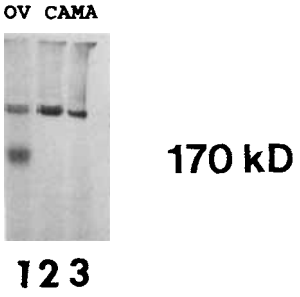

Fig. 5. Biosynthetic labelling of EGFR. The figure shows the fluorographic pattern of OVCAR-3 (Lane 1) and CAMA-1 (Lane 2) as precipitated by extracellular-domain-specific EGFR monoclonal antibodies and CAMA-1 (Lane 3) as precipitated by intracellular-domain-specific monoclonal antibodies. The 170 kD band, shown by an arrow, which represents the mature EGFR, was absent in CAMA-1 cell lysates.

Gene Expression of EGFR

It is known that RNA/PCR procedure offers the most sensitive and rapid means to determine gene expression in cells. We have utilized a method which we termed Intron Differential RNA/PCR (ID RNA/PCR), as shown in Fig. 1, to evaluate the EGFR gene expression in CAMA-1 and OVCAR-3 cells. By design, based on the mobility of the PCR products in the agarose gel electrophoresis, we can distinguish the amplified cDNA copies from the genomic DNA-derived copies by the presence of an intron region in the genomic DNA. This procedure consists of three major steps: a) reverse transcription of extracted RNAs by a down-stream primer, b) PCR run with two primers selected from two adjacent exons, and c) digestion of PCR products with specific restriction enzyme and/or detection of the PCR products by Southern analysis with an internal ^{32}P-probe labelled at the 5′ end. The two primers selected for EGFR were positioned at the N-terminal region. The RNA extracts without reverse transcription were used as negative controls and OVCAR-3 and A431 cells that express the EGFR gene were used as positive controls. Fig. 6A shows that this method is specific for cDNAs of EGFR derived from OVCAR-3 cells, as total RNAs without the reverse transcription process did not show any radioactive bands with the Southern analysis. Similarly, cDNAs from A431 cells were positive for EGFR while those for CAMA-1 cells were negative even after 50 cycles of PCR run. These results show that CAMA-1 cells did not express EGFR under the present experimental conditions and these data are consistent with the foregoing results that CAMA-1 cells are lacking

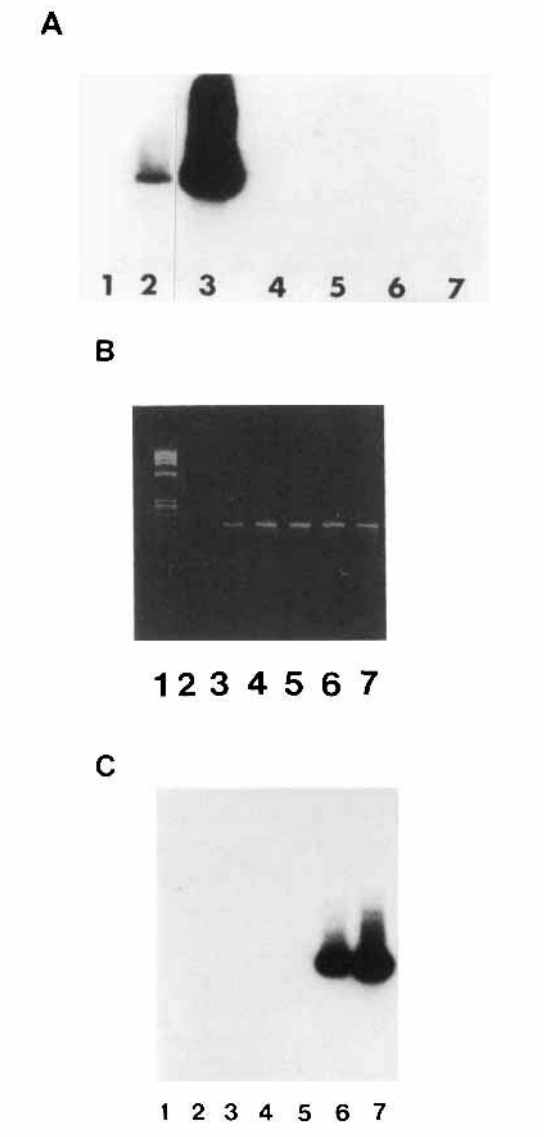

Fig. 6. Analysis of ID RNA/PCR products. **A:** Southern analysis of PCR products. Twenty-five cycles of PCR were performed on OVCAR-3 RNAs (Lane 1), OVCAR-3 cDNA (Lane 2), and A431 cDNA (Lane 3), and 50 cycles for CAMA-1 RNAs (Lane 4), CAMA-1 cDNA (Lanes 5–7). **B:** Agarose gel electrophoresis of c-myc PCR products after 35 cycles. DNA marker ϕX174/Hae III (Lane 1), reagent negative control (Lane 2), CAMA-1 cultured in 0 nM, 5 nM, and 10 nM E_2 (Lanes 3–5), MCF-7 (Lane 6), and CAOV-3 (Lane 7). Primers and region of c-myc have been described [12] and the PCR product is a 191 bp band as shown. **C:** Southern analysis of EGFR PCR product. All conditions were the same as in B, except EGFR primers were used for ID RNA/PCR procedure.

EGFR protein. Following 50 cycles of PCR, this method should be able to detect a few copies of genes represented in 2 μg of total RNAs. Accordingly, if EGFR were present in CAMA-1 cells, it could only occur in an extremely low percentage of cells in the total population. The effect of estradiol-17b on EGFR mRNA was examined under different concentrations of estrogen added to cultures and the extracted RNAs from these cells were subjected to ID RNA/PCR analysis. In each RNA extract, c-myc oncogene was simultaneously analyzed to monitor the efficacy of the procedure. As shown in Fig. 6B, c-myc was present in RNA extracts of CAMA-1, MCF-7, and CAOV-3 cells. Nevertheless, by Southern analysis of the PCR-products (Fig. 6C), EGFR mRNA was only present in MCF-7 and CAOV-3 cells and was absent in CAMA-1 cells regardless of the levels of estradiol added to cultures of CAMA-1 cells. Even at 10 nM of added estradiol, we could not detect EGFR mRNA in CAMA-1 cells.

Determination of Genomic DNA

The presence of EGFR gene in CAMA-1 cells was investigated by Southern analysis (Fig. 7), since a deletion of the gene due to chromosomal aberration could occur. Figure 7 shows that EGFR gene is present in CAMA-1 cells and the Southern bands do not differ in their electromobility pattern from that of MCF-7 or CAOV-3 cells. Furthermore, the EGFR gene is not ampli-

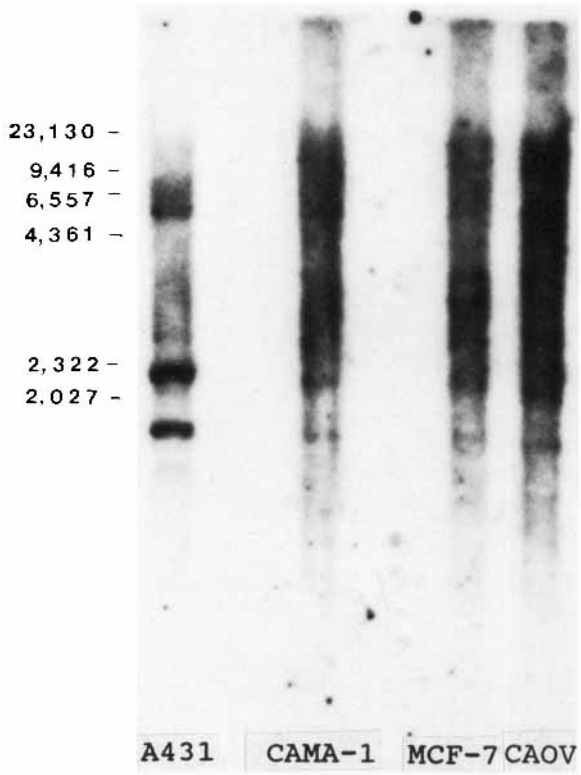

Fig. 7. Southern analysis of EGFR on CAMA-1, A431, MCF-7, and CAOV-3 genomic DNA.

fied in CAMA-1; like MCF-7 and CAOV-3, only one copy of the gene/cell was detected.

DISCUSSION

The mechanism of steroid hormones in breast cancer growth has been a subject of intense study for several decades. It has been well documented that values of estrogen receptor and/or progesterone receptor are related to tumor responsiveness to hormonal therapy [1–3]. Patients with these steroid receptors in tumors out-survived their counterparts lacking the receptors and they generally benefited from antiestrogen therapy, or any mode of treatment which interrupts the estrogenic events in tumors. Despite much effort in studying the mode of estrogen action in various breast cancer models in animals, in monolayer cultures of human-derived breast cancer cell lines, and in human breast cancer patients, the mechanism of estrogen action in tumor growth remains elusive. Accumulated lines of evidence have shown that estrogens or other steroid hormones regulate the production of some GFs or their cognate receptors [4,10,11], and in turn, certain GFs also down- or up-regulate steroid receptors [21,24]. Estrogen modulates the biosynthesis or secretion of several GFs such as insulin-like growth factor-I (IGF-I), EGF, TGF-α, or the EGFR. TGF-α is secreted by MCF-7 cells and this secretion is stimulated by the addition of estrogen, and decreased by antiestrogens [4,10, 11]. Moreover, EGF-like peptides from spent media of estrogen-primed cells were found to partially support the growth of these cells in ovariectomized nude mice [13,14]. The existence of a close relationship between estrogen and EGF system was further implicated by the secretion of three- to fourfold higher levels of TGF-α by an MCF-7 variant transformed by v-Ha-Ras oncogene. While the transformant remained tumorigenic in nude mice it was independent of estrogen for growth [25,26], a finding suggested to be associated with an estrogen-independent growth due to autocrine function of TGF-α in this variant line. Estrogen stimulation of EGFR has also been reported in estrogen-primed rat uterus [27,28], and other cell systems [29]. There is also an inverse quantitative relationship of EGFR to estrogen receptor and/or progesterone receptor [30–32]; thus, tumors with high EGFR behaved clinically in a more aggressive manner and were refractory to hormonal treatment. These findings have led to the general belief that EGF/TGF-α could be a mediator of estrogen action in cell proliferation [13,14]. However, this notion was not supported by a recent report which showed that MCF-7 cell growth induced by estrogen was not suppressed by the blockage of antibody against EGFR [15]. We selected the CAMA-1 cell line to further examine this controversy since this cell line, like MCF-7, has been demonstrated to be estrogen dependent [4–9]. However, our findings herein show that CAMA-1 cells differ from MCF-7 in that they do not respond to EGF or TGF-α in cell proliferation. Furthermore, these cell do not contain detectable level of EGFR.

There are a number of possibilities that might explain the absence of mRNA or protein for EGFR in CAMA-1 cells, including: a) a rapid degradation of these proteins occurred following the synthesis due to EGFR-specific proteases; b) a mutation, deletion, or rearrangement of the EGFR gene such that its biological activity were obscured or its gene product were no longer detectable by EGFR-specific antibodies; c) the EGFR gene were absent in CAMA-1 cells; d) the mRNA in this tumor had an extremely short half-life because of EGFR-specific endonuclease activities; e) EGFR protein might be expressed only in a transient manner at certain phase of the cell cycle; f) the EGFR might be present only in a subtype which constituted a small percentage of the total cell population; g) an aberration of the regulatory mechanism controlling the expression of the EGFR protein so that its gene expression were modified or annulled; and h) the presence of EGFR-specific suppressor which blocks the expression of EGFR. In this study, we have designed experiments to examine some of these possibilities. Our results showed that CAMA-1 cells contain the EGFR gene, and like MCF-7, this gene was not amplified. Although the reason for the lack of gene expression for EGFR remains to be determined, collectively, our results suggest a high probability that it is either contributed by abnormalities at the level of gene regulation, or there is the presence of EGFR-specific suppressor which prevents EGFR expression.

It is worthwhile to note that the cells we harvested for EGFR determination were cultured under conditions in which CAMA-1 cells exhibited estrogen-induced cell proliferation. Therefore, the present findings that no EGF/TGF-α stimulation on CAMA-1 cell growth and the lack of expression for both EGFR mRNA

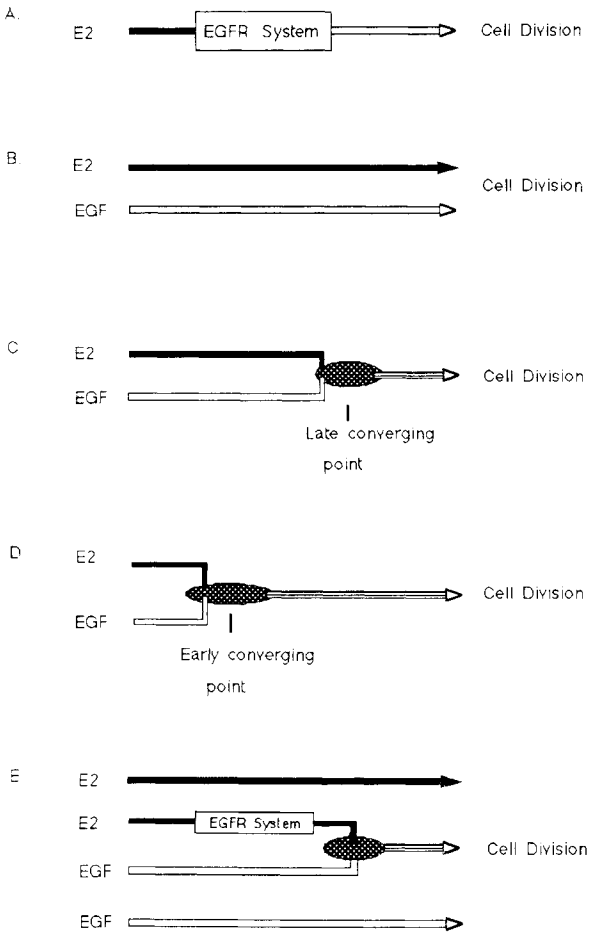

Fig. 8. Diagrammatic illustration of the plausible pathways of EGFR system interaction in estrogen-induced cell proliferation.

and protein are not consistent with the concept of a mediating role of EGF/TGF-α in the pathway of estrogen-induced cell proliferation (Fig. 8A). Furthermore, EGF/TGF-α binding to the EGFR system is not an obligatory event in estrogen-regulated cell proliferation. Our results favor the hypothesis that the action of EGF/TGF-α and that of estrogen work independently in promoting cell proliferation (Fig. 8B). These results do not rule out the possibility that convergent points for EGF and estrogen occur in the proliferation pathway common to both of these growth modulators (Fig. 8C,D). We presume that an EGFR-dependent pathway exists in other cell systems, such as MCF-7 cells and uterine tissues; such a pathway could conceivably function as an alternative route prior to all the mitogenic events, as shown in Fig. 8E.

In this study, we have primarily investigated EGF/EGFR in CAMA-1 cells in relationship to estrogen-induced cell proliferation. Transcripts for TGF-α were detected in CAMA-1 by the ID RNA/PCR [12] and Northern blot [33]. The role of TGF-α in CAMA-1 cells is unknown but it is unlikely that it functions as an autocrine factor in CAMA-1 cells in view of the fact that there is no EGFR for TGF-α binding. In summary, we have presented several lines of information demonstrating the presence of an EGFR-independent pathway for estrogen-induced cell proliferation in CAMA-1 cells. Such a mechanism is likely to occur in vivo since the absence of EGFR has been shown in breast cancer specimens with estrogen receptors [30–32].

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