

Two Independent Mechanisms for Escaping Epidermal Growth Factor-mediated Growth Inhibition in Epidermal Growth Factor Receptor-hyperproducing Human Tumor Cells

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Abstract. Human squamous cell carcinoma cell lines often possess increased levels of epidermal growth factor (EGF) receptor. The growth of these EGF receptor-hyperproducing cells is usually inhibited by EGF. To investigate the mechanism of EGF-mediated inhibition of cell growth, variants displaying alternate responses to EGF were isolated from two squamous cell carcinoma lines, NA and Ca9-22; these cell lines possess high numbers of the EGF receptor and an amplified EGF receptor (EGFR) gene. The variants were isolated from NA cells after several cycles of EGF treatment and they have acquired EGF-dependent growth. Scatchard plot analysis revealed a decreased level of EGF receptor in these ER variants as compared with parental NA cells. Southern blot analysis and RNA dot blot analysis demonstrated that the ER variants had lost the amplified EGFR gene. One variant

isolated from Ca9-22 cells, CER-1, grew without being affected by EGF. CER-1 cells had higher numbers of EGF receptor than parental Ca9-22 but similar EGFR gene copy number. Flow cytometric analysis indicated an increase in ploidy and cell volume which may give rise to the increase in receptor number per cell. The EGF receptors on both Ca9-22 and CER-1 cells were autophosphorylated upon EGF exposure in a similar manner suggesting no obvious alteration in receptor tyrosine kinase. However, very efficient down-regulation of the EGF receptor occurred in CER-1 cells. These data suggest two independent mechanisms by which EGF receptor-hyperproducing cells escape EGF-mediated growth inhibition: one mechanism is common and involves the loss of the amplified EGFR genes, and another is novel and involves the efficient down-regulation of the cell-surface receptor.

EPIDERMAL growth factor (EGF)¹ is a potent mitogen for a variety of cells (Carpenter and Cohen, 1979). The mitogenic signal of EGF is transduced through its membrane-anchored glycoprotein receptor of *M*, 170,000 (Cohen et al., 1982). The binding of EGF to the receptor induces a series of biochemical reactions including autophosphorylation of the EGF receptor (Cohen et al., 1980), clustering of the EGF receptor into coated pits (Schechter et al., 1979) and internalization and intracellular processing of the EGF/EGF receptor complex (Schlessinger et al., 1978; Haigler et al., 1978; Miskimins and Shimizu, 1984). Concurrently, cellular protein phosphorylation (Hunter and Cooper, 1981), *c-fos* and *c-myc* protooncogene expression (Müller et al., 1985) and phosphatidylinositol turnover (Sawyer and Cohen, 1981) are induced.

However, the presence of EGF at the concentrations mitogenic for other cells was found to be markedly inhibitory for the growth of the epidermoid carcinoma cell line A431 (Gill and Lazar, 1981; Barnes, 1982), which possesses extremely high numbers of the EGF receptor (Fabricant et al., 1977)

1. *Abbreviations used in this paper:* EGF, epidermal growth factor; EGFR, epidermal growth factor receptor (genes).

as well as EGF receptor (EGFR) gene amplification (Lin et al., 1984; Ullrich et al., 1984). Similar growth inhibition has been reported for the breast cancer cell line MDA-468 (Filmus et al., 1987), which also possesses high numbers of the EGF receptor. To determine which biochemical response is crucial for growth inhibition of these cells and also for growth stimulation of cells with ordinary numbers of EGF receptor, we and others have isolated EGF-response variants using EGF (Buss et al., 1982; Bravo, 1984), EGF conjugated with the A subunit of ricin (Shimizu et al., 1984) and anti-EGF receptor antibody conjugated with the A subunit of ricin (Behzadian and Shimizu, 1985a). The correlation of growth inhibition with receptor concentration (Kawamoto et al., 1984), receptor tyrosine kinase activity (Buss et al., 1982), cellular protein phosphorylation (Buss et al., 1984), receptor metabolism (Lifshitz et al., 1983), protooncogene induction (Bravo et al., 1985) and phosphatidylinositol turnover (Behzadian and Shimizu, 1985a) was extensively studied in these variants. In most cases, the variants were found to contain reduced levels of EGF receptor (Filmus et al., 1987; Buss et al., 1982; Shimizu et al., 1984; Behzadian and Shimizu, 1985a), which seemed to be essential for escape

from growth inhibition. Moreover, the reduction in EGF receptor numbers accompanied the loss of the amplified EGFR gene which was responsible for receptor hyperproduction (Filmus et al., 1987; Gill et al., 1985).

Recently, many of the cell lines derived from squamous cell carcinoma of the head and neck (Cowley et al., 1984) and the oral cavity and esophagus (Yamamoto et al., 1986; Gamou et al., 1988) were reported to possess elevated levels of EGF receptor accompanied with EGFR gene amplification.

In this report, we describe that EGF-mediated growth inhibition is common in EGF receptor-hyperproducing cell lines. Further, variants which displayed alternate responses to EGF were isolated from two squamous cell carcinoma cell lines, NA and Ca9-22. Our analysis of the EGF-response variants suggested a novel mechanism by which EGF receptor-hyperproducing cells escape EGF-mediated growth inhibition without losing the amplified EGFR gene.

Materials and Methods

Cell Culture

Squamous cell carcinoma cell lines (Yamamoto et al., 1986; Gamou et al., 1988) were kindly provided by Dr. K. Rikimaru (Tokyo Medical and Dental University, Tokyo, Japan). Cell lines were maintained in DME (Gibco, Grand Island, NY) supplemented with 10% FCS (Boehringer-Mannheim), kanamycin (100 µg/ml, Meiji, Tokyo, Japan) and fungizone (1 µg/ml, Squibb, Princeton, NJ) (DME/FCS10) in 5% CO₂ and 100% humidity at 37°C.

Variant Selection

NA and Ca9-22 cells grown in a 90-mm culture dish (~10⁷ cells/dish) were treated with EGF (100 ng/ml; Toyobo [Osaka, Japan]; ultra pure grade) for 3 d and then transferred to 10 90-mm dishes. Fresh media containing EGF (100 ng/ml) were added every 3 d for 1 mo. Several independent colonies which grew in the presence of EGF were isolated. To determine the growth rate of the cells, 5 × 10⁴ cells were plated in 35-mm culture dishes containing DME/FCS10. After 24 h of incubation, the media was replaced with media containing various concentrations of EGF. The cells were detached with trypsin/EDTA and counted with a hemocytometer.

[¹²⁵I]EGF-binding Assay

[¹²⁵I]EGF Binding was assayed as previously described (Gamou et al., 1984). To separate cell surface-bound EGF from intracellular EGF, cells which had been incubated with [¹²⁵I]EGF were treated for 1 min with 0.5 M NaCl/0.2 N acetic acid as described (Haigler et al., 1980). Then the cells were dissolved with 1 N NaOH to determine the amount of intracellular EGF. Degraded EGF in the conditioned medium was separated from intact EGF by adding TCA (final concentration 20%) as described (Gamou et al., 1984).

EGF-Receptor Gene and Its Expression

DNA was isolated as previously described (Hunts et al., 1985), and digested with Hind III (Toyobo). The restriction fragments were separated by agarose gel (0.8%) electrophoresis and transferred to a nitrocellulose filter (Schleicher & Schüll, Keene, NH).

RNA dot blot hybridization was carried out as described (White and Bancroft, 1982). Briefly, cells in 45 µl of Tris buffer (10 mM, pH 7.0)/EDTA (1 mM) were lysed by adding 5 µl of 5% Triton X-100 and mixing on ice for 5 min. After a brief centrifugation to pellet the nuclei, 50 µl of supernatant was mixed with 30 µl of 20× SSC and 20 µl of formalin and incubated at 60°C for 15 min. The cell extract was serially diluted with 15× SSC and applied to a nitrocellulose filter.

The EGF receptor cDNA pE7 (Merlino et al., 1984), kindly provided by Dr. I. Pastan (National Cancer Institute, Bethesda, MD), was labeled with [α-³²P]dCTP and used as a hybridization probe as described (Hunts et al., 1985).

Flow Cytometric Analysis of Cell Volume and EGF Receptor

Cell volume was determined by the intensity of forward scatter light (Mullaney and Dean, 1970) using Toraysphere (Toray Techno Co. Ltd., Otsu, Japan) as a size marker.

To measure the EGF receptor density on the cell surface, cells were first incubated with anti-human EGF receptor monoclonal antibody B4G7 (Behzadian and Shimizu, 1985b) for 40 min at 0°C in DME buffered with Hepes (25 mM, pH 7.4), and washed twice with PBS supplemented with glucose (1 mg/ml). Then the cells were incubated with FITC-labeled sheep anti-mouse IgG antibody (Silenus), washed as described above and resuspended in Hepes-buffered DME. A FACS 440 (Becton Dickinson) was used for the flow cytometric analysis (Maron et al., 1984).

Quantitation of Cellular DNA Content

Detached cells were fixed with 70% ethanol, washed twice with glucose-supplemented PBS and treated with RNase A (1 mg/ml, Sigma Chemical Co., St. Louis, MO) for 30 min at 37°C. Then the cells were washed twice with calcium- and magnesium-free PBS containing EDTA (0.02%) and stained with propidium iodide (50 µg/ml) in the same solution. Flow cytometric analysis was performed as described (Crissman and Steinkamp, 1973).

Chromosome Analysis

Chromosome spreads were prepared after 0.075 M KCl treatment and Carnoy fixation. Karyotypes were analyzed after trypsin/Giemsa banding as described (Gill et al., 1985).

Rate of Receptor Biosynthesis

4 × 10⁵ cells were plated in 35-mm dishes and incubated at 37°C for 2 d. Cells were treated with EGF (100 ng/ml) for the indicated times, washed twice with methionine-free DME (Microbiological Assoc.) and then labeled with [³⁵S]methionine (80 µCi/ml, tran-³⁵S-label, ICN) in methionine-free DME/FCS10 for 30 min. After labeling, the EGF receptor was immunoprecipitated with B4G7 antibody bound to protein A-Sepharose and analyzed on SDS-PAGE as described previously (Gamou et al., 1987).

Receptor Phosphorylation

Confluent cells in 35-mm dishes were washed twice with phosphate-free minimum essential medium (MEM), then labeled with [³²P]orthophosphate (2.5 mCi/ml, Amersham Corp., Arlington Heights, IL) in phosphate-free MEM for 1 h. EGF (200 ng/ml) was directly added to medium at 15 min before lysis. After labeling, the EGF receptors were immunoprecipitated with B4G7 and purified on SDS-PAGE as described before (Gamou et al., 1987). Two-dimensional phosphopeptide mapping and phosphoamino acid analysis were performed as described previously (Beemon and Hunter, 1978; Iwashita and Fox, 1984).

Results

Isolation of EGF Resistant Variants

Growth of the EGF receptor-hyperproducing A431 epidermoid carcinoma cell line was inhibited by EGF. Overproduction of EGF receptor to the same extent as A431 cells was often observed in squamous cell carcinoma lines (Yamamoto et al., 1986; Gamou et al., 1988). Thus, we first examined whether EGF-mediated growth inhibition is common in these EGF receptor-hyperproducing cell lines. As shown in Fig. 1, EGF inhibited the growth of the receptor-hyperproducing cell lines.

To investigate the mechanism of EGF-mediated inhibition of cell growth, variants showing alternate responses to EGF were isolated from two cell lines, NA and Ca9-22. These cell lines have a high number of EGF receptors (Table I) and an amplified EGFR gene; they are the most sensitive cell lines

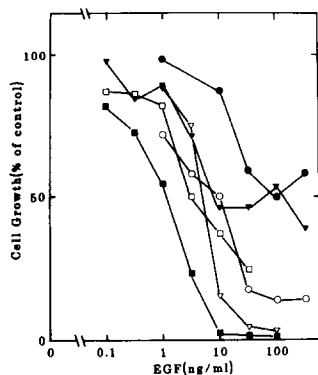


Figure 1. EGF-mediated growth inhibition in EGF receptor-hyperproducing cell lines. 5×10^4 cells of each cell line were plated in 35-mm dishes and incubated for 1 w. EGF was added at various concentrations on 1, 3, and 5 d. Cells were detached with trypsin/EDTA and counted on 7 d using a hemocytometer. (■) NA; (□) Ca9-22; (○) HSC-2; (●) HSC-3; (▼) HSC-4; (▽) A431.

to EGF-mediated growth inhibition. Variants which grew after several cycles of EGF (100 ng/ml) treatment appeared at an approximate rate of 1×10^{-6} in both cell lines, and several clones were isolated from each cell line.

Characteristics of EGF-Resistant Variants from NA Cells

As shown in Fig. 2 A and B, the ER variants, from NA cells, grew in an EGF dose-dependent fashion. The ER variants and parental NA cells showed no difference in their morphology while growing (Fig. 3, A and D). Scatchard plot analysis revealed a decreased level of EGF receptors on their surface as compared with NA cells (Table I). Autophosphorylation of the EGF receptor from membrane preparation indicated a substantial decrease in EGF-responsive protein kinase activity (data not shown), reflecting the decrease in active EGF receptor on their surface.

To examine the reason for the reduced levels of EGF receptor on the cell surface, Southern blot analysis and RNA dot hybridization analysis were performed using the EGF receptor cDNA probe pE7. The ER variants were found to have lost their amplified EGFR gene (Fig. 4 A), accompanied by a decrease in EGF receptor mRNA (Fig. 4 B).

Thus, the mechanism to acquire resistance to EGF-mediated growth inhibition in these variants appears to be through a decrease in EGF receptor level by the loss of the amplified EGFR gene. This situation is similar to the EGF-resistant

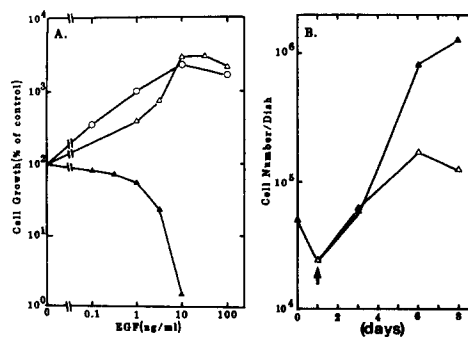


Figure 2. Growth of the ER variants in the presence of EGF. (A) EGF dose-response study. 5×10^4 cells were incubated with various concentrations of EGF. After 1 w of incubation, cells were detached and counted as described in Fig. 1. (▲) NA; (○) ER6-7; (△) ER11. (B) Kinetic study. ER11 (5×10^4 cells) was incubated in the absence (△) or presence (▲) of EGF (50 ng/ml). EGF was added on 1, 3, 5, and 7 d. Cells were detached and counted on every other day as described in Fig. 1.

variants isolated from A431 cells (Gill et al., 1985). However, the ER variants are unique because of their acquired EGF-dependent growth. In contrast, the variants from A431 cells previously reported grew regardless of the presence or absence of EGF (Buss et al., 1982).

Characteristics of EGF-Resistant Variants from Ca9-22 Cells

The CER variants were isolated from Ca9-22 cells in the same manner that ER variants were isolated from NA cells. The CER variants in general appeared to have a flatter morphology than the parental cells (Fig. 3, E and G). Many of the variants grew regardless of the presence of EGF but at retarded rate (Fig. 5). EGF resistance was further analyzed in CER-1 cells, the only variant which grew without being affected by EGF.

Scatchard plot analysis revealed higher numbers of EGF receptor in CER-1 cells than in Ca9-22 cells (Table I). Flow cytometric analysis using anti-EGF receptor antibody also demonstrated the increased EGF receptor level on the CER-1 cell surface ($\sim 150\%$ of Ca9-22, Fig. 6 A). Cytoplasmic

Table I. EGF Receptor Number and Affinity in EGF-Response Variants

Cell	Receptor Number			Dissociation Constant	
	High*	Low*	Total	K_{d1}	K_{d2}
		$\times 10^5$ sites/cell		<i>nM</i>	<i>nM</i>
NA	6	41	47	0.4	8.3
ER6-7	0.4	2.6	3	0.25	16
ER11	0.5	2.5	3	0.25	13
Ca9-22	3	21	24	0.3	7.0
Ca9-22 (EGF)†	ND	21	21(13%)§	ND	7.0
CER-1	4	47	51	0.12	5.5
CER-1 (EGF)†	ND	20	20(61%)§	ND	5.5

* The number of high (K_{d1}) and low (K_{d2}) affinity type EGF receptor was determined by the Scatchard plot analysis.

† Cells were treated with EGF (10 ng/ml) for 10 h before the [125 I]EGF-binding assay.

§ Loss of EGF receptor number after EGF treatment.

ND, Not detected.

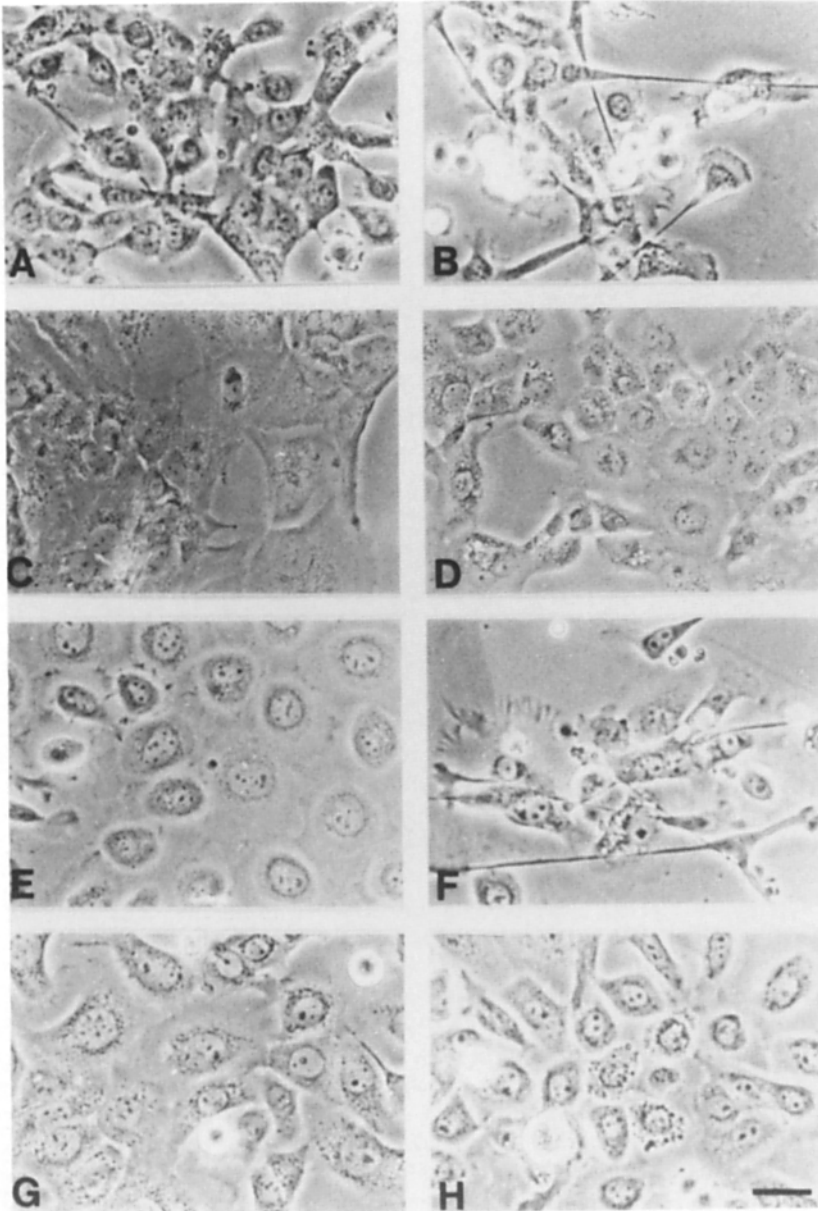


Figure 3. Morphology of the EGF receptor-hyperproducing cells and their variants. (A and B), NA cells; (C and D), ER11 cells; (E and F), Ca9-22 cells; (G and H), CER-1 cells. (A, C, E, and G), cultures in the absence of EGF; (B, D, F, and H), in the presence of EGF (100 ng/ml) for 24 h. Bar, 10 μ m.

RNA dot blot analysis indicated a relatively higher level of EGFR gene expression in CER-1 cells (Fig. 7 B). Southern blot analysis, however, did not indicate an increased amount of EGFR gene (Fig. 7 A). No difference on the rate of receptor protein biosynthesis was observed in CER-1 cells compared to Ca9-22 cells (Fig. 8).

Interestingly, cytogenetic analysis and flow cytometric analysis for DNA content indicated an increase in the ploidy of CER-1 cells. Ca9-22 cells are hyperdiploid (mode of chromosome number, 56–57) (Fig. 9 A) with numerous rearranged chromosomes (Fig. 10), whereas CER-1 cells were hypertriploid (mode of chromosome number, 83) (Fig. 9 A). Flow cytometric analysis confirmed the presence of a larger amount of DNA in CER-1 cells than in Ca9-22 (Fig. 9, B and C). Thus, the increase in ploidy could result in an increase in cell volume (and cell surface) in CER-1 cells, which may accompany the increase in receptor number per cell. As

shown in Fig. 6 B, flow cytometric analysis demonstrated an increased cell volume in CER-1 cells (\sim 116% of Ca9-22).

EGF Receptor Phosphorylation

We have examined the possibility whether the acquired EGF resistance in CER-1 cells correlates with a change in the EGF receptor autophosphorylation. As shown in Fig. 11 A, EGF receptors in both CER-1 and Ca9-22 cells were phosphorylated and their phosphorylation was enhanced by EGF. Phosphopeptide map showed that the phosphorylation of peptide 9 was enhanced by EGF (Fig. 11 B). The phosphorylated amino acid of this peptide was found to be tyrosine (data not shown). Considering the previous observation for A431 cells (Downward et al., 1984), this peptide is likely to correspond to the *in vivo* autophosphorylation site including the Tyr-1173 residue. Thus, EGF receptors in CER-1

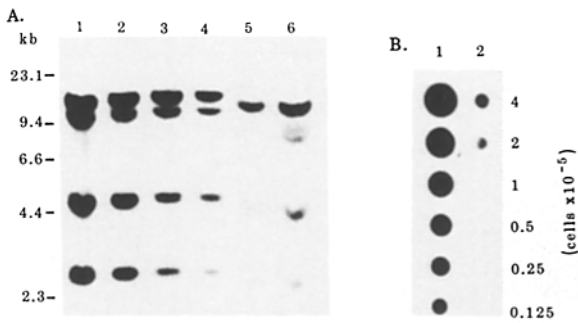


Figure 4. EGFR gene and its expression in the ER variants. DNA (10 μ g) was digested with Hind III, serially diluted, subjected to electrophoresis on an agarose gel (0.8%) and transferred to a nitrocellulose filter. 10^6 cells were lysed with Triton X-100 containing buffer and centrifuged briefly to separate cytoplasmic and nuclear fractions. Cytoplasmic fractions, containing RNA, were denatured with formalin, serially diluted with $15\times$ SSC and applied directly to a nitrocellulose filter equilibrated with $15\times$ SSC. Hybridization was performed using the EGF receptor cDNA probe pE7. (A) Southern blot analysis. (Lane 1) NA (10 μ g); (lane 2) NA (5 μ g); (lane 3) NA (2.5 μ g); (lane 4) NA (1.25 μ g); (lane 5) ER11 (10 μ g); (lane 6) placenta (10 μ g). Size marker used was lambda phage DNA cut with Hind III. (B) RNA dot blot hybridization analysis. (Lane 1) NA; (lane 2) ER11 maintained in the absence of EGF for 2 d.

cells can be autophosphorylated in a manner similar to parental Ca9-22 cells. These results suggest that alterations in EGF receptor kinase may not be involved in the EGF resistance in CER-1 cells.

EGF Internalization and Receptor Down-Regulation

We have examined the possible difference in the internalization and degradation rate of EGF in CER-1 and Ca9-22 cells at 37°C. As shown in Fig. 12, A and B, the amount of [125 I]EGF associated with the intracellular fraction and mem-

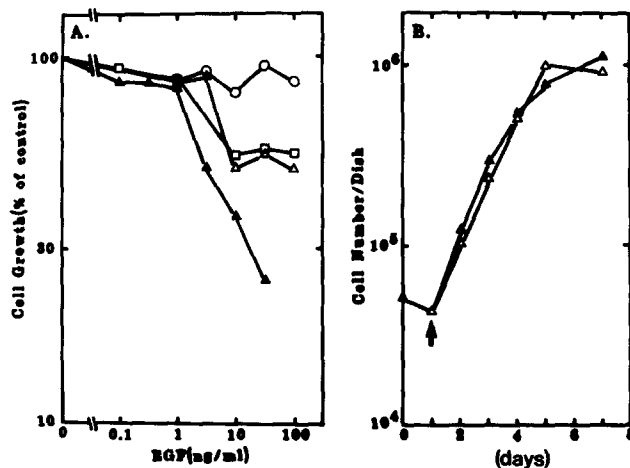


Figure 5. Growth of the CER variants in the presence of EGF. EGF dose-response study and kinetic study were carried out as described in Fig. 2. (A) EGF dose-response study. (▲) Ca9-22; (○) CER-1; (△) CER-4; (□) CER-8. (B) Kinetic study. (Δ) CER-1 in the absence of EGF; (▲) in the presence of EGF.

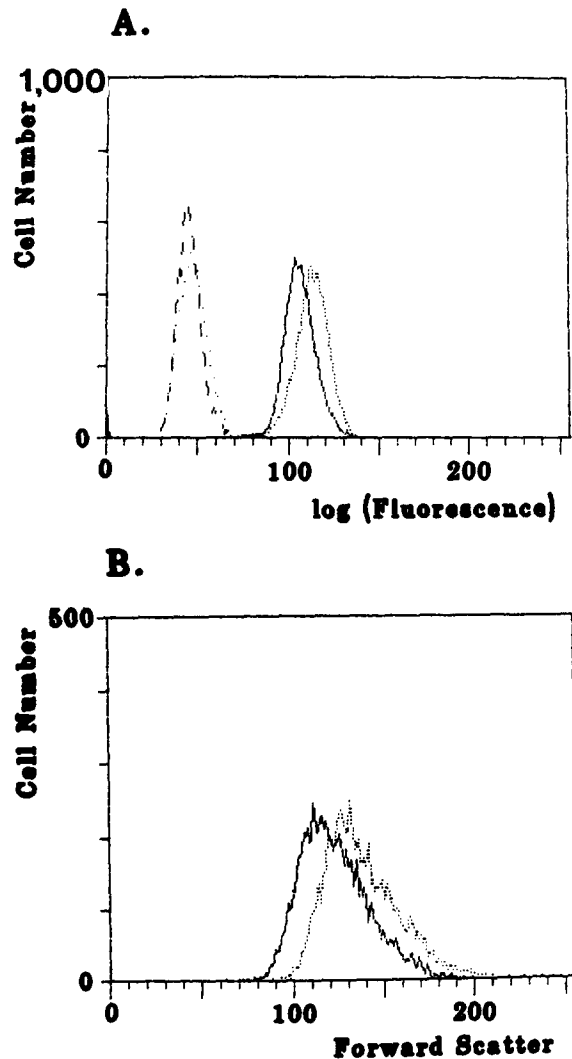


Figure 6. Flow cytometric analysis of relative number of EGF receptors and cell volume in Ca9-22 and CER-1. Cells were incubated with anti-EGF receptor antibody (B4G7) for 40 min at 4°C and then with FITC-conjugated secondary antibody. Analysis was performed on a FACS 440. To determine nonspecific binding of antibodies, cells were first incubated with a nonspecific mouse IgG and then with the secondary antibody. (A) Logarithm of fluorescence intensity. The solid line (—) and dotted line (·····) indicate the B4G7 derived fluorescence in Ca9-22 and CER-1, respectively. The broken line (---) and sparsely dotted line (· · · · ·) indicate nonspecific fluorescence in Ca9-22 and CER-1, respectively. (B) Forward scatter light intensity. The solid line and dotted line indicate the intensity of the forward scatter light of Ca9-22 and CER-1, respectively.

brane fraction of CER-1 cells was lower than Ca9-22 cells despite the elevated EGF receptor levels. Moreover, there was no difference in the secretion rate of degraded EGF (Fig. 12 C). Then, we examined the [125 I]EGF-binding activity after cells were treated with unlabeled EGF (10 ng/ml) for various periods of time. EGF-binding activity of both cell lines was reduced within 3 h to a similar level and the reduced level was kept for at least 10 h (data not shown). Scatchard plot analysis of the binding data revealed that Ca9-22 cells lost 13% of the total EGF receptors by EGF exposure, whereas CER-1 cells lost 61% of the initial surface receptors (Table

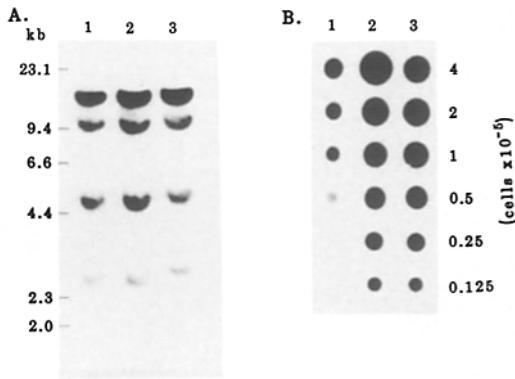


Figure 7. EGFR gene and its expression in Ca9-22 and CER-1. DNA and RNA were prepared and the hybridization was performed as described in Fig. 4. (A) Southern blot analysis and (B) RNA dot blot hybridization analysis. (Lane 1) Ca9-22; (lane 2) CER-1 maintained in the presence of EGF; (lane 3) in the absence of EGF.

I). The decreased EGF binding after exposure to a limited amount of EGF suggests efficient down-regulation of the receptor. Since EGF receptor gene expression was not affected by EGF (Figs. 7 B and 8), the down-regulation may not be caused by transcriptional or translational control. If the receptor internalization occurs only with bound EGF, CER-1 cells with elevated number of receptors should have more internalized EGF than Ca9-22 cells. However, this was not the case. The receptors internalized from CER-1 cells after EGF exposure were far more than the number expected from EGF-binding assay. Therefore, these results suggest that CER-1 cells down-regulate (or internalize) the unoccupied surface receptors more efficiently than Ca9-22 cells. The down-regulation seen in CER-1 cells may involve a mechanism similar to the negative cooperativity or desensitization of the hormone receptor. Although the exact molecular mechanism must await further investigation, this mechanism may give rise to the EGF-resistance of CER-1 cells without loss of the amplified EGF receptor gene.

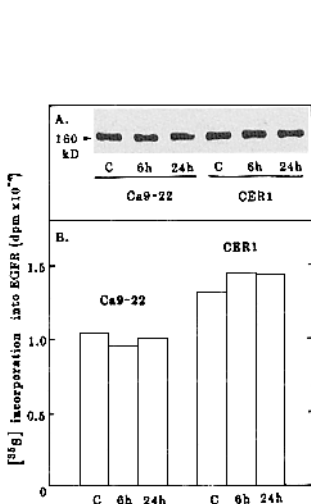


Figure 8. EGF receptor biosynthesis in Ca9-22 and CER-1. Cells were treated with EGF (100 ng/ml) for the indicated times, labeled with [³⁵S]methionine (80 μCi/ml) for 30 min and lysed with RIPA buffer. EGF receptor was immunoprecipitated using anti-EGF receptor antibody and subjected to electrophoresis on SDS-polyacrylamide gel (7%), followed by fluorography (A). The radioactivity in EGF receptor region was determined in a liquid scintillation counter (Beckman LS3800) after solubilizing the gel with 15% H₂O₂/1% NH₃ (B).

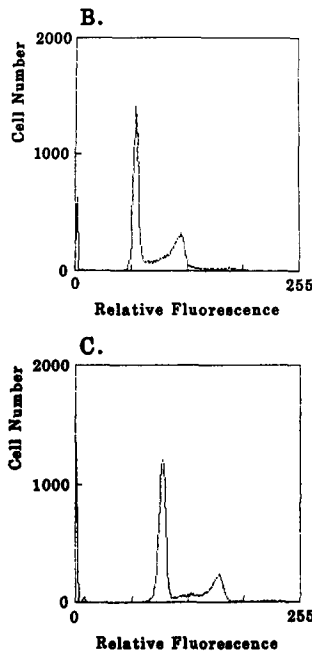
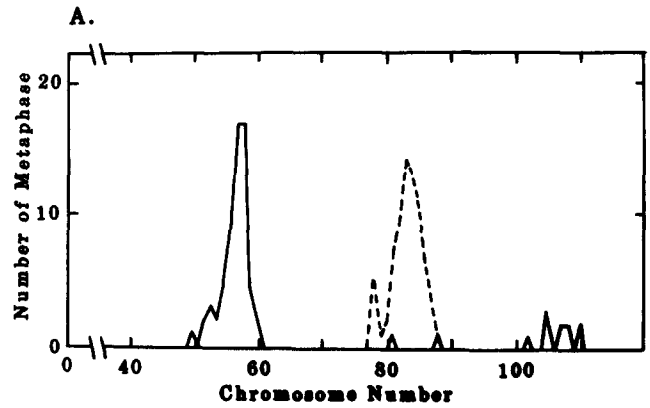


Figure 9. Chromosome number and DNA content in Ca9-22 and CER-1. (A) Chromosome number analysis. Chromosome spreads were prepared after treatment with 0.075 M KCl and Carnoy fixative and stained with Giemsa solution. 76 and 74 metaphases of Ca9-22 and CER-1, respectively, were photographed and chromosome number was determined. The solid line and dotted line represent Ca9-22 and CER-1 cells, respectively. (B) Flow cytometric analysis of DNA content in Ca9-22. Ca9-22 cells were fixed with 70% ethanol, treated with RNase A and stained with propidium iodide (50 μg/ml). Flow cytometric analysis was performed on a FACS 440. (C) Flow cytometric analysis of DNA content in CER-1.

Discussion

Addition of EGF to the culture medium at concentrations that are mitogenic for other cell types results in a marked inhibition of A431 cells' growth (Gill and Lazar, 1981; Barnes, 1982). We extended this analysis to other EGF receptor-hyperproducing cell lines of squamous cell carcinoma origin (Yamamoto et al., 1986; Gamou et al., 1988). We found that growth inhibition by EGF is common in EGF receptor-hyperproducing cell lines. The only exception is the pancreatic carcinoma cell line UCVA-1, possessing $\sim 9 \times 10^5$ receptors/cell (Gamou et al., 1984) with no EGFR gene amplification (Hunts et al., 1986). The growth of UCVA-1 cells was not affected by EGF under either serum-free or serum-containing conditions. UCVA-1 cells have a decreased rate of metabolic turnover of the EGF receptor, which may be responsible for the increased cell surface receptor level (Gamou and Shimizu, 1987). This novel mechanism, however, may not cause EGF-resistance, since the turnover rate in the presence of EGF was similar to other EGF-sensitive, receptor-hyperproducing cell lines.

To investigate the mechanism of EGF-mediated growth inhibition as well as EGF-mediated growth stimulation, we

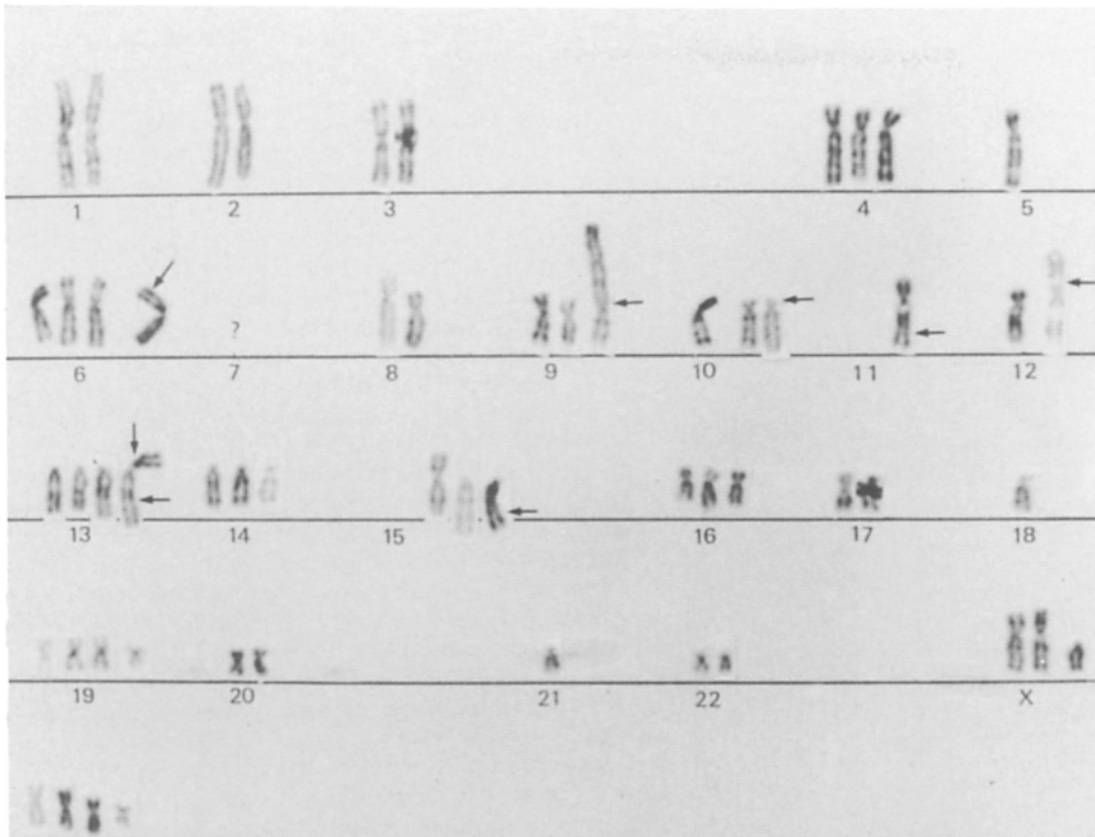


Figure 10. Karyotype of Ca9-22. The chromosome spread was prepared as described in Fig. 9. Karyotype analysis was carried out after G-band staining. Arrows indicate the rearranged chromosomes.

and others have isolated variants displaying alternate responses to EGF (Filmus et al., 1987; Buss et al., 1982; Bravo, 1984; Shimizu et al., 1984; Behzadian and Shimizu, 1985a). In this report, we described two series of variants with altered responses to EGF derived from the receptor-hyperproducing cell lines, NA and Ca9-22. Our results suggest

two independent mechanisms by which the variants escape EGF-mediated growth inhibition. One mechanism is the loss of the amplified EGFR gene responsible for EGF receptor-hyperproduction as demonstrated by the ER variants. This type of variant has also been isolated from the epidermoid carcinoma cell line A431 (Gill et al., 1985) and the breast

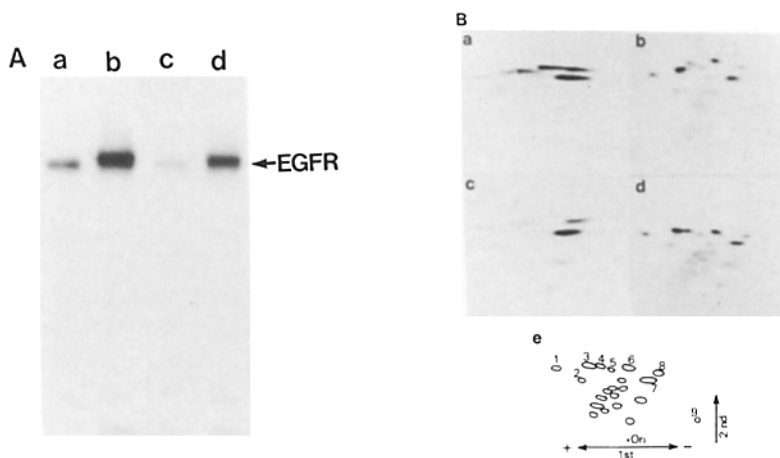


Figure 11. EGF receptor phosphorylation in Ca9-22 cells and CER-1 cells. (A) Cells were labeled with [32 P]orthophosphate for 60 min and treated with EGF (200 ng/ml) for the last 15 min. Immunoprecipitated EGF receptors were separated on SDS-polyacrylamide gel (7%), followed by autoradiography. (B) EGF receptors were eluted from gel and digested with TPCK-trypsin (50 μ g) at 37°C for 24 h. The digests were separated on thin layer plates (20 \times 20 cm): (1st) electrophoresis at pH 8.9 in 1% ammonium bicarbonate; (2nd) chromatography in butanol/pyridine/acetic acid/water (15:10:3:12, vol/vol). 32 P-labeled Peptides were identified by autoradiography for which exposure time was adjusted to keep cpm \times day values constant. The radioactivities loaded on the gels were: (a, 1,814 cpm), (b, 4,653 cpm), (c, 300 cpm), (d, 1,193 cpm). (a and b) Ca9-22 cells; (c and d)

CER-1 cells; (a and c) no EGF stimulation; (b and d) after EGF stimulation; (e) a diagram of tryptic phosphopeptides: phosphorylated amino acids contained in each spot were as follows: (1) Serine; (2) Threonine>Serine, Tyrosine; (3) Serine; (4) threonine; (5) Serine; (6) Threonine>Serine; (7) Serine; (8) Serine, (9) Tyrosine.

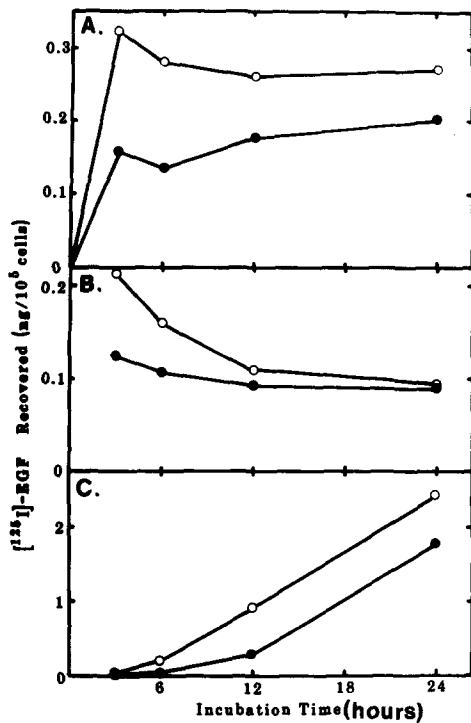


Figure 12. Internalization and degradation of EGF by Ca9-22 and CER-1. Cells were incubated with [¹²⁵I]EGF (100 ng/ml, 3,600 cpm/ng) at 37°C for the indicated times, placed on ice and treated with 0.2 M acetic acid/0.5 N NaCl for 1 min to remove the cell surface fraction of cell-associated [¹²⁵I]EGF (B). Then, the cells were dissolved in 1 N NaOH to determine the intracellular fraction (A). The conditioned medium was collected from each culture. TCA was added to the media (final concentration 20%) to separate degraded EGF (TCA soluble fraction) (C) from intact EGF. (○) Ca9-22; (●) CER-1.

cancer cell line MDA-468 (Filmus et al., 1987). Resistance of these cells was reported to be stable in the absence of EGF for a long period, and seemed to arise from the loss of a certain rearranged chromosome carrying the amplified EGFR gene (Gill et al., 1985). Cytogenetic analysis revealed that the ER variants commonly lost a certain rearranged chromosome seen in NA cells (Gamou, S., M. Kobayashi, and N. Shimizu, unpublished observation). Thus, the examination of whether the amplified EGFR gene is mapped on the rearranged chromosome may be interesting.

The ER variants grew in a strictly EGF-dependent manner. Although the growth of A431 variants was reported to be stimulated by EGF (Buss et al., 1982), they could also grow in the absence of EGF. ER cells showed a flat morphology in the absence of EGF as if they were in the resting stage. We have not been able to identify the molecular basis for the difference in response to EGF in these cells.

Another unique variant, CER-1 cells, acquired EGF-resistance without the loss of the amplified EGFR gene or receptor kinase activity. This type of variant has not been previously described. In contrast to other variants (Filmus et al., 1987; Gill et al., 1985), CER-1 cells gradually regain the sensitivity to EGF-mediated growth inhibition if they are kept in the absence of EGF for several mo.

We described several characteristics that may be related to the mechanism of EGF-resistance in CER-1 cells. One is the

increased cell volume and ploidy. This may allow escape from the growth inhibition by EGF although the relationship of increased cell volume and ploidy to EGF-resistance remains to be elucidated.

Another distinction between CER-1 and parental Ca9-22 cells is the efficient EGF receptor down-regulation which is disjoined from EGF internalization in the variant. This seemed to include receptor desensitization to the ligand. The mechanism of down-regulation reduced the EGF receptor level on the cell surface emulating a loss of the amplified EGFR gene. Thus, this down-regulation may play an important role in uncoupling the excess signals by EGF from the cellular responses.

We previously isolated variants from Swiss-3T3 cells using insulin conjugated with diphtheria toxin A fragment (Miskimins and Shimizu, 1981). One of these variants retained the ability to bind insulin, but accumulated lysosome-like vesicles in their cytoplasm, suggesting an alternative endocytic pathway involved in escaping the cytotoxicity of the chimeric insulin (Miskimins et al., 1981). The alternative endocytic pathway does not appear to be a factor in the present situation because excess accumulation of EGF was not observed in the intracellular fraction of CER-1 cells.

As described above, exclusion of the amplified EGFR gene seems to be the most common mechanism to reduce the EGF receptor level on the cell surface. CER-1 cells may use the down-regulation mechanism instead of losing their EGFR genes because of the possibility that their EGFR genes are rearranged and tightly linked with some gene(s) essential for Ca9-22 cells to survive in vitro. In fact, our karyotype analysis revealed numerous rearranged chromosomes but no intact chromosome 7.

Although the exact mechanism of EGF-resistance remains to be elucidated, these variants will be useful to investigate not only the physiological function of EGF and its receptor, but also the genetic and epigenetic control of EGFR gene expression.

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