Overexpression of Epidermal Growth Factor and Insulin-like Growth Factor-I Receptors and Autocrine Stimulation in Human Esophageal Carcinoma Cells

Shan-Chun Chen, Chen-Kung Chou, Fen-Hwa Wong, Chungming Chang, and Cheng-po Hu

ABSTRACT

The growth-stimulatory effects of epidermal growth factor (EGF), transforming growth factor α (TGF-α), and insulin-like growth factor-I (IGF-I) on the human esophageal carcinoma cell line CE48T/VGH were evaluated. Under serum-free conditions, EGF, TGF-α, and IGF-I promoted 3.6- to 4.1-fold increased cell proliferation. Scatchard analyses and Northern blot hybridization revealed that both the EGF/TGF-α receptor and the IGF-I receptor were overexpressed in CE48T/VGH cells. Furthermore, ligand-dependent autophosphorylation of the EGF receptor and the IGF-I receptor was clearly detected using antireceptor and antiphosphotyrosine antibodies. Autocrine regulation was strongly indicated by the following evidence: (a) CE48T/VGH cells were found to express TGF-α and IGF-I genes, (b) serum-free conditioned medium promoted the growth of CE48T/VGH cells and stimulated the autophosphorylation of the EGF/TGF-α receptor and the IGF-I receptor, and (c) the addition of IGF-I receptor antibodies significantly suppressed CE48T/VGH cell growth under serum-free conditions. Our studies suggest that the overexpression of EGF and IGF-I receptors and autocrine growth regulation may concerently control the proliferation of esophageal carcinoma cells.

INTRODUCTION

The growth and differentiation of normal and transformed cells are controlled by numerous growth factors. The binding of growth factors to their specific receptors on the cell surface may stimulate the receptor-associated tyrosine kinases, which transmit mitogenic signals, and lead to cell proliferation. Neoplastic cells are often found to have altered synthesis of receptors for growth factors. Amplification and overexpression of the EGF receptor gene have been observed in human carcinoma cells (1-6) and sometimes correlated with the invasive and metastatic properties of the tumor cells (3). Furthermore, EGF can stimulate anchorage-independent growth of carcinoma cells with an amplified and overexpressed EGF receptor gene (4). Thus, a close relationship has been suggested between the activation of the EGF receptor gene and carcinogenesis. Additional evidence relating growth factors to oncogenesis comes from the observation that many transformed cells produce growth factors that many stimulate their own growth in an autocrine manner (6-11). This observation suggests that the ability of a cell to produce a factor(s) which can support its own growth might be a general mechanism contributing to the unlimited growth capacity of tumor cells.

Recent studies have shown that human esophageal carcinoma cells overexpress the EGF receptor (2, 5, 6). However, the growth regulation of esophageal carcinoma cells by EGF or by other growth factors remains to be examined. In the present study, we report that EGF, TGF-α, and IGF-I are potent mitogens for a receptor-overexpressed human esophageal carcinoma cell line, CE48T/VGH, under serum-free conditions. Autocrine growth regulation by TGF-α and IGF-I in CE48T/VGH cells is also supported by this study.

MATERIALS AND METHODS

Materials. Mouse EGF (culture grade) and recombinant human TGF-α were purchased from Collaborative Research (Waltham, MA). Recombinant IGF-I was purchased from Bachem (Torrance, CA). Antibodies to EGF receptor and antiphosphotyrosine antibodies were generous gifts of Dr. Fred Kull (Division of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC). Radioactive chemicals, including Na125I, 125I-EGF, [35S]methionine, [32P]dCTP, and [32P]ATP, were from New England Nuclear (Boston, MA). IGF-I was iodinated by the chloramine T method. DMEM and Ham’s F-12 medium were from Gibco (Grand Island, NY). Protease inhibitors aprotinin, antipain, pepstatin A, and PMSF were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. CE48T/VGH cells (12) were maintained in a 2:1 (v/v) mixture of Ham’s F-12 medium and DMEM (F2D1), supplemented with 5% FCS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 250 µg/ml fungizone, 2 mM l-glutamine, and 1 mM nonessential amino acids and buffered with 40 mM sodium bicarbonate and 25 mM HEPES. Human lung carcinoma cell line Calu-1 (13), human hepatoma cell line HA22T/VGH (14), human epidermoid carcinoma cell line A431 (15), and normal human fibroblast line WI-38 (16) were maintained in DMEM containing 10% FCS (complete medium). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. Subculture of cells was performed every 3 to 4 days after the culture had reached confluence, with 0.05% trypsin plus 2 mM EDTA.

Cell Proliferation Assays. CE48T/VGH cells were seeded in 24-well plates, at a density of 5 x 104 cells/well, with F2D1 medium containing 5% FCS. After 18 h, cells were placed in serum-free (SF-F2D1) medium for another 24 h. Triplicate wells were then fed with growth factor-containing SF-F2D1 medium. The media were refreshed once after 2 days. Viable cell numbers were determined 4 days after growth factor treatment, by trypsin blue exclusion.

For proliferation-inhibition studies, various amounts of anti-IGF-I receptor antibody or control antibody were added into SF-F2D1 medium 24 h after serum starvation. The antibody-containing media were refreshed once after 2 days. Viable cell numbers were determined 4 days after the addition of antibodies.

Collection of SFCM. CE48T/VGH cells were plated in 150-mm Petri dishes with F2D1 medium containing 5% FCS. At 90% confluence, the medium was replaced by SF-F2D1 medium, and the conditioned media were refreshed once after 2 days. Viable cell numbers were determined 4 days after the addition of antibodies.

Collection of SFCM. CE48T/VGH cells were plated in 150-mm Petri dishes with F2D1 medium containing 5% FCS. At 90% confluence, the medium was replaced by SF-F2D1 medium and refreshed 1 day later. The conditioned media were collected every 2 days thereafter, pooled, concentrated 100-fold by polyethylene glycol 10,000, dialyzed against fresh SF-F2D1 medium, and stored at -20°C before use.

Ligand Binding Assays. CE48T/VGH cells were seeded in 24-well plates, at a concentration of 1.5 x 104 cells/well, and cultured for 2 days in F2D1 medium containing 5% FCS. The cells were washed twice with HBSS and incubated at room temperature for 90 min with...
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1 ng/ml 125I-labeled ligands in binding buffer (HBSS with 20 mM HEPES, 0.5% BSA, and various concentrations of unlabeled ligands, ranging from 0.5 to 100 ng/ml, at pH 7.6). The cells were washed 5 times with ice-cold phosphate-buffered saline and lysed by 0.2% SDS. The radioactivity was determined with a gamma-counter. The numbers of surface receptors and the affinity constants were calculated by Scatchard analysis. Nonspecific binding was determined in incubations containing 200 ng to 1 µg unlabeled ligands.

RNA Isolation and Northern Blot Analysis. Total RNA was isolated by guanidinium isothiocyanate solubilization and centrifugation through a CsCl cushion. Polyadenylated RNA was enriched by oligo-thymidylic acid-cellulose chromatography, electrophoresed on 1% agarose gels containing 2.2 M formaldehyde, and transferred to Hybond nitrocellulose filters (Amerham, UK) in 20x SSC (3 M NaCl, 0.3 mM trisodium citrate). The RNA blots were hybridized with 32P-labeled probes for 20 h at 42°C, in a solution containing 5x SSC, 50% formamide, 5x Denhardt's reagent, and 0.1% SDS. The filters were washed twice with 0.2x SSC/0.1% SDS, at 55°C and at 60°C for 40 min, and once with 0.1x SSC/0.1% SDS at 60°C for 40 min, followed by autoradiography. The following DNA fragments were used as probes: a 768-base pair EcoRI fragment of human TGF-α cDNA (20), a 1.9-kilobase PstI fragment of human IGF-I cDNA (19), a 1.4-kilobase base pair PstI fragment of human TGF-α cDNA (20), and a 1.9-kilobase fragment of human IGF cDNA from pHGF15 (21).

Preparation of Membrane Fractions. CE48T/VGH cells were grown in Petri dishes (150-mm diameter) until 80% confluent. The monolayers were washed 3 times with ice-cold Ca2+/Mg2+-free HBSS and scraped off into lysis buffer (0.1 M sodium phosphate, 5 mM EDTA, 250 mM sucrose, 10 µg/ml aprotinin, 20 µg/ml antipain, 20 µg/ml peptide A, 2 mM PMSF, pH 7.4), at 5 x 108 cells/ml. All the subsequent steps were carried out at 4°C. Cells were disrupted by sonication with an Artek Ultrasonic 2000 (15 s at 30% power output, 3 times), and the homogenates were centrifuged at 500 x g for 5 min. The supernatants were further centrifuged at 100,000 x g for 1 h. The resulting pellets were resuspended in extraction buffer (50 mM HEPES, 2% Triton X-100, pH 7.4), stirred gently at 4°C for 2 h, and then subjected to another centrifugation at 100,000 x g for 1 h. The solubilized membrane fraction was purified with a wheat germ agglutinin affinity column and stored at -70°C.

Immunoprecipitation of Surface Receptors. For metabolic labeling, subconfluent monolayer cells were washed 3 times with HBSS and labeled for 4 h in methionine-free DMEM containing 50 µCi/ml [35S]-methionine and 10% dialyzed FCS. Crude membrane fractions were prepared as described above and precleared with Protein A-Sepharose coupled with receptor-specific antibodies. After extensive washing with NET buffer (150 mM NaCl, 0.5 mM EDTA, 50 mM Tris, 0.25% Nonidet P-40, 0.1% BSA, 2 mM PMSF, pH 7.4), the immunocomplexes were boiled for 5 min in sample buffer, containing 0.08 M Tris, 15% glycerol, 2% SDS, and 0.1 mM dithiothreitol, and electrophoresed on 10% SDS-PAGE.

In Vitro Autophosphorylation of the Receptors. Forty µg of solubilized membrane fractions of CE48T/VGH cells in kinase buffer (12 mM MgSO4, 3 mM MnCl2, 1 mM diethiothreitol) were treated with EGF (final concentration, 10-7 M), IGF-I (10-7 M), or 10-fold concentrated SFCM, at room temperature. After 15 min, 20 µCi of [32P]ATP (3500 µCi/µg) were added. Phosphorylation proceeded for another 15 min and was terminated by a 10-fold dilution with phosphatase inhibition buffer (1 mM sodium vanadate, 20 mM sodium pyrophosphate, 20 mM sodium fluoride, 100 µM zinc chloride, 1 mM ATP). The samples were immunoprecipitated with either antiphosphotyrosine antibody or antireceptor antibodies. The immunocomplexes were subjected to 10% SDS-PAGE and autoradiography.

RESULTS

EGF, TGF-α, and IGF-I Are Potent Mitogens for CE48T/VGH Cells. The mitogenic effects of EGF, TGF-α, and IGF-I on CE48T/VGH cells were examined (Fig. 1). Under serum-free conditions, the doubling time of CE48T/VGH cells was estimated to be 140 h (data not shown). The exogenous addition of these growth factors resulted in marked proliferation of CE48T/VGH cells, in a dose-dependent manner. The half-maximal stimulatory concentrations were 0.05 nM for EGF and TGF-α and 0.1 nM for IGF-I. The maximal stimulation of cell proliferation by EGF or TGF-α treatment was 4.1-fold, compared with the serum-free control, and that by IGF-I treatment was 3.6-fold. It has been reported that both EGF and TGF-α bind to the EGF receptor and share many biological activities (for review, see Ref. 22). Our results show that EGF and TGF-α are equipotent in the stimulation of CE48T/VGH cell growth and that TGF-α competes equally with EGF for the surface EGF receptor (data not shown).

CE48T/VGH Cells Overexpress EGF and IGF-I Receptors. The EGF and IGF-I receptors on CE48T/VGH cells were characterized by using 125I-ligand binding assay and Scatchard analysis. The number of EGF receptors was estimated to be 6.1 x 105/cell, with a Kd of 0.4 nM, and that of IGF-I receptors on CE48T/VGH cells was estimated to be 2.5 x 105/cell, with a Kd of 0.9 nM (Table 1). Both EGF and IGF-I receptors were overexpressed in CE48T/VGH cells, compared to human lung

![Fig. 1. Dose-dependent stimulation of CE48T/VGH cell proliferation by EGF, TGF-α, and IGF-I. Cell proliferation studies were performed as described in "Materials and Methods." Cell numbers were determined in triplicate after a 4-day treatment with different doses of EGF (•), TGF-α (○), or IGF-I (♦). Data are expressed as the mean ± SD and are representative results from six independent studies. SF, serum-free.](image-url)
chromosomal translocation (17). In CE48T/VGH cells, the control. The mRNA levels in all samples were relatively the corresponding to the reported EGF receptor (24). The IGF-I cells overexpressed EGF and IGF-I receptor genes at the sizes of the EGF receptor gene transcripts were 10.5 and 5.8 transcript in A431 cells was an aberrant form generated by of EGF receptor genes, as reported previously. The 2.8-kilobase cancer cell line CaLu-1, human hepatocellular carcinoma cells were neither amplified nor grossly rearranged at the DNA cell line HA22T/VGH, or normal human fetal WI-38 fibroblasts. The number of EGF receptors on CE48T/VGH cells was about 4 times greater than the number on CaLu-1 and HA22T/VGH cells and 20 times greater than the number on WI-38 cells. However, the number of EGF receptors on CE48T/VGH cells was less than that on A431 cells, a human epidermoid carcinoma cell line which was reported to have 30- to 100-fold amplified EGF receptor genes (17). In addition, CE48T/VGH cells were found to exhibit a 6- to 12-fold greater number of IGF-I receptors than any other cell line examined. In spite of the abundant surface receptors, the affinities (Kd) of both EGF and IGF-I receptors for their specific ligands were similar to those previously reported (23).

When polyadenylated RNA was prepared and subjected to Northern blot analysis, it was clearly shown that CE48T/VGH cells overexpressed EGF and IGF-I receptor genes at the mRNA level, compared to HA22T/VGH and WI-38 cells (Fig. 2). Expression of the β-actin gene was determined as an internal control (Fig. 2). The mRNA levels in all samples were relatively the same (data not shown). A431 cells also showed overexpression of EGF receptor genes, as reported previously. The 2.8-kilobase transcript in A431 cells was an aberrant form generated by chromosomal translocation (17). In CE48T/VGH cells, the sizes of the EGF receptor gene transcripts were 10.5 and 5.8 kilobases, while those of the IGF-I receptor gene were 11.0 and 7.0 kilobases. In addition, the receptor genes of CE48T/VGH cells were neither amplified nor grossly rearranged at the DNA level, as shown by Southern blot analysis (data not shown).

EGF and IGF-I Activate Their Specific Membrane Receptors, as Indicated by in Vitro Autophosphorylation. To confirm that CE48T/VGH cells express EGF and IGF-I receptor proteins, [125I]-labeled EGF receptor cDNA probe (E) or human IGF-I receptor cDNA probe (B), under stringent conditions. Expression of the β-actin gene was probed as an internal control (data not shown). A431 in A was exposed for a shorter time, in order to visualize the 2.8-kilobase aberrant transcript generated by chromosomal translocation (17).

The molecular sizes of the two smaller bands are identical to those of the β- and α-subunits of the IGF-I receptor reported previously (25). The M, 210,000 protein was demonstrated to be the precursor of the IGF-I receptor by a pulse-chase experiment (data not shown).

In order to demonstrate that EGF and IGF-I receptors on CE48T/VGH cells have ligand-specific tyrosine kinase activity, in vitro phosphorylation was performed. Fig. 4 shows the ligand-induced phosphorylation patterns detected by antireceptor antibodies (Fig. 4, lanes 1-4) and antiphosphotyrosine antibody (Fig. 4, lanes 5-8). IGF-I enhanced phosphorylation of a M, 105,000 protein which was detected by anti-IGF-I receptor antibody (αIR-3) (Fig. 4, lanes 1 and 2) and antiphosphotyrosine antibody (Fig. 4, lanes 5 and 7). The size of this protein is identical to that of the β-subunit of the IGF-I receptor.
which was detected by anti-EGF receptor antibody (Fig. 4, lanes 3 and 4) and antiphosphotyrosine antibody (Fig. 4, lanes 5 and 6), corresponding to the EGF receptor identified in Fig. 3, lane 2. The phosphorylation sites were mainly on the tyrosine residues, since the amount of phosphoprotein precipitated by receptor-specific antibodies was not higher than that precipitated by antiphosphotyrosine antibody.

Serum-free Conditioned Medium Promotes Cell Growth and Enhances Autophosphorylation of EGF and IGF-I Receptors. Since CE48T/VGH cells could propagate slowly in SF-F2D1 medium and the growth was dependent on cell density (data not shown), CE48T/VGH cells may produce and respond to endogenous growth factors in an autocrine manner. SFCM was tested for the presence of mitogenic activity. When various concentrations of SFCM were added to fresh SF-F2D1 medium, CE48T/VGH cell growth increased in a dose-dependent manner (Table 2). Furthermore, SFCM enhanced autophosphorylation of two proteins of M, 175,000 and 105,000, corresponding to the EGF receptor and the β-subunit of the IGF-I receptor (Fig. 4, lanes 5 and 6), suggesting the presence of EGF-like and IGF-I-like activities in SFCM.

CE48T/VGH Cells Express TGF-α and IGF-I Genes. In order to identify the growth factors which were produced by CE48T/VGH cells, we examined the expression of growth factor genes, by Northern blot analysis. Fig. 5 clearly demonstrates the expression of TGF-α and IGF-I genes in CE48T/VGH cells, with transcript sizes of 4.5 and 5.0 kilobases, respectively. However, expression of the EGF gene was not detected.

Anti-IGF-I Receptor Antibody Significantly Inhibits CE48T/VGH Cell Growth. To further investigate whether the growth factors synthesized by CE48T/VGH cells indeed stimulate their

Table 2  SFCM stimulation of CE48T/VGH cell proliferation

Preparation of SFCM and assay for cell proliferation were carried out as described in “Materials and Methods.” Concentrated SFCM was diluted 1:40, 1:80, 1:160, and 1:320 with SF-F2D1 medium and added to CE48T/VGH cells 24 h after serum starvation. The SFCM was refreshed once after 2 days. Viable cells were counted 4 days after the addition of SFCM. Values are expressed as the mean ± SD. Percentage increase was determined as the increase in cell growth in the SFCM-treated group, as compared with the serum-free medium group.

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<tr>
<th>Serum-free medium</th>
<th>Dilution of SFCM</th>
<th>Cell number (× 10⁴) Increase (%)</th>
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<tr>
<td>Dilution</td>
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Enhances Autophosphorylation of EGF and IGF-I Receptors. Serum-free conditioned medium, CE48T/VGH cell growth increased in a dose-dependent manner under serum-free conditions, compared to the effect of a control antibody, IgG (26), against a bile canalicular antigen, which was not expressed in CE48T/VGH cells (Table 3). The presence of control IgG in the medium improved the culture conditions for cell growth. Phenomenon was also observed when another control antibody, L243 (anti-MHC class II), or BSA was used (data not shown). At the concentration of 12.5 μg/ml, aIR-3 inhibited CE48T/VGH cell growth by 38%, and 50 μg/ml aIR-3 inhibited cell growth by 68%. A further increase in aIR-3 did not produce greater inhibition of cell growth. The results strongly suggest IGF-I as an autocrine growth factor for CE48T/VGH cells. Since an anti-EGF receptor antibody which can efficiently block ligand binding is currently unavailable, a similar proliferation-inhibition experiment using anti-EGF receptor antibody was not performed.

DISCUSSION

Esophageal carcinoma exhibits striking geographic variations in incidence. Various etiological agents have been suggested as being the causative factors of this disease (27). However, the molecular mechanism of oncogenesis and the biological properties of esophageal carcinoma cells are poorly understood. Cell lines have been established by us (12) and by others (28, 29) from human esophageal tumor specimens to facilitate the examination of the biological properties of this tumor in vitro, thereby contributing to the better understanding of esophageal carcinogenesis as well as cellular regulation and differentiation.

In this study, we found that EGF, TGF-α, and IGF-I were potent mitogens for a human esophageal carcinoma cell line, CE48T/VGH. These growth factors promoted cell proliferation at physiological concentrations, which may reflect their growth-regulatory roles in vivo. We also demonstrated that EGF and TGF-α were equipotent in the stimulation of CE48T/VGH cell growth and that TGF-α competed equally with EGF for binding to the surface EGF receptor. These results indicate that EGF and TGF-α bind to the EGF receptor and may promote similar biological activities in human esophageal carcinoma cells. Following the interaction of EGF or IGF-I with the solubilized cell membrane receptors, the EGF and the IGF-I receptors were found to be autophosphorylated. These results indicate that the binding of EGF and IGF-I to CE48T/VGH cells activates the receptor-associated protein tyrosine kinases and transduces the

Fig. 5. Northern blot analyses of TGF-α and IGF-I gene expression. Five μg of polyadenylated mRNA were subjected to Northern blotting and hybridized with 32P-labeled human TGF-α cDNA (A) and IGF-I cDNA (B) probes.
stimulatory signals of the growth factors, leading to activation of gene expression and, ultimately, cell proliferation (30).

The results of Scatchard and Northern blot analyses indicated that both EGF and IGF-I receptors were overexpressed in CE48T/VGH cells, compared to human fibroblasts, lung carcinoma cells, and hepatocellular carcinoma cells. At the DNA level, neither apparent amplification nor truncation of these receptor genes could be detected. Therefore, the overexpression of EGF and IGF-I receptors is regulated at the mRNA level. Overexpression of the EGF receptor has been suggested to be related to carcinogenesis (1–6). Using epidermal cells that express different amounts of surface EGF receptors, Boonstra et al. (31) have shown that cells possessing high levels of surface EGF receptors tend to have a decreased ability to enter the terminal pathway of differentiation. Recent studies using NIH3T3 cells have also indicated that overexpression of EGF receptors can cause EGF- or TGF-α-induced malignant transformation (32, 33). Thus, the increase in surface EGF receptor levels discovered in this study and others (5, 6) seems to be associated with the development of human esophageal carcinomas.

IGF-I stimulates growth of many types of cells, both in vitro and in vivo (34, 35), through IGF-I receptors. The role of IGF-I and its receptor in promoting tumor growth in vivo is unclear. Recently, Kaleko et al. (36) demonstrated that overexpression of human IGF-I receptors could trigger or potentiate IGF-I-dependent neoplastic transformation of NIH3T3 cells. While overexpression of IGF-I receptors on tumor cell lines has not been documented, we found that CE48T/VGH cells expressed a high level of IGF-I receptors, as demonstrated by Northern blot analysis and ligand binding assay. Our study provides the first example illustrating the role of IGF-I in carcinogenesis of esophageal cells. Furthermore, the finding of overexpression of both EGF receptors and IGF-I receptors on the same cells may add more information on the mechanism of growth regulation of malignant cells.

Alterations in the synthesis of endogenous growth factors may result in uncontrolled growth and neoplastic transformation. TGF-α and IGF-I have been identified as autocrine growth factors in human carcinoma cells (6, 11). In this study, we found that CE48T/VGH cells expressed TGF-α and IGF-I. In addition, SFCM obtained from CE48T/VGH cells promoted cell proliferation and induced autophosphorylation of EGF and IGF-I receptors. Our findings strongly suggest the existence of TGF-α and IGF-I autocrine regulation in CE48T/VGH cells. The observation that antibody to IGF-I receptor was able to suppress cell growth under serum-free conditions further substantiated the autocrine regulation by IGF-I of the growth of CE48T/VGH cells. Therefore, through the overexpression of EGF/TGF-α and IGF-I receptors on the cell surface and autocrine synthesis of TGF-α and IGF-I in CE48T/VGH cells, these growth factors may conversely play an important role in the development and progression of human esophageal carcinoma.

ACKNOWLEDGMENTS

We wish to thank Dr. Steve Rolfe for reading the manuscript, Shiang-lien Lu for preparation of the manuscript, and Dr. Kwen-jen Chang for helping us to obtain oIR-3 antibody.

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