Molecular Evidence for the Lack of Epidermal Growth Factor Receptor Gene Expression in Small Cell Lung Carcinoma Cells

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ABSTRACT

It has been shown that none of the small cell lung carcinoma (SCLC) cell lines possess epidermal growth factor (EGF) binding activity on their surface. We have examined several SCLC cell lines for the possibility that they may have EGF receptors but that the receptors are masked by an EGF-like protein factor(s), which may be produced by an autocrine mechanism. No evidence, however, was found for the production of such factors. We then used an EGF receptor complementary DNA to determine the state of the EGF receptor gene by Southern blot analysis. The receptor gene appears to be present in these cells in an intact, unrearranged form. These cells, however, were found to lack detectable levels of EGF receptor mRNA, suggesting a possible reason for the absence of EGF receptors on the cell surface. Furthermore, karyotype analysis revealed that SCLC cell lines Lul34 and H69 contained a morphologically normal chromosome 7, which carries the EGF receptor gene. Also, these SCLC cells contained the apparently normal chromosome 3 and exhibited the presence of c-ras-1 oncogene in an unrearranged form. Thus, the previously noted partial deletion of chromosome 3 is not necessarily common to the SCLC cells. Instead, the lack of EGF receptor is frequently found in SCLC cell lines and is distinct from the other types of lung cancer. We postulate that SCLC cells have some active regulatory mechanism which prevents the expression of EGF receptor gene.

INTRODUCTION

SCLC is a highly malignant neoplasm with neuroendocrine properties (1). Continuous cell lines have been established from SCLC (2, 3), which allow for in vitro biological studies of this type of human cancer. These SCLC cell lines lack measurable EGF-binding capacity (4) in contrast to other types of lung cancer. Most of the SCLC cell lines in vitro produce growth factors which stimulate anchorage-independent growth of normal rat kidney cells (4). Also, some of these human tumor cell lines release into the culture medium TGF-α (5, 6) which confers the transformed phenotype on untransformed cells. TGF-α interacts with and induces down-regulation of the EGF receptor. However, it is not yet clear whether the production of the growth factor correlates with the absence of EGF receptor in SCLC in culture.

Recently, a partial amino acid sequence of the human EGF receptor has been shown to be strikingly similar to that of the v-erb-B transforming protein of avian erythroblastosis virus (7). DNA sequence analysis of the EGF receptor gene suggests that it is the cellular homologue of the v-erb-B oncogene (8). Furthermore, most of the differentiated cell types possess EGF receptors on their cell surface (9). Therefore, it is worthwhile to examine the mechanisms regulating the EGF receptor gene expression in SCLC cells.

Another interesting aspect of the SCLC is a possible relationship between a specific chromosomal abnormality and oncogene activation. A small deletion is found very often at the short arm of chromosome 3 (3p14p23) (10, 11). In the proximity of this specific deletion site, the c-ras-1 oncogene has been mapped to 3p25 (12). Homogeneously staining regions and double minute chromosomes have also been found in SCLC cells (11, 13). Amplification of the c-myc oncogene often accompanies these chromosome abnormalities (14) and, in fact, amplified c-myc oncogene is found in a variant form of SCLC (13, 15).

In this paper, we provide molecular evidence which suggests that the lack of EGF receptor in the SCLC cells is due not to the autocrine synthesis of an EGF-like growth factor but to the suppression of the EGF receptor gene expression.

MATERIALS AND METHODS

Cell Cultures. Small cell lung carcinoma cell lines Lu134 (3), Lu135, and Lu139 and large cell lung carcinoma cell lines Lu65 (16, 17) and Lu99 (18) were established at National Cancer Center, Japan. Other SCLC cell lines, NCI-H69, H128, and N231, were provided by Dr. J. Minna (National Cancer Institute, Bethesda, MD). Another SCLC line PC-6 was provided by Dr. S. Sakiyama (Chiba Cancer Center, Chiba, Japan). A pancreatic carcinoma cell line, UCVA-1 (19), was provided by Dr. Y. S. Kim (University of California at San Francisco, San Francisco, CA). SCLC and LCLC cell lines were maintained in RPMI 1640 ( Gibco) supplemented with 10% FBS, (Boehringer/Mannheim), kanamycin (Meiji; 100 μg/ml), and Fungizone (Squibb; 1 μg/ml) (RPMI/FBS10) in 5% CO2 and 100% humidity at 37°C. UCVA-1 was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, kanamycin, and Fungizone.

125I-EGF Binding Assay. SCLC and LCLC cells were incubated with 125I-EGF (0.2–0.5 ng/ml) in EBSS with 0.1% BSA for 90 min at 4°C or 37°C (19). These cells were loaded on a Whatman (GF/C) glass fiber filter to separate free 125I-EGF from cell-associated EGF and washed four times with EBSS containing 0.1% BSA. Nonspecific binding was measured in the presence of 200 times excess of unlabeled EGF. In some experiments, prior to binding assay, cells were washed once with the solution containing 0.2 M acetic acid and 0.5 M NaCl and then twice with EBSS-BSA (19).

Coculture Experiments. UCVA-1 cells were cultured together with SCLC or LCLC cells which were removed after 48 h. Then, the EGF-binding capacity of the UCVA-1 cell was measured by incubation with various concentrations of EGF at 4°C for 4 h as described (19).

Conditioned Media. SCLC and LCLC cells grown to saturation density were collected by centrifugation and reincubated and cultured in serum-free RPMI 1640 (or RPMI/FCS10) for 2 days. The conditioned media were collected and clarified by centrifugation at 3000 rpm. UCVA-1 cells were incubated with these conditioned media for 2 h at 37°C, and then a 125I-EGF binding assay was carried out at 4°C for 4 h.

Chromosome Analysis. Metaphase chromosome spreads were prepared in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
pared and stained by an ASG- or trypsin-band technique (20).

Immunoprecipitation of EGF Receptor. Cells were incubated in methionine-free Dulbecco's modified Eagle's medium (Microbiological Associates) containing dialyzed FBS (10%) for 30 min. [35S]Methionine (Amersham; >800 Ci/mmol) was then added to a final concentration of 40 μCi/ml, and incubation was continued for 16 h at 37°C. Cells labeled thus were collected, washed with TBS, and lysed with RIPA buffer (21). Cell lysates were clarified by centrifugation at 40,000 rpm for 1 h with a Beckman 80 Ti rotor. The lysates were incubated with the B4G7 monoclonal antibody-protein A-Sepharose beads (22) for 2 h with gentle agitation. The B4G7 monoclonal antibody specifically reacts with human EGF receptor proteins. The EGF receptor-antibody complex formed on protein A-Sepharose beads was washed twice with RIPA buffer (21) and twice with TBS and then dissolved in Laemmli's 2 x sample buffer. Sodium dodecyl sulfate-polyacrylamide (7%) gel electrophoresis was carried out according to the method of Laemmli (23).

Gels were stained with Coomassie brilliant blue, destained, treated with ENLIGHTNING (New England Nuclear), and dried under vacuum. Fluorograph was obtained at -70°C using Fuji X-ray film.

DNA/RNA Isolation and Hybridization. DNA and RNA were isolated by the published methods (24). Polyadenylate-containing RNA was selected by oligodeoxynucleotide column. DNA was digested with various restriction enzymes (Toyobo) and the restriction fragments were separated by agarose gel electrophoresis and transferred to nitrocellulose membrane (25). Hybridization was carried out using EGF receptor CDNA probe (p7E) (26), actin gene probe (pAM-91) (27), and ras-1 oncogene probe (p28E) (28) under stringent conditions as described (26). RNA was denatured with glyoxal and then applied directly to 20× standard saline citrate-equilibrated nitrocellulose. Removal of probe was done by treating at 100°C for 5 min in 20 mm Tris-HCl, pH 8.0 (29).

RESULTS

The EGF-binding capacity of 7 SCLC cell lines including Lu134 and H69 was examined. As shown in Table 1, no significant EGF binding was observed in any of these SCLC cell lines at 4°C and at 37°C. Contrary to SCLC, Lu65 and Lu99 cells of LCLC origin showed an apparent EGF binding. We assumed that the lack of EGF binding in these SCLC cells might be due to the secretion of a factor(s) which interacts with EGF receptors. If so, the EGF-binding capacity of any EGF receptor-positive cells would decrease when these indicator cells were cocultured with SCLC cells or treated with SCLC-conditioned media. As shown in Fig. 1A, the EGF-binding capacity of UCVA-1 cells, used as an indicator, did not change after treatment with conditioned media of Lu134 cells. Scatchard analysis of the binding data clearly showed no change in either the number (intercepts on the abscissa) or the affinity of the EGF receptor (negative reciprocals of the slope of lines) (Fig. 1B). Similar results were obtained when UCVA-1 cells were cocultivated with another SCLC cell line, H69, or LCLC cell line, Lu65 (Fig. 2A), or treated with their conditioned media (Fig. 2B and C). It has been shown that a brief acid treatment is effective in removing EGF that bound to surface receptor (19). However, acid treatment of H69 and Lu134 cells did not increase [125I]-EGF binding (data not shown). Thus, the SCLC cell lines do not produce a factor which can mask or modulate

Table 1  EGF-binding activity of SCLC cells

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>EGF bound (% of input)</th>
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<tbody>
<tr>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>NCI-H69</td>
<td>0.04 ± 0.04*</td>
</tr>
<tr>
<td>NCI-H128</td>
<td>0.09 ± 0.14</td>
</tr>
<tr>
<td>NCI-N231</td>
<td>0.14 ± 0.29</td>
</tr>
<tr>
<td>Lu134-AHS</td>
<td>0.28 ± 0.34</td>
</tr>
<tr>
<td>Lu135</td>
<td>0.03 ± 0.02</td>
</tr>
<tr>
<td>Lu139</td>
<td>0.10 ± 0.12</td>
</tr>
<tr>
<td>PC-6</td>
<td>0.01 ± 0.02</td>
</tr>
<tr>
<td>Lu65</td>
<td>2.89 ± 0.21</td>
</tr>
<tr>
<td>Lu99</td>
<td>2.03 ± 0.05</td>
</tr>
</tbody>
</table>

* Mean ± SD of three determinations.
* NT, not tested.

Fig. 1. A, EGF binding to UCVA-1 cells at various concentrations of EGF after 2 days of cocultivation with Lu134 cells (O), after treatment with serum-free conditioned medium (A), and after treatment with serum-containing conditioned medium (L). As control, cells were incubated in fresh RPMI/FCS10 for 2 h (C). 125I-EGF binding assay was carried out at 4°C for 4 h. B, Scatchard plot of EGF binding data shown in A. Linear regression lines were shown and in any case a regression coefficient is not more than —0.98.

Fig. 2. Scatchard plot of EGF binding to UCVA-1 cells (A) after cocultivation with H69 (△) and Lu65 (A) for 2 days, (B) after treatment with serum-free conditioned medium, and (C) with serum-containing conditioned media from H69 (△) and Lu65 (A) for 2 h. As control, UCVA-1 cells were incubated in fresh RPMI/FCS10 for 2 h prior to binding assay (O). Linear regression lines were also shown and the regression coefficient is not more than —0.95 in any case.
the EGF receptor binding site and therefore we assumed that the lack of EGF receptor on SCLC cells could be caused by a lesion in the EGF receptor gene or the regulatory mechanism controlling receptor gene expression.

Then we examined the structural integrity and copy number of the EGF receptor gene in SCLC cell lines by Southern blot analysis. DNA was extracted from various SCLC cell lines, cleaved with the restriction enzyme EcoRI, and analyzed with a cDNA probe for the human EGF receptor, pE7. DNA from human placenta was used as control. As shown in Fig. 3A, the EcoRII restriction patterns of Lu134 and H69 DNAs are quite similar to that of placenta. Furthermore, no difference was found in EcoRI patterns of other SCLC cell lines including Lu135, Lu139, and PC-6 and in HindIII patterns of these SCLC DNAs (data not shown). These results suggested no major rearrangement in the EGF receptor gene. We estimated by DNA dot blot analysis that SCLC cells have an average of 2-3 copies of the EGF receptor gene (data not shown).

Next, we analyzed the level of EGF receptor gene expression in these SCLC cells. Two methods were used, an RNA dot blot analysis to test for the presence of EGF receptor-specific mRNA and an immunoprecipitation assay to detect the presence of EGF receptor proteins. For RNA dot hybridization, the nitrocellulose filter loaded with polyadenylate-containing RNA was first hybridized to the EGF receptor gene probe pE7. Then the same filter was used for hybridization to the actin gene probe pAM91. As shown in Fig. 3B, no EGF receptor-specific mRNA was detected in SCLC cells (Lu134 and H69), whereas actin mRNA was equally transcribed in both SCLC and LCLC cells. Similarly, other SCLC cells (Lu139 and PC-6) did not show any EGF receptor mRNA (data not shown).

To analyze the EGF receptor or receptor-related proteins, cells were metabolically labeled with [35S]methionine for 16 h and lysed with RIPA buffer. Immunoprecipitation was performed with an anti-human EGF receptor monoclonal antibody, B4G7 (22), which can recognize the protein moiety of the EGF receptor. The M, 170,000 EGF receptor was immunoprecipitated from Lu65 cells. No significant amount of EGF receptor was detected in any of SCLC cells (Fig. 4). As shown below, no structural abnormality was found for chromosome 7 on which the EGF receptor gene is located. These results together indicate that the EGF receptor gene is probably intact in these SCLC but that it is not transcribed.

The cytogenetic analysis showed that 96% of the H69 cell population are hypodiploid, with 38 to 41 chromosomes. The chromosome mode number was 40. Approximately 4% of the cell population were hypotetraploid having 77 to 81 chromosomes with a mode number of 79. Hypodiploid cells were found to have two copies of morphologically normal chromosome 7 (Fig. 5A) on which the EGF receptor gene has been mapped (30, 31). Several structural abnormalities are seen but none of them involved chromosome 7. Hypotetraploid cells had essen-
Fig. 5. A, karyotype of H69. Cells were treated with colchicine (10 ng/ml) for 3 h, washed with serum-free RPMI 1640, treated with 0.075 M KCl for 15 min at 37°C, and then fixed with Carnoy’s fixative. An air-dried preparation was stained by Giemsa banding technique. Arrows, structural abnormalities. B, karyotype of Lu134.

Fig. 5B). There was no structural abnormality related to chromosome 7. Contrary to the previous report (10, 11), we were unable to find any structural abnormality of chromosome 3 in these SCLC cell lines. Since the c-raf-1 gene has been mapped to the proximity of the reported deletion site (12), we analyzed the state of this gene in these SCLC cell lines and some LCLC...
EGF RECEPTOR IN SMALL CELL LUNG CARCINOMA

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cells neither produce nor release an EGF-competing TGF-α-gene and that the absence of EGF receptor on their cell surface like factor. In this study, we have clearly demonstrated that SCLC unrelated to the absence of EGF receptor on the SCLC cell suggested that the production of this factor was in general related to EGF. EGF receptor is assumed to be a functional TGF-α is one such autocrine growth factor and is structurally same cells often possess functional receptors for these factors. Previously, Sherwin et al. (4) described the absence of both EGF receptor and NGF receptor on SCLC cells and the production of TGF-like "soft agar growth factor" activity. They provided additional evidence that the SCLC cells contain the apparently normal chromosome 3. Thus, the previously noted partial deletion of chromosome 3 is not necessarily common to the SCLC cells.

DISCUSSION

Many types of tumor cells are known to produce and release polypeptide growth factors into culture medium (5, 6) and these same cells often possess functional receptors for these factors. TGF-α is one such autocrine growth factor and is structurally related to EGF. EGF receptor is assumed to be a functional receptor for TGF-α, through which all effects are mediated. Previously, Sherwin et al. (4) described the absence of both EGF receptor and NGF receptor on SCLC cells and the production of TGF-like "soft agar growth factor" activity. They suggested that the production of this factor was in general unrelated to the absence of EGF receptor on the SCLC cell surface. In this study, we have clearly demonstrated that SCLC cells neither produce nor release an EGF-competing TGF-α-like factor.

We have provided molecular evidence which supports the fact that SCLC cells contain an apparently intact EGF receptor gene and that the absence of EGF receptor on their cell surface is due to the lack of EGF receptor gene expression. SCLC is known to exhibit many properties which are common to those of the neutral crest-derived cell or amine precursor uptake and decarboxylation cell (1, 2). Some melanoma cells, which have a distinct embryonal origin in the neural crest, are also known to lack EGF receptor (32). Kudlow et al. (33) found no EGF receptor in TGF-α-secreting melanoma cells even under the condition which prevents the down-regulation and the degradation of the receptor, suggesting that the lack of EGF receptor on the cell surface is independent of TGF-α secretion. Our present study also showed the absence of TGF-α-like growth factors and the complete lack of EGF receptors on the cell surface of SCLC. It is therefore possible to postulate the negative regulatory mechanism of EGF receptor gene expression in both melanoma and SCLC cells. However, the study by Kudlow et al. (33) does not provide molecular evidence for the EGF receptor gene structure and its expression. It is not clear at present whether we and Kudlow et al. are observing a similar phenomenon. Further analysis including the promoter region of EGF receptor gene and the presumptive negative regulatory factor may clarify this important problem.

Recently, we (34) and others (35) found that squamous cell carcinoma of the lung and their cell lines often possess elevated levels of EGF receptor, accompanied by receptor gene amplification. The apparent EGF binding was also seen in LCLC cell lines. In addition, we observed that adenocarcinomas of the lung also possess EGF receptors. Although we have not yet studied fresh SCLC tissues, the absence of EGF receptor appears to be characteristic to SCLC among various types of the lung cancer and may be used for a unique marker.

Cytogenetic analysis has revealed that the c-myc protooncogene amplification is often accompanied with chromosomal abnormalities such as double minute chromosomes and homogeneously staining region (14). SCLC variants are known to have an amplified c-myc gene and an elevated level of expression (12). Our cytogenetic analysis showed the presence of double minute chromosomes in H69 in various sizes and numbers. This cell line is a classic type of SCLC but has been reported to have an amplified N-myc gene instead of a c-myc gene (36). It may be interesting to examine whether N-myc genes are located on double minute chromosomes in this particular cell type.

Deletion of the region 3p14-p23 has been found in a number of SCLC cells (10, 11). However, neither Lu134 nor H69 cells had 3p deletion. Whang-Peng et al. (11) reported that H69 cells are mainly hypotetraploid and contain two intact chromosomes 3 and two types of chromosome 3 aberrations having different break points, del(3)(p21p24) and del(3)(p23q26). The discrepancy between our results and the previous observation may be related to the heterogeneity of H69 cell population. In our H69 cells, less than 5% of the population is hypotetraploid and these cells are apparently derived from a hypodiploid cell through duplication. Although several abnormal chromosomes such as 12p+, 13q+, 17p−, and 19q+ were found in common, each appeared as one copy in our work and as two copies in the previous work. Therefore, it is likely that the hypodiploid H69 cells maintained in our laboratory have kept the original karyotype but that 3p− chromosomes may be lost during the course of cultivation. In this regard, however, Zech et al. (37) recently reported the deletion of chromosome 3 in only two of six SCLC cell lines. They also found the similar 3p deletion in squamous cell carcinoma, large cell carcinoma, and adenocarcinoma of lung. Our analysis of the c-raf-1 gene provided additional evidence that SCLC cells contain the apparently normal chromosome 3.

Thus, the previously noted partial deletion of chromosome 3 is not necessarily common to the SCLC cells. In contrast, the lack of EGF receptor gene expression is almost ubiquitously found in SCLC and is distinct from the other types of lung cancer. Further investigation of the presumptive mechanism which prevents the transcription of the EGF receptor gene may contribute to our better understanding of the process of the onset and development of this type of tumor.

* M. Ueda et al., unpublished results.
EGF RECEPTOR IN SMALL CELL LUNG CARCINOMA

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