Expression of the Epidermal Growth Factor Receptor in Human Small Cell Lung Cancer Cell Lines

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ABSTRACT

Epidermal growth factor (EGF) receptor expression was evaluated in a panel of 21 small cell lung cancer cell lines with radioreceptor assay, affinity labeling, and Northern blotting. We found high-affinity receptors to be expressed in 10 cell lines. Scatchard analysis of the binding data demonstrated that the cells bound between 3 and 52 fmol/mg protein with a Kd ranging from 0.5 x 10^-10 to 2.7 x 10^-10 M. EGF binding to the receptor was confirmed by affinity-labeling EGF to the EGF receptor. The cross-linked complex had a Mr of 170,000-180,000. Northern blotting showed the expression of EGF receptor mRNA in all 10 cell lines that were found to be EGF receptor-positive and in one cell line that was found to be EGF receptor-negative in the radioreceptor assay and affinity labeling. Our results provide, for the first time, evidence that a large proportion of a broad panel of small cell lung cancer cell lines express the EGF receptor.

INTRODUCTION

Epidermal growth factor is a small polypeptide which exerts its main action of growth stimulation and initiation of DNA synthesis after binding to the corresponding high-affinity cell surface receptor. EGF receptors have been demonstrated in head and neck, esophageal, ventricular, breast, bladder, skin, and lung tumors (1-7).

A few reports have disclosed the presence of EGF receptors in lung cancer, a large subset of human malignancies. Most of the experimental data originate from immunoperoxidase assays using EGF receptor antibodies. A monoclonal antibody (EGF-R1) has been raised against the external domain of the receptor (8). Biopsies from patients with NSCLC demonstrated that a high percentage express the EGF receptor, ranging from 125 of 148 (84%) in squamous cell and 22 of 73 (39%) in large cells to 11 of 29 (30%) in adenocarcinomas. No biopsies from 37 patients with SCLC demonstrated any specific EGF receptor staining (data pooled from Refs. 7 and 9-11).

Earlier studies on lung cancer cell lines have demonstrated that in NSCLC a large proportion of the examined cell lines expressed the EGF receptor, whereas SCLC, in a few studies, infrequently expressed the receptor (12-14). The EGF receptor consists of an external ligand binding site and an internal tyrosine kinase complex (15). A close similarity between v-erb-B oncogene and c-Erb-H, a strain of the avian erythroblastosis virus, and the truncated EGF receptor has been established (16). The discrepancy between SCLC and NSCLC could be accounted for if SCLC carried the truncated receptor. However, the monoclonal antibodies EGF-RF4 and EGF-RD10 recognize the internal domain of the EGF receptor (17), and studies with these antibodies have demonstrated that neither SCLC nor NSCLC carried the truncated receptor.

In the present study we have examined the presence of EGF receptor and mRNA for EGF receptor in a panel of 21 SCLC cell lines established in five different laboratories. We have used radioreceptor assay, affinity labeling, and Northern blotting.

We have found that, in contrast to earlier reports, a relatively high proportion of SCLC cell lines carried high-affinity EGF receptors and expressed EGF receptor mRNA.

MATERIALS AND METHODS

Cell Lines. SCLC cell lines were cultured in 150-cm² flasks at 37°C, 5% CO₂, and 80% humidity in medium containing 10% fetal calf serum (Flow Laboratories, Irvine, Scotland) without antibiotics. A total of 21 SCLC cell lines established from 17 patients in five different laboratories were examined. Eight cell lines were established at Dartmouth Medical School (Hanover, NH) (DMS), seven cell lines were established at Groningen Lung Cancer Centre (Groningen, the Netherlands) (GLC), two cell lines were established at the National Cancer Institute (Bethesda, MD) (NCI), and two cell lines were established in Marburg, Germany (24H and 86M1). Two cell lines were established in our own laboratory (Copenhagen, Denmark) (CPH). The origin and establishment of the cell lines have been described elsewhere (18-23). Cells growing as monolayer cultures were passaged twice a week using trypsin/EDTA (Flow Laboratories) to detach the cells. After harvest the cells were subcultured. Cells growing as floating aggregates were precipitated before the medium was removed. After resuspension, the cells were subcultured. Growth morphology and culture medium used for each cell line are given in Table 1. In the present study the breast cancer cell line MDA 231, which is strongly EGF receptor-positive, was used as a positive control cell line. Cells growing as monolayer cultures were plated in 35-mm 6-well tissue dishes for radioreceptor assay and in 100-mm Petri dishes for the affinity-labeling protocol and used within 24 h of plating.

Growth Factors. Human recombinant EGF and recombinant TGF-α were purchased from Bissendorf Biochemicals (Hannover, Germany). Porcine TGF-β was obtained from British Biotechnology, Ltd. (Oxford, England). 125I-labeled EGF with a specific activity of 150-175 μCi/μg was purchased from New England Nuclear (Boston, MA). The 125I-labeled EGF was used within 4 weeks of fresh lot date.

Displacement Assay. In order to evaluate the binding ability of 125I-labeled EGF compared to native EGF, one cell line, DMS 114, was incubated with 125I-labeled EGF at a concentration which saturated the EGF receptor. Native EGF was added (0-30,000 pm). Specific binding was calculated as the percent of maximal binding, 100% being the binding in the absence of displacing EGF. For each concentration of EGF in the incubation media, the theoretical value for the cell-associated radioactivity was calculated as the ratio of 125I-labeled EGF to the total EGF concentration.

Temperature and Time Course of EGF Binding. To evaluate the optimal time and temperature conditions for EGF binding MDA 231 was used. Cells were incubated with 25 pm 125I-labeled EGF at 4°C, 20°C, and 37°C for 20, 40, 60, 120, 180, and 240 min. At the times indicated specific bound EGF was calculated. For 37°C the assay was also performed in the presence of 50 μM of the lysosomal inhibitor ammonium acetate.

EGF Binding in Relation to Protein Concentration. Two cell lines,
DMS 79 and DMS 114, were serially diluted before a radioreceptor assay was performed. The cells were incubated with 125I-labeled EGF and five different concentrations of displacing EGF ranging from 0 to 1000 pM. Nonspecific binding was determined in the presence of 30,000 pM EGF. Binding capacity correlated to the actual protein concentration (ranging from 29 to 600 µg/ml) was determined by Scatchard analysis of the binding data.

**Radioreceptor Assay.** Cells growing as floating aggregates were washed for 60 min with binding buffer [128 mM NaCl-5 mM KCl-5 mM MgSO4-1.2 mM CaCl2-50 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid), pH = 7.2, and 2% bovine serum albumin]. After washing, the cells were mechanically disaggregated by resuspending the cell pellet in a small volume of binding buffer using an 18-gauge x 1/2-inch needle. The resulting single cell suspensions were incubated with 125I-labeled EGF and increasing levels of native unlabeled EGF ranging from 0 to 3000 pM. The assay was performed in 1.5-ml microcentrifuge tubes. The volume in each tube was adjusted to 1 ml with binding buffer. After 2 h of incubation at 20°C with overhead turning, the reaction was stopped by centrifuging at 37°C and resuspending the cell pellet three times with ice-cold binding buffer without albumin. After the final wash, the cell pellet was resuspended in solubilization buffer (128 mM NaCl-0.25 mM EDTA-0.5 mM Tris, pH 7.5-1% v/v Triton X-100). Cells growing as monolayer culture were prepared using the same procedure, except that the assay was performed directly in 35-mm 6-well dishes. After solubilization for 30 min, an aliquot of the supernatant was counted in a Beckmann II gamma counter (70% efficiency). Protein concentration was determined with the BCA protein kit (Pierce Europe, B. V., BA Oud Beijerland, The Netherlands) which utilizes bicinchoninic acid (24). Maximal binding (Bmax) was calculated as fmol/mg protein. Nonspecific binding was determined in the presence of a 300-fold excess of native EGF. Specificity of the binding was determined in specificity experiments with EGF, TGFα, and TGFβ as the displacing agents.

**Affinity Labeling.** Essentially the same procedure as for the radioreceptor assay was used for cells growing as floating aggregates. Cells growing as monolayer cultures were assayed in 100-mm Petri dishes. 125I-labeled EGF was added to a final concentration of 500 pM. For each cell line two parallel experiments were carried out. In one experiment only 125I-labeled EGF was added; in the other 125I-labeled EGF as well as 50 nM native EGF were added. The incubation proceeded for 4 h at 4°C. After the final wash, the cell pellet was resuspended in 950 µl binding buffer without bovine serum albumin before 50 µl 10 mM cross-linking agent disuccinimidyl suberate (Pierce), freshly dissolved in dimethyl sulfoxide, was added. The cross-linking reaction proceeded for 30 min and was stopped by centrifuging and resuspending the pellet in 50 µl solubilization buffer. The resulting supernatant was run on a 7%, 8 x 16 cm SDS-PAGE gel. After staining with Coomasie brilliant blue and destaining, the dried gel was exposed to an X-ray film (Amersham) with an intensifying screen at −80°C for 3 weeks.

**RESULTS**

**Displacement Assay.** One cell line, DMS 114, was saturated with 125I-labeled EGF. Different concentrations of native EGF were added, and specific binding was calculated. At the EGF concentrations used specific binding was identical to the theoretical binding (data not shown). This indicates that the 125I-labeled EGF had the same binding characteristics as the unlabeled compound.

**Temperature and Time Course of EGF Binding.** Binding of EGF was time and temperature dependent (data not shown). At 4°C maximal binding was achieved after 2 h and remained stable for several hours. Binding at 20°C followed a similar course but was slightly higher; it was more complex at 37°C, reaching a maximum after 2 h, but degradation was appreciable after 3 h. When MDA 231 cells were incubated in the presence of the lysosomal inhibitor ammonium acetate, cell-associated radioactivity at 37°C did not decrease over time, suggesting that degradation was mediated by lysosomal enzymes. During the 4-h incubation period EGF binding, in the presence of ammonium acetate, did not reach maximal level. Based on these results all incubations were performed for 2 h at 20°C. Although binding was less than it was at 37°C, it was, however, stable over a long period of time.

**EGF Binding in Relationship to Protein Concentration.** In order to evaluate the minimal protein concentration needed to detect the EGF receptor, the binding capacity of DMS 79 and DMS 114 was evaluated in an assay where different protein concentrations were used. We found proportionality between protein concentration and binding capacity. A doubling of the protein concentration resulted in an approximate doubling of the EGF binding (P < 0.01, Student’s t test) (data not shown).

**Receptor Binding Studies.** Saturation of the receptor was reached with an EGF concentration in the range of 100 to 1500 pM (exemplified in Fig. 1). Nonspecific binding, defined as the cell-associated radioactivity in the presence of a large excess of unlabeled EGF, was in all cases lower than 15% (Fig. 1). Since about one-half of the cell lines grew as floating aggregates and...
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Fig. 1. EGF binding to a SCLC cell line. GLC 2 was incubated with 100 pM 125I-labeled EGF and incubated with increasing concentrations of unlabeled EGF as described in “Materials and Methods.” Inset: Scatchard plot of the receptor specific binding. KD is given by the slope of the curve and Bmx by the x-intercept. N, total binding; O, receptor-specific binding; A, nonspecific binding.

one-half as monolayer cultures, we chose to relate all binding data to protein concentration. Scatchard analysis of the binding data showed that cells bound between 3 and 52 fmol/mg protein (Fig. 1) and had a KD of 0.52–2.73 × 10^−10 M.

The specificity of the EGF receptor/ligand binding was determined using different displacing agents. The specificity of 125I-labeled EGF binding to DMS 153 is shown in Fig. 2. It was found that EGF and TGFα, which both bind to the EGF receptor (29), could displace 125I-labeled EGF, whereas TGFβ, which has a separate receptor (30), was unable to displace EGF.

Affinity Labeling Studies. EGF binding to the receptor was further visualized by affinity-labeling the ligand-receptor complex with the cross-linking agent disuccinimidyl suberate. Fig. 3 illustrates the affinity-labeling results of six DMS cell lines. Following electrophoresis on a SDS-PAGE gel, specific EGF binding was seen as a band with a calculated M, of 170,000–180,000. The presence of excess unlabeled EGF resulted in the disappearance of the band, indicating that the binding to the receptor was specific. All EGF receptor-positive cell lines were positive in the affinity-labeling experiments (Table 2).

Northern Blotting Studies. The cell lines were examined for the production of EGF receptor mRNA. Fig. 4 illustrates a Northern blot analysis of nine cell lines. In all EGF receptor-positive cell lines we also found the corresponding mRNA (Table 2), which is seen as a band of approximately 10 kilobases. Blots were rehybridized with the β-actin probe to demonstrate equal loading in all lanes. The intensity of staining with the pE7 probe therefore semiquantifies the EGF receptor mRNA content.

The results for all binding data, affinity labeling, and Northern blot analysis are summarized in Table 2. For all cells tested and found positive in the radioreceptor assay, binding was in all cases specific and saturable. All affinity labeling and Northern blot analysis in the EGF receptor-positive cells demonstrated similar results, although with different intensity, corresponding to the level of the receptor or receptor mRNA in the cell. In the above each experimental procedure was exemplified by different cell lines.

DISCUSSION

This is, to our knowledge, the first study in which a large representative panel of SCLC cell lines has been studied for the presence of EGF receptors and EGF receptor mRNA. The panel of SCLC cell lines established in five different laboratories and cultured in different media is probably a representative cross-section of SCLC cell lines.

Displacement experiments demonstrated that 125I-labeled EGF had the same binding characteristics as unlabeled EGF. This is essential to obtaining reliable binding data in the radioreceptor assay. Time and temperature course experiments demonstrated different EGF binding at 4°C, 20°C, and 37°C, as described elsewhere (31).
The resulting 7-day autoradiography is shown. Arrow, 10-kilobase EGF receptor β-actin protein concentration was lower than 75 ng/m. It was also mRNA. The β-actin probe indicates that the lanes were loaded equally.

Studies on other receptor systems such as the estrogen receptor have demonstrated that a critical protein level was required to detect the receptor (32). We found that in the SCLC cell lines, EGF receptors could not be demonstrated when the protein concentration was lower than 75 μg/ml. It was also found that the binding capacity was stable in the range of 150–600 μg protein. Therefore, to avoid underestimating binding capacity or falsely classifying a SCLC cell line as EGF receptor-negative, we only concluded about the EGF receptor state in a cell line if the protein concentration was in the range of 200–600 μg/ml.

Scatchard analysis of the binding data demonstrated high-affinity receptors in 10 SCLC cell lines (Fig. 1; Table 2). The dissociation constant was similar in all cases and characteristic for EGF binding (12–14). Maximal binding varied from 3 to 52 fmol/mg protein. Three of the cell lines (24H, DMS 79, and NCI N417) have previously been reported to be EGF receptor-positive, with maximal binding 2–3 times higher than in our results (12). However, binding assays in the report cited were performed at 37°C, where maximal binding is higher. One cell line (NCI H69) previously reported as negative is also EGF receptor-negative in the present study (12–13). Binding of 125I-labeled EGF to the positive cells was specific since only EGF and TGFα, which bind to the EGF receptor (29), could displace the labeled EGF. TGFβ did not influence EGF binding (Fig. 2).

The results obtained from the radioreceptor assay and the displacement studies demonstrated that a large proportion of the examined SCLC cell lines carried the EGF receptor. To ascertain whether EGF was in fact binding to the EGF receptor, the cell lines were tested by affinity labeling. After size fractionation on SDS-PAGE gels, all cell lines found to be EGF receptor-positive in the radioreceptor assay also displayed a specific band with a calculated Mₖ of 170,000–180,000 (Fig. 3). The EGF receptor has previously been reported as having this calculated molecular weight (15). This provides further evidence that EGF binding was to the EGF receptor. We also examined the expression of EGF receptor mRNA in the panel. In 11 of 20 SCLC cell lines mRNA could be detected (Table 2; Fig. 4). One cell line (DMS 92) that was EGF receptor-negative in the radioreceptor assay and affinity labeling expressed low levels of EGF receptor mRNA. We believe this discrepancy may be due to either EGF receptor content below the detection limit of the two other assays or an autocrine production of EGF or TGFα masking the receptor.

In one study (33) the EGF receptor gene was examined. All seven examined SCLC cell lines had an intact and unrearranged EGF receptor gene. Two of these seven cell lines were examined for EGF receptor mRNA and found to be negative. However, our results, based on the investigation of a large panel of cell lines, demonstrated the presence of EGF receptor mRNA in 11 of 20 SCLC cell lines.

The present finding of 50% EGF receptor-positive SCLC cell lines is in agreement with a study by Haeder et al. (12). However, it contrasts a study by Sherwin et al. (13) where only 1 of 11 SCLC cell lines were EGF receptor-positive. Conflicting results were also reported concerning EGF receptors in vivo. Immunocytochemical studies detected no EGF receptors in 37 tumors from patients with SCLC (7–9, 11). Functional EGF receptors have been demonstrated by stimulation with EGF in 80% of 56 primary lung tumors, including seven SCLC (34). Thus, EGF receptors have been demonstrated in vivo, indicating that the expression of the EGF receptor is not a phenomenon induced by in vitro culturing of SCLC cells.

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