Characterization of Epidermal Growth Factor Receptor in Primary Human Non-Small Cell Lung Cancer

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ABSTRACT

Membrane preparations from 36 human non-small cell lung cancers were examined for the expression of epidermal growth factor (EGF) receptors, and comparisons were made between tumor types and stage. Eight normal lung membrane preparations were also examined. The concentrations of EGF receptors were assessed by ligand binding studies using 125I-radiolabeled EGF. In two point saturation experiments using 0.3 nM 125I-EGF incubated with membranes from 35 primary lung tumors, a median of 18 fmol/mg of protein (range, 1.1 to 530) was found. This was significantly greater than binding to eight lung membranes: median, 6.1 fmol/mg of protein (range, 1.0 to 14.5) (P < 0.02). Scatchard binding curves obtained in 21 of the 36 tumors and seven of eight of the normal lung preparations showed high and low affinity sites for EGF receptors on all but two tumor membranes. The dissociation constant of the high affinity sites was similar on tumor and normal lung membranes: range, 0.75 to 30 x 10^18 M/liter. However, the tumors had a significantly higher concentration of these receptor sites: median, 30.4 fmol/mg of protein versus a median of 6.2 fmol/mg of protein on normal lung membranes (P < 0.01). Likewise, there were significantly more low affinity sites on tumors: median, 237 fmol/mg compared to 60.2 fmol/mg on normal lung (P < 0.01). No differences were found in this analysis between tumors of different histological subtypes or clinical stage. It is possible that the high level of expression of high affinity sites on lung tumors could be used as a target for ligand-complexed drugs.

INTRODUCTION

EGF2 is a Mr 6000 protein found in normal human plasma and urine (1) which binds to specific receptors on the cell surface (2). EGFR are characterized by tyrosine protein kinase activity and ligand-stimulated autophosphorylation (3), a property shared with other peptide growth factor receptors, such as those for insulin, platelet-derived growth factor, and insulin-like growth factor I (4). Transformed cells secrete a growth factor, transforming growth factor α, which has extensive homology with EGF and also stimulates tyrosine kinase activity on the EGFR (5).

The physiological role of EGF has not been clearly delineated, but it appears to be involved in both growth and differentiation of cells (6). EGF stimulates differentiation and proliferation of a wide variety of cell types, including keratinocytes and fibroblasts (7). In addition, EGF has been shown to be mitogenic to ectodermal (6) and endodermal cells in vivo (8). The structure of the internal part of the EGFR is similar to the oncogene product of the avian erythroblastosis virus, which also possesses tyrosine-specific protein kinase activity (9). EGFR appears to play an important role in the development and proliferation of certain human malignancies, including those of neuroglia (10), bladder (11), and breast (12). In some tumors, gene amplification is the major mechanism of overexpression of EGFR; in others only a minority of tumors show amplification. In bladder and breast cancers, less than 5% of tumors show amplification, while one-third of tumors show high expression of EGFR (13).

Increased expression of EGFR appears to be particularly common in squamous carcinomas (14). For example, virally transformed keratinocytes express 5 to 50 times more EGFR than normal keratinocytes (15), and A431 cells, derived from an epidermoid carcinoma of vulva, also express greatly increased EGF-R (16). Squamous carcinomas of lung show increased binding of an antibody to EGFR compared to normal skin as demonstrated by an autoradiographic technique (17).

We have also shown previously, by immunoperoxidase studies with a monoclonal antibody to EGFR, that human lung cancers express increased density of epidermal growth factor receptor sites (18) and that more advanced and less differentiated tumors show greater staining for the receptor in comparison with well-differentiated tumors. These results applied to adenocarcinomas and some large cell tumors as well as to squamous tumors (18).

Previous studies of EGFR in lung cancers have used cultured cell lines or qualitative methods (14,17,18). We have therefore quantitated the number of EGFR and their binding properties and affinities in primary human lung tumors, and we have compared these with normal adjacent lung. We have also compared the expression of EGFR in squamous and nonsquamous tumors.

Our results show that lung cancers express increased concentrations of EGFR with high and low affinity binding sites when compared to normal lung tissue. All histological types of non-small cell lung cancer express similar concentrations of EGFR.

PATIENTS AND METHODS

EGF binding was studied in 36 human primary lung tumor membrane preparations, of which 20 were squamous carcinomas, 11 adenocarcinomas, and 5 large cell undifferentiated carcinomas. This distribution of tumor types is due to the collection of sufficient tumors of each type to make valid comparisons of EGFR expression. In addition, eight membrane preparations from apparently normal parts of lungs resected for cancer were studied. This was as far as possible away from the tumor, a minimum of 10 cm.

Tumors were staged by the tumor, nodal involvement, metastasis system on examination of resected material. Tumors with distant metastases or mediastinal nodes were graded Stage III, as also were tumors extending to the parietal pleura or involving a main bronchus less than 2 cm from the carina or any tumor associated with atelectasis or obstructive pneumonitis of an entire lung or with pleural effusion (19).

Tumors were collected fresh at operation and stored in sucrose buffer (20) at -18°C. Membranes were prepared by homogenization of finely cut tissue. The homogenate was centrifuged at 300 x g at 4°C for 10 min, and the supernatant was centrifuged at 110,000 x g at 4°C for 40 min. The pellet obtained formed the membrane preparation. The protein concentration was measured by the Bradford method (21) and standardized to 1000 µg per ml. An increased plasma membrane

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2 The abbreviations used are: EGF, epidermal growth factor; EGFR, epidermal growth factor receptors.

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component was confirmed by 5' nucleotidase activity (22).

the concentration of egfr on the membrane preparation was calculated by competitive ligand binding studies (23) using radio-iodine-labeled egf in competition with varying concentrations of unlabeled ligand. 125 iodine-labeled egf was prepared by the iodogen method (24) with label specific activity of 72 to 102 μci/μg. scatchard binding analysis was performed on the binding data to calculate the concentrations of receptors and their binding affinities (25), using a program designed for a b. b. c. microcomputer by dr. c. s. hetherington.

membrane preparation (0.1 ml) was incubated with 0.1 ml of 125l-labeled egf at a final concentration of 0.3 nm. to the incubation were added 12 to 14 varying concentrations of unlabeled egf (from 0 to 200 nm). the solution was incubated at 26°C for 2 h, conditions which had been established as optimal conditions in preliminary studies. incubation was terminated by the addition of 1 ml of ice-cold buffer and centrifugation at 14,000 x g. the binding reaction was linearly related to protein concentration up to 1.5 mg/ml.

statistical differences were examined using the wilcoxon rank sum test.

results

competitive binding studies. the addition of increasing amounts of radiolabeled egf at constant specific activity showed that 50% of specific binding had occurred with 1.5 nm 125l-egf and that over 90% of maximum specific binding had occurred at 7.5 nm 125l-egf. an example for one tumor is shown in fig. 1.

in order to quantify the amount of receptors on small tumor membrane preparations, the concentration of ligand specifically bound with 0.3 nm radiolabeled egf in incubation was calculated for 35 tumor membranes and 8 lung membranes. the median concentration of ligand bound to tumor membranes at this concentration was 18 fmol/mg of protein compared to 6.1 fmol/mg of protein on lung membranes (P < 0.02). similarly, the amount of egf bound when there was 1.5 nm and 5.0 nm egf in the incubation was 3 times greater on 29 tumor membranes when compared to normal lung membranes (P < 0.01 in both cases) (fig. 2).

displacement kinetics. for 31 tumor membranes and 7 lung membranes, displacement experiments using increasing concentrations of unlabeled ligand were performed. the total concentration of ligand at which 50% of radiolabeled egf binding occurred gave an estimate of the affinity constant (Kₐ) of the high affinity sites (fig. 3). similar results were obtained for normal lung (data not shown). the median and range for tumors were 1.0 nm (0.15 to 25 nm), values similar to those for normal lung membranes: 1.0 nm (0.25 to 5 nm).

scatchard analysis. there was sufficient material for multipoint analysis of binding on 21 tumor membranes and on 7 lung membranes. scatchard binding analysis was performed on
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these data. Nineteen tumors yielded curvilinear Scatchard plots, indicating two classes of binding site on the EGF\textsubscript{r}, a high affinity-low capacity site, and a low affinity-high capacity site (Fig. 4, a and b) (25). Two of the tumor membrane preparations showed linear binding plots with one binding site of high capacity (Fig. 4c). All 7 normal lung membranes analyzed also had high and low affinity sites (Fig. 4d).

The median binding capacity of the low capacity-high affinity sites on 19 tumor membranes was 30.4 fmol/mg of protein, and the median concentration of high capacity-low affinity sites on 21 tumors was 237 fmol/mg of protein (Table 1). There were similar binding affinities for tumor and lung membranes. The high affinity sites had a $K_a$ 10 times that of the low affinity sites (Table 1).

The EGF\textsubscript{r} concentration measured by two different procedures was compared. The concentration of EGF bound specifically when there was 0.5 nM EGF in the incubation (2-point binding assay) correlated well with the concentration of high affinity sites calculated from the Scatchard plots using 10 points (Fig. 5) ($r = 0.9, P < 0.001$). Thus, a two point binding assay using this concentration of $^{125}$I-EGF alone and with 100-fold excess of unlabeled ligand could be used to calculate the concentration of high affinity sites on small specimens.

Comparison of Tumor Types and Lung Tissue. Comparison between tumor membranes and normal lung tissue resected with tumor showed that the $K_a$ values of high and low affinity sites were similar for both tissues but that tumors had a much higher concentration of high and low affinity sites than normal lung membranes (Table 1). Indeed, 75% of the 29 tumor membranes assayed using 1.5 nM EGF had more receptors than any normal lung membrane at that concentration (Fig. 2). These differences were significant ($P < 0.01$). Squamous tumors did not express a greater concentration of high or low affinity sites when compared to adenocarcinoma (Table 1) ($P > 0.1$), nor was there a significant difference when tumor stages were compared (Stages I and II: $n = 25$; median, 18 fmol/mg; range, 2 to 600; Stage III: $n = 10$; median, 8 fmol/mg; range, 1.5 to 70) ($P > 0.5$).

DISCUSSION

We have shown high affinity and low affinity binding sites for EGF in human lung cancer and demonstrated a greater concentration of receptors on tumors than on normal lung tissue membranes.

Sherwin et al. (26) showed that human lung cancer cells in tissue culture express EGF\textsubscript{r} (26). The cell lines which they studied were predominantly from adeno- and large cell carcinomas. Cowley et al. (14), using an antibody to EGF\textsubscript{r} on frozen tissue sections, demonstrated high levels of EGF\textsubscript{r} on squamous carcinomas of lung and nasopharynx. In that study, only 2 of 8
CHARACTERIZATION OF EGFr IN NON-SMALL CELL LUNG CANCER

Table 1 Quantitation and dissociation constants of high and low capacity binding sites for $^{125}$I-EGF on tumor membranes and normal lung membranes

<table>
<thead>
<tr>
<th>Tumor (n = 21): 11 squamous, 7 adenocarcinoma, 3 large cell</th>
<th>Median of $B_{max}$* low capacity sites (fmol/mg protein)</th>
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<td>Normal lung (n = 7)</td>
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<td>Tumor (n = 21): 11 squamous, 7 adenocarcinoma, 3 large cell</td>
<td>29.5 (n = 19') (4.3-1532.6)</td>
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<td>Squamous (n = 11)</td>
<td>19.1 (n = 10') (10.54-256)</td>
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* $B_{max}$, quantity of sites.
* Numbers in parentheses, range.
* Two tumors had high capacity/low affinity sites only.

Fig. 5. Correlation of amount of high affinity sites (calculated by Scatchard ligand binding studies) with concentration of EGF bound at 0.5 nM EGF (2-point binding assays) ($r = 0.9$, $P = 0.001$).

One previous study using ligand binding techniques (31) compared lung cancers and lung tissue from the same five patients. This showed up to a 2.8-fold increase in EGF activity without change in binding affinity. These studies were on receptors partially purified by affinity columns which reduced affinity 10-fold, and they detected only one binding site on the EGF. Our results pertain to receptors that have not been so modified and show two binding sites. This may be more relevant to the in vivo situation, and our studies there was approximately a 5-fold increase in high affinity sites on tumor membranes compared to normal lung membranes.

Various mechanisms can be proposed for the presence of two binding sites of differing affinity. Protein kinase C can interact with EGFr by phosphorylation of threonine 654 (32), which produces a decrease in affinity and receptor down-regulation. Other growth factors interact with protein kinase C and may modulate the EGFr (33). Alternatively, some binding sites may have been occupied by endogenous growth factors and may have undergone autophosphorylation and down-regulation. The presence of two binding sites has been shown in cell lines (34) and also after transfection of the human EGF (35). The results for our membrane preparations are similar to those reported in intact cells by Livneh et al. (35).

Increased expression of EGFr on tumors may be a mediator of growth or may be merely a marker of the malignant state. Retroviral transfection of the EGFr gene, however, leads to increased numbers of EGFr on cells and can contribute to the transformed phenotype (36). There are a number of possible mechanisms by which increased EGFr might mediate the transformation of normal cells. Human cancer cells may produce their own growth factors in vivo (autocrine secretion) to stimulate cell proliferation (37), or the stroma of cancers may produce EGF or TGF-α.

Santon et al. measured the growth rate of several variants of A431 cells in athymic mice and found that the degree of gene amplification and number of EGFr are directly correlated with the growth of these cells as solid tumors (38). The addition of EGF to culture medium led to increased growth by at least 50% in 44 of 56 lung carcinomas (of all cell types) and to a doubling of growth in 21 of these 56 carcinomas (39). Ozawa et al. (40) showed that infusion of EGF into athymic mice with implanted squamous tumors expressing high concentrations of EGFr led to a 3- to 6-fold increase in tumor weight when compared to controls. Recently, it has been reported that overexpression of EGFr in squamous carcinomas from lung, head, and neck is associated with poor survival, and 60% of these tumors had gene amplification (41).

The greater expression of EGFr on tumors may be mediated through amplification of the EGFr gene or abnormal expression of the gene product (42). Berger et al. showed that 2 of 10

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squamous lung cancers had amplification of the EGFr gene (28). However, they reported 4 squamous tumors with increased expression of EGFr without gene amplification. Thus transcriptional factors may be important in high expression of EGFr.

There are a number of possible clinical implications of the high amount of EGFr in lung tumors. Antibodies to EGFr have been shown to inhibit tumor growth (43). Furthermore, ligand-complexed drugs can concentrate in receptor-positive cells by affinity targeting (44). The high affinity sites would be most important for ligand-mediated cytotoxicity, so that in the future, ligand binding studies may be helpful in selecting patients for therapy.

REFERENCES

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