ABSTRACT

The *neu* protooncogene is a recently described transforming gene originally isolated from ethylnitrosourea-induced rat neuroblastomas. We have examined the expression of the *neu* gene in human non-small cell lung carcinomas using immunoprecipitation and immunohistochemistry. The *neu* protein product (p185") was present in eight of 22 non-small cell carcinoma cell lines derived from human lung tumors. Expression of p185" was found in all histological subtypes of non-small cell carcinomas including large cell carcinomas, squamous cell carcinomas, and adenocarcinomas. Extension of these data to biopsy specimens of human lung tumors demonstrated that normal ciliated bronchial epithelium of the peripheral airways expressed p185" at low levels. Neoplastic cells in four of 12 adenocarcinomas and three of five squamous cell carcinomas also expressed p185" at levels higher than the normal ciliated bronchial epithelium. Together these studies indicate that p185" expression is a common feature of human lung tumors.

INTRODUCTION

The most prevalent newly diagnosed tumor in men in the U. S. is lung cancer. In women it is the third most prevalent newly diagnosed cancer (1). In addition, lung cancer is the leading cause of death from all malignancies in both men and women in the U. S. (1). These facts point out the necessity of gaining a more complete understanding of the molecular pathogenesis of lung cancer which may lead to more effective measures of diagnosis, treatment, and prevention.

There are two major classes of lung cancer: small cell lung carcinoma and non-small cell lung carcinoma. The non-small cell lung cancers can be further divided histologically into three types; adenocarcinoma (representing 30% of all lung cancers), large cell carcinoma (15%), and squamous cell carcinoma (25%). As a group, these tumors differ from small cell lung carcinoma in their prognosis, cell of origin, and medical therapy.

Many aspects of the pathogenesis of non-small cell and small cell lung tumors remain unclear. Recently a number of transforming genes (oncogenes) have been found to be expressed in lung cancer and have therefore been implicated in its development. The products of the oncogenes c-myc, N-myc, L-myc, and L-myb are localized to the nucleus and are felt to be involved in the regulation of gene expression and cell replication in small cell lung carcinoma (2, 3). The K-ras oncogene encodes a Mr 21,000 guanosine triphosphate binding protein, related to G-proteins. The Mr 21,000 binding proteins are thought to play a part in growth signal transduction (4) and have been found in lung adenocarcinomas (5). The oncogene c-erb B-1, which encodes the epidermal growth factor receptor, has also been reported as expressed in squamous cell lung carcinomas (6, 7).

Recently a newly described transforming oncogene that has a nucleotide sequence exhibiting overall homology to the epidermal growth factor receptor was identified in ethylnitrosourea-induced rat neuroblastomas (8). This oncogene, called *neu*, encodes a Mr 185,000 transmembrane glycoprotein whose carboxy terminus has features typical of an intracellular tyrosine kinase domain (9-11). Activation of the c-neu gene is mediated by a single amino acid substitution in the transmembrane domain of the protein (12). How this single amino acid dictates transformation is yet to be elucidated but it may involve receptor aggregation and activation (13). The human counterpart to the rat *neu* gene is called c-erb B-2/HER-2. This gene (c-erb B-2/HER-2) is 88% homologous to the rat *neu* gene (11) but differs slightly in the molecular mass of its protein product, Mr 190,000 (14) versus Mr 185,000 for the *neu* gene (8). The difference in molecular weight is most likely due to interspecies differences in posttranslational modification. Due to the high degree of homology, for purpose of clarity, both proteins will be hereafter denoted as p185". Structurally, p185" consists of an extracellular putative ligand binding domain, a transmembrane anchoring domain, and an intracytoplasmic tyrosine kinase domain (8, 11, 15-18). Due to its structural organization and similarity to other growth factor receptors, it has been suggested that p185" is the receptor for an as yet unidentified growth factor (8).

*Neu* gene expression has been reported in human breast carcinoma (10, 19, 20-22), gastric carcinoma (20, 23, 24), salivary gland (9), kidney (20), and colonic polyps (25). However, the point mutation resulting in activation of the protooncogene described in rat neuroglioblastomas has not been found in human tumors. Instead, activation appears to occur through overexpression of the protooncogene (19-22). We hypothesized that since *neu* gene expression has been seen in normal rat and human lungs (11, 26), overexpression may also play a role in human lung carcinogenesis. The present study was therefore undertaken to determine the expression pattern of the *neu* gene in normal and neoplastic tissue of the human lung. Our results show that p185" is expressed in one third of cell lines derived from human lung tumors and human lung cancer biopsy specimens. In addition, p185" is overexpressed in lung cancer biopsy specimens as compared with expression in normal bronchial epithelium.

MATERIALS AND METHODS

Cell Lines. The human breast adenocarcinoma cell line BT474 (gift from S. Aaronson, NCI) and rat B104 cell line were utilized as positive controls in immunohistochemistry and radioimmunoprecipitation analysis. The human lung tumor cell lines, established from biopsy or aspiration specimens as part of approved clinical protocols, included human adenocarcinomas National Cancer Institute numbers H125,
H522, H650, H676, H726, H920, H969, H1355, H1373, H1395, H1437, H1512, and H1581; bronchoalveolar carcinoma H358, H322, H441, H820, and H1404; adenocarcinomas H596 and H647; large cell carcinomas H1155, H1299, and H1334; squamous cell carcinomas H226, and H520; and small cell carcinomas H69, H82, and H249. The cells were cultured in RPMI, 10% fetal bovine serum, modified hydrocortisone-insulin-transferrin-17β-estradiol-selenium medium (27), or ACL-4 medium (28) and incubated at 37°C in a humidified 5% CO2 atmosphere.

**p185** Specific Rabbit Antiserum. Polyclonal anti-p185 antisera (DBW-2), was prepared by immunizing rabbits with a synthetic peptide corresponding to amino acid residues 1240–1255 of the human p185 sequence coupled to keyhole limpet hemocyanin (23). These residues encompass a region of primary sequence shared by the intracytoplasmic carboxy-terminus of the rat and human neu gene products (8, 14, 15). This antisera specifically immunoprecipitates p185 of rat (13), cat, and human (25); and has been used successfully in our laboratory to demonstrate p185 expression by a variety of techniques including immunoblotting (13), and immunohistochemistry of routine paraffin-embedded tissue sections after fixation with paraformaldehyde, formalin, or Bouins (25, 26). Reactivity of the antibody is blocked by preincubation with the immunizing peptide or by passage over a column containing peptide coupled to Sephadex beads.

**Immunohistochemistry.** Paraffin-embedded surgical pathology tissue specimens which had been fixed in Bouins and routinely processed were selected from the files of the Department of Pathology, Hospital of The University of Pennsylvania. Tissue specimens from adult male Sprague-Dawley rats were processed in a manner similar to the human material. Sections (5 μm) were mounted on glass microscope slides, baked at 58°C for 20 min, deparaffinized in xylene, and hydrated through graded alcohols. Endogenous peroxidase was quenched by incubation for 20 min in 0.5% H2O2/methanol at —¿20°C. Subsequent washes and incubations were performed in phosphate buffered saline plus 1% fetal bovine serum at room temperature. Tissue sections were immunostained by the avidin-biotin peroxidase method (29). The sections were incubated for 30 min with a saturating concentration of primary antibody, typically a 1/2000 to 1/4000 dilution of serum. Bound primary antibody was labeled by incubating the sections for 30 min sequentially in biotinylated goat anti-rabbit IgG (Tago, Burlingame, CA) and streptavidin-biotinylated peroxidase (Zymed Laboratories, S. San Francisco, CA). The peroxidase substrate consisted of 1 mg/ml 3,3'-diaminobenzidine and 0.03% H2O2 plus 5 ITIM imidazole, as a color enhancer (30). The sections were lightly counterstained with hematoxylin, dehydrated through graded alcohols and xylene, and mounted in Permount.

**Radioimmunoprecipitation.** Cells were cultured in 60-mm culture plates for 24 h. Following a 15-min incubation in methionine-free high glucose Dulbecco's modified Eagle's media, the cells were labeled for 6 h in HGDMEM supplemented with 2% fetal calf serum previously dialyzed against 0.9% NaCl. For labeling 250 μCi/ml [35S]methionine (New England Nuclear, Boston, MA) was added during the 6-h incubation. The cells were washed with ice-cold phosphate buffered saline, scraped into 500-μl RIPA buffer containing 1 mM ATP, 2 mM EDTA, and 20 mM sodium fluoride. The lysates were precleared with normal rabbit antiserum and precipitated as previously described (31). The samples were suspended in electrophoresis sample buffer (32), and analyzed by electrophoresis on a 0.1% sodium dodecyl sulfate, 7.5% acrylamide, 0.17% bisacrylamide slab gel. The gels were fixed, treated for fluorography, dried, and exposed to Kodak XAR film for 2 days at —¿70°C.

**RESULTS**

**p185 Expression in Human Lung Tumor Cell Lines.** Twenty five cell lines derived from surgically resected human lung tumors were initially screened for p185 expression. After metabolically labeling the cell lines with [35S]methionine, cells were lysed and p185 was immunoprecipitated using 5 μl of DBW-2. As shown in Fig. 1, this antisera reacted with and precipitated a protein with a molecular weight of 190,000 from these cell lines which correlates with the size of p185 reported in human breast and gut carcinomas. We have analyzed in detail the reactivity of the anti-p185 antisera which we have constructed. In immunoprecipitation studies 5 μl of DBW-2 antisera specifically recognizes a protein of M, 185,000 from NIH/3T3 cells that has been molecularly constructed to express the rat p185 gene (Fig. 2A, Lane A). NIH/3T3 cells which do not contain p185 mRNA do not react with the same antisera (Fig. 2A, Lane B). The BT747 cell line which expressed high amounts of the human p185 oncoprotein (M, 190,000) is highly reactive with the same antisera (Fig. 2A, Lane C). In contrast, A431 cells which do not express p185 mRNA but express high amounts of the epidermal growth factor receptor
are not reactive with the DBW-2 antisera (Fig. 2A, Lane D).

The specificity of the antisera was further examined by peptide competition studies (Fig. 2B). Labeled BT474 cells were immunoprecipitated with DBW-2 antisera (Fig. 2B, Lane A), with DBW-2 antisera in the presence of 1 μg of irrelevant peptide (Fig. 2B, Lane B) (33) or relevant DBW-2 peptide (Fig. 2B, Lane C) and with 1 ng of relevant DBW-2 peptide (Fig. 2B, Lane D). These studies demonstrate the specificity of the antisera for the immunizing peptide and specific reactivity with both human and rat p185neu proteins. We have observed no evidence of cross-reactivity with the biochemically related epidermal growth factor receptor protein.

p185neu was found in all histological classes of non-small cell lung carcinomas, but not all lines within a histological type expressed p185neu (Table 1). Expression appeared to be clustered within the adenocarcinoma group, but expression was clearly not lineage specific, as both large cell carcinomas and squamous cell carcinomas also expressed p185neu. Importantly, the three small cell carcinomas examined did not express p185neu. These tumors differ from non-small carcinomas in their cell of origin, arising from bronchial neuroendocrine cells while non-small cell carcinomas arise from ciliated bronchiole epithelial cells.

**neu Expression in Human Lung Biopsies.** To examine the expression levels of p185neu in fresh human tissue, formalin-fixed, paraffin-embedded biopsy specimens of human lung tumors were examined for p185neu expression. Previous work has shown that the antiserum used in this study in conjunction with immunoperoxidase staining techniques identifies p185neu in fixed tissue (25, 26). A total of 24 different lung tumor sections were stained. Correlating histological type with p185neu expression, four of 12 of adenocarcinomas, three of five squamous cell carcinomas, none of three carcinoids, and none of the three small cell carcinomas reacted with the antiserum. This distribution parallels that in studies of the human lung cancer cell lines described earlier.

Fig. 3 shows representative results of immunohistochemical analysis for p185neu. Examination of surrounding histologically normal lung tissue contained in the biopsy specimen demonstrated very faint background reactivity of the antiserum with fibroblasts and collagen. Normal appearing ciliated bronchial mucosa (Fig. 3A) weakly reacted with the antibody at levels clearly above background staining. Alveolar lining cells were unreactive. Fig. 3B is a p185neu expressing adenocarcinoma. Examination of the stained biopsy specimen demonstrated that cells reacting with anti-p185neu antiserum were cells lining the glandular sections of the adenocarcinomas. The antibody reactivity pattern showed localization of p185neu to both the cytoplasm and membrane. Cells lying free in the alveolus occasionally stained and may represent cells naturally sloughed from the main tumor mass or dislodged during sample handling.

Fig. 3C shows the results in a squamous cell carcinoma with the anti-p185neu antiserum. Again, the tumor cells reacted with the antiserum, identifying both membrane and cytoplasmic p185neu. The nonmalignant surrounding tissue did not react. Qualitatively the level of antibody reactivity in the squamous cell carcinomas was not as strong as in the adenocarcinomas yet was stronger than normal bronchial epithelium expression. None of the three small cell carcinomas studied reacted with

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* Number of tumors expressing p185neu per total number of tumors studied within a histological type.

* Number of p185neu expressing or nonexpressing tumors per all tumors studied, regardless of histological type.
the anti-p185E antibody. Therefore, these results support the use of anti-p185E antisera as a differential marker of tumor phenotype in lung pathology.

DISCUSSION

We have investigated the involvement of neu gene expression in human lung cancer and normal lung tissue. Our studies have found that p185E is expressed at low levels in normal adult ciliated bronchiolar epithelium, but appears absent from alveolar tissue. In a significant number of cell lines derived from non-small cell lung carcinomas, and biopsy specimens of human lung cancers, p185E was expressed at high levels.

The immunohistochemical reactivity pattern of the anti-p185E antibody identified both membrane and cytoplasmic p185E. This agrees with previous biochemical localization of p185E performed by Drebin et al. (34), performed on neu-oncogene transformed NIH 3T3 cells but differs slightly from the immunohistochemical pattern seen by Slamon, et al. (22) and van de Vijver et al. (35) in mammary adenocarcinomas where a large amount of cytoplasmic p185E was not identified. As the antiserum raised independently by each laboratory is directed against the carboxy terminus of p185E, the difference in immunohistochemical localization between breast and lung carcinoma may suggest a unique feature of this protein in the lung versus the breast, such as different mechanisms regulating expression or surface localization.

The expression of p185E by nonneoplastic ciliated epithelial cells of the normal bronchiolar mucosa suggests that this receptor may have a role in normal cell proliferation and/or differentiation which must occur to maintain the peripheral airways. In addition, the expression of this receptor in large proportion of lung adenocarcinomas examined suggests that these tumors may arise from ciliated bronchiolar epithelial cells which normally express p185E. The importance of this receptor in pulmonary squamous cell carcinomas, tumors which most commonly arise from central airways, not peripheral airways, is not clear. If p185E is important in tumorigenesis our data would suggest that regardless of progenitor cell type or anatomical location, adenocarcinomas and squamous cell carcinomas may, in some cases, have some mechanisms of tumorigenesis in common, although eventuating as discreet histological entities. Alternatively p185E may represent a particular stage of differentiation of the expressing cells. This would again imply that both adenocarcinomas and squamous cell carcinomas pass through a similar stage during their natural history.

The function of p185E in these tissues remains to be clarified. Several lines of evidence suggest that p185E is a growth factor receptor: its structural homology with epidermal growth factor receptor and related cell surface receptors (11), its developmental and tissue specific expression pattern in rat embryogenesis (26) and the fact that antibody-mediated down-regulation of cell surface p185E leads to reversion of transformation in a variety of cells (35, 36). Other data suggest that p185E plays a role in cell differentiation, as shown by the gradient of expression seen in p185E expression on intestinal villi (25). In preliminary studies examining various developmental stages of human lung for p185E expression, we have yet to identify such a developmental pattern. Therefore, if p185E is a marker of a cellular differentiation state, development of normal lung epithelium does not share the same requirements as intestinal epithelium. In addition, expression in lung tumors suggests that these tumors are not repeating a developmental stage which had been expressed earlier in their natural history.

The mechanism of p185E activation in human lung tumors is presently unknown. Overexpression of p185E has been shown to occur by amplification of the neu protooncogene in many human adenocarcinomas (9, 20, 22, 23) and may be of prognostic value for cancer of the breast (22). In some of the lung cancers studied, a 5-kilobase neu mRNA transcript was found (data not shown). This size transcript is consistent with what has been reported in other human tumors (8-11). The amount of neu mRNA accumulation though was very low in comparison to the B104 cell line, a rat cell line known to have a single copy of the neu gene. The relatively low levels of neu mRNA lung tumors suggest that gene amplification has not occurred. In support of this, amplification of the neu gene has not been found in biopsy specimens of 60 non-small cell human lung cancers (22). Karyotype analysis of two of our p185E expressing cell lines has revealed no structural abnormalities of chromosome 17, the site of the neu locus. Mechanisms other than gene amplification resulting in abnormal expression of p185E have been observed. In human mammary tumor cell lines, p185E overexpression has been found without gene amplification (37). Oncogenic activation of p185E stimulates tyrosine phosphorylation in vivo and results in a protein with a much shorter half-life (38) showing that events beyond the gene level may also influence protein accumulation. The molecular characterization of p185E overexpression in our human lung tumor derived cell lines is currently under investigation.

The importance of this oncogene in carcinogenesis has been recently highlighted in work by Muller et al. (39). These investigators have shown that transgenic mice that carry an activated c-neu oncogene develop multiple mammary tumors. The overexpression of p185E by epithelially derived neoplasms arising in the lung adds to the number of tissues which may utilize a similar mechanism of tumorigenesis. In addition, it extends the list of known oncogenes and protooncogenes (K-ras, c-myc, and c-erB) which may be important in human lung cancer (2, 3, 5-7).

REFERENCES


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Expression of the *neu* Gene-encoded Protein (P185\textsuperscript{neu}) in Human Non-Small Cell Carcinomas of the Lung


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