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Research Article

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CD10/NEP in Non-small Cell Lung Carcinomas

Relationship to Cellular Proliferation

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Abstract

The cell surface metalloproteinase CD10/neutral endopeptidase 24.11 (NEP) hydrolyzes a variety of peptide substrates and reduces cellular responses to specific peptide hormones. Because CD10/NEP modulates peptide-mediated proliferation of small cell carcinomas of the lung (SCLC) and normal fetal bronchial epithelium, we evaluated the enzyme's expression in non-small cell lung carcinomas (NSCLC). Bronchoalveolar and large cell carcinoma cell lines had low levels of CD10/NEP expression whereas squamous, adenosquamous, and adenocarcinoma cell lines had higher and more variable levels of the cell surface enzyme. Regional variations in CD10/NEP immunostaining in primary NSCLC specimens prompted us to correlate CD10/NEP expression with cell growth. In primary carcinomas of the lung, clonal NSCLC cell lines and SV40-transformed fetal airway epithelium, subsets of cells expressed primarily CD10/NEP or the proliferating cell nuclear antigen (PCNA). Cultured airway epithelial cells had the lowest levels of CD10/NEP expression when the highest percentage of cells were actively dividing; in addition, these cells grew more rapidly when cell surface CD10/NEP was inhibited. NSCLC cell lines had receptors for a variety of mitogenic peptides known to be CD10/NEP substrates, underscoring the functional significance of growth-related variability in CD10/NEP expression. (*J. Clin. Invest.* 1994. 94:1784–1791.) Key words: common acute lymphoblastic leukemia antigen • non-small cell lung carcinomas • proliferating cell nuclear antigen • peptide growth factors • growth regulation

Introduction

The cell surface zinc metalloproteinase CD10/neutral endopeptidase 24.11 (NEP)[1] hydrolyzes multiple naturally occurring peptides and functions in a variety of organ systems to downregulate induced responses to peptide hormones (1–7). For example, CD10/NEP is expressed at high levels in the lung where it modulates responses to tachykinins that mediate neurogenic inflammation (8). Inhibition of CD10/NEP dramatically increases both the binding of substance P to bronchial membranes and the resulting tachykinin-induced cough, bronchoconstriction, vasodilation, and neutrophil infiltration (9–12).

Recent studies have also implicated CD10/NEP in the regulation of peptide-mediated proliferation of bronchial epithelial cells (13–15). CD10/NEP hydrolyzes bombesin-like peptides (BLP) (13), which are potent mitogens for fibroblasts and normal bronchial epithelial cells (16–18) and essential autocrine growth factors for many small cell lung carcinomas (SCLC) (19). The enzyme cleaves BLP at two sites within the seven–amino acid conserved carboxy terminus that is required for biological activity (13). The growth of BLP-responsive SCLCs is inhibited by CD10/NEP and potentiated by CD10/NEP inhibition, implicating the enzyme in the regulation of BLP-mediated autocrine tumor cell growth (13). These results are of particular interest because cigarette smoking inactivates CD10/NEP (20), smokers have increased levels of BLP in their bronchoalveolar lavage fluid (21), and SCLCs occur almost exclusively in cigarette smokers (22). Reduced cell surface CD10/NEP enzymatic activity may actually promote BLP-mediated proliferation of pulmonary neuroendocrine cells and facilitate the development of SCLCs (13).

Since CD10/NEP regulates BLP-mediated growth of SCLCs (13) and BLP also stimulate normal fetal lung development (18), we evaluated CD10/NEP expression in normal fetal lungs (14, 15). The temporal and cellular patterns of CD10/NEP expression implicate the enzyme in the regulation of BLP-mediated fetal lung development (14, 15). The enzyme is expressed at the highest levels in the first and early second trimester and is primarily localized to distal undifferentiated airway epithelium and airway-associated mesenchyme (14). In associated in vitro and in vivo assays, the enzyme was directly linked with peptide-mediated airway epithelial cell growth and maturation. Inhibition of cell surface CD10/NEP increased DNA synthesis in human fetal lung organ cultures (14). In related murine studies, the in utero administration of a specific long-acting CD10/NEP inhibitor also increased the proliferation and maturation of developing fetal lungs (15).

Because CD10/NEP has been linked to the peptide-mediated proliferation of SCLCs and normal fetal airway epithelium, we also evaluated its expression and regulation in additional subtypes of human lung cancer (non-small cell lung cancer, NSCLC). Herein, we characterize CD10/NEP expression in NSCLC, determine the enzyme's relationship to cellular proliferation, and identify potential CD10/NEP substrates in these malignancies.

Methods

Cell lines media, and growth conditions. NSCLC cell lines representing major pathological subtypes including squamous (Calu-1, H157, H520,

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1. *Abbreviations used in this paper:* BLP, bombesin-like peptide; GRP, gastrin-releasing peptide; NEP, neutral endopeptidase 24.11; NSCLC, non-small cell lung carcinoma; NMB, neuromedin-B; PCNA, proliferating cell nuclear antigen; SCLC, small cell lung carcinoma; VIP, vasoactive intestinal peptide.

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1752), adenosquamous (H125, H596), adenocarcinoma (A427, Calu-3, H23, H441), bronchoalveolar (A549, H322), and large cell carcinoma (H460, H661, SL6) were obtained from B. Neel (Beth Israel Hospital, Boston, MA) (23) and maintained in either DME or RPMI/10% fetal calf serum (FCS). 56 FHTE, an SV40-transformed human fetal bronchial epithelial cell line, was obtained from D. Gruenert (University of California, San Francisco, CA) and maintained on fibronectin (1 mg/100 ml; GIBCO BRL, Gaithersburg, MD)/vitrogen (1%; Celtrix Laboratory, Palo Alto, CA)-coated plates in DME/10% FCS. CCL-210, normal pulmonary fibroblasts, were obtained from American Type Culture Collection (ATCC) (Rockville, MD) and maintained in DME/10% FCS (24). All cell lines were incubated at 37°C with 5% CO_2. Adherent cells were preincubated with PBS/1 mm EDTA and subsequently detached by manual scraping to prepare single cell suspensions for further analysis.

Enzymatic assay for neutral endopeptidase 24.11 activity. Whole cell suspensions of the bronchial epithelial cell lines were evaluated for cell surface CD10/NEP enzymatic activity using the previously described fluorometric assay (2). In this coupled assay, NEP cleaves the substrate glutaryl-Ala-Ala-Phe 4-methoxy-2-naphthylamide (Enzyme Systems Products, Livermore, CA). The resulting compound, Phe 4-methoxy-2-naphthylamide, is then hydrolyzed to yield the fluorescent product 4-methoxy-2-napthylamide after the addition of aminopeptidase (Sigma Chemical Co., St. Louis, MO). All samples were analyzed in triplicate in the presence or absence of 2 nM of the CD10/NEP inhibitor *N*-(a-rhamnopyranosyloxyhydroxy-phosphinyl)-leu-trp (phosphoramidon; Sigma Chemical Co.) (25). Excitation at wavelength 340 nm and emission at wavelength 425 nm were measured using a spectrofluorometer. Thereafter, peak phosphoramidon-inhibitable fluorescence was calculated and CD10/NEP enzymatic activity (nmol/h per 10^5 cells) determined (2).

Immunofluorescence analysis. Cell suspensions of NSCLC cell lines and fetal airway epithelial cells (56 FHTE) were phenotyped as previously described using the murine anti-CD10 mAb (J5) (26) or a class-matched negative control (3C11, an IgG1 monoclonal antibody directed against a secreted protein [γ-interferon]), and goat anti-mouse FITC (Tago Immunologicals, Camarillo, CA). Immunofluorescence analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA) using a logarithmic scale. The change in mean channel fluorescence (MCF) was determined for each cell sample by subtracting the MCF obtained with the class-matched negative control antibody (3C11) from that obtained with anti-CD10 (J5).

In additional experiments, 2×10^5 56 FHTE cells were plated in 100-mm petri dishes and analyzed at selected time points thereafter for cell numbers, co-expression of CD10/NEP and proliferating cell nuclear antigen (PCNA), and cell cycle. In selected experiments, 56 FHTE cells were also cultured in the presence of 1 μM of the CD10/NEP inhibitor phosphoramidon. Cell suspensions of the adherent 56 FHTE cells were prepared by detaching monolayers that had been previously incubated in PBS/1 mM EDTA. Thereafter, cells were stained with biotinylated anti-CD10 (J5) and FITC-avidin (Tago Immunologicals). Cells were subsequently fixed with 1% paraformaldehyde (4°C) for 30 min and permeabilized with 70% ethanol (−20°C) for 30 min on ice. Cells were then washed twice in PBS and incubated with phycoerythrin-coupled anti-PCNA (Dako Corp., Carpinteria, CA). Immunofluorescence analyses were performed on an Epics Elite Multigraph (Coulter Electronics, Hialeah, FL) using a logarithmic scale. Cell cycle analysis was performed after additional samples of fixed permeabilized cells were incubated with propidium iodide (27) (Sigma Chemical Co.; 50 μg/ml propidium iodide in PBS) and treated with ribonuclease (Sigma Chemical Co.; 50 μg for 30 min at room temperature). The DNA content and associated percentages of cells in the G_0/G_1, S and G_2/M phases of the cell cycle were determined using the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA).

Immunohistochemical analysis. Double immunohistochemical analysis of selected NSCLC cell lines for CD10/NEP and PCNA was performed after paraformaldehyde fixation, permeabilization with 0.3% Triton X-100, and nuclear antigen precipitation with methanol. Cells were initially immunostained for PCNA with a mouse anti-PCNA antibody (PC10; Dako Corp.), swine anti-mouse IgG, horseradish peroxidase immune complexes, and the red peroxidase substrate 3-amino-9-ethylcarbazole (28). Thereafter, cells were stained for CD10/NEP using biotinylated mouse anti-CD10/NEP (J5), avidin biotin complex alkaline phosphatase, and blue alkaline phosphatase substrate (Vector Labs, Burlingame, CA) (28).

Immunohistochemical analysis of primary human NSCLC specimens was also performed as previously described (29). Thin (5 μm) serial sections were individually stained with anti-CD10/NEP (J5), anti-PCNA, or an unreactive murine myeloma protein (MOPC) and subsequently developed with horse anti-mouse IgG and avidin-biotin complex (ABC) immunoperoxidase (29).

Receptor binding assays. Receptor binding assays were performed as previously described (30). Cell suspensions of airway epithelial cells or fibroblasts were prepared and resuspended in binding buffer (DME/10% FCS/100 mM Hepes pH 7.4). Thereafter, 2×10^6 cells were transferred to microfuge tubes and incubated in binding buffer containing 50 pmol of ^{125}I-bombesin, 2,200 Ci/mMol (NEN, Boston, MA) at 37°C for 30 min on a rotator. Cells were subsequently pelleted, resuspended in 250 μl of binding buffer, and spun through 750 μl of FCS × 3. Cell pellets were then transferred to borosilicate counting tubes and analyzed for bound radioactivity. Specific ^{125}I-binding was determined by performing the analysis in the presence and absence of 1 μmol additional unlabeled bombesin.

Reverse polymerase chain reaction (PCR) analysis. Total RNA was prepared from the indicated cell lines as previously described (31). Thereafter, 1-μg aliquots of RNA from each source were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (BRL Life Technologies, Inc. Gaithersburg, MD) and oligo-dT primers (BRL Life Technologies, Inc.).

The quality and quantity of each cDNA pool was initially evaluated by amplifying the cDNA with human β-actin–specific oligonucleotide primers (5′ sense 5′-CAGCCATGTACGTTGCTATCCAG-3′[bp 2120–2140] and 3′ antisense 5′-GTTTCGTGGATGCCACAGGAC-3′ [bp 2656–2634]) and 2.5 U of Taq-DNA-polymerase (Boehringer Mannheim, Indianapolis, IN) for 23 cycles as previously described (32). The cDNAs were then utilized to amplify the receptors for GRP, NMB (33), BRS-3 (34), and VIP (35), and the NMB (36) and VIP (37) peptides, using the indicated sense and antisense oligonucleotide primers (Table I). All amplifications included 40 cycles of denaturation at 94°C (1 min), annealing at the appropriate temperatures (Table I) (1 min), and extension at 72°C (1 min).

Thereafter, one fifth of each PCR reaction was size-fractionated on a 1% agarose gel, transferred to nitrocellulose, and hybridized with an appropriate ^{32}P-labeled internal oligonucleotide probe (Table I).

Results

CD10/NEP in NSCLC cell lines. In a large panel of NSCLC cell lines representing major pathological subtypes including squamous, adenosquamous, adenocarcinoma, bronchoalveolar, and large cell carcinoma, CD10/NEP cell surface protein and enzymatic activity were assessed with flow cytometry and fluorometric assays (Fig. 1); CD10/NEP transcripts were also evaluated by Northern blot (data not shown). There was an excellent correlation between the levels of CD10/NEP cell surface protein and enzymatic activity (Fig. 1). Whereas bronchoalveolar and large cell carcinoma cell lines had low levels of CD10/NEP, squamous, adenosquamous, and adenocarcinoma cell lines had higher and more variable levels of CD10/NEP protein and enzymatic activity. CD10/NEP transcript abundance also correlated with cell surface protein and enzymatic activity (data not shown).

CD10/NEP in primary NSCLCs: relationship to PCNA expression. To further assess CD10/NEP expression in primary NSCLC of the lung, a panel of seven previously well-characterized primary lung cancers (squamous cell ca, 3; adenoma, 1; adenosquamous cell ca, 1; bronchoalveolar cell ca, 1; and poorly

Table I. Oligonucleotide Primers Used in Reverse Polymerase Chain Reactions

Receptor/peptide	Sense primer	Antisense primer	Internal probe	Annealing temperature
Subtype	5′ 3′	5′ 3′	5′ 3′	
GRP receptor	TTCTGAACTTGGAGGTGGACCAC	GAGGCAGATCTTCATCAGGGC	GTGAACGATGACTGGTCC	52°C
NMB receptor	CCTCTCGGTGACCACCGGCGC	GCTACTGATGCGAGCCACTTC	CCTGCCGGCCTCGGAC	56°C
NMB peptide	CCGCTCAGCTGGGATCTCCCG	GCTCAGGATTTACATCCAGATGG	CTGAGGGACCAGCGACTGCA	56°C
BRS-3 receptor	CCTGCACACAGCTTAGAGTC	AGTCTTCAGGATGGCATTGG	TCCTTCTGCAAGGTAGTGAG	50°C
VIP receptor	TGATGTGTCCCAACATGCCCA	TGTTGGTGAAGTAGGTGATGGA	AATATCCAGGCCAAGACCACA	52°C
VIP peptide	GTATCCAGAAATGCCAGGCATGC	TTCACTGGGAAGTTGTCATCAGC	GACAACTATACCCGCCTTAGAA	56°C

differentiated large cell ca, 1) were analyzed for CD10/NEP using immunohistochemical techniques. Although there was good agreement between the analysis of average CD10/NEP expression in NSCLC cell lines and primary NSCLC specimens, there were areas of heterogeneous CD10/NEP expression in certain primary tumors. Since peptide-mediated bronchial epithelial cell proliferation is modulated by cell surface CD10/NEP (13–15), we postulated that the heterogeneity in CD10/NEP expression in specific primary tumors might be correlated with regional variations in tumor cell growth. For this reason, we evaluated selected primary tumors for the co-expression of CD10/NEP and the proliferating cellular nuclear antigen (PCNA). PCNA, which is also known as the DNA polymerase δ auxiliary factor, is necessary for appropriate leading strand DNA synthesis and is primarily expressed by proliferating cells (38–40).

Fig. 2 includes thin serial sections of representative primary NSCLC specimens (poorly differentiated squamous cell carcinoma [*A–C*], and well differentiated adenocarcinoma [*D–F*]), stained with anti-CD10/NEP, anti-PCNA, or a class-matched negative control. As indicated, each tumor contains focal CD10/NEP positive cells (*brown*) with lower levels of PCNA (non-immunostained green nuclei) and CD10/NEP negative cells (*green*) with higher levels of PCNA (*dark brown nuclei*). In certain tumors such as the one shown in the top panel of Fig. 2, PCNA expression was more intense in the less well-differen-

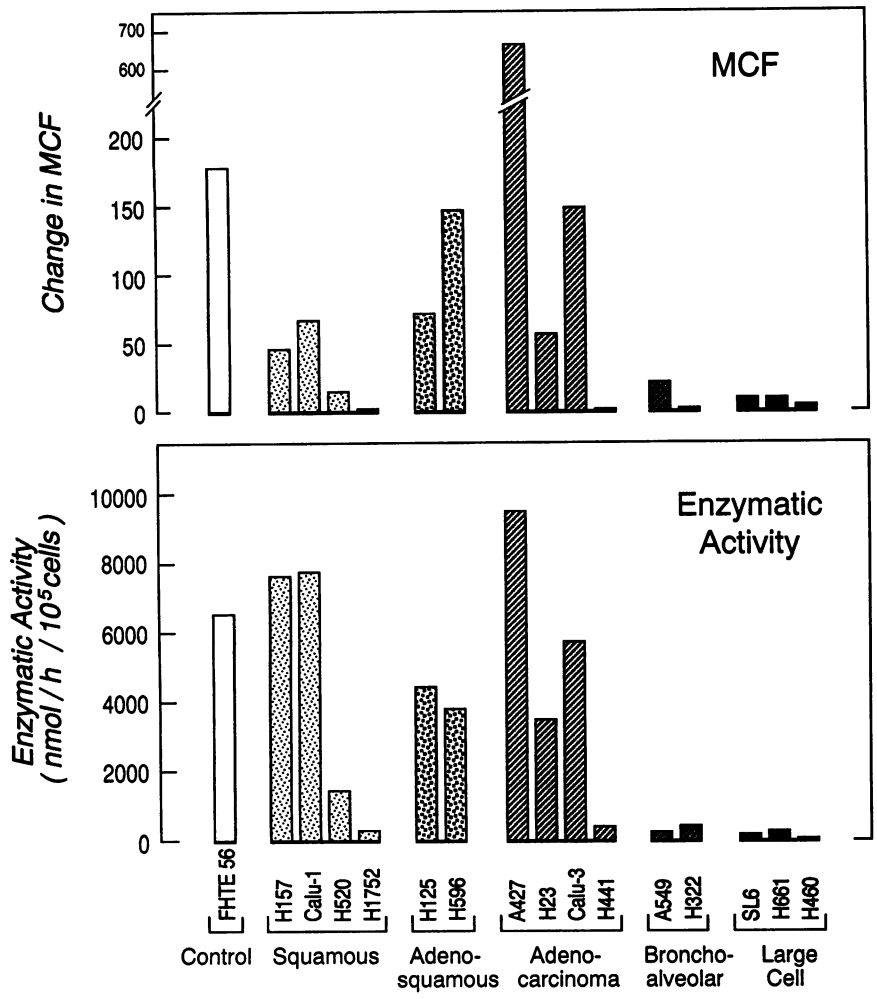

Figure 1. CD10/NEP Cell surface protein and enzymatic activity in NSCLCs. (*Top panel*) CD10/NEP cell surface immunofluorescence. The cell surface expression of CD10/NEP in each of the indicated NSCLC cell lines is represented as the change in mean channel fluorescence resulting from incubation with the anti-CD10 monoclonal antibody rather than the class-matched negative control. The evaluated cell lines include SV40-transformed fetal airway epithelial cells (56 FHTE), and NSCLC cell lines representing major pathologic subtypes including squamous, adenosquamous, adenocarcinoma, bronchoalveolar, and large cell carcinoma. Because of the plasticity of NSCLC subtypes, NSCLC subclassification may be somewhat arbitrary; for this reason, we only included cell lines previously reported to represent the indicated classical pathologic subtypes (23). (*Bottom panel*) CD10/NEP cell surface enzymatic activity. CD10/NEP enzymatic activity for whole cell suspensions of each of the indicated NSCLC cell lines is represented in nmol/h/10^5 cells.

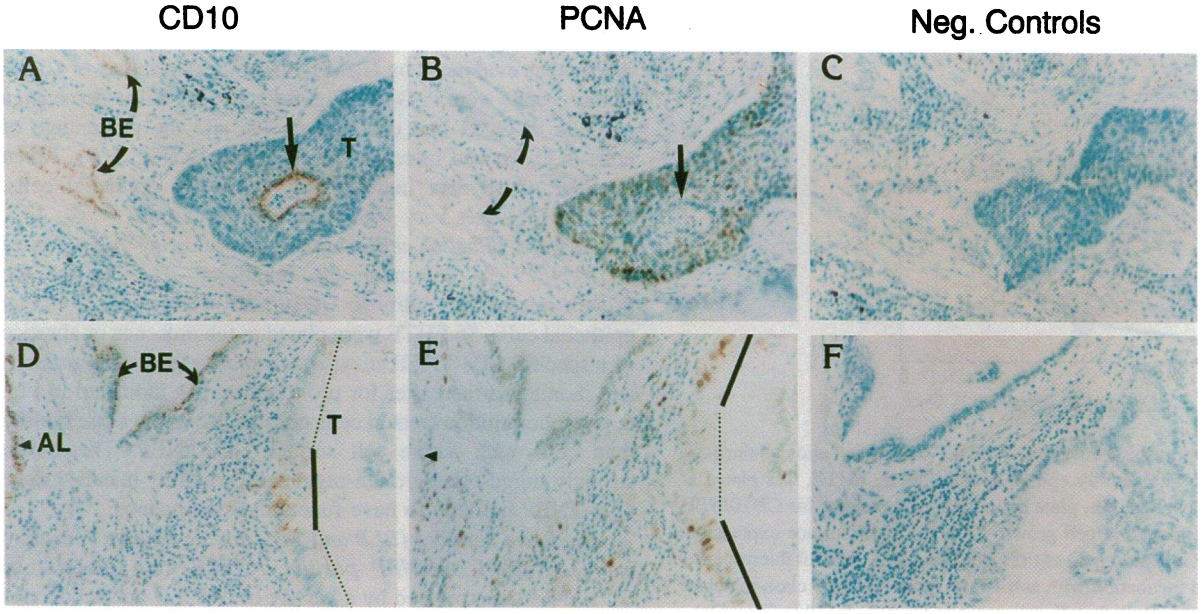

Figure 2. CD10/NEP and PCNA expression in primary NSCLCs. Evaluated primary NSCLC specimens include a poorly differentiated squamous cell carcinoma (*A–C*) and a well differentiated adenocarcinoma (*D–F*) stained with anti-CD10/NEP (*A* and *D*), anti-PCNA (*B* and *E*), or a class-matched unreactive myeloma protein (*C* and *F*). Normal bronchial epithelium in the primary tumor specimens is denoted *BE* and normal alveolar epithelium is indicated *AL* (*A* and *D*). Specific areas of tumor are labelled T (*A* and *D*). Note that the CD10/NEP$^+$ tumor cells in *A* are viable malignant cells lining a small lumen that contains neutrophils.

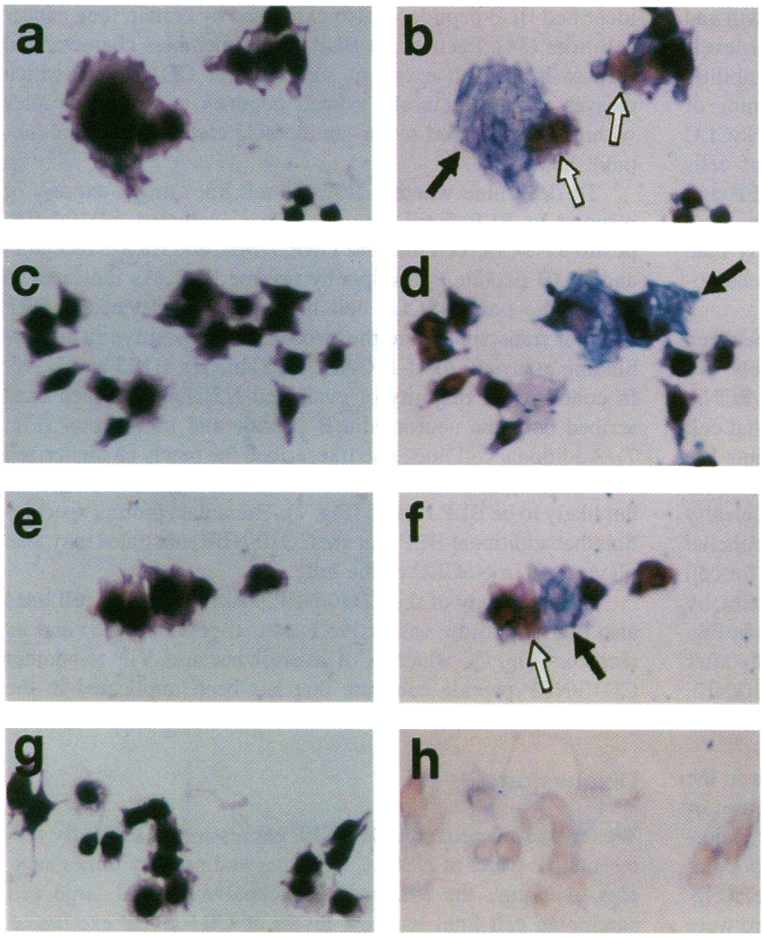

Figure 3. CD10/NEP and PCNA expression in a NSCLC cell line (A427, adenocarcinoma). (*A, C, E,* and *G*) A427 slides (from right panel) restained with hematoxylin and eosin to further delineate cellular morphology and identify all intact nucleated cells. (*B, D,* and *F*) A427 immunostaining with anti-CD10/NEP (*blue*) and anti-PCNA (*red*). As indicated, subsets of cells were identified that expressed low levels of CD10/NEP and high levels of PCNA (*pale blue cells with red nuclei, white arrows*) or high levels of CD10/NEP and low levels of PCNA (*intensely blue cells with pale pink or unstained nuclei, black arrows*); additional cells co-expressed both proteins. (*H*) A427 cells immunostained with class-matched negative controls.

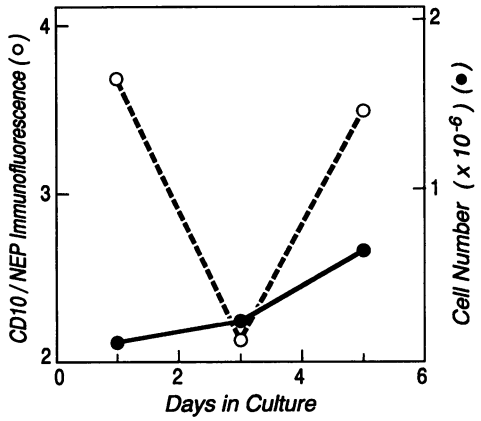

Figure 4. Relationship between CD10/NEP expression and cellular proliferation. The fetal airway epithelial cell line, 56 FHTE, was plated at 2×10^5 cells per 100-mm plate and analyzed thereafter for CD10/NEP expression (Y axis) and cell growth (Y′ axis). Cell surface expression of CD10/NEP is represented as the change in mean channel fluorescence resulting from incubation with anti-CD10 rather than the class-matched negative control. Note that Y axis extends from 2–5 (arbitrary units) to facilitate the display of data from both Y and Y′ axis. The data are derived from one of three representative experiments.

tiated basal epithelial cells whereas CD10/NEP expression was most prominent in more well-differentiated luminal lining cells.

CD10/NEP in bronchial epithelial cells: relationship to cellular proliferation. The inverse relationship between CD10/NEP and PCNA immunostaining in certain primary lung cancers suggested that CD10/NEP might vary with cell growth and that more rapidly proliferating cells might express lower levels of the cell surface enzyme. To further explore this possibility, we also evaluated CD10/NEP and PCNA immunostaining of whole cells from NSCLC cell lines (Fig. 3). In a given NSCLC cell line such as the A427 adenocarcinoma, subsets of cells were identified that expressed low levels of CD10/NEP and high levels of PCNA (pale blue cells with red nuclei) or high levels of CD10/NEP and low levels of PCNA (intensely blue cells with pale pink or unstained nuclei); additional cells coexpressed both proteins (Fig. 3).

To more specifically characterize the relationship between cellular proliferation and cell surface CD10/NEP levels, we also correlated CD10/NEP expression with cell growth, PCNA expression, and DNA content in the fetal airway epithelial cell line, 56 FHTE. 56 FHTE were used in these experiments because we thought that potential relationships between CD10/NEP expression and cellular proliferation would be most clearly delineated in nonmalignant cells. These fetal airway epithelial cells were plated at low density and analyzed thereafter for cell number, CD10 and PCNA expression, and DNA content by flow cytometry. In the representative experiment shown in Fig. 4, cells initially grew slowly and proliferated more rapidly after several days in culture. Of interest, cell surface CD10/NEP expression declined during the first several days in culture and gradually increased thereafter (Fig. 4).

Serial analyses of CD10/NEP and PCNA expression in the airway epithelial cells demonstrated that on day 1, a subset of cells expressed CD10/NEP and no PCNA. On day 3, the majority of cells expressed higher levels of PCNA and a subpopulation of the brightly PCNA-positive cells lacked CD10/NEP (Fig. 5 *A*). By day 5, PCNA expression had declined and there were fewer CD10/NEP negative cells (Fig. 5 *A*). Consistent with these observations, only ~ 0.5% of day 1 cells were actively dividing as evidenced by G_2/M DNA content whereas ~ 10.1% of day 3 cells were in G_2/M (Fig. 5 *B*). By day 5, the percentage of cells in G_2/M had declined to 4% and the percentage of cells in G_0/G_1 had increased proportionately (Fig. 5 *B*). Taken together, these data indicate that in airway epithelial cells, CD10/NEP levels vary with cellular proliferation.

Airway epithelial cell proliferation following inhibition of CD10/NEP. To determine the functional relevance of the growth-related variability in CD10/NEP expression in airway epithelial cells, 56 FHTE were plated in the presence or absence of a specific CD10/NEP enzymatic inhibitor (phosphoramidon) and monitored thereafter for proliferation. As indicated in Fig. 6, phosphoramidon-treated cells consistently grew more rapidly than cells with active cell surface CD10/NEP. These data strongly suggest that growth-regulated variability in cell surface CD10/NEP is likely to have functional significance.

Bioactive peptides (BLP and VIP) and associated receptors expressed by CD10/NEP positive NSCLC cell lines. Since CD10/NEP is expressed by a variety of NSCLC and modulates the effects of bombesin-like peptides (BLP) on SCLC and normal fetal airway epithelium, we also evaluated CD10/NEP-positive NSCLC cell lines for BLP receptors. In a standard ligand-binding assay, only two of the NSCLC cell lines (CALU-1 and CALU-3) bound significant levels of the mammalian bombesin "homolog," (gastrin releasing peptide, GRP) (Table II) (41). However, recent studies have also implicated related BLP family members such as neuromedin B (NMB) and mammalian phyllolitorin (21, 41–43) in bronchial epithelial cell growth. Furthermore, BRS-3, the receptor for an additional as yet unidentified BLP-peptide, is also expressed by certain lung cancer cell lines (34). Each of the BLP family members characterized to date is also an excellent substrate for CD10/NEP, which cleaves these structurally related peptides at analogous sites within the conserved seven–amino acid carboxy terminus (unpublished data).

To determine whether NSCLC cell lines might express or respond to BLP family members, we evaluated CD10/NEP-positive NSCLC cell lines for GRP, NMB, and BRS-3 receptors and NMB peptide transcripts by reverse PCR. As indicated in Fig. 7, the only NSCLC cell lines that had detectable GRP receptor transcripts were the two that were positive in ligand-binding assays (CALU-1, CALU-3, Table II), H125 and H596. In contrast, the majority of evaluated NSCLC cell lines transcribed both the neuromedin B peptide and its receptor (Fig. 7). Additional cell lines also transcribed the newly characterized BRS-3 receptor for which the endogenous ligand is unknown but likely to be BLP-related (Fig. 7). These data prompt speculation that additional BLP that are CD10/NEP substrates may also play a role in NSCLC of the lung.

Of note, many of the CD10/NEP positive NSCLC cell lines also transcribed the vasoactive intestinal peptide (VIP) and its receptor (Fig. 7), which is of interest because VIP is another CD10/NEP peptide substrate that has been implicated in the autocrine stimulation of NSCLC of the lung (44).

Discussion

We have characterized CD10/NEP expression in a broadly representative panel of NSCLC cell lines and primary tumor samples. Although the evaluated bronchoalveolar and large cell carcinoma cell lines had low levels of CD10/NEP expression

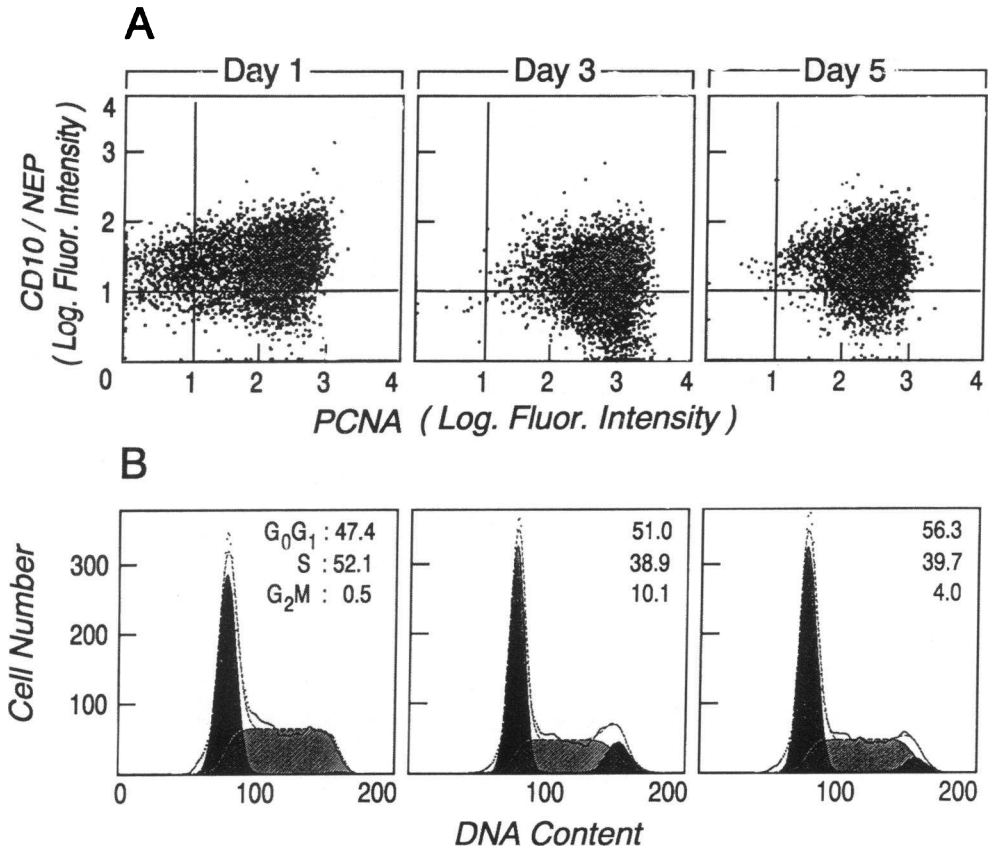

Figure 5. CD10/NEP and PCNA co-expression and cell cycle analysis of airway epithelial cells over time. (*A*) Co-expression of CD10/NEP and PCNA in 56 FHTE fetal airway epithelial cells over time. CD10/NEP expression is indicated on the Y axis and PCNA expression on the X axis. Cells expressing CD10/NEP alone are located in the left upper quadrant, cells expressing both CD10/NEP and PCNA are located in the right upper quadrant, and cells expressing PCNA alone are located in right lower quadrant. (*B*) Cell cycle analysis of 56 FHTE cells over time. Additional samples of the 56 FHTE cells shown in *A* were analyzed for DNA content using propidium iodide stain and the Multicycle Software Program (Phoenix Flow Systems, San Diego, CA) which identifies specific subpopulations of cells in G_0/G_1, S and G_2/M. The percentages of day 1, 3, and 5 cells in each phase of the cell cycle are indicated on each panel. The data are derived from one of three representative experiments. Data shown in Figs. 4 and 5 are from the same representative experiment.

and enzymatic activity, squamous, adenosquamous, and adenocarcinoma cell lines had higher and more variable levels of the cell surface enzyme. There was an excellent correlation between the levels of CD10/NEP cell surface protein and enzymatic activity and transcript abundance in all of the evaluated cell lines; we found no abnormally sized CD10/NEP transcripts and no evidence of cell surface CD10/NEP protein with reduced enzymatic activity. These data are of particular interest because an aberrantly spliced CD10/NEP cDNA from a lung cancer cDNA library was recently reported to encode a protein with reduced enzymatic activity (45).

Although there was good agreement between the analysis of CD10/NEP in NSCLC cell lines and primary NSCLC specimens, there were regional variations in CD10/NEP expression in certain primary tumors. Additional immunohistochemical analysis of CD10/NEP and PCNA in thin serial sections identified an inverse relationship between the PCNA and CD10/NEP expression in the primary malignancies; similar analyses of clonal NSCLC cell lines and cultured fetal respiratory epithelial cells confirmed these observations. Of note, these data are consistent with recent studies which localized CD10/NEP to fetal

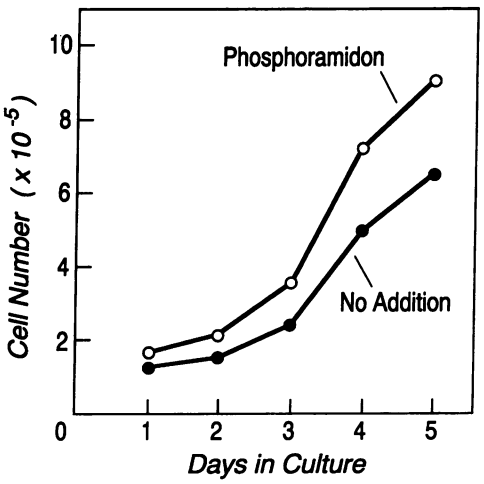

Figure 6. Airway epithelial cell proliferation after inhibition of CD10/NEP. Fetal airway epithelial cells (56 FHTE) were plated at 2×10^5 cells per 100-mm plate in the presence or absence of a specific CD10/NEP inhibitor (phosphoramidon) and subsequently evaluated daily for cell growth. The data are derived from one of two representative experiments.

Table II. Binding of ^{125}I-labeled Bombesin to NSCLC Cell Lines

Cell line	Pathological subtype	Counts/10^6 cells
Calu-1	Squamous	1351
H151	Squamous	< 100
H520	Squamous	< 100
H125	Adenosquamous	< 100
H596	Adenosquamous	< 100
Calu-3	Adenocarcinoma	255
H-23	Adenocarcinoma	< 100
A-427	Adenocarcinoma	< 100
A549	Bronchoalveolar	< 100
CCL-210	Pulmonary fibroblasts	1766
56 FHTE	Fetal airway epithelial cells	2131

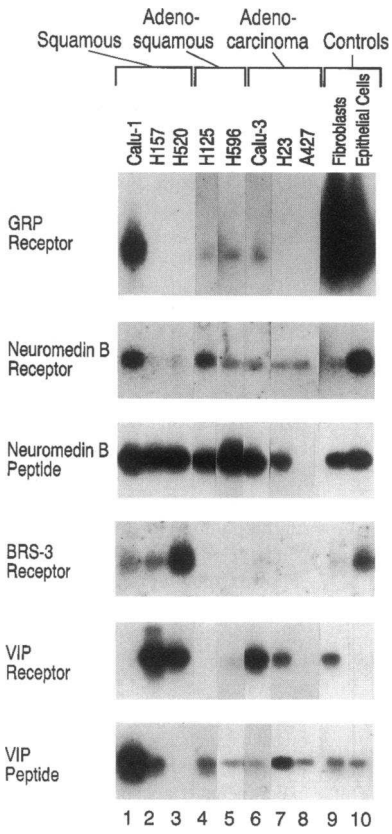

Figure 7. Expression of bioactive peptides (BLP and VIP) and associated receptors in CD10/NEP positive NSCLC cell lines by reverse PCR. The indicated CD10/NEP$^+$ NSCLC cell lines were evaluated for gastrin-releasing peptide (GRP), neuromedin B (NMB), BRS-3 and VIP receptors, and NMB and VIP peptide transcripts by reverse PCR. Sense, antisense, and internal oligonucleotide primers used in these experiments are listed in Table I.

airway epithelium (14) with relatively low proliferative activity (46; unpublished data) and demonstrated increased CD10/NEP expression in confluent epithelial monolayers and dense suspension cultures (47, 48). Taken together, these data support the notion that CD10/NEP is itself regulated by cellular proliferation and prompt speculation that in primary NSCLC of the lung, tumor cells with low levels of CD10/NEP may have a proliferative advantage. That airway epithelial cells treated with an inhibitor of CD10/NEP grow more rapidly underscores the functional relevance of growth-related variability in CD10/NEP expression.

Although the mechanism of CD10/NEP regulation has not been fully elucidated, preliminary studies indicate that CD10/NEP is regulated at both transcriptional and posttranscriptional levels (Ishimaru, F., N. Potter, and M. Shipp, manuscript submitted for publication). It is also of interest that the CD10/NEP cytoplasmic domain contains putative sites for phosphorylation by casein kinase II (CKII) (49) and casein kinase II-mediated phosphorylation of specific cellular proteins has been linked with proliferation (50–52).

CD10/NEP peptide substrates for NSCLC and bronchial epithelium have not been extensively studied; however, receptors for BLP family members are detectable on many of these cells. Although only a subset of the evaluated NSCLC had detectable GRP receptors, additional cell lines expressed both the related neuromedin B peptide and receptor and the recently characterized BRS-3 receptor. Since each of the known BLP-related peptides is an excellent CD10/NEP substrate, it is possible that the enzyme may also regulate the effects of BLP in NSCLC and normal bronchial epithelium. Many CD10/NEP positive NSCLC cell lines also transcribe the vasoactive intestinal peptide (VIP) and its receptor which is of interest because VIP is another CD10/NEP peptide substrate (53) that has been implicated in the autocrine stimulation of NSCLC of the lung (44).

Given the role of CD10/NEP in regulating peptide-mediated growth of bronchial epithelial cells, dynamic changes in the cell surface enzyme may affect cellular responses to peptide stimulation in much the same way as modulation of cell surface peptide hormone receptors. Since CD10/NEP has been implicated in certain bronchial epithelial malignancies, it is possible that these observations may have direct clinical relevance.

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