CD10/Neutral Endopeptidase 24.11 in Developing Human Fetal Lung

Patterns of Expression and Modulation of Peptide-mediated Proliferation

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Abstract

The cell membrane–associated enzyme CD10/neutral endopeptidase 24.11 (CD10/NEP) functions in multiple organ systems to downregulate responses to peptide hormones. Recently, CD10/NEP was found to hydrolyze bombesin-like peptides (BLP), which are mitogens for normal bronchial epithelial cells and small cell lung carcinomas. Growth of BLP-responsive small cell lung carcinomas was potentiated by CD10/NEP inhibition, implicating CD10/NEP in regulation of BLP-mediated tumor growth. BLP are also likely to participate in normal lung development because high BLP levels are found in fetal lung, and bombesin induces proliferation and maturation of human fetal lung in organ cultures and murine fetal lung in utero. To explore potential roles for CD10/NEP in regulating peptide-mediated human fetal lung development, we have characterized temporal and cellular patterns of CD10/NEP expression and effects of CD10/NEP inhibition in organ cultures. Peak CD10/NEP transcript levels are identified at 11–13 wk gestation by Northern blots and localized to epithelial cells and mesenchyme of developing airways by in situ hybridization. CD10/NEP immunostaining is most intense in undifferentiated airway epithelium. In human fetal lung organ cultures, inhibition of CD10/NEP with either phosphoramidon or SCH32615 increases thymidine incorporation by 166–182% ($P < 0.025$). The specific BLP receptor antagonist, [Leu^{13}-psi(CH_2NH)Leu^{14}]bombesin abolishes these effects on fetal lung growth, suggesting that CD10/NEP modulates BLP-mediated proliferation. CD10/NEP expression in the growing front of airway epithelium and the effects of CD10/NEP inhibitors in lung explants implicate the enzyme in the regulation of peptide-mediated fetal lung growth. (*J. Clin. Invest.* 1992. 90:2517–2525.) Key words: bombesin • common acute lymphoblastic leukemia antigen • metalloendopeptidase • respiratory epithelium • growth regulation

Introduction

Molecular cloning and expression studies have shown that CD10 (common acute lymphoblastic leukemia antigen) is identical to the zinc metalloprotease, neutral endopeptidase 24.11 (NEP, "enkephalinase") (1–4). This cell membrane–associated enzyme (CD10/NEP)[1] hydrolyzes a number of naturally occurring peptides, including the endogenous opioid pentapeptides Met- and Leu-enkephalin, substance P, neurotensin, oxytocin, bradykinin, angiotensins 1 and 2, atrial natriuretic factor, endothelin and the chemotactic peptide, fMLP (2, 4–6). CD10/NEP, which is expressed on early lymphoid progenitors, neutrophils, and a variety of nonhematopoietic cell types (5–8), functions in multiple organ systems to downregulate induced responses to peptide hormones (9–16). For instance, CD10/NEP is expressed at high levels in the lung (17–19), where it modulates responses to tachykinins such as substance P that mediate neurogenic inflammation (9–12, 16). Inhibition of CD10/NEP dramatically increases both the binding of substance P to bronchial membranes and the resulting proinflammatory physiological effects (9–11, 16).

An additional family of peptides whose effects are modulated by CD10/NEP are the bombesin-like peptides (BLP) (20). The amphibian peptide bombesin and its homologue gastrin-releasing peptide (GRP) are potent mitogens for normal bronchial epithelial cells (21), fibroblasts (22–24), and certain tumor types including small cell lung carcinomas (SCLCs) (25, 26). In many SCLCs, an autocrine loop exists whereby tumor cells secrete BLP, express BLP receptors, and respond to BLP with increased cellular proliferation (25). CD10/NEP inactivates BLP by cleaving these peptides at two sites within the 7 amino acid (aa) conserved carboxy terminus that is required for biologic activity (27). The growth of BLP-responsive SCLCs was inhibited by CD10/NEP and potentiated by CD10/NEP inhibition, implicating the enzyme in the regulation of BLP-mediated autocrine tumor cell growth (27).

BLP have also been implicated in normal fetal lung development (28, 29). GRP mRNAs were first detected in human fetal lung at 9–10 wk gestation, peaked at 16–30 wk at levels 25-fold higher than in adult lungs, and subsequently declined to near adult levels by 34 wk gestation (28). In situ hybridization analyses of GRP mRNAs in human fetal lung revealed a proximal-to-distal spatial and temporal pattern of gene expression, apparently in parallel with the growth of the airways (28). The high levels of GRP mRNAs found during the pseudoglandular and canalicular phases of pulmonary development suggested that BLP might play an important role in this process. This hypothesis was subsequently confirmed in human and murine fetal lung organ culture studies (29). Bombesin was added to human and murine fetal lung organ cultures and administered to

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1. *Abbreviations used in this paper:* BLP, bombesin-like peptide; BN, bombesin; CD10/NEP, CD10/neutral endopeptidase 24.11; GRP, gastrin-releasing peptide; PF/C, paraformaldehyde-fixed/cryostat; PF/PE, paraformaldehyde-fixed/paraffin embedded; SCLCs, small cell lung carcinomas.

pregnant mice during the respective time periods in gestation when endogenous fetal lung GRP levels were most elevated (29). Bombesin administration resulted in a dose-dependent increase in fetal lung growth and maturation as assessed by biochemical and morphological approaches (29). In addition, an antibombesin (BN) (mAb) blocked murine fetal lung maturation in vivo (30) and in serum-free lung organ cultures (29).

Recently CD10/NEP immunoreactivity has been identified in epithelial cells of both fetal and adult lung (17, 27). Since CD10/NEP modulates BLP effects and BLPs stimulate the growth and maturation of fetal lung, we have analyzed the temporal and cellular expression of CD10/NEP in human fetal lung and compared it to that of BLP. We have also assessed the potential role of CD10/NEP in fetal lung development by evaluating the proliferation of human fetal lung in organ cultures treated with specific CD10/NEP inhibitors.

Methods

Fetal tissue collection. Human fetal lung tissues were obtained within 1 h after elective therapeutic abortions up to ~ 22 wk gestation. The numbers and gestational ages of the lung samples analyzed were: 1, 8 wk; 2, 10 wk, 1, 11 wk; 3, 12 wk; 1, 13 wk; 4, 14 wk; 3, 15 wk; 2, 16 wk; 2, 17 wk; 2, 18 wk; 2, 20 wk; 2, 21 wk; and 2, 22 wk. Of these, three samples at 14 wk gestation and one sample at 15 wk gestation were used for organ cultures. The 8-wk sample and ~ 50% of the remaining specimens were used for histochemical analyses, and the remainder of the samples were used for RNA blots. Appropriate informed consent was obtained from each patient and no records were kept of patients' identification. These studies have been approved in strict accordance with institutional guidelines by the Human Ethics Committee at Brigham & Women's Hospital with yearly renewals consistent with National Institutes of Health guidelines.

RNA preparations and Northern blot analyses. Total RNA was prepared by the method of Chirgwin et al. (31), electrophoresed through 1.5% (wt/vol) formaldehyde-agarose gels, and transferred to nitrocellulose by standard methods (32). Gels were stained with ethidium bromide to visualize the 28S and 18S ribosomal RNA bands to ensure intact RNA recovery. GRP mRNAs were detected by hybridization to a GRP complementary RNA (cRNA) probe which was prepared using 1–2 μg of linearized cDNA template. The 650-bp GRP PstI fragment was cloned into pGEM3, linearized with BamHI, and transcribed with SP6 RNA polymerase (33). The GRP cRNA probe was labeled with [^{32}P]UTP to a specific activity of 10^8 cpm/μg. CD10/NEP mRNAs were detected by hybridization to a 1.6-kb EcoRI fragment from the human CD10/NEP cDNA open reading frame (34) which was labeled with [^{32}P]dCTP using the random priming method (35).

GRP hybridization was carried out in 5× standard saline citrate (SSC) (1× SSC = 0.15 M NaCl, 0.015 M Na Citrate); 5× Denhardt's solution (1× Denhardt's solution = 0.02% [wt/vol] Ficoll-400, 0.02% [wt/vol] BSA, 0.02% [wt/vol] polyvinylpyrrolidone-40); 50% (vol/vol) formamide; 50 mM sodium phosphate, pH 6.5; 0.2% (wt/vol) SDS and denatured salmon sperm DNA (200 μg/ml) at 65°C for 18 h. CD10/NEP hybridization was similarly performed at 42°C. Filters were washed at 65°C for 2 × 15 min in 2× SSC/0.2% SDS followed by 2 × 30 min washes in 0.2× SSC/0.2% SDS, and exposed for 24–48 h at −70°C with Kodak XAR film with an intensifying screen.

Densitometry was carried out by three-dimensional integration of the area of whole bands on autoradiograms using a densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA).

In situ hybridization. In situ hybridization of duplicate fetal lung serial sections was performed as previously described (36). In brief, 5-μm sections were placed on coated slides and baked at 65°C overnight, rehydrated, and treated with 4% paraformaldehyde in PBS for 10 min. After washing, slides were sequentially incubated with Triton X-100 (0.3% in PBS) for 15 min, proteinase K (1 μg/ml in 100 mM Tris, 50 mM EDTA, pH 8.0) at 42°C for 30 min, and 4% paraformaldehyde for 5 min. After washing, tissues were acetylated (acetic anhydride 0.25% in 0.1 M triethanolamine) for 10 min and prehybridized in 50% formamide/2× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate) at 50°C for 20 min. Subsequently, sections were hybridized at 50°C for 16 h in a sealed moist chamber with 20 μl of 5 × 10^4 cpm/μl of ^{35}S-labeled antisense cRNA probe in hybridization buffer (50% formamide, 10% dextran sulfate, 2× SSC, 0.5% BSA, 0.5% Ficoll-400, 0.5% polyvinylpyrrolidone-360, 2.5 μg/ml yeast tRNA, and 0.5% SDS). Additional serial sections were incubated in parallel with an equivalent amount of the corresponding sense cRNA probe. ^{35}S-labeled GRP antisense and sense cRNA probes were prepared as previously described (33). The CD10/NEP cRNA used for in situ hybridization was a 701-bp ClaI/XhoI fragment derived from the open reading frame (1). The CD10/NEP fragment was subcloned into the Bluescript vector (Stratagene Inc., La Jolla, CA), linearized with ClaI, and transcribed with T3 RNA polymerase (antisense orientation) or linearized with XhoI and transcribed with T7 RNA polymerase (sense orientation).

After hybridization, slides were washed in 4× SSC at 42°C for 20 min × 3 and digested in RNAse A (20 μg/ml in 10 mM Tris, 1 mM EDTA, 50 mM NaCl) at 42°C. Subsequently, slides were dehydrated in ethanols containing 0.3 M ammonium acetate, dipped in Kodak NTB-2 photographic emulsion, air dried, and exposed in light-tight boxes with desiccant for 8 d. Slides were then developed with Kodak D-19 and Fix and tissues counterstained with hematoxylin and eosin.

Tissue sections were viewed and photographed using both conventional bright-field and dark-field microscopy. For comparison of proximal to distal airways, we defined proximal airways as bronchi and bronchioles entirely lined by columnar epithelium and associated with closely apposed mesenchymal tissue including desmin-positive cells, and distal airways as bronchioles and primitive airways lined by mostly cuboidal epithelium and associated with only loose mesenchymal tissue that did not contain desmin-positive cells.

Immunoperoxidase analyses. Immunoperoxidase analyses were performed with serial sections of the same fetal lungs that were used for in situ hybridization. The human CD10 monoclonal antibody (mAb) J5 (a gift from Jerome Ritz, Dana-Farber Cancer Institute, Boston, MA) was used as previously described (27). The affinity-purified IgG fraction of polyclonal rabbit anti-NEP (a gift from Catherine Lucas at Genentech, Inc., San Francisco, CA) was used at a working dilution of 1:2,000. mAb to cytokeratins (CAM 5.2) was obtained from Becton Dickinson & Co. (San Jose, CA) and mAb to desmin (D33) was obtained from Dakopatts A/S (Glostrup, Denmark). Both CAM 5.2 and D33 were used at working dilutions of 1:100.

Controls consisted of serial fetal lung sections incubated in parallel with: (*a*) antiserum or mAb preabsorbed with specific antigen (50 μg/ml soluble recombinant CD10/NEP 3.4.24.11 [a gift from Bob Bridenbaugh, Genentech, Inc., San Francisco, CA]); (*b*) a 1:500 dilution of preimmune rabbit serum; or (*c*) a 1:500 dilution of an unreactive myeloma protein of the same IgG subclass as the CD10/NEP monoclonal antibody (MOPC 21; Sigma Chemical Co., St. Louis, MO).

Immunoperoxidase analyses were performed as previously described, using the avidin-biotin complex method (36) without the permeabilization of tissues with 0.3% Triton X-100. In brief, tissues were fixed for 16 h in 4% paraformaldehyde, routinely processed into paraffin, and cut at 5 μm onto coated slides. Tissues on slides were blocked for 20 min with 1:10 dilution of normal goat serum in PBS and subsequently incubated at 4°C overnight with the appropriate dilutions of primary antibody or antiserum. A biotinylated secondary antibody (1:200) was then applied to the slides for 30 min. After endogenous peroxidase was blocked for 30 min in 0.3% H_2O_2/methanol, slides were incubated for 45 min with the standard avidin-biotin complex reagent, developed for 5 min in diaminobenzidine (0.25 gm/100 ml in PBS), and counterstained with 2% aqueous methyl green.

Human fetal lung organ cultures. Fragments of human fetal lung were aseptically excised on ice and cultured in MEM plus 5% fetal calf serum (Gibco-BRL Life Technologies, Inc., Gaithersburg, MD) alone or media with bombesin (Peninsula Laboratories, Inc., Belmont, CA;

100 nM), the specific bombesin receptor antagonist [Leu[13]-psi(CH_2NH)Leu[14]]bombesin (Peninsula Laboratories, Inc., Belmont, CA; L13BN, 100 nM) (37), the specific CD10/NEP antagonists SCH32615 (Schering-Plough Corp., Bloomfield, NJ; 5 μM) (38) or phosphoramidon (cat R6128; Sigma Chemical Co.; 5 μM) (27, 39), or bombesin + L13BN, or SCH32615 + L13BN. Cultures were incubated in 5% CO_2/air at 37°C on a rocking platform at three oscillations/min (29, 40). After 44 h of culture, [^{3}H]thymidine (DuPont Co., New England Nuclear Research Products, Boston, MA; 4 μCi/ml) was added to cultured fetal lungs for the last 4 h before tissues were harvested. [^{3}H]thymidine incorporation into acid-precipitable counts was determined (22) and normalized for DNA content (cpm/μg DNA). DNA content was determined after trichloroacetic acid precipitation by the method of Burton (41). Duplicate or quadruplicate samples were obtained for each culture condition in each of three independent experiments. The thymidine incorporation in untreated control samples from each experiment (mean±SE) was defined as baseline and the percentage change in thymidine incorporation in each of the treated samples determined (mean±SE). The results from the three independent experiments were pooled for final analysis.

Results

Northern analyses. To analyze CD10/NEP transcripts during fetal lung development and compare the temporal expression of CD10/NEP to that of the mammalian BLP, GRP, total RNAs from fetal lung samples (10–22 wk gestation) were prepared, blotted, and probed with both a GRP cRNA and CD10/NEP cDNA. GRP transcripts (~ 900–950 bp) were first detectable at 10–12 wk and subsequently increased and peaked between 15–20 wk gestation (Fig. 1 *A* and [28]). In contrast, the major 5.7- and 3.7-kb CD10/NEP transcripts (1, 2, 13) were barely detectable at 10 wk gestation, peaked at 11–12 wk, and declined thereafter such that they were below the limits of detection on Northern blots of total RNA at 16 wk (Fig. 1 *B*).

GRP and CD10/NEP transcripts were subsequently quantitated by densitometry and normalized for the relative amounts of total RNA by scanning the negative of the photograph from the corresponding ethidium bromide stained gel (Fig. 1 *C*). The normalized densitometric analysis indicates that CD10/NEP transcripts peaked in fetal lung during the first trimester and subsequently declined at about the same time (14–15 wk, early second trimester) that GRP transcript levels rose (Fig. 1 *D*).

In situ hybridization. In situ hybridization was subsequently performed to specifically determine which cells in human fetal lung transcribed CD10/NEP (Fig. 2). Fetal lung sections were examined by light microscopy to characterize cellular morphology (Fig. 2, *a, c, e,* and *g*) and by dark-field microscopy to visualize autoradiographic signals better (Fig. 2, *b, d, f,* and *h*). CD10/NEP transcripts were detectable as early as 8 wk gestation (Fig. 2, *a* and *b*). The strongest hybridization signals were observed in the most primitive distal airways where the majority of grains overlay epithelial cells (Fig. 2, *a* and *b*). However, there was also increased grain density overlying airway-associated mesenchyme and weak hybridization associated with scattered mesenchymal cells in the loose connective tissue in between airways (Fig. 2, *a* and *b*). To confirm the specificity of the CD10/NEP hybridization signals, additional 8-wk fetal lung sections were hybridized with the corresponding CD10/NEP sense probe which is identical, rather than complementary, to CD10/NEP transcripts. As shown in Fig. 2, *c* and *d,* no appreciable signals were detected with this CD10/NEP sense probe.

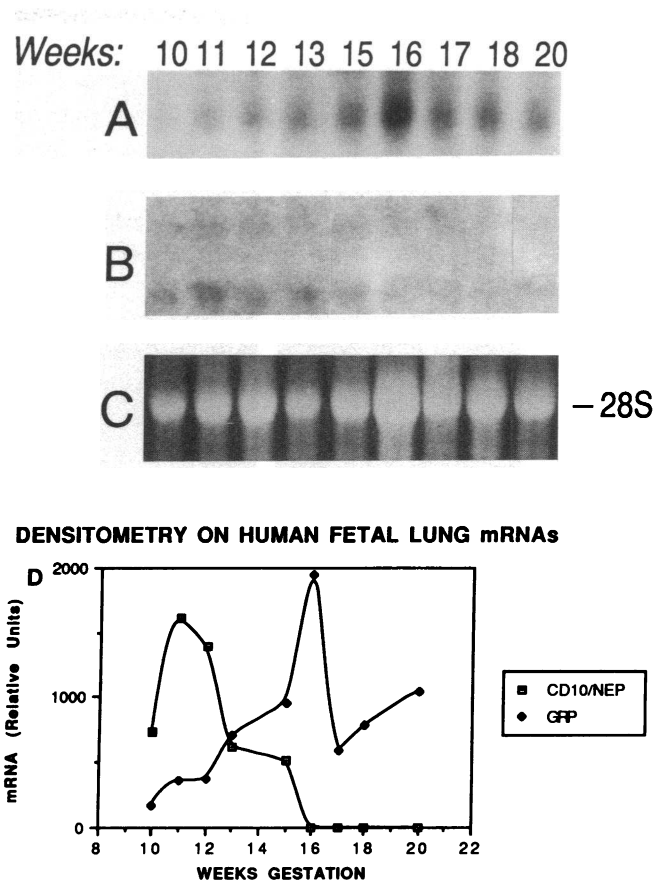

Figure 1. Northern blot analyses of GRP and CD10/NEP transcripts in human fetal lung. Northern blots of total fetal lung RNA (10 μg/lane, 10–20 wk gestation) were hybridized with the human GRP cRNA probe (*A*) or the human CD10/NEP cDNA probe (*B*). Arrows (*right*) indicate the expected sizes of the 900–950-bp GRP and 3.7- and 5.7-kb CD10/NEP transcripts. The 28S ribosomal RNA band from the ethidium bromide–stained RNA gel used in *A* is shown in (*C*). The negative from this ethidium bromide–stained gel was used to normalize relative densitometric units of GRP and CD10/NEP which are shown in (*D*).

By 12 wk gestation, CD10/NEP transcripts were easily detectable in the epithelial cells and airway-associated mesenchyme of distal less differentiated airways (Fig. 2, *e* and *f*). There was a trend towards stronger CD10/NEP hybridization in distal as compared to proximal airways (data not shown) whereas GRP transcripts were only detectable in more differentiated proximal airways that had begun to branch (data not shown and [28]).

At 15 wk (Fig. 2, *g* and *h*) and more weakly at 17 wk gestation (data not shown), CD10/NEP transcripts were predominantly associated with the distal most undifferentiated airways. Additional sections of the same 15- and 17-wk fetal lungs probed with the control CD10/NEP sense cRNA were completely devoid of hybridization signals (data not shown). CD10/NEP transcripts in 18–21-wk gestation human fetal lung were detected only in bronchial-associated glands by in situ hybridization (data not shown).

Immunohistochemical analyses. To correlate CD10/NEP transcripts with cell surface protein expression, immunohistochemical analyses were performed on additional fetal lung samples from the same specimens that were used for in situ hybrid-

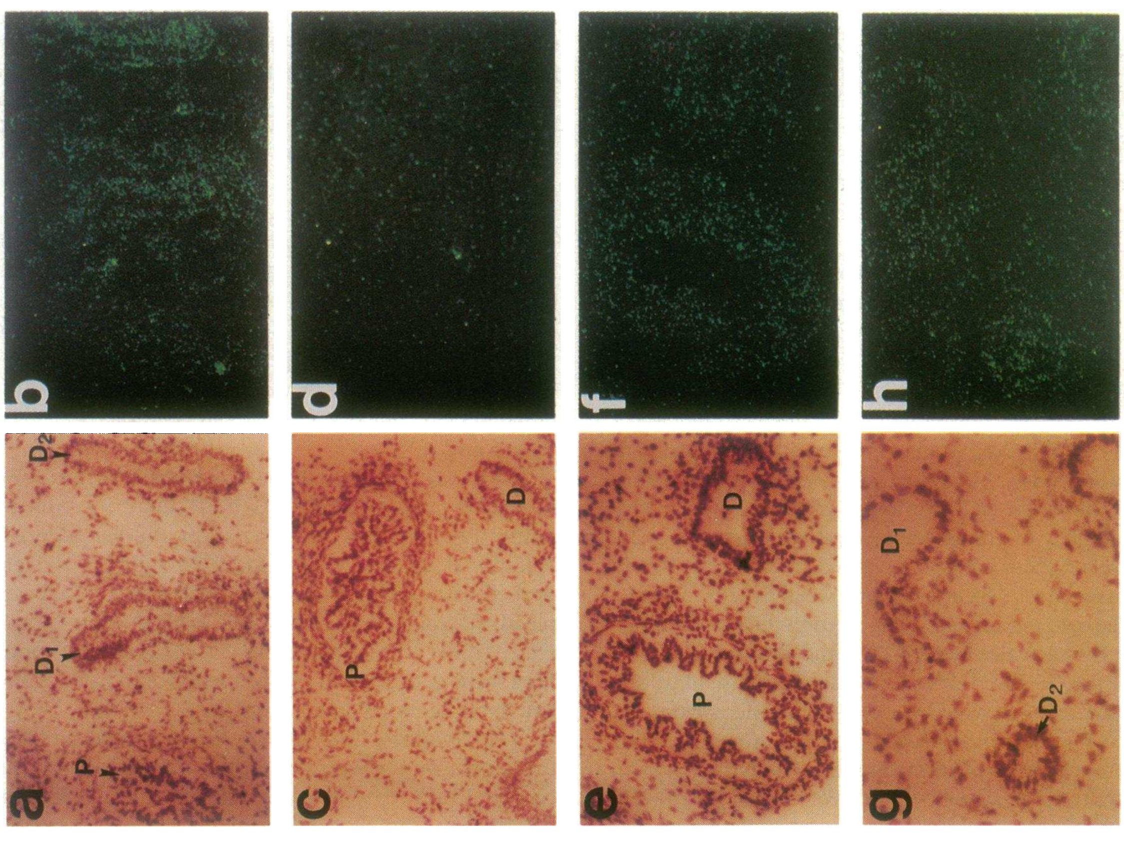

Figure 2.

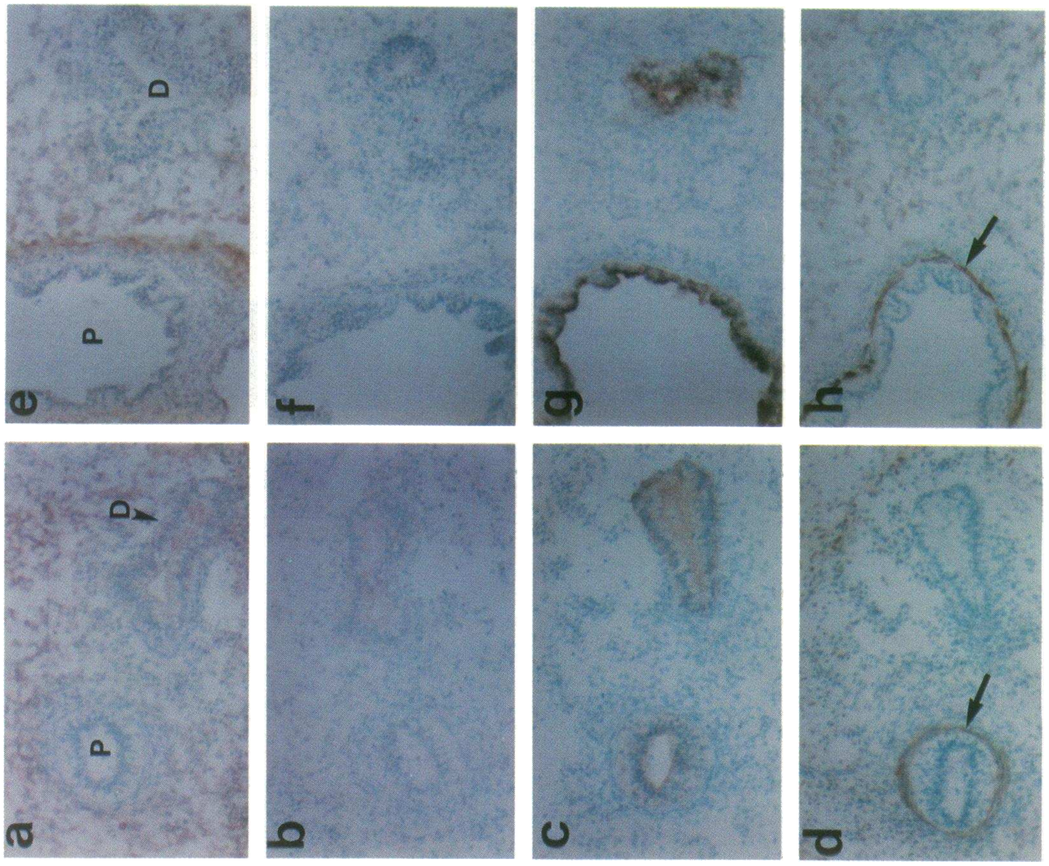

Figure 3.

ization studies. Initially, immunoperoxidase studies were done on paraformaldehyde-fixed/cryostat (PF/C) sections with reagents including anti-CD10/NEP, cytokeratin, and desmin monoclonal antibodies. Consistent with the previous in situ studies, there was CD10/NEP immunostaining of both the epithelium and airway-associated mesenchymal tissue at 8 wk gestation (Fig. 3 *a*). The specificity of CD10/NEP immunostaining was confirmed by demonstrating that serial sections incubated with CD10/NEP mAb that had been preabsorbed with soluble CD10/NEP had markedly reduced signals (Fig. 3 *b*). To compare the localization of CD10/NEP to that of other known markers, additional serial sections were stained with anticytokeratin and antidesmin mAbs. Cytokeratin immunostaining of the same 8-wk fetal lung identifies airway epithelium in proximal (*P*) and distal (*D*) airways (Fig. 3 *c*), whereas desmin immunostaining identifies smooth muscle differentiation which is associated with only the more differentiated proximal airways (Fig. 3 *d*). Comparisons between Fig. 3, *a, c,* and *d,* demonstrate that proximal more differentiated airways have weaker CD10/NEP immunostaining than distal less differentiated airways.

In 12-wk PF/C sections, there was only weak CD10/NEP antigen-specific immunostaining of proximal airway epithelium, with weak to moderate staining of distal airways (Fig. 3 *e*). Both the epithelial cells which were identified by cytokeratin immunostaining and the mesenchyme surrounding desmin-positive differentiated smooth muscle cells expressed CD10/NEP (Fig. 3 *g*). The specificity of CD10/NEP immunostaining at 12 wk was confirmed by incubating additional fetal lung sections with the CD10/NEP mAb following preabsorption with soluble CD10/NEP (Fig. 3 *f*).

Since previous studies suggested that there might be differences between the CD10/NEP immunostaining of PF/C sections and paraformaldehyde-fixed/paraffin-embedded (PF/PE) sections (27), we also evaluated PF/PE fetal lung samples for CD10/NEP expression (Fig. 4). Both the anti-CD10 mAb (Fig. 4, *A–J*) and an anti-CD10 antiserum (data not shown) gave similar patterns of CD10/NEP immunostaining. In the PF/PE sections the epithelial CD10/NEP staining was particularly prominent whereas the mesenchymal staining was less pronounced (Fig. 4). These results suggest that the method of tissue processing may affect CD10/NEP immunoreactivity in specific tissues (42–44). Consistent with our results from the Northern analysis (Fig. 1), in situ hybridization (Fig. 2) and immunostaining of PF/C sections (Fig. 3), CD10/NEP expression in PF/PE sections was most intense at earlier time points (12 wk, Fig. 4, *A* and *B*). In later samples (15–18 wk), CD10/NEP expression was primarily restricted to keratin-positive epithelial cells in the less differentiated distal airways not yet surrounded by desmin-positive mesenchymal cells (Fig. 4, *C–F,* and data not shown). Additional PF/PE-fixed samples incubated with CD10/NEP mAb or antiserum that had been preabsorbed with soluble CD10/NEP had markedly reduced signals, confirming the specificity of the immunostaining (Fig. 4, *B, D,* and *F,* and data not shown).

In previous studies using adult animals, inhibition of CD10/NEP potentiated the bronchoconstriction associated with substance P release (9–11, 16). Since substance P is released from nerve fibers localized to the desmin-positive mesenchymal cells surrounding proximal airways, we examined more mature fetal lung specimens for additional CD10/NEP mesenchymal immunostaining. PF/C fetal lung sections were used because mesenchymal CD10/NEP immunostaining was more prominent with this method of fixation (Fig. 3 versus Fig. 4). The CD10/NEP and desmin immunostaining of serial sections from a 22-wk fetal lung is shown in Fig. 5. Whereas the CD10/NEP immunostaining of airway-associated mesenchyme in younger fetal lungs was external to and distinct from desmin-positive smooth muscle cells (Figs. 3 and 4), the CD10/NEP immunostaining in the older fetal lungs included desmin-positive smooth muscle cells surrounding the proximal airways (Fig. 5), as well as bronchial-associated glands (data not shown).

Human fetal lung organ cultures. To explore the potential function of CD10/NEP in developing lung, fetal lung organ cultures were set up as previously described (29) using three different 14–15-wk gestation human fetal lungs. The 14–15-wk timepoint was chosen because both CD10/NEP and GRP are clearly detectable in human fetal lung during this interval (Fig. 1). Fetal lung organ cultures were incubated with media alone (Neg), bombesin (BN), the specific bombesin receptor antagonist L13BN, BN plus L13BN, the specific CD10/NEP inhibitor SCH32615 (38), SCH32615 plus L13BN, or an unrelated CD10/NEP inhibitor, phosphoramidon (27). Samples were subsequently evaluated for [^{3}H]thymidine incorporation which was normalized for DNA content (Methods, [29]). [^{3}H]thymidine incorporation in samples incubated with media alone was compared to [^{3}H]thymidine incorporation in samples incubated with bombesin, L13BN, SCH32615, phosphoramidon, or the respective combinations and the percent-

Figure 2. In situ hybridization of human fetal lung for CD10/NEP and GRP transcripts. Each pair of photographs (*a,b; c,d; e,f; g,h*) represents the same field viewed by light-field microscopy to demonstrate histology (*left: a, c, e, g*) and dark-field microscopy to demonstrate autoradiographic grains (*right: b, d, f, h*). (*a* and *b*) 8-wk gestation human fetal lung probed with CD10/NEP antisense cRNA. Note hybridization is weak in a proximal (*P*) airway, more intense in an early distal (D_1) airway, and most intense in a further distal (D_2) airway. (*c* and *d*) 8-wk lung probed with CD10/NEP sense cRNA. Note the minimal background and the absence of a specific hybridization signal. (*e* and *f*) 12-wk gestation human fetal lung probed with CD10/NEP antisense cRNA with hybridization signals in both proximal and distal airways. (*g* and *h*) 15-wk gestation human fetal lung probed with CD10/NEP antisense cRNA with weak hybridization in one distal (D_1) airway and stronger hybridization associated with another more distal (D_2) airway.

Figure 3. Immunohistochemical analyses of paraformaldehyde-fixed/cryostat sections of human fetal lung. Human fetal lungs of 8-wk gestation (*a–d*) and 12-wk gestation (*e–h*) were immunostained with the following mAbs: (*a* and *e*) Anti-CD10/NEP mAb J5. CD10/NEP immunostaining is prominent in airway-associated mesenchyme and scattered mesenchymal cells and is also present in the distal (*D*) airway epithelium but not in the proximal (*P*) airway epithelium; (*b* and *f*) J5 preabsorbed with soluble CD10/NEP. Note markedly reduced CD10/NEP immunostaining; (*c* and *g*) Cytokeratin mAb. Cytokeratin is present in both proximal (*P*) and distal (*D*) airway epithelium; (*d* and *h*) Desmin mAb. Desmin is present in the differentiated smooth muscle around more proximal airways.

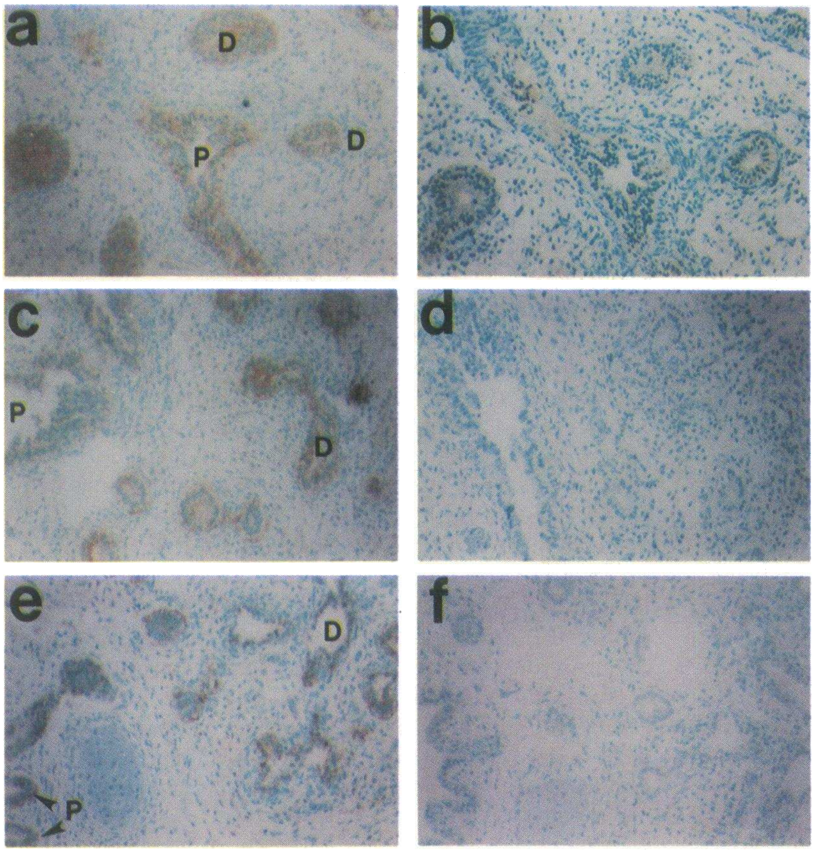

Figure 4.

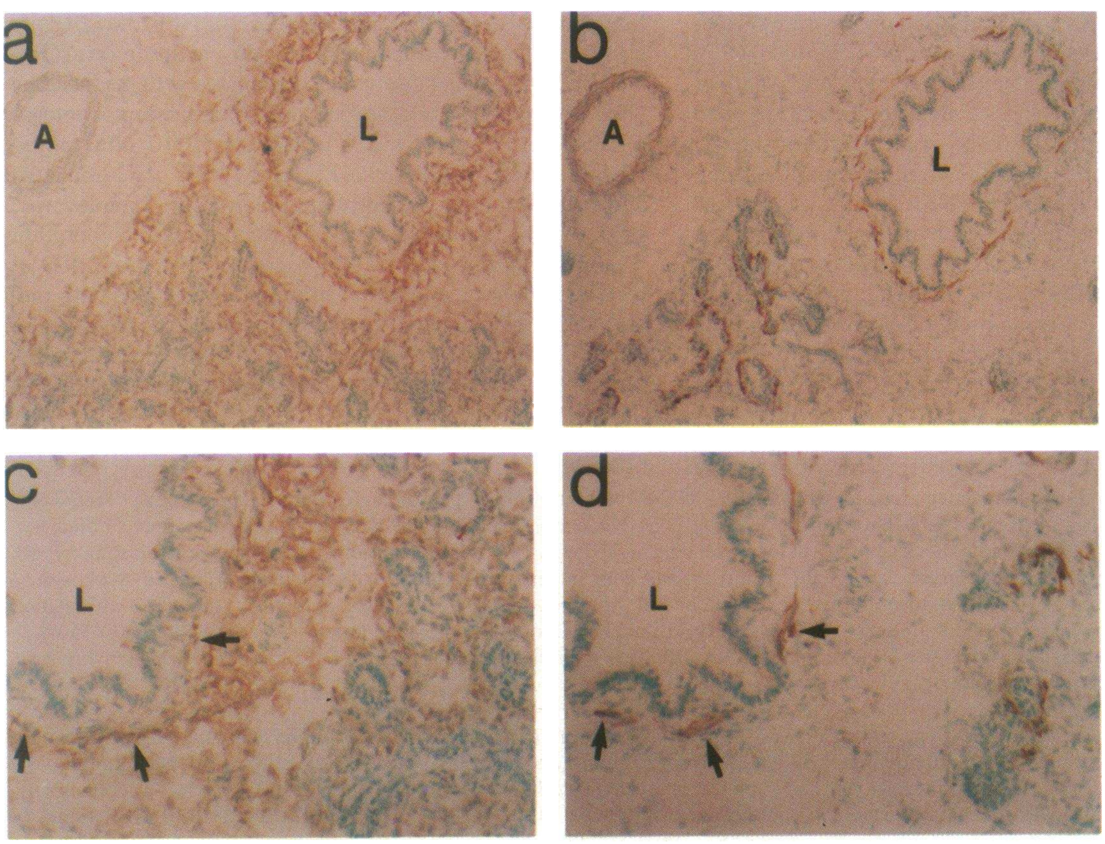

Figure 5.

age change in thymidine incorporation resulting from the additions determined. The results from the three independent experiments are pooled and summarized in Fig. 6. Bombesin significantly increased thymidine incorporation in the fetal lung organ cultures (116% increase, $P < 0.001$) and the addition of L13BN to bombesin-treated cultures reduced [^{3}H]-thymidine incorporation to baseline levels. The CD10/NEP inhibitor SCH32615 also significantly increased thymidine incorporation in fetal lung organ cultures (166% increase, $P < 0.001$). The addition of L13BN to SCH32615-treated cultures similarly reduced thymidine incorporation to baseline levels suggesting that the SCH32615 effect was mediated by BLPs. To obtain additional evidence that the increased thymidine incorporation in SCH32615-treated fetal lung samples was a specific consequence of CD10/NEP inhibition, additional samples were treated with an unrelated CD10/NEP inhibitor, phosphoramidon. As shown in Fig. 6, phosphoramidon-treated fetal lung organ cultures also incorporated significantly more [^{3}H]thymidine (182% increase, $P < 0.025$).

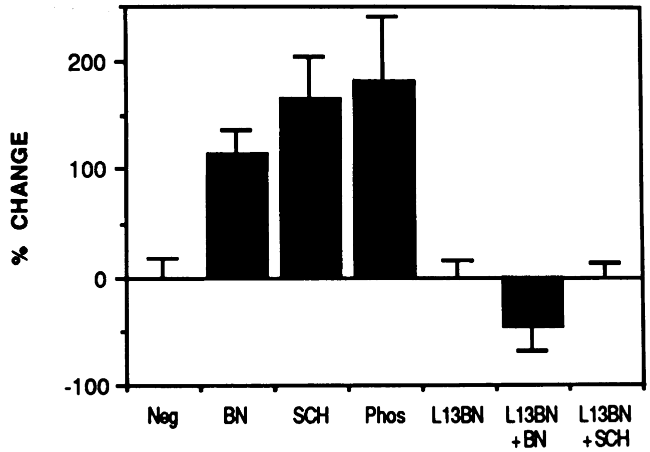

Figure 6. CD10/NEP inhibition stimulates growth in human fetal lung organ culture via endogenous BLPs. Lung organ cultures were set up in duplicate to quadruplicate using 14–15-wk gestation human fetal lung in MEM + 5% fetal calf serum alone (*Neg*) or media with bombesin (*BN*, 100 nM), SCH32615 (*SCH*, 5 μM), phosphoramidon (*Phos*, 5 μM), the specific BN receptor antagonist, [Leu^{13}-psi(CH_2NH)Leu^{14}]BN (L13BN, 100 nM), or BN 100 nM plus L13BN 100 nM, or SCH 5 μM plus L13BN 100 nM. Thymidine incorporation (cpm/μg DNA) in treated samples is represented as percentage change compared to untreated control samples (expressed as mean±SEM). The values shown are the pooled results from three independent experiments. The actual percentage changes with respect to untreated samples are shown below. *P* values comparing untreated samples to experimental samples were calculated using the unpaired Student's *t* test: Neg, 0±17; BN, 116±22, $P < 0.001$; SCH, 166±40, $P < 0.001$; Phos, 182±59, $P < 0.025$; L13BN, 0±16, $P = 1.0$; L13BN + BN, −46±21, $P = 0.140$; L13BN + SCH, 1±12, $P = 0.954$.

Discussion

We have evaluated the expression and function of CD10/NEP in human fetal lung because the enzyme regulates BLP-mediated growth of SCLC and BLPs stimulate the proliferation and maturation of human and murine fetal lung organ cultures (29) and normal pulmonary neuroendocrine cells (45). By in situ hybridization analysis, CD10/NEP transcripts were identified in epithelial cells, airway-associated mesenchyme, and scattered mesenchymal cells as early as 8 wk gestation. The pattern of CD10/NEP immunoreactivity was similar to that of the corresponding transcripts, with prominent epithelial staining primarily of distal undifferentiated airways. Consistent with Northern analyses, CD10/NEP immunostaining was most prominent in the first trimester and declined thereafter. Given the tight correlation between CD10/NEP mRNA levels, cell surface expression and enzymatic activity (1, 2), these results are predictive for high levels of cell surface CD10/NEP enzymatic activity in the least differentiated airways in early fetal lung development. Therefore, the observation that human fetal lung proliferation is increased when CD10/NEP is inhibited with either SCH32615 or phosphoramidon is of particular interest. The fact that a bombesin receptor antagonist abolishes the effects of CD10/NEP inhibitors on human fetal lung suggests that CD10/NEP is regulating local levels of endogenous BLPs and that the enzyme may function to limit BLP effects during the earliest stages of fetal lung development.

In our initial studies, we demonstrated that CD10/NEP was expressed by pulmonary neuroendocrine cells and that the enzyme regulated the BLP-mediated autocrine growth of SCLCs. The present study shows that CD10/NEP is also expressed by undifferentiated epithelial cells in developing airways and that the enzyme modulates the growth of normal fetal lung. Therefore, in addition to modulating the autocrine growth of neuroendocrine cells, another possible role of the enzyme is to regulate the peptide-mediated paracrine growth of bronchial epithelial cells. Pulmonary neuroendocrine cells have recently been implicated in the paracrine stimulation of adjacent bronchial epithelial cell growth in developing hamster lung (46).

That CD10/NEP expression peaks in first trimester fetal lung and is most abundant on epithelial cells lining distal un-

Figure 4. Immunohistochemical analyses of paraformaldehyde-fixed/paraffin-embedded sections of human fetal lung. Human fetal lungs of 12 wk gestation (*a* and *b*), 15 wk gestation (*c* and *d*), and 18 wk gestation (*e* and *f*) were immunostained as follows: (*a, c,* and *e*) Anti-CD10/NEP mAb, J5. CD10/NEP immunostaining is stronger in distal (*D*) airway epithelium than in proximal (*P*) airway epithelium and is more intense in early samples. (*b, d,* and *f*) J5 preabsorbed with soluble CD10/NEP. Preabsorption of J5 with soluble CD10/NEP markedly reduces the immunostaining in 12-wk samples (*b*) and completely eliminates the staining in 15–18-wk samples (*d* and *f*). Note that CD10/NEP epithelial staining is more prominent on PF/PE samples whereas mesenchymal staining is more prominent on PF/C samples (compare Figs. 4 and 3).

Figure 5. Immunostaining of paraformaldehyde-fixed/cryostat sections of 22-wk gestation human fetal lung for CD10/NEP and desmin. Magnification in *a* and *c* is 100 and in *b* and *d* is 200. (*a* and *c*) Anti-CD10/NEP mAb, J5. Note prominent immunostaining of mesenchymal tissue associated with the proximal airway (*L* = airway lumen) and weak immunostaining of loose mesenchyme. Arterial smooth muscle (*A* = artery) is negative for CD10/NEP; (*b* and *d*) Desmin mAb. Well-differentiated smooth muscle cells associated with the proximal airway (*L*) are positively stained for desmin. Note that desmin-positive smooth muscle cells in the proximal airways of 22-wk fetal lung are CD10/NEP positive (compare *a* versus *b* and *c* versus *d;* arrows in *c* and *d* indicate the same smooth muscle cells on serial sections).

differentiated airways is of interest because CD10/NEP is also expressed by immature progenitors of another cellular lineage. CD10 was originally identified as a cell surface glycoprotein on normal and malignant lymphoid progenitors that were either uncommitted or committed to only the earliest stages of B or T cell differentiation (reviewed in reference 8). CD10-positive lymphoid progenitors are abundant early in fetal hematopoietic development but decline in number with subsequent fetal maturation and birth (8). Recent studies indicate that CD10/NEP also modulates the growth of lymphoid progenitors raising the possibility that the enzyme may function in a similar capacity in different organs (47). Since CD10/NEP is predominantly expressed by early lymphoid progenitors in fetal bone marrow and liver and by undifferentiated epithelial cells in fetal lung, there may be common regulatory elements controlling expression of the enzyme in different tissues during fetal development. Of note, several 5′ alternatively spliced CD10/NEP untranslated region cDNA sequences have been identified, raising the possibility that unique regulatory elements control CD10/NEP expression at different developmental stages or in different cell types (3).

CD10/NEP may have different roles in early and late stages of pulmonary development, depending upon the specific cell types expressing the enzyme (16–19) and the localization of relevant CD10/NEP peptide substrates. For this reason, the CD10/NEP immunostaining of desmin positive mesenchymal cells surrounding proximal airways in older fetal lungs is also of interest. CD10/NEP has previously been identified on cultured fibroblasts (8) and multiple tumors of mesenchymal origin (48). The enzyme may also modulate peptide-mediated proliferation of pulmonary mesenchymal cells since BLPs are known mitogens for pulmonary fibroblasts (24, 29). Since additional pulmonary peptides such as substance P and endothelin are also CD10/NEP substrates and fibroblast mitogens (49, 50), the enzyme may regulate fibroblast proliferation mediated by multiple pulmonary peptides. CD10/NEP is also known to modulate substance P–mediated bronchoconstriction in adult animal models (9–11, 16). Substance P, which is released by nerve fibers located in mesenchyme surrounding proximal airways, presumably acts on CD10/NEP positive smooth muscle elements.

Our results indicate that CD10/NEP is primarily expressed by undifferentiated bronchial epithelium in developing airways and that the enzyme modulates the growth of human fetal lung. These studies provide additional insight into the role of the enzyme in regulating pulmonary peptide-mediated proliferative responses.

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