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Localization of neutral endopeptidase (NEP) mRNA in human bronchi

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ABSTRACT: Neutral endopeptidase (NEP) may regulate peptide-induced inflammation in the respiratory tract. It is of interest to determine which respiratory resident cells express NEP.

Trachea and bronchi from seven nonsmoking, nonasthmatic subjects were examined. NEP messenger ribonucleic acid (mRNA) was characterized by Northern blot hybridization of cultured human tracheobronchial epithelial and smooth muscle cells, and reverse transcriptase-polymerase chain reaction (RT-PCR) in trachea and bronchi. *In situ* hybridization with biotin- and ^{35}S-labelled antisense complementary ribonucleic acid (cRNA) probes was used to determine the distribution of NEP mRNA in human bronchial mucosa. NEP-immunoreactive material was detected using MEK10 murine monoclonal antibodies and the immunogold method with silver enhancement.

NEP mRNA was 4.5 kb in size in the cultured human smooth muscle and epithelial cells by Northern blot analysis. No evidence was found by RT-PCR for truncated, alternatively spliced NEP mRNAs, such as *del exon 16* or *del exons 5-18* in human bronchus. NEP mRNA was detected by *in situ* hybridization in epithelial cells, submucosal glands, bronchial smooth muscle and endothelium. NEP-immunoreactive material was identified in the epithelium, submucosal glands, bronchial smooth muscle, and endothelium, demonstrating an excellent correlation between the distribution of NEP mRNA and the cell surface protein. NEP mRNA and immunoreactive material were excluded from epithelial goblet cell and submucosal gland mucous cell vacuoles.

We conclude that the various sites of NEP protein and mRNA expression correlate with the locations of peptide receptors and NEP enzyme function, and are consistent with the hypothesis that NEP may regulate peptide-induced inflammation in human bronchi.

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Neutral endopeptidase (NEP) is a 749 amino acid, zinc-containing, membrane-bound enzyme, which may play a key role in regulating peptide-induced inflammatory events [1–3]. NEP, also known as E.C.3.4.24.11, enkephalinase, common acute lymphoblastic leukaemia antigen (CALLA), CD10, and gp100, has been cloned from human, rat and rabbit tissues [4–9]. Functional studies indicate that NEP activity is present on epithelial, glandular, smooth muscle and vascular cells which possess peptide receptors [1, 2]. Loss of NEP activity may significantly contribute to the hypersecretion, vascular permeability, bronchoconstriction and other pathological changes seen in respiratory inflammation by permitting unopposed, prolonged actions of inflammatory peptides, such as tachykinins and bradykinin.

In the present study, *in situ* hybridization was used to determine the distribution of NEP gene expression in human bronchial mucosa, whilst the identity of NEP messenger ribonucleic acid (mRNA) was confirmed by Northern blotting. NEP immunoreactive material was detected by immunohistochemistry.

Methods

Human Tissue

Human trachea and large, cartilaginous bronchi were obtained from four organ specimens collected at heart transplantation and at autopsy of three victims of motor vehicle accidents. None of the subjects were known to be smokers or asthmatic. Tissue was transported in 4°C Krebs solution, and: 1) frozen in liquid nitrogen for

ribonucleic acid (RNA) extraction and cryostat sectioning; and 2) fixed in 4% paraformaldehyde in pH 7.4 phosphate buffered saline (PBS) for 4 h at 4°C before embedding in paraffin.

Cell culture

Human bronchial epithelial cells [10] and smooth muscle cells [11] were cultured as described previously.

NEP complementary ribonucleic acid (cRNA)

An NEP insert [7] coding for bases 642 to 2223 (1,581 bases) was obtained by *Eco RI* digestion of human NEP complementary deoxyribonucleic acid (cDNA) clones [6, 7] and inserted into a M13+ Bluescript plasmid (Stratagene, San Diego, CA, USA). This insert codes for the region stretching from amino acid 210 in exon 8 to amino acid 735 in exon 24 [6, 8].

Antisense NEP cRNA probes for Northern blot analysis and *in situ* hybridization were prepared by linearizing the plasmid with *Ava II* and transcribing with RNA polymerase T3. The antisense cRNA coded for the region from base 2029 to 2223. Sense cRNA probes were prepared by linearizing with *Pvu II* and transcribing with RNA polymerase T7. The sense cRNA coded for the region between bases 642 and 1099. cRNA probes for Northern blots were labelled by incorporating ^{32}P-uridine triphosphate (UTP) (Amersham, Inc., Amersham, UK) during transcription. Probes for *in situ* hybridization were labelled by incorporation of ^{35}S-UTP (Amersham, Inc., Amersham, UK) for radioactive detection, or biotin-5-UTP (Sigma, Poole, UK) for nonradioactive detection. Probes were purified from unincorporated nucleotides using G-50 Sephadex columns eluted with pH 8, 10 mM Tris (hydroxymethyl) aminomethane (TRIS), 1 mM ethylenediamine tetra-acetic acid (EDTA), 0.1% sodium dodecyl sulphate (SDS). The yield of biotin-5-UTP-labelled probe was determined from the OD_{260}, (optical density at 260 nm).

Northern blots

RNA samples for Northern blot analysis were prepared from cultured cells scraped from culture flasks using the acid, guanidinium thiocyanate, phenol-chloroform extraction method [12]. RNA integrity was confirmed by the appearance of the 28S and 18S recombinant ribonucleic acid (rRNA) bands [13] after denaturing 1% agarose gel electrophoresis in TAE (Tris-Acetic acid-EDTA). The RNA was transferred from the gel to Hybond nylon filters (Amersham, UK) by capillary action with 20× standard sodium citrate (SSC), and fixed by ultraviolet light (UV) for 4 min. The filters were prehybridized in 50% formamide, 5×SSC, 0.1% SDS, 5 mM EDTA, 100 μg·mL^{-1} denatured salmon sperm DNA, 0.5 μg·mL^{-1} yeast total RNA, 5× Denhardt's solution for 16 h at 50°C. ^{32}P-antisense NEP cRNA probe (10^6 cpm per 10 cm^2 in 1 mL hybridization buffer) was added, incubated 16 h at 50°C, and washed in 0.5×SSC at 55°C before exposure to radiographic film. The filter was stripped in 50% formamide, pH 6.5, 10 mM Na phosphate for 60 min at 65°C. After prehybridization, the filter was hybridized with sense NEP cRNA and washed using the same conditions.

In situ *hybridization*

Cryostat tissue sections (10 μm) from seven subjects were thaw mounted onto gelatin-coated slides, allowed to dry for 12 h at 37°C, and then post-fixed in freshly prepared 4% paraformaldehyde in pH 7.4, 0.1 M PBS for 30 min. Paraffin sections were dewaxed and hydrated in PBS for 5 min. Cryostat and paraffin sections were subsequently treated in identical fashion [14, 15]. Sections were permeabilized in 0.3% Triton X-100 in PBS for 10 min and then proteinase K (1 μg·mL^{-1}) in 0.1 M TRIS, 50 mM EDTA for 30 min at 37°C. After treatment in 4% paraformaldehyde in PBS for 5 min, nonspecific binding sites were blocked in 0.25% acetic anhydride, 0.1 M triethanolamine for 10 min. Slides were prehybridized in 0.3 M NaCl, 30 mM Na citrate (2×SSC), 50% formamide for 30 min at 50°C. Slides were hybridized for 16 h at 50°C. ^{35}S-cRNA probe in hybridization buffer was added at 10^6 cpm per slide. Biotin-cRNA probes were added at 200 ng·mL^{-1}. After hybridization, slides were washed in 4×SSC. Unincorporated, single-stranded cRNAs were degraded in 20 μg·mL^{-1} ribonuclease (RNase) A, 0.5 M NaCl, 10 mM TRIS, and 1 mM EDTA for 30 min at 42°C. Washing was continued in decreasing concentrations of SSC to 0.2×SSC at 50°C.

^{35}S-labelled slides were dehydrated in 70% ethanol, air dried, and coated with Ilford K-5 photographic emulsion melted at 42°C. Coated slides were exposed for 14–21 days, developed in Kodak reagents, and stained with haematoxylin.

Biotin-labelled slides were immersed in Lugol's iodine for 2 min, decolourized in 2.5% Na thiosulphate, washed in PBS [15, 16], and nonspecific binding sites blocked with 0.8% bovine serum albumin (BSA), 0.1% gelatin, 5% nonimmune goat serum, 2 mM Na azide in PBS for 1 h at room temperature. Anti-biotin goat serum labelled with 1 nm colloidal gold particles (Amersham, Amersham, UK) diluted 1/10 with blocking solution was added, and the slides incubated overnight at 4°C. Slides were washed twice for 5 min in PBS followed by distilled water. Silver enhancing solution (Amersham, Amersham, UK) was added to pairs of sense and antisense slides and stain development observed under darkfield illumination. Slides were washed in water for 5 min, 2.5% Na thiosulphate for 3 min, and dehydrated.

Primers

NEP primers coding for mRNA splice sites were identified from published cDNA sequences [5, 6, 8] and were synthesized by the Lombardi Cancer Center Core

Laboratory of Georgetown University, Washington, DC. NEP primers were chosen to identify mRNAs containing deletions of exon 16 (*del 16*) [17] and deletion of exons 5–18 (*del 5–18*) [18]. A single antisense primer was used that coded for the exon 19-exon 20 splice site (5'GTTTCTGC/SPLICE\CATTGTCATCGAA). When paired with a sense primer coding for the exon 3-exon 4 splice site (5'ATGCAACCTACGATG/SPLICE\ATGGTAT), several possible NEP mRNA RT-PCR products could be generated, including one from full length mRNA (1,641 nucleotides), one for *del 16* (1,563 nucleotides), and one for *del 5–18* (177 nucleotides). When paired with a sense primer coding for the exon 14-exon 15 splice site (5'AGTAAACATGTG/SPLICE\GTCGAGGAT), full length NEP mRNA would generate a RT-PCR product 477 nucleotides long, and 399 nucleotides long for the *del 16* variant. Deletion of exons 5–18 would generate no product. The annealing temperatures (55°C) were calculated according to MEINKOTH and WAHL [19].

β-actin [20, 21] primers were purchased from Clontech (Palo Alto, CA, USA). They generated an RT-PCR product 661 bases long from mRNA, but 867 bases long from genomic DNA because of the presence of a short intron.

Reverse transcriptase-polymerase chain reaction

RNA (5 μg), reverse transcriptase/antisense primers, and Perkin Elmer RT-PCR reagents (Norwalk, CN, USA) were mixed according to manufacturer's recommendations at 4°C in a Perkin Elmer thermocycler. Mineral Microliter oil (70 μL) was added, and then the temperature increased to 42°C to permit annealing of the RT/antisense primer to specific mRNA sequences. After 60 min at 42°C, the solution was denatured at 99°C for 5 min, and then cooled to 60°C. Perkin Elmer *taq*, other PCR reagents, and sense primers were preheated to 60°C and added to each reversely transcribed tube. In this way, "Hot-Start" conditions that reduce nonspecific priming were produced. To permit efficient annealing and extension for this first cycle of PCR, the temperature was then appropriately adjusted to the annealing temperature (55°C) [19], and maintained for 5 min. This was followed by 2 min at 70°C. Then the thermocycler was set to cycle for a total of 45 cycles at 94°C for 1 min, 55°C for 1 min, and 70°C for 1 min. In preliminary experiments, it was found that most samples generated positive bands after 35 cycles, but some samples with minimal RNA required 45 cycles. Since the additional 10 cycles did not cause generation of superfluous bands, 45 cycles was adopted as a standard method.

PCR products were mixed with TRIS-borate-EDTA (TBE) loading buffer and run in 0.5×TBE on 2% agarose (FMC, Rockland, ME, USA) 1.5 h at 120 V. Clon-Tech (Palo Alto, CA, USA) DNA molecular weight standards (1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118 and 72 nucleotides) were run on each gel. Gels were stained with ethidium bromide, bands visualized by UV-fluorescence, and photographed with Polaroid 667 film.

Immunohistochemistry

Paraffin embedded sections from seven human large bronchi were dewaxed, rehydrated in PBS, and incubated in blocking solution [15]. Murine immunoglobulin G (IgG) monoclonal antibody to NEP (MEK10, courtesy of S. Shak, Genentech, South San Francisco, CA, USA) diluted 1:200 with blocking solution was aliquoted onto the slides and incubated for 20 h at 4°C. After washing in PBS, the monoclonal antibody was detected by the immunogold method with silver enhancement [15]. Nonspecific staining was determined by preadsorption of the antibody with 200 μg·mL^{-1} recombinant human NEP (courtesy of S. Shak, Genentech, South San Francisco, CA, USA).

Results

Northern blot analysis

Cultured human tracheal smooth muscle cells and cultured human bronchial epithelial cells contained a single NEP mRNA band at 4.5 kb (fig. 1).

RT-PCR

A single NEP mRNA band was identified indicating the presence of full length mRNA coded by exons 4–20 (fig. 2). No variant NEP mRNA could be amplified, suggesting that truncated mRNA (*del exon 16* or *del exons 5–18*) were not present in human tracheobronchial tissues from nonasthmatic, nonsmoking subjects.

In situ *hybridization*

The biotin-labelled antisense NEP cRNA probe detected NEP mRNA in epithelial cells and submucosal glands (fig. 3). Staining was less intense, but still present, over bronchial smooth muscle and endothelial cells of subepithelial capillaries/postcapillary venules and deeper, larger venous vessels. This may suggest that the NEP mRNA concentration was lower in smooth muscle and endothelial cells than epithelium and glands.

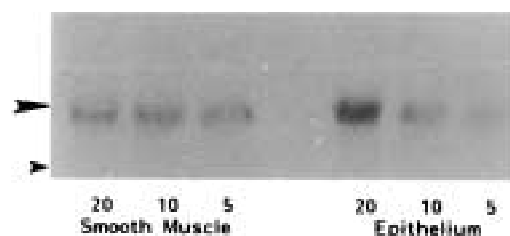

Fig. 1. – Northern blot of ^{32}P-antisense NEP cRNA binding to 20, 10 and 5 μg of total RNA obtained from cultured human smooth muscle [11] and epithelial cells [10]. The bands are at 4.5 kb. The large arrowhead identifies the location of the 28S rRNA band (4.8 kb), and the small arrowhead the 18S rRNA band (1.8 kb) [13]. NEP: neutral endopeptidase; RNA: ribonucleic acid; cRNA: complementary RNA; rRNA: recombinant RNA.

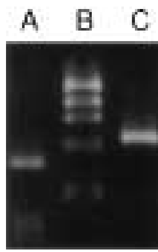

Fig. 2. – Reverse transcriptase-polmerization chain reaction (RT-PCR) for NEP mRNA in human bronchus total RNA. The *exons 19–20* splice site antisense (RT) primer and *exons 14–15* splice site sense primer generated a band at 477 nucleotides corresponding to the full length mRNA coded from the exons 14–20 (Lanes A). No truncated variant NEP mRNAs were found. Lane B shows markers at 1353 (top), 1078, 872, 603 and 310 (bottom) nucleotides. Lane C shows the β-actin mRNA RT-PCR product (661 nucleotides). Lanes A: NEP; B: markers; C: β-actin. mRNA: messenger ribonucleic acid. For further abbreviations see legend to figure 1.

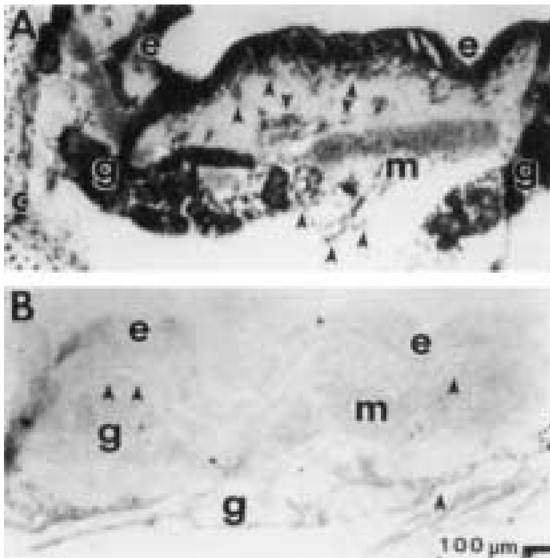

Fig. 3. – Nonradioactive *in situ* hybridization of NEP in human bronchus. A) Biotin-labelled antisense NEP mRNA was detected by the immunogold method with silver enhancement as the intense black stain over the epithelium (e) and submucosal glands (g). Slightly less intense staining was detected over bronchial smooth muscle (m), endothelium of subepithelial capillaries/post-capillary venules and larger vessels (arrowheads), and chondrocytes (c). B) Biotin-labelled sense NEP cRNA did not bind to the sections. For abbreviations see legends to figures 1 and 2. (Internal scale bar=100 μm).

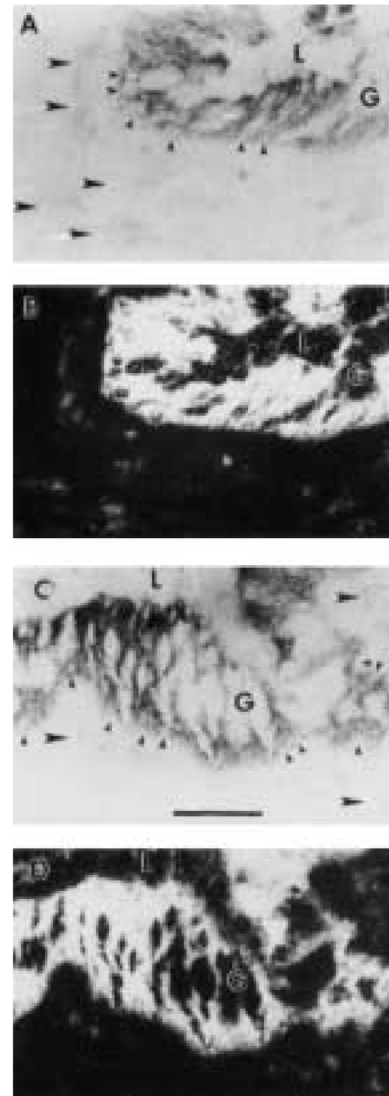

Fig. 4. – NEP *in situ* hybridization in epithelium. Brightfield (A and C) and darkfield (B and D) images of ^{35}S-antisense NEP binding to epithelium shows dense silver grains over epithelial cell cytoplasm excluding nuclei (small arrowheads) and goblet cell vacuoles (G). Superficial vessel endothelial cells (large arrowheads) have a low density of silver grains when viewed with darkfield illumination. The bronchial lumen (L) is shown. Serial sections treated with sense probes had no binding (not shown). Bar line=50 μm.

This distribution of NEP mRNA-containing cells was confirmed using the ^{35}S-labelled cRNA probes. The epithelium, vessels, smooth muscle, and glands contained NEP mRNA. The epithelium had a high silver grain density. The nuclei of basal and other cells, and the vacuoles of goblet cells were devoid of NEP mRNA (fig. 4). The serous cells of submucosal glands were positive (fig. 5). The vacuoles of mucous cells contained no NEP mRNA, but the cytoplasmic rims of mucous cells appeared to contain some silver grains suggesting the presence of NEP mRNA. Endothelial cells of subepithelial capillary/post-capillary venules contained NEP mRNA (figs 3 and 4), as did the smooth muscle of a deeper periglandular artery (not shown). NEP mRNA was detected in bronchial smooth muscle. The distribution was the same in all specimens.

Neither the biotin- nor the ^{35}S-labelled sense probes hybridized with the tissue (figs 3 and 5) indicating the specificity of the antisense probe.

Immunohistochemistry

NEP immunoreactive material was detected in epithelial cells, submucosal glands, smooth muscle and endothelium of human bronchus (figs 6 and 7). In submucosal

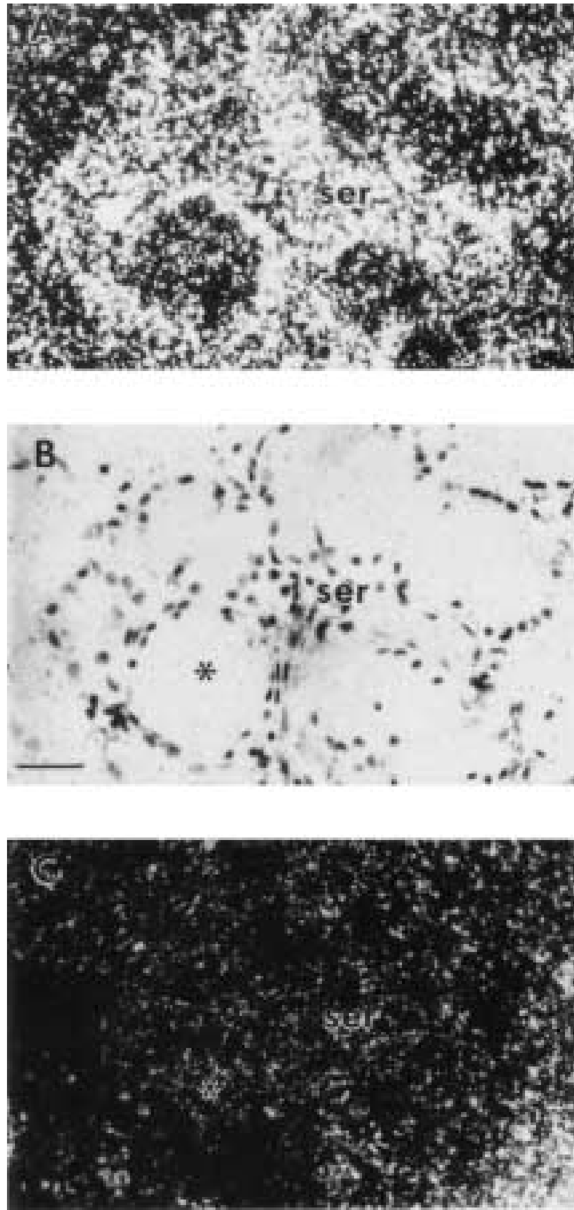

Fig. 5. – *In situ* hybridization using antisense neutral endopeptidase (NEP) ^{35}S-cRNA probe in human bronchial submucosal glands. A) Darkfield showing silver grains over glandular cells. Serous cells, possibly mucous cell cytoplasm, but not mucous cell vacuoles, demonstrate silver grains. B) Brightfield image showing submucosal gland acini stained with haematoxylin. C) The sense probe did not bind to the gland, confirming the specificity of the antisense binding seen in (A). cRNA: complementary ribonucleic acid. Bar line=50 µm.

glands, the NEP appeared to be at the edges of gland cells rather than in secretory granules, suggesting that the NEP was membrane-associated. The same impression is obtained for bronchial smooth muscle. Endothelium of vessels immediately below the epithelium were positive (fig. 6A). The cellular distribution of NEP immunoreactive material was the same as that of NEP mRNA. The distribution was identical in all specimens. Adsorbed NEP antibodies did not stain the tissue, indicating the specificity of the immunolocalization method.

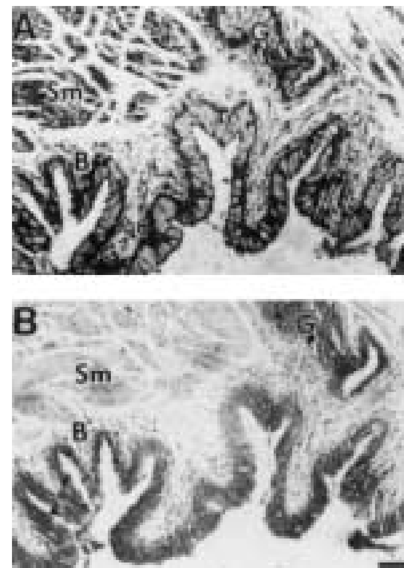

Fig. 6. – Neutral endopeptidase (NEP) immunohistochemistry. A) NEP immunoreactive material was detected with MEK10 antibodies and the immunogold method, and appears as the densely stained black material. Goblet cells and glandular cells appear grey with the methyl pyronin green counterstain. NEP was detected in the basal region of the epithelium (B), and the outer rims of submucosal gland cells (G). Vacuoles of goblet cells and submucosal gland mucous cells did not contain the black stain indicative of NEP, but still appear grey from the counterstain. Smooth muscle (Sm) also contained NEP. The NEP appeared to be localized to the surfaces of these cells in a rim pattern. B) MEK10 antibodies adsorbed with recombinant human NEP did not bind to this serial tissue section. Only methyl pyronin green counterstained cells can be seen. (Internal scale bar=100 µm).

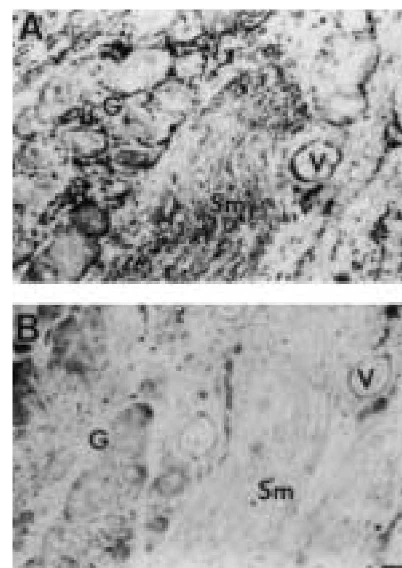

Fig. 7. – Neutral endopeptidase (NEP) immunohistochemistry. A) NEP immunoreactive material (black stain, immunogold method) is shown with methyl pyronin green counterstain. NEP immunoreactive material was detected in the outer rims of glandular acini (G) in serous cells, mucous cells, and possibly myoepithelial cells. Smooth muscle (Sm) and the endothelium of a venule (V) contained NEP. B) MEK10 antibodies adsorbed with recombinant human NEP did not bind to this serial tissue section. Only the methyl green pyronin counterstain (grey) is apparent. (Internal scale bar=40 µm).

Discussion

Cell surface NEP may limit the actions of many of the peptides that are active in human tracheobronchial mucosa [23–25], including tachykinins and calcitonin gene-related peptide (CGRP) released by axon response mechanisms from nociceptive sensory neurones during neurogenic inflammation, vasoactive intestinal peptide (VIP) released by parasympathetic reflexes, bradykinin generated in many types of allergic and nonallergic inflammation, and circulating peptides, such as endothelin and atrial natriuretic peptide (ANP) [26–28]. Decreases in NEP activity [22] may underlie the increased responses of respiratory mucosa found during viral infections [29–31], after exposure to cigarette smoke [32], ozone [33], hypochlorous acid, [34], and high doses of toluene diisocyanate [35]. The release of peptides into areas with reduced NEP activity could lead to enhanced peptide-induced epithelial cell function, glandular secretion, vascular permeability and smooth muscle contraction [1–3, 26, 27]. Each of these proinflammatory processes occurs in sites where NEP mRNA and protein are found: epithelial cells, submucosal gland cells, bronchial smooth muscle, endothelium and arterial smooth muscle (table 1). Apparent differences in the numbers of gland and epithelial cells containing NEP mRNA and immunoreactive materials between specimens were due to different proportions of goblet and mucous gland cells (figs. 3–7). These mucous cell vacuoles did not contain NEP. Other epithelial cells and glandular serous cells did contain NEP mRNA and immunoreactive material.

These locations also correlate with the sites of NEP enzyme activity detected in guinea-pig tracheal epithelium, glands, and vessels by fluorescent zymographic microscopy [36]. Enzyme activity was also detected in the perichondrium and chondrocytes, but was not detected in guinea-pig tracheal smooth muscle cells [36]. Expression of NEP in lung parenchyma has been investigated by Johnson *et al.* [37].

Whilst changes in NEP distribution or expression or decreased activity have been postulated to contribute to changes in airway reactivity to selected stimuli *in vivo* [1, 2], there are as yet few data from humans to confirm this contention. In fact, Roisman *et al.* [38] suggested an increase in NEP activity in lungs of asthmatic subjects. Modulation of NEP activity in disease or after oxidant exposure may be more complex in humans *in vivo* than animal and *in vitro* models [1–3] would lead us to believe. The current investigation of NEP distribution was not designed to determine whether destruction of NEP activity is a primary event in airway inflammation that permits exaggerated neurogenic inflammation, but does indicate that changes in expression by resident cells is a feasible hypothesis.

An additional level of complexity in NEP expression is the regulation of NEP gene transcription. The NEP gene is complex, with 24 miniexons and multiple polyadenylation sites [6, 8]. NEP mRNAs of several sizes are generated by post-transcriptional processing. The use of alternate polyadenylation sites accounts for some of the variation [6]. However, alternate splicing with the deletion of certain exons may also lead to mRNA size and protein product diversity. Iijima *et al.* [17] have detected a 3.2 kb mRNA which lacks exon 16. RT-PCR (fig. 2) identified mRNA coding for exons 14–20, but did not identify any for the *del exon 16* variant. Another truncated NEP gene product detected by PCR in rat thyroid, intestine and whole brain [18] excludes exons 5–18, and is postulated to generate a 255 amino acid protein. No evidence was found to support the expression of this variant (data not shown).

These data indicate that NEP mRNA and immunoreactive materials are widely distributed on airway epithelium, glands, vessels and smooth muscle, sites known to possess receptors for many peptide mediators. The distribution of NEP indicates its critical role in limiting the effects of peptides in human airways *in vivo*.

Table 1. – Distribution and relative intensity of expression of NEP mRNA and immunoreactive material in human trachea and large bronchi

Site	*In situ* hybridization	Immunoreactive material
Epithelium*	++	++
Endothelium	+	+
Submucosal glands*	++	++
Smooth muscle	+	+

*: mRNA and immunoreactive material were excluded from epithelial goblet cell and submucosal gland mucous cell vacuoles. NEP: neutral endopeptidase; mRNA: messenger ribonucleic acid. +: present; ++: intense stain.

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