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Peptidases in human bronchoalveolar lining fluid, macrophages, and epithelial cells: Dipeptidyl (amino)peptidase IV, aminopeptidase N, and dipeptidyl (carboxy)peptidase (angiotensin-converting enzyme)

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The modulation of proteolytic activity is an important factor in regulating the metabolism and function of peptide hormones. In this study, the activities of dipeptidyl (carboxy)peptidase (angiotensin-converting enzyme [ACE]), aminopeptidase N (APN), and dipeptidyl (amino)peptidase IV (DPP IV) were measured in the blood, the human bronchial epithelial and alveolar cells, bronchoalveolar macrophages, and the soluble phase of bronchoalveolar lavage (BAL) samples obtained from normal human volunteers and patients with pulmonary pathologic conditions. BAL fluid expressed ACE activity and very low levels of APN and DPP IV activities in the volunteer population, but higher levels could be measured in samples from patients. In patients, increased APN corresponded to a high granulocyte count, while DPP IV and ACE were associated with a high percentage of lymphocytes. Neither AIDS nor smoking induced an increased level of these enzymes. Immunohistochemical staining of bronchoalveolar smears with anti-human ACE monoclonal antibody showed that only macrophages expressed this enzyme. Enzyme histochemistry for DPP IV and APN showed that all leukocytes expressed these activities. APN, DPP IV, and ACE activities were also found in cell extracts of bronchoalveolar macrophages. In extracts of bronchial epithelial and alveolar cells, only APN and DPP IV activities were detected. Kinetic properties of the soluble enzymes in lavage supernatants were comparable to those of serum enzymes. These results demonstrate that soluble forms of cellular enzymes found in BAL fluid are regulated independently of blood and that different cell types may release these enzymes. (J Lab Clin Med 1997;130:603-14)

Abbreviations: ACE = angiotensin-converting enzyme; AIDS = acquired immunodeficiency syndrome; APN = aminopeptidase N; BAL = bronchoalveolar lavage; DPP IV = dipeptidyl (amino)peptidase IV; γGTP = γ-glutamyltranspeptidase; HIV = human immunodeficiency virus; IC50 = 50% inhibitory concentration; Km = Michaelis-Menten constant; PBS = phosphate-buffered saline solution; Z- = benzylxycarbonyl-
Proteolytic enzymes are involved in the activation, transformation, or degradation of proteins and peptide hormones in various regulatory pathways, during the processing of peptide antigens for presentation by immunocompetent cells or the degradation of extracellular matrix. Thus a tight control of protease activities is necessary to maintain normal cell function. Proteolytic processing of a hormone by a particular enzyme may modulate its biologic function, and peptide hormones can often be processed by several different proteases, depending on both the access of an enzyme to specific hormones and the presence of substances modulating protease activity. Membrane-bound and soluble proteases may have different enzymatic potencies and different access to peptides as a function of the relative locations of the peptide and the enzyme.

Several cell surface antigens defined as leukocyte differentiation markers have been shown to be proteolytic enzymes that are also expressed by epithelial, endothelial, or other tissues, demonstrating that leukocyte differentiation implies a modulation of protease expression. However, factors involved in these differentiation phenomena are not well understood. Under some pathologic conditions, APN—but not DPP IV—is specifically expressed by mononuclear cells, while activation of alveolar macrophages differentially stimulates the expression of cathepsin D and APN. HIV infection also modifies proteolytic activity.

Epithelial cells, mononuclear phagocytes, and lymphocytes are able to express and secrete many proteolytic enzymes, as well as their inhibitors. Although some information has been gained on the protease-antiprotease imbalance in the lung in emphysema, the regulation of peptidases involved in the metabolism of small peptides has not been extensively evaluated in the pulmonary bronchoalveolar space. In the present study, the enzymatic activities of two aminopeptidases (APN and DPP IV) and a dipeptidyl (carboxy)peptidase (ACE) were measured as soluble enzymes in BAL fluid and as cell-bound enzymes in human bronchial epithelial cells (BEAS-2B, a non-tumorigenic cell line with a phenotype close to that of a bronchial cells), alveolar epithelial cells (A549 cells, derived from an alveolar type II carcinoma [ATCC]), and bronchoalveolar macrophages. These enzymes were evaluated in the human BAL fluid obtained from normal human male volunteers and from patients with various lung disorders and were compared with cellular repartition between macrophages, granulocytes, and lymphocytes.

**METHODS**

**Study subjects.** BAL was performed in 23 nonsmoking male volunteers. Subjects were selected after a thorough questionnaire and clinical examination to exclude those with past or present illness as well as those taking any medication. In addition, pulmonary function tests were performed and subjects with abnormal spirometry or positive methacholine challenge test were excluded. All were nonsmokers.

BAL from patients who underwent bronchoscopy for diagnostic purpose were also retrospectively analyzed. Patient populations represented various pathologic conditions including tumors, sarcoidosis, infectious diseases, and AIDS.

The study protocol in the normal volunteers was approved by the ethics committee of our institution. No formal request was made for BAL analysis in the patients group because this part of the study was retrospective and because BAL was done primarily for diagnostic purposes.

**BAL and sample preparation.** Bronchoscopy was performed in normal nonsmoking subjects and patients according to standard procedure. Sterile 0.9% saline solution (150 to 200 ml) was injected in 50 ml aliquots via a fiber optic bronchoscope, was aspirated, and was filtered through sterile gauze. In the volunteers the lavage was done exclusively in a subsegment of the right middle lobe. Because the recovery and time of contact of the injected liquid may be an important parameter that influences the results, these parameters were maintained in as standardized a fashion as possible in the volunteer study. Total recovered cells and differential cell counts were determined on an aliquot sample and treated as cell cultures (below). The remaining cells and fluid were separated by centrifugation. Aliquots of supernatants were frozen at −80°C. In the volunteer study, macrophages were isolated by adherence on plastic for 2 hours in RPMI (Gibco) medium containing 5% FCS. Differential cell counts were performed after concentration by filtration through Millicell (5 μm, SMWP 02500) membranes and hematoxylin-light green or Giemsa staining. Enzymatic activities were measured in 150 μl (APN and DPP IV) or 200 μl (ACE) of supernatants as described below. Blood was withdrawn at the time of lavage in the volunteer study, and aliquots of serum were frozen.

Six broad disorder categories were defined as follows: (1) sarcoidosis of any stage, diagnosed with lung biopsy or with a characteristic BAL cellular profile; (2) interstitial pneumonopathy of any origin, where the diagnosis of sarcoidosis was reasonably excluded; (3) pulmonary infections in immunocompetent patients; (4) pulmonary infections in HIV-positive patients (any stage); (5) primary or secondary lung neoplasm; (6) blood malignancy of the myeloid or the lymphoid lineage.

**In vitro experiments**

**Cell culture.** BEAS-2B cells (obtained from Dr. C. C. Harris, National Cancer Institute, Bethesda, Md.) were grown in collagen-, fibronectin-, and albumin-coated flasks in LCH-9 serum-free medium as described. A549
Table I. Clinical data for volunteers and patients

| Pathologic condition | n | Mean age (range) | Sex (M/F) | Current smokers | BAL profile | Comments |
|----------------------|---|------------------|-----------|-----------------|-------------|----------|
| Normal volunteers    | 23 | 25 (20-38)       | 23/0      | 0               | 72 (13)     | 22 (12)  | 4 (3)    |                |
| ACE patients         |    |                  |           |                 |             |          |          |                |
| Sarcoidosis          | 6  | 36 (26-46)       | 5/1       | 1               | 43 (20)     | 40 (12)  | 12 (17)  |                |
| Int Pneu*            | 10 | 53 (16-76)       | 8/2       | 0               | 49 (26)     | 33 (26)  | 10 (7)   |                |
| HIV                  | 14 | 38 (38-51)       | 11/3      | 7               | 46 (26)     | 21 (20)  | 22 (25)  |                |
| Lung neoplasm        | 11 | 53 (39-72)       | 7/4       | 9               | 48 (27)     | 10 (7)   | 3 (27)   | primary:9      |
| Blood neoplasm       | 7  | 48 (22-76)       | 5/2       | 0               | 33 (20)     | 49 (32)  | 6 (5)    | lymphoid:5     |
| APN/CD13 and DPPIV/CD26 patients | | | | | | |
| Int. Pneu.           | 8  | 63 (50-79)       | 6/2       | 2               | 54 (22)     | 11 (14)  | 23 (14)  |                |
| Infectious†          | 29 | 56 (20-81)       | 20/9      | 6               | 38 (23)     | 40 (32)  | 10 (8)   |                |
| HIV                  | 17 | 34 (3-51)        | 10/7      | 10              | 46 (28)     | 28 (27)  | 14 (12)  |                |
| Lung neoplasm        | 17 | 61 (39-86)       | 8/9       | 9               | 53 (30)     | 16 (24)  | 13 (11)  |                |
| Blood neoplasm       | 6  | 60 (34-66)       | 2/4       | 3               | 46 (35)     | 17 (30)  | 12 (17)  |                |

M, macrophages; L, lymphocytes; PMN, neutrophils; M, L, and PMN in % from total (SD).
*Interstitial pneumopathy (extrinsic alveolitis 1, vasculitis 3, rheumatoid arthritis 1, drug-induced 2, idiopathic 3).
†Lung infection in immunocompetent patients.
‡Pneumocystis carinii pneumonia.

cells were from ATCC and were grown in RPMI medium containing 10% fetal calf serum (Gibco). After reaching confluence, cells were washed twice with PBS, detached in PBS with a rubber policeman, and lysed with 2 cycles of sonication/freezing. Adherent macrophages from normal volunteers were treated as cell cultures. Enzymatic activities were measured in 40 µl (APN and DPPIV) or 100 µl (ACE) cell extracts as described below.

Enzymatic determinations

Aminopeptidase activities. For in situ enzymatic activities, an aliquot of BAL cell suspension from BAL was concentrated by centrifugation onto a glass slide (cytocentrifugation) and incubated for 30 minutes at 37°C with leu-2-methoxy-L-naphthylamide or gly-pro-2-methoxy-L-naphthylamide (Bachem) substrates for APN and DPP IV, respectively, at a final concentration of 1 mmol/L in PBS buffer and 1.4 mmol/L Fast Blue BB (Sigma) as previously described.1° Soluble enzymatic determinations. Leu- and gly-pro-p-nitroanilide derivatives (Bachem) were used at a final concentration of 0.5 mmol/L in 0.02 mol/L Tris, pH 7.5, containing 0.2 mmol/L CoCl2 and 150 µl of lavage solution (10 µl serum) in a final volume of 225 µl. γ-GTP was measured in the same conditions with 1 mmol/L γ-glu-p-nitroanilide as donor and 20 mmol/L gly-gly as acceptor as previously described.10 Conventional methods were adapted for continuous recording of increases in absorbance at 405 nm for 30 minutes in a thermostat-equipped (37°C) microtiter plate reader (96 wells) (Anthos III, Kontron). Analyses were performed in duplicate. Units of enzymatic activity were defined as µmol of substrate hydrolyzed per minute per milliliter of sample, calculated from an ε value of 8800 M⁻¹ x cm⁻¹. Inhibition of APN was attempted with serial dilutions of diprotin A (Novabiochem) and that of DPP IV with serial dilutions of diprotin A (Novabiochem) in human serum and BAL fluid.

ACE and dipeptidase activity determination. Determination of ACE and dipeptidase activity was performed with Z-phe-his-leu or his-leu as substrate and fluorescent derivation of his-leu with o-phenylaldehyde as described for serum or for supernatant of cell culture11,12 with 200 µl of lavage fluid and 1 hour of incubation at 37°C. His-leu (Bachem) was used as standard under the same conditions as for samples. Units of enzymatic activity were defined as nmoles of substrate hydrolyzed per minute per milliliter of BAL fluid, in comparison with the standard. ACE inhibition was evaluated with lisinopril (a gift from Merck, Sharp and Dohme) (final concentration 25 µmol/L). Susceptibility of his-leu to dipeptidase degradation in BAL fluid was controlled by adding his-leu standard to the incubation medium. KM and IC₅₀ determinations in human serum and BAL fluid were performed by serially diluting substrate or inhibitor, respectively.

ACE immunohistochemistry. Macrophage-specific monoclonal anti-CD68 antibody was from a commercial source (Dako) and was used at a 1:200 dilution. The anti-human ACE monoclonal antibody 9B9 was a gift of SM Danilov (INSERM U367, Paris). The characterization of this antibody has been described.13 Lyophilized antibody was dissolved in water (1 mg/ml) and used at a 1:150 dilution in PBS. BAL cells were concentrated by centrifugation, and smears were prepared. Smears were fixed in paraformaldehyde (4%), washed in PBS, blocked for 15 minutes with normal goat serum, and incubated overnight at 4°C with the primary antibody. Peroxidase-conjugated secondary goat anti-mouse immunoglobulin (Dako) was added for 30 minutes, followed by the addition of diaminobenzidine (0.035%). For control slides, the primary antibody was omitted.
**Table II.** Enzymatic activities in BAL fluid and plasma in normal samples

| Enzyme | Substrate | U/ml BAL fluid | U/ml plasma | SEM |
|--------|-----------|----------------|-------------|-----|
| APN    | Leu-p-NA  | <0.11         | 9.39        | 0.40|
| DPP IV | Gly-pro-p-NA | <0.11     | 15.80       | 0.66|
| ACE    | Z-phe-his-leu | 0.39   | 26.2        | 2.1 |

Activities were measured in BAL fluid and plasma taken at the time of lavage (n = 23). Results are expressed as means of units of enzymatic activity/ml.

*One supernatant 0.11 U/ml, 1 supernatant 0.14 U/ml, 1 supernatant 0.15 U/ml, 1 supernatant 0.27 U/ml.

**Table III.** Enzymatic activities in BAL fluid from patients

| Enzyme | Substrate | n (mean) | U/ml | SEM |
|--------|-----------|----------|------|-----|
| APN    | Leu-p-NA  | 165      | 0.42 | 0.06|
| DPP IV | Gly-pro-p-NA | 147  | 0.29 | 0.03|
| ACE    | Z-phe-his-leu | 83  | 0.62 | 0.08|
| Dipeptidase | His-leu | 83   | <0.05 | — |

Specific substrates were added to aliquots of BAL fluid (n = number of different samples), and enzymatic activities were measured as described under Methods. Results represent combined data from different patient populations.

**Protein content.** Protein content was measured with the BCA kit (Pierce) with bovine serum albumin as standard.

**Statistical evaluation.** Results were expressed as the mean ± SEM. Statistical significance was assessed by calculating the correlation coefficient (r) with a two-tailed unpaired Student t test (t) or a one-way analysis of variance, followed by Bonferroni, with post-test correction.

For comparisons between normal volunteers and selected pathologic states, the non-parametric Kruskal-Wallis test was used, followed by Dunn’s Q analysis, where applicable.

**RESULTS**

In this study, 23 normal male volunteers and a collective of randomly selected frozen BAL samples were retrospectively studied without preliminary selection of patients and pathologic states. For further analysis from this collective, known smokers, non-smokers, and patients with AIDS were selected. In addition, a selection of patients in whom a well-documented diagnostic was available was specifically analyzed. Clinical data from the normal volunteers and these selected patients are summarized in Table I. Cell repartition between macrophages, lymphocytes, and polymorphonuclear neutrophils was within the normal values in the normal volunteers.

BAL supernatants obtained from normal volunteers (Table II) and from patients with various pathologic conditions (Table III) specifically expressed ACE, APN, and DPP IV activities. In the patient population, dipeptidase activity on his-leu was controlled to ensure that low levels of ACE activity were not caused by his-leu degradation (Table III).

To compare ACE, APN, and DPP IV kinetic properties in BAL fluid and plasma, the effect of the specific inhibitors lisinopril, actinonin, and diprotin A, respectively, was analyzed. Lisinopril inhibited the enzymatic activity of the soluble ACE in two BAL and two blood samples (Fig. 1, A). The respective IC50 values were 0.2 μmol/L for the enzyme from the blood and 0.5 μmol/L for the enzyme from the BAL fluid. However, the Km values (0.08 mmol/L) measured in controlled Cl- concentration from the x-axis intercept of the Lineweaver and Burk plot and substrate inhibition (starting at 0.5 mmol/L substrate concentration), a characteristic of many carboxypeptidases including ACE,14 were identical for the enzymes of blood and bronchoalveolar origin. The amount of inhibitor necessary to inhibit half of the activity (IC50) of both serum and BAL ACE activities was higher than postulated by the inhibition constants. This can be explained by the high substrate concentration necessary for evaluation of ACE in vitro, because it has been shown that inhibition decreases with a high S/Km ratio.12,15

ACE activities between serum and BAL supernatants (n = 23, r = 0.08) and between BAL supernatants and BAL cell extracts (n = 22, r = 0.08) from the normal volunteers were not correlated. In the patient population, soluble ACE activity was increased when lymphocyte content in the BAL was increased (p < 0.05) (Table IV), independent of the number of granulocytes.

Actinonin and diprotin A similarly inhibited blood and BAL enzymes (Fig. 2, A and B). APN activity was inhibited by actinonin in BAL fluid (1.12 μU/ml) and serum (9.88 μU/ml) (Fig. 2, A), and DPP IV activity was inhibited by diprotin A in BAL fluid (1.14 μU/ml) and serum (20.43 U/ml) (Fig. 2, B) with IC50 values of 0.5 μmol/L and 7 μmol/L, respectively.

In the patient population, soluble APN activity was markedly elevated when the relative proportion of granulocytes was increased (Table IV), and that of DPP IV was slightly elevated when an increased proportion of lymphocytes was observed (Table IV), independent of the number of granulocytes, whereas these activities were not measurable in samples with normal cellular repartition.

However, no strict correlation between BAL cellular composition and supernatant enzyme activities could be observed. In some BAL fluid containing a
very high proportion of either granulocytes or lymphocytes, the activities of APN, ACE, and DPP IV were not elevated. In some samples with high levels of these enzymatic activities, the respective proportions of the corresponding cells were not increased. Some samples with high levels of APN activity also expressed increased levels of DPP IV, but in other samples, only one of these enzymatic activities was elevated. Thus the relative proportion, or even the absolute number of the three classes of cells evaluated, was not the exclusive factor determining relative levels of enzymatic activity, but other undetermined factors were likely involved.

To examine potential clinically relevant factors, we evaluated several distinct groups (clinical data in Table I) of disorders (Fig. 3, A, B, and C). Patients
Fig. 2. Comparison of IC₅₀ values for inhibition of APN/CD13 by actinonin and DPP IV/CD26 by diprotin A in human BAL fluid and blood serum. Normal serum (B) or lavage supernatant ([2) was preincubated for 10 minutes at 37 ° with decreasing concentrations of either actinonin or diprotin A; specific substrates were then added, and either APN or DPP IV activity was measured. A, APN/CD13. B, DPP IV/CD26.

with sarcoidosis (Fig. 3, A, group B) had significantly elevated levels of ACE activity, but an elevated level of this enzyme was also found in patients with blood malignancy (Fig. 3, A, group G). A normal level of ACE activity was found in the other pulmonary disorders, including interstitial pneumopathy (sarcoidosis excluded), infection, neoplasia (groups C to F) when comparison was made with normal volunteers (group A). No specific condition could be found for patients with ACE activity values below the normal range. APN activity was increased in some samples of pulmonary infections (Fig. 3, B, groups D and E) and DPP IV activity in some samples in all groups, except for samples from patients with blood malignancies (Fig. 3, C). In BAL samples from a small group of patients with sarcoidosis (n = 4), APN activity was found to be in the normal range (0.15 U/ml), whereas DPP IV activity was increased (0.55 U/ml) in accordance with results obtained with high lymphocyte counts (Table IV).

Table IV. Comparison of soluble ACE, DPP IV, and APN activities with differential cell counts in BAL fluid from patients

| PMN | LYM |
|-----|-----|
|     |     | Norm | 10%-25% | >25% | 20%-50% | >50% |
| n   | 20  | 20   | 10     | 0     | 0      | 0    |
| DPP IV (U/ml) | <0.11 | 0.27* | 0.39* | 0.44* |
| SEM | --  | 0.05 | 0.06   | 0.08  |
| n   | 23  | 18   | 50     | 34    | 5      | 3    |
| APN (U/ml)    | <0.11 | 0.40* | 0.87* | 0.21* |
| SEM | --  | 0.12 | 0.17   | 0.05  |
| n   | 12  | 17   | 18     | 27    | 8      |     |
| ACE (U/ml)    | 0.25 | 0.63 | 0.60   | 0.93  | 1.59†  |
| SEM | 0.06 | 0.11 | 0.15   | 0.18  | 0.43   |

Lavage was performed and differential cell counts and enzymatic activities determined as described under methods.
Norm, Normal cellular distribution; PMN, increased proportion of polymorphonuclear cells; LYM, increased proportion of lymphocytes.
*Different from Norm values for APN and DPP IV activities (below detection limit in normal samples)
†Different from Norm (p < 0.05) by one-way analysis of variance, followed by Bonferroni after tests.

To analyze the effects of two known modulators of the immune system, the effect of AIDS pathology or of current smoking (more than 10 cigarettes per day) was evaluated (Table V). It was concluded that neither condition alone importantly altered the level of the proteolytic enzymes studied. However, smokers did exhibit a lower ACE activity in BAL fluid when compared with nonsmokers (p < 0.05).

When the aminopeptidase activities were evaluated in situ in cells from BALs, APN and DPP IV (Fig. 4, A and B) were detected. Immunohistochemical staining with monoclonal anti-ACE (9B9) and anti-macrophage (CD68) antibodies demonstrated that ACE was expressed by bronchoalveolar macrophages (Fig. 5). However, the number of cells that stained positive for ACE in smears was not correlated with ACE activity in cell extracts (results not shown). ACE immunostaining was also observed exclusively in macrophages in bronchoalveolar cells obtained from a sample with a high lymphocyte ratio (45% macrophages, 50% lymphocytes, CD4+/CD8⁻ = 0.3) (not shown).

Human epithelial cells both from bronchial (BEAS-2B) and alveolar origin (A549) expressed APN and DPP IV activities as well as γ-GTP (a specific marker of epithelial cells used as an internal control) but not ACE activity. BAL macrophages expressed APN, DPP IV, and ACE activities but not γ-GTP activity (Table VI).
Fig. 3. ACE, APN, and DPP IV activity in the BAL fluid of patients as a function of pathologic condition. In this diagram, only patients with well-documented pathologic conditions were included. A, Normal nonsmoker volunteers; ACE, n = 23; APN and DPP IV, n = 23. B, Sarcoidosis; ACE, n = 6; APN and DPP IV, 0. C, Interstitial pneumopathy (sarcoidosis excluded); ACE, n = 10; APN and DPP IV, n = 8. D, Pulmonary infection (HIV–); ACE, n = 21; APN and DPP IV, n = 29. E, Pulmonary infection (HIV+); ACE, n = 14; APN and DPP IV, n = 17. F, Pulmonary neoplasia; ACE, n = 11; APN and DPP IV, n = 17. G, Blood malignancy; ACE, n = 7; APN and DPP IV, n = 6. On the diagram, the median, dispersion, and standard deviation are indicated. Bar, median; box, interquartiles; whisker, adjacent values. Statistical analysis was performed with a one-way non-parametric Kruskal-Wallis test, p = 0.013 for patients with sarcoidosis as compared with normal subjects.
Table V. Comparison of soluble ACE, DPP IV, and APN activities in BAL fluid from smokers, non-smokers, and patients with AIDS with the combined data from the patient population

|            | Nonsmokers | Smokers | AIDS | All Samples |
|------------|------------|---------|------|-------------|
| n          | 29         | 40      | 49   | 147         |
| DPP IV (U/ml) | 0.27      | 0.24*   | 0.29 | 0.29        |
| SEM        | 0.05       | 0.04    | 0.06 | 0.04        |
| n          | 29         | 40      | 48   | 165         |
| APN (U/ml) | 0.27       | 0.26*   | 0.45 | 0.42        |
| SEM        | 0.07       | 0.06    | 0.13 | 0.06        |
| n          | 23         | 20      | 13   | 83          |
| ACE (U/ml) | 0.95       | 0.39†   | 0.50 | 0.62        |
| SEM        | 0.23       | 0.10    | 0.16 | 0.08        |

Results are expressed as units of enzymatic activities/ml BAL fluid. Nonsmokers, Patients who said they never smoked; smokers, more than 10 cigarettes per day; AIDS, patients with a recognized stage III-IV disease. *Not significant, smokers versus nonsmokers.

DISCUSSION

The role of membrane-bound proteases is not well understood, but it might be linked to hormone metabolism and possibly to cell adhesion and migration. Protease shedding from the membrane is a regulatory mechanism for the degradation of peptides in biologic fluids and in the regulation of cell adhesion. Membrane-bound proteases, although active on small synthetic substrates, are not as efficient on larger natural peptide substrates, which are better hydrolyzed when the enzyme has been released from the membrane.

The regulation of proteases involved in the metabolism of peptide hormones in the bronchoalveolar space has not been extensively evaluated in normal situations and in lung disorders. The expression of peptidases either in pulmonary cells or in the supernatants of BALs had been previously shown for a few enzymes. For example, in a rat model, carboxypeptidase M and neutral endopeptidase 24.1116 were released into BAL fluid. However, the evaluation of enzyme activities in bronchoalveolar cells, in supernatants, and in the blood in normal human studies has been performed only for ACE.17 Our results demonstrate that ACE, APN, and DPP IV were secreted in the human epithelial lining fluid as soluble enzymes and were expressed by specific cells of the bronchoalveolar surface, macrophages (ACE, APN, and DPP IV) and epithelial cells (APN and DPP IV). The absence of correlation between ACE enzymatic activity in BAL supernatants and blood under normal situations suggested an independent regulation in both biologic compartments. This is further confirmed by high levels of APN and DPP IV activities measured under normal situations in the blood and almost undetectable activities in BAL fluid. In normal volunteers and in patients, the activity of ACE was detectable in all samples; however, the activities of APN and DPP IV were very low under normal situations but were dramatically increased in some pathologic samples.

ACE (EC 3.4.15.1) is a ubiquitous membrane-bound ectopeptidase of wide specificity that is able to remove dipeptides and some tripeptides without processing from the carboxyl end of proteins. Its potential substrates include bradykinin, substance P, angiotensin I, and enkephalins. The use of ACE inhibitors in the treatment of hypertension is sometimes linked to cough, possibly because of an increased bronchial reactivity and a decreased degradation by this enzyme of the proinflammatory peptides bradykinin or substance P.18 Interestingly, we found a lower ACE activity in BAL fluid from smokers than in that from other groups. Because ACE is involved in bradykinin degradation, this might have potentially important implications for the effect of smoking on peptide metabolism.

Serum ACE activity is increased in active sarcoidosis, HIV, and hyperthyroidism.5,19-21 We did not find increased levels of this enzyme in the BAL fluid from patients with HIV, confirming the differential regulation of this enzyme in both compartments. The level of ACE activity was significantly increased in samples with high lymphocyte counts and in the BAL from patients with sarcoidosis, confirming previous results.22 or lymphoid neoplasm, a new finding. In normal samples, ACE antigenicity was expressed only in macrophages.

Several of the cell-surface antigens of the cluster differentiation system have emerged as cell-surface peptidases with a wide distribution.1 DPP IV (EC 3.4.14.5), a peptidase specific for the release of xaa-
pro and xaa-ala dipeptides from the N-terminal end of peptides, is analogous to CD26. DPP IV/CD26 is involved in T cell activation, mainly CD4+ cells, in vivo and in vitro, but is also expressed by epithelia, certain endothelia, and platelets.\textsuperscript{1,23,24} The expression of DPP IV has been shown in bronchoalveolar macrophages\textsuperscript{25} but not in BEAS-2B cells.\textsuperscript{26} In the present study we found low levels of DPP IV activity in the same cell line and in A549 cells. This discrepancy may be explained by different methods of dosage or by differences in the confluence of cultures, because we found that the expression of DPP IV activity was dependent on cell confluence in various epithelial cell lines (unpublished experiments).

The number of CD26\textsuperscript{+},CD4\textsuperscript{+} cells is decreased during HIV infection; however, the activity of DPP IV/CD26 is unchanged in the blood of patients with AIDS.\textsuperscript{27} We observed the same lack of relationship in the BAL fluid. DPP IV activity was slightly increased, and independently of the presence of poly-
Fig. 5. Immunocytochemical staining of ACE in bronchoalveolar cell smears and comparison with ACE activity in cell extracts in normal volunteers. Histochemical staining of bronchoalveolar cell smears. A, ACE-specific 9B9 monoclonal antibody was used for this experiment. B, Identity of cells was controlled with macrophage-specific CD68 antibody. C, Negative control obtained without primary antibodies.
morphonuclear cells, when the lymphocyte proportion was increased in BAL, but neither AIDS nor smoking by themselves importantly modified DPP IV activity in BAL fluid. Although no physiologic substrate for DPP IV has yet been formally identified, many important peptides including substance P and several cytokines or peptides derived from collagen are all potential substrates.

APN (EC 3.4.11.2) is analogous to CD13 and is expressed by monocytes, macrophages, and granulocytes; intestinal, pulmonary, hepatic, and renal epithelial tissue; some endothelia; and fibroblasts. This exopeptidase can release neutral amino acids from the N-terminal end of peptides, and a function has been postulated for APN in the modulation of activity of peptides and in tumor invasion and collagen degradation. APN expression is regulated by biologically active peptides or by the state of cell differentiation. T lymphocytes appear to be involved in the induction of APN in monocytes in a manner similar to that with ACE. CD13-positive cells are permissive for cytomegalovirus infection. Human APN has been shown to be a receptor for two human corona viruses from the gastrointestinal tract and human lung fibroblasts. In BAL fluid, we show that soluble APN activity was linked to an increased proportion of polymorphonuclear cells.

However, actinonin, a relatively specific inhibitor of APN, could not completely inhibit the hydrolysis of leu-p-nitroanilide in BAL samples and in serum. Several explanations can be postulated. First, several enzymes act on the substrate, and each has a different sensitivity to actinonin inhibition. Second, the kinetic of inhibitor binding to enzyme(s) may be complex, which may explain an incomplete inhibition. Third, the hydrolysis of small substrates is still possible for some enzymes, even in the presence of bound inhibitors. The present approach did not allow us to differentiate these possibilities, and more detailed enzymatic analysis would be necessary to answer these questions.

In the normal volunteer population, only very low levels of soluble APN and DPP IV activities could be detected, whereas bronchial BEAS-2B and alveolar A549 epithelial cells and bronchoalveolar macrophages expressed high levels of APN. However, in some pathologic states, such as infectious states, much higher amounts of active enzymes were present in soluble form in the epithelial lining fluid, indicating that shedding of these proteases from cell membrane occurs in disease. From the specific expression of these enzymes, APN and DPP IV activities may originate from both macrophages or epithelial cells, as well as endothelial cells, whereas ACE may originate only from macrophages or endothelial cells, where the expression of this enzyme is established. However, in some diseases, nonspecific diffusion from serum cannot be excluded.

In conclusion, our results demonstrated that specific aminopeptidases and carboxypeptidases were released as soluble enzymes in high amounts in some pulmonary pathologic states, either from bronchoalveolar structures or from immunocompetent cells of the bronchoalveolar layers, or from both. The observation that increased expression of these enzymes can be measured in some samples of any pathologic disorder limits their usefulness as diagnostic tools. Drugs able to modulate the APN/CD13 and DPP IV/CD26 activities, in addition to inhibitors of ACE activity, may be potentially interesting in studying the metabolism of important peptide hormones.

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