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Temporal dependence of ectopeptidase expression in alveolar epithelial cell culture: implications for study of peptide absorption

B. Forbes [a,*], C.G. Wilson [b], M. Gumbleton [c]

[a] *Department of Pharmacy, King's College London, Manresa Road, London, SW3 6LX, UK*
[b] *Department of Pharmaceutical Sciences, University of Strathclyde, Glasgow, G1 1XW, UK*
[c] *Department of Pharmacy, UWC, Cardiff, CF1 3XF, UK*

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Abstract

There is little data available regarding the extent of peptide metabolism encountered following inhalation to the lung. We have studied the activity of five ectopeptidases in primary rat alveolar epithelial cells, A549 cells and pulmonary macrophages. Peptidase activity was assayed in the plasma membrane fractions (PMF) of primary type II alveolar epithelial cells (ATII cells) after 2 days in culture and after 7 days in culture when they had formed monolayers of type I-like cells (ATI-like cells). Dipeptidyl peptidase IV (DPP) activity fell from 36.65 mU/mg protein to 16.29 mU/mg protein between day 2 and day 7 in culture, aminopeptidase N (AMN) activity increased from 16.16 mU/mg protein to 23.99 mU/mg protein, angiotensin-converting enzyme (ACE) activity was lost (4.29 mU/mg protein at day 2, not detected at day 7), and carboxypeptidase M (CPM) activity was acquired (not detected at day 2, 5.20 mU/mg protein at day 7). The profile of exopeptidase expression in A549 cells was similar to that of primary rat alveolar cells at day 7 in culture (DPP 24.24 mU/mg protein, AMN 47.74 mU/mg protein, CPM 4.28 mU/mg protein, ACE not detected). Macrophages expressed high levels of aminopeptidases (DPP 46.85 mU/mg protein, AMN 28.28 mU/mg protein) but carboxypeptidase activity was not detected. Low neutral endopeptidase 24.11 (NEP) activity was found in all cell types studied (0.96–2.41 mU/mg protein). The qualitative and quantitative changes in the peptidase activity of primary cultured rat alveolar cells between day 2 and day 7 in culture has implications for the use of alveolar cell monolayers as drug absorption models to investigate peptide absorption from the lung. Ectopeptidase activity in cultured alveolar cells can be used to infer the peptide-metabolising capacity of the surface of the alveolar epithelium. The aminopeptidase activity in particular, if representative of enzyme activity in vivo, would offer a significant metabolic barrier to systemic delivery of peptides via the lung.

* Corresponding author. Tel.: +44-171-333-4823; fax: +44-171-351-5307.

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1. Introduction

The pulmonary route has been proposed as one of the more promising routes for the systemic delivery of peptides and proteins (Wall, 1995; Patton, 1996; Yu and Chien, 1997). The attraction of the pulmonary route lies in the thin absorption barrier, large surface area for drug absorption, and high rate and volume of blood supply of the lung. It has also been suggested that the lung may possess relatively low proteolytic activity (Zhou, 1994; Sayani and Chien, 1996), but the inaccessibility of the peripheral lung has restricted the amount of relevant data regarding the extent of peptide metabolism encountered following inhalation.

Unlike protein absorption which is restricted by the permeability barrier of the lung, pulmonary peptide availability is largely limited by the activity of peptide-degrading enzymes in the lung (Kobayashi et al., 1996). Enhancement of insulin and calcitonin absorption by the coadministration of protease inhibitors (Morita et al., 1994; Fukuda et al., 1995; Yamamoto et al., 1996) supports the notion that pulmonary peptide absorption is restricted by peptidase activity. However, assays of proteolytic activity in lung homogenates (Orawski et al., 1989; Choi and Wang, 1993; Fukuda et al., 1995) are of limited significance; the lung is a complex organ and homogenates will contain peptidases from non-respiratory regions of the lung and intracellular peptidases that have been decompartmentalised.

Peptides are absorbed across the alveolar-capillary barrier by passive paracellular processes and are unlikely to encounter intracellular peptidases. The good correlation between the enhancement of pulmonary calcitonin absorption in vivo and the inhibition of proteases in lung membrane fractions, but not cytosolic fractions, provides experimental evidence that membrane-bound enzymes (ectoenzymes) play a major role in the inactivation of inhaled peptides (Kobayashi et al., 1994). The rate-limiting absorption barrier encountered by peptides deposited in the peripheral lung is the alveolar epithelium, which comprises a mixed monolayer of type I (ATI) and type II (ATII) alveolar epithelial cells and is patrolled by alveolar macrophages. The ectopeptidases of these cells are ideally located to threaten the stability of peptides deposited in the alveolar lining fluid.

ATII cells differentiate into ATI cells in vivo; a mechanism which constitutes the normal replacement and repair mechanism (Adamson and Bowden, 1974). Isolated ATII cells can be cultured to form monolayers of ATI-like cells possessing morphological and certain biochemical characteristics of ATI cells in vivo (Dobbs et al., 1988; Cheek et al., 1989; Danto et al., 1992). ATI-like monolayers have been widely used to model the alveolar epithelium for drug transport studies (review: Mathias, 1996). In this study we have investigated the peptidase activity of cultured alveolar epithelial cells as a function of time in culture, i.e. as the cells changed from an ATII phenotype (day 2) to an ATI-like phenotype (day 7). Peptidase activity was also measured in A549 cells (an ATII adenocarcinoma cell line; Smith, 1977), and in rat alveolar macrophages obtained by bronchoalveolar lavage.

The five ectopeptidases selected for study represent a battery of widely distributed peptidases with a broad combined substrate specificity. They constitute over a third of the known mammalian ectopeptidases (Kenny and Hooper, 1991), and provide a substantial combined barrier to peptide absorption in the gastrointestinal tract (Bai, 1994). The ectopeptidase expression in the alveolar epithelial cells determined in the present study will enable us to (i) infer the peptide-metabolising capacity of the alveolar epithelium and (ii) characterise the alveolar epithelial monolayers which are used for drug transport and metabolism studies.

2. Materials and methods

2.1. Materials

Buffer solutions were prepared using reagent grade chemicals obtained from BDH (Lutterworth, UK), elastase was obtained from Lorne Diagnostics (Reading, UK), Percoll was obtained from Pharmacia (Amersham, UK), Dulbeccos Modified Eagle's medium (DMEM) and fetal

bovine serum (FBS) were from Gibco (Paisley, UK). Leu^5(D)Ala^2-enkephalinamide (TAGPL), Leu-(*p*)nitroanilide (LN), Gly–Pro-(*p*)nitroanilide GPN), Hippuryl-His-Leu (HHL), Hippuryl-Lys (HL), pentobarbital, dexamethasone, penicillin, gentamycin, glutamine, trypsin 0.25%, phosphate buffered saline, Lowry reagent, Folin and Ciocalateu's Phenol Reagent, bovine serum albumin, *p*-nitrophenylphosphate, 2-amino-2-methyl-1-propandiol, *p*-nitrophosphate, succinic acid, potassium cyanide, 2-6-dichlorophenolindophenol, nicotinamide adenine dinucleotide phosphate (NADPH), and DNase I type IV were obtained from Sigma Chemical Company (Poole, UK). Whole lungs and lung cells were from pathogen-free male CD rats (University of Wales, Cardiff), A549 alveolar cells were obtained from the European Collection of Animal Cell Cultures.

2.2. Preparation of lung cells

ATII cells were isolated from rat lungs by the method of Richards et al. (1987) with slight modification. Macrophages were obtained by repeated bronchoalveolar lavage with NaCl 0.15 mM as part of the ATII cell isolation procedure, then washed twice by centrifugation $250 \times g$ for 20 min (Brain and Frank, 1968). The ATII cells were seeded at 1×10^6 cells/cm^2 in 12-well tissue culture plates and incubated at 37°C in 95% air:5% CO_2. Cell culture medium (DMEM supplemented with 10% FBS, glutamine 8 mM, penicillin 100 U/ml gentamycin 50 μg/ml, and dexamethasone 0.1 μM) was replaced every second day. The cultured alveolar cells were used for experiments on day 2 (ATII cells) and day 7 (ATI-like cells). A549 cells (passage number 88–90) were seeded in 25 cm^2 flasks at a density of 4×10^4 cells/cm^2 and incubated at 37°C in 95% air:5% CO_2. Cell culture medium (DMEM supplemented with 10% FBS) was replaced every second day, and cells were used for experiments at day 10. To prepare whole rat lung homogenate, the trachea and main bronchi were dissected away from the lung, the remaining tissue was finely chopped then homogenised using a Dounce homogeniser and filtered through a 30 μm nylon filter.

2.3. Preparation of plasma membrane fractions

The cultured epithelial cells (ATII cells, ATI-like cells and A549 cells) were harvested from tissue culture plastic using a cell scraper. The cells were suspended in homogenisation buffer (NaCl 133 mM, KCl 5.2 mM, NaH_2PO_4 6.0 mM, Na_2HPO_4 1.0 mM, sucrose 0.3 mM, Tris 10.0 mM) and rinsed into 50-ml centrifuge tubes. Cells were disrupted by two × 20-s bursts of an ultrasonic disintegrator (MSE, Crawley, UK) 60% power × 150 Watts at 20 kHz to produce the homogenate. Cell disruption was confirmed microscopically.

Plasma membrane fractions (PMF) were prepared from the cells by (i) centrifugation at $300 \times g$ for 10 min, (ii) centrifugation of the supernatant at $2{,}000 \times g$ for 20 min, (iii) layering of the pellet onto a discontinuous sucrose gradient (consisting of 0.7 ml each of 0.5, 0.7, 0.9 mM sucrose, and a 1.4 ml cushion of 1.2 mM sucrose) and centrifugation at $95{,}000 \times g$ for 60 min. The plasma membrane fraction was collected from the interface of the 0.9 and 1.2 mM sucrose solutions. The entire procedure, as illustrated in Fig. 1, was conducted at 4°C.

Protein concentration and marker enzyme recovery was assessed in each fraction produced

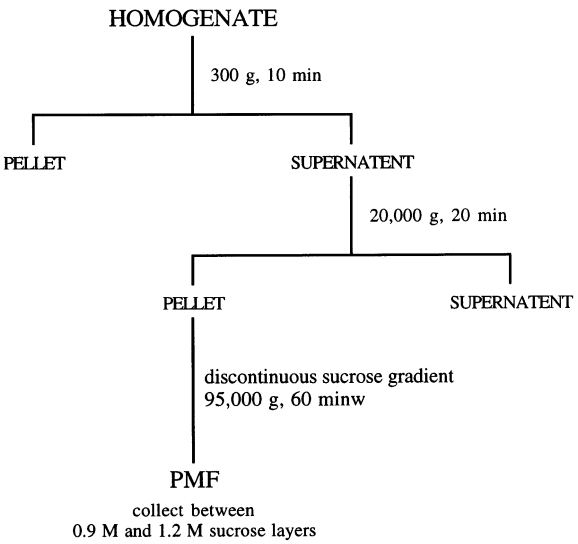

Fig. 1. Procedure for preparing PMF from cell homogenate.

during the preparation of the plasma membranes and marker enzyme enrichment was assessed in the final PMF. Protein was measured using the micro-Lowry method using bovine serum albumin as the standard (Peterson, 1977). Adaptations of reported methods were used to monitor the distribution of marker enzymes: alkaline phosphatase (AP) was used as a marker for the plasma membranes (Sigma Kit 104-LS), succinic dehydrogenase (SD) was used as a marker for mitochondria (Green et al., 1955), and NADPH cytochrome c reductase (NCCR) was used as a marker for endoplasmic reticulum (Phillips and Langdon, 1962). Marker enzyme assays used 50 μl plasma membrane fraction (protein content 4–40 μg) in a final incubation volume of 250 μl.

2.4. Peptidase enzyme assays

Peptidase substrates were assayed using selective substrates in a method adapted from Howell et al. (1992). Concentrations of peptidase substrates in the final incubation mixture were 0.12 mM TAGPL for neutral endopeptidase 24.11 (NEP) activity, 1.2 mM LN for aminopeptidase N (AMN) activity, 1.2 mM GPN for dipeptidylpeptidase IV (DPP IV) activity, 1.8 mM HL for carboxypeptidase M (CPM) activity, and 1.2 mM HHL for angiotensin-converting enzyme (ACE) activity. Cobalt chloride was added to the CM assay at a final concentration of 1 mM. All determinations were initial velocity calculations based on the linear portion of the enzyme reaction with the substrate utilisation restricted to the first 12% of the initial concentration.

Incubation mixtures consisted of 50 μl of plasma membrane fraction (4–40 μg protein) and 200 μl of peptidase substrate in Hanks Balanced Salt Solution. Activities of peptidases were studied at 37°C, aliquots were taken periodically for up to 15 h according to the activity of the peptidase enzyme. Samples were treated by the immediate addition of 100 μl of ice-cold acetonitrile, followed by centrifugation at $1000 \times g$ for 10 min. Substrate concentrations in the sample supernatants were analysed by HPLC. Each assay was performed in triplicate and controls consisted of PMF which had been boiled for 10 min before the assay. Enzyme activity was expressed as mU/mg protein.

2.5. Analytical methods

Peptidase substrate concentrations were determined by HPLC using assay conditions reported previously (Forbes et al., 1995). HPLC analysis was carried out using a P2000 binary pump, UV1000 detector, and Datajet integrator (Spectra-Physics, UK) on a spherisorb ODS2 C18 column (25 cm $\times$ 4.6 i.d., 5 μm). Marker enzyme and protein micro-assays were analysed using a microtitre plate-reader with absorbance determined at 405 nm for the AP assay, 620 nm for the SD and NCCR assays and 690 nm for the protein assay.

3. Results

The isolation of ATII cells resulted in a yield of $10.3 \pm 3.3 \times 10^6$ macrophage cells per rat lung, with a purity of $> 90\%$. In addition, each lung yielded $7.6 \pm 0.6 \times 10^6$ cells recovered in the bronchoalveolar lavage fluid. The purity of ATII cells was 95% at day 2 of culture. Confluence was reached in 4–5 days and the morphology of the cells changed from ATII to ATI cell-like between days 2 and 7 in culture as previously described (Dobbs et al., 1988; Cheek et al., 1989; Danto et al., 1992). The A549 epithelial cells proliferated rapidly in culture. After seeding at 5×10^4 cells/cm^2 the A549 cells reached confluency in 75–80 h (cell density 1.1 cells/cm^2, lag time 18 h, doubling time 18 h) and resembled type II cells with their cuboidal shape and lamellar bodies.

No intact cells were visible following disruption of the cell suspensions by sonication. Approximately 5% of the total protein content of the crude homogenate was recovered in the PMF. Mass balance of the marker enzymes calculated from all the fractions of the purification process was of the order AP 85–118%, SD 62–85%, NCCR 99–133%. Within experimental error there was no major problem with loss, deactivation, or activation of marker enzymes during the fractionation procedure. The levels of AP in the PMF

Table 1
Marker enzyme activity of plasma membrane preparations (mean ± SEM)

Cell type	Marker enzyme	Enzyme activity (mU/mg protein)		
		Homogenate	Plasma membrane	Enrichment factor
Lung homogenate ($n = 2$)	Alkaline phosphatase	994.5	914.3	9.2
	Succinic dehydrogenase	9.3	18.5	2.0
	NADPH cytochrome c reductase	5.7	1.8	0.3
Macrophages ($n = 3$)	Alkaline phosphatase	2.2 ± 1.0	24.4 ± 6.4	12.0
	Succinic dehydrogenase	5.1 ± 1.2	7.1 ± 2.1	1.4
	NADPH cytochrome c reductase	3.8 ± 0.8	1.1 ± 0.6	0.3
A549 cells ($n = 3$)	Alkaline phosphatase	125.8 ± 21.3	1397.6 ± 127.3	11.0
	Succinic dehydrogenase	6.3 ± 2.2	8.2 ± 2.3	1.3
	NADPH cytochrome c reductase	4.9 ± 1.3	2.0 ± 0.7	0.4
Alveolar type I cells ($n = 3$)	Alkaline phosphatase	22.2 ± 4.4	217.8 ± 22.4	9.9
	Succinic dehydrogenase	3.8 ± 0.9	7.6 ± 2.0	2.0
	NADPH cytochrome c reductase	5.3 ± 0.9	1.1 ± 0.6	0.2
Alveolar type II cells ($n = 3$)	Alkaline phosphatase	246.6 ± 29.4	1777.5 ± 16.1	7.2
	Succinic dehydrogenase	5.7 ± 1.2	7.4 ± 2.2	1.3
	NADPH cytochrome c reductase	5.4 ± 1.4	3.2 ± 1.4	0.6

(Table 1) indicated that this fraction contained plasma membrane material enriched approximately ten times compared to the initial homogenate, while activities of the other marker enzymes indicated some contamination by the endoplasmic reticulum, but very little mitochondrial contamination. The actual level of AP activity declined from 0.247 U/mg protein at day 2 to 0.022 U/mg protein at day 7, in accordance with the use of AP as a differentiation marker of type II cells as proposed by Edelson et al. (1988).

Initial rates of substrate utilisation were linear with respect to time for all membrane preparation-substrate incubations. The substrate utilisation-time plots which were used to calculate initial reaction velocities were all within the first 12% of substrate utilisation. The lower limits of detection for activity were 0.69 mU/mg protein for NEP, 3.08 mU/mg protein for ACE, 3.20 mU/mg protein for CPM, 0.83 mU/mg protein for DPP, and 0.41 mU/mg protein for AMN. High levels of aminopeptidase (DPP and AMN) activity were present in all PMF (Table 2). NEP also showed activity in the PMF from each cell types, but differential carboxypeptidase (ACE and CPM) expression was found according to the cell phenotype. ACE activity was lost and CPM activity was acquired during the transition from ATII to ATI-like phenotype. In parallel with these these changes DPP IV activity was reduced and AMN activity increased (Fig. 2), i.e. both the level and type of peptidase activity changed between day 2 and day 7 of primary alveolar culture. Intriguingly, the profile of peptidase activities in A549 cells was closer to that of the primary cultured alveolar cells at day 7 (i.e. ATI-like) than at day 2 when possess the ATII cell phenotype. Macrophages possessed relatively high aminopeptidase activity, but low NEP activity and no detectable carboxypeptidase activity.

4. Discussion

The lung contained the activities of all five peptidases under investigation. However, the membrane-bound peptidases of the alveolar epithelial surface represent the most relevant enzymatic threat to inhaled peptides, and are suggested to be the source of most of the proteolytic activity of alveolar cells (Bai and Kowsar, 1993). The use of PMF in this study confined the

peptidase assays to the measurement of ectopeptidase activity. The mild homogenisation procedure (cell scraping followed by two short bursts of ultrasonic energy of strictly controlled duration, frequency and intensity) was used to favour the production of large membrane sheets which are more easily recovered and purified and are less prone to vescicularisation than smaller fragments. Damage to organelles is also minimised which favours subsequent separation of subcellular components and minimises contamination of the final preparation. The PMF of each cell type was monitored for (i) the extent of mitochondrial or endoplasmic reticular contamination, (ii) the proportion of total plasma membrane recovered in the final fraction, and (iii) enrichment of plasma membranes relative to the starting material (Table 1). AP is a selective marker for the apical membrane domain, but the apical and basolateral membrane domains have similar physical properties and are generally co-isolated unless special techniques are used to separate them. No attempt was made to separate the apical and basolateral membranes in this study. For the peptidase assays, prolonged incubation times were used to increase assay sensitivity and minimise inaccuracies caused by inadequate mixing, temperature control, and sample handling (Howell et al., 1992).

The activity of plasma membrane-bound peptidases in primary cultured alveolar epithelial cells was different at day 2 and day 7 in culture. In common with epithelial cells of the bronchi and gastrointestinal epithelium (Howell et al., 1992; Proud et al., 1994), the highest activities in each cell type were those of the aminopeptidases. AMN is recognised to be so widely expressed that it has been used as a marker for epithelial cell membranes and DPP activity has also been found in a wide range of absorptive mucosae (Zhou, 1994). However, specific measurement of the levels of either aminopeptidase in alveolar epithelial cell membranes has not been reported. Low levels of NEP activity were associated with each of the alveolar epithelial cell preparations. Carboxypeptidase activities were lower than those of the aminopeptidases, with ACE and CPM differentially expressed by the alveolar epithelial cells. Immunohistochemistry supports the expression of CPM by ATI cells (Nagae et al., 1993), and the acquisition of CPM activity over time in culture is consistent with the differentiation of ATII cells into ATI cells between day 2 and day 7.

It is difficult to make direct quantitative comparisons between peptidase activities measured in different studies because of methodological differences. The activities of some of the peptidases targeted in this study have been measured previously in whole lung homogenates. DPP activity was 359 mU/mg protein in rat lung using the substrate Gly-Pro-2-naphthylamide (Krepela et al., 1985), activities for ACE and NEP in a fetal human lung homogenate were 2.7 and 0.6 mU/mg protein, respectively (Johnson et al., 1985), and CPM activity assayed in human lung membrane fraction using 5-diamethylamino-1-naphthalenesulfonyl-Ala-Arg was 3.3 mU/mg protein (Nagae et al., 1993). Peptidase activities have also been

Table 2
Peptidase activity of plasma membrane preparations

Peptidase enzyme	Peptidase activity mU/mg protein				
	Whole lung	Macrophages	A549 cells	Alveolar type I cells	Alveolar type II cells
Neutral endopeptidase 24.11	0.99	1.69 ± 0.80	2.41 ± 0.54	0.96 ± 0.37	1.81 ± 0.89
Angiotensin-converting enzyme	10.80	nd[a]	nd	nd	4.29 ± 1.28
Carboxypeptidase M	4.63	nd	4.28 ± 0.93	5.20 ± 0.82	nd
Dipeptidyl peptidase IV	69.26	46.85 ± 4.67	24.24 ± 2.49	16.29 ± 2.35	36.65 ± 3.14
Aminopeptidase N	24.77	28.28 ± 1.47	47.74 ± 6.11	23.99 ± 3.91	16.16 ± 3.30

[a] nd, not detected (mean ± SEM).

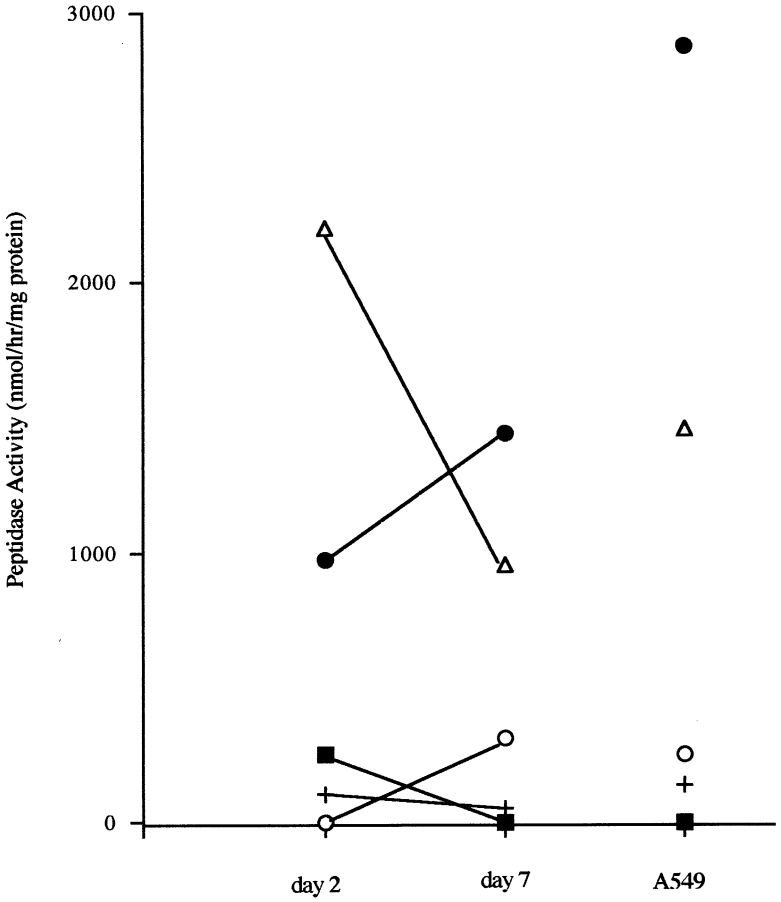

Fig. 2. Change in enzyme activity between day 2 (ATII cells) and day 7 (ATI-like cells) in culture compared with A549 cells. Neutral endopeptidase 24.11(+), angiotensin converting enzyme (n), carboxypeptidase M (o), dipeptidyl peptidase IV (Δ), aminopeptidase N (•).

measured in cells obtained by bronchoalveolar lavage. Consistent with data obtained using primary rat alveolar macrophages in this study, aminopeptidase activities have been reported to be much higher than NEP activity in human macrophages (Jackman et al., 1995) and high levels of AMN and DPP activity have also been reported in the cellular component of rat and canine lavage samples (Wall and Lanutti, 1993).

This study represents the first attempt to systematically study the activity of individual peptidases in the alveolar epithelium. The results can be compared with ectopeptidase activities measured in Caco-2 cells using similar methodology to the present study. Both Caco-2 cells and primary rat ATII cells at day 2 in culture a showed similar pattern of peptidase expression (i.e all of the peptidases except CPM), and the activities of the ectopeptidases were quantitatively similar (Howell et al., 1992).

As ATI cells comprise $>93\%$ of the alveolar epithelial surface, even taking into account the ATII cell microvilli, there is a clear rationale for using ATI-like cell monolayers as drug absorption models for the alveolar epithelium. The peptidase expression measured in alveolar epithelial cells in monolayer culture can help to explain degradation reported during transport studies using these models. The degradation profile of enkephalins by ATI-like cell monolayers after 5–7 days in culture

(Wang et al., 1993) showed abundant AMN activity, little ACE activity, and no detectable NEP activity. Aminopeptidase activity has also been reported to limit the absorption of dipeptides (Morimoto et al., 1993), insulin (Yamahara et al., 1994a), and vasopressin (Yamahara et al., 1994b). Degradation of LHRH by cultured alveolar epithelial cells has also been reported (Yang et al., 1996, 1997). The A549 lung derived epithelial cells have also been used as a drug absorption model of the alveolar epithelium (Kobayashi et al., 1996; Foster and Audus, 1996), but little peptide or protein degradation was detected during transport studies (Kobayashi et al., 1995).

A major implication for pulmonary peptide absorption is that peptides which are susceptible to exopeptidase activity will undergo significant metabolism in the lung. Our results indicate that aminopeptidase-susceptible peptides are likely to be faced with a substantial metabolic barrier during pulmonary absorption. High aminopeptidase activity at the endothelial side of the air–blood barrier will also contribute to the first pass metabolism of peptides during absorption (Forbes et al., 1995). In contrast, low levels of NEP (the major human membrane-bound endopeptidase) in the alveolar–capillary barrier help to explain the high systemic availability which can be achieved for exopeptidase-resistant compounds following inhalation. Unlike the gut which has high levels of soluble endopeptidase activity, the endopeptidase activity in lung lining fluid is low (Wall and Lanutti, 1993) and a bioavailability of approximately 50% of the respirable fraction has been achieved in man for the exopeptidase-resistant peptide leuprolide acetate following delivery to the lung (Adjei and Garren, 1990).

The time-dependant changes during cell culture represent the acquisition of many characteristics of the ATI cell phenotype (Dobbs et al., 1988; Cheek et al., 1989; Danto et al., 1992). Concurrently, we have shown that the pattern of peptidase expression changes. As a drug absorption model it is desirable that the enzyme activity and other physiological systems in cultured cells are typical of the epithelium in vivo. However, the significance of peptidase expression in the alveolar epithelium extends beyond pharmaceutical drug delivery to include physiological roles such as the regulation of autocrine and paracrine factors, processing of proproteins, modification of surfactant, and processing of antimicrobial peptides (Funkhouser et al., 1991).

In conclusion, we have measured peptidase activities in alveolar epithelial cells which we consider to be relevant to pulmonary peptide delivery. Although, individual peptides will have different affinities for enzymes, we have shown the expression of exopeptidase activity in alveolar epithelial cells which is capable of providing a substantial barrier to systemic delivery of peptides via the lung.

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