International Journal of Pharmaceutics, 76 (1991) 247–255

ADONIS 0378517391003545

IJP 02575

Aminopeptidases of newborn bovine nasal turbinate epithelial cell cultures

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(Received 27 December 1989)
(Modified version received 3 August 1990)
(Accepted 22 November 1990)

Key words: Aminopeptidase; Turbinate; Nasal epithelium; Cell culture; 4-Methoxy-2-naphthylamide; Bestatin; Puromycin

Summary

Newborn bovine nasal turbinate epithelial cell cultures were grown to confluent monolayers and characterized by electron and light microscopy. The passaged nasal turbinate cells retain some similar but not identical morphological characteristics (e.g., microvilli, cuboidal shape, dome formation, apparent mucin granules) exhibited by parent tissue. Morphological properties, however, were consistent with other nasal cell culture systems that have been used to elucidate transport and metabolic features of nasal mucosa. The activity of aminopeptidases in homogenates of the turbinate cell cultures was assayed with 4-methoxy-2-naphthylamides of leucine, alanine, and arginine as substrates. The observed pattern of hydrolysis, apparent kinetic parameters, and inhibition by typical aminopeptidase inhibitors, puromycin and bestatin, were consistent with reported characteristics of aminopeptidases associated with excised nasal epithelial cells. Results here form the basis for consideration of the continuous newborn bovine nasal turbinate epithelial cell line in examining the role of aminopeptidases in determining the metabolic fate of intranasally administered peptides and proteins.

Introduction

Intranasal delivery of some therapeutic peptides and proteins into the systemic circulation is a potentially important alternative to oral administration (Su, 1986; Lee and Longenecker, 1988). Although advantageous over oral delivery in several respects, systemic delivery of intranasally administered peptides and proteins is substantially restricted by biological barriers. Intranasal barriers to systemic delivery of peptides may include, as a minimum, the permeability characteristics of nasal mucosa, mucociliary clearance, infections, and metabolic degradation. Collectively, all of these factors may contribute in some way to the typically observed low bioavailability of intranasally administered peptides and proteins (Su, 1986; Lee and Longenecker, 1988).

Although metabolic degradation of peptides and proteins administered by the intranasal route is considered significant, neither the exact role in regulating systemic peptide delivery nor the localization of peptidases in nasal epithelial cells has

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been clearly defined. Part of the difficulty in resolving these issues is the availability of sufficient quantities of nasal epithelia, either human or animal, with which to conduct conveniently the appropriate experimentation. On this basis, a stable commercially available cell line, normal bovine nasal turbinate epithelial cells (BT; McClurkin et al., 1974), was examined for peptidase expression in this study.

Aminopeptidases, principal enzymes in peptide and protein degradation (Burbach et al., 1982; Schwartz et al., 1983; Palmieri et al., 1985), were assayed in turbinate cell homogenates with 4-methoxy-2-naphthylamide substrates (Stratford and Lee, 1986) to provide an indication of the extent to which peptidase expression is retained in the cell line. Neither the aminopeptidases nor other metabolic properties of this bovine nasal turbinate epithelial cell line have been previously characterized.

Materials and Methods

Materials

Newborn bovine turbinate cells (BT; ATCC CRL 1390), passage 24, were obtained from the American Type Culture Collection, Rockville, MD. Culture medium was purchased from Hazelton Biologics, Lenexa, KS. Equine serum was purchased from HyClone, Logan, UT. Aminopeptidase substrates, 4-methoxy-2-naphthylamides, and inhibitors, bestatin and puromycin, were purchased from Sigma Chemical Co., St. Louis, MO. All other reagents were of the highest grade commercially available.

Bovine nasal turbinate cultures

Newborn bovine turbinate cells were grown and passaged in T-75 flasks (Corning Glass Works, Corning, NY). The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with high glucose, 10% equine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 3.7 g/l sodium bicarbonate. The culture medium was changed twice per week and the cells subcultivated 1:3. Seeded at a density of 9×10^5 cells/T-75 flask, confluence was observed in 7–10 days.

Electron microscopy

Confluent monolayers were grown on rat-tail collagen coated 3 μm polycarbonate filters placed in 100 mm culture dishes. Monolayers were rinsed with phosphate-buffered saline, pH 7.4 (PBS) three times and fixed for at least 4 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Samples on filters were then post-fixed in 1% osmium tetroxide, ethanol dehydrated, Spurr's resin embedded, and thin sectioned by standard electron microscopic techniques. Thin sections were stained with 2% uranyl acetate and lead citrate and examined under a Philips EM 300 microscope (Tavakoli-Saberi and Audus, 1989).

Homogenate preparations

Confluent monolayers of turbinate epithelial cells, passages 30–38, grown in T-75 flasks, were rinsed three times with PBS, pH 7.4 scraped from the flasks and collected in 2–3 ml of PBS containing 0.32 M sucrose. The suspension was homogenized for 1 min and then centrifuged at $1000 \times g$ for 5 min. The resulting supernatant was used immediately for both aminopeptidase assays described below and protein determination by the method of Lowry et al. (1951).

Aminopeptidase assays

The method of Stratford and Lee (1986) was used to assay for aminopeptidases in the tissues. Briefly, 4-methoxy-2-naphthylamides (leucine, alanine, arginine) were added to a reaction mixture consisting of 150 μl tissue supernatant and 2.8 ml of 0.05 M Tris-maleate buffer, pH 7.4, in duplicate 3 ml quartz cuvets. Before addition of the substrate to initiate the reaction, the tissue-buffer suspension was preincubated at 37 °C for 15 min. The reaction mixture was incubated at 37 °C, the sample excited at 342 nm, and the increase in fluorescence emission observed at 426 nm for 10 min. Fluorescence measurements were made with an SLM-Aminco 4800 Spectrofluorometer (Urbana, IL). Blanks consisted of a reaction mixture prepared exactly as above with tissue supernatant that had been boiled to inactivate

associated enzymes. In experiments where enzyme inhibitors were employed, 0.1 or 0.01 M concentrations of either bestatin or puromycin were added to tissue-buffer mixtures 15 min prior to addition of a 4-methoxy-2-naphthylamide substrate.

Enzyme velocities in nmol/mg per min were calculated from standard curves for fluorescence intensity vs mol β-naphthylamide and from plots of fluorescence intensity vs time. For experiments where the substrate concentration was varied, Michaelis-Menten kinetic parameters, K_m and V_{max}, were estimated with a nonlinear regression data analysis program, ENZFITTER (Elsevier-Biosoft, Cambridge, U.K.).

Results

Growth of the newborn bovine nasal turbinate epithelial cells under the conditions described above resulted in confluent monolayers after approx. 7–10 days in culture. Due to the described stability of the cell line under those conditions, manipulation of culture conditions was not considered in this study. Typically, the cells exhibited a cuboidal shape with appearance of dome structures suggesting some ion transport systems were retained in the cell line. The light microscope image in Fig. 1 represents the appearance of a typical confluent monolayer of nasal epithelial cells observed in these studies. This appearance was consistent for up to 1 month in culture (not shown). At the electron microscope level, the cells were flattened with numerous mitochondria and occasional microvilli as shown in Fig. 2. The appearance of microvilli was reproducible from culture to culture but not every cell expressed microvilli. Cilia were rarely observed in the cultures. Cells containing an abundance of granules presumed to be mucin or surfactant-filled were occasionally and consistently observed in the monolayers as shown in Fig. 3. The biochemical composition of their contents was not determined.

To facilitate comparisons between aminopeptidase expression in the cell line and that informa-

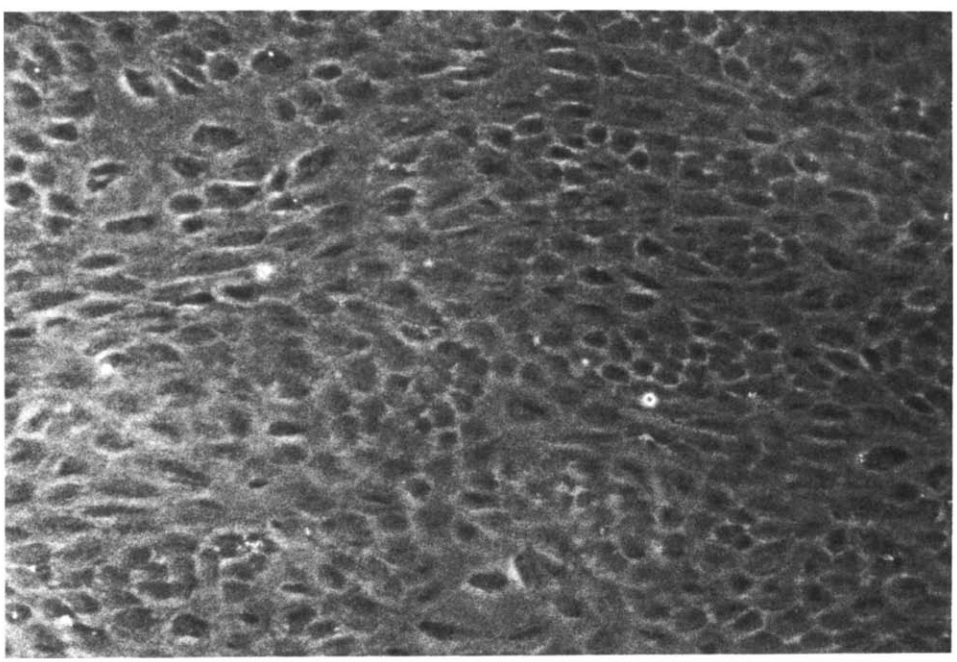

Fig. 1. 10-day-old confluent monolayer of newborn bovine nasal turbinate epithelial cells. Cells were grown as described in Materials and Methods in T-75 flasks (magnification 200 ×).

tion on aminopeptidases currently in the literature, homogenate preparations were used throughout this study. Aminopeptidases of the bovine nasal turbinate homogenates hydrolyzed the 4-methoxy-2-naphthylamide substrates in a manner consistent with Michaelis-Menten kinetics. Fig. 4 illustrates the typical relationship between release of naphthylamine and concentration of 4-methoxy-2-naphthylamide substrate. For convenience, kinetic parameters for aminopeptidase activity were assayed in mixtures of 10–14-day-old confluent monolayers. Quadruplicate

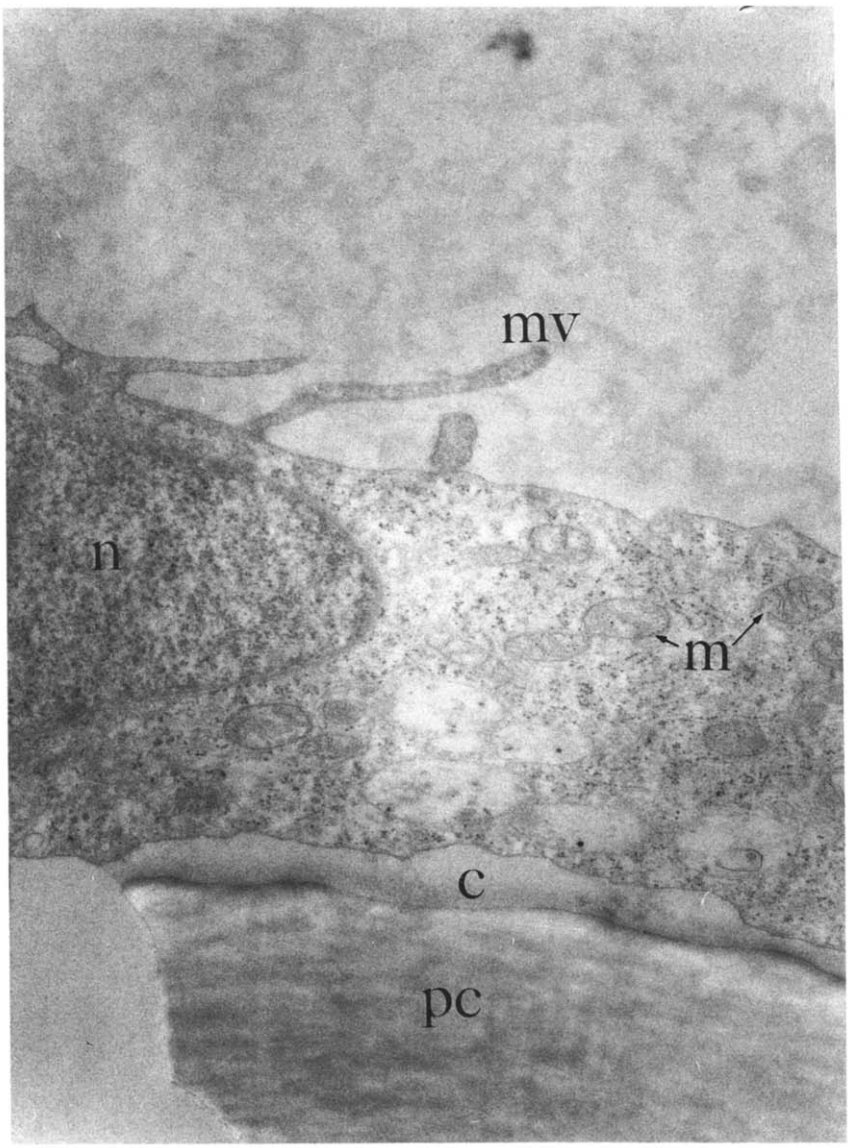

Fig. 2. Transmission electron micrograph of a cross-section through 14-day-old confluent monolayers of newborn bovine nasal turbinate epithelial cells. Shown here are the nucleus (n), microvilli (mv), mitochondria (m), collagen matrix (c), and polycarbonate membrane (pc) for a typical cross-section through a monolayer. Cells were grown on polycarbonate membranes and prepared for electron microscopy as described in Materials and Methods. (Magnification 20000 × .)

TABLE 1

Apparent Michaelis-Menten kinetic parameters for selected 4-methoxy-2-naphthylamide aminopeptidase substrates in newborn nasal turbinate epithelial cell homogenates

Substrate	Enzyme	$K_m \pm SD$ [a] (μM)	$V_{max} \pm SD$ [a] (nmol/mg per min)
Leucine	Leucine aminopeptidase (EC 3.4.11.1)	5.0 ± 1.0	16.6 ± 1.1
Alanine	Aminopeptidase N (EC 3.4.11.2)	25.9 ± 3.5	14.1 ± 1.0
Arginine	Aminopeptidase B (EC 3.4.11.6)	7.1 ± 1.0	13.0 ± 1.4

[a] Data represent the means of at least quadruplicate determinations ± SD from different cultures of passages 36–38.

samples were collected from passages 36–38 (different cultures), combined, and analyzed for appropriate kinetic parameters. According to the kinetic parameters listed in Table 1, apparent V_{max} determinations were similar for the three substrates with the leucine substrate exhibiting a slightly higher value. According to the apparent K_m determinations, the leucine and arginine substrates were observed to have about 3.5–5-times higher affinities than the alanine substrate for their respective enzymes. Statistically significant, age-dependent trends in enzyme kinetic parameters over the 10–14-day-old monolayers were not observed (not shown).

The hydrolysis of leucine- and alanine-4-methoxy-2-naphthylamide substrates by amino-

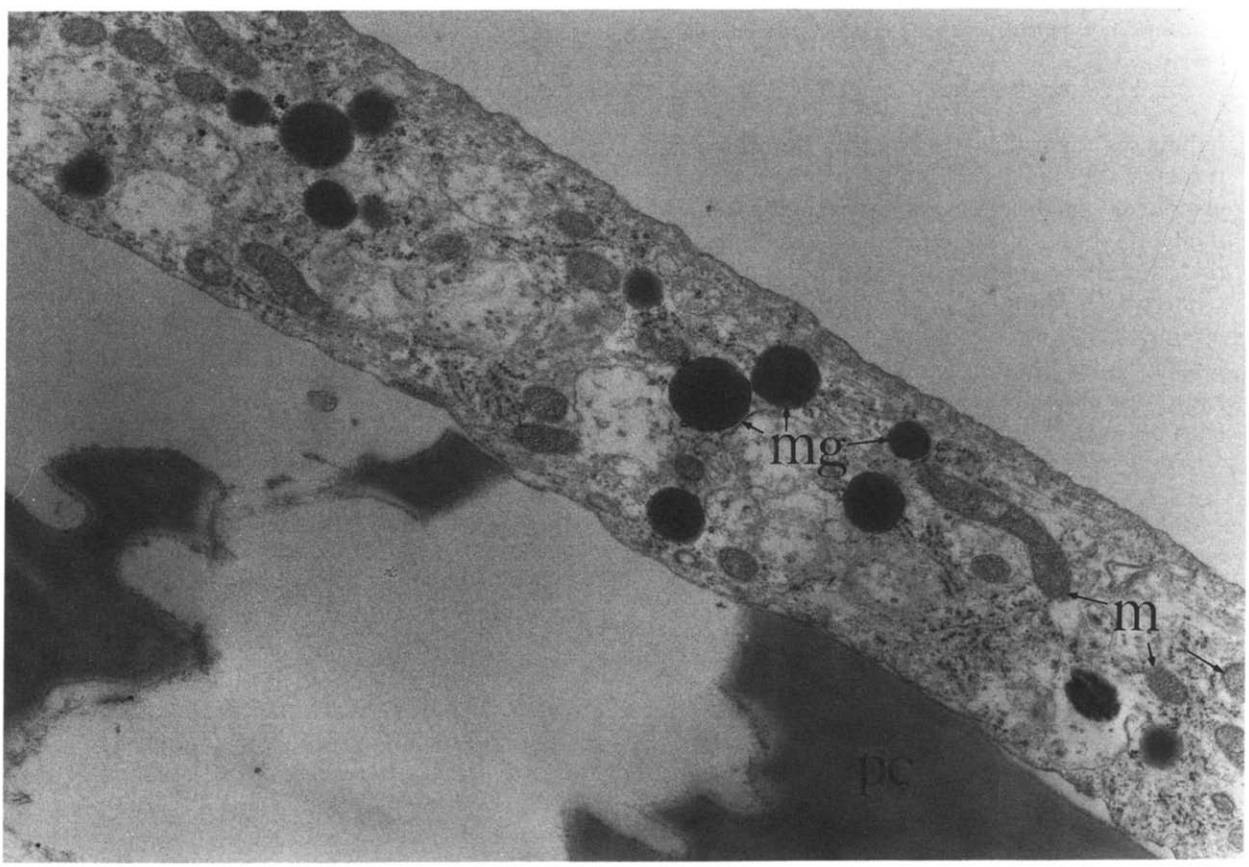

Fig. 3. Transmission electron micrograph of a cross-section through 14-day-old confluent monolayers of newborn bovine nasal turbinate epithelial cells. Shown here are the mucin granules (mg), mitochondria (m), and polycarbonate membrane (pc) observed in a typical cross-section through a monolayer. Cells were grown on polycarbonate membranes and prepared for electron microscopy as described in Materials and Methods. (Magnification 8000 × .)

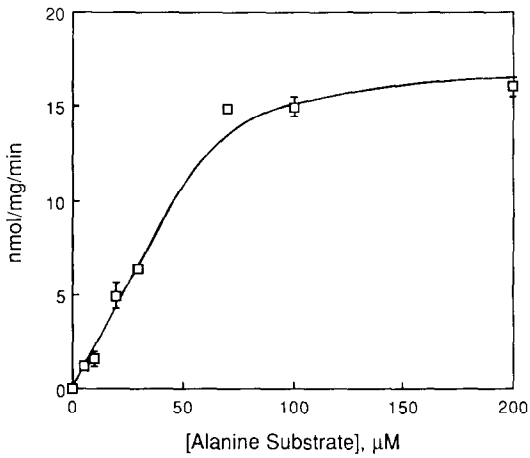

Fig. 4. Concentration dependence of the hydrolysis of a representative 4-methoxy-2-naphthylamine substrate to naphthylamine (nmol/mg per min) in homogenates of bovine nasal turbinate epithelial cells. Data points represent the means of quadruplicate determinations ± SD from different cultures of passages 36–38.

peptidases associated with the bovine nasal turbinate epithelial cells was strongly but not completely inhibited by typical aminopeptidase inhibitors, bestatin and puromycin. Hydrolysis of the arginine-4-methoxy-2-naphthylamide substrate was moderately inhibited by bestatin and puromycin. Table 2 contains a summary of data for the inhibition of aminopeptidase substrate hydrolysis in the presence of two concentrations each of bestatin and puromycin.

TABLE 2

Inhibition of newborn bovine nasal turbinate epithelial cell aminopeptidases assayed with 4-methoxy-2-naphthylamide substrates

	% Inhibition [a] of substrate hydrolysis		
	Leucine	Alanine	Arginine
[Puromycin]			
10 μM	16.7 ± 6.7	41.6 ± 1.8	6.1 ± 0.5
100 μM	58.6 ± 3.0	46.9 ± 4.7	29.7 ± 8.8
[Bestatin]			
10 μM	31.8 ± 5.6	39.8 ± 1.4	21.2 ± 5.6
100 μM	37.4 ± 1.4	50.0 ± 6.8	24.4 ± 2.6

[a] Data represent the means of at least quadruplicate determinations ± SD from different cultures of passages 36–38.

Discussion

The availability of substantial amounts of either human or an appropriate animal source of tissues for characterizing biochemical features of the nasal epithelia has been limited. The continued need for generally limited nasal tissues to study both normal and diseased conditions of nasal epithelia at the biochemical or cellular level can be overcome by expanding the available tissue through development of appropriate tissue culture systems (Wu et al., 1985). Commercially available nasal epithelial cell lines have been derived from tumor tissues, and several primary culture systems of nasal tissues from normal and diseased turbinate, septal, and tracheal origin have been developed. Most of these culture systems retain properties similar, but not identical, to parent tissues (Audus et al., 1990). Despite the absence of characteristics identical to those of the parent tissue, all of these tissue culture systems have found utility in the investigation of a variety of basic biological features of nasal epithelium including ion transport (Schoppa et al., 1989; Willumsen and Boucher, 1989; Willumsen et al., 1989b), bioelectric properties of normal and diseased tissue (Coleman et al., 1984; Yankaskas et al., 1985), growth factor production (Ohnishi et al., 1989), and chronic cytotoxicity and carcinogenesis (Newton et al., 1980; Brittebo et al., 1983; Steele and Arnold, 1985; Hood et al., 1987; Rutten et al., 1988).

The continuous bovine nasal turbinate epithelial cell line described here represents one of the few normal nasal cell lines available for examining the biochemistry of nasal epithelia. Appealing features of this cell line include the lack of morphological changes on up to 81 passages and the consistency of genetic composition except at high passage number (McClurkin et al., 1974). This cell line has been available for a number of years to grow viruses for vaccine development. Based on reported stability and availability, the cell line was chosen for examination of nasal aminopeptidases.

Cilia, microvilli, mucin granules, and the biochemical composition of the mucin have been considered as markers for differentiation of air-

way mucosa (Wu et al., 1985). Bovine nasal turbinate epithelial cell monolayers exhibited some of the typical characteristics of the epithelial cell morphology including microvilli, apparent mucin granules, cuboidal shape, and domes. These features were consistent, but not identical, with those of the pseudostratified epithelium representing the drug absorption sites of the nasal cavity (Jafek, 1983; Petruson et al., 1984; Lee and Longenecker, 1988). For instance, the flattened appearance and minor numbers of microvilli were in contrast to expectations for a turbinate epithelial cell. Cilia were rare in these cultures whereas normally they occur on about 15–20% of turbinate epithelia in vivo. On the other hand, morphological features of this cell line were consistent with observations in other primary and passaged culture systems derived from animal and human nasal turbinate epithelial cells (Steele and Arnold, 1985; Wu et al., 1985; Yankaskas et al., 1985; Schoppa et al., 1989). Only recently have more fully differentiated nasal epithelial cell cultures been described. Nasal epithelial cells isolated from the tracheal region differentiate to a morphology similar to that of the parent tissue in primary culture (Schoppa et al., 1989; Willumsen et al., 1989a). The functional and biochemical differences between epithelia from turbinate and tracheal regions of the nasal cavity have not yet been determined. Further manipulations of the culture conditions for the turbinate cell line described here, to promote further morphological differentiation seen in tracheal cultures, are currently under evaluation.

The aminopeptidase activity associated with the nasal cavity has been observed to be significant in both rat (Su et al., 1985) and tissue homogenates from rabbit (Stratford and Lee, 1986). We employed as substrates, 4-methoxy-2-naphthylamides, derivatives of which are considered rather specific for aminopeptidases (Sylven and Bois, 1962; Stratford and Lee, 1986). In our studies, aminopeptidase activities (apparent V_{max} values) in bovine nasal turbinate epithelial cells as assayed with the 4-methoxy-2-naphthylamide substrates were similar to those described by Stratford and Lee (1986). Enzyme activities were also in good agreement with preliminary work in our laboratory with homogenates of adult bovine nasal septal epithelium. While apparent K_m values have not previously been determined for nasal epithelial cell aminopeptidases, our results indicate that the apparent K_m values in the micromolar concentration range closely parallel typical findings in other epithelial and endothelial cells (Baranczyk-Kuzma and Audus, 1987; Kenny et al., 1987).

Hydrolysis of all three of these 4-methoxy-2-naphthylamide substrates by the cultured turbinate cells was sensitive to puromycin, an arylamidase inhibitor, and bestatin, a general aminopeptidase inhibitor (Hui et al., 1983). That a given aminopeptidase activity may represent the presence of one or more enzyme systems can be illustrated with the inhibitors. For example, leucine aminopeptidase has been shown to be strongly inhibited by bestatin but not by puromycin (Stratford and Lee, 1986). The hydrolysis of the leucine 4-methoxy-2-naphthylamide substrate by both bestatin and puromycin in this system was observed. Therefore, the aminopeptidase activity determined with the leucine substrate here likely represents the existence of additional peptidases capable of hydrolyzing the substrate. These results were in good agreement with the findings of Stratford and Lee (1986) who demonstrated that leucine aminopeptidase was not a predominant peptidase in various mucosal cells including nasal epithelium. Additionally, the lack of absolute specificity of the 4-methoxy-2-naphthylamide substrates used in this study precludes definitive identification of how many aminopeptidases may be present in this cell line. Similar conclusions regarding the possible number of aminopeptidases represented in the rabbit nasal homogenates were also reached by Stratford and Lee (1986).

Homogenate preparations were used in this study to facilitate comparisons of the cell line's expression of aminopeptidase activity with existing published information on excised nasal tissues. Although homogenates were used in their studies, Stratford and Lee (1986) have noted that these homogenate preparations disrupt normal cell configurations and do not provide for subcellular localization of the enzymes. As a result,

observed peptidase action on a particular peptide or protein substrate may occur in homogenates that may not be typically observed in an intact cell system. The cell line offers the opportunity to conduct peptidase studies with intact monolayers of nasal epithelium. Unfortunately, however, information on intact parent tissue (e.g., newborn tissue here) aminopeptidases has been lacking. In fact, the work of Stratford and Lee (1986) remains one of the few reports to have surveyed, to some extent, peptidase activity of the nasal mucosa. Accordingly, direct correlations of this and previous works with intact nasal tissue, particularly from the human nasal cavity, remain to be established before the potential of these systems in studying nasal degradation of peptides may be realized.

To conclude, morphological characteristics of a continuous bovine nasal turbinate epithelial cell line were not identical to those of the parent tissue. This observation was consistent with reported observations for available turbinate cell culture systems. Our findings indicate that the nasal turbinate cell line retains expression of aminopeptidases with properties representative of excised nasal tissue homogenates described by others. These results form the basis for considering the nasal turbinate cell line as a potential tool to evaluate the role of nasal aminopeptidases in degrading peptides. Provided that a better understanding of factors eliciting appropriate morphological differentiation is established, the cell line may find utility in characterization of the cellular localization of nasal aminopeptidases.

Acknowledgements

This work was supported by a grant from INTERx Research Corporation, Lawrence, KS and a Life Sciences Contacts Award (K.L.A.) from Lilly Research Laboratories, Indianapolis, IN.

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