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In-vitro nasal drug delivery studies: comparison of derivatised, fibrillar and polymerised collagen matrix-based human nasal primary culture systems for nasal drug delivery studies

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Abstract

The aim of this study was to establish a collagen matrix-based nasal primary culture system for drug delivery studies. Nasal epithelial cells were cultured on derivatised (Cellagen membrane CD-24), polymerised (Vitrogen gel) and fibrillar (Vitrogen film) collagen substrata. Cell morphology was assessed by microscopy. The cells were further characterised by measurement of ciliary beat frequency (CBF), transepithelial resistance (TER), permeation of sodium fluorescein, mitochondrial dehydrogenase (MDH) activity and lactate dehydrogenase (LDH) release upon cell exposure to sodium tauro-24, 25 dihydrofusidate (STDHF). Among the three collagen substrata investigated, the best epithelial differentiated phenotype (monolayer with columnar/cuboidal morphology) occurred in cells grown on Cellagen membrane CD-24 between day 4 and day 11. Cell culture reproducibility was better with Cellagen membrane CD-24 (90%) in comparison with Vitrogen gel (70%) and Vitrogen film (< 10%). TER was higher in cells grown on Vitrogen gel than on Cellagen membrane CD-24 and Vitrogen film. The apparent permeability coefficient ($P_{app} \times 10^{-7}$cm s^{-1}) of sodium fluorescein in these conditions was 0.45 ± 0.08 (Vitrogen gel) and 1.91 ± 0.00 (Cellagen membrane CD-24). Except for LDH release, CBF and cell viability were comparable for all the substrata. Based on MDH activity, LDH release, CBF, TER and permeation studies, Cellagen membrane CD-24- and Vitrogen gel-based cells were concluded to be functionally suitable for in-vitro nasal drug studies. Vitrogen film-based cultures may be limited to metabolism and cilio-toxicity studies.

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Introduction

Many research groups are actively involved in developing and validating nasal cell culture systems to serve as alternatives to animal investigations for nasal drug delivery studies. We have recently demonstrated the relevance of primary cultures of human nasal epithelium for in-vitro nasal toxicological investigations (Agu et al 1999, 2000; Ugwoke et al 2000a, b). Efforts to develop and characterise nasal culture systems for drug metabolism and permeation studies are still in their infancy. This emphasises the need for development of new culture systems or improvement of already existing ones.

Specialised epithelial functions such as barrier formation and vectorial transport of solutes depend on formation of tight junctions that separate the plasma

Table 1 Summary of culture conditions and physical characteristics of collagen preparations investigated.

Parameters for comparison	Collagen substrata[a]		
	Fibrillar collagen (Vitrogen film)	Polymerised collagen (Vitrogen gel)	Derivatised collagen (Cellagen membrane CD-24)
Method of collagen preparation	Polymerisation/drying of 3 mg mL^{-1} solution (100 μL cm^{-2})	Polymerisation of 3 mg mL^{-1} solution (100 μL cm^{-2})	Patented derivatisation/stabilisation method
Visual appearance when wet	Thin, non-pliable film coated to filter membrane	Thick hydrated soft gel coated on filter membrane	Hydrated, thin, pliable stabilised film
TEM appearance	Collapsed structure due to drying	Thickness varies widely across the entire surface	Flat and interwoven with very few surface defects
SEM appearance	Coils of several fibrils in random orientations without alignment and with loose strands on the surface	More coils of several fibrils in random orientations without alignment and with more loose strands on the surface than in fibrillar film	Uniform layer of collagen with few fibrils (on the surface). The fibrils are colinear with long uninterrupted longitudinal sections
Culture medium	DMEM-F12 supplemented with Ultroser G (2%) + choleratoxin (10 ng mL^{-1}) + streptomycin (50 μg mL^{-1})/penicillin (50 IU mL^{-1})	DMEM-F12 supplemented with Ultroser G (2%) + choleratoxin (10 ng mL^{-1}) + streptomycin (50 μg mL^{-1})/penicillin (50 IU mL^{-1})	DMEM-F12 supplemented with Ultroser G (2%) + choleratoxin (10 ng mL^{-1}) + streptomycin (50 μg mL^{-1})/penicillin (50 IU mL^{-1})
Culture method	Air–liquid interface	Air–liquid interface	Air–liquid interface
Seeding density	10^6 cells cm^{-2}	10^6 cells cm^{-2}	10^6 cells cm^{-2}
Incubation condition	95% O_2 − 5% CO_2	95% O_2 − 5% CO_2	95% O_2 − 5% CO_2
Days of cell morphology assessment	Day 7 and Day 12	Day 7 and Day 12	Day 7 and Day 12

TEM, transmission electron microscopy; SEM, scanning electron microscopy. [a]Description of physical characteristics based on microscopy conducted in our laboratory, Grinnell & Bennett (1982), Macklis et al (1984) and product information provided by ICN Biomedicals, Belgium.

membrane into apical and basolateral membranes. Such differentiation is expectedly dependent on the cell-support matrices (e.g. collagen, laminin, extracellular matrix) (Yankaskas et al 1985; Baeza-Squiban et al 1994). Homologous cell–cell interaction, presence of soluble factors (nutrients, Ca^{2+}, O_2/CO_2), matrix interaction, polarity and shape of the cells are also important variables that determine the extent of epithelial cell differentiation in-vitro (Folkman & Moscona 1978; Strom & Michalopoulos 1982; Shannon et al 1987). These variables are reasonably influenced by the architecture of the cell-support matrices. Baeza-Squiban et al (1994) reported that dried collagen film induced cell monolayer formation in cultured rabbit tracheal epithelium, while collagen gel caused cell multi-layering. Similarly, floating hydrated collagen gel after cell attachment resulted in formation of pseudostratified and predominantly ciliated nasal epithelium (Hanamure et al 1994).

To use nasal epithelial cells cultured on collagen for routine drug delivery studies, additional improvement with respect to cell culture reproducibility, reduction of media components, improvement of time to attain confluence and better epithelial differentiation are important considerations (Schmidt et al 1998).

The objective of this study was to establish a collagen matrix-based human nasal primary culture system for in-vitro drug delivery studies using a simple culture medium. Based on literature information Vitrogen gel, Vitrogen film and Cellagen membrane CD-24 were selected for this study. These collagen substrata are chemically identical (type I collagen), but physically different. The physical differences stem from the method of collagen gel, film and membrane preparation.

The various collagen substrata were classified according to their physical characteristics and method of preparation as follows: derivatised collagen (Cellagen membrane CD-24), polymerised collagen (Vitrogen gel) and fibrillar collagen (Vitrogen film). The physical characteristics and the cell culture conditions investigated are summarised in Table 1. The influence of these collagen substrata on human nasal epithelial cell differentiation, function and culture reproducibility was investigated and compared. The implications of these attributes for the selection of optimal collagen preparation to grow nasal epithelial cells for in-vitro drug

delivery studies (permeability, metabolism, mucosal and cilio-toxicity) were demonstrated.

Materials and Methods

Chemicals

Dimethyl sulfoxide (DMSO) and sodium tauro-24, 25 dihydrofusidate (STDHF) were obtained from Riedel-Haen (Seelze, Germany) and Leo Pharmaceuticals (Copenhagen, Denmark), respectively. Sodium bicarbonate (S8875), Triton X-100 (T6878), NADH (N8129), pyruvic acid (P2256) and (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolinium bromide (MTT)) were supplied by Sigma (St Louis, MO). DMEM F12 1/1, phosphate buffered saline, Hanks' balanced salt (HBBS), Ultroser G and fetal calf serum (FCS) were obtained from Life Technologies (Paisley, UK). Vitrogen solution was provided by Cohesion (Palo Alto, CA). Transwell polycarbonate inserts, Millicel-CM inserts and Cellagen membrane CD-24 were provided by Corning (NY), Millipore (Bedford, MA), and ICN Biomedical (Costa Mensa, CA), respectively.

Cell culture procedure

The cell culture protocol used for this study was based on the selection, modification and combination of optimal conditions of the cell culture methods described for human nasal and tracheal epithelia (Jorissen et al 1989; Yamaya et al 1992; Blank et al 1995).

Ultrastructurally normal human nasal epithelial tissues obtained from 8 patients undergoing elective surgery were dissociated enzymatically overnight at 4°C using 0.1 % pronase (Sigma, St Louis, MO). The pronase was competitively inhibited with 10 % FCS and the cells were filtered through 60 or 70-μm nylon mesh or polypropylene filters (Pall, Portsmouth, UK) to remove cell debris and to obtain a homogenous cell suspension. Subsequently, the cells were washed three times in DMEM-F12 1/1 supplemented with FCS 5 %, streptomycin 50 μg mL^{-1} and penicillin 50 IU mL^{-1}, before counting with a Coulter Multisizer counter (Northwell, UK). Incubation of the cells on plastic for 1 h reduced fibroblast contamination. Cells were then plated at a density of 5.0×10^5 to 1.0×10^6 cells cm^{-2} to grow on various supports. Millicel-CM inserts (0.45 μm pore size, 0.6 cm^2 area; Millipore, Bedford, MA) and Transwell polycarbonate inserts (0.4 μm pore size, 1 cm^2 area; Corning, NY) were either not coated or coated with Vitrogen gel and Vitrogen film. Cells were also seeded on Cellagen membranes, CD-24 (effective growth surface area 0.785 cm^2, 4000 MW cut off; ICN Biomedicals, Costa Mensa, CA). Vitrogen gel and film were cross-linked with 25 % ammonia vapour for 30 min. Drying of Vitrogen gel to form Vitrogen film was done under laminar workstation at room temperature overnight. Before cell plating, the substrata were conditioned by incubation with DMEM-F12 supplemented with 5 % FCS (0.5–1.0 mL, added to the basolateral compartment) for 30–60 min at 37°C. Cells were maintained in this medium for 24 h to allow maximum attachment. Thereafter, the medium was changed to DMEM-F12 1/1 supplemented with Ultroser G (2 %), streptomycin 50 μg mL^{-1}, penicillin 50 IU mL^{-1} and choleratoxin 10 ng mL^{-1}. The medium was subsequently changed every day and the culture maintained at air–liquid interface until used for experiments (up to day 12). The volume of medium added to the basolateral compartment ranged from 0.3 to 0.5 mL depending on the inserts used to avoid the detrimental effect of high hydrostatic pressure on cell attachment and proliferation. The cells were carefully washed every two days to reduce the accumulation of cell metabolic products. Cell culture reproducibility was calculated as the number of inserts developing adequate transepithelial electrical resistance ($\geqslant$ 150 Ω cm^2 with respect to total inserts seeded with cells). A total of 48 inserts (for each type of substratum) was used to estimate the culture reproducibility.

Morphological studies

Ultrastructural organisation and the level of epithelial cell differentiation were assessed using phase contrast and scanning electron microscopy. The cells for scanning electron microscopy were fixed in glutaraldehyde 3.0 % in 0.1 M sodium cacodylate buffer pH 7.4 for 2 h and were dehydrated in graded ethanol series. The dehydration process was completed by a critical point drying (E300-polaron) with CO_2. Subsequently, specimens were mounted in aluminium stubs, sputter-coated with gold (E5100-polaron), and viewed with a scanning electron microscope (Philips XL20, The Netherlands).

Biochemical, functional and toxicological studies

MTT assay

MTT assay was used to monitor the viability of the cells grown on the various collagen substrata.

The MTT assay protocol used for this study was adapted from the method described by Hovgaard & Brondsted (1995), with some modifications. Briefly, MTT (2 mg mL^{-1}) was dissolved in DMEM-F12 1/1 on the assay day. Subsequently, 1 mL of the solution was added to the cells and incubated for 2 h at 37°C.

Formazan crystals were extracted from the cells using 1 mL DMSO on a rotor (80 rev min^{-1}) for 1 h. Formazan absorbancy was measured using a multi-well spectrophotometric scanner (Beckman, Irvine, CA) at excitation and emission wavelengths of 540 and 650 nm, respectively, using DMSO as a blank. The MTT assay was conducted on days 7 and 12. The assay conducted on day 1 with 1.0×10^6 cells (number of cells seeded per insert, n = 3) served as control to monitor the change in cell viability with time. Mitochondrial dehydrogenase (MDH) enzyme activity, as reflected by formazan formation, was used to assess the absolute viability of the cells (number of viable cells per square cm of insert) (Wadell et al 1999).

Lactate dehydrogenase assay

The cells were treated with either STDHF (0.5%), an absorption enhancer known to cause cellular damage (positive control), or with PBS (negative control) for 30 min on days 7 and 12. LDH activity was assayed using a kinetic method (Roesems et al 1997). To determine the amount of lactate dehydrogenase (LDH) inside the cells, Triton X-100 in PBS was used to disrupt the cell following 1 h incubation. LDH activity in the lysate or supernatant was assayed using a Beckman DU-Spectrophotometer (Beckman, Irvine, CA) at a wavelength of 390 nm. The LDH substrate consisted (per 80 mL PBS) of pyruvic acid (sodium salt 18.32 mg, P2256), NADH (disodium salt 21.28 mg, N8129), $NaHCO_3$ (31.76 mg, S8875). The rate of reduction of pyruvic acid (within 4 min at 10–15-s intervals) due to the presence of LDH was calculated using Soft Pac kinetic module (Beckman, Irvine, CA). Only the linear part of the curve with a minimum correlation coefficient of 0.990 was used. The % $LDH_{release}$ was calculated as the relative decrease in rate of absorbancy using equation 1:

$$\%\ LDH_{release} = (LDH_{supernatant}/(LDH_{supernatant} + LDH_{cell})) \times 100 \quad (1)$$

Ciliary beat frequency (CBF) studies

The number of ciliated cells and their CBF were qualitatively and quantitatively assessed every day for a period of 12 days.

The CBF of the cells was measured using computerised microscope photometry as described by Jorissen et al 1989. The CBF of 15 different cells, selected each day from three different inserts, was measured for 12 days to monitor the rate of CBF degeneration. The CBF of cells measured a day after seeding (to allow equilibration) served as control.

Measurement of epithelial cell resistance and permeability

The development of transepithelial (TER) electrical resistance was followed every 2 days for 12 days using Millicel ERS (Millipore, Bedford, MA). The TER values were obtained by subtracting blank filter and collagen resistance from the cell resistance and multiplying the result by the effective growth surface area. The formation of tight junctions was confirmed by investigating the transport of sodium fluorescein. The transport was performed by initially incubating the cells in transport medium (HBBS, pH 7.4, supplemented with 25 mM glucose and HEPES 15 mM) for 1 h at 37°C. Transport was initiated by placing sodium fluorescein (1 mg mL^{-1}, 0.25 mL) on the apical side and 0.75 mL of transport medium in the basolateral compartment. The transport of the dye to the basolateral compartment was monitored every 10 min (collagen preparations without cells) and at 15-min intervals (collagen preparations with cells) for a period of 60 and 120 min, respectively. The amount of sample withdrawn was immediately replaced with an equivalent volume of transport medium. The concentration of sodium fluorescein was determined spectrophotometrically at 490/520 nm wavelength using a multiple well scanner (Beckman, Irvine, CA). Permeation studies were conducted with 7–9-day-old cultures.

The apparent permeability coefficient, P_{app} (cm s^{-1}) was calculated using equation 2:

$$P_{app} = (dQ/dt) \times (V/A) \times Co \quad (2)$$

where, dQ/dt (μg s^{-1}) is the steady rate of appearance of sodium fluorescein to the basolateral side, Co (μg mL^{-1}) is the initial concentration in the apical chamber, A is the effective growth surface area of the inserts (0.6 cm^2 Millicell-CM; 0.785 cm^2 Cellagen membrane CD-24).

Data presentation and statistical analysis

Unless stated otherwise, three different inserts were used for each experiment and the results expressed as mean ± s.d., n = 3. Differences between control and treated groups with respect to LDH release, MDH activity and CBF were compared using one-way analysis of variance. The permeation of sodium fluorescein across collagen substrata with and without cells was determined using Student's *t*-test. $P < 0.05$ was considered significant.

Results and Discussion

Morphological and functional studies

We could observe human nasal epithelial cells with

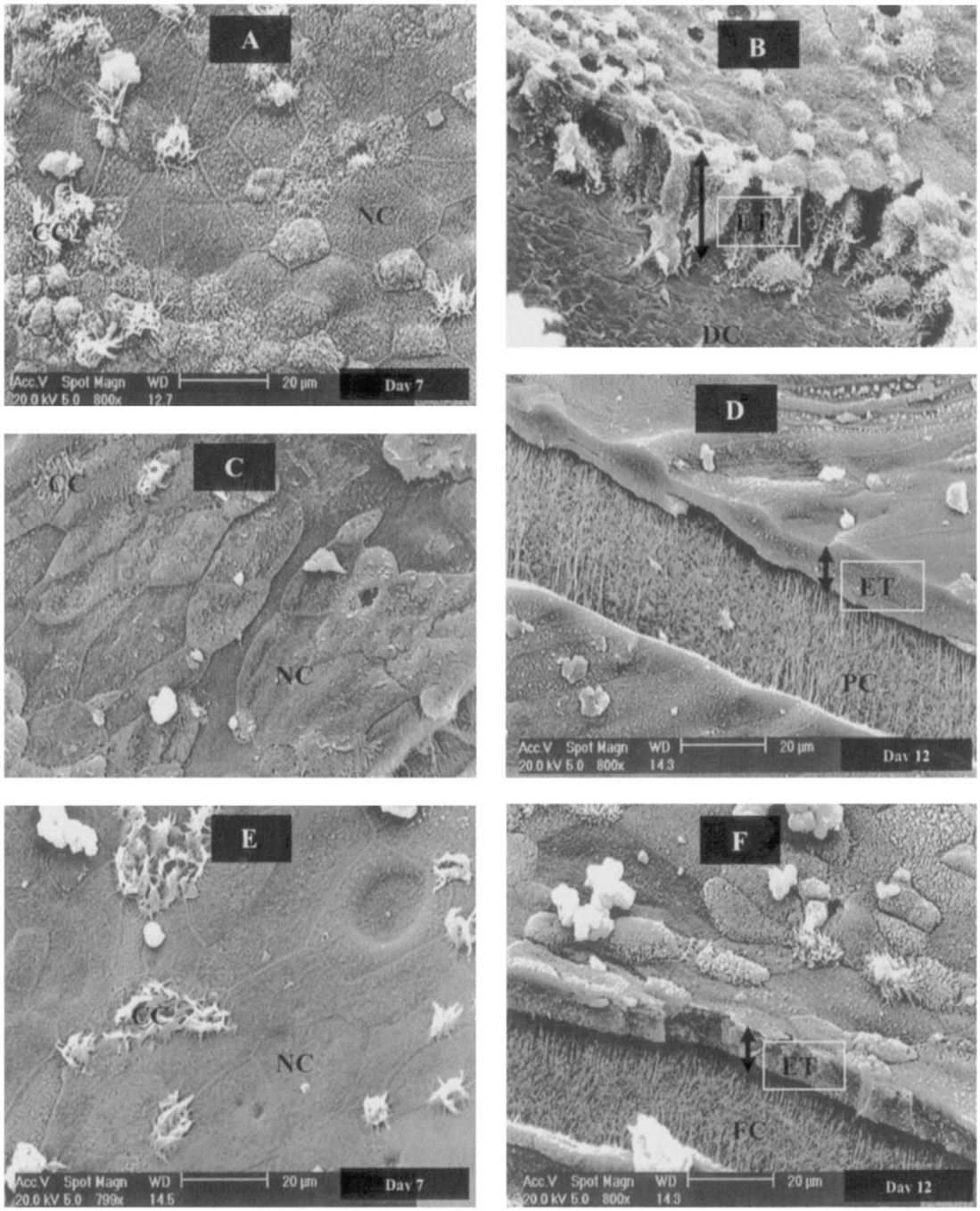

Figure 1 Scanning electron micrographs of human nasal epithelium cultured on Cellagen membrane CD-24 (A, D), Vitrogen gel (B, E) and Vitrogen film (C, F). Note the remarkable morphological differences between the surface morphology of the cells and epithelial thickness. CC, ciliated cells; DC, derivatised collagen surface; ET, epithelial thickness; FC, fibrillar collagen surface; NC, non-ciliated cells; PC, polymerised collagen surface. Note: cell surface roughness in D occurred during cell processing for microscopy.

functional polarisation, better differentiation and significant TER only when collagen cell-support matrices were used. Cell culture reproducibility was better with Cellagen membrane CD-24 (90%) than with Vitrogen gel (70%) or Vitrogen film (< 10%).

Literature information suggests that nasal epithelial

cells cultured on surfaces not coated with a biological matrix (e.g. plastic) may multiply rapidly. Nevertheless, the cells have the tendency to detach or become squamous within a few days (Wasilenko & Marchok 1984). Cell detachment frequently occurs in such surfaces because nasal epithelial cells are predominantly columnar cells, which do not have hemidesmosomes, and attach to the basement membrane only by cell-adhesion molecules (e.g. laminin and fibronectin) (Mygind & Dahl 1998). Consequently, biological matrices (e.g. collagen, laminin, extracellular matrix, etc.) are important to obtain a stable, differentiated and reproducible nasal culture system. However, Werner & Kissel (1995) reported the development of a differentiated nasal culture system consisting of highly differentiated columnar-shaped ciliated, non-ciliated and mucous-producing epithelial cells. Their observation is surprising and difficult to explain given the context of their culture condition (10% FCS, absence of a biological matrix and no preliminary fibroblast removal before cell plating). Though serum may be an important source of extracellular matrix (e.g. laminin), it contains transforming growth factor β-1, which is implicated in induction of squamous metaplasia in respiratory epithelial cells in-vitro (Lechner et al 1984).

The morphological features of cells cultured on the various collagen substrata are shown in Figure 1. Domes (picture not shown) were frequently observed in cells cultured on Cellagen membrane CD-24, but rarely on Vitrogen gel and film. The best epithelial differentiated phenotype (monolayer with columnar/cuboidal ciliated and non-ciliated cells) occurred in cells grown on Cellagen CD-24 (Figures 1A and 1B). The cells had a typical cobble stone appearance. In contrast, cells grown on Vitrogen gel and film were squamous (Figures 1C and 1E). For these substrata, cell cross-section revealed multilayered cells (Figures 1D and 1F), with Vitrogen gel-based cells being more densely packed in comparison with Vitrogen film. Cell piling may result in a tortuous network of aqueous pores, which may culminate in greater resistance for molecules that traverse via these pores. The epithelial thickness of cells grown on Cellagen membrane CD-24 was remarkably different from the thicknesses in other collagen preparations (Cellagen membrane CD-24, $\approx$ 20 μm of monolayer; Vitrogen gel and film, $\approx$ 10 μm of multi-layers; Figures 1B, 1D and 1F).

The collagen preparations we investigated are biochemically identical (type I collagen), but structurally they have a different scaffolding that may affect cell shape and orientation to nutrient. While collagen fibres in Vitrogen gel and film exist as loose coils with random orientations, the fibres in Cellagen membrane CD-24 manifest as uniform layers of fibrils arranged in collinear uninterrupted longitudinal sections. In addition, Vitrogen gel and film are hydrated non-flexible thick gel and film, respectively, while Cellagen membrane CD-24, when wet, are hydrated thin pliable collagen films. When used to grow epithelial cells at the air–liquid interface, Cellagen membranes CD-24 may literally be described as floating collagen films. Floating of collagen substratum after cell attachment is known to induce better nasal epithelial cell differentiation (Hanamure et al 1994).

CBF studies

It was necessary to determine the culture period within which the cells retained their ciliary beating at a physiological level (7–20 Hz) because concurrent studies involving drug transport, metabolism and mucosal or ciliary toxicity is an advantage. The number of ciliated cells in Cellagen membrane CD-24 and Vitrogen gel and film was more than 10% up to day 6. Thereafter, ciliated cells in all the culture conditions progressively dropped to less than 10% and disappeared before day 21. The CBF of the cells decreased significantly ($P < 0.05$) from control (day 1) in all the collagen preparations after 7 days, with Vitrogen film-grown cells showing more rapid decline in CBF. CBF of the cells decreased from 11.4 ± 1.7 Hz to 4.6 ± 0.9 Hz (Vitrogen gel), 10.6 ± 2.1 Hz to

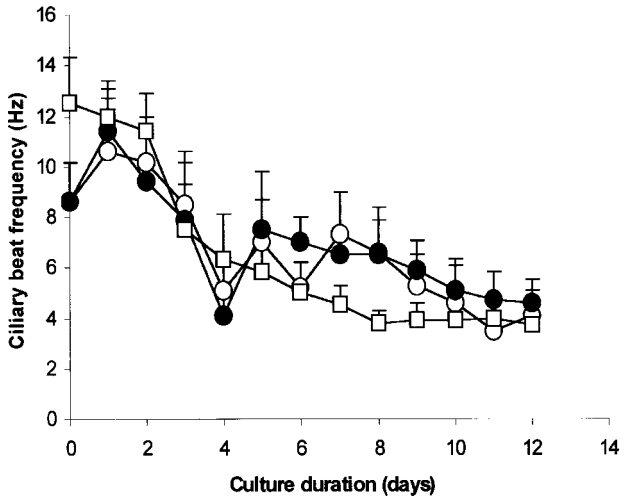

Figure 2 Ciliary beat frequency of human nasal epithelium (mean $\pm$ s.d., n = 15 cells) cultured on Cellagen membrane CD-24 (○), Vitrogen gel (●) and Vitrogen film (□).

4.1 ± 1.0 Hz (Cellagen membrane CD-24) and 12.5 ± 1.8 to 3.7 ± 0.4 Hz (Vitrogen film) on day 12 (Figure 2). The percentage CBF decrease within this period for all the substrata was 60–70%. However, within 7 days in culture, the cells maintained a co-ordinated ciliary beating pattern, an indication of intercellular communication.

In all the collagen substrata investigated, the observed CBF decrease agreed with literature information (Rautiainen et al 1993; Agu et al 1999). The progressive decline in the number and frequency of ciliated cells may be explained by the loss of the cells' three-dimensional geometry upon attachment (Jorissen et al 1989). Despite this decline, a sufficient number of ciliated cells with CBF within physiological range (7–20 Hz) could be seen in all the substrata.

Biochemical and toxicological studies

The MDH activity of 1.0×10^6 cells (number of cells seeded per insert) was determined before plating the cells (day 1) to serve as a reference to monitor the change in cell viability with culture duration. Trypan blue exclusion assay routinely conducted after releasing cells with pronase showed that the cells were more than 95% viable. Therefore, a statistically significant decrease in MDH activity on days 7 and 12, respectively (relative to day 1), implied a decline in cell viability. Days 7 and 12 were chosen for the study because the optimal morphological and functional features of cells grown on the three collagen substrata were observed between days 4 and 11. The results of the MDH assay are summarised in Table 2. There was a statistically significant ($P < 0.05$) increase in the MDH activity of cells grown on the various collagen supports on days 7 and 12.

Considering the fact that higher metabolic activity in a particular collagen substratum may be due to the presence of more cells per insert, the absolute viability (formazan absorbancy based on number of viable cells per cm^2 of insert) was determined. The absolute viability of cells grown on the various substrata on day 7 followed the rank order Vitrogen gel > Vitrogen film > Cellagen membrane CD-24. On day 12 the rank order changed to become Vitrogen gel > Cellagen membrane > Vitrogen film.

The higher absolute viability for cells cultured on Vitrogen gel on day 7, in comparison with Cellagen membrane and Vitrogen film, may be explained by the extent of cell multi-layering (i.e. more cells per cm^2) in gel (Figures 1B, 1D and 1F). The higher MDH activity of Cellagen membrane CD-24-based culture on day 12 in comparison with Vitrogen film might be due to better epithelial cell differentiation or time-dependent loss of viability in cells grown on Vitrogen film. Also, the number of mitochondria per cell (which may vary from cell to cell) or metabolic differences, as well as cell size, may account for differences in cell viability (Saxton et al 1994). Given the fact that the viability of the cells grown on the different collagen substrata were statistically higher ($P < 0.05$) on days 7 and 12 (relative to day 1) the cells were considered to retain their viability and thus be suitable for metabolism studies.

To validate the suitability of the cells grown on the different collagen substrata for assessment of mucosal damage following exposure to pharmaceutical compounds, LDH release by STDHF was measured as an index of cell membrane damage and intracellular toxicity

Table 2 Absolute MDH activity of human nasal epithelium cultured on Cellagen membrane CD-24, Vitrogen gel and Vitrogen film.

Substrata	Absolute MDH activity (absorbance cm^{-2})			Change in MDH activity between culture days (expressed as a ratio)		
	Day 1	Day 7	Day 12	Day 7 : Day 1	Day 12 : Day 1	Day 12 : Day 7
Control (1.0×10^6 cells)	1.31 ± 0.17[a]	–	–	–	–	–
Cellagen membrane CD-24	–	2.67 ± 0.30*	4.78 ± 0.56*	2.03↑	3.64↑	1.79↑
Vitrogen gel	–	5.58 ± 0.31*	7.87 ± 0.10*	4.25↑	6.00↑	1.41↑
Vitrogen film	–	4.68 ± 0.17*	2.99 ± 0.15*	3.57↑	2.28↑	0.64↓

Absolute MDH activity is expressed as mean ± s.d., n = 3. *$P < 0.05$ relative to control; [a]MDH activity of 1.0×10^6 cells (number of cells seeded per insert) determined on day 1 to serve as reference. ↑, Increase in MDH activity; ↓, Decrease in MDH activity. Increase in MDH activity relates to cell multiplication and maintenance of viability.

Table 3 LDH release following exposure to STDHF.

Substrata	LDH release					
	Day 7			Day 12		
	$LDH_{rate\ medium}$	$LDH_{rate\ cell}$	$\%\ LDH_{release}$	$LDH_{rate\ medium}$	$LDH_{rate\ cell}$	$\%\ LDH_{release}$
Cellagen membrane						
PBS (control)	0.0120 ± 0.0044	0.1570 ± 0.0079	6.9 ± 3.4	0.0063 ± 0.0062	0.1687 ± 0.0420	4.4 ± 5.8
0.5% STDHF	0.1520 ± 0.0096	0.0530 ± 0.0262	75.4 ± 9.6*	0.1857 ± 0.0468	0.0750 ± 0.0134	70.8 ± 2.5*
Vitrogen gel						
PBS (control)	0.0035 ± 0.0025	0.1235 ± 0.0005	2.7 ± 2.7	0.0012 ± 0.0001	0.1760 ± 0.0160	0.7 ± 0.8
0.5% STDHF	0.0647 ± 0.0200	0.0420 ± 0.0193	62.5 ± 7.9*	0.1160 ± 0.0187	0.0667 ± 0.0269	65.3 ± 9.2*
Vitrogen film						
PBS (control)	0.0010 ± 0.0001	0.2240 ± 0.0010	0.44 ± 0.1	0.0190 ± 0.0040	0.0985 ± 0.0225	17.1 ± 8.6
0.5% STDHF	0.3150 ± 0.1449	0.0270 ± 0.0094	91.4 ± 2.6*	0.3257 ± 0.1367	0.0250 ± 0.0088	92.5 ± 1.4*

LDH release is expressed as mean ± s.d., n = 3. *$P < 0.05$ relative to control. PBS, phosphate buffered saline; STDHF, sodium tauro-24, 25 dihydrofusidate.

using 7- and 12-day-old cultures. The results of the LDH assay are summarised in Table 3. A statistically significant difference ($P < 0.05$) was observed between control cells (treated with PBS) and cells treated with STDHF for 30 min in all the collagen substrata. In terms of degree of LDH release, similar percentages of release were seen for cells grown on Cellagen membrane CD-24 and Vitrogen gel. The percentage LDH release was much higher in cells cultured on Vitrogen film. In all the culture conditions investigated, the percentage LDH release did not vary significantly between day 7 and day 12. Thus the assay was not affected by the culture duration (within the period investigated), an indication that within this period the cells are suitable to study mucosal or cytotoxicity of pharmaceutical compounds. Cells cultured on Vitrogen film exhibited much higher LDH release compared with other conditions. Consequently, cells grown on this collagen preparation may not be suitable for nasal mucosal toxicity studies due to the possibility of overestimating the toxicity of drugs or excipients.

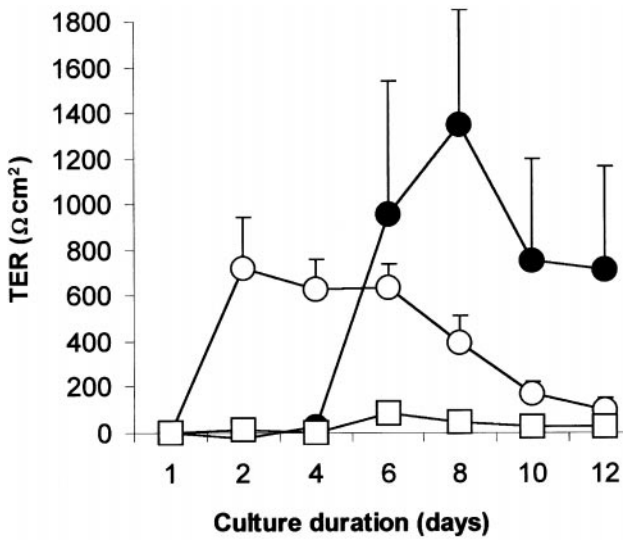

Figure 3 Development of TER (mean ± s.d., n = 12 inserts) in human nasal epithelium cultured on Cellagen membrane CD-24 (○), Vitrogen gel (●) and Vitrogen film (□).

Permeation studies

The development of electrical resistance over cell layers cultured on Cellagen membrane CD-24 and Vitrogen collagen gel and film is highlighted in Figure 3. The TER for cells grown on Cellagen membrane CD-24 was 200–650 Ω cm^2 (days 2–10). This value is within the 200–600 Ω cm^2 range reported in literature for cultured nasal epithelium (Werner & Kissel 1995; Leuba et al 1996). The relatively higher TER seen in Vitrogen gel (1349 ± 508 Ω cm^2) also agreed with published data (Yankaskas et al 1985; Yamaya et al 1992). The lower TER observed in cells grown on Cellagen membrane CD-24 in comparison with Vitrogen gel was not due to the flow of outward current in the domes as cells without domes had similar TER values. The higher TER values in cells grown on Vitrogen gel can be linked to cell piling (Figure 1F).

Given the insignificant TER, poor culture reproducibility and leaky nature of cells cultured on Vitrogen

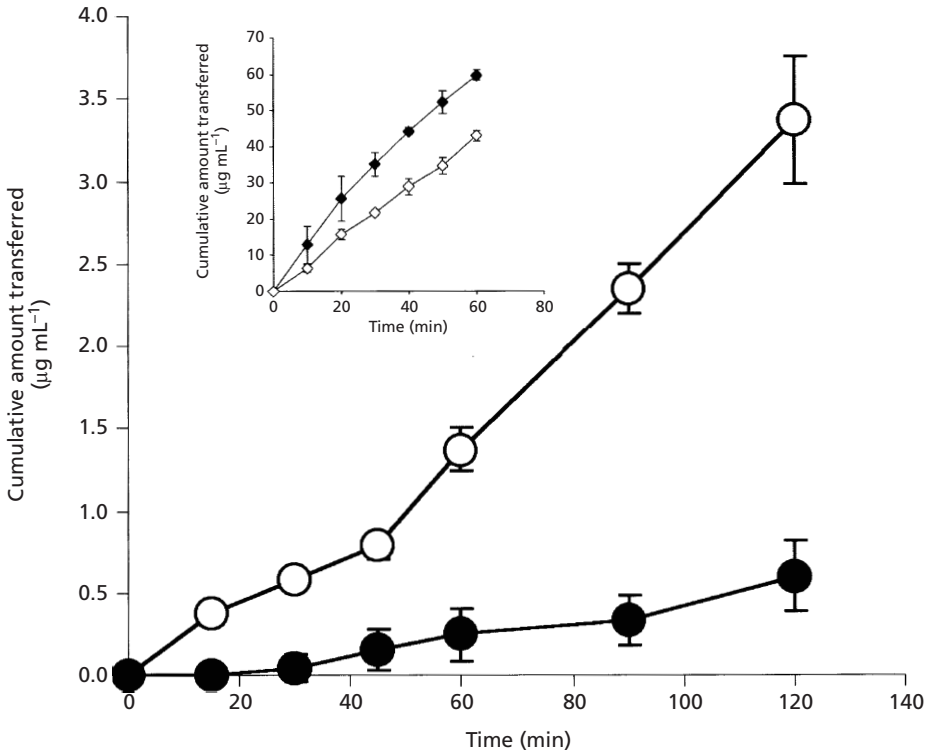

Figure 4 Sodium fluorescein permeation across nasal epithelium cultured on Cellagen membrane CD-24 (○) and Vitrogen gel (●). Inset: permeation of sodium fluorescein across collagen substrata without cells (◇, Cellagen membrane; ◆, Vitrogen gel). The cells were cultured for 7 and 9 days for Cellagen membrane CD-24 and Vitrogen gel, respectively.

film, these cells were not used for permeation studies. Results of the permeation studies are summarised in Figure 4. The flux of sodium fluorescein, a paracellular marker, through collagen preparations without cells was linear, $r^2 = 0.990 \pm 0.003$, $n = 3$ (Cellagen membrane) and 0.990 ± 0.009, $n = 3$ (Vitrogen gel), indicating that these collagen preparations did not form a significant barrier to diffusion of sodium fluorescein. The apparent permeability coefficients (P_{app} cm s^{-1}) of sodium fluorescein across the collagen preparations were $40.70 \pm 2.30 \times 10^{-7}$ (Cellagen membrane CD-24) and $61.70 \pm 6.00 \times 10^{-7}$ (Vitrogen gel). For Cellagen membrane CD-24 and Vitrogen gel with cells grown on them, the flux of fluorescein was statistically ($P < 0.05$) reduced to $1.91 \pm 0.00 \times 10^{-7}$ cm s^{-1} (Cellagen membrane CD-24) and $0.45 \pm 0.08 \times 10^{-7}$ cm s^{-1} (Vitrogen gel). For both substrata the diffusion of sodium fluorescein was $\leqslant 2\%$ of the amount added to the apical side within 2 h, thus confirming the formation of tight junctions between the cells.

The permeability coefficients of sodium fluorescein across the cells were lower, though comparable, than that of $5.86 \pm 0.48 \times 10^{-7}$ obtained for a Caco-2 model. It is however, important to mention that the comparison of the absolute P_{app} values obtained using different experimental set-ups should be done with some reservation as some variables affect these values. Some of the experimental factors that directly affect the absolute values of P_{app} include pH gradients, additional diffusion barriers (i.e. unstirred water layer, type of filter support), analyte concentration, detection method, cell culture variation (Caldwell et al 1998) and, possibly, the formula used to calculate the P_{app}.

Conclusion

This study highlighted the fact that collagen is important as a cell-support matrix to establish a stable, reproducible and differentiated primary cell culture system for nasal drug delivery studies. In terms of cell morphology, Cellagen membrane induced better epithelial cell differentiation than Vitrogen gel or film. For all the collagen substrata investigated, cell morphology was optimal between days 4 and 11. Therefore, the physical structure of the collagen matrix and time of investigation are important considerations when using collagen-based

human nasal primary culture systems for in-vitro drug delivery studies. Based on MDH activity, LDH release, CBF, TER and permeation studies, human nasal epithelial cells cultured on derivatised collagen (Cellagen membrane CD-24) and polymerised collagen (Vitrogen gel) were concluded to be functionally suitable for in-vitro nasal drug delivery studies. Due to their leaky nature and comparatively higher LDH release upon exposure to STDHF, cells cultured on fibrillar collagen (Vitrogen film) may be limited to metabolism and ciliotoxicity studies. Based on better culture reproducibility and epithelial cell differentiation, further characterisation of the permeation profile of cells cultured on Cellagen membrane CD-24, using compounds of different molecular weights and absorption characteristics, is recommended.

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