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The Development and Characterisation of an Organotypic Tissue Engineered Human Oesophageal Mucosal Model[a]

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Abstract

There is demand for a reliable 3D tissue engineered model of the oesophageal mucosa for use as an experimental platform for investigating oesophageal epithelial biology and the pathogenesis of oesophageal neoplasia and precursor lesions such as Barrett's Metaplasia. A number of models have been described but there has been little systematic assessment of the different approaches, making selection of a preferred platform difficult. This study assesses the properties of organotypic cultures using four different scaffolds (human and porcine oesophageal matrix; human dermal matrix; collagen) and two different epithelial cell types (primary human oesophageal squamous cells and the Het-1A oesophageal squamous cell line). Human oesophageal matrix and dermis did not give consistent results, but porcine oesophageal matrix and collagen proved more reliable and were studied in greater detail. Both matrices supported the formation of a mature stratified epithelium which was similar to that of normal human oesophagus, demonstrated by Ki67, CK4, CK14 and involucrin staining. However, collagen showed reduced epithelial adherence whilst fibroblast penetration into the porcine matrix was poor. Composite cultures using Het-1A cells formed a hyperproliferative epithelium with no evidence of differentiation. We propose HOS cells seeded onto porcine oesophageal matrix as the preferred model of normal human oesophagus.

Introduction

The oesophagus is a hollow muscular tube serving to propel food from the pharynx to the stomach. Its inner lining is formed by non-keratinised stratified squamous epithelium. This squamous epithelium is analogous to that of the epidermis, but lacks many of the specialised structures found in the skin (i.e. hair follicles, melanocytes, specialised immune cells, etc). The oesophagus does, however, contain mucus-producing glands in the lamina propria and submucosa.

A number of groups have described organotypic culture systems that recapitulate some elements of the native oesophageal micro-anatomy. Some have been developed in an attempt to form functional substitute tissue for surgical use in the repair or replacement of damaged tissue. For example, porcine oesophageal epithelial cells have been cultured on a biodegradable electrospun synthetic scaffold of poly(L-lactide-co-caprolactone) (PLLC)[1], and oesophageal grafts have been introduced into rats using an acellular gastric matrix[2]. Researchers have also described a mature, stratified human oesophageal epithelium grown on collagen containing human fibroblasts[3] and more recently human smooth muscle cells were also incorporated into the model[4]. However these models were developed for transplantation purposes and no detailed histological characterisation was described. Demand for such replacement tissue is relatively modest because resection of the distal oesophagus (e.g. during cancer surgery) can usually be compensated by formation of a neo-oesophagus from the proximal stomach, whilst colon can be interposed to compensate for longer defects.

A more compelling case for the generation of tissue-engineered oesophageal tissues is the need for experimental platforms for the investigation of oesophageal mucosal disorders, in particular oesophageal cancer. There are marked geographic differences

in the incidence of different histological forms of the oesophageal cancer[5]. In regions such as China and the Middle East there is a high incidence of oesophageal squamous carcinoma (OSC). Exposure to dietary carcinogens seems particularly implicated in the development of this form of the disease. In the Western world there is a rapidly increasing incidence of oesophageal adenocarcinoma (OAC)[6]. In this form the development of cancer is preceded by the metaplastic replacement of the squamous epithelium by a glandular epithelium resembling, to a variable extent, that which is found in the large bowel – a phenomenon known as Barrett's oesophagus[7]. Conventional cell culture systems are of limited use in understanding the early pathogenesis of oesophageal cancer and its precursor lesions for a variety of reasons. Firstly, they fail to capture potentially important cell-cell and cell-matrix interactions. Secondly, work with transformed cells lines derived from established tumours may be of limited relevance to the study of the very early stages of the disease, when potential for therapeutic intervention may be highest.

Animal models have frequently been employed for the artificial induction of gastro-oesophageal reflux disease[8-11], known to be a critical factor in the development of oesophageal adenocarcinoma. These do in principle provide a more realistic cellular microenvironment, but controlled manipulation of this environment can be technically challenging. Furthermore there is a dietary impact on the composition of refluxate resulting in interspecies variations and consequently animal experiments still may not accurately reflect the events occurring within the environment of the human oesophagus.

A number of in vitro models have been described for experimental purposes including a collagen/Matrigel based model that incorporates primary human oesophageal squamous epithelial cells and primary human oesophageal fibroblasts[12]. This

approach results in a mature stratified epithelium that is morphologically similar to the native oesophagus and which has been incorporated into a number of experimental procedures[13-16]. However there is little published characterisation of this model itself and the approach does not include a basement membrane (BM). Experiments on human skin have shown the importance of the presence of a basement membrane in the degree of epithelial cell attachment, the differentiation of the epithelium and the subsequent remodelling of the BM in vitro[17]. A rat-derived de-epithelialised acellular oesophagus with an intact BM has been used for the culture of oesophageal cells[18], however in these experiments the cells used were also derived from the rat. A model has been developed that uses human oesophageal epithelial cells seeded onto a de-epithelialised, acellular rat trachea matrix. The epithelium produced has been shown to reproduce many of the features of a normal oesophagus[19]. However this system requires that the culture be placed subcutaneously under the dorsal skin of immuno-deficient mice for cell growth to occur. As a result manipulation of the model during this time becomes significantly more complex and a system that does not require the use of animals in this way would face fewer ethical and practical barriers.

Published descriptions of individual tissue-culture approaches are often limited, and there has been little systematic comparison of the properties of different approaches. Moreover, individual platforms are frequently described by one group but seldom appear to be replicated by others, suggesting a high degree of variability and complexity within these protocols.

In this study we performed a side-by-side comparison of the histology of oesophageal composites produced using four different scaffolds (human and porcine oesophageal matrix, human dermal matrix and collagen I gel) and two different epithelial cell types (primary human oesophageal squamous cells and the Het-1A oesophageal squamous

cell line). In all cases each experimental protocol was performed a minimum of three times and composite cultures were produced in duplicate within each experiment. Cultures showing the most realistic morphology were further characterised with immunohistochemistry to highlight cell proliferation (Ki67), differential cytokeratin expression (CK14 & CK4), involucrin and collagen IV.

Materials and Methods

All materials were purchased from Sigma Aldrich unless otherwise stated.

Human oesophageal squamous (HOS) epithelial cell isolation and culture

Oesophageal tissue samples were obtained with informed consent from patients undergoing gastric or oesophageal surgery, with appropriate ethical approvals (SSREC 165/03 & 07/1309/138). Approximately 2 cm^2 samples of disease-free background oesophageal squamous mucosa showing no gross pathology were dissected from oesophagectomy specimens, transported to the laboratory in sterile PBS containing 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B and stored overnight at 4˚C prior to cell isolation. Samples were then cut into 0.5 cm strips and incubated in 0.1% w/v trypsin (Gibco, Carlsbad, CA) for 1 hour at 37˚C. Fetal Calf Serum (FCS) was then added to inhibit the trypsin and the epithelial cells removed by gentle scraping with a scalpel blade. This removed the uppermost terminally differentiated epithelial cells along with the underlying proliferative epithelial cells. For standard culture the cells were collected into Greens medium[20] (consisting of DMEM and Ham's F12 in 3:1 ratio, supplemented with 10 % FCS, 2 x 10^{-3}M glutamine, 1 x 10^{-10}M cholera toxin, 10 ng/mL EGF, 0.4 µg/mL hydrocortisone, 1.8 x 10^{-4} M adenine, 5 µg/mL insulin, 5 µg/mL transferrin, 2 x 10^{9}

M triiodothyronine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B). The resulting cell suspension was centrifuged (200g, 5 min), the cells resuspended in 12 mL Green's medium and cultured in the presence of a feeder layer of lethally irradiated mouse 3T3 fibroblasts (1 x 10^6 per 12 mL culture). Cell counts were performed using a haemocytometer and trypan blue exclusion was used to assess cell viability. HOS cells were used between passage 1 and 4.

Human oesophageal fibroblast (HOF) cell isolation and culture

Oesophageal stromal tissue resulting from HOS isolation was finely minced with a scalpel and incubated overnight at 37˚C in 10 mL 0.5 % (w/v) collagenase A solution. The digest was then centrifuged (200g, 10 min) and the pellet resuspended in 10 – 15 mL fibroblast culture medium (DMEM (Glutamax), 10 % FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B). Fibroblasts were used between passages 4 and 9.

Het-1A cell culture

An SV-40 immortalised human oesophageal epithelial cell line, Het-1A (ATCC-LGC, Middlesex, UK) was cultured in serum-free BRFF-EPM2 media (Axxora, Nottingham, UK). The medium was replaced every 3 to 4 days and cells were passaged once they had reached 80% confluency.

Quantification of gland density in porcine oesophagus

To identify those regions of the oesophagus with the lowest gland density, nine full-length porcine oesophagi were retrieved freshly from the abattoir and fixed in 4% formalin. Histological sections were taken at each of nine equidistant levels from rostral to caudal, and stained for mucins using a combined Alcian-Blue/Periodic Acid-Schiff (AB/PAS) stain. Sections were digitised on a flat-bed scanner at a resolution of 0.0729 mm^2 per square-pixel. The Image-J software package was used to measure the

cross-sectional area of glands, the lumen and the squamous epithelium, and the total tissue area. Gland density (defined as gland area/total area) was calculated at each level, averaged for the 9 oesophagi and compared between levels using analysis of variance (ANOVA).

Production of oesophageal composites

A number of different scaffolds, cells, seeding regimes and culture conditions were evaluated during the production of the oesophageal composites, as outlined in Table 1. Those methods that were found to be the most successful are described in more detail below.

Preparation of the oesophageal scaffold

Human oesophagi were obtained at post-mortem, with ethical approval (SSREC 165/03) and the informed consent of the deceased's relatives. Porcine oesophageal tissue from Landrace pigs was obtained from an abattoir. Tissue from both sources was treated identically in the initial stages. The freshly removed oesophagus was placed into Betadine Antiseptic Solution (10% povidone-iodine solution, Mölnlycke Health Care Limited, Manchester, UK) for 5 minutes to reduce microbial growth. The tissue was then placed in sterile PBS containing 200 IU/mL penicillin, 200 µg/mL streptomycin and 1.25 µg/mL amphotericin B for transport. On arrival at the laboratory the tissue was handled using aseptic technique to minimise microbial contamination. The oesophagus was opened longitudinally and rinsed in sterile PBS. The muscularis propria was removed, by dissecting longitudinally along the lamina propria, and discarded. The most proximal and distal portions of the oesophagus were also discarded because of a greater number of submucosal glands and a thicker lamina propria in those areas respectively. The remaining tissue was cut into 5 cm^2 and washed in PBS.

Production of human de-epithelial acellular oesophagus (hDEO)

Pieces of human oesophagus prepared as described above were incubated in sterile 1M NaCl for 16 hours at 37°C, causing the epithelium to separate from the underlying stroma. The stromal tissue was retained and washed in PBS prior to sterilisation in 0.1% (w/v) peracetic acid in PBS, pH 7.0 for 3 hours at room temperature.

Production of porcine de-epithelial acellular oesophagus (pDEO)

Pieces of porcine oesophagus prepared as described above were incubated in sterile 1M NaCl, 200 IU/mL penicillin, 200 µg/mL streptomycin, 1.25 µg/mL amphotericin B for 72 hours at 37°C. The tissue was washed in PBS and the epithelium peeled off using forceps and discarded. The remaining tissue was washed x3 in PBS and placed into 80% glycerol solution for 24 hours, transferred to 90% glycerol for 24 hours and stored in 100% glycerol at room temperature for a minimum of 4 months in order to sterilise the tissue; previous research having shown that this was a reliable method for the storage and sterilisation of skin whilst preserving tissue integrity[21]. When required the tissue was re-hydrated by multiple washing in PBS and sterility tested by incubating in DMEM at 37 °C.

Preparation of de-epithelialised acellular dermis (DED)

Normal split thickness human skin was obtained from routine abdominoplasty and breast reduction operations, with appropriate ethical approval (Tissue Bank Licence No. 12179). Tissue was placed in PBS containing 100 IU/mL penicillin, 100 µg/mL streptomycin, 0.625 µg/mL amphotericin B for 1 week at 4°C. The epithelium was removed by 24 h incubation in sterile 1M NaCl at 37 °C; the tissue was then washed three times in PBS and stored in DMEM at 4 °C until required. It was found that no further sterilisation of the DED was required for our purposes.

Production of an oesophageal composite on collagen

This method was modified from that described by Andl et al.[12]. Aliquots (0.5 mL) of a base gel mixture (3 mg/mL rat tail collagen type I, 0.54 mM glutamine, 0.04 % (w/v) $NaHCO_3$, 7 % (v/v) FCS in MEM solution) were placed into wells of a 24 well tissue culture plate and allowed to set (room temp, 10 minutes). HOF cells (5×10^5 per gel) were then resuspended in FCS and incorporated into a second gel mixture (1.5 mL containing 2 mg/mL rat tail collagen type I, 0.62 mM glutamine, 0.04 % (w/v) $NaHCO_3$, 6 % (v/v) FCS in MEM solution). This second gel was placed on top of the base gel, allowed to harden (45 minutes, 37 °C) and covered with DMEM containing 10% FCS, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.625 µg/mL amphotericin B. After 24 hours the gels were released from the sides of the wells to allow contraction to occur, fresh media added and the gels returned to the incubator for a further 5 days, replacing the media as necessary every 3 to 4 days. The gels were then pre-equilibrated for 1 hour at 37 °C in Composite Media I (DMEM:Hams F12 (3:1 ratio v:v), 4 mM L-glutamine, 0.5 µg/mL hydrocortisone, 0.1 mM O-phosphorylethanolamine, 20 pM triiodothyronine, 0.18 mM adenine, 1.88 mM $CaCl_2$, 4 pM progesterone, 10 µg/mL insulin, 10 µg/mL transferrin, 10 ng/mL selenium (Lonza, Wokingham, UK), 10 mM ethanolamine, 0.1 % v/v chelated newborn calf serum) before removing the media and adding 5×10^5 epithelial cells (HOS or Het-1A) in 20 µl Composite Media I to the top of each gel and incubating at 37 °C. After 2 hours the gels were fully submerged in Composite Media I and incubated for 2 days. The media was then replaced with Composite Media II (as Composite Media I except serum replaced with 0.1 % v/v non-chelated newborn calf serum) and the cells allowed to grow for a further 2 days. The gel was then moved to the air-liquid interface by placing on the surface of a sterile stainless grid and the media replaced by Composite Media III (as Composite Media I except without

progesterone and serum increased to 2% newborn calf serum). The media was replaced with fresh Composite Media III every 3 to 4 days and the composite maintained at the air-liquid interface for 10 to 14 days.

Production of an oesophageal composite on hDEO, pDEO or DED

The method was adapted from that already in use in our laboratory for making skin composites[22]. Initial experiments used Greens with 10% FCS as the culture medium following addition of epithelial cells; however for better control of oesophageal cell proliferation and differentiation, the same three-step culture medium described above when using the collagen scaffold was later employed. HOF cells (5×10^5 in 0.5 mL DMEM, 10% FCS) were first seeded on the submucosal side of the DEO or DED using a sterile stainless seeding ring. After 24 hours the ring was removed and fresh culture medium was added. After one week the HOF seeded scaffold was inverted and epithelial cells (HOS or Het-1A) were seeded on the mucosal side at a density of 1×10^6 in 0.5 mL Composite Media I. After 48 hours the medium was replaced by Composite Media II and after a further 2 days the constructs lifted to an air-liquid interface and the media changed to Composite Media III. The media was replaced every 3 to 4 days and the composites maintained at the air-liquid interface for 10 days.

Histology and immunohistochemistry

Samples were fixed in 10% formalin, sectioned and stained with haematoxylin and eosin (H&E). For immunohistochemistry (IHC) the following primary antibodies were used: Ki67 (1:100, clone MM1; Novocastra, Newcastle on Tyne, UK), cytokeratin 14 (1:200, clone LL002; Novocastra), cytokeratin 4 (1:200, clone 6B10; Abcam, Cambridge, UK), and involucrin (1:100, clone SY5; Novocastra). Mouse IgG and TBS buffer were used as controls. Antigen retrieval was performed by incubating slides in 10mM citrate buffer (pH 6) for 20 mins at 95 °C. A biotinylated horse anti-

mouse secondary antibody (Vector Labs, Peterborough, UK) and the streptavidin/HRP ABC detection system (Vector Labs) were used. Samples were counterstained with haematoxylin. The morphology and immunohistochemical patterns in all samples were reviewed by a histopathologist. In addition tissue morphology was formally assessed blind by five independent, experienced observers using a previously published semi-quantitative scoring system[23,24], where each image was given an integer score for each of four separate parameters. Parameters assessed were (i) formation of a multilayered epithelium, (ii) stratification and maturation within the epithelium, (iii) epithelial attachment to the matrix and (iv) penetration of fibroblasts into the scaffold.

Statistics

Comparison of gland density data was performed using ANOVA. The Mann-Whitney test was used to compare scores for the different composite methodologies.

Results

Quantification of gland density in porcine oesophagus

In keeping with our subjective assessment, there was a marked difference in gland density at different sites of the oesophagus. Gland density was significantly higher in the proximal third of the oesophagus relative to the distal two-thirds (Figure 1, $p=0.0001$; ANOVA).

Evaluation of the matrices

Composites incorporating human de-epidermised dermal (DED) scaffold produced very variable results. Some failed to develop beyond a single epithelial layer whilst others produced multilayered stratified epithelia (results not shown). Similar issues of variability arose when using the hDEO. As a result, no further characterisation

experiments were performed using these scaffolds and attention was focused upon the pDEO and collagen I matrices.

H&E staining of pDEO and collagen I matrices is shown in Figure 2. The porcine derived DEO is shown prior to the re-addition of cells, in order to demonstrate the absence of residual porcine cells. It can be seen that in the process of de-cellularisation the oesophageal mucosa was completely removed, but the structure of the connective tissue in the submucosa was maintained with retention of the papillae/rete ridges. The collagen I gel is shown after the addition and culture of HOF cells since these cells modify the gel prior to the addition of epithelial cells by causing considerable contraction of the scaffold. However even following this contraction the collagen gel did not possess the same convoluted surface observed with the DEO.

Characterisation of the oesophageal composites

Initial studies were performed to optimise the growth conditions for the composites. We first evaluated the requirement for the culture to be maintained at an air/liquid interface. Figure 3 shows the epithelium formed when cultures were exposed to an air/liquid interface for 0, 6 or 13 days. As the H&E images show, there was an increase in the number of layers and a degree of differentiation, indicated by the flattening of cells and loss of the nuclei in the upper layers, with an increased period of growth at the air/liquid interface. Despite some variability in the quality of the epithelium produced when using the DED as a scaffold, the requirement for an air/liquid interface was reproducible and a mature epithelium was never produced unless the cells were exposed to an air/liquid interface. It was also observed that the use of Greens plus 10% FCS medium resulted in less reproducibility in the formation of a mature, stratified and differentiated epithelium. Instead a three-step protocol using media which were altered to induce proliferation and then differentiation was

employed as described by Andl et al.[12]. This gave a reproducibly better mature, stratified epithelium when compared to that grown on DED with Greens medium, with improved epithelial stratification ($p=0.0228$) organisation ($p=0.0253$) and attachment ($p=0.0083$) scores.

Both the pDEO and collagen I matrices supported the regeneration of a multilayered epithelium from either HOS or Het-1A cells (Figure 4). The epithelium attached well to the pDEO matrix but was significantly less well adherent to the collagen I gel ($p=0.0036$). Both scaffolds were seeded with identical numbers of fibroblasts, although the actual seeding methodology differed, with the fibroblasts being either incorporated into the collagen gel or seeded onto the pDEO reticular surface. Consequently the fibroblasts could be clearly observed throughout the collagen gel, however using the pDEO there was a significant reduction in the level of fibroblasts observed within the scaffold ($p=0.0014$) with cells observed instead on the reticular seeding surface. Despite the poor penetration, the presence of HOF cells still appeared to be important in the production of a good stratified epithelium, since when composites were grown in the absence of HOF cells the epithelium was significantly thinner ($p<0.0001$) and less well stratified ($p<0.0001$), although no significant difference in attachment of the epithelium was observed ($p=0.1146$).

IHC staining for collagen IV, a major component of the basement membrane, confirmed the presence of this protein both within and on the surface of the pDEO matrix (Figure 5). Interestingly, composite cultures grown on collagen I gel also demonstrated collagen IV staining in small foci around fibroblasts and at the interface between the stroma and epithelium. It is likely that the collagen IV seen in the pDEO simply reflects the retention of native protein, but its presence in the collagen I gel

indicates that the cells were able to produce, at least in part, this component of the basement membrane themselves.

When using either scaffold, the HOS cells produced a mature, stratified epithelium, with the cells becoming progressively flatter and finally anuclear as they migrated towards the surface. There were no significant differences in stratification ($p=0.9498$) or organisation ($p=0.9177$) scores between the two scaffolds. IHC staining confirmed the similarities between the newly formed epithelia and that of the normal oesophagus. As expected expression of both the proliferation marker Ki67 (Figure 6) and basal cytokeratin CK14 (Figure 7) was restricted to basal and immediately suprabasal layers of the native oesophagus, and HOS composites grown on either pDEO or collagen I gel showed identical staining patterns within the epithelia for both markers. Similarly involucrin, expression of which is tightly linked to the onset of differentiation, was detected in the native oesophagus and both HOS-containing composites in cells from the suprabasal layer upwards, with no staining observed in the most basal layer (Figure 7). Finally Cytokeratin 4 (CK4), a squamous differentiation marker found in the cytoplasm of suprabasal squamous cells, was observed in the more superficial cells upwards with no staining in the two most basal layers in the native oesophagus; again this expression pattern was recapitulated in both HOS-containing composite models (Figure 7).

Composites that incorporated Het-1A cells showed very different epithelial morphology. A multilayered epithelium was still produced (Figure 4) but there was no evidence of stratification or maturation occurring within these layers. Ki67 staining demonstrated proliferation throughout all layers (Figure 6) and neither CK4 nor involucrin was detected, suggesting cells were not undergoing any level of differentiation (Figure 7).

Integrating the results of the various experiments indicates that both the scaffold and the cell type have a profound impact on the morphology of the composite cultures and the extent to which they recapitulate different aspects of the native oesophagus. A summary of the requirements for a successful system and the methods that most reliably fulfilled these requirements is presented in Table 2, and a summary of the advantages and disadvantages of the four key methods that we have more fully characterised is presented in Table 3.

Discussion

Our goal was to develop an in vitro system for studying the pathogenesis of oesophageal cancer. Such a model needs to incorporate both epithelial and stromal elements, but the muscular elements of the oesophagus (the muscularis mucosa and muscularis propria) are not thought to play a role in the development of oesophageal cancer and are thus not a requirement in this context. A small number of organotypic tissue-culture systems have been used in studies of oesophageal carcinogenesis, and our aim in this study was to conduct a systematic comparison of some of the key approaches and assess the impact of some of the major variables on the resultant tissue morphology. We compared four sources of tissue matrix: normal human oesophageal matrix, human skin dermal matrix, porcine oesophageal matrix and collagen I. Two epithelial cell types were also compared; human oesophageal squamous epithelial cells and Het-1A cells.

These experiments demonstrated that primary human oesophageal squamous cells form a microanatomically realistic epithelium when seeded on either collagen I gel or porcine oesophageal matrix. Tissue derived from human oesophagus or human skin proved unreliable as scaffolds, with some of the resultant cultures showing limited cell growth and no evidence of differentiation. Accordingly we did not characterise

these in any further detail. Such material is also less attractive as a scaffold in practice given the logistical and ethical issues associated with the use of human tissues.

A critical initial event in the pathogenesis of oesophageal adenocarcinoma is the metaplastic transformation of the oesophageal squamous epithelium into a glandular epithelium (Barrett's oesophagus), in response to exposure to gastric refluxate. Given the potential role of this model in supporting investigation into this phenomenon, it is desirable to avoid any theoretical confounding effect caused by any residual material associated with the native oesophageal submucosal glands. We have demonstrated that gland density is greatest in the proximal region of the porcine oesophagus and suggest that tissue from this region should be avoided in pDEO production.

Few detailed studies have been published which characterise an in vitro human oesophageal model to the extent that we are reporting. Bhrany and co-workers employed Ki67, CK14 and loricrin (which targets a range of cytokeratins) to characterise a model using rat primary oesophageal epithelial cells[18], observing staining which was similar but not identical to the normal human oesophagus. Other studies that employed collagen I gel as a scaffold for oesophageal epithelial cells have published H&E characterisation of the resulting models[12,13,16]. These images appear similar to those we have achieved with primary HOS cells. These publications do not comment on the adherence of the epithelial layer to the scaffold, although the published images do not appear to show reduced adhesion.

Primary human oesophageal cells, both squames and fibroblasts, present practical challenges as they require isolation from freshly resected surgical tissue. Moreover, limited passages are possible which may restrict the number and duration of the experiments that can be performed using them. Immortalised cell lines such as the Het-1A line represent an attractive alternative. Het-1A cells are a non-tumourigenic,

SV40 immortalised cell line derived from human oesophageal squames[25]. They have been shown to retain the characteristics of proliferating basal epithelial cells rather than the differentiated cells of the upper layers of the epithelium when grown in conventional culture. It was hoped that their incorporation into the 3-D system used here, with preservation of extracellular signals from the basement membrane and fibroblasts, might still lead to some degree of stratification. Unfortunately, although seeding on either collagen I or porcine oesophageal tissue resulted in a multilayered epithelium, there was no evidence of maturation or stratification. Similar hyperproliferation and lack of differentiation has been described when another immortalised cell oesophageal cell line was incorporated into an oesophageal model, although in this instance further characterisation of the composite was not described in detail[16]. Human oesophageal primary epithelial cells meanwhile produced a mature, stratified epithelium that stained in a similar manner to normal oesophagus for a number of proliferative and differentiative markers. Despite the practical obstacles, therefore, primary HOS cells must therefore remain the model of choice when studying phenomena in which cell differentiation may be of influence.

Oesophageal tissue appears to make a more reliable and effective scaffold than dermal tissue, possibly due to the variations in the composition of the basement membrane which can be specific to a given tissue type[26]. It is known that the basement membrane can play an important role in the growth and differentiation of a variety of epithelial cells[17,26]. Porcine tissue makes an attractive alternative to human oesophageal tissue as a source of matrix, as there are recognised physiological similarities between porcine and human oesophageal tissue and the size of the animal and ready availability of the tissue allows reasonably large areas of matrix to be isolated. In contrast there are significant ethical and practical difficulties in obtaining

normal healthy human oesophageal tissue for such studies. Porcine oesophageal matrix did support good adhesion of human oesophageal squamous cells, and the formation of a multilayered squamous epithelium - a key feature of a good model system.

Previous research has shown the importance of interactions between epithelial cells and fibroblasts in the formation and behaviour of organotypic models[27,28], and we accordingly incorporated HOF cells into the methodologies for both the porcine DEO, where the cells were seeded onto the reticular surface and collagen I, where the cells were incorporated into the gel. The presence of HOF cells was seen to cause contraction of the collagen I gel prior to the addition of epithelial cells. No such contraction was observed for the pDEO scaffold and H&E staining of the composites indicated that penetration of HOFs into the pDEO matrix was poor. Nevertheless the HOF cells positively influenced the formation of an organised, stratified multilayered epithelium. Previous studies have demonstrated changes to the composition of conditioned media produced by composites containing fibroblasts and/or epithelial cells[22] and our data indicate that even when predominantly located on the reticular surface of the model HOF cells produce the soluble factors required to support the epithelial cells. Variable penetration of fibroblasts into a de-epithelialised dermal scaffold has been described previously[29] but no explanation for this was reported. The increased thickness of the pDEO relative to DED suggests that the reduced penetration seen in our experiments may simply reflect the thickness of the scaffold. Studies using synthetic scaffolds have shown that the rate of cell ingrowth correlates with matrix porosity and pore size[30,31], although the optimal values vary with cell type[32]. In addition, fibroblast penetration into acellular dermal scaffolds has been

shown to be affected by the matrix porosity[33]. Consequently it is also possible that the porosity of the pDEO may be limiting HOF ingrowth.

In contrast to the pDEO regime, the collagen I gel seeding regime ensured a good fibroblast presence was observed, but adhesion of the epithelium to this substrate was reduced compared to the oesophageal matrix. The lack of adhesion may reflect the absence of specific basement membrane proteins and microanatomical structures (e.g. papillae) which were preserved in the porcine tissue. The formation of collagen IV by fibroblasts cultured within the gel is notable in this respect and it may be that longer incubation times would lead to further production of this and other proteins critical to epithelial adhesion and thus provide improved results.

The most suitable model for a given experiment will depend on the scientific question being asked. For example, studies of interactions between squamous cells and fibroblasts might be best undertaken using collagen I as the scaffold. Het-1A and human oesophageal fibroblasts give an abnormal epithelium irrespective of the substrate used. Studies focusing on the regulation of maturation of squamous cells would obviously require non-transformed squamous cells which have retained the ability to differentiate. Here our study clearly shows seeding HOS (together with HOF) on porcine oesophagus gives an epithelium which mimics many of the features of the normal oesophagus at both the gross histology and immunohistochemistry level and we recommend this as a robust model of the normal oesophagus. We have also recently maintained cultures grown in this way for up to 32 days (21 days at the air liquid interface), a time frame that should facilitate a wide range of studies into pathogenesis of oesophageal cancer and other diseases.

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