Urothelial cell transplantation using biodegradable synthetic scaffolds

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Reconstruction of the urinary bladder with bowel to restore storage capacity is associated with significant complications arising from substituting an absorptive, mucus-producing intestinal epithelium for the barrier urothelium of the bladder. To overcome these problems, we are developing a ''composite enterocystoplasty'' procedure to replace the epithelium of the bowel with autologous *in vitro*-propagated normal urothelial cells. The aims of this study were to evaluate synthetic biomaterials as delivery vehicles for the cultured urothelial cells and provide support during transfer and cell adherence to the de-epithelialized bowel wall. The surgical compliance of 12 biomaterials was evaluated, along with their ability to support urothelial cell attachment. Transfer of urothelial cells onto biomaterials as single cell suspensions or intact cell sheets was investigated. Seeding of a single cell suspension on to non-woven mesh resulted in poor cell attachment. Seeding onto woven mesh was more efficient, but the most effective transfer method involved producing an intact cell sheet that could be combined with woven, knitted and non-woven biomaterials. Transfer of the cell sheet : mesh complexes onto a de-epithelialized bladder stroma produced a stratified epithelium incorporating the strands of the mesh and expressing urothelial-associated antigens after 48 h in organ culture. Thus, we have developed and evaluated a suitable transfer method for *in vitro* propagated urothelial cells to be used in ''composite enterocystoplasty''.

1. Introduction

Reconstructive surgery is commonly used to manage the structural or functional loss of the urinary bladder caused by a number of pathological conditions [1–5]. Contemporary reconstructive methods use segments of the gastrointestinal tract as an alternative material for bladder replacement (enterocystoplasty). However, a number of complications arise from using a material that is lined with a secretory and absorptive epithelium, which makes the use of this approach less than ideal [6–11].

Alternatives to enterocystoplasty for bladder reconstruction have been investigated for many years and have included the use of non-biodegradable synthetic materials, such as Teflon, polyvinyl alcohol, terylene, nylon and silicone rubber (reviewed in Gleeson and Griffith [12]). Because of problems of biocompatibility, the use of such synthetic materials has largely been abandoned and more recently, several natural biodegradable materials have been used with limited success in animal models. These include bladder acellular matrix grafts [13], small intestinal submucosa [14] and a trilayer urothelial cell : scaffold : smooth muscle composite [15]. With the recent advances in tissue engineering a number of artificial biodegradable materials have become available and several have been investigated for their ability to support urothelial cell attachment with a view to developing a bladder substitute [16–19].

Our approach has been to develop a ''composite enterocystoplasty'' procedure, taking advantage of the compliance of the intestine wall, but replacing the intestinal epithelium with a normal bladder urothelial cell lining that can function as a urinary barrier. This approach has been made feasible by the development of techniques for the *in vitro* propagation of urothelium, so that adequately large areas of urothelium can be produced by culture of urothelial cells isolated from biopsies taken by cystoscopy [20, 21]. Critical parts of

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TABLE I Biomaterials

Biomaterial	Configuration	Source
Hyaluronic Acid derivatives		
$H_{11}P_{75}$	Membrane	Fidia
H_{11}	Membrane	Fidia
Hyaloskin	Membrane	Fidia
H_7	Non-woven mesh	Fidia
H_{11}	Non-woven mesh	Fidia
$H_{11}P_{75}$	Non-woven mesh	Fidia
Alginate	Non-woven mesh	P.Frey
Chitosan	Non-woven mesh	P.Frey
Polyglactin 910	Woven mesh	Ethicon
	Knitted mesh	Ethicon
	laminin coated	modified
	collagen-embedded	modified
	etched with NaOH, H_2SO_4, H_2O_2	modified
PDS/Polyglactin composite	mesh	Ethicon
PDS/Prolene composite	mesh	Ethicon
Zenoderm		Ethicon

this procedure are (a) to develop an efficient method of cell transfer from *in vitro* to *in vivo* and (b) identify a suitable biocompatible substratum to support urothelial cell attachment to a de-epithelialized bowel stroma. Here, we describe the results obtained with a number of candidate synthetic biomaterials.

2. Materials and methods

2.1. Biomaterials

Twelve biocompatible synthetic materials were studied (Table I). Polyglactin 910 was either used in its manufactured state, coated with laminin ($1\,\mu g\,ml^{-1}$) or etched by soaking in NaOH, or H_2SO_4 (1, 10 or 100 mM), or H_2O_2 (10%, 25%, and 50%) for 1, 5, and 10 min and rinsed in sterile phosphate-buffered saline (PBS).

2.2. Urothelial cell isolation and culture

Urothelial tissues were collected from surgical patients with no history of urothelial malignancy. The urothelium was detached from the stroma by incubation in 0.1% (w/v) ethylene di-amino tetra-acetic acid, di-sodium salt (EDTA) in PBS and the de-epithelialized stroma used for organ cultures (below).

The urothelium was used to establish normal human urothelial (NHU) cell lines, as previously described [21]. NHU cells were maintained as adherent monolayer cultures in Keratinocyte Serum-Free Medium (KSFM; Gibco BRL, Paisley, UK) containing bovine pituitary extract and epidermal growth factor at the manufacturer's recommended concentrations and $30\,ng\,ml^{-1}$ Cholera toxin (Sigma Chemical Company, Poole, UK). For serial passage, just-confluent cultures were incubated in 0.1% (w/v) EDTA in PBS for 5 min, detached from the substratum by incubation in 0.25% (w/v) trypsin and 0.02% (w/v) EDTA and collected into medium containing $0.5\,mg\,ml^{-1}$ soybean trypsin inhibitor (Sigma). For the experiments described here, NHU cell lines were used between passages 2 and 5.

2.3. Growth of normal human urothelial (NHU) cells on biomaterials

NHU cells were harvested and seeded as a single cell suspension onto the different materials at a seeding density of 0.75×10^5 cells cm^{-2} on membranes and 3×10^5 cells cm^{-2} on meshes. Cultures were monitored, where possible, by phase-contrast microscopy and harvested after one week. The cell : substratum complexes were fixed in a 1 : 1 mixture of methanol and acetone, and stained with haematoxylin and eosin or with acridine orange ($10\,\mu g\,ml^{-1}$) and examined by transmitted or epifluorescent light microscopy, respectively.

2.4. Preparation of urothelial cell sheets

NHU cell cultures were grown to post-confluence and the medium replaced with growth medium to which sterile $CaCl_2$ solution was added so as to increase the final calcium concentration from 0.09 mM to 1.15 mM. After 16 h culture, the medium was replaced with 3 ml of 2% (w/v) dispase (Boehringer, Bracknell, UK) in Hank's balanced salt solution, which resulted in the detachment of the urothelium as an intact sheet, commencing from the edges of the culture vessel.

For combination with meshes, the material was cut to approximately 80% the surface area of the culture vessel and placed on the center of the detaching cell sheet. This prevented excessive contraction of the sheet and maintained the correct polarization of the urothelium for subsequent transfer. Representative samples of the urothelial sheet : mesh complex were fixed in 10% formalin in PBS and embedded in paraffin wax for sectioning or stained with acridine orange and examined by epifluorescent microscopy.

2.5. Transfer of urothelial cell sheets to de-epithelialized organ cultures

Urothelial sheets attached to mesh were cut to size and placed mesh-side up onto 1 cm^2 fragments of autologous bladder stroma from which the urothelium had been removed. The tissue combination was maintained for 48 h at an air : liquid interface on a permeable membrane, as previously described [22]. Cultures were formalin-fixed, processed into paraffin wax, sectioned and examined by histology or immunochemistry.

2.6. Immunochemistry

A panel of antibodies against cytokeratins, cell adhesion molecules and basement membrane components were used as detailed in Table II. Immunochemistry was performed as previously described [23], using an indirect streptavidin ABC immunoperoxidase method (Dako, Ely, UK). Where necessary, the immunoreactivity of antigens masked by tissue processing was restored by boiling in 10 mM citrate buffer pH 6.0 for 10 min, digestion for 10 min in 0.1% (w/v) trypsin (Sigma), or a combination of the two (Table II). Endogenous peroxidase activity on tissue sections was blocked by incubation with 3% (v/v) hydrogen peroxide solution and endogenous avidin-binding sites were blocked using an avidin/biotin kit (Vector Laboratories, UK).

TABLE II Antibodies

Antibody	Specificity	Antigen retrieval	Source
LP1K	CK 7	Microwave	ICRF, London UK
LE 41	CK 8	Microwave	ICRF, London UK
E3	CK 17	Trypsin	Sigma, Poole, Dorset, UK
CY 90	CK 18	Microwave	Sigma, Poole, Dorset, UK
LP2K	CK 19	Microwave	ICRF, London, UK
2F10	CD 44v 6	Microwave	R&D Systems, Abingdon, Oxford, U.K
B115	β_1 integrin subunit	Trypsin	Serotec Kiddling, Oxford, UK
HECD 1	E-Cadherin	Microwave	Takara Biomedicals supplied by R&D Systems
Anti-laminin	Laminin	Trypsin	Sigma, Poole, Dorset, UK
CIV 22	Collagen IV	Trypsin	Dako, High Wycombe, Bucks, UK

Antibodies were titrated on appropriate positive control tissues and omission of primary antibody was included as negative control.

3. Results

3.1. Surgical compliance of biomaterials

Composites of PDS/Polyglactin, and PDS/Prolene were not malleable enough to be manipulated during surgery or to conform to the contours of the underlying tissue. Zenoderm was flexible and surgically manipulatable. The non-woven and woven materials were surgically compliant and conforming.

3.2. Attachment of cells as suspension to biomaterials

Only limited cell attachment was observed when single cell suspensions of NHU cells were seeded onto non-woven meshes H_7, H_{11}, $H_{11}P_{75}$, Alginate and chitosan (Fig. 1a). The large interfiber distance resulted in the majority of cells passing between the fibers and seeding on the surface of the culture vessel below (Fig. 1b). Laminin coating or etching of the polyglactin 910 with NaOH, H_2SO_4, or H_2O_2 had no effect on cell attachment from single cell suspensions. After one week in culture, isolated attached cells showed little evidence of growth.

Cells seeded as a suspension onto the woven mesh, polyglactin 910, which had a negligible interfiber distance, resulted in the majority of cells attaching to the surface (Fig. 1c).

NHU cells attached to PDS/Polyglactin and PDS/Prolene, but as stated above, these materials were too rigid to be of any surgical use. Monitoring of cell attachment and growth by phase contrast microscopy was not possible on Zenoderm due to the density of the matrix fibers. However, staining with hematoxylin and eosin showed evidence of only limited cell attachment. These materials were therefore not studied further.

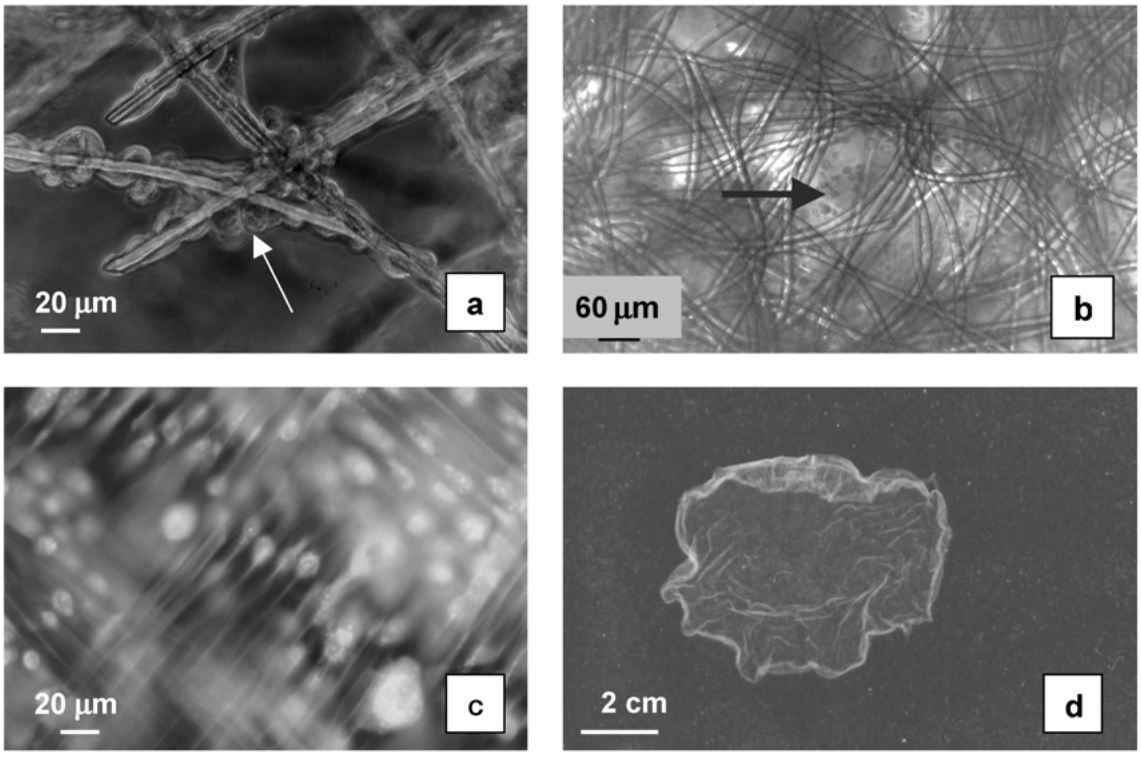

Figure 1 Phase contrast micrograph of a single cell suspension of urothelial cells seeded onto Hyaluronate mesh. Cells attach sparsely to individual fibers (a). The majority of cells pass between adjacent fibers and seed onto the floor of the culture dish below (→) (b). Staining with acridine orange reveals a high attachment of urothelial cells seeded onto woven polyglactin 910 mesh (c). Note the orientation of the cells with their long axis parallel to the direction of the mesh fibers. Phase contrast micrograph of an intact urothelial cell sheet detaching from the floor of culture dish (d).

3.3. Production and characterization of urothelial cell sheets

The transfer of superconfluent urothelial cell cultures to a high calcium medium resulted in a coherent cell sheet that could be harvested with dispase as an intact sheet of cells (Fig. 1d). Transverse sectioning and immunolabeling of the cell sheets revealed a closely-packed monolayer of cells that expressed cytokeratins CK7, CK17, CK18 and CK19, characteristic of normal urothelial cells (Fig. 2). Although these cells did not stratify, they exhibited evidence of polarity, with the nuclei situated basally (Fig. 2).

Combination of the detaching urothelial cell sheet with either a non-woven, woven or knitted mesh allowed 100% transfer efficiency and resulted in a robust cell : biomaterial complex. In this way, materials of any configuration could be used to support the transfer of urothelial cells from monolayer culture to host tissue.

3.4. Transfer of urothelial cell sheets to stromal tissues

In order to evaluate the use of a non-woven mesh as a urothelial cell delivery vehicle and to assess the potential of *in vitro* generated urothelial cell sheets to integrate with a de-epithelialized stroma, urothelial cell sheets were transferred via polyglactin 910 mesh onto de-epithelialized stroma in organ cultures. Organ cultures analyzed after 48 h showed the development of a stratified epithelium incorporating mesh fibers (Fig. 3a). Immunohistochemical analysis of the cell : mesh stromal composite showed that although the stratified epithelium did not display all the morphological or antigenic characterisitcs of fully differentiated urothelium within this time period, the epithelium did express cytokeratins characteristic of urothelium (CK7, CK8, CK17, CK18 and CK19) (Fig. 3). In addition, there was evidence of E-Cadherin localization to the intercellular plasma membrane (Fig. 3d). The cell adhesion molecules, CD44 and β_1 integrin, were expressed within the cytoplasm. All layers of the urothelium expressed intracellular laminin.

4. Discussion

It is critical that the transplantation of urothelial cells is as efficient as possible in order to reduce the quantity of cells required for transplant per unit area of scaffold, as this will reduce the initial harvest of urothelium from the patient, keep the culture time to a minimum and limit the number of cell doublings required to obtain sufficient numbers of cells. This is important as normal cells can undergo only a finite number of divisions before becoming senescent and transplanted cells will require some regenerational capacity. Therefore, the choice of both the cell transfer technique and the delivery scaffold plays an important part in determining the ultimate efficiency.

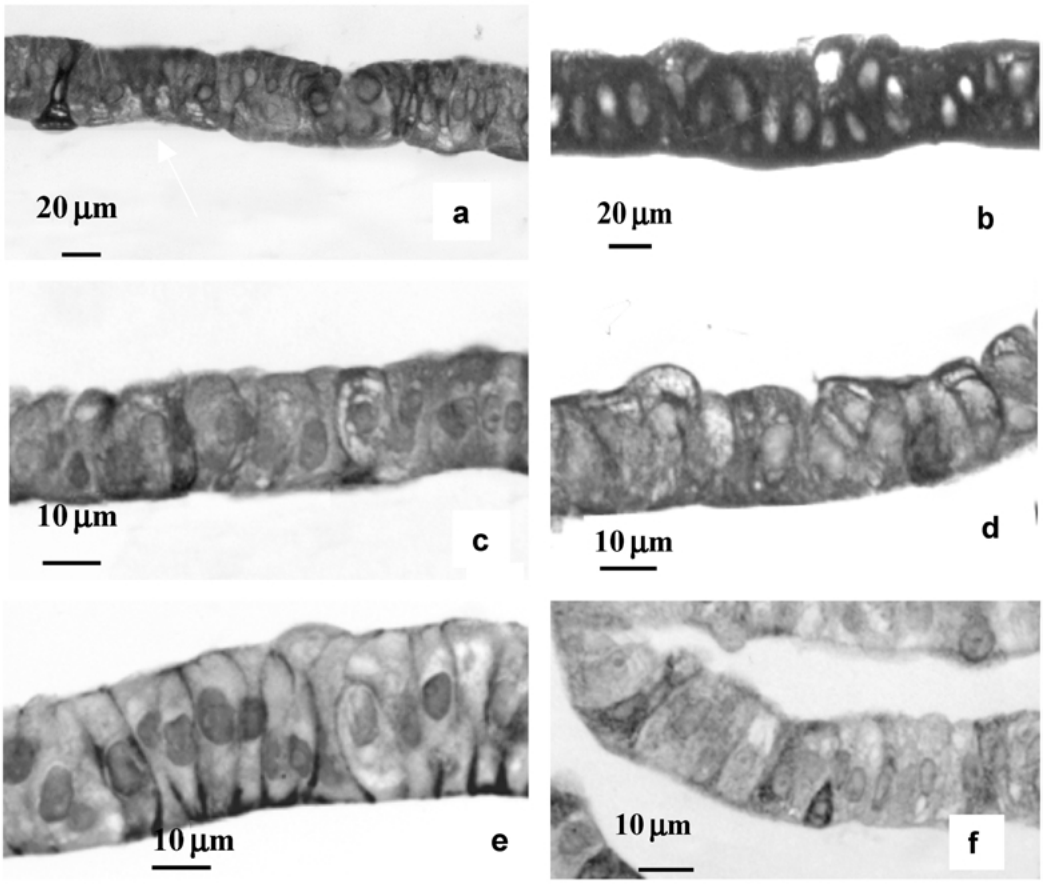

Figure 2 Immunohistochemical labeling of transverse sections through an intact urothelial cell sheet reveals that it is composed of a monolayer of cells expressing CK7 (a), CK17 (b), CK18 (c) and CK19 (d), characteristic of transitional urothelium. The cell adhesion molecule E-cadherin is localized to the intercellular borders (e) whilst there is evidence of cytoplasmic laminin expression in all cells (f).

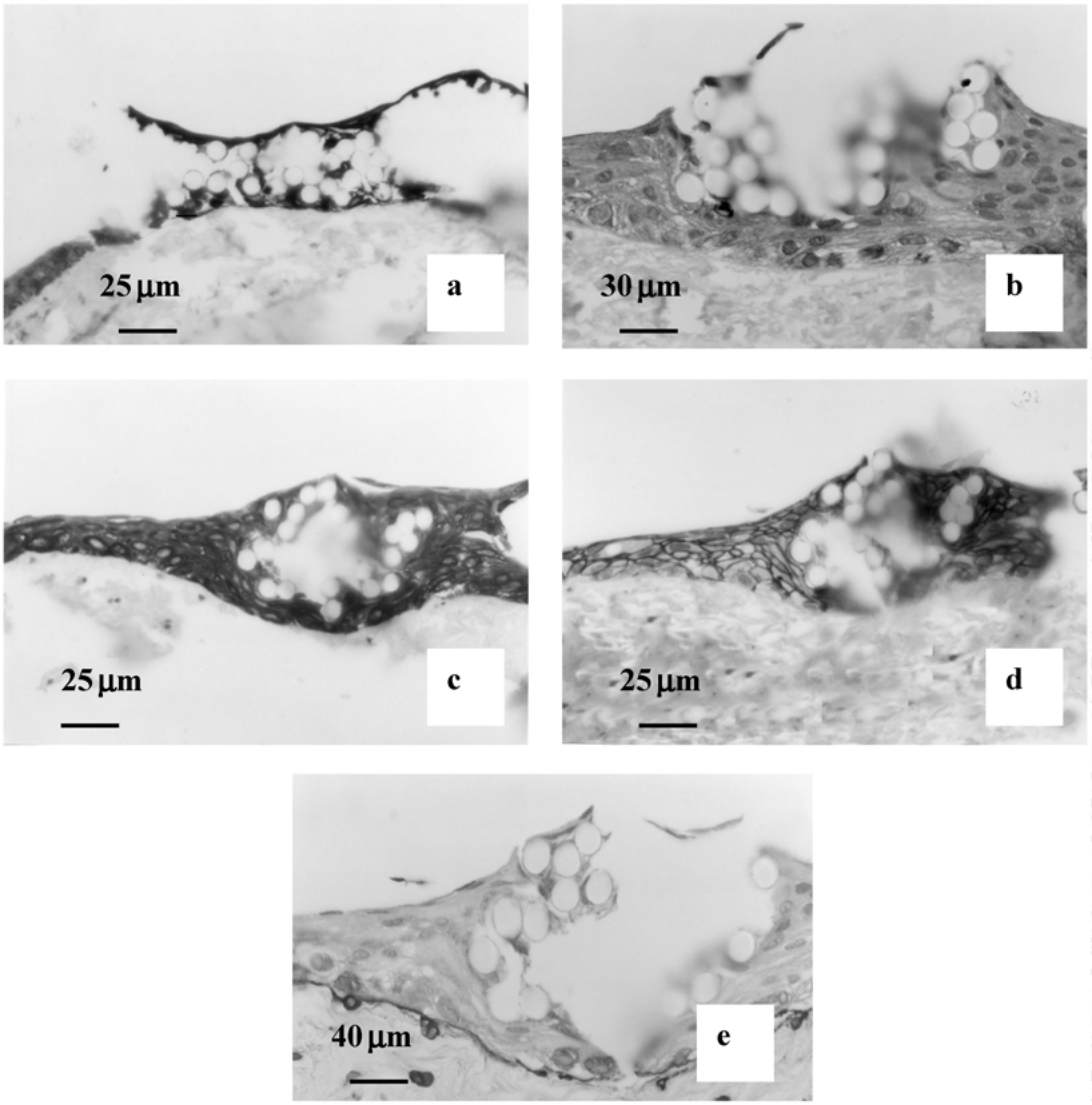

Figure 3 Immunolabeling of the urothelial cell : mesh stromal composite: Within 48 h the monolayer cell sheet forms a stratified epithelium that has incorporated the mesh fibers (a). Expression of cytokeratins CK17 (a), CK18 (b) and CK19 (c) is evident. E-Cadherin is localized to the intercellular borders (d) and intra-cytoplasmic laminin is seen within the urothelial cells (e).

The ability to produce intact urothelial cell sheets for urothelial transfer is a major step forward. First, it allows the transfer of urothelial cells with 100% efficiency, thus substantially reducing the initial quantity of cells required to establish cultures. This is an extremely important factor as the patients who are likely to undergo this type of surgery have very little native urothelium in the first instance. Second, it allows combination of cultured urothelial cells with a wider range of biomaterial configurations, which is likely to be of importance when this work is extended to larger scale pre-clinical studies. It also allows the use of meshed materials, which may be important to allow escape of inevitable exudate from de-epithelialized stromal surfaces and hence allow more rapid cellular attachment. Finally, it maintains cell polarity and allows contact between basal cell surface and exposed stroma.

The choice of scaffold must take into account the need for a material that is compliant surgically and will conform to the underlying tissue shape. Polyglactin 910 is one such material and *in vitro* evaluation of its properties as a vehicle to deliver cells to the transplant site, supported its use for this purpose. Cytokeratin analysis of the epithelium formed following transfer onto a stroma showed that the cells retained urothelial characteristics and had reconfigured to a multilayered epithelium. The rapid stratification indicates that once transplanted *in vivo* a neo-urothelium should develop quickly, whereas localization of E-cadherin at inter-cellular borders indicates cell : cell cohesion. The finding of intracellular laminin supports our previous work suggesting this component of the basement membrane is produced by the epithelial cells [21, 22]. The rapid incorporation of the mesh fibers indicates the development of an intricate relationship between cells and fibers, so that the mesh will need to remain *in situ* until the fibers biodegrade naturally. One of the advantages of using a synthetic biodegradable material is that its structure and hence physical properties can be altered during manufacture, thereby increasing or decreasing its half-life *in situ*. If it is found that polyglactin 910 degrades more quickly than anticipated when in contact in urine, it should be a relatively straightforward issue to address. Further studies are required to determine the optimum life of the mesh.

Following this work it will be necessary to ascertain

whether urothelial transplantation onto de-epithelialized bowel stroma occurs with the same avidity. Recent co-culture studies of urothelial cells with intestinal fibroblasts suggest that these cells do not support urothelial cell growth to the same extent as their bladder or ureteric counterpart [24]. Unfortunately intestinal stromal organ cultures are extremely difficult to maintain in organ culture and therefore, it is likely that these studies will have to be conducted *in vivo*. Work addressing this issue is currently being undertaken.

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