Comparison of a poly-L-lactide-co- ϵ -caprolactone and human amniotic membrane for urothelium tissue engineering applications

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The reconstructive surgery of urothelial defects, such as severe hypospadias is susceptible to complications. The major problem is the lack of suitable grafting materials. Therefore, finding alternative treatments such as reconstruction of urethra using tissue engineering is essential. The aim of this study was to compare the effects of naturally derived acellular human amniotic membrane (hAM) to synthetic poly-L-lactide-co- ϵ -caprolactone (PLCL) on human urothelial cell (hUC) viability, proliferation and urothelial differentiation level. The viability of cells was evaluated using live/dead staining and the proliferation was studied using WST-1 measurement. Cytokeratin (CK)7/8 and CK19 were used to confirm that the hUCs maintained their phenotype on different biomaterials. On the PLCL, the cell number significantly increased during the culturing period, in contrast to the hAM, where hUC proliferation was the weakest at 7 and 14 days. In addition, the majority of cells were viable and maintained their phenotype when cultured on PLCL and cell culture plastic, whereas on the hAM, the viability of hUCs decreased with time and the cells did not maintain their phenotype. The PLCL membranes supported the hUC proliferation significantly more than the hAM. These results revealed the significant potential of PLCL membranes in urothelial tissue engineering applications.

Keywords: urothelium tissue engineering; poly-L-lactide-co- ϵ -caprolactone; amniotic membrane; urothelial cell characterization

1. INTRODUCTION

Urothelial defects are fairly common and these defects are mainly caused by congenital malformation, trauma or stricture. For instance, the incidence of congenital hypospadias is approximately 1 of 200 male births [1,2]. The urothelial defects are nowadays repaired by traditional reconstructive surgery using, for instance, the patient's own genital tissue. The more severe cases, when the patients' genital tissue is inadequate, are repaired using non-urolurgical tissue, such as buccal mucosal grafts [1–3]. However, these operations, particularly those where non-urolurgical grafts are used, are prone to complications, such as fistula formation or urethral strictures [3,4]. Thus,

alternative methods are needed and tissue engineering may be a potential method for remedying severe urothelial defects in the future.

Native urethra has a tubular structure; therefore an ideal biomaterial for urothelial tissue engineering should be elastic to form the tube-like structure; further, the basement membrane of urothelium is elastic and, therefore, the elastic biomaterial would mimic the natural growth surface of human urothelial cells (hUCs). Additionally, the biomaterial should be biodegradable, biocompatible, promote urothelial tissue regeneration and degrade without disadvantageous tissue reactions [5–7]. Furthermore, the ideal tissue-engineered urothelium should have a urothelial-specific surface structure: the cells in the superficial cell layer of native urothelium are large and frequently binucleated umbrella cells characterized by compact tight junctions

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and the presence of defined plaques of asymmetric unit membrane (AUM). The native urothelium expresses intermediate filament proteins, cytokeratins (CKs) and uroplakins (UPs) and these proteins should also be present in the ideal tissue-engineered urothelium.

Various natural biomaterials, such as collagen, small intestinal submucosa, human amniotic membrane (hAM) and different synthetic biomaterials, such as polyglycolide (PGA), polylactide (PLA) and polycaprolactone, have proven to be suitable for urothelial tissue-engineering applications [5,7–9]. Additionally, Wunsch *et al.* [7] demonstrated that porcine urothelial cells on natural matrices were more similar to native urothelium when compared with synthetic matrices. Furthermore, a recent study showed that the hAM was a potential matrix for mouse urothelial cells (mUCs) when compared with collagen, peritoneum and omentum [10]. The major problems concerning natural biomaterials have been their poor mechanical properties, xenograft origin and fabrication difficulties [5,9]. These problems may be avoided using synthetic biomaterials, but the absence of bioactive molecules makes the utilization of synthetic biomaterials in cell culture more challenging compared with natural biomaterials [5]. PGA and PLA have shown to possess an excellent biocompatibility for hUCs [11]; however, the disadvantage of using PGA and PLA is their hardness and inelasticity. Additionally, PGA degrades and loses its mechanical properties rapidly [12,13]. Even though many different biomaterials have been tested with promising results, the optimal biomaterial has not yet been found.

In this study, we compared the effects of synthetic poly-L-lactide-co-ε-caprolactone (PLCL) membrane to natural hAM matrix on hUC proliferation and differentiation. We hypothesized that especially PLCL would serve as a suitable matrix for hUCs because of the elasticity of PLCL and the potential of PLA-based polymers in various tissue-engineering applications [5,9].

2. MATERIALS AND METHODS

2.1. Materials

The hAM was separated from the chorion, rinsed with 0.9 per cent NaCl and incubated in $2.5 \mu\text{g ml}^{-1}$ amphotericin B, $5.0 \mu\text{g ml}^{-1}$ penicillin, $50 \mu\text{g ml}^{-1}$ streptomycin and $100 \mu\text{g ml}^{-1}$ neomycin in Dulbecco's phosphate-buffered saline (DPBS, Sigma-Aldrich). The epithelial cells on hAM were removed with 4 per cent dispase (Invitrogen, Paisley, UK) solution. The hAM was rinsed to completely remove dispase traces and the acellularity of hAM was confirmed with a microscope.

The copolymer, poly-L-lactide-co-ε-caprolactone (70/30) (PURAC biochem bv, Gorinchem, The Netherlands), used for manufacturing the PLCL membranes, was medical grade and highly purified (residual monomer <0.5%) with an inherent viscosity of 1.58 dl g^{-1} . The PLCL membranes were fabricated by compression moulding of PLCL granules, which was performed with NIKE (Hydraulics Ab, Eskilstuna, Sweden) device as follows. First, preliminary moulding was performed at 10 MPa, 110°C for 90 s followed by

the final moulding into $150 \mu\text{m}$ thick membranes at 20 MPa, 130°C for 30 s. The membranes were cut into samples with a diameter of 16 mm, washed with ethanol and sterilized at 25 kGy before the cell culture experiments. Cell culture plastic (PS) wells (Sigma–Aldrich/Corning CellBIND, St Louis, MO, USA) served as a control material.

2.2. Material characterization

Material characterization was performed to the raw 70/30 PLCL material and to the fabricated samples after sterilization. Differential scanning calorimeter (DSC; DSC Q 1000, TA Instruments, New Castle, Delaware, USA) was used to determine the glass-transition temperature (T_g) of the material.

Inherent viscosity (IV) measurements were performed with Lauda PVS viscometer (Lauda Dr R. Wobser GmbH&co, Königshofen, Germany). Samples were prepared by dissolution of the sample in chloroform with a concentration of about 1 mg ml^{-1} . The IV was determined with capillary viscometer (Ubbelohde type 0c, Schott-Geräte, Mainz, Germany).

2.3. Cell isolation and culture

Cell isolation was performed as represented by Southgate *et al.* [14] with minor modifications. Briefly, the tissue samples were cleaned, cut and incubated in stripping solution containing 0.01 per cent HEPES buffer (1 M, Sigma–Aldrich), 4×10^{-3} per cent aprotin ($1 \text{ KIU } \mu\text{l}^{-1}$, Sigma–Aldrich), 0.1 per cent EDTA (Sigma–Aldrich), 0.01 per cent penicillin/streptomycin (Lonza, Verviers, Belgium) in Hanks' balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+} (Invitrogen). The urothelial sheets were separated from tissue samples and incubated in 0.1 per cent trypsin (Lonza). To inactivate trypsin, 10 per cent human serum of the clot type AB (HS, PAA Laboratories, Pasching, Austria) in HBSS was added. The solution was centrifuged and the resulting pellet was suspended in a defined urothelium medium (EpiLife, Invitrogen). The hUCs were cultured in cell bind T75 flasks (Sigma–Aldrich) at 37°C in a humidified atmosphere of 5 per cent CO_2 in air. Overall, hUCs from three patients, passages 2 and 3, were used in the experiments.

2.4. Flow-cytometric surface marker expression analysis of human urothelial cells

The hUCs were harvested and analysed after primary culture by a fluorescence-activated cell sorter (FACS) (FACSaria: BD Biosciences, Erembodegem, Belgium). Monoclonal antibodies against CD44-PE, CD73-PE, CD105-PE, CD133-PE, CD166-PE (BD Biosciences), CD326-APC (Miltenyi Biotech, Bergisch Gladbach, Germany) and Keratin 8/18 (Cell Signaling Technology, Danvers, MA, USA) were used. MAb Keratin 8/18 was conjugated with IgG-alexa488 (Molecular Probes, Eugene, OR, USA). A total of 10 000 cells per sample was analysed; positive expression was defined as a level of fluorescence which was 99 per cent of the corresponding unstained cell sample.

2.5. Cell seeding

Before cell seeding, PLCL membrane and hAM were attached to the membrane fixation devices, cell crowns (CellCrown24, Scaffoldex, Tampere, Finland); later, these were attached to the wells of a 24-well plate. The sample materials were preincubated in urothelium medium at 37°C for 48 h. On to each membrane, previously supplied with 1 ml of medium, 20 000 cells were seeded. The hUCs were cultured at 37°C in a humidified atmosphere until analysis.

2.6. Cell viability evaluation

Cell viability was studied using qualitative live/dead fluorescent staining after 3, 7 and 14 days of cell culture. Briefly, the cells were rinsed with DPBS and incubated at room temperature for at least 30 min with a mixture of 0.25 µM Calcein AM (green fluorescence, Molecular Probes) and 0.3 µM Ethidium homodimer-1 (red fluorescence, EthD-1, Molecular Probes). A fluorescence microscope (Olympus, IX51S8F-2, camera DP71) was used to image the viable cells (green fluorescence) and dead cells (red fluorescence). PLCL, hAM and PS without cells were used to exclude false-positive staining caused by materials alone.

2.7. Cell proliferation assay

After 3, 7 and 14 days of culture, the proliferation of hUCs was determined by WST-1 analysis by measuring the mitochondrial activity. The cells were incubated at 37°C for 4 h with 50 µl of premix WST-1 cell proliferation assay system (Takara Bio Inc, Otsu, Shiga, Japan) and 500 µl of DPBS. The absorbance was measured with a microplate reader (Victor 1420 Multilabel Counter, Wallac, Turku, Finland) at 450 nm.

2.8. The phenotype characterization of human urothelial cells using immunostaining

The phenotype of hUCs was verified by immunostaining after 7 and 14 days using CK7/8 (1:200, Lab Vision, Fremont, CA, USA), CK19 Ab-1 (1:500, Lab Vision) and UPIII (1:200, Santa Cruz Biotechnology, Heidelberg, Germany) as primary antibodies. The cells were fixed with 4 per cent paraformaldehyde fixative (Sigma-Aldrich) and incubated in primary antibody dilutions. Thereafter, secondary antibodies from mouse and goat (1:400, Alexa-488, green fluorescence, Molecular Probes) were conjugated to primary antibodies. Finally, the cells were mounted with Vectashield (DAPI; blue fluorescence, Vector Laboratories, Peterborough, UK) and imaged with the fluorescence microscope (Olympus). False-positive staining caused by materials alone was excluded using membrane materials without cells.

2.9. Statistical analysis

Statistical analyses were performed with SPSS, v. 13 (SPSS, Chicago, IL, USA). After verifying normal distribution and homogeneity of variance, the effects of different materials were compared using one-way analysis of variance (ANOVA). Post hoc (Bonferroni) tests

were performed to detect significant differences between the materials. The effect of culturing period (3 versus 7 days and 7 versus 14 days) was also studied using one-way ANOVA and *post hoc* tests. Data were reported as mean $\pm$ s.d., $p < 0.05$ was considered significant and $p < 0.01$ highly significant.

3. RESULTS

3.1. Material characterization

Material characterization studies showed minor changes in the PLCL properties after processing and sterilization. The T_g of PLCL samples was reduced from 18.1°C to 15.7°C, which indicated that there was a certain degree of polymer degradation during membrane manufacturing. Also, IV measurements supported these results as they decreased from 1.58 to 1.03 dl g⁻¹.

3.2. Flow-cytometric surface marker expression analysis of human urothelial cells

Based on the FACS analysis, the isolated hUC population was homogeneous. The hUCs expressed the extracellular matrix adhesion marker CD44, endothelial markers CD73 and CD105, and epithelial markers CD133, CD166 (ALCAM) and CD326 (EpCAM). The hUCs also expressed the intracellular marker Keratin 8/18, which is a specific marker for epithelial cells (figure 1).

3.3. Cell viability

Live/dead staining illustrated that the majority of cells adhered to all the studied materials and were viable at the 3 day timepoint (figure 2). After a week of cell culture, more dead cells were detected on the hAM and PS than after 3 days but on the PLCL there were very few dead cells. Additionally, after two weeks of cell culture, the majority of cells were viable on the PLCL. On the PS, more dead cells were detected, although the majority of cells were viable. However, on the hAM, the majority of cells were dead after 2 weeks of culture. The visual qualitative analysis revealed that the number of cells attached on the PS and PLCL membranes was notably higher than on the hAM at the 7th and 14th days of culture. In addition, on the PLCL and PS, the number of cells increased from the 3rd day of culture to the 14th day of culture as opposed to the hAM.

3.4. Cell proliferation

WST-1 analysis revealed significant differences between the material types and culturing periods (figure 3). On the PLCL membranes, the cell number significantly increased in all timepoints, and after 14 days of culture the cell number was significantly higher than on the other materials. Only on the hAM, the cell number did not increase after 7 days of culture when compared with 3 days. Also, the hAM supported hUC proliferation less well at the 7 and 14 day timepoints when compared with the PLCL and PS. Interestingly, the

Figure 1. Surface marker expression characteristics of human urothelial cells (hUCs) as analysed by flow cytometry. Dot plot demonstrating homogeneity of cell size (FSC) and surface (SSC) complexity of unstained control cells. Histograms depicting relative cell number (*y*-axis) and fluorescence intensity (*x*-axis), with unstained control cells (empty histograms) and cells stained with antibodies against the surface proteins (filled histograms).

hUCs reached maximum proliferation at the 7 day time-point when cultured on the PS and hAM, and the cell number significantly decreased at the 14 day timepoint when compared with the 7 day timepoint.

3.5. The phenotype characterization of human urothelial cells using immunostaining

The phenotype of hUCs was assessed after 7 and 14 days of cell culture by determining the expression of CK7/8, CK19 and UPIII proteins. Moderate expression of CK7/8 and CK19 (green fluorescence) was detected after 7 and 14 days of cell culture on the PS and PLCL (figure 4). The phenotype of hUCs on the PLCL and PS was unchanged at 14 days when compared with 7 days. However, on the hAM, the CK19 and CK7/8 expression of hUCs decreased during the assessment period. UPIII was not expressed by hUCs on any material or at any timepoint (data not shown).

4. DISCUSSION

In this study, we used hUCs taken from the ureter during routine surgery. These cells are fairly similar to cells in the proximal part of the urethra, as the whole urinary tract except the distal part of urethra is covered by the urothelium and therefore these cells can be used in future urethral reconstruction applications [15]. Urothelial cells have also been widely studied *in vitro* for urothelial tissue engineering purposes; further, Fossum *et al.* [16] used bladder urothelial cells for clinical urethra reconstruction. Moreover, urethral stratified squamous epithelial cells could not be used for this since it would have been unethical to take tissue from the urethra because the urethra is susceptible to scarring [17].

The identity of hUCs was confirmed using FACS analysis. The markers CD44, CD73, CD105, CD133 and Keratin 8/18 were selected for this study as they have been previously used to characterize hUCs.

Figure 2. Representative images of viable (green fluorescence) and dead (red fluorescence) hUCs attached to (a) hAM, (b) PLCL and (c) PS after (i) 3, (ii) 7 and (iii) 14 days of culture. Scale bar, 100 μm .

Figure 3. The number of hUCs cultured for 3, 7 and 14 days on the hAM (white), PLCL (grey) and PS (black). Results are expressed as means + s.d. cell number in two experiments ($n = 5-6$). * $p < 0.05$ with respect to hAM; ** $p < 0.01$ with respect to hAM and PS. The number of cells increased significantly ($p < 0.05$) on PLCL in all timepoints. On PS, the cell number increased significantly ($p < 0.01$) after 14 days of culture when compared with 7 days. Cell number on PS and hAM decreased significantly ($p < 0.05$) at day 14 when compared with day 7.

Our characterization results were consistent with previous results for urothelial cells [18,19]. In addition to these previously tested markers, we wanted to test CD166 and CD326, as those markers are known to be expressed in epithelial cells [20,21], which was also demonstrated for hUCs in our study.

The immunostaining results demonstrated that on PLCL and PS, CK7/8 and CK19 staining was evident

during the whole cell culture period as expected, because those markers are present in all layers of normal urothelium [14,22–24]. In contrast, on the hAM, the CK19 expression of hUCs decreased with time. Although UPIII is expressed in native urothelium, previous studies have demonstrated that UPIII is not expressed in human urothelium primary cultures, as our results also indicated [14,25,26]. Only one study, carried out with mouse cells, has demonstrated the expression of UPIII in urothelial primary cultures [10]. The lack of expression may have been because UPIII is present only in terminally differentiated umbrella cells in AUM [14,24]. After the isolation of hUCs, the umbrella cells may not attach and grow as well as the other hUCs from the intermediate and basal layers.

To our knowledge, this is the first study comparing the capacity of PLCL membrane and hAM to maintain the viability, proliferation and phenotype of hUCs. Choosing a suitable biomaterial is critical for successful urothelial tissue engineering application. We selected PLCL, a copolymer of lactic acid and ϵ -caprolactone, owing to the application requirements, and because the ϵ -caprolactone makes the material softer, more elastic and easier to suture and construct as a tube-like structure [27,28]. Furthermore, PLCL was a safe choice since synthetic lactic acid-based polymers are widely used in different tissue engineering applications, including urology, with promising results [6,9,11].

Our WST-1 measurement results showed that the PLCL membranes supported the proliferation of hUCs during the 14 day culture period, as expected. In addition, according to the live/dead staining, the majority of hUCs were viable and the cell morphology corresponded to the native hUCs with compact and round shapes at all timepoints. The live/dead staining

Figure 4. The immunofluorescence images of hUCs at the 7th and 14th day when cultured on hAM, PLCL and PS. Antibodies (a) CK7/8 and (b) CK19 (green fluorescence) were used to confirm urothelial phenotype. Scale bar, 100 μm .

and WST-1 measurement results were consistent, showing the increase in cell number on the PLCL with time. These results are also concordant with studies where PLCL has proven to be a biocompatible material for different tissue engineering applications [29,30].

The second material, acellular hAM, was chosen for this study as the previous reports have shown the hAM to be a potential matrix material for corneal epithelial cells [31] and mUCs [10]. hAM is a highly collagenous natural biomaterial, containing mainly collagen I and III; collagenous biomaterials are generally known to facilitate cellular adhesion [5,32]. Furthermore, Koizumi *et al.* [33] showed that epithelial-based cells grew better on denuded hAM than on cellular hAM. As expected, the attachment of hUCs on the hAM was good. However, in contrast to the previous study of mUCs [10], our live/dead staining and WST-1 measurement both verified that the cell number significantly decreased after 7 days, demonstrating that hAM is not a suitable biomaterial for hUC growth. Furthermore, the cell morphology on the hAM differed notably from the normal hUC morphology on the PLCL membranes and on the PS; the cells were large, irregularly shaped and formed projections. Results contradicting those of Sharifiaghdas *et al.* [10] may be due to the differences between the species and cell types, as in this study we used urothelial cells isolated from human tissue.

5. CONCLUSIONS

The results of this work demonstrated that hUCs preferred to proliferate on the synthetic PLCL

membranes. On the other hand, hAM appeared to be an unsuitable matrix material for the hUCs. Based on these results, the PLCL membranes show significant potential in future urothelial tissue engineering applications. Additionally, owing to the promising results, PLCL will be further studied in an *in vivo* model.

Amniotic membranes were obtained following routine caesarean section at Tampere University Hospital in accordance with the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (R06045). Human urothelial tissue samples were isolated from dilated ureters, which were resected or tapered for neoinplantation from child patients in Tampere University Hospital with the approval of the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland (R071609).

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