Electrospun collagen/poly(L-lactic acid-co-ε-caprolactone) scaffolds for conjunctival tissue engineering

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Abstract. Conjunctival injuries are general but intractable ocular surface diseases, the sequelae of which are particularly challenging to treat. A promising therapy for conjunctival injuries is to employ biodegradable scaffolds to deliver conjunctival epithelial cells for repairing damaged or diseased conjunctiva. In the present study, an ultrathin porous nanofibrous scaffold was fabricated by using collagen and poly(L-lactic acid-co-ε-caprolactone) (PLCL) and displayed a thickness of 20 μm, with a high porosity and an average fiber diameter of 248.83±26.44 nm. Conjunctival epithelial cells seeded on the scaffolds proliferated well and had a high cell viability. Reverse-transcription quantitative PCR showed the expression of conjunctival epithelial cell-specific genes; in addition, there was no significant difference in the inflammatory gene expression between cells grown on collagen/PLCL scaffolds and tricalcium phosphate scaffolds. After co-culture for 2 weeks *in vitro*, epithelial cell stratification was observed using hematoxylin and eosin staining, exhibiting three to four epithelial-cell layers. In conclusion, these results suggested that collagen/PLCL scaffolds have potential application for repairing conjunctival epithelial coloboma.

Introduction

The conjunctival epithelium and the corneal epithelium form the outer surface of the eye, and injury to one part may result in system-wide secondary dysfunction (1). Normal function of the conjunctiva is critical for supporting the normal function of the ocular surface and ensuring clarity of the corneal epithelium, as it provides the mucin (MUC) component of the tear film (2,3). Therefore, reconstruction of the conjunctiva is the prerequisite for successful ocular surface reconstruction. Upon injury, conjunctival epithelium spontaneously re-epithelializes. However, this is usually accompanied with a range of complications, particularly if an extensive area is affected, such as in patients with chemical or thermal burns, Stevens-Johnson syndrome or ocular cicatricial pemphigoid (4,5). In these situations, an appropriate substitute needs to be applied for repairing the coloboma tissue.

The ideal conjunctival scaffolds should be a stable, biocompatible and easy to manipulate, and importantly, mimic the structure and biological function of the natural extracellular matrix (ECM) (6). Although various therapies have been used in clinical studies or in animal models, such as autologous conjunctiva, human amniotic membrane (hAM) (7) and artificial membranes (8,9), they are limited due to numerous reasons. For instance, hAM is widely used as a substitute for conjunctival reconstruction due to the ability to reduce scarring, anti-microbial, anti-angiogenic and anti-inflammatory properties, but concerns regarding the possible transmission of infectious diseases are the main drawbacks (10). Thus, there is a need for artificial scaffolds with well-defined composition and mimicking the ECM for ocular surface application.

Electrospinning has recently attracted increased attention as a method of fabricating nanofibrous scaffolds with high porosity and high surface area to resemble the topographic features of the ECM. The unique nanofibrous structure facilitates cell growth and differentiation, and allows for efficient exchange of nutrients and metabolic wastes between the scaffolds and their environment (11,12).

Type I collagen, the most abundant stromal protein conjunctiva, is biocompatible and biodegradable and possesses low immunogenicity (13). Therefore, collagen, as the main component of ECM, is appropriate for use in the formation of a tissue-engineered conjunctival scaffold, but its poor mechanical properties have hampered its applications (14). Poly(L-lactic acid-co-ε-caprolactone) (PLCL), a co-polymer of poly(L-lactic acid) and poly-ε-caprolactone, is one of the most common biodegradable polyesters for tissue engineering due to its favorable mechanical properties. However, the drawbacks of PLCL, such as its strong hydrophobicity and lack of natural cell recognition sites, have greatly limited its

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application as scaffolds in tissue engineering (15). Therefore, the present study hypothesized that blending the bioactive functions of collagen with the good mechanical properties of PLCL may generate a novel material with the desired cell adhesion, degradation rate and mechanical properties for conjunctival reconstruction.

The present study attempted to employ collagen/PLCL scaffolds to engineer a conjunctival equivalent containing proliferative cells and goblet cells that is an essential indication of a functional conjunctival epithelium. Characteristics of collagen/PLCL scaffolds, such as the diameter of the nanofibers, wettability, mechanical properties and cell viability were determined. To study the cyto-compatibility of the nanofibrous structure for applications in conjunctival tissue engineering, conjunctival epithelial cells were seeded onto the scaffolds. Cell morphology, phenotypes, viability and proliferation were evaluated. Furthermore, histological analysis of the cell-scaffold complexes was performed by hematoxylin and eosin (H&E) staining.

Materials and methods

Fabrication of collagen/PLCL scaffolds by electrospinning. 1,1,1,3,3,3,-hexafluoro-2-propanol (HFIP) and a copolymer of PLCL with a composition of 50% w/w L-lactide and 50% w/w ε-caprolactone monomers were obtained from Donghua University (Shanghai, China) (12). Type I collagen (Mingrang Biotechnology Co., Chengdu, China) and PLCL were dissolved separately in HFIP at a concentration of 8% w/w and stirred vigorously at room temperature for 24 h. Prior to electrospinning, Type I collagen solution and the PLCL solution were mixed at a 25:75 volume ratio, followed by stirring at room temperature for 1 h. The electrospinning conditions were as follows: Injection rate, 1.0 ml/h; voltage, 16 kV and distance, 12 cm. Collagen/PLCL scaffolds were dried in a vacuum oven for 1 week at room temperature to remove residual solvent and stored in desiccators until use.

Characterization of collagen/PLCL scaffolds. The morphology of collagen/PLCL scaffolds was observed by scanning electron microscopy (SEM; JSM-6701; JEOL, Tokyo, Japan). Prior to imaging by SEM, the samples were sputter-coated with gold for 50 sec to increase conductivity. The mean diameter of the nanofibers was measured by Image Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). At least 50 nanofibers from the SEM image were analyzed.

The hydrophilicity of collagen/PLCL scaffolds was determined by water contact angle measurement as described previously (16). The contact angle was measured by a video contact angle instrument (Attension Theta; Attension, Espoo, Finland). Droplets of 1.0 μl were dropped onto the scaffolds. The contact angle indicating the wetting ability of the scaffolds was automatically calculated.

The mechanical properties of collagen/PLCL scaffolds were determined using an uniaxial material testing machine (Instron-3343; Instron, Norwood, MA, USA) equipped with a 10-N load cell (17). Rectangular-shaped specimens (30x10 mm) were stretched at a constant crosshead speed of 10 mm/min. For each specimen, the greatest slope in the linear region of the stress-strain curve corresponding to a strain of 0-20% was used to calculate the Young's modulus. At least five samples were tested.

To evaluate whether collagen/PLCL scaffolds altered the cell cycle, the B4G12 human corneal endothelial cell line (Creative Bioarray, Shirley, NY, USA) was seeded onto the scaffolds in a 24-well plate and cultured in a humidified environment at 37˚C with 5% CO_2 for 3 days (18). The cells were then harvested using 0.25% trypsin (Thermo Fisher Scientific, Inc., Waltham, MA, USA), followed by washing with pre-cooled (4˚C) PBS, and then fixed in pre-cooled (4˚C) 70% ethanol for 1 h. Fixed cells were treated using propidium iodide (PI, Abcam, Cambridge, MA, USA). The total number of cells in different phases of the cell cycle was detected using flow cytometry (Cytomics™ FC500 flow cytometer; Beckman Coulter Ltd., Brea, CA, USA).

Cell isolation and culture. Conjunctival epithelium was isolated and cultured as previously described (19). All animal experiments were approved by the Medical Ethics Committee of Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine. In brief, after sacrification, the conjunctiva was carefully dissected from BALB/c mice (age, 8 weeks; weight, 20±2 g; 10 male and 10 female; purchased from the Animal Centre of Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine) with the underlying connective tissue removed. The sheet was rinsed three times with PBS (1X; 130 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) containing 100 U/ml penicillin and was then incubated with dispase II (2.4 units/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 4˚C for 10 h. The detached epithelial layer was then scattered into single cells with 0.05% trypsin/EDTA for 10 min at 37˚C. Then cells were seeded on a cell culture dish in Dulbecco's modified Eagle's medium/Ham's nutrient mixture F12 (1:1 DMEM/F12; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (HyClone; GE Healthcare, Little Chalfont, UK), 5 μg/ml insulin (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), 5 μg/ml transferrin (Santa Cruz Biotechnology, Inc.), 5 ng/ml selenium (Santa Cruz Biotechnology, Inc.), 1% penicillin/streptomycin, 10 ng/ml human epidermal growth factor (R&D Systems, Inc., Minneapolis, MN, USA) and 100 ng/ml nerve growth factor (R&D Systems). After 2 days of culture, the non-adherent cells were removed by washing with PBS. When reaching confluence, conjunctival epithelial cells were passaged with 0.05% trypsin/EDTA and subcultured for further experiments.

Cell morphology and phenotype on collagen/PLCL scaffolds. The morphology of conjunctival epithelial cells on collagen/PLCL scaffolds was observed by SEM. Conjunctival epithelial cells were seeded on collagen/PLCL scaffolds at a density of $2x10^6$ cells/well in 24-well plates. Two days after cell seeding, the samples were fixed with 0.25% glutaraldehyde (Merck KGaA) overnight at 4˚C. Samples were rinsed and dehydrated with graded concentrations of ethanol (30, 50, 70, 80, 90 and 100% v/v) for 10 min each. Subsequently, the samples were critical-point dried. After drying, the samples were coated with gold sputter and examined by SEM.

Immunofluorescence staining was performed using standard procedures as described previously (19). Briefly, two

days after cell seeding the cell-scaffold complexes were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. The samples were incubated at 4˚C overnight in the primary antibodies [rabbit monoclonal anti-cytokeratin (CK) 19 (ab52625, Abcam, 1:200), rabbit monoclonal anti-CK4 (ab183329, Abcam, 1:200) and mouse monoclonal anti-MUC 5, subtypes A and C (MUC5AC) (ab24071, Abcam, 1:200)]. After washing with PBS, Alexa Fluor^{488} goat anti-mouse/rabbit (BD Biosciences, San Jose, CA, USA, BD5002) was diluted 1:500 in PBS and applied for 1 h at room temperature. A control sample was prepared by omitting the primary antibody. Nuclei were stained with Hoechst 33342 (Invitrogen; Thermo Fisher Scientific, Inc.). Images were captured under a confocal laser scanning microscope.

Cell proliferation and viability on collagen/PLCL scaffolds. To detect the effect of collagen/PLCL scaffolds on cell proliferation, a Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) was performed (20). In brief, conjunctival epithelial cells were seeded onto collagen/PLCL scaffolds at a density of $2x10^4$ cells/well in 24-well plates. After 1, 3, 5 or 7 days of cell seeding, the cells were washed with PBS and incubated with 10% CCK-8 in DMEM/F12. After incubation for 3 h, the absorbance of each well was measured at 450 nm with a microplate reader (Bio-Tek ELx800; Bio-Tek, Winooski, VT, USA). At least six samples were measured at each time-point. For the cell viability study, viability staining was performed using a calcein-acetoxymethylester (CAM) /ethidium homodimer 2 (EthD-2) (Invitrogen; Thermo Fisher Scientific, Inc.) assay, which is based on differential permeability of live and dead cells. When the cells reached confluence, live cells were stained with green-fluorescent CAM, and dead cells were stained with red-fluorescent EthD-2. A fluorescent microscope (Olympus BX51; Olympus, Tokyo, Japan) was used to capture images of the cell staining.

Gene expression detection of the cell-scaffolds complexes. The cell-scaffold complexes were taken from coverslips by using microscope forceps and immersed in TRIzol reagent (Thermo Fisher Scientific, Inc.). The complexes were ground using a Bio-gen pro200 Homogenizer (PRO Scientific Inc., Oxford, CT, USA). Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. The concentration and purity of the total RNA were determined spectrophotometrically at optical density at 260 and 280 nm. DNase I was used to eliminate genomic DNA contamination. The complementary (c)DNA was synthesized from 1 mg total RNA using a PrimeScript™ RT reagent kit (Takara Bio Inc., Otsu, Japan). Real-time polymerase chain reaction (PCR) was conducted using a 7500 Real-Time PCR Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and activated at 95˚C for 10 min and 40 cycles of amplification (15 sec at 95˚C and 1 min at 60˚C). The efficiency of the reaction was measured with primers using serial dilutions of the cDNA (1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125). The primer sequences used for Real-time PCR were as follows: CK4 (192 bp) forward, 5'-TTGAGCAATGACAAGGTCGCC-3' and reverse, 5'-AAGGCTTTCCATCTTGGCCTCT-3'; CK19 (143 bp) forward, 5'-CAGGTCAGTGTGGAGGTGGATT-3' and reverse, 5'-TTCAGCTCCTCAATCCGAGCAA-3'; MUC5AC (150 bp) forward, 5'-ACCACTTTCTCCTTCTCCACAC-3' and reverse, 5'-AACAGGGCTCTTCACAGACAATA-3'; interleukin 4 (IL-4; 160 bp) forward, 5'-CGTCCTCACAGCAACGAAGAAC-3' and reverse, 5'-GCATCGAAAAGCCCGAAAGAGT-3'; IL-5 (128 bp) forward, 5'-ATACTCCCTCCCCCTCATCCTC-3' and reverse, 5'-GTATGTGATCCTCCTGCGTCCA-3'; IL-6 (103 bp) forward, 5'-TTGCCTTCTTGGGACTGATGCT-3' and reverse, 5'-TAGACAGGTCTGTTGGGAGTGG-3'. The relative mRNA levels were expressed as the fold change relative to the control sample [cells cultured on a tricalcium phosphate scaffold (TCPS; Corning Life Sciences, Amsterdam, The Netherlands)] after being normalized to the expression of GAPDH. Relative gene expression was analyzed using the Pfaffl method (21).

Histological findings. After culture for 1 or 2 weeks *in vitro*, the complexes were fixed in 4% (w/v) paraformaldehyde, embedded in Tissue-Tek Optimal Cutting Temperature compound (Sakura Seiki, Tokyo, Japan) and then cut into 10-μm sections. H&E staining was performed to assess epithelial cell stratification.

Statistical analysis. SPSS 18.0 software was used for statistical analysis (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard deviation. The statistical analysis was performed using the Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Characterization of collagen/PLCL scaffolds. Under light, ultrathin scaffolds composed of collagen and PLCL were successfully produced by electrospinning (Fig. 1A). As displayed in Fig. 1B and C, the scaffolds were composed of randomly oriented, defect-free fibers and thoroughly interconnected pore structures, and the average fiber diameter was 248.83±26.44 nm.

The tensile strength was measured to confirm the operability of the scaffolds in tissue engineering. A typical tensile stress-strain curve of collagen/PLCL scaffolds is presented in Fig. 1D, exhibiting considerable tensile strength.

Surface wettability is an important parameter affecting the attachment, proliferation, migration and viability of cells. To determine the wettability of the scaffolds, the water contact angle was measured. It was observed that the water drop was immediately absorbed into the scaffolds, indicating that collagen/PLCL scaffolds were hydrophilic (Fig. 1E).

As shown in Fig. 1F, there was no marked difference in the amount of B4G12 cells in the active cell cycle (G2/M+S) between those on collagen/PLCL scaffolds and those on TCPS, indicating that collagen/PLCL scaffolds did not have any adverse effect on cell proliferation.

Morphology and phenotypes of conjunctival epithelial cells on collagen/PLCL scaffolds. Ideal scaffolds for tissue engineering should maintain a normal morphology and differentiation of the cells. At two days after conjunctival epithelial cells were seeded onto collagen/PLCL scaffolds, cells with polygonal shape adhered and spread on the scaffolds (Fig. 2A).

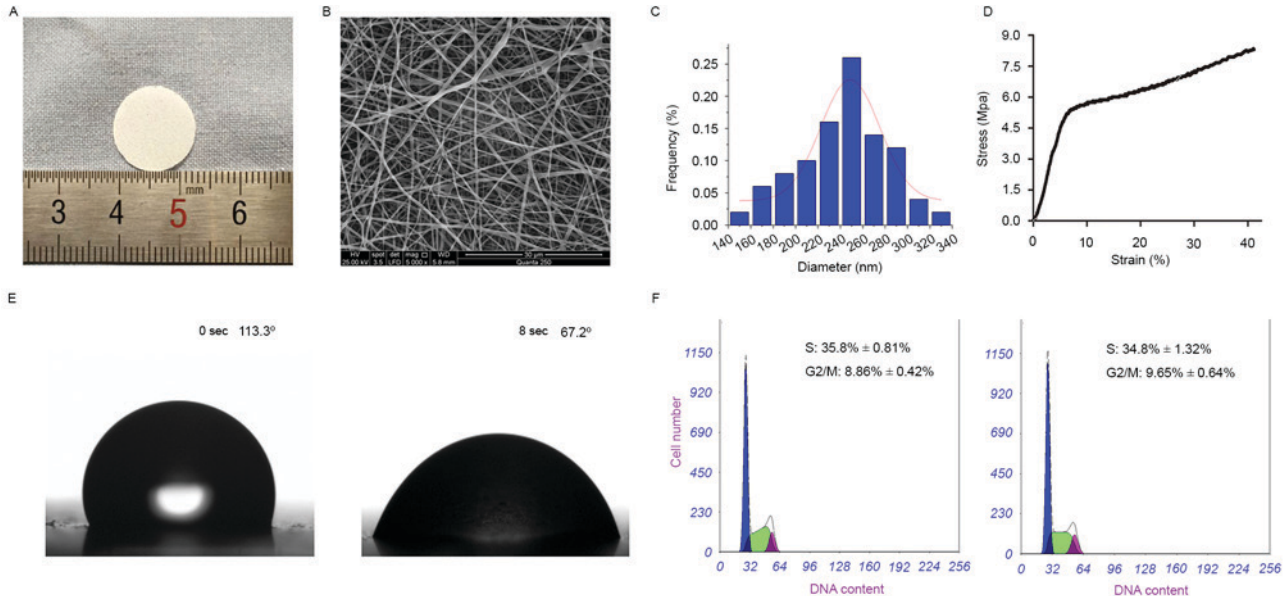

Figure 1. Characterization of collagen/PLCL scaffolds. (A) General appearance of collagen/PLCL scaffolds. (B) Scanning electron microscopy image of electrospun nanofiber scaffolds (scale bar, 30 μm). (C) Diameter distributions of collagen/PLCL scaffold. (D) Typical stress-strain curve of collagen/PLCL scaffolds. (E) Water contact angles at 0 and 8 sec. (F) Cell cycle analysis of B4G12 cells grown on the scaffolds after 3 days of culture. PLCL, poly(L-lactic acid-co-ε-caprolactone).

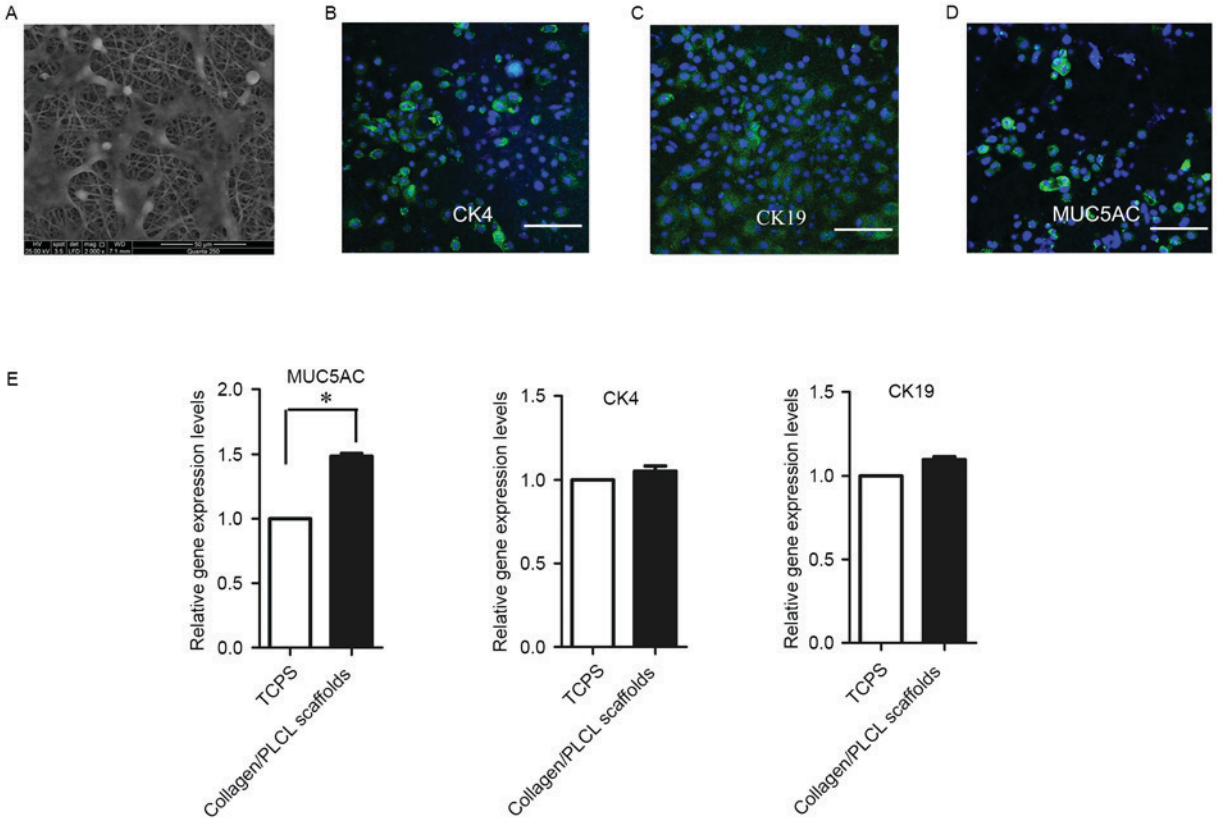

Figure 2. Characterization of conjunctival epithelial cells on collagen/PLCL scaffolds. (A) Morphology of conjunctival epithelial cells on collagen/PLCL scaffolds after 2 days of culture (scale bar, 50 μm). (B-D) Immunofluorescence staining of conjunctival epithelial cells on collagen/PLCL scaffolds (scale bar, 100 μm). (E) Reverse-transcription quantitative polymerase chain reaction analysis of the expression of conjunctival epithelial cell-specific genes. Values are expressed as the mean ± standard deviation. *P<0.05. PLCL, poly(L-lactic acid-co-ε-caprolactone); TCPS, tricalcium phosphate scaffold; CK, cytokeratin; MUC5AC, mucin 5, subtypes A and C.

To study the cell phenotype, the putative differentiation marker proteins of conjunctival epithelial cells were identified (Fig. 2B-D). The cells were positive for CK4 and CK19, specific markers of conjunctival epithelial cells. Furthermore, staining

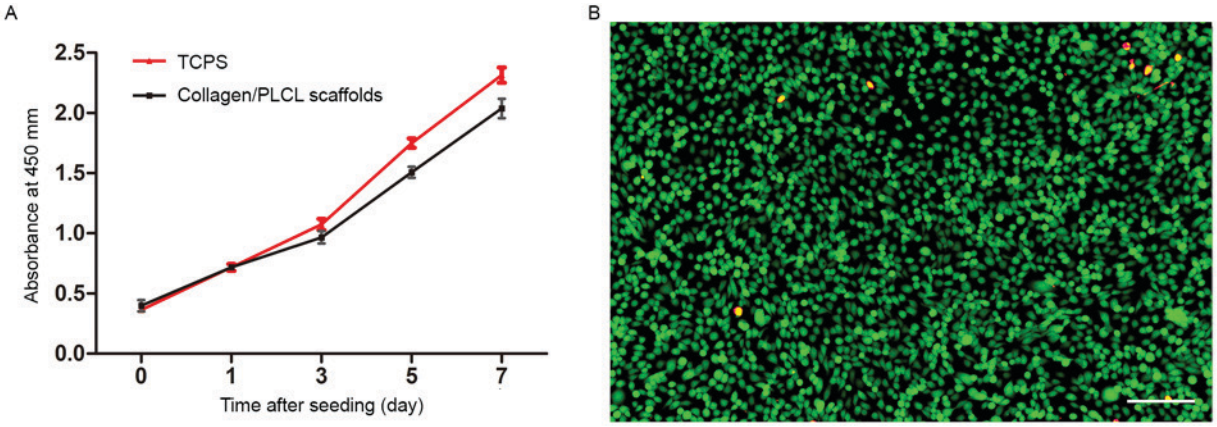

Figure 3. Proliferation, viability of conjunctival epithelial cells on collagen/PLCL scaffolds. (A) Proliferation of conjunctival epithelial cells on TCPS and collagen/PLCL scaffolds at 1, 3, 5 and 7 days after cell seeding determined by a Cell Counting Kit-8 assay. Values are expressed as the mean ± standard deviation. (B) Viable cells on the collagen/PLCL scaffolds were evaluated using a live/dead staining assay. Live cells stained green and dead cells stained red (scale bar, 100 μm). PLCL, poly(L-lactic acid-co-ε-caprolactone); TCPS, tricalcium phosphate scaffold.

for specific secretory conjunctival MUC5AC was positive, indicating the presence of goblet cells.

Reverse-transcription quantitative (RT-q)PCR was also performed to characterize gene expression of conjunctival epithelial cells cultured on the collagen/PLCL scaffolds and TCPS (Fig. 2E). There was no significant difference in CK4 and CK19 expression between cells grown on the collagen/PLCL scaffolds and TCPS; however, MUC5AC transcripts exhibited a significant, 1.5-fold increase in cells grown on collagen/PLCL scaffolds in comparison with those in cells grown on TCPS.

Proliferation and viability of conjunctival epithelial cells on collagen/PLCL scaffolds. A CCK-8 assay was used to quantify cell proliferation on collagen/PLCL scaffolds and TCPS (Fig. 3A). Starting from the same seeding density, one day after cell seeding, cell proliferation was not significantly different between the scaffolds and TCPS. Although the proliferation was lower than that of cells on the TCPS, conjunctival epithelial cells proliferated well and the number of cells increased with culture time, indicating that the scaffolds were non-toxic.

The live/dead staining was performed, with live cells staining green and red color indicating cell death, revealing only a minor proportion of dead cells on the scaffolds (Fig. 3B). This result further confirmed that the scaffolds were non-toxic.

Expression of inflammatory cytokines by conjunctival epithelial cells on collagen/PLCL scaffolds. It is known that numerous scaffolds induce elevated expression of inflammatory genes. Using RT-qPCR analysis, is was demonstrated that IL-4, IL-5 and IL-6 expression between conjunctival epithelial cells cultured on collagen/PLCL scaffolds and those cultured on TCPS was not obviously different (Fig. 4), suggesting that the scaffolds may not elicit any obvious inflammatory responses.

Histological analysis. After 1 or 2 weeks of culture *in vitro*, cell stratification of the cell-scaffold complexes was examined by H&E staining (Fig. 5). The cells adhered tightly to the upper

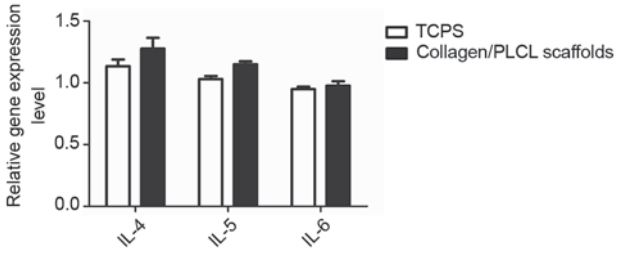

Figure 4. mRNA expression levels of inflammatory factors by cells grown on collagen/PLCL scaffolds and TCPS. There were no significant differences in IL-4, IL-5 and IL-6 expression between cells grown on collagen/PLCL scaffolds and those grown on TCPS. Values are expressed as the mean ± standard deviation. PLCL, poly(L-lactic acid-co-ε-caprolactone); TCPS, tricalcium phosphate scaffold; IL, interleukin.

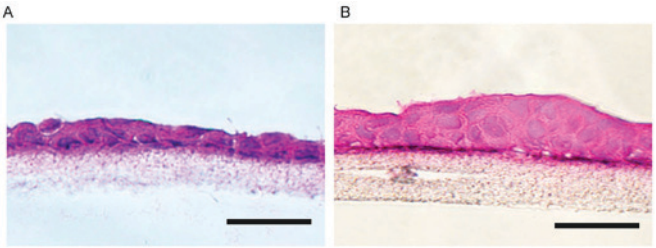

Figure 5. Histological staining of cell-scaffold complexes at different time-points with hematoxylin and eosin. Conjunctival epithelial cells formed multi-layers on collagen/poly(L-lactic acid-co-ε-caprolactone) scaffolds at (A) one week and (B) two weeks (scale bar, 100 μm).

surface of the scaffolds, and 1-2 stratified epithelial layers were present on the surface of the scaffolds after 1 week of culture. With the extension of culture time (2 weeks), cell stratification of the cell-scaffold complexes (3-4 layers) became more similar to the native conjunctiva.

Discussion

Conjunctiva-associated injuries compromise the homeostasis and functionality of the ocular surface (22). Therefore,

conjunctival tissue engineering is the prerequisite for successful ocular surface reconstruction. In the present study, collagen/PLCL scaffolds fabricated by electrospinning were used for conjunctival reconstruction, and it was demonstrated that conjunctival epithelial cells cultured on the scaffolds formed a stratified epithelium containing proliferative cells and goblet cells.

Cell differentiation largely depends on the surrounding microenvironment (23). Therefore, to achieve optimal outcomes in tissue engineering, the applied biomaterials should imitate the structure and biological functions of the natural ECM, which is most favorable for tissue engineering. Recently, electrospinning has attracted increasing interest for application in fabricating biomimetic nanofibrous scaffolds due to their structural resemblance to topographic features of the ECM (24). In the present study, collagen/PLCL scaffolds were successfully prepared by electrospinning. SEM analysis revealed that the collagen/PLCL scaffolds were composed of defect-free nanofibers with a high porosity to mimic the topographic features of the ECM.

Goblet cells, one of the hallmarks of conjunctival epithelium, are responsible for the secretion of large gel-forming mucins in the tear film (25). Mucin component alterations or goblet cell loss are always found in patients with conjunctival disorders (26). Therefore, functional restoration of goblet cells may be a critical procedure for the reconstruction of the ocular surface. It is commonly accepted that if conjunctival epithelium is cultured *in vitro*, is difficult to maintain the differentiation of goblet cells (5). However, the results of the present study revealed that collagen/PLCL scaffolds successfully supported the growth and differentiation of goblet cells. The epithelium formed on top of the scaffolds expressed markers of goblet cells detected by gene expression and immunocytochemical analysis. It was speculated that the beneficial effect of collagen/PLCL scaffolds in maintaining the differentiation of cells may be attributed to their structure, which closely mimics the ECM.

Previous studies have examined whether scaffolds altered the secretion of inflammatory factors by conjunctival epithelial cells. IL-4, IL-5 and IL-6 are important molecules in conjunctival inflammation (3,27,28). The present study found that conjunctival epithelial cells on collagen/PLCL scaffolds did not increase the expression of IL-4, IL-5 and IL-6 compared with those obtained under TCPS culture conditions.

The present study generated collagen/PLCL scaffolds by electrospinning. The scaffolds showed desirable mechanical properties, wettability and the ability to promote cell proliferation. The cultured stratified epithelium displayed a cell stratification similar to that of native conjunctival epithelium. The present study suggested that collagen/PLCL may be a desirable scaffold for the regeneration of conjunctival epithelium.

Acknowledgements

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