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Construction of tissue-engineered full-thickness cornea substitute using limbal epithelial cell-like and corneal endothelial cell-like cells derived from human embryonic stem cells

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ABSTRACT

The aim of this study was to construct a full-thickness artificial cornea substitute *in vitro* by coculturing limbal epithelial cell-like (LEC-like) cells and corneal endothelial cell-like (CEC-like) cells derived from human embryonic stem cells (hESCs) on APCM scaffold. A 400 μm thickness, 11 mm diameter APCM lamella containing Bowman's membrane was prepared as the scaffold using trephine and a special apparatus made by ourselves. LEC-like cells and CEC-like cells, derived from hESCs as our previously described, were cocultured on the scaffold using a special insert of 24-well plates that enabled seeding both sides of the scaffold. Three or four layers of epithelium-like cells and a uniform monolayer of CEC-like cells could be observed by H&E staining. The thickness, endothelial cell density, and mechanical properties of the construct were similar to that of native rabbit corneas. Immunofluorescence analysis showed expression of ABCG2 and CK3 in the epithelium-like cell layers and expression of *N*-cadherin, ZO-1 and Na+/K + ATPase in the CEC-like cells. The corneal substitutes were well integrated within the host corneas, and the transparency increased gradually in 8-week follow-up after transplantation in the rabbits. These results suggest that the strategy we developed is feasible and effective for construction of tissue-engineered full-thickness cornea substitute with critical properties of native cornea.

1. Introduction

Corneal disease (or injury) as a major cause of blindness in the world today remains second only to cataract, affecting more than 10 million people worldwide [1]. Keratoplasty is still the only procedure performed to transplant tissue with the goal of treating corneal diseases [2]. However, there is a significant lack of donor corneal tissue for transplantation in the world [3]. In this situation, tissue engineering and regenerative medicine become the focus of public attention and hold considerable promise for solving the shortage of donor graft for transplantation through the development of a suitable alternative graft material [3,4].

It was reported that acellular porcine corneas and recombinant human collagen as graft replacement could be used for lamellar keratoplasty or stromal replacement [5–8]. However, penetrating keratoplasty (PK) that involves the replacement of the full thickness cornea remains the most widely used corneal transplantation technique for a variety of corneal disorders [9]. Due to the global shortage of donor corneas, the full-thickness cornea substitute suitable for penetrating keratoplasty is desperately needed as an alternative to the donor grafts. Moreover, cornea is avascular and less exposed to the immune system, and therefore cornea transplantation, different from other solid organ transplantation, shows a low risk of graft rejection [10]. Cornea contains relatively few cell types mainly including corneal epithelial cells, keratocytes and corneal endothelial cells. Given the above situation, cornea may be a better choice for tissue engineering [10]. In recent years, tissue-engineered corneal equivalents have experienced major progress. Some researchers have constructed full-thickness corneal substitutes using collagen or extracellular matrix scaffold together with primary cultured corneal cells or corneal cell lines [11–15], but due to the poor biomechanical properties, these corneal

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replacements have been limited to *in vitro* applications.

Acellular porcine cornea matrix (APCM) possessed the key properties of cornea, such as optical clarity, the toughness to withstand surgical procedures, good biocompatibility, and could be used in the construction of tissue engineering cornea [16–18]. It was reported that corneal stromal cells could be found in APCM after 3 weeks of transplantation, and APCM remained stable and retained the thickness they had at surgery for a year [16,19]. Based on this, the keratocytes were not seeded in the construction of the full thickness tissue engineering corneal substitute. Recently, Zhang et al. [10] constructed a bioengineered multilayered human cornea that included corneal epithelium and endothelium using discarded human corneal tissue, and concluded that the constructs could be suitable for full-thickness corneal transplantation, but the shortage of donor corneas limited the generalization of this construct. Compared with human donor corneas, porcine cornea can provide a more sufficient source of scaffold materials. Meanwhile, hESCs have a much stronger proliferation capacity than primary cultured human corneal epithelial cells and endothelial cells. Given this situation, APCM together with the corneal epithelial cells and endothelial cells derived from hESCs may be a better choice for the construction of the tissue-engineered cornea.

In this study, we developed a tissue-engineered multilayered corneal graft that included corneal epithelium and endothelium using APCM together with limbal epithelial cell-like (LEC-like) cells and corneal endothelial cell-like (CEC-like) cells derived from hESCs. The construct possessed critical features of cornea, and showed a good function in penetrating keratoplasty in rabbits. Furthermore, an APCM lamella with similar thickness to native cornea were fabricated as scaffold using a special apparatus made by ourselves, which might be conducive to solve the thickness mismatch between the graft and host in PK.

2. Materials and methods

2.1. Animals

Fresh pig eyes were obtained from a slaughterhouse (Jinan Welcome Food Co. Ltd., Jinan, China). The whole globes were kept at 4 °C in moist chambers, and the central corneas were excised from these eyes within 1 h of receival with a trephine (11 mm diameter). The decellularization procedure was performed at once. Twenty New Zealand white rabbits (Xilingjiao experimental animal breeding center, Jinan, China) weighing 2.0–2.5 kg were used for animal transplantation. All animals were treated in accordance to the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. All animal experiments were approved by the Medical Ethics Committee of Qilu Hospital of Shandong University, China.

2.2. Preparation of scaffold

It was reported that corneas processed with sodium chloride plus nucleases could successfully remove all cellular material and keep the epithelial basement membrane completely intact [20]. Therefore, this method was used to prepare the corneal scaffolds as previously described [10,20]. Briefly, porcine corneas were immersed in 1.5 M sterile sodium chloride solution for 48 h with a change after 24 h, followed by treating the corneas with DNAse 5 U/mL and RNAse 5 U/mL (Sigma-Aldrich) for 48 h. Then, the samples were washed with sterile PBS supplemented with 200 U/ml penicillin and 200 U/ml streptomycin for 72 h and changed every 24 h. The decellularization procedure was carried out at 4 °C under continuous agitation. Rabbits were used as experimental animal, and the thickness of rabbit cornea was 407 ± 20 μm [21]. Therefore, APCMs were fabricated to 400 μm-thickness APCM lamellas containing Bowman's membrane as the scaffolds. The fabrication process was shown in Fig. 1 (Fig. 1A), as follows: 1) The full-thickness APCM was placed into the hole of the right column made by stainless steel. 2) The column is pushed to the left of the cutting apparatus. 3) Twist the flat head screw, which is in the same hole with the full-thickness APCM but in the opposite side of the column, to push the APCM into the hole of the left column, which share the same height and diameter with the hole of the right column. The left column had been fixed to the base before use. 4) Twist the flat head screw of the left column to the 400 μm-scale. At this moment, the right column has been slightly pushed to the right. Adjust the right screw, and the two columns were pressed together. Then the right column was fixed, and the screw was tightened. 5) APCM was cut through the gap between the two columns with a thin blade. 6) Relieve the fixation of the right column, and take out the APCM scaffold (AS). Then, the scaffolds were stored at −20 °C before use. All steps were conducted under sterile conditions.

2.3. Isolation and culture of human limbal epithelial cells (LECs) and corneal fibroblast cells (CFCs) from human limbus tissue

LECs and CFCs were isolated and cultured as previously described methods [22–24]. The isolated LECs were seeded on the plates coated with 2% growth factor reduced Matrigel (354230, BD Biosciences). The isolated stromal cells were cultured in corneal fibroblast medium to form corneal fibroblast cells (CFCs). The hLEC medium consisted of DMEM/F12 (Invitrogen), 10% fetal bovine serum (FBS; Gibco), 5 ng/mL human epidermal growth factor (hEGF; R&D), and human corneal growth supplement (HCGS) containing 0.18 mg/mL hydrocortisone, 5 mg/mL insulin, 5 mg/mL transferrin, 1 ng/mL EGF, and 0.2% bovine pituitary extract (Invitrogen). CFC medium contained DMEM/F12 supplemented with B27 (Invitrogen), 10% FBS (Gibco), 20 ng/mL EGF (R&D), and 40 ng/mL bFGF (Sigma).

2.4. Preparation of LEC-conditioned medium and corneal endothelial cell (CEC) differentiation medium

LEC-conditioned medium and corneal endothelial cell (CEC) differentiation medium were prepared as our previously reported, but with some modifications [22,23]. When cultured hLECs reached 70%–90% confluence, the media were collected daily. The collected medium mixed with hLEC medium at a ratio of 3:1 as LEC-conditioned medium (LM).

SV-40-transformed human lens epithelial cells (Shanghai ay Biological Technology Co. Ltd., Shanghai, China) were cultured in DMEM/F12 containing 20% FBS. Lens epithelial cell-conditioned medium was obtained by collecting the medium from these cultured cells at 70%–90% confluence every 12 h, and mixed with CFC medium at a ratio of 3:1 as CEC differentiation medium (EM). All collected conditioned medium was centrifuged for 5 min at 1000 rpm, filtered through a 0.22-mm filter (Millipore), and stored at - 80 °C before use.

2.5. Culture and induction of hESCs

The hESC line H1 (WiCell Research Institute Inc., Madison, WI) was grown on hES-qualified Matrigel (Corning®) coated plates with mTeSR1 medium (StemCell Technologies, Canada) as previously described [25]. To obtain EBs, hESCs were detached with 2 mg/mL Dispase (Gibco) and grown in suspension in EB medium: DMEM/F-12 supplemented with 20% serum replacement without bFGF [26].

The differentiation of LEC-like and CEC-like cells was performed