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Construction of tissue-engineered cornea composed of amniotic epithelial cells and acellular porcine cornea for treating corneal alkali burn

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ABSTRACT

Although acellular corneas have been reported to be a potential substitute for allogeneic cornea transplantation to treat corneal injury, severe corneal injury is hard to repair due to inflammation and neovascularization. The use of the amniotic membrane as a graft in ocular surface reconstruction has become widespread because of the anti-inflammatory and anti-angiogenic properties of amniotic epithelial cells (AECs). Our objective was to construct a tissue-engineered cornea (TEC) composed of an acellular porcine cornea (APC) and AECs to repair severe corneal injury. Corneal cells were completely removed from the prepared APC, and the microstructure, mechanical properties, and stability of a natural porcine cornea (NPC) was maintained. In vitro, MTT and flow cytometry analyses showed that the APC did not negatively affect cell viability and apoptosis. In vivo, corneal pocket and subcutaneous transplantation demonstrated that the APC was incapable of trigging accepted immune response. AECs isolated from the human amniotic membrane have proliferation potential and present healthy morphology before 6 passages. After 7 days of culture on the surface of the APC, the AECs were stratified into 5–6 layers. We found that the AECs reconstituted the basement membrane that had been disrupted by the decellularization process. ELISA results showed that after culturing the TEC, the culture medium contained anti-inflammatory and anti-angiogenic growth factors, such as MIF, IL6, Fas-L, and PDEF. Finally, the results of lamellar keratoplasty to treat an alkali burn showed that the transplanted TEC was transparent and completely inoculated into the host cornea. However, the transplanted APC was degraded due to host rejection. Therefore, we conclude that a TEC composed of AECs and an APC holds great potential for the repair of severe corneal injury.

1. Introduction

The cornea is the refractive system of and a barrier for the eye. Corneal trauma and ulceration are major contributors to corneal blindness worldwide and are estimated to be responsible for 1.5–2 million new cases of monocular blindness per year [1]. Lamellar keratoplasty is one of the main forms of therapy for many disorders of the cornea that lead to blindness. However, there is a chronic worldwide shortage of donor tissue for clinical use. Therefore, many researchers have attempted to fabricate corneal equivalents for the treatment of corneal blindness [2,3]. Collagen fibrils in the corneal stroma are arranged into a stacked array of approximately 250 layers or lamellae, which are thinner and more interwoven in the anterior cornea [4]. To mimic the components and microstructure of the natural cornea, numerous studies of corneal equivalents have focused on artificial corneas synthesized from natural or synthetic polymers [5]. Although these corneal grafts possess good biocompatibility, the microstructure of the natural cornea is not yet imitated completely [6]. Thus, very few synthesized artificial corneas have been used to successfully treat corneal disease due to a deficiency in specialized corneal physical, chemical, and biological characteristics.

Acellular ECM, a biological scaffold derived from natural tissue and organs, has been commonly used in a variety of

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reconstructive surgical applications and is increasingly used in regenerative medicine for tissue and organ replacement [7]. Due to its preservation of natural structural and elementary components, acellular ECM serves many functions, including the provision of structural support and tensile strength and attachment sites for cell surface receptors. This material also serves as a reservoir for signaling factors that modulate such diverse host processes as angiogenesis and vasculogenesis, cell migration, cell proliferation and orientation, inflammation, immune responsiveness, and wound healing [8]. Several research groups have succeeded in preparing an acellular corneal stroma using detergent and/or several enzymes [9–11]. Our laboratory has also prepared an acellular cornea from porcine tissue [12]. Although the acellular cornea that we prepared possesses good biocompatibility and a retained macrostructure, the physical and chemical properties evidently changed due to the disruption of collagen fibrosis by a decellularization agent [7]. These changes can lead to the failure of risk improvement by acellular cornea transplantation. Furthermore, in vivo studies of acellular corneas have reported a positive therapeutic effect only in the corneal pocket [9,11] and LPK with an acute defect [10]. The successful treatment of severe corneal injury, such as an alkali burn or ulcer, has rarely been reported. This phenomenon may be due to the disruption of immune privilege and avascularity in severe corneal injury, resulting in corneal graft rejection.

In the clinic, many severe diseases of the cornea are a result of chemical or thermal burns that cause intractable inflammation and limbal epithelial stem cell (LESC) deficiency (LSCD) [13]. One of the therapies currently being used to treat this disorder is the transplantation of cultivated autologous epithelial cell sheets [14]. However, this treatment strategy is often unable to suppress inflammation. In the acute phase of a corneal burn, inflammation is the most common complication, which is an important element during corneal wound healing [15]. Although mesenchymal stem cell transplantation has been reported to be a potential therapeutic strategy for corneal inflammation [16,17], the success of clinical treatment has not yet been reported. Therefore, there is still a need for an alternative approach to improving the treatment of patients with corneal inflammation.

The use of the amniotic membrane as a graft in ocular surface reconstruction has become widespread because of the membrane's availability and convenience [18]. Clinical observation has shown that amniotic membrane transplantation decreases vascularization on the ocular surface. The anti-angiogenic effect of the amniotic membrane is one of the foremost reasons for this tissue's therapeutic application [19]. In recent years, much attention has been given to the cell types isolated from the amniotic membrane. Amniotic epithelial cells (AECs) arise from the embryonic epiblast and form a monolayer lining the membrane. AECs express the pluripotency-associated Nanog 3, Sox-2, Tra-1-60, and Tra-1-80 genes and can differentiate into all three germ layers [20]. In addition, AECs have been shown to produce a variety of anti-angiogenic and anti-inflammatory factors in vitro [21]. Based on the characteristics of AECs, which are immune-privileged [22] and augment regeneration, AECs have been widely used in tissue repair, such as cell transplantation to the liver [23], heart muscle [24], pancreas, and ocular surface [25–27].

The goal of this study was to fabricate an AEC-containing tissue-engineered cornea (TEC) to repair severe corneal injury. We first prepared an acellular cornea using a porcine cornea as a scaffold for the TEC. We then analyzed the structure and composition and evaluated the cyto- and immunocompatibility of the acellular porcine cornea (APC). Next, we isolated AECs, which we used as seed cells to construct the TEC. Finally, we tested the possibility that the constructed TEC could repair a corneal alkali burn.

2. Materials and methods

2.1. Preparation of APC

Pig eyes were harvested at the local abattoir. The eyes were enucleated immediately after death and thoroughly washed with phosphate-buffered saline (PBS). Whole corneas containing part of the sclera were cut from the eyes and soaked in ultrapure water to allow swelling for 12 h. The corneal stroma then underwent agitation in 2 M NaCl for 30 min, followed by ultrapure water for 30 min. The process repeated 3 times. Next, 0.2% Triton X-100 was used to wash the corneas for 6 h. After a thorough washing in PBS, the APCs were dehydrated in glycerol to the normal thickness of a native cornea. Finally, sterilization was performed by 15 K ^{60}Co.

2.2. Evaluation of structure and composition

The APCs were fixed in 4% paraformaldehyde overnight before embedding in paraffin. Sections with a thickness of 5 μm were stained with hematoxylin and eosin (H&E) and observed under a light microscope (OlympusIX71, Japan).

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were performed to evaluate the APC ultrastructure after decellularization. For SEM, the grafts were fixed for 24 h in 4% glutaraldehyde in 0.1 M PBS. The APCs were further rinsed in DI water and dehydrated with a graded series of ethanol solutions (50%, 70% 90%, and 100%) for 10 min each. Finally, each APC was treated with hexamethyldisilazane and air-dried in a fume hood overnight. The specimens were mounted on stubs, sputter-coated with gold, loaded into an SEM (JEOL S3400N, Japan), and viewed under an accelerating voltage of 5 kV. To analyze collagen fiber arrangement, samples around the pupil were fixed in 2.5% glutaraldehyde/0.1 M phosphate buffer, embedded in resin, and cut into ultrathin sections. The sections were observed with a TEM (JEOL 1010, Japan).

Immunostaining was performed as previous described, with moderate modification [28]. For immunostaining analysis, specimens were cut into 6 mm sections and mounted on glass slides. The specimens were deparaffinized by treatment with xylene, followed by exposure to a graded series of ethanol solutions (70–100%). Following deparaffinization, the slides were placed in citrate antigen-retrieval buffer, which was then brought to a boil (95–100 °C) for 20 min. The buffer was allowed to cool, and the slides were then washed 3 times in PBS/Tween 20 (PBST) solution (pH 7.2). Following H_2O_2 treatment for 45 min at room temperature, the slides were washed 3 times in PBST. The sections were incubated in 1.5% goat serum (Millipore, US) for 1 h at 37 °C in a humidified chamber to inhibit nonspecific binding of the primary antibody. Following incubation in blocking serum, the sections were incubated with primary antibody in a humidified chamber for 2 h at 37 °C. The primary antibody was a mouse anti-collagen I and mouse anti-collagen V antibody (Santa Cruz Biotechnology, US). Two hours later, the slides were washed three times in PBS. The sections were then incubated in FITC-labeled goat anti-mouse secondary antibody (Zhongshanjingqiao, China) for 30 min in a humidified 37 °C chamber. After washing three times in PBST or incubating in 4% diaminobenzidine substrate solution at room temperature, images were acquired from a fluorescence microscope (Olympus BX-51, Japan).

2.3. Cytotoxicity of APC

To evaluate APC cytotoxicity, APCs were extracted using 5 ml Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C for 48 h. The fibroblast L929 cell line was seeded in each well of a 96-well plate and cultured in leaching liquor for 3 days in a humidified 37 °C environment with 5% CO_2. Three days later, 50 ml 0.5% MTT was added to the culture system. Following 4 hof culture, the supernatant was discarded, and the pellet was dissolved in DMSO. Following 10 min of shaking, the absorbance value was detected by spectrophotometry (Bio-tek PowerWave 200, US) at 490 nm. L929 cells were cultured in non-leached 10% FBS DMEM as negative control.

To evaluate the apoptotic effect of the APC, fibroblast L929 cells were seeded in a 24-well plate and cultured in DMEM with 10% FBS in a humidified 37 °C environment with 5% CO_2. When the cells were 60% confluent, the DMEM was substituted with APC leaching liquor. After 3 days of culture, the cells were harvested using 0.25% trypsin and resuspended in binding buffer. The cells were then incubated with Annexin-V-FITC and PE-Texas Red BD Bioscience) in the dark for 15 min. An apoptosis analysis was performed with a FACScan flow cytometer (BD Bioscience, US).

2.4. Mechanical properties of APC

The suture retention strength and tear resistance of the APCs were measured using an Instron materials-testing system (Instron, USA). Specimens of APC were loaded into a custom test fixture. A stainless-steel wire with a diameter of 0.36 mm was passed through each specimen at a distance of 20 mm from the bottom edge of the APC. The diameter of the wire was chosen to simulate a 10-0 polypropylene suture with a diameter of 0.35 mm. A suture retention test then was conducted in tension at a rate of 300 mm/min until the suture tore through the APC material. The maximum load sustained by the APC was recorded as the suture retention strength in units of newtons (N).