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Vitrified collagen-based conjunctival equivalent for ocular surface reconstruction

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ABSTRACT

The main functions of the conjunctiva, an essential part of the ocular surface, are to maintain the equilibrium of the tear film and to protect the eye. Upon injuries, the prerequisite to successful ocular surface repair is conjunctival reconstruction. Tissue engineering techniques, including transplantation of autografts, amniotic membranes and numerous synthetic/natural materials, have been developed. However, none of these strategies is completely satisfactory due to lack of goblet cell repopulation, poor mechanical properties or non-standardized preparation procedure. Here, we cultured conjunctival epithelial cells on vitrified collagen membranes and developed a tissue equivalent for repairing damaged conjunctiva. Optimized vitrified collagen has superior mechanical and optical properties to previous biomaterials for ocular surface application, and its unique fibrillar structure significantly benefited conjunctival epithelial cell growth and the phenotypic development *in vitro*. In a rabbit model, vitrified collagen greatly promoted conjunctival regeneration with rapid re-epithelization, sufficient repopulation of goblet cells and minimized fibrosis and wound contracture, proved by gene expression analyses and histological staining. In conclusion, we have demonstrated the potential suitability of utilizing vitrified collagen-based tissue equivalent in ocular surface reconstruction.

1. Introduction

The primary function of the ocular surface system is to protect the refractive surface on the eye. It is composed of continuous epithelia, including the cornea and conjunctiva, and injury to one part may result in system-wide secondary dysfunction [1]. The conjunctiva, starting from the corneoscleral limbus and lining the inner surface of the eyelids, is a very important component of the ocular surface. It is a natural mechanical barrier against pathogens, and its main functions include maintaining the equilibrium of the tear film by secreting mucins [2,3]. Therefore, conjunctival repair is the prerequisite for successful ocular surface reconstruction. The conjunctiva consists of a stratified non-keratinized epithelium with goblet cells resting on a basement membrane, and has the capacity to spontaneously re-epithelialize upon injury [4]. However this is usually accompanied with a certain amount of fibrosis and wound contracture, especially in extensive disorders, such as cicatricial pemphigoid, Stevens-Johnson syndrome and chemical/thermal burns [2]. In these cases, an appropriate tissue substitute needs to be applied for optimal wound healing after the excision of diseased tissue.

The general principle of tissue engineering is to replace tissue lost from disease or trauma using biomaterial scaffolds, in combination with cells and/or biological cues to accelerate the regeneration [5]. The ideal conjunctival substitute should be a stable, thin and elastic matrix that is not rejected by the patient's immune system [2]. Although use of autologous tissue substitutes (conjunctiva and oral/nasal mucosa) has made progress in clinical studies [3], and synthetic matrices based on fibrin [6,7], keratin [8], collagen [9] and poly (lactide-co-glycolide) (PLGA) [10] were also tested in animal models, they are limited for numerous reasons. For example, available donor grafts are very limited in patients

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suffering from systemic autoimmune diseases. Donor site morbidity could be a concern if large grafts are required. Furthermore, none of the synthetic matrices is sufficiently elastic for ideal conjunctival reconstruction. So far, the most widely accepted substitute is human amniotic membrane (AM) [3]. AM is epithelium harvested from the inner layer of human placenta and contains a basement membrane with underlying stroma. Clinically, AM-based conjunctival equivalents have been transplanted to treat ocular diseases with promising results. However, the availability, cost and standardization in the preparation of AM still remain issues. Therefore, there is a need for a biomaterial scaffold with well-defined composition and tunable structure that has excellent mechanical and biological properties for ocular surface application.

Type I collagen is the most abundant component in the extracellular matrix (ECM) of conjunctival stroma, where collagen fibrils synthesized by fibroblasts form a random network. Vitrified collagen (collagen vitrigel, CV) is a vitrified type I collagen membrane and has densely packed and randomly aligned collagen fibrils [11]. It is transformed from a normal collagen gel (CG) through a vitrification process to become a thin, elastic and transparent membrane, and, most importantly, its fibril density is tremendously increased, which has been proven to be a significant factor in maintaining cell phenotypes [12]. The vitrification process has been optimized by systematically varying the conditions (temperature, humidity and time) to yield the best CV that is highly transparent, mechanically strong and elastic, and also has a denaturing temperature well above body temperature [13]. *In vitro*, CV has been used for the cultivation of various ocular cells, including corneal epithelial cells, without the use of a feeder layer or other substrates [14]. In summary, CV was designed for ocular surface application with well-defined composition and controllable structure, and it closely imitates the architecture and properties of native conjunctival stroma. Therefore, we considered utilizing CV as a tissue substitute for cell transplantation in conjunctival reconstruction.

In this study, we attempted to employ the optimized CV to engineer a conjunctival equivalent containing goblet cells, a distinctive phenotype of conjunctival epithelium responsible for the secretion of large gel-forming mucins in the tear film. The presence of goblet cells is an essential indication of a functional conjunctival epithelium, as destruction to the conjunctiva could result in decreased numbers of goblet cells. If left uncorrected, this defect may cause severe dysfunction of the ocular surface or even blindness [3]. Rabbit conjunctival epithelial cells (rCjECs) were isolated and grown on optimized CV, normal CG (without the vitrification process) or tissue culture plates (TCP) (control) to determine the best culture substrate. Cell morphology, proliferation and phenotypes were inspected and compared among them. Furthermore, CV-based conjunctival equivalents were transplanted to repair conjunctival defects in a rabbit model. The outcomes were evaluated both anatomically and physiologically. Conjunctival scarring and contraction were assessed and inspected histologically; re-epithelialization, cell integration and goblet cell re-population in the defect sites were detected by gene expression and immunohistochemistry studies. Overall, the feasibility of CV transplantation as a strategy for treating conjunctival defects and restoring a balanced, healthy ocular surface post-damage was demonstrated.

2. Materials and methods

At all times in this study, the animals involved were housed and treated in accordance with the guidelines in the ARVO Statement for the Use of Animals in Ophthalmic and Visual Research and also with the approval of the Animal Care and Use Committee at Johns Hopkins University.

2.1. Collagen vitrigel preparation

The CV membrane was prepared as previously described [11,13,14]. Briefly, the procedure includes three main stages: gelation, vitrification and rehydration. Preceding these steps, acid collagen solution (0.25% v/v, Cosmo Bio, Tokyo, Japan) was prepared in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS, Thermo Scientific, Rockford, IL) and 20 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, Life Technologies) on ice. Collagen solution was then incubated at 37 °C for 2 h. After complete gelation, the opaque collagen gel was vitrified under 40% relative humidity (RH) at 40 °C, resulting in its conversion into a glassy material. After 1 week, the gel membrane was rinsed with phosphate buffered saline solution (PBS, Life Technologies) to remove the phenol red pH indicator in DMEM solution. Finally, the vitrigel was rehydrated to obtain a regenerated and stable membrane. Normal CG was prepared similarly to CV except that it was only kept in the humidifier for one day (40% RH, 40 °C) before rehydration.

2.2. Transmission electron microscopy (TEM)

The fibrillar structures of CV and CG were revealed by TEM, according to our previous study [12]. In short, samples were fixed in 3% paraformaldehyde (PFA), 1.5% glutaraldehyde, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 2.5% sucrose and 0.1% tannic acid in 0.1 M sodium cacodylate buffer at pH 7.2 overnight at 4 °C, followed with post-fixation on

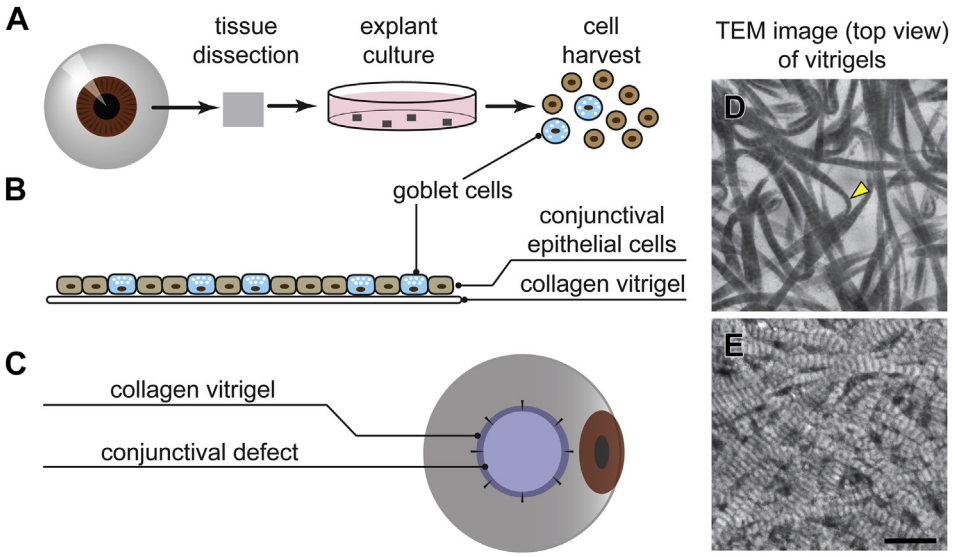

Fig. 1. Schematic view of experimental design. (A) Rabbit conjunctival epithelial cells (rCjECs) isolation and harvest. (B) Cultivation of rCjECs on CV with the specific goblet cell phenotype. (C) Reconstruction of conjunctival defect using CV based conjunctival equivalent. (D, E) TEM images showing the fibrillar density and organization of normal CG and optimal CV, respectively. Arrowhead showing the bending of collagen fibril. Scale bar: 500 nm.

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