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Recent Advances in Biosynthetic Corneal Substitutes

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Abstract

Purpose of Review Regeneration of the corneal epithelium and endothelium has been achieved, but regeneration of the corneal stroma has not, because it has several remarkable properties, including high tensile strength, thrust resistance, and high transparency. We introduce several biosynthetic approaches to creating stromal substitutes closely resembling the native human corneal stroma are currently being studied.

Recent Findings Currently, there are five approaches to regenerate corneal stroma: (1) decellularization, (2) the use of human recombinant collagen (HRC), (3) optical clarification (and lamination), (4) cell-based regeneration, and (5) organoid generation with induced pluripotent stem (iPS) cells.

Summary Immediate next steps for this area of research include clinical trials of decellularized stromal scaffolds created from porcine corneas or RHC. While these methods have both advantages and disadvantages, their refinement and clinical use, as well as the use of other methods, promise to lead to the continuing development of new approaches.

Keywords Corneal substitutes · Decellularization · Human recombinant collagen · Optical clarification · Cell-based regeneration · Organoid generation

Generally, allograft keratoplasty uses donor corneas obtained from eye banks. Keratoplasty can restore visual acuity to patients suffering from various vision-threating corneal diseases. Allograft keratoplasty is a safe and established surgical procedure, but it is not yet possible to completely avoid chronic endothelial cell loss and graft rejection, and the long-term prognosis after keratoplasty therefore remains unsatisfactory. Furthermore, a shortage of donor corneas is a worldwide problem that has not yet been resolved. In response to these clinical and social challenges, new regenerative medicine approaches have attracted attention. These approaches involve the creation and transplantation of biosynthetic corneal graft tissue (e.g., epithelial, stromal, and endothelial tissue). Biosynthetic corneal epithelial tissue is already widely used all over the world; cultivated corneal epithelium transplantation (CLET) [1] is used for patients with unilateral limbal deficiency, and cultivated oral mucosal epithelium transplantation (COMET) [2] is used for patients with bilateral limbal deficiency. Regeneration of the corneal endothelium has also seen success, with reports of cultivated corneal endothelial tissue being successfully used in animal models, and clinical applications expected soon [3].

On the other hand, regeneration of the corneal stroma, the majority of which comprises collagen fibers, has not yet been achieved. The corneal stroma has several remarkable properties, including high tensile strength, thrust resistance, and high transparency, that make it extremely difficult to replicate. However, several biosynthetic approaches that aim to produce stromal substitutes that are very similar to the native human corneal stroma are currently under investigation [4, 5•, 6••, 7•, 8]. The ideal corneal stromal substitute would be usable for penetrating keratoplasty (i.e., full-thickness corneal transplantation). However, full-thickness transplantation is very demanding, because it requires stromal substitutes with high strength and transparency, and because it is difficult to produce a tissue that is simultaneously compatible with the corneal epithelium, corneal endothelium, and the recipient cornea. Therefore, most attempts to develop biosynthetic corneal

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stromal substitutes have strictly targeted lamellar keratoplasty (i.e., partial-thickness corneal transplantation). In addition to the physical characteristics of substitute tissues, the role of keratocytes in the corneal stroma is also a very important consideration. Keratocytes mediate interactions between the corneal epithelium and endothelium, and it is therefore essential that corneal stromal substitutes are compatible with the proliferation of keratocytes. Additionally, nerve formation in cornea stromal substitutes, branching off the trigeminal nerve, is critical for their long-term survival, as it is necessary for a satisfactory tear reflex loop to prevent dryness of the ocular surface.

In this article, we introduce five approaches to the biosynthesis of corneal stromal substitutes, based on (1) decellularization, (2) human recombinant collagen (HRC), (3) optical clarification (and lamination), (4) cell-based regeneration, and (5) organoid generation from iPS cells.

Decellularization

Firstly, we will discuss decellularized corneal stromal scaffolds. Decellularization is performed to delete all interstitial cells, diminish the host reaction, and eliminate infectious agents. This technique has been already applied in heart valve [9], dermis [10], and ligaments [11]. These decellularized scaffolds maintain the naive stromal structure but possess no stromal cells, such as keratocytes or inflammatory cells. Therefore, the mechanical strength of these stromal substitutes is equivalent to the normal cornea, and they are difficult targets for the immunological reaction that causes corneal rejection. Decellularization of the corneal stroma can be achieved with various methods, such as freeze-drying [12], applying high hydrostatic pressure [13, 14], and by various chemical methods [4, 5•]. Recently, Yam GHF et al. prepared corneal scaffolds be decellularizing human corneas extracted during femtosecond laser-assisted refractive surgery (small incision lenticule extraction; SMILE) with SDS-based chemical treatment [4]. The authors found that their SDS-based procedure resulted in scaffolds that retained their stromal structure and preserved components such as collagens and glycosaminoglycan. Furthermore, keratocytes proliferated finely throughout these SDS-treated decellularized lenticules. When these human decellularized lenticules were implanted into the corneal stroma of rabbits, the grafts were stable and were not rejected.

Hashimoto et al. decellularized porcine corneas with high hydrostatic pressurization (HHP). The corneas were subjected to hydrostatic pressure at 980 MPa at 10 °C for 10 min and then washed with EGM-2 medium to remove remaining cells [14]. The HHP-treated corneas were trimmed to a 300-μm thickness and 6.0-mm diameter and implanted into rabbits. After 3 months, complete re-epithelialization was achieved, and after 4 months, the transplants were fully transparent. Zhang et al. developed a method to produce acellular porcine corneal stromal (APCS) tissue with chemical treatment (2 M NaCl, 0.2% Triton X-100, and glycerol) and irradiation. The tissue was transplanted into 47 eyes of 47 patients suffering from fungal keratitis [5•]. Interestingly, 87% (41/47) of these APCS grafts steadily gained transparency, and in 72% (34/47) of the patients, BCVA improved by more than two lines.

Stromal Substitutes with Recombinant Human Collagen

Decellularized corneal stromal scaffolds are unique in that they retain the normal stromal structure and preserve the ability to accept the migration of keratocytes. However, the use of these scaffolds presents ethical problems if the original corneal tissue is obtained from healthy human subjects. On the other hand, scaffolds based on xenogenic corneal tissue have potentially risk of transfer of infectious agents with the cornea: porcine endogenous retroviruses to the recipient and possibly to the recipient's contacts. By contrast, decellularized corneal stromal scaffolds based on recombinant human collagen avoid these problems.

Liu W et al. developed a recombinant human collagen (RHC) hydrogel implant by syringe-mixing RHC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysuccinimide (NHS), and incubating the mixture on a curved plastic mold [15]. These RHC hydrogels had high transparency, sufficient tensile strength, and were elastic enough for transplantation. The hydrogels also had high biocompatibility due to their excellent epithelization and innervation. Fagerholm et al. built on these findings by reinforcing the strength of the hydrogels with cross-linking, and after performing an animal experiment, launched an initial in-human phase I clinical trial that included ten patients (9 with keratoconus and 1 with a central corneal scar) [16, 17]. The implanted hydrogels were completely covered by the corneal epithelium, and the barrier function of this epithelium was comparable to that of the normal human cornea. The peripheral nerves of the trigeminal nerve extended into the implanted hydrogels with directionality that was close to normal, and corneal perception was restored to near that of a normal cornea. Confocal microscopy revealed that there were no dendritic (antigen-presenting) cells around the implanted hydrogels, whereas there were many of these cells in and around allografted corneas. Thus, the RHC hydrogels did not give rise to an immune response, avoiding corneal rejection, and the patients were able to taper the use of corticosteroids over a short period (6–8 weeks). Even 4 years after this phase I clinical trial, the implanted hydrogels survived in the patients' corneas with a striking lack of problems [6••]. Thus, RHC stromal scaffolds may seem to be the currently most-advanced clinical method for biosynthesizing stromal

substitutes. However, in a rabbit alkaline burn model, new blood vessels, which can reduce visual acuity in human subjects, invaded the implanted hydrogels due to severe chronic inflammation [17]. Therefore, Yumoto et al. added a second network of 2-methacry-loyloxyethyl phosphorylcholine (MPC; a biosynthesized anti-inflammatory phospholipid) to these RHC hydrogels to reduce inflammation [18]. Furthermore, in a pilot study, lamellar transplantation of RHC stromal scaffolds (specifically, RHCIII-MPC hydrogels) was performed in three patients. This study found that the RHC hydrogels were fragile and easily broken by surgical needles, and therefore had to be implanted with overlying sutures, not direct sutures. Moreover, these overlying sutures caused surface irregularities and deterioration in visual acuity.

Optical Clarification (and Lamination)

We investigated laminated atelo-collagen [19], clarified sclera [20], clarified skin [21], and clarified amniotic membrane (AM) [22] as corneal stromal substitutes. All these tissues resulted in substitutes with sufficient transparency, while the clarified AM also had high strength, suitable for direct suturing with surgical needles. Hariya et al. laminated the AM by alternating steps of hydration and dehydration [7•]. Additionally, Hariya cross-linked the AM chemically, with a 1-Ethyl-3-(3-dimethylaminopropyl) carbodimide (EDC)/N-hydroxy succimide (NHS) solution, followed by a final dehydration step. Finally, the chemical cross-linking reaction was arrested with a 100-mM glycine solution (Fig. 1). Cross-linking did not increase transparency or light transmittance in single-layer and 2-layer samples, but did improve these optical properties when the laminates had 4, 6, or 8 layers. Moreover, the final dehydration step after cross-linking further improved these properties in the 8-layer laminates. The cross-linked AM samples had high resistance to piercing damage, and this resistance increased linearly with the number of layers. Furthermore, the cross-linked AM samples were resistant to damage caused by soaking them in balanced salt solution (BSS; an intraocular irrigation solution) with collagenase, while the non-cross-linked AM became degraded. This suggests that the cross-linked AM samples effectively tolerated the presence of collagenase, a substance that is secreted from a variety of bacteria (such as Pseudomonas aerugnosa), Thus, they may be effective as patch grafts for corneal perforation induced by bacterial infection. When cross-linked AM samples were transplanted into a pocket created in the corneal stroma in rabbits, the samples showed no invasion of inflammatory cells or neovascularization, and did not cause any defects in the corneal epithelium or endothelium of the rabbit recipient corneas. Furthermore, it was possible to suture the cross-linked, 8-layer AM laminates directly onto the rabbit cornea. Full epithelization over the laminates was observed 7 days after transplantation. Moreover, these transparent AM laminates could be molded to any shape with a casting mold, and may therefore be useful for other types of ocular surface surgery (such as scleral patch grafting and bleb formation during filtering surgery for glaucoma). Finally, long-term, room-temperature storage of these AM laminates was possible, making them easily and quickly available for surgery. However, the thickness of these laminates was at most 80 μm, making them suitable only for lamellar grafts. Thus, the development of thicker substitute materials for deep-lamellar keratoplasty or penetrating keratoplasty is needed.

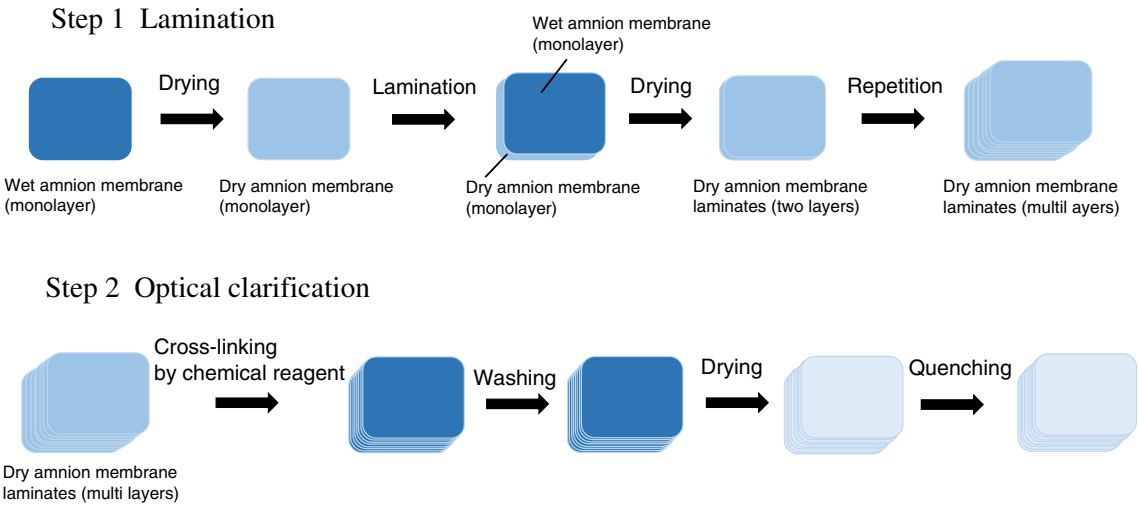

Fig. 1 In the first step, amnion membrane was laminated by alternating steps of hydration and dehydration. In the second step, the laminated amnion membrane was cross-linked chemically, followed by a final dehydration step. Finally, the chemical cross-linking reaction was arrested with a glycine solution

Cell-Based Regeneration

Finally, we will discuss the cell-based regenerative approach. In this method, the formation of keratocytes is induced from stromal fibroblasts or other progenitor cells. When stromal fibroblasts are cultured in a medium with serum and then harvested from the medium without serum, in this order, they transform into keratocytes [23]. As a second step, Gouveia and Connon et al. cultured stromal fibroblasts in a serum-free medium containing ascorbic acid and retinoic acid [24]. Interestingly, they found that these keratocytes secreted a number of proteins, including keratocan, lumican, and decorin, that are needed to construct the extra-cellular matrix (ECM). In the original procedure, this secreted ECM was very thin and weak, making it unsuitable as a stromal substitute. In a follow-up study, Gouveia et al. improved their method, allowing the production of thicker and more pliable ECM sheets [25].

In addition to stromal fibroblasts, keratocytes can be induced from adipose tissue stem cells [26], umbilical cord stem cells [27], stromal progenitor cells [28], and corneal limbal stem cells [29]. Adipose tissue stem cells and umbilical cord stem cells can transform to keratocytes, but these keratocytes cannot secrete ECM that has adequate thickness and strength [26, 27]. However, stromal progenitor cells can also differentiate into keratocytes, and these keratocytes can secrete ECM that resembles the normal corneal stroma [28]. Basu et al. inserted limbal stromal stem cells in a fibrin gel and transplanted them into a damaged mouse cornea [29]. The stromal stem cells differentiated into keratocytes, and the corneal wound healed without scarring.

Recently, Greene CA et al. developed a new approach based on gene reprogramming [8]. Normally, the postnatal corneal stroma is composed of type I and V collagens, whereas the embryonic corneal stroma is composed of type I, II, and V collagens. When the cornea is damaged, keratocytes are activated and secrete collagens, but when the cornea is scarred, cornea opacification can occur. In previous research, stromal keratocytes were reliably transformed into the neuronal phenotype with a neuronal lineage-specifying growth factor. An evolution of this method cultured keratocytes in a chondrogenic differentiation medium for 3 weeks. The medium consisted of Dulbecco's modified eagle medium (DMEM) supplemented with 10 ng/ml TGFβ3, 10^{-7} M dexamethasone, 1% glutamax, and 1% anti-anti. Generally, the TGF-β family is an exogenous chondrogenic factor, but TGFβ1 and 2 can transform keratocytes into myofibroblasts, which induce corneal scarring. Therefore, this study used TGFβ3 (and dexamethazone) to reprogram the keratocytes. To administer the transformed keratocytes, they were mixed with a water-soluble poly-saccharide (gellan gum) and used as a 0.5% ophthalmic solution. After administration to damaged rat corneas, the transformed keratocytes secreted type II collagen and the wound healed without scarring, fibrosis, or opacity. Furthermore, the strength and elasticity of the cornea increased. Interestingly, keratocytes with the neuronal phenotype disappeared completely when TGFβ3 in the eye drops was quenched. Thus, this method may be effective to treat corneal diseases with stromal fragility, such as keratoconus and keratoectasia.

Organoid Generation with iPS Cells

iPS cells have the potential to solve various problems related to ethics, transplant rejection, and donor shortages, because of their ability to differentiate into keratocytes and generate corneal stromal ECM. However, it is technically difficult to differentiate pluripotent stem cells (including both embryonic stem cells and iPS cells) into three-dimensional tissue structures on a flat dish. Therefore, for a long time, there were no attempts to produce corneal stromal tissue in vitro. However, in 2011, a new approach was reported for three-dimensional cell culturing and tissue generation, which used primordial eye cell clusters from mouse embryonic stem cells. These clusters developed into self-organized, three-dimensional, miniature retinal structures [30]. In 2017, James WF et al. [31] and Susaimanickan PJ et al. [32] used human iPS cells to develop three-dimensional, miniature corneal organoids. These corneal organoids showed anatomical features and molecular marker expression profiles that were similar to the adult corneal epithelium, endothelium, and stroma. In addition, collagen micro fibrils in these corneal organoids were thin and uniform, and when accumulated were presumed to form a packed lamella resembling the native corneal stroma [31]. This technique requires further improvement, particularly in the size of the organoids, before it can be used to create corneal substitutes that can replace donated corneas. However, in the near term, it presents possibilities for the generation of materials, such as corneal epithelial cell sheets and endothelial cell sheets, that could serve as substitutes for various different layers of the cornea.

Here, we have introduced several approaches to the biosynthesis of corneal stromal substitutes. Immediate next steps for this area of research include clinical trials of decellularized stromal scaffolds created from porcine corneas or RHC. While these methods have both advantages and disadvantages, their refinement and clinical use, as well as the use of other methods, promise to lead to the continuing development of new approaches.

Compliance with Ethical Standards

Conflict of Interest Shunji Yokokura and Yuji Tanaka declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article contains studies with human and animals. These studies followed the tenets of the Declaration of Helsinki and all study protocols were approved by the Institutional Ethics Committees of Tohoku University hospital

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