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Preserved Corneal Lamellar Grafting Reduces Inflammation and Promotes Wound Healing in a Scleral Defect Rabbit Model

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Purpose: To investigate the effect of preserved corneal lamellar grafting on inflammation and wound healing and to compare its effect with that of preserved scleral grafting in a scleral defect rabbit model.

Methods: New Zealand White rabbits were assigned to a corneal lamellar grafting group ($n = 5$) or a scleral grafting group ($n = 5$). After lamellar dissection of superotemporal sclera using 6.0-mm trephine, the same sizes of preserved human corneal or scleral grafts were transplanted with 10-0 nylon interrupted sutures. The grafted areas were photodocumented at 3 to 21 days after surgery to evaluate epithelial wound healing index (%), neovascularization and presence of filaments. The existence of $CD3^+$ T cells and $CD34^+$ cells at the grafted areas was analyzed at 21 days.

Results: Epithelial wound healing index was significantly higher in the corneal grafting group at 9 days ($P < 0.05$). Scleral grafts showed copious formation of filaments adherent to the engrafted area from 9 to 14 days, whereas the corneal grafts were free of filaments. The numbers of inflammatory cells were significantly higher in the scleral grafts ($P < 0.05$), and $CD3^+$ T cells and $CD34^+$ cells were populated within inflammatory cells at graft–recipient junctions in both groups. The mean areas of the estimated perigraft and intragraft neovascularization tended to be higher in scleral grafts.

Conclusions: Preserved corneal lamellar grafting enhances epithelial wound healing and alleviates inflammation in a scleral defect rabbit model.

Translational Relevance: This work suggests that the preserved corneal graft may be considered as a favorable alternative option for repairing scleral defects.

Introduction

Scleromalacia or necrotizing scleritis may occur after pterygium excision, trauma, infectious scleritis, or surgery for retinal detachment repair and may also occur in patients with systemic autoimmune diseases such as rheumatoid arthritis, Wegener's granulomatosis, and collagen vascular disease.[1] To reinforce thinned sclera, various materials have been attempted for grafting including allogeneic sclera, amniotic membrane (AM), fascia lata, dura mater, pericardium and periosteum.[2–6] However, an ectopic donor tissue such as periosteum, dura mater, or pericardium is hard to obtain; therefore, clinical experience with these has been limited. Among these materials, preserved donor scleral grafts have been preferred for a long time and have produced acceptable outcomes.[7–11] Because epithelial wound healing is delayed on a scleral graft, a permanent AM transplantation or a conjunctival graft is usually combined with scleral grafting, which consumes lots of surgical time.[9]

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Currently, both human preserved donor corneas and scleras are obtainable from a number of eye banks and are readily available for surgery. Corneas and scleras are histologically similar in that they are organized mostly from type I collagen fibers.[12] However, compared with the sclera, corneal collagen fibers are highly uniform in diameter, and they are regularly and compactly arranged. Moreover, cornea has a subepithelial basement membrane consisting of collagen type IV, laminin, and fibronectin that may affect wound healing, whereas the external surface of the sclera is bound to the overlying episcleral only by very thin bands of collagen.[13,14] Several reports have stressed the favorable effects of scleral grafts, which were well-organized in the perforated corneal area corneal perforated area; however, it is worrying that the collagen fibrils of the scleral graft were broken down by fraying into microfibrils, then shrank and became rapidly cleared.[15–17]

Our hypothesis is that a composition of basement membrane and compact and regular collagen fibrils of the preserved cornea may promote wound healing and may prolong survival as a graft in scleromalacia. Therefore, in the present study, we produced a scleral thinning model by lamellar dissection of the scleras in rabbit eyes. Using this model, we then investigated the effect of preserved corneal grafting on wound healing and inflammation and compared the effects of corneal grafting with those of preserved scleral grafting.

Methods

Study Design

Our study design was outlined as follows:

1. Prepare scleral defect rabbit models.
2. Transplant preserved human corneas and preserved human scleras.
3. Observe and evaluate the wound healing rate, vascularization, and filament formation over 3 weeks.
4. Histologically evaluate the inflammatory cells or pro-angiogenic stem cells in the engrafted donor–recipient complex.

Animals and Animal Models

Animal experiments were performed in accordance with the ARVO Statement for Use of Animals in Ophthalmic Vision and Research, and the protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University Biomedical Research Institute (IACUC No. 18-0138-S1A0).

Eight-week-old female New Zealand White rabbits (total 10 eyes) weighing 2.0 to 3.0 kg (Orient Bio, Inc., Seongnam-si, Gyeonggi-do, Korea) were used in this study. Five eyes were assigned to each of two groups: the corneal grafting group and the scleral grafting group. A combination of tiletamine and zolazepam-mixed agent (10 mg/kg; Zoletil, Virbac, Fort Worth, TX, USA) and xylazine (2 mg/kg; Rompun, Bayer, Leverkusen, Germany) was injected intramuscularly for anesthesia. Corneolimbal bridle suture was done using 8-0 Vicryl suture to expose the superotemporal sclera for lamellar dissection. Both the conjunctiva and sclera were trephined using a 6.0-mm Barron vacuum trephine (Katena Products, Denville, NJ) with approximately 250 µm of partial scleral thickness at the superotemporal area, 2 mm apart from the corneal limbus and temporal margin of superior rectus muscle. Thereafter, roundly cut scleral tissue was subjected to lamellar dissection using a #69 Beaver Mini-Blade (Beaver-Visitec, Waltham, MA) as a scleral defect rabbit model.

Preserved Human Donor Cornea and Scleras

Preserved human donor full-thickness corneas contained in glycerol were obtained from Eversight Eye Bank (Cleveland, OH). Preserved human donor scleras contained in 95% ethanol were obtained from Central Ohio Lions Eye Bank (Columbus, OH). The usage of human donor tissues was approved by the Institutional Review Board of Seoul National University Biomedical Research Institute (IRB No. E-1808-028-963; Jongno-gu, Seoul, Korea).

Transplantation of the Graft

Representative photos of serial surgical procedures are shown in Figure 1. The preserved donor corneas and scleras were washed and soaked in BSS sterile irrigating solution (Alcon, Fort Worth, TX), then Descemet's membrane was peeled off from each cornea and remnant uveal tissues were completely removed from each sclera before use. For grafting, corneal and scleral tissues were trephined into 6.0-mm diameter pieces using a 6.0-mm Barron vacuum trephine (Katena Products). Prepared corneal grafts with the basement membranes facing up or scleral graft tissues were placed on the scleral defect areas, and were then fixated by eight simple interrupted sutures using 10-0 nylon. Levofloxacin eye drops (Cravit; Santen Pharmaceutical Co., Ltd., Osaka, Japan) were instilled to prevent infection at the end of the procedure.

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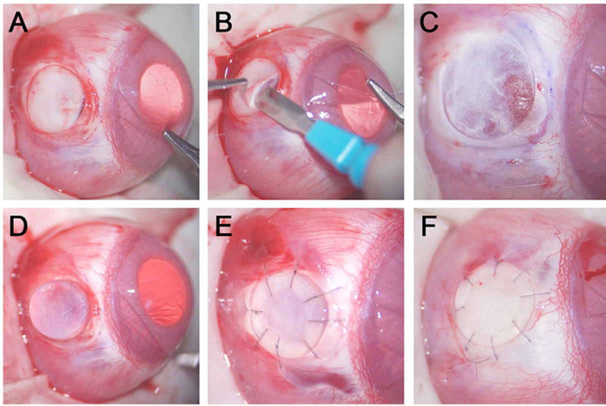

Figure 1. Representative photographs of serial surgical procedures of lamellar corneal and scleral grafting in scleral defect rabbit models. After outlining a circle of 6.0-mm diameter (A), lamellar scleral dissection was performed (B, C). Then, the cut preserved cornea or sclera of the same diameter was placed and sutured using nylon sutures (D, E).

Observations and Outcome Measurements

After surgery, we performed time–serial observations of the surgical wounds for 3 weeks, especially on postoperative days 3, 5, 7, 9, 14, and 21. When a sutured area became epithelized, the relevant sutures were removed over the follow-up period. Every observation was done under anesthesia by the intramuscular injection of a combination of tiletamine and zolazepam-mixed agent and xylazine. Surgical wounds were photodocumented in all eyes with and without fluorescein dye staining using a digital camera under a surgical microscope. At postoperative day 21, all rabbits were sacrificed, and the circular cornea- or sclera-engrafted areas were excised 2 mm wider than the circular junction of the donor tissue and the recipient conjunctivas and scleras. We established four outcome measurements, including the wound healing index, surface filament formation, vascularization (perigraft and intragraft), and histologic evaluation of the whole graft, to evaluate the postsurgical wound healing and inflammation at the area of engraftment.

Wound Healing Index

The epithelization rate was analyzed to evaluate wound healing over the surface at the engrafted area. The total graft area and deepithelized area (i.e., fluorescein dye-stained area) were estimated based on the acquired photos using the ImageJ software ver. 1.46 (National Institutes of Health, Bethesda, MD; http://rsbweb.nih.gov/ij/). The extent of wound healing was determined by the proportional area of epithelization relative to the total graft area and was presented as a wound healing index (%) equal to the epithelized wound area divided by the total graft surface area.

Estimations of Perigraft and Intragraft Vascularization

We quantified the densities of intragraft and perigraft vasculature using ImageJ software to investigate the vascularization over the graft and the vascular hyperemia near the graft, respectively. First, the photos used for estimation were opened in ImageJ and were changed into eight-bit images. Then, we measured the estimated area of vascularization by thresholding the vasculature with a cutoff value of 160 for perigraft analysis and with a cutoff value of 180 for intragraft analysis. For perigraft analysis, we drew an imaginary ring of 1.5-mm thickness around the circular graft in a photo. Then, only the quarter-ring from 2 to 4 o'clock adjacent to the corneal limbus was included in the perigraft analysis. We excluded the vasculature within the opposite half of the ring because it includes the vessels from the extraocular muscle and conjunctiva. For intragraft analysis, the vessel density was estimated only from the inner circle with a 4-mm diameter, not including the peripheral vessels at the graft, to determine whether there were vascularized vessels over the graft surface or perigraft hyperemia.

Histologic Evaluation

The excised transplanted graft-recipient bed complexes were fixed in 10% formaldehyde and embedded in paraffin. The formalin-fixed graft was cut into 4-µm thicknesses and placed on microscope slides. After deparaffinization, tissue sections were stained with hematoxylin or incubated with rat monoclonal antibody against CD3 (1:100; #14017.7, Abcam, Cambridge, MA) and rabbit polyclonal CD34 (1:100; #ABIN676898, Antibodies-online GmbH, Aachen, Germany) overnight at 4°C, and were then incubated with secondary antibodies conjugated with rhodamine (1:500; #AP136R, Merck Millipore, Burlington, MA) and Alexa Fluor 488 (1:500; #A11034, Invitrogen), respectively, for 1 hour at room temperature. After washing with phosphate buffered solution, cover slips were mounted using fluorescent mounting medium with DAPI (#E19-18, GBI Labs, Bothell, WA). The sections were examined under

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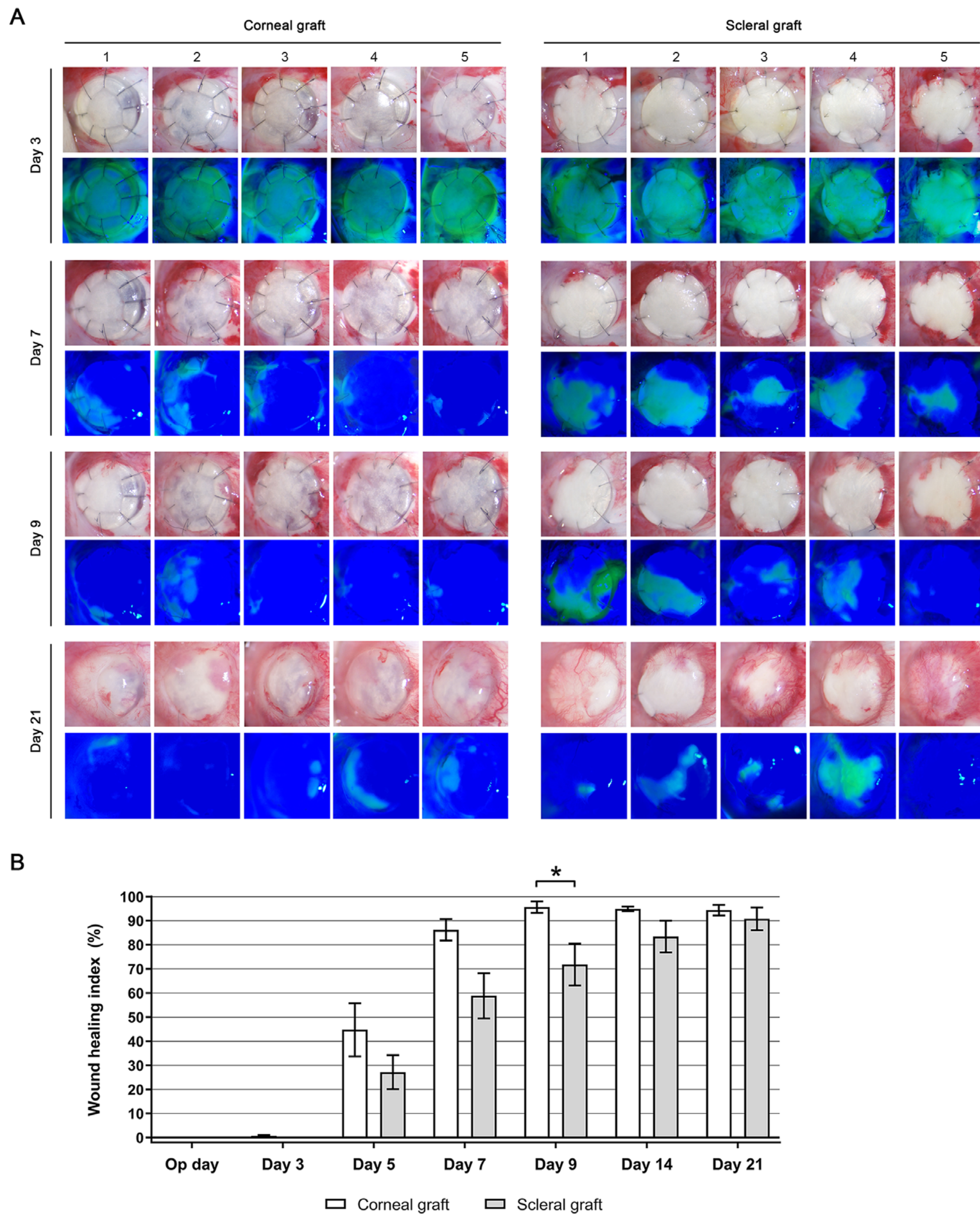

Figure 2. Time-serial photographs of wound area after lamellar corneal and scleral grafting in scleral defect rabbit models and the wound healing indices during the whole follow-up period. (A, B) The entire epithelial defect was noted in all cases from both groups after 3 days. Corneal grafts revealed more rapid surface epithelial wound healing than did the scleral grafts, and such differences were statistically significant at 9 days. Grafts in both groups achieved nearly full epithelization at the final follow-up date. *$P < 0.05$. Values represent the mean ± standard error of the mean.

a microscope (BX53; Olympus, Tokyo, Japan) and photodocumented.

To count inflammatory cells and $CD3^+$ or $CD34^+$ cells, respectively, we selected five square spots (0.1 mm^2 area per spot) in the section of hematoxylin and eosin stain for inflammatory cells and three square spots (0.1 mm^2 area per spot) in the section of immune stain for CD3 and CD34. Then, the average numbers of the relevant cells were calculated in each eye.

Statistical Analysis

The data are presented as the mean ± standard error measurement. Statistical analyses were performed using the SPSS software version 20.0 (SPSS, Inc., Chicago, IL) and GraphPad Prism v.8.1.2 (GraphPad Software, La Jolla, CA). Differences between two values were analyzed by Mann–Whitney *U* test, and categorical data were analyzed using McNemar's test.

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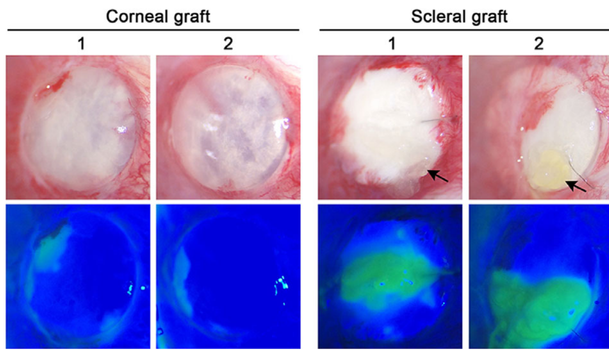

Figure 3. Photographs of two representative cases each from the lamellar corneal and scleral grafting groups to verify the filament formation at the surfaces of wounds 14 days after surgery in scleral defect rabbit models. Unlike the surfaces of the corneal grafts, which were smooth and free of filaments, the scleral grafts exhibited filaments (arrows) with staining by fluorescein dye.

A P value of less than 0.05 was considered statistically significant.

Results

Epithelial Wound Healing

The surface of the grafts became nearly full epithelized by 21 days in both groups, although the wound healing index in the corneal grafting group was constantly higher throughout the follow-up period, with significance especially at 9 days after surgery (P = 0.032, Mann–Whitney U test; Fig. 2). The standard deviation of the wound healing indices between objects was markedly lower in the corneal grafting group after 7 days. There was no filament formation in the surfaces of corneal grafts during any of the follow-up period. Although without statistical difference between the two groups, four eyes (80 %) of the total five eyes with scleral grafts had copious filaments over the graft surface at day 14 (Fig. 3 and Supplementary Table S1).

Vascularization of Grafts

At the end of the follow-up period, the mean area of perigraft vascularization adjacent to the corneal limbus and the vascularized area on the graft surface tended to be higher in the scleral grafting group (Fig. 4), although the difference was not significant. However, in some of the cases with scleral grafts, the perigraft vessels seemed highly dense, edematous and engorged extending to the margins of the grafts.

Histopathology of Grafts

In sections, engrafted donor corneas still nearly held their original shapes, while the graft junctions of the engrafted scleras became inconspicuous (Fig. 5A). The average number of inflammatory cells at the graft areas was significantly higher in the scleral grafting group (63.7 ± 16.1 cells/0.01 mm^2) than in the corneal lamellar grafting group (6.8 ± 4.1 cells/0.01 mm^2; P = 0.016, Mann–Whitney U test; Fig. 5B). Although the inflammatory cells were mostly confined to the graft–recipient junction in the corneal grafting group, the inflammatory cells were found not only at the graft junction, but also throughout the entire graft in the scleral grafting groups. The outermost layers of the engrafted corneas were composed of three to four cell layers of stratified squamous epithelial cells with constant thickness, whereas copious inflammatory cells were observed throughout the surface epithelial layers in the engrafted scleras to disrupt the intact epithelial layers, resulting in uneven outermost surfaces (Fig. 5C). The stromal layers were loosened in the scleral grafts, and their bitemporal margins were replaced with inflammatory cells and appeared melted. In the engrafted corneas, compact collagen layers were well-preserved and junctional structures were well-retained. Unlike the corneal grafts, there were a number of inflammatory cells at the graft–recipient junctions in the scleral grafts associated with the marginal graft melting.

Next, we investigated the phenotypical features of CD3$^+$ T cells and CD34$^+$ endothelial progenitor cells in the inflammatory area at the graft-recipient junctions in both groups. CD3$^+$ T cells and CD34$^+$ cells were identified at the margins of the grafts, and they were colocalized mostly in the same area (Figs. 6A and 6B), which suggests that perigraft vascularization may be a secondary phenomenon accompanied by inflammatory reaction. The mean number of CD3$^+$ T cells tended to be slightly higher in the scleral grafting group (55.9 ± 7.0 cells/0.01 mm^2) than in the corneal lamellar grafting group (47.4 ± 7.8 cells/0.01 mm^2; P = 0.841, Mann–Whitney U test; Fig. 6C), although it was not significantly different. There was no difference in the numbers of CD34$^+$ cells between the two groups (36.1 ± 6.0 cells/0.01 mm^2 in the corneal grafting group and 36.1 ± 5.3 cells/0.01 mm^2 in the scleral grafting group; P = 0.841, Mann–Whitney U test).

Discussion

In this study, we newly applied the preserved human cornea in lamellar grafting in a scleral defect rabbit

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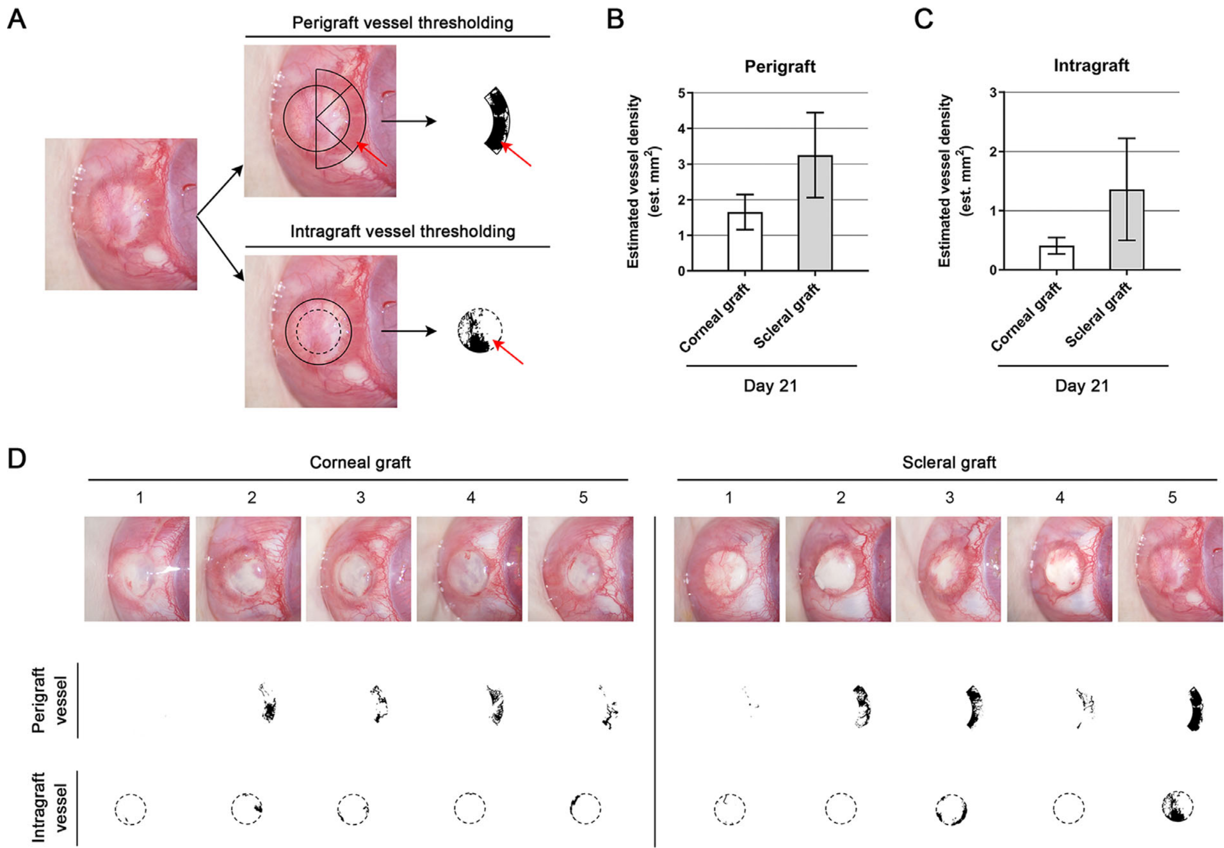

Figure 4. Quantification of the vascularization within the margin of (intragraft) and around (perigraft) the corneal and scleral grafts 21 days after surgery in scleral defect rabbit models. (A) Representative photographs defining the areas of perigraft and intragraft vascularization within the sectorial rim and the dotted circle (both are indicated as red arrows) and the relevant thresholding outcomes. (B, C) The estimated perigraft and intragraft vascularized areas after thresholding using photographs in both groups. The average areas of the two indices were higher in the scleral grafts than in the corneal grafts, although such a difference was not significant. Values represent the means ± standard error of the mean.

model and evaluated the surgical usefulness of the preserved cornea compared with the preserved sclera. We found that wound healing was more rapid, less inflammatory, and caused less vascular engorgement in corneal lamellar grafting, compared with scleral grafting. In addition, the corneal grafts maintained their original structure of the graft-recipient complex at 3 weeks postoperatively, and they had a compact stromal layer with a homogenous graft thickness, unlike scleral grafts, suggesting the possible long-term durability of the graft. For these reasons, we suggest that the preserved corneal lamellar tissue may be a favorable alternative as a tectonic graft material in scleral melting diseases, including scleromalacia perforans or necrotizing scleritis.

In fact, the lamellar corneal grafting had been attempted in patients with severe scleral melting after pterygium excision in Singapore.[18] It was effective; however, they did not use preserved corneas but rather fresh corneas in unscheduled surgeries. This is because whole-eye donations are not accepted in their country owing to religious reasons, so only in situ corneal removals were performed. Moreover, the study was not a controlled trial, so the effect of tectonic lamellar corneal grafting was not compared with scleral grafting. Afterward, a few studies reported cases with lamellar grafting surgery using preserved corneas in patients with scleral necrosis after radiotherapy or pterygium excision or during limbal dermoid surgery.[19–22] Moreover, a recent study analyzed the surgical effect of the irradiated corneal versus scleral patch on the rates of erosion in glaucoma drainage device surgery. Although there was no erosion event in either group, the corneal graft was greatly to be preferred.[23] Despite these interests, to the best of our knowledge, there has been no interventional study designed to investigate the comparative effects of tectonic lamellar corneal grafting and scleral grafting on wound healing and inflammation.

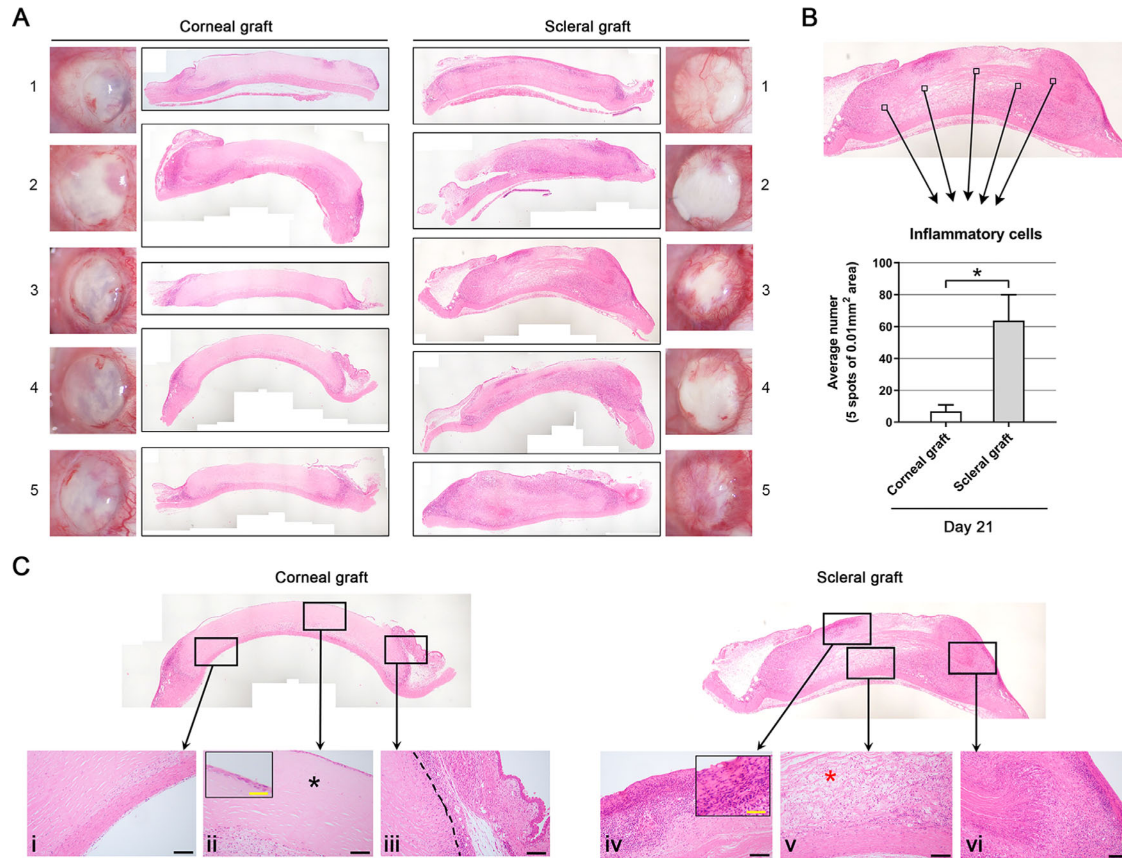

Figure 5. The histopathologic hematoxylin and eosin stains of all the corneal and scleral grafts after surgery in scleral defect rabbit models and the evaluation of the number of inflammatory cells at the graft areas. (A) In sections, the engrafted donor corneas had almost an even thickness among cases 21 days after surgery, whereas graft thickness was quite varied in the scleral grafts, with severe inflammation. (B) The number of inflammatory cells at the graft areas was significantly higher in the scleral grafting group. $^*P < 0.05$. Values represent the mean ± standard error of the mean. (C) Representative photographs showing the detailed structures of the corneal and scleral grafts. Corneal grafts had clear borders between the grafts and the recipient scleras (dotted line, i and iii) with three to four cell layers of stratified squamous epithelial cells (small rectangle, ii) and a compact stromal layer (black asterisk, ii). In the scleral grafts, the stromal layer was loosened (red asterisk, v) and the depth of epithelial layer was ambiguous due to the existence of inflammatory cells underneath the outmost layer (iv). In addition, the morphologic structure at the sclera donor–recipient junction became indistinct with copious inflammatory cells (vi). Scale bars = 50 μm (black) and 20 μm (yellow).

In general, intact basement membranes are of great importance in enhancing reepithelization by enabling the proliferation of viable epithelial cells at the margins of wounds. Moreover, corneal basement membrane proteins are known to play roles in epithelial differentiation and polarization that could affect antimicrobial barrier function as well as epithelial growth.[24] It is, therefore, possible to speculate that the friable epithelium seen at places in a few cases after scleral grafting could be attributed to the absence of basement membrane at the area of the wound. In addition, the irregular and rough surfaces of scleral grafts without basement membranes might have attracted mucin and detached conjunctival epithelial cells to produce filaments, as shown in this study. In this regard, it would be better to incorporate the additional AM transplantation over scleral grafts to serve as a basement membrane, in so far as preserved scleras are used for tectonic lamellar grafting in scleras.[9,25,26] However, this requires additional medical expenses[27] and extra surgical time for surgeons.

It is not clear whether the enhanced vascularization over and adjacent to the scleral grafts in our results is the product of healthy wound healing or of an inflammatory reaction. CD34 has been regarded as being ubiquitously related to hematopoietic cells, and accumulating evidence has demonstrated that CD34 expression is present on several other cell types, including vascular endothelial progenitor cells and fibroblastic cells for angiogenesis.[28–30] The number of $CD3^+$ T cells or $CD34^+$ cells was not significantly different between the two groups, probably

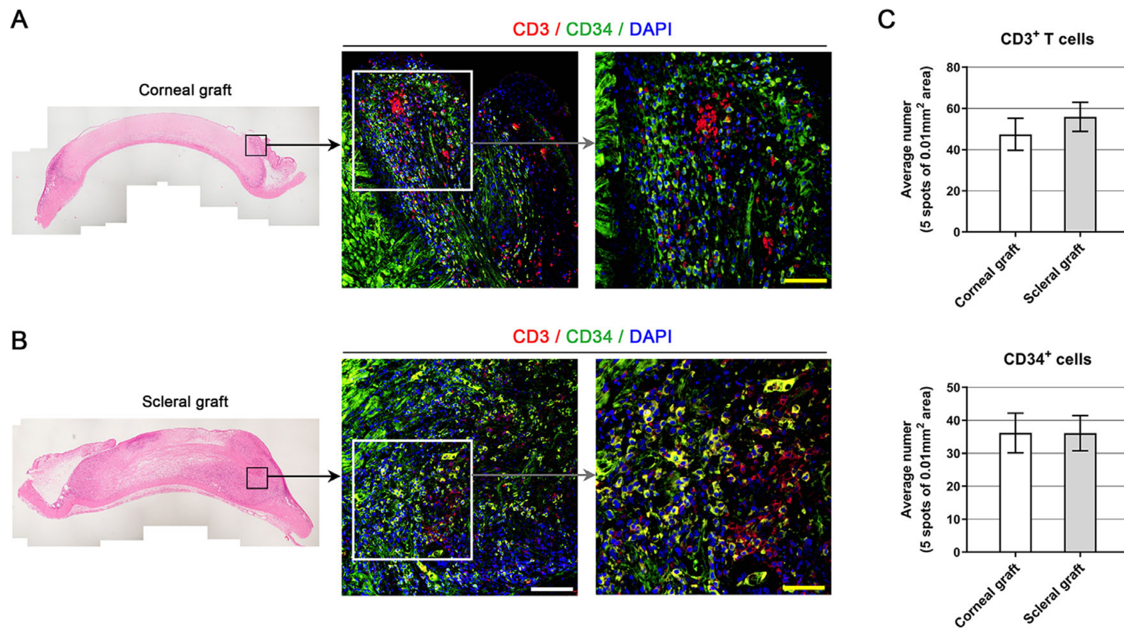

Figure 6. Representative images of CD3 and CD34 staining of corneal and scleral grafts in rabbit scleral defect models and the evaluation of the number of $CD3^+$ T cells or $CD34^+$ cells in the inflammatory area at graft–recipient junctions. (A, B) In both the corneal and the scleral grafts, the areas of inflammation were composed of $CD3^+$ cells and $CD34^+$ cells in the same area. Scale bars = 100 µm (black) and 50 µm (yellow). (C) The number of $CD3^+$ T cells or $CD34^+$ cells at graft–recipient junctions in the corneal graft and scleral graft groups. Values represent the mean ± standard error of the mean.

owing to the effect of biased sampling, because the focused areas for immunophenotyping were definitely inflamed in both groups. In addition, there is a possibility that a 3-week period may be short to provoke strong activation of adaptive immune system; therefore, the recruited inflammatory cells near grafts might be composed of innate immune cells other than T lymphocytes. However, the colocalization of $CD3^+$ T cells and $CD34^+$ cells in both corneal and scleral grafts suggests that neovascularization may be accompanied by inflammation. Similarly, a previous report supported that inflammation accompanies lymphangiogenesis or vasculogenesis in dry eye.[31] Although more inflammation was observed in the scleral grafting group than in the corneal grafting group, the neovascularization index did not show a statistically significant difference, although a higher tendency toward neovascularization was seen in the scleral grafting group. Given that neovascularization is a phenomenon that may have high intercase variation, it is thought that small-sized analyses have a decreased ability to detect statistical significance. Moreover, neovascularization at the inner layer of opaque scleral grafts might have been omitted from analyses, because photodocumented neovascularization mostly reflects surface area.

Scleral melting is a clinically serious problem because it threatens the integrity of the eye. Necrotizing scleritis may occur after infection or the exposure to chemicals, beta irradiation or mitomycin C use during surgery, and may frequently be associated with underling systemic diseases such as rheumatoid arthritis, systemic lupus erythematosus and Wegener's granulomatosis.[32] The intrinsic melting tendency in those patients with immune-related vasculitis may continue after grafting surgery[33]; thus, the necessity for more durable and less inflammatogenic graft material can be an important issue. Accordingly, compact collagen fibers in the corneal stromal layer may be another advantage over sclera with regard to the preservation of shapes of the graft structure. In contrast, we sometimes face suture loosening, graft melting, inflammation, and delayed epithelization after lamellar scleral grafting in patients with necrotizing scleritis or scleromalacia. We believe that the irregular and loosened arrangement of scleral stromal collagen fibers and the lack of basement membrane may be linked to the drawbacks of lamellar scleral grafting according to the present study. In our experience, we performed a tectonic lamellar corneal graft using a preserved human cornea successfully in a 73-year-old woman who underwent pterygium excision 15 years ago and recently suffered from scleromalacia with exposed calcium deposits (Supplementary Fig. S1). The surface of the graft became rapidly epithelized by more than 80% within 1 week; moreover, there was no observed vascular engorgement in the perigraft area.

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The preserved cornea is easy to store and is available from several eye banks. Given that the preserved lamellar corneal grafting can be applied when the transparency is required in surgical wounds such as postuveal melanoma excision and when a firm graft is needed, it merits surgical usefulness. However, the cost of the preserved corneal tissue is high (up to $2,000 USD) and even much higher than preserved sclera and pericardium tissues. A cost-effectiveness analysis would be required in the future study.

Our preclinical study is limited by a small number of subjects and lacks a group combining scleral graft with AM transplantation or conjunctival flap. Therefore, the study results might not represent real clinical scenario where scleral grafting is often accompanied by conjunctival or AM grafting. Nevertheless, this is the first comparative study on graft materials between preserved corneas and scleras in scleral defect models. Our results may provide the proof of principle for the future use of preserved corneal lamellar grafts in patients with scleromalacia or necrotizing scleritis.

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