Biosynthetic Corneal Implants for Replacement of Pathologic Corneal Tissue: Performance in a Controlled Rabbit Alkali Burn Model

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PURPOSE. To evaluate the performance of structurally reinforced, stabilized recombinant human collagen-phosphorylcholine (RHCIII-MPC) hydrogels as corneal substitutes in a rabbit model of severe corneal damage.

METHODS. One eye each of 12 rabbits received a deep corneal alkali wound. Four corneas were implanted with RHCIII-MPC hydrogels. The other eight control corneas were implanted with either allografts or a simple cross-linked RHIII hydrogel. In all cases, 6.25 mm diameter, 350 μm thick buttons were implanted by anterior lamellar keratoplasty to replace damaged corneal tissue. Implants were followed for nine months by clinical examination and in vivo confocal microscopy, after which implanted corneas were removed and processed for histopathological and ultrastructural examination.

RESULTS. Alkali exposure induced extensive central corneal scarring, irregular surface irregularity, and neovascularization in one case. All implants showed complete epithelial coverage by four weeks postoperative, but with accompanying suture-induced vascularization in 6 out of 12 cases. A stable, stratified epithelium with hemidesmosomal adhesion complexes regenerated over all implants, and subbasal nerve regeneration was observed in allograft and RHCIII-MPC implants. Initially acellular biosynthetic implants were populated with host-derived keratocytes as stromal haze subsided and stromal collagen was remodeled. Notably, RHCIII-MPC implants exhibited resistance to vascular ingrowth while supporting endogenous cell and nerve repopulation.

CONCLUSIONS. Biosynthetic implants based on RHC promoted cell and nerve repopulation in alkali burned rabbit eyes. In RHCIII-MPC implants, evidence of an enhanced resistance to neovascularization was additionally noted. (Invest Ophthal Vis Sci. 2011;52:651–657) DOI:10.1167/iovs.10-5224

Damage to the cornea caused by disease or injury is the second largest cause of blindness in the world after cataracts, affecting an estimated 10 million people worldwide. Currently, corneal transplantation by full-thickness penetrating keratoplasty (or partial-thickness lamellar keratoplasty where recipient endothelium is spared) with human donor tissues is the only widely accepted treatment. Although corneal transplantation with allogeneic tissue has high success rate, the major problem in most countries is a shortage of high quality donor tissue. In addition, in so-called high-risk cases where the recipient corneal bed is inflamed and/or neo-vascularized, prognosis is poor and graft failure rates are high.

When multiple human donor grafts fail or are contra-indicated, the only current clinical option is to use a fully synthetic keratoprosthesis, which provides for light transmission into the eye. However, biological integration with surrounding recipient tissue is still a major problem, and there is no restoration of sensory or physiologic corneal function. Alternatively, a tissue engineered corneal substitute that allows regeneration of endogenous cells and nerves but has the structural stability and resistance to biodegradation that the keratoprostheses offers could provide a fully-integrated, biologically functional cornea that would supplement the use of human donor tissue as a primary choice for implantation, and thereby address the issue of tissue shortages. We have now shown that reinforced interpenetrating networks of collagen and 2-methacryloyloxyethyl phosphorylcholine (MPC) can be molded into acellular corneal substitutes and be implanted into eyes in animal models, where they remain anchored into the host corneas and permit regeneration of functional corneal nerves as different, active nerve sub-types within the implants. Unlike purely collagen implants, which enabled cell and nerve regeneration in human clinical trials, the MPC-reinforced implants also show enzyme resistance in vitro. However, this resistance has not been confirmed in vivo, nor have the implants been evaluated for use within corneas with severe pathologies; that is, in a high-risk transplantation setting. Chemical burns and corneal alkali burns in particular, are characterized by corneal opacification, recurrent epithelial erosions, and neovascularization leading to permanent visual impairment, necessitating transplantation. The persistent inflammation and resulting complications, however, lead to a poor prognosis and a subsequently high graft failure rate. Hence, controlled alkali burns in animals such as rabbits are commonly used to model severe corneal pathologies that would constitute high-risk transplantation cases (without concomitant limbal stem cell deficiency). Here, we use the alkali wounded corneal model to assess the performance of structurally reinforced, stabilized recombinant human collagen-phosphorylcholine (RHCIII-MPC) hydrogels.
drogals as corneal substitutes in a rabbit model of severe corneal damage.

**METHODS**

**Fabrication of Collagen Hydrogels**

The biosynthetic implants were fabricated from freeze-dried recombinant human collagen type III (RHCIII), either with or without the incorporation of MPC, as previously described. Briefly, for RHCIII substitutes, a 13.7% (w/w) aqueous solution of RHCIII was prepared and mixed thoroughly with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC; Sigma-Aldrich, Ontario, Canada) and N-hydroxysuccinimide (NHS; Fluka, Buchs, Switzerland) using a syringe mixing system. For RHCHIII-MPC substitutes, the RHCIII was mixed with MPC (Biocompates, UK) and poly(ethylene glycol) diacrylate (575 mw-PEG; Sigma-Aldrich) using an ammonium persulfate (APS; Sigma-Aldrich) and N,N,N',N'-tetramethyl ethylene diamine (TEMEDA; Sigma-Aldrich) solution as a chemical initiator followed by the addition of EDC and NHS to cross-link the collagen. The homogenous collagen solutions were then dispensed into polypropylene molds and cured at 100% humidity at 21°C for 16 hours, and then at 37°C for five hours. After removal from the molds, the substitutes were washed thoroughly with 10 mM phosphate buffered saline (PBS).

The resulting implants were cornea-shaped, 12 mm in diameter, and 350 µm thick. Implants were stored in 1% chloroform in 0.1 M PBS (pH 7.2-7.4) to maintain sterility until used. Before use, the implants were washed well in PBS to remove all chloroform traces and incubated overnight in PBS.

**Alkali Burn Model and Corneal Implantation**

After approval by the research ethics committee at Linköping University (Application No. 47-45), 16 New Zealand White rabbits (3.5–4 kg) were used in this study. Twelve rabbits were subjected to alkali corneal wounds, while four untouched rabbits served as allogeneic cornea donors. All procedures were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. A standardized corneal alkali wound model was used. Rabbits were anesthetized with xylazine (Rompun; Bayer, Gothenburg, Sweden) and ketamine (Ketalar; Parke-Davis, Taby, Sweden). Additionally, local anesthetic was given (tetracaine hydrochloride eye drops 1%, Chauvin Pharmaceuticals Ltd., Surrey, UK). A 3-mm diameter filter paper, soaked in 1 N NaOH solution (pH 13.6), was placed over the central cornea in the right eye of each animal. Any surplus liquid was removed by brief contact with clean absorbent filter paper. The cornea was exposed for one minute, the filter paper was removed, and the ocular surface was subsequently rinsed with a balanced salt solution. Chloramphenicol eye ointment (Chloromycetin, Pfizer AB, Sollettuna, Sweden) was then applied to the cornea, and the eyelid was taped shut for one hour.

Lamellar keratoplasty was performed in all 12 corneas two months after alkali wounding. Using a 6-mm lamellar keratoplasty trephine, damaged corneal tissue was removed to a depth of 350 µm. Animals were divided into three groups of four animals each. The first group received allogeneic implants (allografts), taken from the four non-wounded donor rabbits. The second and third groups received tissue-engineered corneal substitutes 6.25 mm in diameter and 350 µm thick, composed of either RHCIII only or RHCIII-MPC, respectively. Untouched left eyes served as controls. All implants were placed into the recipient stromal bed and were retained using overlying sutures (10-0; Ethicon, Edinburgh, UK) with their knots buried into the recipient stroma. After surgery, animals received viscous eye drops (Fucithalmic; Leo Pharma AB, Malmö, Sweden) three times daily for the first week, but no postoperative steroids were administered. Sutures were removed one month postoperatively.

**Clinical Evaluation**

Corneas were followed for nine to ten months postoperatively. The general health and eyes of the rabbits were monitored daily for the first postoperative week, bi-monthly for the first month, and monthly thereafter. Slit-lamp biomicroscopy was used to examine the corneas for signs of inflammation or neovascularization, and to assess optical transparency, and photographs were taken. From the photographs, vascular infiltration of the implanted region was assessed by estimating the percentage area of the implanted tissue covered by vessels. Transparency of the central cornea was assessed using a numerical grading scale, with 0 representing transparent, 1 indicating mild haze not obscuring the pupil, 2 indicating moderate haze partially obscuring the pupil, and 3 indicating an opaque area totally obscuring the pupil. Sodium fluorescein staining was used to assess the epithelial integrity and barrier function. Immediately before euthanatization, in vivo confocal microscopy (IVCM) examination (Heidelberg Retina Tomograph 3 with Rostock Corneal Module, HRT3-RCM, Heidelberg Engineering, Heidelberg, Germany) was used to assess corneal morphology, including cell and nerve in-growth. Typical in vivo images of the epithelium, sub-epithelial region, mid-implant region, and endothelium were selected from each cornea by a single observer (NL) blinded to the identity of the implant type by coding image data. All rabbits with corneal substitutes were killed at nine months postoperatively, while rabbits with allografts were killed at 10 months. The corneas (control and operated) were excised with 1 mm of the scleral rim for further processing.

**Histology and Immunohistochemistry**

Implanted and control contralateral untreated corneas were cut into pieces for light and electron microscopy. Some pieces were fixed in 4% paraformaldehyde in 0.1 M PBS, parafin embedded, and then sectioned. Hematoxylin and eosin (H&E) staining was performed for routine histopathologic examination. For transmission electron microscopy (TEM), samples were fixed in Karnovsky fixative and osmium tetroxide, stained with uranyl acetate, and embedded in epoxy resin. Thin and ultrathin sections were cut and stained to visualize corneal morphology.

Cellular activity was further evaluated by immunohistochemical staining for α-smooth muscle actin (SMA). Dewaxed and rehydrated samples were blocked for non-specific staining with 4% PBS and 1% BSA in PBS, and incubated in the primary antibody (1:100; Cell Marque Corp) overnight at 4°C. Samples were then incubated with a Cy3 conjugated secondary antibody (1:400; Sigma) and 4',6-diamidino-2-phenylindole (DAPI; Sigma) before light microscopy examination (Zeiss Axioskop 2).

**Statistical Analysis**

Differences in corneal transparency between implant types were assessed at the final postoperative examination by the nonparametric Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks. Normality of the data were determined by the Kolmogorov-Smirnov test. For all statistics, a two-tailed value of < 0.05 was considered significant. Statistics were performed with statistic software (Sigma Stat 3.5 for Windows; Systat Inc., Chicago, IL).

**RESULTS**

**Clinical Results**

Two months after the infliction of alkali wounds, all injured corneas exhibited stromal opacities of varying depth in the central 2 mm, while one cornea was vascularized (Fig. 1). Lamellar keratoplasties were completed without complications (Fig. 2). The surface of all implants stained positive with fluorescein one week postoperatively. Complete epithelial coverage of implants, however, was noted on repeated fluorescein testing at the time of suture removal at one month postoperatively. When sutures were removed, three of four RHCIII implants, one of four RHCIII-MPC, and two of four allograft implants were vascularized. Neovascularization appeared to be
stimulated by the buried suture knots, and ranged from mild to severe. Notably, in the single vascularized RHCIII-MPC implant, the implant appeared to form an early barrier to vascular invasion (Fig. 3). At this time, all corneas were centrally hazy. No additional medication was given despite vascularization. At six and nine months postoperative, a reduction in the number of vessels and level of haze was noted relative to earlier examinations (Figs. 3, 4). Quantitative analysis of central corneal transparency revealed a marked reduction in haze from ‘3’ at one month postoperative to ‘1’ at six months postoperative in all implanted corneas (Fig. 5). No differences in transparency were detected among the groups at nine months postoperative (P = 0.99). Analysis of vascularization revealed a lower level of vascular invasion in RHCIII-MPC implants relative to the other implant types (Fig. 5). Vessels in RHCIII implants and allografts subsided over the first six months, while a slight increase in vessel coverage occurred at six months in the single vascularized RHCIII-MPC implant, possibly indicating a delayed vascularization response. Statistical analysis of implant vascularization was not possible due to the small number of vascularized corneas in the groups.

IVCM, Histology, and Immunohistochemistry

By IVCM, all implanted eyes exhibited a morphologically-normal, stratified epithelium (Fig. 6), comprised of superficial, wing, and basal cell layers. H&E sections and DAPI-labeled epithelial cell nuclei confirmed regeneration of stratified epithelium resembling the native cornea in all implant types (Fig. 7). The stroma in all implanted eyes had a slightly hazy appearance (Figs. 4, 6) which by IVCM appeared as increased ECM reflectivity, primarily localized to the sub-epithelial region and the posterior implant-to-stroma interface. Immunohistochemically, only a few α-SMA positive cells were observed centrally, with staining confined to peripheral interface regions, with the bulk of the central stroma α-SMA negative (Fig. 7). The gross morphology of central H&E sections revealed epithelial and stromal compartments in RHCIII and RHCIII-MPC implants that were similar to the corresponding control and allograft corneas (Fig. 7). Fine, parallel-running nerve fiber bundles at the level of the basal epithelium (comprising the subbasal nerve plexus) were noted by IVCM in allografts and RHCIII-MPC implanted corneas, but were not observed in RH CIII implants (Fig. 6). Mid-implant nerves were observed in allografts only. Stromal cell nuclei (presumed keratocytes) were observed by IVCM in the anterior and mid-implant region in all implant types. The appearance of stromal cells in allografts, however, differed from the appearance of cell-repopulated implants, likely due to elevated stromal reflectivity in implants (Fig. 6). Stromal cells were additionally observed by DAPI staining in the central cornea of all implant types (Fig. 7), and their presence extended through the full stromal thickness. The endothelium in corneas with all implant types appeared morphologically normal by IVCM. In seven of 12 implanted eyes, however, posterior stromal haze was observed by IVCM and extended to the endothelium, consistent with a full-thickness alkali wound.

Transmission Electron Microscopy

TEM images of the epithelial-stromal boundary (Fig. 8) indicated the presence of hemidesmosomal complexes in all samples. Additionally, the presence of anterior stromal cells (keratocytes) was confirmed by TEM in all implants. Sub-epithelial nerves were additionally observed in TEM sections from bio-
implants (duration in one case. Post-implantation, delayed epithelialization of corneal scarring, an irregular ocular surface, and vascularization also subsided over time for RHCIII and allograft implants. In RHCIII-MPC implants delayed but low-level vascularization was noted.

Epithelial regeneration is critical for survival of the implant and the maintenance of corneal transparency, integrity, and function. The RHCIII-MPC implants allowed for regeneration of a morphologically normal, stratified epithelium. The epithelium was stably attached to the underlying implant, as evidenced by regeneration of hemidesmosomes and anchoring fibrils at the epithelium-implant interface. The delayed epithelial coverage of implants was noted in this study to be associated with epithelial topographic irregularity taking the form of imprinted suture lines, which became visible on suture removal. We noted a similar phenomenon of delayed epithelialization in a previous study, in which overlying sutures had hindered the centripetal migration of epithelium to cover the implant region. However, despite the severity of alkali injury and the surgical trauma, all corneas in this study regenerated a stable, morphologically normal stratified epithelium to cover the implant. In previous studies in healthy porcine eyes, RHCIII implants were fully epithelialized by one week, while in humans, full epithelial coverage of RHCIII implants took longer than one month, completing only after the removal of retaining sutures. Although the alkali burns in the present study may have induced corneal changes that slowed the course of epithelial regeneration, it is expected that with a modified implant retention technique, epithelial closure over the implant would proceed more rapidly.

Corneal innervation, particularly of the subbasal nerve plexus, is an important consideration as it gives rise to the sensory innervation of the epithelium which in turn provides the protective blinking reflex and serves to maintain the pre-corneal tear film and epithelial function. Nerves in the sub-epithelial region were observed by TEM in all implant types in this study, but regenerated nerves of the subbasal plexus could only be detected by IVCM in allograft and RHCIII-MPC implants. Sub-epithelial haze in RHCIII implants, however, may have masked subbasal nerve presence during in vivo examination. When evaluated in porcine corneas, RHCIII implants exhibited subbasal nerve regeneration as early as six months postoperatively, while the first regenerating human subbasal nerves were observed overlying RHCIII implants as early as seven months postoperatively. The regeneration of long, parallel-running subbasal nerve fibers observed in the central cornea in this study within nine months is consistent with our earlier studies, and is markedly more rapid than human allograft re-innervation as observed by IVCM in several human studies. Subbasal nerve regeneration in the implanted corneas in this study thus appeared to be unhindered by the initial alkali wound, and occurred to an equivalent degree in both allografts and RHCIII-MPC implants.

Stromal cells (or keratocytes) migrated to repopulate the initially acellular biosynthetic implants, and by nine months after surgery, collagen fibrils were imprinted by regenerating stromal cells and fibroblasts as a result of the surgical procedure. The collagen fibrils were arranged into distinct lamellae in the new corneal stroma, with vessels covering the entire graft in a number of cases. While sutures are known to induce vascular ingrowth, with vessels skirting around the implant periphery and only later invading the implant itself in one of four corneas. This behavior was not observed in allograft or RHCIII implants (which were extensively vascularized). We postulate that the increased enzymatic resistance of RHCIII-MPC implants shown in vitro likely contributed to in vivo resistance of extracellular matrix degradation and remodeling normally preceding vascular invasion. In allografts and RHCIII implants, vascular coverage of implants and vessel size gradually decreased over the postoperative period, while RHCIII-MPC implants exhibited a delayed, low-level vascularization. By 9 to 10 months postoperatively, only fine vessels could be detected in implants (Fig. 3). This result indicates that in the absence of a sustained inflammatory stimulus (or steroid treatment), the biosynthetic implants could support the return of corneal transparency—or in the case of enzyme-resistant implants, the maintenance of initial transparency—in a pathologic corneal milieu.
postoperatively, keratocytes occupied the entire implanted region from the sub-epithelial space to the posterior implant-to-stromal interface. Individual keratocytes were observed in vivo, by immunofluorescence, and in TEM sections in all implant types. Since allograft tissue in this study was freshly harvested before surgery, live, functional, and presumably quiescent keratocytes were implanted in these cases. By contrast, acellular matrix repopulation by host stromal keratocytes was accompanied by the development of stromal “haze,” observed both clinically and microscopically. While this haze may have been the result of keratocyte activation, stromal remodeling, or new collagen production, its exact origin remains unclear. Arrangement of stromal collagen in lamellae within biosynthetic implants (Fig. 9) suggests possible remodeling of the implants had occurred. This may have contributed to the transient haze observed in the implants. Stromal haze, however, gradually subsided in implants, an effect which was observed clinically (Figs. 4, 5), and was confirmed by α-SMA negativity in the central implant region (Fig. 7) indicating an absence of the myofibroblast phenotype. IVCM provided a further confirmation of stromal clearing, with only a mild background haze present in the implant enabling the visualization of stromal keratocytes. This result suggests that both biosynthetic implant types were equally ‘cell friendly’ and did not impede the migration and/or proliferation of stromal cells or the return to stromal transparency. Moreover, at nine months, we could not detect any feature in the implanted stroma at the morphologic level that could be attributed to the initial alkali burn injury. The only indications of the initial burn were a residual stromal haze in some recipient stromal beds observed both clinically and by IVCM. It is expected that if such deep stromal scars were instead treated by deep anterior lamellar keratoplasty (DALK) (to replace the entire stroma to the level of Descemet’s membrane) with an appropriately thick biosynthetic replacement, improved corneal transparency would result. In a previous study where RHCIII matrices were implanted into porcine eyes, keratocyte repopulation of acellular matrices was noted as early as the first few preoperative months, however, quiescence of cells and the surrounding matrix was observed only at 12 months. Repopulation of acellular RHCIII implants by quiescent keratocytes in humans was not observed at seven months, only fibroblast-like stromal cells within hyper-reflec-

FIGURE 6. Images from the native rabbit cornea (column 1) and implanted corneas (columns 2–4) taken before animal kill. Row 1: clinical appearance of non-vascularized corneas from each group. Rows 2–5: corneal micromorphology by IVCM. Row 2: epithelial cells, with similar morphology in all groups. Row 3: region immediately below the epithelium containing parallel nerves of the subbasal nerve plexus (arrows), which were not detected in collagen-only implants. Row 4: stromal images at a depth of 200 μm below the corneal surface. Stromal cell nuclei were observed in all implant types, with allograft tissue exhibiting the lowest level of microscopic haze. Mid-stromal nerves (arrows) were only observed in control corneas and allografts. Row 5: corneal endothelium with a normal morphologic appearance in all groups. All images represent a 400 × 400 μm area of the central cornea.
tive fibrotic tissue occupied the implants. The first indications of cell migration into implants in humans occurred at 12 months (unpublished result), representing a slower stromal renewal process (in keratoconus patients) than in previous animal studies. In the context of these previous studies, stromal repopulation of both implant types in this study proceeded relatively quickly. We could not determine, however, exactly when the first recipient keratocytes entered the implants since IVCM was only performed at the final examination. Nevertheless, neither the alkali burn injury nor the RHCIII-MPC implant composition appeared to negatively impact keratocyte migration.

In summary, we tested for the first time, the use of tissue-engineered biosynthetic corneal implants as a primary alternative to implantation with allogeneic tissue for the treatment of corneal pathology in an animal model. Despite the suboptimal preoperative condition of the corneas and the avoidance of postoperative steroid use, there were no apparent graft fail-
ures. Instead, the biosynthetic implants allowed population by host cells and re-generated nerves, mimicking components found in the allograft tissue. Although some residual stromal reflectivity was apparent in implants microscopically, this was likely the result of cell migration into the initially acellular implant. This initial acellularity minimizes the possibility of host immune reaction to foreign cell-borne antigens, a risk inherent in the implantation of allograft tissue. Significant postoperative vascularization in some allografts and RHCIII implants was observed, although a modification of the surgical technique may avoid this complication. Unexpectedly, however, RHCIII-MPC implants exhibited a resistance to vascular ingrowth, while retaining the ability to allow epithelial attachment and regeneration, nerve regeneration, and keratocyte repopulation. This study thus provides the first evidence that enzyme-resistant biosynthetic substitutes for allogeneic tissue may be a viable option for implantation in severely scarred and vascularized cases requiring treatment by keratoplasty, but with a preserved stem cell niche. Further animal studies with enhanced materials and improved surgical techniques are required to investigate the potential for endogenous corneal regeneration after severe disease or trauma, as facilitated by the use of tissue-engineered, biosynthetic corneal implants. Extending the clinical indications of applicability and demonstrating a suitable performance of biosynthetic implants relative to the clinical indications of applicability and demonstrat-

References