

Tissue-Engineered Recombinant Human Collagen-Based Corneal Substitutes for Implantation: Performance of Type I versus Type III Collagen

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PURPOSE. To compare the efficacies of recombinant human collagens types I and III as corneal substitutes for implantation.

METHODS. Recombinant human collagen (13.7%) type I or III was thoroughly mixed with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide. The final homogeneous solution was either molded into sheets for in vitro studies or into implants with the appropriate corneal dimensions for transplantation into minipigs. Animals with implants were observed for up to 12 months after surgery. Clinical examinations of the cornea included detailed slit lamp biomicroscopy, in vivo confocal microscopy, and fundus examination. Histopathologic examinations were also performed on corneas harvested after 12 months.

RESULTS. Both cross-linked recombinant collagens had refractive indices of 1.35, with optical clarity similar to that in human corneas. Their chemical and mechanical properties were similar, although RHC-III implants showed superior optical clarity. Implants into pig corneas over 12 months show comparably stable integration, with regeneration of corneal cells, tear film, and nerves. Optical clarity was also maintained in both implants, as evidenced by fundus examination.

CONCLUSIONS. Both RHC-I and -III implants can be safely and stably integrated into host corneas. The simple cross-linking methodology and recombinant source of materials makes them potentially safe and effective future corneal matrix substitutes. (*Invest Ophthalmol Vis Sci.* 2008;49:3887-3894) DOI:10.1167/iov.07-1348

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The current and projected shortages of high-quality donor human corneas for transplantation in many countries have prompted the development of viable, long-term alternatives to human donor tissue. As the cornea is the main refractive element of the eye and serves as a protective barrier, it has several key properties that need to be replicated in any artificial replacement. These include high optical clarity, appropriate refractive index and toughness, and the ability to integrate into host tissues seamlessly and remain avascular. To date, corneal substitutes that have been tested clinically are prostheses that address replacement of the cornea's function with synthetic polymers that are either compatible with or non-harmful to host tissue. More recent approaches, however, have been to develop biointeractive materials that would allow some host tissue regeneration, such as reconstitution of a healthy epithelium¹⁻³ or epithelium, stroma, and nerves.^{4,5}

We have developed optically clear cornea stromal matrix substitutes based on cross-linked porcine or bovine collagen, that when implanted into minipigs, promote regeneration of corneal cells, nerves, and tear film mucin.^{4,5} However, these animal collagen-based corneal substitutes gave low level, but observable, immunogenic reactions when implanted into mice as xenogeneic grafts.⁶ Furthermore, as we move toward use in human clinical implantation, identification of an implant material that is safe as well as efficacious is essential to overcome safety concerns around the use of animal-source collagen, including the potential for transmission of disease⁷ and immune response within the host.⁸ Recombinant human collagen or artificial collagens based on repeats of Gly-X-Y would circumvent these obstacles. However, most artificial collagen chains reported to date are short (<10 nm) or use chemical synthesis methods⁹ that are not as readily adapted to sequence modification and glycosylation as they would be if made recombinantly.

In this study, we compared the efficacy of two recombinantly produced human collagens, types I and III, for use in tissue-engineering corneal substitutes. These highly purified, recombinant collagens have been fully characterized and resemble natural fibrils in their ultrastructure, in their formation of triple helices, and in Western blot analyses. While collagen I is the species most commonly found within the human cornea, collagen III has several properties, including smaller fibril diameter¹⁰ that may allow for a more tightly packed hydrogel that is more robust. In the present study, despite differences between type I and III fibrils, hydrogels fabricated from both collagens were found to share many similar properties, and implants of both collagens gave similar results in vivo.

METHODS

Fabrication of Corneal Implants

Recombinant human collagens I and III (RHC-I and -III, respectively) produced in yeast cells (*Pichia pastoris*) were purchased from FibroGen, Inc. (South San Francisco, CA), freeze-dried, and reconstituted.

To fabricate collagen hydrogels, 250- μ L aliquots of either 13.7% (wt/wt) of RHC-I or -III in aqueous solution were each loaded into a syringe mixing system free of air bubbles.⁵ Calculated volumes of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and *N*-hydroxy-succinimide (NHS) solutions were added and thoroughly mixed at 4°C (ice water). The final solution was immediately dispensed as flat sheets or into curved polypropylene contact lens molds (500 μ m thick, 12 mm diameter) and cured at 100% humidity at 21°C for up to 24 hours and then at 37°C for up to 24 hours. The implants were washed three times with fresh 1 \times PBS and then stored in PBS containing 1% chloroform to maintain sterility.⁵ Optimal RHC-I and -III hydrogels were prepared using EDC to collagen-NH₂ ratios of 0.5:1 and 0.4:1, respectively.

Physical, Chemical, and Mechanical Characterization

Optical Properties. Refractive indices of fully hydrated RHC-I and -III hydrogels were recorded with an Abbe refractometer (model C10; VEE GEE Scientific Inc., Kirkland, WA) at 21°C with bromonaphthalene as the calibration agent. Light transmission and back-scattering were measured by using a custom-built instrument as described previously (Priest D, et al. *IOVS* 1998;39:ARVO Abstract 1634). Briefly, transmission and back scattering measurements were made for both white light (quartz-halogen lamp source) and for the narrow spectral regions centered at 450, 500, 550, 600, and 650 nm. Differences in the optical properties between the hydrogels were analyzed statistically with one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple-comparisons test. Statistical significance was set at $P < 0.05$.

Mechanical Properties. The tensile strength, elongation at break, and elastic moduli of the hydrogels were determined on an electromechanical universal tester (model 3342, with Series IX/S software; Instron Corp., Norwood, MA). Statistical analyses were the same as for optical properties.

Diffusion Permeability. Glucose permeability of hydrogels was determined as described previously⁵ at the cornea's normal physiological temperature (35°C) by using a modified Ussing chamber (Warner Instruments, Hamden, CT) with air-lift mixing. Measurements were made colorimetrically at 540 nm, with a glucose assay kit (GAGO20; Sigma-Aldrich, St. Louis, MO) with a spectrophotometer (model UV-1601; Shimadzu, Columbia, MD). FITC-labeled bovine serum albumin (BSA; 66 kDa; Sigma-Aldrich) was used for measuring albumin diffusion. A side-by-side diffusion chamber, with magnetic stirring in each chamber (to avoid foaming problems (PermeGear, Bethlehem, PA), set at 35°C was used. Both the receptor and permeation chambers were 3 mL in volume, with 0.5 mM albumin used in the permeation chamber. Sampling was performed as in the glucose measurements. Receptor chamber albumin concentrations were determined by fluorophotometry (Gilford Fluoro IV; CIBA-Corning Diagnostics Corp., Park Ridge, IL), fitting the resulting values to a regression line of standards of known concentration. Diffusion coefficients were calculated with these values.

Toxicology

Toxicological tests were performed as we have previously described⁵ by North American Science Associates (NAMSA, Northwood, OH) in accordance with International Organization for Standardization (ISO) protocols. These tests included agarose overlay for cytotoxicity and the cytotoxicity test specific for extractables that may leach out of the materials (ISO 10993-5),¹¹ genotoxicity tests with a bacterial reverse-mutation study (ISO 10993-3)¹² and systemic toxicity tests (ISO 10993-11).¹³

In Vitro Biocompatibility

Immortalized human corneal epithelial cells (HCECs) were used to evaluate epithelial coverage as we have described elsewhere.^{5,14} Briefly, HCECs were seeded on top of 1.5-cm² flat hydrogels of either

RHC-I or -III and supplemented with the serum-free medium containing epidermal growth factor keratinocyte serum-free medium (KSEF; Invitrogen, Burlington, ON, Canada) and grown until confluent. They were then switched to a serum-containing modified supplemented hormonal epithelial medium (SHEM) for the next 2 days, followed by maintenance at an air-liquid interface. Time taken by the cells to attain confluence was compared with plasma-treated, tissue culture plastic controls, and the ability of hydrogels to support epithelial stratification was evaluated.

To determine the ability of the hydrogels to support nerve growth, dorsal root ganglia from chick embryos (E8.0) positioned onto gels with a collagen-based adhesive. Neurite outgrowth was observed for up to 7 days, after which the gels were fixed in 4% paraformaldehyde in 0.1 M PBS (pH 7.2-7.4) and stained for neurofilaments using mouse anti-NF200 antibody (Sigma-Aldrich), diluted 1:40 in Tris-buffered saline (TBS) containing 4% fetal bovine serum (FBS), overnight at 4°C. They were visualized the following day by immunofluorescence after conjugating with a donkey anti-mouse-Cy2 neurofilament secondary antibody (1:200 dilution in TBS-FBS; GE Healthcare, Baie D'Urfe, QC, Canada).

In Vivo Biodegradation

RHC-I and -III gels, 5 mm \times 5 mm \times 500 μ m, were implanted subcutaneously into the backs of male Sprague-Dawley rats of at least 300 g (*Rattus norvegicus*, Hla (SD)CVF; Hilltop Laboratory Animals, Inc., Scottsdale, PA) for 30, 60, 90, and 180 days at NAMSA, to evaluate the subchronic and chronic effects of implantation according to ISO 10993-6.¹⁵ This test also provides a measure of the biodegradation rate of implanted materials. For surgery, the animals were anesthetized with intraperitoneal injection of ketamine hydrochloride and xylazine (66 mg/kg + 9 mg/kg) at 2.25 mL/kg and subcutaneous injection of 0.05 mg/kg buprenorphine. Small incisions (7 mm in length) were made paravertebrally in the thoracic and lumbar region (2 cm away from the vertebral column) for up to four samples per rat. After sample insertion, incisions were closed with wound clips that were removed 11 days later. At each time point, the animals were euthanized, and the skin over each implant site was incised and reflected to expose the implant sites. One sample each was randomly selected for macroscopic evaluation, whereas the other four samples were processed for histopathologic examination.

Corneal Implantation and Clinical Evaluation

Procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and with ethics approval from the University of Ottawa (Protocol EI-5), as we have published elsewhere.⁵ Briefly, in a blinded study, the pigs were anesthetized (mixture of 4.4 mg/kg Telazol [Wyeth, Carolina, Puerto Rico] and 2.2 mg/kg xylazine [Rompun; Bayer Inc., Etobicoke, ON, Canada]) were implanted with one of either RHC-I or -III hydrogels (6.26 mm diameter, 500 μ m thick), after removal of the equivalent host corneal tissue. The implants were held in place by overlying sutures that were removed four weeks later. All animals received steroids (Prednisolone; Sandoz, Quebec, Canada) and antibiotics (Zymar; Allergan, Irvine, CA) four times daily. The nonsurgical, contralateral corneas served as the control. Additional control subjects consisted of four animals that received allograft transplants.

Follow-ups were performed daily on each pig up to 7 days after surgery, then weekly for a month, followed by detailed examinations at 2, 6, and 12 months. Examinations included slit lamp and fundus biomicroscopy (to image the retina) to assess implant optical clarity, Schirmer's to assess tear film regeneration, and sodium fluorescein staining to assess integrity and barrier function. Intraocular pressure measurements were taken to ensure that the implants were not blocking aqueous humor flow.

In vivo confocal microscopy (IVCM; ConfoScan3; Nidek, Gamagori, Japan) was used to assess cell and nerve ingrowth, as well as to measure corneal thickness in live animals. Corneal touch sensitivity

TABLE 1. Primary Antibody Specifications

Primary Antibody	Dilution	Company	Specificity
AE-5	1:100	Chemicon, Temecula, CA	Corneal epithelial cell-specific marker
<i>Ulex europaeus</i> agglutinin	1:200	Sigma-Aldrich, Oakville, ON, Canada	Mucin of tear film
Collagen VII	1:100	Sigma-Aldrich	Hemidesmosome complex marker
SP8-D1 (procollagen I)	1:60	Developmental Studies Hybridoma Bank, Iowa City, IA	De novo collagen synthesis
Smooth muscle actin (SMA)	1:100	Cell Marque Corp, Rocklin, CA	Myofibroblasts
F4/80	1:400	Serotec USA, Raleigh, NC	Pan macrophage marker
CD45	1:40	BD Biosciences, San Diego, CA	Bone marrow-derived cells
Type III collagen-biotin	1:100	Southern Biotech Co., U.S.A.	Type III collagen

was measured with a Cochet-Bonnet aesthesiometer (Handaya Co., Tokyo, Japan). The density of nerve fibers within the central 5 mm of all corneas was quantified by analysis of in vivo confocal images. Full details of the nerve density quantification methodology are given in a paper by Lagali et al.¹⁶ Nerve density was reported as the total length of all nerve fibers detected within a corneal depth zone divided by the zone volume (confocal microscope image cross-sectional area multiplied by the corneal zone depth of field), expressed in micrometers per cubic millimeter. Central corneal nerve density 12 months after surgery was compared within the various graft types by corneal depth zone,¹⁶ by using the Kruskal-Wallis analysis of variance (ANOVA) on ranks method. In addition, for each graft type, nerve density was compared to levels in control corneas for the same depth zone with either a *t*-test (where data was normally distributed) or a Mann-Whitney rank sum test (for non-normally distributed data). For all nerve density comparisons, normality was determined with the Kolmogorov-Smirnov test, and for all statistical tests, $P < 0.05$ was considered statistically significant. All statistics were calculated with commercial software (SigmaPlot v9.0 with SigmaStat integration; Systat Software Inc., Point Richmond, CA).

Histopathologic Evaluation

Corneas with implants and control corneas were cut into four pieces for light and electron microscopy. Two were fixed in 4% paraformaldehyde in 0.1 M PBS, processed, paraffin embedded, and sectioned at 10 μ m. One quarter of each cornea was also prepared for cryosectioning. Sections were stained with H&E for histopathologic examination. Immunofluorescence was performed as described earlier on deparaffinized sections for type VII collagen, a hemidesmosome marker,¹⁷ procollagen I, smooth muscle actin (SMA), and type III collagen. On frozen sections, samples were stained with *Ulex europaeus* agglutinin (UEA), AE-5, CD45, and F4/80. Antibody details are in Table 1. Slides used for procollagen I, SMA, and type III collagen staining were deparaffinized and treated with 3% H₂O₂. Samples were then blocked for nonspecific staining with 4% FBS+1% BSA in PBS followed by

antigen retrieval performed by reacting with proteinase K (2 mg/mL) for 20 minutes at 37°C primary antibody incubation overnight at 4°C. The sections were incubated in a 1:400 dilution of the biotinylated anti-mouse IgG antibody (GE Healthcare) after washing followed by a 1:400 dilution of avidin-horseradish peroxidase (GE Healthcare) and visualized with diaminobenzidine (Roche, Mannheim, Germany). Sections stained for type VII collagen, UEA, CD45, AE-5, and F4/80 were blocked with 4% FCS+1% BSA in PBS and incubated in primary antibody overnight at 4°C. They were then incubated with 1:400 FITC-conjugated secondary antibody (Sigma-Aldrich) and visualized by microscope (Axioskop 2; Carl Zeiss Meditec, Inc., Dublin, CA).

For ultrastructural examination, one fourth of each sample was fixed in Karnovsky fixative, postfixed in osmium tetroxide, and processed for routine transmission electron microscopy.

RESULTS

RHC-I hydrogels cross-linked with an EDC-collagen ratio of 0.5 and RHC-III hydrogels with EDC-collagen ratios of 0.4 had optimal physical and mechanical properties. These were therefore the hydrogels used for further comparison in the study.

Physical and Mechanical Characterization

The physical and mechanical properties of the RHC hydrogels are summarized in Table 2. Both had water content of >90%, which most likely correlates with a refractive index of 1.35, that of water. Light transmission through RHC-III gels was significantly higher than that through RHC-I gels at 89.8% \pm 0.9% versus 80.7 \pm 2.6% ($P < 0.01$), but backscatter for both gels was very low (<1%).

There were no statistically significant differences in tensile strength, elongation at break, and moduli between RHC-I and -III gels. No significant differences were recorded in the ther-

TABLE 2. Comparison of Key Properties of Optimized EDC/NHS Cross-Linked RHC-I and -III Corneal Substitutes to Excised Human Eye Bank Corneas

	Human Cornea	RHC-I (EDC Ratio = 0.5)	RHC-III (EDC Ratio = 0.4)
Water content	80% ¹⁸	91%	90%
Optical properties			
Refractive index	1.3375 ¹⁹	1.35	1.35
Transmission (%)	>85 ¹⁴	80.7 \pm 2.6	89.8 \pm 0.9
Backscatter (%)	6-8	0.54	0.81
Mechanical properties			
Tensile strength (MPa)	3.8 ²⁰	1.126 \pm 0.154	1.700 \pm 0.205
Elongation at break (%)	—	15.33 \pm 1.45	13.89 \pm 0.69
Elastic modulus(MPa)	3-13 ^{21,22}	16.7 \pm 1.49	20.26 \pm 2.04
Thermodynamic properties T_d (°C)	65.1	59.2	58.6
Permeability			
Glucose (cm ² /s)	2.5 \times 10 ⁻⁶ (23)	1.4 \pm 0.083 \times 10 ⁻⁶	1.2 \pm 0.069 \times 10 ⁻⁶
Albumin (cm ² /s)	10 ⁻⁷ (23)	1.5 \times 10 ⁻⁷	8.5 \times 10 ⁻⁸

$n \geq 3$ samples for each test. T_d , dissolution temperature.

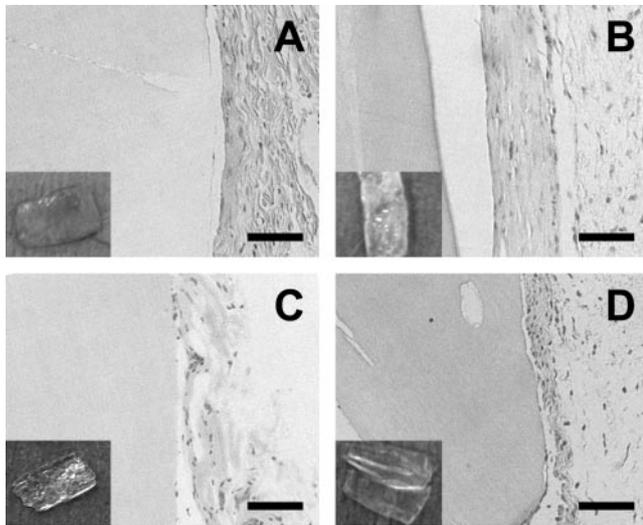


FIGURE 1. H&E-stained sections through RHC-I and -III hydrogels implanted subcutaneously into rats. After 90 days, both RHC-I (A) and RHC-III (B) gels had smooth, continuous edges, with no signs of biodegradation. *Insets:* intact samples retrieved after implantation. After 180 days, there was evidence of loss of the smooth, continuous edge, showing the beginning of possible biodegradation of the RHC-I (C) and -III (D) implants. Bar, 100 μm .

modynamic properties. However, neither hydrogel was as thermally stable as the human cornea.

The glucose diffusion coefficients for types I and III were $1.39 \times 10^{-6} \text{ cm}^2/\text{s}$ and $1.19 \times 10^{-6} \text{ cm}^2/\text{s}$, respectively, in agreement with that of the human corneal stroma at $2.4 \times 10^{-6} \text{ cm}^2/\text{s}$.²³ The bovine serum albumin diffusion coefficient for type I and III implants were 1.456×10^{-7} and 8.512×10^{-8} , respectively. RHC-I gels were within the same range as

the diffusion coefficient of albumin in human corneal posterior stroma at $1.10 \times 10^{-7} \text{ cm}^2/\text{s}$ ²³ but RHC-III had a diffusion coefficient that was slightly lower.

Toxicology

Both RHC-I and -III were nonreactive or negative in all the in vitro toxicology tests performed. They were nontoxic in in vivo tests, as none of the mice treated with hydrogel extracts showed any mortality or evidence of systemic toxicity.

Biodegradation

Subcutaneous implants in rats up to 12 weeks (90 days) are considered short-term survivors, whereas those beyond 12 weeks are considered long-term. Results showed no evidence of any significant inflammation or fibrosis of host tissues at the implantation sites, indicating good biocompatibility over both the short- and long-term. The polymers were intact at all time points tested at macroscopic levels. Microscopic examination, however, showed sections of implants with continuous, smooth edges at 90 days (Figs. 1A, 1B), but macrophage attachment to the cut surfaces at 180 days (Figs. 1C, 1D). The latter are possible indications of early degradation.

In Vitro Biocompatibility

Both RHC-I and -III gels supported attachment and proliferation of corneal epithelial cells, reaching confluence over 4 days and stratifying after air-lifting. Nerve overgrowth and ingrowth were also observed, but there were no differences observed between RHC-I and -III gels (data not shown).

Implantation and Clinical Evaluation

No adverse inflammatory or immune reaction was observed after implantation of either RHC matrices or allografts. Epithelial coverage of implants was complete by 4 days after surgery. Slit lamp examinations showed mild haze up to 6 months for

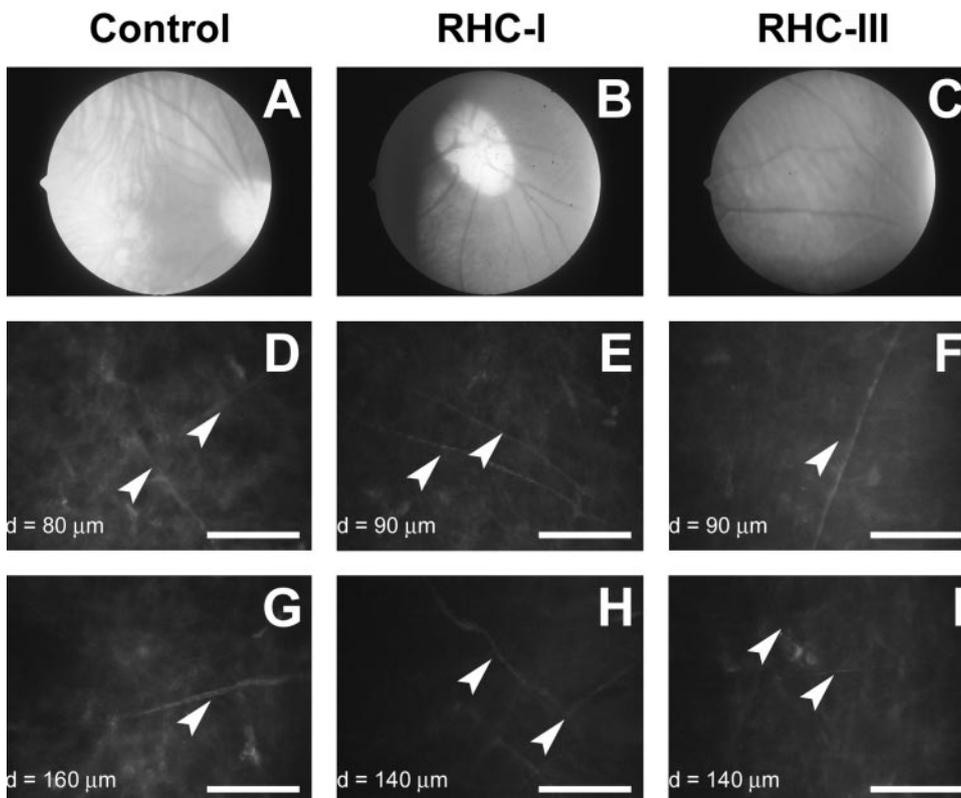


FIGURE 2. Fundus and IVCN images at 12 months from pigs. Fundus images of the retina. Proper morphology of the retinal vessels are seen in surgical eyes of RHC-I (B) and -III (C) implants compared with the nonsurgical corneas (A). IVCN images showed fine, regenerated subepithelial nerves in both RHC-I (E) and -III (F) implanted corneas. Larger, stromal nerves were also observed in both RHC-I (H) and -III (I) implants. Corresponding nonsurgical control corneas are shown in (D) and (G). Bar, 50 μm .

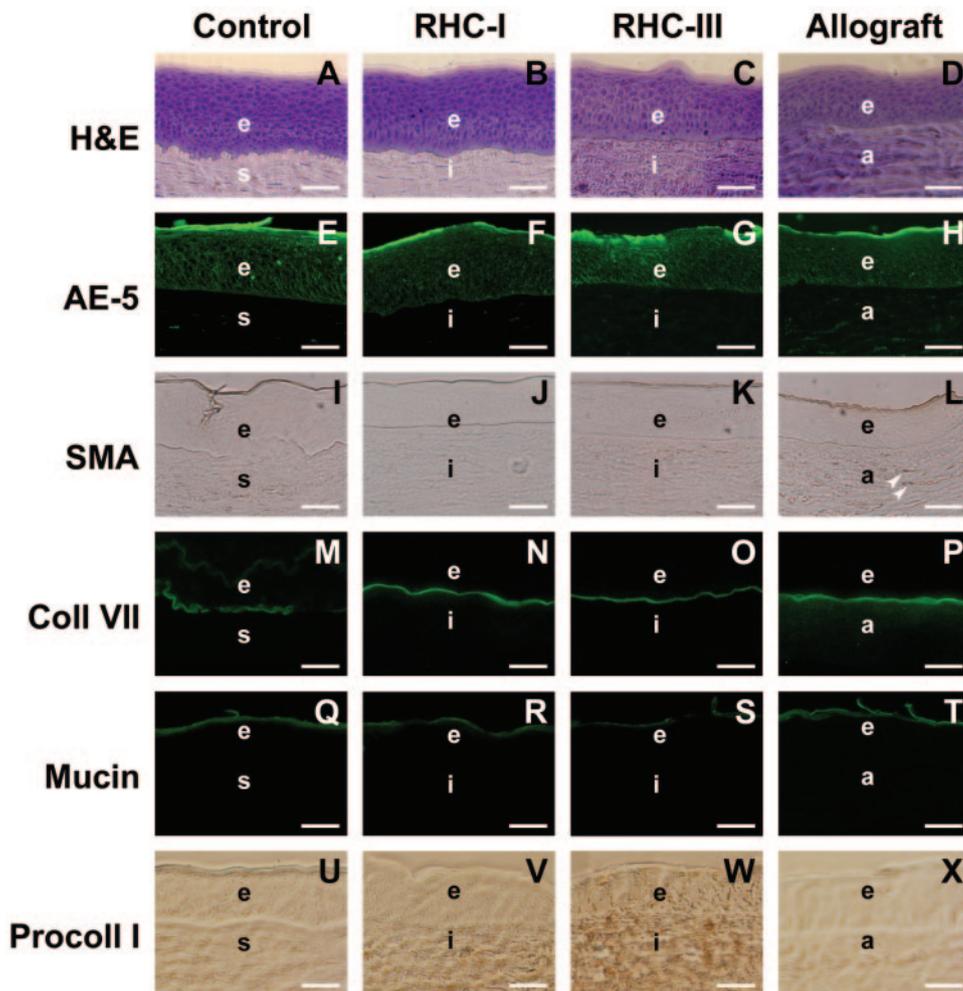


FIGURE 3. Postsurgical corneal regeneration at 12 months. (A–D) H&E sections show stratified epithelia (e) over the stroma (s), implants (i), and allograft (a). Both RHC-I and -III implants were populated by stromal cells. (E–H) Regenerated epithelia over RHC implants were AE5-positive as were the control corneas. (I–L) Smooth muscle actin staining, indicative of activated stromal cells or myofibroblasts, was found in only a few cells (*arrowheads*) in the allograft (L). (M–P) Type VII collagen staining seen at the epithelium–implant interface of corneas with RHC-I (N) and -III (O) implants is comparable to that observed in nonsurgical control corneas (M) and allograft (P). (Q–T) UEA staining indicating presence of tear film mucin was seen in all samples. (U–X) Procollagen I staining was more pronounced in RHC-III (W) implants than in the others. Bars, 50 μ m.

both RHC-I and -III. Very mild haze (+0.5) was observed in the corneas one of four pigs implanted with RHC-I gels at 12 months after surgery. A diffuse +1 haze was observed in the corneas of one of four pigs in the corresponding RHC-III group. Animals that had received allografts had corneal haze scores of +1 for two animals and +2 for a third animal, out of four implant recipients. All control, untreated corneas were haze free (0). The regenerated epithelium showed exclusion of sodium fluorescein dye, indicating that the epithelium was intact and had re-established barrier properties. Schirmer's tests indicated comparable tear production in all surgical and nonsurgical corneas. Intraocular pressures were unchanged, showing that the implants did not block the flow of aqueous humor within the eye. Fundus camera images of the retinal vessels (Figs. 2B, 2C) were comparable with those of nonsurgical corneas (Fig. 2).

Clinical IVCM of the implanted stromal matrices at 6 weeks after surgery showed the presence of fine subepithelial nerves (Figs. 2E, 2F) and corresponding stromal cells and nerves (Figs. 2H, 2I) with morphology mimicking that of nonsurgical control animals (Figs. 2D, 2G). All surgical corneas showed comparable touch sensitivity to nonsurgical control corneas by esthesiometry. Touch sensitivity was extinguished by application of lidocaine.

Histopathologic Evaluation

H&E sections through control corneas (Fig. 3A), RHC implants (Figs. 3B, 3C) or allografts (Fig. 3D) showed typical corneal morphology. The epithelium was stratified and overlies a ma-

trix containing stromal cells. All epithelia were fully differentiated as indicated by positive AE5 staining (Figs. 3E–H). The allografts contained a small number of smooth muscle actin-positive cells (Fig. 3L) but both RHC samples were unstained (Figs. 3J, 3K). All samples were positive for type VII collagen, a marker for hemidesmosomes¹⁷ at the basement membrane–epithelium interface (Figs. 3M–P) and UEA staining showed the presence of a tear film in all samples (Figs. 3Q–T). All samples were negative for F4/80 and CD45 (data not shown), indicating that the implants had minimal or no macrophage activity and very few or no marrow-derived cells. Samples of corneas receiving RHC-III hydrogel implants for 12 months were negative for type III collagen, whereas control, nonimplanted RHC-III hydrogels were intensely stained (data not shown). Observable procollagen I staining, however, was present in RHC-III implants (Fig. 3W).

TEM observations of the epithelial–stromal interphase region (Fig. 4) confirmed the presence of stromal cells in RHC-I and -III implants. Subepithelial nerves were also observed in implants, allograft, and control corneas (Figs. 4E–H). The epithelium–implant interface showed the presence of hemidesmosomes in all samples (Figs. 4I–L).

Analyses of central corneal nerve density are shown in Figure 5. No significant nerve density differences were found among the allografts or the RHC-I and -III grafts in any corneal depth zone ($P = 0.80, 0.74,$ and 0.06 for zones 1, 2, and 3, respectively). All graft types, however, had a significantly reduced nerve density in corneal depth zone 1 relative to nonsurgical control corneas ($P < 0.01$ for all graft types). In

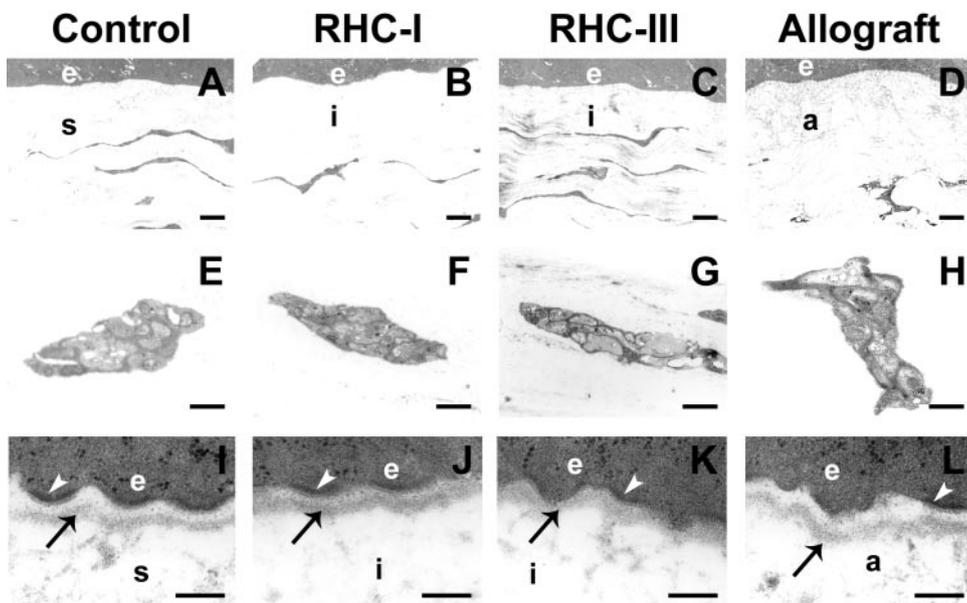


FIGURE 4. (A-D) TEM of epithelial-stromal compartment interface. Epithelial cells overlay either a stroma (s), implant (i), or allograft (a), all containing stromal cells. (E-H) Higher magnification of regenerated nerve, showing the staining of regenerated nerve, showing of clear and dense vesicles. (I-L) Hemidesmosome plaques (*arrowheads*) and basement membrane (*arrows*) have formed within the ECM between the epithelial cells and underlying RHC implants, emulating the structures normally found at the epithelial-stromal interface of non surgical corneas. Bars: (A-D) 5 μm ; (E-H) 1 μm ; (I-L) 0.1 μm .

addition, in RHC-III grafts and allografts, nerve density in depth zone 3 were significantly increased over that in untreated corneas ($P = 0.04, 0.03$ for RHC-III and allograft, respectively).

DISCUSSION

The human corneal stromal matrix comprises 71% collagen by dry weight.²⁴ The predominant collagen is type I, but smaller amounts of types III and V are present.²⁵ All are fibrillar collagens with structural supporting roles. Type I collagen is a

heterotrimer comprising two $\alpha 2(\text{I})$ and one $\alpha 1(\text{I})$ chain, whereas type III is a homotrimer of $\alpha 1(\text{III})$.²⁶ In vivo, collagen cross-linking allows for the tensile strength of tissues, since it increases the resistance of the fibers against proteolysis.²⁷ It is believed that both type I and III collagens undergo similar cross-linking, although the lysine aldehyde pathway in the cornea and heterotrimers of both collagens exist. However, type III collagen chains are characterized by the disulphide bonds²⁵ that form between the three chains in the triple helical domain and are more resistant to digestion by collagenases

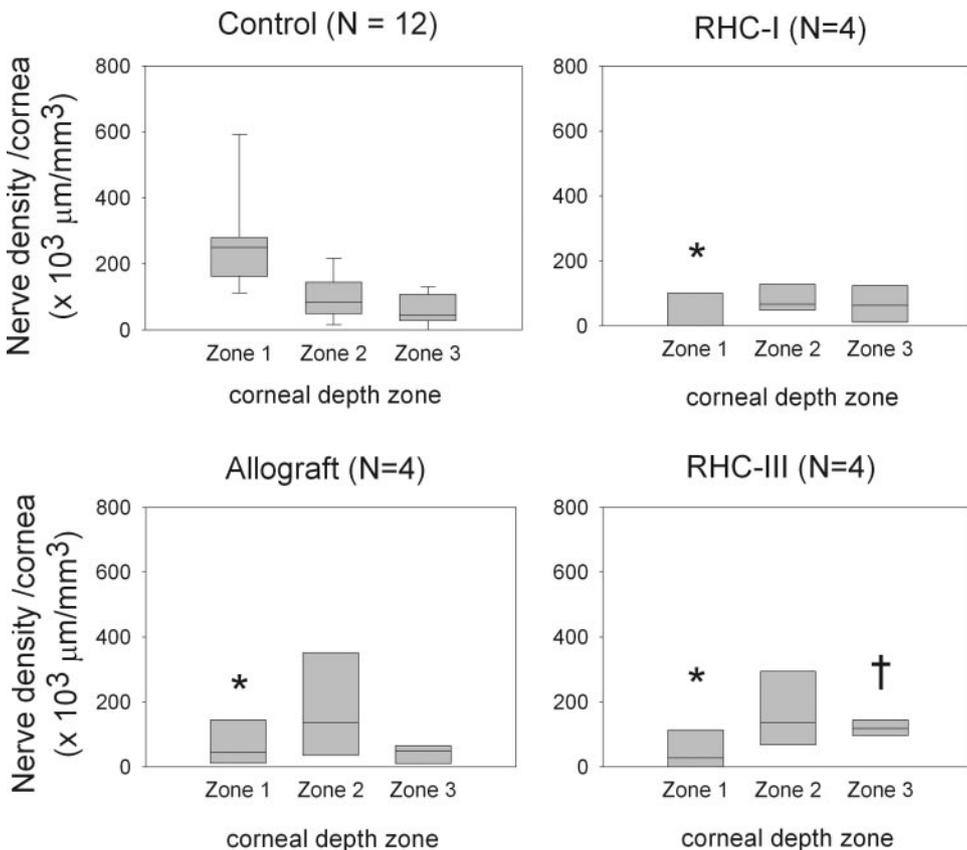


FIGURE 5. Nerve density per central cornea 12 months after surgery in nonsurgical control corneas and in various corneal graft types. For control corneas, box plots indicate medians, 1st and 3rd quartiles, and 5th and 95th percentiles (whiskers). For the grafts, only the medians and 1st and 3rd quartiles are shown. *Significant reduction in nerve density from control levels; †significant increase relative to control corneas.

secreted by granulocytes.²⁸ This suggests that type III collagen is likely to be more resistant to degradation when used as implants. Furthermore, Beuerman et al.²⁹ reported that the inclusion of type III collagen in hydrated matrices significantly increased the optical transmittance and reduced contraction of the hydrogels by stromal cells. Their results suggest that type III collagen gels may be more robust and optically clear than type I gels.

As reported by Beuerman et al.,²⁹ with extracted collagens, RHC-III gels had significantly superior light transmission. Although there were no significant differences in tensile strength, elongation at break, and moduli between optimized RHC-I and -III gels, we noted that "optimized" gels were cross-linked using an EDC/coll-amine ratio of 0.5 and 0.4, respectively. This finding shows that type III collagen can be cross-linked at lower EDC ratios than can type I collagen, with no significant loss in mechanical properties. Albumin diffusion was decreased, suggesting that the thinner type III collagen fibrils may allow for tighter packing of the hydrogel.

Both RHC-I and -III hydrogels supported corneal epithelial cell proliferation and stratification at similar rates in vitro. Neurite extensions on these two hydrogels were also similar. These in vitro results were predictive of the in vivo results as implants.

Both implanted RHC-I and -III gels were optically clear at 12 months, as evidenced by clear fundus images of retinal vessels and minimal or no haze compared with allograft control corneas. Both hydrogels were equally effective in allowing re-epithelialization and stromal cell and nerve ingrowth. Hemidesmosomes and positive type VII collagen immunohistochemistry (as well as manipulations during fundus and in vivo confocal imaging) demonstrated stably anchored epithelia.

There were no significant differences in the patterns of reinnervation between the RHC-I and -III gels. Nerve density in corneal depth zone 1 (representing subepithelial and subbasal nerves); however, was significantly reduced after 12 months in both RHC gels and in allografts relative to the untreated control samples. While the recovery of zone 1 nerve density to control levels is expected to take longer than 12 months, the rate of nerve recovery in RHC-based gels did not significantly differ from that of allograft tissue. At 12 months after surgery, both RHC-I and -III implanted corneas were quiet (i.e., there was no noticeable number of macrophages). Our sections were also negative for CD45 marrow-derived cells in either implants or stromas of controls, in contrast to previous reports by Yamagami et al.,³⁰ perhaps because of either a difference in sample preparation or a species difference. Procollagen I staining was found, in particular, in RHC-III implanted corneas, suggesting that there is synthesis of type I collagen, which in turn suggests that corneal stromal cells are probably laying down type I collagen, but in a very gradual manner, since optical clarity was unaffected. Although we were unable to determine definitively that both RHC-I and -III implants had been completely remodeled, the lack of type III collagen staining in RHC-III implanted corneas at 12 months after surgery compared with that in positive controls of unimplanted hydrogels together with the increased procollagen I staining very strongly suggest remodeling and turnover of the collagen scaffold.

We have therefore shown that although type III collagen is a minor component in the human cornea, it is nevertheless a suitable alternative to type I collagen in the development of stromal matrix substitutes that allow for both corneal cell and nerve regeneration after implantation. The use of recombinant materials allows for control over batch homogeneity and, since it is fully synthetic, circumvents the safety concerns associated with animal source proteins. In addition, as corneal nerves are

regenerated, such synthetic, biomimetic corneal implants could circumvent potential problems, such as dry eye that result from lack of corneal nerve regeneration after transplantation.³¹

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