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Stable corneal regeneration four years after implantation of a cell-free recombinant human collagen scaffold☆, ☆☆

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ABSTRACT

We developed cell-free implants, comprising carbodiimide crosslinked recombinant human collagen (RHC), to enable corneal regeneration by endogenous cell recruitment, to address the worldwide shortage of donor corneas. Patients were grafted with RHC implants. Over four years, the regenerated neo-corneas were stably integrated without rejection, without the long immunosuppression regime needed by donor cornea patients. There was no recruitment of inflammatory dendritic cells into the implant area, whereas, even with immunosuppression, donor cornea recipients showed dendritic cell migration into the central cornea and a rejection episode was observed. Regeneration as evidenced by continued nerve and stromal cell repopulation occurred over the four years to approximate the micro-architecture of healthy corneas. Histopathology of a regenerated, clear cornea from a regrafted patient showed normal corneal architecture. Donor human cornea grafted eyes had abnormally tortuous nerves and stromal cell death was found. Implanted patients had a 4-year average corrected visual acuity of 20/54 and gained more than 5 Snellen lines of vision on an eye chart. The visual acuity can be improved with more robust materials for better shape retention. Nevertheless, these RHC implants can achieve stable regeneration and therefore, represent a potentially safe alternative to donor organ transplantation.

1. Introduction

In 2012, the World Health Organization estimated that 285 million people worldwide are visually impaired, while 39 million are blind [1]. Corneal blindness accounts for 5.1% of cases, with 1.5–2 million new cases of unilateral blindness reported annually [2]. While transplantation can cure corneal blindness, there is a severe shortage of donor corneas [2,3]. Although two-year success rates for corneal transplantation are 85% in developed nations like Sweden [4], those for a developing country (e.g. South India) start at 69% [5]. Data from the Australian Corneal Graft Registry shows that the success rate for corneal transplantation is 73% at 5 years and 62% at 10 years [6], which is even lower than for kidney transplantation [7].

As an alternative to donor cornea transplantation, in Fagerholm et al. [8], we showed the regeneration of corneal tissues and nerves after implantation of cell-free, biointeractive corneal implants made from 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) crosslinked recombinant human collagen type III (RHCIII). These RHCIII corneal implants were designed as simple mimics of the largely collagenous extracellular matrix of the cornea stroma to stimulate *in situ* regeneration of pathologic corneas.

In the present study, we provide details of the RHCIII implants and report on their four year, long-term progress within the

☆ Four years after grafting of recombinant human collagen-based corneal implants into patients, the overall shape of the patient's regenerated corneas remained stable, while corneal stromal cells and nerves continue to actively regenerate without immunosuppression or immune cell recruitment, confirming the potential of such implants as substitutes for donor human corneas.

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corneas of patients. Specifically, we analysed the stability of the regenerated neo-corneas and the time course of the regenerative events. We also compared the immune compatibility of such cell-free, biointeractive implants over four years without immunosuppression against corneas grafted with human donor corneas by penetrating keratoplasty, which is considered the "gold standard" in corneal transplantation [9].

2. Materials and methods

2.1. Biosynthetic corneal implants

Implants were produced under Class 100 conditions in a cleanroom following Good Manufacturing Practice guidelines. Clinical grade recombinant human collagen type III (RHCIII) produced in yeast (*Pichia pastoris*) was purchased from FibroGen, Inc. (San Francisco, CA), dia-filtrated, freeze-dried, and reconstituted to a 10% (w/w) optically clear solution. EDC was supplied by Sigma–Aldrich (St. Louis, MO). NHS was supplied by Fluka (Buchs, Switzerland). Phosphate buffered saline (PBS, pH 7.4) was prepared from the tablet form (Calbiochem Corp., Darmstadt, Germany). Aliquots of 500 μl of RHCIII solution were carefully loaded into a syringe mixing system we previously developed [10], to ensure that the RHCIII solution was free of air bubbles. A predetermined quantity of cross-linker was added based on a molar equivalent ratio of crosslinker: collagen-NH_2 (collagen-NH_2 denotes the ε-amine groups on collagen molecules) of 0.4:1. After thorough mixing at 4 °C, the solution was dispensed into curved polypropylene contact lens moulds (500 μm thick, 10 mm diameter) and cured at 100% humidity at ambient temperature for 24 h. The implants were washed thoroughly in sterile phosphate buffered saline (PBS) and then immersed in PBS containing 1% chloroform to maintain sterility and stored at 4 °C. The EDC and NHS are not incorporated into the final implant [11], circumventing the possibility of toxic breakdown products.

2.2. Characterization of implants

Quality control criteria used to determine release for patient use were visual inspection using a zonometer to ensure smooth, defect-free inner and outer surfaces, and the optical properties of the implant. Briefly, the refractive indices of each fully hydrated implant equilibrated in PBS were measured using an Abbe refractometer (Model C10, VEE GEE Scientific Inc., Kirkland, Washington) at 21 °C with bromonaphthalene as the calibration agent to ensure refractive indices of ≥1.35. A custom-built instrument was used to measure the light transmission of individual samples at room temperature as compared to open beam intensity [12]. The relative percent of light back scattered from the collimated beam by the sample was measured with a circular array of eight photodiodes, 30° off axis. For clinical use, only samples showing light transmission >90% were selected.

Other properties of the implants were also recorded, with all samples tested in triplicate as we previously described [13]. The mechanical properties, tensile strength, moduli and elongation at break, were determined with an Instron mechanical universal tester (Model 3342, Instron, Canton, MA) equipped with a 0.01 kN load cell and Instron Series IX/S software. The crosshead speed was 10 mm min^{-1} and the sampling rate was 10 points s^{-1}. Implants were not pre-stressed. Measurements were taken at room temperature in a >75% humidity chamber.

Thermal stability of PBS-equilibrated hydrogel samples was determined using differential scanning calorimetry (DSC-2C, Perkin Elmer, Waltham, MA; with Thermal Analysis Software system, Instrument Specialists Inc., Spring Grove, IL). Heating scans were recorded in the range of 8–80 °C at a scan rate of 5 °C min^{-1}. PBS equilibrated samples (5–10 mg) were surface-dried with filter paper and then hermetically sealed in aluminium pans to prevent water evaporation. A resulting heat flux vs. temperature curve was then used to calculate the denaturing temperature (T_d) and the enthalpy (ΔH_d). The denaturing temperature is given by the maximum point of the endothermic peak. Enthalpy was determined by integrating the endothermic peak to determine the peak area. The enthalpy of transition, $\Delta H = KA$, where K is the calorimetric constant and A is the area under the curve. The calorimetric constant was pre-determined by the manufacturer.

The water content of PBS-equilibrated hydrogels was measured as follows. Hydrogels were removed from the solution, gently blotted dry with filter paper and immediately weighed on a microbalance to record the wet weight of the sample. Hydrogels of known weight were then dried at room temperature under vacuum to constant weight. The total equilibrated water content of the hydrogels (W_t) was calculated according to the equation:

$$W_t = (W - W_o)/W_w \times 100\%,$$

where W and W_o denote the wet and dry weights of the samples, respectively.

2.3. Clinical study design and treatment

This study was approved by the Linköping Regional Ethical Review Committee and Swedish Medical Products Agency, registered (EudraCT no. 2006-006585-42) and conformed to the Declaration of Helsinki. Subjects were comprised of 10 consecutive patients on the waiting list for corneal transplantation, 8 males and 2 females aged 18–75 years at time of surgery, with keratoconus (9 cases) or central scarring (1 case) [8]. They were grafted with biosynthetic implants by anterior lamellar keratoplasty and retained using overlying sutures, as they were not sufficiently robust for stabilization with running sutures. An additional 9 patients with similar corneal pathologies, 6 males and 3 females aged 40–79 years, with keratoconus (5 cases), endothelial decompensation (2 cases), a deep central scar (1 case) and pseudophakic bullous keratopathy (1 case) were grafted with human donor allograft corneas by full-thickness penetrating keratoplasty, stabilized with peripherally located running sutures. A tenth allografted patient was excluded after a retinal detachment occurred two months postoperatively. Twenty volunteers with healthy corneas, aged 15–88 years, were examined as normal benchmarks.

2.4. Postoperative assessments

Patients were assessed at 1, 3, 6 and 9 months, and 1, 2, 3, and 4 years postoperatively, while healthy volunteers were examined once. Examinations included slit-lamp microscopy, corneal surface sensitivity measurement by Cochet-Bonnet contact esthesiometry, anterior segment optical coherence tomography, and laser-scanning in-vivo confocal microscopy.

2.5. Optical coherence tomography and topographical mapping to measure implant stability

The stability of the implants and regenerated neo-corneas was assessed by examining the changes in thickness and shape over time.

Anterior segment optical coherence tomography was used to monitor the change in corneal thickness. Two-way ANOVA analysis was used with a general linear model to compare central corneal thickness with respect to group and post-operative time. The nonparametric Mann–Whitney Rank Sum test was used where data did not satisfy equal variance testing for the comparison of central corneal thickness at four years in operated groups versus normal healthy corneas. Statistics were performed using statistical software (SigmaStat 3.5 for Windows, Systat Software Inc., Chicago IL).

To map changes in the shape, primarily of the anterior surface over time, corneal topography analyses were performed using a Serial Orbscan II (Bausch and Lomb, Rochester, New York) on all eyes over 4 years. Difference maps and statistics maps were generated according to a protocol we previously developed [14].

2.6. Analysis of antigen presenting dendritic cells as an indication of immune compatibility

In vivo confocal microscope images of the central basal and sub-epithelial areas were analysed to identify the 2 reported morphological types of dendritic cells, the resident antigen presenting cells of the cornea [15]. The 'immature' cells have reflective cell bodies only, while the 'mature' antigen-presenting type have reflective cell bodies bearing one or more short processes termed 'dendrites' [16,17]. In randomized and coded images, the number of each type of dendritic cell per image was manually counted and converted to a density value. Two-way ANOVA analysis was used to compare dendritic cell density with respect to cell subtype and group.

2.7. Histopathology

One patient underwent regrafting since contact lenses required for good visual acuity could not be fitted. A clear corneal button was obtained from this patient and was routinely processed for histopathological examination. Paraffin-embedded sections were stained with haematoxylin and eosin for visualization.

3. Results

3.1. Implants

Properties of the EDC/NHS crosslinked RHCIII implants are shown in Table 1. Optical clarity exceeded that of the average human cornea, which is above 87% transmission [18]. However, the mechanical strength was significantly lower than that of the human cornea [19–21], and implants were much softer, resulting in the need for overlying sutures instead of interrupted sutures for retention. At four years post-operation, examination by slit lamp biomicroscopy showed that the implants were well integrated within the corneas of all 10 patients (Fig. 1).

3.2. Implant stability

Anterior segment optical coherence tomography showed that the shape, thickness, and border areas of engraftment of the implanted corneas remained constant from one to four years post-