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Immunological responses in mice to full-thickness corneal grafts engineered from porcine collagen

Lei Liu[a,1], Lucia Kuffová[a,1], May Griffith[b,c], Zexu Dang[a], Elizabeth Muckersie[a], Yuwen Liu[d], Christopher R. McLaughlin[b,c], John V. Forrester[a,*]

[a]Department of Ophthalmology, Institute of Medical Science, Foresterhill, University of Aberdeen, Aberdeen AB25 2ZD, Scotland, UK
[b]University of Ottawa Eye Institute, Ottawa Health Research Institute-Vision Centre, 501 Smyth Road, Ottawa, ON, Canada K1H 8L6
[c]Department of Cellular and Molecular Medicine, University of Ottawa, 451 Smyth Road, Ottawa, ON, Canada K1H 8M5
[d]National Research Council Canada, 1200 Montreal Road, Ottawa, ON, Canada K1A 0R6

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Abstract

Tissue-engineered (TE) corneas were fabricated from porcine collagen cross-linked with 1-ethyl-3-(3-dimethyl aminoproplyl)carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS), and were transplanted into BALB/c mice orthotopically using a full-thickness penetrating keratoplasty (PKP) procedure. The biocompatibility was evaluated by assessing both local and systemic immune responses. Myeloid cells including granulocytes and macrophages were the main infiltrating cells in recipient cornea and in retro-TE corneal membrane which developed 7–10 days post surgery. Sodium citrate was found to be effective in reducing fibrin accumulation in anterior chamber post grafting at early time points, but it did not prevent formation of the retro-TE corneal membrane. No significant T cell activation was observed in the submandibular draining lymph nodes (SMDLN) by flow cytometry. Anti-porcine type I collagen IgG antibodies were detected in the serum of grafted mice from 2 weeks post grafting and the concentration of antibodies increased with time. Overall, porcine collagen-EDC/NHS TE corneas were tolerated well in murine recipients, causing mainly a self-limiting local innate immune response and a low-grade humoral response with little evidence of sustained T cell activation. Retro-TE corneal membrane formation was the main complication and barrier to clarity.
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1. Introduction

Corneal transplantation is the main therapeutic intervention for many corneal diseases producing opacification. Its success rate in uncomplicated cases at 1 year is high but in the longer term overall acceptance of grafts declines [1,2]. This is particularly so for "high risk" grafts i.e. grafts performed in patients with corneal opacification associated with ocular surface disease or extensive corneal vascularization. Such cases include severe keratoconjunctivitis sicca, ocular cicatricial pemphigoid, severe corneal alkali burns, herpes zoster keratitis, or cases of previous corneal graft failure [3]. These cases depend on the supply of donor grafts but there is a world-wide chronic shortage of donor tissue for clinical use. Therefore, many researchers have attempted to fabricate corneal replacements including keratoprosthesis and tissue-engineered (TE) corneal equivalents with the dual aims of providing a plentiful supply of graft material which is also less likely to be rejected.

Early studies were based on use of keratoprostheses. A keratoprosthesis is a biomaterial device which fulfils the corneal functions of maintaining globe integrity and protection, while permitting light transmission and refraction [4,5]. However, none of the current keratoprosthesis used clinically combines all the desirable features within one model and severe complications including infection, tissue necrosis, implant extrusion, retro-prosthesis membrane

*Corresponding author. Tel.: +44 1224 553782; fax: +44 1224 551237.
E-mail address: j.forrester@abdn.ac.uk (J.V. Forrester).
[1]First authors.

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forming and secondary glaucoma are frequent complications [4,6].

Other approaches to restoring sight in these cases have therefore been adopted. The term "engineered corneal equivalent" refers to three-dimensional artificial corneal tissue developed in vitro by culturing corneal cells in polymeric scaffold materials [7–9]. The principle of tissue engineering is to seed and expand cells within a synthetic matrix or scaffold which would model normal tissue. Several "artificial corneas" have been developed, using three-dimensional collagen matrices with three layers of various bovine, mouse, porcine, and human cells, but these have been limited to in vitro applications [10–16]. Collagen-based acellular scaffolds, however, have been tested by implanting the matrices into rabbits in corneal stromal pockets [9,17] or as supra-descemetic corneal substitutes mini-pigs by lamellar keratoplasty [9,17]. In pigs, both collagen-synthetic and collagen only implants, fabricated with porcine collagen appeared to be well tolerated, allowing host epithelialization, and stromal cell and nerve axon ingrowth [9,17,18]. TE corneas grafted in this way appeared useful for biomechanical and toxicology research, but their optical quality, refractive power, biocompatibility and stability in vivo required further study [19].

In this study we have tested a very simple implant comprising only crosslinked porcine type I collagen. These matrices were cross-linked with a water soluble carbodiimide (WSC), 1-ethyl-3-(3-dimethyl aminoproplyl)carbodiimide (EDC) along with *N*-hydroxysuccinimide (NHS). WSCs are protein crosslinking reagents that themselves do not become incorporated into the final structures. So, all TE corneas that were implanted into mice using a full-thickness PKP procedure in the present study comprise type I porcine collagen only. Type I collagen is an extracellular matrix protein that is widely used in biomedical applications such as drug delivery and tissue engineering [20,21]. Our data show that TE corneas are well tolerated as PKP in mice, inducing predominantly a local inflammatory (innate immune) reaction and a low-level systemic anti-porcine antibody response. However, corneal clouding and opacity occur mainly due to retro-TE corneal membrane formation, a type of "foreign body reaction", which recedes, but does not resolve, as the local inflammatory response declines.

2. Materials and methods

2.1. Collagen-EDC/NHS preparation

Lyophilized acidic type I porcine collagen (Nippon Ham, Tokyo, Japan) were dissolved in water to 10% (w/w) concentration. A uniform solution was prepared by stirring for a minimum of 24 h at 4 °C. The resulting acidic collagen solution was loaded into a plastic syringe and air bubbles removed by centrifugation at 4 °C to give clear, bubble-free viscous solution, ready for dispensing and for mixing with the cross-linking reagents. To cross-link the collagen, 0.5 ml collagen solution was loaded into a syringe mixing system, also free of air bubbles. The pH of the collagen solution was adjusted to 5 ± 0.5 (optimum for EDC chemistry) by injection of microliter quantities of 1.0 M aqueous NaOH, followed by thorough mixing under ice water. The 20 μl EDC (Sigma-Aldrich) and 6 μl NHS (Sigma-Aldrich) solutions (both at 10 wt%/vol) were added from a second syringe through a septum in the syringe mixing system and mixed thoroughly with the collagen solution, cooled in an ice bath. This homogeneous solution was immediately dispensed into polypropylene lens moulds (7.5 mm diameter, 70 μm spacing) and cured at 100% humidity (at 21 °C for 24 h and then at 37 °C for 24 h). The cross-linked, cornea-shaped hydrogel samples were removed from the moulds after soaking in PBS (pH = 7.2) for 2 h or longer before prizing the moulds apart. The gels were washed for 4 h in three batches of PBS at 4 °C. These fully hydrated hydrogels were then stored in chloroform saturated PBS to maintain sterility.

2.2. Transplantation of the collagen-based TE cornea

The procedures conformed to the regulations of the Animal License Act (UK) and to the ARVO Statement for the use of Animals in the Ophthalmic and Vision Research. The surgical procedure has been established in our lab [22–24]. Briefly, TE artificial corneas of 2 mm in diameter were trephined and placed in balanced salt solution (BSS) (Alcon Laboratories Inc., Forth Worth, TX, USA). The recipient mice (BALB/c, H-2^{d}) were anesthetized by intramuscular injection of a mixture of 20 mg/ml Ketamine (Fort Dooge Animal Health LDT, Southampton, UK) and 2 mg/ml Xylazine (KVP Pharma und Veterinaer-Produkte GmbH, Kiel, Germany) diluted in saline. To dilate the pupil a combination of 1% Tropicamide and 2.5% Phenylephrine drops were used. The recipient right cornea was marked with a 1.5 mm trephine and excised using Vannas scissors after penetrating the anterior chamber with a 25 G sharp needle. Viscoelastic material Microvisc® (Vision Matrix Ltd, Harrogate, UK) was used to maintain the anterior chamber throughout the procedure. The artificial corneas were sutured into the recipient bed using a running continuous suture (11-0 Ethilon). After grafting, 1% chloramphenicol ointment was applied to the eye. Nine groups with 4–5 animals per group were transplanted and observed for periods up to 120 days post grafting. The mice were sacrificed at the different time points post transplantation (6 h, 2, 6, 12, 24, 48, 60, 90 and 120 days).

2.3. Sodium citrate treatment

TE cornea was transplanted in BALB/c mice of four groups. For each group, 3–4 mice were treated with 10% sodium citrate diluted in PBS and an identical number of mice were treated with PBS as control. The anterior chamber was washed with sodium citrate or PBS as control during the operation. Post-operatively, 10% sodium citrate eye drops were applied once a day in treated groups. The eyes were collected for immunohistochemistry after 1, 3, and 6 days.

2.4. Clinical evaluation

The corneal grafts were examined twice a week under an operating microscope. Corneal epithelial integrity, opacity grade, oedema, infiltration, and vascularization, were recorded as well as anterior chamber activity, and iris vessel engorgement. In conventional corneal allografts, corneal opacity grade has previously been shown to correlate well with graft rejection. In the current experiments, opacification of the cornea after placement of the artificial corneas was also accompanied by changes in the clarity of the cornea, which correlated with the overall immunological response. We have therefore used a grading system, which is similar to that for allograft. The scale of opacity grades range from 0 to 4, where grade 2 (vessels of the iris still distinguishable through a hazy cornea) or more is considered to represent a significant immunological response [23,24].

2.5. Immunohistochemistry

The eyes were enucleated, immersed in OCT medium (Sakura Finetek Europe, Zoeterwounde, NL) and snap frozen in liquid nitrogen cooled isopentane. Cryostat sections (5–6 μm) of the eyes were immunostained with

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