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Research article

Keratocytes are induced to produce collagen type II: A new strategy for in vivo corneal matrix regeneration

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ABSTRACT

The stroma, the middle layer of the cornea, is a connective tissue making up most of the corneal thickness. The stromal extracellular matrix (ECM) consists of highly organised lamellae which are made up of tightly packed fibrils primarily composed of collagens type I and V. This layer is interspersed with keratocytes, mesenchymal cells of neural crest origin. We have previously shown that adult corneal keratocytes exhibit phenotypic plasticity and can be induced into a neuronal phenotype. In the current study we evaluated the potential of keratocytes to produce collagen type II via phenotypic reprogramming with exogenous chondrogenic factors. The cornea presents a challenge to tissue engineers owing to its high level of organisation and the phenotypic instability of keratocytes. Traditional approaches based on a scar model do not support the engineering of functional stromal tissue. Type II collagen is not found in the adult cornea but is reported to be expressed during corneal development, raising the possibility of using such an approach to regenerate the corneal ECM. Keratocytes in culture and within intact normal and diseased tissue were induced to produce collagen type II upon treatment with transforming growth factor Beta3 (TGFβ_3) and dexamethasone. In vivo treatment of rat corneas also resulted in collagen type II deposition and a threefold increase in corneal hardness and elasticity. Furthermore, the treatment of corneas and subsequent deposition of collagen type II did not cause opacity, fibrosis or scarring. The induction of keratocytes with specific exogenous factors and resulting deposition of type II collagen in the stroma can potentially be controlled by withdrawal of the factors. This might be a promising new approach for *in vivo* corneal regeneration strategies aimed at increasing corneal integrity in diseases associated with weakened ectatic corneal tissue such as keratoconus.

1. Introduction

It has become increasingly important to develop new therapeutic interventions for tissue engineering and regeneration as current tissue regeneration technology is yet to deliver in the clinical setting. The introduction of cell reprogramming via the production of induced pluripotent stem cells [33] has heralded a new revolution and has driven the search for the ultimate tissue regeneration treatment. However, before genetically induced cell reprogramming based methods can be successfully translated into therapeutic treatments, problems such as the low efficiency and safety issues associated with such technology first need to be dealt with.

The stromal layer of the cornea is composed of highly organised lamellae which are made up of tightly packed collagen fibrils of collagen types I and V [22] interspersed with keratocytes, mesenchymal cells of neural crest origin. Keratocytes are quiescent, their main function being the repair of the cornea. This is possible as they can transform into an activated phenotype in response to growth factors and cytokines at the site of injury. The activated keratocytes have morphological characteristics of fibroblasts and myofibroblast [5,12]. The biosynthetic activity of these repair phenotypes is mostly limited to the production of fibrotic repair extracellular matrix different in composition to that produced by the normal keratocytes and the persistence of myofibroblasts results in scarring and loss of transparency of the cornea [1,30]. Populations of stem cells have been identified in many mesenchymal tissues and in the corneal stroma stem cells are thought to reside in the limbal region, the transition zone between the cornea and the sclera [4]. Specialised features of the limbal microenvironment such as the presence of a dense blood supply is

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thought to help maintain the stem/progenitor character of corneal stromal stem cells *in vivo* [27]. However there are no definitive markers for the stem cell population within the stroma. In previous studies we have shown that all keratocytes from adult human and rat corneas differentiate into a neuronal phenotype when treated *ex vivo* and *in vivo* with neuronal lineage specifying growth factors [7]. During this phenotype switch genes and proteins linked to multipotency and neuronal differentiation were upregulated. We have shown that, in the cornea, stemness is not limited to a sub-population of cells found near the corneal limbus. Although a limbal niche might provide the required environment for cells to be maintained in a particular differentiation state, all cells in the cornea are amenable to phenotype reprogramming.

The cornea, in particular, presents a challenge to tissue engineers. After cataracts, corneal damage and diseases are the largest cause of vision loss, affecting more than 10 million people worldwide [35]. Currently, the most successful treatment for corneal blindness is replacement of damaged corneal tissue with human donor corneal tissue. Apart from drawbacks such as complications arising from immune rejection, donor-derived infection, and expensive pretransplant screening, the major problem with this approach is the severe shortage of donor tissue. Significant progress has been made in both the generation of artificial corneas and stem cell therapy [20]. However, corneal transplantation using cadaveric donor corneas remains irreplaceable. Keratoconus, an ectatic corneal dystrophy, affects approximately 1 in 2000 individuals worldwide. The progressive thinning of the corneal stroma typically occurs over decades and results in a conical shaped cornea that then impairs vision due to irregular astigmatism and myopia [18]. The corneal thinning is a result of the loss of corneal stromal extracellular components such as collagen [15,29,32]. Currently there are no treatments which stop corneal thinning. Interventions are mainly focussed on stiffening a weakened cornea or improving the visual acuity rather than stopping disease progression.

The goal of this study was to investigate the potential of keratocytes to switch to a phenotype that secretes collagen type II. The reason being that type II collagen has been reported to be expressed during cornea embryonic development [21] and its re-expression might be induced for corneal repair. The most suitable driving factors for differentiation were first identified in literature. These factors were then used to drive keratocytes to differentiate into a cell phenotype that secretes collagen type II thereby leading to deposition *in vitro* in cell culture and *ex vivo* in organotypic slice cultures. When choosing growth factors, cytokines and chemicals that might bring about collagen deposition in the corneal stroma it was important to consider known effects of exogenous growth factors. For example, the TGFβ family of growth factors are known to be the most potent inducers of chondrogenic differentiation [8]. TGFβ proteins stimulate the synthesis of collagens and fibronectin [11]. However, $TGF\beta_1$ and $TGF\beta_2$ are known to cause ECM deposition associated with scarring, possibly due to conversion of keratocytes into the myofibroblast phenotype [6]. For this study, $TGF\beta_3$ was chosen as previous studies have shown that ECM deposition elicited by it does not cause fibrosis or deposition of scar tissue [14]. A combination of TGFβ and dexamethasone has been routinely used to induce progenitor cells to differentiate into chondrocytes in vitro [3,13,36]. Therefore a combination of $TGF\beta_3$ and dexamethasone was used to drive the differentiation of keratocytes towards a chondrocyte phenotype.

The induction of collagen type II could potentially be used to strengthen a weakened keratoconic cornea and for this research to be of therapeutic value it would first needed to be tested on diseased keratoconic corneas. An important aim, therefore, was to determine whether keratocytes in keratoconic tissue were amenable to this method of cell reprogramming and subsequent production of collagen type II rich ECM. The effect of type II collagen deposition on the biomechanical properties of the *in vivo* and *ex vivo* treated corneas was evaluated by using nanoindentation testing, a bioengineering approach that enables analysis of hardness and elastic modulus. The final aim was to establish whether collagen type II deposition can be induced *in vivo* in the corneas of rats without an adverse effect on the optical properties of the corneas, and whether it also led to corneal stiffening.

2. Methods and materials

2.1. Tissue collection

Cadaveric whole human corneas and keratoconic corneas obtained at the time of transplant surgery were obtained from donors sourced through the New Zealand National Eye Bank (Auckland, New Zealand). Prior to the use of tissue, research ethics approval and consent was obtained from the Northern X Regional Human Ethics Committee. Ethics approval for animal studies was obtained from the University of Auckland Animal Ethics Committee. Eyes from 6 to 8 week old adult male Wistar rats were obtained and the corneas were carefully dissected out using surgical scissors with the aid of a dissecting microscope.

2.2. Isolation and culture of corneal keratocytes and organotypic culture of intact cornea

The corneal epithelium and endothelium were gently scraped off with a keratome and discarded. The stromal tissue was digested in 0.4% type II collagenase (Sigma-Aldrich), in Hanks Balanced Salt Solution (GIBCO, Life Technologies) at 37 °C with gentle mixing on an orbital shaker. A variety of digestion times were tried with 5 h being the time required for optimal tissue digestion and cell viability. Isolated keratocytes were cultured in either 12 or 24 well cluster plates (Falcon) on glass coverslips in 2–3 ml of cell culture media. Cells were kept in a humidified incubator at 37 °C with 5% CO_2. Culture media was changed after 24 h and then every two days subsequently or more frequently if required. For cell pellet culture, corneal fibroblasts were pelleted by centrifuging at 300 g for 7 min at 20 °C in a plastic conical tube. Culture media was added to the tubes. After 24 h of incubation at 37 °C, the cells had contracted and formed a pellet which did not adhere to the walls of the tube. For *ex vivo* experiments, corneas were placed in an organotypic air-liquid interphase culture system. Briefly, the explants of healthy tissue were cultured on 0.4 μm pore size cell culture inserts (Millicell) at the interface between culture medium and a CO_2 rich environment. The tissue was placed epithelium side up on cell culture plate inserts with 3 ml of culture medium. The culture media was changed every other day.

2.3. Chondrogenic differentiation of keratocytes

Chondrogenic differentiation medium consisted of Dulbecco's Modified Eagle Medium (DMEM) medium supplemented with 10 ng/ml $TGF\beta_3$, 10^{-7} M Dexamethasone, 1% Glutamax 1% Anti-Anti. Fibroblast induction medium consisted of DMEM supplemented with 10% foetal bovine serum (FBS), 1%Anti-anti, 1% Glutamax. In the control medium the addition of FBS was omitted. For obtaining a monolayer of cells, keratocytes were seeded on glass coverslips at a density of 15×10^4 per cm^2. Cultures were maintained for up to 3 weeks. For *in vivo* treatment of rat corneas, eye drops were formulated using Gellan gum, a water soluble polysaccharide produced by the bacterium, Pseudomonas elodea. The use of gel base formulation allows a prolonged corneal residence time and increased ocular bioavailability of the therapeutic agent.