Acta Biomaterialia 22 (2015) 50–58

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Development of human corneal epithelium on organized fibrillated transparent collagen matrices synthesized at high concentration

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ARTICLE INFO

Article history:
Received 7 December 2014
Received in revised form 6 March 2015
Accepted 14 April 2015
Available online 27 April 2015

Keywords:
Collagen
Liquid-crystal
Transparent matrices
Human corneal epithelium
Limbal stem cell

ABSTRACT

Several diseases can lead to opacification of cornea requiring transplantation of donor tissue to restore vision. In this context, transparent collagen I fibrillated matrices have been synthesized at 15, 30, 60 and 90 mg/mL. The matrices were evaluated for fibril organizations, transparency, mechanical properties and ability to support corneal epithelial cell culture. The best results were obtained with 90 mg/mL scaffolds. At this concentration, the fibril organization presented some similarities to that found in corneal stroma. Matrices had a mean Young's modulus of 570 kPa and acellular scaffolds had a transparency of 87% in the 380–780 nm wavelength range. Human corneal epithelial cells successfully colonized the surface of the scaffolds and generated an epithelium with characteristics of corneal epithelial cells (i.e. expression of cytokeratin 3 and presence of desmosomes) and maintenance of stemness during culture (i.e. expression of ΔNp63α and formation of holoclones in colony formation assay). Presence of cultured epithelium on the matrices was associated with increased transparency (89%).

1. Introduction

Cornea, the outer layer of the eye, is a transparent tissue. Its high refractive power contributes to 2/3 of the eye optical power. It consists of several distinct layers, including corneal epithelium and stroma [1]. The stroma accounts for approximately 90% of the corneal thickness. It is mainly composed of collagen type I, with minor quantities of collagen type V and other types of collagen and biomolecules such as proteoglycans and glycosaminoglycans [2,3]. Distinctive features of the collagen fibrils are the narrow size distribution of around 30 nm in diameter, the regular spacing and the layer structure that is reminiscent of plywood – all of them assumed to contribute to the transparency of the tissue and its mechanical properties [4–8]. Stromal keratocytes show high metabolic activities accounting for a renewal of the extracellular matrix by synthesizing collagens and keratan sulfate proteoglycans [4,9]. Keratocytes show a dendritic morphology thus minimizing light diffusion [4]. They are supplied with nutrients by molecular diffusion across the corneal stroma. The lack of vascularization contributes to the relative immune privilege of the cornea, meaning that antigens introduced at this site are tolerated to a certain degree without provoking an immune response [10]. For this reason, allogeneic corneal transplantation is the standard treatment of corneal stromal diseases that lead to a loss of vision such as: keratoconus, stromal scars after infectious keratitis or trauma, stromal dystrophies. In about 20% of cases, transplantation of allogenic donor tissue induces an inflammatory reaction, despite the corneal immune privilege, that may eventually lead to rejection and destruction of the donor cells [11]. In these cases, and for the treatment of severe inflammatory ocular surface diseases, such as Stevens–Johnson syndrome or ocular cicatricial pemphigoid, alternative treatment options such as synthetic corneal implants provide higher success rates [12]. These medical devices include keratoprosthesis made of polymethylmethacrylate or polyhydroxyethylmethacrylate and artificial cornea substitutes also derived from synthetic polymers. Limits of these therapeutic approaches include lack of true integration to the surrounding tissue that may lead to rejection of the implanted material. To overcome the disadvantages of synthetic materials for corneal repair,

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http://dx.doi.org/10.1016/j.actbio.2015.04.018
1742-7061/

collagen-based implants are being developed by several research groups across the world [13–19]with promising results [20,21].

Current collagen corneal implants are generally made of collagen I or III at low concentration together with additional components such as glycosaminoglycan to ensure transparency and chemical cross-linkers such as 1-Ethyl-3-(3-dimethylaminopropy l)carbodiimide and N-Hydroxysuccinimide to improve the mechanical properties of the artificial scaffold [20]. The key idea of our approach is to propose a new type of acellular dense collagen matrix that could be either seeded with stem cells, or used for direct implantation allowing the recipient peripheral corneal cells to migrate and proliferate into the implanted matrix and leading to reconstruction of the original native corneal structure. It is known that lyotropic liquid crystal properties of acidic collagen solution can be used to generate opaque dense organized matrices [22–26]. We describe here, for the first time, the process to get transparent dense collagen matrices using only collagen I without any cross-linkers or additional biological molecules. Ruberti et al. used a similar procedure but with the use of additional molecules [27]. The transparent collagen I scaffolds are then checked for the potentiality to host human limbal explants. Cell viability and growth as well as maintenance of stemness were assessed after 14 days of culture. The scaffolds have been analyzed before and after cell culture by transmission electron and light microscopies and mechanical tests were conducted on acellular matrices.

2. Materials and methods

2.1. Collagen I purification

Type I collagen was extracted from young Wistar or Sprague-Dawley rat-tails and purified following published protocols [26]. Collagen solutions purified in 500 mM acetic acid were kept at 4 °C and centrifuged at 41,000g for 4 h before use. Sample purity was assessed by SDS-PAGE electrophoresis. Collagen concentration was determined from the acidic solution by assessing the amount of hydroxyproline [28].

2.2. Sample preparation

Collagen solution (3.16 mg/mL) prepared in 500 mM acetic acid was dialyzed to obtain a final solution in 75 mM acetic acid and 1.25 mM hydrochloric acid (pH 2.55). The solution was subsequently concentrated at fixed concentrations (15, 30, 60 and 90 mg/mL) by centrifugation–filtration using Vivaspin (30 kD cut-off) on a variable angle Beckman-Coulter J26-XP centrifuge (4500 rpm – 4922 g, 10 °C). The concentrated solutions have a gel-like consistency. They are transferred into a sterile syringe and centrifuged to withdraw air bubbles. Approximately 200 µl of the concentrated solution is put into a ceramic mold that roughly mimics the anterior shape of the cornea. A plastic counterpart roughly mimicking the shape of the posterior part of the cornea is set on the small collagen ball and pressed on to it (Fig. 1). The molds were then submitted to ammonia vapor at saturation for 17 h at 20 °C to induce fibrillogenesis of collagen [29]. After 17 h, the samples were collected and immersed in Milli-Q water and stored at 20 °C until use.

2.3. Cell culture

This study was carried out according to the tenets of the Declaration of Helsinki and it followed international ethic requirements for human tissues. Human organ-cultured donor corneas and amniotic membrane were obtained from the EFS – Ile-de-France tissue bank (Paris, France).

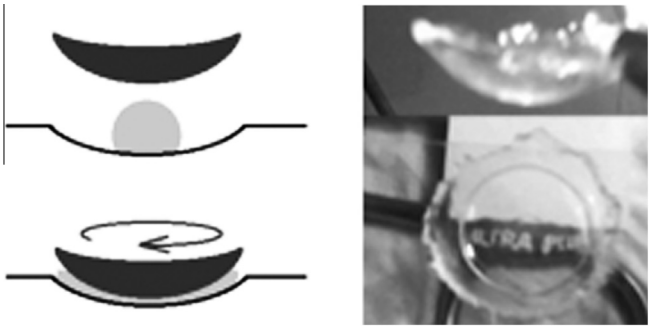

Fig. 1. Transparent collagen matrix at 90 mg/mL. Left side illustrates the molding of the collagen dense solution prior setting into to ammonia vapors. Right side shows the collagen matrix after fibrillogenis.

Six human corneas were used in this study. The average donor age was 77 years (range 68–83 years). Time from donor death to tissue procurement ranged from 10 to 48 h (mean, 27 h). Donor corneas were organ-cultured in CorneaMax medium (Eurobio, Courtaboeuf, France) at 31 °C for 14–35 days (mean, 21 days) as previously described [30,31]. The central part of the donor cornea was transplanted to the scheduled recipient and the remaining corneoscleral rim was used for cell culture.

Amniotic membranes were obtained from two female donors undergoing cesarean section. Donors featured negative serology for hepatitis B, hepatitis C, HTLV, HIV, and syphilis. The placenta was first washed with saline solution containing antibiotics for two hours and rinsed with sterile water. The membrane was separated from the placenta after removal of its border with scissors and placed in nitrocellulose surface, cut into pieces and stored in a 1:1 mixture of RPMI medium (Eurobio) and glycerol (Haemonetics, Lyon, France) at −80 °C for 2 years. Before culture amniotic membrane grafts were thawed at room temperature and rinsed with phosphate buffered saline. There was no selection of the amniotic membrane area used for the experiments.

Superficial limbal explants were prepared as previously described [32]. Six human limbal rims were divided into 2 parts. Each semi-rim was divided into 3 explants. For each donor cornea, five explants were cultured for 14 days on the surface of five different carriers (i.e. 15, 30, 60, and 90 mg/mL collagen matrices and human amniotic membrane as control). Collagen scaffolds were rinsed first with Milli-Q water and culture medium to remove all ammonia residues before cell proliferation test. Human donor cornea explants were then stitched on top of the scaffolds, epithelial side up, to induce close contact to the scaffold and allow for cell migration from explants to matrix as described in Fig. SI-1. The setup was immersed entirely in Green's medium composed of a 3:1 mixture of calcium-free Dulbecco's Modified Eagle's Medium (Dutscher, Brumath, France) and Ham F12 medium (Invitrogen, Cergy Pontoise, France) with 10% fetal bovine serum (Invitrogen), 1 mM/ml HEPES buffer (Invitrogen), 5 µg/ml human recombinant insulin (Actrapid®; Novo Nordisk, Paris, France), 0.4 µg/ml hydrocortisone (Pharmacia, Pfizer, Paris, France), 4 µM/ml L-glutamine (Invitrogen), 2 pM/ml tri-iodo thyronine (Sigma, Saint Quentin en Yvelines, France), 200 nM/ml adenine (Sigma), 100 IU/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 0.25 µg/ml amphotericin B (Invitrogen), and 10 ng/ml human recombinant Epithelial Growth Factor (EGF; Sigma) and incubated for 14 days at 37 °C in the presence of CO2 [30]. These culture conditions were shown to be optimal for expansion of progenitors [31,32,35]. The culture medium was changed three times a week. At the end of the culture period the scaffolds were used for further investigations. Samples were not pooled.