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ScienceDirect
Biomaterials 28 (2007) 4268–4276
Biomaterials
www.elsevier.com/locate/biomaterials

Orthogonal scaffold of magnetically aligned collagen lamellae for corneal stroma reconstruction

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Received 6 March 2007; accepted 25 May 2007
Available online 5 July 2007

Abstract

The creation of 3D scaffolds that mimic the structure of physiological tissue required for normal cell function is a major bioengineering challenge. For corneal stroma reconstruction this necessitates the creation of a stroma-like scaffold consisting of a stack of orthogonally disposed sheets of aligned collagen fibrils. This study demonstrates that such a scaffold can be built up using magnetic alignment. By allowing neutralized acid-soluble type I collagen to gel in a horizontal magnetic field (7 T) and by combining a series of gelation–rotation–gelation cycles, a scaffold of orthogonal lamellae composed of aligned collagen fibrils has been formed. Although initially dilute, the gels can be concentrated without noticeable loss in orientation. The gels are translucent but their transparency can be greatly improved by the addition of proteoglycans to the gel-forming solution. Keratocytes align by contact guidance along the direction of collagen fibrils and respect the orthogonal design of the collagen template as they penetrate into the bulk of the 3D matrix. The scaffold is a significant step towards the creation of a corneal substitute with properties resembling those of native corneal stroma.

Keywords: Corneal stroma reconstruction; Collagen; Proteoglycans; Keratocytes; Stroma-like scaffold; Magnetic alignment

1. Introduction

The cornea consists of three distinct cellular layers: the outer epithelium, the central stroma composed of keratocytes embedded in a dense, highly organized extracellular matrix of collagen fibrils and proteoglycans, and an inner endothelium. The stroma is delimited by specialized acellular structures, Bowman and Descemet membranes, lying at the interface between the epithelium and endothelium, respectively. In view of the world-wide shortage of donors, the increasing risk of transmissible diseases, the widespread use of corrective surgery that renders corneas unsuitable for grafting, and the limitations of currently available synthetic polymer-based artificial corneas (keratoprostheses), there is an urgent need to develop new therapeutic strategies for corneal repair. A multi-disciplinary tissue-engineering approach involving bioactive scaffolds that support and guide cell growth and development is beginning to show promise [1–12]. Scaffolds can be used as stand-alone implants, or be seeded with cells to create cell-scaffold composites, which might be allowed to mature in vitro until tissue-like properties develop. In addition to their potential clinical uses as 3D tissue mimics, tissue-engineered corneas should facilitate the in vitro study of the complex physiology of living tissue and would also serve as alternatives to animal models for pharmacotoxicity testing [3,4,13]. While the two in vitro human corneal models currently commercially available for the latter purpose, from SkinEthic Laboratories (Nice, France) and MatTek Corporation (EpiOcular™; Ashland, MA, USA), provide a cornea-like epithelium, the key stromal and

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doi:10.1016/j.biomaterials.2007.05.024

endothelial components are entirely absent. The validity of toxicity testing can only be enhanced by the introduction of more complete models resembling more closely normal human cornea in both composition and organization.

Recent advances in tissue engineering, stem cell technology and nano-engineering have been successfully used to repair human corneas and even recreate cornea-like tissue de novo. Grafts of human cornea-limbal epithelial sheets, cultured on fibrin, show long-term clinical efficacy in the regeneration of the damaged corneal surface [2,7]. Damaged eye tissue has also been restored using epithelial cells cultured on amniotic membranes [5,6] and temperature-responsive cell culture surfaces [9]. Scaffolds preloaded with stromal cells [10] or used as acellular supports for natural self-healing [8,12] have given encouraging results in animal models. The in vitro reconstruction of fully functional cornea replacement tissues is a long-term goal. It has already been demonstrated that the three main corneal layers [1,3,4] can be created in vitro using collagen-based scaffolds. Griffith et al. [4] have claimed that Bowman and Descemet membrane-like structures form spontaneously in their in vitro system. Despite this progress, the matrices produced so far lack the highly organized 3D collagen-based architecture essential for the biomechanical and optical properties of native stroma.

The stroma makes up some 90% of the corneal volume and 70% of the cornea dry weight. During embryogenesis, the adult stroma is preceded by a highly structured acellular primary stroma produced by the epithelial cells. In the avian system, this primary stroma is believed to dictate the pattern of organization of the adult stroma [14]. Within adult stroma the collagen fibrils, composed of types I and V collagen, have a uniformly narrow diameter ($\approx$30 nm) and are arranged in lamellae, within which fibrils are parallel and separated by a proteoglycan-rich matrix [15]. The collagen fibrils are stabilized by intra- and intermolecular covalent cross-links, which confer tensile strength and stabilize fibrils against proteolytic degradation. Transparency is believed to be largely dependent upon the ordered 3D architecture of thin collagen fibrils [16]. Electron microscopy [14,15,17,18] and X-ray diffraction [19] studies indicate that fibrils in adjacent stromal lamellae are predominantly orthogonal. The interlacing of stromal lamellae endows the cornea with highly non-linear biomechanical properties—becoming increasingly stiffer under high intra-ocular pressure, which enables the cornea to survive abnormal influences such as impact, injury and surgery without bursting. The construction of a scaffold having orthogonal lamellae of aligned collagen fibrils as in native stroma, would be an important asset in the development of bio-engineered corneal implants.

Here, we use magnetic orientation to create a corneal stroma-like scaffold consisting of multiple intermeshed orthogonal layers of oriented collagen I fibrils. Addition of proteoglycans substantially improves gel transparency most likely by reducing fibril diameter and eliminating lateral fusion of fibrils. Human corneal keratocytes seeded onto scaffolds are aligned by contact guidance both on the surface and within the bulk of the orthogonal collagen template.

2. Materials and methods

2.1. Oriented 3D collagen scaffolds

Acid-soluble rat-tail type I collagen (0.02 N acetic acid; $\approx$4 mg/ml; from BD Biosciences) and bovine skin type I collagen ($\approx$5 mg/ml. gift from Symatèse Biomatériaux) were used. Both of these collagens behaved similarly so only the results obtained with rat-tail collagen are reported in this article. The collagen solution was well mixed at 4 °C with an equal volume of neutralizing buffer (60 mM Na phosphate, 60 mM HEPES, 270 mM NaCl, pH = 7.4; [20]). Aliquots were then pipetted onto the precooled $\approx$4 °C sample holder then positioned horizontally in the central region of the magnet bore at 20 °C. After a few minutes the temperature was raised progressively over a period of about 10 min to 30 °C. The passage in the magnet lasted for a total of approximately 30 min. During this period the collagen molecules assemble into oriented fibrils transforming the solution into a viscous gel. In order to create orthogonal lamellae, gels that had already undergone orientation were removed from the magnet, and covered with an aliquot of cold neutralized collagen solution and replaced in the magnet after having been rotated by the desired angle with respect to the previous orientation. The final collagen gel concentration was sometimes increased by neutralizing three volumes of collagen solution with one volume of double-strength phosphate buffer. The thickness of the initial hydrated gels was calculated by dividing volume by surface area.

Sample holders were either glass-bottomed plastic culture dishes (MatTek Co.) or Transwell® semi-permeable polycarbonate supports (Corning Co.). The culture dishes (outer diameter 35 mm) had a shallow (1.5 mm deep) central glass well (14 mm diameter). Transwell plates were cut to produce individual wells. The wells (12 mm inner diameter) were placed in standard 35 mm plastic Petri dishes. The pore diameter of the semi-permeable membrane was 0.4, 3 or 12 μm. The volume of neutralized collagen solution added to the wells varied from 80 to 200 μl, which correspond to a fully hydrated thickness of 0.5 to 1.3 mm.

The split-coil superconducting magnet used (Thor Cryogenics) had a horizontal (5 cm diameter, 40 cm long) room temperature bore. The field profile rose to a flat plateau extending $\pm$5 cm about the bore center making it possible to simultaneously place up to three 35 mm diameter samples in maximum field. The temperature of the sample space was controlled by circulating water from a temperature-controlled bath. The field used for processing was near the maximum possible (7 T). This magnet and the necessary infrastructure were provided by the Grenoble High Magnetic Field Laboratory.

In some experiments, aliquots of transglutaminase solution were added following neutralization of the collagen solution using an equal volume of calcium containing phosphate-free buffer (120 mM HEPES, 5 mM $CaCl_2$, 270 mM NaCl, pH 7.4). Transglutaminase, from guinea pig liver (Sigma-Aldrich), was dissolved (2.26 mg/ml protein; 8.1 units/ml) in buffer (10 mM Tris-acetate, 160 mM KCl, 1 mM EDTA, 2 mM DTT, pH 8.0) and stored at −80 °C [19,21]. Transglutaminase had no effect on orientation.

Scaffolds, dehydrated either by blotting or evaporation, could be conserved for several months at 4 °C; up to 7 days before use they were rehydrated with Dulbecco's Modified Eagle's Medium (DMEM) with double-strength antibiotic concentration, i.e. Penicillin G 200 UI/ml, Gentamycin 40 μg/ml and Amphotericin B 2 μg/ml.

2.2. Proteoglycans

Proteoglycans were extracted from calf corneas [22] using 4 M GuHCl, 50 mM Na acetate, pH 5.8. Extracts were diluted in 7 M urea, 50 mM Tris, pH 6.7 and proteoglycans were purified by DEAE-ion exchange chromatography. From 10 corneas, 41 mg lyophilized material containing