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Corneal stromal bioequivalents secreted on patterned silk substrates

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ABSTRACT

Emulating corneal stromal tissue is believed to be the most challenging step in bioengineering an artificial human cornea because of the difficulty in reproducing its highly ordered microstructure, the key to the robust biomechanical properties and optical transparency of this tissue. We conducted a comparative study to assess the feasibility of human corneal stromal stem cells (hCSSCs) and human corneal fibroblasts (hCFs) in the generation of human corneal stromal tissue on groove-patterned silk substrates. In serum-free keratocyte differentiation medium, hCSSCs successfully differentiated into keratocytes secreting multilayered lamellae with orthogonally-oriented collagen fibrils, in a pattern mimicking human corneal stromal tissue. The constructs were 90–100 μm thick, containing abundant cornea-specific extracellular matrix (ECM) components, including keratan sulfate, lumican, and keratocan. In contrast, hCFs tended to differentiate into myofibroblasts that deposited less organized collagen in a pattern resembling that of corneal scar tissue. RGD surface coupling coupling was an essential factor in enhancing cell attachment, orientation, proliferation, differentiation and ECM deposition on the silk substratum. These results demonstrated that an approach of combining hCSSCs with an RGD surface-coupled patterned silk film offers a powerful tool to develop highly ordered collagen fibril-based constructs for corneal regeneration and corneal stromal tissue repair.

1. Introduction

The cornea provides essential features to the visual system, including optical transparency, biological and mechanical protection, and light refraction. Once the cornea loses transparency due to scarring or disease, corneal transplantation (penetrating keratoplasty, PK) is the prevailing option to correct visual impairment. In USA, there are 30,000–40,000 PK operations performed every year [1]. Although short-term success of this procedure is high, long-term (i.e. >10 year) graft survival is similar to that of other organ transplants, 64% [2]. Once a transplanted cornea is rejected, a subsequent graft is at high risk for rejection. The increasing use of Laser-Assisted In Situ Keratomileusis (LASIK) surgery for refractive correction has reduced the availability of donor corneas and in many parts of the world, donor tissues are in short supply or not available. This shortage of healthy corneal donor tissue has stimulated efforts to develop biological human corneal equivalents employing a tissue engineering strategy [3–5].

Synthetic corneal prostheses are in limited use and recombinant collagen is in clinical trials for partial thickness keratoplasty [6], but there are currently no clinically viable cellularized human corneal equivalents produced by tissue engineering methods. A major challenge in bioengineering a cornea is in producing a biological equivalent of the corneal stroma. This tough, optically transparent tissue is made up of 300–500 lamellae composed of tightly-packed, highly aligned collagen fibrils with uniform fibril size and interfibril spacing. This complex, hierarchical microstructure is principally responsible for optical transparency and biomechanical properties of human cornea [7–9] and is challenging to recapitulate *in vitro*.

Silk fibroin, mainly produced from *Bombyx mori* silkworm cocoons, has been extensively introduced as biomaterial scaffolds for tissue engineering and regenerative medicine due to its biocompatibility [10,11], controllable degradability [12,13], tunable mechanical properties [14,15] and low immunogenicity [11,16]. Because of its optical transparency, silk fibroin film has previously been used in ocular tissue reconstruction [17,18]. Silk fibroin membranes have been shown to support the growth of corneal epithelial cells [19–21], corneal endothelial cells [22], and retinal pigment epithelial cells [23]. Preclinical *in vivo* studies in a rabbit

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model demonstrated that the transparent porous silk membranes are a promising carrier for cultivated epithelial sheets in the regeneration of corneal epithelium [24]. Coupled with Arginine-Glycine-Aspartic acid (RGD) peptide cell-receptor motif and groove-patterned surface, silk films efficiently support corneal fibroblast attachment, orientation, proliferation, enhanced corneal stroma gene expression and deposition of aligned fibrillar collagen [25–27]. RGD-coupled silk films also improve attachment and differentiation of mesenchymal stem cells [28].

Keratocytes are native resident cells of the corneal stroma, principally responsible for the maintenance of the transparent stromal tissue by secreting a spectrum of unique matrix molecules [29–32]. Expansion of keratocytes *in vitro* inevitably leads to their differentiation into corneal fibroblasts [29,30,32,33]. Corneal fibroblasts exhibit a wound-healing phenotype and secrete disorganized extracellular matrix (ECM) typically found in corneal scars [29,30,32]. The discovery and isolation of human corneal stromal stem cells (hCSSCs) [34–37] make it possible to mimic the developmental process and generate stromal tissue *in vitro*. Du et al. demonstrated that hCSSCs maintain the potential to become keratocytes through a large number of population doublings [36]. Different from corneal fibroblasts, hCSSCs can produce abundant ECM containing cornea stroma-specific components, keratan sulfate and keratocan [35]. In this study, we compared the applicability of human corneal stromal stem cells (hCSSCs) versus human corneal fibroblasts (hCFs) in the generation of human corneal stromal tissue on patterned silk fibroin membranes.

2. Materials and methods

2.1. Preparation of regenerated silk fibroin solution

Silk fibroin solution was prepared according to previously reported methods [38]. Briefly, 5 g of *B. mori* silkworm cocoons (Tajima Shoji Co., LTD, Japan) were degummed in 2 L of boiling 0.02 M sodium carbonate for 30 min to remove the sericin protein from the fiber. The degummed fibers were dissolved in a 9.3 M lithium bromide solution (20% wt/v) at 60 °C for 4 h. The dissolved silk solution was dialyzed against 4 L of ultrapure water in dialysis cassettes with a 3500 molecular weight (MW) cutoff (Pierce Biotechnology, Rockford, IL). Water was changed three times per day for three days. The dialyzed silk solution was centrifuged twice at 8800 rpm for 20 min and the supernatant collected at 4 °C.The concentration of the final silk solution (6–8% wt/v) was determined by gravimetric analysis.

2.2. Preparation of PDMS substrates

Patterned polydimethylsiloxane (PDMS, Sylgard 184 Silicone Elastomer Kit, Dow Corning, Midland, MI) substrates were prepared by casting PDMS on a reflective diffraction grating surface with linear 3.5 μm wide and 500 nm deep grooves (Edmund Optics, Inc, Barrington, NJ). The substrates were cut into 40 × 40 mm squares, washed in 70% v/v ethanol and thoroughly rinsed in distilled water before casting silk solution on the substrates to generate the patterned films.

2.3. Preparation of silk films

A 1.2 mL aliquot of 1% w/v silk solution was cast upon grooved PDMS molds resulting in 3 μm thick films after drying. The films were covered with a venting lid and allowed to dry overnight at room temperature. The as-cast films were water annealed in a vacuum oven with a 200 mL water tray at the bottom at 25 °C, 20 mmHg vacuum for 2 h. Dry silk films were wrapped in aluminum foil and autoclaved at 121 °C for 20 min to sterilize. The films were hydrated in distilled water, punched into 12 mm (diameter) constructs and placed in 24-well plates for cell seeding.

2.4. RGD surface modification

RGD coupling to silk film surface was carried out as previously described [27] under sterile conditions. The silk films were presoaked in BupH™ MES buffered saline, pH 6.0 (Thermo Scientific, Waltham, MA). The –COOH groups in silk fibroin were activated with 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC–HCl)/N-hydroxysuccinimide (NHS) solution (0.5 mg/mL of EDC and 0.7 mg/mL of NHS in MES buffer, pH 6.0) for 30 min at room temperature, generating amine-reactive NHS-esters on the silk film surface. The activated silk films were washed with MES buffer three times and subsequently incubated in 1 mg/mL Glycine-Arginine-Glycine-Aspartic acid-Serine (GRGDS) peptide (Bachem, Torrance, CA) in MES buffer pH 6.0 for 2 h. After the GRGDS coupling reaction, the surface modified silk films were washed twice in MES buffer and five times in distilled water.

2.5. Culture of human corneal stromal stem cells and human corneal fibroblasts

Human corneal stromal stem cells (hCSSCs) were isolated from collagenase-digested limbal stromal tissue of human corneas unsuitable for transplant obtained from the Center for Organ Recovery & Education (Pittsburgh, PA), as previously described [36]. Cells at passage six were used for the experiments [39]. To prepare human corneal fibroblasts (hCFs) as a comparison, the isolated human corneal stromal keratocytes were expanded in DMEM/F-12 (Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS, Life Technologies). Passage 6 fibroblasts were used for the experiment [37].

Discs of sterile patterned silk film were cut and anchored in wells of 24-well culture plates using Silicone Rubber O-rings. hCSSCs and hCFs were seeded on the substratum at 5.0×10^4 cells/cm^2. hCSSCs were incubated with 1.0 mL of stem cell growth medium containing DMEM/MCDB-201 with 2% fetal bovine serum, 10 ng/mL epidermal growth factor, 10 ng/mL platelet-derived growth factor (PDGF-BB), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenous acid (ITS), lipid-rich bovine serum albumin (Albumax, Life Technologies, Grand Island, NY) 0.1 mM ascorbic acid-2-phosphate, 10^{-8} M dexamethasone, 100 IU/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, 100 ng/ml cholera toxin until confluent (usually 3 days) [39,40]. hCFs were incubated in DMEM/F12 containing 10% FBS until confluence.

At confluence, cells were incubated in keratocyte differentiation medium (KDM) consisting of Advanced DMEM (Life Technologies) supplemented with 1.0 mM L-ascorbic acid-2-phosphate (Sigma–Aldrich, St Louis, MO), 2 mM L-alanyl-L-glutamine (GlutaMax™-1, Life Technologies), 50 μg/mL gentamicin (Life Technologies), 100 μg/mL penicillin, 100 μg/mL streptomycin (Mediatech, Manassas, VA), 10 ng/mL basic fibroblast growth factor (FGF-2, Sigma–Aldrich) and 0.1 ng/mL transforming growth factor-beta3 (TGF-β3, Sigma–Aldrich) [40]. The medium was changed twice per week for up to 9 weeks.

2.6. Two-photon fluorescent microscopy

Multiphoton imaging was performed employing an Olympus FV1000 multiphoton microscope (Center Valley, PA). It is an upright fixed stage microscope equipped with a large area motorized stage. Samples were set in a custom built imaging chamber, and three-dimensional image sets collected with a 25 × 1.0 NA objective specifically designed for multiphoton microscopy. The microscope laser with wavelength of 830 nm was utilized to collect the second harmonic generation (SHG) signal of collagen fibrils. The images were collected as three-dimensional data sets (section spacing 2 μm), with the number of sections in each stack varying from specimen to specimen. Image stacks once collected were processed into three-dimensional stacks using Imaris (Bitplane, South Windsor, CT).

2.7. Electron microscopy

Specimens for electron microscopy imaging were prepared as described previously [37,39]. Briefly, all of the specimens were fixed in cold 2.5% glutaraldehyde (EM grade, Taab Chemical, Aldermaston, England) in 0.1 M PBS, pH 7.3. The specimens were rinsed in PBS, post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences, Hatfield, PA) with 0.1% potassium ferricyanide (Fisher Scientific, Pittsburg, PA), and dehydrated through a graded series of ethanol washes.

For the imaging of transmission electron microscopy (TEM), the dehydrated samples were embedded and cured in Epon (Energy Beam Sciences, East Granby, CT). Sections were cut perpendicular to the alignment of the underlying grooved substrates. Semi-thin sections (300 nm) were stained with 0.5% Toluidine Blue (Fisher) and examined under optical microscope equipped with CCD camera. Ultra-thin sections (65 nm) were stained with 2% uranyl acetate (Electron Microscopy Sciences) in a 1:1 mixture of water and methanol, and then with aqueous 1% phosphotungstic acid (Sigma–Aldrich), pH 3.2. The sections were examined and photographed with a Jeol 1011 transmission electron microscope (JEOL Ltd., Tokyo, Japan) working at 80 kV. For scanning electron microscopy (SEM), the dehydrated samples were chemically dried with hexamethyldisilazane for 15 min. The samples were imaged at 5 kV by a Jeol JSM-6330F Scanning Electron Microscope.

2.8. Gene expression

Gene expression was examined by quantitative RT-PCR (qPCR) as described previously [39]. In brief, DNAse-treated total RNA (400 ng) isolated from constructs (RNeasy, Qiagen, Valencia, CA) was transcribed to cDNA by SuperScript II reverse transcriptase (Life Technologies) using priming with random hexamers. Quantitative RT-PCR of cDNA equivalent to 20 ng RNA was performed with direct dye binding (SYBR Green; Applied Biosystems, Carlsbad, CA) according to the manufacturer's instruction. A dissociation curve for each SYBR-based reaction was generated to confirm that there was no nonspecific amplification. Amplification of 18S rRNA was conducted for each cDNA (in triplicate) for normalization of RNA content. Relative mRNA abundance was calculated as the cycle threshold (Ct) for amplification of a gene-specific cDNA minus the average Ct for 18S expressed as a power of 2 ($2^{-\triangle Ct}$). Three individual gene-specific values thus calculated were averaged to obtain a mean ± SD. Primer sequences were as previously published [36,41].

Six genes were selected which have previously been used to identify keratocyte phenotype. Keratocan (KERA) [33] and prostaglandin D2 synthase (PTGDS) [42] are keratan sulfate core proteins, unique molecular markers of the corneal stroma.