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Keratocyte behavior in three-dimensional photopolymerizable poly(ethylene glycol) hydrogels

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Abstract

The goal of this study was to evaluate three-dimensional (3-D) poly(ethylene glycol) (PEG) hydrogels as a culture system for studying corneal keratocytes. Bovine keratocytes were subcultured in DMEM/F-12 containing 10% fetal bovine serum (FBS) through passage 5. Primary keratocytes (P0) and corneal fibroblasts from passages 1 (P1) and 3 (P3) were photoencapsulated at various cell concentrations in PEG hydrogels via brief exposure to light. Additional hydrogels contained adhesive YRGDS and nonadhesive YRDGS peptides. Hydrogel constructs were cultured in DMEM/F-12 with 10% FBS for 2 and 4 weeks. Cell viability was assessed by DNA quantification and vital staining. Biglycan, type I collagen, type III collagen, keratocan and lumican expression were determined by reverse transcriptase–polymerase chain reaction. Deposition of type I collagen, type III collagen and keratan sulfate (KS)-containing matrix components was visualized using confocal microscopy. Keratocytes in a monolayer lost their stellate morphology and keratocan expression, displayed elongated cell bodies, and up-regulated biglycan, type I collagen and type III collagen characteristic of corneal fibroblasts. Encapsulated keratocytes remained viable for 4 weeks with spherical morphologies. Hydrogels supported production of KS, type I collagen and type III collagen matrix components. PEG-based hydrogels can support keratocyte viability and matrix production. 3-D hydrogel culture can stabilize but not restore the keratocyte phenotype. This novel application of PEG hydrogels has potential use in the study of corneal keratocytes in a 3-D environment.
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1. Introduction

Tissue engineering has recently emerged as a strategy to recreate corneal tissue. Several groups have successfully reproduced the general morphology and organization of the cornea with a fully stratified epithelium, continuous basement membrane, collagenous cellular stroma, and intact endothelium [1–6]. These in vitro reconstructions are able to support cell adhesion, proliferation, differentiation and deposition of extracellular matrices important for use as replacement cornea tissue [4,5,7]. In addition, the constructs have been shown to be functional, maintaining transparency and responding to physical and chemical injuries in a manner similar to the native cornea [3,7]. While the majority of these research efforts have focused on the creation of a replacement cornea for therapeutic purposes, the goal of this study was to create a three-dimensional (3-D) in vitro model for studying corneal keratocyte populations in an environment that substantially mimics their in vivo milieu, which would be applicable

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for the study of stromal wound healing as well as the evaluation of keratocyte response to therapeutic agents.

Keratocytes are known to readily differentiate into corneal fibroblasts, losing their native morphology and phenotype, when cultured using traditional serum-based monolayer systems. 3-D culture systems have been applied to other cell types and have demonstrated retention of in vivo cell morphologies and phenotypes [8], as well as the restoration of native phenotypes that have been lost or altered in serial monolayer cultures [8,9]. Hydrogel culture systems can provide a 3-D environment, the cellular and spatial arrangement of which more closely resembles the in vivo tissue than do monolayer cultures. In addition, hydrogels have sufficient water content and porosity to allow the adequate diffusion of nutrients, growth factors and cellular by-products [10]. It is hypothesized that a 3-D hydrogel system can promote or restore the normal keratocyte phenotype, thus delaying or avoiding the fibroblastic transition that occurs in traditional monolayer cultures and frustrates the practical study of keratocytes in their native state.

The objective of this study was to assess the suitability of photopolymerizable poly(ethylene glycol)-based (PEG) hydrogels as a model system for supporting keratocyte populations and stabilizing their native phenotype. To that end, bovine keratocytes were photoencapsulated in both adhesive and non-adhesive (PEG) hydrogels and evaluated for cell viability, gene expression and protein deposition. In addition, the spatial features and porosity of the resulting hydrogel constructs were characterized.

2. Materials and methods

2.1. Cell isolation and subculture

Bovine eyes were purchased from Research 87, Inc. (Marlborough, MA) within 36 h of slaughter. Keratocytes were isolated using a modified sequential, collagenase digestion [11]. Central corneas were removed and quartered with a scalpel, rinsed and collected on ice in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco, Grand Island, NY) containing antibiotics. Physical scraping of superficial layers was avoided due to its potential induction of underlying keratocyte apoptosis [12]; histological analysis confirmed the removal of epithelial and endothelial layers with the first digestion. Cornea quarters were transferred to a 50 ml centrifuge tube containing 3.3 mg ml^{-1} collagenase (Sigma, St. Louis, MO) in DMEM/F-12, and incubated on a rotary shaker (150 rpm) at 37 °C for 30 min. After vortexing briefly, undigested tissue was separated using a 70 μm cell strainer (BD Falcon, Franklin Lakes, NJ) and transferred to a fresh collagenase solution for a second 60-min digestion. This process was repeated for a final 180-min digestion. Second and third digestion cells were collected through the 70 μm cell strainer and centrifuged at 1400 rpm for 10 min to pellet the keratocytes before the collagenase solution was removed. The cell number and viability from each digestion was determined using a hemacytometer and trypan blue exclusion. Primary cells were pooled from the second and third digestions, plated for subculture, encapsulated in hydrogels, or pelleted for RNA extraction.

Cells were plated in tissue culture flasks at a density of 5000 cells cm^{-2}. Primary cells were cultured in DMEM/F-12 containing 1% platelet-poor horse serum (Sigma) for 2 days, followed by DMEM/F-12 containing 10% FBS (Gibco), 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin (Gibco), 50 μg ml^{-1} gentamicin (Quality Biological, Inc., Gaithersburg, MD) and 1.25 μg ml^{-1} amphotericin B (Gibco) for the remaining culture period. The medium was changed every 2–3 days. Upon confluency, the cells were trypsinized and subcultured, encapsulated or pelleted for RNA extraction. Monolayers were routinely subcultured through passage 5.

2.2. Hydrogel polymer preparation and characterization

Poly(ethylene glycol) diacrylate (PEGDA) macromer (mol. wt. = 3400) was purchased from Nektar Therapeutics (Huntsville, AL). The adhesive peptide sequences RGD and YRGDS and the non-adhesive control peptides YRDGS (Department of Biological Chemistry, Johns Hopkins University) were covalently attached to PEGDA macromers by reacting raw peptide with an excess of acryl-poly(ethylene glycol)-*N*-hydroxysuccinimide (PEG-NHS) (Nektar) in 50 mM Tris buffer and lyophilizing the reaction product overnight [13].

The following five polymers were used in encapsulations: 10% w/v PEGDA, 15% w/v PEGDA, 15% PEGDA + 2.5 mM RGD, 15% PEGDA + 2.5 mM YRGDS and 15% PEGDA + 2.5 mM YRDGS. Polymer concentrations for peptide-decorated hydrogels were increased to ensure a sufficient number of cross-linking sites and also allowed a comparison of hydrogel porosity. These and plain PEGDA polymer solutions were prepared by dissolving macromers in sterile phosphate-buffered saline (PBS) containing antibiotics.

Additional acellular gel constructs ($n = 5$) were prepared from 10% PEGDA, 15% PEGDA and 15% PEGDA + 2.5 mM YRGDS in PBS to characterize hydrogel properties. Wet and dry weights of gels were obtained to calculate porosity and average pore size via the Peppas–Merrill equation [14].

2.3. Cell encapsulation in hydrogels

Primary keratocytes were encapsulated in 10% PEGDA at 1.25, 3.75, 6.25, 12.5 and 17.5 million cells ml^{-1} to determine the optimal cell concentration for encapsulations. A concentration of 12.5 million cells ml^{-1} was chosen for all further encapsulations.

Pelleted primary keratocytes and corneal fibroblasts from P1 and P3 cultures were mixed with each polymer solution. Irgacure 2959 photoinitiator (Ciba Specialty

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