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Ocular biocompatibility of gelatin microcarriers functionalized with oxidized hyaluronic acid

Jui-Yang Lai [a,b,c,d,e,*], David Hui-Kang Ma [c,d,f]

[a] Institute of Biochemical and Biomedical Engineering, Chang Gung University, Taoyuan 33302, Taiwan, Republic of China
[b] Biomedical Engineering Research Center, Chang Gung University, Taoyuan 33302, Taiwan, Republic of China
[c] Center for Tissue Engineering, Chang Gung Memorial Hospital, Taoyuan 33305, Taiwan, Republic of China
[d] Department of Ophthalmology, Chang Gung Memorial Hospital, Taoyuan 33305, Taiwan, Republic of China
[e] Department of Materials Engineering, Ming Chi University of Technology, New Taipei City 24301, Taiwan, Republic of China
[f] Department of Chinese Medicine, Chang Gung University, Taoyuan 33302, Taiwan, Republic of China

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ABSTRACT

Given that the presence of aldehyde groups on the oxidized sugar residues may pose toxicity concerns, it is necessary to examine the safety of gelatin microcarriers (GMC) functionalized with oxidized hyaluronic acid (oHA) for potential ophthalmic applications. In this study, the ocular biocompatibility of biopolymer microcarriers was investigated in vitro using primary rabbit corneal cell cultures and in vivo using the anterior chamber of the rabbit eye model. Our results showed that different types of corneal cells including epithelial, stromal, and endothelial cells remain viable and actively proliferate following 2 and 4 days of exposure to test materials. In addition, similar interleukin-6 gene expression levels and comet tail lengths were seen in the presence and absence of biopolymer microcarriers, suggesting no cellular inflammation and genotoxicity. After 7 and 14 days of intracameral injection in the rabbit eyes, both the GMC samples and their counterparts functionalized with oHA were well tolerated in the ocular anterior chamber as demonstrated by slit-lamp biomicroscopy. Clinical observations including specular microscopic examinations, corneal topography, and corneal thickness measurements also showed that the rabbits bearing biopolymer microcarriers exhibit no signs of corneal edema and astigmatism as well as endothelial damage, indicating the absence of tissue response. It is concluded that the GMC materials functionalized with oHA (oxidation level: 10.4 ± 0.9%) are compatible toward corneal cells and ocular anterior segment tissues at a concentration of 10 mg/ml. The information about the effect of coupling of aldehyde-functionalized HA to gelatin on in vitro and in vivo biocompatibility of biopolymer composites can be used as further development of corneal stromal cell microcarriers for tissue engineering applications.

1. Introduction

In the human body, the extracellular matrix (ECM) surrounding the cells is responsible for construction of tissue framework. The ECM components are mainly biomolecules (i.e., proteins and polysaccharides). Therefore, both protein-origin and polysaccharidic polymers are important biomaterials as carriers and scaffolds for cell delivery in tissue engineering applications [1]. Harkin et al. have reported that the silk fibroin is a promising proteinaceous matrix in the reconstruction of various ocular tissues including corneoscleral limbus, corneal stroma and endothelium, and outer blood-retinal barrier [2]. Due to their excellent biocompatibility, ease of processing and availability at low cost, gelatin-based materials have also been widely investigated for ocular tissue engineering and regenerative medicine [3]. A recent review paper by Feng et al. has summarized the researches on the cell carriers made of different materials such as collagen, fibrin, gelatin-chitosan, silk fibroin, and keratin for the treatment of limbal stem cell deficiency [4]. The use of protein-origin and polysaccharidic polymers alternative to human amniotic membrane for cultivation and transplantation of limbal epithelial cells may overcome the limitations of potential disease transmission risk, limited transparency, and low mechanical strength of biological tissue materials [5].

The stromal layer is known to be the major structural element of the cornea that is responsible for eye protection and light refraction. In vivo, corneal keratocytes are interspersed between the layers of the stromal lamellae. Given that the collagen and glycosaminoglycans constitute the ECM of the corneal stroma, the production of biomimetics has attracted much attention. Chemically cross-linked collagen-chitosan composite hydrogels have been studied with respect to their function as corneal implants [6]. To develop a reconstructed cornea model, the collagen-chondroitin sulfate foams are fabricated for in vitro cultivation of human corneal cells [7]. In another study, Tonsomboon et al. have

* Corresponding author at: Institute of Biochemical and Biomedical Engineering, Chang Gung University, Taoyuan 33302, Taiwan, Republic of China.
E-mail address: jylai@mail.cgu.edu.tw (J.-Y. Lai).

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proposed the use of composite electrospun gelatin fiber-alginate hydrogels as mechanically robust scaffolds for corneal tissue engineering [8]. In our laboratory, the cross-linked porous gelatin-chondroitin sulfate scaffolds are also prepared and employed in the engineering of corneal stroma [9]. Although the modification of gelatin materials with chondroitin sulfate may enhance the water content, light transmittance, and nutrient permeability, the stromal cell phenotype is not adequately maintained by the incorporation of chondroitin sulfate into the porous scaffolds.

To provide an ideal hospitable environment for corneal keratocyte cultivation, our group has recently proposed the use of biopolymer microcarrier as a possible framework for development of three-dimensional cell scaffolds [10]. Biofunctionalization of gelatin microspheres with oxidized hyaluronic acid (oHA) can effectively promote the growth of corneal keratocytes on the microcarriers and enhance the biosynthetic capacity of cultured cells. Although the three-dimensional microcarrier culture system may have a great potential in the modulation of corneal keratocyte behavior and regeneration of stromal tissue function, it is necessary to evaluate the safety of biomaterial carriers composed of proteins and oxidized polysaccharides before their use for corneal stromal regenerative medicine. Following oxidation, the HA molecules carrying aldehyde groups can be used for further reaction with amine-containing gelatin molecules. However, the aldehydes are known to have very high reactivity and tend to interact with electron-rich biological macromolecules, thereby inducing cytotoxicity [11]. Draye et al. have demonstrated that the dextran dialdehyde exhibits a negative effect on the replication of human skin fibroblasts and bovine endothelial cells after in vitro culture for 6 days [12]. In another study, Boontheekul et al. have reported the presence of aldehyde groups on the sugar residues may decrease the biocompatibility of oxidized alginate [13].

Therefore, the aim of this work was to examine the ocular biocompatibility of gelatin microcarriers (GMC) functionalized with oHA in vitro and in vivo. The cellular responses to the biopolymer microcarriers were analyzed using corneal epithelial, stromal, and endothelial cell cultures. The viability, proliferation, pro-inflammatory gene expression, and DNA damage degree were investigated to give insight into the in vitro compatibility of chemically modified microcarrier samples. On the other hand, the present study was undertaken to evaluate the tissue responses to the biopolymer microcarriers after their injection into the anterior chamber of rabbit eyes. The animals were monitored by clinical observations including slit-lamp and specular microscopic examinations, corneal topography, and corneal thickness measurements. The obtained data were used to assess the in vivo compatibility of chemically modified microcarrier samples.

2. Materials and methods

2.1. Materials

Hyaluronic acid sodium salt was obtained from Kewpie (Tokyo, Japan) as a dry powder. It was made by fermentation method and was highly purified. According to information from the supplier, the HA samples had a weight-average molecular weight of around 1100 kDa. Gelatin (type A; 300 Bloom) and 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *N*-hydroxysuccinimide (NHS) was supplied by Acros Organics (Geel, Belgium). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Phosphate-buffered saline (PBS, pH 7.4) was acquired from Biochrom AG (Berlin, Germany). Collagenase type II was purchased from Worthington Biochemical (Lakewood, NJ, USA). Dispase type II was obtained from Roche Diagnostics (Indianapolis, IN, USA). Medium 199 (M199), gentamicin, Hanks' balanced salt solution (HBSS, pH 7.4), 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (DMEM/F12), trypsin-ethylenediaminetetraacetic acid (EDTA), and TRIzol reagent were purchased from Gibco-BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) and the antibiotic/antimycotic (A/A) solution (10,000 U/ml penicillin, 10 mg/ml streptomycin and 25 μg/ml amphotericin B) were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). All the other chemicals were of reagent grade and used as received without further purification.

2.2. Preparation of oHA-functionalized GMC

The oHA and GMC were prepared according to the protocols as described elsewhere [10]. In brief, an aqueous solution was obtained by dissolution of 1 g of HA in 100 ml deionized water. Then, 5 ml of a 0.4 M solution of sodium periodate was gradually added to the HA solution. The reaction was allowed to proceed at 25 °C in the dark for 2 h. In order to inactivate unreacted periodate, ethylene glycol was added to the above solution under stirring and reacted for additional 1 h. The resulting samples were exhaustively dialyzed (MWCO 3500, Spectra/Por®Dialysis Membrane, Rancho Dominguez, CA, USA) for 3 days against deionized water. The purified product was lyophilized at −50 °C. The oxidation level of oHA samples determined using *tert*-butyl carbazate assay was 10.4 ± 0.9%. An aqueous solution of 10 wt% gelatin was also obtained by dissolution of gelatin powder in 20 ml of deionized water at 50 °C. The resulting solution was added slowly to 100 ml of olive oil preheated to 50 °C. The two phases were mixed and stirred with a rate of 350 rpm for 40 min to form a water-in-oil (w/o) emulsion. Then, the emulsion system was cooled to 5 °C in an ice bath and stirred for a further 60 min to obtain the samples. After dehydration in chilled acetone, the microspheres were isolated from the suspension by filtration, rinsed again with acetone to remove the residual oil, and dried in vacuo for 24 h. The gelatin microspheres were further processed for chemical cross-linking. An amount of 1 g of microsphere sample was suspended in an ethanol/water mixture (8:2, v/v, pH 5.0) containing 10 mM EDC and 5 mM NHS. The cross-linking reaction was allowed to proceed at 25 °C for 24 h. The reaction mixture was subsequently centrifuged at 1000 rpm for 3 min to collect the pellet of cross-linked microspheres. Samples were thoroughly washed with ethanol to remove excess EDC and urea byproduct, and were dried in vacuo for 24 h. All the microspheres were sieved through stainless steel sieves (U.S. Standard Testing Sieve, Advantech Manufacturing, New Berlin, WI, USA). Under light microscopic observations, the carbodiimide cross-linked gelatin microsphere (GMC) had an average diameter of 73.0 ± 8.1 μm. The cross-linking index of the EDC/NHS-treated GMC samples determined by ninhydrin assay was 71.6 ± 2.2%.

In this study, the preparation of GMC-oHA samples involves in the formation of Schiff base between free amino groups in protein and aldehyde groups in oxidized polysaccharide. 300 mg of GMC was suspended in an ethanol/water mixture (8:2, v/v, pH 9.0) containing 160 mg of oHA, followed by reaction at 25 °C for 24 h. Then, the reaction mixture was centrifuged and rinsed twice with 95% ethanol to obtain GMC-oHA materials. The resulting microcarrier samples were dried in vacuo for 24 h. The grafting yield of oHA onto GMC determined by alcian blue staining assay was 26.9 ± 0.1% [10].

2.3. In vitro studies

All animal procedures were approved by the Institutional Review Board and were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Twenty adult New Zealand white rabbits (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan, ROC) weighing 2.5–3.0 kg were used for in vitro biocompatibility studies. Corneal epithelial, stromal, and endothelial cells were prepared according to previously published methods [14]. The Descemet's membrane with the attached endothelium was aseptically stripped from the stroma under a dissecting microscope (Leica, Wetzlar, Germany). After digestion of the Descemet's membrane-corneal endothelium complex using 2 mg/ml collagenase