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Photo-cross-linking of amniotic membranes for limbal epithelial cell cultivation

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ABSTRACT

In the present study, we developed photo-cross-linked amniotic membrane (AM) as a limbal stem cell niche. After ultraviolet (UV) irradiation for varying time periods, the biological tissues were studied by determinations of cross-linking structure, degradability, and nutrient permeation ability. Our results showed that the number of cross-links per unit mass of AM significantly increased with increasing illumination time from 5 to 50 min. However, the cross-link formation was inhibited by longer irradiation time (i.e., 150 min), probably due to the scission of tissue collagen chains through irradiation. The biological stability and matrix permeability of photo-cross-linked AM materials strongly depended on their cross-linking densities affected by the UV irradiation. In vitro biocompatibility studies including cell viability and pro-inflammatory gene expression analyses demonstrated that, irrespective of the irradiation time employed, the physically cross-linked biological tissues exhibited negligible cytotoxicity and similar interleukin-6 (IL-6) mRNA levels. The data clearly indicate that these AM matrices do not cause potential harm to the corneal epithelial cells. After the growth of limbal epithelial cells (LECs) on AM substrates, Western blot analyses were conducted to examine the expression of ABCG2. It was found that the ability of UV-irradiated AM to maintain the undifferentiated precursor cell phenotype was significantly enhanced with increasing extent of photo-cross-linking. In summary, the UV irradiation time may have a profound influence on the fabrication of photo-cross-linked AM matrices for LEC cultivation.

1. Introduction

The human amniotic membrane (AM) is a very important biological material and has been clinically used in the treatment of several ocular surface disorders, such as thermal or chemical burns, corneal ulcers, ocular cicatricial pemphigoid, and Stevens–Johnson's syndrome [1]. A previous study from Grueterich et al. reports that the AM can serve as a niche for the ex vivo expansion of limbal epithelial stem cells [2]. In addition, the AM may play a critical role in the enhanced generation of anti-angiogenic/anti-inflammatory factors in human limbo-corneal epithelial cells that lead to rapid restoration of corneal avascularity following cultivated epithelial stem cell transplantation [3]. However, the application of AM is limited by its relatively rapid degradation and resorption in vivo [4]. To overcome these drawbacks, a strategy based on cross-linking of AM has been proposed for the reinforcement of biomaterial structures. As reported in the literature, various cross-linking agents such as glutaraldehyde [5], carbodiimide [6], and tissue transglutaminase [7] are used to improve the stability of this tissue membrane. Nevertheless, a method involving chemical modification with cross-linker probably raises concerns of safety issues. According to our earlier observations, the extent of cross-linking of biopolymers has a profound influence on the compatibility of retinal pigment epithelial cells with carbodiimide-treated gelatin membranes [8]. When the cross-linker concentration is higher than 0.1 mmol carbodiimide/mg gelatin membrane, the chemically modified tissue delivery carriers cause significant cell death. More recently, the effect of glutaraldehyde treatment time on the fabrication of robust AM materials for limbal epithelial stem cell cultivation has been studied in our laboratory [9]. Results of Live/Dead bioassays indicate that although the aldehyde groups introduced in the AM matrices are quenched with glycine, the exposure of human corneal epithelial cell cultures to test samples cross-linked for a longer duration (i.e., 24 h) leads to more intense toxicity. It is highly desirable to adopt alternatives to enhance the resistance of collagenous tissue materials to enzymatic digestion.

Gamma-ray irradiation is usually an approach to sterilize medical products. For retinal sheet encapsulation and transplantation, the gelatin carrier materials are disinfected with gamma-ray irradiation [10,11]. In general, the irradiation process may also simultaneously form bridges between the molecular chains, thereby cross-linking adjacent proteins. Spira et al. have previously demonstrated that gamma-ray irradiation is able to completely sterilize and physically cross-link human amnion collagen as an injectable biomaterial for soft tissue augmentation [12].

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Later, Fujisato et al. have investigated the influence of radiation cross-linking on the physicochemical and biodegradation properties of AM [4]. A study from Singh et al. has reported that once the gamma-irradiated AM is placed on the burn wound, the sterilized graft biomaterial may promote a favorable effect on skin epithelialization [13]. Although the gamma-ray method displays some advantages for the preparation of physically cross-linked AM, many investigators do not have access to such a valuable research facility. In terms of the choice of sterilization procedure, ultraviolet (UV) irradiation is considered to be more easily applied on a laboratory scale than gamma irradiation [14]. Our group has employed UV irradiation technique for cleaning poly(*N*-isopropylacrylamide)-grafted culture surfaces that allow for noninvasive harvest of bioengineered human corneal endothelium [15]. More recently, in order to fabricate a transparent and bioinert keratoprosthesis, we have copolymerized the 2-hydroxyethyl methacrylate monomer with acrylic acid by UV photoinitiation [16,17].

For a UV photoinitiation system of radical polymerization, the reaction time is found to be one of the most important factors. With increasing UV illumination time from 10 to 50 min, the Young's modulus and suture strength of poly(2-hydroxyethyl methacrylate)-*co*-poly(acrylic acid) hydrogel membranes significantly increased, indicating the enhancement of mechanical stability [16]. Given that the performance of photopolymerized hydrogels for artificial cornea applications is closely linked to the UV irradiation-mediated change in their cross-linked structure, we therefore hypothesize that the reaction time will influence the extent of photo-cross-linking of AM and its potential use in limbal epithelial cell (LEC) cultivation. To the best of our knowledge, the development of UV-irradiated cross-linked AM as a LEC scaffold is not yet available. The aim of this work was to optimize the procedure of photo-cross-linking of AM materials. After UV irradiation for varying time periods, the biological tissues were studied by determinations of cross-linking structure, degradability, and nutrient permeability. Human corneal epithelial cell line cultures were used for the assessment of in vitro biocompatibility of physically cross-linked AM. Cell viability assays and pro-inflammatory gene expression analyses were performed. After cultivation of LECs on AM substrates, Western blot analyses were conducted to examine the expression of ABCG2. The understanding of stem cell–biomaterial interactions would facilitate the engineering of an appropriate extracellular microenvironment.

2. Materials and methods

2.1. Materials

This study followed the tenets of the Declaration of Helsinki involving human subjects and received approval from the Institutional Review Board of our institution. Human AM tissues (i.e., the innermost layer of the placental membranes) were obtained with informed consent at the time of elective cesarean section from mothers when human immunodeficiency virus, syphilis, and hepatitis B and C had been excluded by serologic tests. The separation of AM was carried out using blunt dissection to cut the tissue samples approximately 2 cm from the placental disc. The average thickness of AM samples used in this work was 100 μm. Riboflavin-5-monophosphate, dextran, collagenase (type I *Clostridium histolyticum*, EC 3.4.24.3), and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water used was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Phosphate-buffered saline (PBS, pH 7.4) was purchased from Biochrom (Berlin, Germany). Balanced salt solution (BSS, pH 7.4) was obtained from Alcon Laboratories (Fort Worth, TX, USA). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). Dispase II was purchased from Roche Diagnostics (Indianapolis, IN, USA). FNC Coating Mix (i.e., a fibronectin/collagen mixture) was obtained from Athena ES (Baltimore, MD, USA). Dulbecco's modified Eagle's medium (DMEM), keratinocyte serum-free medium (KSFM), Ham's F-12 nutrient mixture (Ham's F-12), gentamicin, 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). 24-well tissue culture polystyrene (TCPS) plates (Falcon 353047) were purchased from Becton Dickinson Labware (Franklin Lakes, NJ, USA). All the other chemicals were of reagent grade and used as received without further purification.

2.2. Preparation of photo-cross-linked amniotic membranes

The AM samples were aseptically washed three times with PBS containing 1% A/A solution and 50 μg/ml of gentamicin according to the protocols reported previously [18]. The membranes were immersed with sequential concentrations of DMSO, followed by freezing and storing at −80 °C in DMEM containing 50% glycerol. After a further incubation with 0.02% EDTA at 37 °C, the AM was denuded of its amniotic epithelial cells by gentle scraping. For photo-cross-linking of biological tissues, the riboflavin-5-monophosphate was dissolved in PBS containing 20% dextran at a concentration of 1 mg/ml. Then, the riboflavin-treated AM materials were subjected to irradiation using the Blak-Ray high intensity UV lamp (UVP, Upland, CA, USA) with 365 nm. Samples were placed at a distance of 25 cm from the radiation source and exposed for different time periods (i.e., 0–150 min). In this study, the AM photo-cross-linked by UV irradiation for 150 min was designated as m150.

2.3. Cross-linking density measurements

The cross-linked structure of the UV-irradiated AM such as degree of cross-link and average molecular weight of polymer chains between two consecutive junctions was analyzed according to the method reported previously [16]. After immersion in deionized water for 12 h at 25 °C, the photo-cross-linked membranes (20 mm × 10 mm) were mounted between two clamps of an Instron Mini 44 universal testing machine (Canton, MA, USA). The lower clamp was then adjusted downward until the sample was just in tension and the unstressed length was noted. Following the determination of mechanical properties, the test specimens were removed from the clamps and blotted with tissue paper, and the density was determined by the specific gravity bottle method. A graph of σ against $(\alpha-\alpha^{-2})$ would be a straight line with the slope giving $RT\rho V^{1/3}/M_c$, where σ = the force per unit area of the swollen unstretched sample; α = extension ratio; R = gas constant; T = absolute temperature; ρ = density of sample; V = volume fraction; and M_c = average molecular weight of the chains between cross-links. The number of cross-links per unit mass would be given by $(2M_c)^{-1}$. Results were the average of five independent measurements.

2.4. In vitro degradation tests

To measure the extent of degradation, each test AM (1×1 cm^2) was first dried to constant weight (W_i) in vacuo and was immersed in 1 ml of BSS containing 12 μg collagenase at 37 °C with reciprocal shaking (50 rpm) in a thermostatically controlled water bath. After 3 days, the membrane samples were taken out and washed with deionized water. The degraded samples were further dried in vacuo and weighed to determine the dry weight (W_d). The percentage of weight remaining (%) was calculated as $(W_d/W_i) \times 100$ [19,20]. Results were the average of four independent measurements.