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Preparation of TGF-β1-conjugated biodegradable pluronic F127 hydrogel and its application with adipose-derived stem cells

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ABSTRACT

In this study, a composite hydrogel using Pluronic F127 derivatives and crosslinked hyaluronic acid (X-HA) was investigated, exploring the benefits in the induction of chondrogenic differentiation of human adipose-derived stem cells (ASCs). F127 was chemically modified through a series of reactions that produced multiple F127 derivatives. A chondrogenic growth factor, transforming growth factor-beta 1 (TGF-β1) was then coupled to the heparin-conjugated F127. X-HA was used as a physical stabilizer of the composite hydrogel (X-HA/F127). The chemical structures of F127 derivatives were analyzed using ^{1}H-NMR and ATR-FTIR. Sol–gel transition of the composite hydrogel was identified at body temperature. The conjugated TGF-β1 was moderately released *in vitro* from the composite hydrogel. Cell viability of human ASCs in the hydrogels was about 50% after *in vitro* culture for 3 days. As the ASCs/hydrogel were injected into nude mice subcutaneously, DAPI staining of the retrieved constructs showed that ASCs were dispersed through the hydrogel matrix. From the immunofluorescent staining of type II collagen, the TGF-conjugated group exhibited more active green signals than the others. In addition, when those constructs were loaded into the full-thickness defect of rabbit knee articular cartilage, Alcian blue staining identified the formation of cartilaginous matrix from the TGF-conjugated hydrogel. The present work indicated that X-HA/F127 composite hydrogel was thermoreversible and biodegradable, and that the TGF-conjugated hydrogel could be effective in inducing chondrogenesis of human ASCs.

1. Introduction

Thermosensitive and injectable hydrogels are of particular interest in clinics, due mainly to their minimally invasive nature for human applications. Hydrogels have a structural similarity to the natural macromolecules found in the body and they are thus generally recognized biocompatible. Therefore, hydrogels have been a very useful tool in drug delivery and tissue engineering. Specifically focused on tissue regeneration, stem cells are often encapsulated in hydrogels and these constructs were either cultured *in vitro* or implanted for *in vivo* test [1–3]. In this study, Pluronic® F127 was chemically modified in order to directly couple a chondrogenic growth factor, transforming growth factor-beta 1(TGF-β1) with F127 hydrogel. If properly worked, this system could be effective for the induction of *in situ* stem cell differentiation and resultant cartilaginous tissue formation *in vivo*. Because synthetic hydrogels are rather inert, they are often modified in order to add specific functionality to nascent ones. For example, both hydrogel and cells are physically mixed with some growth factors in order to elicit specific cellular responses. When insulin-like growth factor (IGF)-1 and/or TGF-β2 were included in a thermoreversible polymer, poly(N-isopropylacrylamide-co-n-butyl methacrylate) (polyNIPAAm-co-BMA) with bovine chondrocytes, the presence of growth factors significantly boosted the production of cartilage-specific matrices after 16 weeks of *in vitro* culture [4]. As bioactive molecules-loaded microparticles are encapsulated in hydrogels, the slowly released molecules are intended to manipulate cellular behaviors. Park et al. reported that the use of TGF-β3-loaded nanoparticles with fibrin hydrogel prompted *in vitro* and *in vivo* chondrogenesis of rabbit bone marrow-derived stromal cells [5]. In an oligo(poly(ethylene glycol) fumarate) (OPF) hydrogel, gelatin microparticles containing TGF-β1 were also effective for bovine chondrocytes in upregulating cell proliferation and matrix production [6].

F127 is a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymer that can undergo sol–gel transition, depending on its concentration and ambient temperature. It has water-solubility and carries a low toxicity. Approved by the FDA for use in the human body, F127 has been investigated as a delivery vehicle of indomethacin and interleukin-2, and as a wound healing dressing for thermal burns [7]. In addition, F127 is also utilized for tissue engineering applications: tissue-engineered lung using F127/somatic lung progenitor cell constructs, adipose differentiation of MSCs using F127 hydrogel *in vitro*, and bone repair using hydroxyapaite and biphasic calcium phosphate combined with F127 [8–10]. In case of using F127 in making tissue-engineered cartilage, F127 was served as an

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injectable cell carrier to regenerate a cartilage in the nude mouse model [11]. Despite the multiple benefits of F127, F127 itself is rather weak in mechanical properties and thus the maintenance of physical shape is barely possible during a long-term *in vitro* culture of F127-encapsulated cells. To reinforce the mechanical stability of F127, crosslinked hyaluronic acid (X-HA) was introduced as a constituent of composite hydrogel. HA is a long linear chain of non-sulfated glycosaminoglycan (GAG), serving as a backbone of cartilage extracellular matrix (ECM).

In this work, we hypothesize that conjugated TGF-β1 with a biodegradable F127 polymer derivative may play a role in triggering chondrogenesis of ASCs whereas a physical stability of X-HA/F27 composite hydrogel is maintained. In fact, there are few examples, in which hydrogel is chemically modified to directly conjugate biomolecules for stem cell differentiation. Once a composite hydrogel was prepared, its thermoreversibility was evaluated in varying concentrations of F127. Furthermore, after human ASCs-encapsulated hydrogel constructs were subcutaneously injected into nude mouse or implanted in the full-thickness defect of rabbit knee articular cartilage, the effects of the conjugated TGF-β1 on the induction of chondrogenesis of ASCs were evaluated.

2. Materials and Methods

2.1. Materials

Pluronic F127 (Mw: 12,600) and hyaluronic acid (HA, Mw: 1,600 K) were purchased from Sigma-Aldrich (St. Louis, MO). Heparin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES), and dexamethasone (Dex) were obtained from Sigma (St. Louis, MO). Recombinant human TGF-β1 and enzyme linked immunosorbent assay (ELISA) kit were purchased from R&D Systems (Minneapolis, MN). Dulbecco's modified eagle's medium (DMEM-HG), fetal bovine serum (FBS), and penicillin-streptomycin were obtained from Gibco (Gaithersburg, MD). Ascorbate-2-phosphate, ITS™$^+$ premix, sodium pyruvate, proline, and Histopaque®-1077 were obtained from Sigma-Aldrich. Other chemicals were of reagent grade and used as received.

2.2. Synthesis of Pluronic F127 Derivatives

Pluronic F127 was subjected to a series of chemical reactions to introduce glycolide (G), 4-methacryloxyethyl trimellitic anhydride (META), and heparin, respectively. After both F127 (4.5 g) and glycolide (0.43 g) were dissolved in toluene (50 ml), they were added with Tin(II) 2-ethylhexanoate, a catalyst that was dissolved in toluene. The mixture was then gently stirred at 140 °C for 6 h in reflex. The reactants were transferred in chloroform and precipitated in n-hexane with a vigorous stirring. This process was repeated twice for purity. The product was then filtered and the residual solvent was evaporated under vacuum at room temperature for 2 days. The obtained F127-glycolide (FG) (4.12 g) was dissolved again in toluene (50 ml) with the addition of 4-META (0.228 g), followed by the addition of anhydrous pyridine as another catalyst. As the mixed solution was stirred under a nitrogen gas for 12 h, the resultant products were precipitated in n-hexane and filtered, then evaporated under vacuum for 2 days. For the grafting of heparin to F127-glycolide-META (FGM), FGM (1.63 g) was dissolved in MES buffer (pH 4.6) and a carboxyl group of META was then activated using EDC at room temperature for 4 h under shaking. Heparin was then added and stirred in the activated FGM solution for 24 h. The reactants including both FGM and heparin were dialyzed by using a membrane (Mw cut-off: 16,000; Spectrum Medical Industries) for 5 days to remove the unreacted byproducts. After the dialysis, the product was freeze-dried for 3 days at −60 °C to obtain FGM-hep (referred to as FH hereafter). The final product, FH was stored in the refrigerator for future use, because it was sensitive to the ambient temperature.

2.3. Characterization of F127 Derivatives

The chemical structures of the synthesized F127 derivatives were analyzed using attenuated total reflection-Fourier transform infrared (ATR-FTIR) and ^{1}H Nuclear Magnetic Resonance (NMR), respectively. For the measurement of thermosensitivity, the sol–gel transition of F127 derivative hydrogels was also examined using a tube tilting method in varying polymer concentrations and in different temperatures.

2.4. Preparation of TGF-β1 Conjugated Hydrogel and Release Test

When the FH hydrogel was physically mixed with 400 ng of TGF-β1 in PBS solution, they were allowed to react for 24 h at 4 °C, rendering TGF-β1 to be coupled with heparin domain. The obtained product, TGF-β1-conjugated F127 hydrogel (FH-TGF), was freeze-dried for 3 days at −60 °C and then kept in the refrigerator, because the product easily melted at room temperature. Meanwhile, HA was dissolved at a concentration of 4 wt% in 0.1 N NaOH for 2 h in refrigerator and then chemically crosslinked using glycerol diglycidyl ether (GDE). To make the crosslinked HA (XH), GDE (200 mM) in ethanol solution was prepared and 100 μl of the solution was added in 2% HA solution for crosslinking. The final concentration of GDE was adjusted to 20 mM. It was sonicated for 30 min at 40 °C and poured in 300 ml of 1 M NaCl and 700 ml EtOH, then subjected to neutralization using 0.1 N HCl for 2 days. The X-HA was dialyzed with distilled water for 2 days, autoclaved, and finally lyophilized. For a composite hydrogel, the final concentration (w/v) of X-HA and F127 was adjusted to 10 and 20%, respectively. For an *in vitro* release test of TGF-β1, the composite hydrogels were put in the 12-well plate and incubated for 10 min at 37 °C for gellation. As the hydrogel samples ($n=3$) remained for up to 20 days under static condition, the phosphate buffer saline (PBS) solution was collected at the specific time points and replenished with a fresh one. The amount of the released TGF-β1 was quantitatively determined using ELISA. The growth factor mixed with X-HA hydrogel was also tested for a comparison under the same protocol as mentioned above.

2.5. Isolation of Human ASC and In Vitro Culture

Human adipose-derived stem cells (ASCs) were harvested from the subcutaneous adipose tissue of a female donor during liposuction. This procedure was carried out under her voluntary consent. After the collagenase digestion and differential centrifugation, the isolated cells were cultured in the incubator at 37 °C with 5% CO_2 supply in DMEM supplemented with 10% FBS and 1% antibiotics. When ASCs were confluent at passage 4, they were collected using 0.25% Trypsin and total cell number was counted using a hemacytometer. For cell viability test, the suspended ASCs were mixed with the composite hydrogels in 12-well plate, where the cell/hydrogel mixtures were thoroughly blended using pipettes at room temperature. The final concentration of polymer solution was 20 wt% (w/v) for F127. The ASC/hydrogel constructs were immediately incubated for gellation at 37 °C and then cultured *in vitro* for up to 3 days. The viable cells were visualized using a live/dead assay.

2.6. Nude Mouse Implantation of ASC/Hydrogel Constructs

ASCs (1×10^6) were blended with the composite hydrogels, in a serum-free DMEM supplemented with 1% penicillin-streptomycin, 50 mg/mL ITS™$^+$ premix, 50 μg/mL ascorbate-2-phosphate, 100 μg/mL sodium pyruvate, and 40 μg/mL proline. The total injection volume was 250 μl, containing the cell suspension (20 μl) and composite hydrogel (230 μl). A free form of Dex (D), another chondrogenic factor, was also included in the hydrogels. For the subcutaneous injection of hydrogels, the nude mice ($n=10$) were anesthetized using ketamine and Rompun. Each carried two samples on its back. Test groups were X-HA/F (control),