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Bone regeneration using hyaluronic acid-based hydrogel with bone morphogenic protein-2 and human mesenchymal stem cells

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Abstract

Acrylated hyaluronic acid (HA) was used as a scaffold for bone morphogenic protein-2 (BMP-2) and human mesenchymal stem cells (hMSCs) for rat calvarial defect regeneration. HA was acrylated by two-step reactions: (1) introduction of an amine group using adipic acid dihydrazide (ADH); (2) acrylation by *N*-acryloxysuccinimide. Tetrathiolated poly(ethylene) glycol (PEG-SH_4) was used as a cross-linker by a Michael-type addition reaction and the hydrogel was formed within 10 min under physiological conditions. This hydrogel is degraded completely by 100 U/ml hyaluronidase *in vitro*. hMSCs and/or BMP-2 was added during gelation. Cellular viability *in vitro* was increased up to 55% in the hydrogels with BMP-2 compared with the control. For *in vivo* calvarial defect regeneration, five different samples (i.e., control, hydrogel, hydrogel with BMP-2, hydrogel with MSCs, and hydrogel with BMP-2 and MSCs) were implanted for 4 weeks. The histological results demonstrated that the hydrogels with BMP-2 and MSCs had the highest expression of osteocalcin and mature bone formation with vascular markers, such as CD31 and vascular endothelial growth factors, compared with the other samples. This study demonstrated that HA base hydrogel can be used for cell and growth factor carriers for tissue regeneration.

Keywords: Hyaluronic acid; Hydrogel; Bone regeneration; Bone morphogenic protein; Mesenchymal stem cells

1. Introduction

Hyaluronic acid (HA) is one of the major components of the extracellular matrix (ECM) and is found in all connective tissues of the body. It is a naturally derived, linear, high molecular weight polymer with visco-elastic properties [1]. HA is involved in biological processes, such as morphogenesis, wound repair, inflammation, and metastasis through cellular receptors [2–5]. Due to its visco-elastic properties and biological functions, HA has been used predominantly as a raw material for hydrogel [6,7]. The carboxyl group of HA is one of the major targets for derivatizing HA. Prestwich et al. [7] modified the carboxyl groups of HA with mono- and poly-functional hydrazides. Bulpitt and Aeschlimann [8] derivatized HA with amino or aldehyde functional groups. Leach et al. [9,10] modified HA with methacrylate groups and prepared glycidyl methacrylate-HA (GMHA) conjugates, which were subsequently photo-polymerized to form cross-linked GMHA hydrogels. Park et al. [11] produced a HA derivative with methacrylic esters via photopolymerization for the preparation of the hydrogels.

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For the regeneration of bone defects, tissue-engineering studies combine cells with an appropriate scaffolds and osteogenic signals to stimulate bone repair. Shea et al. [12] engineered bone development from a pre-osteoblast cell line on a three-dimensional scaffold using copolymers of D,L-lactide and glycolide (PLG). Kim et al. [13] investigated the effect of three-dimensional silk fibroin scaffold preparation methods (aqueous and solvent) on osteogenic responses by human bone marrow stem cells (hMSCs). Hydrogels have been used also for bone tissue repair with fully differentiated cells or adult and embryonic stem cells. Chen et al. [14] studied the generation of new bone in the cell–hydrogel complex using temperature-dependent polymerizing polyethylene oxide hydrogel as a vehicle to deliver bone marrow mesenchymal cells by injection in six nude mice. Nuttelman et al. [15] developed a dexamethasone-releasing poly(ethylene glycol) (PEG)-based hydrogel scaffold to deliver dexamethasone to encapsulated human mesenchymal stem cells in a sustained manner and demonstrated that encapsulated hMSCs are capable of osteogenic differentiation in response to released dexamethasone.

In the previous study, HA was methacrylated and hydrogel was prepared by photopolymerization [11]. Michael-type addition reaction was used for gelation of hydrogel [16,17]. In addition, this reaction was applied to the methacrylate HA-based hydrogels [18]. Due to its relatively lower reactivity of the methacryl group, more than 8 h are required for gelation. Even though methacrylated HA can be used for drug delivery [19], this approach has limitations as a delivery vehicle of sensitive materials, such as cells, and for in situ polymerization, which requires a shorter gelation time for higher viability.

In this study, we developed novel HA-based hydrogel with faster gelation time. We evaluated *in vivo* the bone-forming activity of hydrogels with stem cells and/or bone morphogenic protein-2 (BMP-2) and this hydrogel successfully deliver the stem cells and BMP-2 to the bone defect and regenerated the new bones. Bone forming activity is highly dependent on the delivering materials, such as stem cells and BMP-2.

2. Materials and methods

2.1. Materials

HA (MW 170,000 Da) was purchased from Lifecore Biomedical Co. (Chaska, MN, USA) and adipic acid dihydrazide (ADH) and 1-hydroxybenzotriazole hydrate (HOBT) were purchased from Fluka Chemical Co. (Buchs, Switzerland). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodimide and triethanolamine were acquired from Sigma-Aldrich, Inc. (St. Louis, MO, USA). PEG tetra-thiols (PEG-SH_4) (MW10,000 Da) were purchased from Sun Bio, Inc. (Orinda, CA, USA). *N*-acryloxysuccinimide (NAS) was purchased from Polyscience, Inc. (Warrington, PA, USA).

Carrier-free recombinant human BMP-2 was purchased from R & D Systems (Minneapolis, MN, USA), as were anti-VEGF and anti-osteocalcin antibodies. Anti-human CD 31 antibody was obtained from DAKO (Glostrup, Denmark) and human-specific anti-nucleolin antibody was purchased from Stressgen Biotechnologies Corp. (Victoria, BC, Canada). vectastain® kit and DAB was obtained from Vecta Laboratories (Burlingame, CA, USA). Dexamethasone, alkaline phosphatase (ALP) assay kit, ascorbic acid, and β-glycerophosphate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ficoll-paque plus was obtained from Amersham Biosciences (Uppsala, Sweden). Fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were purchased from GIBCO BRL (Carlsbad, CA, USA). Low- and high-glucose Dulbecco's Modified Eagle's Medium (DMEM) was purchased from JBI (Daegu, Korea) or Gibco BRL (Carlsbad, CA, USA).

2.2. Acrylation of HA and preparation of hydrogel

HA (0.25 mmol, based on the repeating unit MW) was dissolved in 40 ml of distilled water and EDC (0.24 g, 1.25 mmol), HOBT (0.17 g, 1.25 mmol) and adipic acid dihydrazide (ADH) (2.2 g, 12.5 mmol) were added to the solution [20]. The EDC mediated coupling reaction between the carboxyl group of HA and the hydrazide group of ADH continued, with stirring, at room temperature for 8 h. HA–ADH was dialyzed against 100 mM NaCl for 2.5 days and distilled water for 1 day, using a dialysis membrane (MWCO14,000, SpectraPor; Rancho Dominguez, CA, USA). NAS (0.5 g, 3 mmol) was subsequently added to the HA–ADH solution. The reaction continued, with stirring, at room temperature for 12 h. HA–ADH–NAS was dialyzed extensively against 100 mM NaCl for 2.5 days and distilled water for 1 day. The product was then lyophilized for 3 days to obtain solid acrylated HA (HA-Ac). The NMR spectra were obtained on a Mercury 200 NMR (200 MHz) from Varian (Lake Forest, CA, USA). D_2O was used as a solvent for all the samples and the reported spectra represented an average of 64 scans. The degree of acrylation was calculated by comparing peaks from the acryl and methyl groups from the HA residue. For gel preparation, acrylated HA was dissolved in a triethanolamine-buffered solution (TEA; 0.3 M, pH 8). PEG-SH_4 (MW 10,000) was added as a cross-linker with the same molar ratio of acryl and thiol groups. HA-based hydrogel was formed via a Michael-type addition reaction [17]. The reaction mixture was incubated at 37 °C for gelation. This hydrogel (5% wt of HA and PEG-SH_4) was used in both *in vitro* and *in vivo* experiments (Table 1).

2.3. Evaluation of the mechanical properties of hydrogels

Rheological behaviors of HA-based hydrogels were analyzed with a Rotational Rheometer Gemini (Bohlin Instruments Ltd.; Pforzheim, Germany). The gelation process occurred over the 1 ml mixture solution of HA-Ac and the cross-linker, PEG-SH_4, on the sandblast parallel plate (diameter 15 mm) under the conditions of a time sweep at 37 °C, with a 500 μm gap, 0.1% strain, and frequency sweep at 0.1–10 rad/s and a strain of 0.1% at 20 °C. Gelation was monitored for 1 h by observing the viscous modulus and the elastic modulus.

In order to measure the swelling properties of hydrogel, it was incubated in PBS overnight at room temperature. The swelling ratio was measured by comparing the change of the wet weight of hydrogel before and after incubation. The percentage of water absorbed was calculated by the following formula:

$$\text{Swelling ratio } (\%) = \{(W_w - W_i)/W_i\} \times 100\%,$$

where W_w is the wet weight of hydrogel; W_i the initial weight of hydrogel.

Degradation of the hydrogel by hydrolysis was measured by incubating pre-swollen hydrogels in PBS up to 2 days. The weight loss of the hydrogel was measured for 20 h. Degradation by hyaluronidase was also monitored by measuring the weight loss of the hydrogel. Hyaluronidase was added to PBS buffer solution to a final concentration of 100 U/ml. The pre-swollen hydrogel was added to the buffer and incubated at 37 °C. The weight loss of the hydrogel was measured after 2, 4, and 20 h.

2.4. In vitro cell culture and cellular viability evaluation

Human mesenchymal stem cells were purified from bone marrow with the permission of the donors. Mononuclear cells were purified using Ficoll density gradients. The cells were cultured on the tissue culture plates and