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Heparin-hyaluronic acid hydrogel in support of cellular activities of 3D encapsulated adipose derived stem cells

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ABSTRACT

We have developed stem cell-responsive, heparin-hyaluronic acid (Hep-HA) hydrogel, crosslinked by thiolated heparin (Hep-SH) and methacrylated hyaluronic acid (HA-MA) *via* visible light mediated, thiol-ene reaction. Physical properties of the hydrogel (gelation time, storage modulus, and swelling ratio) were tunable by adjusting light intensity, initiator/polymer concentration, and precursor pH. Culture of human adipose derived mesenchymal stem cells (ADSCs) using this hydrogel was characterized and compared with the control hydrogels including Hep-PEG hydrogel, PEG-HA hydrogel. Sufficient initial adhesion and continuous proliferation of ADSCs in 2D were observed on both heparin-containing hydrogels (Hep-HA and Hep-PEG hydrogel) in contrast to no adhesion of ADSCs on PEG-HA hydrogel. On the other hand, in the case of 3D culture of encapsulated ADSCs, efficient cellular activities such as spreading, proliferation, migration, and differentiation of ADSCs were only observed in soft Hep-HA hydrogel compared to Hep-PEG or PEG-HA hydrogel with the similar modulus. The upregulated expressions of hyaluronidases in ADSCs encapsulated in Hep-HA hydrogel compared to the control hydrogels and effective degradation of the hydrogel by hyaluronidase imply that the degradation of hydrogel was necessary for 3D cellular activities. Thus, Hep-HA hydrogel, where heparin acts as a binding domain for ADSCs and HA acts as a degradation site by cell secreted enzymes, was efficient for 3D culture of human ADSCs without any additional modification using biological/chemical molecules.

Statement of Significance

Stem cell-responsive hydrogel composed of heparin and hyaluronic acid was prepared by visible light-mediated thiol-ene reaction. Without additional modification using functional peptides for cell adhesion and matrix degradation, ADSCs encapsulated in this hydrogel showed efficient cellular activities such as spreading, proliferation, migration, and differentiation of ADSCs whereas control hydrogels missing heparin or hyaluronic acid could not support cellular activities in 3D. In this hydrogel, heparin mainly acts as a binding domain for stem cells and hyaluronic acid mainly acts as a degradation site by ADSC secreted enzymes, but interrelated synergistic functions of heparin and HA were observed. Therefore, we speculate that this hydrogel can serve as a promising carrier for stem cell based therapy and various tissue engineering applications.

1. Introduction

Cell therapy using human mesenchymal stem cells has been widely investigated in tissue engineering and regenerative medicine due to their characteristics of self-renewal, immune tolerance, and pluripotency [1]. Especially, adipose derived mesenchymal stem cell (ADSC) is a major cell source which has least ethical problem and abundant cell source donation [2]. For the effective therapeutics, successful carrier of living cells is necessary to replace defective, degenerated, or damaged tissues [3].

Hydrogels are widely used as a scaffold for cell therapy or tissue engineering due to their mechanical properties, multi-tunability, intrinsic biocompatibility, and easy fabrication [4]. Hydrogel can potentially mimic the native ECM with similar soft/flexible structures and high water content, so it can be designed to allow enhanced cell viability, proliferation [5], and retention [6].

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The works in 2D culture system have provided significant information on how stem cells feel and respond to their microenvironment in terms of migration, proliferation, survival, and fate decision [7]. However, there are significant differences in stem cell functions when cultured in 3D compared to 2D including cell morphology, proliferation rate, and differentiation as well as gene and protein expression profiles [8]. In terms of cell morphology, cell attachment occurs on one side of the cell in 2D culture, whereas cell attachment occurs around the entire cell surface in 3D culture [9]. In terms of proliferation rate [10], initial cell attachment and spreading [11] on 2D substrates occur rapidly, whereas cell attachment and spreading take place slowly due to their physical environment surrounded by hydrogel in 3D culture. Furthermore, in most cases, 2D culture models support limited cell differentiation and *in vivo*-like functionality compared to 3D culture model [12]. Collectively, 3D cell culture systems can provide various degrees of cell complexity and functionalities that are observed *in vivo* due to improved cell–cell interactions and cell-matrix interactions. Therefore, various 3D hydrogel systems have been developed to mimic the native ECM by incorporating ECM components (for e.g., collagen, fibrin, elastin, hyaluronic acid) into the hydrogel for better understanding of stem cell functions *in vitro* and for potential *in vivo* applications [13].

Stem cells require a lot of environmental cues for their cellular activities [14]. Environmental cues implemented inside hydrogels include cell adhesion site, matrix degradation, topography, mechanical strength, and presentation of growth factors [15,16]. Among them, cell adhesion site is the most basic cue since it is essential for survival of adherent cells including mesenchymal stem cells [17]. Typically, cell adhesive ligands such as short peptide including RGD and cell adhesive biomolecules including collagen/gelatin are incorporated into hydrogel to provide cell adhesion, proliferation, survival as well as control of stem cell fate [18]. In addition, degradability of hydrogel is also considered to be essential for achieving desired cellular activities of stem cells to proliferate and migrate inside the hydrogel as well as to deposit matrix components toward the formation of functional tissue structures [19,20].

Several water soluble polymers have been applied to form hydrogel for 3D stem cell culture. Among them, hyaluronic acid (HA) has been most extensively studied, which is one of the major components of glycosaminoglycans in many tissues and can be enzymatically degraded by hyaluronidase secreted from various cells including stem cells [21]. It has been reported that MSCs interact with HA *via* surface receptors (CD44) and enhanced hyaluronidase expression by HA coating [22]. However, there are many reports showing that proper cellular activities of HA hydrogels were obtained only after modification with peptide or ECM materials. MSC cultured in the RGD and degradation site-incorporated hydrogels showed dramatic cell spreading compared to non-functionalized HA hydrogels [23]. Similarly, RGD and/or MMP-sensitive peptide-conjugated HA hydrogel showed that migration and spreading of MSCs were observed in both functional peptides modified HA hydrogel only [18]. Fibronectin fragment incorporated HA hydrogel also showed the significantly enhanced stem cell attachment and spreading compared to non-functionalized HA hydrogel [24]. Encapsulation of hMSCs into RGD/MMP peptide incorporated HA hydrogel resulted in increased viability and directed cell fates by degradation-mediated tractions in contrast to control HA hydrogel [25]. Photocrosslinked hybrid HA/gelatin hydrogels were designed for the potential application of stem cell transplantation, showing the importance of bioactive signal in the hydrogel for the cellular functions of encapsulated cells [26]. Thus, HA hydrogel alone was not enough to provide sufficient cellular activities for stem cells, requiring further modification of cell adhesive ligand and degradation site to HA hydrogels.

In our previous study, we demonstrated that soft heparin-based hydrogel, composed of heparin (Hep) and poly(ethylene glycol) (PEG) linker was excellent for 2D stem cell cultivation [27]. Selective adhesion, proliferation, and maintenance of stemness of MSCs as well as desired differentiation in an induction media on this Hep-PEG hydrogel were observed, showing the effectiveness of heparin as a binding site for stem cells. However, as previously discussed and will be presented later in the results of this study, this Hep-PEG hydrogel was not suitable for 3D cultivation of encapsulated stem cells. Thus, in this study, we prepared hydrogel composed of heparin and hyaluronic acid using thiolated heparin (Hep-SH) and methacrylated hyaluronic acid (HA-MA) *via* thiolene photopoylmerization reaction without additional functional peptide such as RGD or MMP degradable peptide. We employed eosin Y (EY) as an initiator and visible light [28] for photopolymerization to achieve better biocompatibility of gel forming chemical reaction instead of hydrophobic initiator with a low water solubility and UV light which can impair cytocompatibility due to the production of cytotoxic free radicals that could damage cellular proteins and DNA [29]. Human ADSCs were encapsulated and cultured in 3D inside the Hep-HA hydrogel and cellular activities of encapsulated cells were compared with those cultured in the control hydrogels including Hep-PEG and PEG-HA hydrogel to elucidate the roles of each component. We also analyzed the expression of hyaluronidases in ADSCs encapsulated in the hydrogels to seek the origin of the hydrogel degradation.

2. Experimental

2.1. Materials

Heparin (sodium salt, from porcine intestinal mucosa, Mw 12 kDa) was obtained from Celsus Ins. (Cincinnati, OH, USA). 6-arm poly(ethylene glycol) sulfhydryl (6-arm PEG-SH, Mw 10 kDa) and 4-arm poly(ethylene glycol) acrylate (4-arm PEG-AC, Mw 10 kDa) were purchased from Sunbio Inc. (Anyang, Korea). Hyaluronic acid (HA, Mw 43 kDa) was purchased from Bloomage Freda Biopharm Co., Ltd. (Jinan, CN). Methacrylic anhydride (MA), acridine orange (AO), propidium iodide (PI), and tetramethylrhodamine B isothiocyanate (TRITC)-labeled phalloidin were obtained from Sigma-Aldrich (St. Louis, MO, USA). Eosin Y (D&C Red 22, code no. 25DA0500) was purchased from Emerald Hilton Davis (Cincinnati, OH, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were all purchased from GIPCO (Grand Island, NY, USA). Cell proliferation reagent (WST-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Mounting medium with 4,6-diamidino-2-phenylindole (DAPI), human adipose derived stem cell (ADSC), MesenPRO RS™ medium, and StemPro Adipogenesis Differentiation Kit were purchased from Invitrogen (Carlsbad, CA, USA). AccuPower RocketScript™ Cycle RT PreMix, AccuPower 2× GreenStar qPCR Master Mix, and PCR primers were obtained from Bioneer (Daejeon, Korea).

2.2. Synthesis of polymer precursors

Two polymer precursors, methacrylated hyaluronic acid (HA-MA) and thiolated heparin (Hep-SH), were synthesized for the preparation of photocrosslinked hydrogel. To synthesize HA-MA, 1% (w/v) HA solution was reacted with 3-fold molar excess amount of methacrylic anhydride (MA) for 12 h in the dark at 4 °C while maintaining pH between 8 and 11 using 1 N NaOH [30]. The final product (HA-MA) was precipitated in cold ethanol and purified by dialysis against deionized water (DIW) using a dialysis membrane (3.5 kDa Mw cut-off) to remove unreacted reagents. Purified