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Synthesis and characterization of matrix metalloprotease sensitive-low molecular weight hyaluronic acid based hydrogels

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Abstract Hyaluronic acid is a naturally derived glycosaminoglycan (GAG) involved in biological processes. A low molecular weight hyaluronic acid (50 kDa)-based hydrogel was synthesized using acrylated hyaluronic acid. Matrix metalloproteinase (MMP) sensitive hyaluronic acid-based hydrogels were prepared by conjugation with two different peptides: cell adhesion peptides containing integrin binding domains (Arg-Gly-Asp: RGD) and a cross-linker with MMP degradable peptides to mimic the remodeling characteristics of natural extracellular matrices (ECMs) by cell-derived MMPs. Mechanical properties of these hydrogels were evaluated with different molecular weights of acrylated hyaluronic acid (10 kDa and 50 kDa) cross-linked by MMP sensitive peptides by measuring elastic modulus, viscous modulus, swelling ratio and degradation rate. The MMP sensitive hydrogel based on the 50 kDa hyaluronic acid showed a 31.5-fold shorter gelation time, 4.7-fold higher storage modulus and 0.51-fold smaller swelling ratio than those of the hydrogel based on the 10 kDa. Degradation rate was dependent on MMP sensitivity of the peptide cross-linker. MMP sensitive hyaluronic acid based hydrogels were degraded faster than MMP insensitive-hyaluronic acid-based hydrogels. Human mesenchymal stem cells (MSCs) were cultured in MMP-sensitive or insensitive hyaluronic acid-based hydrogels (50 kDa hyaluronic acid) and/or immobilized cell adhesive RGD peptides. Cells cultured in the MMP-sensitive hydrogel with RGD peptides showed dramatic cell spreading compared with that of the control, which remained round. This MMP-sensitive low molecular weight hyaluronic acid-based hydrogel could be useful in tissue engineering by improving tissue defect regeneration and tissue remodeling.

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1 Introduction

Hyaluronic acid is a high molecular weight (up to 1–2 million Da) unmodified glycosaminoglycan (GAG) in the extracellular matrix (ECM) and is found in all connective tissues [1]. Hyaluronic acid is visco-elastic and is a linear copolymer of D-glucuronic acid and *N*-acetyl-D-glucosamine [2]. This acid is an important element in major biological processes, such as cell motility and proliferation [3], tissue organization [4, 5], wound healing [6, 7] and angiogenesis [8, 9]. Cellular interactions with hyaluronic acid occur through cell surface receptors (CD44, RHAMM, ICAM-l) and influence processes such as morphogenesis, wound repair, inflammation and metastasis [10–14]. In particular, the biological activity of hyaluronic acid differs depending on the molecular weight. Lower molecular weight hyaluronic acid has a higher angiogenic activity than higher molecular weight hyaluronic acid. Using low molecular weight hyaluronic acid as a building block for scaffolds could modulate the biological functions in tissue regeneration in vivo [15]. Hyaluronic acid has been chemically modified with mono- and polyfunctional hydrazides [16], amino or aldehyde functional groups [17] and methacrylate groups [18–20] to form stable cross-linked networks that can be used for scaffolds.

The qualities of hyaluronic acid as a scaffold for tissue regeneration was previously confirmed, particularly for bone tissue engineering [21]. In a previous study, a synthetic polymer, polyethylene glycol (PEG)-tetra thiols was used as a cross-linker. Although hyaluronic acid can be degraded by hyaluronidase [22], the synthetic polymer cross-linker had limitations for tissue demand, degrading the smart system. An active degradation system by biological signals is essential for in vivo regeneration of tissue defects using tissue engineering constructs.

Especially, MMPs (matrix metalloproteinases) and TIMPs (tissue specific inhibitors) are important for the tissue regeneration and remodeling process. Mesenchymal stem cell (MSC) function is controlled by MMP activity. Thus, MMP/TIMP balance appears to play an essential role in transferring mechanical signals into MSCs function [23]. Lutolf et al. fabricated PEG-based synthetic hydrogels that have been molecularly engineered with a combination of integrin binding sites and substrates for MMPs [24]. Zisch et al. presented VEGF-conjugated PEG-peptide hydrogels with pendant RGD-containing peptides by cross-linking MMP substrate peptides [25]. Seliktar et al. prepared VEGF and/or TGF β-conjugated PEG hydrogel matrices modified by conjugation with the cell adhesive peptide motif RGD by cross-linking peptide sequences for cleavage by MMP-2 into the polymer backbone [26]. Park et al. prepared MMP-sensitive poly(ethylene glycol)-based hydrogels and cultured bovine primary chondrocyte in that hydrogel [27]. Girotti et al. designed that a elastin-like protein polymer degraded by elastase via incorporating elastase target sequences as a cross-linker [28].

In this study, scaffolds were developed that can assist tissue regeneration by mimicking the natural ECM using low molecular weight hyaluronic acid MMP-sensitive peptides and RGD peptides via Michael-type conjugate addition reactions. The formation of biomimetic hydrogels, mechanical characterizations, and in vitro biological responses with varying adhesiveness and sensitivity to MMPs of the microenvironment were examined. Specifically, 3-dimensional adhesion and proliferation of human MSCs cultured in hyaluronic acid-based hydrogels with MMP sensitive peptides were evaluated.

2 Materials and methods

2.1 Materials

Hyaluronic acid (MW 50,000 Da and 10,000 Da) was purchased from Lifecore Biomedical Co. (Chaska, MN, USA) and 1-hydroxybenzotriazole hydrate (HOBT) was purchased from Fluka Chemical Co. (Buchs, Switzerland). 1-ethyl-3-(3-dimethylaminopropyl) carbodimide and adipic acid dihydrazide (AAD), triethanolamine (TEA), collagenase, and hyaluronidase were acquired from Sigma-Aldrich Inc. (St. Louis, MO, USA). *N*-acryloxysuccinimide (NAS) was purchased from Acros organics (New Jersey, USA). RGD peptides and MMP-sensitive and MMP-insensitive peptides were purchased from AnyGen. Co. Ltd (Gwangju, Korea). Fetal bovine serum (FBS), penicillin/streptomycin, trypsin, and low-glucose Dulbecco's Modified Eagle's Medium (DMEM) were purchased from GIBCO BRL (Grand Island, NY, USA).

2.2 Preparation of MMP sensitive hyaluronic acid-based hydrogel

Hyaluronic acid based hydrogel was prepared by acrylated hyaluronic acid as described previously [21]. For gel preparation, acrylated hyaluronic acid was dissolved in a TEA-buffered solution (0.3 M, pH 8). The cross-linker peptides, MMP-sensitive peptides (GCRDGPQGIWGQDRCG) and MMP-insensitive peptides (GCRDGDQGIAGFDRCG) in 0.3 M TEA buffered solution [24] were added as a cross-linker with the same molar ratio of acryl and thiol groups. The reaction mixture was incubated at 37°C for gelation. The hyaluronic acid-based hydrogel was formed via a Michael-type addition reaction [29]. This hydrogel (10% wt) was used for the in vitro experiments.

2.3 Evaluation of the mechanical properties of MMP sensitive hyaluronic acid-based hydrogels

Rheological behaviors of hyaluronic acid-based hydrogels were analyzed with a Rotational Rheometer Gemini (Bohlin Instruments Ltd.; Pforzheim, Germany). Four samples were evaluated: 10% wt hydrogel with 10 kDa hyaluronic acid + MMP insensitive peptides (A), 10 kDa hyaluronic acid + MMP sensitive peptides (B), 50 kDa hyaluronic acid + MMP insensitive peptides (C) and 50 kDa hyaluronic acid + MMP sensitive peptides (D). The gelation process occurred over the 1-ml mixture solution of hyaluronic acid-acryl and the peptide cross-linkers, 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 2 h by observing the viscous and the elastic modulus.

In order to measure the swelling properties, the hydrogel was incubated in PBS at room temperature for 3 days ($n = 4$ for each group). The swelling ratio was measured by comparing the change of the wet weight of the hydrogel before and after three days of incubation. The percentage of water absorbed (W_a) was calculated by the following formula; Swelling ratio $(\%) = ((W_w - W_i)/W_i) \times 100\%$ (Ww: wet weight of hydrogel; W_i: initial weight of hydrogel).

Degradation of the hydrogel by collagenase and hyaluronidase was measured. Hyaluronidase was added to the PBS buffer solution to a final concentration of 50 U/ml. And collagenase was added to the PBS buffer solution to a final concentration of 0.5 U/ml, 1 U/ml and 2 U/ml. The hydrogel sample was swelled for 3 days before degradation test. The pre-swollen hydrogels were added to the enzyme solutions and incubated up to 24 h at 37°C ($n = 4$ for each group). The weight loss of the hydrogel was measured every hour.

2.4 In vitro cell culture and cellular activity evaluation

Human MSCs 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 adherent cells were cultured further for passage. MMP sensitive or insensitive hyaluronic acid based hydrogels (50 kDa hyaluronic acid; 10% wt) and/or immobilized cell adhesive RGD peptides were mixed with the human MSC suspensions (1×10^5 cells/construct; 5μl) and produced a final volume of 50 μl ($n = 3$ for each group). The samples were cross-linked for 10 min at 37°C. The cells in the hydrogels were cultured in DMEM with 10% (v/v) FBS and 1% (v/v) antibiotics, containing penicillin and streptomycin in a humidified incubator (5% CO_2, 37°C) for three days. Cellular morphology in the hydrogel was documented by actin staining with Alexa 594 phalloidin (Molecular Probes; Eugene, OR, USA) under fluorescence microscope. The proliferation of human MSCs in the hydrogels was evaluated by the Cell Counting Kit-8 assay (Dojindo Laboratories; Kumamoto, Japan) and the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Molecular Probes; Eugene, OR, USA) was used to measure the viability. Live cells (green fluorescence) and dead cells (red fluorescence) were observed under fluorescence microscope. All experiments followed the manufacturer's protocols.

3 Results and discussion

Hyaluronic acid (50 kDa and 10 kDa) was used in this study because low molecular weight hyaluronic acid has different biological functions from those of high molecular weight hyaluronic acid. Moreover, the solubility of high molecular weight (~1 million Da) hyaluronic acid is low because of its highly hygroscopic and viscous properties [30]. Low molecular weight hyaluronic acid has been used in this study because it has different biological activities compared with higher molecular weight hyaluronic acids. In preparation of the MMP sensitive hyaluronic acid-based hydrogels, the acryl group of hyaluronic acid reacts selectively with thiol groups of Cys in the peptide cross-linkers at physiological conditions via a Michael-type addition reaction (Fig. 1). Mechanical properties of these hydrogels were evaluated with different molecular weights

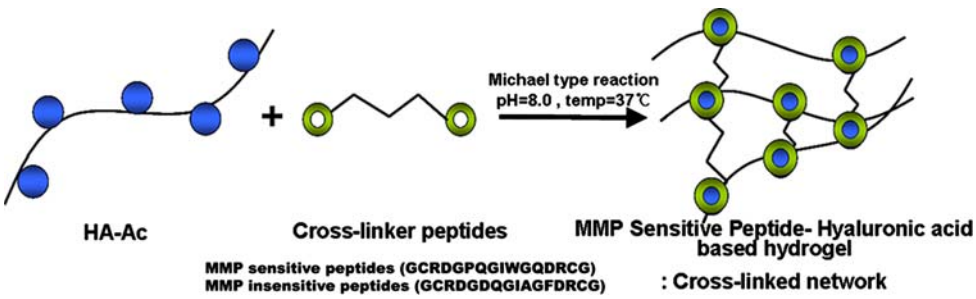

Fig. 1 Schematic representations of the formation of injectable hydrogels by acrylated hyaluronic acid and peptide cross-linkers. Cross-linker peptides: MMP-sensitive peptides (GCRDGPQGIWGQDRCG) and MMP-insensitive peptides (GCRDGDQGIAGFDRCG)

of acrylated hyaluronic acid (10, 50 kDa) and two types of cross-linker peptides of hydrogel (MMP sensitive, MMP insensitive peptides). Gelation time was determined by measuring time point where elastic modulus (G′) is larger than viscous modulus (G″) during gelation. The elastic modulus was shown to be higher than the viscous modulus at 1.3 min with 10% wt hydrogel with MMP sensitive 50 kDa hyaluronic acid-acryl and at 40.9 min with MMP sensitive 10 kDa hyaluronic acid-based hydrogel from the rheological data (Fig. 2(a) B, D). This hydrogel based on 50 kDa hyaluronic acid can be directly applied to tissue defects by injection due to the shorter gelation time. Measuring the elastic modulus and swelling ratios evaluated the mechanical properties of hydrogels. During frequency sweep, the average elastic modulus was compared for the evaluation strength of hydrogel. The elastic modulus of MMP-insensitive hyaluronic acid based hydrogels with 50 kDa and MMP-sensitive hyaluronic acid hydrogels with the same MW (Fig. 2(b) C, D) were 1,386 and 1,342 Pa respectively. Swelling ratios also showed the similar values; 330% and 314% (Fig. 3C, D). Mechanical properties of MMP-sensitive and insensitive hydrogels is not different because the length of the peptide sequences and moles of the peptide added is not different. It is clear that 50 kDa hyaluronic acid-based hydrogels were stronger than the 10 kDa hyaluronic acid-based hydrogels.

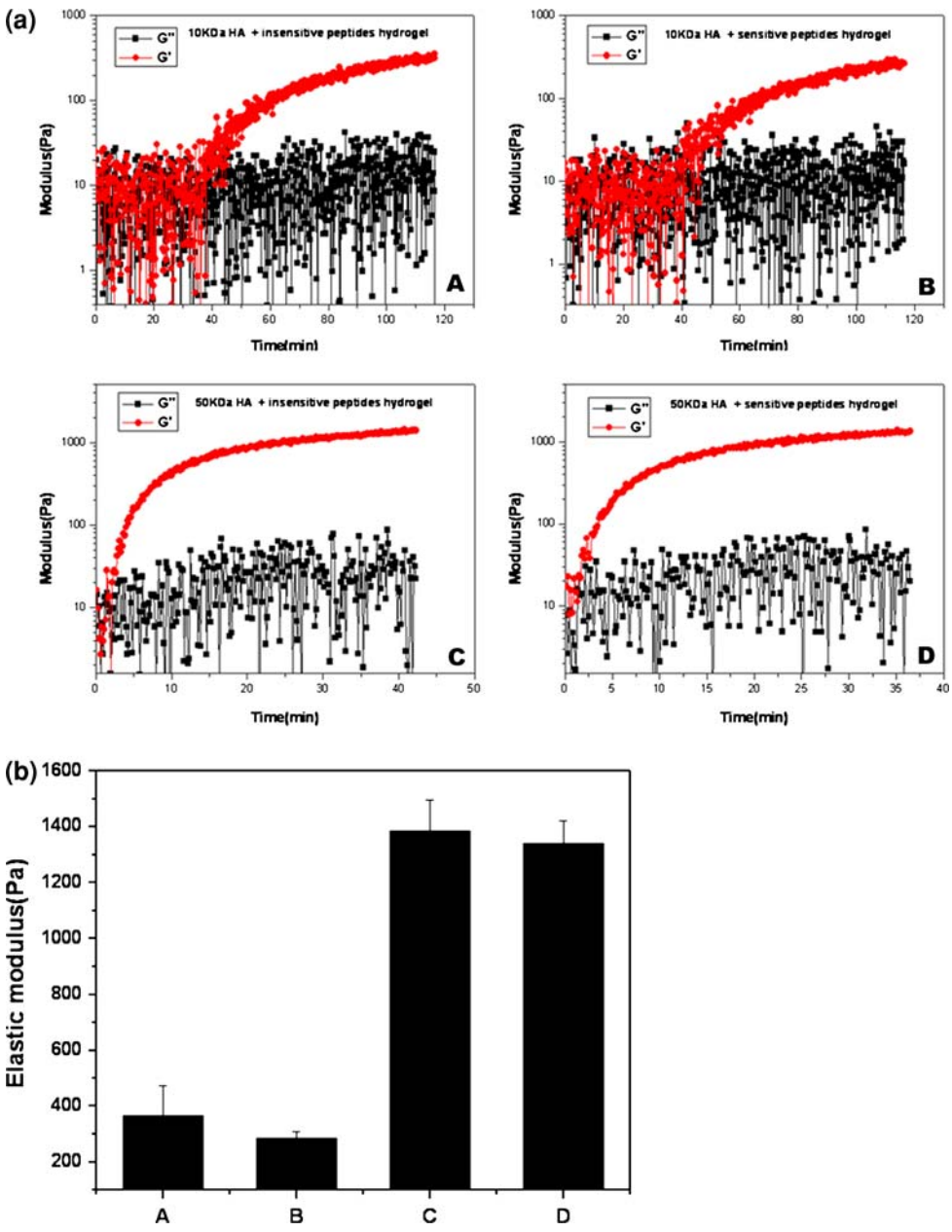

Fig. 2 Comparison of mechanical properties of peptide—hyaluronic acid-based hydrogels (**a**) Gelation Kinetics (time sweep) of peptide—hyaluronic acid based hydrogels. Elastic (G′) and viscous (G″) modulus evolution during cross-linking. (**b**) Elastic modulus during frequency sweep (mean ± SD); 10% wt hydrogel with A: 10 kDa hyaluronic acid+ MMP-insensitive peptides, B: 10 kDa hyaluronic acid + MMP sensitive peptides, C: 50 kDa hyaluronic acid + MMP insensitive peptides, D: 50 kDa hyaluronic acid + MMP sensitive peptides

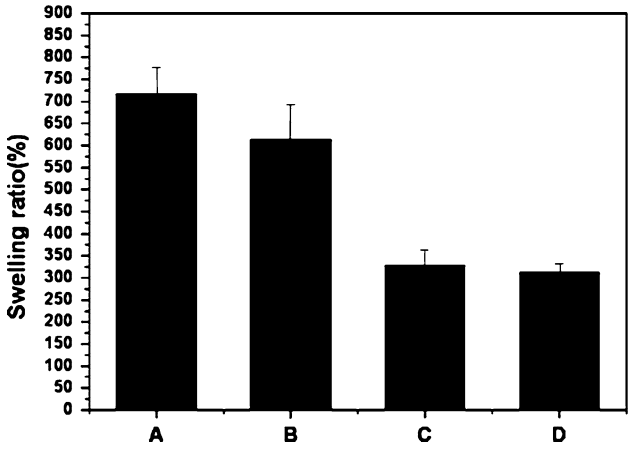

Fig. 3 Influence of peptide—hyaluronic acid-based hydrogel composition on the swelling ratio. The hydrogels were soaked in PBS for 3 days. The swelling of the hydrogels was measured as a degree of cross-linking; 10% wt hydrogel with A: 10 kDa hyaluronic acid + MMP-insensitive peptides, B: 10 kDa hyaluronic acid + MMP-sensitive peptides, C: 50 kDa hyaluronic acid + MMP-insensitive peptides, D: 50 kDa hyaluronic acid + MMP-sensitive peptides ($n = 4$ for each group; mean ± SD)

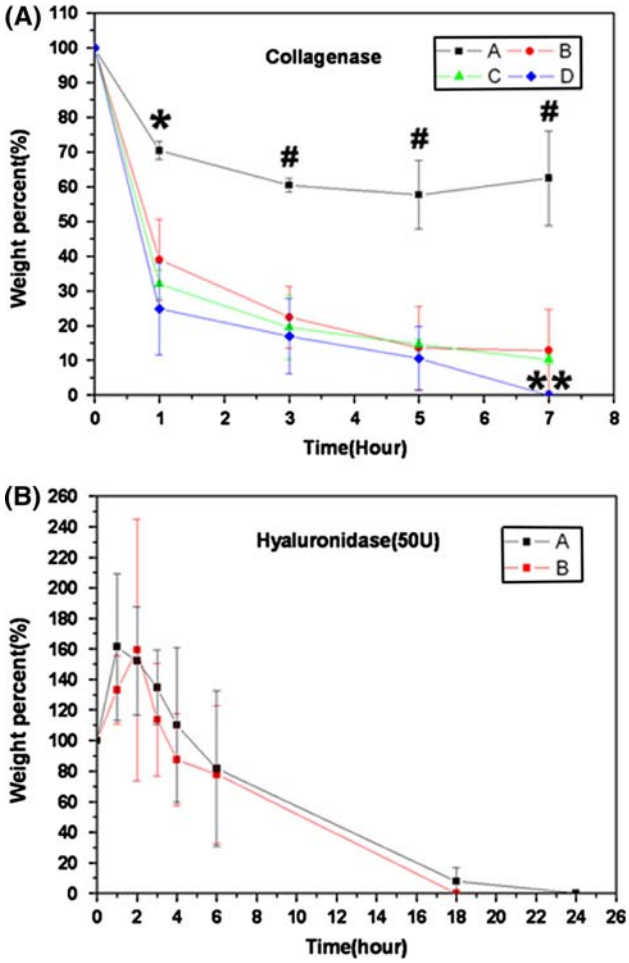

Fig. 4 In vitro degradation rate of peptide—hyaluronic acid-based hydrogels. (**a**) Degradation by collagenase 0.5 U/ml, 1 U/ml, 2 U/ml at 37°C; A: MMP-insensitive peptide hyaluronic acid-based hydrogel: collagenase 0.5 U/ml, B: MMP-sensitive peptide hyaluronic acid-based hydrogel; collagenase 0.5 U/ml, C: MMP-sensitive peptide hyaluronic acid-based hydrogel: collagenase 1 U/ml, D: MMP-sensitive peptide hyaluronic acid-based hydrogel: collagenase 2 U/ml ($n = 4$ for each group; mean ± SD; * $P < 0.05$ and # $P < 0.01$ vs. samples of B, C and D; ** $P < 0.1$ vs. sample B; ** $P < 0.01$ vs. sample C). (**b**) Degradation by 50 U/ml hyaluronidase at 37°C; A: MMP-insensitive peptide hyaluronic acid-based hydrogel, B: MMP-sensitive peptide hyaluronic acid-based hydrogel All samples are peptide-50 kDa HA-based hydrogels ($n = 4$ for each group; mean ± SD)

The degradation of scaffolds in vivo is an important factor in tissue remodeling and regeneration. Stimulus responsive degradation is ideal because the degradation rates of the scaffolds can be modulated by the tissue regeneration rate [31]. Hyaluronic acid-based hydrogels with MMP-sensitive peptides as a cross-linker can be degraded by two independent mechanisms. One is the degradation of hyaluronic acid by hyaluronidase that is secreted from the tissues, and another mechanism is the degradation of MMP-sensitive peptide cross-linkers by MMP secreted from cells. Additionally, the hyaluronic acid-based hydrogel was slowly degraded by hydrolysis in vitro due to ester bonds. The gel was completely degraded by 50 U/ml hyaluronidase within 24 h and the MMP sensitive peptide cross-linker was degraded by collagenase (Fig. 4). Different amounts of collagenase were used to check the dose-dependent degradation of MMP-sensitive hydrogels. As the concentration of collagenase increased, the degradation rate of peptide hydrogels increased (Fig. 4a). The amount of hyaluronidase and collagenase added to the solution was much higher than that of physiological conditions [32–34]. However, hyaluronidase could clearly recognize the gelated and derivatized hyaluronic acid and degrade gels in vitro, although hyaluronic acid was modified by the acryl group and cross-linked in the gel (Fig. 4b).

Human MSC cultured for three days in different hydrogel compositions showed dramatic differences in cell morphology (Fig. 5) Cells in MMP sensitive hydrogels spread inside the gel by degrading surrounding hydrogels with MMPs whereas cells in the control, insensitive hydrogel remained round (Figs. 5, 6). When cells cultured in RGD-immobilized, MMP-insensitive hydrogels, cell spreading was not dramatic as shown in MMP-sensitive hydrogels samples. It means that cell spreading in 3D environments requires remodeling of matrices. Cells in RGD-immobilized, MMP sensitive hydrogels showed more spindle-like, shapes sprouting filopodia into the hydrogels (Fig. 5d). Actin filament staining of cells in MMP-sensitive hydrogels shows that tip of cells were divide into two or three part and started to invading into the hydrogel matrix by degrading MMP-sensitive cross-linkers (Fig. 6d). Cell

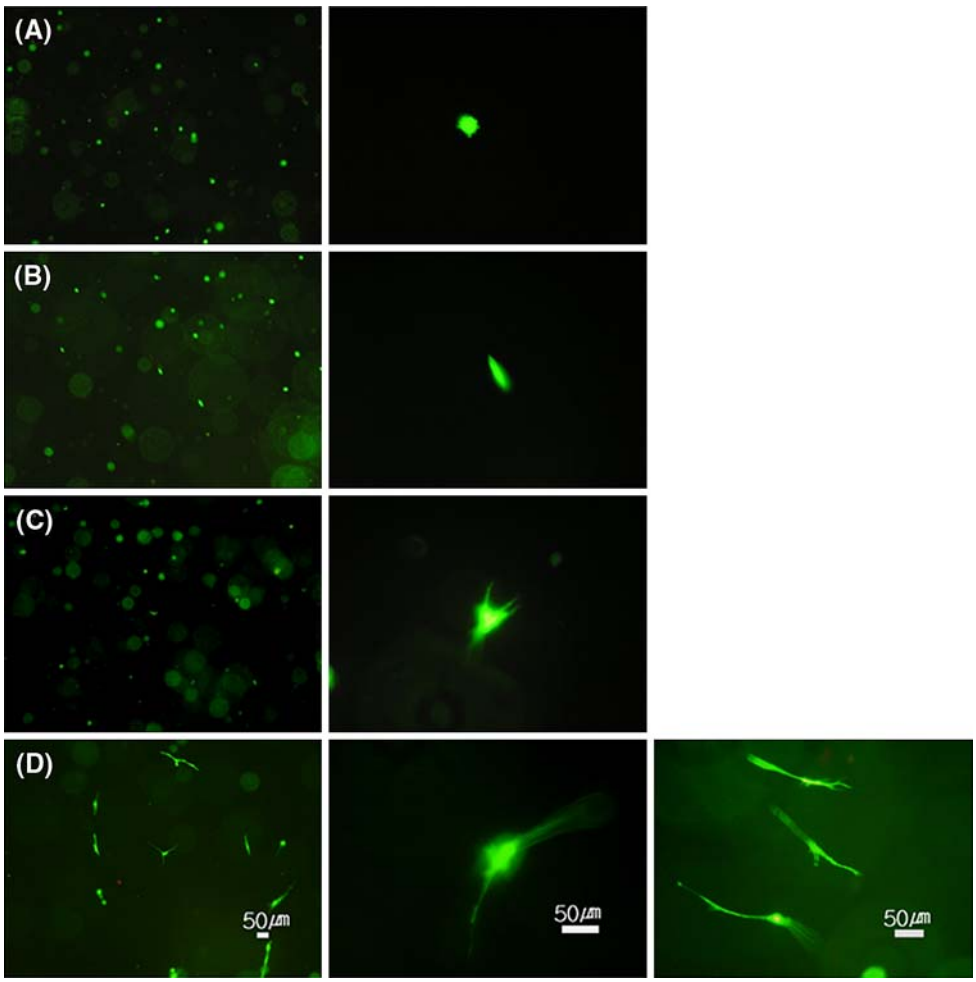

Fig. 5 Cell viability test of human MSCs in the peptide—hyaluronic acid-based hydrogels by Live and Dead assay: Photographs of human MSCs in the hyaluronic acid-based hydrogel. Human MSCs were cultured for 3 days in hyaluronic acid-based hydrogels; 1×10^5 human MSCs per construct. The membranes of live cells were stained with green fluorescence and the nuclei of dead cells with red; (**a**) MMP-insensitive peptide hyaluronic acid-based hydrogel, (**b**) MMP-insensitive peptide hyaluronic acid-based hydrogel + RGD peptides, (**c**) MMP-sensitive peptide hyaluronic acid-based hydrogel, (**d**) MMP-sensitive peptide hyaluronic acid-based hydrogel + RGD peptides; All samples are peptide-50 kDa HA-based hydrogels. Scale bar = 50 μm

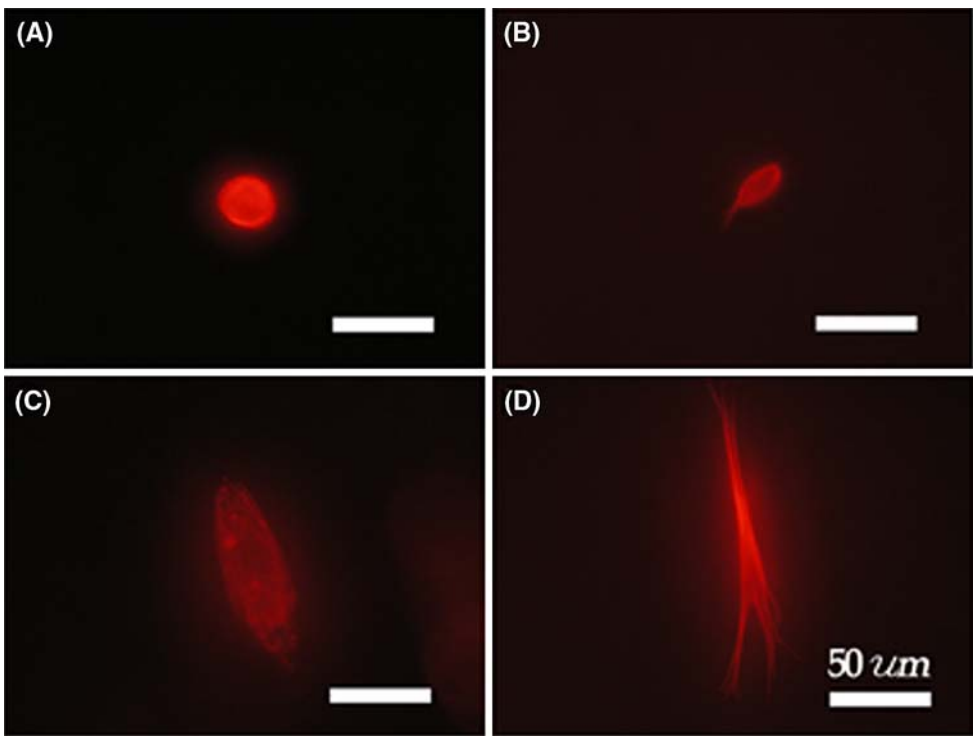

Fig. 6 Cell morphology evaluation of human MSCs in the peptide—hyaluronic acid-based hydrogels by actin staining: Photographs of human MSCs in the hyaluronic acid-based hydrogel. Human MSCs were cultured for 3 days in hyaluronic acid-based hydrogels; 1×10^5 hMSCs per construct; (**a**) MMP-insensitive peptide hyaluronic acid-based hydrogel, (**b**) MMP-insensitive peptide hyaluronic acid-based hydrogel + RGD peptides, (**c**) MMP-sensitive peptide hyaluronic acid-based hydrogel, (**d**) MMP-sensitive peptide hyaluronic acid-based hydrogel + RGD peptides; All samples are peptide-50 kDa HA-based hydrogels. Scale bar = 50 μm

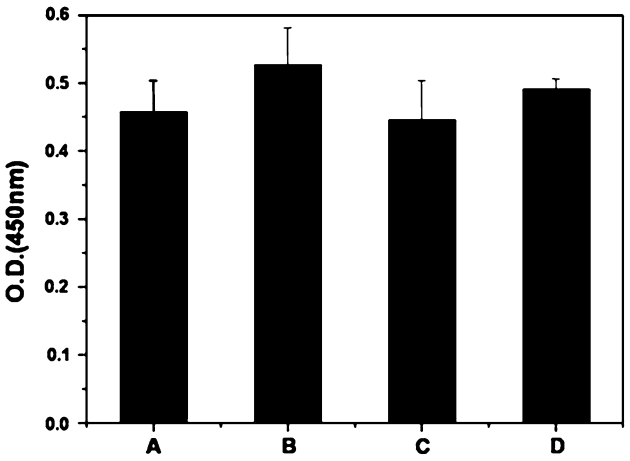

Fig. 7 Cell proliferation test by Cell Counting Kit-8 assay of human MSCs in hyaluronic acid-based hydrogel. Human MSCs cultured for 3 days in HA-based hydrogels; 1×10^5 human MSCs per construct. A: MMP-insensitive peptide hyaluronic acid-based hydrogel, B: MMP-insensitive peptide hyaluronic acid-based hydrogel + RGD peptides, C: MMP-sensitive peptide hyaluronic acid-based hydrogel, D: MMP-sensitive peptide hyaluronic acid-based hydrogel + RGD peptides; All samples are peptide-50 kDa HA-based hydrogels. ($n = 3$ for each group; mean ± SD)

viability in the gel is an important issue because synthetic gels showed relatively lower viability [35]. Cell viability cultured in the higher molecular weight hydrogel (200 kDa) demonstrated lower than 70% viability in a previous study [21]. In the previous study, cells cultured in the low molecular weight hyaluronic acid-based hydrogel using a PEG tetra thiols cross-linker showed that more than 90% cells were alive [36]. Cells in the MMP-sensitive, low MW hyaluronic acid based hydrogel also showed the higher viability (Fig. 5). Cells in hydrogels without RGD peptides showed lower proliferation rates compared to hydrogels with RGD peptides (Fig. 7). However, cell proliferation assay in the different hydrogels showed that there were no significant differences among samples (Fig. 7). If cells were cultured for a longer period, cells in the MMP-sensitive hydrogels might show the higher proliferation rates compared to those in MMP-insensitive hydrogels.

During tissue remodeling and regeneration, most cells locally activate MMPs [37]. Therefore, MMP-sensitive characteristics of scaffolds are necessary for smart degradation system in tissue regeneration. In the present study, low molecular weight hyaluronic acid based smart hydrogel systems were prepared incorporated with MMP-sensitive peptides and integrin adhesive peptides (RGD) via covalent bonding. These hydrogels are sensitive to cellular enzymes, such as hyaluronidase and MMPs. The differential cellular behaviors of stem cells cultured in these biological responsive hydrogels, especially in morphology and cytoskeletons, were observed. Thus, these biomimetic hydrogels can be a useful tool in future tissue engineering.

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