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Therapeutic effects of neuropeptide substance P coupled with self-assembled peptide nanofibers on the progression of osteoarthritis in a rat model

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ABSTRACT

Osteoarthritis (OA) is a progressively degenerative disease that is accompanied by articular cartilage deterioration, sclerosis of the underlying bone and ultimately joint destruction. Although therapeutic medicine and surgical treatment are done to alleviate the symptoms of OA, it is difficult to restore normal cartilage function. Mesenchymal stem cell (MSC) transplantation is one of the therapeutic trials for treating OA due to its potential, and many researchers have recently reported on the effects of MSCs associated with OA therapy. However, cell transplantation has limitations including low stem cell survival rates, limited stem cell sources and long-term ex vivo culturing. In this study, we evaluated the efficacy of neuropeptide substance P coupled with self-assembled peptide hydrogels in a rat knee model to prevent OA by mobilizing endogenous MSCs to the defect site. To assess the effect of the optimal concentration of SP, varying concentrations of bioactive peptides (substance P (SP) with self-assembled peptide (SAP)) were used to treat OA. OA was induced by unilateral anterior cruciate and medial collateral ligament transection of the knee joints. Forty rats were randomly allocated into 5 groups: SAP-0.5SP (17.5 μg of SP), SAP-SP group (35 μg of SP), SAP-2SP group (70 μg of SP), SAP-SP-MSC group, and control group. At 2 weeks post-surgical induction of OA, each mixture was injected into the joint cavity of the left knee. Histologic examination, immunofluorescence staining, quantitative real time-polymerase chain reaction and micro-computed tomography analysis were done at 6 weeks post-surgical induction. As shown by our results, the SAP-SP hydrogel accelerated tissue regeneration by anti-inflammatory modulation shown by an anti-inflammation test using dot-blot *in vitro*. Additionally, the treatment of OA in the SAP-SP group showed markedly improved cartilage regeneration through the recruitment of MSCs. Thus, these cells could be infiltrating into the defect site for the regeneration of OA defects. In addition, from the behavioral studies on the rats, the number of rears significantly increased 2 and 4 weeks post-injection in all the groups. Our results show that bioactive peptides may have clinical potential for inhibiting the progression of OA as well as its treatment by recruiting autologous stem cells without cell transplantation.

1. Introduction

Osteoarthritis (OA) is characterized by synovial inflammation, cartilage destruction, and subchondral bone sclerosis, and several inflammatory cytokines including matrix metalloprotease are involved in these phenomena [1,2]. Many treatments have been used in patients with OA, but no definitive treatment is available to modify the progression of the disease although several candidates

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have been tried [3]. Clinical medication is universally prevalent for OA, but this therapy has a limited role in relieving symptoms and improving joint function. Surgery is performed in cases of severe symptoms from OA, but it has drawbacks such as infection, implant wear and loosening, and co-morbidities from the procedures [4] and has a great risk due to general or spinal anesthesia in elderly patients.

Currently, stem cell therapy is generating a great deal of interest in its clinical application in various diseases such as cardiac, corneal, and neurodegenerative diseases, OA, diabetes mellitus and so on [5–10]. However, treatment using stem cell transplantation has drawbacks such as low stem cell survival rates, limited stem cell sources and long-term ex vivo culturing [11,12]. Therefore, recent stem cell research has focused on recruiting circulating endogenous host cells to the necessary site for tissue regeneration and enhancement of tissue functions using endogenous homing/mobilization factors instead of injection or implantation of exogenous stem cells [13–17].

SP, an 11 amino acid small endogenous neuropeptide is known to have an important role in cell proliferation, bone marrow fibrosis and regulation of wound healing. In addition, SP is known to function as a chemotactic factor by mobilizing MSCs to the circulation [18]. Recently, it was reported that SP can have an anti-inflammatory role in rheumatoid arthritis [19]. The anti-inflammatory action of SP occurs by a change in the cytokine profile, in which the level of Interleukin (IL)-10 increases and tumor necrosis factor-α (TNF-α) decreases [20]. Furthermore, SP is thought to increase the proliferation rate of chondrocytes dose-dependently [21] and to enhance tissue regeneration through endogenous stem cell recruitment and angiogenesis [22]. Hong et al. identified that SP has a novel role as an injury-inducible factor by activating the recruitment of mobilized $CD29^{+}$ stromal like cells to damaged tissues for the wound healing process [18]. Ko et al. investigated the dual drug delivery method, in which a combined systemic injection and local release of SP and stromal-derived factor-1α (SDF-1α) were used to enhance the recruitment of endogenous stem cells without exogenous stem cells [15]. Noh et al. also investigated a bone regeneration strategy by dual delivery of SP and growth factor (BMP-2) to calvarial defects [23].

Despite this therapeutic potential of SP, SP is an important element in pain perception and reduces the pain threshold [24]. Previous studies have revealed that antagonists of the SP receptor improved the reduction of arthritic pain and swelling [25]. Seidel commented that blocking SP can reduce pain but increase the progression of arthritic changes in OA [26]. Therefore, for treating OA, SP can have dual opposing roles (OA treatment by anti-inflammatory action and recruitment of endogenous stem cells *vs* evolution of pain by reducing the pain threshold). This contradictory potential of SP for therapeutic application in OA can be resolved by the adjustment of the SP dose, the continuity of SP release, and the use of an adequate conjugate to modify the properties of SP. One study showed that continuous infusion of SP alleviates pain by decreasing tonic nociception rather than facilitating pain [27].

A self-assembled peptide (SAP) is a biomaterial composed of alternating hydrophilic and hydrophobic side groups, which allow self-assembly into aligned nanostructures [28]. SAPs are used for biomedical applications in regenerative medicine as scaffolds, tissue engineering and drug delivery as well as defined cell culture matrices because SAPs closely mimic the porosity and gross structure of extracellular matrices from cells which enable them to reside and migrate in a microenvironment [29–31]. SAPs also have the ability to support cell attachment and differentiation of various cells. Furthermore, they have advantages in designing and modifying their peptide sequence easily, so SAPs have been applied in the delivery of individual proteins, biologically active domains and molecules including growth factors and functionalized motifs such as RGD, YIGSR, and CMPs [32–35]. In addition, because SAP is a solution before being injected into the body and it is converted to a gel in the body, it can be used with an injectable system. Direct treatment through open surgery is accompanied by considerable morbidity in patients with OA. Therefore, our SAP has a clinical potential as a minimally invasive injection into the articular cavity during a substantially short time.

KLD-12 (Ac-KLDLKLDLKLDL-NH_2), one of the SAPs, has a role in promoting cartilage regeneration and in continuously releasing the conjugated materials without inducing a significant immune response as a scaffold for bone marrow stromal cells or stem cells in OA [36–40]. KLD-12 is known to retain a chondrocyte phenotype and support the growth and differentiation of chondrocytes along with stimulating the accumulation of the extracellular matrix. KLD-12 also has a functional role in delaying OA progression by itself [37].

Mesenchymal stem cells (MSCs) have potential as therapeutic factors in treating various cellular damages because of their self-renewal ability and multi-potency with the ability to differentiate to a variety of cells such as bone, cartilage, adipose tissue, muscle and so on. MSCs also have a paracrine effect through the secretion of growth factors, cytokines and antifibrotic or angiogenic mediators [41,42]. Among the secreted factors from MSCs, indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), IL-10, and transforming growth factor β1 (TGF-β1) are immunomodulatory factors which could be involved with the immunosuppression function of MSCs [11]. Based on their immunomodulatory effects, MSCs may have potential to treat inflammatory diseases such as joint diseases [43]. For these reasons, we examined the effects of SP-induced endogenous recruitment of MSCs on OA.

Furthermore, we expected to achieve anti-inflammatory effects in an OA model because SP and MSCs facilitate clinical therapeutic effects including alleviation of synovial inflammation and a reduction of pro-inflammatory cytokines. This anti-inflammatory effect can reduce the apoptosis of cells, which is part of the OA mechanism [44]. SP is also known to counteract intracellular death signals by activation of the neurokinin-1 receptor, which may suggest another anti-apoptotic role of SP [45].

In this study, we assumed that SP conjugated with SAP would promote cartilage regeneration, reduce the inflammatory reaction, and subsequently delay the progression of OA without facilitating arthritic pain. Finally, the aim of this study was to establish the effect of the SAP-SP conjugates on cartilage regeneration and inhibition of apoptosis in OA.

2. Materials and methods

2.1. Preparation of the SAP–SP complex

KLD12 and KLD12-SP (Ac-KLDLKLDLKLDLGGRPKPQQFFGLM-NH_2) were synthesized (Peptron, Daejeon, Korea) and dissolved in 295 mM sucrose solution to prepare a 1% (wt/vol) peptide gel. The peptide gel was mixed with PBS to make a 0.5% working peptide gel and then sonicated the gel with an ultrasonic cleanser for 30 min. KLD12 and KLD12-SP were mixed with different concentrations for the experimental groups. The experimental groups were as follows: i) saline, ii) mixture of KLD12 and KLD12-SP (200 μl:3.5 μl) [SAP-0.5SP], iii) mixture of KLD12 and KLD12-SP (200 μl:14 μl) [SAP-2SP], iv) mixture of KLD12 and KLD12-SP (200 μl:7 μl) [SAP-SP], and v) mixture of KLD12, KLD12-SP (200 μl:7 μl) and mesenchymal stem cells [SAP-SP-MSCs].

2.2. In-vitro anti-inflammatory tests

To confirm the anti-inflammatory effects of the bioactive KLD12 (KLD12/KLD12-SP), *in vitro* anti-inflammatory tests were performed using dot-blot (Group 1: non-stimulated, Group 2: stimulated, Group 3: KLD12 (200 μl of KLD12), Group 4: KLD12/KLD12-SP (200 μl of KLD12 and 7 μl of KLD12-SP), Group 5: 35 μg of soluble SP and Group 6: 0.4 μg/ml of dexamethasone). Granulocyte macrophage-colony stimulating factor (GM-CSF), IL-2, IL-17A, Interferon-γ (IFN-γ), TNF-α, Macrophage inflammatory protein (MIP), and MIP-1α, β were evaluated as pro-inflammatory factors because they cause the migration of a variety of inflammatory cells, cellular transformation of fibroblasts and immune cells in the tissue, and finally tissue destruction in the joint. Human peripheral blood mononuclear cells (PBMCs) were purchased from LONZA (Basel, Switzerland). The PBMCs were plated at a density of 1.5×10^6 cells/ml in supplemented culture medium RPMI 1640 with the peptide gels, soluble SP or dexamethasone. Then, they were stimulated or not with 10 μg/ml of Con A for 48 h [46]. After incubation, supernatants were harvested by centrifugation, and cytokines were confirmed with the Human XL Cytokine Array Kit (Proteome Profiler, R&D Systems, Minnesota, USA). Additionally, the pixel densities of the dots were calculated by the Image J Program (NIH, Maryland, USA).

2.3. Surgical induction of the OA model and injection of the SAP–SP complex

All animals were treated in accordance with standard operating protocols by the Institutional Animal Care and Use Committee at our Biomedical Research Institute. All protocols for Animal Experiments were approved by the Institutional Review Board of Animal Experiments at our institutes.

For the OA model, 40 rats were anesthetized with an intraperitoneal injection of a 1:1 mixture of tiletamine and zolazepam (Zoletil 50, Virbac, France) with xylazine (Rompun, Bayer Vital GmbH, Leverkusen, Germany) at a dose of 30 mg of the tiletamine and zolazepam mixture and 10 mg of xylazine per kg of body weight. The anterior portion of the left hind limb was shaved with an electric clipper, and cleansed with povidone iodine. The skin around the knee cap was vertically incised along the medial border of the knee cap. The patella was displaced laterally to expose the anterior cruciate ligament. Then, the anterior cruciate and medial collateral ligaments were cut and the medial meniscus was completely extracted with surgical scissors without injury to the cartilage of the tibia. The patella was then replaced back, and the fascia and skin were closed with a 3-0 polydioxanone suture. A single dose of antibiotic cream was applied to prevent postoperative infection. To induce the OA model, all the rats were made to run on the treadmill 20 min per day, 5 days per week, for 6 weeks.

2.4. Injection of the SAP–SP complex into the joint cavity

Forty rats were randomly allocated into 5 groups the SAP-0.5SP group (n = 9), SAP-SP group (n = 7), SAP-2SP group (n = 7), SAP-SP-MSC group (n = 10), and control group (n = 7) according to the injected materials mentioned above. At 2 weeks post-surgical induction of OA, 0.2 ml of each mixture was injected into the left knee joint cavity using a 26-gauge needle after palpating the patella with the knee bent. The injection technique is described elsewhere [37].

2.5. Immunofluorescent analysis

At 6 weeks post-surgical induction, caspase-8 (Santa Cruz Biotechnology, Texas, USA), TIMP-1 (Santa Cruz Biotechnology, Texas, USA), and collagen type II (Chemicon, Darmstadt, Germany) were detected by immunofluorescence staining. Alexa Fluor 488 goat anti mouse IgG (Invitrogen, Oregon, USA), Alexa Fluor 594 donkey anti rabbit IgG (Invitrogen, Oregon, USA) and Alexa Fluor 594 rabbit anti mouse IgG (Invitrogen, Oregon, USA) were used for secondary antibodies. Nuclei were also counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes), and the stained tissues were examined with fluorescence microscopy (Eclipse TE2000U; Nikon, Tokyo, Japan) in 4 random fields at 200 × magnification with a blinded rater in the border zone (n = 4 in each group). The areas of positive stained sections were analyzed as the mean per unit area (1 mm^2) with the image J program. For immunofluorescence analysis, the sections were double stained with caspase-8 and TIMP-1 antibody, and the resulting images were merged with DAPI stained images (n = 4 in each group). Additionally, each positive area (caspase-8 and TIMP-1 expression area) was quantified with the image J program. Caspase-8 is a factor that is associated with cell apoptosis, and TIMP-1 is known to be an inhibitor of MMP-9 during the progression of OA.

Moreover, to assess the retained SP by using SAP in the knee joints of rats, SP was stained with the SP antibody (Santa Cruz Biotechnology Texas, USA). Alexa Fluor 488 chicken anti goat IgG (Invitrogen Oregon, USA) was used for the secondary antibody. After the staining with the anti-SP antibody, the samples were examined under a microscope (200 × magnification, n = 4 in each group).

2.6. Quantitative real-time polymerase chain reaction

For gene expression of inflammatory mediators (caspase-8 and IL-1β) and chondrogenesis markers (type II collagen and SOX-9), rats were euthanized at 6 weeks post-surgical induction. Articular cartilage was obtained from specimens of the knee joints in the rats and closely minced with fine scissors (n = 3–5). Chondrocytes were isolated with 0.1% collagenase type II in PBS supplemented with penicillin-streptomycin and incubated at 37 °C overnight for enzymatic digestion. The digested articular cartilage was centrifuged and passed through a 100 μm filter (BD Biosciences, San Jose, CA, USA) to remove cellular debris three times. The isolated cells were washed with PBS, and RNA was extracted from the isolated chondrocytes according to the manufacturer's instructions using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). The RNA of each sample was then evaluated with the A260/280 absorbance ratio, and then, 2 mg of RNA were reversed transcribed into cDNA in a 20 mL reaction using the Omniscript System Kit (QIAGEN, Hilden, Germany). Real-time PCR was done with an Applied Biosystems 7500 Real-Time PCR System with the Power SYBR® Green PCR Master Mix (Applied Biosystems, Massachusetts, USA). Real-time PCR was done in a final volume of 25 μl, and the cDNA was amplified by 45 cycles of 95 °C for 15 s and 55 °C for 60 s. To normalize the samples, glyceraldehyde-3-phosphate dehydrogenase (GAPDH; a house keeping gene) primers were used. The sequences of the primers for this reaction are listed in Table 1.

2.7. Micro-CT analysis

At 6 weeks post-surgical induction, the knee joints of all the rats were extracted for micro-CT analysis. A Siemens Inveon Micro-PET/CT scanner was used, and this was designed as an *in-vivo* system (Siemens Medical Solutions, Knoxville, USA). All samples were scanned with a 1.5 mm aluminum filter, using the following settings: 360° total rotation and 720 rotation steps, 0.5° step size, 70 kV and 400 μA source setting, and 800 ms exposure time per step. And pixels were binned by 2, resulting in an effective pixel size or resolution of approximately 20.26 μm.

Table 1
List of primers used in quantitative real-time PCR analysis of isolated chondrocyte from specimens of knee joints in rats.

Primer name	Forward sequence	Reverse sequence	Product Size (bp)
GAPDH	GACATGCCGCCTGGAGAAAC	AGCCCAGGATGCCCTTTAGT	92
Type II collagen	CCCCTGCAGTACATGCGG	CTCGACGTCATGCTGTCTCAAG	60
Caspase-3	AATTCAAGGGACGGGTCATG	GCTTGTGCGCGTACAGTTTC	67
IL-1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC	79
Sox-9	CTGAAGGGCTACGACTGGAC	TACTGGTCTGCCAGCTTCCT	140

For each scan, the dataset was reconstructed with a down sample factor of 2 using the Inveon Acquisition Workplace software package (Siemens Medical Solutions, Knoxville, Tennessee, USA), implementing the modified Feldkamp filtered back projection algorithm (Shepp-Logan filter). The reconstructed images were then imported using the Inveon Research Workplace into the accompanying two-dimensional (2D) and three-dimensional (3D) biomedical image analysis software package (CT Bone Visualization and Analysis, Siemens Medical Solutions, Knoxville, TN, USA) for visualization and analysis. Additionally, the knee joint was divided into four compartments (medial femur (MFC), lateral femur (LFC), medial tibia (MTP), and lateral tibia (LTP)), each containing the anterior, central, and posterior cylindrical regions of interest (ROIs), providing a total of 12 sample locations per knee joint. The cylindrical ROIs were created with a diameter of approximately 0.8 mm and a length of 0.8 mm, giving a ROI volume of approximately 0.45 mm^3. BMD measurements were calculated by scanning a phantom (same acquisition settings as the femurs) containing rods of known density values in g/cm^3 and creating a linear calibration curve ($R^2 = 0.9987$) relating these densities to their measured Hounsfield (HU) values upon scanning.

2.8. Histological analysis

The knee joints were stained as described by Appleton et al. [47] for histological analysis at 6 weeks post-surgical induction. The collected samples were fixed in 10% (v/v) buffered formalin, decalcified by hydrogen chloride ethylenediaminetetraacetic acid solution, and then embedded in paraffin. The specimens were sectioned in the sagittal plane under the midline at 6 μm thicknesses (n = 4, 5). To observe the collagen and sulfated glycosaminoglycan, we conducted Masson's trichrome and Alcian blue staining. Furthermore, sections were stained with hematoxylin and eosin to identify the nucleus and cytoplasm.

Modified Mankin scoring system was used to evaluate the OA changes. The items of the scoring system were the cartilage structure, cartilage cells, Alcian blue staining, and tidemark integrity. A detailed description is given in a previous article [37].

2.9. Recruitment of intrinsic MSCs

The expression of MSCs verified by double staining was observed at 6 weeks post-surgical induction in the cartilage. To investigate the evidence of MSCs recruitment by SAP-SP, sections were stained with MSC markers (CD90, CD105). CD90 (Thy-1 antibody, Santa Cruz Biotechnology, Texas, USA) and CD105 (Anti-Endoglin monoclonal antibody, Millipore, Temecula, CA) were detected by immunofluorescence staining. Alexa Fluor 594 donkey anti rabbit IgG and Alexa Fluor 488 goat anti mouse IgG were used for secondary antibodies. Samples were also counterstained with DAPI to confirm the location of the nuclei, and the specimens were observed with fluorescent microscopy (Eclipse TE2000U). After the staining, the sections were observed in 4 random fields at 200 × magnification with a blinded rater in the border zone (n = 4 in each group). The areas of positive stained sections were analyzed as the mean per unit area (mm^2/mm^2) with the image J program.

2.10. TUNEL assay

After euthanization to examine apoptosis at 6 weeks post-surgical induction, apoptotic cell death was assessed with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay using a commercially available apoptosis detection kit (Millipore, Temecula, CA) according to the manufacturer's instructions. Briefly, after routine deparaffinization, rehydration, and blocking of endogenous peroxidase with 3% hydrogen peroxide in PBS for 10 min at room temperature, the tissue sections were digested with 20 μg/ml proteinase K in PBS for 15 min at room temperature. After washing in PBS buffer, equilibration buffer was applied to the sections for 1 min at room temperature, and the sections were then incubated with working strength terminal deoxynucleotidyl transferase (TdT) enzyme for 60 min at 37 °C in a humidity chamber. The reaction was terminated in a working strength stop/wash buffer for 30 min at room temperature. After washing in PBS, the sections were covered with anti-digoxigenin-peroxidase for 30 min at room temperature, and the color reaction was developed using DAB substrate chromogen solution for 5 min and then washed with distilled water. Sections were lightly counterstained with Mayer's hematoxylin for 30 s. All sections were also stained with H&E for histologic evaluation.

For the quantitative analysis of the TUNEL assay, we adopted the apoptotic index which is determined by the percentage of apoptotic cells counted in 5 fields randomly chosen at 400 × magnification (n = 4 in each group).

2.11. Behavioral analysis

To evaluate the pain induced by SP injection or inflammation from the OA, the number of rears by the rats was checked before injection, and at 2 and 4 weeks post-injection. In an open field acrylic box in which the floor was covered with foam stamp pads, and the 4 sides were covered with white paper, a rat touched the walls of the box with their fore limbs stained with ink from the foam stamp pads and the number of touches above 5 cm from the bottom of the paper was counted (n = 3, 4). The method in detail is described elsewhere [37,38]. Larger numbers of touches (rears) represent less pain.

2.12. Statistical analysis

Statistical analysis by SPSS 20.0 (IBM, New York, USA) was done to evaluate the correlation between the various treatments and the experimental results. For the data from the behavioral analysis, repeated measures ANOVA was conducted to determine whether

there were significant differences between the treatment groups and the different time points. For other experiments, Kruskal–Wallis and post-hoc Mann–Whitney U tests with Bonferroni correction were used to determine the differences between the groups. The threshold p-value for statistical significance was set as $p < 0.05$.

3. Results

3.1. In vitro anti-inflammatory tests

When tissue has damage, an immune response occurs in the injury site to treat the tissue. To determine whether SAP-SP conjugates are related to an anti-inflammation effect, we investigated the anti-inflammatory response using dot-blot *in vitro*. The over-expression of pro-inflammatory factors, such as TNF-α, IL-1, IL-6, IL-8, IL-12, and IL-17, causes the migration of a variety of inflammatory cells, the cellular transformation of fibroblasts and immune cells in the tissue, and finally tissue destruction in the joint. Fig. 1 shows that soluble SP, KLD12, and KLD12/KLD12-SP inhibited the expressions of GM-CSF, IL-2, IL-17A, IFN-γ, TNF-α, MIP, and MIP-1α, β. In addition, they improved the secretion of anti-inflammatory factor IL-4. These results mean KLD12 and SAP-SP had anti-inflammatory effects, as did dexamethasone. This result suggests that our peptide gel decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines, which may be contributed to not only the alleviation of synovial inflammation but also the regeneration of cartilage in the rat OA model. Therefore, it was considered that our peptide gel accelerated tissue regeneration by anti-inflammatory modulation.

3.2. Quantitative real-time polymerase chain reaction

To examine the gene expression of cartilage according to each treatment in the OA model, quantitative real-time polymerase chain reaction (qRT-PCR) was performed. RNA was extracted from the chondrocyte of the rat knee joint and then the gene expression of the marker was identified, which is related to the chondrogenic marker (type II Collagen, Sox-9) and inflammatory mediators (Caspase 3, IL-1β). The gene expression was normalized to a house keeping gene, GAPDH. All the gene expression data except for type II collagen showed a significant difference. The gene expressions of IL-1β and caspase-3 decreased as the SP amount increased, which

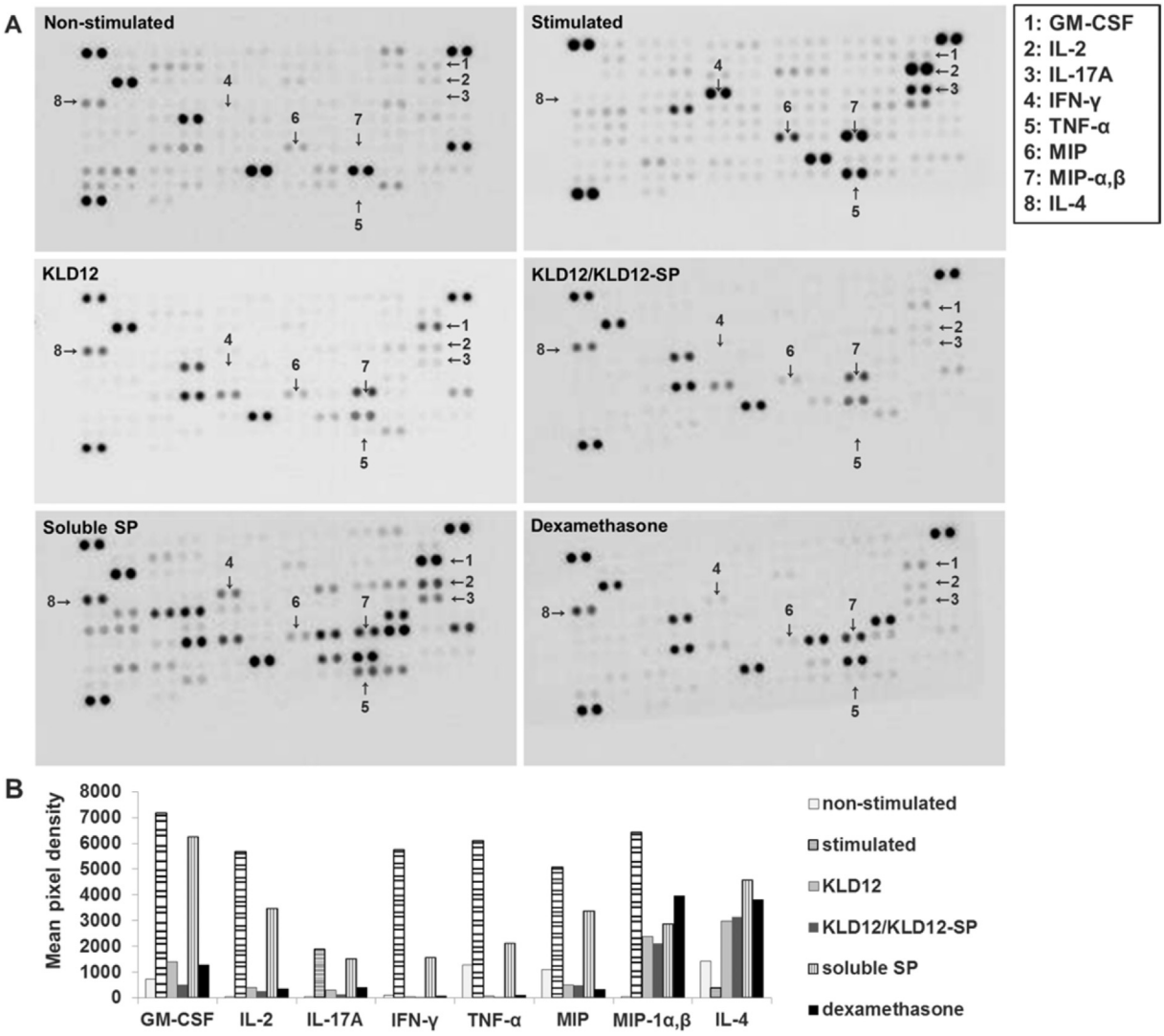

Fig. 1. Effect of anti-inflammation of bioactive peptides using human peripheral blood mononuclear cells *in vitro*. (A) Anti-inflammation tests for a dot blot. (1–7: Pro-inflammatory cytokines, 8: anti-inflammatory cytokine.) (B) Semi-quantitative analysis of the dot blots by image J program. Soluble SP, KLD12, and KLD12/KLD12-SP inhibited the expression of pro-inflammatory cytokines, but the anti-inflammatory cytokine expression was increased.

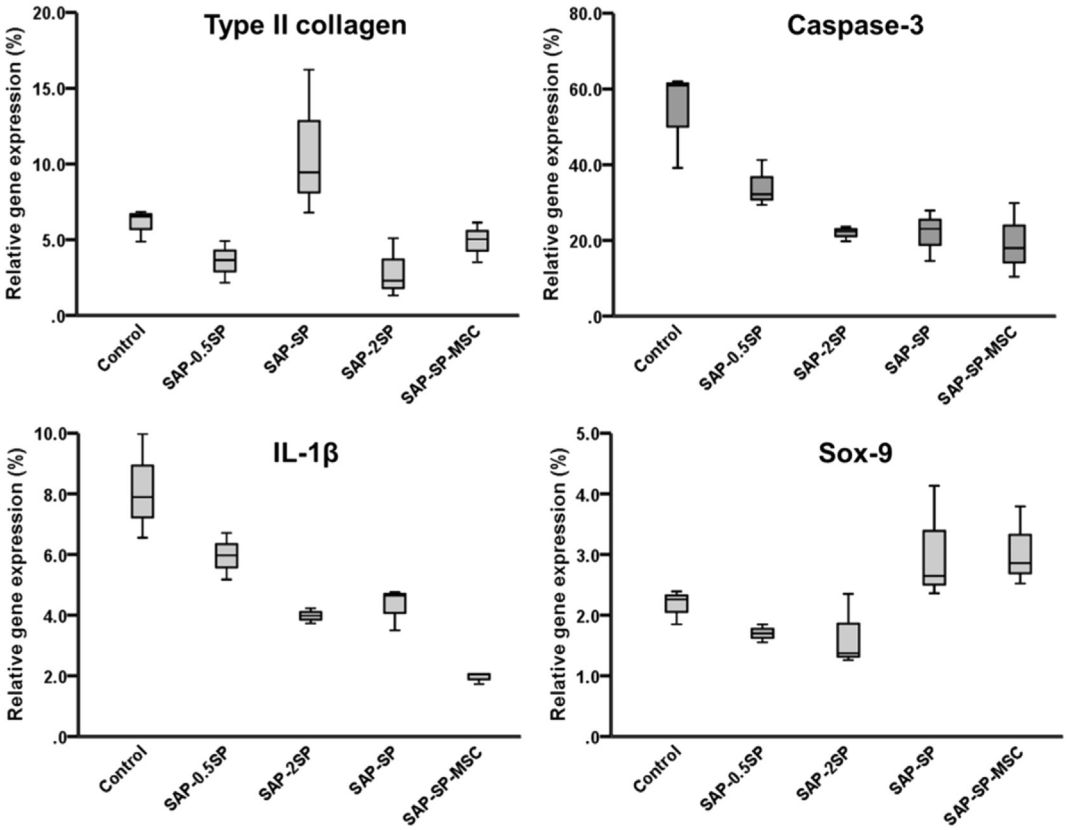

Fig. 2. Quantitative real-time PCR analysis of the chondrogenic marker (type II Collagen, Sox-9) and inflammatory mediators (Caspase 3, Interleukin-1β) expressions of chondrocytes from specimens of the knee joint. The gene expression was normalized to a house keeping gene, GAPDH.

showed the anti-inflammatory action of SP in a dose-dependent manner ($P = 0.04$ and $P = 0.01$) (see Fig. 2). SAP-SP-MSC group also showed the anti-inflammatory effect with its lower gene expression of IL-1β and the lowest expression of caspase-3. Expression of Sox-9 was higher in the SAP-SP and SAP-SP-MSC groups than in the other groups, which was statistically different ($p = 0.04$).

3.3. Immunofluorescent analysis

The immunofluorescence staining results of caspase-8 and TIMP-1 are shown in Fig. 3. The mean area of the positive stained section decreased as the SP amount increased, which represented the anti-inflammatory action of SP in a dose-dependent manner. SAP-SP-MSC also had the smallest area of positive stained section for TIMP-1, and the second smallest area for caspase-8 compared to the SAP-2SP group.

Additionally, the sections were also double stained with type II collagen and anti-substance P antibody, and the resulting images were merged with the DAPI staining im ages. Type II collagen was strongly expressed in all treatment groups except in the control group (Fig. 3). The quantity of substance P increased as the SP amount increased, which retained substance P in a dose-dependent manner. These results indicate that SAP is suitable for the sustained delivery of SP into the joint defect region as a hydrogel scaffold.

3.4. Micro-CT analysis

To estimate the cartilage regeneration from the treatment of the SAP complex, the knee joints were examined by micro-CT, and the bone mineral density (BMD) was measured based on the micro-CT analysis. Representative micro-CT 3D images are shown in Fig. 4. The BMD value was 575.0 ± 102.2 g/cm^3 in the SAP-0.5SP group, 368.2 ± 94.1 g/cm^3 in the SAP-SP group, 523.0 ± 36.9 g/cm^3 in the SAP-2SP group, and 458.4 ± 55.8 g/cm^3 in the control group. The Kruskal–Wallis test showed a significant difference between the groups ($p = 0.001$), and post-hoc analysis revealed that this difference was found between SAP-SP and the other groups.

3.5. Histological analysis

H&E staining of the cytoplasm and nuclei revealed well-formed cartilaginous tissues and normal looking synovial tissue in the SAP-SP group (Fig. 5). In contrast, severe erosion and a thin layer of superficial cartilage with synovial tissue were observed in the control group. Alcian blue staining indicated an accumulation of sulfated GAGs. The sufficient amount of GAGs in the SAP-SP group revealed the regenerated cartilage in the surrounding cartilage. However, incomplete cartilage that appeared to be weakly stained for GAGs and a thin layer of superficial cartilage were observed in the control and SAP-0.5SP groups. Furthermore, Masson's trichrome staining showed an abundant accumulation of collagen in the SAP-SP and SAP-SP-MSC groups. To analyze the histological images in a semi-quantitative manner, the modified Mankin scoring system was used. The modified Mankin score was 4.5 ± 1.3 in the SAP-0.5SP group, 3.8 ± 1.7 in the SAP-SP group, 6.5 ± 0.6 in the SAP-2SP group, and 10.3 ± 1.3 in the control group. The Kruskal–Wallis test showed a significant difference between the groups ($p = 0.01$), and the post-hoc Mann–Whitney U analysis with Bonferroni correction showed that this difference was found between the SAP-0.5SP and control groups and between the SAP-SP and control groups.

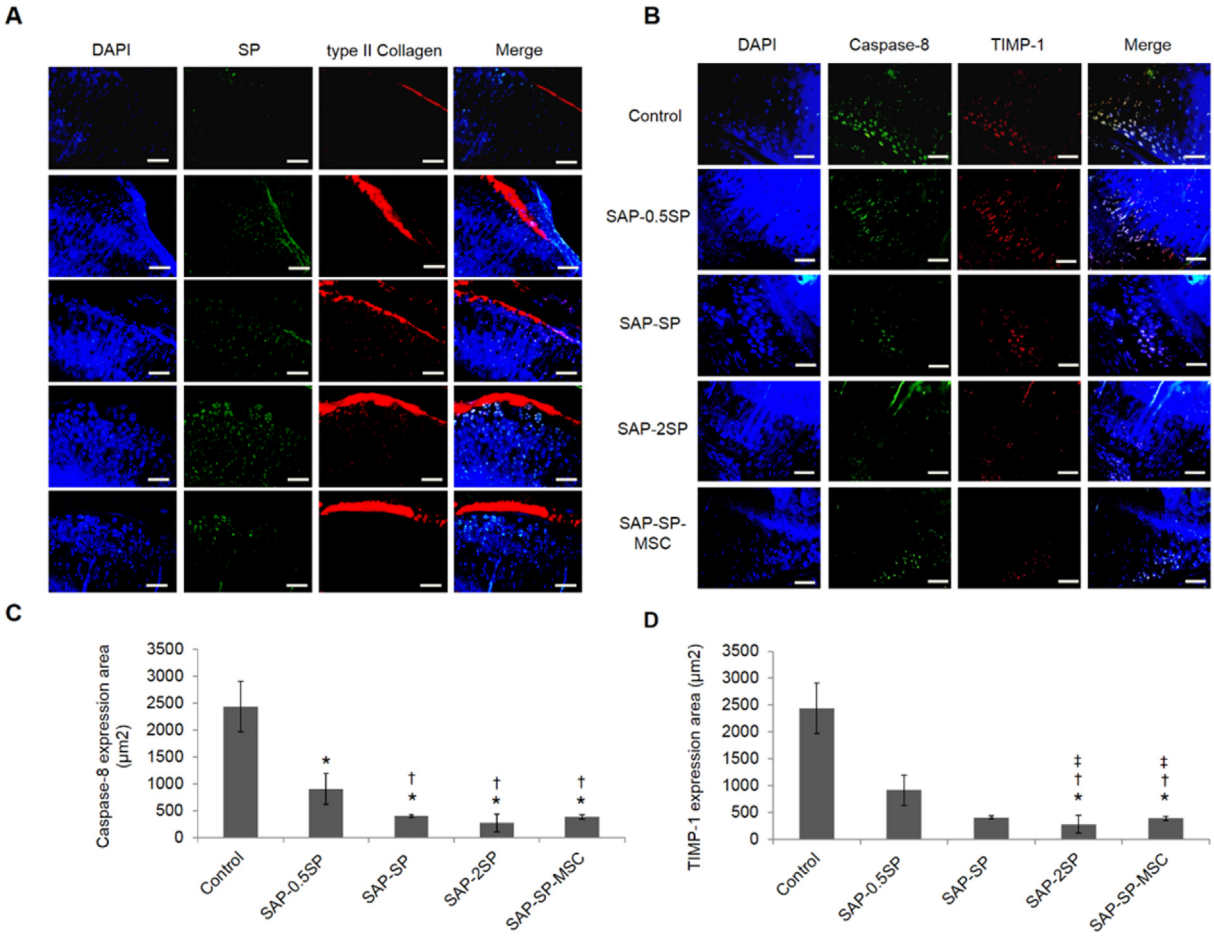

Fig. 3. Immunofluorescence anlaysis. (A) Substance P and type II collagen were detected by immunofluorescence staining. (DAPI: blue, Substance P: green, type II collagen: red). The expressed positive area of substance P means that retained substance P into knee joint defect region until 28 days after the injection. SP was more expressed as injected concentration of SP in a dose-dependent manner. Type II collagen was detected in all treatment groups without the control group. Scale bars: 100 μm. (B) Caspase-8 and TIMP-1 were detected by immunofluorescence staining (DAPI: blue, caspase-8: green, TIMP-1: red). Scale bars: 100 μm. (C) Quantification of caspase-8 positive expression area (μm^2). $^*p < 0.05$ with control group, $^\dagger p < 0.05$ with SAP-0.5SP group. D) Quantification of TIMP-1 positive expression area (μm^2). $^*p < 0.05$ with control group, $^\dagger p < 0.05$ with SAP-0.5SP group, $^\ddagger p < 0.05$ with SAP-SP group. Positive staining for caspase-8 and TIMP-1 was found in the control group, whereas less staining was observed in the other groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. Recruitment of intrinsic MSCs

To identify the MSC recruiting ability of SP-conjugated peptides, immunofluorescence analysis was performed for CD90 and CD105 which were MSCs specific markers at six weeks after surgery. After staining with anti-CD90 and CD105 antibody, stained area was quantitatively analyzed ($\mu m^2/\mu m^2$, n = 3 in each group). In the results, all SAP-SP groups had significantly higher recruitment of intrinsic MSCs than that of the control group (198.1 ± 15.6 μm^2/ 171.1 ± 15.6 μm^2 in control group) (Fig. 6). Additionally, CD90+/ CD105 + cells were enhanced in the SAP-SP group (1170.7 ± 291.4 μm^2/999.6 ± 71.5 μm^2) compared to the other concentrations of SAP-SP conjugates (423.3 ± 179.9 μm^2/ 522.3 ± 31.2 μm^2 in SAP-0.5SP group and 891.5 ± 38.2 μm^2/ 1008.6 ± 229.8 μm^2 in SAP-2SP group).

3.7. TUNEL assay

The TUNEL assay was done in chondrocytes in cartilage that underwent apoptosis, and the results are expressed as the Apoptotic index (%) shown in Fig. 7.

$TUNEL^+$ cell density (%) revealed a significant difference between the groups ($p = 0.04$) in the Kruskal–Wallis test, which was due to the difference between the SAP-SP (45.0 ± 19.1%) and control groups (80.0 ± 10.0%) in the post-hoc analysis. Additionally, the percentage of apoptotic cells in the SAP-SP-MSC group (37.5 ± 9.6%) was 2.13% lower than that of the control group which was significantly different. Moreover, there was no significant difference between the SAP-SP group and the SAP-SP-MSC group. Based on this result, the SAP-SP group was effective in preventing cell apoptosis, which is similar compared to the SAP-SP-MSC group.

3.8. Behavioral analysis

A behavioral test of the rat OA model was investigated by measuring the number of rears, which is related to pain and spontaneous activity. The number of rears before the injection, and at 2 and 4 weeks post-injection according to the groups is shown in Fig. 8. The number of rears significantly increased at 2 and 4 weeks post-injection in all the groups (F_{time} ($df = 1$) = 196.3, $p < 0.001$) although this was not statistically significant between the groups (F_{group} ($df = 3$) = 1.21, $p = 0.33$).

4. Discussion

Various studies have extensively investigated the treatment of

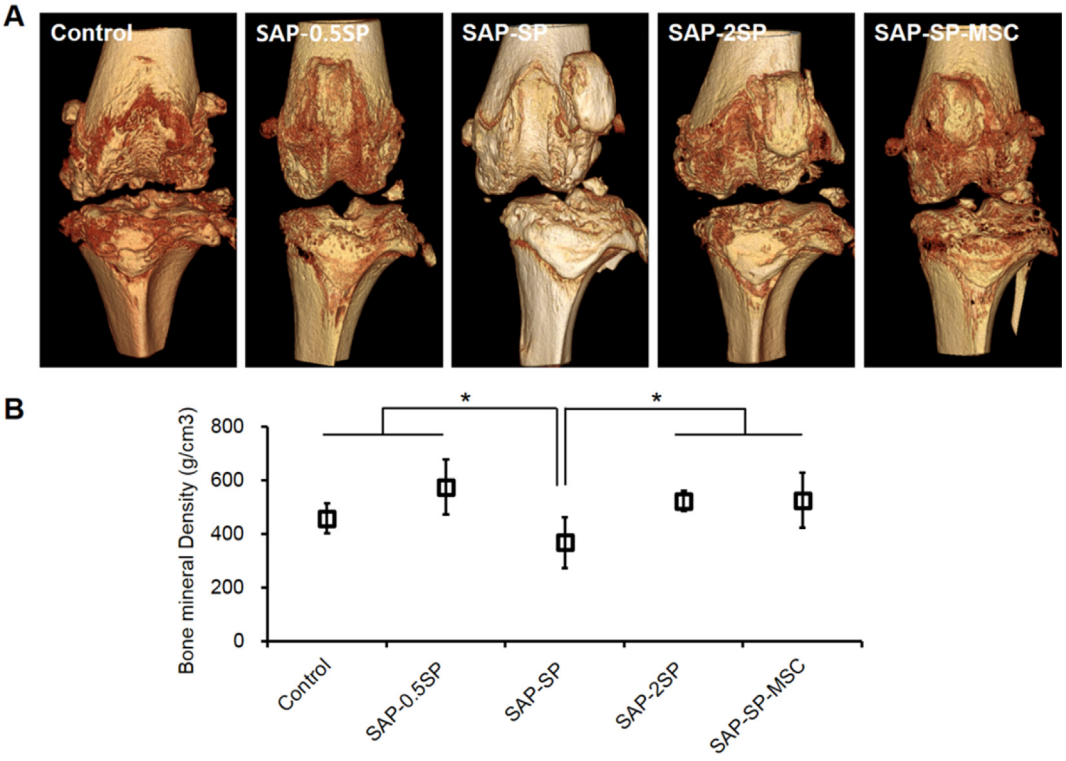

Fig. 4. Micro-computed tomography images of rat knee joints. (A) At 6 weeks post-surgical induction, the knee joints of the rats were extracted for a micro-CT analysis. (B) Bone mineral density (BMD) was calculated by scanning a phantom from micro-CT results. The represented picture means that the BMD was lower in the SAP-SP group than in the other groups. A significant difference was found between the SAP-SP group and the other groups ($^*p = 0.001$).

OA. Especially, stem cell-based approaches, including MSC injections have focused on regenerative cellular therapeutic materials because they have the potential to proliferate and differentiate rapidly to musculoskeletal lineages such as bone and cartilage [48]. Yang et al. have studied the effect of injecting allogenic MSCs intra-articularly in a rat OA model and evaluated the effect on immobility [49]. Furthermore, several groups also have used autologous chondrocyte or bone marrow stem cells (BMSCs) to avoid allogenic cell transplantation for OA treatment [50–52]. However, they have a limit like a slow process and it often leads to insufficient results due to the poor self-renewal and regeneration potentials [53]. In addition, the medications for the treatment of OA are available through an intra-articular injection of steroids or hyaluronans [54]. However, they are unable to induce the regeneration of the injured site but simply bring about arthritic pain relief. In this study, we have focused on the endogenous regeneration system, which is provoked by the mobilization of circulating MSCs without exogenous cell transplantation, and we confirmed that this system would promote cartilage regeneration and restore articular joint function in an OA rat model.

Also, we confirmed that our peptide gel as a therapeutic agent has an anti-inflammatory activity. It was shown that the SAP-SP conjugates affect the action of cytokines in anti-inflammation by increasing IL-4 and reducing the levels of pro-inflammatory cytokines such as GM-CSF, IL-2, IL-17A, IFN-γ, TNF-α, MIP-α, and MIP-β. In addition to the anti-inflammatory activity, the SAP-SP conjugates were found to induce the recruitment of intrinsic MSCs and to inhibit apoptosis in chondrocytes.

Hong et al. reported that SP has anti-inflammatory role in the collagen II–induced arthritis model [19]. In addition, SP induces M2-type macrophages after spinal cord injury [20]. SP skewed the systemic cytokine profiles toward anti-inflammation by reducing the levels of TNF-α and IL-17 and increasing that of IL-10. Moreover, SP could increase the percentage of Treg and M2 type macrophages in the circulation after SP administration. Anti-inflammatory cytokines act by inhibiting the synthesis of pro-inflammatory cytokines. Particularly, IL-4 is known to have a strong chondroprotective effect not only by inhibiting the degradation of proteoglycans by inhibiting the secretion of metalloproteinases (MMPs) but also by reducing the variation in the production of proteoglycans in the articular cartilage. In addition to the anti-inflammatory actions, cytokines have additional positive effects such as inhibiting apoptosis in chondrocytes and decreasing the level of PGE2. PGE2 is reported to decrease proteoglycan synthesis and to enhance the degradation of aggrecans and type II collagen which are the main constituents of articular cartilage [55]. As shown by our results, SAP-SP conjugates increase anti-inflammatory cytokines, which result in decreased synthesis of pro-inflammatory cytokines (Fig. 1). Therefore, we anticipate that the anti-inflammatory response has a role in delaying the progression of OA by inhibiting apoptosis in the chondrocytes and the degradation of cartilage. Our findings show that the identified cell apoptosis in the defect site was reduced in the SAP-SP group, which suggests that SAP-SP can protect chondrocytes from apoptosis. Furthermore, it is believed that cell apoptosis is affected by the nanofibrous structural characteristics and 3D microenvironment of the SAP-SP conjugates. Several previous studies have reported that SAP supplied the 3D microenvironment because their porosity and gross structures are very similar to the highly hydrate structures of human extracellular matrices. Because of this characteristic, SAP may surely be involved in cell attachment, proliferation, migration and differentiation. In our previous study, we showed that KLD12, a kind of SAP, could inherently inhibit cell apoptosis by itself. Considering that both KLD-12 and KLD-12-SP have similar stable β-

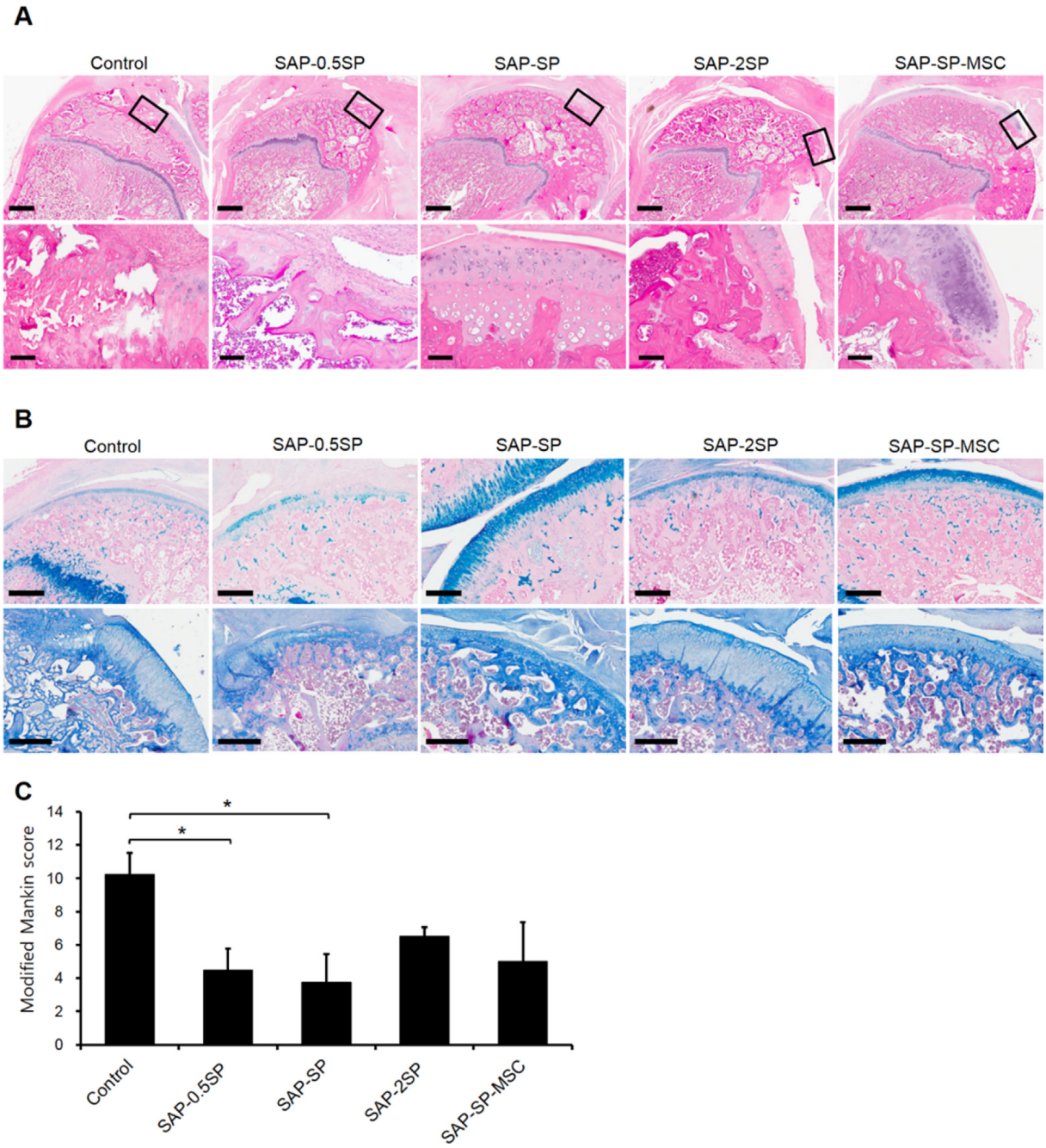

Fig. 5. Histological studies of knee joints from rat at 6 weeks after surgical induction. (A) Macroscopic images of hematoxylin and eosin staining. Boxed areas in panels (upper line, Scale bars: 1 mm) are shown at higher magnification in panels (lower line, Scale bars: 100 μm). (B) Alcain blue (upper line) and Masson's trichrome staining (lower line) of the retrieved knee joints from rat at 6 weeks after surgical induction. Scale bars: 100 μm. (C) Evaluation of Modified Mankin score according to cartilage structure, cartilage cells, Alcian blue staining, and tidemark integrity. (*p value is less than 0.05.) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sheet structures based on structural analysis, the SAP-SP conjugates will have a similar anti-apoptotic function. Moreover, Koon et al. reported that SP also has an anti-apoptotic effect by binding to its neurokinin-1 receptor (NK-1R) which phosphorylates and activates Akt [56]. Therefore, it is suggested that SAP-SP conjugates have the potential to protect chondrocytes from apoptosis and to enhance tissue recovery and regeneration.

In this study, to determine whether the SAP-SP conjugates has different therapeutic potentials according to the ratio of SAP to SP, we used three different ratios of SAP-SP concentrations. As shown by our results, treatment of OA with 200 μl of SAP mixed with 7 μl of SP improved cartilage regeneration but not significantly different from that of the other mixtures. This cartilage regeneration was similar in the SAP-SP-MSC group, which means that irrespective of the dose of SP, the effectiveness of the SAP-SP conjugates was as good as the exogenous MSC implanted group for tissue regeneration. Because we focused on endogenous stem cell mobilization following chondrogenic differentiation for the treatment of OA to overcome the limitations of stem cell implantation, we adopted SP as one of the therapeutic factors to induce the homing effect of

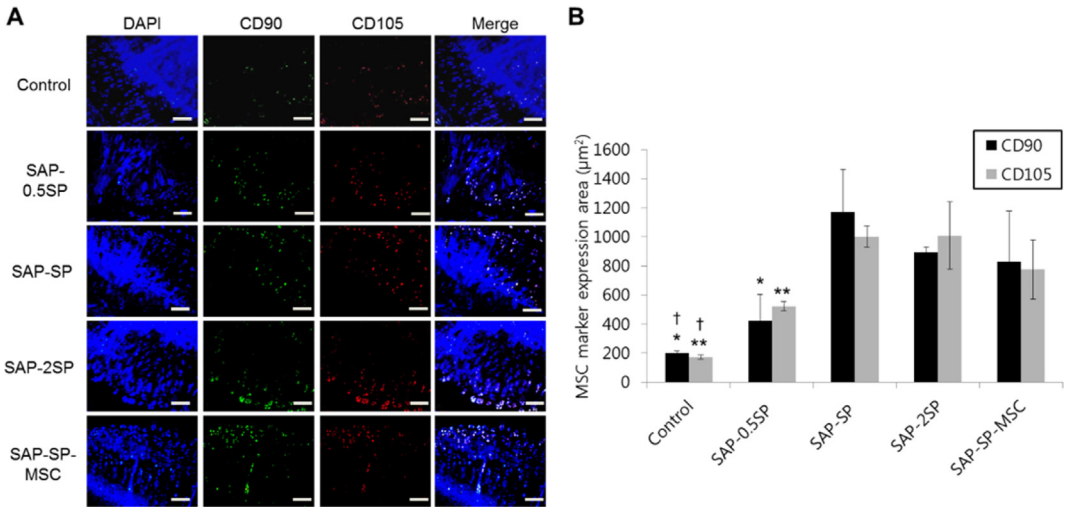

Fig. 6. Mesenchymal stem cells (MSCs) recruitment of SAP-SP conjugates. SAP-SP conjugates are effective for the recruitment of MSCs into arthritic defect region. (A) Representative images of articular cartilage defect site from each group after CD90, CD105 staining. Scale bars: 100 μm. (B) Quantification of MSCs expression area of each group (μm^2). $^*p < 0.05$ with SAP-SP group, $^{**}p < 0.001$ with SAP-SP group, $^{\dagger}p < 0.05$ with SAP-SP-MSC group.

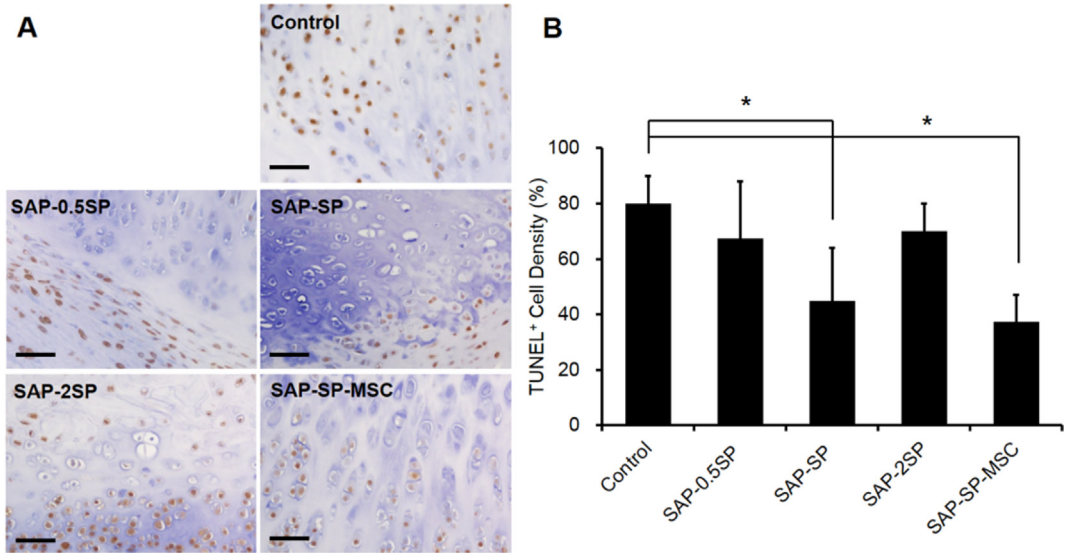

Fig. 7. Apoptosis of chondrocytes from each group was detected by apoptosis detection kit at 6 weeks post-surgical induction. (A) TUNEL staining in the articular cartilage regions. Apoptotic cells: brown. (B) The ratio of TUNEL-positive to total nuclei was quantified each group (expressed as a %). Scale bars: 25 μm ($^*p$ value is less than 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

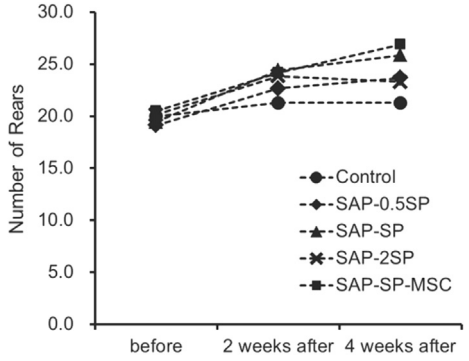

Fig. 8. Number of rears result before, 2 weeks after, and 4 weeks after the injection were described in all groups. Behavioral results showed that all groups were increased at 2 and 4 weeks, but this was not a significant difference between the groups.

endogenous MSCs. SP, an injury-inducible messenger, was reported to mobilize CD29$^+$ stromal-like cells and has been used to recruit stem cells and to regenerate injured tissues as a chemotactic agent. For efficient and successful *in situ* tissue regeneration, it is important that the appropriate numbers of host stem cells are recruited to the injured site. SAP has been actually studied for the controlled delivery and release of functional molecules such as functional proteins, therapeutic macromolecules, and bioactive motifs. In our micro-CT results, the BMD values of the control, SAP-0.5SP, and SAP-2SP groups did not show significant differences, while the BMD of the SAP-SP group was significantly low compared to the other groups. It was considered that the SAP-0.5SP treatment showed enough deficient effects to induce the endogenous healing process. Regarding the SAP-2SP group, we observed the saturated effect over the dose of SAP-SP in histological studies. However, it was thought that excess SP could lead to negative effects on

healing, as it was shown that the apoptosis from TUNEL assays was increased in SAP-2SP. Therefore, we will study the side effects and safety test of SAP-SP for various doses as a further study.

In our immunofluorescence data, SP remained in the joint cavity until 28 days post-injection. This result suggests that the retention and sustained delivery of SP were improved by physically adsorbing and covalently binding to SAP up to 28 days. Furthermore, we confirmed that CD90 and CD105 (MSC markers) were 5.90 and 5.84 times greater than those of the control group due to the effect of SP remaining for 28 days in the SAP-SP group. Likewise, the SAP-0.5SP, SAP-2SP and SAP-SP-MSC groups exhibited a higher expression of SP than that of the control group. The reason for these results could be that SP stimulation via its sustained release from the SAP-SP conjugates would result in continuous recruitment of MSCs and more effective interactions between the cells in the adjacent defect site, thereby facilitating the upregulation of chondrogenic markers including Sox-9 and collagen type II, the chondrogenic differentiation of mobilized MSCs, and the inhibition of inflammatory mediators such as IL-1β and caspase-3. However, judging from the strongest effect of the SAP-SP group, we emphasize that 200 μl of SAP mixed with 7 μl of SP is the optimal concentration ratio of SAP to SP with therapeutic potential for OA. In particular, this tendency was also supported by the histologic results. The SAP-SP group showed that the lacunae surrounded the collagen in the cartilage region remarkably. Conversely, an incomplete cartilage shape including a thin cartilage layer and less chondrocytes in the lacunae were observed in the control group. In terms of the modified Mankin scoring system, the cartilage structure, cartilage cells, Alcian blue staining and tidemark intensity for regeneration were improved in the SAP-SP group more than that of the other groups. We speculate that SP has the optimal mobilization activity for the present ratio in the SAP-SP group via recruitment of MSCs; thus, these cells may infiltrate into the defect site for regeneration of the OA defects. Compared with the SAP-SP-MSC and SAP-SP groups, the effect of endogenous stem cell mobilization seems to be similar to exogenous stem cell injection based on our total results. Even though MSCs were exogenous injected into the knee joint cavity, the injected MSCs did not partially engraft but instead diffused into the surrounding area of the defect site and subsequently could not be participate in cartilage regeneration. Therefore, we showed that treatment with our bioactive SAP that included SP might be enough for cartilage regeneration due to the homing of endogenous cells without the need of exogenous MSCs and all their inherent difficulties in the OA rat model. In summary, we showed that SP conjugated with SAP provides the proper microenvironment for MSC differentiation by recruiting MSCs and can be an efficient tool for tissue regeneration and for the therapeutic treatment of OA. In this study, our hydrogel is a potentially excellent injectable therapeutic treatment for OA by recruiting autologous stem cells. With the goal of clinical application, we will do further studies using a middle-sized animal, such as rabbits or Dunkin Hartley pigs, in an OA model.

5. Conclusion

We developed injectable bioactive peptides which could enhance endogenous stem cell recruitment that delay the progression of cartilage destruction in OA in a rat knee model. In this study, SAP facilitated the long term release of SP and subsequently, provided the proper environment for tissue regeneration at the defect site of the articular joint. SAP-SP can prevent apoptosis, increase the extracellular matrix involved chondrogenesis, promote chondrogenic differentiation, and alleviate the inflammation in OA by secreting anti-inflammatory cytokines. Consequently, SAP-SP could delay the progression of OA and restore articular joint function without cell transplantation. Therefore, we respectfully believe that our bioactive peptide SAP-SP can be a potentially treatment as an injectable type of therapy for OA through the homing of endogenous stem cells.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.09.040.

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