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Original Article

Role of vasoactive intestinal peptide in the progression of osteoarthritis through bone sclerosis and angiogenesis in subchondral bone

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ABSTRACT

Objective: Osteoarthritis (OA) is a progressive joint disorder, with abnormal remodeling of subchondral bone linked to the disruption of cartilage metabolism. Nerves also play an important role in bone remodeling in OA progression, and vasoactive intestinal peptide (VIP), one of the neuropeptides, plays an important role in bone metabolism. The aim of this study was to analyze the expression pattern of VIP in subchondral bone, and its potential as a therapeutic target for OA progression.

Design: The pattern of VIP expression in the human tibia was histologically evaluated. The effect of VIP on angiogenesis was investigated using human umbilical vein endothelial cells (HUVECs). Knee OA was induced by the resection of the medial meniscotibial ligament in C57BL/6 mice. A VIP receptor antagonist was intraperitoneally administered postoperatively, and therapeutic effects were analyzed at 4 and 8 weeks.

Results: VIP expression in the subchondral bone increased as OA progressed in human tibia. VIP was also expressed in the vascular channels into the cartilage layer. The total length and branch points were significantly increased, due to the VIP receptor agonist in HUVECs. In OA mice, the VIP receptor antagonist could prevent cartilage degeneration and subchondral bone sclerosis. The Osteoarthritis Research Society International score in the VIP receptor antagonist group was significantly lower than in the control group.

Conclusion: VIP is involved in the progression of OA through its effect on subchondral bone sclerosis and angiogenesis. Inhibition of VIP signaling has the potential to be a therapeutic target to prevent OA progression.

1. Introduction

Osteoarthritis (OA) is a progressive joint disorder that affects both cartilage and the subchondral bone [1,2]. Studies have focused on the remodeling of subchondral bone with OA progression, due to the important role that subchondral bone plays on cartilage metabolism [2]. However, the mechanism by which subchondral bone is changed in the pathogenesis of OA has not been completely elucidated. Several animal studies have demonstrated a relationship between cartilage degeneration and the change in subchondral bone during OA progression, such as bone sclerosis and absorption [3]. Although these reports indicated that subchondral bone could be a feasible therapeutic target to prevent the progression of OA, the effectiveness of targeting molecules is still unclear.

The neurological system also plays an important role in bone metabolism. Neuropeptides, such as substance P, calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and neuropeptide Y, are synthesized in unmyelinated sensory neurons and sympathetic nerves, and released from their peripheral terminals located in the bone and periosteal tissue [4]. Neuropeptides have been implicated in the regulation of local bone metabolism, in addition to nociception, inflammation, angiogenesis, and cellular

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proliferation [5]. It is reported that spinal cord injury causes abnormal bone mass, structure and metabolism in the sublesional area, both in animal models and humans [6], with these changes being possibly caused by a change in the level of neuropeptides in the microenvironment [7]. VIP is a 28-amino acid neuropeptide that belongs to a glucagon/secretin superfamily, namely the ligand of class II G protein-coupled receptors: VIP receptor type 1 (VPAC1), VIP receptor type 2 (VPAC2) and the pituitary adenylatecyclase-activating polypeptide (PACAP) type 1 (PAC1) receptor [8,9]. VIP is expressed in the tissue of the lungs, small intestine and neurons of the central nervous system, and plays important roles in various biological functions, including stimulation of the contractility of the heart, vasodilation, promotion of neuroendocrine-immune communication, increasing glycogenolysis, and lowering of the arterial blood pressure [9]. VIP has anti-inflammatory and immune-modulatory activities, such that modulation of the VIP level has been considered as a potential candidate for the treatment of inflammatory and autoimmune diseases, such as pancreatitis, septic shock, inflammatory bowel disease, lipopolysaccharide (LPS) induced acute inflammation, and arthritis [9]. VIP also assists in promoting osteoblast differentiation and decreasing osteoclastogenesis via its effect on the regulation of the receptor activator of nuclear factor kappa B ligand, osteoprotegerin and macrophage colony stimulating factor (M-CSF) [4,10,11]. Regarding the pathogenesis in OA, the plausible role of VIP in OA is supported by findings of a negative correlation between VIP levels in synovial fluid and cartilage and the severity of OA disease [8].

It is reported that VIP is expressed in subchondral cancelous bone in OA, but the details of its pattern of expression in patients with OA and the role of VIP in the progression of OA have not been elucidated [12]. Subchondral bone maintains the metabolism and homeostasis of articular cartilage, and abnormal turnover of subchondral bone causes OA progression [13]. However, the precise mechanism of subchondral bone change in OA has not been elucidated. Therefore, to investigate the important factor of remodeling of the subchondral bone, we focused on neuropeptides, based on previous evidence of the important role that nerves play in bone remodeling [14]. In a previous study, neuropeptides such as substance P were detected in the subchondral bone of patients with OA [15]. Another previous report demonstrated that the CGRP receptor antagonist BIBN4069 could attenuate the degeneration of articular cartilage in mice with surgically induced OA [16]. Therefore, neuropeptides appear to influence the pathogenesis of OA. As VIP is involved in the regulation of bone metabolism, its expression in subchondral bone might contribute to the pathogenesis of OA through promotion of sclerosis in the subchondral bone, subsequently leading to cartilage degeneration. We hypothesized that blocking VIP signaling could inhibit sclerotic changes in the subchondral bone, thus subsequently preventing cartilage degeneration. Therefore, the purpose of this study was to analyze the pattern of expression of VIP in the subchondral bone in patients with OA, and to examine the therapeutic effect of blocking VIP signaling on the progression of OA in a mouse model with surgically induced OA.

2. Materials and methods

2.1. Study group

For analysis of VIP expression in human OA, bone samples were obtained from 7 patients who had previously undergone total knee arthroplasty for the treatment of knee OA. The study group included one man and six women, with a mean age of 73 years (range: 66–86 years). All patients were treated conservatively using celecoxib and orthosis for at least 1 year before surgery. Knee OA was diagnosed from standing radiographs, using the American Rheumatism Association's criteria for OA, with Kellgren–Lawrence (KL) grades III (5 cases) and IV (2 cases) included in our study group. Patients with a history of knee trauma and those with systemic diseases, such as rheumatoid arthritis, were excluded. The whole proximal tibia, obtained using standard surgical techniques for total knee arthroplasty, was used to analyze the expression of VIP. To evaluate the relationship between VIP expression in the cartilage and subchondral bone, the proximal tibia was divided into 6 regions, as degeneration of cartilage and subchondral bone in OA varies across the different regions of the proximal tibia. Specifically, the medial and lateral tibial plateau was subdivided into 3 regions each, with 33 samples obtained for analysis from each proximal tibia specimen.

For the animal study, 30 male 10-week-old C57BL/6 mice were used. The animals were housed with free access to food and water, and were allowed unrestricted weight bearing prior to the surgical intervention. For the OA model, the medial meniscotibial ligament of the bilateral knee joints was resected and evaluated the right knee according to the method described in a previous report [17]. The other mice were used for sham operation which skin incision was applied. To examine the effect of the VIP receptor antagonist on the progression of OA (the VIP receptor antagonist group), an intraperitoneal injection of a VIP receptor antagonist (N-terminal Lys-Pro-Arg-Arg-Pro-Tyr, followed by 7–28 residues VIP: Bachem Holding AG, Bubendorf, Switzerland), at a dose of 10 nmol/animal, was administered immediately after the surgery [18]. In the control group, phosphate-buffered saline (PBS) was injected after surgery, at a dose of 100 μl/animal. Mice were sacrificed at 0, 4 and 8 weeks, and knee joints ($n = 5$) were harvested. All samples were fixed in 4% paraformaldehyde (PFA) and were subsequently analyzed using micro-computed tomography (mCT). After this, paraffin-embedded sections were prepared.

2.2. Micro-computed tomography

Samples were analyzed under high-resolution mCT (Sky-Scan1176, Toyo Corporation, Tokyo, Japan), using the following parameters: source voltage, 40 kV; source current, 580 μA; pixel size, 12.47 μm; and spatial resolution, 9 μm. Images were reconstructed (NRecon, Toyo Corporation, Tokyo, Japan) for analysis (CT-analyzer, Toyo Corporation, Tokyo, Japan). For human samples, cylindrical regions (0.5 μm in diameter × 0.5 μm in height) were obtained from the region of interest (ROI), defined as the area of greatest sclerotic change in the subchondral bone. In mice, ROI was the subchondral bone of the medial tibial plateau, with the bone volume/tissue volume (BV/TV, %) ratio measured as previously described [19].

2.3. Histological analysis

After being fixed in 4% PFA, samples were decalcified in 20% ethylenediaminetetraacetic acid for 2 weeks and were subsequently embedded in paraffin. Coronal sections of human samples and sagittal sections of mouse samples, 4 μm in thickness, were prepared for histological analysis. Sections were stained using safranin-O fast-green and hematoxylin-eosin (HE). The pathological changes in the tibial plateau, for both human and mice samples, were scored using the Osteoarthritis Research Society International's (OARSI) scoring system for OA [20,21]. The average OARSI grade and score were calculated. Human samples were classified into three groups, as follows: the mild OA group (OARSI grade: 1.0–2.5); the moderate OA group (OARSI grade: 3.0–4.5); and the severe OA group (OARSI grade: > 5.0) groups. Other sections were used for the immunohistochemistry and tartrate-resistant acid phosphatase (TRAP) staining.

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2.4. Immunohistochemical analysis

Each section was immunostained with an anti-VIP antibody (1:500 dilution, Abcam, Cambridge, MA), anti-osteocalcin (1:100 dilution, Santa Cruz Biotechnology, Dallas, TX), anti-matrix metallopeptidase 13 (MMP 13) (1:20 dilution, Neomarkers, Fremont, CA), anti- A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5; 1:100 dilution, Gene Tex, Irvine, CA), and type II collagen (1:10 dilution, The Developmental Studies Hybridoma Bank, IA), using a 3,3′-diaminobenzidine substrate, as described previously [22]. For immunofluorescence staining, anti-VIP antibody, anti-CD-31 antibody (1:20 dilution, BD Bioscience Pharmingen, San Jose, CA) and anti-neurofilament antibody (1:200 dilution, Abcam, Cambridge, MA) were used. The secondary antibodies were Alexa Fluor 488-conjugated anti-rabbit for VIP, Alexa Fluor 568-conjugated anti-mouse for CD-31 and anti-chicken for neurofilament (1:500 dilution, Molecular Probes; Invitrogen, Carlsbad, CA). The negative control group was treated with the isotype IgGs to replace the primary antibodies, using a 4′,6-diamidino-2-phenylindole solution (Dojindo Laboratories, Kumamoto, Japan) for nuclear staining.

Regarding the human samples, VIP-positive cells in the subchondral bone were counted and the area of the subchondral was measured using Image J (National Institution of Health) and were used to report the cell count per unit area of 0.25 mm^2. With the mouse samples, TRAP staining was performed using a commercially available kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer's protocol. TRAP-positive multinucleated cells, containing more than three nuclei, were recognized as osteoclasts and counted in the subchondral bone using Image J. To analyze the expression of VIP, bone formation and osteoclasts, the total number of VIP- and osteocalcin-positive cells and osteoclasts in the subchondral bone were counted and corrected by unit area (0.04 mm^2). In the subchondral bone, lining cells along with the trabecular bone in marrow cavity were defined as osteoblast, and cells in the lacunae of the trabecular bone were defined as osteocyte. The number of VIP positive osteoblasts and osteocytes were counted, and the ratio of total VIP positive cells and osteoblasts (%) were calculated. To evaluate the expression of MMP 13 and ADAMTS-5, the number of MMP 13-positive cells and ADAMTS-5-positive cells were counted according to the method of a previous report [23].

2.5. Angiogenesis

Human umbilical vein endothelial cells ([HUVECs], Lonza Group AG, Basel, Switzerland) were pre-cultured in an endothelial basal medium-2 (Lonza, Basel, Switzerland) and reseeded at a density of 1.0 × 104 cells/well in 96-well plates that were pre-coated with Matrigel Matrix (Merck MillipoFre, Billerica, MA). VIP receptor agonist (Bachem Holding AG, Bubendorf, Switzerland) was added to the wells at doses of 10 and 100 nM, and PBS was added to the control solution. The cells were incubated at 37 °C with 5% CO2, and the total tube length was measured using Image J. Branch points were counted at 12 h after incubation, according to the method previously described [24]. All experiments were performed at least three times.

2.6. Isolation and culture of human articular chondrocytes

Articular cartilage samples were obtained from lateral femoral condyle of patients who underwent total knee arthroplasty for varus osteoarthritis of the knee. These patients consisted with two men and two women, with a mean age of 73 years (range, 67–84 years). Chondrocytes were isolated by sequential treatment with trypsin and collagenase Type 2 (Worthington, Lakewood, NJ, USA) in DMEM/Ham's F12. Isolated chondrocytes were cultured in medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in 12-well plates until the cells became confluent. An in-vitro chondrocytes utilizing interleukin (IL)-1β-stimulation (1 ng/ml; PeproTech, Rocky Hill, NJ, USA) to induce OA inflammation was used to assess the chondroprotective effects of VIP receptor antagonist against OA. Chondrocytes were treated with or without VIP receptor antagonist (10 nM/L or 100 nM/L) in each well for 24 h. Then, Quantitative real-time polymerase chain reaction (PCR) was used to evaluate the effect of the VIP receptor antagonist on chondrocyte.

2.7. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from chondrocytes using Isogen. Complementary DNA (cDNA) was synthesized using 500 ng of total RNA with the SuperScript VILO cDNA Synthesis Kit (Invitrogen). A real-time PCR assay was performed using TaqMan Gene Expression Assay probes (Applied Biosystems, Foster City, CA, USA) MMP-13 (Hs00233992_m1), IL-6 (Hs00985639_m1), and GAPDH (Hs02758991_g1) was used as the internal control to normalize the sample differences. Relative expression was calculated using the ΔΔCt values, and results were expressed as 2-ΔΔCt.

2.8. Glycosaminoglycan release assay

Based on prior reports characterizing the inflammatory degradation of mouse cartilage, a femoral head cartilage explant model was used [25]. Briefly, hips from C57/BL/6 male 4-week old mice were disarticulated to expose the femoral head and the cartilage layer was separated from underlying bone with the use of thin forceps. Cartilage samples were cultured for 72 h in 96 well plate at 37 °C. Each well contains 500 μl medium (DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin). Cartilages were stimulated with IL-1β (1 ng/ml; PeproTech, Rocky Hill, NJ, USA) and treated with VIP receptor antagonist (10 nM/L or 100 nM/L) in each well for an additional 72 h. The Cell-conditioned media was collected and measured glycosaminoglycan (GAG) concentration using Blyscan Glycosaminoglycan Assay Kit (Biocolor).

2.9. Statistical analysis

All results were expressed as the mean and standard deviation. Three or four groups were compared using Tukey–Kramer's post hoc test, and Spearman's correlation coefficients were used to explore the relationship between the OARSI grade, the number of VIP and BV/TV ratio of subchondral bone. A p-value <0.05 was considered statistically significant.

3. Results

3.1. The expression pattern of VIP in human samples

The distribution of OA severity in human samples included in the analysis was as follows: mild OA: 12 samples; moderate OA: 10 samples; and severe OA: 11 samples. The average OARSI grade in each of the 3 groups was as follows: 1.8 ± 0.6 in the mild OA group; 3.6 ± 0.5 in the moderate OA group; 5.4 ± 0.3 in the severe OA group. All samples were in the range of 3.6 ± 1.5 points. VIP was expressed along the surface of the trabecular bone and in the bone marrow cavities, as well as in bone cells. In mild OA, the bone marrow cavity was well maintained, and VIP was expressed mainly along the surface of the trabecular bone and in the bone marrow cavities. The greater the OA progression and reduction in size of the bone marrow cavity, the greater the tendency for VIP expression in bone cells. In samples with severe OA, the size of the bone marrow

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cavity was reduced, and the bone volume was increased, with VIP expressed in the trabecular bone, and around the blood vessels and cystic lesion (Fig. 1A).

mCT analysis was conducted in 28 samples (7 mild OA, 10 moderate OA and 11 severe OA). 5 samples were corrupted and could not be included. A positive correlation was identified between the BV/TV ratio of subchondral bone and the OARSI grade ($R = 0.63$; Fig. 1B), with the number of VIP-positive cells being significantly higher in samples of severe rather than mild OA ($p < 0.05$; Fig. 1C). As OA progressed, invasion of the blood vessels from the subchondral bone into the calcified zone of the cartilage layers increased (Fig. 2A). In these lesions, the immunohistochemistry of CD31 (Fig. 2B), neurofilaments (Fig. 2C) and VIP revealed that VIP was expressed along with neurofilaments and blood vessels during cartilage degeneration.

3.2. The effect of VIP on angiogenesis

To examine the effect of VIP on angiogenesis, a tube formation assay was performed. The branch points in the VIP 10 nM group and 100 nM group were significantly greater than in the control group. ($p < 0.05$; Fig. 3A, B). The total length was significantly greater in the VIP 100 nM than in the control group ($p < 0.05$; Fig. 3A, C). There was no significant difference in the branch points and total length between the VIP 10 nM and the VIP 100 nM group.

3.3. The effect of the VIP receptor antagonist on the inhibition of OA

All mice survived the experimental procedures, without experiencing any adverse events. The control group exhibited progressive degenerative OA change, with cartilage degeneration being attenuated in the VIP receptor antagonist group (Fig. 4A). The OARSI score in the VIP receptor antagonist group was significantly lower than in the control group at 4 and 8 weeks ($p < 0.05$; Fig. 4B). Analysis of mCT images showed that the BT/VT ratio of the medial plateau's subchondral bone in the control group was significantly higher than in the VIP receptor antagonist group at 4 weeks ($p < 0.05$; Fig. 4C, D). However, there was no significant difference between the two groups at 8 weeks.

Immunohistochemistry revealed that the VIP receptor antagonist could suppress the expression of MMP 13 and ADAMTS-5, while the control OA mice exhibited the expression of MMP 13 and ADAMTS-5 intensely, and they also displayed decreased type 2 collagen in cartilage as it degenerated (Fig. 5A–C). The rate of MMP 13 and ADAMTS-5 positive cells/total cells of articular cartilage in the control group was significantly higher than in the VIP receptor antagonist group at 4 and 8 weeks ($p < 0.05$; Fig. 5D, E). The expression of VIP was observed in the trabecular bone and bone marrow cavity in the subchondral bone, and its expression increased as OA progressed (Fig. 6A). The number of VIP-positive cells in subchondral bone was significantly higher in the VIP receptor antagonist group at 8 weeks than in the sham group and the control group at 4 weeks ($p < 0.05$; Fig. 6B). There was no significant difference between the sham group and the control group at 4 weeks and between the control group at 4 weeks and the VIP receptor antagonist group at 4 weeks ($p < 0.05$; Fig. 6B). The expression of VIP was suppressed in the VIP receptor antagonist group at 8 weeks, although the expression of VIP was not significantly different between the 2 groups at 4 weeks ($p < 0.05$; Fig. 6B). There was a mild correlation between the number of VIP positive cells and BV/TV ($rs = 0.425$). In addition, the VIP positive cell ratio of osteoblasts in the subchondral bone decreased from $53.6 \pm 4.8\%$ at 4 weeks to $45.7 \pm 4.1\%$ at 8 weeks. There was a significant difference between 4 and 8 weeks ($p < 0.05$). Osteocalcin was intensely expressed in the subchondral bone in the control group compared to in the VIP receptor antagonist group. In contrast, the VIP receptor antagonist group showed abundant TRAP-positive cells in the subchondral bone. The ratio of osteocalcin-positive cells to bone marrow cells in mice in the control group was significantly higher than in mice in the VIP receptor antagonist

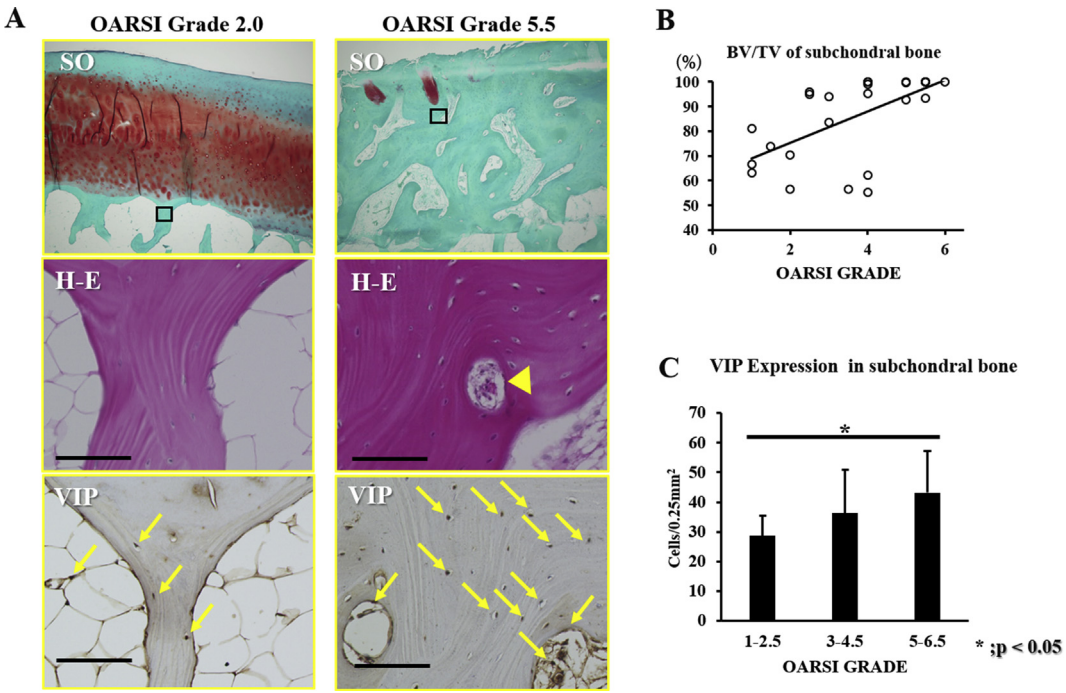

Fig. 1. A. Hematoxylin and eosin (HE), safranin-O (SO) staining and vasoactive intestinal peptide (VIP), B. Correlation between the Osteoarthritis Research Society International (OARSI) grade and the bone volume/tissue volume (BT/VT) ratio, C. The expression of VIP. Bar: 100 μm.

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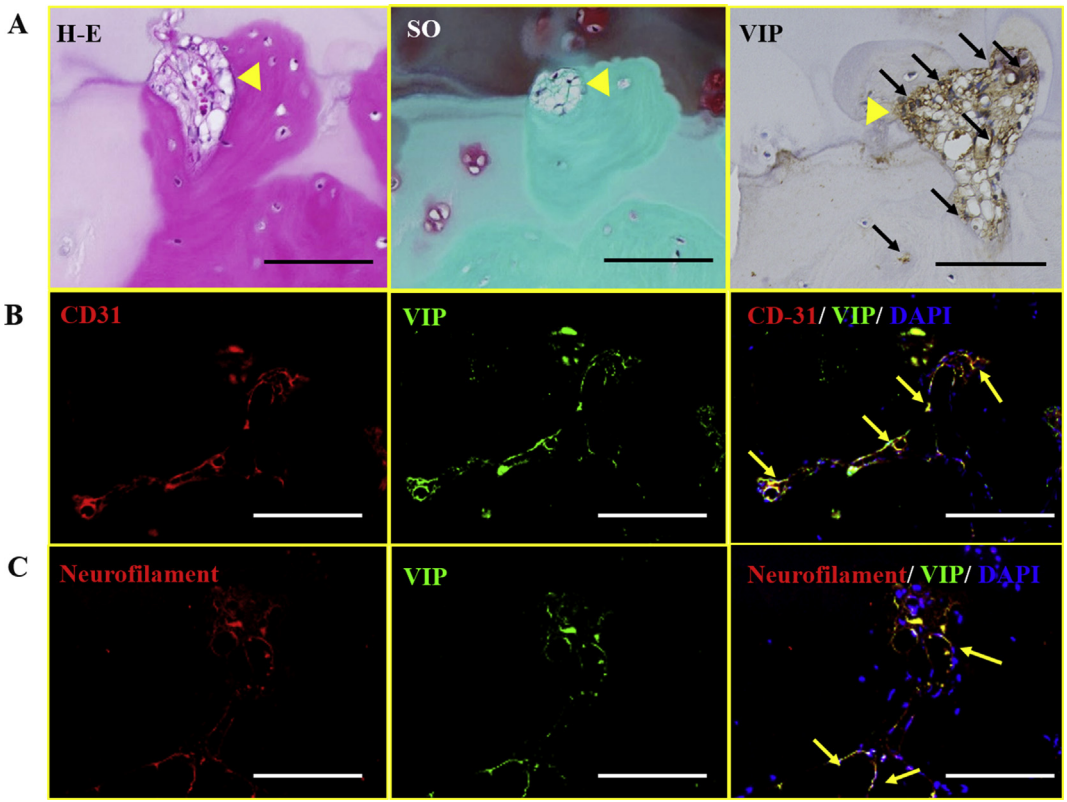

Fig. 2. A. Hematoxylin and eosin (HE), safranin-O (SO) staining and vasoactive intestinal peptide (VIP). Subchondral bone and blood vessel penetrate the tidemark. B. CD-31 and vasoactive intestinal peptide (VIP). C. Neurofilaments and VIP. Bar: 100 μm.

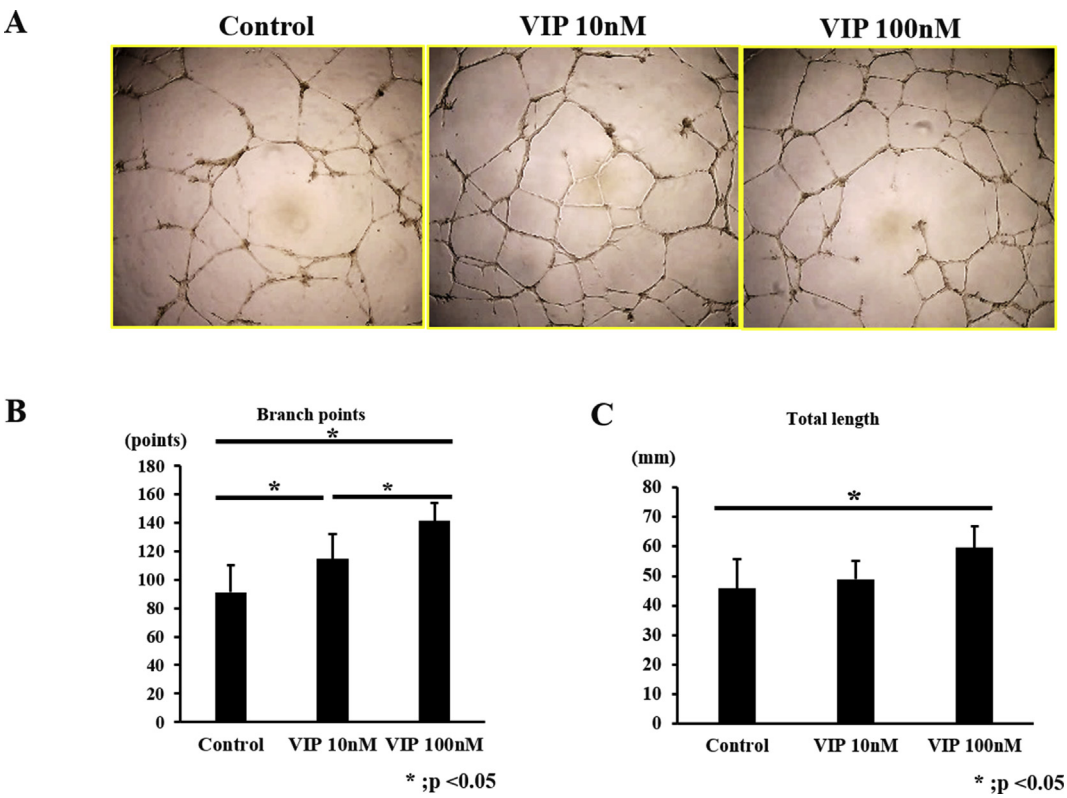

Fig. 3. A. Tube formation of human umbilical endothelial cell (HUVECs) at 12 h. B. The branch points of HUVECs. C. The total length of the HUVECs. VIP: vasoactive intestinal peptide.

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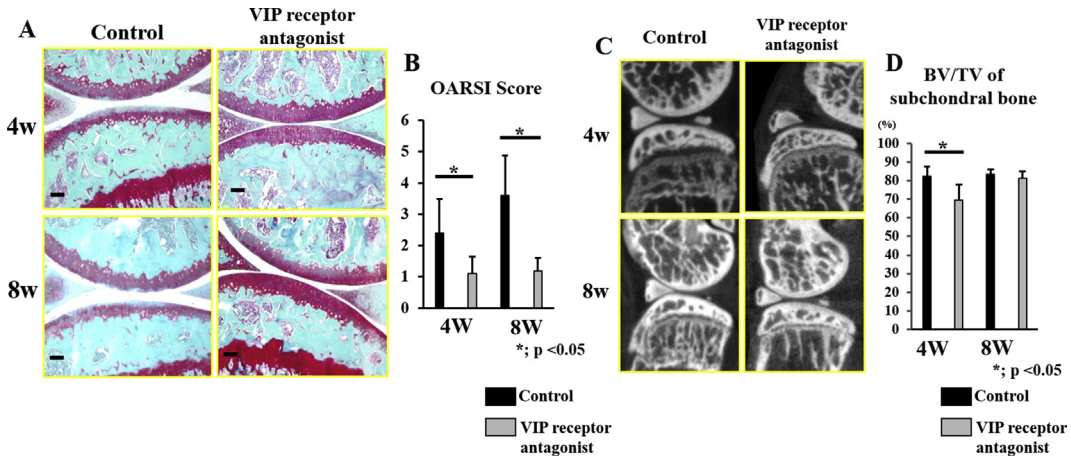

Fig. 4. A. Safranin O (SO) staining of the knee joint, B. The Osteoarthritis Research Society International (OARSI) score. C. Micro CT findings. D. The bone volume/tissue volume (BT/TV) ratio of the subchondral bone. Bar: 100 μm.

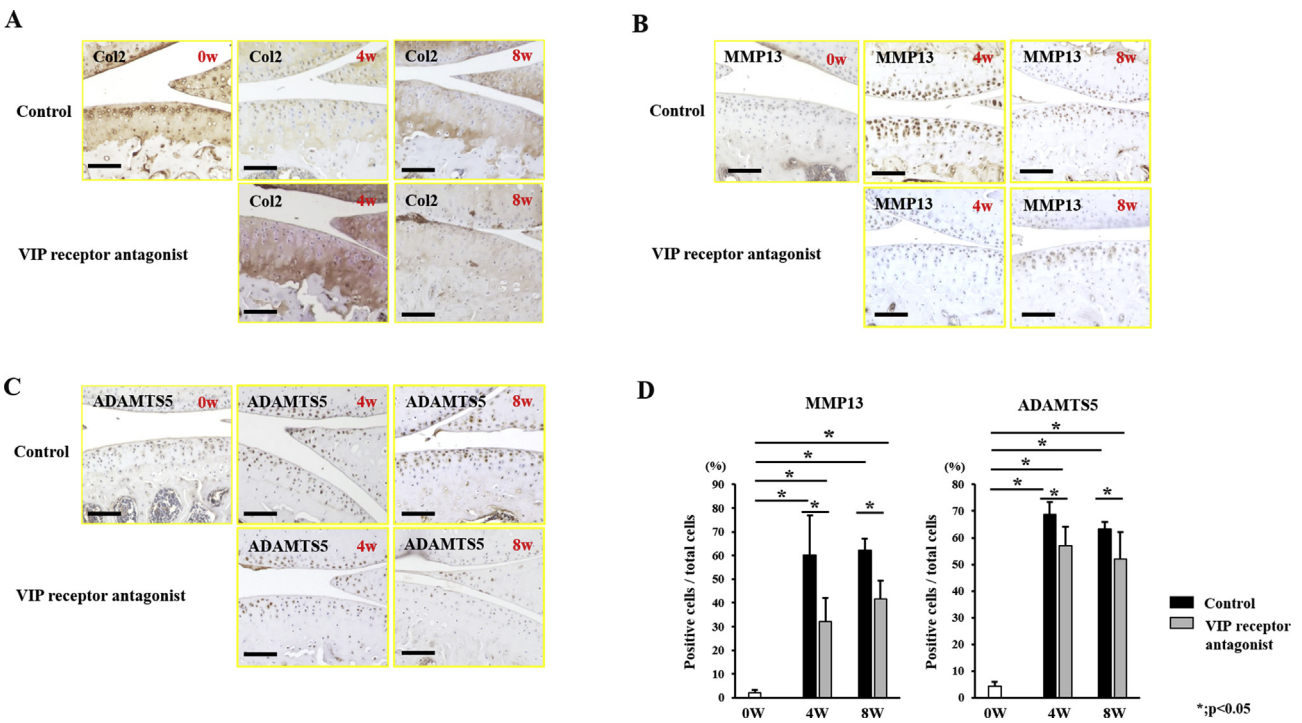

Fig. 5. A. Type 2 collagen (Col 2). B. Matrix metallopeptidase 13 (MMP 13). C. A disintegrin and metalloproteinase with thrombospondin motifs 5 (ADAMTS-5). D. The ratio of MMP13 or ADAMTS5 positive cells and total cells. Bar: 100 μm. VIP: vasoactive intestinal peptide.

group ($p < 0.05$; Fig. 7A, B), and the number of TRAP-positive cells in mice in the control group was significantly lower than in mice in the VIP receptor antagonist group ($p < 0.05$; Fig. 7C, D).

3.4. The effect of VIP receptor antagonist on cartilage

The results of real time PCR showed that VIP receptor antagonist could not prevent decreasing Col2a1 and aggrecan gene expression induced by IL-1β stimulation, and it could not decrease the MMP13 and IL-6 expression (Fig. 8A). In GAG release assay, treatment of VIP receptor antagonist for cartilage degeneration induced by Il-1β could not reduce the GAG loss on cartilage (Fig. 8B).

4. Discussion

In this study, we have shown that the expression of VIP increases in subchondral bone with OA, and VIP receptor antagonist could ameliorate OA progression in DMM mice. In general, the volume of subchondral bone increases as OA progresses, which is indicative of a sclerotic change. In this study, VIP was expressed along the trabecular bone and in the bone marrow cavity in mild OA cases, with the expression of VIP increasing and being distributed into the subchondral bone as OA progressed. There was a mild correlation between the number of VIP positive cells and BV/TV, and VIP positive osteoblasts significantly decreased from 4 to 8 weeks, but VIP positive

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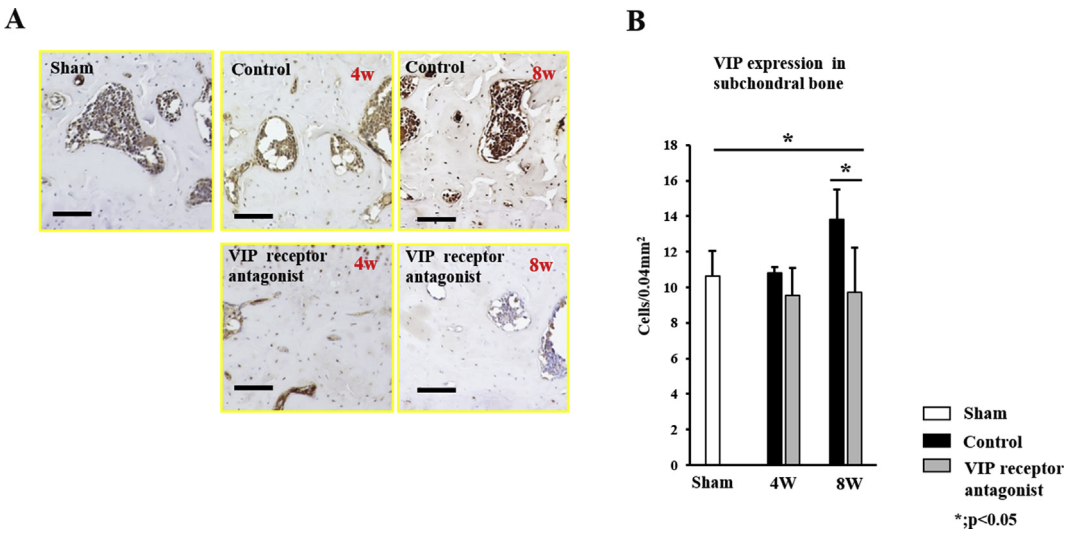

Fig. 6. A. Vasoactive intestinal peptide (VIP) of the knee joint. B. VIP expression in the subchondral bone. Bar: 100 μm.

osteocytes increased in the subchondral bone. These findings suggest that VIP might play a role in sclerosis of the subchondral bone by promoting the differentiation of osteoblasts. However, there was no previous report about the VIP expression and function in osteocytes, therefore, the role of VIP in osteocytes in OA pathogenesis would be elucidated.

In our animal model, expression of osteocalcin, which is released from osteoblasts and is involved in bone formation, was suppressed, and TRAP positive cells were increased by VIP receptor antagonist injection, indicating that the VIP receptor antagonist suppresses osteogenesis. VIP activation of VPAC2 receptors in osteoblasts enhances the RANKL/OPG ratio via mechanisms mediated by cyclic AMP and ERK pathways. Activation of these pathways stimulates the biosynthesis of osteoblastic alkaline phosphatase [10] and assists in decreasing osteoclast formation and activity via regulation of the receptor activator of nuclear factor kappa B ligand/osteoprotegerin ligand pathway [4,11]. Therefore, with osteoclast expressed VPAC-1 receptors, VIP induces construction and ceased

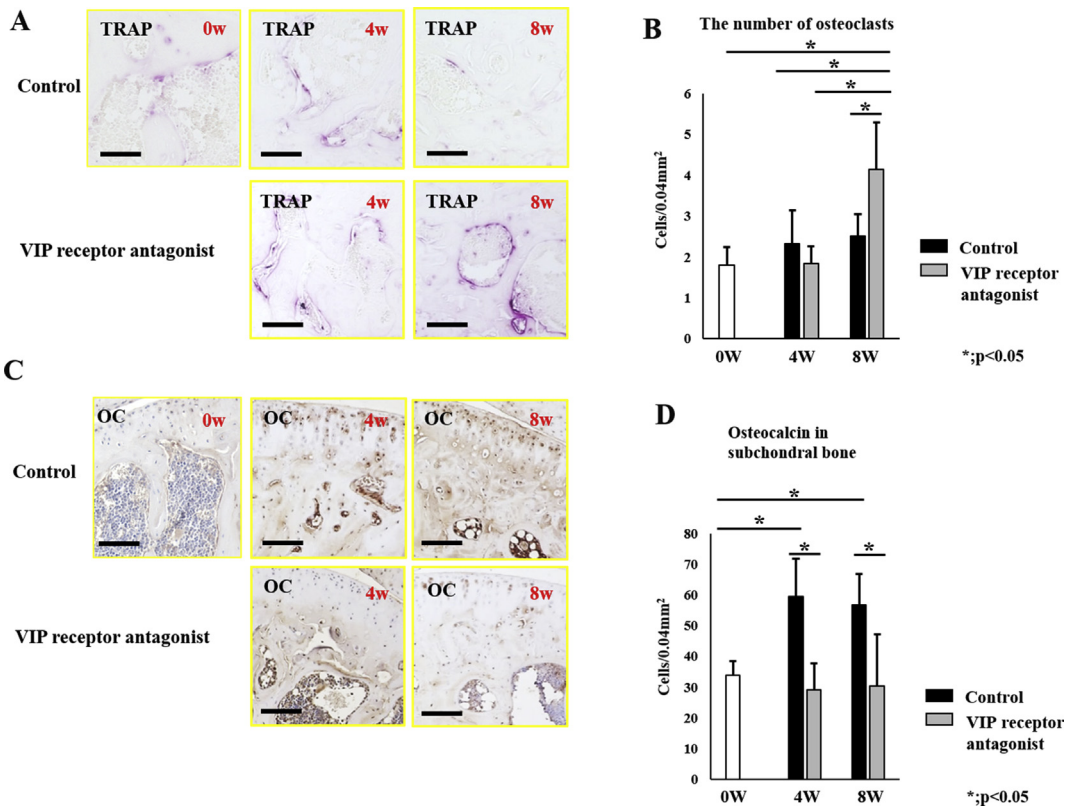

Fig. 7. A. Tartrate-resistant acid phosphatase (TRAP) staining, B. The number of osteoclasts in subchondral bone. C. Osteocalcin (OC) of the knee joint. D. The expression of OC in subchondral bone. Bar: 100 μm. VIP: vasoactive intestinal peptide.

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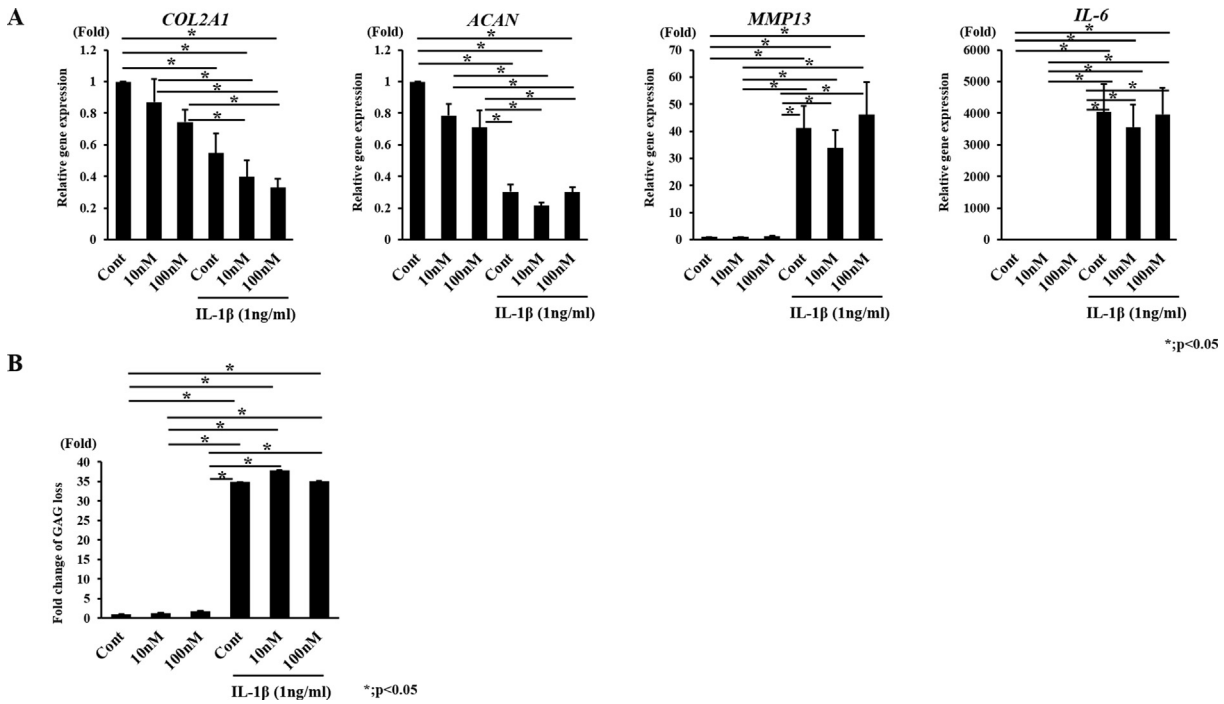

Fig. 8. A. Real time PCR of relative expression of COL2A1, ACAN, MMP13 and IL-6. B. GAG release assay.

motility of osteoclasts, and decreases the osteoclast genesis [26]. VIP is intensely expressed in the subchondral bone, especially osteoblasts in early phase of DMM mice, and VIP receptor antagonist might block the VIP signaling in osteoblasts, subsequently prevent the subchondral bone sclerosis.

The invasion of the vascular channel from subchondral bone to the non-calcified cartilage layers has been recognized as an important change in the subchondral plate [27]. Therefore, we investigated the expression pattern of VIP in vascular channels in OA and the role of VIP on angiogenesis. VIP is expressed in these vascular channels, as well as being expressed in both blood vessels and nerves in the subchondral bone in OA. VIP may affect the invasion of these vascular channels into the layers of cartilage; this finding is supported by the results of our *in vitro* study using HUVECs, and provides further evidence of the therapeutic possibility of using VIP (Fig. 9).

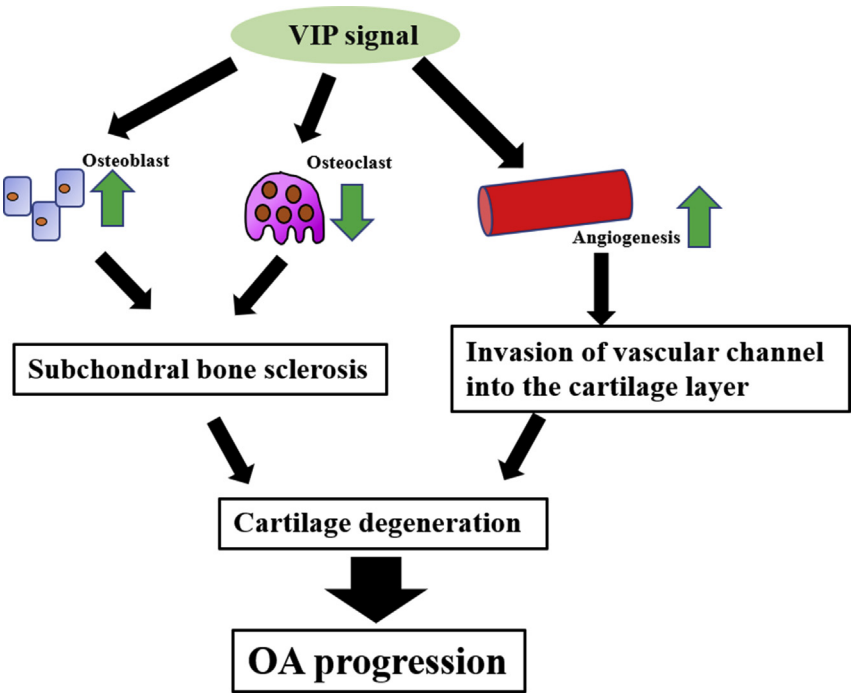

Fig. 9. Schema of OA progression through VIP signaling to bone metabolism and angiogenesis in the subchondral bone.

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Although administration of VIP receptor antagonist successfully ameliorated OA progression of DMM mice, it was unclear whether it has the effect of articular cartilage in OA as anabolic factor. From *in vitro* study, VIP receptor antagonist could not work as anabolic factor to articular cartilage, therefore, it may have the effect on the subchondral bone sclerosis and vascular invasion into the cartilage layer.

This study has several limitations that should be acknowledged. First, only a single dose of the VIP receptor antagonist was injected into mice in which the medial meniscus was destabilized. The onset of OA in these mice was sudden because of the severe instability of the knee joint. There was no significant difference in the BV/TV ratio at 8 weeks. Multiple doses of the VIP receptor antagonist may be more effective to prevent OA progression than a single dose. Moreover, VIP receptor antagonist was administered just after surgery, which has the possibility to affect the bone volume of the subchondral bone because bone volume decreases temporarily in early phase of OA before cartilage degeneration [16,28,29]. VIP receptor antagonist might accelerate the bone loss in early phase of DMM mice, which affect the results. It is also necessary to administer at different time point.

Second, the effect of the VIP receptor antagonist on pain relief was not evaluated. VIP plays an important role in treating pain because VIP sensitizes afferent nerve fibers leading to the generation of joint pain, and previous studies have reported that the VIP receptor antagonist VIP6-28 reduces nociception in OA [12,30]. Inhibiting VIP signaling may be an effective method of preventing the progression of OA and of relieving OA-related pain. Third, the anti-inflammatory effect of VIP was not analyzed; this will need to be done, since the effect of VIP on inflammation are still controversial. In our study, we focused on the osteogenesis effect of VIP, and the fact that a systematic injection of the VIP receptor antagonist could prevent OA progression by inhibiting subchondral bone sclerosis, and ameliorating subsequent cartilage degeneration. In our study, the amelioration of OA progression by the positive effect of VIP on the subchondral bone, seems to have been greater than the effect of VIP on inflammation. We used a DMM model mouse, in which OA was caused by mechanical stimulation rather than inflammation. The VIP receptor antagonist in this model was most effective by suppressing subchondral bone sclerosis caused by mechanical stress, and by maintaining cartilage metabolism and preventing OA. Finally, the adverse effects of the VIP receptor antagonist were not evaluated. VIP is expressed in various tissues and organs, and thus the impact of systemically administering a VIP receptor antagonist on these tissues should be investigated, including the appropriate dose and method of administration.

In conclusion, VIP contributes to the progression of OA through sclerosis of subchondral bone and angiogenesis, and administration of VIP receptor antagonist could prevent the progression of OA. Inhibition of VIP signaling has a potential to be a novel treatment for OA.

Ethical approval

The study was reviewed and approved by the Ethics Committee of Hiroshima University, and informed consent was obtained from all patients.

Authors' contribution

Munekazu Kanemitsu, M.D.: Conception and design of the study, data acquisition, analysis and interpretation, drafting the article, and final approval of the version to be submitted.

Tomoyuki Nakasa, M.D., Ph.D.: Conception and design of the study, data acquisition, analysis and interpretation, drafting the article, final approval of the version to be submitted, provision of study material, and obtention of funding.

Yoshiko Shirakawa, M.D.: Data acquisition, analysis and interpretation, and final approval of the version to be submitted.

Masakazu Ishikawa, M.D., Ph.D.: Conception and design of the study, data acquisition, analysis and interpretation, drafting the article, final approval of the version to be submitted, and provision of study material.

Shigeru Miyaki, Ph.D.: Data acquisition, analysis and interpretation, drafting the article, and final approval of the version to be submitted.

Nobuo Adachi, M.D., Ph.D.: Conception and design of the study, data acquisition, analysis and interpretation of data, drafting the article, and final approval of the version to be submitted.

Declaration of Competing Interest

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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