Local intra-articular injection of vascular endothelial growth factor accelerates articular cartilage degeneration in rat osteoarthritis model

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Abstract. In the pathophysiology of osteoarthritis (OA), articular cartilage degeneration exhibits a significant role. Vascular endothelial growth factor (VEGF) is considered to be an effective angiogenic factor and a crucial regulator of articular cartilage degeneration in the development of OA. Therefore, the present study aimed to investigate the underlying influences of exogenous VEGF on articular cartilage degeneration in OA model rat. A total of 24 male Sprague-Dawley rats were randomly allocated into 3 groups. In the normal saline (NS) and VEGF groups, animals received bilateral anterior cruciate ligament (ACL) transection to establish the OA model; at 4 weeks post-surgery, the rats received local intra-articular injections of 100 μl NS or VEGF solution, respectively, every week for 4 weeks. The Control group received neither surgery nor injections. All animals were sacrificed at 12 weeks following surgery. Prominent cartilage degeneration was observed in rats in the NS- and VEGF-injected groups. The extent and the grade of cartilage damage in the VEGF-injected group were notably more severe compared with those in the NS-treated group. Western blotting results demonstrated that the expression levels of aggrecan and type II collagen were significantly reduced in OA model rats that were treated with VEGF. In addition, the expression levels of matrix metalloproteinase (MMP)-3, MMP-9, MMP-13, a disintegrin and metalloproteinase with thrombospondin motifs (a disintegrin and metalloproteinase; ADAMTS)-4, -5 and -12, type III collagen and transforming growth factor-β1 were significantly increased following VEGF administration. Results from the present study indicated that VEGF may exhibit a promoting role in the development of OA by destroying articular cartilage matrix.

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Abbreviations: ACL, anterior cruciate ligament; ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MMP, matrix metalloproteinase; TGF, transforming growth factor; ADAM, a disintegrin and metalloproteinase; OA, osteoarthritis; VEGF, vascular endothelial growth factor

Key words: vascular endothelial growth factor, osteoarthritis, matrix metalloproteinase-3, matrix metalloproteinase-9, a disintegrin and metalloproteinase with thrombospondin motifs-4, cartilage degeneration

Introduction

Osteoarthritis (OA) is a common disease of the joints that is characterized by chronic and progressive loss of articular cartilage. Articular cartilage is a type of hyaline cartilage comprising highly differentiated chondrocytes and extracellular matrix (ECM). The major structural components of ECM include type II collagen and aggrecan. Aggrecanases belong to the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, comprising ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, ADAMTS-9 and ADAMTS-15. ADAMTS-4 and ADAMTS-5 mediate the degradation of aggrecan. In addition, matrix metalloproteinase (MMP)-3, MMP-9 and MMP-13 cleave the telopeptide region of type II collagen, which significantly influences OA development. Although the mechanisms of cartilage degradation have been widely studied, the pathogenesis of OA is unclear (1). The etiology of OA involves mechanical, biochemical and genetic factors (2).

Vascular endothelial growth factor (VEGF) is an influential angiogenic factor and a crucial regulator of angiogenesis during skeletal development and bone remodeling. A number of previous studies have reported on the possible role of VEGF in OA and have demonstrated that OA chondrocytes produce VEGF and VEGF receptor (3-5), and it is hypothesized that VEGF exerts considerable influence on the pathogenesis of OA (6,7). As previously reported, VEGF affects endochondral bone formation by inducing endochondral angiogenesis (8), which also leads to the upregulated expression levels of MMPs and other catabolic mediators that may degrade the cartilage matrix and to the downregulated expression levels of tissue inhibitors of metalloproteinases (TIMPs) in immortalized chondrocytes (9). VEGF expression is associated with the Mankin score and the degree of cartilage destruction (10); injection of VEGF into knee joints was reported to induce OA

in mice (11). Our previous study demonstrated an increase in VEGF and VEGFR-2 mRNA expression in the cartilage of a rabbit OA model (12).

To the best of our knowledge, no systematic study has been conducted on the biological activity of exogenous VEGF in articular cartilage degeneration, and the potential molecular mechanisms are still under investigation. As a result, the present study aimed to investigate the effects of local intra-articular injection of exogenous VEGF and the subsequent changes in expression levels of catabolic mediators in the cartilage of OA model rats. The results findings of the present study revealed the macroscopic- and microscopic-morphology changes in rat osteoarthritis model following surgery and injection treatment. The changes were validated and illustrated by reverse transcription-quantitative polymerase chain reaction analysis and western blotting. The results may provide new ideas for the roles of VEGF in cartilage degeneration in OA progression, and offer valuable diagnostic indicators and therapeutic targets for the treatment of the disease.

Materials and methods

Animals. This study was approved by the Institutional Animal Care and Use Committee of Wuhan University (Wuhan, China) and followed the 1996 Guide for the Care and Use of Laboratory Animals. A total of 24 male Sprague-Dawley rats (age, 8-10 weeks; weight, 250±20 g), were obtained from the Center of Experimental Animals of Wuhan University Medicine College (Wuhan, China). Rats were housed under specific pathogen-free (SPF) conditions at a temperature of 20-26˚C, 50±10% humidity under a 12 h light/dark cycle. The noise is below 60 decibels, and the concentration of ammonia is below 14 mg/m^3. Ventilation is greater than or equal to 15 times per hour. Then rats were provided access to conventional chow and tap water ad libitum. With the aim to minimize suffering, all rats were anesthetized with ketamine (30 mg/kg) intraperitoneally, maintained with 1% isoflurane and placed on a homoeothermic table to retain a body temperature of 37˚C during surgery.

Materials. Recombinant murine VEGF165 was purchased from PeproTech (Rocky Hill, NJ, USA) and dissolved in normal saline (NS; Baxter, Shanghai, China) to reach a final concentration of 0.1 mg/ml. Mouse anti-collagen II monoclonal antibody (sc-52658) and rabbit anti-aggrecan polyclonal antibody (sc-166951) were purchased from Santa Cruz Biotechnology. Inc. (Dallas, TX, USA); rabbit anti-MMP-3 polyclonal antibody (ab53015), rabbit anti-MMP-9 monoclonal antibody (ab76003) and rabbit anti-ADAMTS-4 polyclonal antibody (ab185722) were purchased from Abcam (Cambridge, MA, USA); and mouse anti-GAPDH monoclonal antibody (60004) was purchased from ProteinTech Group, Inc. (Chicago, IL, USA).

Experimental design. Rats were randomly assigned to one of three groups (n=8/group): i) Control group; ii) NS-injected group; and iii) VEGF-injected group. Prior to surgery, animals in NS- and VEGF-treated groups were anesthetized with ketamine (30 mg/kg body weight), and thereafter maintained with 1% isoflurane. Animals were placed on a homoeothermic table to maintain the body temperature at 37˚C, and bilateral anterior cruciate ligament (ACL) transection was conducted in SPF conditions. Briefly, medial arthrotomy was performed using a medial parapatellar approach that incised the skin. Following dislocation of the patella and positioning the knee in full flexion, the ACL was visualized and carefully transected without damaging the articular cartilage. The knee was irrigated with NS, followed by separate closure of the capsule and skin.

At 4 weeks post-surgery, rats in the NS and the VEGF groups were anesthetized as aforementioned, the knees were shaved to expose the patellar ligament and access from the lateral side of the knee was visualized using a microsyringe. A 100 μl solution containing either 9 mg/ml NS or 0.1 mg/ml VEGF was injected into the intra-articular space beneath the patellar ligament through the microinjection needle. This injection was performed every week for a total of four weeks in the NS and the VEGF groups. The rats were maintained under SPF conditions for an addition four weeks without any disposal. Conversely, rats in the Control group did not undergo any surgical procedures nor received any treatment. At the termination of the study, 12 weeks post-surgery, all the animals were sacrificed with an overdose injection of pentobarbital sodium (200 mg/kg). Subsequently, both knee joints of each rat were excised and fixed in 4% paraformaldehyde, or immediately frozen and stored at -80˚C until subsequent analyses.

Macroscopic morphological assessment. Animals were sacrificed 12 weeks post-surgery as aforementioned. Blinded assessment of macroscopic cartilage injury was conducted, and the degree of cartilage injury was evaluated according to a well-defined semi-quantitative grading table comprising a five-grade scale: 0, surface smooth with normal color; 1, surface rough with minimal fibrillation or a slight yellowish discoloration; 2, cartilage erosion extending into the superficial or middle layers; 3, cartilage erosion extending into the deep layers; 4, complete cartilage erosion with subchondral bone exposed (13).

Microscopic morphology. Following evaluation of macroscopic morphology, knee joint tissues were excised and fixed in neutral buffered solution (4% formaldehyde in 0.1 M PBS pH 7.4) for 48 h, followed by decalcification in 10% EDTA for 3-4 weeks at room temperature, dehydration in graded ethanol and embedding in paraffin. Paraffin sections (5 μm) were mounted on glass slides and stained with hematoxylin and eosin (H&E) for general morphological evaluation. Tissue sections were also stained with Safranin-O and Fast Green at a concentration of 0.1% to illustrate sulfated proteoglycans. For either staining method, two sections from each animal in all groups were used. The stained slides were analyzed using a computerized morphometric system (cellSens, Olympus Corporation, Tokyo, Japan) connected to OLYMPUS BX53 upright microscope; ≥10 fields of view per slide were analyzed.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cartilage tissues from the medial femoral condyle were harvested from rats in each group and frozen in liquid

Table I. Gene-specific primer sequences.

Genes	Sequence (5' → 3')
MMP-3	F: GGCCATCTCTTCCTTCAG
	R: GTCACTTTCTTTGCATTTGG
MMP-9	F: CTTCTGGCGTGTGAGTTTCC
	R: GCACGGTTGAAGCAAAGA
MMP-13	F:CCTGGACAAGTAGTTCCAAAGG
	R: AGGGATAAGGAAGGGTCACAT
ADAMTS-4	F: GCAACGTCAAGGCTCCTCTT
	R: CTCCACAAATCTACTCAGTG
ADAMTS-5	F: CCTGCCCACCCAATGGTAAATC
	R: CGGCCTACATTCAGTGCCATC
Type III collagen	F: ATGGTGGCTTTCAGTTCACC
	R: TGGGGTTTCAGAGAGTTTGG
TGF-β1	F: TGAGTGGCTGTCTTTTGACG
	R: GTITGGGACTGATCCCATTG
ADAM-12	F: GCTGATGAAGTTGTCAGTGC
	R: GAGACTGACTGCTGAATCAG
GAPDH	F: ATCACTGCCACCCAGAAGAC
	R: ATGAGGTCCACCACCCTGTT

ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; F, forward; MMP, matrix metalloproteinase; R, reverse; TGF, transforming growth factor.

nitrogen. The samples were ground into a powder in liquid nitrogen based on the hand milling technique. Total RNA was extracted using the TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's protocol; RNA samples were quantitated at an absorbance of A260. Subsequently, RNA was reverse-transcribed to cDNA using an PrimeScript One Step RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol; cDNA was used immediately or stored at -20˚C. qPCR was conducted using the SYBR Green Realtime PCR Master Mix (Toyobo Life Science, Osaka, Japan) and an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.); primer sequences are listed in Table I. The final volume of the qPCR reaction was 10 μl, which comprised 2X SYBR Premix Ex Taq (5 μl), primer solution (0.2 μl; 10 μmol/l), 50X ROX Reference Dye (0.2 μl), cDNA template (1 μl), adjusted to 10 μl with distilled water. qPCR thermocycling conditions included an initial denaturing step at 95˚C (10 sec) followed by 40 cycles of 95˚C (5 sec), 60˚C (30 sec) and 72˚C (30 sec). A standard curve was established using 5-fold decrements of synthesized oligonucleotides that resembled cDNA fragments as a template. GAPDH expression was used as an endogenous control and to normalize target gene expression, from which the fold-change in gene expression level was determined. Specificity of each reaction was controlled by melting curve analysis. Data were analyzed by $2^{-\Delta\Delta Cq}$ quantitation method (14). A blank PCR control containing water rather than cDNA was also carried out. RT-qPCR was conducted on three independent biological replicates.

Western blotting. Protein was extracted from cartilage tissues using the M-PER Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc.) and the Protease Inhibitor Cocktail Set III (EMD Millipore, Billerica, MA, USA) with 5 mmol/l EDTA. A total of 20 μg protein [quantitated by a Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology, Haimen, China)] was separated by 10% SDS-PAGE and subsequently transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% non-fat milk in TBS containing 0.05% Tween-20 (TBST) for 1 h at room temperature, and incubated with primary antibodies (1:1,000) in TBST overnight at 4˚C. The membranes were washed three times with TBST and incubated with horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (IgG; 1:10,000; A0216, Beyotime Institute of Biotechnology) or goat anti-rabbit IgG (1:10,000, A0208, Beyotime Institute of Biotechnology) in TBST for 1 h at room temperature. Protein bands were visualized with an Enhanced Chemiluminescence System kit (Pierce; Thermo Fisher Scientific, Inc.) and densitometric analysis was performed with ImageJ software (v1.48u, National Institutes of Health, Bethesda, MD, USA). The GAPDH from the cartilage tissues were applied as endogenous control and the relative expression levels of the target gene mRNA were calculated based on the determination of its copy numbers. The associated fold changes were determined, with the value set equal to one for each experiment; all tests were repeated in triplicate.

Statistical analysis. All values were expressed as the mean ± standard deviation of at least three independent experiments. SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was used to conduct statistical analyses, and significant differences were analyzed by one-way analysis of variance followed by Student-Newman-Keuls post hoc q-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

VEGF increases articular cartilage injury. To evaluate the extent of articular cartilage injury, pathological changes of the femoral condylar cartilage were examined macroscopically, and the degree of damage was evaluated and scored according to a semi-quantitative five-grade scale. The condylar cartilage of rats in the Control group was macroscopically normal, without any signs of destruction (Fig. 1A). Conversely, different degrees of degenerative lesions of the cartilage, such as ulceration, erosion, fibrocartilage proliferation and osteophyte formation, were observed in NS-injected and in VEGF-injected OA model rats (Fig. 1B and C, respectively). However, the extent and grade of cartilage damage in the VEGF-treated group were obvious and more severe than those in the NS-treated group. The 16 condyles examined from the 8 rats in Control group presented at Grade 0 (surface smooth with normal color). All 16 specimens within each of the NS and VEGF groups exhibited complete transection of the ACL, which indicated successful establishment the OA model. In the NS group, 2 out of 16 condyles were classified as Grade 1 (surface rough with minimal fibrillation or a slight yellowish discoloration), whereas none of the VEGF-treated rats presented Grade 1. In addition, 6 out of 16 rats in the NS

Table II. Classification of condyles in three groups.

	Grade					
Group (n)	0	1	2	3	4	Mean[a]
Control (16)	16	0	0	0	0	0
NS (16)	0	2	6	5	3	2.56
VEGF (16)	0	0	3	6	7	3.25

[a]Average mean values.

group and 3 out of 16 rats in the VEGF group were classified as Grade 2; 5 out of 16 rats in the NS group and 6 out of 16 in the VEGF group were Grade 3 (cartilage ulceration extending into the deep layers); and 3 out of 16 in the NS group and 7 out of 16 in the VEGF group were Grade 4 (cartilage depletion with subchondral bone exposed). The statistical frequency of distribution of grade classifications in the different groups were calculated (Fig. 1D; Table II), and the average scores of the NS and VEGF injection groups were 2.56 and 3.25, respectively.

VEGF induces structural component loss in articular cartilage. A series of microscopic morphology tests were conducted to examine the effects of VEGF on articular cartilage in the OA rat model. H&E staining was used to assess general morphology, and the condylar cartilage of rats in the Control group was healthy, as indicated by the clear and ordered arrangement of the transitional layer, the surface layer the calcified layer and the radiation layer, as well as round, regularly distributed and evenly dyed chondrocytes (Fig. 2A). Cartilage tissues exhibited obvious degenerated changes in the NS and VEGF-injected group (Fig. 2B and C, respectively). In particular, the layer structure is absent, and fibrillations and fissures were observed in most the examined cartilage samples. At the cellular level, chondrocytes were irregularly distributed, swollen, and uneven staining. Notable differences in H&E staining were observed among these groups, and more severe injury to cartilage structures were noted in the VEGF-injected group compared with rats in the NS and Control groups. In the VEGF-injected group, there was a notable reduction in average thickness, including calcified cartilage layers, subchondral bone, as well as the total joint thickness. Notably, vessels invaded the tidemark in NS-injected and in VEGF-injected groups, but not in normal joints.

In addition, Safranin-O/Fast Green staining indicated a loss of Safranin-O stain in the cartilage tissues from rats in the NS-treatment group and in the VEGF-treatment group compared with the Control group (Fig. 3). The number of chondrocytes labeled with Safranin-O/Fast Green was notably different in the three groups. Similar to the results from H&E staining, more severe injuries to the cartilage structures were observed in the VEGF-injected group compared with the NS-injected and the Control groups.

Results from these two staining methods indicated that more severe lesions were observed in the condylar cartilage from rats in the VEGF-injected group compare with rats in the NS and Control groups, which was mostly due to a decline in chondrocyte number as well as the loss of structural components.

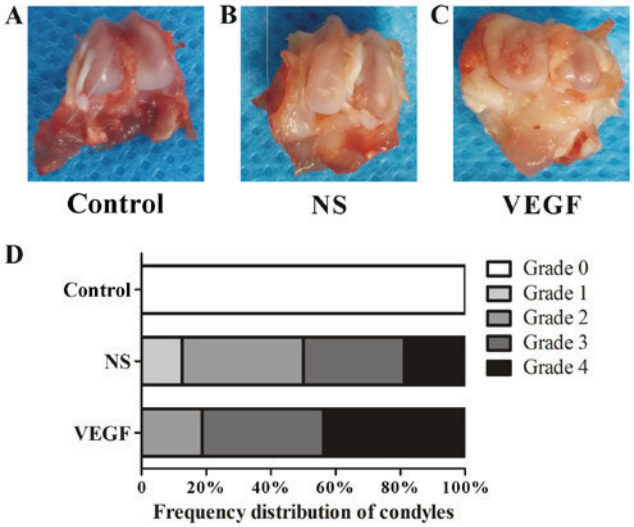

Figure 1. Representative macroscopic images of the femoral condyles at 12 weeks post anterior cruciate ligament transection. (A) The articular surface of the Control group was smooth and lustrous, with complete and homogeneous cartilage. (B) The articular surface of the NS-injected group appeared rough and yellow, with scattered ulcers, moderate fibrillation and osteophyte formation. (C) The articular surface of the VEGF-injected group appeared rough, with extensive erosion, overt fibrillation and severe osteophyte formation. (D) The grade distribution from each group was calculated; n=8/group. NS, normal saline; VEGF, vascular endothelial growth factor.

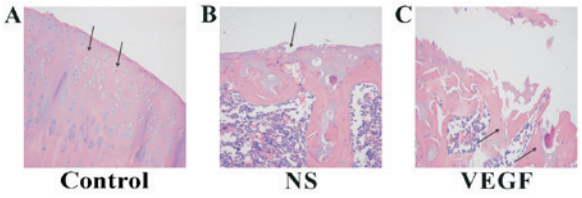

Figure 2. Representative sections of rat condylar cartilage at 12 weeks following anterior cruciate ligament transection. Hematoxylin and eosin staining; magnification, x200). (A) Representative sections of the Control group, which presented round, regularly distributed and evenly dyed chondrocytes (arrows). (B) Representative sections of NS-injected group revealed fissure (arrows). (C) Representative sections of VEGF-injected group presented empty lacuna (arrows). NS, normal saline; VEGF, vascular endothelial growth factor.

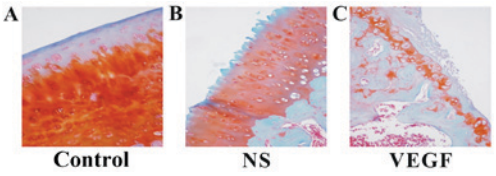

Figure 3. Representative sections of rat condylar cartilage at 12 weeks following anterior cruciate ligament transection Safranin-O and Fast Green staining; magnification, x200). (A) Representative sections of the Control group. (B) Representative sections of NS-injected group. (C) Representative sections of VEGF-injected group. NS, normal saline; VEGF, vascular endothelial growth factor.

VEGF downregulates aggrecan and type II collagen expression. Protein expression levels of aggrecan and type

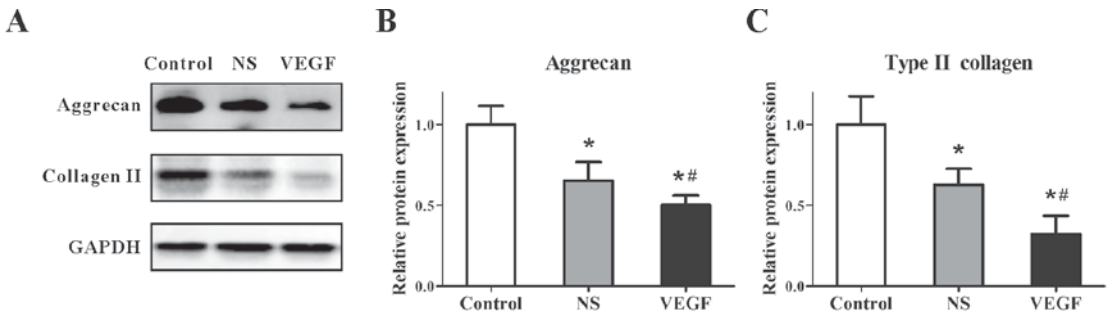

Figure 4. Protein expression levels of aggrecan and type II collagen in rat condylar cartilage at 12 weeks after anterior cruciate ligament transection. (A) Representative western blot analyses of aggrecan and type II collagen protein expression; GAPDH was used as a loading control. (B and C) Densitometric analysis of protein expression of (B) aggrecan and (C) type II collagen. Data were normalized to the Control group and presented as the mean ± standard deviation; *P<0.05 vs. Control; #P<0.05 vs. NS. NS, normal saline; VEGF, vascular endothelial growth factor.

II collagen were examined by western blotting (Fig. 4A). GADPH was used as an internal reference, and densitometric analyses of the bands were conducted by computerized laser densitometry, which demonstrated that the protein expression levels of aggrecan (Fig. 4B) and type II collagen (Fig. 4C) were significantly reduced in the NS group compared with the respective expression levels in the Control group (0.65±0.14 vs. 1.00±0.11 and 0.61±0.09 vs. 1.00±0.16, respectively; P<0.05). In addition, protein expression levels were further reduced in model rats treated with VEGF compared with rats in the NS-treated group (aggrecan at 0.48±0.05 vs. 0.65±0.14 and type II collagen at 0.32±0.11 vs. 0.61±0.09; P<0.05). These results indicated that the protein expression levels of aggrecan and type II collagen were significantly inhibited by VEGF injection in the rat model of OA.

VEGF upregulates the expression of cartilage-degradation and fibrogenic factors. In a previous study, eight cartilage-degradation and fibrogenic factors were examined to be associated with collagenase-mediated OA pathogenesis (15). Therefore, the present study examined the mRNA expression levels of eight related cartilage-degradation and fibrogenic proteins to see whether they were influenced by exogenous VEGF in our model. RT-qPCR was used to determine the relative mRNA expression levels of cartilage degradation factors, including MMP-3, MMP-9, MMP-13, ADAMTS-4 and ADAMTS-5 and of fibrogenic factors, including type III collagen, transforming growth factor (TGF)-β1 and ADAM-12, which were normalized to the relative mRNA expression of the housekeeping gene GAPDH (Fig. 5). The relative mRNA expression levels were significantly increased in the NS-injected group compared with the Control group (MMP-3, 1.46±0.11 vs. 1.00±0.04; MMP-9, 1.52±0.11 vs. 1.00±0.11; MMP-13, 2.75±0.63 vs. 1.00±0.25; ADAMTS-4, 1.47±0.09 vs. 1.00±0.10; type III collagen, 2.35±0.55 vs. 1.00±0.29; TGF-β1, 1.34±0.23 vs. 1.00±0.19; and ADAM-12, 2.22±0.47 vs. 1.00±0.13; all P<0.05), whereas no significant difference was identified for the mRNA expression levels of ADAMTS-5 between the NS-treated group and the untreated Control group (1.14±0.11 vs. 1.00±0.12; P>0.05). There was a significant increase in the mRNA expression levels in the VEGF-injected group compared with the NS-injected group (MMP-3, 2.38±0.42 vs. 1.46±0.11; MMP-9, 3.08±0.38 vs. 1.52±0.11; MMP-13, 5.50±1.13 vs. 2.75±0.63; ADAMTS-4, 3.43±0.75 vs. 1.47±0.09; ADAMTS-5, 1.39±0.18 vs. 1.14±0.11; type III collagen, 3.62±0.57 vs. 2.35±0.55; TGF-β1, 1.88±0.31 vs. 1.34±0.23; and ADAM-12, 4.67±1.01 vs. 2.22±0.47; all P<0.05). The data demonstrated that the mRNA expression levels of these eight genes were significantly promoted by VEGF injection in OA model rats.

To further validate the influence of VEGF on these deleterious factors at the translation level, MMP-3, MMP-9 and ADAMTS-4 were selected as the representatives, since the three proteins have been greatly studied as representative cartilage degradation-associated factors (16-18), and protein expression levels were examined by western blotting (Fig. 6A); GADPH was used as an internal reference and densitometric analyses of the bands were conducted by computerized laser densitometry. The results revealed that the protein expression levels of MMP-3 (Fig. 6B), MMP-9 (Fig. 6C) and ADAMTS-4 (Fig. 6D) were significantly increased in the NS group compared with expression levels in the Control group (MMP-3, 1.24±0.10 vs. 1.00±0.13; MMP-9, 2.71±0.16 vs. 1.00±0.17; ADAMTS-4, 3.50±0.55 vs. 1.00±0.19; all P<0.05). The expression levels of these proteins were further enhanced in the VEGF-injected group compared with the NS group (MMP-3, 1.55±0.06 vs. 1.24±0.10; MMP-9, 3.43±0.28 vs. 2.71±0.16; ADAMTS-4, 5.75±1.05 vs. 3.50±0.55; all P<0.05). Consistent with the mRNA expression results, the protein expression levels of MMP-3, MMP-9 and ADAMTS-4 from cartilage tissue were significantly promoted by VEGF injection in OA model rats.

Discussion

OA is a degenerative joint lesion that progresses to the articular cartilage and causes intra-articular inflammation, as characterized by synovitis (8). The pathogenesis and pathophysiology of OA remain unclear, which creates obstacles in studying the development and progression of OA (9). Numerous studies have reported on the involvement of VEGF in the degenerative progression of cartilage (19-21). Angiogenic inhibitors are produced by normal articular cartilage, which, consequently, lack vascular tissue (4). VEGF is a well-studied angiogenic factor that serves an important role in skeletal bone growth and remodeling by regulating angiogenesis, and is hypothesized to be involved in pathogenesis of OA (22). Neve's research suggested the involvement of osteoblast-derived VEGF in the pathogenesis of bone diseases (23). Hence, the present study

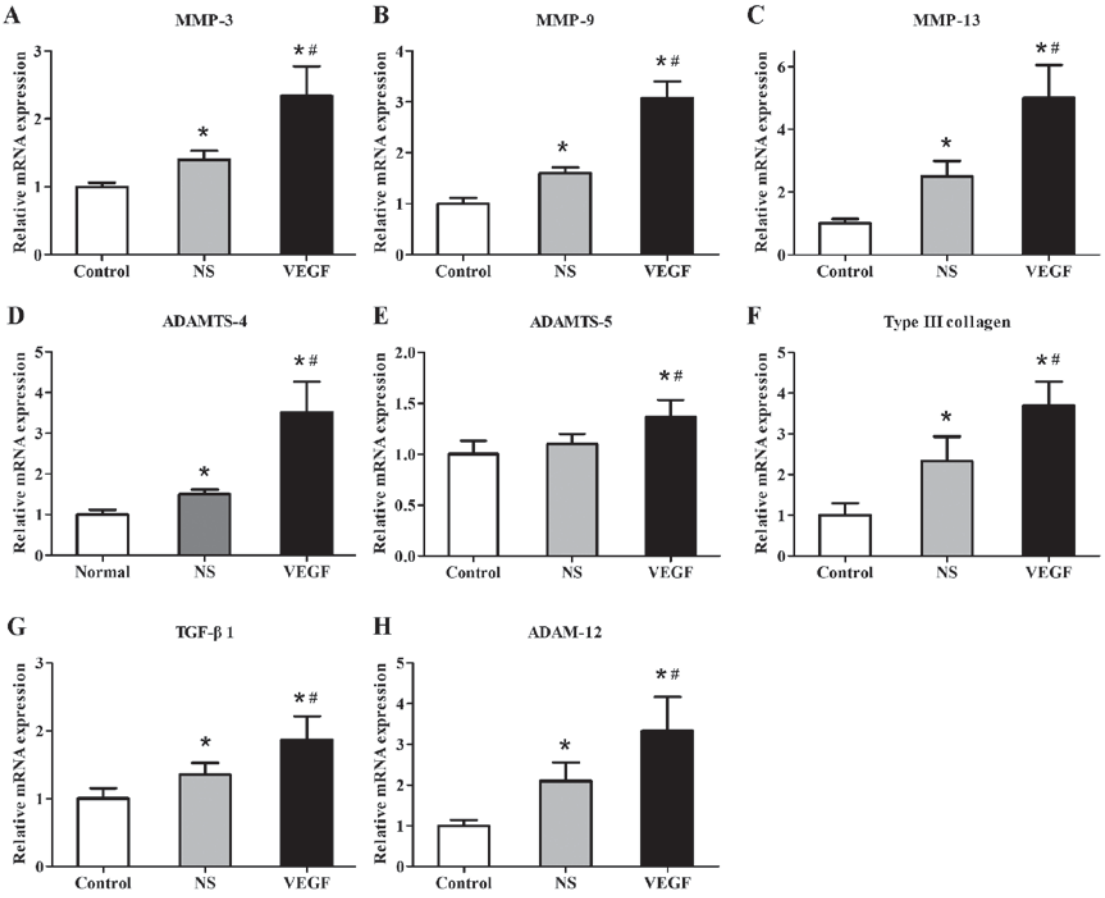

Figure 5. mRNA expression levels of cartilage degradation factors and fibrogenic factors in rat condylar cartilage at 12 weeks following anterior cruciate ligament transection. Reverse transcription-quantitative polymerase chain reaction was used to detect the mRNA expression levels of cartilage degradation factors (A) MMP-3, (B) MMP-9, (C) MMP-13, (D) ADAMTS-4 and (E) ADAMTS-5, and the expression levels of fibrogenic factors (F) type III collagen, (G) TGF-β1 and (H) ADAM-12. Data are presented as the mean ± standard deviation; *P<0.05 vs. Control; #P<0.05 vs. NS. ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MMP, matrix metalloproteinase; NS, normal saline; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

speculated that VEGF may be a potent biomarker for diagnosis and a potential therapeutic target of OA.

Previous studies have reported an elevated expression level of VEGF in osteoarthritic cartilage compared with the normal tissue, which had a direct association with the Mankin score for OA (3,5,10). Our previous studies demonstrated a strong association between the levels of VEGF expression and OA pathogenesis (12,24,25). VEGF is a proangiogenic factor in numerous tissues and affects angiogenesis associated with cartilage (8). Its expression is regulated by interleukin (IL)-1β, hypoxia-inducible factor-1α, tumor necrosis factor (TNF)-α and IL-6, as well as growth factors under inflammatory environment associated with OA (26-28).

Articular cartilage, of hyaline nature, comprises ECM and highly differentiated chondrocytes (29). ECM is made of type II collagen and aggrecan, the former of which is major structural component, constituting 30-60% of the dry weight of the cartilage ECM. Collagen fibrils interact with other components of ECM, especially aggrecans in articular cartilage (30). Aggrecans expanded due to water and alter their structure formation to confront compressive force. Hence, a lack of type II collagen as well as aggrecan may induce articular cartilage degeneration. MMP-3 is demonstrated to involve in the cleavage of telopeptide region of type II collagen. Besides, ADAMTS-4, a type of aggrecanase, is capable to induce aggrecan degradation. Altogether, these two proteins interplay with OA development.

MMP expression levels are enhanced, whereas the secretion of TIMPs is blocked by VEGF in immortalized chondrocytes (9). In addition, the expression of IL-6, IL-1β, nitric oxide, and TNF-α were reported to be induced by VEGF, and subsequently stimulate the proliferation of chondrocytes, which may lead to accelerative articular cartilage degeneration (9). VEGF production, in turn, is activated by TNF-α in osteoarthritic chondrocytes (31). OA in the temporomandibular joint was reported to be induced by VEGF, which destroyed subchondral bone and cartilage (32). In a mouse model, OA was induced following VEGF injection into knee joints (11). Articular cartilage degeneration in OA model rats was delayed following local injections of the anti-VEGF monoclonal antibody bevacizumab (22).

Collectively, VEGF may be considered as a promising biomarker to assess disease severity in osteoarthritis, and may be the basis for targeted treatments in the future; however, further investigation regarding the detailed mechanism is required.

However, it was not clear if exogenous VEGF served a role in joint degeneration. The expression of cartilage-degradation and fibrogenic factors as well as histological changes of

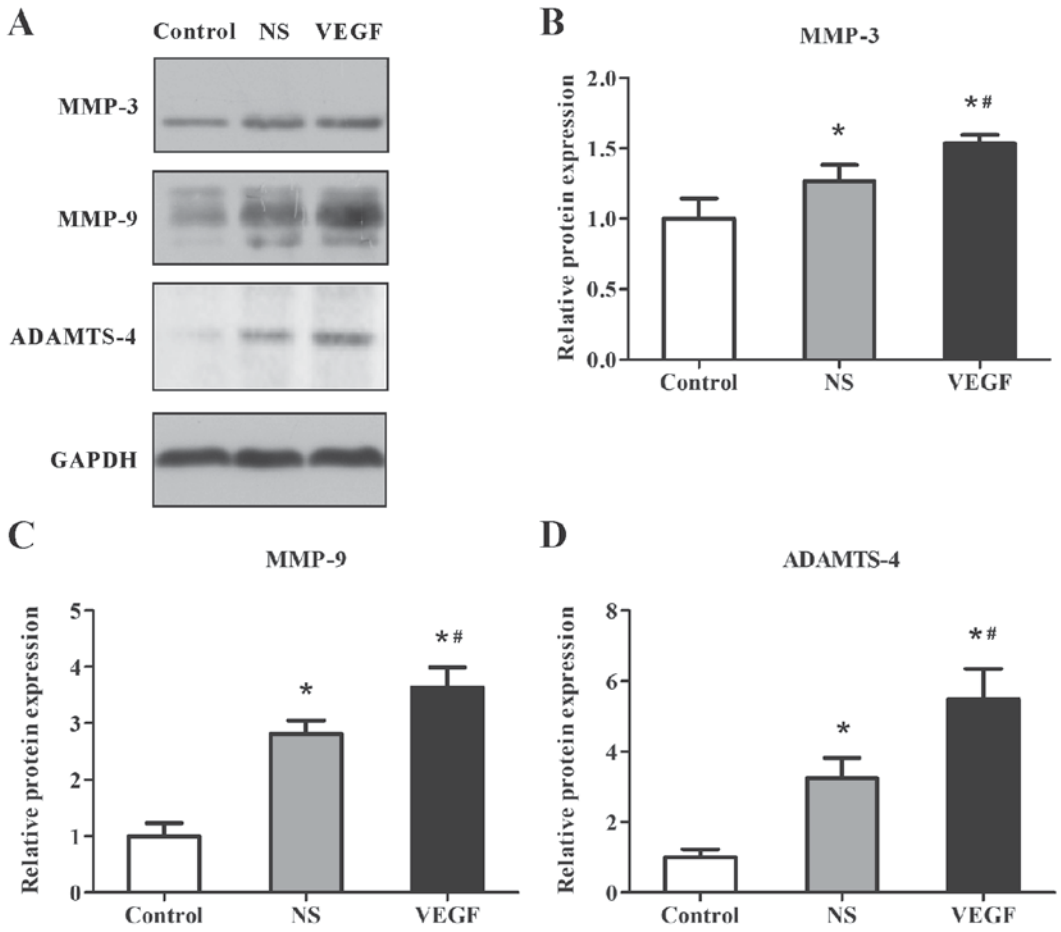

Figure 6. Protein expression levels of MMP-3, MMP-9 and ADAMTS-4 in rat condylar cartilage at 12 weeks following anterior cruciate ligament transection. (A) Representative western blotting images of the protein expression levels of MMP-3, MMP-9 and ADAMTS-4; GADPH was used as a loading control. (B-D) Densitometric analyses from part (A) on the expression levels of (B) MMP-3, (C) MMP-9 and (D) ADAMTS-4 were normalized to the Control group. Data are presented as the mean ± standard deviation; *P<0.05 vs. Control: #P<0.05 vs. NS. ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MMP, matrix metalloproteinase; NS, normal saline; VEGF, vascular endothelial growth factor.

osteoarthritic joint tissues were assessed by the present study following injections of exogenous VEGF. The results suggested that exogenous VEGF may accelerate OA progression: Cartilage degradation was more severe in rats in the VEGF-injected group compared with the NS-injected group, as defined by erosive cartilage, deep fibrillation and increased loss of Safranin O.

Similar to a previous report (24), results from the present study suggested that exogenous VEGF may induce degeneration of articular cartilage by blocking the synthesis of and inhibiting the expression of type II collagen and aggrecan. In immortalized chondrocytes, MMPs were reported to be upregulated, whereas TIMPs were downregulated by VEGF (9). To the best of our knowledge, the present study was the first to reveal the effects of locally injected, exogenous VEGF on the expression levels of ADAMTS-4, MMP-3 and MMP-9 in OA joints. The results demonstrated that mRNA and protein expression levels of ADAMTS-4, MMP-3 and MMP-9 were elevated in VEGF-injected and NS-injected OA model rats, and these increased in expression levels were highest in the VEGF-injected group. These data suggested a potential role for ADAMTS-4, MMP-3 and MMP-9 in cartilage degeneration in response to exogenous VEGF, which may degrade type II collagen and aggrecan as well. In addition, MMP-3 was involved in pathological degradation of collagen and MMP-1 activation (33). Increases in MMP-3 and MMP-9 expression levels have been reported to be related with decreasedTIMP-1 and TIMP-2 (9). The role of VEGF in articular cartilage destruction has been confirmed by various studies, which, induces ADAMTS-4 (15), ADAMTS-5 (34) and MMPs expressions (32) in return, and degrades major ECM molecules, such as type II collagen (24), aggrecan (22). Type III collagen, TGF-β1 and ADMS-12 have been associated with synovial fibrosis in OA knees (15). However, little is known about these fibrosis-related proteins in the progression of cartilage degeneration. In the present study, the increased expression levels of type III collagen, TGF-β1 and ADAM-12 have positive association with the severity of cartilage degeneration as demonstrated by macroscopic-morphological assessments and analyses of mRNA expression levels. It remains to be elucidated whether these proteins are involved in cartilage degeneration through fibrosis or through some other mechanism.

In conclusion, the present study demonstrated the effects of VEGF in OA progression through cartilage degeneration, which indicated that VEGF may be a

valuable candidate as a diagnostic biomarker as well as therapeutic target. Additional studies are warranted to reveal the mechanism of OA.

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