Intraperitoneal injection of thalidomide alleviates early osteoarthritis development by suppressing vascular endothelial growth factor expression in mice

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Abstract. Vascular endothelial growth factor (VEGF) is expressed in articular cartilage and increases in expression levels have been associated with the progression of osteoarthritis (OA). Thalidomide is a drug that has been reported to inhibit angiogenesis and reduce VEGF production by down-regulating VEGF expression. The objective of the present study was to determine whether intraperitoneal administration of thalidomide may attenuate early OA development in mice. Male C57BL/6 mice (10-weeks-old) were randomly assigned into the destabilization of the medial meniscus (Dmm) with thalidomide treatment (Dmm+Th), Dmm and Sham groups equally. An OA model was induced surgically in Dmm+Th and Dmm groups, and mice of the Dmm+Th group were subsequently treated with an intraperitoneal injection of thalidomide (200 mg/kg/day). At 2 and 4 weeks following surgery, the pathological alterations in cartilage samples were assessed qualitatively by hematoxylin and eosin staining and Safranin O/Fast green staining, and quantitatively by the Osteoarthritis Research Society International scoring system. The mRNA expression levels of matrix metalloproteinase-13 (MMP-13) and VEGF were measured by reverse transcription-quantitative polymerase chain reaction. The protein expression levels of MMP-13 and VEGF were detected by immunofluorescence and immunohistochemistry, respectively. The production of VEGF in serum was evaluated via an ELISA assay. Pathological scores were significantly higher in the Dmm and the Dmm+Th groups than those in the Sham group; however, the Dmm+Th group exhibited markedly less severe pathological changes compared with the Dmm group. Compared with the Sham group, the mRNA and protein expression levels of VEGF and MMP-13 in the Dmm and the Dmm+Th groups were significantly increased. The Dmm+Th group exhibited significantly decreased expression levels of VEGF and MMP-13, as well as significantly decreased serum VEGF concentration compared with the Dmm group. Thus, the results of the present study demonstrated that intraperitoneal administration of thalidomide may alleviate the development of early OA by suppressing VEGF expression in mice and may have potential as a novel therapy for the treatment of OA.

Introduction

Osteoarthritis (OA) is the most prevalent form of joint disease, with a high prevalence in the elderly, and an increasing social and economic healthcare burden (1,2). The etiology of OA is complex and has been associated with multiple factors of mechanical stress, gender, age, environment and genes, which may interact to cause its initiation and progression (3-6). Its primary clinical symptoms are joint pain and restricted movement, with the main pathologies of synovitis, cartilage degeneration, subchondral bone sclerosis, and osteophyte formation (7). The pathogenesis of OA has been well reported and has been associated with decreases in chondrocyte numbers and degradation of the extracellular matrix (ECM) in cartilage (8). Matrix metalloproteinase-13 (MMP-13) is one of the main enzymes responsible for ECM degradation (9).

Vascular endothelial growth factor (VEGF) has previously been reported to be expressed in osteoarthritic articular cartilage (10-12); VEGF has the capacity to increase expression levels of MMPs and to decrease expression levels of their inhibitors (tissue inhibitors of metalloproteinases) (13,14),
VEGF appears to serve an important role in the development of OA. In addition, a wide range of studies suggest that increased VEGF expression levels are associated with the progression of OA (15,16) and the inhibition of VEGF expression may alleviate the development of OA (17). Therefore, downregulation of MMP-13 and VEGF expression levels may be considered to be a promising therapy for OA.

Thalidomide, a drug first used to treat pregnant women for nausea or vomiting, was retracted from administration due to its teratogenic effects exerted on newborns (18). It was reported to be an angiogenic inhibitor in 1994 (19) and its first clinical success in treating multiple myeloma was reported in 1999 (20). Thalidomide was later reported to decrease VEGF expression, at the mRNA and protein levels (21); Mercurio et al (22) proposed that thalidomide may affect VEGF expression levels via downregulation.

At present, thalidomide is used in erythema nodosum leprosum, multiple myeloma, prostate cancer, glioblastoma, glioma, renal cell carcinoma, colon cancer and advanced breast cancer, diabetic retinopathy, rheumatoid arthritis and lupus, as well as in other pathological conditions where inflammation and angiogenesis co-exist (23-27).

To the best of our knowledge, the therapeutic effects of thalidomide on OA in vivo have not been investigated. Therefore, the present study utilized surgically-induced OA mice to investigate the hypothesis that thalidomide may attenuate the early development of OA by suppressing VEGF expression.

**Materials and methods**

**Reagents.** Thalidomide, dimethylsulfoxide (DMSO), and a Safranin-O/Fast Green Staining kit were obtained from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Thalidomide was dissolved in DMSO at 200 mg/ml as a 50 mM stock solution and then stored at 4˚C; the DMSO concentration was <0.1%. Primary rabbit polyclonal antibodies against MMP-13 and VEGF were purchased from Abclonal Biotech Co., Ltd. (Boston, MA, USA). Secondary anti-rabbit antibody was purchased from Beijing Ray Antibody Biotechnology (Beijing, China). Fluorescein isothiocyanate goat anti-rabbit immunoglobulin G (IgG) antibody was purchased from OriGene Technologies, Inc. (Beijing, China). Hematoxylin and eosin (HE) staining kit, a diaminobenzidine tetrahydrochloride (DAB) kit, and a DAPI staining kit were purchased from Abclonal Biotech Co., Ltd. (Beijing, China). TRIzol total RNA reagent, HiScript® II Q RT SuperMix for quantitative polymerase chain reaction (qPCR; +gDNA wiper), and ChamQ™ SYBR® qPCR Master Mix were purchased from Vazyme (Piscataway, NJ, USA). An ELISA kit of VEGF (E-EL-M1292c) was purchased from Elabscience Biotechnology Co., Ltd., Wuhan, China.

**Mice model of OA.** A total of 48 male C57BL/6 mice (10-weeks-old, 27±0.8 g) were purchased from the Laboratory Animal Center of Sun Yat-Sen University (Guangzhou, China), were randomly divided into experimental (Dmm+Th), control (Dmm), and Sham groups (n=16 in each). The mice were housed under clean conditions with controlled temperature (25±1˚C), humidity (50±10%), and a 12 h light/dark cycle, allowed free access to water and food, and received humane care in the Laboratory Center of The Third Affiliated Hospital of Southern Medical University (Guangzhou, China). In the Dmm+Th and the Dmm groups, to induce experimental OA, each mouse was anesthetized and then subjected to a destabilization of the medial meniscus (DMM) procedure as previously described (28). In the Sham group, only the skin of the right knee joint was resected. The skin of each mouse in the three groups was then sutured in layers, and the mice were allowed to move, eat and drink freely following surgery. All the surgical procedures performed herein were performed under sterile conditions and general anesthesia with 3% pentobarbital sodium (40 mg/kg) via intraperitoneal injection. The Dmm+Th group was injected intraperitoneally daily (8 mice for 2 weeks and another 8 mice for 4 weeks) with thalidomide (200 mg/kg body weight) the day following surgery (29); the Dmm and Sham groups were injected intraperitoneally daily with normal saline according to the same standard. At 2 and 4 weeks following surgery, 8 mice of each group were first anesthetized to collect blood samples from the heart vein via cardiac puncture approach and then sacrificed to harvest the right knees for histological assessments (n=4) and gene expression (n=4). All efforts were made to minimize suffering. All animal experiments were approved by and conducted according to the guidelines of The Third Affiliated Hospital of Southern Medical University Animal Healthcare Ethics Committee (Guangdong, China) (30).

**Histological analysis.** The specimens of the right knees were fixed in 4% paraformaldehyde for 48 h at 4˚C and then decalcified with 14.5% EDTA at pH 7.3 for 3 weeks. Following embedding in paraffin, the specimens were sectioned via the medial tibial plateau at a thickness of 5 μm in the sagittal plane. Then, the sections were dewaxed in xylene and hydrated with a graded ethanol series. To assess cartilage destruction, staining with HE and Safranin O/Fast Green staining was performed according to the manufacturer’s protocols. The hyaline cartilage/calcified cartilage (HC/CC) and Osteoarthritis Research Society International (OARSI) cartilage OA grading system (31) was employed to determine the extent of cartilage degeneration. Image-Pro Plus version 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA) (32) was used to analyze the HC/CC of the Safranin O/Fast Green-stained sections. A total of 3 different areas from the medial tibial plateau were randomly selected to calculate and evaluate the OARSI score. The sections were examined blindly by two musculoskeletal researchers respectively, and the scores were averaged to minimize observer bias. The OARSI score used for the present study was presented in Table I.

**Reverse transcription (RT)-qPCR.** RT-qPCR was employed to quantify the mRNA expression levels of MMP-13 and VEGF in the medial articular cartilage. At weeks 2 and 4, a total of four mice in each group were sacrificed to harvest the right knees, which were then stored at -80°C until use. The frozen samples constituted collected articular cartilage from the medial tibial plateau, as published previously (33) and crushed in a mortar under liquid nitrogen. Subsequently total RNA extraction from articular cartilage from the medial tibial plateau was performed using TRIzol® (Thermo Fisher Scientific, Inc.,
Waltham, MA, USA) according to the manufacturer's protocols to extract total RNA. The concentration and purity of total RNA were determined with a NanoDrop spectrophotometer (NanoDrop; Thermo Fisher Scientific, Inc., Wilmington, DE, USA); total RNA was reversed transcribed into cDNA using HiScript® II Q RT SuperMix for qPCR(+gDNA wiper) according to the manufacturer's protocols. Subsequently, qPCR was performed in triplicate using ChamQ™ SYBR qPCR Master Mix according to the manufacturer's protocols. Relative gene expression levels were calculated using the 2^−ΔΔCq method (34). Target mRNA expression levels were normalized to the reference gene GADPH, which served as an internal control. The specific primer sequences (Sangon Biotech, Co., Ltd., Shanghai, China) employed in the present study were listed in Table II.

Immunohistochemistry (IHC) and immunofluorescence (IF). In the articular cartilage of the medial tibial plateau, IHC was used to locate the protein expression of VEGF; IF was employed to locate the protein expression of MMP-13, which was conducted to detect cartilage lesions. For IHC, 5-µm paraffin-embedded sections were dewaxed and hydrated using a graded ethanol gradient, and incubated with rabbit polyclonal antibody anti-VEGF (A12303) at dilution of 1:100 at 4°C overnight. Following staining with Goat anti-Rabbit IgG(H+L)-HRP secondary antibody of anti-VEGF (RM3002) at dilution of 1:100 for 1 h at room temperature, DAB was used for 2 min at room temperature, followed by counterstaining with hematoxylin for 30 sec at room temperature. For IF, the sections were prepared and incubated with rabbit polyclonal antibody anti-MMP-13 (A1606) at dilution of 1:100 at 4°C overnight. Then, the sections were incubated with fluorescein-conjugated goat anti-rabbit IgG antibody (TA130022) at dilution of 1:100 for 1 h at room temperature and were mounted in medium containing DAPI for 10 min at room temperature. The sections were viewed with a FluoView FV1000 confocal laser scanning microscope (magnification, x400; Olympus Corporation, Tokyo, Japan). The percentage of positive cells were calculated in arbitrary three slices.

ELISA. The VEGF expression levels in serum were measured via ELISA. Immediately after collection, blood samples were allowed to coagulate at 4°C overnight and were then centrifuged at 1,000 x g at room temperature for 20 min. The supernatant was collected and stored at -80°C until use. The concentration of VEGF in serum of the different groups were analyzed via ELISA, according to the manufacturer's protocols. In order to calculate the concentration of VEGF in serum, the standard curve was constructed by plotting the mean absorbance (Y) of standards against the known concentration (X) of standards in logarithmic scale, using the four-parameter algorithm.

Statistical analysis. All experiments were performed independently at least three times. All quantitative data were expressed as the mean ± standard deviation. For three group comparisons, one-way analysis of variance followed by Tukey's post-hoc test was performed. All statistical data were analyzed using SPSS software (version 19.0, IBM Corp., Armonk, NY, USA). P<0.05 was considered to indicate a statistically significant difference.

Results
Thalidomide treatment may attenuate the progression of OA in a mice model. As presented in Fig. 1A and B respectively,
HE and Safranin O/Fast Green staining demonstrated within the Sham group the articular cartilages had smooth and intact surface and almost no fibrillation was observed. Conversely, in the Dmm group, articular cartilage was easily observed with superficial fibrillation at 2 weeks post-surgery, notable loss of proteoglycan, as well as vertical clefts and erosion to the calcified layer lesion at 4 weeks post-surgery. Compared with in the Dmm group, articular cartilage in the Dmm+Th group had rough surface at 2 weeks post-surgery, with less proteoglycan loss, cartilage fibrillation, and loss of surface lamina at 4 weeks post-surgery. Additionally, compared with in the Sham group, the hyaline cartilage layer appeared thinner and aberrant subchondral bone structure in the Dmm group was observed, while the hyaline cartilage layer relatively restored its normal thickness in the Dmm+Th group at 4 weeks following surgery.

As presented in Fig. 1C, consistent with the results of Safranin O/Fast Green staining, compared with the Sham group, the OARSI scores were significantly higher in the Dmm group and the Dmm+Th group at 2 and 4 weeks post-surgery (P<0.05). However, the Dmm+Th group revealed significantly lower OARSI scores than those of the Dmm group at 2 and 4 weeks post-surgery (P<0.05).

As presented in Fig. 1D, HC/CC values in the Dmm group were slightly lower at 2 weeks post-surgery (P<0.05) and were significantly lower at 4 weeks post-surgery (P<0.05) compared with the Sham group. In addition, the HC/CC value in the Dmm+Th group increased significantly compared with the Dmm group at 4 weeks (P<0.05). In addition, no significant difference between the Sham group and the Dmm+Th group was observed at 2 weeks post-surgery (P>0.05).

Thalidomide treatment downregulates VEGF and MMP-13 mRNA expression levels in early OA mice. As presented in Fig. 2A and B, the results obtained from RT-qPCR indicated that the mRNA expression levels of VEGF and MMP-13 in the Dmm and Dmm+Th groups significantly increased with
the development of OA compared with in the Sham group at 2 and 4 weeks post-surgery (P<0.05). Compared with the Dmm group, the mRNA expression levels of VEGF and MMP-13 were significantly downregulated by varying degrees in the Dmm+Th group at 2 and 4 weeks post-surgery (P<0.05), with the exception of VEGF mRNA expression levels between the Dmm+Th group and the Dmm group at 2 weeks post-surgery (P>0.05).

Thalidomide treatment inhibits VEGF protein expression in early OA mice. As presented in Fig. 3A, immunohistochemical staining revealed that the VEGF-positive cells were detected mainly in the superficial layers of the articular cartilage among the three groups. Compared with in the Sham group, the percentage of VEGF-positive cells increased as OA progressed and were significantly higher in the Dmm group and the Dmm+Th group at 2 and 4 weeks post-surgery (P<0.05; Fig. 3B). However, the Dmm+Th group demonstrated significantly lower percentages of VEGF positive cells than those of the Dmm group at 2 and 4 weeks post-surgery (P<0.05).

Thalidomide treatment reduces MMP-13 protein expression in early OA mice. As presented in Fig. 4A, immuno-positive cells were detected in the areas of cartilage breakdown. In the Dmm and Dmm+Th groups, the majority of these cells were observed in the superficial layers and very few in the middle layers of the articular cartilage; however, in the Sham group, where the articular cartilage had a smooth and intact surface, almost no such staining was observed. The percentages of MMP-13-positive cells in the articular cartilage were significantly higher in the Dmm group and the Dmm+Th group as compared with the Sham group at 2 and 4 weeks post-surgery (P<0.05; Fig. 4B). The Dmm+Th group demonstrated a significant decrease in the number of VEGF-positive cells in the articular cartilage compared with the Dmm group at 2 and 4 weeks post-surgery (P<0.05).

Thalidomide treatment reduces serum VEGF concentration in early OA mice. As presented in Fig. 5, compared with in the Sham group, serum VEGF concentration in the Dmm and the Dmm+Th groups were significantly increased by varying

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**Figure 2.** mRNA expression levels of VEGF and MMP-13 in the knee articular cartilage of mice among the Sham, Dmm and Dmm+Th groups (n=4 in each group). (A) Relative mRNA expression levels of VEGF in the medial articular cartilage. (B) Relative mRNA expression levels of MMP-13 in the medial articular cartilage. The values are presented as the mean ± standard deviation. *P<0.05 compared with the Sham group; †P<0.05 compared with the Dmm group. Dmm, destabilization of the medial meniscus; MMP-13, matrix metalloproteinase-13; Th, thalidomide; VEGF, vascular endothelial growth factor.

**Figure 3.** Immunohistochemical analysis of VEGF expression in the knee articular cartilage of mice among the Sham, Dmm and Dmm+Th groups (n=4 in each group). (A) Immunohistochemistry staining of VEGF in the articular cartilage of the medial tibial plateau (magnification, x400, scale bar=100 µm). (B) Quantification of VEGF positive cells, based on the results of immunohistochemistry staining. The values are presented as the mean ± standard deviation. *P<0.05 compared with the Sham group; †P<0.05 compared with the Dmm group. Dmm, destabilization of the medial meniscus; Th, thalidomide; VEGF, vascular endothelial growth factor.

**Figure 4.** Immunohistochemical analysis of MMP-13 expression in the knee articular cartilage of mice among the Sham, Dmm and Dmm+Th groups (n=4 in each group). (A) Immunohistochemistry staining of MMP-13 in the articular cartilage of the medial tibial plateau (magnification, x400, scale bar=100 µm). (B) Quantification of MMP-13 positive cells, based on the results of immunohistochemistry staining. The values are presented as the mean ± standard deviation. *P<0.05 compared with the Sham group; †P<0.05 compared with the Dmm group. Dmm, destabilization of the medial meniscus; Th, thalidomide; MMP-13, matrix metalloproteinase-13; VEGF, vascular endothelial growth factor.
degrees; however, compared with in the Dmm group, the serum VEGF concentration was significantly lower within the Dmm+Th group at 2 and 4 weeks following surgery (P<0.05).

Discussion

Thalidomide was first developed as a sedative in the 1950s in European countries, and was later reported to be useful in the treatment of morning sickness in pregnant women, but was withdrawn from the market globally due to its teratogenic effects (35). Then thalidomide, which was researched by scientists for numerous years, was reported to exhibit antiangiogenic properties and suppress VEGF expression (19,23). In addition, in the present study, thalidomide was observed to inhibit the expression of VEGF at the mRNA and protein levels, detected via RT-qPCR and IHC. Additionally, the concentration of VEGF in serum was also significantly reduced in the Dmm+Th group compared with in the Dmm group. The results of the present study revealed that thalidomide may be effective in reducing the production of VEGF. Furthermore, Alberto et al (36) revealed that thalidomide may decrease serum levels of VEGF in patients with severe intestinal bleeding.

In the present study, the method of intraperitoneal injection of thalidomide was employed. Thalidomide may not be suitable for intra-articular injection as the administration of dosing is greater than the volume of the articular cavity of the C57BL/6 mouse. In addition, intravenous administration has been reported to have some relevant complications, including delayed wound healing, hemorrhage, thromboembolism, proteinuria (37). The administration of intraperitoneal injection of thalidomide is rather mature and has been used in previous studies for numerous years (38,39), and has been reported to be more effective in inhibiting VEGF than oral administration of thalidomide (29,40).

OA is a widely prevalent and degenerative disease of the joints and occurs mainly in elderly people. The pathology is mainly characterized by the degeneration of cartilage. Chondrocytes, unique cells in articular cartilage, may synthesize ECM components, which mainly comprises aggrecans and type II collagen (Collagen II), and serve a vital role in maintaining the dynamic balance of ECM metabolism (8). Destruction of articular cartilage, involving the destruction of aggrecans and Collagen II, is mainly caused by proteolytic enzymes of aggrecanase families and MMPs (9). Xia et al (41) published a review suggesting that disruptions of the ECM dynamic equilibrium are pivotal events in the pathogenesis of OA. MMPs constitute a family of proteolytic enzymes, which regulate a variety of functions in tissue remodeling, such as ECM degradation (42). Among the MMPs, MMP-13 has been reported to serve an important role in the progression of OA because it irreversibly and efficiently breaks down Collagen II (43), which has made it an attractive therapeutic target for OA. In addition, the expression levels of several MMPs have been observed to be raised within cartilage tissues of patients with OA (44). Studies have revealed that selective inhibitors of
The results of the present study also indicated that the superficial layers of cartilage in surgically-induced OA mice, which were characterized by various degrees of fibrillations and cartilage erosion, contained a high proportion of cells positive for MMP-13 as observed via immunostaining; however, almost no immuno-positive cells of MMP-13 were detected in the Sham group. These observations were consistent with previous findings (44). Baragi et al (45) and Janusz et al (47) reported that MMPs or MMP-13 inhibitors may protect cartilage from degeneration (48).

VEGF, which was discovered and isolated in 1989, is a protein that stimulates blood vessel growth (49). Kim et al (50) demonstrated the significant inhibition of VEGF-induced angiogenesis, as well as the progression of diverse forms of tumors by a means of specific monoclonal antibodies. Yuan et al (16) reported a meta-analysis of the association between OA and VEGF expression levels, and indicated that higher VEGF expression levels were strongly correlated with the pathogenesis of OA.

From the results of IHC analysis of VEGF expression, VEGF was also expressed in normally growing mice and decreased progressively over time in the Sham group. This phenomenon was similar to the study of Tibesk et al (15) who reported an initial VEGF-increase in sham-operated New Zealand rabbits which decreased after 6 weeks. In the present study VEGF expression levels increased along with the progression of early OA, and may be inhibited by the intraperitoneal injection of thalidomide in the Dmm+Th group. Nagai et al (17) suggested that intravenous administration of an antibody against VEGF may contribute to articular cartilage repair in an osteochondral defect model. Furthermore, the findings of the present study indicated that the expression of VEGF at the mRNA and protein levels were downregulated in the Dmm+Th group and that the degree of cartilage damage in OA mice model, which would lead to cartilage degeneration and loss of proteoglycans, was significantly improved by suppression of VEGF expression within the Dmm+Th group, compared with the Dmm group. The results of present study supported the hypothesis that intraperitoneal administration of thalidomide may alleviate early OA development by suppressing VEGF expression in mice; to the best of our knowledge, this has not been previously reported.

There were some limitations to the present study: i) The OA model was surgically-induced via the Dmm method. This is a classical and effective method to establish an animal OA model for study in vivo (51); however, the method differs from the processes by which OA develops clinically. The etiology of OA is multifactorial, as the disease is caused by aging, as well as trauma (52). ii) The present study focused mainly on the effects of thalidomide on chondrocytes and cartilage, but did not investigate its effects on other types of cell (meniscus cells, osteoblasts/osteoclasts, and fibroblasts) or tissues (meniscus, subchondral bone, and synovium). iii) Only a single dose of thalidomide (200 mg/kg/day) for the treatment of early-stage OA. Thus, the effects of various doses and longer treatment courses on the different stages of OA requires further investigation. Additionally, the findings of the present study indicated that treatment with thalidomide may alleviate early OA development by suppressing VEGF expression; however, the exact molecular mechanisms associated with the interaction between thalidomide, VEGF, MMP-13 and chondrocytes requires further investigation.

In conclusion, the results of the present study suggested that intraperitoneal injection of thalidomide may alleviate the development of early OA by suppressing VEGF expression in mice. Thalidomide may be a promising treatment for OA; however, additional comprehensive studies to elucidate the exact molecular mechanisms and the clinically potential treatments for OA are required.

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Availability of data and materials
The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author's contributions
JLS, HF and DZC conceived and designed the study. JLS and DLL performed the experiments. JLS, HF and DLL collected, analyzed and interpreted the data. All authors drafted and edited the manuscript, and gave final approval of the article.

Ethics approval and consent to participate
The present study was approved by and conducted according to the guidelines of The Third Affiliated Hospital of Southern Medical University Animal Healthcare Ethics Committee (Guangdong, China).

Consent to participate
Not applicable.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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