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Effects of Vascular Endothelial Growth Factor (VEGF) on the Progression of Osteoarthritis in the Mouse Temporomandibular Joint

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Abstract: Osteoarthritis (OA) is a chronic degenerative joint disease with a multifactorial etiology including inflammatory mediators. The effects of vascular endothelial growth factor (VEGF) on OA have been studied widely in the field of orthopedics. This study aimed to evaluate whether VEGF could affect the progression of OA in the mouse temporomandibular joint (TMJ). C57BL/6J mice (n = 54) were assigned to three groups, namely, the VEGF+Discectomy, Discectomy, and Sham groups. OA was induced with a discectomy performed on the TMJ in 12-week-old mice in the VEGF+Discectomy and Discectomy groups. Mice in the VEGF+Discectomy group underwent intra-articular VEGF administration after discectomy. For the mice of the Sham group, the joint space was opened surgically, but the disc was not removed. At 4, 8, and 16 weeks after the induction of TMJ OA, the animals were sacrificed. Condylar dimensions and cartilage thickness were measured. Histological changes of the cartilage were assessed using a modified Mankin scoring system. The VEGF+Discectomy group showed a marked reduction of cartilage thickness at 16 weeks post-surgery. According to the modified Mankin scoring system, the VEGF+Discectomy group exhibited the highest scores for the severe reduction of safranin O staining, hypocellularity, and clefts in deep cartilage zones at 16 weeks post-surgery. In the surgically induced TMJ OA mouse model, the VEGF+Discectomy group exhibited highly progressive OA changes in articular cartilage. The detrimental effects of VEGF on TMJ OA may be via its role in the promotion of degradation.

Key words: Vascular endothelial growth factor, Osteoarthritis, Temporomandibular joint

Introduction

Osteoarthritis (OA) is a slowly progressive degenerative joint disease that affects articular cartilage, subchondral bone, and synovial tissue, leading to pathological matrix changes. Temporomandibular joint (TMJ) OA is clinically characterized by joint pain, limited mouth opening, and joint noise. Radiological studies of TMJ OA have demonstrated osseous changes such as bone erosion, osteophyte formation, sclerosis, and subchondral cysts. Risk factors for OA include age, sex, obesity, diabetes, diet, mechanical stress, injury, and genes. Vascular endothelial growth factor (VEGF) is associated with the development of OA in the knee joint, TMJ, and so on. A previous study noted that VEGF administration to the knee joint leads to OA in rats. As for the TMJ, animal studies have not examined the progressive OA changes following the administration of VEGF. Well-designed small animal studies on TMJ OA are required to understand the effects of VEGF on degenerative changes.

The purpose of the present study was to evaluate the effect of VEGF on a surgically induced TMJ OA model in C57BL/6J mice.

Materials and Methods

Animals

The animals used were wild-type adult male C57BL/6J mice (n = 54; Japan SLC, Inc., Hamamatsu, Japan) housed in an air-conditioned room maintained at a constant temperature (23 ± 2°C) with a controlled light/dark cycle. The mice had ad libitum access to food and water. Four-week-old C57BL/6J mice were placed on a standard diet (CLEA Rodent Diet CE-2; CLEA Japan, Inc., Tokyo, Japan). Animal care and the experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee at Aichi Gakuin University (approval number: AGUD397).

Surgical induction of OA (Figs. 1 and 2)

At 12 weeks of age, the mice of the VEGF+Discectomy (n = 18) and Discectomy (n = 18) groups underwent OA model surgery. For the mice of the Sham group (n = 18), the joint space was opened surgically, but the disc was not removed, and the incision site was sutured.

General anesthesia was induced with an intraperitoneal injection of mixed anesthetic agents including medetomidine hydrochloride (Domitor; Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Japan), midazolam (Sandoz KK, Tokyo, Japan), and butorphanol (Vetorphale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Local anesthesia was applied to the TMJ area using 2% lidocaine HCl with 1:80,000 epinephrine (Showa Yakuhin Kako Co., Ltd., Tokyo, Japan). The preauricular region was shaved and draped in a sterile manner. Discectomy was performed uni-

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laterally using a microsurgical scope. A vertical preauricular skin incision was made over the lateral capsule of the left TMJ. The joint space was opened by a horizontal incision through the joint capsule. The disc was removed gently using a scalpel blade and periosteal elevator. In the VEGF+Discectomy group, a biodegradable gel (MedGel II™; Nitta Gelatin, Inc., Osaka, Japan) containing 20 μg VEGF was placed into the joint space following disc removal. The wound was closed in layers.

Histological processing and staining
At the completion of the experiment, at 4, 8, and 16 weeks after OA model surgery, the mice were euthanized with CO₂ asphyxiation. The TMJ was harvested en bloc and fixed in 10% neutral buffered formalin. The specimens were decalcified using 10% ethylene diamine tetraacetic acid and embedded in paraffin wax. Samples containing the TMJ were serially sectioned at 5 μm from anterior to posterior in the frontal plane. The sections were mounted on glass slides. The slides were stained with hematoxylin and eosin or safranin O-fast green for quantification and to evaluate the histological state of the TMJ. Photographs of the slides were taken with an optical microscope and a DP22 digital camera (Olympus Corporation, Tokyo, Japan).

Quantification of the condyle (Fig. 3)
The width and height of the condyle and the thickness of the articular cartilage were determined as follows. Linear measurements of the condyle were performed on the computer screen of the DP2-SAL system (Olympus Corporation, Tokyo, Japan) using cellSens imaging software (Olympus Corporation, Tokyo, Japan). The width of the condyle was measured by a straight line between the lateral and medial poles of the condyle. To measure the height of the condyle and the thickness of the articular cartilage, a straight line was drawn using the same line between the poles used for the previous measurements of the width. Then, the height of the condyle was measured by a perpendicular line that was drawn to the point on the articular surface furthest from the line between the poles. Articular cartilage thickness was also measured using the same perpendicular line.

Histological assessment of articular cartilage with a modified Mankin scoring system
Images of the joint from all groups were digitized and analyzed. The osteoarthritic state of condylar cartilage was graded histologically with a modified Mankin scoring system. The degree of articular cartilage degradation was determined using a scoring system that measured safranin O-fast green staining on a scale of 0–6, chondrocyte loss on a scale...
of 0–4, and structure on a scale of 0–8. Sum totals of the scores range from 0 (normal cartilage) to 18 (severe degradation). The median of the data with the ordinal score scales was used to determine statistical significance.

**Statistical analysis**

All data are presented as the median. The data (quantification and modified Mankin scores) were compared between the VEGF+Discectomy and Discectomy groups and between the VEGF+Discectomy and Sham groups. Statistical analysis was carried out using BellCurve for Excel software (Social Survey Research Information Co., Ltd., Tokyo, Japan). The Kruskal-Wallis test was used to examine the differences of the data. A probability of less than 0.05 was considered statistically significant.

**Results**

**Quantification of the condyle (Figs. 4–9)**

The width of the condyle was not significantly different at 4, 8, and 16 weeks post-surgery between the VEGF+Discectomy (1256.5, 1324, and 1292 µm, respectively) and Discectomy (1137.5, 1279, and 1223.5 µm, respectively) groups. Condyle width was significantly increased at 4, 8, and 16 weeks post-surgery in the Discectomy group (1137.5, 1279, and 1223.5 µm, respectively) compared to the Sham group (854.5, 855.6, and 876.2 µm, respectively).

The height of the condyle was not significantly different at 4, 8, and 16 weeks post-surgery between the VEGF+Discectomy (528, 561, and 426.8 µm, respectively) and Discectomy (552, 494.8, and 434.1 µm, respectively) groups. Condyle height at 4, 8, and 16 weeks post-surgery was significantly increased in the VEGF+Discectomy group (528, 561, and 426.8 µm, respectively) compared to the Sham group (median: 468.1, 428.6, 420.2 µm, respectively).

Cartilage thickness was significantly decreased at 16 weeks post-surgery in the VEGF+Discectomy group (95.2 µm) compared to the Discectomy group (197.6 µm). However, cartilage thickness was not significantly different at 4 and 8 weeks post-surgery between the VEGF+Discectomy (224.7 and 185.2 µm, respectively) and Discectomy (228.8 and 144.5 µm, respectively) groups. Cartilage thickness was significantly decreased at 16 weeks post-surgery in the VEGF+Discectomy group (95.2 µm) compared to the Sham group (206.5 µm). However, there was no significant difference in cartilage thickness at 4 and 8 weeks post-surgery between the VEGF+Discectomy (224.7 and 185.2 µm, respectively) and Sham (193.8 and 218.7 µm, respectively) groups.

**Histologic analysis of articular cartilage (Figs. 4–6, 10–12)**

In hematoxylin and eosin- and safranin O-fast green-stained sections, condylar cartilage exhibited considerable OA changes in the VEGF+Discectomy and Discectomy groups. For histologic evaluation of the severity of OA lesions of articular cartilage, the modified Mankin scoring system was applied to safranin O-fast green-stained sections.

**Safranin O-fast green staining intensity (Figs. 4–6, 10–12)**

The cartilage staining intensity score was significantly higher at 4 and 8 weeks post-surgery in the VEGF+Discectomy group (2 and 4, respectively) than in the Discectomy group (1 and 2, respectively). However, there was no significant difference in the cartilage staining intensity score at 16 weeks post-surgery between the VEGF+Discectomy (4) and Discectomy (3.5) groups. Cartilage staining intensity of the condyle was significantly higher at 4, 8, and 16 weeks post-surgery in the VEGF+Discectomy group (528, 561, and 426.8 µm, respectively) compared to the Sham group (median: 468.1, 428.6, 420.2 µm, respectively).

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Figure 5. 8 weeks post-surgery histopathology of the temporomandibular joint. (a-c) Hematoxylin Eosin staining, (d-i) Safranin O-fast green staining. (a-f) Scale bars = 200 μm, (g-i) Scale bars = 100 μm. Haematoxyline and Eosin staining (top) showing the condyle showing structural changes of articular cartilage in the VEGF+Discectomy, the Discectomy, and the Sham groups. Safranin O-fast green staining (bottom) showing OA changes in the VEGF+Discectomy, the Discectomy, and the Sham groups.

Figure 6. 16 weeks post-surgery histopathology of the temporomandibular joint. (a-c) Hematoxylin Eosin staining, (d-i) Safranin O-fast green staining. (a-f) Scale bars = 200 μm, (g-i) Scale bars = 100 μm. Haematoxyline and Eosin staining (top) showing the condyle showing structural changes of articular cartilage in the VEGF+Discectomy, the Discectomy, and the Sham groups. Safranin O-fast green staining (bottom) showing OA changes in the VEGF+Discectomy, the Discectomy, and the Sham groups.
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Figure 7. 4 weeks post-surgery width, height and cartilage thickness of the condyle of the VEGF+Discectomy, the Discectomy, and the Sham groups. The data are presented as the median of the score. **P<0.01.

Figure 8. 8 weeks post-surgery width, height and cartilage thickness of the VEGF+Discectomy, the Discectomy, and the Sham groups. The data are presented as the median of the score. *P<0.05, **P<0.01.

Figure 9. 16 weeks post-surgery width, height and cartilage thickness of the VEGF+Discectomy, the Discectomy, and the Sham groups. The data are presented as the median of the score. *P<0.05, **P<0.01.
VEGF+Discectomy group (2, 4, and 4, respectively) than in the Sham group (0, 0.5, and 0.5, respectively).

**Chondrocyte loss (Figs. 4–6, 10-12)**

There was no significant difference in the chondrocyte loss score at 4 and 8 weeks post-surgery between the VEGF+Discectomy (1 and 1.5, respectively) and Discectomy (1 and 1, respectively) groups. However, the chondrocyte loss score was significantly higher at 16 weeks post-surgery in the VEGF+Discectomy group (4) than in the Discectomy group (2). The chondrocyte loss score was significantly higher at 4, 8, and 16 weeks post-surgery in the VEGF+Discectomy group (1, 1.5, and 3.5, respectively) than in the Sham group (0, 0, and 0, respectively).

**Cartilage structure (Figs. 4–6, 10-12)**

The condylar cartilage appeared to be damaged and clefts of the cartilage were commonly recognized in the VEGF+Discectomy and Discectomy groups. However, no cartilage damage was observed in the Sham group at 4, 8, and 16 weeks post-surgery. There was no significant difference in the cartilage structure score at 4, 8, and 16 weeks post-surgery between the VEGF+Discectomy (4, 4.5, and 7.5, respectively) and Discectomy (4, 7, and 5, respectively) groups. The cartilage structure score was significantly higher at 4, 8, and 16 weeks post-surgery in the VEGF+Discectomy group (4, 4.5, and 7.5, respectively) than in the Sham group (0, 0, and 0, respectively).

**Total modified Mankin score (Figs. 4–6, 10–12)**

There was no significant difference in the total modified Mankin score at 4, 8, and 16 weeks post-surgery between the VEGF+Discectomy (7, 10, and 15, respectively) and Discectomy (6, 10.5, and 9, respectively) groups, but the scores of the VEGF+Discectomy group at 4, 8, and 16 weeks post-surgery were significantly higher than those of the Sham group (0, 0.5, and 0.5, respectively). The most advanced OA changes to articular cartilage were observed in the VEGF+Discectomy group.

**Discussion**

**Effect of VEGF on the progression of OA**

The local application of VEGF significantly increased the modified Mankin scores and decreased condylar height. The Discectomy group also showed a decrease of condylar cartilage thickness. This study utilized a TMJ OA pathologic mouse model to evaluate the effect of VEGF on the progression of OA, compared to mice who received discectomy or sham surgery. At 16 weeks after OA model surgery, we observed highly progressive OA lesions in the VEGF+Discectomy group and moderate OA lesions in the Discectomy group, compared to the Sham group. VEGF administration to the mice led to a severe state of

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Figure 10. The 4 weeks post-surgery modified Mankin Scoring of the VEGF+Discectomy, the Discectomy, and the Sham groups. The data are presented as the median of the score. *P<0.05, **P<0.01.

Figure 11. The 8 weeks post-surgery modified Mankin Scoring of the VEGF+Discectomy, the Discectomy, and the Sham groups. The data are presented as the median of the score. *P<0.05, **P<0.01.

Figure 12. The 16 weeks post-surgery modified Mankin Scoring of the VEGF+Discectomy, the Discectomy, and the Sham groups. The data are presented as the median of the score. *P<0.05, **P<0.01.
cartilage degradation. As a whole, the outcome of TMJ OA in the VEGF+Discectomy group indicates the effect of VEGF on cartilage degradation. The modified Mankin scores had a substantial association with the VEGF+Discectomy group. The loss of safranin O staining intensity in articular cartilage is a strong indicator of cartilage degradation\(^{(13)}\). According to the histopathologic results of this study, it was considered that VEGF administration promoted the progression of TMJ OA in a mouse model.

**VEGF**

OA is a progressive disease characterized by articular cartilage degeneration with the loss of aggrecan and type II collagen\(^{(8)}\). In the physiological state, articular cartilage is avascular tissue, but once inflammation is provoked in the joint, the cartilage begins to vascularize under the influence of VEGF, an angiogenic growth factor\(^{(3)}\). VEGF is detected at high levels within the synovial fluid of the human knee in advanced stage OA\(^{(17)}\). VEGF is speculated to play an essential role in an animal knee OA model by increasing the activity of collagenase\(^{(9)}\). In regard to TMJ, high levels of VEGF are observed in synovial cells during TMJ internal derangement\(^{(9)}\). Overloading the TMJ, that is, posterior tooth loss\(^{(20)}\) and malocclusion with lateral shift\(^{(21)}\), upregulates the expression of VEGF and some biologic factors. Such excessive mechanical loading causes hypoxia within the joint\(^{(22)}\), which induces the expression of cytokines and eventually leads to VEGF expression\(^{(23)}\). VEGF is an angiogenic factor that consists of multiple family members; VEGF\(_{165}\) is highly expressed in articular cartilage of the human OA knee joint\(^{(23)}\). We used VEGF\(_{165}\) in this study to determine whether VEGF could contribute to the progression of TMJ OA.

**Utilization of a gelatin hydrogel**

In the current study, a biocompatible and biodegradable gelatin hydrogel was impregnated with VEGF. At the initial stage of this study, VEGF was prepared as an aqueous solution for administration to the TMJ following discectomy. However, this solution instantly leaked out from the wound and was mostly lost from the TMJ space. Gelatin hydrogels have been utilized as delivery carriers for drugs and biochemical factors\(^{(24,25)}\). The cross-linked gelatin molecules can contain drugs and slowly release them, degrading over a few weeks. Gelatin hydrogels are now clinically applied to deliver bioactive growth factors, such as fibroblast growth factor-2, to heal limb ischemia and knee OA without major harm\(^{(26,27)}\). Gelatin hydrogels do not appear to exert any adverse effects on the TMJ.

**Animals**

In the current study, the TMJ OA C57BL/6j mouse model was used to evaluate the effect of VEGF on OA progression. Discectomy in mice is known to induce reproducible articular cartilage degradation in the TMJ\(^{(12)}\). According to previous animal research\(^{(7,27)}\), the local application of VEGF into the knee joint of C57BL/6 mice results in progressive OA caused by injury to ligaments and the meniscus.

**Limitations**

The major limitation of the current study was the use of an invasive surgical approach to induce OA in mice. The surgical TMJ OA induction method might complicate the results of this study because of the unintentional impact of the procedure, which could evoke an inflammatory reaction instead of disturbing the joint itself. The use of non-invasive methods might be necessary to produce a natural osteoarthritic reaction in the TMJ. However, the inflammatory reaction is limited to the initial period following OA model surgery. In the current study, the specimens were obtained at 4, 8, and 16 weeks after surgery and the inflammatory reaction seemed to disappear early in the course of this experiment. According to the literature for the OA model surgery\(^{(13)}\) used in this study, the surgically induced osteoarthritic response is consistent among the animals.

In conclusion, VEGF has detrimental effects on the TMJ OA mouse model. The VEGF+Discectomy group exhibited the most severe progressive OA changes in this model of surgically induced TMJ OA. These results might help to clarify the relationship between VEGF and TMJ OA. However, the mechanism of the effects of joint loading is still unclear. Future studies should examine the relationship between VEGF expression and mechanical loading in the TMJ.

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**Conflict of Interest**

The authors declare no conflicts of interest.

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