Expression of VEGF-A Signaling Pathway in Cartilage of ACLT-induced Osteoarthritis Mouse Model

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Abstract:

Background: The clear understanding of the underlying mechanism of Osteoarthritis(OA) remains elusive. Researches have shown that Vascular Endothelial Growth Factor(VEGF) induced angiogenesis and inflammation were important processes in the pathophysiology of OA. Now, Anterior Cruciate Ligament Transection surgery (ACLT) induced OA model was often used to investigate the molecular mechanism of OA, but till now the angiogenesis and inflammation reaction in different pathological stages of ACLT-induced OA model has never been revealed.

Methods: Moderate OA model was established by ACLT, and 1, 2, 4, 8, and 12 weeks after surgery, Hematoxylin–eosin(HE) and Safranin-O(S-O) staining were used to detect the pathological changes in mouse knee cartilage, and the matrix biomarkers A Disintegrin and Metalloproteinase with Thrombospondin Motifs 5(ADAMTS5), Collagen II(COL-II) were detected using Immunohistochemistry (IHC), CD31 was detected by Immunofluorescence(IF) to show the vascular invasion in cartilage, and proteins expression of VEGF-A pathway were detected by Western blot(WB). Meanwhile the inflammatory biomarkers Cyclooxygenase-2 (COX-2) and inducible Nitric Oxide Synthase(Inos) in cartilage were detected by WB.

Results: ACLT surgery can lead to degeneration of cartilage in mice, and the characteristics of the lesion were time dependent. The ADAMTS5 positive cells increased while COL-II decreased in OA cartilage with time, new blood vessel labeled by CD31 can been seen from 1 week in OA cartilage, and increased in 8 and 12 weeks. The expression of VEGF-A, VEGFR2, COX-2 and iNOS were higher than control groups, which were basically consistent with the degree of osteoarthritis.

Conclusions: VEGF-A related signaling pathway played an irreplaceable role in the occurrence and development of ACLT model, and the underlying mechanism may be related to the angiogenesis and inflammation in cartilage.

Keywords: osteoarthritis; aclt; vegf-a/vegfr2; angiogenesis; inflammation

1.Introduction

Osteoarthritis(OA) is an irreversible degenerative arthritis disease[1,2], OA-associated degeneration of articular cartilages, synovitis, and the formation of osteophyte have become the main reasons underlying adult disability, especially in the elderly, and OA is estimated to be the fourth leading cause of disability by 2020[3-5]. It is well known that increased age, obesity, joint
injuries and lifestyle are all risk factors for OA[6], and these factors are closely related to the mechanical loading to the joints, so it is assumed that a large part of OA is induced by accumulated mechanical stress[7]. ACLT-induced OA model is a normal stress-induced OA models in mice[7]. As previously reported, the ACLT mouse model demonstrate great similarities with human osteoarthritis, including subchondral change, articular cartilage damage and synovitis[8-10], made this model ideal for the research of OA. But the molecular mechanisms in the process of this OA model remain undefined. Clarification of the postoperative biomarkers changes may provide theoretical support for this model as a vector of OA pathogenesis study.

Nowadays, although the clear understanding of the underlying mechanism of OA remains elusive[11], researches have shown that angiogenesis and inflammation are important processes in the pathophysiology of OA[12, 13]. Which can cause joint damage, endochondral ossification and pain. VEGF was a potent stimulator of angiogenesis, which can also contributed to inflammation[14]. Till now the angiogenesis and inflammation reaction in different pathological stages of ACLT-induced OA model has never been revealed. Clarify the correlation of VEGF, angiogenesis and inflammation reaction in the OA pathological process may reveal the mechanism of OA to a certain extent.

Therefore, in this study, we established a moderate OA model by ACLT surgery, and visualized the angiogenesis and inflammation reaction in cartilage, and their relationship with OA proceeding in ACLT-induced OA mouse model. We also tried to explore the expression of VEGF-A/VEGFR2 signaling pathway to further study on OA mechanism. Our studies have confirmed that the expression of VEGF-A/VEGFR2 was basically consistent with the degree of OA, and VEGF induced angiogenesis and inflammation may be the potential mechanism of OA.

2. Materials and methods

2.1Animas models

Eighty eight C57BL/6 mice (12-week-old) of both sexes were purchased from Shandong skobas Biotechnology Co, Ltd (Shandong, China). Mice were housed in Laboratory Animal Center of Nanjing University of Chinese medicine under specific pathogen-free (SPF) and maintained at 27°C under a 12-hr light/dark cycle with 50% of humidity throughout the experiments. Experiments performed in this study were all approved by the Animal Experiment Committee of Nanjing University of Chinese Medicine (ethics No:201904A009).

Eight mice served as control animals (B), and the others were randomly divided into two groups, namely the model group (n=40) and the sham group (n=40). Each group was further divided into five subgroups depending on when the animals were killed (n=8). We established a surgically induced moderate OA model by Anterior Cruciate Ligament Transection surgery (ACLT) which was described in previously researches[7, 15], and the groups were named as M1, M2, M4, M8, M12 (1, 2, 4, 8, 12 weeks after ACLT). In the sham group, only a 1.5 cm incision in the same position was made, but the ligament was not cut, the groups were named as S1, S2, S4, S8, S12 (1, 2, 4, 8, 12 weeks after sham surgery).

2.2Hematoxylin–eosin(HE) and Safranin-O(S-O) staining

Knee tissues of each group were fixed in 4% paraformaldehyde for 24 h, then decalcified in 10% EDTA for 8 weeks. After dehydrated in graded ethanol, tissues were embedded in paraffin. 4μm sections were stained with hematoxylin eosin and Safranin O-Fast Green. Three to five fields were randomly selected from each section, and observed under a 400x light microscope. The pathological changes were evaluated thrice and graded by 3 independent researchers using the Mankin histological criteria, which was scored according to structural intergrit, cells, Safranin-O staining and tidemark intergrity[16].
2.3 Immunofluorescence and immunohistochemistry

Knee joint sections (4μm) were deparaffinized at 37 °C for 30 min, then hydrated with xylene, graded alcohol; After antigen retrieval was performed, blocked for 10 min with 3% H2O2 methanol solution at room temperature; Sections were then incubated with anti CD31 (1:500, abcam ab182981), anti-ADAMTS5 (1:100, Bioss) and anti COL-II (1:100, Affinity AF0135), incubated overnight at 4°C, 50 μL of Sheep anti rabbit polymer was added for 20 min at room temperature; DAB was added for color development haematoxylin counterstaining for 10 min. The protein expression was observed under a light microscope, and three areas with high expression were taken and photographed for storage (all pictures were taken at 400X).

2.4 Western blot

Proteins of knee cartilage from each group were extracted and collected using RIPA Lysis Buffer (KGP250, China), mixed with 10μl phosphatase inhibitor, 1μl protease inhibitor and 5μl 100mM PMSF. Protein concentrations of each group were determined by BCA kit (KGA902, China). Then, electrophoresis was performed, blocking with 10% milk powder for 2h, incubated with primary antibodies anti-iNOS (diluted 1:500 affinity AF0199), anti-COX2 (diluted 1:1000 UK Abcam plc ab179800), VEGFA (diluted 1:500, UK Abcam plc ab1316), VEGFR-2 (diluted 1:1000, UK Abcam plc ab39638). After washing again, secondary antibodies were added and incubated for 2h at room temperature. Image-J software was used to analysis the Gray scale.

2.5 Statistical analysis

The results were displayed as mean±SD. All data were analyzed with GraphPad Prism8 statistical software. Differences among three groups were analyzed by one-way analysis and P<0.05 indicated statistical significance.

3. Result

3.1 Histopathological changes in cartilage by HE and Safranin-O staining

Hematoxylin–eosin (HE) and Safranin-O (S-O) staining were utilized to evaluate the changes of Histological examination of cartilage, as shown in Fig.1(A B), the HE and S-O staining showed that the cartilage surface was smooth and intact in the sham groups, similar to those of normal knee cartilage of mice in Fig.1(C). However, the model groups exhibited cartilage superficial destruction, but limited to the superficial layers in 1 week. At 2 weeks, Safranin-O staining in the middle zone decreased. The defect of cartilage was developed to the calcified cartilage layer below the tidemark by 4 weeks, and gradually extended to the full thickness of cartilage at 8 and 12 weeks. Next, we performed a modified Mankin score on cartilage in different groups in Fig.1(D). The results showed that the Mankin scores of the experimental groups generally showed an increasing trend, and the difference between model group and sham group was statistically significant (P<0.01). Indicating this surgery can lead to degeneration of cartilage in mice. And the characteristics of the lesion were time dependent.
Figure 1. Histopathological analysis of the cartilage tissues obtained from each group (×200) to show the development in the ACLT model. A: After fixation, decalcification and embedding, 4 mm frontal sections were cut from the knee joints, and were stained with HE staining. B: Safranin-O-fast green staining images of cartilage in each group. C: HE and Safranin-O-fast green staining of control group. D: Mankin score. The data in the figures represent mean values ± SD, *p < 0.05, **p < 0.01 compared with the control group, #p < 0.05, ##p < 0.01 compared with the sham group at the same point.

3.2 Changes of matrix biomarkers ADAMTS5, COL-II in knee joint cartilage

Immunohistochemistry was used to detect the expression of ADAMTS5 and COL-II, both important biomarkers to demonstrate ECM degradation of cartilage. The protein expression of ADAMTS5 in the sham groups showed weakly expressed. In the model groups, significant expression of ADAMTS5 was observed in the first week after surgery, and the location of positive cell extended to a deeper layer from 4 weeks. Because of the destruction of cartilage from 8 weeks, the chondrocytes was hardly to been seen, especially in 12 weeks, so it was hard to calculate the number of positive cells as shown in Fig. 2.(A). And the change was basically consistent with the histopathological results. COL-II was strongly stained in all zones of the articular cartilage both above and below the tidemark in sham groups. In the model groups, the expression of COL-II showed gradually decreased with the extension of experiment time, and dramatically decreased from 4 weeks in Fig. 2.(B).
The expression of cartilage matrix biomarkers ADAMTS5, COL-II of knee joint. A: Immunohistochemistry staining of ADAMTS5 (scale bar: 20 μm). B: Immunohistochemistry staining of COL-II (scale bar: 20 μm). C: Immunohistochemistry staining of ADAMTS5 and COL-II in control group (scale bar: 20 μm).

3.3 The protein expression of inflammatory biomarkers COX-2 and iNOS in cartilage

The expression of COX-2 and iNOS in cartilage of each group was detected by western blot. As shown in Fig. 3(A B), the expression of COX-2 and iNOS gradually up-regulated after ACLT surgery. The expression pattern was correlated with the degree of osteoarthritis. The results in this chapter indicate that inflammation in cartilage was involved in the development of osteoarthritis.

3.4 Angiogenesis in the cartilage of ACLT-induced mouse

Immunostaining of CD31 was used to visualize the vascular invasion, as Fig. 4 described. CD31-positive cells in sham groups
were rare, and new blood vessel was not found. In the cartilage of OA mice, new blood vessel can been seen from 1 week at the osteochondral junction and cartilage. The number of blood vessel increased in 8 and 12 weeks. This indicated that vascular invasion was involved in the development of OA.

**Sham Group**

**Model Group**
3.5 The pattern of protein expression of VEGF-A and VEGFR2 in the articular cartilage

To further explore the underlying mechanism of the angiogenesis, we next investigated the protein level of VEGF-A and VEGFR2 in cartilage at different pathological stages of OA with western blot (Fig5A B). VEGF was a well-known angiogenic factor; according to our study, expression of VEGF-A was a particular feature in OA, and demonstrated that VEGF-A and VEGFR2 was associated with vascular invasion at cartilage. The expression pattern of VEGF-A was basically consistent with the Mankin score. But the expression of VEGFR2 showed decrees within two weeks, then increased from 4 weeks, significantly at 8 weeks in Fig.5., which need to be further studied.

4. Discussion

In order to explore the pathogenesis of OA, animal models are often used as study subjects. Due to rapid progress of mouse genomics and the availability of transgenic and knockout mice, the mouse is now the most ideal animal model for the study of molecular backgrounds of physiological and pathological conditions[7]. Among which, ACLT induced OA mouse model, a stress-induced model was often used[7]. Here, we made a moderate OA model based on the description of Kamekura, it was described that the moderate model seemed suitable to follow the entire process including early stage changes. After the surgery, HE and safranin O Fast Green staining was used to observe the pathological changes after surgery, and make sure the model is successful. According
to the Histopathological analysis and Mankin scores, the experiment indicated that the severity of cartilage degeneration in the experiment group was closely related to the time after surgery. COL-II and aggrecan are considered as the most important structural components forming the normal Extracellular Matrix (ECM) in cartilage[17,18]. The ECM maintains the balance due to the metabolism of chondrocytes, synoviocytes and subchondral bone cells, and once the balance is broken, cartilage damaged thus causing OA[19]. ADAMTS5, an important cartilage matrix-degrading enzymes, growing evidence showed it was involved in the pathogenesis of aggrecan cleavage of OA[20]. So we use COL-II and ADAMTS5 as matrix biomarkers in this research to reveal the damage of cartilage. In this experiment, immunohistochemistry results showed that the expression of ADAMTS5 increased from 1 week, while Collagen II decreased with the extension of experiment time.

The research of OA pathogenesis showed that, normal articular cartilage is avascular, but angiogenesis at the osteochondral junction and in non-calcified cartilage was observed in OA[21-23]. Loss of resistance to vascular invasion distinguishes OA cartilage from normal articular cartilage, which may be important in the pathogenesis of OA[24]. CD31 is a marker of endothelial cells expressed in vascular development, was often used to identify newly formed blood vessels[25]. Here we investigate the CD31 by immunostaining to visualize the vascular invasion. As shown, the blood vessels haven’t seen in Sham group. In model groups new blood vessel can been seen from 1 week and the number of blood vessel increased in 8 and 12 weeks, this result was nearly correlated with the degree of osteoarthritis. Confirmed that angiogenesis played a significant role in the degeneration of cartilage.

The mechanism of angiogenesis involves a coordinated signaling axis, among which the VEGF played an important role. VEGF expression has been found to be increased in the articular cartilage, subchondral bone, synovium, synovial fluid, and serum[26-30]. VEGF-A is the founding member of the VEGF family, VEGF-A binds to VEGF receptor-2 (VEGFR2) and play the most important role[31]. Previous studies have confirmed that VEGF-mediated vascular invasion plays an important role in OA, which could led to increased production of matrix metalloproteinase (MMP) 1, MMP-3, and MMP-13, induce chondrocyte apoptosis and inflammatory reaction as well as increased expression of Nerve Growth Factor (NGF)[32-34]. Assessment of VEGF as a biomarker in patients with OA showed that increased VEGF in synovial fluid was correlated with grade of OA severity[35]. Our previous study has confirmed that Chinese medicine can decrease the VEGF expression thus delay the progression of OA, Blocking VEGF signaling pathways and angiogenesis has emerged as a promising approach in recent preclinical studies in OA[36]. But the change of VEGF-A/VEGFR2 expression in ACLT-induced OA model was still unknown. In the current study, we found cartilage expression of VEGF-A was basically consistent with angiogenesis, this could indicate VEGF-A was a particular feature in OA and more fully demonstrated that VEGF-A and VEGFR2 was associated with vascular invasion at cartilage.

Previous studies indicated that VEGF may have specific roles in inflammation, which are closely related processes in OA. Although OA is commonly described as a non-inflammatory disease in order to distinguish it from ‘inflammatory arthritis’, such as rheumatoid arthritis (RA) or the seronegative spondyloarthropathies[12]. Many studies showed that inflammation triggered by factors like biomechanical stress is involved in the development of osteoarthritis[37]. Researches have confirmed that a variety of inflammatory mediators involved in the pathological process of OA, COX-2 plays an important role in joint destruction[38]; iNOS can produces high levels of NO, which can inhibiting the synthesis and secretion of ECM in chondrocytes, leading to cartilage degradation[39]. To confirm the inflammation reaction in this model, inflammatory biomarkers were tested by Western blot. Here, we detected the expression of COX-2 and iNOS in cartilage. Our results showed expression of COX-2 and iNOS increased in model group, which is closely related to the Mankin scores and with VEGF-A.

5. Conclusion

Taken together, we successfully established a surgical induced OA model with Anterior Cruciate Ligament Transection surgery (ACLT). The protein expression of cartilage matrix biomarkers after surgery were revealed, and can basically revealed the degree of OA. Aside from this, our study confirmed that the angiogenesis and inflammation played critical roles in the whole stage of ACLT-induced OA model, and VEGF-A/VEGFR2 single pathway may be the key action factor. However, as far as we
concerned, the expression changes were tested in protein level, changes in gene level needed to be further investigated. In summary, these findings provide theoretical support for this model as a vector for the study of OA pathogenesis, and further highlight the importance of preventing angiogenesis and inflammation as targeted therapeutic approach for OA.

List of Abbreviations

OA Osteoarthritis
ACLTL Anterior Cruciate Ligament Transection
VEGF Vascular Endothelial Growth Factor
VEGF-A Vascular Endothelial Growth Factor-A
VEGFR2 Vascular Endothelial Growth Factor Receptor 2
HE Hematoxylin-eosin
S-O Safranin-O
IHC Immunohistochemistry
WB Immunofluorescence
ADAMTS-5 A Disintegrin and Metalloproteinase with Thrombospondin Motifs 5
COL-II Collagen II
COX2 Cyclooxygenase-2
iNOS Inducible Nitric oxide synthase
MMP matrix metalloproteinase
ECM Extracellular Matrix
NGF Nerve Growth Factor

Declarations:

Ethics approval and consent to participate
The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of Animal Experiment Committee of Nanjing University of Chinese Medicine (ethics No:201904A009). Written informed consent was obtained from individual or guardian participants.

Consent for publication
Not applicable.
Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

Huang G.C. conceived and designed the study and critically revised the manuscript. Qian J.J. and Xu Q performed the experiments and drafted the manuscript. Xu W.M. participated in study implementation, and manuscript revision. Cai R. participated in analyzed and interpreted the data. The authors read and approved the final manuscript.

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