Egr-1 increases angiogenesis in cartilage via binding Netrin-1 receptor DCC promoter

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Background: Osteoarthritis (OA) is an age-dependent, chronic, incurable, and destructive joint disease characterized by degradation of cartilage. The etiology of OA is still unclear. Vascular endothelial growth factor (VEGF) plays a key role of angiogenesis in the pathogenesis of OA and contributes to the angiogenesis of NT-1/DCC. Whether or not NT-1/DCC and VEGF interact in regulating angiogenesis of OA cartilage is not known.

Methods: Histological studies for CD34, VEGF, and safranin-O staining were performed to determine angiogenesis and cartilage tissue injury. ELISA indicated the level of pro-inflammation cytokines. Immunoblotting, immunoprecipitation, and electrophoretic mobility shift assay (EMSA) were performed to assay the expression and function of NT-1/DCC-VEGF signaling pathway.

Results: Our data indicated that VEGF expression was increased in cartilage tissue from OA rats, while the chondrocytes were disorganized, and cartilage degeneration was increasing in OA rats. The inflammation factors in articular cavity fluid were higher in the OA rats than in the sham. The protein expression of NT-1, DCC, and VEGF were increased in osteoarthritic cartilage. DCC was involved in the positive regulation of osteoarthritic angiogenesis by VEGF. Egr-1 expression was higher in OA rats than in sham rats. Egr-1 is a regulator of DCC promoter activity, and the binding is higher in OA rats than in sham rats.

Conclusion: Our present study provides a mechanism by which Egr-1 induced angiogenesis via NT-1/DCC-VEGF pathway.

Keywords: Egr-1, Angiogenesis, Osteoarthritis, Netrin-1 receptor, DCC promoter
vascularization and remodeling hypertrophic cartilage, which finally leads to endochondral bone formation [13]. Whether or not NT-1/DCC and VEGF interact in regulating angiogenesis of OA cartilage is not known. And the upstream signaling that regulates NT-1/DCC-VEGF to angiogenesis in cartilage is still unclear.

Early growth response-1 (Egr-1), also called NGFI-A, is a zinc finger transcription factor and immediate early gene, which plays an important role in angiogenesis [14, 15]. Meanwhile, Egr-1 may be involved in the pathogenesis of OA [16, 17]. Few studies report the effect of Egr-1 to angiogenesis in osteoarthritic cartilage. Thus, our present study provides a mechanism by which Egr-1 induced angiogenesis via NT-1/DCC-VEGF pathway.

Methods

Animals

Lewis rats (260–280 g) were purchased from Chengdu Da Shuo Biotech Co. Ltd. (Chengdu, China) and randomized to the sham group and OA group, n = 8 per group. The rat OA model was performed as anterior cruciate ligament transection (ACLT). The ACLT was conducted as described in the previous study [18], which induced mechanical instability-associated OA. The rats were euthanized 2 months after ACLT. And siRNA or recombinant netrin 1 were injected into knee joints 48 h before euthanasia. The limbs of OA rats were dissected for staining, and articular cavity fluid were collected for further research.

All experiments conformed to the guidelines of the ethical use of animals, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Histological study

Cartilage tissues from rats in each group were fixed with 4% paraformaldehyde for 24 h and decalcified for 2 weeks with 20% EDTA at 4 °C. After embedding in paraffin, the tissues were sectioned to 4-μm slices and mounted on slides. The slides were incubated with anti-CD34 (1:100) antibody (Santa Cruz Biotechnology, CA) overnight at 4 °C followed by immunofluorescence and immunohistochemistry, or stained with 0.1% safranin-O for 30 min (Sigma Aldrich, St. Louis, MN). To assess microvessel density in epiphysis tissues, the number of new blood vessels visualized by CD34+ vascular endothelial cells was counted per high-power field (× 400) [19].

ELISA and immunoblotting

The concentration of IL-1β and TNF-α in articular cavity fluid was determined by the ELISA kit (R&D Systems) according to the manufacturer’s instructions. And tissues were washed three times with PBS, pulverized in liquid nitrogen, and lysed [20]. The homogenates (20 μg of protein) were separated by 8% SDS-PAGE and blotted on polyvinylidene fluori (PVDF) membranes (Bio-Rad Laboratories). Transblots were probed with the rabbit anti-Egr-1 (Cell Signaling technology Inc., 1:1000), rabbit anti-VEGF antibody (Santa Cruz Biotechnology, 1:400), rabbit anti-DCC antibody (Santa Cruz Biotechnology, 1:400), or rabbit anti-Netrin-1 antibody (Santa Cruz Biotechnology, 1:400) to examine the protein expression in the lysates. The amount of protein transferred onto the membranes was verified by immunoblotting for GAPDH (Santa Cruz Biotechnology, 1:500).

Immunoprecipitation

Equal amounts of homogenates (400 μg of protein) were incubated with affinity-purified anti-DCC antibody for 1 h and protein-G agarose at 4 °C for 12 h. The immunoprecipitates were suspended in a sample buffer and subjected to immunoblotting with the VEGF antibody. To determine the specificity of the bands found on the immunoblots, IgG (negative control) and anti-DCC antibody (positive control) were used as the immunoprecipitants.

Small interfering RNA

Small interfering RNA (siRNA) against rat DCC (NM_012841) and Egr-1 (NM_012551) mRNA was synthesized and purified by Qiagen (Hilden, Germany). The effects of 50-nM siRNA were compared with scrambled RNA (negative control, Qiagen). Briefly, 10-nM siRNA or control RNA were mixed with 6 μL of oligofectamine in OptiMem medium (Invitrogen Life Technologies) and incubated with cells grown in six-well plates for 24 h and then switched to growth medium and incubated for another 24 h.

EMSA

Electrophoretic mobility shift assays (EMSAs) were performed as described before [21, 22], by the Light-shift Chemiluminescent EMSA Kit (Pierce Chemical Co., Rockford, IL). A synthetic DNA double-stranded oligonucleotide probe (5′-biotin-CCTGACAGCACACAG GCTGAC-5′) containing the sequence of the rat DCC gene promoter between nucleotides -101 and -91 bp (5′-CCAGCTCGCA-3′) was labeled with biotin and incubated with the nuclear extracts. Rabbit anti-Egr-1 (Cell Signaling technology Inc.) was used for supershift experiments. A chemiluminescent detection method with a luminal/enhancer solution and stable peroxide solution (Pierce Chemical Co., Rockford, IL) was used, as described by the manufacturer, and the membranes were exposed to X-ray films for 30 s to 5 min before development.
Statistical analysis
The data are expressed as mean ± SEM. Comparison within groups was made by t test or one-way ANOVA for repeated measures. A value of $P < 0.05$ was considered significant.

Results

Angiogenesis in cartilage of OA rat
To check the angiogenesis in OA rat, the microvessel density in epiphysis tissues of the proximal epiphysis of the tibia was quantified via CD34 staining, and found microvessel density significantly increased in OA cartilage tissue (Fig. 1a, b). The data showed that VEGF expression was increased in cartilage tissue from OA rats (Fig. 1c), indicating that proliferation and migration of endothelial cell were promoted in OA. Moreover, the chondrocytes were disorganized, and cartilage degeneration was increasing in OA rats (Fig. 1d). The TNF-α and IL-1β levels in articular cavity fluid were higher in the OA rats than in the sham (Fig. 1e). These data suggest that angiogenesis could contribute to the pathogenesis of OA.

DCC regulates the angiogenesis in OA via VEGF
Previous study demonstrated that NT-1 and its receptor, DCC, stimulate growth of umbilical vein endothelial cells and vascular smooth muscle [23]. However, the role of NT-1/DCC in angiogenesis of osteoarthritic cartilage is still unclear. Thus, our studies checked the expression of NT-1 and DCC in cartilage and found that the protein expression of NT-1 and DCC were increased in osteoarthritic cartilage (Fig. 2a, b).

Besides, VEGF protein expression was significantly higher in the OA rats than in the sham, while the DCC siRNA decreased the increasing VEGF expression (Fig. 2c), indicating DCC was involved in the positive regulation of osteoarthritic angiogenesis by VEGF.

An additional study found a co-immunoprecipitation (Fig. 2d) between DCC and VEGF; the co-immunoprecipitation of DCC and VEGF was high in OA rats than in sham rats, which could be a factor in the increased expression of VEGF in OA rats.

The binding of Egr-1 at DCC promoter regulates the angiogenesis
To investigate whether or not Egr-1 was involved in the regulation of DCC on angiogenesis, we measured the Egr-1 expression in cartilage firstly. Egr-1 protein expression was higher in OA rats than in sham rats (Fig. 3a). The Egr-1 siRNA decreased the increasing DCC expression in osteoarthritic cartilage (Fig. 3b) and decreased the NT-1-induced VEGF expression (Fig. 3c).

![Fig. 1](image.png)
Angiogenesis in cartilage of OA rat. a, b Immunofluorescence staining of CD34. CD34 immunofluorescence showed the stained microvessels. The number of new blood vessels visualized by CD34 + vascular endothelial cells was counted per high-power field (× 400). (*$P = 0.0004$ vs. sham, $n = 5$, scale bar = 20 μm). c, d Immunohistochemical staining of VEGF. The severity of proteoglycan depletion in OA cartilage was demonstrated by safranin-O staining. e The levels of IL-1β and TNF-α were measured by ELISA (*$P = 0.0001$ vs. others, $n = 5$).
To identify the hypothesis that Egr-1 is a regulator of DCC promoter activity, we measured Egr-1 binding to the DCC promoter and found it higher in OA rats than in sham rats (Fig. 3d), indicating that the regulation of DCC by Egr-1 occurred at transcriptional level.

Discussion

The balance between angiogenic and anti-angiogenic factors is an important regulator of cartilage homeostasis [24–26]. And healthy adult human articular cartilage is avascular, and blood vessels could penetrate newly formed cartilage at the joint margins in OA and increase osteophyte formation, which may contribute to symptoms and joint damage [5, 27, 28]. In the present study, we found that the endothelial cell proliferation, vascular density, and VEGF abundance were increased in osteoarthritic cartilage which lead to the cartilage degeneration. Besides the stimulation of angiogenesis, VEGF may also contribute to inflammation [29, 30]. Indeed, our data show a significant upregulation in osteoarthritic articular cavity fluid of cytokines such as TNF-α and IL-1β. The pro-inflammatory factors are the major mediators of the catabolism of OA cartilage and stimulator for osteoarthritic chondrocyte [31–33]. Thus, VEGF seems to induce osteoarthritis via angiogenesis and inflammatory. And whether or not NT-1/DCC and VEGF interact in regulating angiogenesis of OA cartilage is not known.

Further studies identified the increasing expression of NT-1/DCC in osteoarthritic cartilage and increasing co-immunoprecipitation between DCC and VEGF. Meanwhile, the DCC siRNA decreased the increasing VEGF expression in OA. These data indicated DCC was involved in the positive regulation of osteoarthritic angiogenesis by VEGF directly in OA. The mechanisms of DCC in the regulation of VEGF are still unclear. Previous studies found that DCC, on NT-1 binding, activates the extracellular signal-regulated kinase-1/2 (ERK-1/2) and mitogen-activated protein kinase (MAPK) [34, 35]. And DCC was also shown to activate the ERK-1/2-eNOS pathway [36]. It was reported earlier that NO mediates VEGF-induced angiogenesis [37]. MAPK/ERK signaling pathway also plays an important role in the regulation of VEGF [38, 39]. We infer that the DCC
could upregulate VEGF via MAPK/ERK or eNOS/NO signaling pathway. However, more data from further studies are necessary to support the hypothesis.

As an active transcription factor, Egr-1 regulates promoter activity of various proteins. We believe the Egr-1 could be an upstream regulator of DCC/VEGF pathway. The expression of Egr-1 in OA cartilage has been controversial. Despite Wang et al. found the Egr-1 expression in OA cartilage is decreased [40], we demonstrated that Egr-1 protein expression was higher in OA rats than in sham rats. This is consistent with previous studies showing that Egr-1 expression increases in OA cartilage [17]. The Egr-1-mediated regulation of DCC expression might be complicated, as in our present study, we found that Egr-1 siRNA decreased the increasing DCC expression in osteoarthritic cartilage and NT-1-induced VEGF expression. And our data uncover a possible mechanism: The activity of Egr-1, a regulator of DCC promoter activity, is increased, accompanied by an increase in its binding to the DCC promoter in OA cartilage. And the pathway leading to the higher binding of Egr-1 with DCC promoter is not known, which needs to be elucidated in the future.

The current study has several limitations. First, chromatin immunoprecipitation (ChIP) may be used to identify the increased binding of Egr-1 to the DCC promoter in OA cartilage, rather than EMSA. Second, while our present study focused on the role of Egr-1/DCC pathway, whether or not Egr-1 or DCC could be a therapy target is another topic of future study.

**Conclusion**

In conclusion, the present study reinforces the role of Egr-1 in OA and shows that an increased expression of Egr-1 increases cartilage DCC expression, which may be involved in the abnormalities of angiogenesis in OA. The results imply that Egr-1 may be an effective therapeutic target for OA.

**Abbreviations**

ACLT: Anterior cruciate ligament transection; DCC: Deleted in Colorectal Cancer; Egr-1: Early growth response-1; ERK-1/2: Extracellular signal-regulated kinase-1/2; MAPK: Mitogen-activated protein kinase; MVD: Determining microvessel density; NT-1: Netrin-1; OA: Osteoarthritis; PVDF: Polyvinylidene fluoride; VEGF: Vascular endothelial growth factor

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Availability of data and materials

All data and materials were in full compliance with the journal’s policy.

Authors’ contributions

JS and DL designed, directed, and carried out the experiments; analyzed the data; and wrote the manuscript. XK and YC carried out the experiments and analyzed the data. JX provided important advice on the experimental design. WZ designed the experiments, directed the project, and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval

Ethical approval was obtained by the Institutional Animal Care and Use Committee at Chengdu Military General Hospital. Mice were maintained in an enclosed, pathogen-free facility, and experiments were performed in accordance with Chengdu Military General Hospital Animal Care Committee regulations. All experiments conformed to the guidelines of the ethical use of animals, and all efforts were made to minimize animal suffering and to reduce the number of animals used.

Competing interests

The authors declare that they have no competing interests.

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