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Original Article

Soluble Flt-1 improves the repair of ankle joint injury in rats

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Abstract: The ankle injuries create great pain to a great number of patients worldwide. Past studies have focused on the development of practical treatments to relieve pain and improve recovery, but the molecular mechanisms underlying the ankle injuries, especially the local inflammation in the damaged ankle joint, have been rarely studied. Moreover, although reduction of production and secretion of pro-inflammatory cytokines may reduce the pain and promote the recovery, a practical approach is currently lacking. Here, we detected significantly higher levels of placental growth factor (PLGF) and pro-inflammatory cytokines in the joint fluid from the patients of acute ankle joint injury (AAJI). Interestingly, the levels of PLGF and pro-inflammatory cytokines in the joint fluid strongly correlated. In order to examine whether PLGF may regulate the production and secretion of pro-inflammatory cytokines in the injured joint, we used a rat carrageenan-induced ankle injury model for AAJI in humans. We injected soluble Flt-1 (sFlt-1) into the articular cavity of the injured ankle joint to block PLGF signaling and found that injection of sFlt-1 significantly improved the rat behavior in activity wheels test, which appeared to result from reduced secretion of the pro-inflammatory cytokines in the ankle joint. Thus, our study suggests that blocking PLGF signaling may be a novel therapeutic approach for treating AAJI in humans.

Keywords: Soluble Flt-1 (sFlt-1), acute ankle joint injury (AAJI), inflammation, placental growth factor (PLGF)

Introduction

Ankle injuries often result from sports injuries, and can cause a painful, debilitating sprain [1]. The ankle injuries include sprains, ligament and bone fractures and joint damage, which create great pain to the patients [2]. Past studies have focused on development of practical treatments to relieve pain and to improve recovery [3], but the molecular mechanisms underlying the ankle injuries, especially the inflammatory events in the damaged ankle joint, have been rarely studied.

Animal models have been used to study ankle joint injuries. However, testing manipulation in animal models appears to be difficult due to the association of endpoint with a number of osteopathic treatments and the lack of objective methods to measure relevant parameters [4, 5]. Cobos et al. has developed a method called activity wheels to measure locomotion of the animals for evaluation of the pain after ankle joint injury and as an indicator for recovery [6]. This relatively inexpensive method is able to yield objective and recordable endpoints, and was thus chosen to be applied in the current study.

Although the mechanisms underlying the effects of inflammation on the symptoms and recovery of an injured ankle joint remain incompletely elucidated, the involvement of inflammatory cells and their production and secretion of a set of pro-inflammatory cytokines, e.g. Interleukin 6 (IL-6), tumor necrosis factor α (TNFα) and Interferon ɣ (IFNɣ), have been acknowledged [7-10]. Reduction of production and secretion of these pro-inflammatory cytokines may reduce the pain and promote the recovery [11], while a practical approach is currently lacking.

Vascular endothelial growth factor (VEGF) family has six secreted proteins: placental growth factor (PLGF), and VEGF-A~E [12-16], among which VEGF-A is most potent for inducing endothelial cell proliferation and increasing endothe-

lial cell permeability [17-20]. On the other hand, PLGF appears to regulate pathological vessel formation [21, 22]. VEGF receptor I (VEGFR1) is the only receptor for PLGF. Soluble fms-like tyrosine kinase-1 (sFlt-1) is secretory, and function as a decoy receptor for VEGF ligands to compete with VEGF receptors in target cells [23]. However, the application of sFlt-1 in treating ankle injury has not been reported before.

Here, we detected significantly higher levels of PLGF and pro-inflammatory cytokines in the joint fluid from the patients of acute ankle joint injury (AAJI). Interestingly, the levels of PLGF and pro-inflammatory cytokines in the injured joint fluid strong correlated. In order to examine whether PLGF may regulate the production and secretion of pro-inflammatory cytokines in the joint, we used a rat carrageenan-induced ankle injury (CIA) model for AAJI in humans. We injected sFlt-1 into the articular cavity of the injured ankle joint to block PLGF signaling and found that injection of sFlt-1 significantly improved the rat behavior in activity wheels test, which appeared to result from reduced secretion of the pro-inflammatory cytokines in the injured ankle joint.

Materials and methods

Animals

All experiments were approved by the local Animal Care and Use Committee and were carried out in accordance with guidelines from the Care and Use of Laboratory Animals, issued by Shenyang Military Region General Hospital, and proved by the research committee of Shenyang Military Region General Hospital. Male Sprague Dawley rats (250-350 g, Jackson Laboratories, Bar Harbor, ME, USA) were housed on a 12-hour light cycle in polypropylene cages with cedar bedding and were fed with Laboratory Rodent Diet (Jackson Laboratories). On arriving at the animal facility, rats were allowed to acclimatize 2 days to 2 weeks before being placed in polycarbonate cages that contained activity wheels connected to digital display counters to analyze running (Thermo Fisher Scientific, Rockford, IL, USA). Rats were singly housed in wheel cages. Opaque barriers were placed between cages to minimize the influence of an adjacent rat's activity on each rat's running behavior. Because virtually all running occurs during the dark cycle, 14 wheel revolutions were recorded daily within 4 hours after the beginning of the light cycle for the convenience of the investigators, as very little running occurred after the end of the dark cycle. Each wheel revolution measured 1 meter.

Induction of carrageenan-induced ankle injury model and sFlt-1 injection

To induce inflammatory injury, rats were anesthetized with 2% to 5% isoflurane mixed with 95% O_2/5% CO_2 and placed in the left lateral recumbent position. With the right foot in neutral position, the junction of the tibia and fibula just superior to the talus was identified. At this location, a 0.5-inch, 27-gauge needle was advanced in a posterior medial direction through the skin and subcutaneous tissue and into the ankle joint capsule. After the needle entered the joint space, 50 µl 3% carrageenan (Sigma Aldrich, St. Louis, MO, USA) in 0.9% saline (pH 7.4) or saline alone (as a control; CTL) was injected.

Intra-articular injection of sFlt-1

Rats were anesthetized. The right ankle joint of the rats was punctured once with a 27-gauge needle below the patella to induce a hemorrhage. Subsequently a 50 µl solution, containing 1 mg recombinant rat sFlt-1 (R&D systems, Minneapolis, MN, USA) or vehicle only (0.9% saline (pH 7.4)), was injected through the needle in rats for each treatment. All intra-articular injections were performed between 6 am and 8 am.

Histology

The right hind paws from the rats were fixed in a 10% formalin solution, decalcified in 15% ethylenediaminetetraacetic acid-PBS, and embedded in 4% paraffin for 4 hours for histological analysis. The fixed tissue was then cut into 5 µm sections, mounted on glass slides, and stained with hematoxylin and eosin (H&E) using a H&E staining kit (Sigma-Aldrich).

Western blot

The protein was extracted from the ankle joint tissue and homogenized in RIPA lysis buffer (1% NP40, 0.1% SDS, 100 µg/ml phenylmethylsulfonyl fluoride, 0.5% sodium deoxycholate, in

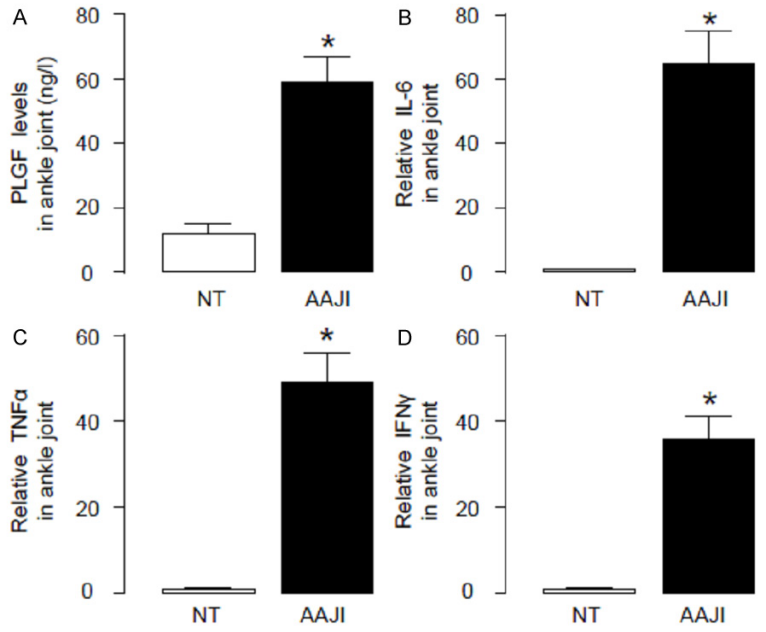

Figure 1. High PLGF and pro-inflammatory cytokines are detected in ankle joint from AAJI patients. We randomly chose 20 AAJI patients with ankle joint injury at one side for this study. The ankle joint fluid was taken from both ankle joints of the patients and analyzed for the levels of PLGF and pro-inflammatory cytokines, IL-6, TNFα and IFNɣ. The uninjured site was used a control (NT). (A-D) ELISA for the levels of PLGF (A), IL-6 (B), TNFα (C) and IFNɣ (D) in the joint fluid from the injured ankle joint of the AAJI patients, compared to NT. N=20. *P<0.05.

PBS) on ice. The supernatants were collected after centrifugation at 12000×g at 4°C for 20 min. Protein concentration was determined using a BCA protein assay kit (Bio-rad, China), and whole lysates were mixed with 4×SDS loading buffer (125 mmol/l Tris-HCl, 4% SDS, 20% glycerol, 100 mmol/l DTT, and 0.2% bromophenol blue) at a ratio of 1:3. Protein samples were heated at 100°C for 5 min and were separated on SDS-polyacrylamide gels. The separated proteins were then transferred to a PVDF membrane. The membrane blots were first probed with a primary antibody. After incubation with horseradish peroxidase-conjugated second antibody, autoradiograms were prepared using the enhanced chemiluminescent system to visualize the protein antigen. The signals were recorded using X-ray film. Primary antibodies for Western Blot are rat anti-phosphorylated VEGFR1 (pVEGFR1), anti-VEGFR1 (detect total VEGFR1 protein) and anti-GAPDH (Cell Signaling, San Jose, CA, USA). The secondary antibody was HRP-conjugated anti-rabbit (Jackson ImmunoResearch Labs, West Grove, PA, USA). Images shown in the figures were representative of 5 individuals.

ELISA

ELISA was performed, using a human PLGF, human or rat tumor necrosis factor α (TNFα), human or rat interleukin 6 (IL-6), and human or rat interferon ɣ (IFNɣ) ELISA Kit (R&D System, Los Angeles, CA, USA). Briefly, the protein solution was added to a well pre-coated with polyclonal antibody, and then immunosorbented by biotinylated monoclonal anti-human antibody at room temperature for 2 hours. The color development catalyzed by horseradish peroxidase was terminated with 2.5 mol/l sulfuric acid and the absorption was measured at 450 nm. The protein concentration was determined by comparing the relative absorbance of the samples with the standards.

Statistical analysis

All statistical analyses were carried out using GraphPad prism 6.0 (GraphPad Software Inc. La Jolla, CA, USA). All values are depicted as mean ± SD and are considered significant if $P<0.05$. All data were statistically analyzed using one-way ANOVA with a Bonferroni correction, followed by Fisher's Exact Test for comparison of two groups. Bivariate correlations were calculated by Spearman's Rank Correlation Coefficients.

Results

High PLGF and pro-inflammatory cytokines are detected in ankle joint from AAJI patients

Here, we aimed to study the mechanisms underlying the regulation of inflammation in the ankle joint after AAJI. Thus, we randomly chose 20 AAJI patients with ankle joint injury at one side for this study. Since PLGF has recently shown to regulate pathological inflammation and angiogenesis, the ankle joint fluid was taken from both ankle joints of the patients and analyzed for the levels of PLGF and pro-inflammatory cytokines, IL-6, TNFα and

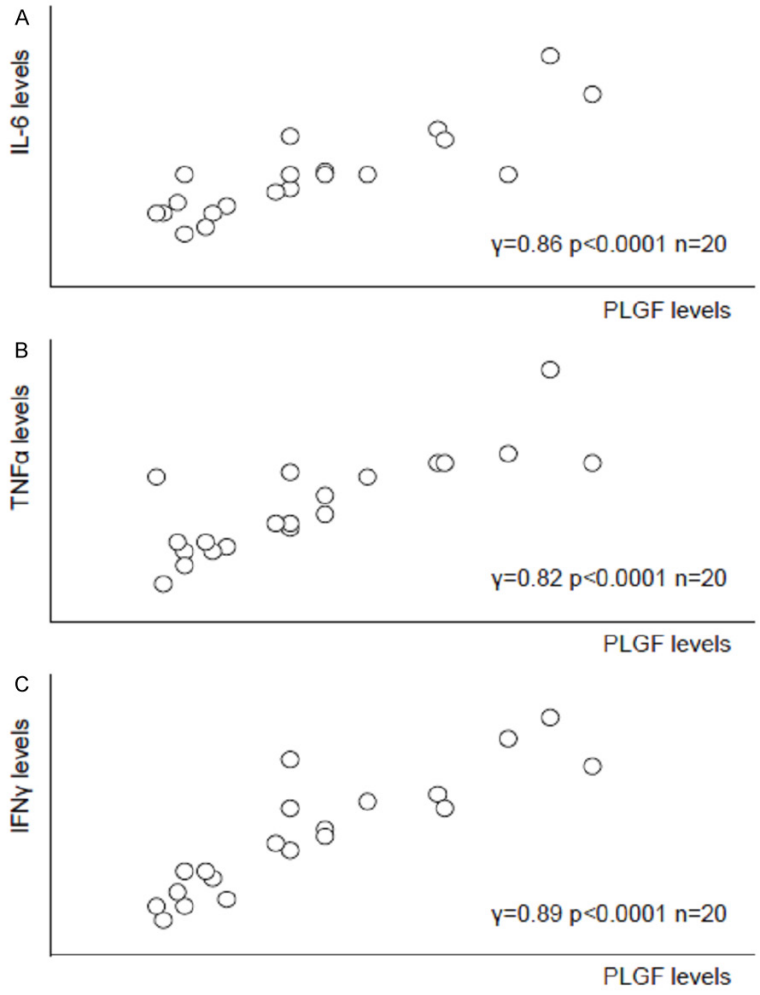

Figure 2. PLGF correlates with IL-6, TNFα and IFNɣ levels in the injured ankle joint from AAJI patients. A: We detected a strong correlation between PLGF and IL-6 in the joint fluid from the injured ankle joint of the AAJI patients (ɣ=0.86, P<0.0001, N=20). B: We detected a strong correlation between PLGF and TNFα in the joint fluid from the injured ankle joint of the AAJI patients (ɣ=0.82, P<0.0001, N=20). C: We detected a strong correlation between PLGF and IFNɣ in the joint fluid from the injured ankle joint of the AAJI patients (ɣ=0.89, P<0.0001, N=20).

IFNɣ. The uninjured site was used a control (NT). Interestingly, we found that the levels of PLGF (**Figure 1A**) and 3 pro-inflammatory cytokines [IL-6 (**Figure 1B**), TNFα (**Figure 1C**) and IFNɣ (**Figure 1D**)] all significantly increased in the joint fluid from the injured ankle joint of the AAJI patients, compared to NT.

PLGF correlates with IL-6, TNFα and IFNɣ levels in the injured ankle joint from AAJI patients

Next, we examined the relationship between PLGF and pro-inflammatory cytokines, IL-6, TNFα and IFNɣ. We detected a strong correlation between PLGF and IL-6 in the joint fluid from the injured ankle joint of the AAJI patients (**Figure 2A**, ɣ=0.86, P<0.0001, N=20). Moreover, we also detected a strong correlation between PLGF and TNFα in the joint fluid from the injured ankle joint of the AAJI patients (**Figure 2B**, ɣ=0.82, P<0.0001, N=20). Further, we detected a strong correlation between PLGF and IFNɣ in the joint fluid from the injured ankle joint of the AAJI patients (**Figure 2C**, ɣ=0.89, P<0.0001, N=20). These data suggest presence of a regulatory relationship between PLGF and these pro-inflammatory cytokines in the injured ankle joints.

Suppression of PLGF signaling alleviates severity of ankle joint inflammation in rats

In order to examine whether PLGF may regulate the production and secretion of pro-inflammatory cytokines in the injured ankle joint, we used a rat carrageenan-induced ankle injury model for AAJI in humans. Since sFLT-1 compete with VEGFR1 for binding with PLGF, we injected sFlt-1 every other day, since the day of the injection of carrageenan, into the articular cavity of the ankle joint to block PLGF signaling (**Figure 3A**). We found that while carrageenan significantly increased the hindlimb volume (**Figure 3B**) and diameter (**Figure 3C**), injection of sFlt-1 significantly reduced the increases in hindlimb volume (**Figure 3B**) and diameter (**Figure 3C**) by carrageenan. Thus, suppression of PLGF signaling alleviates severity of ankle joint inflammation in rats.

Suppression of PLGF signaling improves rat behavior in activity wheels test

In order to evaluate the effects of suppression of PLGF signaling on pain in rats with injured ankle joint, the rat behavior in activity wheels

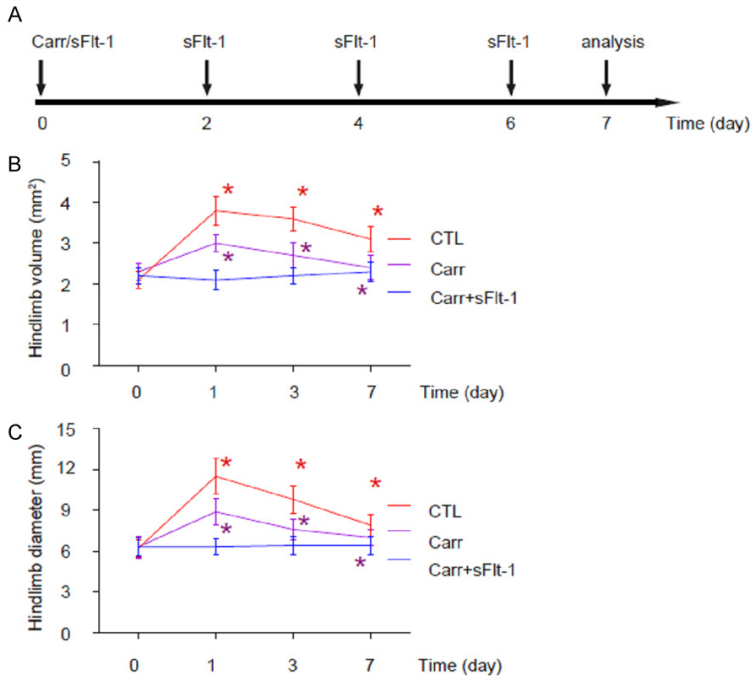

Figure 3. Suppression of PLGF signaling alleviates severity of ankle joint inflammation in rats. (A) A rat carrageenan-induced ankle injury model for AAJI in humans. We injected sFlt-1 every other day, since the day of the injection of carrageenan, into the articular cavity of the ankle joint to block PLGF signaling. At day7, rats were analyzed. CTL: no Carr injection. (B, C) The hindlimb volume (B) and diameter (C) in rats that received carrageenan (Carr), and Carr and sFlt-1 (Carr+sFlt-1), compared to CTL. N=10. *(red) P<0.05 (Carr vs CTL). *(purple) P<0.05 (Carr+sFlt-1 vs Carr).

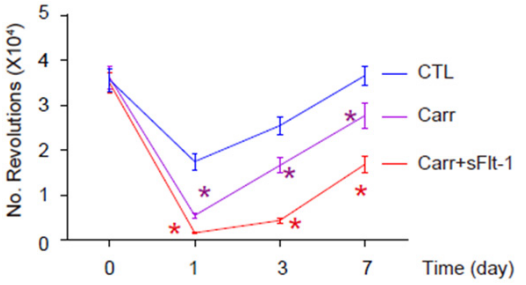

Figure 4. Suppression of PLGF signaling improves rat behavior in activity wheels test. In order to evaluate the effects of suppression of PLGF signaling on pain in rats with injured ankle joint, the rat behavior in activity wheels test was performed. The number of revolutions in rats was analyzed in rats that received carrageenan (Carr), and Carr and sFlt-1 (Carr+sFlt-1), compared to control (CTL, no Carr injection). N=10. *(red) P<0.05 (Carr vs CTL). *(purple) P<0.05 (Carr+sFlt-1 vs Carr).

test was performed. We found that while carrageenan significantly reduced the number of revolutions in rats, injection of sFlt-1 significantly attenuated the reduction in revolution number by carrageenan (**Figure 4**). Hence, suppression of PLGF signaling improves rat behavior in activity wheels test.

Suppression of PLGF signaling reduce pro-inflammatory cytokines in the injured ankle joint by carrageenan in rats

Finally, we studied the underlying mechanisms. The histology of the injured ankle joints showed significant increases in local inflammation, compared to CTL, while sFLT-1 reduced the carrageenan-induced inflammatory (**Figure 5A**). Phosphorylation of VEGFR1 was analyzed by Western blot, showing that VEGFR1 phosphorylated was induced in the injured joint by carrageenan, but was attenuated by sFLT-1 treatment (**Figure 5B**). Moreover, carrageenan significantly increased the levels of IL-6 (**Figure 5C**), TNFα (**Figure 5D**) and IFNɣ (**Figure 5E**), which were significantly attenuated by sFlt-1 treatment (**Figure 5C-E**), possibly as a basis of reduction of ankle joint inflammation. Thus, our study suggests that blocking PLGF signaling may be a novel therapeutic approach for treating AAJI in humans.

Discussion

AAJI is a high-incident, inflammatory disease that affects numerous people worldwide. So far, most treatments have been developed to reduce pain, while a specific strategy that my also control local inflammation is practically lacking. PLGF has recently been shown to regulate pathological angiogenesis and inflammation in various diseases [24-33]. For example, Zhou et al. has nicely shown that PLGF regulates expression of matrix metalloproteinase 3 (MMP3) via ERK/MAPK signaling pathway in larynx carcinoma. Moreover, MMP9 upregulated in larynx carcinoma and the source of MMP9 was mainly M2 macrophages. PLGF in

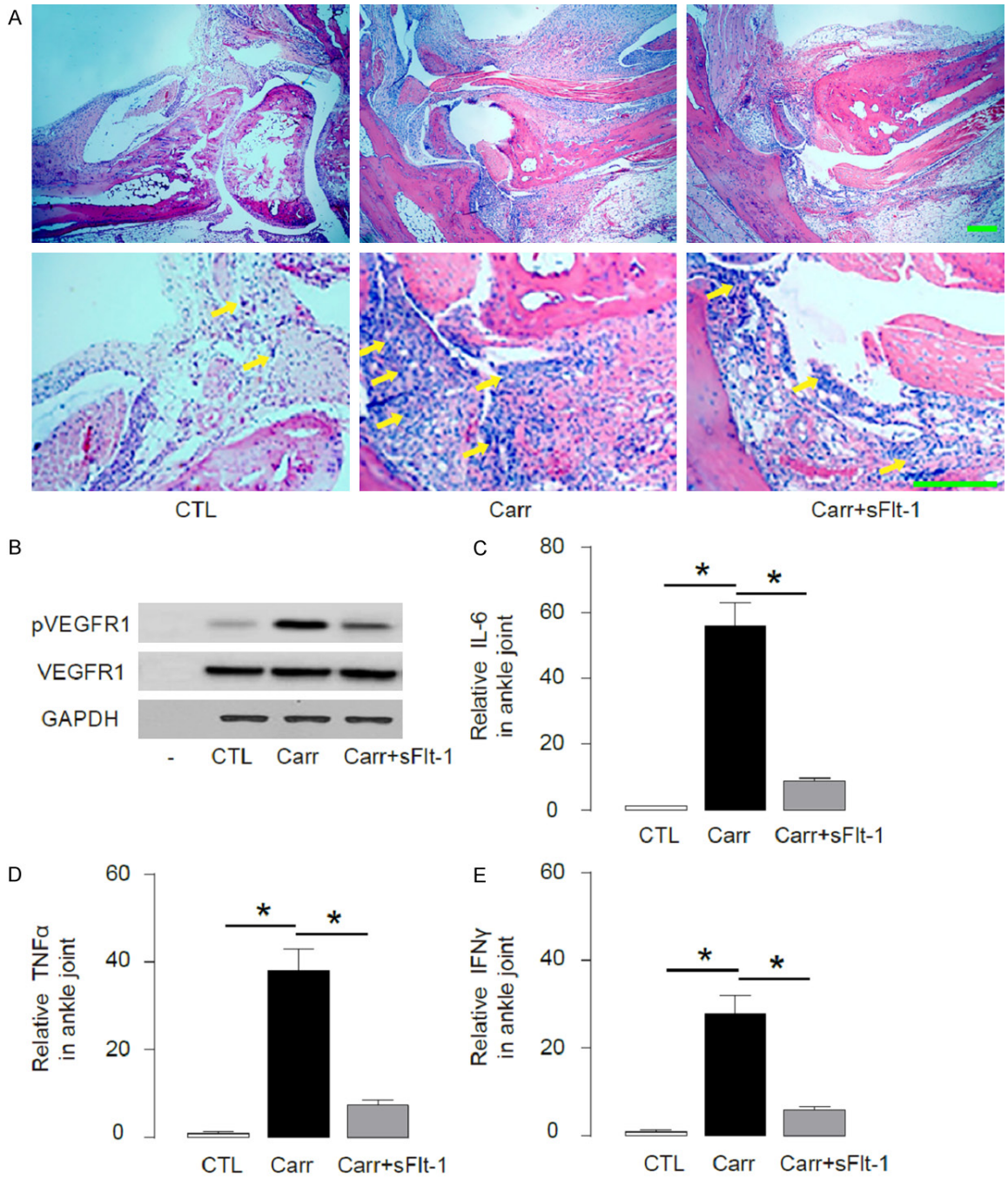

Figure 5. Suppression of PLGF signaling reduce pro-inflammatory cytokines in the injured ankle joint by carrageenan in rats. (A) The histology of the injured ankle joints showed significant increases in local inflammation, compared to CTL, while sFLT-1 reduced the carrageenan-induced inflammatory. The Yellow arrows point to inflammatory cells, which appeared to be stained strongly for hematoxylin. CTL: no Carr injection. Carr: rats that received carrageenan. Carr+sFlt-1: rats that received both Carr and sFlt-1. (B) Phosphorylation of VEGFR1 was analyzed by Western blot, showing that VEGFR1 phosphorylated was induced in the injured joint by carrageenan, but was attenuated by sFLT-1 treatment. (-) negative control of Western blot, in which no protein was loaded. (C-E) The levels of IL-6 (C), TNFα (D) and IFNɣ (E) in the injured ankle joint were analyzed by ELISA. N=10. *P<0.05. Scale bars are 100 μm.

larynx carcinoma cells induced macrophage polarization in vivo and in vitro, and significantly promoted the growth of larynx carcinoma in mice [24, 28].

The application of sFlt-1 has recently been used to suppress angiogenesis and tumor metastases [34-42]. For example, Zhang et al. has shown that a Chinese medicine significant-

ly inhibited hepatic metastasis of cancer cells after removal of primary cancer. In addition, the soluble Flt-1 in the medicine neutralized the secreted PLGF to inhibit the cancer neovascularization promoted growth of metastatic cancer [23]. This study encouraged us to use sFlt-1 to block PLGF signaling in rat AAJI model.

Here, we first showed significantly higher levels of PLGF and pro-inflammatory cytokines in the joint fluid from the patients of AAJI. Interestingly, the levels of PLGF and pro-inflammatory cytokines in the joint fluid strong correlated. These clinical data strongly support our hypothesis, and are bases for our following studies using rat model.

In order to examine whether PLGF may regulate the production and secretion of pro-inflammatory cytokines in the joint, we used a rat carrageenan-induced ankle injury model for AAJI in humans. This model is easy and economic, and has been shown to be a reliable rat model for human AAJI. We injected sFlt-1 into the articular cavity of the ankle joint to block PLGF signaling, since we injected carrageenan. We did not wait for one day to start sFlt-1 since the inflammation was induced immediately after carrageenan treatment and our experimental period was only 7 days which may result in failure to detect the effects of sFlt-1 if applied late. We found that injection of sFlt-1 significantly improved the rat behavior in activity wheels test, which appeared to result from reduced the secretion of the pro-inflammatory cytokines in the ankle joint.

To summarize, here we provide compelling data to highlight PLGF signaling as a regulator of ankle joint inflammation after injury. Thus, blocking PLGF signaling may be a novel therapeutic approach for treating AAJI in humans.

Disclosure of conflict of interest

None.

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