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Fibroblast Growth Factor-2 Determines Severity of Joint Disease in Adjuvant-Induced Arthritis in Rats

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Rheumatoid arthritis (RA) is a chronic disease of unknown etiology. It affects systemic organs, RA is basically a severe form of progressive synovitis in peripheral joints, and destruction and ankylosis follow. Histopathologically, synovial tissue shows papillary projection and consists of hyperplastic and hypertrophic synovial cells and a vascular-rich supportive layer infiltrated by monocellular cells and neutrophils and proliferation of histiocytes (1–4). Later in the course of the disease, inflamed synovial and granulation tissue (so-called pannus formation) erodes and destroys the articular surface, and invades subchondral bone tissue partly by osteoclast-type giant cell-induced bone absorption (2, 3, 5–8).

Angiogenesis, the formation of new vessels, may also critically affect the disease progression in RA (9–12). Various angiogenic factors, including vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs), have been noted in synovial tissue or the joint fluid of human RA, suggesting that these angiogenic growth factors may contribute to disease initiation/progression of RA (12–21). FGF-2 is a member of the family of heparin-binding growth factors, which show specifically enhanced expression in RA synovial fluid, compared with that in osteoarthritis in human subjects (13). In addition, FGF-2 stimulates angiogenic events partly by up-regulation of VEGF (22), and FGF-2 directly accelerates osteoclast maturation to promote bone resorption, as well as osteoclastogenesis in murine bone marrow cultures (23–26), suggesting its disease-modulatory role in the progression of RA. However, information regarding direct evidence for the role of FGF-2 in RA in vivo is scanty.

To determine the exact role of overexpression of FGF-2 in the RA joint in vivo, we conducted in vivo gene transfer of FGF-2 using a recombinant Sendai virus vector (SeV), which has shown effective transfer potential to various organs (27, 28). We show in this study that the endogenous FGF-2 level is elevated in rat joints of adjuvant-induced arthritis (AIA), as well as in human RA fluid. Furthermore, the SeV-mediated overexpression of FGF-2 enhanced, and inversely, inhibition of endogenous FGF-2 via administration of neutralizing Ab attenuated the disease severity with regard to joint swelling and destruction in AIA. To our knowledge, this is the first direct in vivo evidence indicating the disease-modulatory role of FGF-2 in AIA, and we conclude that FGF-2 deserves further attention as a possible therapeutic target in care of human RA.

Materials and Methods

Human synovial fluid samples

Synovial fluid was obtained from knee joints of 12 RA Japanese patients with severe joint edema, who were under the care of Showa Central Hospital (Kitakyushu, Japan). The diagnosis of RA was based on the American Rheumatism Association (1987) criteria for RA (29). All the patients were classified grade IV or V in accordance with Larsen and Toen classification in terms of the degree of knee joint destruction seen on plain x-ray photographs (30). Synovial fluids were aspirated under aseptic conditions using 18-gauge needles, and were treated with or without 25% hyaluronidase...
for 1 h at 37°C before measurements. Quantitation of human FGF-2 and human VEGF proteins was done by ELISA using commercially available systems (R&D Systems, Minneapolis, MN). The lower limit of sensitivity of the systems was consistently ~3 pg/ml. Written informed consent for the study was obtained from all the patients.

**Rat model of AIA**

One hundred and twenty-four inbred male Lewis rats, which were Charles-River grade (8 wk of age; purchased from KBT Oriental, Tosu, Saga, Japan), were used. Eighty-two rats were immunized s.c. at the base of the tail with 1 mg *Mycobacterium Butyricum* desiccated (MBD; Difco, Detroit, MI) dissolved in 100 μl mineral oil (NACALAI TESQUE, Kyoto, Japan) on day 0 to prepare the AIA model (31). The AIA model of AIA was approximately 10 days after the adjuvant treatment. These experiments were reviewed by the Committee of the Ethics on Animal Experiment in Faculty of Medicine, Kyushu University, and conducted under the control of the Guideline for Animal Experiment in Faculty of Medicine, Kyushu University, and The Law (number 105) and Notification (number 6) of the Government.

**Recombinant SeVs**

Recombinant SeVs were constructed, as described (32, 33). In brief, 18 bp of spacer sequence 5′(G)-GGGCGGCGAGATCTTCACG-3′ with a NotI restriction site were inserted between the 5′ untranslated region and the initiation codon of the nucleoprotein (N) gene. This cloned SeV genome also contains a self-cleaving ribosome site from the antigenomic strand of the hepatitis B virus. The entire DNA-coding *Escherichia coli lacZ* with a nuclear localizing signal (for SeV-NLS-lacZ), luciferase (for SeV-luciferase), and murine FGF-2 (for SeV-FGF-2) were amplified by PCR, using primers with a NotI site and new sets of SeV E and S signal sequence tags for an exogenous gene, then inserted into the NotI site of the cloned genome. The entire length of the template SeV genomes, including exogenous genes, was arranged in multiples of six nucleotides (so-called “rule of six”) (34). Template SeV genomes with an exogenous gene and plasmids encoding N, P, and L proteins (plasmids pGEM-N, pGEM-P, and pGEM-L) were complexed with commercially available cationic lipids, then cotransfected with vaccinia virus vTF7-3 into CV-1 or L-Mullck cells. Forty hours later, the cells were disrupted by three cycles of freezing and thawing and injected into the chorioallantoic cavity of 10-day-old embryonated chicken eggs. Subsequently, the virus was recovered and the vaccinia virus was eliminated by a second propagation in eggs. Virus titers were determined using chicken RBCs in hemagglutination assay (35), and viruses were kept frozen at –80°C until use.

**SeV-mediated reporter gene transfer to rat joints**

Characterization of SeV-mediated gene transfer was determined in AIA ankle and knee joints, using SeVs encoding reporter genes (n = 8 joints for lacZ, and n = 36 joints for luciferase). The ankle joints were held at 20° of planter flexion, and a 30-gauge needle on a plastic syringe was inserted anterolaterally at the ankle joint line –1 mm distal to the lateral malleolus. The knee joints were held at 90° of flexion, and the needle was inserted at the femorotibial joint line –1 mm medial to the patellar tendon. Four rats for AIA were treated with 1 mg MBD on day 0, and injected intraarticularly with SeV-NLS-lacZ (5 × 10^6 PFU) into the ankle and the knee joints 7 days after the adjuvant treatment. Two days later, each joint was dissected out on bloc, incised transversally to expose the joint space. These en bloc were incubated with the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) for 3 h at room temperature (36–38). All samples were photographed using a stereoscopic dissecting microscope (Carl Zeiss, Oberkochen, Germany).

Sixteen rats were intraarticularly injected with 5 × 10^6 PFU of SeV-luciferase into bilateral ankle joints, and eight, four, and four rats were killed on 2, 7, and 14 days after the virus injection, respectively. Two naïve rats, i.e., four ankle joints were prepared for negative control. Each ankle joint was dissected out and incised transversally to expose the joint space, and incubated with 500 μl 1× cell culture lysis buffer (Promega, Madison, WI) for 5 min. Twenty microliters of the supernatant were exposed to 100 μl luciferase assay buffer (Promega), and the luciferase activity was measured using a luminometer (model LB9507; EG&G Berthold, Bad Wildbad, Germany). Data were expressed as relative light units/mg protein. Protein concentrations were determined by Bradford’s method with a commercially available protein assay system (Bio-Rad, Hertfordshire, U.K.).

**Measurement of FGF-2 and VEGF in rat joint extracts**

Twenty-four rats were immunized with 1 mg MBD on day 0 for AIA rats. Among them, 12 rats were intraarticularly injected with 5 × 10^5 PFU of SeV-FGF-2 into bilateral ankle joints for AIA + FGF-2 group; the others were injected with 50 μl PBS into bilateral ankle joints on day 7 for AIA. Each ankle joint was dissected out and incised transversally to expose the joint space, then incubated with 500 μl 1× cell culture lysis buffer (Promega) for 5 min on day 9. Protein levels of murine FGF-2 and VEGF were measured using commercially available ELISA systems (R&D Systems). Data were expressed as FGF-2 or VEGF protein ng/mg protein. The lower limit of sensitivity of the FGF-2 and VEGF ELISA was consistently ~3 pg/ml.

**Treatment protocol**

Experimental groups and experimental protocols for FGF-2 overexpression are summarized in Fig. 1. Rats were divided into two groups: control rats (C rats; n = 24) and AIA rats (n = 36). C rats were injected with 100 μl mineral oil at the base of the tail, and AIA rats were immunized with MBD on day 0. The C rats were further divided into two groups: C + FGF-2 group injected SeV-FGF-2 (n = 12) and C + luciferase group injected SeV-luciferase (n = 12). AIA rats were further separated into three groups: AIA group given PBS injection, AIA + FGF-2 group given SeV-FGF-2, and AIA + luciferase group given SeV-luciferase (eight joints for each group). Twelve rats of AIA group were killed on days 14, 21, and 28 (four rats, i.e., eight joints for each day). Twenty-four rats of AIA + FGF-2 and AIA + luciferase groups were intraarticularly injected with SeV-FGF-2 or SeV-luciferase into the right or left ankle joint, respectively, on day 7, and killed on days 14, 21, and 28 (eight rats for each day, i.e., eight joints for each group per each day).

**Clinical assessment of arthritis**

The extent of swelling in the hind paw was measured using a volume meter (MK-550; Muromachi Kikai, Tokyo, Japan) at each time point by two individual examiners (A. Yamashita and T. Iriya) in a blinded fashion. After macroscopic examinations, the hind paws were amputated at the middle of the leg and imaged on industrial x-ray film (Fuji, Tokyo, Japan) to evaluate bone and joint destruction radiologically (31), using Softex imaging equipment (CMB-2; Softex, Tokyo, Japan). The severity of bone and joint destruction was scored for each ankle joint, according to the Bariberi et al. (31, 39) method, but with some modifications. Briefly, based on soft tissue swelling, joint space narrowing, extent of bone atrophy (osteoporosis), erosion, and bone destruction due to osteolysis of each ankle joint was scored, using a scale of 0–4 termed the radiologic index.

**Histopathological assessment of arthritis**

Ankle joints were resected en bloc and fixed in 4% paraformaldehyde for 4 days. After decalcification with 10% EDTA for 14 days, the ankle joints were sectioned on the sagittal plane through the center of the joint line. Samples were embedded in paraffin, and 3-μm sections were prepared for H&E staining. Whole sections were histopathologically analyzed for the degree of synovitis, proliferation of synovial granulation tissue known as a pannus, and associated destruction of bone and cartilage. For an objective evaluation of synovitis, infiltrating granulocytes were counted at the adjacent synovium to talo-tibial joint in a high power field (×400) by two individual pathologists (A. Yamashita and Y. Yonemitsu) in a blinded fashion.

**FIGURE 1.** Experimental design for FGF-2 overexpression. Arrows indicate each time point for injection. C, Nonarthritis control. MBD, Injection of MBD dissolved in mineral oil. MO, Injection of mineral oil only as the nonarthritis control. PBS, As a mock transfection control. s, Time points of sacrifice. n, Number of ankle joints for this study at the time points.
**Immuno histochemical study**

To detect vascular endothelial cells, an immunohistochemical examination for rat von Willebrand factor (vWF) was done using dextran polymer conjugate two-step visualization, so-called the EnVision system (40). In brief, deparaffinized sections were digested by 0.1% trypsin for 30 min at 37°C and incubated with 10% normal goat serum for 10 min to minimize non-specific binding of the primary Ab, then were incubated with the primary rabbit anti-vWF (DAKO, A/S, Glostrup, Denmark) Ab overnight at 4°C in a moisture chamber. To inhibit endogenous peroxidase activity, the sections were incubated with 1% H2O2 in absolute methanol for 30 min. Thereafter, the sections were incubated with EnVision (EnVision+, rabbit/HRP, Dako) for 30 min. The EnVision was a polymeric conjugate consisting of a large number of secondary Abs (goat anti-rabbit) bound directly to a dextran backbone containing HRP. Visualization of a positive reaction was developed with a peroxidase substrate solution containing 0.02% (w/v) H2O2 and 0.1% (w/v) 3,3-diaminobenzidine tetrahydrochloride (Merck, Darmstadt, Germany) in PBS to give the reaction product a brown color, then the sections were lightly counterstained with hematoxylin. Small vessels consisting of vWF-positive endothelial cells were counted at three optional lesions for every ankle joint by two individual pathologists (A. Yamashita and Y. Yonemitsu) in a blinded fashion, and numbers of vessels in each ankle joint were expressed as the average value.

**Tartrate-resistant acid phosphatase stain for osteoclasts**

The tissue sections were further subjected to tartrate-resistant acid phosphatase (TRAP) staining. TRAP staining was done using a leukocyte acid phosphatase kit, a cell staining kit for the detection of TRAP (Sigma, St. Louis, MO). Multinucleated macrophages and mononuclear cells showing a positive reaction in TRAP staining were defined as osteoclasts or precursor cells (41). TRAP-positive osteoclasts and their precursor cells were counted at three optional pannus sites for every ankle joint by two individual pathologists (A. Yamashita and Y. Yonemitsu) in a blinded fashion, and the number of osteoclasts and precursor cells in each ankle joint were expressed as the average value.

**Administration of anti-FGF-2 Ab**

Eighteen rats were prepared to evaluate the suppressive effect of the FGF-2 neutralization in AIA, right ankle joints were used for AIA + anti-FGF-2 Ab group, and ipsilateral left ankle joints were used for AIA + nonimmunized IgG group, as the control. These 18 rats were treated on day 0 with MBD, as described above. Right ankle joints were intraarticulately injected with 50 μg anti-FGF-2 Ab (anti-FGF basic Ab; R&D Systems), while left ankle joints were also intraarticulately injected with 50 μg nonimmunized rabbit IgG on days 7, 10, 14, and 17, respectively. Thus, the total amount of administered anti-FGF-2 Ab and nonimmunized rabbit IgG was 200 μg/rat (Fig. 8A). Anti-FGF-2 Ab was produced in rabbits immunized with bovine FGF-2, and neutralized the biological activity of bovine and recombinant human FGF-2. Measurements of hind paw volume and macroscopic examination were made on days 0, 7, 14, 21, and 28. On day 28, these rats were killed, and bilateral ankle joints were removed. Under methods described above, radiological examination and histological evaluations were made.

**Statistical analysis**

Data were expressed as the mean ± SEM, and for statistical analysis we used the one-way ANOVA or the nonparametric Mann-Whitney U test, as appropriate, according to data form. Differences were considered significant at a level of p < 0.05.

**Results**

**Characterization of SeV-mediated gene transfer into rat joint synovium in vivo**

As this is apparently the first attempt to apply SeV to joint gene transfer, we first assessed the in vivo gene transfer efficiency using reporter genes. Two days after 5 × 107 PFU of SeV-NLS-lacZ injection, knee and ankle joints were subjected to in situ X-gal staining. As shown in Fig. 2, all rats macroscopically had representative diffuse and widespread blue staining, in the synovial surface of knee (data not shown) and ankle joint (Fig. 2, A and B). Surface of the cartilage did not show blue stain, indicating that this cartilage was resistant to SeV-mediated gene transfer (Fig. 2, A and B). These findings were also noted in joints of naïve rats (data not shown). Histological examination revealed gene expression of NLS-lacZ in the nuclei of synoviocytes and sublining cells (Fig. 2C). Control rat joints received SeV-luciferase for X-gal staining showing negative results (data not shown).

Next, we examined the time course of gene expression transferred by SeV, using the luciferase transgene. Mean luciferase activity in ankle joints of rats injected with SeV-luciferase intraarticulately was 2,699,012 (±77,536 SD); 24,621 ± 4,650; and 14,522 ± 5,134 relative light units/mg protein on days 2, 7, and 14, respectively (Fig. 2D). A time course study demonstrated a significant transgene expression that persisted for 14 days after injection of the virus.

**Expression of angiogenic growth factors in the synovial fluids**

To assess the relevance of angiogenic gene transfer, we first measured levels of VEGF and FGF-2 using ELISAs. As shown in Fig. 3A, the expression level of VEGF in human synovial fluids was 1.2 ± 0.4 and 1.1 ± 0.5 ng/ml in original samples and hyaluronidase-digesting samples, respectively. Marked elevation of FGF-2 (2.3 ± 0.5 ng/ml) was also noted in human RA synovial fluid digested with hyaluronidase, while no significant FGF-2 protein was detected in undigested fluid, suggesting that FGF-2 may be stabilized by interaction with colloids present in the fluid.

Next, we made similar measurements to assess the elevation of endogenous and vector-mediated FGF-2 and VEGF in AIA rat joints. Although FGF-2 protein in synovial extract of naïve rats given PBS injection was out of range of detection (n = 4), FGF-2 in the ankle joint of AIA rats was detected (3.9 ± 0.5 ng/mg protein, n = 12). Two days after transfer of the FGF-2 gene (5 × 107 PFU), marked elevation of FGF-2 approximately 18 times over the endogenous level was observed (70.2 ± 7.7 ng/mg protein, n = 12, p < 0.001), thus indicating the efficient gene transfer ability of SeV vector to the rat synovium (Fig. 3B).
VEGF expression was also shown to be enhanced by FGF-2 to achieve synergistic angiogenic properties (22), and we examined VEGF protein concentrations, using the same synovial extract samples. As shown in Fig. 3B, endogenous VEGF in AIA joints was detected (0.3 ± 0.1 ng/mg protein, n = 12), while negative results were seen in naïve rat joints. In contrast, AIA + FGF-2 rats showed a marked elevation of endogenous VEGF, approximately 16 times greater than that of AIA-related expression (4.9 ± 0.6 ng/mg protein, n = 12, p < 0.0001), thus indicating a rough parallel increase with FGF-2. These observations suggest that FGF-2 enhances VEGF expression in AIA joints.

**Progressive effect of FGF-2 overexpression on AIA**

**Hind paw volume.** In another set of time course experiments, we examined macroscopic paw swelling and hind paw volume (Fig. 1). Although neither macroscopic findings of arthritis such as paw swelling, redness, nor ulceration of the skin were found in C + FGF-2 and C + luciferase groups throughout the examination (Fig. 4A), these untoward events were recognized in AIA (Fig. 4B), AIA + FGF-2 (Fig. 4C), and AIA + luciferase (Fig. 4C) groups, and were accentuated from 10 days after the adjuvant treatment. Macroscopic joint swelling in AIA + FGF-2 group was the most severe compared with findings in AIA and AIA + luciferase groups (Fig. 4A–C).

Hind paw volume in C + FGF-2 and C + luciferase groups increased somewhat, due to natural course of growth. Hind paw volumes in AIA and AIA + luciferase groups markedly increased from 14 days after immunization, but without any significant difference. On the other hand, the hind paw volume in the AIA + FGF-2 group was greatly enhanced following FGF-2 gene transfer (p < 0.001) (Fig. 4D).

**Radiological assessment of joint destruction.** Next, radiological examination was made when we killed the rats to assess effects of FGF-2 gene transfer on joint and bone destruction. No apparent radiological abnormality was seen in ankle joints both of C + FGF-2 and C + luciferase groups in throughout the experimental course (data not shown). On day 14, radiological findings of soft tissue swelling and joint space narrowing were similar in ankle joints in all of AIA, AIA + FGF-2, and AIA + luciferase groups (Fig. 5, A, D, and G). On day 21, radiological findings mainly of joint space narrowing and bone atrophy were found in ankle joints of AIA and AIA + luciferase groups, while more destructive findings such as bone erosion and osteolysis were found in ankle joints of AIA + FGF-2 group (Fig. 5, B, E, and H). On day 28, disappearance of joint spaces, total bone atrophy, bone erosion, and focal and mild osteolysis was similar in AIA and AIA + luciferase group (Fig. 5, C and F). More severe destructive findings, including marked osteolysis, periostal reaction, and disappearance of joint structure, were seen in the AIA + FGF-2 group (Fig. 5I). We then divided ankle joints of AIA, AIA + FGF-2, and AIA + luciferase groups into five subclasses radiologically on the basis of degree of joint destruction (31, 39). As shown in Fig. 5J, the radiological index of AIA + FGF-2 group was higher (p < 0.05) than that of any other group on days 21 and 28.
Histopathological analysis for arthritis. No apparent active synovitis is noted in the ankle joints of C + FGF-2 and C + luciferase groups on days 14–28 (data not shown). In AIA, AIA + luciferase, and AIA + FGF-2 groups, on day 14, active synovitis showing synoviocytes hyperplasia, increase of vascularity, and moderate to severe infiltration of neutrophils and mononuclear cells was noted (data not shown). On day 21, in AIA and AIA + luciferase groups, the active synovitis continued, and fibrinopurulent exudate was noted in the joint spaces, occasionally associated with mild and focal erosion of articular cartilages (Fig. 6, A–F). On the other hand, in the AIA + FGF-2 group, histological joint structures such as demarcation of the bone cortex and joint line composed of articular cartilages were unclear in low power field (Fig. 6, G–I). In the high power field, articular cartilages were totally destroyed by marked erosion; moreover, inflamed granulomatous tissue associated with aggregates of osteoclast-like multinucleated giant cells had invaded subchondral bone tissue (Fig. 6I). On day 28, proliferative synovitis was dominant, and tibial distal end, talus, and other tarsal bones were largely invaded by inflamed granulation tissue so-called pannus formation; these findings were most severe in the AIA + FGF-2 group (data not shown).

FIGURE 5. Radiological assessment of effect of FGF-2 gene transfer on bone and joint destruction. A–I, Demonstrate typical representative x-ray photographs of rat ankle joints of AIA (A–C), AIA + luciferase (D–F), and AIA + FGF-2 (G–I) groups on days 14, 21, and 28. On day 14, soft tissue swelling and joint space narrowing were found to be equal in the three groups (A, D, G). On days 21 and 28, joint space disappearance and mild (B and E) to moderate (C and F) osteoporosis were found in AIA and AIA + luciferase groups. In contrast, the most severe osteolysis and following joint destruction were found in AIA + FGF-2 joints on days 21 (H) and 28 (I). J, Demonstrates the radiological index of ankle joints at each time point. FGF-2 gene-transferred joints showed significantly severe radiological joint destruction on days 21 and 28. Data at each time point are the mean ± SEM of eight hind paws. *, p < 0.01; #, p < 0.05 with one-way ANOVA.

FIGURE 6. Histopathological assessment of effect of FGF-2 gene transfer. Rats were immunized with adjuvant and were given intraarticular injection of PBS, SeV-luciferase, or SeV-FGF-2, as described in Materials and Methods, A–C, D–F, and G–I, Show representative histological findings in AIA, AIA + luciferase, and AIA + FGF-2 groups, respectively, on day 21. Exudative synovitis and focal and mild erosion of articular cartilage were found in AIA (A–C) and AIA + luciferase (D–F) groups. In contrast, in the AIA + FGF-2 group (G–I), synovial tissue invades into subchondral bone tissue through the eroded orifices, and numerous osteoclast-type multinucleated giant cells were present in the border of granulation tissue and bone trabeculae. Ti, tibia; Ta, talus; C, calcaneus; JS, joint space. *, Erosion. A, D, and G was scheme of B, E, and H, respectively. Original magnification, ×3.1 (B, E, and H), ×20 (C and F), and ×27 (I).

FIGURE 7. Effects of FGF-2 gene transfer on inflammatory infiltration, pannus invasion, angiogenesis, and osteoclastogenesis during the development of AIA. A, Number of granulocytes infiltrating to the synovium adjacent tali-tibial joint. The count was done in a high power field (×400). B, vWF-positive vessel density per 1 mm². C, Demonstrates osteoclasts and its precursor cells showing positive reaction for TRAP staining in AIA + FGF-2 group on day 21. D, Number of osteoclasts in three individual granulation tissues. TRAP-positive multinucleated and mononuclear cells were counted in a high power field (×400). Data at each time point are the mean ± SEM of eight hind paws. *, p < 0.01 with one-way ANOVA.
The number of infiltrated granulocytes in AIA + FGF-2 group was greater than in the other two groups, with a statistical significance (p < 0.01) on day 14 (Fig. 7A), but not on day 21, probably due to decline in cell number during repair process.

In the immunohistochemical study, mean numbers of small vesicles that reacted positively for vWF on days 14 and 21 were greater than in the other two groups, with a statistical significance (p < 0.01) (Fig. 7B), which suggests that SeV-mediated overexpression of FGF-2 in ankle joints promotes neovascularization.

Furthermore, to determine whether FGF-2 gene transfer would affect osteoclastogenesis in vivo, we determined the number of infiltrating TRAP-positive osteoclasts. The number in the AIA + FGF-2 group on days 21 and 28 was larger than in the other two groups, with a statistical significance (p < 0.01) (Fig. 7C and D), suggesting that overexpression of FGF-2 in ankle joint also promotes osteoclastogenesis, especially in late phase.

**Effects of neutralization by anti-FGF-2 IgG on AIA development**

To obtain further in vivo evidence that FGF-2 is a key mediator in AIA, we assessed the suppressive effect of neutralizing anti-FGF-2 Ab (Fig. 8A). As shown in Fig. 8B, administration of anti-FGF-2 Ab significantly ameliorated both joint swelling and hind paw volume (p < 0.05) as compared with rat joints with the nonimmunized rabbit IgG in AIA rats at days 14 and 21 of the experimental course. In radiological assessment of ankle joints injected with anti-FGF-2 Ab showed less bone and joint destruction, and a lower radiological index than was observed in ankle joints treated with control IgG (Fig. 8C). Moreover, in histopathological examinations, administration of anti-FGF-2 Ab also significantly reduced vWF-positive vessel density (Fig. 9A) and the number of osteoclasts (Fig. 9B) on day 28.

**Discussion**

Our in vivo evidence indicates the critical role of FGF-2 in the pathogenesis of AIA. Key findings obtained in this study were: 1) endogenous FGF-2 was elevated in a rat model of arthritis as induced by adjuvant administration, as well as in human RA joint fluid; 2) endogenous VEGF expression in joint synovial extract, which may be an important modulator of RA, was enhanced following FGF-2 gene transfer, suggesting that FGF-2 may enhance VEGF expression in AIA joint in vivo; 3) SeV vector-mediated FGF-2 overexpression accelerated disease severity of AIA, namely enhanced joint swelling, inflammatory reactions, synovial angiogenesis, and osteoclastogenesis, but had no effect on joints of naive animals, suggesting that FGF-2 may affect disease progression, but not disease initiation; and 4) administration of anti-FGF-2-neutralizing Ab attenuated disease severity. These findings indicate the first in vivo evidence for a significant and important biological role of FGF-2 during progression of arthritis in rats.

Recently, VEGF and FGF-2 have received much attention from the viewpoint of the therapeutic control of RA. VEGF is considered to be a vascular endothelial cell-specific mitogen, as well as a vascular permeability factor. Recent studies showed that the level of VEGF protein in joint fluids of RA patients was significantly higher than that of non-RA fluids with osteoarthritis patients (14, 15), and its receptors, Flt-1 and KDR, were also induced in the active phase of RA (15). Administration of soluble VEGF receptor (17) or VEGF antiserum (18, 19) after the onset of collagen-induced mice arthritis ameliorated not only joint inflammation, but also bone destruction, suggesting that the control of VEGF function can be an effective strategy to treat RA.
FGF-2 is an alternative angiogenic growth factor acting on not only vascular endothelial cells, but also various mesenchymal cells. FGF-2 was specifically up-regulated in joint fluid from RA human subjects, as well as in synovial fluid extracted from animal models, suggesting that FGF-2 has a crucial role in joint destruction of RA, affecting joint inflammation and bone destruction.

In this study, we compared VEGF and FGF-2 protein content levels from human RA joint fluid treated with or without hyaluronidase. Joint fluid from RA patients is hyaluronic acid rich, resulting in increased viscosity and in gel formation. As shown in Fig. 3A, no FGF-2 was detected in the solution phase of RA fluid without hyaluronidase treatment, while a high level of FGF-2 was recovered in the same fluid sample treated with hyaluronidase. These findings suggest that FGF-2 may be bound to and stabilized by substances with hyaluronic acid in human RA fluid. The value of FGF-2 detected in this study was 2 to 3 logs higher than in a previous report (13), and we concluded that this discrepancy was due to hyaluronidase treatment.

Although little knowledge is available regarding the importance of these angiogenic factors, our current findings that FGF-2 over-expression resulted in VEGF up-regulation in the synovial tissue suggest hierarchy in the regulatory process of angiogenesis. Recent reports indicated that FGF-2 stimulates VEGF expression in some mesenchymal cells, and these two act synergistically for an efficient angiogenic response (22), which supports our current findings. Furthermore, we recently found that neutralization of endogenous VEGF activity completely abolished FGF-2-mediated angiogenesis (43), thereby suggesting that in vivo angiogenic properties of FGF-2 completely depend on VEGF. On the other hand, recent studies suggested that the nonangiogenic action of FGF-2 might even worsen RA. FGF-2 accelerated osteoclastogenesis and activated bone resorption by osteoclasts through activation of FGFR1 and p42/p44 mitogen-activated protein kinase and stimulated bone resorption at physiological or pathological concentrations in vitro (26).

Since the pathophysiology of RA has been thought to involve synovial proliferation and angiogenesis as well as bone destruction and absorption, blockade of VEGF, dominantly resulting in suppression of angiogenesis and fluid collection, might not be sufficient to prevent RA disease from the viewpoint of clinical treatment. Our current results suggest that FGF-2 seems to be a better molecular target of RA, because it might indirectly control VEGF expression via regulating FGF-2. Clinical prognosis of RA, for example necessity of surgical treatment and deterioration of quality of life, almost always depends on the degree of bone and joint destruction brought by pannus formation and bone absorption due to osteoclastogenesis. Thus, functional control or neutralization of FGF-2 would be a meaningful treatment approach to suppress RA progression and deteriorate RA severity. However, the limitation of the current study is that little is known regarding the initial events of RA and the relationship between FGF-2 and other proinflammatory cytokines specifically expressed in RA, including IL-17 (43–48). Further studies on the understanding of RA pathophysiology are called for to clarify this complexed process of the disease to establish more effective therapeutic approaches.

In conclusion, FGF-2 appears to have an important role in the progression of AIA, namely, exacerbation of joint swelling and bone destruction. The control of FGF-2 may prove to be an effective therapeutic approach to modify RA in humans.

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