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

Akihisa Yamashita,*† Yoshikazu Yonemitsu,1ª Shinji Okano,* Kazunori Nakagawa,* Yutaka Nakashima,* Takahiko Irisa,† Yukihide Iwamoto,† Yoshiyuki Nagai,* Mamoru Hasegawa,§ and Katsujo Sueishi*†

Rheumatoid arthritis (RA), a systemic inflammatory disease of unknown etiology, mainly affects synovial joints. Although angiogenic growth factors, including fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF), may play a critical role in the development and progression of RA joint disease, little information is now available regarding their exact role in initiation and/or progression of RA. In this study, we show that both polypeptides were up-regulated in the rat joint synovial tissue of an adjuvant-induced model of arthritis (AIA), as well as human subjects with RA. FGF-2 overexpression via Sendai virus-mediated gene transfer significantly worsened clinical symptoms and signs of rat AIA, including hind paw swelling and radiological bone destruction, as well as histological findings based on inflammatory reaction, synovial angiogenesis, pannus formation, and osteocartilaginous destruction, associated with up-regulation of endogenous VEGF. FGF-2 gene transfer to non-AIA joints was without effect. These findings suggested that FGF-2 modulated disease progression, but did not affect initiation. Reverse experiments using anti-FGF-2-neutralizing rabbit IgG attenuated clinical symptoms and histopathological abnormalities of AIA joints. To our knowledge, this is the first report indicating direct in vivo evidence of disease-modulatory effects of FGF-2 in AIA, as probably associated with endogenous VEGF function. FGF-2 may prove to be a possible therapeutic target to treat subjects with RA. The Journal of Immunology, 2002, 168: 450–457.

Rheumatoid arthritis (RA) is a chronic disease of unknown etiology. Although 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 mononuclear 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 blood 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-regulating 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 Thoen 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 intraarticularly injected with 5 × 10⁷ PFU of MBD dissolved in 100 µl mineral oil (NACALAI TESQUE, Kyoto, Japan) on day 0 to prepare the AIA model (31). The onset 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′-GCGCGCGCATCTTACG-3′ with a NorI 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-clearing ribosome site from the antigenic strand of the hepatitis B virus. The entire DNA-coding *Escherichia coli lacZ* with a nuclear localizing signal (for SeV-NLS-luc), luciferase (for SeV-luciferase), and murine FGF-2 (for SeV-FGF-2) were amplified by PCR, using primers with a NorI site and new sets of SeV E and S signal sequence tags for an exogenous gene, then inserted into the NorI site of the cloned genome. The entire length of the template SeV genomes, including exogeneous 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 vT7-3 into CV-1 or LLMCK 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 titer was determined using chicken RBCs in hemagglutination assay (35), and virus titers 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 plantar 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-luc (5 × 10⁷ PFU) into the ankle and the knee joints at 7 days after the adjuvant treatment. Two days later, each joint was dissected out en bloc, incised transversally to expose the joint space, and then cotransfected with vaccinia virus vT7-3 into CV-1 or LLMCK 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 titer was determined using chicken RBCs in hemagglutination assay (35), and virus titers were kept frozen at −80°C until use.

**Ankle joint biopsy and 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.

**Histopathological assessment of arthritis**

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

Twenty-four rats were intracuticularly injected with 1 mg MBD on day 0 for AIA rats. Among them, 12 rats were intraarticularly injected with 5 × 10⁷ 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; Muramachi Kikai, Tokyo, Japan) at each time point by two individual examiners (A. Yamashita and T. Irisa) 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 Barbier 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 radiological index.

![FIGURE 1](http://www.jimmunol.org/) 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.
Immunohistochemical 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 nonspecific 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/HPD, 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 0.025% (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 intra-articularly injected with 50 µg anti-FGF-2 Ab (anti-FGF basic Ab; R&D Systems), while left ankle joints were also intra-articularly 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 resected. 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 naive rats (data not shown). Histological examination revealed gene expression of MLS-lucZ 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 intraarticularly was 2,699,012 ± 7.7 ng/mg protein, 7.7 ng/mg protein, 5.134 relative light units/mg protein on days 2, 7, and 14, respectively (Fig. 2D). A time course study determined 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.

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FIGURE 2. Characterization of rSeV-mediated gene transfer using reporter genes. Seven days after adjuvant injection, 50 µl SeV-NLS-lacZ (A–C) or SeV-luciferase (D) was injected into the rat ankle joint, as described in Materials and Methods. A and B. A typical representative result of in situ X-gal staining in an ankle joint, 2 days after NLS-lucZ gene transfer. Macroscopically, synovial surface was diffusely and intensely stained blue in both posterior (A) and posterolateral (B) views. AT, Achilles tendon. Original magnification, ×6 (A and B). C. Demonstrates histological examination of the same joint demonstrated in A and B. Intense blue nuclei of synovial cells are shown. Counterstained with hematoxylin. Original magnification, ×175. The four animals subjected to NLS-lucZ gene transfer showed similar results. D. Demonstrates course of SeV-mediated transgene expression assessed by firefly luciferase gene transfer. Following vector administration, joint extracts were collected, and luciferase activity was measured, as described in Materials and Methods. Once peaking on day 2, transgene expression decreased in a time-dependent manner, but was detected at about day 14. Data are expressed as mean ± SEM, and the value of each sample is standardized, depending on the protein concentration. Note log scale.

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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, periosteal 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.

FIGURE 4. Effect of FGF-2 gene transfer on hind paw volume. Twenty-four control (Cont. or C) rats were injected with 100 µl mineral oil, and 36 AIA rats were injected with adjuvant on day 0. Seven days after mineral oil or adjuvant administration, C + FGF-2 and AIA + FGF-2 groups, C + luciferase and AIA + luciferase groups, and AIA group were intrarticularly injected with 50 µl SeV-FFG-2 (5 × 10⁷ PFU), SeV-luciferase (5 × 10⁷ PFU), and PBS into the ankle joints, respectively (eight joints for each group). Hind paw volume was measured at each time point. A–C, Demonstrate typical representative macroscopic photographs of rat ankle joints on day 21. Significant swelling was found symmetrically in AIA joints (B), but in neither luciferase nor FGF-2 gene-transferred joints, without adjuvant injection (A). In contrast, the FGF-2-injected joint with AIA showed marked swelling (C, right ankle joint), compared with luciferase injection (C, left ankle joint). D, Demonstrates time course measurement of hind paw volume. FGF-2 gene-transferred joints showed significantly severe swelling on days 14, 21, and 28. Data at each time point are the mean ± SEM. n, Number of ankle joints measured the hind paw volume for each group at the time points, *p < 0.01 with one-way ANOVA.
Histopathological analysis for arthritis. No apparent active synovitis was 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 and H). In the high power field, articular cartilages were totally destroyed by marked erosion; moreover, inflamed granulation tissue associated with aggregates of osteoclast-like multinucleated giant cells had invaded subchondral bone tissue (Fig. 6I).

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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 talo-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 cells 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.

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The number of infiltrated granulocytes in \( \text{AIA} + \text{FGF-2 group} \) exceeded that in 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 with 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 \( \text{AIA} + \text{FGF-2 group} \) on days 21 and 28 was larger than in the other two groups, with a statistical significance \((p < 0.01)\) (Fig. 7, C 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 \( \text{Ab} \) (Fig. 8A). As shown in Fig. 8B, administration of anti-FGF-2 \( \text{Ab} \) significantly ameliorated both joint swelling and hind paw volume \((p < 0.05)\) as compared with rat joints with the nonimmunized \( \text{IgG} \) in AIA rats at days 14 and 21 of the experimental course. In radiological assessment of ankle joints injected with anti-FGF-2 \( \text{Ab} \) showed less bone and joint destruction, and a lower radiological index than was observed in joint ankles treated with control \( \text{IgG} \) (Fig. 8C). Moreover, in histopathological examinations, administration of anti-FGF-2 \( \text{Ab} \) 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 \( \text{Ab} \) 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 antisera (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 (13, 21), as well as in synovial extract from animal models (42), thus 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 overexpression 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 complicated 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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