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Gene Therapy Targeting the Tie2 Function Ameliorates Collagen-Induced Arthritis and Protects Against Bone Destruction

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Objective. **In a previous study, we demonstrated that Tie2 regulates angiogenesis in arthritis. The current study was performed to determine whether systemic delivery of a soluble Tie2 receptor (ExTek) using an adenoviral vector (AdExTek) as a Tie2 inhibitor affects arthritis development and progression in an animal model.**

Methods. **We used a collagen-induced arthritis (CIA) mouse model to study the outcome of treatment with either AdExTek or a control vector. The onset, incidence, and severity of arthritis were quantified. Immunohistologic analysis of endothelium obtained from the paws was performed. Bone destruction in paws was analyzed using phase-contrast radiography.**

Results. **The data showed that systemic delivery of ExTek before disease development significantly inhibited the onset, incidence, and severity of arthritis. When AdExTek was given after disease onset, the severity of disease in mice treated with AdExTek was significantly lower than that in the control group at 35 days postimmunization, which correlated with significantly diminished angiogenesis in mouse paws. Strikingly, AdExTek treatment protected bone from erosion in the CIA model and reduced levels of RANKL. No differences in collagen-specific antibodies were detected between these 2 groups.**

Conclusion. **We demonstrated that blocking Tie2 receptor activation inhibits angiogenesis and arthritis development and protects against bone destruction in a CIA mouse model. These findings identify Tie2 as a therapeutic target for arthritis treatment and imply that interventions designed to target the Tie2 pathway could be clinically beneficial.**

Rheumatoid arthritis (RA) is a chronic inflammatory disease associated with intense angiogenesis (1). Formation of new blood vessels (angiogenesis) is one of the earliest histopathologic findings in RA and appears to play a critical role in the generation and prolonged survival of the hypercellular synovial membrane and invasive pannus in RA (2,3). First, neovascularization increases the nutrient supply to tumorlike synovium. Second, the expanded endothelial cell surface area in an inflamed joint maximizes the routes of ingress that allow immune and inflammatory cells to adhere and migrate into the synovium. Finally, activated endothelial cells provide a potent source of proinflammatory cytokines, chemokines, and growth factors (4). Therefore, activated endothelium and angiogenesis should be good targets for therapeutic intervention in RA. Reduced inflammation arising from the diminished angiogenesis could further ameliorate new blood vessel formation, because many mediators of inflammation are, themselves, proangiogenic.

Although no therapy specifically targeting angiogenesis in RA is currently available, many existing treatments for RA have been shown to possess some degree of antiangiogenic activity. For example, methotrexate, a common treatment in patients with RA, was shown to inhibit endothelial cell proliferation in vitro (5). Anti–tumor necrosis factor α (anti-TNFα) antibody treatment significantly decreased serum levels of vascu-

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lar endothelial growth factor (VEGF) and reduced vascularity in RA joints (6,7). In addition, AGM-1470 (a low molecular weight synthetic analog of fumagillin), D-penicillamine, paclitaxel, gold-containing compounds, sulfasalazine, cyclosporine, and glucocorticoids also exhibit angiostatic properties (8–12). More recently, several antiangiogenic agents targeting VEGF and $\alpha v\beta 3$ integrins have been shown to be effective in attenuating arthritis in animal models (13–17).

Tie2 is an endothelium-specific receptor tyrosine kinase that is required for embryonic vascular development (18). Tie2 also plays a critical role in pathologic angiogenesis and disease development (19–25). Several studies have demonstrated the presence of both Tie2 and its ligands, angiopoietin 1 (Ang-1) and Ang-2, in RA synovium (26–31). Recently, we showed that levels of Tie2 and Ang-1 were elevated in human RA synovium, and that blocking Tie2 activation using a soluble Tie2 receptor (ExTek) inhibited arthritis-induced angiogenesis in a synovium vascular window model (32). The current study was designed to determine whether neutralization of Tie2 has a therapeutic benefit in arthritis development. Our results show that systemic delivery of a Tie2 inhibitor using a gene therapy approach delayed disease onset, reduced disease severity, and protected against bone erosion in a collagen-induced arthritis (CIA) mouse model. Thus, our study has identified a new target for arthritis treatment.

MATERIALS AND METHODS

Mice. DBA/1j mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Male mice, 6–7 weeks old, were used in the study. The animals were housed in pathogen-free units at Vanderbilt University Medical Center, in compliance with Institutional Animal Care and Use Committee regulations.

Induction and assessment of CIA. Disease-susceptible DBA/1j mice develop polyarthritis 3–4 weeks after primary immunization with bovine type II collagen (CII). Arthritis was induced following the standard immunization protocol (32,33). Beginning on day 22, mice were examined every other day in a blinded manner by 2 independent examiners for signs of arthritis and the date of disease onset. Clinical arthritis was assessed by using the following scoring system: grade 0 = no swelling, grade 1 = paws with swelling in a single digit, grade 2 = paws with swelling in multiple digits, and grade 3 = severe swelling and joint rigidity. Each limb was graded, and the maximum possible score per mouse was 12.

Histologic analysis. Mouse paws were removed postmortem, fixed in 4% paraformaldehyde, and decalcified in Immunocal (Decal Chemical, Congers, NY). The paws were then embedded in OCT compound (Sakura, Torrance, CA). Frozen sections (8 μm) were cut, fixed in ice-cold acetone for 10 minutes, and blocked with 1% goat serum and avidin–biotin blocking reagents (Vector, Burlingame, CA). The sections were then incubated with monoclonal antibodies against the endothelial marker CD31 (PharMingen, San Diego, CA) and RANKL (Santa Cruz Biotechnology, Santa Cruz, CA) in a humidified chamber overnight at 4°C. A biotinylated secondary antibody was applied for 30 minutes, followed by another 30-minute incubation with streptavidin-conjugated horseradish peroxidase (HRP). Peroxidase activity was localized with diaminobenzidine. The serial sections were also stained with hematoxylin and eosin for histologic analysis.

Measurement of serum anti-CII antibody levels. Sera were collected from blood samples at 3 and 6 weeks after immunization and stored at −20°C. Individual sera were analyzed for anti-CII antibodies using an isotype-specific enzyme-linked immunosorbent assay (ELISA), as previously described (34). Briefly, ELISA plates (Dynex Technologies, Chantilly, VA) were coated with 10 μg/ml of native bovine CII in 0.1*M* $NaHCO_3$ (pH 9.6) overnight at 4°C. After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20, the plate was blocked with PBS containing 1% bovine serum albumin for 1 hour at room temperature. All serum samples were tested in serial dilutions from 1:100 to $1:10^5$. After overnight incubation at 4°C, detection of IgG subclasses was performed with alkaline phosphatase–conjugated goat anti-mouse IgG, IgG1, and IgG2a (Southern Biotechnology Associates, Birmingham, AL) at room temperature for 1 hour. Quantification of bound enzyme was performed with *p*-nitrophenol (Sigma, St. Louis, MO) in 1*N* NaOH. The optical density at a wavelength of 420 nm was measured using a microplate reader and Delta 3 software (BioTek Instruments, Winooski, VT).

Propagation, purification of adenoviral vectors, and systemic delivery of a soluble Tie2 inhibitor (ExTek) in vivo. Plaque-purified adenoviral vectors directing the expression of either a soluble Tie2 receptor (AdExTek) (35) or a *lacZ* gene (AdPacβ-gal) were used to generate high-titer virus stocks by infecting forty 150-mm plates of confluent 293 cells at a multiplicity of infection of 0.1. The viruses were purified from the infected cell lysates as described previously (36). The virus was stored in aliquots in a virus storage buffer (20 m*M* Tris [pH 7.4], 150 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$) containing 1 mg/ml of mouse albumin (Sigma) and 10% glycerol at −80°C. Titers of viral stocks were determined by plaque assay on 293 cells using standard techniques. The adenoviral vectors used in the study are adenovirus serotype 5.

In vivo detection of ExTek was carried out in DBA/1j mice, which received intravenous injections of AdExTek (1×10^9 plaque-forming units per mouse). Plasma was collected at various days after viral injection and analyzed by Western blotting using a monoclonal anti-Tie2 antibody (MoAb33.1) as previously described (21). Arthritic joints were also homogenized in lysis buffer. Cell lysates (50 μg) were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by transfer to nitrocellulose membranes. After blocking, the membrane was incubated with an anti-Tie2 antibody (MoAb33.1), followed by incubation with an HRP-conjugated secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescence Western blotting analysis system (Perkin Elmer, Boston, MA). The same membrane was reprobed with an anti–β-actin antibody as a loading control. The plasma concentration of ExTek was measured by

ELISA as previously described (35). Purified recombinant ExTek protein was used as a control (20).

Phase-contrast radiography. Detailed imaging procedures have been described previously (37,38). After the paws were disarticulated from the legs, phase-contrast radiography was performed. Radiologic evaluation of the bones in mouse paws was performed by an investigator who was blinded to the treatment protocol. A score was assigned based on periarticular demineralization, periosteal new bone formation, and joint deformity. Each of these 3 categories was given a score of 0–2, with a higher score indicating more severe symptoms. A combined score of 0–6 was assigned to each paw.

Reverse transcription–polymerase chain reaction (RT-PCR). Total RNA from fresh-frozen joint tissue was extracted using the RNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. For RT-PCR, 10 μg of total RNA was reverse transcribed to first-strand complementary DNA (cDNA) using oligodeoxythymidylic acid primers and the SuperScript preamplification system (Invitrogen, San Diego, CA). Mouse RANKL and β-actin cDNA were amplified from single-stranded cDNA by PCR using TaqDNA Polymerase (Invitrogen) and 0.4 μM of each primer. The RANKL PCR primers were as follows: forward, 5′-CCAGCATCAAAATCCCAAGTT-3′; reverse, 5′-TCAAGGTTCTCAGTGGCACAT-3′. The PCR product of RANKL is 568 bp. The mouse β-actin PCR primers were as follows: forward, 5′-GACAACGGCTCCGGCATGTGC-3′; reverse, 5′-TGGCTGGGGTGTTGAAGGTC-3′. The PCR product of β-actin is 363 bp.

Statistical analysis. Results are reported as the mean ± SEM for each group. The chi-square test (for disease incidence) and Student's unpaired 2-tailed *t*-test (for arthritis scores) were used to analyze statistical differences between the control group and the treated group. *P* values less than 0.05 were considered significant.

RESULTS

Amelioration of CIA in the AdExTek-treated mice. In a previous study, we showed that levels of Tie2 and Ang-1 are elevated in human RA synovium, and that blocking the action of Tie2 with a soluble Tie2 protein (ExTek) inhibits neovascularization in arthritis (32), indicating a critical role of Tie2 signaling in arthritis development. Here, we evaluated the therapeutic benefits of systemic delivery of ExTek in arthritis development, using a gene therapy approach. Arthritis was induced by immunizing DBA/1j mice with intradermal injections of bovine CII in a CIA model. The AdExTek virus and the control AdPacβ-gal virus were injected in the tail vein on day 20 postimmunization. Plasma samples from the mice were obtained 3 days after the viral injection and were analyzed for AdExTek expression by Western blotting. High levels of ExTek protein were easily detected in the mice treated with AdExTek virus but not in the mice receiving the control virus (Figure 1A). Protein expression in the circulation lasted up to 10 days (Figure 1B), and tissue deposition of ExTek lasted even longer (Figure 1C).

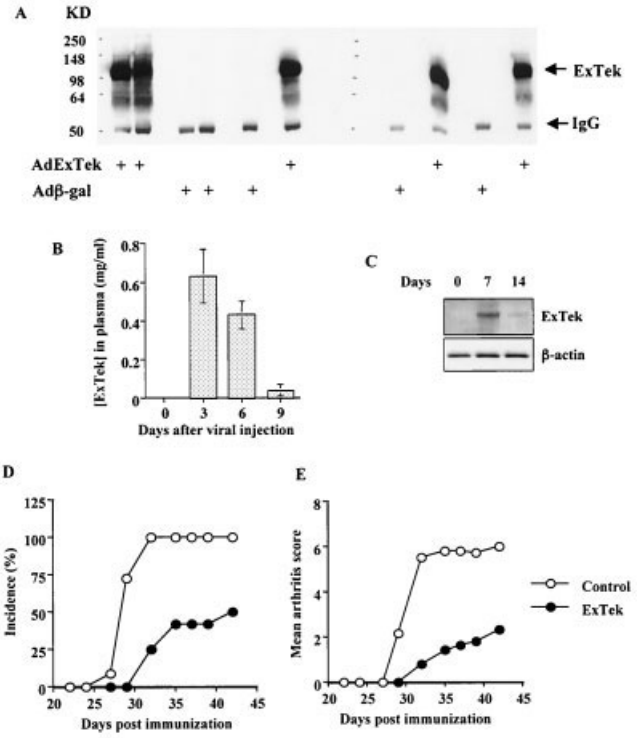

Figure 1. Systemic delivery of soluble Tie2 receptor in a collagen-induced arthritis (CIA) mouse model. DBA/1j mice were immunized with bovine type II collagen to induce arthritis. Twenty days after the primary immunization, the mice were randomly divided into 2 groups, and adenoviral vectors directing the expression of either a soluble Tie2 receptor (AdExTek) or a *lacZ* gene (Adβ-gal) were injected intravenously. Plasma was collected 3 days after injection and subjected to Western blot analysis. ExTek expression was detected by incubation with an anti-Tie2 antibody (**A**). Plasma was also collected at various time points after the AdExTek injection, followed by measurement of the ExTek concentration using an enzyme-linked immunosorbent assay. Purified recombinant ExTek protein was used as a standard to calculate the ExTek concentration in the circulation (n = 3 mice) (**B**). Protein lysates were prepared from arthritic joints after the viral injection. The ExTek concentration in joint tissues was detected by Western blotting. The same membrane was reprobed with an anti–β-actin antibody to assure equal loading (**C**). The disease onset, incidence, and severity of arthritis were examined by 2 independent investigators in a blinded manner. Results from 3 independent experiments were pooled (summarized in Table 1) and graphed as the disease incidence (**D**) and mean arthritis score (**E**) in each group during the course of CIA (n =11 control mice, n = 12 ExTek-treated mice). Values in **B** are the mean ± SEM.

We began to monitor the clinical signs of disease on day 22 after the primary immunization. The results of 3 separate experiments are summarized in Figures 1D

Table 1. Collagen-induced arthritis in AdExTek-treated and control mice*

Experiment/group	Incidence, %	Day at onset	Arthritis score
Experiment 1			
Control	100	27	4
AdExTek-treated	0	NA	0
Experiment 2			
Control	100	29	6
AdExTek-treated	40	32	2
Experiment 3			
Control	100	29	6.8
AdExTek-treated	80	32	3.8
Experiment 4			
Control	100	29	6 ± 0.72
AdExTek-treated	50	32†	2.3 ± 0.83‡

* Mice were immunized with bovine type II collagen. Values for day at onset are the median. Values for the arthritis score (maximum possible score = 12) are the mean (mean ± SEM of 3 experiments in experiment 4). In experiment 4, n = 11 control mice, and n = 12 treated mice. NA = not applicable.
† $P < 0.05$ versus control littermates, by chi-square analysis.
‡ $P < 0.01$ versus nontransgenic littermates, by Student's *t*-test.

and E and Table 1. The incidence of disease in animals in each group, and their mean arthritis score, were calculated from pooled data from these experiments. We observed a significant inhibition of arthritis development, as measured by a decrease in disease severity and incidence, as well as delayed disease onset in the AdExTek-treated group. The incidence of disease in the AdExTek-treated mice (50% [6 of 12 mice]) was significantly lower than that in the control littermates (100% [11 of 11 mice]) ($P < 0.05$). The severity of disease, measured as the mean ± SEM arthritis score (total clinical score per group/number of animals in each group), was also significantly decreased in the AdExTek treatment group compared with the control group (2.33 ± 0.83 versus 6 ± 0.72; $P < 0.01$). In addition, there was a delay in disease onset in the AdExTek-treated mice: the median day of disease onset was day 32 in treated mice and day 29 in control mice (Figure 1 and Table 1). Taken together, these results clearly demonstrate that blocking Tie2 function inhibits arthritis development in the CIA model.

Reduced neovascularization in AdExTek-treated mice. To investigate the molecular mechanism by which Tie2 inhibition alleviates disease, we examined whether application of AdExTek affected arthritis-associated angiogenesis. The mouse paw joints from both groups were harvested 6 weeks postimmunization and processed. The cryosections were stained with an anti-CD31 antibody for endothelium. There were fewer blood vessels in synovium obtained from the AdExTek-treated mice compared with control synovium (Figures 2A and B). After quantification of CD31-positive vessels in syno-

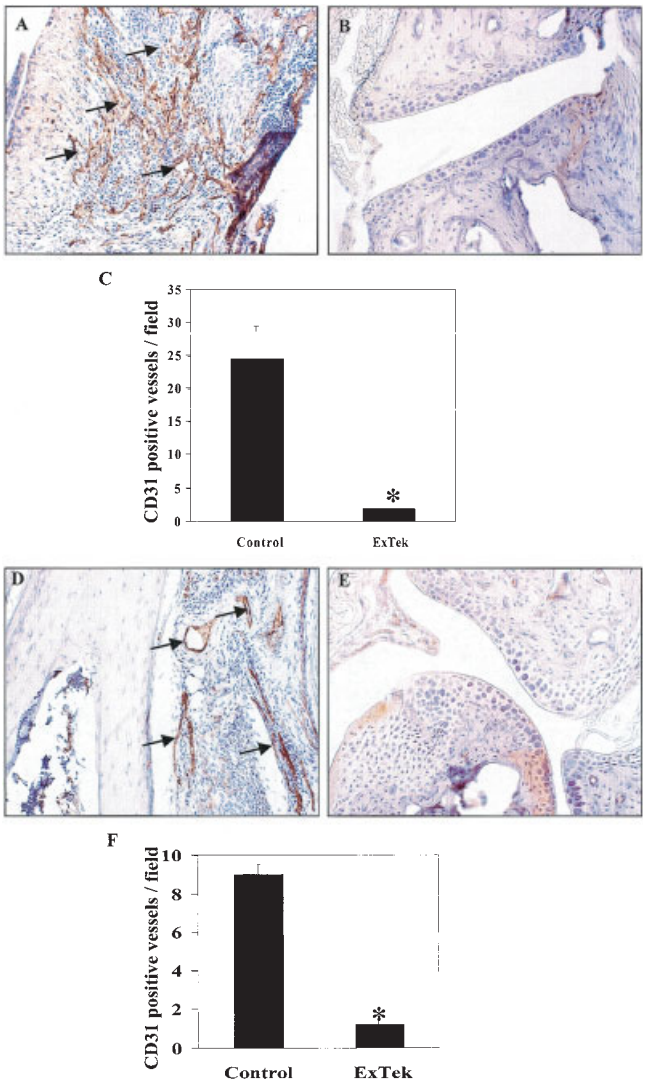

Figure 2. Attenuation of angiogenesis in the CIA model. DBA/1j mice were immunized with bovine type II collagen to induce CIA. The mice were randomly divided into 2 groups and received AdExTek or AdPacβ-gal injections 20 days after the initial immunization. Paw joints were harvested from the mice 6 weeks after the initial immunization. The joint tissues were processed for immunohistochemical staining with an anti-CD31 antibody for blood vessels. Representative joint sections from the control littermates (**A**) and the AdExTek-treated mice (**B**) are shown (original magnification × 200). The number of CD31-positive blood vessels was counted in 8 randomly selected high-power fields (200×) and plotted (**C**). We also examined the angiogenic responses at an early time point prior to appearance of any clinical signs of disease. Mouse paws from both groups were harvested 26 days postimmunization, and joint neovascularization was evaluated by immunohistochemical staining with an anti-CD31 antibody. Representative joint sections from the control group (**D**) and the AdExTek-treated mice (**E**) are shown (original magnification × 200). The number of CD31-positive blood vessels was counted in 8 randomly selected high-power fields (200×) and plotted (**F**). **Arrows** indicate neovascularization. Values in **C** and **F** are the mean and SEM. * = $P <$ 0.01. See Figure 1 for definitions.

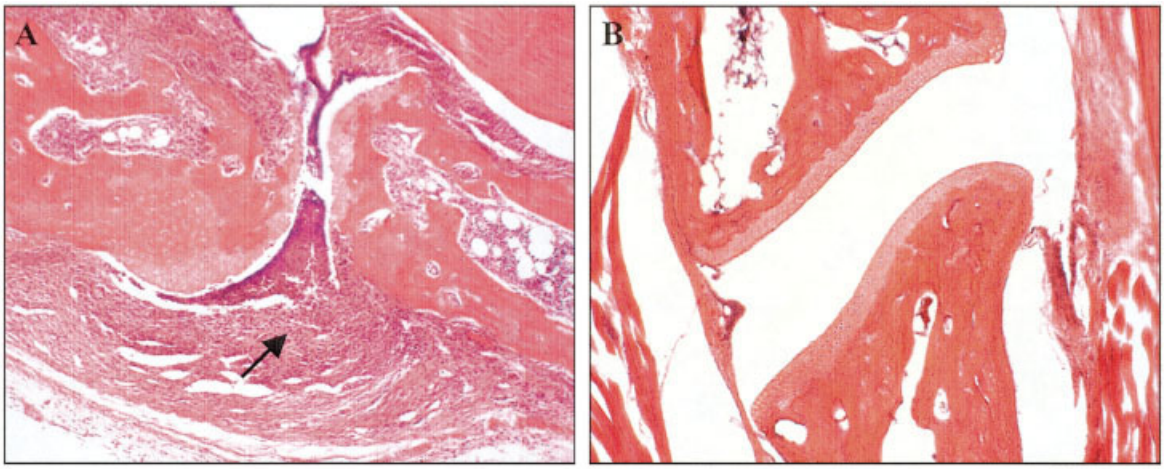

Figure 3. Inhibition of inflammatory cell infiltration in the synovium. Paw joints were harvested 6 weeks after the initial immunization and processed for hematoxylin and eosin staining. Histologic examination of affected joints from the control littermates (11 mice analyzed) showed marked inflammatory cell infiltration in the synovium (**A**). In contrast, most joints from the AdExTek-treated mice (12 mice analyzed) displayed either mild infiltration or no sign of inflammation (**B**). Representative joint sections are shown (original magnification × 200). **Arrow** indicates infiltrated cells. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

vium, we observed a significant reduction in vascular density in the AdExTek-treated mice compared with controls (Figure 2C) ($P < 0.01$).

To further confirm the antiangiogenic function of AdExTek in arthritis development, we harvested the joints at a very early time point (day 26 after the primary immunization, which is just before disease onset). As shown in Figure 2D, abundant angiogenesis, as indicated by CD31-positive blood vessels, was already initiated in the control group, even before the clinical signs of arthritis. In contrast, angiogenesis either was not visible or was greatly reduced in the AdExTek-treated mice (Figure 2E). Quantification of CD31-positive vessels in synovium confirmed significantly less vascular density in the AdExTek-treated mice compared with controls (Figure 2F). A significant reduction of angiogenesis in the early stage of arthritis is associated with inhibition of the development and progression of the disease. These results imply that amelioration of arthritis by AdExTek is attributable to inhibition of angiogenesis.

Reduction of synovial inflammatory cell infiltration in AdExTek-treated mice. We reasoned that inhibition of angiogenesis should reduce accessibility of the tissue to leukocytes. Therefore, we examined whether blocking Tie2 activation affected synovial inflammatory cell infiltration in animals. Mouse paws were harvested at 6–7 weeks postimmunization and processed. Histologic examination of the joints revealed marked synovial inflammatory cell infiltration in joints from control mice (11 mice analyzed) (Figure 3A). In contrast, most joints from the AdExTek-treated mice (12 mice analyzed) exhibited either mild infiltration or no sign of infiltration, except for some joints that developed high-grade arthritis and therefore displayed visible synovial infiltration (Figure 3B). The ameliorated inflammation correlated well with the reduced angiogenesis in the AdExTek-treated mice, which further supports a beneficial role of targeting Tie2 function in arthritis treatment.

Disease attenuation not attributable to inhibition of collagen-specific immunity. Given that the CIA model of RA is also an autoimmune disease in which antibody production is an active component, we examined the effects of AdExTek on T cell–dependent antibody production in vivo by measuring bovine CII–specific IgG, IgG1, and IgG2a antibodies. Serum samples were collected at 3 weeks and 6 weeks postimmunization. Antibodies in sera were measured by ELISA. As shown in Figure 4, there was no significant difference in the levels of all 3 CII-specific antibodies between the AdExTek-treated group and the control group. The results reveal that inhibition of disease by AdExTek is not attributable to suppressed antibody production. This finding supports our claim that ameliorated arthritis in the AdExTek-treated group is secondary to inhibition of angiogenesis.

Inhibition of CIA progression by AdExTek. Our data showed that systemic delivery of ExTek prior to development of the disease phenotype significantly inhibited the onset, incidence, and severity of arthritis (Figures 1–3). We next explored whether blocking Tie2

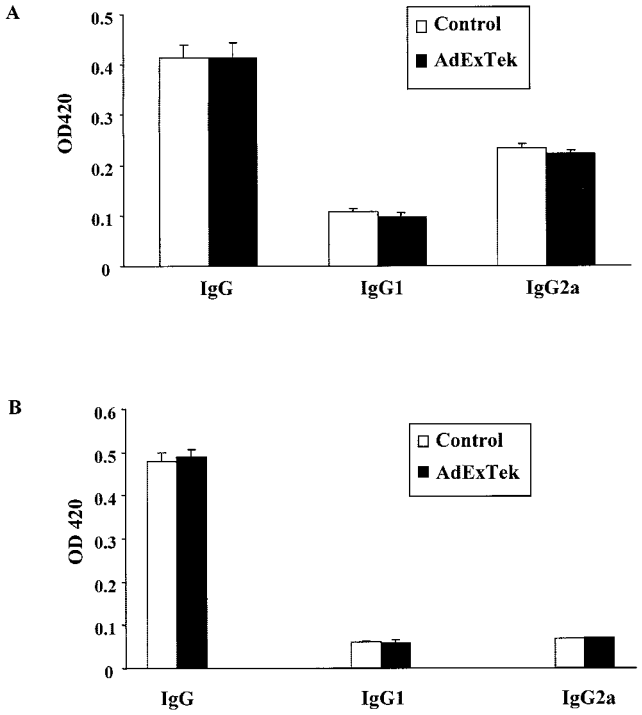

Figure 4. Type II collagen (CII)–specific antibody production in AdExTek-treated and control vector–treated groups. Serum samples were collected 3 weeks (**A**) and 6 weeks (**B**) after the initial immunization with bovine CII. Anti-CII–specific IgG, IgG1, and IgG2a levels were measured by enzyme-linked immunosorbent assay. Values are the mean and SEM from 3 independent experiments. OD = optical density.

with AdExTek could inhibit disease progression after disease onset. On day 28 after the primary immunization, at which time some of the mice had first developed clinical signs of arthritis, we monitored the clinical signs of arthritis in each mouse, and then divided the mice into 2 groups according to the similarity of disease severity at baseline. Next, the mice were treated with intravenous injections of AdExTek or control AdPacβ-gal, respectively. ExTek protein expression was easily detected in the circulation in the AdExTek-treated mice 3 days after injection (Figure 1). Arthritis development was monitored every other day, as previously described. As shown in Figure 5, both groups displayed similar baseline characteristics on day 28 (mean $\pm$ SEM arthritis score 0.75 $\pm$ 1.76 in both groups), and AdExTek treatment inhibited disease progression. The severity of CIA in mice treated with AdExTek (n = 12) was significantly lower than that in the control group (n = 12) at 35 days postimmunization (mean $\pm$ SD arthritis score 6.08 $\pm$ 4.1 in the control group and 1.73 $\pm$ 2.69 in the AdExTek group; $P < 0.05$). However, the difference diminished thereafter; this decrease was possibly attributable to diminished ExTek expression in vivo. The disease incidence was also reduced in the AdExTek-treated group compared with controls, but the difference was not statistically significant.

Protection against bone destruction by AdExTek. Bone erosion is a pathognomonic feature of RA that is not observed in other forms of arthritis. The resulting deformities represent a major reason for the progressive disability and impairment of patients with RA (39). Therefore, radiographs of the paws were obtained to examine erosive changes as a surrogate for joint destruc-

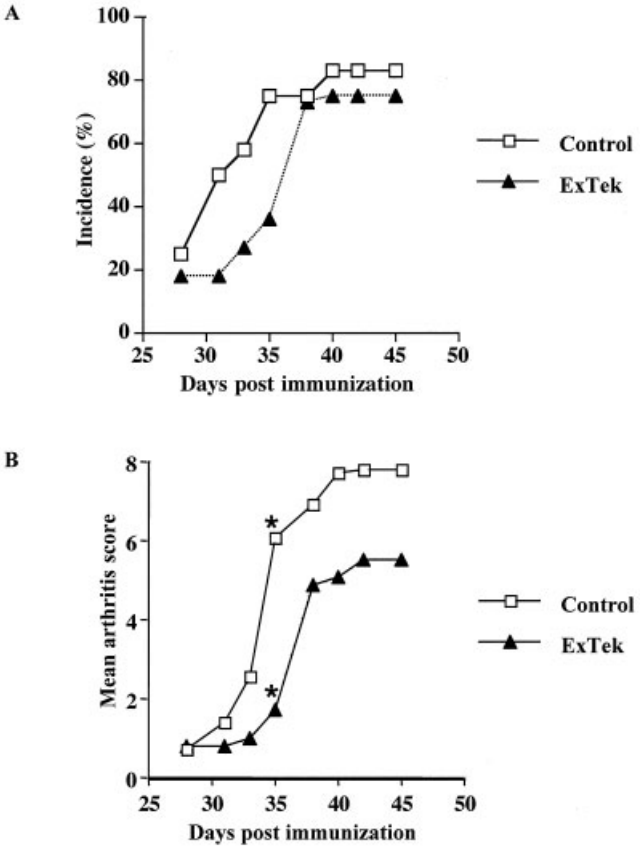

Figure 5. Graphs showing inhibition of CIA progression even when treatment was given after disease onset. DBA/1j mice were immunized with bovine type II collagen to induce arthritis. On day 28, mice that had developed clinical signs of arthritis were divided into 2 groups of 12 mice each. The mice were treated with intravenous injections of AdExTek or AdPacβ-gal, respectively. The incidence and severity of arthritis were examined during the course of CIA by 2 independent investigators in a blinded manner. Results from 2 independent experiments were pooled and are presented as the incidence (**A**) and the mean arthritis score (**B**). $* = P < 0.05$. See Figure 1 for definitions.

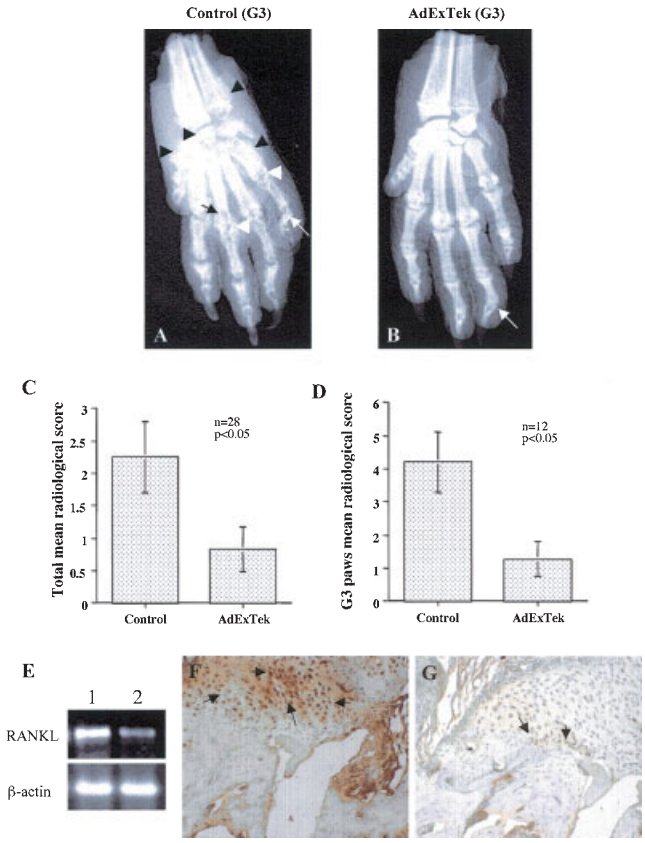

Figure 6. AdExTek treatment protected against bone damage. Six weeks postimmunization with bovine type II collagen, paws from the AdExTek-treated group and the control group were imaged for bone damage using phase-contrast radiography. Representative paws with clinical grade 3 (G3) from the control group (**A**) and the AdExTek-treated group (**B**) are shown. In **A, black arrowheads** indicate periosteal new bone formation, **black arrows** indicate demineralization, and **white arrowheads** indicate joint space narrowing and deformity. **White arrow** in **A** and **B** indicates subluxation. Joint damage was quantified (mean ± SEM) in total joints from both groups (13 control mice and 15 ExTek-treated mice) (**C**) and in grade 3 paws from both groups (7 control mice and 5 ExTek-treated mice) (**D**) based on periarticular demineralization, periosteal new bone formation, and joint space narrowing/deformity. Reverse transcription–polymerase chain reaction was used to measure RANKL levels in grade 3 control (lane 1) and ExTek-treated (lane 2) joints (**E**). β-actin was used as a loading control. Immunohistochemical analysis for RANKL expression in grade 3 control (**F**) and ExTek-treated (**G**) joint tissues is shown. In **F** and **G**, **arrows** indicate RANKL-positive cells. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

tion. We adopted state-of-the-art phase-contrast radiography with very high–resolution digital radiographs (37,40), which allowed us to image the bone changes in all 4 paws of the mice. Mouse paws at 6 weeks postimmunization were examined by phase-contrast radiography, and joint damage was quantified in a blinded manner based on periarticular demineralization, periosteal new bone formation, and joint space narrowing/deformity (Figures 6A and B). Our data showed that the mean radiographic score for all mouse paws was significantly lower ($P < 0.05$) in the AdExTek treatment group (n = 15) than in the control group (n = 13) (Figure 6C). Most interestingly, even among paws with equivalent degrees of clinical signs, especially clinical grade 3, those in the AdExTek treatment group (n = 5) displayed significant protection against bone damage ($P < 0.05$) compared with those in the control group (n = 7) (Figure 6D). Collectively, these data demonstrated that systemic delivery of ExTek inhibits RA progression even after disease onset and lessens bone damage in the joints.

Osteoclasts are responsible for bone erosion. Therefore, we performed tartrate-resistant acid phosphatase staining to determine the number of osteoclasts on sections obtained from joints with grade 3 arthritis. We did not observe any significant difference in the number of osteoclasts between the control group and the AdExTek-treated group (data not shown), which indicates that targeting Tie2 action does not affect production of osteoclasts. Next, we examined the expression of RANKL, an important mediator of bone erosion, in joint tissues. We isolated total RNA from both ExTek-treated and control joints with grade 3 arthritis and performed RT-PCR for RANKL. Interestingly, we observed a decrease of RANKL in the AdExTek-treated group compared with controls (Figure 6E). We also performed immunohistochemical analysis for RANKL expression in joint tissue sections from both groups. The results were consistent with the RT-PCR results showing higher levels of RANKL in the control joint sections than in the ExTek-treated sections (Figures 6F and G). Taken together, our results suggest that inhibition of Tie2 function in endothelium affects RANKL expression, which may contribute to bone protection.

DISCUSSION

Angiogenesis is an essential step in the development of arthritis. In this study, we demonstrate that systemic delivery of a Tie2 inhibitor (ExTek) using a gene therapy approach (AdExTek) significantly inhibited disease onset, as well as the incidence and severity of arthritis in a CIA model. AdExTek treatment was able to inhibit arthritis progression even after initiation of the disease. Reduced disease development and progression correlated with a significant reduction of neo-

vascularization in arthritic synovium, suggesting that inhibition of the disease is secondary to inhibited angiogenesis. Most strikingly, we show that neutralization of Tie2 protects against bone destruction in the CIA model, possibly through inhibition of RANKL production, which reveals a significant clinical benefit of targeting this signaling pathway in arthritis therapy.

Angiogenesis is a multistep process that requires sequential activation of different growth factors and their cognate receptors. Of the molecular mechanisms identified to date, activation of endothelial receptor tyrosine kinases (RTKs) by polypeptide growth factors appears to play a pivotal role in blood vessel growth and differentiation (41,42). Importantly, RTKs for 2 families of angiogenic growth factors, the VEGF receptor (VEGFR) family and the Tie2 family, are expressed predominantly on vascular endothelial cells, making them attractive targets for antiangiogenic therapy (41,42). VEGF and its receptors are present in arthritic synovium, and neutralization of VEGF function inhibited arthritis development in animal models (13,16,17). In a previous study, we observed significantly elevated levels of Tie2 and its ligand, Ang-1, in human RA synovium (32). Consistent with our report, several studies have also shown the presence of Tie2 and Ang-1 in arthritic synovium (26–29). We further demonstrated, using a synovium vascular window model, that blocking Tie2 action with AdExTek significantly inhibited angiogenesis in arthritis (32). Results of our current study further illustrate the therapeutic potential of targeting the Tie2 signaling pathway in arthritis treatment.

Results of genetics studies indicate that the Tie2 and VEGFR pathways work in a coordinate and collaborative manner in regulating angiogenesis, with VEGFR acting during the early stages of vessel development and Tie2 acting later to promote angiogenic remodeling as well as vessel maturation and stabilization (41,42). We have shown that by blocking Tie2 function using ExTek, adult angiogenesis and tumor angiogenesis were inhibited, even in the presence of VEGF (20,22). Neutralization of either the Tie2 pathway or the VEGFR pathway for a mammary tumor could significantly block tumor angiogenesis and tumor growth (20,22). These results suggest that the Tie2 and VEGFR pathways are 2 independent mechanisms essential for tumor angiogenesis. This observation was further confirmed by another study of a human melanoma, A375v (24). The ability of AdExTek to inhibit angiogenesis despite the presence of VEGFR suggests that disrupting the angiogenic program at stages distal to endothelial activation will provide effective and perhaps more globally useful inhibitors of pathologic neovascularization. In the setting of arthritis development, it would be of great interest to compare the effects of blocking both Tie2 and VEGFR signaling with those of blocking the signaling of either one of them.

The significant inhibitory effect of AdExTek on arthritis supports the notion that angiogenesis is an essential step in arthritis development and progression. Indeed, we observed robust angiogenesis at a very early stage, days before appearance of the clinical signs of disease onset (Figure 2), which is consistent with a report that angiogenesis starts very early and may even antedate the specific clinical and histologic signs of inflammation in human RA (43). Furthermore, the therapeutic effects were more profound when the treatment was given before disease onset rather than after its initiation, which implies that it might be easier to inhibit vascular formation than to induce vascular regression after the establishment of matured vessels. Antiangiogenic therapy requires long-term delivery of inhibitors in vivo. Although the adenoviral vector is very efficient in transducing genes in vivo, transgene expression ceases after 1 week to 10 days, due to host immune surveillance. It appeared that ExTek expression correlated with inhibition of the disease. The significant differences in disease development and progression between the treated group and the control group diminished at a later time. With the fast development of better viral vectors that are much less immunogenic or not immunogenic at all, this limitation should be solved in the near future.

Bone destruction in later stages of arthritis is one of the major reasons for the progressive disability and morbidity in patients with RA (39). Surprisingly, we observed significant protection against bone damage in the AdExTek-treated joints compared with controls, even when the mice showed identical clinical signs. This protection most likely results from reduced angiogenesis, which evidently would limit inflammatory cell infiltration. Reduced angiogenesis also means a limited nutrient supply and a reduction in the levels of inflammatory cytokines and growth factors that could be either transported by the blood vessels or directly produced by the vascular endothelium.

The molecular mechanism responsible for bone destruction in RA is less clear. Several lines of evidence support a role for osteoclasts in the pathogenesis of bone erosion in RA (29). Therefore, we examined the effects of ExTek on osteoclasts in joint tissues. However, we did not observe any significant difference in the number of osteoclasts between the control and ExTek-treated

groups, indicating that targeting Tie2 action does not affect the production or recruitment of osteoclasts. Additionally, we measured RANKL levels, an important mediator of bone destruction, in grade 3 joint tissues from both groups. Interestingly, we observed a significant decrease in RANKL levels in ExTek-treated joint tissues compared with those from controls. These findings imply that blockade of Tie2 activation by ExTek inhibited RANKL expression and thus might contribute to bone protection. TNFα plays a central role in RA, not only in inflammation but also in bone destruction. TNFα-transgenic mice develop a spontaneous and destructive polyarthritis with rapidly progressive destruction of bone, which mimics human RA in many aspects (44). Anti-TNFα therapies not only effectively block synovial inflammation but also affect tissue destruction (7,45). Results of our previous study showed that TNFα up-regulates Tie2 signaling in multiple ways. Collectively, these findings indicate that TNFα might regulate RANKL production in endothelial cells via Tie2 activation.

Quantitative radiographic scoring of RA in humans has been in use for decades, providing unique information that is not available by clinical examination (46). However, there is a lack of similar approaches in animal studies, especially in mouse models. In this study, we adapted a method of phase-contrast radiography, which is a new class of plain radiography based on the shifts of x-rays as they pass through an object (47). Conventional radiography is based on the absorption of x-rays rather than on their displacement. A phase-contrast radiograph contains all of the absorption information of a conventional radiograph plus the additional information related to x-ray shifts (evident predominantly at the edges of objects). It offers very high resolution and a digital image. Recent advancements indicated that phase-contrast radiography might have a role in arthritis imaging in the near future (37,40,48). Because of the availability of different transgenic mouse strains for arthritis studies, this technique should be very valuable.

In conclusion, this study clearly shows the clinical, histologic, and radiologic benefits of specific Tie2 blockade on disease onset, progression, and bone destruction in the CIA mouse model. These findings identify a new molecular target for arthritis treatment. It opens up new possibilities for RA treatment strategies and warrants clinical trials of specific Tie2 inhibitors for the treatment of arthritis.

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