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Arginine-Rich Anti-Vascular Endothelial Growth Factor (Anti-VEGF) Hexapeptide Inhibits Collagen-Induced Arthritis and VEGF-Stimulated Productions of TNF-α and IL-6 by Human Monocytes[1]

Seung-Ah Yoo,* Dong-Goo Bae,‡ Jae-Woong Ryoo,* Hae-Rim Kim,* Gyeong-Sin Park,† Chul-Soo Cho,* Chi-Bom Chae,‡ and Wan-Uk Kim[2]*

Vascular endothelial growth factor (VEGF) has been suggested to play a critical role in the pathogenesis of rheumatoid arthritis (RA). We previously identified a novel RRKRRR hexapeptide that blocked the interaction between VEGF and its receptor through the screening of peptide libraries. In this study, we investigated whether anti-VEGF peptide RRKRRR (dRK6) could suppress collagen-induced arthritis (CIA) and regulate the activation of mononuclear cells of RA patients. A s.c. injection of dRK6 resulted in a dose-dependent decrease in the severity and incidence of CIA and suppressed synovial infiltration of inflammatory cells in DBA/1 mice. In these mice, the T cell responses to type II collagen (CII) in lymph node cells and circulating IgG Abs to CII were also dose-dependently inhibited by the peptides. In addition, VEGF directly increased the production of TNF-α and IL-6 from human PBMC. Synovial fluid mononuclear cells of RA patients showed a greater response to VEGF stimulation than the PBMC of healthy controls. The major cell types responding to VEGF were monocytes. Moreover, anti-VEGF dRK6 inhibited the VEGF-induced production of TNF-α and IL-6 from synovial fluid mononuclear cells of RA patients and decreased serum IL-6 levels in CIA mice. In summary, we observed first that dRK6 suppressed the ongoing paw inflammation in mice and blocked the VEGF-induced production of proinflammatory cytokines. These data suggest that dRK6 may be an effective strategy in the treatment of RA, and could be applied to modulate various chronic VEGF-dependent inflammatory diseases. ***The Journal of Immunology,*** **2005, 174: 5846–5855.**

Rheumatoid arthritis (RA)[3] is characterized by a tumor-like expansion of the synovium, which is composed of proliferating synoviocytes, infiltrating leukocytes, and new blood vessels (1). Angiogenesis, the process of new blood vessel formation, is highly active in RA, particularly at the early onset of the disease (2). The newly formed vessels can maintain the chronic inflammatory state by transporting the inflammatory cells to the site of synovitis, as well as supplying nutrients and oxygen to the pannus. Angiogenesis is tightly regulated by many inducers and inhibitors. A number of angiogenic factors have been suggested for their involvement in the neovascularization in RA joints (as reviewed in Refs. 3 and 4). These include acidic and basic fibroblast growth factors, TGF-β, angiogenin, placenta growth factor, and vascular endothelial growth factor (VEGF).

VEGF is a dimeric glycoprotein that induces the proliferation and migration of endothelial cells to form new blood vessels and increases the vascular permeability (3, 4). Interestingly, recent studies have demonstrated that VEGF directly activates NF-κB, and induces IL-8 and MCP-1 production by endothelial cells (5, 6), suggesting it promotes leukocyte recruitment into the site of neovascularization. In support of this notion, VEGF has been implicated in a variety of chronic inflammatory diseases including RA. In RA, VEGF appears in increased amounts in the sera, synovial fluid (SF), and inflamed synovium of patients (7–9) and, thus, constitutes a potential candidate for therapeutic modulation. For example, treatment with anti-VEGF Ab has been shown to attenuate collagen-induced arthritis (CIA) in mice (10). Again, specific inhibition of VEGF by soluble VEGFRs reduced the disease severity in murine CIA (11).

VEGF exerts its activity through binding to its identified receptors, Flt-1 and Flk-1, which are both present on endothelial cells. Flk-1 has strong intrinsic tyrosine kinase activity and is the major mediator of endothelial cell proliferation in response to VEGF (as reviewed in Refs. 12 and 13). However, unlike Flk-1, Flt-1 is also present on inflammatory cells, such as macrophages and monocytes. Moreover, a recent study has shown the increased expression of cytochemokines, including MCP-1, IL-8, TNF-α and IL-1β, in peripheral blood monocytes treated with placenta growth factor (14), which has a strikingly similar three-dimensional structure to VEGF (15). Therefore, it is possible that the VEGF-Flt-1 complex may activate inflammatory cells in the RA joints through the induction of proinflammatory cytokines, although the direct effect of VEGF on human monocytes remains to be defined.

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; VEGF, vascular endothelial growth factor; CIA, collagen-induced arthritis; SF, synovial fluid; SFMC, SF mononuclear cell; Dexa, dexamethasone; FLS, fibroblast-like synoviocyte; CII, type II collagen; LN, lymph node.

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Through the screening of peptide libraries, we have identified a soluble arginine-rich hexapeptide sequence (e.g., RRKRRR), which could bind to the VEGF and thereby block the interaction between $VEGF_{165}$ and the VEGFR (16). The present study shows that ongoing paw inflammation in CIA animals was inhibited by the RRKRRR peptide (dRK6), synthesized with D-amino acids to enhance its stability, in a dose-dependent manner. VEGF directly increased the production of TNF-α and IL-6 from human monocytes via a receptor-coupling event. Synovial fluid mononuclear cells (SFMC) of RA patients showed a greater response to VEGF stimulation than the PBMC of healthy controls. Moreover, dRK6 inhibited the VEGF-induced production of TNF-α and IL-6 from SFMC of RA patients and decreased serum IL-6 levels in CIA mice. These results suggest that anti-VEGF RRKRRR peptides, or their analogues, may be effective agent(s) for the treatment of RA through the suppression of the VEGF-stimulated production of TNF-α and IL-6.

Materials and Methods

Reagents

The dRK6 was synthesized at the Pohang University of Science and Technology in Korea, as described previously (16). The human recombinant $VEGF_{165}$ was purchased from R&D Systems. The anti-Flt-1 mAb and isotype control mAb were obtained from R&D Systems. For the flow cytometry analysis, PE-conjugated anti-CD14 mAb, PE-conjugated anti-CD3 mAb, FITC-conjugated anti-TNF-α mAb and FITC-conjugated anti-IL-6 mAb were obtained from BD Pharmingen. For ELISA, the recombinant human TNF-α, IL-6, and VEGF, together with the mAbs to these cytokines, were also purchased from R&D Systems. PHA, PMA, ionomycin, LPS, and dexamethasone (Dexa) were purchased from Sigma-Aldrich. The reagents used for the culture were found to contain <200 pg/ml endotoxin, as determined by the *Limulus* amebocyte cell lysate assay.

Effect of dRK6 on the interaction of ^{125}I-VEGF$_{165}$ with its receptors on HUVEC

HUVEC (Cambrex) were cultured, as described previously (17). The cells from passages 3 to 6 were used in this study. Seeded HUVEC (5×10^4 cells/well) incubated at 37°C overnight and washed with warm binding buffer containing 25 mM HEPES (pH 7.4), 0.1% BSA in serum-free Medium 199 at 37°C for 2 h. The cultures were then preincubated with various concentrations of dRK6 in 200 μl of binding buffer at 37°C for 1 h, and then transferred to 4°C on an oscillating platform set at 1 cycle/s. Radiolabeled VEGF (1903 Ci/mM, 20 nCi/well; Amersham Biosciences) with or without antagonists were incubated at 4°C for 3 h. Nonspecific binding was determined by binding experiments in the presence of 100-fold excesses of nonlabeled VEGF. After washing the cells twice with cold binding buffer, followed by washing in cold PBS/0.1% BSA, the cells were solubilized by the addition of 0.25 ml of 20 mM Tris-HCl (pH 7.4) containing 1% Triton X-100 at room temperature for 20 min on an oscillating platform set at 2 cycles/s, and the receptor-bound radioactivity was determined in a gamma counter. Experiments were conducted in duplicate and more than twice. The variation between duplicate determinations in an experiment was <10%.

Stability of RK6 and dRK6 in rat serum

Blood from animals (16-wk-old male Sprague Dawley rats) was collected in sterile centrifugation tubes, allowed to clot at 4°C, and then centrifuged twice at $1,500 \times g$ for 10 min at 4°C and $20,000 \times g$ for 20 min at 4°C. The supernatant (serum) was filtered with a sterile filter (0.22 μm; Millipore) and stored in small aliquots at −70°C until used. For estimating the stability of each peptide in serum, RK6 or dRK6 peptide (100 μg in 50 μl of PBS) was incubated with 50 μl of filtered rat serum at 37°C for the indicated time. The incubated sample was fractionated by C18 reverse phase HPLC with linear gradient of acetonitrile (Vydac protein and peptide C18, 0.1% trifluoroacetate in H_2O for equilibration, and 0.1% trifluoroacetate in acetonitrile for elution). The amount of peptide was estimated by comparing the area and height of each peak between the incubated serum and nonincubated peptide, and also serum incubated without peptide. To confirm the identity of the peak from the profiles of C18 reverse phase HPLC, each peak was collected, lyophilized, and analyzed by mass spectrometry. Original RK6 (100 pM), dRK6 (100 pM), and lyophilized samples from column peak were analyzed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (TOF/TOF; Applied Biosystems). All mass spectroscopy spectra were recorded in positive reflector mode. For mass spectroscopy data, 2000 shots were accumulated for each spectrum obtained from the TOF/TOF.

Determination of the effect of dRK6 in CIA

Male DBA/1 mice, obtained from The Jackson Laboratory, were maintained in groups of three to five in polycarbonate cages and fed standard mouse chow (Ralston Purina) and water ad libitum. The environment was made specifically pathogen-free for the mice. Neonatal mice were obtained by breeding the mice in our facility. The mice were immunized with native bovine type II collagen (CII), a generous gift from Dr. A. H. Kang (University of Tennessee, Memphis, TN), at 8 to 12 wk of age, as described previously (18). Briefly, CII was dissolved in 0.05 N acetic acid at 2 mg/ml, and emulsified (1:1 ratio) with CFA at 4°C. The mice received 0.1 ml of the emulsion, containing 100 μg of CII, in the base of the tail as a primary immunization. Booster injections were given into the footpad with 50 μg of CII, similarly dissolved and emulsified with CFA (1:1), 14 days after the primary immunization.

To determine the therapeutic potential of dRK6 peptides in RA, we tested whether the peptides would inhibit disease progression in mice. In a preliminary study, CIA developed as early as 3 wk, peaked at 5–7 wk, and thereafter spontaneously resolved to 10 wk (data not shown). Therefore, from 3 wk after the primary immunization, we injected s.c. 25 or 50 μg of dRK6, dissolved in PBS, in the CIA animals every other day for 3 wk. The control mice received PBS alone. The incidence and severity of arthritis in the two groups of mice were determined by visual inspection. The mice were observed two to three times a week for the onset, duration, and severity of joint inflammation over a period of 10 wk after the primary immunization. Each limb was assessed on a 0- to 4-point scale, as described earlier (18). The hindfoot that received the booster immunization was excluded from the evaluation. Therefore, the maximum arthritis score possible was 12. The mean arthritic index and incidence of arthritis (%) were used for data comparison between the experimental groups.

Histological examinations

At day 42, mice were killed for histological analysis. All three paws and ankles, except the hindfoot that received the booster immunization, were harvested from each mouse and fixed overnight in 10% formalin, decalcified in 30% citrate-buffered formic acid for 3 days at 4°C, dehydrated in a graded series of methanol and xylene, and then embedded in paraffin. Thin sections (4-μm thick) were stained with H&E, and histopathologic scoring was performed under the light microscope by a blinded observer. The degree of synovial hyperplasia, inflammation, and pannus formation in the joints was analyzed using a standard scoring protocol (19), where the severity was scored on a scale from 0 to 3; score 0, absent; score 1, weak; score 2, moderate; score 3, severe. The maximal total score possible was 9.

Assay for IgG Abs to CII

Sera were collected from each group of mice on day 42 after the primary immunization, and stored at −20°C until assayed. The IgG anti-CII levels in the sera were determined by a commercially available ELISA kit (Chondrex), as previously described (18). The OD of the standard serum, which was serially diluted 2-fold, was expressed as 200, 100, 50, 25, 12.5, and 6.25 arbitrary units, respectively. The relationship of the OD measured in the standard serum, diluted serially, and the arbitrary units showed good linear correlation in all determinations ($r > 0.98$, data not shown). The IgG anti-CII concentrations in the sera, diluted 1/10,000 to 1/40,000, are presented as relative values (arbitrary units) compared with the OD of the standard sera.

Assessment of T cell proliferation

Inguinal and popliteal lymph nodes (LN) were removed from each group of mice 42 days after the primary immunization, and washed in RPMI 1640. Tissues were pooled from four to five mice, minced into single cell suspensions in RPMI 1640, and washed three times with RPMI 1640. Cells (5×10^5/well) were cultured in 96-well microtiter plates (Nunc) with or without 40 μg/well CII in 0.3 ml of Click's medium (Irvine Scientific) (20), supplemented with 0.5% mouse serum, at 37°C in a 5% humidified CO_2 for 4 days. To assess the rate of proliferation, 1 μCi of [^{3}H]thymidine was added to each well, and the cells were harvested 18 h later onto glass fiber filters and counted on a Matrix 96 direct ionization beta counter (Packard Instrument). The data are presented as the stimulation indices, which was calculated as the ratio of cpm in the presence of CII, divided by cpm without CII.

Isolation and culture of mononuclear cells

Heparinized peripheral blood (100 ml) was sterilely collected from 15 healthy controls (10 females and 5 males), after obtaining their informed consent, and diluted 1/1 with RPMI 1640. SF from 11 patients, who fulfilled the revised criteria of the American Rheumatism Association for RA (21), was collected by arthrocentesis into sterile tubes, diluted 1/5 with PBS immediately after collection, and passed through sterile gauze, as previously described (22). The mean age of the patients (9 females and 2 males) was 42.6 years. The mean disease duration was 23.7 mo. Eight of 11 patients had a positive rheumatoid factor.

Mononuclear cells in the peripheral blood or SF were isolated by density gradient centrifugation on Ficoll-Hypaque (SG 1077). The cell viability was >95% by trypan blue exclusion. Mononuclear cells were resuspended in complete medium, consisting of RPMI 1640 supplemented with 10% FCS (Invitrogen Life Technologies), 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM L-glutamine. Each culture was performed in triplicate at a density of 1×10^6/well in 96 microtiter wells (Nunc) at 37°C in a 5% CO_2 atmosphere, after which variable concentrations of $VEGF_{165}$ (0.1–10 ng/ml; R&D Systems) were added to the wells. The mitogens, including 1 μg/ml PHA, 50 ng/ml PMA plus 5 μg/ml ionomycin and 5 μg/ml LPS, were used as positive control Ags to stimulate the mononuclear cells. Anti-Flt-1 mAb was added to the mononuclear cells 1 h before the VEGF treatment for the determination of the specificity of the VEGF action. Again, to block the interaction of the VEGF and its receptor, various concentrations of dRK6, ranging from 0 to 100 μM, were pre-incubated with 10 ng/ml VEGF for 1 h at room temperature, and then the VEGF and dRK6 mixture was added to the mononuclear cells. In some experiments, the adherent cells were separated from the SFMC by incubation of the mononuclear cells in a petri dish for 2 h, as described previously (23), and then cultured in RPMI 1640 medium at a density of 0.6×10^5/well. The adherent cells isolated by this procedure had a surface marker of CD14 in the range of ~75%. After 24 h of incubation (unless otherwise stated), the cell-free supernatants were collected, and stored at −20°C until assayed.

Isolation and culture of RA synoviocytes

The fibroblast-like synoviocytes (FLS) were prepared from the synovial tissues of five RA patients that were undergoing total joint replacement surgery. Isolation of the FLS from the synovial tissues was performed according to the procedure described previously (24). Briefly, tissues were minced into 2- to 3-mm pieces, and treated for 4 h with 4 mg/ml type I collagenase (Worthington Biochemical) in DMEM at 37°C in a 5% CO_2 atmosphere. Dissociated cells were then resuspended in DMEM, supplemented with 10% FCS, 2 mM glutamine, penicillin, and streptomycin, and then plated in 75-cm^2 flasks. After overnight culturing, the nonadherent cells were removed, and adherent cells were cultivated in DMEM plus 10% FCS. Cultures were kept at 37°C in a 5% CO_2 atmosphere, and the medium was replaced every 3 days. At confluence, the cells were passed by diluting 1/3 with fresh medium and recultured until used.

Synoviocytes, from passages 4 through 8, were used for each experiment. The cells were morphologically homogenous and had the appearance of FLS with typical bipolar configuration under inverse microscopy. The purity of the cells was tested by flow cytometry analysis (>95% CD90, <2% CD14, <1% CD3, and <1% CD19 positive). The FLS cell lines were seeded in triplicate into 24-well plates (Nunc) at a density of 2×10^4 cells/well in DMEM, supplemented with 5% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. After 24 h of incubation (unless otherwise stated), cell-free media were collected and stored at −20°C until assayed.

ELISA for TNF-α, IL-6, and $VEGF_{165}$

The amounts of TNF-α and IL-6 released into the culture supernatants were measured by ELISA, as previously described (25). Also, the levels of TNF-α, IL-6, and $VEGF_{165}$ were measured by ELISA in the SF of the RA patients. Recombinant human TNF-α, IL-6, and $VEGF_{165}$ (R&D Systems), diluted in culture medium, were used as a calibration standard, ranging from 5 to 2000 pg/ml. A standard curve was drawn by plotting the OD vs the log of the concentration of the above recombinant cytokines.

Flow cytometry analysis

The surface-Ag expressions and intracellular production of TNF-α and IL-6 were measured with a BD Cytofix/Cytoperm kit manual (BD Pharmingen). Briefly, after stimulation of the mononuclear cells with different concentrations of VEGF (0.1 to 10 ng/ml) or mitogens for 12 h, 1 μM monensin (GolgiStop; BD Pharmingen) was added to the cultures during the last 2 h of culturing. Viable cells were harvested, incubated for 20 min on ice in blocking buffer (PBS with 10% normal rat serum and 0.02% 1 M sodium azide), and subsequently stained with PE-conjugated anti-CD14 mAb (BD Pharmingen), or PE-conjugated anti-CD3 mAb (BD Pharmingen), for 30 min at 4°C in the dark. The cells were washed twice in staining buffer (PBS containing 2% FCS and 0.02% 1 M sodium azide) and resuspended in 100 μl of fixation buffer (Cytofix/Cytoperm) for 20 min on ice. The fixed cells were washed twice in permeabilization buffer and stained with FITC-labeled anti-TNF-α mAb (BD Pharmingen), or FITC-labeled anti-IL-6 mAb (BD Pharmingen), for 30 min at 4°C in the dark. Finally, the

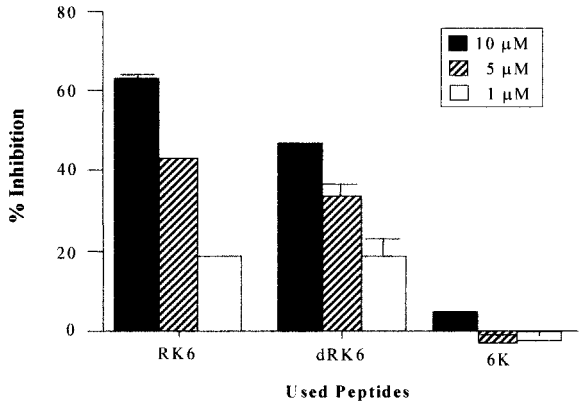

FIGURE 1. Effects of RK6 and dRK6 on the binding of VEGF to cell surface receptor. *A*, Effect of dRK6 on the binding of VEGF to cell surface receptor. HUVECs were incubated with ^{125}I-$VEGF_{165}$ and three different kinds of peptide, RRKRRR (L- or D-amino acids) and KKKKKK. Activity of each peptide was compared for inhibitory effect on the binding of radiolabeled $VEGF_{165}$ to its receptors on the surface of HUVEC. The *y*-axis shows the percentage of inhibition of ^{125}I-$VEGF_{165}$ binding to its receptors on the surface of HUVEC. Total binding and nonspecific binding were 3566 ± 73 cpm and 145 ± 1 cpm, respectively. RK6, dRK6, and 6K denote RRKRRR, rrkrrr, and KKKKKK, respectively, where capitals represent L-amino acids and small characters D-amino acids.

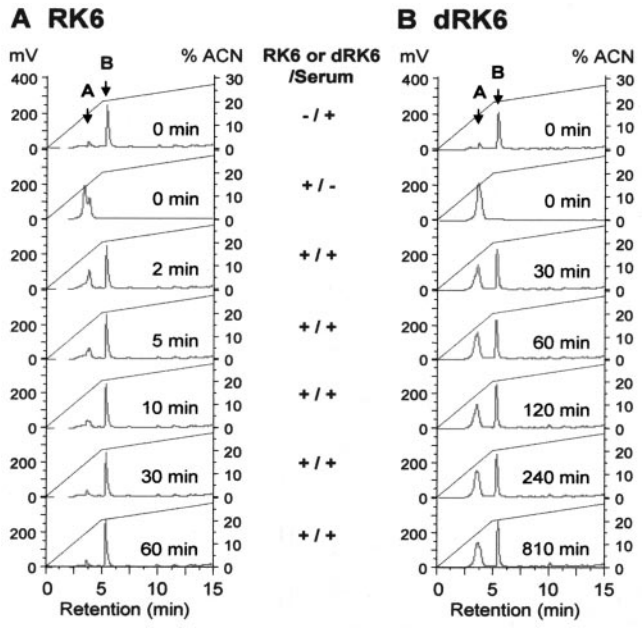

FIGURE 2. Stability of RK6 and dRK6 in rat serum. RK6 (100 μg) or dRK6 peptide (100 μg) was incubated with rat serum at 37°C for the indicated time of each panel, and the incubated sample was fractionated by C18 reverse-phase HPLC, and the amount of each peptide was estimated as described in *Materials and Methods*. For each HPLC profile, solid and dashed lines represent the absorbance (left *y*-axis) and percentage of acetonitrile (%ACN; right *y*-axis), respectively.

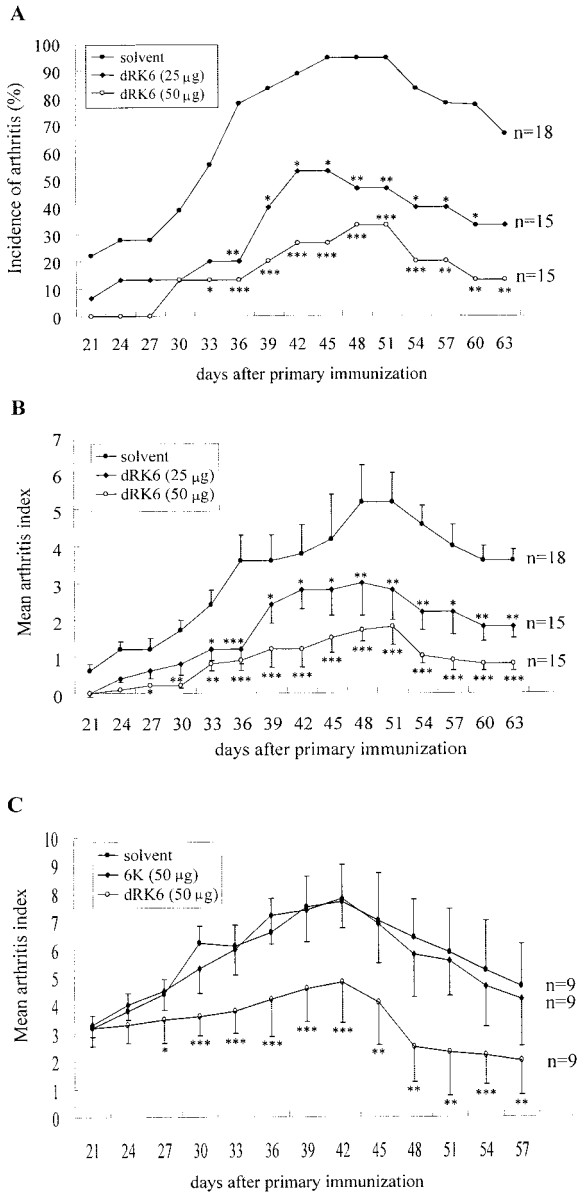

FIGURE 3. Suppression of CIA by dRK6. From 3 wk after the primary immunization, 25 or 50 μg of dRK6 dissolved in PBS were injected in the CIA animals every other day for 3 wk. The control mice received PBS (solvent) alone. The severity and incidence of arthritis were determined in the three groups of mice by a visual inspection. *A*, Reduction in the incidence of arthritis in peptide-treated mice during the course of CIA. *B*, Decrease in the mean arthritis index in mice injected with the peptides. *C*, No effect of hexalysin (6K), positively charged control peptides, on the development of arthritis. The data is presented as the mean ($\pm$ SD) values. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs mice treated with PBS alone.

cells were washed twice and resuspended in staining buffer, and then analyzed on a FACScan cytometer (BD Biosciences).

Statistical analysis

Data are expressed as the mean $\pm$ SD. Comparisons of the numerical data between groups were performed by paired or unpaired Mann-Whitney *U* test and of the categorical data by a χ^2 test or Fisher's exact probability test, as appropriate. Correlations between two variables were performed using Spearman's rank correlation coefficient. Values of $p < 0.05$ were considered statistically significant.

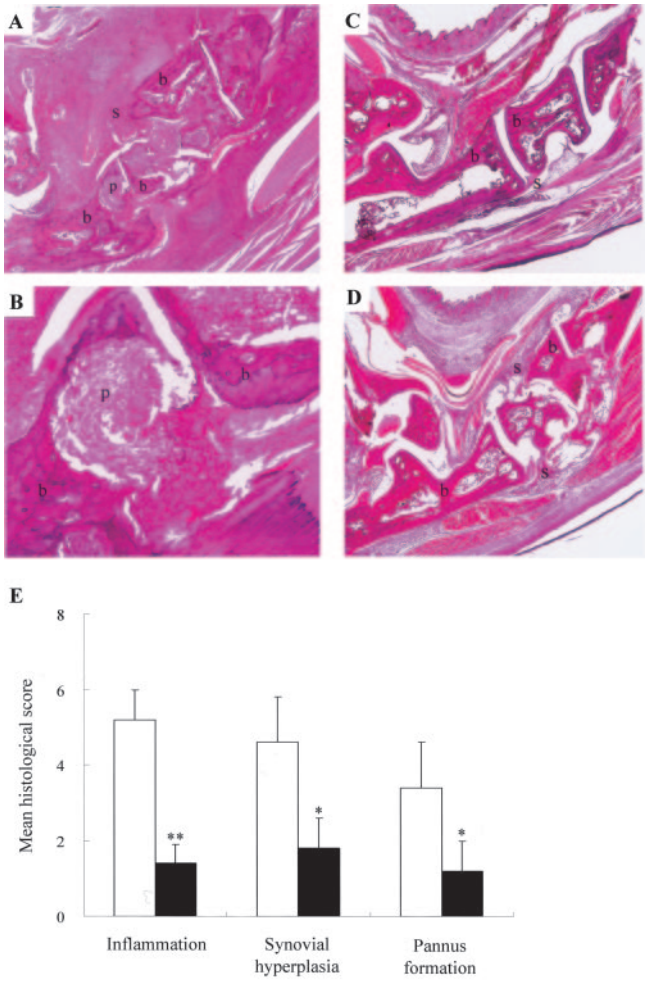

FIGURE 4. Histological examinations of joints from mice treated with PBS or dRK6. Mice were killed on day 42 after primary immunization. All paws and ankles except the hindfoot that received the booster immunization were harvested from each mouse and stained with H&E. *A* and *B*, Joints from a mouse treated with PBS; *A* (magnification, $\times$40) shows erosive and destructive arthritis in tarsal joints. Articular spaces are filled with inflammatory cells and a rheumatoid-like pannus (*B*, magnification, $\times$200). *C* and *D*, Joints from two mice administered 50 μg of dRK6. Mice show milder arthritis in ankle and tarsal joint (magnification, $\times$40) with less erosion, inflammation, and pannus formation. *E*, Histological score of the degree of synovial hyperplasia, inflammation, and pannus formation in mice treated with PBS ($n = 5$) vs 50 μg of dRK6 ($n = 5$). *, $p < 0.05$; **, $p < 0.01$ vs PBS-treated mice. *A–D*, b, bone; p, pannus; s, synovium.

Results

dRK6, a D-form derivative of RK6, inhibits VEGF binding to its cell surface receptor with similar activity to RK6

We previously identified a novel RRKRRR hexapeptide (RK6) that blocked the interaction between VEGF and its receptor through the screening of peptide libraries (16). However, the hexapeptide with L-amino acids will be unstable in the in vivo environments, because of various kinds of circulating proteases. For this, dRK6 (a D-amino acid derivative of RK6) was synthesized and the inhibitory activity was compared with RK6 for VEGF/VEGFR interactions. Both RK6 and dRK6 showed concentration-dependent inhibitory activity for the VEGF binding to HUVEC, but another positively charged peptide, hexalysine (6K) did not affect it (Fig. 1).

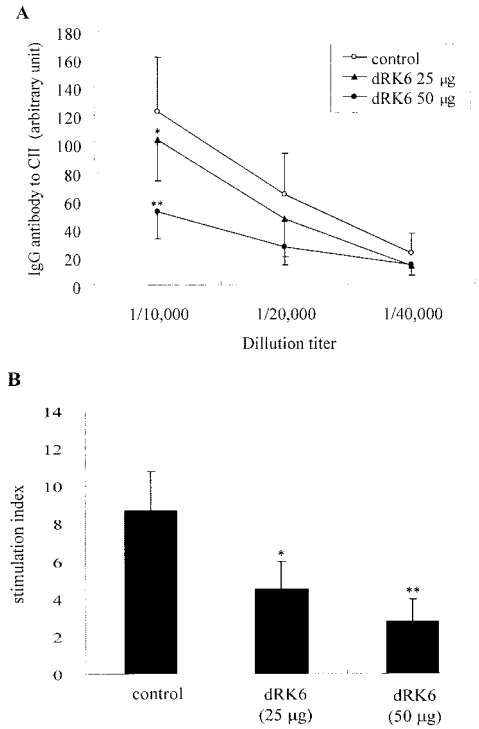

FIGURE 5. Suppression of the autoimmune responses to CII by dRK6. *A*, Levels of circulating IgG Abs to CII in mice treated with PBS alone ($n = 18$), 25 μg of dRK6 ($n = 15$), and 50 μg of dRK6 ($n = 15$), as determined at 6 wk after primary immunization. The mean levels of IgG anti-CII Abs in the sera diluted 1/10,000 were compared among the three groups for statistical analysis. *B*, T cell responses to CII in LN cells. Inguinal and popliteal LN were removed from similar groups of mice 6 wk after primary immunization. Pooled LN cells from four or five mice were cultured with or without 40 μg/well CII. Mean background cpm without CII was 3250 ± 773. T cell responses are presented as the mean (± SD) stimulation indices of five independent experiments. *, $p < 0.05$; **, $p < 0.01$ vs PBS-treated mice.

Stability of RK6 and dRK6 in rat serum

Because short peptides are usually low immunogenic, the stability of dRK6 was determined by a HPLC method. After incubation of dRK6 or RK6 with rat serum for the indicated time, the amount of peptides remaining in the serum was analyzed by C18 reverse phase HPLC. As shown in Fig. 2, both RK6 and dRK6 showed the same retention time (peak A, 4.0 min or 16% acetonitrile). The serum samples showed a peak lagging behind peak A (peak B, 5.5 min, 21% acetonitrile) as well as a minor peak overlapping with peak A (the two top panels of Fig. 2). Therefore, an excess amount of each peptide (100 μg) was used for the assay. Half-life of RK6 at 37°C in rat serum was ~3 min, and the peptide had nearly completely disappeared after 30 min (Fig. 2*A*). In contrast, dRK6 was stable for 13.5 h in the serum (Fig. 2*B*), which is consistent with earlier reports that the change of L-form to D-form increased the in vivo stability of the synthetic peptides (26, 27). The molecular identity of peak A was identical with original RK6 or dRK6, because peak A prepared from HPLC showed exactly the same molecular mass as the loaded peptide (100 pM) in the mass spectrometric analysis (data not shown). Therefore, more stable dRK6 in the serum was used for in vivo biologic experiments, instead of its L-form counterpart, RK6.

Suppression of CIA by dRK6

In a previous study, the anti-VEGF dRK6 showed a potent antitumor effect at a concentration of 50 μg/day (16). In this study, the dRK6 was tested to see if it could suppress the chronic inflammatory response in CIA animals. The result showed that s.c. injections of dRK6 (25 and 50 μg), every other day for 3 wk, reduced the incidence and severity of CIA in a dose-dependent manner (Fig. 3, *A* and *B*). The maximal incidence of arthritis, which was evident during weeks 5–7 after the primary immunization, was 94.4% for the vehicle-treated mice ($n = 18$), 53.3% for mice treated with 25 μg of dRK6 ($n = 15$) and 33.3% for mice treated with 50 μg of dRK6 ($n = 15$) ($p < 0.01$ for 25 μg of dRK6 vs the vehicle alone, $p < 0.001$ for 50 μg of dRK6 vs the vehicle alone).

Joint cartilage surfaces are negatively charged due to chondroitin sulfate and other moieties. Thus, the positively charged dRK6 might have caused nonspecific suppression of arthritis by binding to cartilage surfaces. To test this possibility, we investigated the effect of 6K, positively charged control peptide, on the development of arthritis in another set of experiment. As shown in Fig. 3*C*, the severity of arthritis was not significantly decreased by the treatment with 50 μg of 6K, compared with PBS treatment,

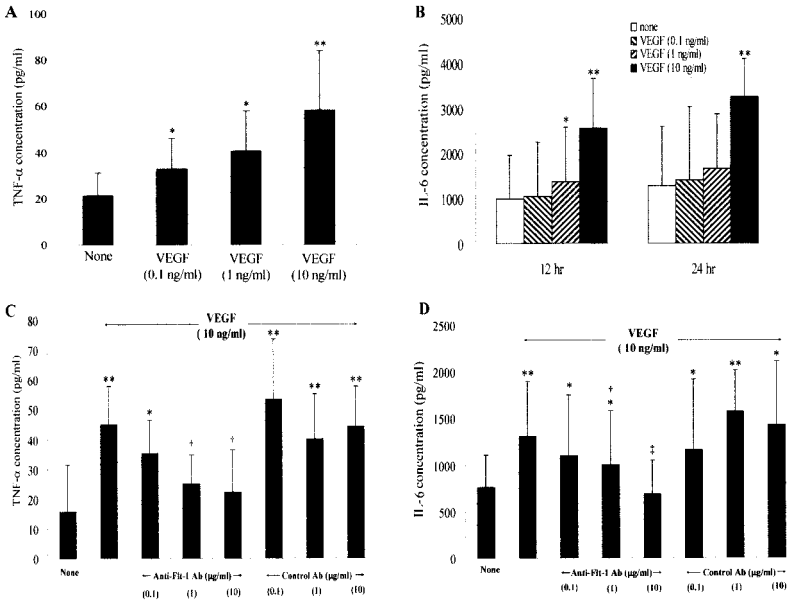

FIGURE 6. VEGF specifically induces the production of TNF-α and IL-6 by PBMC. PBMC were isolated from healthy volunteers and cultured in triplicate with various concentrations of VEGF, ranging from 0.1 to 10 ng/ml. The levels of TNF-α and IL-6 were measured in the culture supernatants by ELISA. *A* and *B*, Dose-dependent increase in the production of TNF-α and IL-6 by PBMC. Data are presented as mean ± SD of seven independent experiments. *C* and *D*, The specificity of VEGF-stimulated production of TNF-α and IL-6. PBMC were cultured with 10 ng/ml VEGF in the presence or absence of various concentrations (0.1–10 μg/ml) of blocking Abs to Flt-1. The equivalent concentration of isotype Abs were used for a negative control. Each value represents the mean ± SD of five separate experiments. *, $p < 0.05$; **, $p < 0.01$ vs medium alone. †, $p < 0.05$; ‡, $p < 0.01$ vs cells treated with 10 ng/ml VEGF.

whereas injection of 50 μg of dRK6 markedly reduced the mean arthritis index. This result indicates that the effect of dRK6 on arthritis suppression is specific and eliminates the concern about charge-related inhibition of arthritis by dRK6.

On histological examination of the joints, it was also found that the paws and ankles of the dRK6-treated (50 μg every other day) mice exhibited lower degrees of inflammation, synovial hyperplasia, bone destruction, and pannus formation compared with the PBS-treated mice, as determined on day 42 after immunization (Fig. 4, *A–D*). Mean histological scores of above three variables were reduced by >2.5-fold in dRK6-treated mice ($n = 5$), when compared with PBS-treated mice ($n = 5$) (Fig. 4*E*).

Inhibition of autoimmune responses to CII by dRK6

To investigate the effect of the dRK6 on the CII-specific immune responses, we measured the IgG Abs to CII in the sera and the T cell proliferative responses to CII in the draining LN, 6 wk after immunization. As expected, the serum levels of the IgG Abs to CII were significantly lower in the mice treated with 25 and 50 μg of dRK6 than in those treated with solvent alone, as determined 6 wk after the primary immunization (Fig. 5*A*) ($p < 0.05$ and $p < 0.001$ vs the solvent-treated mice, respectively). The inhibitory effect of dRK6 on the anti-CII autoantibody production was dose-dependent. In addition, the assay for the T cell responses to CII in the draining LN of similar groups also showed that mice injected with dRK6 had lower stimulation indices to CII compared with the control mice (mean background cpm without CII, 3250 ± 773; stimulation index, 8.7 ± 2.1 for the solvent-treated mice, 4.5 ± 1.5 for mice treated with 25 μg of dRK6, and 2.8 ± 1.2 for mice treated with 50 μg of dRK6) (Fig. 5*B*). These observations show that anti-VEGF dRK6 effectively suppresses the systemic CII-specific immune responses in CIA animals.

VEGF specifically induces the production of TNF-α and IL-6 from PBMC

Based on the finding that the anti-VEGF peptide treatment reduced ongoing paw inflammation in mice, we next investigated whether $VEGF_{165}$ could directly induce an inflammatory response in vitro. For this, human PBMC from healthy volunteers were cultured with purified VEGF, and then the levels of TNF-α and IL-6, the two most important proinflammatory mediators implicated in RA pathogenesis, were measured in the culture supernatants. As shown in Fig. 6*A*, there was a dose-dependent stimulatory effect of the $VEGF_{165}$ (0.1–10 ng/ml) on the production of TNF-α by the PBMC compared with the spontaneous level. The stimulatory effect of $VEGF_{165}$ on TNF-α production was observed over 12– 48 h of incubation (data not shown). Similarly, treatment of PBMC with $VEGF_{165}$ resulted in dose- and time-dependent increases in the production of IL-6 (Fig. 6*B*). The maximal increases on VEGF stimulation were 2.7- and 2.6-fold for TNF-α and IL-6 compared with their basal levels. The specificity of VEGF in TNF-α and IL-6 production was demonstrated by an inhibition study using the anti-VEGFR Ab. Treatment of the PBMC with 0.1–10 μg/ml anti-Flt-1 mAb dose-dependently blocked the VEGF-induced production of TNF-α and IL-6 to near their constitutive levels but not with the equivalent concentrations of isotype control mAb (Fig. 6, *C* and *D*). These data suggest that VEGF, an angiogenic factor, is able to specifically activate the mononuclear cell fractions composed of monocytes and lymphocytes.

VEGF stimulates the production of TNF-α and IL-6 from SFMC

In the RA inflamed joints, the SFMC are exposed to various proinflammatory cytokines and chemokines, which are interdependently linked to promote and maintain the inflammatory environments. An experiment was conducted to determine the effect of VEGF on the production of TNF-α and IL-6 by the SFMC. In agreement with the PBMC findings, treatment with VEGF caused an increase in the production of TNF-α and IL-6 from the SFMC of five RA patients in a dose-dependent manner (Fig. 7, *A* and *B*). Compared with the PBMC of healthy controls, the TNF-α and IL-6 concentrations spontaneously produced were significantly higher in the RA SFMC (2.0- and 1.8-fold, respectively) ($p < 0.05$). On stimulation with VEGF (0.1–10 ng/ml), the SFMC also secreted higher amounts of TNF-α and IL-6 than the PBMC of the healthy controls ($p < 0.01$). The maximal stimulatory production of TNF-α and IL-6 by the SFMC were 2.9- and 1.9-fold over those of the PBMC of the healthy controls, respectively (Fig. 7, *A* and *B*). The VEGF

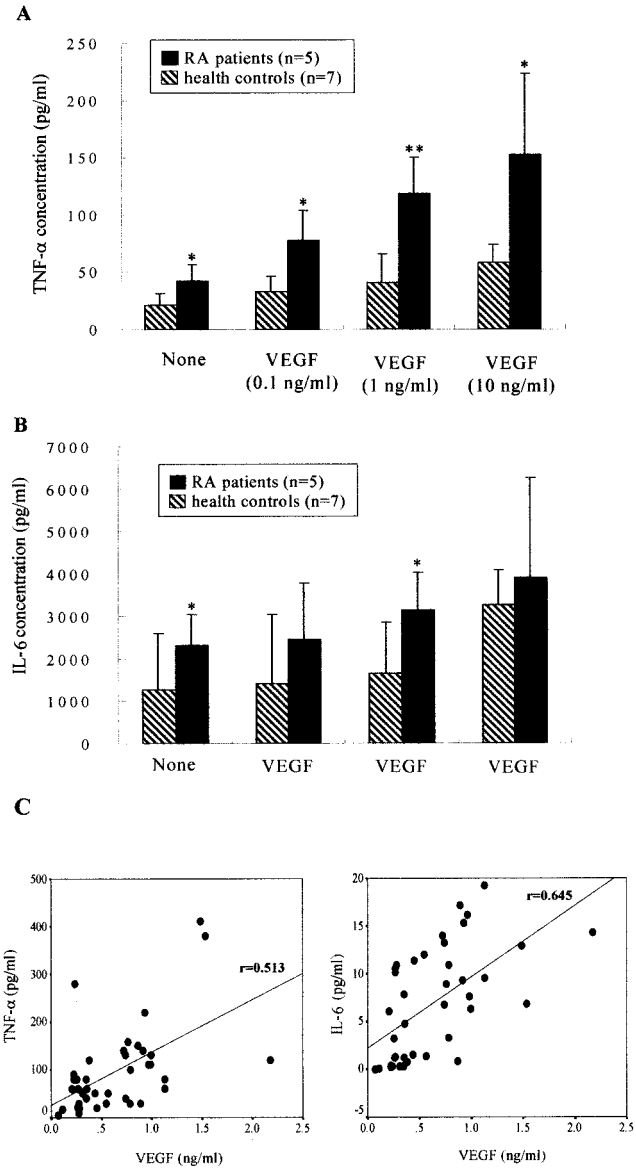

FIGURE 7. Effect of VEGF on the production of TNF-α and IL-6 by SFMC. SFMC were isolated from five RA patients and then incubated with VEGF for 24 h. The production of TNF-α and IL-6 from SFMC were determined by ELISA in the culture supernatants, and compared with those from PBMC of healthy controls. *A* and *B*, Dose-dependent increase in the production of TNF-α and IL-6 by SFMC. Data are expressed as the mean ± SD of five independent experiments. *, $p < 0.05$; *, $p < 0.01$ vs PBMC of seven healthy controls. *C*, Correlation of VEGF levels with TNF-α and IL-6 concentrations measured in synovial fluid from 40 RA patients.

concentrations, which were measured in the SF from 40 RA patients, correlated well with the TNF-α and IL-6 levels ($r = 0.513$ and $p = 0.001$, $r = 0.645$ and $p < 0.001$, respectively) (Fig. 7*C*).

Cell types responding to VEGF stimulation

Our next goal was to analyze the main cell types responsible for the production of TNF-α and IL-6 on VEGF stimulation. As shown in Fig. 8*A*, when the PBMC of the healthy controls were stimulated with various concentrations of VEGF, the proportion of TNF-α-producing cells was dose-dependently increased in the $CD14^+$ cells (1.7% for medium alone, 4.4% for 1 ng/ml VEGF, and 18.4% for 10 ng/ml VEGF), as with the LPS (5 μg/ml). The proportion of $CD14^+$ and IL-6^+ cells was also instigated by the addition of VEGF in a dose-dependent fashion (11.3% for medium alone, 31.3% for 1 ng/ml VEGF, and 43.8% for 10 ng/ml VEGF) (Fig. 8*B*). However, $CD3^+$ cells showed no change in the TNF-α and IL-6 production with VEGF stimulation (data not shown).

The macrophages/monocytes and synovial fibroblasts represent the most abundant cell populations in inflamed RA joints and are major sources for the production of VEGF, IL-6, and TNF-α (7, 8). As shown in Fig. 8*C*, in cultured adherent cells (primarily monocytes) separated from SFMC, the TNF-α production was dose-dependently increased by treatment with VEGF. The incubation of adherent cells with 10 ng/ml VEGF in the presence of Dexa (2 and 200 nM) resulted in the dose-dependent repression of the TNF-α production to the basal level (Fig. 8*C*). Similarly, VEGF treatment elevated the IL-6 production by the adherent cells (data not shown) and synovial fibroblasts (Fig. 8*D*). However, the nonadherent cells (primarily lymphocytes) did not respond to VEGF stimulation. Together, these results suggest that VEGF activates monocytes/macrophages and synovial fibroblasts to produce TNF-α and IL-6.

dRK6 inhibits the VEGF-stimulated production of TNF-α and IL-6 from mononuclear cells and decreases serum IL-6 levels in arthritic mice

To determine the therapeutic relevance of dRK6 in human RA, the question as to whether dRK6 may regulate the activation of mononuclear cells stimulated with VEGF, as with anti-Flt-1 mAb, was addressed. As shown in Fig. 9, *A* and *B*, the dRK6 inhibited the production of TNF-α and IL-6 from RA SFMC stimulated with VEGF in a concentration-dependent manner. The VEGF-stimulated IL-6 production by synovial fibroblasts was also blocked by the addition of dRK6 (data not shown). However, the basal production of TNF-α and IL-6, without VEGF, were unaffected by the

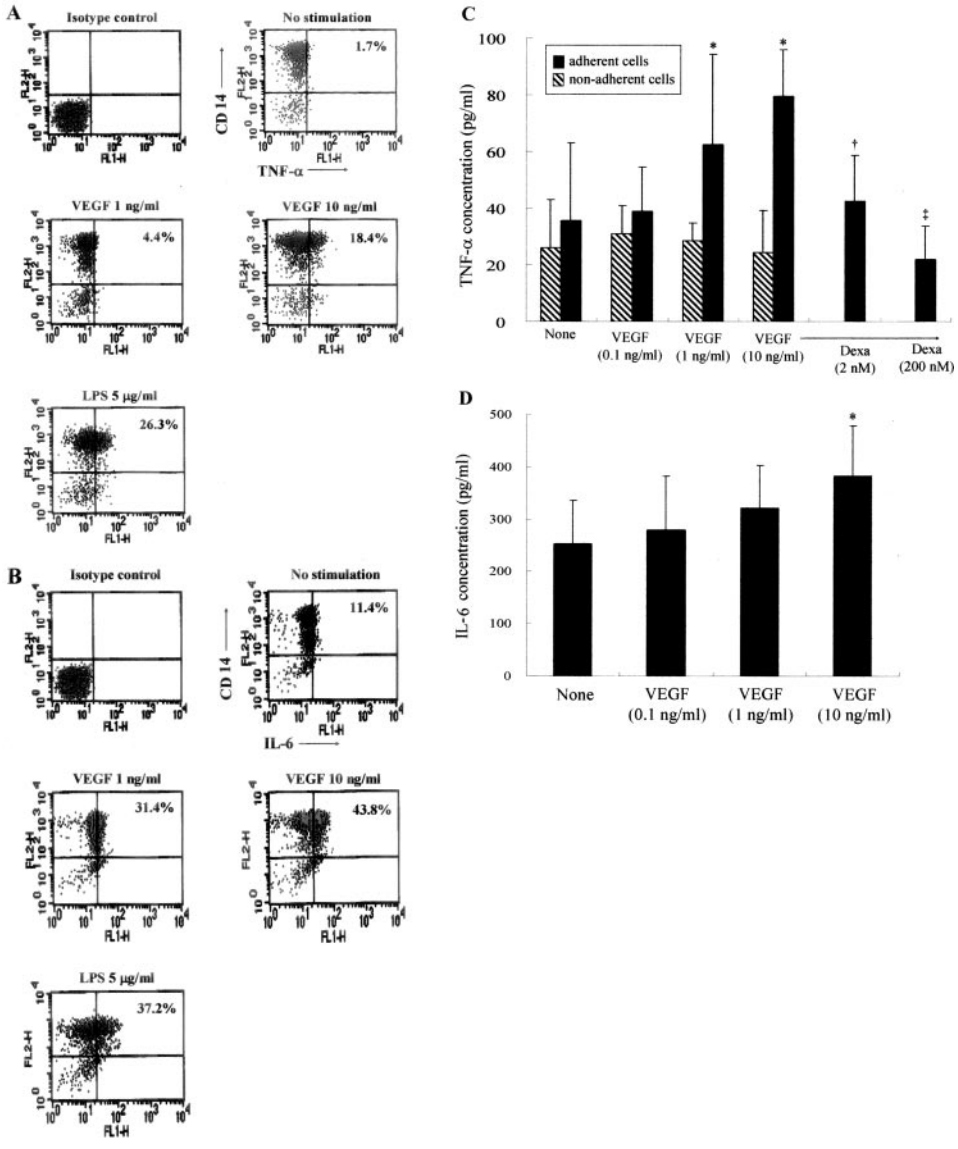

FIGURE 8. Monocytes and synovial fibroblasts are major cell types responding to VEGF stimulation. *A* and *B*, Flow cytometry analysis for TNF-α and IL-6 production by PBMC of healthy controls. PBMC were stimulated with different concentrations of VEGF for 12 h, and then stained with PE-conjugated anti-CD14 mAb, PE-conjugated anti-CD3 mAb, FITC-conjugated anti-TNF-α mAb, and FITC-conjugated anti-IL-6 mAb. LPS (5 μg/ml) was used as a positive control Ag to stimulate monocytes. A representative result from three independent experiments using different cells is shown. *C*, VEGF-triggered TNF-α production by the adherent vs nonadherent cells. The adherent and nonadherent cells were separated from SFMC, and then each type of cell was cultured apart with VEGF (0.1–10 ng/ml) for 24 h. Additionally, adherent cells were incubated with various concentrations of Dexa in the presence of 10 ng/ml VEGF. The TNF-α concentration was measured by ELISA in the culture supernatants. Data represent the mean $\pm$ SD of three independent experiments. $*$, $p < 0.05$; $*$, $p < 0.01$ vs medium alone. $\dagger$, $p < 0.05$; $\ddagger$, $p < 0.01$ vs adherent cells stimulated with 10 ng/ml VEGF. *D*, IL-6 production by FLS of RA patients. FLS were stimulated with various concentrations of VEGF for 24 h. The IL-6 concentration was measured by ELISA in the culture supernatants. Data are presented as mean $\pm$ SD of three independent experiments. $*$, $p < 0.05$ vs medium alone.

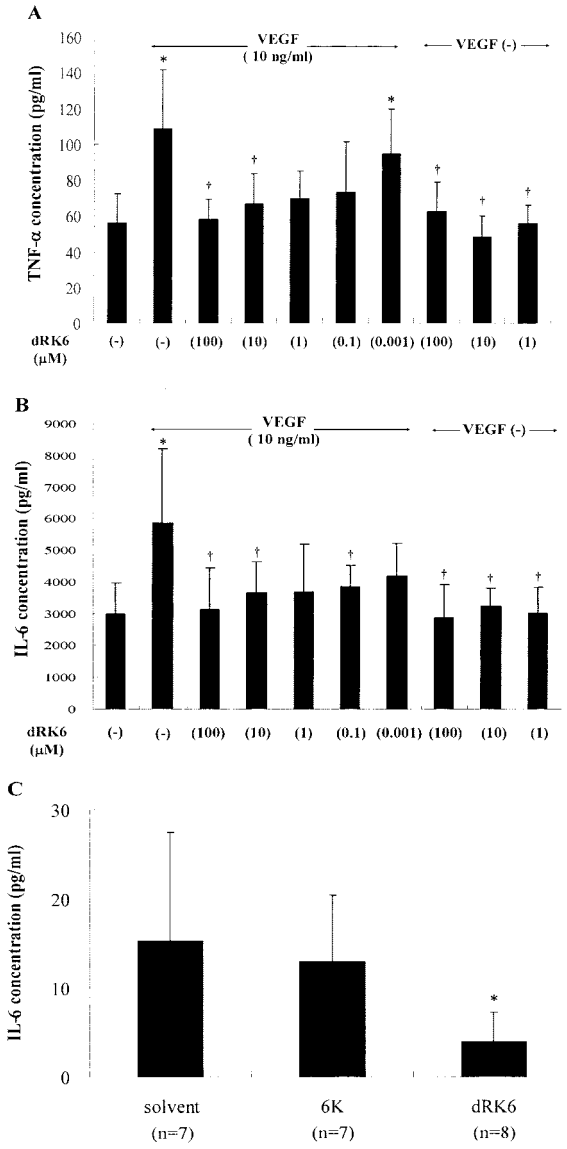

FIGURE 9. Inhibition of TNF-α and IL-6 production by dRK6. *A* and *B*, Suppression of VEGF-stimulated production of TNF-α (*A*) and IL-6 (*B*) from SFMC by dRK6. Various concentrations of dRK6, ranging from 0 to 100 μM, were preincubated with 10 ng/ml VEGF for 1 h at room temperature and then the VEGF and dRK6 mixture was added to SFMC. The TNF-α and IL-6 concentration was measured by ELISA in the culture supernatants. Data represent the mean $\pm$ SD of three independent experiments. *, $p < 0.05$ vs medium alone. †, $p < 0.05$; ‡, $p < 0.01$ vs cells stimulated with 10 ng/ml VEGF. *C*, Decrease of serum IL-6 levels in mice treated with dRK6. From 3 wk after the primary immunization, 50 μg of dRK6 (RRKRRR, $n = 8$) were injected in the CIA animals every other day for 3 wk. The control mice received 50 μg of 6K (KKKKKK, $n = 7$) dissolved in PBS or solvent alone ($n = 7$). The IL-6 concentration was measured by ELISA in the sera of the mice 8 wk after primary immunization. *, $p < 0.01$ vs solvent- or 6K-treated mice.

peptides themselves in the SFMC (Fig. 9, *A* and *B*), suggesting that the inhibition was not caused by blocking effects of other inflammatory mediators or by the nonspecific cytotoxicity of the peptides. In addition, the viability of the SFMC and FLS, determined by the MTT (tetrazolium) assay, was not influenced at any of the dRK6 concentrations tested (Table I). Based on these results, it can be concluded that dRK6 has strong inhibitory activity on the VEGF-stimulated production of proinflammatory cytokines by blocking the VEGFR interactions.

To examine the in vivo effect of dRK6 on the production of proinflammatory cytokine, we measured IL-6 concentrations in the sera of CIA mice administered PBS alone ($n = 7$), 50 μg of 6K ($n = 7$), or 50 μg of dRK6 ($n = 8$) every other day for 3 wk. The result showed that the circulatory levels of IL-6, determined at day 56 after primary immunization, were significantly lower in dRK6-treated mice than the other groups of mice, suggesting that dRK6 may have anti-inflammatory activity in vivo by blocking IL-6 production (Fig. 9*C*).

Discussion

Neovascularization is necessary for the continual proliferation of synovial tissue and thus believed to play an important role in the development and progression of RA (1, 2, 7–9). A number of angiogenic factors have been described in RA, of which VEGF is one of the most potent molecules. Thus, blocking agents against VEGF have been potential candidates for RA treatment (10, 11). There are several possible mechanisms of anti-angiogenesis treatment in RA (28). These include: 1) decrease in the nutrient supply to the tumor-like synovium, 2) inhibition of leukocyte adhesion and migration by decreasing the endothelial cell surface area, and 3) decrease in the chemokine and cytokine production by the activated endothelial cells.

In a previous study, dRK6 showed significant inhibition of VEGF-induced angiogenesis, and also retarded the growth and metastasis of colon carcinoma cells without direct cytotoxicity (16). The RA synovium can be viewed as a tumor-like mass in that it invades local environments and exhibits characteristics of tumor cells such as somatic mutations in H-*ras* and p53 (29, 30). Given the high efficacy of dRK6 in the suppression of colon tumors, it would be highly relevant to test its efficacy in a RA animal model. In the present study, anti-VEGF dRK6 strongly inhibited the severity and incidence of CIA in mice, and suppressed synovial infiltration of inflammatory cells on histological examination of the joints, indicating that the peptides have anti-inflammatory activity in vivo. Since the small peptides are relatively safe, inexpensive, and easily made, this form of treatment can be used for the purpose of blocking VEGF in animal experiments, and might be applicable for the treatment of various VEGF-dependent human diseases including RA.

CIA is mediated by the synergistic actions of both CII-reactive T cells and Abs to CII (31–33). In this study, dRK6 reduced the levels of circulating IgG anti-CII Abs and the T cell responses to CII in LN cells compared with those in control mice, which was

Table I. *Effect of dRK6 on the viability of SFMC and FLS from RA patients*[a]

	Concentration of dRK6 (μM)			
	0.1	1	10	100
SFMC ($n = 3$), %	102.9 $\pm$ 0.5	102.6 $\pm$ 12.8	109.7 $\pm$ 11.3	113.7 $\pm$ 4.9
FLS ($n = 3$), %	99.4 $\pm$ 0.6	106.7 $\pm$ 1.4	107.8 $\pm$ 3.6	108.9 $\pm$ 3.1

[a] Cell viability was determined by MTT (tetrazolium) assay 24 h after culturing with various concentrations of dRK5. Data are the mean $\pm$ SD of three independent experiments in triplicate and presented as the relative value (%) compared with untreated cells.

consistent with the data obtained from the visual inspection of the arthritic joints. Our data, together with earlier reports (31–33), suggest that dRK6 may protect against arthritic joint destruction by suppressing the formation of CII-reactive T cells and/or IgG Abs to CII. Because the autoimmune responses against CII were determined in the extra-articular regions devoid of synovium, angiogenesis-independent mechanisms, such as the inactivation of anti-CII Ab-producing B cells, are possibly involved in this inhibition. Based on this hypothesis, an experiment was performed to measure the direct effect of VEGF, or its blockade, on the production of proinflammatory mediators (cytokines) associated with the activation of immune cells and joint destruction.

Proinflammatory cytokines, such as TNF-α and IL-6, are known to play crucial roles in the pathophysiology of RA (34–39). TNF-α stimulates the production of several other cytokines and chemokines as well as other inflammatory mediators such as prostaglandin (34, 35). A significant level of TNF-α is detected in the sera and SF of patients with RA, which contributes to the signs and symptoms of the disease (36). IL-6 is another key cytokine, produced mainly by the macrophages and monocytes, early during the inflammatory response, and is also responsible for tissue destruction in RA (37). IL-6 has potent actions in promoting B cell growth and activation, and in the synthesis of Ig (38, 39). In this study, we demonstrated that VEGF directly increased the production of TNF-α and IL-6 from human mononuclear cells, which was almost completely repressed by treatment with anti-Flt-1 mAb. The SFMC of RA patients showed a greater response to VEGF stimulation than the PBMC of the healthy controls. The major cell types responding to VEGF were monocytes and synovial fibroblasts. Moreover, the concentration of VEGF used in our culture system was physiologically relevant in that the levels of VEGF measured in the SF of 40 RA patients were mostly within the range of 0.1–10 ng/ml (Fig. 7*C*). Collectively, our data suggest that VEGF may be directly involved in the activation of RA monocytes and synoviocytes, producing TNF-α and IL-6, via a receptor-coupling event.

The current model of RA pathogenesis favors complex interactions between immune cells, via cytokine secretion and cell-to-cell contact. VEGF is produced by infiltrating synoviocytes, near endothelial cells, and acts on them via interactions with its receptors (7, 8). It has recently been documented that VEGF activates endothelial cells to induce the production of chemokines such as MCP-1 and IL-8 (5, 6). These chemokines may recruit monocytes around the endothelial cells in the synovial membrane, where newly used macrophages, in addition to the resident synoviocytes, can produce TNF-α and IL-6 by VEGF stimulation, or through cell contact with activated endothelial cells. TNF-α and IL-6, in turn, further enhance the capacity of macrophages and synoviocytes to secrete VEGF, and also stimulate the endothelial cells to induce the cell contact-mediated macrophage activation, generating a positive feedback loop. Moreover, VEGF showed a similar stimulatory action on the TNF-α production by cultured HUVEC (data not shown). Thus, VEGF may provide the mutual activation link between the endothelial cells and macrophages/synoviocytes. If this assumption is correct, the autoamplification loop could be broken off by treatment with Dexa, which showed a potent down-regulatory effect on the VEGF production (Fig. 8*C*).

According to a recent study, immunostaining of the inflamed synovium revealed that Flk-1 was only present on new blood vessels, whereas Flt-1 was expressed by both inflammatory and endothelial cells (40). Treatment with anti-Flt-1 mAb reduced the incidence of CIA in mice and protected against bone destruction, whereas anti-Flk-1 mAb failed to block arthritis (40, 41). This suggests that direct suppression of inflammation, rather than anti-angiogenesis, may be the primary mechanism responsible for the efficacy of anti-VEGF treatment in RA. In this study, dRK6 suppressed the VEGF-stimulated production of TNF-α and IL-6 from the SFMC and FLS, indicating the anti-inflammatory potential of the peptide. Considering that blocking Abs to TNF-α or to IL-6 receptor ameliorates experimental arthritis (42, 43), our data suggest that anti-VEGF dRK6 might mitigate the arthritis severity in mice and autoimmune responses against CII, by blocking the VEGF-triggered production of proinflammatory cytokines. This assumption is supported by the findings that serum IL-6 was decreased in dRK6-injected mice, but not 6K-treated mice (Fig. 9*C*). Of note, dRK6 did not affect the basal cytokine production and viability of mononuclear cells and FLS in the absence of VEGF stimulation. This implies that dRK6 selectively interferes with the binding of VEGF and its receptor on the RA monocytes and synoviocytes, which are abnormally activated. Therefore, our data provide a rationale for evaluating dRK6 as a novel strategy for selectively blocking the uncontrolled inflammation triggered by VEGF without toxicity.

It should be addressed that the induction of TNF-α and IL-6 by VEGF is relatively small throughout the experiments (e.g., maximum increase of 2.7- and 2.6-fold, respectively, with PBMC in optimized in vitro conditions), considering the clear effects of dRK6 on the development of CIA in mice. There are many more cytokines or enzymes involved in the pathogenesis of RA that have not been measured in this study. Given that a variety of pro- and anti-inflammatory mediators coordinately influence on the arthritis progression, the ultimate effect of dRK6 might be determined by suppressing combinatory action of VEGF on these mediators, as well as its anti-angiogenic activity during RA progression. Further studies are underway to evaluate these possibilities.

In conclusion, the anti-VEGF dRK6 peptide suppressed the ongoing paw inflammation in mice, and blocked the VEGF-induced production of proinflammatory cytokines from the monocytes and synoviocytes of RA patients. Our data suggest that dRK6 may be effective in the treatment of RA and could be applicable to the modulation of various chronic VEGF-dependent inflammatory diseases. Work is in progress to improve the activity and safety of dRK6 by modifying the structure of the peptide.

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Disclosures

The authors have no financial conflict of interest.

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