Interaction of Vascular Endothelial Growth Factor 165 with Neuropilin-1 Protects Rheumatoid Synoviocytes from Apoptotic Death by Regulating Bcl-2 Expression and Bax Translocation

Wan-Uk Kim, Soon Suk Kang, Seung-Ah Yoo, Kyung-Hee Hong, Dong-Goo Bae, Mi-Sook Lee, Seung Woo Hong, Chi-Bom Chae and Chul-Soo Cho

*J Immunol* 2006; 177:5727-5735;
doi: 10.4049/jimmunol.177.8.5727
http://www.jimmunol.org/content/177/8/5727

References

This article cites 45 articles, 22 of which you can access for free at:
http://www.jimmunol.org/content/177/8/5727.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Interaction of Vascular Endothelial Growth Factor 165 with Neuropilin-1 Protects Rheumatoid Synoviocytes from Apoptotic Death by Regulating Bcl-2 Expression and Bax Translocation

Wan-Uk Kim,* Soon Suk Kang,* Seung-Ah Yoo,* Kyung-Hee Hong,* Dong-Goo Bae,†
Mi-Sook Lee,‡ Seung Woo Hong,‡ Chi-Bom Chae,§ and Chul-Soo Cho*‡

Rheumatoid arthritis (RA) synoviocytes are resistant to apoptosis and exhibit a transformed phenotype, which might be caused by chronic exposure to genotoxic stimuli including reactive oxygen species and growth factors. In this study, we investigated the role of vascular endothelial growth factor165 (VEGF165), a potent angiogenic factor, and its receptor in the apoptosis of synoviocytes. We demonstrated here that neuropilin-1, rather than fms-like tyrosine kinase-1 and kinase insert domain-containing receptor, is the major VEGF165 receptor in the fibroblast-like synoviocytes. Neuropilin-1 was highly expressed in the lining layer, infiltrating leukocytes, and endothelial cells of rheumatoid synovium. The production of VEGF165, a ligand for neuropilin, was significantly higher in the RA synoviocytes than in the osteoarthritis synoviocytes. The ligation of recombinant VEGF165 to its receptor prevented the apoptosis of synoviocytes induced by serum starvation or sodium nitroprusside (SNP). VEGF165 rapidly triggered phospho-Akt and phospho-ERK activity and then induced Bcl-2 expression in the rheumatoid synoviocytes. The Akt or ERK inhibitor cancelled the protective effect of VEGF165 on SNP-induced synoviocyte apoptosis. Moreover, VEGF165 blocks SNP-induced Bcl-2 down-regulation as well as SNP-induced Bax translocation from the cytosol to the mitochondria. The down-regulation of the neuropilin-1 transcripts by short interfering RNA caused spontaneous synoviocyte apoptosis, which was associated with both the decrease in Bcl-2 expression and the increase in Bax translocation to mitochondria. Collectively, our data suggest that the interaction of VEGF165 with neuropilin-1 is crucial to the survival of rheumatoid synoviocytes and provide important implications for the abnormal growth of synoviocytes and therapeutic intervention in RA.

Copyright © 2006 by The American Association of Immunologists, Inc. 0022-1767/06/$02.00

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication November 29, 2005. Accepted for publication July 28, 2006.

Address correspondence and reprint requests to Dr. Chul-Soo Cho, Department of Internal Medicine, School of Medicine, Catholic University of Korea, Seoul, Korea; Division of Molecular and Life Science, Pohang University of Science and Technology, Pohang, Korea; Laboratory of Cell Cycle Control, Cancer Research Institute, Seoul National University College of Medicine, Seoul, Korea; and Institute of Biomedical Science and Technology, Konkuk University, Seoul, Korea.

The Journal of Immunology, 2006, 177: 5727–5735.

**Abbreviations used in this paper: RA, rheumatoid arthritis; FLS, fibroblast-like synoviocytes; HBD, heparin-binding domain; NP-1, neuropilin-1; SNP, sodium nitroprusside; VEGF, vascular endothelial growth factor; Flt-1, fms-like tyrosine kinase; KDR, kinase insert domain-containing receptor; OA, osteoarthritis; ITSA, insulin-transferrin-selenium A pAkt, phospho-Akt; pERK, phospho-ERK.**
tyrosine kinase activity, and both are expressed in the majority of vascular endothelial cells (10). KDR is a primary mediator of endothelial cell proliferation in response to VEGF165. Unlike KDR, Flt-1 is present in inflammatory cells, including macrophages and monocytes (10, 11). NP-1 has been demonstrated to function as a non-tyrosine kinase receptor for VEGF165 and, specifically, for the heparin-binding domain (HBD) of VEGF165 (12, 13). This molecule was initially characterized as a receptor for semaphorin 3A, which mediates the guidance of neuronal cells (14). In the endothelial cells, NP-1 also functions as a coreceptor for VEGF and has been shown to regulate KDR-dependent angiogenesis (12, 13). Moreover, NP-1 mediates the antiapoptotic activity of VEGF165 in breast cancer cells (15).

Although three VEGF165 receptor subtypes, Flt-1, KDR, and NP-1, are expressed in the RA synovium (16), the expression and function of the VEGF165 receptors on synovial fibroblasts remain to be clearly elucidated. In our previous study, VEGF165 was shown to directly increase the production of IL-6 by RA synovial fibroblasts, and this effect was inhibited by blocking the interactions between VEGF165 and its receptor (17), suggesting that synovial fibroblasts harbor their own VEGF165 receptor. In this study, we have attempted to identify the major VEGF165 receptor in the synovial fibroblasts of RA patients and then to characterize its functions in RA pathogenesis. We first demonstrated that NP-1, rather than Flt-1 and KDR, is the principal VEGF165 receptor in the synovial fibroblasts. Although it exerted no effects on cell proliferation, the ligation of VEGF165 to its receptor prevented the synovioocyte apoptosis induced by serum starvation and sodium nitroprusside (SNP). In parallel, the expressions of phospho-Akt (pAkt), phospho-ERK (pERK), and Bcl-2 were increased by adding VEGF165 to cultured synovioocytes, whereas SNP-induced Bax translocation from the cytosol to the mitochondria was blocked by VEGF165 treatment. Moreover, the down-regulation of NP-1 transcripts by short interfering RNA (siRNA) led to spontaneous synovioocyte apoptosis, which was associated with the modulation of Bcl-2 expression and Bax translocation. These findings indicate that the interaction of VEGF165 with NP-1 is crucial to the survival of rheumatoid synovioocytes and offer a new possibility that NP-1 may be a potential target for the therapeutic intervention of chronic inflammation.

Materials and Methods
Preparation of VEGF165, VEGF121, and the heparin-binding domain of VEGF165
Human recombinant VEGF165 and VEGF121 were generated in Escherichia coli and purified as previously described (18). To prepare HBD, VEGF165 was digested by the addition of plasmin (Roche) at a 1:200 (w/w) ratio, with respect to VEGF165, then incubated for 24 h at 25°C. The digested VEGF165 was then applied to a heparin column (Amersham Biosciences) which had been equilibrated with PBS, and the HBD was eluted with 1 M NaCl in 50 mM phosphate, pH 7.4. The eluted protein was then further purified with reverse phase HPLC (Vydac; Hesperia).

Isolation and culture of fibroblast-like synoviocytes (FLS)
The FLS were prepared from the synovial tissues of RA or osteoarthritis (OA) patients, who had undergone total joint replacement surgery. The isolation of the FLS from the synovial tissues was performed according to the previously described protocol (17). The purity of the cells was verified by flow cytometric analysis (>95% CD90, <2% CD14, <1% CD3, and <1% CD19). The FLS, from passages 3 through 7, were seeded in 24-well plates (Nunc) or in 100-mm culture dishes in DMEM supplemented with 10% FCS (Invitrogen Life Technologies) and then cultivated for 24 h at 37°C. In the experiments conducted to determine the effects of VEGF165 and its associated molecules on apoptosis, the FLS were washed in DMEM and then incubated for an additional 48 h in serum-free DMEM supplemented with insulin-transferrin-selenium A (ITSA; Invitrogen Life Technologies). The medium was exchanged with fresh DMEM-ITSA, and VEGF165 was then added to the cells for the predetermined times.

Determination of [125I]-VEGF165 binding to its receptors
The seeded FLS (5 × 10⁴ cells/well) were incubated overnight at 37°C and then washed for 2 h with a warm binding buffer (25 mM HEPES (pH 7.4), 0.1% BSA in serum-free DMEM) at 37°C. The cultures were then transferred at 4°C to an oscillating platform, set at 1 cycle/second. [125I]-VEGF165 (1723 Ci/mmol, 20 nCi/well; Amersham Biosciences) was added to the cells, and the binding was allowed to proceed for 3 h at 4°C. In some of the experiments, the cells were preincubated for 1 h with various VEGF receptor-specific competitors in 200 µl of binding buffer at 37°C and then transferred onto an oscillating platform. For hypoxic stimulation, FLS maintained under normoxic conditions (20% O₂) were placed in a 1% O₂ atmosphere for 24 h. The nonspecific binding of [125I]-VEGF165 to the cells was determined in the presence of a 100-fold excess of nonlabeled VEGF165. After two washings 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. The receptor-bound radioactivity was then determined using a gamma counter.

RR-PCR for VEGF receptor subtypes
Total RNA was prepared from the FLS and HUVECs (2 × 10⁶ cells) using TRI reagent (Molecular Research Center), in accordance with the manufacturer’s instructions. The cDNA was reverse transcribed from 50 to 300 ng of total RNA in the presence of random hexaprimers (Promega) by Moloney murine leukemia virus-reverse transcription (Promega), after which PCR amplification was conducted for 30–35 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (polymerization). The following sense and antisense primers were used for the detection of three kinds of transmembrane VEGF receptors, such as Flt-1, KDR, and NP-1 (5’–3’): Flt-1 sense, CAGCGCGTTTTTGGAAGACT CAC; Flt-1 antisense, ACACTCTGGTGACTCTTGGAC; KDR sense, TGGAACCCCTGGCATAAGCGTCTT; KDR antisense, GCCCTCAAGGTCAAGAGCTTAT; NP-1 sense, GAGACAGGaCTAGGCGG; and NP-1 antisense, TCATGCCTCCGAATAAGTACT. The quantity of total RNA utilized in each reaction was normalized with the results of β-actin (Invitrogen Life Technologies) before conduction of saturation PCR amplification (23 cycles). The PCR products were separated via 1% agarose gel electrophoresis, and the expression patterns of each VEGF receptor of the FLS were compared with the results of HUVEC, in which all three of the VEGF receptors are known to be expressed. The identities of the PCR products were confirmed by direct DNA sequencing.

Western blot analysis for VEGF receptor subtypes
The cells were lysed in isus buffer (20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.25% Triton X-100, protease inhibitor mixture, 2 mM PMSF, and 1 mM DTT), and the resulting lysates were cleared by centrifugation. The quantity of cellular proteins in the clarified supernatant was determined by Bradford protein assays (Bio-Rad). Fifty micrograms of proteins were resolved on 8% SDS-PAGE. After transfer to a nylon membrane, the Flt-1, KDR, and NP-1-specific bands were visualized by ECL (Amersham Biosciences), using a secondary anti-goat Ab coupled with HRP.

NP-1 receptor immunocytochemistry
For the immunocytochemical testing of the NP-1 receptor, the FLS of RA patients were cultured for 24 h on Lab-Tek chamber slides (Nalge; Nunc) at a density of 2 × 10³/well in DMEM supplemented with 10% FCS and then fixed for 15 min in 2% paraformaldehyde. After air drying, the cells were incubated for 30 min in 8% BSA in PBS to eliminate nonspecific binding sites. After being washed with PBS, the cells were incubated for 24 h with goat polyclonal anti-NP-1 Abs (1/10 diluted; Santa Cruz Biotechnology) at room temperature. After three more washing with PBS, Cy3-conjugated secondary Ab (Sigma-Aldrich) was added to the cells. A negative control was prepared by incubating the cells with 1% BSA in PBS in the absence of the secondary Ab. The slides were mounted with mounting medium containing 15% Vinol 205, 30% glycerol, and 0.1% sodium azide in PBS and examined by fluorescence microscopy.

Immunohistochemical analysis for NP-1
Immunohistochemical staining of the synovium was performed on 5-μm sections of formalin-fixed, paraffin-embedded blocks. The sections were...
mounted on Superfrost glass slides, deparaffinized in xylene, and rehydrated in a graded series of ethanol, followed by microwave Ag retrieval. The endogenous peroxidase activity was blocked by 3% hydrogen peroxide. After blocking the nonspecific binding by treating the slides with 10% normal goat serum at 37°C for 60 min, the slides were incubated with anti-human NP-1 Ab (Santa Cruz Biotechnology) at a 1/100 dilution overnight at 4°C. The sections were washed and incubated with the secondary Ab, biotinylated goat anti-mouse IgG (DakoCytomation). After the sections were washed and incubated with peroxidase-conjugated streptavidin (DakoCytomation) at room temperature for 30 min, 3,3-diaminobenzidine was added to reveal the Ag. The sections were counterstained with Mayer hematoxylin, dehydrated, cleared, and mounted. The negative control tissue was prepared in the same manner described above, except that primary Ab was omitted or replaced by isotype control Ab (IgG1; R&D Systems).

ELISA of VEGF

The VEGF in culture supernatants was quantified by sandwich ELISA, as described previously (17).

Synoviocyte proliferation assay

The synoviocyte proliferation rates were assessed by [3H]thymidine incorporation assay. Briefly, the FLS were incubated for 48 h in serum-free DMEM supplemented with ITS+. The medium was replaced with fresh DMEM-ITS+. The VEGF165, ranging from 0 to 100 ng/ml, was then added to the cells (3 × 10⁵) for the indicated time. Before the final 6 h of culturing, 1 μCi of [3H]thymidine (NEN) was added to each of the wells. The cells were harvested onto nitrocellulose membranes, and the incorporated radioactivity was counted with a scintillation counter.

Apoptosis assay

Apoptosis was induced in FLS by 5 days of serum deprivation or by the administration of SNP (1 mM). The amount of FLS (3 × 10⁵ cells) that underwent apoptosis was determined by ELISA for the level of cellular DNA fragmentation (Roche Applied Science). The degree of apoptosis was also evaluated by flow cytometric analysis for annexin V. In brief, the cells were transferred from the culturing wells to a staining tube, washed twice in cold PBS, and incubated for 10 min at room temperature with annexin V-FITC (BioSource) at a final concentration of 2.5 µg/ml in annexin V buffer (10 mM HEPES-NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl₂). After two washings in annexin V buffer, the samples were resuspended in the same buffer and then analyzed with a FACSCalibur (BD Biosciences) flow cytometer. Immediately before analysis, propidium iodide (BioSource) was added, at a final concentration of 5 µg/ml.

Determination of pAkt and pERK activity

The RA FLS were incubated for 24 h in DMEM without FCS before the administration of VEGF₁₆₅ and were then treated with VEGF₁₆₅ (50 ng/ml) for various times. The expressions of Bcl-2, caspase-3, and Bax proteins were detected by Western blot analysis. In addition, the subcellular localization of the Bax protein in FLS was investigated. Briefly, to prepare the cytosolic and mitochondrial fractions, the cells were resuspended in an isotonic buffer containing 10 mM HEPES (pH 8.0), 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 2 mM PMSF (Sigma-Aldrich), 100 µg/ml leupeptin (Sigma-Aldrich), and 10 µg/ml pepstatin (Sigma-Aldrich) and then homogenized with the needle of a 30-gauge syringe. The cell homogenates were spun at 1000 × g to remove any unbroken cells, nuclei, or heavy membranes. The supernatant was then spun for 30 min at 14,000 × g to collect the mitochondrial (pellet) and cytosolic (supernatant) fractions. The mitochondrial fraction was washed once in extraction buffer and then resuspended in radioimmunoprecipitation buffer. Aliquots of each fraction were resolved by SDS-PAGE, and then immunoblotted for Bax. The following Abs were used for Western blot analysis: anti-Bax, anti-beta-actin, anti-tubulin, and anti-HSP-60 Abs (Santa Cruz Biotechnology).

Transfection of siRNA for NP-1 transcripts

For the down-regulation of NP-1 transcripts, 50 DNA template oligonucleotides were designed, yielding 21-nucleotide siRNA corresponding to 807–828 of NP-1 cDNA (GenBank Accession No. NM_003873), using an online siRNA sequence selector tool (Invitrogen Life Technologies). To generate double-stranded hairpin RNAs specific for NP-1 from the human U6 promoter, the pENTR-U6 vector (Invitrogen Life Technologies) was used. The sequences of the following oligonucleotides were used to create the pENTR-NP-1: 5'-CACCGCTACGCCGCCGCTAGAAATCTCCTGAAAGATTTCTAGCC-GTTGATACGTCGTAAG-3' and 5'-AAAAAGCTACGACGCGTCAAGATATCCTGGATGATGACGTCGTAAG-3'. The negative control plasmids were provided by Invitrogen Life Technologies. They expressed a hairpin siRNA with limited homology to all known sequences in the human, mouse, and rat genomes. Each of the oligonucleotides was mixed in equimolar amounts, heated for 5 min at 95°C, gradually cooled to room temperature in annealing buffer (10 mM Tris-HCl, 1 mM EDTA, and 100 mM NaCl), and finally cloned into the pENTR-U6 vector. These clones were confirmed by DNA sequencing (ABI Prism 310; Applied Biosystems). For the siRNA transfection, the FLS immortalized with SV40 Tag were used as previously described (19). The day before the transfection, the cells were trypsinized and seeded in 6-well culture plates at 3 × 10⁴ cells/well. The cells were then transfected with 0.8 µg of pENTR-U6-NP1 or pENTR-U6-control using Lipofectamine 2000 reagent (Invitrogen Life Technologies), according to the manufacturer’s instructions. Twenty-four hours after transfection, the cells were harvested, and the NP-1 mRNA and protein expression levels were determined by RT-PCR and Western blot analysis, respectively.

Results

The binding motif of VEGF₁₆₅ to its receptor in the RA synoviocytes is the HBD

To characterize the VEGF₁₆₅ binding sites, ¹²⁵I-VEGF₁₆₅ binding experiments were conducted with the FLS isolated from the RA (n = 3) and OA patients (n = 3). There were specific binding sites for ¹²⁵I-VEGF₁₆₅ on the surface of all of the FLS from RA or OA patients, as shown by the reduction of binding of labeled VEGF₁₆₅ in the presence of excess of nonlabeled VEGF₁₆₅ (Fig. 1A). No

FIGURE 1. Characteristics of VEGF-binding sites on the surface of synoviocytes from arthritis. A, ¹²⁵I-VEGF₁₆₅ binding to the cell surface was verified by the procedures described in Materials and Methods. A, Specific binding of ¹²⁵I-VEGF₁₆₅ was observed in all of six FLS from RA and OA patients. B, Total binding of ¹²⁵I-VEGF₁₆₅, nonspecific binding, which was determined by binding in the presence of excess of cold VEGF₁₆₅. C, Time-dependent increase in ¹²⁵I-VEGF₁₆₅ binding to the cell surface of FLS. E, Effect of hypoxic (H) stimulation on the binding of VEGF to FLS. D, Inhibition of ¹²⁵I-VEGF₁₆₅ binding to the cell surface of FLS is not altered by hypoxic stimulation, as compared with normoxic (N) conditions. D, Inhibition of ¹²⁵I-VEGF₁₆₅ binding to the FLS by exogenous VEGF₁₆₅ and its HBD, but not by VEGF₁₂₁, V₁₆₅, VEGF₁₆₅/VEGF₁₂₁, VEGF₁₂₁.
NP-1 is the major VEGF<sub>165</sub> receptor on the synoviocytes

To confirm that NP-1, but not KDR or Flt-1, is the major VEGF<sub>165</sub> receptor on the FLS, we examined the Flt-1, KDR, and NP-1 expression in the FLS of RA and OA patients by RT-PCR and Western blot analysis. As expected, the HUVEC, which were used as a control, evidenced expression of the mRNA and protein of all three of the VEGF<sub>165</sub> receptors. The FLS showed different expression levels for each VEGF<sub>165</sub> receptor subtype. All of the FLS expressed the NP-1 mRNA and protein at high level (Fig. 2, A and B). The Flt-1 was also expressed in the majority of the FLS, but its protein expression level was much lower than the NP-1 protein level. The KDR mRNA was detected only in some of the FLS, but no KDR protein was noted in OA and RA samples tested, indicating that KDR is not a major receptor in the FLS, although it is in the HUVEC line. There were no differences in the pattern and expression level of VEGF<sub>165</sub> receptor subtypes between the RA and OA FLS, which is consistent with the data obtained from the receptor binding assay using <sup>125</sup>I-VEGF<sub>165</sub>. In our immunocytochemical tests for the NP-1 protein, the FLS stained strongly with the anti-NP-1 Ab, as did the HUVEC, thereby verifying the presence of the NP-1 protein on the surfaces of these synoviocytes (Fig. 2C). Collectively, these results clearly indicate that NP-1 is the principal binding site for VEGF<sub>165</sub> on the surface of the FLS, and Flt-1 or KDR is the minor one.

The FLS are the major cell types consisting of the lining layer of synovium. To investigate the distribution and localization of NP-1 in synovial tissues, we performed immunohistochemical staining of the synovium of three RA patients and two OA patients using an Ab to NP-1. The NP-1 expression was observed in all three RA synovial tissue sections. The positive staining was mainly seen in the lining layer (Fig. 3, A, B, D, and E), infiltrating leukocytes (Fig. 3, B and D), and endothelial cells in the sublining (Fig. 3E) of RA synovium, highlighting the ubiquitous expression of NP-1. NP-1 was also expressed in the lining layer of two OA synovium (Fig. 3, F–H). As a negative control, isotype Ab did not

significant difference in binding affinity of VEGF<sub>165</sub> to its receptor was found among patients or between the RA and OA patients. <sup>125</sup>I-VEGF<sub>165</sub> binding was increased over time and reached the saturation at ~16 h (Fig. 1B). It has been documented that Flt-1 is induced by hypoxia but KDR and NP-1 are not (20). Thus, we attempted to determine whether any hypoxia-responsive VEGF-binding sites were present on the surfaces of the synoviocytes. Our findings revealed no differences in the binding ability of VEGF<sub>165</sub> between the hypoxia-stimulated and normoxia-maintained FLS (Fig. 1C), suggesting that Flt-1 does not significantly contribute to the binding of VEGF<sub>165</sub> to the FLS. NP-1 interacts with the HBD of VEGF<sub>165</sub> but Flt-1 and KDR do not (13). We next conducted blocking experiments using VEGF isoforms to further characterize the binding site of <sup>125</sup>I-VEGF<sub>165</sub>. The binding of <sup>125</sup>I-VEGF<sub>165</sub> to the FLS was abolished completely by the excess amount of nonlabeled VEGF<sub>165</sub> or its HBD (Fig. 1D). In contrast, the VEGF<sub>121</sub> isoform, which harbors no HBD, had no effect on the binding of <sup>125</sup>I-VEGF<sub>165</sub> to the FLS, indicating that the binding motifs of VEGF<sub>165</sub> and its receptors on the synoviocytes are HBD and NP-1, respectively. Together, these findings suggest that the primary VEGF<sub>165</sub>-binding site on the surfaces of the FLS is NP-1, rather than Flt-1 or KDR.

FIGURE 2. Expression of VEGF receptor subtypes in the synoviocytes of arthritis patients. A, RT-PCR for VEGF<sub>165</sub> receptor subtypes in FLS. The mRNA was extracted from OA FLS (lanes 1–3), RA FLS (lanes 4 and 5), and from HUVECs (HU) as described in Materials and Methods. Flt-1, KDR, and NP-1 expressions were determined by reverse transcription-PCR, using β-actin as a reference gene. Values are representative results of more than five experiments for each VEGF receptor. All experiments yielded similar results. B, Western blot analysis for VEGF<sub>165</sub> receptor subtypes. The proteins were extracted from HU and the FLS of three RA and three OA patients and were subjected to SDS-PAGE in 8% acrylamide gel. VEGF receptor subtypes and β-actin were detected by immunoblotting. All of the HU and FLS were different from the cells used in RT-PCR. Data are representative of three independent experiments. C, Immunocytochemistry was conducted on the RA FLS and HU using Abs to NP-1, as was described in Materials and Methods. The RA FLS stained strongly with anti-NP-1 Abs (red), as did the HU. Original magnifications: upper panel, ×100; lower panel, ×400.

FIGURE 3. Expression of NP-1 in the synovium of patients with RA and OA. Tissue sections from the synovium of patients with RA and OA were stained with the anti-NP-1 Ab. A–E, The NP-1 immunoreactivity in the synovium obtained from three different patients with RA. Rectangular area in A is magnified in B. Cells stained with anti-NP-1 Abs are shown in brown. Intense staining in the RA synovium was observed in the lining layer (pink arrowheads), endothelial surface of the synovial vessels (black arrows) and infiltrating leukocytes. In contrast, isotype control Ab did not stain the RA synovium (C). F–H, NP-1 expression in the synovium of two OA patients. G, Magnified image of the rectangular area in F. The positive staining was also observed in the lining layer of the OA synovium (pink arrowheads).
VEGF<sub>165</sub> protects rheumatoid synoviocytes from apoptotic death

A number of cytokines have been detected in the synovial fluid and synovial tissues of RA patients, including IL-1β and TGF-β, both of which are well-known stimulators of VEGF production (9, 21). As is shown in Fig. 4A, the RA synovial fibroblasts secreted more VEGF<sub>165</sub> than did the OA synoviocytes when stimulated with medium alone, IL-1β, or TGF-β, a finding consistent with earlier reports (21). VEGF<sub>165</sub> has been shown to stimulate the proliferation of endothelial cells via KDR and/or Flt-1 (4, 10, 11), and also protects tumor cells from apoptotic death via NP-1 (15). To characterize the role of VEGF<sub>165</sub> and its receptor in the biology of the synoviocytes, we investigated the effects of VEGF<sub>165</sub> on the proliferation and apoptosis of rheumatoid synoviocytes. Exogenous VEGF<sub>165</sub> (10 and 100 ng/ml) resulted in no increases in the uptake of [³H]thymidine in the RA FLS (Fig. 4B), suggesting that VEGF<sub>165</sub> does not contribute to the proliferation of synoviocytes. However, VEGF<sub>165</sub> effected a dose-dependent inhibition of the starvation-induced apoptosis of RA FLS (Fig. 4, C and F). The synoviocyte apoptosis induced by SNP, a NO donor, was also blocked by treatment with VEGF<sub>165</sub> in a dose-dependent manner (Fig. 4D). Considering that the FLS secreted a large quantity of VEGF<sub>165</sub> by themselves, our data suggest that VEGF<sub>165</sub> provides a survival advantage for the FLS in an autocrine manner, regardless of its angiogenic activity.

**Regulation of Bcl-2 expression and Bax translocation by VEGF<sub>165</sub>**

Our next experiment was to determine how VEGF<sub>165</sub> prevents SNP-induced synoviocyte apoptosis. It is well known that Bcl-2 family members are critical with regard to the regulation of survival via the modulation of mitochondrial integrity. In RA, the enhanced expression of antiapoptotic Bcl-2 family members, but not proapoptotic members including Bad and Bax, has been implicated in pathogenesis (22). As shown in Fig. 5A, the expression of Bcl-2 in the RA FLS was elevated 4 h after the treatment of VEGF<sub>165</sub> (50 ng/ml), and these levels remained elevated for up to 36 h. In contrast, treatment with VEGF<sub>165</sub> effected no change in the expression of Bax, the cell death activator, or in the expression of cyclin D, the signal molecule associated with cellular proliferation. The addition of SNP (1 mM) to the FLS culture resulted in a reduction in Bcl-2 expression, and this reduction was blocked by the addition of 50 ng/ml of VEGF<sub>165</sub> (Fig. 5B). Moreover, the SNP-induced translocation of Bax from the cytosol to the mitochondria, as well as the SNP-mediated increase in caspase-3 activity, was abrogated almost completely by VEGF<sub>165</sub> treatment (50 ng/ml) (Fig. 5C).

The activation of Akt-1 and ERK by growth factors maintains mitochondrial integrity via the up-regulation of Bcl-2 expression (23, 24) or via the inhibition of the damage promoted by members of the proapoptotic Bcl-2 family (22, 25). Given that Akt and ERK activation are both critical for the survival of RA FLS (22, 26, 27), we attempted to determine whether VEGF<sub>165</sub> would regulate the phosphorylation of Akt and ERK in the synoviocytes. Our findings indicated that the activities of pAkt and pERK 1/2 were rapidly triggered, as early as 5 min after stimulation with VEGF<sub>165</sub> (50 ng/ml; Fig. 5D). Moreover, the protective effect of VEGF<sub>165</sub> on SNP-induced FLS apoptosis was completely blocked by treating cells with LY294002 (20 μM) and PD98059 (20 μM), which are the Akt and ERK inhibitors, respectively (Fig. 5E). These findings, together with the results of earlier studies (23–27), appear to show that VEGF<sub>165</sub> promotes synoviocyte survival by increasing Bcl-2 expression and also by blocking Bax translocation from the cytosol to the mitochondria, as well as the SNP-mediated increase in caspase-3 activity.

**NP-1 is essential for the survival of synovial fibroblasts**

On the basis of the data provided in Figs. 1–5, we predicted that NP-1 would transmit an antiapoptotic signal to the rheumatoid synoviocytes via interference with the Bcl-2/Bax pathway upon VEGF<sub>165</sub>-stimulation. To address this hypothesis, we conducted a blocking experiment using pENTR-U6 vector-mediated siRNA for NP-1 transcripts. As shown in Fig. 6, A and B, the levels of NP-1 mRNA and protein expression were reduced in the FLS transfected with NP-1 siRNA, as compared with the levels observed in the control siRNA-transfected or untransfected cells. The knockdown of NP-1 mRNA in the FLS resulted in the spontaneous apoptosis.

**FIGURE 4.** VEGF<sub>165</sub> rescues the synoviocytes from apoptotic death induced by serum starvation or SNP. **A,** VEGF<sub>165</sub> production by RA vs OA FLS. The FLS (3 × 10⁴ cells) were cultured in serum-free DMEM supplemented with ITSa for 24 h in the absence or presence of 10 ng/ml TGF-β or 10 ng/ml IL-1β. The concentration of VEGF<sub>165</sub> in the supernatants was determined by ELISA. The results are presented as the mean (± SD) of three independent experiments, using different cells. **B,** Synoviocyte proliferative responses to VEGF<sub>165</sub>. The RA FLS were incubated for 24 h in DMEM supplemented with ITSa and treated with VEGF (10 and 100 ng/ml) for the indicated times. FLS proliferation rate was determined by [³H]thymidine incorporation assays. Data are expressed as means ± SD from three independent experiments. **C** and **D,** Inhibition of synoviocyte apoptosis by VEGF<sub>165</sub>. The apoptosis of FLS (3 × 10⁴ cells) was induced by 4 days of serum deprivation or by treating the cells with SNP (1 mM). The degree of apoptosis in the absence or presence of VEGF<sub>165</sub>, ranging from 1 to 50 ng/ml, was assessed by cellular DNA fragmentation ELISA. Data are expressed as the means ± SD of five independent experiments. **E,** Phase contrast microscopy of FLS apoptosis induced by serum starvation. The starved FLS (middle) became spherical, shrunken, and detached from the bottom of the culture plates, in contrast to the appearance of the cells treated with 10% FCS (left) or 50 ng of VEGF<sub>165</sub> (right) with a typical bipolar and attached configuration.
of the cells, as determined by DNA fragmentation ELISA (Fig. 6C). A 5-fold increase in the number of annexin V cells was also observed 24 h after the NP-1 siRNA transfection (Fig. 6D). Exogenous VEGF165 did not rescue the spontaneous apoptosis of the FLS transfected with NP-1 siRNA (Fig. 6D), implying that the VEGF165-induced increase in FLS survival is dependent primarily on the NP-1. To determine whether NP-1 regulates the mitochondrial apoptotic pathway, we finally investigated the changes in Bcl-2/Bax expression in the NP-1 knockdown cells. As shown in Fig. 7, A and B, the down-regulation of NP-1 resulted in a decrease in Bcl-2 expression, but a slight increase in Bax expression in the FLS, as compared with the untransfected or control siRNA-transfected cells (Fig. 7, A and B). Moreover, translocation from the cytosol to the mitochondria was strongly increased after NP-1 knockdown (Fig. 7C). Taken together, our results show that NP-1 regulates both Bcl-2 expression and Bax translocation and thereby protects the synoviocytes from apoptotic cell death.

**Discussion**

The rheumatoid synovium can be viewed as a tumor-like mass, which can destroy the bone and cartilage (1–3). Within the synovium, synovial fibroblasts actively participate in the chronic inflammatory responses, producing matrix-degrading enzymes, as well as several cytokines, including IL-1β, IL-6, and IL-8 (2, 28). Moreover, they proliferate abnormally, invading local environments and exhibiting characteristics reminiscent of tumor cells (1–3, 29–32). The RA-associated change in the synovial fibroblasts from normal to aggressive behavior may be attributable to the up-regulation of antiapoptotic genes. Synovial fibroblasts from RA patients harbored several oncogenes, including H-ras and p53 with somatic mutations, at higher abundance than were seen in normal synovial fibroblasts (29, 30). Such fibroblasts also abundantly express several anti-apoptotic proteins, including FLIP (31) and Bcl-2 (32), both of which protect against apoptosis initiated via death receptor- or mitochondria-dependent pathways. Moreover,
FIGURE 6. Down-regulation of NP-1 by RNA interference induces the spontaneous apoptosis of synoviocytes. A and B, Down-regulation of NP-1 mRNA and protein by siRNA. NP-1 knockdown cells were established via transfection of the NP-1 siRNA into the immortalized synoviocytes with SV40 T Ag (M7HA cells) using the pENTR-U6 vector system, as described in Materials and Methods. The mRNA and protein expression levels for NP-1 were determined by RT-PCR (A) and Western blot analysis (B), respectively. NP-1 expression was reduced by >80% in the cells transfected with NP-1 siRNA (lane 2) as compared with those transfected with control siRNA (lane 3) or untransfected cells (lane 1). Data are representative of three independent experiments yielding similar results. C, Induction of spontaneous synoviocyte apoptosis by NP-1 knockdown. The degree of apoptosis was determined via DNA fragmentation ELISA 24 h after the transfection of NP-1 siRNA: lane 1, untransfected; lane 2, control siRNA; and lane 3, NP-1 siRNA. Data are the means ± SD of three separate experiments. D, The percentage of apoptosis was determined by flow cytometric analysis for annexin V 24 h after transfection with NP-1 siRNA: top, untransfected; middle, control siRNA; bottom, NP-1 siRNA. Values are the percentage of annexin V<sup>+</sup> propidium iodide<sup>−</sup> cells, indicating early synoviocyte apoptosis. Data represent four independent experiments with similar results.

FIGURE 7. Changes in the expression of Bcl-2 and Bax translocation by NP-1 knockdown. A and B, NP-1 regulates Bcl-2 expression and Bax translocation. The immortalized synoviocytes were transfected with control siRNA (lane 2) or NP-1 siRNA (lane 3), and the expressions of NP-1, Bcl-2, and Bax were determined by Western blot analysis. Lane 1, Data obtained from the untransfected cells. The ratios of NP-1 to β-actin (left axis) and Bcl-2/Bax (right axis) expression are presented as the means ± SD of three separate experiments, in B, C, NP-1 mediates the translocation of Bax from the cytosol to the mitochondria. The synoviocytes were fractionated as described in Materials and Methods. The amounts of Bax protein in the cytosol or mitochondria were determined by Western blot analysis 24 h after the transfection of NP-1 siRNA. Hsp60, heat shock protein 60. Values are representative of three independent experiments: lane 1, untransfected; lane 2, control siRNA; and lane 3, NP-1 siRNA.

the chronic exposure of rheumatoid synoviocytes to growth factors and cytokines within the joints, including TGF-β1, fibroblast growth factor, and IL-13 (33–35), protects the synoviocytes from apoptosis, thereby maintaining a unique transformed phenotype. In this sense, the induction of synoviocyte apoptosis, either through the inhibition of antiapoptotic molecules or via cytokine modulation, might prove therapeutically beneficial in RA.

NP-1 is a receptor for members of the class III semaphorin subfamily, which are known to mediate axon guidance (14). Recent studies have indicated that NP-1 mediates a variety of biological functions in extraneuronal systems. NP-1 modulates the functions of VEGF<sub>165</sub> during angiogenesis (12, 13). NP-1 also appears to be essential for the initiation of the primary immune response and is also likely to mediate contacts between the dendritic cells and the T lymphocytes, via homotypic interactions (36). Moreover, the ligation of VEGF<sub>165</sub> to NP-1 enhances both the survival and invasion of breast cancer cells in vitro (15, 37). Given that RA synovial fibroblasts show similar characteristics with metastatic cancer cells (1–3, 29–32), it would be highly relevant to test the role of NP-1 in the survival of rheumatoid synoviocytes. In this study, we have demonstrated that NP-1 is highly expressed in the rheumatoid synovium and is the major VEGF<sub>165</sub> receptor in the synovial fibroblasts of RA and OA patients. The ligation of VEGF<sub>165</sub> to its receptor was shown to protect the synoviocytes against apoptosis. Moreover, the siRNA-induced down-regulation of NP-1 resulted in spontaneous apoptosis of synoviocytes. Collectively, these data indicate that the interaction between VEGF<sub>165</sub> and NP-1 is critical for the survival of the rheumatoid synoviocytes.

Our work also underscores the importance of VEGF<sub>165</sub> as the driving force in the perpetuation of synovial proliferation. There exist several potential mechanisms whereby VEGF might enhance the survival of the synoviocytes. First, as was evidenced in this study, VEGF<sub>165</sub>, which is primarily generated by macrophages and synoviocytes, can hamper synoviocyte apoptosis via binding to NP-1 and thus can function as a survival factor, in an autocrine or paracrine manner. Second, VEGF<sub>165</sub> diminishes the growing burden of synoviocytes by supplying oxygen and nutrients for tissue metabolism. Third, VEGF<sub>165</sub> may indirectly stimulate the proliferation of synoviocytes. For example, the VEGF<sub>165</sub>-stimulated increase in cytochemokine generation may recruit leukocytes into the synovial membrane (17, 38, 39), in which newly used leukocytes might induce synoviocyte proliferation via cell-to-cell contact. If this is indeed the case, the development of synovial inflammation, hyperplasia, and angiogenesis may be regulated by a common cue, VEGF<sub>165</sub>, in the joints of RA patients.

The unique transformed phenotype of the RA synoviocytes has been associated with a genotoxic environment containing reactive oxygen species, growth factors, and proinflammatory cytokines (1–3). In this study, the ability of VEGF<sub>165</sub> to bind to its receptor and the levels of NP-1 expression did not differ between the FLS from RA and OA patients. Therefore, the finding that NP-1 functions as a survival factor appears not to be unique to RA FLS but rather may be universal in synoviocytes, regardless of the underlying diseases. VEGF<sub>165</sub> can be detected with greater abundance in the sera, synovial fluid, and inflamed synovial tissues of RA patients than in those of OA patients (5–8). Therefore, the synoviocytes of RA patients may have more opportunity to be stimulated by VEGF<sub>165</sub> than do the OA synoviocytes. Under these circumstances, the synoviocytes in the RA joints might be hyperplastic...
and may in turn secrete more VEGF165, generating positive feedback for survival, ultimately resulting in their transformation into aggressive cells. The hypothesis regarding an autocrine mechanism of transformation has been bolstered by recent findings that the constitutive expression of platelet-derived growth factor, a family of growth factors, resulted in malignant transformations in mice (40, 41).

The Bcl-2 gene family encodes for several proteins that regulate apoptosis in mammalian cells. Bcl-2, Bcl-XL, and Mel-1 are known to inhibit apoptosis, whereas others, including Bax, Bik, Bak, Bad, and Bcl-XD, appear to promote apoptosis (22). In particular, Bcl-2 expression in the synovial fibroblasts is crucial for the maintenance of mitochondrial homeostasis and cell viability (32). Although VEGF165 has been shown to promote cell survival via the induction of Bcl-2 in leukemic and endothelial cells (42, 43), the exact role of NP-1 in the regulation of the Bcl-2/Bax pathway has yet to be clearly elucidated. In this study, VEGF165 treatment resulted in an increase in the expression of Bcl-2 in rheumatoid synoviocytes, and also elevated levels of pAkt and pERK, both of which are known to be located upstream of the Bcl-2 signaling pathway (23–25). The antiapoptotic effect of VEGF165 was cancelled by the Akt or ERK inhibitor, suggesting that both signals mediate the VEGF165-induced increase in synoviocyte survival. In addition, the SNP-induced translocation from the mitochondria to the cytosol was blocked completely by the ligation of VEGF165 to its receptor. Moreover, the siRNA-induced selective inhibition of NP-1 transcripts resulted in a suppression of Bcl-2 expression but also caused the translocation of Bax from the cytosol to the mitochondria, thereby protecting the synoviocytes from apoptosis.

The biological relevance of our data requires comment. We examined synovial fibroblasts from RA patients undergoing knee replacement. These patients represent far advanced disease with secondary degenerative changes that may affect the synovial cell behavior and not reflect changes occurring at an earlier phase (44). In addition, we failed to consider the pivotal role of T cells in the pathogenesis of RA. T cells have a primary pathogenic role in RA and the fact that activated T cells secrete VEGF is highly relevant to this study (45). Finally, moderate reduction (~40%) in the level of NP-1 expression led to the total abolishment of the effect of VEGF165 on synoviocyte survival. This lack of VEGF165 effect may represent a more essential and critical role of NP-1 in cell survival than VEGF165. NP-1 appears to serve a hub receptor for different ligands, such as semaphorin and placenta growth factor-2. Other ligands for NP-1, as well as VEGF165, might be present in the RA joints, and display a similar mode of action to VEGF165. We are currently investigating such a possibility.

In conclusion, our data indicate that NP-1 is the principal VEGF165 receptor on the RA synovial fibroblasts and that it plays a crucial role in the protection of the synoviocytes from apoptosis. The binding of VEGF165 to NP-1 blocked the SNP-induced down-regulation of Bcl-2 and the translocation of Bax, which may be responsible for the cytoprotective activity of VEGF165. Our data point to the novel function of VEGF165 and NP-1 in the pathogenesis of RA and also explain how normal synoviocytes acquire the hyperplastic phenotype. In addition, the results of this study underline the importance of NP-1 as a potential candidate for the therapeutic modulation of chronic inflammatory arthritis.

Acknowledgments
We thank Dr. Seung-Pil Yang for help with preparation of heparin-binding domain and Dr. Deug Y. Shin for constructive comments on this study.

Disclosures
The authors have no financial conflict of interest.

References


