Angioinhibitory Action of NK4 Involves Impaired Extracellular Assembly of Fibronectin Mediated by Perlecan-NK4 Association*§

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NK4, a fragment of hepatocyte growth factor (HGF), exerts bifunctional action as a competitive antagonist against HGF and its receptor c-Met and an angiogenesis inhibitor. Here we studied the anti-angiogenic mechanism of NK4. In cultured human endothelial cells, NK4 inhibited DNA synthesis induced not only by HGF but also by either basic fibroblast growth factor or vascular endothelial growth factor. Even if c-Met expression was diminished by small interference RNA, NK4 inhibited basic fibroblast growth factor-induced DNA synthesis, indicating that anti-angiogenic action of NK4 is c-Met-independent. Affinity purification with NK4-immobilized beads revealed that NK4 binds to perlecan. Consistent with this, NK4 colocalized with perlecan in endothelial cells. Perlecan is a multidomain heparan sulfate proteoglycan that interacts with basement membrane components such as fibronectin. NK4 inhibited extracellular assembly of fibronectin, by which fibronectin-dependent endothelial cell spreading was inhibited by NK4. Knockdown of perlecan expression by small interference RNA significantly abrogated the inhibitory effect of NK4 on fibronectin assembly and cell spreading. In NK4-treated endothelial cells, tyrosine phosphorylation of focal adhesion kinase and Rac activation were reduced, whereas overexpression of activated Rac recovered the DNA synthesis in NK4-treated endothelial cells. These results indicate that the association between NK4 and perlecan impairs fibronectin assembly, thereby inhibiting anchorage-dependent signaling. The identified mechanism for angioinhibitory action provides further proof of significance for NK4 in the treatment of cancer and potentially for vascular regulation as well.

The manipulation of angiogenesis has potential therapeutic value for the treatment of a variety of diseases including cancer, arthritis, and cardiovascular disease (1, 2). In addition to endothelial cell migration and proliferation, angiogenesis is a process involving dynamic matrix transition (3). During angiogenesis, the vascular basement membrane undergoes proteolytic degradation and transit to the provisional matrix consisting of fibronectin, etc., followed by an intermediate and mature new vascular basement membrane. Growing evidence has shown that such an extracellular matrix (ECM)2 not only provides mechanical support to the cells but also essentially regulates cell growth, migration, and survival. The fact that a number of endogenous inhibitors of angiogenesis have been identified from proteolytic fragments of ECM molecules also highlights the important regulatory roles of ECM in angiogenesis (3).

NK4 is a proteolytic fragment of hepatocyte growth factor, HGF (4), consisting of an N-terminal hairpin domain and four kringle domains of the α-chain of HGF (5). By competitively binding to HGF receptor c-Met, NK4 acts as an HGF antagonist (5, 6). The NK4 fragment seems to be physiologically generated by mast cells and neutrophils peptidases during inflammation (7). Because HGF regulates malignant behavior in a variety of tumors by inducing invasive, angiogenic, and metastatic responses (8, 9), the blockade of HGF-c-Met signaling by NK4 is a strategy to inhibit tumor invasion and metastasis (6, 9–11). During investigation of a therapeutic approach with NK4 in experimental cancer models, we unexpectedly found that NK4 functions as an angiogenesis inhibitor (12). Based on the bifunctional characteristic as HGF antagonist and angiogenesis inhibitor, NK4 suppressed malignant behavior of cancers, including invasion, metastasis, and angiogenesis-dependent tumor growth (9–12).

The angiostatic activity of NK4 is probably independent of its original activity as an HGF antagonist because an anti-HGF antibody capable of preventing HGF-c-Met association did not inhibit human endothelial cell growth stimulated by either bFGF or VEGF (12). However, the mechanism by which NK4 inhibits angiogenic responses in endothelial cells remains to be addressed. In the present study we newly identified perlecan to be an NK4 binding molecule and found that in vascular endothelial cells the association of NK4 with perlecan inhibited extracellular fibronectin assembly, fibronectin-dependent cell

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2 The abbreviations used are: ECM, extracellular matrix; HGF, hepatocyte growth factor; bFGF, basic fibroblast growth factor; VEGF, vascular endothelial growth factor; HSPG, heparan sulfate proteoglycan; siRNA, small interference RNA; FAK, focal adhesion kinase; BrdUrd, 5-bromo-2′-deoxyuridine; PAK, p21-activated kinase; ERK, extracellular signal-related protein kinase; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; BSA, bovine serum albumin; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PBS, phosphate-buffered saline; Rb, retinoblastoma 1.
spreading, and the subsequent anchorage-dependent signals. Together with our finding that c-Met/HGF receptor is not required for the inhibition of DNA synthesis by NK4, we propose that the association of NK4 with perlecan plays a key role in angiogenesis inhibition by NK4.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant NK4 was prepared as described elsewhere (12). VEGF-A165 was obtained from R&D Systems. bFGF was a gift from Kaken Pharmaceutical. Fibronectin-related peptides (GRGDSP and GRGESP) were obtained from Takara Bio. Cycloheximide was obtained from Calbiochem. Human plasma fibronectin (Sigma) was biotinylated with NHS-sulfo-LC-LC-biotin (Pierce) according to the manufacturer’s instructions. Anti-HGF antibody used for NK4 detection was prepared as described previously (13). Other antibodies were obtained as follows: anti-Rb (G3-245), anti-cyclin D1 (DCS-6), anti-paxillin (349), and anti-5-bromo-2′-deoxyuridine (BrdUrd) (B44) were from BD Biosciences; anti-c-Met (C-12), anti-PAK (N-20), anti-c-Myc (A-14), and anti-phosphotyrosine (PY99) were from Santa Cruz Biotechnology; anti-PAK (N-20), anti-c-Myc (A-14), and anti-phosphotyrosine (PY99) were from Santa Cruz Biotechnology; anti-β-actin (AC-15) and anti-α-tubulin (B-5-1-1) were from Sigma; anti-phospho-ERK1/2 (E10) was from Cell Signaling; anti-ERK1/2 was from Upstate Biotechnology; anti-phospho-FAK (Tyr(P)-397) was from BIOSOURCE; anti-fibronectin (3E3) and anti-laminin γ1 (A5) were from Chemicon; anti-perlecan (7B5) was from Zymed Laboratories Inc.; anti-heparan sulfate (C58–10E4) was from Seikagaku; anti-syndecan-1 was from BioVision; anti-syndecan-2 was from R&D Systems.

**Cell Culture and Preparation of ECM**—Human umbilical vein endothelial cells (HUVECs) were purchased from Cambrex and grown in MCDB131 medium supplemented with 5% fetal bovine serum (FBS), 2 mm l-glutamine, and 20 ng/ml bFGF. Culture plates for endothelial cells were coated with 0.1% gelatin (Sigma). For cell spreading assay, the cover glass was coated with 0.1% gelatin, 5 μg/ml fibronectin (Sigma), 5 μg/ml vitronectin (Sigma), 5 μg/ml collagen type I (BD Biosciences), 5 μg/ml laminin (Sigma), or 10 μg/ml collagen type IV (Sigma) at room temperature for 5 h. After washing with PBS twice, the cover glass was blocked with 3% BSA in PBS for 1 h. Human dermal fibroblasts were prepared as described elsewhere (12). ECM was prepared according to Hedman et al. (14). Conditioned medium was concentrated by Centricon YM-100 (Millipore).

**BrdUrd Labeling**—HUVECs were synchronized in G0 by growth factor and serum deprivation for 24 h. G0-synchronized HUVECs were re-plated and cultured with MCDB131 medium supplemented with 5% FBS, 2 mm l-glutamine, 10 μm BrdUrd with and without 20 ng/ml bFGF, VEGF, or HGF, and NK4 for 24 h. The cells were stained with anti-BrdUrd antibody, and nuclei were stained with TO-PRO-3 (Invitrogen).

**Western Blot and Immunoprecipitation**—Cells were lysed on ice with 50 mm Tris-HCl (pH 7.5), 150 mm NaCl, 25 mm β-glycerophosphate, 50 mm NaF, 1 mm Na3VO4, 1% Triton X-100, 10% glycerol, and protease inhibitors (1 mm phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, pepstatin A, and leupeptin). Protein concentration was determined by DC protein assay reagent (Bio-Rad). Immunoprecipitation and Western blotting was performed as described previously (12).

**Immunostaining**—For immunofluorescent staining, cells on cover glass were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked with 3% BSA in PBS for 1 h at room temperature. For BrdUrd staining, the cells were treated with 2 M HCl and neutralized with 0.1 M sodium tetaborate before blocking. The cells were incubated with primary antibodies followed by secondary antibodies conjugated to Alexa Fluor 488 or 546 (Invitrogen). Biotinylated fibronectin was stained with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Actin was stained with phalloidin-rhodamine (Cytoskeleton). Nuclei were stained with TO-PRO-3. Cells were imaged at room temperature in Perma Fluor (Shandon Immunon) using a laser-scanning confocal microscope (LSM5 PASCAL; Carl Zeiss). Images were adjusted for brightness and contrast and cropped using Photoshop 6.0 (Adobe) software. The fluorescence intensity of the phospho-FAK (a), the area (b in pixel) in each focal adhesion, and the total number of focal adhesions per single cell (c) were determined by image analysis using adobe Photoshop 6.0. The fluorescence intensity of the phospho-FAK per cell was obtained by multiplying a × b × c. Each value in the data represents the means ± S.D. calculated from more than 20 cells in random fields.

**Identification of Proteins Bound to NK4**—NK4 was biotinylated with NHS-sulfo-LC-LC-biotin. Plasma membrane from HUVECs was prepared by sucrose gradient centrifugation and was lysed with 20 mm Tris-HCl buffer (pH 7.5) containing 1% Triton X-100, 150 mm NaCl, 5 mm MgCl2, 5 mm CaCl2, and protease inhibitors as above. The lysate was incubated with 200 nm biotin-NK4 for 2 h at 4 °C and applied to streptavidin-agarose (Sigma). After washing with lysis buffer, proteins were eluted with 20 mm Tris-HCl (pH 7.5) containing 500 mm NaCl, concentrated by Centricon YM-10 (Millipore), separated by SDS-PAGE, and visualized by silver staining. Gels were cut out, and proteins were reduced, S-carboxymethylated, and digested with trypsin. Peptides were analyzed by MALDI-TOF mass spectrometry (Bruker Daltonics).

**Binding Assay**—ELA/RIA 96-well plates (Coster, #9018) were coated with 0.2% gelatin in PBS overnight at 4 °C and blocked with 3% BSA in PBS at room temperature for 2 h. The wells were washed with PBS and incubated with the indicated concentrations of biotin-fibronectin with or without 300 nm NK4 in PBS at room temperature for 2 h, washed with PBS, and incubated with 2 μg/ml Alexa Fluor 488-conjugated streptavidin in PBS at room temperature for 1 h. After washing with PBS, fluorescent intensity was measured with a Multi-Detection Microplate Reader, POWERSCAN HT (Dainippon Pharmaceutical).

**Tubulogenesis in Collagen Gel**—HUVECs were suspended in 1 mg/ml type I collagen (BD Biosciences) at 1 × 106 cells/ml. Cells were cultured in MCDB131 medium supplemented with 5% FBS with and without 20 ng/ml bFGF and 500 nm NK4 for 24 h. The cells were fixed with 4% paraformaldehyde in PBS and stained with 0.4% crystal violet. For detection of pericellular fibronectin, each 20-μl aliquot of cell suspension in collagen was dropped on cover glass. Cells were cultured for 24 h, fixed
with 4% paraformaldehyde in PBS, and blocked with 3% BSA in PBS. The cells were incubated with primary antibody and subjected to 3,3′-Diaminobenzidine staining (Dako Cytomation). Between each step, samples were extensively washed 3 times with PBS for 1 h.

Small Interference RNA (siRNA)—siRNA oligonucleotides were obtained from Nippon EGT. siRNAs for c-Met were siMet#1 (sense, 5′-CAUACACAUUGCUAGAUGUUtt-3′; antisense, 3′-GUAGCAGCGUGAGGAACAGAu-5′) and siMet#2 (sense, 5′-CUUCUUUAGGCAUACCUUtt-3′; antisense, 3′-ttGAAGAAACAUCGUAAUGG-5′) and the siRNAs for perlecan were 5′-GUUGAGAGCGCCGACAUUt-3′ (sense) and 3′-tCAACCUCGUCGCGCUAUAU-5′ (anti-sense). Random siRNA was used as a control. HUVECs were transfected with 100 nm siRNA by Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions and recovered for 16 h in complete medium before each assay.

PAK Kinase Assay and Electroporation of Active Rho Family Proteins—PAK was immunoprecipitated, and PAK activity was determined by measuring phosphorylation of myelin basic protein as described previously (15). cDNAs encoding Myc-tagged-Rho families (gifts from Dr. Y. Takai, Osaka University) were cloned into pcDNA3.1(-)HisA. Cells (2 × 10⁶) resuspended in 400 μl of Opti-MEM (Invitrogen) were transfected with 20 μg of each vector by electroporation at 240 V and 950 microfarads and recovered for 16 h in complete medium before each assay.

RESULTS

c-Met-independent Inhibition of bFGF-induced DNA Synthesis by NK4—We previously demonstrated that NK4 inhibited endothelial cell growth and migration induced not only by HGF but also by either bFGF or VEGF (12). We first examined whether NK4 exhibits angioinhibitory action in other assays. In an endothelial tube formation assay on Matrigel, NK4 inhibited tube formation induced not only by HGF but also by bFGF (supplemental Fig. S1). When we examined DNA synthesis by BrdUrd incorporation, NK4 inhibited DNA synthesis induced not only by HGF but also by either bFGF or VEGF (Fig. 1A). Thus, NK4 showed angioinhibitory action in different in vitro assays.

To address the mechanism(s) by which NK4 inhibits angiogenesis, we evaluated intracellular signaling involved in DNA synthesis. NK4 dose-dependently inhibited the increase in cyclin D1 expression and Rb phosphorylation induced by bFGF in endothelial cells (Fig. 1B, left panels), suggesting that NK4 inhibits cell cycle progression at the G₁ phase. Interestingly, NK4 did not inhibit bFGF-induced up-regulation of cyclin D1 and Rb phosphorylation in fibroblasts (Fig. 1B, right panels). This was consistent with our previous finding that NK4 did not inhibit proliferation of fibroblasts enhanced by bFGF (12).

To clarify whether c-Met is required for the angioinhibitory action of NK4, we examined the effect of NK4 on DNA synthesis of endothelial cells in which c-Met expression was reduced by siRNA. Transfection of two different sequences of siRNA for c-Met remarkably inhibited c-Met expression in endothelial cells (Fig. 1C, right panel). In a comparable profile to that of control siRNA, bFGF-induced DNA synthesis was inhibited by NK4 in cells transfected with siRNA for c-Met (Fig. 1C, left graph). These results indicate that c-Met is not required for the inhibition of bFGF-induced DNA synthesis by NK4.
Identification of perlecan as an NK4-interacting protein. A, identification of NK4-binding proteins by affinity purification and mass spectrometry analysis. Plasma membranes prepared from HUVECs were solubilized, incubated with or without biotinylated-NK4, and subjected to affinity purification using a streptavidin-agarose column. Bound proteins were subjected to SDS-PAGE and subsequent silver staining (upper panel). Each band was identified by trypsin digestion and MALDI-TOF MS analysis. Immunoblotting with anti-perlecan antibody confirmed perlecan in the NK4-bound fraction (lower panel). B, colocalization of NK4 with perlecan in endothelial cell culture. HUVECs were re-plated and cultured with 100 nM NK4 and 20 ng/ml bFGF for 2 h. Cells were fixed, permeabilized, and stained for NK4 (red) and perlecan (green). Scale bar, 20 μm.

Identification of Perlecan as a NK4-binding Protein—To identify proteins mediating the angiostatic action of NK4, plasma membrane proteins prepared from endothelial cells were affinity-purified using beads immobilized with NK4. Proteins bound to the NK4 column were eluted, separated by SDS-PAGE, and subjected to protein staining. A mass spectrometry analysis of peptides revealed that these proteins were perlecan, laminin γ1 chain, and thrombospondin-1 (Fig. 2A, upper panel). Western immunoblot of a purified fraction confirmed perlecan association (Fig. 2A, lower panel). We further examined whether NK4 is colocalized by immunostaining with perlecan on endothelial cells (Fig. 2B) and human fibroblasts (supplemental Fig. S2A). NK4 significantly colocalized with perlecan both in endothelial cells and fibroblasts. However, the amount of NK4 colocalized with perlecan was much less because of very low expression of perlecan in fibroblasts (supplemental Fig. S2, A and B). In contrast, significant colocalization of NK4 with thrombospondin-1 or laminin γ1 was not observed.3

Inhibition of Fibronectin-dependent Endothelial Cell Spreading on Gelatin by NK4—Colocalization of NK4 with perlecan let us consider the effect of NK4 on the interaction between endothelial cells and ECM because perlecan participates in the assembly of basement membrane components through its ability to bind ECM proteins including fibronectin (16–20). When endothelial cells were cultured on gelatin, NK4 induced morphological changes characterized by elongation and insufficient spreading (Fig. 3A). Because endothelial cell spreading on gelatin largely depended on the presence of serum, we speculated that the spreading on gelatin depends on the presence of either fibronectin or vitronectin in serum. To test this possibility, we examined the effect of GRGDSP peptides, blocking cell adhesion to fibronectin and vitronectin, on endothelial cell spreading on gelatin. GRGDSP peptides prevented endothelial cell spreading on gelatin, whereas control GRGESP peptide had no effect (Fig. 3B). Next, to deplete the fibronectin and vitronectin that was either in serum or produced by endothelial cells, endothelial cells were cultured with cycloheximide under serum-free conditions. Under these conditions, endothelial cells did not spread on gelatin, whereas the addition of purified fibronectin restored the spreading (Fig. 3C). Importantly, NK4 inhibited this fibronectin-dependent endothelial cell spreading (Fig. 3C). We considered three possible mechanisms by which NK4 inhibited fibronectin-dependent endothelial cell spreading, 1) inhibition of integrin-mediated endothelial cell adhesion on fibronectin, 2) inhibition of interaction between gelatin and fibronectin, and 3) inhibition of extracellular assembly of fibronectin. The possibilities of 1 and 2 were unlikely because (i) endothelial cell adhesion on precoated fibronectin was not prevented by NK4 (Fig. 3D), and (ii) the interaction between fibronectin and gelatin was not prevented by NK4 in the binding assay (Fig. 3E).

When we examined the effect of NK4 on endothelial cell spreading on other ECM substrates, NK4 did not inhibit endothelial cell spreading on either collagen IV, laminin, or vitronectin, whereas it moderately inhibited spreading on type I collagen (supplemental Fig. S3). Thus, the inhibition of cell spreading by NK4 is most obvious on gelatin and moderately on type I collagen; both are major adhesive substances of fibronectin.

Inhibition of Extracellular Fibronectin Assembly—Fibronectin molecules are assembled by cells and exist as fibrillar extra-

3 K. Sakai, T. Nakamura, K. Matsumoto, and T. Nakamura, unpublished data.
fibronectin assembly. Immunostaining of perlecan and fibronectin in endothelial cell culture showed colocalization of these molecules in extracellular assembly (Fig. 5A). Endothelial cells express other heparan sulfate proteoglycans (HSPGs), such as syndecans and glypicans (23), and syndecan-2 is involved in fibronectin assembly (21, 24). However, we did not observe colocalization of either syndecan-1 or -2 with fibronectin in endothelial cells (supplemental Fig. S4). To test whether binding of NK4 to perlecan is required for its angiostatic action, we examined the effect of NK4 on endothelial cells in which perlecan expression was reduced by siRNA. Western immunoblot of cell lysates prepared after transfection of control-siRNA or perlecan-siRNA showed efficient knockdown of perlecan expression by perlecan-siRNA (Fig. 5B). NK4 significantly inhibited fibronectin assembly and cell spreading of endothelial cells transfected with control-siRNA (si-Control, Fig. 5, C and D). In contrast, the inhibitory effect of NK4 on fibronectin assembly and cell spreading was significantly reduced in endothelial cells transfected with perlecan-siRNA (si-Perlecan, Fig. 5, C and D). Taken together, these data suggest that perlecan-NK4 association is required for mediating the inhibitory effect of NK4 on fibronectin assembly and cell spreading. Although we also examined whether the knockdown of perlecan can restore DNA synthesis and proliferation of endothelial cells treated with NK4, knockdown of perlecan resulted in defective DNA synthesis induced either by bFGF or by VEGF. This might be because of the essential role of perlecan as a co-receptor for heparin binding growth factors (19, 20, 25).

Because NK4 has a heparin binding feature (26), the heparan sulfate side chain of perlecan was a possible NK4 binding site. This was suggested because the addition of heparin almost completely prevented the binding of NK4 to the cells (supplemental Fig. S5A), and this was associated with an abrogation of the inhibitory effects of NK4 on the fibronectin assembly (supplemental Fig. S5A) and on endothelial cell spreading (supplemental Fig. S5B).

Reduced Formation of Focal Adhesions by NK4 and Abrogation of the Inhibitory Effect of NK4 by Active Rac—Cooperation of signals from integrins and growth factor receptors, known as anchorage dependence, is growth factor proliferation of normal cells including endothelial cells (27). Inhibition of the extracellular fibronectin assembly and cell spreading strongly suggests that anchorage-dependent signal transduction was inhibited by NK4. Immunostaining of focal cellular matrix in tissues (21, 22). To address the possibility that NK4 may affect fibronectin assembly, we examined the extracellular fibronectin assembly by immunostaining (Fig. 4A). Endothelial cells were replated and cultured for 5 h in the presence or absence of NK4. Extracellular fibronectin assembly was observed in control cells, but it was mostly undetectable in the presence of NK4. Western immunoblot of fibronectin in deoxycholate-insoluble ECM or conditioned medium showed efficient deposition of fibronectin into ECM in the absence of NK4 (Fig. 4B). In the presence of NK4, fibronectin deposition in ECM was remarkably reduced, and unassembled fibronectin was detected in conditioned medium (Fig. 4B). Immunoblotting for laminin γ1 (LN) showed that the amount of LN in conditioned medium was not changed by NK4. These results indicate that NK4 inhibited fibronectin assembly into ECM without preventing the synthesis and secretion of fibronectin.

When endothelial cells were cultured in three-dimensional collagen gels, bFGF induced tubular network formation over a period of 24 h, whereas this was significantly inhibited by NK4 (Fig. 4C). Under these conditions, NK4 inhibited extracellular fibronectin assembly (Fig. 4D), which suggests that the impaired fibronectin assembly was associated with the inhibition of endothelial tube formation by NK4.

Perlecan Is Required for the Inhibition of Fibronectin Assembly and Cell Spreading by NK4—We speculated that the binding and colocalization of NK4 with perlecan might affect
Angioinhibitory Mechanism of NK4

Adhesion by paxillin and focal adhesion kinase (FAK) phosphorylated at Tyr 397 showed reduced numbers of focal adhesions with significantly thinner and shorter morphology in NK4-treated cells (Fig. 6A). Reduced tyrosine phosphorylation of FAK and paxillin in NK4-treated cells were also detected by Western immunoblot (Fig. 6B). Activation of Rac plays a critical role in integration of signaling pathways from growth factor receptors and integrin-mediated cell-ECM interaction in human endothelial cells (28). To analyze change in the activity of Rac, the activity of p21-activated kinase (PAK), a downstream target of Rac, was measured by Western immunoblot (Fig. 6C). Compared with cells kept in suspension, PAK activity increased in cells attached to a culture plate for 2 h, suggesting the activation of Rac by integrin-mediated anchoring of cells. However, NK4 treatment decreased PAK activity even when the cells were attached to a culture plate.

Next, we tested whether the expression of dominant-active Rac restores the endothelial cell DNA synthesis inhibited by NK4. For this purpose, activated forms of the Rho-family proteins Rac V12, Rho V14, and Cdc42 V12 in endothelial cells were expressed in endothelial cells, and the cells were subjected to measurement of DNA synthesis (Fig. 6, D and E). Similar expression levels of Myc-tagged Rac V12, Rho V14, and Cdc42 V12 in endothelial cells were confirmed by Western immunoblot (Fig. 6E, upper panel). In addition to nuclear staining of endogenous Myc seen in most cells, the cells expressing Myc-tagged Rac V12 were obvious from their cytoplasmic localization (Fig. 6E, lower graph). Expression of Rac V12 efficiently canceled the inhibitory effect of NK4 on bFGF-induced BrdUrd incorporation. In contrast, expression of activated forms of Rho and Cdc42 did not cancel the inhibitory effect of NK4. These results strongly suggest that the inhibitory effect of NK4 on anchorage-dependent Rac activity, which may be caused by impaired fibronectin assembly by NK4, plays a definitive role in the endothelial cell growth inhibition by NK4.

DISCUSSION

The HGF-c-Met pathway plays an important role in tumorigenesis, invasion metastasis, and drug resistance (8, 9, 29–31). Likewise, anti-angiogenesis therapy has become standard for treatment of several types of advanced cancers. Therefore, the simultaneous inhibition of tumor angiogenesis and the HGF-c-Met pathway by NK4 seems to be a unique strategy for blocking the highly aggressive behavior of cancers. In experimental models, NK4 inhibited tumor growth, angiogenesis, and metastasis and prolonged life span in several models (9–12). Together with a finding that NK4 seems to be generated in physiological or pathological conditions (7), understanding of the mechanism by which NK4 inhibits angiogenesis seems to provide not only a rationale for anti-cancer therapy by NK4 but also a possible physiological significance of NK4 in vascular regulation.

Both soluble growth factors and integrin-mediated adhesion to ECM are required for many cellular processes such as growth, survival, and migration (27). In endothelial cells, the Rho-family GTPase Rac plays a critical role in the integration of signals from growth factor receptors and integrins required for cell cycle progression (28). We previously demonstrated that NK4 did not inhibit VEGF-induced activation of VEGF receptor 2, and VEGF- and bFGF-induced activation of extracellular signal-related protein kinase (ERK) (12). Consistent with this,
NK4 did not inhibit short term and long term activation of ERK1/2 induced by bFGF or VEGF (supplemental Fig. S6). These results suggest that NK4 inhibits growth factor-induced cyclin D1 expression and Rb phosphorylation without preventing growth factor-induced receptor activation and ERK activation. Instead, NK4 reduced phosphorylation of FAK in focal adhesions and inhibited adhesion-dependent PAK kinase activity, and dominant expression of activated-Rac canceled inhibition of endothelial cell proliferation by NK4 (Fig. 6). Considering these results, we propose that NK4 inhibits anchorage-dependent signals, leading to Rac activation that is required for endothelial cell proliferation.

Angiogenesis is a process involving dynamic ECM transition (3). During angiogenesis, the vascular basement membranes undergo proteolytic degradation and transit to the provisional matrix followed by intermediate and mature new vascular basement membranes. Fibronectin is a major component of the provisional matrix, and its expression is up-regulated on blood vessels during wound healing (32) and vascular sprouting (33). Furthermore, genetic or pharmacological inhibition of fibronectin-integrin α5β1 interaction disrupts angiogenesis in both developmental and pathological conditions (34–38), indicating an essential proangiogenic role of fibronectin and its main receptor integrin α5β1. Considering this essential proangiogenic role of fibronectin, we propose that the inhibition of extracellular fibronectin assembly in endothelial cells accounts for a mechanism by which NK4 inhibits angiogenesis.

Cells that have impaired proteoglycan synthesis exhibit a defective fibronectin matrix assembly (39). Among proteoglycans, the syndecans, transmembrane HSPGs that can bind to fibronectin, play a role in fibronectin assembly (40, 41). Syndecan-2, the major syndecan in fibroblasts, appears to have a regulatory effect on matrix assembly (24). However, significant colocalization of fibronectin with syndecan-1 and -2 was not observed in HUVECs (supplemental Fig. S4). Rather, colocalization of perlecan with fibronectin assembly was observed in HUVECs (Fig. 5A). Perlecan is expressed in basement membranes and is the major extracellular HSPG associated with blood vessels (19, 20). Perlecan provides binding sites for a number of ligands, such as basement membrane components including collagen type IV, nidogen, laminin, and fibronectin (16–20). The presence of perlecan in the basement membrane and its ability to interact with other basement membrane components suggests that it is involved in basement membrane assembly. Indeed, mice lacking perlecan showed reduced basement membrane integrity (42, 43). However, perlecan is not essential for fibronectin assembly in endothelial cells, because we observed that reduction of perlecan expression by siRNA did not prevent fibronectin assembly (Fig. 5C). We speculate that perlecan may provide...
FIGURE 6. Reduced anchorage-dependent signals by NK4 and abrogation of NK4-induced growth inhibition by dominant-active Rac. A, changes in distribution of focal adhesions and phosphorylated FAK. HUVECs were re-plated and cultured in medium containing 5% FBS and 20 ng/ml bFGF with and without 300 nM NK4 for 3 h. Focal adhesions were stained for FAK phosphorylated at Tyr-397 (pFAK, red) and paxillin (green). Scale bar, 50 μm. The right graph indicates change in fluorescence intensity of pFAK in focal adhesions per cell. The value was quantified as described under “Experimental Procedures.” The differences in values indicated by the double asterisk are statistically significant (**, p < 0.01). B, immunoblotting of tyrosine phosphorylation in FAK and paxillin. Total cell lysates (400 μg of protein) were immunoprecipitated with anti-FAK antibody or anti-paxillin antibody. Immunoprecipitates (IP) were subjected to SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody (pY). C, decrease in adhesion-induced PAK activity by NK4. Overnight serum-starved HUVECs were resuspended in serum-free medium containing 0.2% BSA and 20 ng/ml bFGF and kept in suspension (Sus.) or attached (Att.) for 2 h in the presence or absence of NK4. Total cell lysates (300 μg of protein) were immunoprecipitated with anti-PAK antibody in the presence or absence of PAK epitope blocking peptides (BLK-pep) and subjected to kinase assay using myelin basic protein as a substrate (PAK activity) or immunoblotting using anti-PAK antibody (PAK protein). D, changes in DNA synthesis by expression of dominant-active Rac. HUVECs were transfected with Myc-tagged Rac V12 expression vector and subjected to measurement of bFGF-induced DNA synthesis by BrdUrd incorporation in the presence or absence of 300 nM NK4. Cells were stained for Myc (red) and BrdUrd (green). Arrows indicate the cells expressing Rac V12 detected by cytoplasmic red staining. Nuclear red staining is because of endogenous c-Myc localization. Note that NK4 treatment reduced BrdUrd incorporation in cells lacking Rac V12 but not in cells expressing Rac V12. Scale bar, 20 μm. E, effect of Rac V12, Rho V14, or Cdc V12 expression on NK4-induced inhibition of DNA synthesis. Change in BrdUrd incorporation was determined in cytoplasmic Myc-positive cells under three conditions; that is, the basal medium without bFGF (open bar), with bFGF (closed bar), and with bFGF and 300 nM NK4 (hatched bar). Each value represents the mean ± S.D. in a representative experiment from three independent experiments. The upper panel shows expression levels of Myc-tagged Rac V12, Rho V14, or Cdc42 V12 as determined by Western immunoblot using anti-Myc antibody.

NK4 inhibits proliferation of endothelial cells but not fibroblasts nor canine renal epithelial cells (12). Here we show that inhibition of Rb phosphorylation and cyclin D1 expression by NK4 occurs in endothelial cells but not in fibroblasts (Fig. 1B). Because perlecan is a major component of the vascular basement membrane and is highly expressed in endothelial cells but not in fibroblasts (supplemental Fig. S2), preferential expression of perlecan might be attributable to the cell type-specific effect of NK4. Another possibility is the differences of ECMs that are required for cell proliferation in each cell type. For example, fibroblast proliferation was supported by integrin α5β1-mediated adhesion to collagen (44), whereas endothelial cell proliferation was supported by integrin α5β1 or αVβ3-mediated adhesion to either fibronectin or vitronectin (28).

NK4 consists of an N-terminal hairpin domain and four kringle domains of HGF. We have reported that only kringle domains of NK4 (K1–4) have anti-angiogenic effects; however, inhibition of endothelial cell growth by K1–4 was less potent than that by NK4 (45). N-terminal and second-kringle domains are required for binding to heparin (26), and we show here that the anti-angiogenic effects of NK4 require binding to HSPGs. These results suggest the importance of the N-terminal domain, which might mediate the association of NK4 with HSPGs for the inhibitory effect of NK4 on endothelial cell proliferation. The recombinant N-terminal domain of HGF inhibited endothelial cell proliferation by preventing the binding of bFGF and VEGF to endothelial cells (46).

a multidomain scaffold required for the proper organization of basement membrane components during remodeling from a fibronectin-rich provisional matrix to a mature basement membrane. The association of NK4 with perlecan may recruit NK4 to the site of cell-associated fibronectin assembly and may interfere with fibronectin assembly. The mechanism by which the specific interaction between NK4 and perlecan inhibits extracellular fibronectin assembly remains to be addressed.

This is likely because of the N-terminal domain simply occupying the glycosaminoglycan chain of HSPGs required for high affinity binding of these heparin binding growth factors to growth factor receptors. However, this scenario is unlikely in the case of NK4, because NK4 inhibits endothelial proliferation, spreading, and fibronectin assembly without inhibiting growth factor signaling. Thus, although both the kringle and N-terminal domain of NK4 have independent
Angioinhibitory Mechanism of NK4

Angioinhibitory effects, both domains are required for the efficient inhibition of fibronectin assembly and proliferation by NK4.

In summary, we suggest that NK4 inhibits endothelial cell proliferation by interfering with fibronectin assembly and anchorage-dependent Rac activation required for cyclin D1 expression. Our proposal is that this process is dependent on the interaction of NK4 with HSPG, especially perlecann. HGF-c-Met and angiogenesis participate strongly in malignant progression of cancers. Thus, together with a newly identified mechanism for the angioinhibitory action of NK4, its bifunctionality (targeting both HGF-c-Met and angiogenesis) indicates a unique therapeutic value of NK4 in molecular-targeted therapy for cancer.

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REFERENCES
1. Folkman, J. (1995) *Nature Med.* 1, 27–31
2. Carmeliet, P., and Jain, R. K. (2000) *Nature* 407, 249–257
3. Kalluri, R. (2003) *Nat. Rev. Cancer* 3, 422–433
4. Nakamura, T., Matsumoto, K., Tano, Y., and Nakamura, T. (2001) *Int. J. Cancer* 94, 575–580
5. Date, K., Matsumoto, K., Shimura, H., Tanaka, M., and Nakamura, T. (1998) *Oncogene* 17, 3045–3054
6. Raymond, W. W., Cruz, A. C., and Caughey, G. H. (2006) *Cancer Res.* 66, 8177–8185
7. Engelman, J. A., Zeijnullahu, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J. O., Lindeman, N., Gale, C. M., Zhao, X., Christensen, J., Kosaka, T., Holmes, A. J., Rogers, A. M., Cappuzzo, F., Mok, T., Lee, C., Johnson, B. E., Cantley, L. C., and Janne, P. A. (2007) *Science* 313, 1039–1043
8. Clark, R. A., DellaPelle, P., Manseau, E., Lanigan, J. M., Dvorak, H. F., and Colt, V. S. (1982) *Nat. Med.* 3, 274–276
9. Yano, S., Wang, W., Li, Q., Matsuhita, H., Kamurako, T., Ono, H., Kakuishi, S., Taniguchi, M., Nishiiga, Y., Uehara, H., Mitsuomizu, T., Takeda, Y., Nakamura, T., and Sone, S. (2008) *Nat. Genet.* 40, 115–123
10. Yablonski, D., Kane, L. P., Qian, D., and Weiss, A. (1998) *EMBO J.* 17, 5647–5657
11. Isemura, M., Sato, N., Yamaguchi, Y., Akaiwa, J., Munakata, H., Hayashi, N., Yoshizawa, Z., Nakamura, T., Kubota, A., and Arakawa, M. (1987) *J. Biol. Chem.* 262, 8926–8933
12. Heremans, A., Van den Berghe, H., and David, G. (1990) *J. Biol. Chem.* 265, 8716–8724
13. Hof, M., Gohring, W., Kohfeldt, E., Yamada, Y., and Tiimpf, R. (1999) *Eur. J. Biochem.* 259, 917–925
14. Knox, S. M., and Whitelock, J. M. (2006) *Cell Mol. Life Sci.* 63, 2345–2445
15. Farach-Carson, M. C., and Carson, D. D. (2007) *Glycobiology* 17, 897–905
16. Wierzbicka-Patynowski, I., and Schwarzbaumer, J. E. (2003) *J. Cell Sci.* 116, 3269–3276
17. Heremans, A., De Cock, B., Cassiman, I. J., Van den Berghe, H., and David, G. (1990) *J. Biol. Chem.* 265, 8716–8724
18. Hopf, M., Gohring, W., Kohfeldt, E., Yamada, Y., and Tiimpf, R. (1999) *Eur.
J. Biochem.* 259, 917–925
19. Arikawa-Hirasawa, E., Wilcox, W. R., Le, A. H., Silverman, N., Govindraj, P., Hassell, J. R., and Yamada, Y. (2001) *Nat. Genet.* 27, 431–434
20. Mettouche, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J. K., and Giancotti, F. G. (2001) *Curr. Opin. Genet. Dev.* 11, 48–53
21. Comoglio, P. M., Giordano, S., and Trusolino, L. (2008) *Nat. Rev. Drug Discov.* 7, 504–516
22. Francis, S. E., Goh, K. L., Hodivala-Dilke, K., Bader, B. L., Stark, M., Davidsen, D., and Hynes, R. O. (1993) *Cell* 119, 1079–1091
23. Clark, R. A., DellaPelle, P., Manseau, E., Lanigan, J. M., Dvorak, H. F., and Colt, V. S. (1982) *Nat. Med.* 7, 274–276
24. Yano, S., Wang, W., Li, Q., Matsuhita, H., Kamurako, T., Ono, H., Kakuishi, S., Taniguchi, M., Nishiiga, Y., Uehara, H., Mitsuomizu, T., Takeda, Y., Nakamura, T., and Sone, S. (2008) *Nat. Genet.* 40, 115–123
25. Yablonski, D., Kane, L. P., Qian, D., and Weiss, A. (1998) *EMBO J.* 17, 5647–5657
26. Isemura, M., Sato, N., Yamaguchi, Y., Akaiwa, J., Munakata, H., Hayashi, N., Yoshizawa, Z., Nakamura, T., Kubota, A., and Arakawa, M. (1987) *J. Biol. Chem.* 262, 8926–8933
27. Heremans, A., De Cock, B., Cassiman, I. J., Van den Bergh, H., and David, G. (1990) *J. Biol. Chem.* 265, 8716–8724
28. Mettouche, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J. K., and Giancotti, F. G. (2001) *Curr. Opin. Genet. Dev.* 11, 48–53
29. Mettouche, A., Klein, S., Guo, W., Lopez-Lago, M., Lemichez, E., Westwick, J. K., and Giancotti, F. G. (2001) *Cell* 8, 115–127
30. Comoglio, P. M., Giordano, S., and Trusolino, L. (2008) *Nat. Rev. Drug Discov.* 7, 504–516
31. Engel, J. A., Zeijnullahu, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J. O., Lindeman, N., Gale, C. M., Zhao, X., Christensen, J., Kosaka, T., Holmes, A. J., Rogers, A. M., Cappuzzo, F., Mok, T., Lee, C., Johnson, B. E., Cantley, L. C., and Janne, P. A. (2007) *Science* 316, 1039–1043
32. Mercuri, K., and Hoss, K. (1988) *Exp. Cell Res.* 179, 269–276