The N-terminal Region of NTAK/Neuregulin-2 Isoforms Has an Inhibitory Activity on Angiogenesis*

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NTAK (neural- and thymus-derived activator for ErbB kinases), also known as neuregulin-2, is a member of the epidermal growth factor (EGF) family, which binds directly to ErbB3 and ErbB4 and transactivates ErbB2. Because ErbB signaling has been implicated in various angiogenic mechanisms, the effect of NTAK (which has at least nine isoforms due to alternative splicing) in angiogenesis is explored. One isoform, NTAKγ, inhibited cell growth in terms of DNA synthesis and cell numbers in vascular endothelial cells specifically, whereas NTAKα and β had no activity. On the other hand, NTAKγ secreted by transfected MDA-MB-231 cells inhibited endothelial cell growth, and NTAKγ expressed in endothelial cells by adenovirus infection suppressed cell growth in a dose-dependent manner. The EGF-like domain of NTAKγ did not have this activity. The NTAKδ isoform, which had the Ig-like domain but not the EGF-like domain, inhibited proliferation of endothelial cells. NTAKδ prevented hyper-phosphorylation of the retinoblastoma tumor suppressor protein and caused G1 arrest in endothelial cells. Both NTAKγ and δ isoforms displayed anti-angiogenic activity in the chick embryo chorioallantoic membrane in vivo. These results suggest that the active site of NTAK is localized outside of the EGF-like domain but within the N-terminal region, including the Ig-like domain, of NTAK.

Members of the ErbB family of receptor tyrosine kinases, including epidermal growth factor receptor (EGFR or ErbB1), ErbB2 (neu), ErbB3, and ErbB4 (1–4), are important mediators of cell growth, differentiation, and survival (5, 6). In an earlier report, we described the purification and cloning of a novel member of the epidermal growth factor (EGF) family that was named NTAK (for neural- and thymus-derived activator for ErbB kinases) (7). NTAK has at least nine alternatively spliced isoforms, which are derived from the same gene as neuregulin-2 (NRG2) and divergent of neuregulin 1 (Don-1) (8–10). Like NRG1, NTAK binds directly to ErbB3 and ErbB4 and transactivates ErbB1 and ErbB2 via heterodimerization with ErbB3 or ErbB4 (7). Two other member of NRG1-like ligands, NRG3 and NRG4, bind to ErbB4 but not ErbB1, ErbB2, or ErbB3 (11, 12).

The human NTAK gene comprises 12 exons spanning >55 kilobases (13). Among the products of alternative splicing, the α isoform of the NTAK gene is expressed in all tissues including the brain, and the β isoform is restricted to the brain. The γ isoform is expressed in a rat pheochromocytoma cell line, PC-12. NTAKδ is an isoform missing the EGF-like domain and is expressed in a human neuroblastoma cell line, SK-N-SH. NTAKα and β preferentially induce phosphorylation in ErbB3 and ErbB4, respectively, transactivate ErbB2, and stimulate growth of human breast cancer cells (14). However, differences in the biological roles and the functions of the various NTAK isoforms are still unknown.

The roles of ErbB overexpression in cellular transformation and tumor metastasis have been elucidated by a line of in vitro and clinical studies. Furthermore, targeted deletion of ErbB2, ErbB3, ErbB4, or NRG1 in mice leads to developmental abnormalities that are severe in the nervous system and to lethality due to cardiovascular system failure (5, 6). The cardiac abnormalities include aborted development of the endocardial cushion, which is dependent on mesenchymal cell growth and development of the endocardial endothelium.

Angiogenesis is the process of new vascular formation from preexisting blood vessels and is tightly regulated by the balance of angiogenic factors and inhibitors (15). Under normal conditions, vascular endothelial cells are quiescent due to the dominance of angiogenic inhibitory factors, including angiotatin (16), endostatin (17), and NK4 (18). Angiogenesis occurs during pathological events such as solid tumor growth and metastasis, diabetic retinopathy, atherosclerosis, and rheumatoid arthritis. Angiogenic inhibitors are capable of preventing tumor growth and metastasis, and, in fact, a number of angiogenic inhibitors are being tested in clinical trials for cancer treatment.

ErbB signaling has also been implicated in angiogenesis. Neutralizing antibodies against ErbB1 and ErbB2 down-regulate VEGF and inhibit tumor growth and angiogenesis in vivo (19). NRG1 has been reported to activate ErbBs in endothelial cells and induce angiogenesis (20). NRG1 binds to heparan sulfate proteoglycan (HSPG) via the Ig-like domain, and NRG1-HSPG interaction potentiates ErbB phosphorylation by the EGF-like domain of NRG1 (21). Targeted deletion of the Ig-like domain of NRG1 in mice leads to the embryonic lethality associated with a deficiency of ventricular myocardial trabec-
ulation and impairment of cranial ganglion development (22). The Ig-like domain of NTAK is 38.2% identical to the corresponding domain of NRG1 (7). However, the function of the Ig-like domain of NTAK remains unknown.

We scrutinized the angiogenic effects of NTAK isoforms and report herein that the N-terminal region of NTAK, including the Ig-like domain but not the EGF-like domain, inhibits angiogenesis and that NTAKδ causes G1 arrest in vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Materials—Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA). HUVECs were maintained in EBM-2 medium (Clonetics) and, in the assays, were grown in MCDB-131 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 10 ng/ml fibroblast growth factor-2 (FGF-2), 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (PS). Bovine aortic endothelial cells (BAECs) were isolated from bovine thoracic aorta using methods described previously (23). MDA-MB-231 cells were purchased from the American Type Culture Collection. MDA-MB-231 cells were maintained in Dulbecco’s modified Eagle’s medium, 10% FCS, and PS. The anti-NTAK antibody, #N1-1, was raised in rabbits against a synthetic peptide corresponding to the 308–327 amino acid sequence (7). The anti-Rb antibody was purchased from Santa Cruz Biotechnology. The anti-Rb antibody was raised in rabbits against a synthetic peptide corresponding to the 308–327 amino acid sequence (7). The anti-Rb antibody was purchased from Santa Cruz Biotechnology.

Preparation of Recombinant Proteins from Escherichia coli—The 1.2-kbp cDNA fragments corresponding to the extracellular regions of NTAKδ, NTAKδ, NTAKδ, and NTAKδ were inserted into pASK-IBA6 vectors (Genosys Biotechnologies, The Woodlands, TX). The resulting plasmids were then used to transform E. coli DH5α, and the recombinant proteins were purified using a StrepTactin affinity column according to the manufacturer’s instructions (Genosys Biotechnologies). Establishment of NTAKδ Gene-Transfected MDA-MB-231 Cells—An expression vector of NTAKδ was constructed by inserting the full sequence of NTAKδ cDNA into pRC/CMV. The vector was transfected into MDA-MB-231 cells by the electroporation method, and selection was performed with G418 (Sigma-Aldrich). The conditioned media from each clone were concentrated using heparin-Sepharose (Amersham Biosciences), and NTAKδ expression was examined by Western blotting analyses using the anti-NTAK antibody, #N1-1 (7). Three highly expressing clones, 231γ, 231γ, and 3, were independently selected.

Cell Growth Assay—For the cell number assay, cells were resuspended in the maintenance medium and then seeded onto collagen-coated 24-well microplates (2 x 10^4 cells/well). The plates were incubated for 24 h at 37 °C and then re-fed medium containing 5% FCS. After 5 h, the samples or growth factors to be tested for inhibitory activity were added. After a 24-h incubation, 10 μl (37 kBq) of [3H]thymidine was added, and incubation was continued for another 6 h. The [3H]thymidine incorporation into DNA was determined by liquid scintillation counting (1450 MicroBeta TRI-LUX; PerkinElmer Life Sciences). To determine HUVEC growth, HUVECs were plated at a density of 1 x 10^4 cells/well in collagen-coated 12-well microplates. After 12 h, the plates were re-fed MCDB-131/FCS (5%/PS, followed by the addition of 20 ng/ml NTAKδ or NTAKα (Day 0). The plates were incubated for 48 h (Day 2) and then re-fed fresh MCDB-131/FCS (5%/PS) without NTAKs.

Co-culture Assay—HUVECs were resuspended in the maintenance medium and then seeded onto collagen-coated 24-well microplates (2 x 10^4 cells/well). The plates were incubated for 6 h at 37 °C and re-fed with MCDB-131 supplemented with 5% FCS and 5 ng/ml FGF-2. The inner cup (Intercell ST, Kurabo, Neyagawa, Japan) containing MDA-MB-231 cells or transfectants (1 x 10^4 cells/1 ml/well). The plates were incubated for 6 h at 37 °C and re-fed with MCDB-131 supplemented with 5% FCS and 5 ng/ml FGF-2. The inner cup (Intercell ST, Kurabo, Neyagawa, Japan) containing MDA-MB-231 cells or transfectants (1 x 10^4 cells/1 ml/well) was placed. After a 24 h incubation, DNA synthesis and cell number were measured as described above.

Inhibitory Effect of Adenoviral Vectors—We used an adenovirus expression vector kit (Takara, Kusatsu, Shiga, Japan) that carries a cytomegalovirus-driven cDNA for full-length NTAKδ. For the cell growth assay, we measured DNA synthesis in HUVECs infected by adenovirus for 24 h, as described above.

Immunoprecipitation and Western Blotting Analyses of Retinoblas
toma Protein—To synchronize the cells in a quiescent state (G0), the cells were cultured in starvation medium containing 0.5% FCS for 16 h and re-fed fresh medium with or without 50 ng/ml NTAKs. Cells were harvested and lysed with lysis buffer (1% Triton X-100, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 5 mM EDTA, 0.1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation at 15,000 rpm for 10 min, the supernatant was incubated with anti-Rb antibody for 2 h at 4 °C and then with 20 μl of protein A-Trisacryl (50% suspension; Pierce) for 2 h at 4 °C. The samples were analyzed by electrophoresis on a 6% polyacrylamide gel. Proteins in the gel were transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) in 150 mM CAPS buffer, pH 10.5, containing 20% methanol. The membrane was blocked with 5%
briefly, fertilized white Leghorn chicken eggs were incubated at 37°C and assayed using the chick chorioallantoic membrane (CAM) method (24). NTAKs. NTAKs analyzed the DNA synthesis of BAECs stimulated by recombinant structures differ in their EGF-like domain (Fig. 1). We analyzed the DNA synthesis of BAECs stimulated by recombinant NTAKs. NTAKα and β dose-dependently induced phosphorylation of ErbBs in MDA-MB-453 and T47D cells within the 1–10 ng/ml range (14). At these levels, NTAKα and β had neither stimulatory nor inhibitory effects on [3H]thymidine incorporation by BAECs, but NTAKγ inhibited it in a dose-dependent manner (Fig. 2A). Similarly, NTAKα and β isoforms had no effects on the proliferation of BAE cells, whereas NTAKγ was inhibitory (Fig. 2B). The inhibitory activity of NTAKγ was comparable with that of a typical angiogenic inhibitor, TNF-α (25). However, NTAKγ had no effects on the growth of smooth muscle cells (SMCs), T47D cells, MDA-MB-453 cells, or SK-N-SH cells (Fig. 2C). Thus, it is suggested that the growth inhibitory activity of NTAKγ is specific for vascular endothelial cells.

Growth Inhibition of Endothelial Cells Co-cultured with NTAKγ Transfectants—Human breast cancer-derived MDA-MB-231 cells are undifferentiated cells that are invasive by nature and secrete angiogenic factors such as VEGF and FGF-2 (26, 27). We examined whether NTAKγ was capable of antagonizing these angiogenic factors. We transfected a plasmid containing the full-length NTAKγ cDNA and the neomycin resistance gene into MDA-MB-231 cells. Three cell lines, 231γ-1, -2, and -3, secreting high levels of NTAKγ, were obtained and co-cultured with HUVECs. These transfectants secreted VEGF and FGF-2 as verified by an enzyme-linked immunosorbent assay (data not shown). The [3H]thymidine incorporation into HUVECs in the lower chambers was increased by the angiogenic factors secreted by MDA-MB-231 cells in the upper chambers. When three transfectants were co-cultured in the upper chambers, [3H]thymidine incorporation into HUVECs was reduced to the basal level (Fig. 3A), and the expected increase in HUVEC number was inhibited (Fig. 3B). Thus, NTAKγ was able to reduce the growth of endothelial cells to the basal level, probably by antagonizing angiogenic factors.

Growth Inhibition of Adenovirus-infected HUVEC—We then evaluated potential therapeutic actions via NTAK delivery. HUVECs were infected with adenoviral vectors encoding NTAKγ or a control. The expressions of vectors were verified histochemically by β-galactosidase activity. The DNA synthesis of HUVECs infected by the adenoviral vector encoding NTAKγ was inhibited in a dose-dependent manner, and the half-maximal effectiveness was ~5 × 10^5 multiplicities of infection (Fig. 4). The cells showed no morphological changes, and the control vector had no effect on HUVEC proliferation. Thus, growth inhibition of HUVECs was not due to the cytotoxic effect of viral infection but to NTAKγ expressed by adenoviral vectors. This suggested that NTAKγ has a potential therapeutic use for angiogenesis-related diseases.

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* N. Nakano and S. Higashiyama, unpublished observations.
The Active Site of Angiogenic Inhibition in NTAK—NTAKα and β isoforms have complete EGF-like domain motifs, which have consensus sequences containing three disulfide bonds. On the contrary, the NTAKγ isoform has an incomplete EGF-like domain, which does not have the third cysteine loop (Fig. 1B). To investigate the active site of NTAK involved in angiogenic inhibition, we focused on the EGF-like domain of NTAKγ and synthesized a 33-amino acid-long peptide of γEGF corresponding to the region. In the assays of cell number and DNA synthesis, this γEGF peptide had no effect on the growth of HUVECs or BAECs at concentrations up to 3.3 nM (Fig. 5A and data not shown), whereas the NTAKγ protein showed an inhibitory activity at 10 ng/ml or 0.22 nM, as shown in Fig. 2, A and B. Detectable levels of ErbB phosphorylation were not induced by the NTAKγ or the γEGF peptide in Western blotting analysis. It was suggested, therefore, that the active site for the angiogenic inhibitory effect of NTAKγ existed in a region other than the EGF-like domain. Subsequently, we examined the angiogenic inhibitory effect of NTAKδ, which has the same N-terminal part, including the Ig-like domain, as the other isoforms but is missing the EGF-like domain (Fig. 1A). NTAKδ decreased [3H]thymidine incorporation into HUVECs in a dose-dependent manner (Fig. 5B) with no accompanying ErbB phosphorylation (data not shown). It is suggested that the anti-angiogenic activity of NTAK resides in the N-terminal region of NTAK, including the Ig-like domain, but not in the EGF-like domain of NTAKγ.

NTAKδ Reduces Retinoblastoma Protein Phosphorylation in HUVEC—We investigated the mechanism underlying the angiogenic inhibitory effects of NTAKδ. Retinoblastoma protein (pRb) is the most important protein regulating cell cycle progression into the S phase, and hyper-phosphorylation of pRb inactivates its growth inhibitory function (28). We investigated whether NTAKδ affected pRb hyper-phosphorylation. When serum-starved HUVECs were re-fed fresh medium, the hyper-phosphorylation of pRb was increased. However, treatment of HUVECs with NTAKδ inhibited hyper-phosphorylation of pRb, suggesting that the G1 arrest was induced by NTAKδ (Fig. 6).

To determine whether the inhibition of angiogenesis by NTAKδ is reversible, we analyzed HUVEC growth in response to with NTAKα or δ. HUVECs treated with 20 ng/ml NTAKδ became spindle-shaped on Day 1 and cell growth was reduced, whereas HUVECs treated with NTAKα proliferated normally (Fig. 7). The NTAKδ-treated cells did not show apoptotic or necrotic changes, and growth was restored when the medium was replaced, on Day 2, with medium not containing NTAKδ. Thus, it was suggested that NTAKδ blocks cell cycle progression and causes G1 arrest in HUVECs.

In Vivo Anti-angiogenic Activity of NTAK—The in vivo angiogenic inhibitory activity of NTAKs was examined using the CAM assay. NTAKα and control bovine serum albumin neither induced nor inhibited angiogenesis of chick microvessels on CAMs (Fig. 8, A and B). In contrast, the microvascular network formation was inhibited by NTAKγ and, more markedly, by NTAKδ (Fig. 8, C and D). Avascular areas were clearly formed surrounding the disks containing NTAKγ or NTAKδ. Thus, NTAKγ and NTAKδ, but not NTAKα, inhibited angiogenesis in vivo as well as in vitro.

**DISCUSSION**

NTAK represents at least nine alternative splicing isoforms derived from the same gene as NRG2 (7–10, 13), and belongs to the EGF family. In our previous study, NTAKα and β bound directly to the ErbB3 and ErbB4 receptors, but not to ErbB1 or ErbB2 (7). It has been reported that targeted deletion of ErbB2, ErbB3, ErbB4, or NRG1 in mice is lethal due to developmental abnormalities involving the cardiovascular system. Effects of NTAK in angiogenesis have been suggested, because endothelial cells express ErbB2, ErbB3, and ErbB4 (20). Recombinant NTAKα and β isoforms using an *E. coli* expression system stimulated breast tumor cell growth and differentiation at physiological concentrations ranging from 1 to 10 ng/ml (14). However, NTAKα and β neither stimulated nor inhibited endothelial cell growth. NTAKγ did not stimulate the phosphorylation of ErbBs in MDA-MB-453 and T47D cells (data not shown) but did inhibit endothelial cell growth in terms of DNA synthesis and increase of cell number (Fig. 2, A and B). Furthermore, NTAKγ, as well as NTAKα and β, had no effect on the proliferation of SMCs, breast cancer cell lines, and neuro-
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Loop. Unexpectedly, the \( \gamma \text{EGF} \) peptide, corresponding to the EGF-like domain of NTAK\( \gamma \), had no effect on the growth of HUVECs and BAECs at concentrations up to 3.3 nM, although NTAK\( \gamma \) exerted an inhibitory effect at 10 nM (0.22 nM). Otherwise, NTAK\( \delta \), which has the Ig-like domain but not the EGF-like domain, inhibited angiogenesis. The \( \gamma \text{EGF} \) peptide and the NTAK\( \gamma \) and \( \delta \) isoforms did not stimulate ErbB phosphorylation (data not shown). The results suggest that the EGF-like domain of NTAK\( \gamma \) is not involved in the anti-angiogenic activity and that the active site is in the Ig-like domain.

The function of the Ig-like domain of NTAK remains unknown. NTAK is structurally homologous to NRG1, and the Ig-like domain of NRG1 contains a heparin-binding site and increases ligand-receptor affinity (29). Targeted deletion of the Ig-like domain of NRG1 leads to embryonic lethality. The Ig-like domain of NTAK may also interact with glycosaminoglycans on the cell surface and affect the interaction of NTAK with ErbB3s. The structures of both the Ig-like and EGF-like domains of NTAK may be essential for ErbB interaction and the
NTAK isoforms presumably stimulate the growth of endothelial cells, because endothelial cells have ErbB2, ErbB3, and ErbB4. We can speculate that NTAKα and β have both angiogenic activities because of ErbB phosphorylation via the EGF-like domain and anti-angiogenic properties associated with other regions, including the Ig-like domain, which results in no net effects on cell growth.

It is now well known that some of endogenous angiogenic inhibitors are fragments of proteins whose biological functions are unrelated to angiogenesis. For example, angiotatin and endostatin are proteolytic fragments of plasminogen (16) and collagen XVIII (17), respectively. NK4 is a internal fragment of hepatocyte growth factor (HGF) and is considered to be bifunctional, as it is a hepatocyte growth factor antagonist and an endogenous angiogenic inhibitor (18). NK4 inhibits angiogenesis through another pathway than the c-Met receptor. It is also possible that NTAK proteins with the full EGF-like domain have no effects on angiogenesis, and that only fragments of NTAK with the truncated EGF-like domain have the anti-angiogenic properties.

The mechanism underlying the angiogenic inhibitory effects of NTAK isoforms remains unknown. NTAKγ and δ isoforms did not induce ErbB phosphorylation (data not shown). We have searched for a ligand binding NTAKγ on the endothelial cell surface but have not, to date, found it. NTAKδ inhibited hyper-phosphorylation of pRb in HUVECs, thus providing molecular confirmation of G1 arrest induced by NTAKδ. In addition, NTAKδ did not induce apoptotic or necrotic cell death of endothelial cells. We also investigated the effect of NTAKδ on other molecules involved in cell cycle regulation such as the p16, p21, and p27 proteins, but their expressions were unchanged on Western blotting analysis (data not shown).

The therapeutic potential of angiogenic inhibitors for angiogenic diseases, such as malignant tumors and diabetic retinopathy, have been proposed. Pathological angiogenesis could be triggered either by up-regulation of angiogenic factors or down-regulation of endogenous angiogenic inhibitory factors. Angiogenic inhibitors have been shown to inhibit tumor growth and metastasis, and some angiogenic inhibitors are currently being tested in clinical trials for cancer treatment. We found that the NTAKγ and δ isoforms had angiogenic inhibitory activities, and these isoforms of NTAK have therapeutic potential for angiogenic diseases. The EGF-ErbB family is involved in tumor growth, and EGF family members generally act as stimulators of tumor growth. This is the first report showing that molecules belonging to the EGF family inhibit angiogenesis.

In summary, NTAKα and δ isoforms, but not α and β isoforms, have angiogenic inhibitory activities in vitro and in vivo. The active sites are localized in the N-terminal region of NTAK containing the Ig-like domain but not the EGF-like domain. Further studies are needed to identify the target of NTAK and δ on the endothelial cell surface to delineate the mechanism underlying anti-angiogenesis.

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