Neprilysin is a cell surface peptidase that catalytically inactivates neuropeptide substrates and functions as a tumor suppressor via its enzymatic function and multiple protein-protein interactions. We investigated whether neutral endopeptidase could inhibit angiogenesis in vivo utilizing a murine corneal pocket angiogenesis model and found that it reduced fibroblast growth factor-2-induced angiogenesis by 85% (P<0.01), but had no effect on that of vascular endothelial growth factor. Treatment with recombinant neprilysin, but not enzymatically inactive neprilysin, resulted in a slight increase in basic fibroblast growth factor electrophoretic mobility from proteolytic cleavage between amino acids L135 and G136, which was inhibited by the neutral endopeptidase inhibitor CGS24592 and heparin. Cleavage kinetics were rapid, comparable to that of other known neprilysin substrates. Functional studies involving neprilysin-expressing vascular endothelial cells demonstrated that neutral endopeptidase inhibition significantly enhanced fibroblast growth factor-mediated endothelial cell growth, capillary array formation and signaling, while exogenous recombinant neprilysin inhibited signaling. Recombinant constructs confirmed that cleavage products neither promoted capillary array formation nor induced signaling. Moreover, mutation of the cleavage site resulted in concomitant loss of cleavage and increased the potency of FGF-2 to induce capillary array formation. These data indicate that neprilysin proteolytically inactivates fibroblast growth factor-2 resulting in negative regulation of angiogenesis.

Neprilysin (Neutral endopeptidase 24.11, CD10) is a 90-110 kD cell surface peptidase normally expressed by a variety of tissues, including epithelial cells of the prostate, kidney, intestine, endometrium, adrenal glands and lung. This enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active peptides, including atrial natriuretic factor, substance P, bradykinin, oxytocin, Leu- and Met-enkephalins, neurotensin, bombesin, endothelin-1 (ET-1), and beta-amyloid. Loss or a decrease in neprilysin expression has been reported in a variety of malignancies, including renal cancer, invasive bladder cancer, poorly differentiated stomach cancer, small cell and non-small cell lung cancers, endometrial cancer and prostate cancer (1,2). Reduced expression of cell surface peptidases such as neprilysin results in the accumulation of higher peptide concentrations that mediate neoplastic progression (3).

Using prostate cancer as a model to study the involvement of neprilysin in malignancy, we have demonstrated the following: 1) Neprilysin protein expression is absent in nearly 50% of primary PCs (2); 2) Neprilysin inhibits neuropeptide-mediated cell growth, cell migration, and ligand-independent activation of the insulin-like growth factor-1 receptor (IGF-1R) leading to Akt phosphorylation (1,4); 3) Neprilysin can inhibit
cell migration independently of its catalytic activity, via protein-protein interaction of its cytoplasmic domain with tyrosine-phosphorylated Lyn kinase, which then binds the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) resulting in an neprilysin-Lyn-PI3-K protein complex. This complex competitively blocks the interaction between focal adhesion kinase (FAK) and PI3-K (5); 4) Neprilysin directly binds to ezrin/radixin/moesin (ERM) proteins resulting in decreased binding of ERM proteins to the hyaluronan receptor CD44 such that cells expressing neprilysin demonstrate decreased cell adhesion and cell migration (6); 5) Neprilysin directly interacts with the PTEN tumor suppressor protein, recruiting endogenous PTEN to the cell membrane, leading to prolonged PTEN protein stability and increased PTEN phosphatase activity, and resulting in a constitutive downregulation of Akt activity (7); and 6) Neprilysin expression inhibits tumorigenicity in an animal model of PC (8). Taken together, these studies have demonstrated that neprilysin protein functions to suppress and inhibit many processes that contribute to neoplastic progression. Enzymatically active neprilysin is also expressed by vascular endothelial cells of venous and arterial origin (9). The neprilysin substrate ET-1 has previously been shown to act directly on endothelial cells via the ET_B receptor to modulate different stages of neovascularization, including proliferation, migration, invasion, protease production and morphogenesis, resulting in neovascularization in vivo (10). Based on these observations, we investigated whether neprilysin also functions as an antagonist of angiogenesis. We report here that neprilysin is indeed anti-angiogenic in vivo, significantly inhibiting angiogenesis. Surprisingly, we demonstrate that neprilysin catalytically inactivates the potent angiogenic factor, fibroblast growth factor-2 (FGF-2). This is the first report of an enzyme that specifically cleaves and inactivates FGF-2 resulting in inhibition of angiogenesis in vivo, further demonstrating the potent tumor suppressive function of neprilysin.

Experimental procedures

Cell lines and Reagents - LNCaP cells were maintained in RPMI 1640 media with 10% fetal calf serum (FCS), supplemented with penicillin (100 IU/mL) and streptomycin (100 μg/mL); Human umbilical vein endothelial cells (HUVEC) were isolated as previously described (11) and maintained in M199 media (Gibco) supplemented with 10 U/mL heparin sodium, 10% FCS (Gemini), 2mM L-glutamine, 100 μg/μL ECGS (Biomedical Technology) supplemented with penicillin and streptomycin. SV40-transduced human bone marrow microvascular endothelial cells (hHBMEC, kindly provided by Dr. Babette Weksler, Weill Medical College) were maintained in Dulbecco Modified Eagle’s Medium supplemented with 5% FCS as previously described (12). Vascular endothelial growth factor (VEGF) and growth factor-reduced matrigel were purchased from BD Bioscience and recombinant fibroblast growth factor-2 was purchased from Research Diagnostics, Inc.

Proteolysis and Mass Spectrometry - Commercially available recombinant FGF-2 was incubated at a concentration of 5-13 μM with recombinant neprilysin (rNEP; Arris Pharmaceutical, Inc.) at a concentration of 0.4-1.0 μM in 100 mM Tris-HCl, pH 7.6 buffer for 1 hour at 25°C in the presence or absence of 10 μM the specific neprilysin inhibitor CGS24592 (Novartis Pharmaceutical, Inc.) or 12 U/mL heparin sulfate. Reaction aliquots were analyzed by 14% SDS-PAGE with Coomassie blue staining or by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOFMS, Micromass, UK) in the mass spectroscopy core facility of Weill Medical College.

GST-FGF-2 and MBP-FGF-2 fusion proteins - Full length human FGF-2 cDNA (kindly provided by Dr. Daniel Rifkin, New York University Medical Center) was used as template to amplify the entire FGF-2 cDNA which was then subcloned into pGEX -2T (Amersham) and pMAL-2Cx (New England Biolabs) vectors using PCR primers containing restriction sites to enable directional cloning. The following amplimers were used: 5’-
ACCATGGCAGCCGGGAGCATC-3’ (sense) and ATATGAATTCTCAGCTCTTAGCAGAC ATGGAAGAAAG (antisense) for glutathione-S-transferase (GST) fusion proteins and 5’-ATGGCAGCCGGGAGCATC-3’ (sense) and 5’-CCCCAAGCTTTAGCTCTTAGCAGAC AT-3’ (antisense) for maltose binding protein (MBP) fusion proteins, as previously described (13). For GST constructs, the PCR product was then purified and digested with EcoRI and BamHI, generating the BamHI-EcoRI fragment corresponding to amino acids 136-155, and a BamHI-BamHI fragment corresponding to amino acids 1-135 of the FGF-2 protein. The restriction fragments were subcloned into pGEX-2T to generate GST fusion proteins with FGF-2 amino acids 1-155 (full length), 1-135 (N-terminal neprilysin cleavage product) and 136-155 (C-terminal neprilysin cleavage product) and DNA sequencing was performed to confirm their accuracy. For MBP constructs, the PCR product was digested with HindIII and XmnI and subcloned into the pMAL-2Cx vector. Fusion proteins of GST and maltose binding protein with FGF-2 were expressed and purified from *E. coli* BL21 cells using glutathione-agarose beads (Sigma) or amylase beads (New England Biolabs) as described (14). Protein content of the beads and 10 mM glutathione eluants were determined by densitometric analysis of Coomassie-stained SDS-PAGE gels against BSA standards (NIH ImageJ software). In some experiments FGF-2 was cleaved from amylose beads (1 mg total fusion protein) by digestion with 2U Factor Xa in Tris-buffered saline buffer and affinity purified using heparin-sepharose chromatography (15). Site-directed mutants (L135A, G136A, and L135A/G136A) of the neprilysin cleavage site on FGF-2 were generated using a Quick-change mutagenesis kit (Stratagene) according to the manufacture’s instructions (mutagenic primers available upon request).

**Matrigel capillary array formation assay** - Transduced human bone marrow microvascular endothelial cells plated in 96 well plates at a density of 15,000 cells/well in DMEM media containing 5% FCS, penicillin, streptomycin, and L-glutamine over 50 μL polymerized growth factor reduced matrigel (10 mg/mL). CGS24592 at a concentration of 0.5 nM-10 nM, phosphoramidon (30 μM, Sigma), or an equivalent volume of dimethylsulfoxide vehicle (DMSO) was added to inhibit neprilysin activity for two hours, followed by FGF-2 proteins at 0.3 -50 nM concentrations. Cells were photographed after 4-18 hours and measurements of capillary cord length obtained for multiple fields using ImageJ software and expressed as mean +/- SEM. Statistical analysis of replicates were performed using unpaired 2-tailed t testing (Prism Graph, Graphpad software).

**MTT growth assay** - Transduced human bone marrow microvascular endothelial cells plated in 96 well plates at a density of 1000 cells/well in DMEM containing 5% FCS, penicillin, streptomycin, L-glutamine and 600 pg/mL FGF-2 were treated with increasing concentrations of CGS24592. Growth assays with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were performed after 48-96 hours as described (16).

**Corneal Pocket Assay** - Hydron (Hydro Med Sciences, Cranbury, New Jersey, USA) and Sucralfate (Teva Pharmaceuticals, North Wales, Pennsylvania) pellets of <1 μL were formulated with combinations of FGF-2 (10 ng or 50 ng/pellet), VEGF (200 ng/pellet) and rNEP (50 ng or 100 ng/pellet) and implanted into corneas of C57BL/6 mice 0.5-1.0 mm from the limbus as described (17). Angiogenesis was assessed by slit-lamp microscopy five days after implantation (18). Statistical analysis of replicates was performed using a 2-tailed unpaired t-test (Prism Graph, Graphpad software). Studies were approved by the Institutional Animal Care Use Committee of Weill Medical College of Cornell University.

**Neprilysin enzyme assays** - Neprilysin enzyme activity determinations were performed as previously described (1). Briefly, subconfluent cells were rinsed in cold lysis buffer (50 mm Tris pH 7.0, 150 mm NaCl) and lysed in lysis buffer containing 0.5% CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propane-sulfonate). Protein concentrations were measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Total neprilysin activity was assayed colorimetrically, and specific activities representing an average of
six independent measurements and expressed in units of pmol/μg protein/minute.

**Western Blotting for GST, total and phosphorylated extracellular regulated kinase (ERK)** - Subconfluent monolayers of hHBMEC, HUVEC, and LNCaP cells were lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 1% v/v NP-40 0.25% w/v sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of aprotinin, leupeptin, pepstatin, 2 mM sodium orthovanadate) following two hour pretreatment with 10 nM CGS24592 or vehicle, and then treated for twenty minutes with various combinations of FGF-2, GST or GST-FGF-2 fusion proteins, and rNEP as indicated. Lysates (50 μg each) were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes, blocked for 0.5 hours and blotted in 3% BSA Tris-buffered saline with 0.1% Tween-20 using either anti-GST antibody (B14, Santa Cruz, CA 1:1000) or anti-phospho-ERK (Cell Signaling Technology, 197G2 1:500) as indicated for one hour. This was followed by appropriate horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse for B14 or donkey anti-rabbit for 197G2, Amersham) at 1:4000 dilution for 0.5 hours, enhanced chemoluminescence (Amersham) and exposure to Kodak Biomax XAR film. Anti-phospho-ERK blots were stripped in 62.5 mM Tris-Cl pH 6.8, 100 mM β-mercaptoethanol, 2% SDS and reprobed with anti-ERK (C14, Santa Cruz 1:1000) followed by anti-rabbit horseradish peroxidase secondary antibody. Films were scanned using an HP scanner and subjected to densitometric analysis using ImageJ and Graphpad software.

**RESULTS**

**Nepriylsin cleaves FGF-2 protein between residues leucine 135 and glycine 136** - To test the hypothesis that nepriylsin could regulate angiogenesis in vivo, we used the murine corneal pocket assay to study the effect of recombinant nepriylsin (rNEP) on neovascularization induced by either FGF-2 or VEGF. In this assay, hydron pellets containing various concentrations of FGF-2 were implanted in the cornea ~1 mm from the limbus and neovascularization measured five days later. As shown in Figure 1, rNEP significantly inhibited FGF-2 induced neovascularization (P<0.01), but had no effect on that of VEGF. These results suggested the possibility that FGF-2 was inactivated catalytically by nepriylsin. Basic FGF is a potent proangiogenic, heparin-binding growth factor, with a primary translation product of 155 amino acids. Nepriylsin hydrolyzes peptide bonds on the amino side of neutral residues, however, a protein of 155 amino acids is theoretically too large to be a substrate for nepriylsin as previous identified substrates are less than 43 amino acids (19,20). To test whether nepriylsin could hydrolyze FGF-2, we incubated rNEP with recombinant FGF-2 for one hour and separated the products on a 14% polyacrylamide gel. Recombinant vascular endothelial growth factor (VEGF) was used as control. As shown in Figure 2A (lane 1, arrow), the molecular weight of FGF-2 protein but not VEGF protein was appreciably lower following rNEP incubation. The increased electrophoretic mobility of FGF-2 incubated with rNEP was blocked by the specific nepriylsin inhibitor CGS24592 (21), indicating that nepriylsin and not a contaminating protease cleaved FGF-2 (Figure 2B, lane 4). To confirm nepriylsin specifically cleaves FGF-2, we performed the same digestion using either immunoprecipitated wildtype nepriylsin (WT5) or enzymatically inactive nepriylsin (M22) expressed using tetracycline-repressible promoter (4) and demonstrated that an intact enzyme activity is both necessary and sufficient to observe FGF-2 cleavage (Figure 2C).

To confirm that FGF-2 cleavage occurs rapidly under physiologically relevant conditions, we assessed the kinetics of FGF-2 proteolysis by nepriylsin, using an enzyme:substrate ration of 1:30 and monitoring reaction progression using SDS-PAGE. As shown in figure 2D, 50% of FGF-2 was cleaved within the first 5-10 minutes, indicating rapid reaction kinetics.

Next the nepriylsin cleavage site on FGF-2 was localized. Fibroblast growth factor-2 and rNEP were combined with or without the nepriylsin inhibitor CGS24592 and analyzed.
using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. This identified a specific 2019 Da band produced in the absence of CGS24592 that corresponded precisely to a 20 amino acid peptide located at the C-terminus of the FGF-2 protein (Figure 2E). Examination of the FGF-2 amino acid sequence confirmed the potential neprilysin cleavage site between leucine 135 and glycine 136 (22). Correlation with the 3-dimensional structure of FGF-2 (23) indicated that the neprilysin recognition site was located at the outer edge of the FGF-2 protein suggesting that it could fit into the neprilysin active site (Figure 2F).

Basic FGF is primarily stored in the extracellular matrix and basement membrane associated with heparan sulfate proteoglycan (HSPG). Activity of FGF-2 is controlled in part by a low-affinity but high-capacity interaction with HSPG. Free FGF-2 may be proteolytically degraded, as suggested by in vitro reactivity of the C-terminal portion of FGF-2 to trypsin and chymotrypsin (24,25). We hypothesized that HSPG binding could protect FGF-2 from degradation by neprilysin since leucine 135 and glycine 136 of the FGF-2 protein lie within a basic region where heparin-derived tetra- and hexasaccharides have been reported to complex with FGF-2 (26). Incubation of rNEP and FGF-2 plus heparin (12 units/ml) showed that heparin completely inhibited the ability of neprilysin to cleave FGF-2 (Figure 2G, lane 2 compared to lane 3). Together, these data suggest that FGF-2 is a neprilysin substrate, and that HSPG protects FGF-2 from neprilysin cleavage.

Endogenous neprilysin expressed on human vascular endothelial cells negatively regulates FGF-2-induced angiogenesis - Previous studies indicate that neprilysin is expressed by human vascular endothelial cells (9,27). Analyses of SV-40 transduced human bone marrow endothelial cells (tHBMEC)(12) and HUVEC revealed neprilysin enzyme specific activities of 197 pmol/μg/min and 36 pmol/μg/min, respectively (data not shown). To assess the functional effect of endogenous neprilysin on FGF-2-induced angiogenesis in vitro, we used a capillary array formation assay to measure the effects of FGF-2 with and without the neprilysin inhibitor CGS24592 in tHBMEC cells plated on matrigel coated plates. As shown in Figure 3A, cells grown in the presence of FGF-2 and CGS24592 demonstrated significantly more arrays compared to cells grown in FGF-2 alone, CGS24592 alone or the untreated negative control (P<0.05). We next assessed endothelial cell growth in tHBMEC treated with 600 pg/mL FGF-2 and with increasing concentrations of CGS24592. Transduced HBMEC incorporated more methyl-thiazol-tetrazolium (MTT) as a function of CGS24592 concentration, suggesting that inhibition of endogenous neprilysin activity results in increased FGF-2-induced cell growth (P<0.03, Figure 3B). Similar results were obtained for HUVEC cells (data not shown). These data show that neprilysin expressed on vascular endothelial cells regulates FGF-2-induced angiogenesis.

FGF-2 cleavage products do not induce angiogenesis - To confirm that neprilysin cleavage blocks FGF-2 function, we produced and purified glutathione-S-transferase (GST) proteins fused to full-length FGF-2, FGF-2 cleavage products corresponding to amino acids 1-135 and 136-155, and as negative control, GST alone, and tested their ability to promote capillary array formation in primary HUVEC cultures in the presence of the neprilysin inhibitors phosphoramidon (PPA, Figure 3C) and CGS24592 (data not shown). Recombinant and full length GST-FGF-2 protein promoted similar amounts of array formation. However, neither the 1-135 nor the 136-155 FGF-2 cleavage products demonstrated any biologic activity in this assay.

To establish that neprilysin anti-angiogenic activity is a direct consequence of FGF-2 cleavage, we examined whether cleavage of FGF-2 abrogated its ability to signal through the fibroblast growth factor receptor (FGFR). Upon engaging FGF-2, FGFR undergoes dimerization, autophosphorylation and then signals by way of the mitogen-activated protein kinase pathway resulting in ERK phosphorylation (28). Therefore, we first assessed rNEP inhibition of FGF-2 signaling by blotting HUVEC lysates for phosphorylated ERK (P-ERK) and total ERK (T-ERK) prepared
following treatment with either FGF-2 (100 ng/mL) alone, or FGF-2 preincubated with rNEP. As shown in Figure 4A, rNEP treatment decreased FGF-2-induced ERK phosphorylation by ~50%. As a complimentary approach, we next examined the effect of neprilysin inhibition on ERK phosphorylation following treatment with a tenfold lower concentration of FGF-2 (10 ng/mL). At this concentration neither FGF-2 nor inhibition of endogenous neprilysin with CGS24592 resulted in appreciable stimulation of ERK phosphorylation (Figure 4B), however CGS24592 treatment led to a FGF-2-stimulated increase in ERK phosphorylation.

Next we sought to identify the mechanism, either loss of receptor binding or receptor antagonism, by which FGF-2 cleavage results in its inactivation. This was done by simultaneously examining cell surface binding of FGF-2 cleavage products and their ability to induce ERK phosphorylation. On incubation of GST-FGF-2 fusion proteins with intact HUVEC and tHBMEC, FGF-2 cleavage products but not full length FGF-2 constructs failed to signal through FGF-R (Figure 4C) and coincidently failed to bind to cultured vascular endothelial cells (Figure 4D), implying that neprilysin cleavage reduces FGF-2 signaling by rendering FGF-2 incapable of binding cell surface receptor. Ten percent input standards were included on right side of 4D as controls.

Mutagenesis of the neprilysin cleavage site residues to alanines resulted in decreased neprilysin cleavage, with the double mutant L135A/G136A demonstrating a near complete loss of cleavage by neprilysin (Figure 5A). Consistent with neprilysin normally cleaving N-terminal to a hydrophobic residue, the G136A mutant, which adds hydrophobicity to this position, largely retains its ability to be cleaved by neprilysin. When tested in a matrigel array formation assay, the neprilysin-resistant L135A/G136A FGF-2 was capable of inducing arrays to an extent comparable to that seen with a 10-fold higher concentration of FGF-2 or with CGS24592 treatment (Figure 5B). Taken together, these data suggest that endogenously expressed neprilysin on vascular endothelial cells regulates FGF-2-induced angiogenesis.

DISCUSSION

Our previous studies have demonstrated that neprilysin possesses multiple properties similar to those of tumor suppressor proteins. These inhibitory effects derive from both neprilysin’s catalytic action on peptide substrates and from direct protein-protein interactions between neprilysin’s short cytoplasmic domain and lyn kinase, PTEN and ERM proteins. In the current study, we demonstrate that neprilysin is capable of cleaving FGF-2 resulting in its inactivation and inhibition of angiogenesis in vivo. With a primary sequence of 155 amino acids, FGF-2 is the largest reported neprilysin substrate, indicating that substrate specificity is not restricted to peptides less than 43 amino acids, as previously believed, and raising the possibility that neprilysin may inactivate other large proteins with sterically permissive tertiary structures. With regard to FGF-2, the external position of the cleavage site permits the generation of a 20 amino acid C-terminal clipped product of FGF-2. These findings also support a novel mechanism by which FGF-2 signaling is attenuated prior to receptor engagement by a cell-surface peptidase. While this mechanism had been proposed previously as the C-terminal portion of FGF-2 has been shown to be cleaved by limited proteolysis in vitro with trypsin and chymotrypsin (24), this is the first example of a protease that cleaves FGF-2 resulting in decreased angiogenesis both in vitro and in vivo.

Cleavage of FGF-2 by neprilysin was rapid in vitro, with 50% cleavage occurring in 5-10 minutes. While we cannot exclude that neprilysin in vivo may be activating another enzyme which in turn cleaves FGF-2, the reaction kinetics using purified proteins are comparable to those observed for other neprilysin substrates; for example amyloid-beta at a one micromolar concentration was observed to be fifty percent degraded by 150 nM neprilysin in about five minutes (29).

Regulation of the interaction between neprilysin and FGF-2 likely occurs through the actions of extracellular matrix and cell surface HSPG, as suggested by the ability of heparin to protect FGF-2 from cleavage by neprilysin.
The putative neprilysin cleavage site is one of the sites where heparanoids are reported to complex with FGF-2 (26), potentially explaining why heparin binding prevents FGF-2 from neprilysin cleavage. Heparan sulfate proteoglycan augments FGF signaling by interacting with both FGF-2 and its receptors, with evidence emerging that FGF-2 may signal through HSPG independently of FGFR (30). It is possible that augmentation of signaling may be due in part to protecting FGF-2 from proteolytic cleave by neprilysin, resulting in higher local concentrations of FGF-2 and the cell surface. The FGF-2 cleavage products produced by neprysin do not have any detectable receptor binding activity, but the 1-135 fragment retains binding to heparin (data not shown) and would be predicted to interact with the plasmin substrate fibrinogen (31), both events associated with enhanced angiogenesis (32), raising the possibility that it may modulate the FGF-2 activity. Likewise, it is possible that the twenty amino acid fragment may possess some as yet undetermined biologic activity and that neprilysin functions to release this product similar to how the neprilysin homologue endothelin converting enzyme 1 (ECE-1) cleaves proendothelin to form the biologically active ET-1 (33).

Fibroblast growth factor-2 has been studied extensively in prostate cancer. Of note, neprilysin-expressing LNCaP cells do not produce measurable amounts of FGF-2, in contrast to non-neprilysin expressing, androgen-independent PC-3 and DU-145 cells (34). Fibroblast growth factor-2 is highly expressed in prostate cancer tissues (35-37), and expression correlates with neovascularization in PC tissue specimens (38). Patients with prostate cancer have significantly elevated serum FGF-2 levels compared to healthy controls, which increase on progression to androgen-independent disease (35). Importantly, androgen-independent prostate cancer is frequently accompanied by loss of tumor cell neprilysin expression (1), and therefore increased serum FGF-2 in these patients may be explained by our findings. Introduction of FGF-2 into prostate epithelial cells induces a neoplastic phenotype (39), and a recent study showed that FGF-2-mediated angiogenesis promotes tumor progression in the TRAMP animal model of PC (40).

Neprilysin may possess anti-angiogenic properties in addition to cleaving FGF-2. Neuropeptide substrates of neprilysin implicated in prostate cancer progression such as ET-1 (41) and bombesin (42) are capable of inducing FGF-2 as well as VEGF expression. Furthermore, neprilysin stabilizes PTEN protein (7), resulting in reduced levels of phosphorylated Akt, known to induce VEGF expression in vascular endothelial cells (43). Thus neprilysin appears capable of negatively regulating angiogenesis via multiple signaling pathways.

Our findings implicate the enzymatic activity of neprilysin as anti-angiogenic, and may be therapeutically applicable beyond malignant neoangiogenesis. Neprilysin levels are reported to be elevated in the diabetic ulcers (44), raising the possibility that impaired wound healing in diabetic patients may result in part from neprilysin’s inhibitory effect on FGF-2. Use of a neprilysin inhibitor administered topically in diabetics in conjunction with recombinant FGF-2 potentially would represent a novel therapeutic approach in these patients. Alternatively, the use of a neprilysin-resistant form of FGF-2 could circumvent the need for an neprilysin inhibitor and provide a novel therapy to target ischemia, where robust angiogenesis is required. Neprilysin loss has also been implicated in the pathogenesis of Alzheimer disease, where its substrate beta-amyloid is increased (45). In addition, FGF-2 levels are also elevated in amyloid plaques in association with HSPG in the affected brain relative to control, a phenomenon which may be explained by this study (46).

In summary, our studies identify the cell-surface peptidase neprilysin as a protease that cleaves and inactivates FGF-2. In addition to inhibiting cell growth and migration via multiple mechanisms including neuropeptide inactivation and protein-protein interactions, neprilysin functions to inhibit FGF-2-mediated angiogenesis. As an anti-angiogenic protein, neprilysin fulfills a previously unrecognized novel tumor suppressive function.
REFERENCES

FOOTNOTES

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The abbreviations used are: AR, androgen receptor; FGF-2, fibroblast growth factor-2; ECE, endothelin converting enzyme; ET-1, endothelin-1; ERK, extracellular regulated kinase; ERM, ezrin/radixin/moesin; FAK: focal adhesion kinase; FGFR, FGF receptor; GST, glutathione-S-transferase; HSPG, heparan sulfate proteoglycan; IGF-1R, insulin-like growth factor-1 receptor; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; MBP, maltose binding protein; MTT, Methyl-thiazol-tetrazolium; PC, prostate cancer; PPA, phosphoramidon; P-ERK, Phospho-ERK; PI3-K, phosphatidylinositol 3-kinase; rNEP, recombinant neprilysin; T-ERK, total ERK; tHBMEC, SV40-transduced human bone marrow microvascular endothelial cells; VEGF, vascular endothelial growth factor.

FIGURE LEGENDS

Fig. 1. Recombinant neprilysin inhibits FGF-2-induced but not VEGF-induced angiogenesis. A, Hydron pellets containing 50 ng FGF-2 (positive control), 10 ng FGF-2, 10 ng FGF-2 + 50 ng rNEP, 200 ng VEGF, 200 ng + 50 ng rNEP, 200 ng + 100 ng rNEP, or 50 ng rNEP alone (negative control) were implanted in the cornea of C57/B6 mice and new vessel formation at 5 days measured by slit lamp ophthalmoscopy. Statistical analysis of six eyes (3 mice) in two independent experiments (a total of 6 mice for each group) was performed (* denotes P<0.01 compared to 10 ng FGF-2, 2-tailed unpaired t-test). B, Photograph of representative corneas: 10 ng FGF-2 alone on left, 10 ng FGF-2 + 50 ng rNEP on right.

Fig. 2. Neprilysin enzymatic activity cleaves FGF-2 Protein. A, Basic FGF or VEGF was incubated with or without rNEP for 1 hour at 25 °C in 100mM Tris-HCl, pH 6.6 buffer, and the samples separated by 14% SDS-PAGE. Lane 1: 12.5 μM FGF-2 + 1 μM rNEP; Lane 2: 12.5 μM FGF-2 alone; Lane 3: 12.5 μM VEGF alone; Lane 4: 12.5 μM VEGF + 1 μM rNEP. Note faster migration of FGF-2 protein treated with rNEP (arrow). B, 12.5 μM of FGF-2 was incubated with or without rNEP and the neprilysin inhibitor CGS24592 for 1 hour at 25 °C, and the samples separated by 14% SDS-PAGE. Lane 1: DMSO vehicle (control); Lane 2: 3 μM CGS24592; Lane 3: 1 μM rNEP + 12.5 μM FGF-2; Lane 4: 1 μM rNEP + 12.5 μM FGF-2 + 3 μM CGS24592. Note that the addition of CGS24592 blocks the faster migration of FGF-2 protein treated with rNEP (Lane 4 compared with Lane 3). C, Lysates from TSU-Pr1-derived WT5 and M22 cells cultured in tetracycline-free media for 48 hours containing 500 μg total protein was subjected to immunoprecipitation with J5 antibody and incubated with 100 ng of FGF-2 for 4 hours at 37°C. Samples were analyzed by SDS-PAGE and Western blotted for both FGF-2 and neprilysin. Note the increased electrophoretic mobility seen with wildtype neprilysin (WT5) but not enzymatically deficient neprilysin (M22). D, FGF-2 (12 μM) and neprilysin (400 nM) were incubated in 50 mM Hepes pH 7.4, 100 mM NaCl and aliquots removed at the various times (1-230 minutes), quenched with SDS sample buffer, and analyzed by SDS-PAGE. Coomassie-stained intact FGF-2 (upper arrow on right) was then quantified densitometrically (NIH ImageJ software) and expressed graphically as the percentage of intact FGF-2, with 100% defined as the intensity of the intact FGF-2 band at time 0, just prior to addition of rNEP. E, Recombinant neprilysin was incubated with FGF-2 with (upper panel) or without (lower panel) CGS24592 and analyzed by MALDI-TOF mass spectrometry. Note the presence of a 2019 Da band, which corresponds to amino acids 136-155 of the FGF-2 protein in the absence of CGS24592. F, Crystal structure of the 155-amino-acid form of recombinant FGF-2 (PDB ID: 1BFF from ref 23). The neprilysin
cleavage site is highlighted. G, Fibroblast growth factor-2, rNEP and heparin were incubated for 1 hour at 25 °C in 100 mM Tris-HCl, pH 7.6 and subjected to 14% SDS-PAGE analysis with Coomassie blue staining. Lane 1: 1 μM rNEP alone; Lane 2: 12.5 μM FGF-2 + 1 μM rNEP +12 U/mL heparin; Lane 3: 12.5 μM FGF-2 + 1 μM rNEP; Lane 4: 12.5 μM FGF-2 alone. Note heparin inhibits the faster migration of FGF-2 protein treated with rNEP (Lane 2). Lanes 1 and 4 are controls.

Fig. 3. Endogenous neprilysin inhibition enhances FGF-2-induced angiogenesis. A, Transduced human bone marrow microvascular endothelial cells were assayed using a matrigel capillary array formation assay. Cells were plated on growth factor reduced matrigel in the presence (+) or absence(-) of CGS24592 (30 nM) for 2 hours followed by the addition of FGF-2 (5 ng/mL) for 4 hours as indicated. Representative photographs were then taken. B, Transduced human bone marrow microvascular endothelial cells were incubated with 600 pg/mL FGF-2 in DMEM supplemented with 1% FCS and increasing concentrations of CGS24592. After 48 hours, media was exchanged with that containing MT at a concentration of 0.5 mg/ml and incubation continued for four more hours. Incorporated MTT was liberated with DMSO, and expressed as OD_{570nm} (* denotes P<0.03 relative to no CGS24592). Results are representative of two independent experiments with similar results performed in triplicate. C, 15,000 HUVEC cells were plated on growth factor reduced matrigel in the presence or absence of 30 μM phosphoramidon (PPA), and the indicated commercially available FGF-2 or GST-FGF-2 fusion protein (included FGF-2 amino acids designated in parentheses) added to a final concentration of 50 nM. Array formation was quantified after 18 hours by measuring total length of capillary cords formed per 500X field using ImageJ software. Results are representative of 3 independent experiments with similar results performed in triplicate (* denotes P<0.05 relative to GST control).

Fig. 4. Neprilysin inhibition of FGF-2 signaling through MAP-kinase. A, Neprilysin attenuation of FGF-2 signaling through ERK. FGF-2 (8 μg/mL) was incubated in the presence or absence of rNEP (60 μg/mL) for 1 hour at 25°C in 100 mM Tris-HCl pH 7.6 and then added to subconfluent HUVEC monolayers for a FGF-2 concentration of 100 ng/mL, incubated for 20 minutes, harvested with RIPA lysis buffer and 50 μg of protein subjected to Western blotting analysis using anti-total ERK antibody (T-ERK) or anti-phosphorylated ERK antibody (P-ERK). As a negative control, buffer alone was used. Results are representative of three independent experiments. The inset shows densitometric analysis of the included experiment. B, Neprilysin inhibition potentiates FGF-2 signaling through ERK. As in 4A above, FGF-2 and rNEP were incubated and then added to subconfluent HUVEC monolayers which were pretreated with 10 nM CGS24592 or vehicle for two hours, for a final FGF-2 concentration of 10 ng/mL, incubated for 20 minutes, harvested with RIPA lysis buffer and 50 μg of protein subjected to Western blotting using T-ERK or P-ERK antibodies. The inset shows densitometric analysis of the included experiment. C, FGF-2 cleavage products are unable to induce MAP kinase phosphorylation. Subconfluent HUVEC monolayers were treated with 5 nM of recombinant proteins as indicated for 20 minutes and washed 3 times with 2 mL PBS. RIPA cell lysates were analyzed for total and phosphorylated ERK as in 4A. Recombinant commercially purchased FGF-2 (5nM) was used as positive control. D, Testing of FGF-2 cleavage products for binding to intact cells. Lysates from C above were analyzed by SDS-PAGE, and blotted with anti-GST antibody. As an in input standard, ten percent of each FGF-2 cleavage product included in the experiment was analyzed on the same gel (four right lanes).

Fig. 5. Mutagenesis of the putative neprilysin cleavage site results in abrogation of cleavage and increases FGF-2 potency. A, One microgram each of wildtype FGF-2 (Lanes 1-2) and the FGF-2 mutants L135A (Lanes 3-4), G136A (Lanes 5-6), and L135A/G136A (Lanes 7-8) was incubated with 2 μg rNEP (even numbered lanes) or vehicle (odd numbered lanes) at 25 degrees for 12 hours in a final volume of 25 μL of 100 mM Tris-HCl pH 7.2 and subjected to SDS-PAGE analysis. A lower band corresponding to the major cleavage product is observed for wildtype FGF-2 reaction and in varying degrees for the FGF-2 mutants. Below is the densitometric analysis (ImageJ software) for each cleavage product expressed as
percentage of total FGF-2 (intact + cleaved band) and corrected by subtracting the corresponding pixels obtained in the absence of neprilysin. These data are representative of three individual experiments with similar results. B, 15,000 HUVEC cells were plated on growth factor reduced matrigel in the presence or absence of 30 nM CGS 24592, and the indicated FGF-2 protein (5-50 ng/mL). Array formation was quantified after 18 hours by measuring total length of capillary cords formed per 500X field in five different fields using ImageJ software. Results are representative of 3 independent experiments with similar results performed in duplicate (*P<0.01 relative to control and wildtype FGF-2).
1A

Neovascularized area (mm²)

50 ng FGF-2
10 ng FGF-2
10 ng FGF-2 + 50 ng rNEP
200 ng VEGF
200 ng VEGF + 50 ng NEP
200 ng VEGF + 100 ng NEP
50 ng rNEP

Corneal Implant
1B

FGF-2  FGF-2+rNEP
2B

1  2  3  4

rNEP

FGF-2
2D

time (min) 0 1 5 10 30 110 230 0

rNEP

FGF-2

intact cleaved

% of remaining intact full length FGF-2

0 10 20 30 40 50 60 70 80 90 100 110 120

time (min)
4D

|-----|-----|-------------------|---------------------|-------------------|---------------------|

Bound 10% input
Tube length (pixels)

- Control
- 5 ng/mL FGF-2 WT
- 5 ng/mL FGF-2 WT + 30 nM CGS
- 50 ng/mL FGF-2 L135Al/G136A
- 50 ng/mL FGF-2 WT

* indicates significant difference from control.
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