Syndecan-4 modulates basic fibroblast growth factor 2 signaling in vivo

Yufeng Zhang,1 Jianyi Li,2 Chohreh Partovian,1 Frank W. Sellke,2 and Michael Simons1

1Angiogenesis Research Center and Section of Cardiology, Dartmouth-Hitchcock Medical Center and Dartmouth Medical School, Lebanon, New Hampshire 03756; and 2Department of Surgery, Beth Israel Deaconess Medical Center, Boston, Massachusetts 02215

Submitted 30 October 2001; accepted in final form 20 January 2003

Zhang, Yufeng, Jianyi Li, Chohreh Partovian, Frank W. Sellke, and Michael Simons. Syndecan-4 modulates basic fibroblast growth factor 2 signaling in vivo. Am J Physiol Heart Circ Physiol 284: H2078–H2082, 2003. First published January 23, 2003; 10.1152/ajpheart.00942.2001.— Syndecan-4 is one of the principal heparan sulfate-carrying proteins on the cell surface. Unlike other members of syndecan family, syndecan-4 mediates phosphatidylinositol 4,5-bisphosphate 2 (PIP2)-dependent PKC-O activation, and overexpression of syndecan-4 in vitro results in enhanced FGF2 signaling. The present study was designed to test the functional effect of increased syndecan-4 expression in endothelial cells in transgenic mice. Several transgenic mice lines expressing syndecan-4 cDNA under control of human endothelial nitric oxide (NO) synthase (eNOS) promoter were generated. Exogenous syndecan-4 was mainly expressed in the heart, brain, and lungs. In particular, the heart demonstrated the greatest increase in the ratio of transgenic-to-native syndecan-4 gene expression. Vessels from the eNOS-syndecan-4 mice demonstrated more pronounced vasodilation to FGF2 but not to VEGF-A165, sodium nitroprusside, and A 23187 compared with wild-type mice. To elucidate the mechanism of this effect, we measured NO release from primary cardiac endothelial cells isolated from transgenic or wild-type adult mice. Cells from the eNOS-syndecan-4 transgenic mice had a significant increase in FGF2- and VEGF-A165-induced NO release compared with endothelial cells from the wild-type mice. However, the absolute magnitude of this increase was higher for FGF2 than VEGF-A165. In conclusion, enhanced syndecan-4 expression in mouse cardiac endothelial cells results in preferential augmentation of FGF2 but not VEGF-A165-induced NO release.

sodium nitroprusside; angiogenesis; heparan sulfate; nitric oxide; vascular endothelial growth factor

Heparan sulfate (HS) proteoglycans can mediate both heparin-binding growth factor receptor interaction at the cell surface and accumulation and storage of these growth factors in the extracellular matrix (27). The presence of HS is required for basic FGF2-dependent activation of cell growth in vitro (18, 29), and removal of HS chains from the cell surface by enzymatic digestion greatly impairs FGF2 activity and inhibits neovascularization in vivo (16). Therefore, any alteration of HS chain composition on the cell surface or in the ECM by means of altered synthesis, degradation, or modification of glycosaminoglycan chains can conceivably affect growth factor signaling.

Cell surface HSs are found on two principal classes of core proteins: transmembrane syndecans and membrane-anchored glypicans. Of these, syndecan-1, syndecan-4, and glypican-1 are the primary core proteins found in endothelial cells (19). Recent studies from our laboratory (8, 9, 28) suggest that syndecan-4, in addition to its contributions to the composition of the cell surface and extracellular matrix, may function as a specific basic FGF (FGF2) signaling molecule. In particular, overexpression of syndecan-4 (but not syndecan-1 or glypican-1) in ECV304 cells resulted in enhanced migration and proliferation in response to FGF2 (28). The present study was designed, therefore, to test whether altered syndecan-4 expression in vivo would have similar specific effect on FGF2 signaling.

FGF2 is a pluripotent mitogen capable of variety of biological activities (23, 25). One of the more interesting and least studied actions of FGF2 is its ability to induce nitric oxide (NO) release from a number of cell types, including endothelial cells (12). Previous investigations have documented the profound hemodynamic effect of FGF2-induced NO release (1) and suggested that one of the factors regulating tissue responsiveness to FGF2-induced NO release is the level of FGF receptor 1 expression (21, 22). Therefore, we have used FGF2-induced NO release as a marker to assess FGF2 activity in mice carrying a syndecan-4 transgene-directed high level of core protein expression in endothelial cells. We find that these mice demonstrate enhanced NO release in response to FGF2 but not VEGF-A165, which leads to significantly greater vasodilation of coronary microvasculature.

Materials and Methods

Generation and analysis of syndecan-4 transgenic mice. A 1600-bp promoter fragment of the human endothelial NO synthase (eNOS) gene (6) was used to drive expression of the rat syndecan-4 cDNA. The construct was injected into C57/BL6 mouse embryos, and transgenic founders and offspring...
were identified by the PCR of mouse-tail genomic DNA. Expression levels of exogenous and endogenous syndecan-4 were checked by Northern blot analysis of mouse tissues. All functional experiments were performed on the second generation of mice from founder. Heart and body weights were recorded for all mice in the study.

Northern blot analysis of mouse heart gene expressions. Total RNA was extracted from mouse heart tissues with the Tri Reagent (Sigma). Total RNA (20 μg) was fractionated on 1.2% formaldehyde-agarose gel and transferred onto GeneScreen Plus membrane. cDNA probes for different genes were labeled with [α-32P]dCTP by using random priming kit (Boehringer-Mannheim) and hybridization was carried out in Quickhyb solution (Stratagene). The quantitative analysis of signals was carried out with PhosphoImager (Molecular Dynamics) by using ImageQuant software using cardiac actin probe to adjust expression levels.

Immunohistochemical staining of syndecan-4 in mouse heart tissue. The anti-syndecan-4 antibody was raised against the extracellular domain of rat syndecan-4 in chickens. Before incubation with the primary antibody, tissue sections were blocked with 10% of normal goat serum for 30 min at 37°C and 1:10 of BlockHen solution (Aves Labs) for 30 min at 37°C. The sections were incubated with the anti-syndecan-4 antibody (20 μg/ml) diluted with 1.5% normal serum/PBS overnight at 4°C. Fluorescein-labeled goat anti-chicken IgG (AvesLabs) (5 μg/ml) diluted with 1.5% normal serum/PBS was then added for 1 h at room temperature. The sections were washed with PBS between each step.

Isolation of microvascular endothelial cells from adult mice hearts. Hearts were rapidly extracted from anesthetized adult eNOS-syndecan-4 (eNOS-S4) transgenics and control C57/BL6 mice and quickly dipped into ice-cold 70% ethanol for 20 s and then transferred to ice-cold HBSS. Atria were discarded and the remaining ventricular part was quickly minced into ~1 mm³ pieces. The minced tissue was then digested with 2 mg/ml of collagenase II (Worthington Biochemical) in Ca²⁺- and Mg²⁺-free HBSS with agitation for 30 min at 37°C and then trypsin was added to a final concentration of 0.3 mg/ml for 30 min. Cells released after this digestion cells were plated onto laminin-coated dishes for 1 h and unattached cells were discarded. After three washes with PBS, the remaining cells were cultured in DMEM supplemented with 20% FBS. Cells in the second passage were used for all experiments.

Measurement of NO release from cultured endothelial cells. The primary endothelial cells in the second passage were plated in 24-well tissue culture plates and cultured in 20% FBS-DMEM until confluency. Cells were then changed to 0.5% FBS-DMEM overnight, and, after PBS washes, the media was replaced with Krebs-Henseleit buffer solution containing (in mM) 120 NaCl, 4.6 KCl, 1.5 CaCl₂, 0.5 MgCl₂, 1.5 NaH₂PO₄, 0.7 Na₂HPO₄, 10 HEPES and 10 glucose, pH 7.4, and supplemented with 8 U/ml superoxide dismutase (Sigma) just before the experiment. FGF2 (Chiron) or VEGF (R&D Systems) was added to the middle of wells to a final concentration of 50 ng/ml as gently as possible and then NO was measured with a commercially available NO meter (Isorex-World Precision Instruments, Sarasota, FL), as detailed previously (26). The probe was calibrated with a standard chemical method by using KNO₂ (0.05 mM) as a NO generator in the KI (0.1 M) and H₂SO₄ (0.1 M) mixture, based on the following equation: 2 KNO₂ + 2KI + 2H₂SO₄ → 2 NO + I₂ + 2H₂O + 2K₂SO₄. All measurements were performed at room temperature; the results are shown as means ± SD. Each preparation contained cells from four to six mice and two separate preparations were performed for each study group.

In vitro microvascular study protocols. Microvessels (70–180 μm internal diameter) from mouse coronary artery branches were dissected under anesthesia and mounted in a microvessel chamber as described previously (13). Baseline vascular diameter was measured first, and then the vessels were preconstricted with the thromboxane A₂ analog U-46619. The relaxation responses of microvessels to various agents were examined by using the optical system. Vessels were incubated with FGF-2 (10⁻¹⁵–10⁻¹⁰ mol/l), VEGF-A₁₆₅ (10⁻¹⁵–10⁻¹⁰ mol/l), sodium nitroprusside (SNP) (10⁻⁹–10⁻⁴ mol/l), and A 23187 (10⁻⁹–10⁻⁵ mol/l). All drugs were added extraluminally and measurements were performed 2–3 min after the drugs were administered. Vessels were washed three times with Krebs buffered solution for 15–30 min between interventions. Relaxation responses were expressed as percent relaxation of the U-46619-induced vascular contraction (means ± SE) of the microvessels.

RESULTS

eNOS-S4 transgenic mice. Four independent eNOS-S4 transgenic lines were identified by genomic DNA screening. All transgenic lines grew and bred normally compared with the wild-type mice, and no gross abnormalities were observed in any organs. The line with the highest cardiac expression of syndecan-4 transgene was selected for further studies. Exogenous syndecan-4 was highly expressed in the heart and lung. Less robust expression was seen in other organs including the brain, kidney, ovary, intestine, and skin. No expression was seen in the liver, spleen, stomach, and colon (Fig. 1). The highest increase in exogenous compared with endogenous syndecan-4 gene expression was observed in the heart. Immunohistochemical staining confirmed increased expression of syndecan-4 protein in cardiac microvascular endothelial cells in eNOS-S4 transgenic compared with the wild-type mice (Fig. 2).

Syndecan-4 transgenic mice were no different from controls with regard to the weight of the animals [wild-type, 25.5 ± 5.89 g; eNOS-S4, 25.8 ± 5.3 g; P = not
significant (NS)) or their hearts (wild-type, 143.3 ± 13.3 mg; eNOS-S4, 143.1 ± 14.2 mg; P = NS). No gross anatomic differences were noted. The effect of syndecan-4 transgene expression on the expression of other genes potentially involved in NO signaling was studied in whole hearts of transgenic mice. Northern blot analysis showed a mild increase in expression of VEGF-A165, its receptor Flt-1, and eNOS. There were no significant differences with regard to FGF2 or its receptor FGF-R1 expression (Fig. 3).

Vasodilatory response of coronary microvessels. FGF2 has been shown to induce vasodilation of resistance vessels via its effect on NO release. To study the functional effect of increased endothelial syndecan-4 expression on FGF2 signaling, we analyzed vasodilatory responses of preconstricted coronary microvessels isolated from eNOS-S4 and control mice. SNP and a Ca²⁺ ionophore A 23187 had equally potent vasodilatory effect on microvessels from both eNOS-S4 and C57BL/6 mice. Likewise, VEGF-A₁₆₅, another heparin-binding growth factor, also had similar effects on relaxation of microvessels from control and transgenic mice. However, FGF-2 induced significantly greater vasodilation of eNOS-S4 mice microvessels (Fig. 4).

Increased sensitivity of microvessels from eNOS-S4 mice to FGF2-induced vasodilation may reflect greater release of NO in response to FGF2 in eNOS-S4 mice. To test this directly, we measured NO release during incubation with FGF2 and VEGF-A₁₆₅ from primary cardiac endothelial cells isolated from both sets of mice. Endothelial expression of syndecan-4 increased both FGF2 and VEGF-A₁₆₅-induced NO release (Fig. 5) that was fully blocked by NG-nitro-L-arginine methyl ester (not shown). However, the magnitude of this increase was much higher for FGF2 than VEGF-A₁₆₅ (Fig. 5).

DISCUSSION

The principal finding of this study is that endothelial expression of syndecan-4 in mice results in significant enhancement of FGF2-dependent release of NO and consequent vasodilation of microvascular resistance vessels. Whereas the increase in endothelial cell syndecan-4 expression with its accompanying increase in endothelial HS mass resulted in increased production of NO in response to another heparin-binding growth factor, VEGF-A₁₆₅, the effect was much more pronounced for FGF2. Furthermore, FGF2, but not VEGF-A₁₆₅, induced significantly greater vasodilation in eNOS-S4 mice microvessels compared with C57Bl/6 mice.

The role of syndecan-4 in cell signaling has recently attracted considerable attention due to its unique ability among other syndecans to activate PKC-α in a phosphatidylinositol 4,5-bisphosphate (PIP₂)-dependent manner (7, 8, 17). A prior study from our laboratory (28) demonstrated that overexpression of syndecan-4 in cells leads to enhanced migration and prolif-
eration in response to FGF2. Furthermore, these effects can be attributed to the presence of syndecan-4 cytoplasmic domain because expression of a chimera composed of extracellular domain of syndecan-4 linked to GPI linker had no effect on cell FGF2 responsiveness, whereas expression of a chimera constructed of glypican extracellular domain linked to the syndecan-4 cytoplasmic domain augmented cell growth and migration in response to FGF2 (28). At the same time, expression of other members of the syndecan family members, such as syndecan-1, lacking the ability to bind PIP2 and activate PKC-\(\alpha\), actually inhibits FGF2-induced proliferation (14, 28). It should be noted that increased cell surface HS mass secondary to increased syndecan-4 expression (28) may potentially increase activity of other heparin-binding growth factors such VEGF-A165.

However, the apparently preferential response to FGF2 and not to other heparan binding growth factors may, in part, be explained by the ability of FGF2 to activate a type I/IIa serine phosphatase that promotes dephosphorylation of Ser\(^{183}\) in the cytoplasmic domain of syndecan-4, thereby facilitating syndecan-4-PIP2 binding, oligomerization, and activation of PKC-\(\alpha\) (9). VEGF-A165, in contrast to FGF2, does not demonstrate such an activity (A. Horowitz and M. Simons, unpublished observations).

The ability of FGF2 to induce NO release is well documented (2, 11). Whereas in the case of VEGF, NO release involves AKT-1-dependent activation of eNOS on Ser\(^{1177}\) (3, 5, 15), no similar mechanism for FGF2 has been established. Given syndecan-4 involvement in PKC-\(\alpha\) activation and a requirement for PKC in FGF2-mediated activation of endothelial cell proliferation (10), the results of the present study may suggest that PKC-\(\alpha\) may play a role in FGF2-dependent NO release.

Whereas syndecan-4, as well as HS carrying core proteins, may shed their chains into the extracellular matrix (4, 24), it is unlikely that this may have affected the enhanced NO release and vasodilation in response to FGF2 in eNOS-S4 mice. In particular, microvessels from both mice groups were equally responsive to an endothelium-dependent vasodilator calcium ionophore A-23187 and to endothelium-independent direct NO donor SNP (20, 21).

In summary, endothelial expression of syndecan-4 selectively enhances microvessel responsiveness to FGF2 due to increased generation of NO. Syndecan-4...
expression, therefore, serves to preferentially regulate FGF2 signaling.

This study was supported in part by National Heart, Lung, and Blood Institute Grants R01-HL-53793, HL-62289, P50-HL-63609 (to M. Simons), HL-46716 and HL-69024 (to F. W. Sellke).

Present address of Y. Zhang: Dept. of Medicine, Boston University School of Medicine, 715 Albany St., Boston, MA 02118.

REFERENCES