Syndecan-4 Is Required for Thrombin-induced Migration and Proliferation in Human Vascular Smooth Muscle Cells

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Thrombin is a mitogen and chemoattractant for vascular smooth muscle cells (SMCs) and may contribute to vascular lesion formation. We have previously shown that human SMCs, when stimulated with thrombin, release basic fibroblast growth factor (bFGF), causing phosphorylation of extracellular signal-regulated receptor (ERK)-1. Treatment with bFGF-neutralizing antibodies or with heparin inhibits thrombin-induced DNA synthesis. We concluded that thrombin may stimulate entry into the cell cycle via bFGF release and FGFR-1 activation. In the present study, we demonstrate a requirement for not only FGFR-1 but also syndecan-4, a transmembrane heparan-sulfate proteoglycan. Inhibition of syndecan-4 expression using small interfering RNA (siRNA) resulted in reduced DNA synthesis by human SMCs after stimulation with thrombin (10 nmol/liter). Anti-bFGF antibody, which inhibits DNA synthesis in control cells, had no inhibitory effect when syndecan-4 expression was reduced by siRNA. Thrombin- or bFGF-induced SMC migration, determined in Boyden chamber assays, was reduced in cells treated with syndecan-4 or FGFR-1 siRNA or by anti-bFGF. Thrombin induced phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 in a biphasic pattern. Although thrombin-mediated ERK phosphorylation at 5 min was not affected by syndecan-4 or FGFR-1 siRNA, ERK phosphorylation at later time points was reduced. We conclude that thrombin-released bFGF binds to syndecan-4 and FGFR-1, which is required for thrombin-induced mitogenesis and migration.

Vascular smooth muscle cell (SMC) proliferation and migration are key events in atherosclerosis and restenosis after vascular injury (1, 2). The G-protein-coupled receptor agonist thrombin may contribute to disease progression through stimulation of mitogenic signaling. In a previous study with cultured human SMCs, we demonstrated that thrombin releases basic fibroblast growth factor (bFGF), which is bound to the pericellular matrix and causes phosphorylation of the FGF receptor-1 (FGFR-1) (3). Preventing FGFR-1 phosphorylation by bFGF-neutralizing antibodies or by heparin inhibits thrombin-induced mitogenesis. Since heparin binds bFGF and inhibits bFGF-FGFR-1 interactions, we hypothesized that an endogenous heparan sulfate proteoglycan (HSPG) may be involved in mediating the effects of thrombin-released bFGF and thus be required for thrombin-induced mitogenesis.

Syndecan-4 is a transmembrane HSPG with a core protein of 35 kDa carrying mainly heparan sulfate side chains (4). Syndecans and the HSPG glypican have been shown to bind bFGF and increase bFGF-FGFR-1 interactions (5). Of the family of syndecans (syndecan-1 to -4), syndecan-4 is the only member with an intracellular domain that is capable of binding phosphatidylinositol 4,5-bisphosphate and protein kinase C (PKC). It binds bFGF and increases bFGF-FGFR-1 interactions (5). Syndecan-4 is expressed in endothelial cells, fibroblasts, macrophages, and pericytes, and may mediate growth factor cell signaling and a cofactor for thrombin-induced mitogenesis and migration (9–13). Since thrombin activates FGFR-1 by releasing bFGF into the pericellular matrix and syndecan-4 functions as a mediator for bFGF signaling and a cofactor for FGFR-1 (14), we tested the possibility that thrombin-induced mitogenesis and migration require syndecan-4 for binding thrombin-released bFGF.

**EXPERIMENTAL PROCEDURES**

**Materials—**Antibodies (Abs) against syndecan-4 and FGFR-1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Ab against phospho-ERK1/2 was from Cell Signaling Technology (Beverly, MA). Ab against heparin was from American Diagnostica (Greenwich, CT). Recombinant bFGF, heparin (porcine intestinal mucosa), and heparitinase III digestion (anti-HS, clone 3G10), was from Seikagaku Corp. (Tokyo, Japan). Protein A-agarose was from Roche Diagnostics. Neutralizing Ab against human bFGF was a generous gift from Dr. Michael A. Reedy (University of Washington) (14). ERK1/2 antisera was a generous gift from Dr. Karen Bornfeld (University of Washington). Human α-thrombin was from American Diagnostics (Greenwich, CT). Recombinant bFGF, heparin was from Tokyo, Japan. Protein A-agarose was from Roche Diagnostics. Neutralizing Ab against human bFGF was a generous gift from Dr. Michael A. Reedy (University of Washington) (14).

**Cell Culture—**Human aortic SMCs were prepared by the explant technique as described previously (15). Cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 15% fetal bovine serum, 200 units/ml penicillin, and 200 μg/ml streptomycin. Cells at passage 5–12 were used for experiments.

**Gene Silencing with Small Interfering RNA (siRNA)—**The following sequences were chosen to generate siRNAs for syndecan-4, 5′-AAGGC-CCATACTTCTCCCGAGCAGTA-3′ (sense), and 5′-AAGGCCATCTCCCGAGCAGTA-3′ (complementary); for FGFR-1, 5′-AAGTCGAGCGCAAGAAAGAAAC-3′ (sense), and 5′-AACAGAAGGATCAGGAGCCAA-3′ (complementary). SiRNAs were generated in vitro using a siRNA construction kit (Ambion, Austin, TX) according to the manufacturer’s instructions.
Cells were transfected with siRNAs (final concentration 10 nmol/liter) by calcium phosphate precipitation for 15 h, as described (16). Cells were washed three times with phosphate-buffered saline, once with medium containing 15% fetal bovine serum, and allowed to recover for at least 9 h. Cells were detached with trypsin, counted, and reseeded for experiments in 15% serum.

**Immunoprecipitation and Western Blotting**—Immunoprecipitation of FGFR-1 was carried out as described previously (3). For detection of syndecan-4 or HS-PG neo-epitope (ΔHS), cells were lysed with 8 mM urea buffer (25 mM Tris-HCl, 8 mM urea, pH 7.5, 2 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were frozen at −80 °C to disrupt syndecan-4 dimers (13). Cell extracts were dialyzed against 0.1 M NaOH at 4 °C followed by dialysis against 0.01 M sodium acetate, pH 7.0, 0.1 mM calcium acetate. Samples were concentrated using centrifugal filter tubes (Amicon®, cut-off 10,000, Millipore, MA) and resuspended in 100 μl of digestion buffer (10 mM Tris-HCl, pH 7.4, 2 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride). Samples were incubated with heparitinase I–III (10 units/ml heparitinase I and II, 2.5 units/ml heparitinase III) for 4 h at 37 °C to digest HS side chains. Protein concentrations were determined to ensure equal protein loading. To determine ERK phosphorylation, cells were seeded into 6-well plates (80,000–100,000 cells/well) and incubated in serum-free medium for 48 h with a change of medium 24 h before stimulation. For Western blot analysis, samples were boiled in Laemmli sample buffer and subjected to SDS-PAGE followed by transfer to nitrocellulose. Primary antibodies used were monoclonal Ab against syndecan-4 (clone 5G9, Santa Cruz Biotechnology), anti-actin (Sigma), anti-ERK1/2 (Cell Signaling), anti-β-catenin (clone 5G9, Santa Cruz Biotechnology), anti-actin (Sigma), anti-β-catenin (clone 5G9, Santa Cruz Biotechnology), anti-actin (Sigma), and anti-β-catenin (clone 5G9, Santa Cruz Biotechnology). Protein was visualized by enhanced chemiluminescence (kit from Amersham Biosciences) according to the manufacturer’s protocol. Results were quantified by densitometry (Amersham Biosciences) or by phosphorimaging (ImageQuant software (Amersham Biosciences)).

**Semiquantitative RT-PCR**—Untreated SMCs or those transfected with 10 nmol/liter siRNA were subjected to SDS-PAGE and subjected to electrotransfer to nitrocellulose. Primary antibodies used were monoclonal Ab against syndecan-4 (clone 5G9, Santa Cruz Biotechnology), anti-actin (Sigma), anti-β-catenin (clone 5G9, Santa Cruz Biotechnology), anti-actin (Sigma), anti-β-catenin (clone 5G9, Santa Cruz Biotechnology), anti-actin (Sigma), and anti-β-catenin (clone 5G9, Santa Cruz Biotechnology). Protein was visualized by enhanced chemiluminescence (kit from Amersham Biosciences) according to the manufacturer’s protocol. Results were quantified by densitometry (Amersham Biosciences) or by phosphorimaging (ImageQuant software (Amersham Biosciences)).

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**Statistics**—All experiments were performed at least three times in duplicate, triplicate, or quadruplicate. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s multiple comparison test or by a paired two-tailed t test as indicated. Values of p < 0.05 were considered significant.

**RESULTS**

Inhibition of Syndecan-4 and FGFR-1 Expression by siRNA in Human Vascular SMCs—Specific siRNA significantly reduced RNA levels for syndecan-4 and FGFR-1 in human SMCs, respectively, but had no effect on RNA of syndecan-2 or glypi-
can, two proteoglycans that can both bind bFGF (5). RNA oligonucleotides with a scrambled sequence derived from syndecan-4 or FGFR-1 siRNA had no effect on RNA levels (Fig. 1).

Syndecan-4 protein levels were determined in extracts from human aortic SMCs by Western blotting. Although the specific siRNA inhibited syndecan-4 protein expression by over 90%, a matched scrambled RNA oligonucleotide had no effect on syndecan-4 expression (Fig. 2A). The blot contains duplicates of each sample. B, the effect of 10 nmol/liter siRNA to syndecan-4 on syndecan-4 protein. The blot contains duplicates of each sample. C, the effect of 10 nmol/liter siRNA to FGFR-1 on FGFR-1 protein. Values are mean ± S.E. of 4–5 independent experiments; *, p < 0.05 versus CaHPO$_4$ and scrambled RNA (ANOVA).

Cell Spreading Is Impaired in SMCs with Reduced Syndecan-4 Expression—Because syndecan-4 is involved in the organization of the cytoskeleton (9, 21), we investigated whether syndecan-4 siRNA affects cell spreading. When compared with controls, syndecan-4 siRNA-treated cells spread less at 1 and 3 h in serum-free medium. However, by 6 h, all cells were equally spread on collagen (Fig. 3). Cell spreading in 15% serum appeared to be normal, and also, the number of cells that attached in 15% serum overnight was not decreased in syndecan-4 siRNA cells (Fig. 3B) when compared with control cells. These data suggest that the lack of syndecan-4 delays cell spreading on collagen. Delayed spreading was not observed in cells with decreased FGFR-1 expression, indicating distinct functions of syndecan-4 and FGFR-1.

Syndecan-4 and FGFR-1 Are Required for Thrombin- and bFGF-induced Migration—In Boyden chamber assays, thrombin- or bFGF-induced migration of SMCs transfected with syndecan-4 siRNA was impaired when compared with the migration of scrambled RNA-treated control cells (Fig. 4A). In contrast, serum-induced migration was not affected by syndecan-4 siRNA. SMCs transfected with FGFR-1 siRNA also migrated less toward thrombin than control cells, and unlike control cells, bFGF-neutralizing antibodies had no inhibitory effect on thrombin-induced migration (Fig. 4B). The migration pattern of untreated SMCs was not different from control cells treated with scrambled RNA matched for either syndecan-4 or FGFR-1 siRNA (data not shown).

Syndecan-4 and FGFR-1 Are Required for Thrombin-induced bFGF-dependent DNA Synthesis—SMCs transfected with syndecan-4 or FGFR-1 siRNA or with matched scrambled RNA were stimulated with thrombin or bFGF. In cells treated with syndecan-4 siRNA, DNA synthesis in response to thrombin or bFGF was reduced (Fig. 5A). A bFGF-neutralizing antibody inhibited thrombin-induced DNA synthesis in control cells but had no inhibitory effect when syndecan-4 expression was reduced by siRNA. Also of interest, inhibition of thrombin-induced DNA synthesis by heparin was only significant in control

![Figure 2](image2.png)

**Figure 2.** Inhibition of syndecan-4 (Syn-4) and FGFR-1 protein expression by siRNA. Syndecan-4 core protein and FGFR-1 were determined by Western blotting as described under “Experimental Procedures”; α-actin in total cell lysates is shown for loading control. A, the effect of 10 nmol/liter siRNA to syndecan-4 on syndecan-4 protein. The blot contains duplicates of each sample. B, the effect of 10 nmol/liter siRNA to syndecan-4 on cell layer-bound HSPGs. HSPGs were isolated and digested with heparitinase as described. HS stubs antibody (anti-HSPGs, clone 3G10) was used to detect HSPG core proteins. A similar pattern with bands of ~35–37, 48, and 70 kDa and some high molecular bands has been described by others (20). The 35–37-kDa band presumably corresponds to syndecan-4 core protein. scr RNA, scrambled RNA. C, the effect of 10 nmol/liter siRNA to FGFR-1 on FGFR-1 protein. Values are mean ± S.E. of 4–5 independent experiments; *, p < 0.05 versus CaHPO$_4$ and scrambled RNA (ANOVA).

![Figure 3](image3.png)

**Figure 3.** Cell spreading, but not adhesion, is impaired in SMCs with reduced syndecan-4 expression. A, SMCs were seeded in serum-free medium on glass slides coated with monomeric collagen. Cells, treated with CaHPO$_4$, alone, with syndecan-4 siRNA (Syn-4 siRNA), or with matched scrambled RNA (scr RNA), were allowed to spread for 1, 3, or 6 h. Pictures are shown at ×200 magnification after fixation and staining. B, SMCs (10,000/well) were seeded in 12-well plates overnight in 15% serum, and cells were washed with phosphate-buffered saline, detached with trypsin, and counted. Values are mean ± S.E. of 4 independent experiments.
FIG. 4. Syndecan-4 and FGFR-1 are required for thrombin- and bFGF-induced migration. SMCs were transfected with siRNA (grey bars) or with matched scrambled RNA (black bars). 48 h after transfection, Boyden chamber assays were performed in 48-well chambers. After 6 h of stimulation, filters were removed from the chambers, and cells on the lower side of the filters were stained. The cell number is shown as -fold over non-stimulated controls.

FIG. 5. Syndecan-4 and FGFR-1 are required for thrombin-induced bFGF-dependent DNA synthesis. SMCs were transfected with siRNA (grey bars) or with matched scrambled RNA (black bars) as described under "Experimental Procedures." A, SMCs transfected with syndecan-4 siRNA or matched scrambled RNA were stimulated with bFGF (1 ng/ml) or thrombin (10 nmol/liter) in the absence or presence of anti-bFGF (α-bFGF, 30 μg/ml), nonspecific IgG (30 μg/ml), or heparin (100 μg/ml). B, SMCs transfected with FGFR-1 siRNA or matched scrambled RNA were stimulated with thrombin (100 ng/ml) or bFGF (1 ng/ml) in the absence or presence of anti-bFGF, IgG, or heparin. Values are mean ± S.E. of 7 (in A) or 4 (in B) independent experiments; *, p < 0.05 for siRNA versus scrambled RNA, #, p < 0.05 for cells pretreated with anti-bFGF or heparin versus thrombin alone (ANOVA).

DISCUSSION

In previous studies, we and others have shown that thrombin-induced migration and proliferation require the activation of a secondary ligand-receptor system (15, 22). In rat SMCs, thrombin transactivates the EGF receptor by releasing heparin-binding EGF-like growth factor (HB-EGF) from the cell surface (15). In contrast, we recently demonstrated that in human SMCs, thrombin does not transactivate the EGF receptor. Instead, thrombin causes rapid release of bFGF into the pericellular matrix with subsequent FGFR-1 phosphorylation, which can be blocked by bFGF-neutralizing antibodies and by heparin (3). In the present study, we have demonstrated that the cell surface heparan sulfate proteoglycan syndecan-4 is required for thrombin-induced mitogenesis and migration by using syndecan-4 siRNA to specifically decrease syndecan-4 core protein (Fig. 2A). We found that human SMCs with reduced syndecan-4 or FGFR-1 migrate and proliferate less in response to thrombin or bFGF (Figs. 4 and 5). The magnitude of this effect is similar to the level of inhibition of thrombin-mediated mitogenesis obtained by treating normal cells with bFGF-neutralizing antibodies (3). In contrast, the bFGF-neutralizing antibody did not alter thrombin- or bFGF-mediated migration and proliferation of FGFR-1 knockdown SMCs. These data indicate that the thrombin-induced bFGF-dependent pathway is mediated by both syndecan-4 and FGFR-1.

Syndecan-4, as well as syndecan-1 and -2 and glypicans, can bind bFGF and increase bFGF-FGFR-1 interactions (5). In addition, syndecan-4 is known to play a unique role in bFGF-dependent signal transduction (9, 13, 23). The cytoplasmic tail of syndecan-4 forms a complex with phosphatidylinositol 4,5-bisphosphate and PKCα, which promotes PKCα activation (6–8). It has been proposed that a serine/threonine protein phosphatase becomes activated after bFGF binds to its tyrosine kinase receptor. This phosphatase dephosphorylates the cytoplasmic tail of syndecan-4, increasing its affinity for phosphatase...
Thrombin Signaling Requires Syndecan-4

Fig. 6. Late phase of thrombin-induced ERK1/2 phosphorylation requires syndecan-4 and FGFR-1. A and B, non-treated SMCs (white bars), SMCs transfected with syndecan-4 siRNA (A) (black bars) or FGFR-1 siRNA (B) (black bars) or with the respective matched scrambled RNA (gray bars) were seeded in 6-well plates overnight and then incubated in serum-free medium for 48 h with a medium change 24 h before stimulation. Cells were stimulated with thrombin (10 nmol/liter) or bFGF (1 mg/ml) for the indicated times and lysed with Laemmli sample buffer. ERK 1/2 phosphorylation was determined with phospho-specific ERK antibody (p-ERK 1/2). Blots were stripped and reprobed with phospho-specific PKC antibody (p-PKCα). Bars represent mean + S.E. of 4 independent experiments and are shown as -fold over non-stimulated controls; *, p < 0.05 for siRNA versus non-treated cells and scrambled RNA (ANOVA).

Dylinositol 4,5-bisphosphate and promoting dimerization (and possibly higher order multimers) of the syndecan-4 cytoplasmic tail. This in turn increases the binding and activation of PKCa (24). It is known that thrombin-induced signaling involves activation of PKC isoforms and translocation of PKCa into focal domains (25). Therefore, since syndecan-4 activates PKCa (6–8), it is possible that thrombin may exert its effects on migration and proliferation, in part, via promoting bFGF and syndecan-4 dependent PKCa activation.

Thrombin also induces signal transduction through the MAPK pathway, and prolonged ERK activation is required for cell cycle progression induced by various mitogens, including thrombin (26). In rat SMCs, the EGF receptor co-stimulatory pathway was required to sustain ERK activity beyond 30 min (15). In human SMCs, we found that this sustained activation of ERK by thrombin required syndecan-4 and FGFR-1 (Fig. 6), in part explaining the role of syndecan-4 and FGFR-1 in thrombin-mediated SMC proliferation (Fig. 5). It would be of interest to further investigate the mechanisms involved in syndecan-4 dependent bFGF-mediated ERK activation.

Our observation of impaired spreading of syndecan-4 siRNA cells on collagen-coated slides (Fig. 3) is in agreement with previous reports that syndecan-4 is involved in cell spreading through the assembly of focal contacts (9, 13). This may explain the reduced migration of these SMCs (Fig. 4). Although we did not directly investigate focal adhesion formation, the observation that syndecan-deficient cells were fully spread by 6 h suggests that human SMCs have sufficient compensatory mechanisms to form focal adhesions. This is consistent with observations using fibroblasts from syndecan-4-deficient animals. Although these cells are able to form normal focal adhesions (13, 27), focal adhesion formation is impaired when the cells are cultured on the cell binding fragment of fibronectin in the presence of culture medium with the heparin-binding fragment of fibronectin (27). Of interest, these syndecan-4-deficient mice develop normally, but when challenged, they exhibit impaired wound healing and decreased angiogenesis in the granulation tissue (28, 29). Thus, the specific role of syndecan-4 for focal adhesion formation, especially in human cells, is uncertain and requires further study.

In summary, we demonstrate a requirement for syndecan-4 and FGFR-1 for thrombin-induced bFGF-dependent migration, mitogenesis, and sustained ERK activation in human vascular SMCs.

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