

Biglycan Intensifies ALK5–Smad2/3 Signaling by TGF- β_1 and Downregulates Syndecan-4 in Cultured Vascular Endothelial Cells

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ABSTRACT

Proteoglycans are macromolecules that consist of a core protein and one or more glycosaminoglycan side chains. A small leucine-rich dermatan sulfate proteoglycan, biglycan, is one of the predominant types of proteoglycans synthesized by vascular endothelial cells; however, the physiological functions of biglycan are not completely understood. In the present study, bovine aortic endothelial cells in culture were transfected with small interfering RNAs for biglycan, and the expression of other proteoglycans was examined. Transforming growth factor- β_1 signaling was also investigated, because the interaction of biglycan with cytokines has been reported. Biglycan was found to form a complex with either transforming growth factor- β_1 or the transforming growth factor- β_1 type I receptor, ALK5, and to intensify the phosphorylation of Smad2/3, resulting in a lower expression of the transmembrane heparan sulfate proteoglycan, syndecan-4. This is the first report to clarify the function of biglycan as a regulatory molecule of the ALK5–Smad2/3 TGF- β_1 signaling pathway that mediates the suppression of syndecan-4 expression in vascular endothelial cells. *J. Cell. Biochem.* 118: 1087–1096, 2017. © 2016 The Authors. *Journal of Cellular Biochemistry* published by Wiley Periodicals, Inc. This is an open access article under the terms of the Creative Commons Attribution–NonCommercial–NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modification or adaptations are made.

KEY WORDS: BIGLYCAN; SYNDECAN-4; TGF- β ; PROTEOGLYCAN; ENDOTHELIAL CELL

Vascular endothelial cells cover the inner surface of blood vessels and are involved in the regulation of the blood coagulation–fibrinolytic system by synthesizing and secreting not only procoagulants, including tissue factor [Maynard et al., 1977] and plasminogen activator inhibitor type 1 [van Mourik et al., 1984], but also anticoagulants, including prostacyclin [Weksler et al., 1977], thrombomodulin [Esmon and Owen, 1981], and tissue plasminogen activator [Levin and Loskutoff, 1982]. The cells also synthesize and secrete anticoagulant proteoglycans, macromolecules that consist of a core protein and one or more glycosaminoglycan side chains [Ruoslahti, 1988]. Vascular endothelial cells express two types of proteoglycans. One type is the heparan sulfate proteoglycans, including a large heparan sulfate

proteoglycan, perlecan [Saku and Furthmayr, 1989]; members of the syndecan family of transmembrane proteoglycans, such as syndecan-1 and syndecan-4 [Kojima et al., 1992]; and the cell-associated proteoglycan, glypican [Mertens et al., 1992]. The other type is the dermatan sulfate proteoglycans, such as small leucine-rich dermatan sulfate proteoglycans, biglycan [Yamamoto et al., 2005] and decorin [Schönherr et al., 1999].

Vascular endothelial proteoglycans have various physiological functions, such as permeability, lipid metabolism, hemostasis, thrombosis, and extracellular assembly [Camejo, 1981; Berenson et al., 1984]. Proteoglycans are also involved in regulating the activity of growth factors and cytokines, such as fibroblast growth factor-2 (FGF-2) and transforming growth factor- β (TGF- β), to

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which some proteoglycans bind. When a monolayer of vascular endothelial cells is injured, FGF-2 leaks from the damaged cells and stimulates the migration and proliferation of cells near the damaged site to repair the monolayer [Rifkin and Moscatelli, 1989]. During repairment of the damaged endothelial monolayer, perlecan promotes the binding of FGF-2 to its receptor [Aviezer et al., 1994]. In addition, the heparan sulfate chains of endothelial heparan sulfate proteoglycans exhibit heparin-like activity and contribute to the anticoagulant properties of vascular endothelial cell monolayers [Mertens et al., 1992]. On the other hand, biglycan and decorin activate heparin cofactor II via the dermatan sulfate chains to inhibit a coagulation factor, thrombin [Whinna et al., 1993], and decorin is bound to TGF- β via the core proteins to inactivate this cytokine in vitro [Hildebrand et al., 1994]. However, the physiological functions of dermatan sulfate proteoglycans, especially biglycan, are not fully understood.

TGF- β is a multifunctional cytokine involved in various vascular events and diseases [Ruiz-Ortega et al., 2007]. The activity of TGF- β_1 is mediated by type I transmembrane serine/threonine kinase receptors that are activated by type II receptors bound to TGF- β_1 [Wrana et al., 1994]. There are two kinds of type I TGF- β receptors: one is activin receptor-like kinase 5 (ALK5), which is expressed in most cell types, and the other is activin receptor-like kinase 1 (ALK1), which is specifically expressed in vascular endothelial cells [Goumans et al., 2002]. In vascular endothelial cells, ALK5 and ALK1 transduce different signals from TGF- β_1 . Cell migration and proliferation are inhibited by the pathway mediated by transcriptional factors Smad2 and Smad3, which are phosphorylated by ALK5, whereas they are stimulated by the pathway mediated by Smad1, Smad5, and Smad8, which are phosphorylated by ALK1 [Derynck et al., 1998; Goumans et al., 2003].

The synthesis of endothelial proteoglycans is regulated by growth factors and cytokines, such as VEGF165 [Kaji et al., 2006] and connective tissue growth factor [Kaji et al., 2004]. TGF- β_1 also regulates endothelial perlecan and biglycan synthesis in a cell density-dependent manner [Kaji et al., 2000]. We hypothesized that biglycan may be involved in regulating the expression of other types of proteoglycans by TGF- β_1 in vascular endothelial cells. These data indicated that biglycan intensifies ALK5–Smad2/3 signaling with TGF- β_1 as a co-receptor and then downregulates the expression of syndecan-4 in cultured vascular endothelial cells.

MATERIALS AND METHODS

MATERIALS

Bovine aortic endothelial cells were obtained from Cell Applications (San Diego, CA). Dulbecco's modified Eagle medium and Ca²⁺- and Mg²⁺-free phosphate-buffered saline were obtained from Nissui Pharmaceutical (Tokyo, Japan). Tissue culture dishes and plates were from Iwaki (Chiba, Japan). Fetal bovine serum was obtained from HyClone Laboratories (Waltham, MA). Recombinant human TGF- β_1 and ALK5 inhibitor LY364947 were purchased from Wako (Osaka, Japan). Chondroitinase ABC (EC 4.2.2.4, derived from *Proteus vulgaris*), heparinase II (derived from *Flavobacterium heparinum*), heparinase III (EC 4.2.2.8, derived from *Flavobacterium heparinum*),

and Diethylaminoethyl–Sephacel (DEAE–Sephacel) were purchased from Sigma–Aldrich (St Louis, MO). Rabbit polyclonal antibodies against TGF- β_1 (V) or ALK5 (V–22), and goat polyclonal antibodies against biglycan (L–15), syndecan–4 (N–19), or ALK1 (C–20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–Smad2/3 (D7G7) and anti–phospho–Smad2/3 (D27F4) rabbit monoclonal antibodies, and horseradish peroxidase (HRP)–conjugated anti–rabbit IgG antibody (#7074) were obtained from Cell Signaling Technology (Beverly, MA). HRP–conjugated anti–goat IgG antibody (ab6885) was obtained from Abcam (Bristol, UK). Lipofectamine RNAiMAX, Lipofectamine LTX, Opti–MEM, and Mammalian Expression System with Gateway Technology were obtained from Invitrogen (Carlsbad, CA). His Mag Sepharose Ni was obtained from GE Healthcare Bio–Sciences AB (Björkgatan, Sweden). Synthetic small interfering RNAs (siRNAs) were purchased from Cosmo Bio Co. (Tokyo, Japan). Other reagents of the highest grade available were from Wako Pure Chemical Industries (Osaka, Japan).

CELL CULTURE AND TREATMENT

Vascular endothelial cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics (5,000 IU/mL penicillin and 5 mg/mL streptomycin) until confluent. The cells were transfected with siRNAs for knockdown of biglycan, TGF- β_1 , ALK1, ALK5, or both biglycan and TGF- β_1 . After this, the expression of syndecan–4 or other proteoglycans was determined by real-time reverse transcription polymerase chain reaction (RT–PCR) or Western blot analysis as described below. In another experiment, cultures of confluent cells were treated with LY364947 or TGF- β_1 and then the expression of syndecan–4 was determined by real-time RT–PCR or Western blot analysis.

siRNA TRANSFECTION

Transient transfection of siRNAs was performed using Lipofectamine RNAiMAX, according to the manufacturer's protocol. Briefly, annealed siRNA duplex and Lipofectamine RNAiMAX were dissolved in Opti–MEM in separate tubes and incubated for 5 min at room temperature. They were then mixed and incubated for 20 min at room temperature. Vascular endothelial cells were grown to about 80% confluence in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and then incubated for 4 h at 37°C in fresh Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and the siRNA/Lipofectamine RNAiMAX mixture. The final concentrations of siRNA and Lipofectamine RNAiMAX were 40 nM and 0.2%, respectively. After that, the medium was changed to Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum alone and incubated further. The sequences of sense and antisense siRNAs were as follows: bovine biglycan siRNA–1 (siBGN–1), 5'–CCAUCCA–GUUUGGCAACUAdTdT–3' (sense), and 5'–UAGUUGCCAAACUG–GAUGGCC–3' (antisense); bovine biglycan siRNA–2 (siBGN–2), 5'–GCUCCGACCGGUCUGAAAdTdT–3' (sense), and 5'–UUCA–GACCCAGGUCGGAGCAC–3' (antisense); bovine TGF- β_1 siRNA (siTGF β_1), 5'–GCGUGCUAAUGGUGGAAUAdTdT–3' (sense), and 5'–UAUCCACCAUAGCAGCGG–3' (antisense); bovine ALK1 siRNA (siALK1), 5'–CCAGCUUUGAGGACAUGAAAdTdT–3' (sense),

and 5'-UUCAUGUCCUCAAAGCUGGGG-3' (antisense); and bovine ALK5 siRNA (siALK5), 5'-CCAUCGAGUGCCAAAUGAAAdTdT-3' (sense) and 5'-UUCAUUUGGCACUCGAUGGUG-3' (antisense). Negative Control siRNA (siCont) (Qiagen, Valencia, CA) was used as a non-specific sequence.

REAL-TIME RT-PCR

Total RNA was extracted using an RNeasy Lipid Tissue Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from the mRNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA). Real-time PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA) with 1 ng/ μ L cDNA and 0.1 μ M primers (Table I) on a StepOnePlus Real-Time PCR System (Applied Biosystems). Levels of biglycan, decorin, perlecan, syndecan-1, syndecan-2, syndecan-3, syndecan-4, glypican-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNAs were quantified by the comparative CT method. The fold changes for each gene were normalized by the intensity value of GAPDH.

PROTEOGLYCAN CORE PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

Proteoglycans that accumulated in the cell layer and conditioned medium of vascular endothelial cells were extracted under dissociative conditions. Specifically, the conditioned medium was harvested and solid urea was added at a concentration of 8 M. The cell layer was washed twice with Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline and lysed with 8 M urea cell extract solution (pH 7.5) containing 120 mM 6-aminohexanoic acid, 12 mM benzamidine, 10 mM N-ethylmaleimide, 2 mM EDTA, 0.1 M phenylmethanesulfonyl fluoride, 0.1 M NaCl, 50 mM Tris base, and 2% Triton X-100. The extracts were applied to DEAE-Sephacel (0.3 mL of resin) columns and washed four times with 0.25 M NaCl 8 M urea buffer (pH 7.5), containing 2 mM EDTA, 0.5% Triton X-100, and 50 mM Tris base. Proteoglycans were eluted with 0.9 mL 3 M urea buffer (pH 7.5) containing 2 mM EDTA, 0.5% Triton X-100, and 50 mM Tris base, and precipitated with 3.5 volumes of 1.3% potassium acetate in 95% ethanol for 2 h at -20°C ; this precipitation step was repeated three times. Precipitated proteoglycans were digested with heparinase II/III in 100 mM Tris-HCl buffer (pH 7.0) containing 10 mM calcium acetate and 18 mM sodium acetate or with chondroitinase ABC in 50 mM Tris-HCl buffer (pH 8.0) containing 1 mg/mL bovine serum

TABLE I. Bovine Gene-Specific Primers for Real-Time Polymerase Chain Reaction

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Biglycan	GCTGCCACTGCCATCTGAG	CGAGGACCAAGGCGTAG
Decorin	CTGCGGTTGACAATGGC	CTCACTCTGAATAAGAAGCC
Perlecan	GCTGAGGGCGTACGATGG	TGCCAGGCGTCGGAAC
Syndecan-1	CAGTCAGGAGACAGCATCAG	CCGACAGACATTCATACC
Syndecan-2	CCAGATGAAGAGGACACAAACG	CCAATAACTCCGCCAGCAA
Syndecan-3	CAAGCAGGCGAGCGTC	GGTGGCAGAGATGAAGTGG
Syndecan-4	TTGCCGTCTTCTCTGTGC	AGGCGTAGAACTCATTGGTGG
Glypican-1	GAAGGTCGGCAGGAAGAG	CCAGGAGCAGCAGAGGA
GAPDH	AACACCCCTAAGATTGTCAGCAA	ACAGTCTTCTGGGTGGCAGTGA

albumin and 3 mM sodium acetate for 3 h at 37°C to determine core proteins of syndecan-4 and biglycan, respectively. Separately, total proteins from vascular endothelial cells were prepared by lysis in sodium dodecyl sulfate (SDS) sample buffer (50 mM Tris-HCl buffer solution containing 2% SDS and 10% glycerol, pH 6.8) followed by incubation at 95°C for 10 min. The protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) before addition of 2-mercaptoethanol and bromophenol blue to the samples. The proteoglycans or cellular proteins were separated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel and then transferred onto a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA, USA) at 2 mA/ cm^2 for 1 h. Membranes were blocked with 5% skim milk in 20 mM Tris-HCl buffer solution (pH 7.5) containing 150 mM NaCl and 0.1% Tween 20, and then incubated overnight with a primary antibody against biglycan, syndecan-4, ALK1, ALK5, Smad2/3, or phosphorylated Smad2/3 at 4°C . The membranes were washed and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence Western blotting detection reagents (Chemi-Lumi One L, Nacalai, Kyoto, Japan) and scanned with a LAS 3000 Imager (Fujifilm, Tokyo, Japan). Representative blots are shown from three independent experiments.

Ni^{2+} PULL-DOWN ASSAY

To prepare $6 \times$ His-tagged biglycan, the pDEST26-BGN (NM_001711.3) plasmid vector was constructed using a Mammalian Expression System with Gateway Technology, and the vector was transfected into vascular endothelial cells with Lipofectamine LTX and PLUS reagent. One microgram per microliter of plasmid vector and PLUS reagent in Opti-MEM and Lipofectamine LTX in Opti-MEM were prepared in separate tubes and mixed; the mixture was then incubated for 5 min at room temperature. Vascular endothelial cells at about 80% confluence were incubated in the mixture for 1 h at 37°C ; the final concentration of the vector, PLUS reagent, and Lipofectamine RNAiMAX were 1.7 $\mu\text{g}/\text{mL}$, 0.17%, and 0.35%, respectively. The pDEST26 vector was used as a control. After incubation, the medium was changed to Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and the cells were further incubated for 24 h. After incubation, proteoglycans that had accumulated in the conditioned medium were extracted, concentrated, and digested with chondroitinase ABC as described above in "Proteoglycan core protein extraction and Western blot analysis." The binding of biglycan to either TGF- β_1 or ALK5 was analyzed by a Ni^{2+} pull-down assay as follows: 40 μL of His Mag Sepharose Ni was equilibrated with a binding/wash buffer, 25 mM Tris-HCl containing 150 mM NaCl, and 5 mM imidazole (pH 7.5). Chondroitinase ABC-digested proteoglycans were mixed with either 500 ng recombinant human TGF- β_1 or 50 μg of membrane proteins extracted from endothelial cells using the ProteoExtract Native Membrane Protein Extraction Kit (Merck KGaA, Darmstadt, Germany) and incubated with the beads for 30 min. After incubation, the beads were washed four times with binding/wash buffer and boiled with elution buffer, 50 mM Tris-HCl containing 8% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.005%

bromophenol blue, and 500 mM imidazole (pH 6.8) at 95°C for 3 min. The supernatant was collected and used to determine complexes of biglycan with TGF- β_1 or ALK5 by Western blot analysis.

STATISTICAL ANALYSIS

Data were tested for statistical significance by analysis of variance and Student's *t*-test or Tukey's method, as appropriate. *P* values of less than 0.05 were considered statistically significant differences.

RESULTS

BIGLYCAN SUPPRESSES SYNDECAN-4 EXPRESSION

To examine the effects of biglycan knockdown on the expression of messenger RNAs (mRNAs) coding for other types of proteoglycans, either a negative control siRNA (siCont) or bovine biglycan siRNA-1 (siBGN-1) was transfected into vascular endothelial cells, and then the mRNA expression levels were analyzed (Fig. 1). Suppression of biglycan mRNA expression resulted in the induction of mRNAs for decorin (1.50-fold), syndecan-1 (1.65-fold), and syndecan-4 (1.90-fold). An siRNA for bovine biglycan, siBGN-2, also induced the expression decorin and syndecan-4 mRNAs, but failed to induce syndecan-1 mRNA expression (data not shown). We have investigated the relationship between the expression of biglycan and that of syndecan-4 in vascular smooth muscle cells and found that siRNA-mediated knockdown of biglycan expression results in a higher expression of syndecan-4 mRNA (Fig. S1). This suggests that biglycan suppression of syndecan-4 expression occurs in not only vascular endothelial cells but also vascular smooth muscle cells. Since the expression level of syndecan-4 in vascular smooth muscle cells was much lower than that in vascular endothelial cells, we could not show the expression of syndecan-4 core protein.

When the cells were transfected with siBGN-1, the level of biglycan mRNA consistently decreased during a 24-h incubation (Fig. 2A, upper panel), while the syndecan-4 mRNA level increased during a 6-h incubation (Fig. 2A, lower panel). Expression of syndecan-4 increased with suppression of biglycan expression

during a 24-h incubation (Fig. 2B). These results suggest that biglycan suppresses the expression of syndecan-4 in vascular endothelial cells.

TGF- β_1 SUPPRESSES THE EXPRESSION OF SYNDECAN-4

We next examined the effects of exogenous TGF- β_1 on the expression of syndecan-4 in vascular endothelial cells (Fig. 3), because an interaction between biglycan and TGF- β_1 has been reported [Hildebrand et al., 1994]. TGF- β_1 elevated the expression levels of syndecan-4 mRNA after a 3-h treatment at 1 and 5 ng/mL or after a 6-h treatment at 5 ng/mL (Fig. 3A). The expression of syndecan-4 increased after a 6-h incubation (Fig. 3B) and then decreased after a 24-h incubation with TGF- β_1 at 5 ng/mL (Fig. 3C). A nonspecific band observed at the top of the images in Figure 3B did not increase and that in Figure 3C did not decrease by TGF- β_1 , suggesting that the cytokine specifically modulates the expression of syndecan-4.

To determine the effects of endogenous TGF- β_1 on endothelial syndecan-4 expression, we assessed the expression of syndecan-4 in vascular endothelial cells in which the expression of TGF- β_1 was suppressed by bovine TGF- β_1 siRNA (siTGFB1). As shown in Figure 4, both siBGN-1 and siBGN-2 suppressed the expression of biglycan mRNA in the presence or absence of siTGFB1. Similarly, siTGFB1 suppressed the expression of TGF- β_1 in the presence or absence of siBGN-1/siBGN-2 (Fig. 4A). The expression of syndecan-4 mRNA was elevated by siBGN-1, siBGN-2, and siTGFB1; such an elevation was also observed at the protein level (Fig. 4B), suggesting that endogenous biglycan and TGF- β_1 each suppress syndecan-4 expression. The effect of siBGN-1/siBGN-2 and siTGFB1 on syndecan-4 expression was additive, indicating that not only endogenous biglycan but also endogenous TGF- β_1 suppresses syndecan-4 expression in vascular endothelial cells.

TGF- β_1 RECEPTOR TYPE-I, ALK5, INHIBITS THE EXPRESSION OF SYNDECAN-4

To identify the TGF- β receptor involved in the suppression of syndecan-4, siALK1, and siALK5 were transfected into vascular

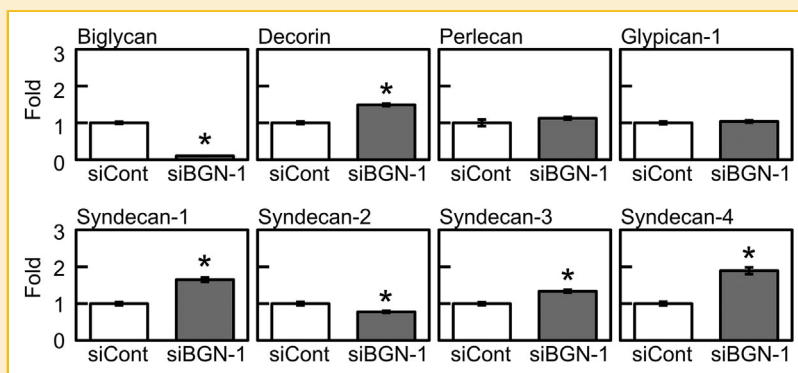


Fig. 1. Effects of siRNA-mediated knockdown of biglycan on the expression of proteoglycan mRNAs in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont or siBGN-1 for 24 h and the expression of biglycan, decorin, perlecan, glypican-1, syndecan-1, syndecan-2, syndecan-3, and syndecan-4 mRNAs was analyzed by real-time RT-PCR. Values are means \pm SE of three independent experiments performed in duplicate. Significantly different from the corresponding siCont, **P* < 0.01.

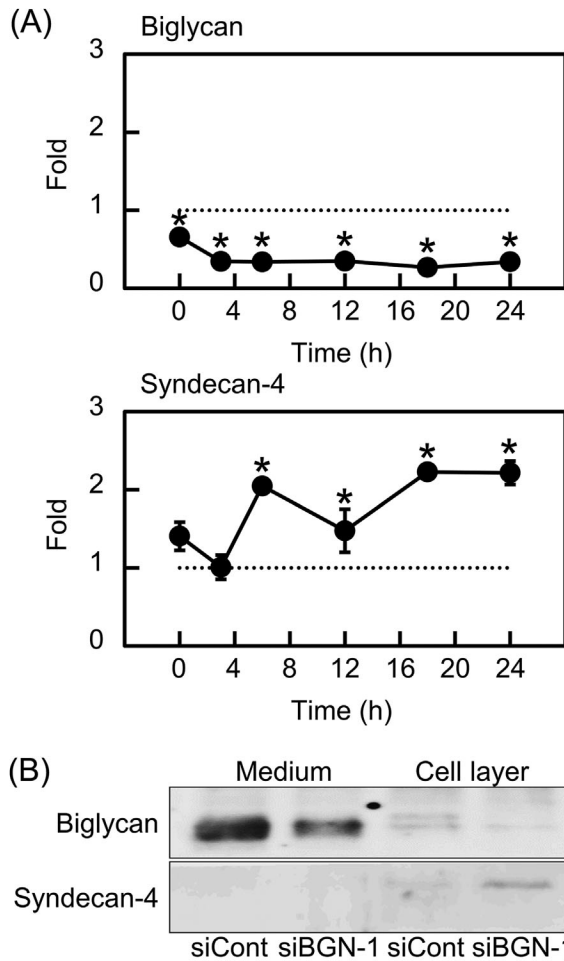


Fig. 2. Effects of siRNA-mediated knockdown of biglycan on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont or siBGN-1 at 37°C for 3, 6, 12, 18, and 24 h and the expression of mRNAs coding for biglycan and syndecan-4 was analyzed. Separately, the cells were transfected with siCont or siBGN-1 for 24 h and the expression of syndecan-4 core protein was determined. (A) Expression of biglycan (upper panel) and syndecan-4 (lower panel) mRNAs. Vascular endothelial cells transfected with siRNAs for 4 h and then incubated in a fresh medium. The time in (A) indicates the incubation time after transfection. The mRNA expression was normalized to the corresponding GAPDH mRNA level and presented as the fold induction relative to the corresponding control (siCont treatment). Values are means \pm SE of three experiments performed in duplicate. Significantly different from the corresponding siCont, * $P < 0.01$. (B) Accumulation of biglycan and syndecan-4 core proteins in the conditioned medium and the cell layer transfected with siCont or siBGN-1 for 24 h.

endothelial cells and the expression of syndecan-4 was evaluated (Fig. 5). The expression of ALK1 and ALK5 was suppressed by siALK1 and siALK5, respectively (Fig. 5A and B, left and middle panels), but was not influenced by siALK5 and siALK1 (data not shown). Under such conditions, the expression of syndecan-4 mRNA was elevated by either siALK1 or siALK5 (Fig. 5A, right panel); however, the expression of syndecan-4 core protein was increased by siALK5 but not by siALK1 (Fig. 5B), suggesting that ALK5 is involved predominantly in negative regulation of syndecan-4 expression

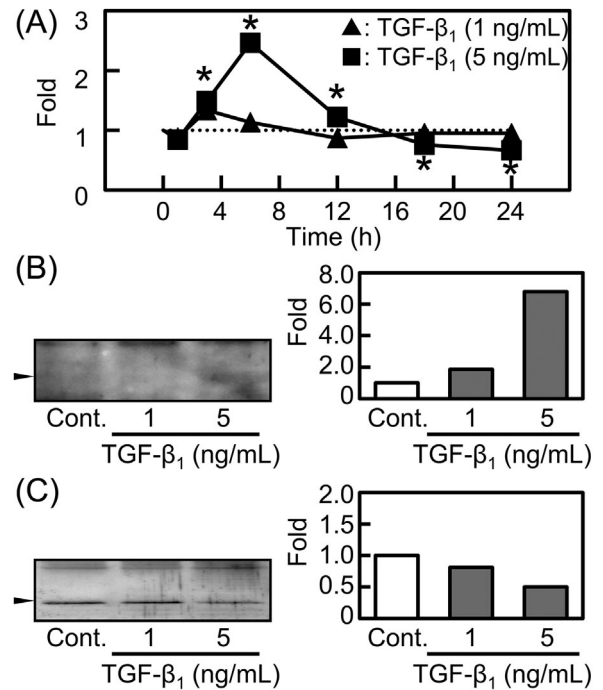


Fig. 3. Effects of TGF- β_1 on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were treated with TGF- β_1 at 1 (\blacktriangle) and 5 (\blacksquare) ng/mL at 37°C for 1, 3, 6, 12, 18, or 24 h. (A) Expression of syndecan-4 mRNA. The mRNA expression was normalized to the corresponding GAPDH mRNA level and presented as the fold induction relative to the corresponding control (siCont treatment). Values are means \pm SE of two experiments performed in duplicate. Significantly different from the corresponding siCont, * $P < 0.01$. (B) Accumulation of syndecan-4 core protein in the cell layer treated with TGF- β_1 at 1 or 5 ng/mL for 6 h. Western blot analysis (left panel) and its quantitative analysis (right panel). Arrow head in the left panel indicates the position of syndecan-4. (C) Accumulation of syndecan-4 core protein in the cell layer treated with TGF- β_1 at 1 or 5 ng/mL for 24 h. Western blot analysis (left panel) and its quantitative analysis (right panel). Arrow head in the left panel indicates the position of syndecan-4.

by endogenous TGF- β_1 . In fact, a selective inhibitor of ALK5, LY364947, increased the expression of syndecan-4 at both the mRNA and protein levels (Fig. 5C and D).

BIGLYCAN CORE PROTEIN IS BOUND TO EITHER TGF- β_1 OR ALK5

Since the data suggested that TGF- β_1 -ALK5 signaling negatively regulates the expression of syndecan-4 in vascular endothelial cells, we analyzed the interaction of biglycan with either TGF- β_1 or ALK5. As shown in Figure 6A, biglycan core protein appeared after digestion with chondroitinase ABC, suggesting that the cells synthesized biglycan as a proteoglycan molecule that bears chondroitin/dermatan sulfate chains. The band of TGF- β_1 that had been pulled down by the His-tagged biglycan core protein was detected when recombinant human TGF- β_1 reacted with biglycan core protein after digestion with chondroitinase ABC, suggesting that TGF- β_1 is bound to biglycan core protein, as previously reported

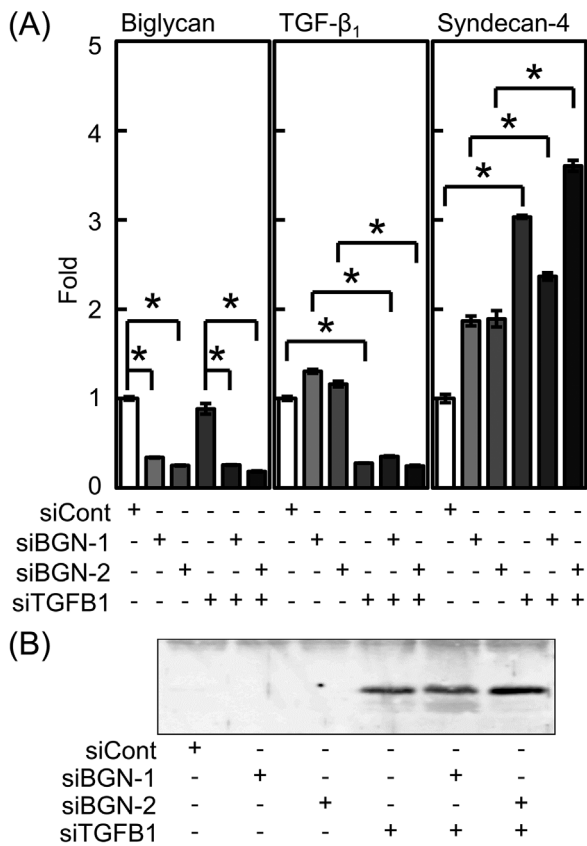


Fig. 4. Effects of siRNA-mediated knockdown of biglycan or TGF- β_1 or both on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont, siBGN-1, or siBGN-2 combined with or without siTGF β_1 at 37°C for 24 h. (A) Expression of biglycan (left panel), TGF- β_1 (middle panel), and syndecan-4 (right panel) mRNAs. Values are means \pm SE of three experiments performed in duplicate. Significantly different from the corresponding siCont or siTGF β_1 , * P < 0.01. (B) Accumulation of syndecan-4 core protein in the cell layer transfected with siCont, siBGN-1, and siBGN-2 combined with or without siTGF β_1 for 24 h.

by Hildebrand et al. [1994]. Figure 6B shows the interaction of biglycan core protein with ALK5. The ALK5 band was identified by Western blot analysis as an immunoreactive band that was decreased by siRNA-mediated knockdown. ALK5 was increased only when the receptor reacted with the His-tagged biglycan core protein obtained from the conditioned medium of the cells and prepared by digestion with chondroitinase ABC. This suggests that ALK5 is also bound to biglycan core protein. Taken together, these results suggest that the biglycan core protein potentiates TGF- β_1 -ALK5 signaling by binding to both TGF- β_1 and ALK5.

BIGLYCAN ENHANCES PHOSPHORYLATION OF Smad2/3

TGF- β_1 is bound to ALK5 and phosphorylates the Smad2/3 C-terminus, which transduces the TGF- β_1 signal to the nucleus. The effect of siBGN-1/2 on the phosphorylation of Smad2/3 was examined to clarify the significance of the formation of a complex of biglycan with TGF- β_1 and ALK5. Although Smad2/3

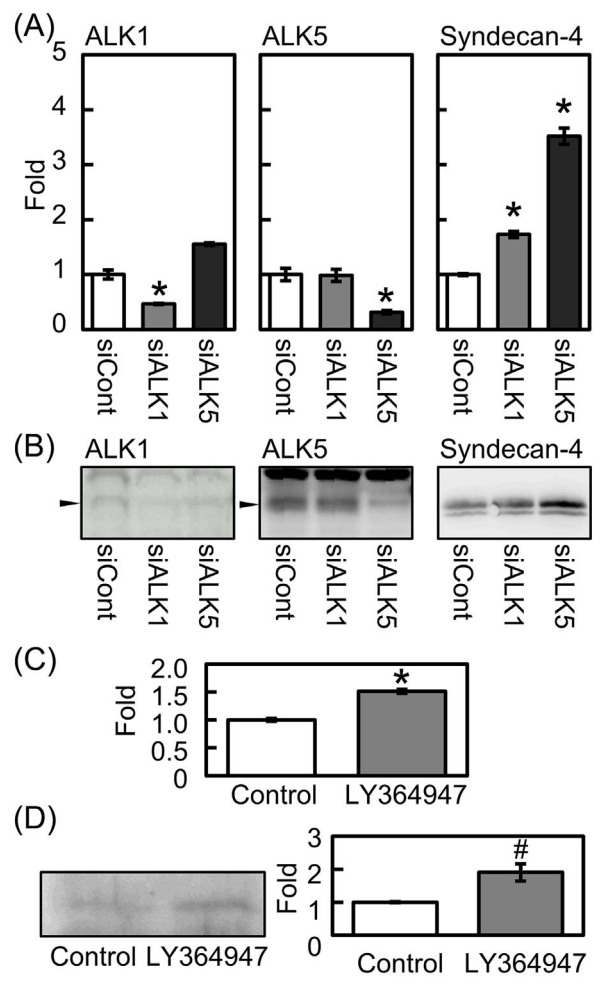


Fig. 5. Effects of siRNA-mediated knockdown of ALK1 and ALK5 and an ALK5 inhibitor, LY364947, on the expression of syndecan-4 in vascular endothelial cells. Bovine aortic endothelial cells were transfected with siCont, siALK1, or siALK5 at 37°C for 24 h (ALK1 and ALK5) or 36 h (syndecan-4). Separately, the cells were treated with an ALK5 inhibitor LY364947 at 1 μ M at 37°C for 24 h. (A) Expression of ALK1 (right panel), ALK5 (middle panel), and syndecan-4 (right panel) mRNAs. Arrow heads indicate the position of ALK1 (left panel) or ALK5 (center panel). Values are means \pm SE of three experiments performed in duplicate. Significantly different from the corresponding siCont, * P < 0.01. (B) Expression of ALK1 (right panel), ALK5 (middle panel), and syndecan-4 (right panel) proteins. (C) Expression of syndecan-4 mRNA. Values are means \pm SE of three experiments performed in duplicate. Significantly different from the control, * P < 0.01. (D) Expression of syndecan-4 core protein in the cell layer. Western blot analysis (left panel) and its quantitative analysis (right panel). Values are means \pm SE of three experiments performed in duplicate (right panel). Significantly different from the control, # P < 0.05.

phosphorylation was observed when the cells were treated with TGF- β_1 , phosphorylation was not detected in either siCont- or siBGN-1/2-transfected cells in the absence of TGF- β_1 . When the cells were stimulated with TGF- β_1 at 1 ng/mL for 30 min, Smad2/3 phosphorylation was detected. This phosphorylation decreased with siBGN-1/2 (Fig. 7), indicating that biglycan potentiates TGF- β_1 -ALK5 signal transduction by activating Smad2/3 phosphorylation.

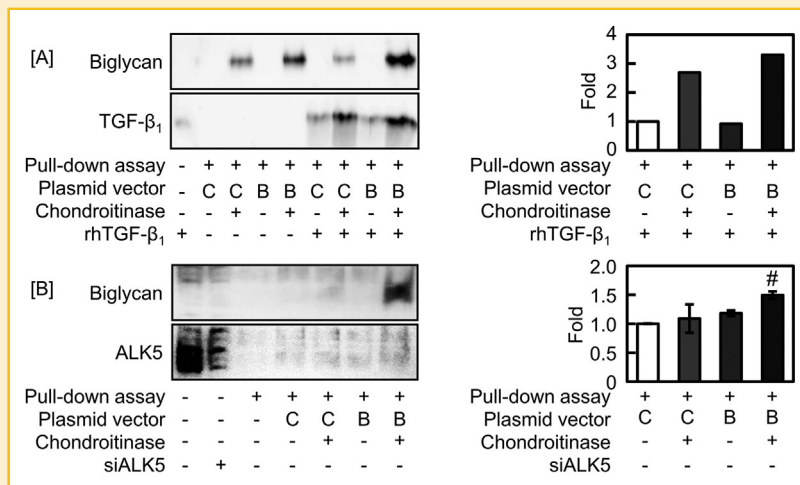


Fig. 6. Binding of biglycan core protein to TGF-β₁ and ALK5. **[A]** Binding of endothelial biglycan core protein to TGF-β₁. Bovine aortic endothelial cells transfected with the pDEST26 (C) or pDEST26-BGN (B) plasmid vector were incubated at 37°C for 24 h to obtain 6 × His-tagged biglycan. The conditioned medium was concentrated and dermatan sulfate chains of biglycan were digested with chondroitinase ABC (+, digested; -, not digested). The biglycan core proteins were subjected to a Ni²⁺ pull-down assay (+, performed; -, not performed) with (+) or without (-) recombinant human TGF-β₁ (rhTGF-β₁). Western blot analysis (left panel) and its quantitative analysis (right panel). **[B]** Binding of endothelial biglycan core protein to ALK5. Bovine aortic endothelial cells were transfected with siCont at 37°C for 24 h, and the membrane proteins were extracted. Separately, the cells were transfected with the pDEST26 (C) or pDEST26-BGN (B) plasmid vector to obtain 6 × His-tagged biglycan. The conditioned medium was concentrated and dermatan sulfate chains of biglycan were digested with chondroitinase ABC (+, digested; -, not digested). The membrane proteins and the biglycan core proteins were subjected to a Ni²⁺ pull-down assay (+, performed; -, not performed). The position of ALK5 was confirmed by siRNA-mediated knockdown of ALK5 (+, siALK5; -, siCont). Western blot analysis (left panels) and its quantitative analysis (right panels). Values are means ± SE of three experiments performed in duplicate. Significantly different from without chondroitinase ABC, #*P* < 0.05.

DISCUSSION

The physiological functions of biglycan in vascular endothelial cells are not fully understood. The present data, however, indicate that (1) the expression of endothelial biglycan induces lower levels of syndecan-4 expression, (2) both exogenous and endogenous TGF-β₁ downregulate syndecan-4 expression, (3) syndecan-4 downregulation is mediated by TGF-β₁-ALK5 signaling, (4) biglycan is bound to both TGF-β₁ and ALK5, and (5) biglycan potentiates the phosphorylation of Smad2/3, which is induced by TGF-β₁. Taken together, these results indicate that biglycan acts as a co-receptor in the TGF-β₁-ALK5-Smad2/3 system and is involved in the downregulation of syndecan-4 expression in vascular endothelial cells. Biglycan has

been reported to bind not only TGF-β₁ [Hildebrand et al., 1994] but also other cytokines such as tumor necrosis factor-α [Tufvesson and Westergren-Thorsson, 2002] and receptors [Schaefer et al., 2005]. The present study, for the first time, revealed that biglycan serves as a co-receptor in TGF-β signaling and this signaling downregulates the expression of syndecan-4 in vascular endothelial cells.

Biglycan and TGF-β₁ were shown to suppress the expression of syndecan-4 via the TGF-β₁-ALK5 signaling pathway. Activation of the TGF-β₁-ALK5 pathway induces phosphorylation of not only Smad2/3, part of the canonical Smad pathway, but also ERK1/2, JNK1/2/3, and p38 MAPK in a non-Smad pathway. Although the involvement of the non-Smad pathway in the downregulation of endothelial syndecan-4 expression cannot be excluded, the present results indicate that the Smad pathway is certainly involved in this downregulation. There are previous examples of phosphorylated Smads inhibiting gene expression through the formation of complexes with transcription factors E2F4 and DP1 to stabilize the TGF-β inhibitory element (TIE) [Chen et al., 2002; Suzuki et al., 2004], which includes a TTGG sequence [Kerr et al., 1990; Chen et al., 2001]. There are consensus TTGG sequences about -1.5 kb from the promoter regions of the syndecan-4 gene in humans, mice, and bovines, according to NCBI data. These TTGG sequences may serve as TIE elements, and some of them are involved in the downregulation of endothelial syndecan-4 expression.

The syndecan family is a group of transmembrane-type heparan sulfate proteoglycans. In this family, syndecan-4 is a type of syndecan that is essential for focal adhesion; the core protein of syndecan-4 forms focal adhesions and stress fibers in fibronectin

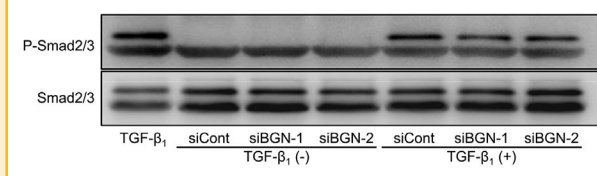


Fig. 7. Effects of siRNA-mediated knockdown of biglycan on the phosphorylation of Smad2/3 by TGF-β₁ in vascular endothelial cells. Bovine aortic endothelial cells were transfected with or without siCont, siBGN-1, or siBGN-2 at 37°C for 24 h and then incubated with or without TGF-β₁ at 1 ng/mL for 30 min.

substrates [Woods and Couchman, 2001]. In fact, degradation of cell surface heparan sulfate chains with heparinase weakens cell-fibronectin adhesion and reduces focal adhesions in vascular endothelial cells [Moon et al., 2005]. Heparan sulfate chains of syndecan-4 promote the construction of larger focal adhesions [Gopal et al., 2010]. Syndecan-4 is required for the alignment of vascular endothelial cells along the luminal surface of normal blood vessels, and a syndecan-4 deficiency results in the activation of atherosclerotic plaques in *ldlr*^{-/-} and *ApoB100/100* mice fed high-fat diets [Baeyens et al., 2014]. In addition, an increase in dermatan sulfate chains together with a decrease in heparan sulfate chains is observed in atherosclerotic intima [Stevens et al., 1976]. Excess biglycan activates TGF- β_1 -ALK5 signaling and reduces syndecan-4, leading to a disturbance in the normal structure of the extracellular matrix in a vascular endothelial monolayer, which may weaken the barrier function of these cells. Therefore, it is suggested that increased biglycan downregulates endothelial syndecan-4 expression, and consequently affects the adhesion of vascular endothelial cells to the extracellular matrix in atherosclerotic vascular tissue, promoting lesions. Since TGF- β_1 induces the synthesis of biglycan with elongated dermatan sulfate chains in vascular endothelial cells [Kaji et al., 2000], and since TGF- β_1 -induced biglycan synthesis in aortic cells is involved in the progression of atherosclerosis [Tang et al., 2013] via lipid accumulation in the vascular wall [Hayashi et al., 2012], an assumption can be made that TGF- β_1 and biglycan participate in a positive feedback loop to enhance endothelial biglycan expression and contribute to the accumulation of lipids in the atherosclerotic vascular wall.

Neovascularization and atherosclerosis progression are interrelated. Plaque neovascularization mainly occurs in the ruptured site of atherosclerotic lesions [Moreno et al., 2004], and the inhibition of neovascularization reduces the progression of advanced atherosclerosis [Moulton et al., 2003]. In addition, cultured human umbilical vein endothelial cells highly express ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs 4), an extracellular matrix metalloproteinase that digests biglycan during tube formation in the collagen gel [Melching et al., 2006]. At that time, the expression of biglycan is reduced [Obika et al., 2014], suggesting that ADAMTS-4 and its substrate biglycan are involved in angiogenesis by vascular endothelial cells. On the other hand, it has been reported that TGF- β can suppress the expression of ADAMTS-4 [Salter et al., 2011; Ashlin et al., 2013; Wang et al., 2013]. Furthermore, the present data showed that decreased biglycan expression inhibits the TGF- β signaling pathway, which can suppress ADAMTS-4 expression. Therefore, it is postulated that the TGF- β signaling pathway, which was reduced by lower expression of biglycan, mediates higher expression of ADAMTS-4 in vascular endothelial cells during angiogenesis. This positive feedback loop by biglycan, the TGF- β signaling, and ADAMTS-4 may be involved in angiogenesis by vascular endothelial cells in atherosclerotic lesions. The involvement of syndecan-4 in angiogenesis, which is increased by the inhibition of the TGF- β signaling pathway, remains to be elucidated.

Cellular proliferation is promoted by decreasing syndecan-4 in vascular smooth muscle cells in atherosclerosis-susceptible

pigeons [Bortoff and Wagner, 2005]. On the other hand, the effect of biglycan on proliferation depends on the cell type. For example, biglycan promotes the proliferation of vascular smooth muscle cells but not of vascular endothelial cells [Shimizu-Hirota et al., 2004]. Biglycan activates TGF- β_1 -ALK5 signaling as a co-receptor as shown in this study. Since ALK5 is ubiquitously expressed in most cell types, including vascular endothelial cells, we postulate that biglycan potentiates TGF- β_1 signaling in vascular endothelial cells as well as other cell types, although the regulation that results may depend on the cell type. Interrelated regulatory mechanisms in the synthesis of different types of proteoglycans have been suggested [Tang et al., 2014]; however, little was known about these mechanisms. The present report is the first to reveal the molecular mechanism by which the expression of one proteoglycan type influences that of another proteoglycan type. The present study provides a partial molecular explanation for the histopathological studies on atherosclerosis that show the abundance of proteoglycans varies depending on the progression of atherosclerosis. In addition, the present data suggest the significance of an excess accumulation of biglycan in atherosclerotic vascular walls. Specifically, biglycan contributes not only to the change in proteoglycan types expressed during the progression of atherosclerosis, but also to the accumulation of low-density lipoprotein that increases with biglycan [Evanko et al., 1998; O'Brien et al., 1998]. There may be crosstalk among different types of proteoglycans in vascular endothelial and smooth muscle cells. Clarification of this system is essential to understanding the complex histopathological changes associated with variation in the proteoglycan types in atherosclerotic vascular walls.

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