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Insulin-like growth factor binding protein-7 (IGFBP7) blocks vascular endothelial cell growth factor (VEGF)-induced angiogenesis in human vascular endothelial cells

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ABSTRACT

Insulin-like growth factor binding protein-7 (IGFBP7) and vascular endothelial growth factor (VEGF) are expressed in vascular endothelial cells in several tumor types. In this study, we examined the effect of IGFBP7 on VEGF-induced tube formation in cultured human umbilical vein endothelial cells (HUVECs) and its potential action in the modulation of VEGF signaling in vascular cells. IGFBP7 treatment suppressed VEGF-induced tube formation, proliferation, and the phosphorylation of mitogen-activated protein kinase kinase (MEK) and extracellular signal-regulated kinase (ERK) 1/2 in HUVECs. IGFBP7 attenuated VEGF-enhanced cyclooxygenase (COX)-2 and VEGF mRNA expression, and prostaglandin E_2 secretion. Knocking down endogenous IGFBP7 enhanced COX-2 and VEGF mRNA expression. A significant increase in IGFBP7-induced caspases was not observed in the presence of VEGF. These findings indicate that IGFBP7 can modulate the stimulatory effect of VEGF on angiogenesis by interfering with VEGF expression as well as VEGF signaling and not by inducing apoptosis.

1. Introduction

Angiogenesis is the development of new microvessels from preexisting capillaries and post capillary venules (Carmeliet, 2003; Folkman and D'Amore, 1996). Angiogenesis is seen in pathological conditions such as malignancy and chronic inflammatory disorders including rheumatoid arthritis and diabetic retinopathy, as well as physiological processes during wound healing, fetal growth and development, and the cyclic changes of the female reproductive tract. In response to angiogenic stimuli, endothelial cells degrade the extracellular matrix, migrate into the perivascular space, proliferate, and align themselves into new blood vessels. Vascular endothelial growth factor (VEGF) is essential for endothelial proliferation and migration resulting in both physiological and pathological angiogenesis (Neufeld et al., 1999). Endothelial cells, which are derived from vascular cells near tumor or endothelial progenitor cells in the vessels, form continuously vascular network in cancer tissues under the environment which angiogenic factors, especially VEGF and angiopoietin-2 commit intentionally (Marcus et al., 1978). In addition, endothelial cells produce prostaglandins in response to various stimuli (Camacho et al., 1998), and prostaglandins, particularly PGE_2, promotes angiogenesis (Leahy et al., 2000; Salcedo et al., 2003). Several inhibitory factors for angiogenesis including thrombospondin-1 (TSP-1), angiostatin, and vasohibin have been identified and shown to play pivotal roles in the regulation of angiogenesis in a coordinated manner (Carmeliet, 2003).

Insulin-like growth factor-binding protein-7 (IGFBP7) is a secreted 31 kDa protein which is also called IGFBP-related protein 1 (IGFBP-rP1), mac25, TAF, and angiomodulin (Hwa et al., 1999). IGFBP7 is one of the IGFBP-related proteins (IGFBP-rP) that exhibits a low affinity for IGF; however, it shares high homology with the IGFBPs and binds IGF-I and insulin, but its binding affinity for IGF-I is lower than those of IGFBP-1 to -6 (Collet and Candy, 1998). This protein is expressed in endothelial cells together with type IV collagen and accumulates in the capillary-like tubes of vascular endothelial cells *in vitro* (Akaogi et al., 1996). IGFBP7 was also found to be abundant in the female reproductive organs such as ovarian blood vessels in the follicular wall (Wandji et al., 2000), the corpus luteum (Casey et al., 2004), and the human uterus (Dominguez et al., 2003; Kutsukake et al., 2007). Interestingly, IGFBP7 is highly elevated in tumor-associated endothelium relative to normal blood vessels (Akaogi et al., 1996; Croix et al., 2000; Pen et al., 2007); however, the nature of this difference still remains unknown. It has been reported that IGFBP7 is an inducible marker for activated endothelial cells and it has been shown to stimulate endothelial production of PGI_2 (Usui et al., 2002). In this study we sought to determine whether IGFBP7 affects VEGF-stimulated formation of new blood vessels and the gene expression associated with vascular prostaglandin metabolism in

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humans. In order to achieve this we have focused our investigation on the effect of IGFBP7 on *in vitro* tubular formation by normal endothelial cells.

2. Materials and methods

2.1. Reagents

MCDB131 medium and endothelial cell growth supplement (ECGS) were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human IGFBP7 and VEGF165 were obtained from R&D Systems, Inc. (Minneapolis, MN). Growth factor-reduced Matrigel (BD Biosciences, MA) in 24-well plates was gelatinated at 37 °C in a CO_2 incubator for 30 min before the Matrigel assay. WST-1 reagent (Cell Counting Kit, Dojindo, Tokyo) was used for evaluating the proliferation of HUVECs. Poly(A)$^+$ RNA was isolated using a QuickPrep micro mRNA purification kit (GE Healthcare, Buckinghamshire, UK). The phospho-extracellular signal-regulated kinase (Erk) 1/2 pathway sampler kit and anti-mitogen-activated protein kinase kinase (MEK) 1/2 antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Apoptosis-related protein antibodies (Caspase-2/7, DFF45) and the caspase-Glo 3/7 assay kit were obtained from BD Biosciences and Promega Co. (Madison, MI), respectively. IGFBP7-specific small interfering RNA (siRNA) (siGENOME SMARTpool reagent) was synthesized by Darmacon, Inc. (Chicago, IL), and the irrelevant control siRNA (sc-37007) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2. Endothelial cell culture

HUVECs (Cell Systems, Kirkland, WA) were grown on a 0.1% gelatin-coated dish in MCDB131 medium containing 10% fetal bovine serum (FBS, JRH Biosciences, ACSL, Lenexa, MD), ECGS (50 μg/ml), and antibiotics (the passage medium), as described previously (Tamura et al., 2006). Experiments were performed using subcultured cells between the third and sixth *in vitro* passages. Cells were treated with VEGF and/or IGFBP7 or IGFBP7 siRNA in serum-free MCDB131 medium without ECGS.

2.3. In vitro endothelial tubular formation on Matrigel

HUVECs were resuspended at a density of 4×10^4 cells/ml in serum-free MCDB131 medium without ECGS, and seeded in gelatinated Matrigel. In order to examine the effect of VEGF and/or IGFBP7 on tubular formation in HUVECs, cells were treated for 24 h with various doses of IGFBP7 in the presence or absence of VEGF (10 ng/ml). In the second experiment, HUVECs that were pretreated for 6 h with IGFBP7 (160 ng/ml) were seeded onto the Matrigel and then incubated for 24 h with VEGF. Culture plates were photographed and the formation of tubular structures was analyzed. HUVECs were fixed in 4% paraformaldehyde for 15 min at room temperature in order to allow the number of junctions or joint-forming cell–cell networks and the total length of tube-like cells to be analyzed. The Matrigel was dehydrated at −20 °C with 75% ethanol for 1 h and then with 95% ethanol for 3 min at room temperature. Cells were stained with 0.1% trypan blue for 2 min and were then washed twice with phosphate-buffered saline. Plates were photographed and the number of junctions and the total length of the tubular endothelial cells were measured using KSW-500U software (Kurabo, Tokyo). The results are expressed as the means of each parameter/three fields at ×10 original magnification. The experiments for tubular formation were repeated at least three times.

2.4. Cell proliferation assay

To determine the effect of IGFBP7 on the proliferation of HUVECs, changes in cell number after IGFBP7 treatment were monitored using a proliferation bioassay. HUVECs (3×10^3 cells) were seeded in 48-well culture plates in serum-free MCDB medium without ECGS and were treated with IGFBP7 in the presence or absence of VEGF (10 ng/ml) for 24 h before the evaluation of cell proliferation. The proliferation bioassay was performed by incubating the cells with WST-1 for 20 min at 37 °C. The staining intensity was determined by measuring the absorbance at 450 nm with a microtiter plate reader. The data are expressed as ratios of the control value.

2.5. Western blot analysis

Subconfluent HUVECs were treated with IGFBP7 in serum-free MCDB medium without ECGS in the presence or absence of VEGF. After 20 min, cells were lysed with Chaps Cell Extract Buffer (Cell Signaling Technology, Inc.) and the lysates were prepared for immunoblotting. Samples were subjected to a 10–20% gradient SDS-polyacrylamide gel (PAG) (Daiichi Pure Chemicals Co., Ltd., Tokyo) electrophoresis and were then electrotransferred onto PVDF membranes (Millipore, Bedford, MA). As specified, the membranes were incubated with rabbit anti-human phospho-c-Raf antibody, phospho-MEK1/2 antibody, phospho-p42/44 MAPK (Erk-1/2) antibody (1:1000) from a phospho-Erk1/2 pathway kit, and anti-human MEK1/2 antibody (1:1000). To detect changes in the levels of apoptosis-related molecules, the cells were lysed with Chaps Cell Extract Buffer 6 or 12 h after treatment of the cells with VEGF and/or IGFBP7. The cell lysates were subjected to SDS-PAGE, and blotting membranes were prepared as described above. The membranes were incubated with primary antibodies against Caspase-2, -7, and DFF-45. After the detection of target molecules, the same membranes were incubated in stripping solution [75 mM Tris–HCl (pH 6.7) containing 2% (w/v) SDS and 0.7% (v/v) β-mercaptoethanol] and were reprobed for β-actin as a loading control using a mouse monoclonal anti-β-actin antibody (1:10,000; Sigma-Aldrich). Goat anti-rabbit or anti-mouse IgG (Vector Lab. Inc., Burlingame, CA) (0.5 μg/ml) conjugated with horseradish peroxidase served as the secondary antibody for each analysis. The immunoreactive bands were detected by enhanced chemiluminescence (PerkinElmer Life Science, Wellesley, MA). All blotting experiments were repeated at least twice and representative data are shown.

2.6. Measurement of PGE_2 in culture media

HUVECs were cultured in 12-well tissue culture plates and were treated with IGFBP7 in the presence or absence of VEGF for 24 h before the cell culture medium was collected. The levels of PGE_2 in the culture media were determined using the Prostaglandin E_2 Enzyme Immunoassay Kit (Assay Designs, Inc., Ann Arbor, MI), as described previously (Sakurai et al., 2004).

2.7. RT-PCR for the VEGF receptors, VEGF, and cyclooxygenase (COX)-2

Subconfluent HUVECs were prepared in a 6-well culture plate in serum-free MCDB medium and were treated for 2 h with various doses of IGFBP7. Poly (A)$^+$ RNA (0.1 μg) was subjected to RT-PCR using specific primer pairs and the one step RNA PCR Kit (AMV, TaKaRa), according to the manufacturer's instructions. The sequences of the primers for COX-2 were 5′-TTCAAATGAGATTGT GGGAAA ATTGCT-3′ (sense) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (antisense). The primers for human VEGF were 5′-ATGAACTTTCTGTCTTGGG-3′ (sense) and 5′-CACCGCCTCGGCTTGTCA CAT-3′ (antisense). The predicted lengths of the fragments were 304 bp for COX-2, 442 bp for VEGF-121, and 574 bp for VEGF-165, 646 bp. The primers for the VEGF receptors (VEGFR-1 and VEGFR-2) were 5′-AGGAGAGGACCT-GAAACTGTC TT-3′ (sense) and 5′-ATTCCTGGCTCTGCAGGCATAG-3′ (antisense), and 5′-GTGATTGCCATGTTCTTCTGGC-3′ (sense) and

5′-TCAGACA TGAGAGCTCGATGCT-3′ (antisense), respectively. The predicted lengths of the fragments were 113 bp for VEGFR-1 and 337 bp for VEGFR-2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. The primer sequences for GAPDH were 5′-ACCACAGTCCATGCCAT CAC-3′ (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (antisense) and the expected size of the amplified product was 452 bp. The PCR protocols used were as follows: 28 cycles with annealing at 57 °C (COX-2), 32 cycles with annealing at 60 °C (VEGF), and 30 cycles with annealing at 61 °C (VEGFR-1 and VEGFR-2). The PCR products were separated on a 1.5%-agarose gel containing ethidium bromide and were visualized with UV light.

2.8. Small interfering RNA transfection

Transfection with IGFBP7 siRNA (10 pmol/24-well culture plate) and irrelevant control siRNA was performed by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. HUVECs at 50% confluency were treated with siRNA for 24 h in MCDB131 medium lacking serum and antibodies. Inhibition of

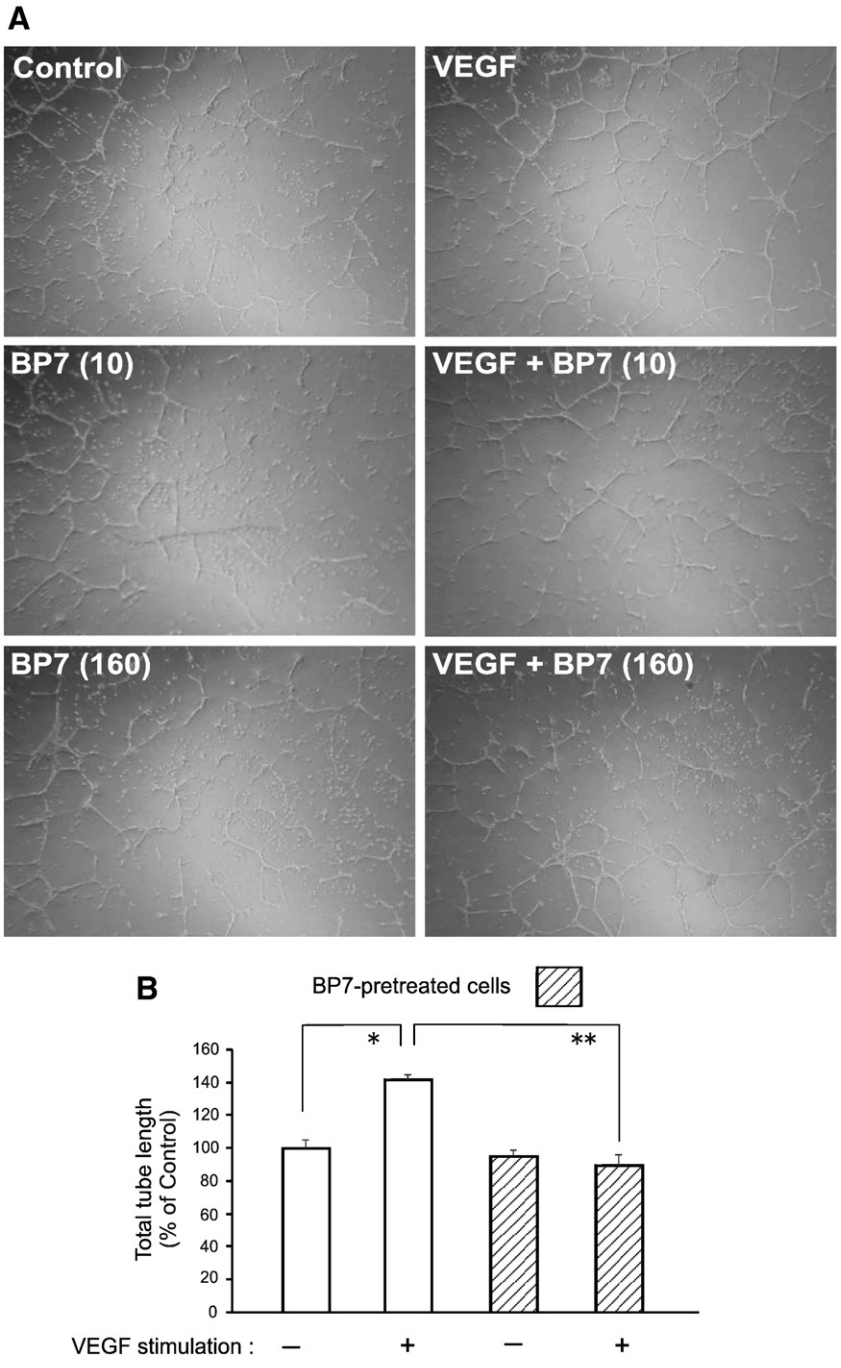

Fig. 1. Representative photograph of *in vitro* angiogenesis of human umbilical vein endothelial cells (HUVECs) in the presence of VEGF and/or IGFBP7 (A) and the effect of IGFBP7 pre-treatment on VEGF-induced tubular formation (B). (A) HUVECs were plated on Matrigel and treated for 24 h with VEGF (10 ng/ml) and/or IGFBP7 (10 or 160 ng/ml). Tube formation was evaluated as described in Table 1. Original magnification ×40. (B) HUVECs were pre-incubated for 6 h with IGFBP7 (160 ng/ml). Cells that had been seeded onto the Matrigel were cultured in the absence or presence of VEGF (10 ng/ml) for 24 h. Tubular formation was measured by measuring the total tube lengths in three randomly selected fields and the data were expressed as ratios of the control value. The results are expressed as means +/− S.E.M. *$P<0.05$, **$P<0.01$ BP7; IGFBP7, figure in parentheses; ng/ml of IGFBP7.

Table 1
Effects of IGFBP7 on VEGF-stimulated tube formation in HUVECs.

Group	The number of junctions		Total tube length % of control	
Control	100.0 ± 5.2	b	100.0 ± 4.1	b
BP7 (10 ng/ml)	102.8 ± 7.3		101.4 ± 6.4	
BP7 (160 ng/ml)	98.9 ± 7.4		98.9 ± 7.4	
VEGF (10 ng/ml)	131.6 ± 19.4		122.0 ± 3.5	
VEGF + BP7 (10 ng/ml)	117.2 ± 9.7	a	105.7 ± 1.8	a
VEGF + BP7 (160 ng/ml)	86.4 ± 8.4		95.8 ± 1.4	

HUVECs were grown on a Matrigel and treated as described in the legend to Fig. 1A. Tube formation was determined by counting the number of connected cells (junctions), and the total tube length in three randomly selected fields. The average of the values obtained in the three fields was calculated for each experiment. Each value is the mean +/− S.E.M. from three independent experiments. [a]$P<0.05$, [b]$P<0.01$. BP7; IGFBP7.

IGFBP7 mRNA expression was observed within 24 h and knockdown was maintained for at least 72 h after removal of the medium containing the siRNA. IGFBP7 siRNA-treated cells were incubated for 2 h in the absence or presence of VEGF (10 ng/ml).

2.9. Assay of caspase-3 activity

HUVEC were cultured in 96-well tissue culture plates and were treated with IGFBP7 in the presence or absence of VEGF. After 24–48 h, cells were lysed with Caspase-Glo 3/7 Reagent (Promega) and the contents of wells were gently mixed with a plate shaker, according to the manufacturer's instructions. After incubation at room temperature for 1.5 h, the activity of each sample was determined by measuring the luminescence at $485_{Ex}/527_{Em}$ nm with a microtiter plate reader.

2.10. Immunoprecipitation of recombinant IGFBP7 and VEGF assay

To determine whether physical binding between VEGF and IGFBP7 in media blocks the access of VEGF to the VEGF receptor in HUVECs, the unbounded form of VEGF on HUVECs was measured after incubating IGFBP7 with VEGF. Subconfluent cells in 96-well tissue culture plates were treated with IGFBP7 in the presence or absence of VEGF. After 4 h, culture media were collected and anti-IGFBP7 antibody (Santa Cruz) has been added to the media. The complex between IGFBP7 and VEGF was immunoprecipitated with protein G-magnetic beads (New England Biolabs, Inc., Bevery, MA) according to the manufacturer's instructions and the supernatant was assayed for VEGF content using the Quantikine Human VEGF Elisa kit (R&D Systems).

2.11. Statistical analysis

All experiments were carried out at least three times. Values represent means +/− S.E.M. except for immunoblot and RT-PCR analyses. The statistical significance of the results was tested using Dunnett's test for multiple comparisons. Differences with a P value <0.05 were considered to be significant.

3. Results

3.1. The effects of recombinant IGFBP7 on VEGF-stimulated tube formation

HUVECs were treated for 24 h with IGFBP7 and/or VEGF, and representative pictures are shown in Fig. 1. The values obtained from three independent experiments are summarized in Table 1. IGFBP7 alone (10 or 160 ng/ml) did not significantly affect the number of junctions or the total tube length; however, VEGF treatment served as a positive control and promoted tube formation. IGFBP7 treatment (160 ng/ml) attenuated the VEGF-induced stimulation of tube formation. No morphological change related to apoptosis was observed in IGFBP7-treated cells. The effect of IGFBP7 pretreatment on VEGF-stimulated tube formation of HUVECs was examined (Fig. 1B). Cells were treated for 6 h with IGFBP7 before treatment with VEGF in the *in vitro* Matrigel assay. The tube formation induced by VEGF treatment was completely blocked by IGFBP7 pretreatment.

3.2. The effects of IGFBP7 on PGE_2 production and the proliferation in HUVECs

VEGF increases PGE_2 levels in the culture media of HUVECs (Tamura et al., 2006). The effect of IGFBP7 on VEGF-induced PGE_2 production was examined (Fig. 2). IGFBP7 decreased the VEGF-induced enhanced secretion of PGE_2 into the medium in a dose-dependent manner (Fig. 2A) and suppressed VEGF-stimulated COX-2 mRNA expression at concentrations of 10 and 160 ng/ml (Fig. 2B).

We next examined the effect of IGFBP7 on the proliferation of HUVECs in the presence or absence of VEGF (10 ng/ml) (Fig. 2C). Treatment with VEGF for 24 h stimulated proliferation of the HUVECs. IGFBP7 (160 ng/ml) inhibited the VEGF-stimulated cell proliferation, although IGFBP7 alone (10 or 160 ng/ml) did not influence proliferation. When the activation of MAP kinases was evaluated (Fig. 2D),

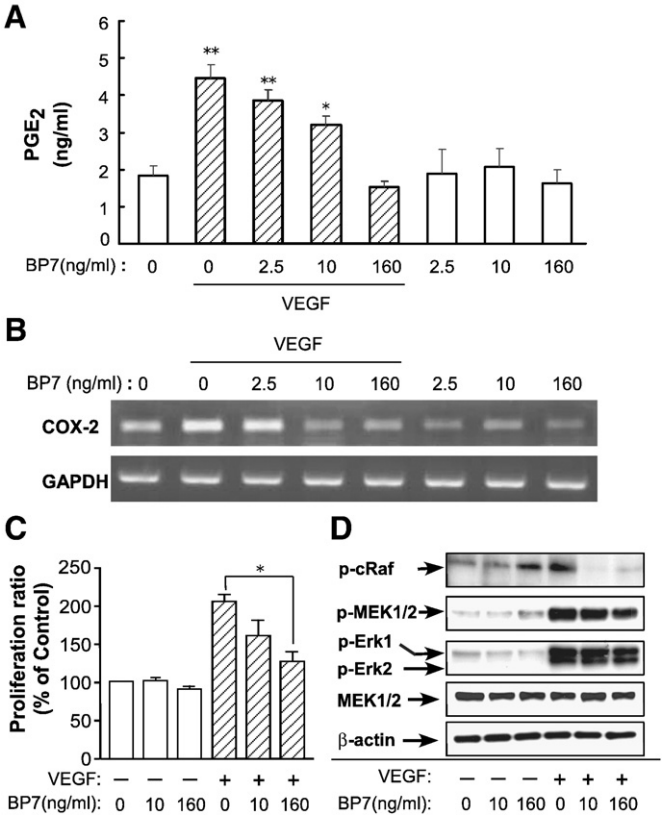

Fig. 2. Effects of IGFBP7 on VEGF-stimulated PGE_2, and cyclooxygenase-2 expression (A, B), VEGF-induced cell proliferation (C), the phosphorylation of MAP kinases (D) in HUVECs. HUVECs (4×10^4 cells/well) were incubated for 24 h with IGFBP7 (2.5–160 ng/ml) in the absence or presence of VEGF (10 ng/ml). The culture medium was collected and used in the PGE_2 assay. Three independent sets of experiments were performed in triplicate, and the results are expressed as means +/− S.E.M. *$P<0.05$, **$P<0.01$; *vs* BP7(0 ng/ml) (B) After cells were incubated for 2 h with IGFBP7 (2.5–160 ng/ml) in the absence or presence of VEGF (10 ng/ml), RNA was isolated and subjected to semi-quantitative RT-PCR. (C) Cells were incubated for 24 h with IGFBP7 (10 or 160 ng/ml) in the absence or presence of VEGF (10 ng/ml). The number of cells was evaluated using the WST-1 proliferation assay. Three independent sets of experiments were performed in triplicate, and the results are expressed as means +/− S.E.M. (D) Cells were incubated with IGFBP7 (10 or 160 ng/ml) in the absence or presence of VEGF (10 ng/ml) for 20 min, before the proteins were isolated using Chaps Cell Extract Buffer. Protein (8 μg) was subjected to Western blot analysis using the following primary antibodies: anti-phospho-cRaf (Ser338), anti-phospho-MEK1/2 (Ser217/221), anti-phospho-p44/42(Th202/Tyr204) and anti-MEK1/2. Representative blots are shown. BP7; IGFBP7.

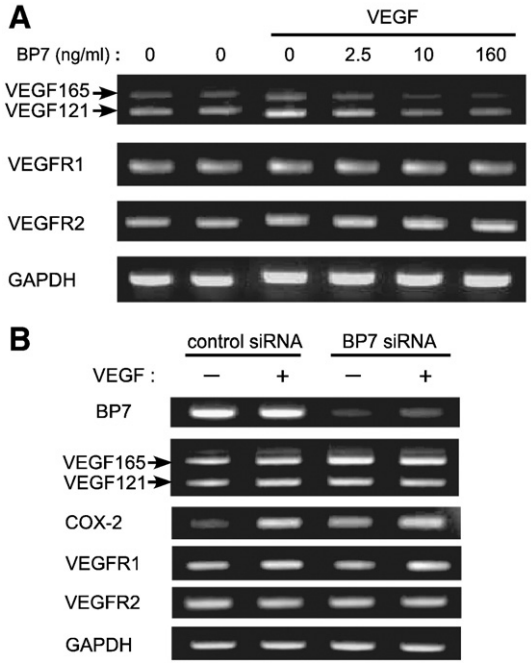

Fig. 3. Effects of IGFBP7 (A) and knocking down of IGFBP7 (B) on the expression of VEGF and VEGF receptor (VEGFR-1/2) mRNA in HUVECs. (A) HUVECs were incubated with IGFBP7 (2.5–160 ng/ml) and VEGF (10 ng/ml) for 2 h, and then poly $(A)^+$ RNA was isolated from the cells. Semi-quantitative RT-PCR was performed using specific primers for VEGFR-1, VEGFR-2 and VEGF. GAPDH primers were used as a control. The products were subjected to electrophoresis on a 1.5% agarose gel and cDNA was visualized by ethidium bromide staining. (B) Cells were transfected with control or IGFBP7 siRNAs for 24 h to knockdown IGFBP7 expression, and then incubated for 2 h in the absence or presence of VEGF (10 ng/ml). Total RNA was isolated and subjected to semi-quantitative RT-PCR for detecting VEGF, COX-2, VEGF receptor (VEGFR-1/2), and GAPDH. BP7; IGFBP7.

VEGF treatment increased the levels of phosphorylated cRaf, MEK, and Erk1/2; however, VEGF-stimulated phosphorylation of these molecules was suppressed by treatment with IGFBP7. VEGF or IGFBP7 did not affect the total MEK1/2 and β-actin levels.

3.3. The effects of IGFBP7 on VEGF-stimulated VEGF expression and induction of apoptosis in HUVECs

In order to determine the effect of IGFBP7 on VEGF production and VEGF signaling, the mRNA expression of VEGF (VEGF121 and 165) and two types of VEGF receptor (VEGFR-1 and VEGFR-2) were examined (Fig. 3A). IGFBP7 treatment inhibited VEGF-induced expression of both isoform-specific VEGF transcripts in HUVECs (top panel in A). But, IGFBP7 did not alter the expression of the VEGF1/2 receptors.

Table 2
The concentration of VEGF in the culture media after co-incubation of VEGF and IGFBP7 in the presence of HUVECs.

Group	VEGF (pg/0.1 ml)
Control	4.5 ± 3.0
BP7 (160 ng/ml)	0.1 ± 7.7
VEGF (10 ng/ml)	885 ± 59.4
VEGF + BP7	903 ± 94.7

HUVECs (4×10^4 cells/well) were incubated for 4 h with VEGF (10 ng/ml) in the absence or presence of IGFBP7 (160 ng/ml). The culture medium was collected and immunoprecipitated with anti-IGFBP7 antibody, followed by incubation with protein G-magnetic beads. The content of VEGF in the supernatant was measured by using a VEGF Elisa kit. The results of three independent wells for each group are expressed as means +/− S.E.M. BP7; IGFBP7.

Table 3
Effect of IGFBP7 on caspase-3 in HUVECs.

Group	Caspase-3 activity (% of control)			
	24 h		48 h	
Control	100.0 ± 36.77	a	100.0 ± 27.43	b
BP7 (160 ng/ml)	268.4 ± 18.69		304.2 ± 53.56	
VEGF (10 ng/ml)	148.8 ± 34.13		128.7 ± 50.60	
VEGF + BP7	158.2 ± 34.80		234.5 ± 54.22	

HUVECs (1×10^4 cells/well) were seeded into a 96 well-culture dish. Cells were incubated for 24 and 48 h with IGFBP7 (160 ng/ml) in the absence or presence of VEGF (10 ng/ml). The activity of caspase-3 was measured using a luminescent assay (the Caspase-G10 3/7 assay). Three independent sets of experiments were performed in triplicate, and the results are expressed as mean +/− S.E.M. of three independent experiments. $^aP<0.05$, $^bP<0.01$. BP7; IGFBP7.

The effect of knocking down endogenous IGFBP7 expression on VEGF mRNA levels was examined in the presence or absence of VEGF stimulation (Fig. 3B). IGFBP7 levels were suppressed following a 24 h treatment with siRNA (data not shown). The cells were then treated with or without VEGF for another 2 h. Representative data showed that IGFBP7 siRNA-treated cells had enhanced basal VEGF and COX2 mRNA levels in the absence of VEGF stimulation. However, VEGFR-1/2 mRNA levels were not influenced by IGFBP7 siRNA treatment. Higher VEGF mRNA levels in IGFBP7 siRNA-treated cells were not observed in the presence of VEGF.

As there existed the possibility that IGFBP7 could interact with VEGF in the culture media and prevent VEGF binding to its receptor, recombinant IGFBP7 and/or VEGF were co-incubated for 4 h in the HUVEC culture system and the VEGF content of the supernatant was analyzed after performing immunoprecipitation to remove any VEGF–IGFBP7 complexes (Table 2). However, there was no difference in the concentration of VEGF between the VEGF alone group and the VEGF and IGFBP7 group, indicating that IGFBP7 does not bind to VEGF in culture media.

To further explore the induction of apoptosis by IGFBP7, we examined the activity of caspase-3 (Table 3) and the levels of caspase-2, -7, and DFF45 protein (Fig. 4). IGFBP7 alone increased caspase-3 activity and apoptosis-related factor levels in HUVECs, although VEGF had no effect on basal caspase levels and IGFBP7 did not change caspase levels in the presence of VEGF.

4. Discussion

Our data clearly indicate that exogenous IGFBP7 blocks VEGF-stimulated tube formation and proliferation in human endothelial cells. VEGF-stimulated MAP kinase activation is associated with endothelial cell proliferation; this was also inhibited by IGFBP7 treatment. Thus, IGFBP7 interferes with VEGF-triggered intracellular signaling in vascular endothelial cells. The previous data showing

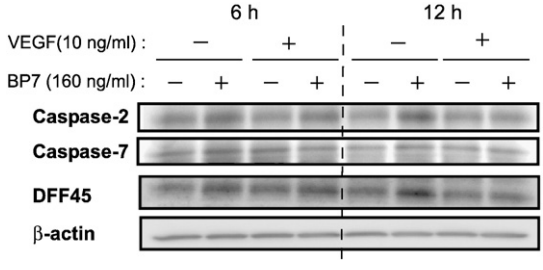

Fig. 4. Effects of IGFBP7 on the apoptosis pathway in VEGF-untreated or -treated HUVECs. HUVECs were incubated with IGFBP7 (160 ng/ml) in the absence or presence of VEGF (10 ng/ml) for 6 or 12 h, before the proteins were isolated using Chaps Cell Extract Buffer. Protein (5 μg) was subjected to Western blot analysis using the following primary antibodies; anti-caspase-2/ICH-1L, anti-caspase-7/MCH-3, and anti-DFF45 antibodies. Representative blots are shown. BP7; IGFBP7.

specific binding of IGFBP7 to VEGF (Usui et al., 2002) prompted us to examine IGFBP7 binding to VEGF in culture medium and the effect of this binding on VEGF binding to its receptor, which stimulates endothelial cell activation. However, IGFBP7 did not bind VEGF in the culture medium, and IGFBP7 suppressed both VEGF-stimulated HUVEC tube formation and HUVEC proliferation even when the cells were pretreated with IGFBP7 for 6 h before VEGF stimulation. These results indicate that the block in VEGF action induced by IGFBP7 is not due to the direct binding of IGFBP7 to VEGF in the culture medium.

COX-2 is a rate-limiting enzyme in the biosynthetic pathway of PGE_2 and is inducible by mitogens, proinflammatory cytokines, and growth factors such as VEGF (Hla et al., 1999). Inhibition of COX-2 results in the suppression of neovascularization (Marcus et al., 1978; Chuchman et al., 2007). Several studies have indicated that there is a positive correlation between VEGF and COX-2 activity and microvessel density; these observations are strongly associated with angiogenesis (Cianchi et al., 2001; Gallo et al., 2001). VEGF stimulates COX-2 mRNA expression in HUVEC (Murphy and Fitzgerald, 2001) and membrane-associated PGE synthase and COX-2 mRNA expression in rat ovarian cells (Sakurai et al., 2004). The proangiogenic effects of COX-2 are mediated primarily by three products of arachidonic acid metabolism: TXA_2, PGE_2, and PGI_2 (Gately and Li, 2004). VEGF-induced PGE_2 may directly promote angiogenesis in endothelial cells (Tamura et al., 2006). In the present study, the VEGF mRNA expression was suppressed by addition of IGFBP7, and VEGF-stimulated PGE_2 production and COX-2 expression decreased after IGFBP7 treatment; thus, IGFBP7 inhibited VEGF-enhanced angiogenesis-related products. The selective COX-2 inhibitor has been shown to induce apoptosis and prevent tube formation in HUVECs (Chuchman et al., 2007). However, the reduction of VEGF-induced tube formation by IGFBP7 could be mainly mediated by inhibition of MAP kinase cascade through c-Raf, because the elevation of caspases was not significant in the presence of VEGF, and IGFBP7 alone did not affect basal levels of tube formation when caspase-3 activity increased in HUVECs. Thus, the induction of apoptosis probably does not contribute to the inhibitory effect of IGFBP7 on angiogenesis in this study.

Our data, together with previous reports showing that several endothelial cell lines express IGFBP7 mRNA (Akaogi et al., 1996; Ono et al., 1994), and new evidence that knocking down of IGFBP7 enhances the basal levels of endothelial VEGF and COX-2 expression, supports the hypothesis that IGFBP7 negatively regulates the constitutional expression of vascular angiogenic factors. In our HUVEC culture system, the level of secreted IGFBP7 in the conditioned medium of a 24 h control culture was ~40 ng/mL (data not shown). The IGFBP7 activity we observed could therefore be effective under physiological conditions. Recently, Pen et al. (2008) reported that TGF-β1 secreted from glioblastomas stimulates IGFBP7 expression and IGFBP7 enhances capillary-like tube formation of human brain endothelial cells (HBEC) *in vitro*. However, we did not observe a stimulatory effect of IGFBP7 alone on tube formation. This discrepancy could be due to differences in the doses of IGFBP7 used and/or in cell types, because HBEC tube formation is significantly stimulated by 1.5 μg/ml of IGFBP7 (but not by 0.3 μg/ml) in their report, which was approximately ten times the IGFBP7 concentration used in the present study. We also observed that the same dose of IGFBP7 (1.5 μg/ml) increased HUVECs tube formation (data not shown). Thus, IGFBP7 alone might have dose-dependent biphasic effect on tube formation via unknown mechanisms.

The gene encoding IGFBP7 was originally identified as a gene that shows reduced mRNA expression in meningioma cell lines as compared to normal cells (Murphy et al., 1993). The IGFBP7 protein was identified as tumor-derived cell adhesion factor (TAF), tentatively also called angiomodulin, in human bladder carcinoma cells (Ono et al., 1994), and as a PGI_2-stimulating factor in human fibroblast (Yamauchi et al., 1994). The addition of recombinant IGFBP7 to the culture medium of human tumor cells has been shown to significantly suppress their growth, resulting in an increased number of cells in the G1 phase of the cell cycle (Hata et al., 2000). IGFBP7 inhibits tumor proliferation via a senescence-like mechanism (Burger et al., 2005) and by insulin/IGF-independent mechanism *in vivo* (Sato et al., 2007). Furthermore, it has been recently shown that IGFBP7 may play a central role in an activated homo sapiens v-raf murine sarcoma viral oncogene homolog B1(BRAF) oncogene-mediated senescence and apoptosis in human fibroblasts (Wajapeyee et al., 2008). The authors proposed a new model for how secreted IGFBP7 could be involved in senescence and apoptosis. Although BRAF stimulates BRAF-MEK-ERK signaling for transient proliferation, in the second phase, BRAF expression causes the synthesis of IGFBP7, which acts through an autocrine/paracrine pathway to inhibit BRAF-MEK-ERK signaling and activate a senescence program. The inhibition of MAP kinase cascade by IGFBP7 treatment in the present study might be associated with the inhibition of BRAF-MEK-ERK signaling. Understanding of BRAF-mediated signals and the relationship between BRAF and IGFBP7 will be a next focus to explore.

Tumor angiogenesis is essential for tumor development. IGFBP7 is highly expressed in the blood vessels of various human cancer tissues, suggesting that it might suppress the pathological action of VEGF which is mainly derived from tumor cells. These data suggest that IGFBP7 in the blood vessels of tumors may lead to a unique tumor vasculature with characteristics significantly different from those of normal vasculature. The inhibitory effect of IGFBP7 on tumorigenicity might be partially mediated by its ability to suppress VEGF-stimulated angiogenesis, although there is so far no direct evidence to explain if IGFBP7 affects tumor blood vessels. Further experiments will be needed to determine the mechanism of IGFBP7-induced inhibition of biological action of VEGF and the role of IGFBP7 in vascular function.

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