Original Article

Big Mitogen-Activated Protein Kinase 1 (BMK1)/ Extracellular Signal Regulated Kinase 5 (ERK5) Is Involved in Platelet-Derived Growth Factor (PDGF)–Induced Vascular Smooth Muscle Cell Migration

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Big mitogen-activated protein kinase 1 (BMK1), also known as extracellular signal–regulated kinase 5 (ERK5), is a newly identified member of the mitogen-activated protein (MAP) kinase family. Recently, several studies have suggested that BMK1 plays an important role in the pathogenesis of cardiovascular disease. To clarify the pathophysiological significance of BMK1 in the process of vascular remodeling, we explored the molecular mechanisms of BMK1 activation in vascular smooth muscle cells (VSMCs). From the results of co-immunoprecipitation and immunoblotting analyses, it was found that platelet-derived growth factor (PDGF), a known potent mitogen, activated BMK1 and triggered the Gab1–SHP-2 interaction in rat aortic smooth muscle cells (RASMCs). The abrogation of SHP-2 phosphatase activity by transfection of the SHP-2-C/S mutant suppressed PDGF-stimulated BMK1 activation. Infection with an adenoviral vector expressing dominant-negative MEK5α, which can suppress PDGF-stimulated BMK1 activation to the control level, inhibited PDGF-induced RASMC migration. Moreover, we observed an increase of BMK1 activation in injured mouse femoral arteries. From these findings, it is suggested that BMK1 activation leads to VSMC migration induced by PDGF *via* Gab1–SHP-2 interaction, and that BMK1-mediated VSMC migration may play a role in the pathogenesis of vascular remodeling. (*Hypertens Res* 2007; 30: 1107–1117)

***Key Words*: big mitogen-activated protein kinase 1/extracellular signal regulated kinase 5, platelet-derived growth factor, vascular smooth muscle cells, Src homology 2–containing protein tyrosine phophatase-2, vascular remodeling**

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Introduction

Big mitogen-activated protein kinase 1 (BMK1), also known as extracellular signal regulated kinase 5 (ERK5) is the fourth and most recently identified mitogen-activated protein (MAP) kinase family member (*1*). Recently, studies using gene-targeted mice lacking BMK1 itself or components related to the BMK1 cascade have revealed that BMK1 signaling is critical for angiogenesis, cardiac development, and the maintenance of vascular integrity (*2*, *3*). Our previous study showed that BMK1 is activated in the glomeruli of type 2 diabetes mellitus model rats, and that BMK1 activation mediates the rat mesangial cell proliferation induced by high glucose stimulation (*4*). These findings suggest that BMK1 is involved in the progression of diabetic nephropathy. We also reported that BMK1 activation was implicated in cardiac dysfunction in mice loaded with angiotensin II (*5*). On the other hand, several *in vitro* studies have demonstrated that BMK1 is activated by serum, oxidative stress, osmotic stress, and growth factors in various cells (*4*, *6*, *7*), and the activation of BMK1 can lead to cell proliferation, morphogenesis, antiapoptosis, and so on (*8–11*). From these observations, BMK1 is strongly suspected to play a role in the development of cardiovascular diseases. However, the specific pathophysiological role of BMK1 remains to be elucidated.

The primary cause of many fatal cardiovascular diseases is believed to be atherosclerosis (*12*). During atherogenesis and the progression of the disease, chronic inflammatory responses induce vascular wall remodeling or the generation of neointima and thickening of the tunica media, which leads to the development of plaque and artery stenosis (*13*). The neointima and thickened media are primarily composed of abnormally proliferating and migrating vascular smooth muscle cells (VSMCs) (*14–17*). VSMC migration is induced by various inflammatory cytokines and chemokines. Platelet-derived growth factor (PDGF) is a known potent mitogen abundant in the area of inflammation, and also stimulates VSMC migration (*18*). It has been reported that PDGF-induced VSMC migration is mediated by MAP kinases, phosphatidylinositol 3-kinase (PI3K), and many other kinases (*19*, *20*). Although BMK1 activation is expected to be involved in the cell migration induced by PDGF, a relationship between BMK1 and VSMC migration has not been demonstrated yet.

The binding of PDGF to its receptor triggers autophosphorylation on multiple tyrosine residues. Following that, several Src homology (SH) 2–containing signal transducers, such as Crk, PI3K, Src, and so on, are recruited to start the activation of multiple signaling cascades, and various cellular responses occur (*21*). SH2-containing protein tyrosine phophatase-2 (SHP-2) is known to be a key molecule in the PDGF-stimulated intracellular signaling pathway (*22*). It has been reported that PDGF-induced ERK1/2 activation is mediated by SHP-2 phosphorylation (*23*). Furthermore, PDGF promotes the association between SHP-2 and Grb2-associated binder 1 (Gab1), and formation of the complex has been shown to mediate ERK1/2 activation (*23*). Various other forms of stimulation also have been found to induce binding of SHP-2 to Gab1 in a variety of cells (*23–26*). Gab1 is an adapter protein with an amino-terminal pleckstrin homology domain, several proline-rich sequences, and multiple potential tyrosine phosphorylation sites for the binding of SH2- and SH3-containing proteins (*27*). SHP-2 is recruited to these tyrosyl-phosphorylated sites on Gab1 and transmits downstream signaling (*22*). From these findings, it is conceivable that these two molecules mediate the activation of other MAP kinase family members induced by PDGF in VSMCs. Therefore, we focused on the Gab1–SHP-2 complex as the upstream regulator of PDGF-induced BMK1 activation in VSMCs.

In the present study, we explore the involvement of BMK1 in PDGF-induced VSMC migration and demonstrate a relationship between SHP-2 and BMK1 activation.

Methods

Cells and Materials

Rat aortic smooth muscle cells (RASMCs) were isolated from male Sprague-Dawley rats weighing 200 to 250 g and maintained in 10% fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) as described previously (*28*, *29*). For the experiments, cells from passages 3 to 8 were used after 24–48 h of serum depletion. Platelet derived growth factor-BB (PDGF; human, recombinant) was purchased from Sigma (St. Louis, USA). Since phosphorylation of Thr218 and Tyr220 represents BMK1 activation (*1*), BMK1 tyrosine phosphorylation in the cell lysate or tissue was determined using phospho-specific BMK1 antibody purchased from Cell Signaling Technology Inc. (Beverly, USA). Fluorescein isothiocyanate (FITC)–coupled donkey antirabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, USA). Antibodies for phosphotyrosine (clone 4G10) and Gab1 were from Upstate Biotechnology, Inc. (Lake Placid, USA). MEK5 antibody and SHP-2 antibody were from BD Biosciences (San Jose, USA) and Santa Cruz Biotechnology Inc. (Santa Cruz, USA), respectively. For gene transfer, an Adeno-X™ Expression System from Clontech Laboratories, Inc. (Palo Alto, USA), a Nucleofector kit for human aortic smooth muscle cells (AoSMC) (Amaxa Biosystems, Köln, Germany), and *Trans*IT® -293 transfection reagent (Mirus Corp., Madison, USA) were used. BIOMOL GREEN and pp60$^{c\text{-}src}$ C-terminal phosphoregulatory peptide (TSTEPQpYQPGENL) for phosphatase activity assay were from BIOMOL (Plymouth Meeting, USA). All other chemicals were of reagent grade and obtained from commercial sources, and were used without further purification.

Adenoviral Infection and Transfection

The MAP kinase kinase (MEK) 5 plasmids, pcDL-SRαMEK5A (dominant-negative; DN-MEK5) and pcDL-SRαMEK5D (constitutively active; CA-MEK5) were a gift of Dr. Eisuke Nishida (Kyoto Univ.) (*30*). Construction of the adenoviral vectors pAdeno-X–DN-MEK5 and pAdeno-X–CA-MEK5 was performed using the Adeno-X™ Expression System according to the instructions of the manufacturer. Viruses were used for infection of RASMCs plated on 6-well plates, 12-well plates or 60 mm dishes. As a control, a lacZ gene–carrying adenoviral vector was used. Twenty-four hours after infection, the medium was depleted of serum for 24 h, followed by sequential experiments. For SHP-2 gene transfer, a Nucleofector® device and Human AoSMC Nucleofector™ Kit were used according to the optimized protocol for human AoSMC. The mammalian expression vectors of pJ3-hSHP-2 (WT) and pJ3-hSHP-2 (C459S), hereinafter referred to as SHP-2-WT and SHP-2-C/S, respectively, were provided by Dr. Ben Neel (Beth Israel Deaconess Medical Center, Boston, USA). As a control, pmax-GFP was transfected. Twenty-four hours after transfection, the medium was depleted of serum for 24 h, followed by sequential experiments.

Preparation of Cell Lysate for Immunoprecipitation, Immunoblotting, and Phosphatase Activity Assay

Quiescent cells were treated with or without PDGF at the indicated concentrations for the indicated times. Then, incubation media were discarded, and the cells were lysed with cell lysis buffer for immunoprecipitation or immunoblotting as described previously (*28*). For phosphatase activity assay, cells were lysed with cell lysis buffer without the protein phosphatase inhibitor sodium orthovanadate. The protein concentrations of the supernatants were measured with a protein assay kit (BIO-RAD, Hercules, USA) and subjected to co-immunoprecipitation, immunoblotting and the phosphatase activity assay.

Co-Immunoprecipitation and Immunoblotting

Immunoprecipitation using anti-Gab1 or anti–SHP-2 antibody was performed as described previously (*28*). Immunoblotting using anti–phospho-BMK1 antibody, anti-phosphotyrosine (4G10), anti-MEK5, anti-Gab1, or anti–SHP-2 antibody was also performed as described previously (*28*). Immunoreactive bands were visualized using enhanced chemiluminescence (ECL; GE Healthcare UK Ltd., Buckinghamshire, UK) and were quantified by densitometry in the linear range of film exposure using a UMAX Astra 2200 scanner (UMAX Technologies, Fremont, USA) and imageJ 1.31v software.

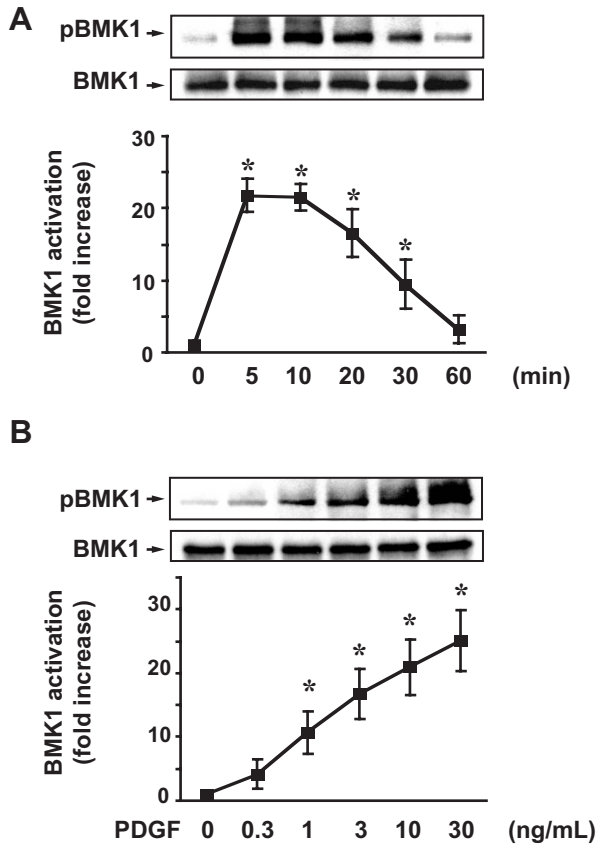

Fig. 1. *Time-course (A) and concentration-response curve (B) of PDGF-induced BMK1 activation in RASMCs. Cells were stimulated with 10 ng/mL PDGF for the indicated periods of time (A) or with the indicated concentrations of PDGF for 5 min (B). Values were normalized by arbitrarily setting the densitometry of control cells (time = 0 and without PDGF) to 1.0 (values are the mean ± SD, n = 5). The asterisks represent significant differences compared to the control (*p < 0.01).*

Modified Boyden Chamber Assay

To assess cell migration, especially invasive displacement, a modified Boyden chamber assay was performed by minor modification to the method described previously (*31*). The assay was performed using Transwell chambers (6.5 mm, model 3422; Costar, Cambridge, USA) with an 8-μm pore polycarbonate membrane. After 6 h of incubation with or without PDGF, the membrane was fixed with methanol and stained with Diff-Quik solution (Baxter, McGaw Park, USA). Cells that had migrated from the upper to the lower side of the membrane were counted at 400× magnification in 10 microscope fields per filter.

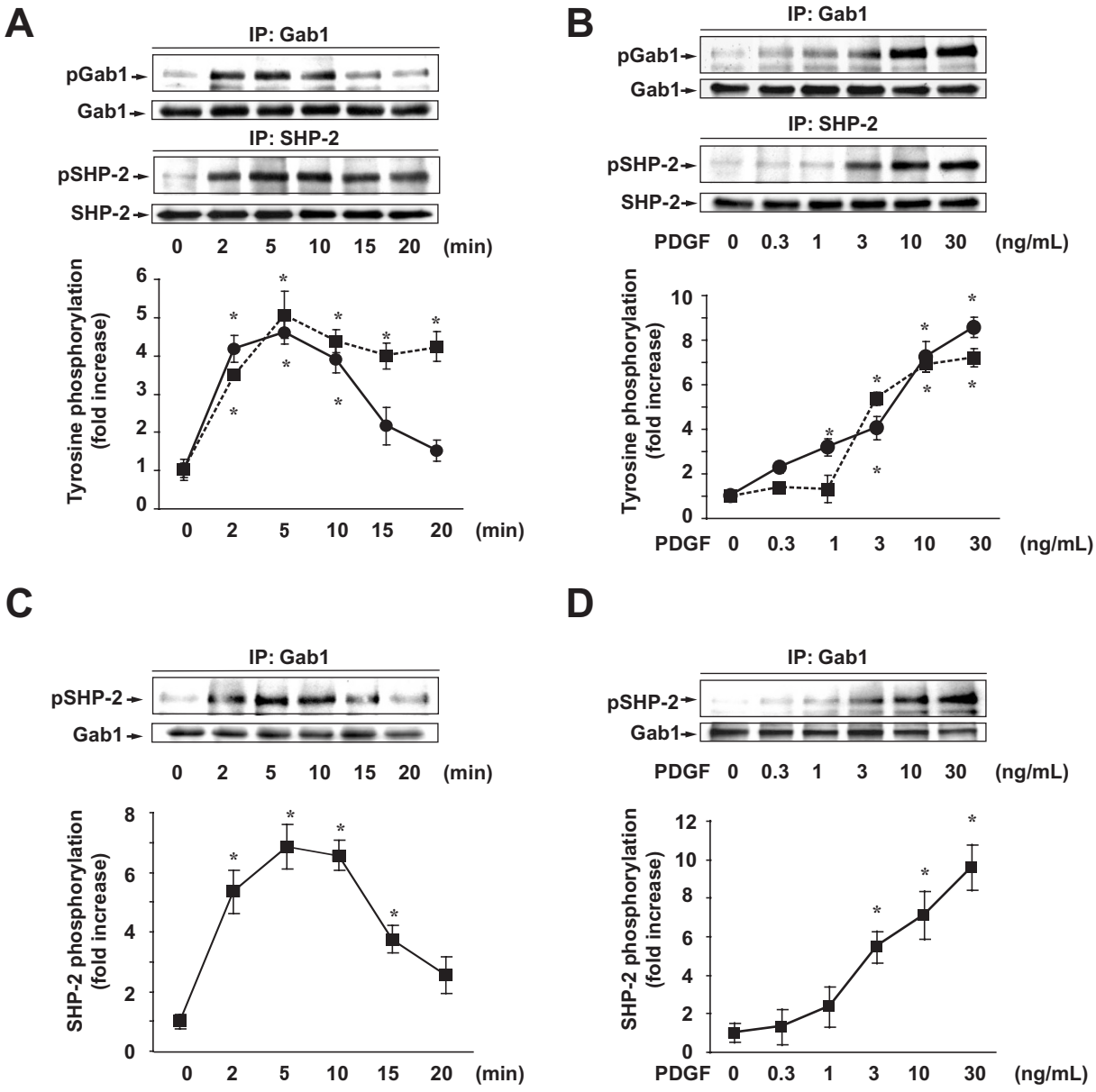

Fig. 2. *PDGF-induced Gab1 and SHP-2 phosphorylation (A and B) and Gab1–SHP-2 association (C and D) in RASMCs. Cells were stimulated with 10 ng/mL PDGF for the indicated periods of time (A and C) and were stimulated with the indicated concentrations of PDGF for 5 min (B and D). Circle, pGab1; square, pSHP-2. Values were normalized by arbitrarily setting the densitometry of control cells (time = 0, without PDGF) to 1.0 (values are the mean ± SD, n = 5). The asterisks represent significant differences compared to the control (*p < 0.01).*

Wound Healing Assay

For assessing cell migration, especially transverse displacement, a wound healing assay was conducted. RASMCs were seeded and attached on 12-well plates, and infected with adenovirus carrying lacZ, CA-MEK5, or DN-MEK5 as described above. Twenty-four hours later, confluent monolayer cells were scratched by a yellow tip (0 h), and then cultured in the absence or presence of PDGF for 15 h. At 0 h and 15 h, the scratched monolayer cultures were photographed.

Phosphatase Activity Assay

For the measurement of SHP-2 phosphate activity, an *in vitro* protein tyrosine phosphatase activity assay was conducted based on a protocol previously published by Taghibiglou *et al*. (*32*). Cell lysates were immunoprecipitated with anti–SHP-2 antibody, then washed four times with sodium ortho-

vanadate-free lysis buffer and once with 54 μL of protein tyrosine phosphatase (PTP) assay buffer (100 mmol/L HEPES, pH 7.6, 1 mmol/L dithiothreitol, 2 mmol/L EDTA, 150 mmol/L NaCl, 0.5 mg/mL BSA). SHP-2 in PTP assay buffer was incubated with 200 μmol/L of $pp60^{c\text{-}src}$ C-terminal phosphoregulatory peptide for 1 h at 30°C. Free phosphates, which were dephosphorylated from $pp60^{c\text{-}src}$ C-terminal phosphoregulatory peptide by activated SHP-2, were detected using 100 μL of BIOMOL GREEN.

Cuff-Injured Intimal Thickening of the Murine Femoral Artery

Femoral artery cuff placement was performed based on previous reports (*33*, *34*) with some modification. All experimental procedures were performed in accordance with the guidelines of the Animal Research Committee, The University of Tokushima Graduate School. Adult male C57BL/6J mice ($n=10$) were given a standard diet (Oriental Yeast Co., Ltd., Tokyo, Japan) and water ad libitum. The left femoral artery was isolated from surrounding tissues, loosely sheathed with a 2.0-mm polyethylene cuff made of PE-50 tubing (Becton Dickinson, Mountain View, USA) and tied in place with an 8-0 suture. The right femoral artery was dissected from surrounding tissues (sham-operated), but a cuff was not put in place. The femoral arteries were replaced, and the wounds were sutured. Four weeks after cuff placement, animals were sacrificed and their vessels were fixed *in situ* with 10% formalin. Both sides of the harvested arteries were fixed in 10% formalin and subjected to Elastica van Gieson staining, and immunostaining for phosphorylated BMK1 or α-smooth muscle actin (SRL Inc., Tokyo, Japan). For immunohistochemical staining of phosphorylated BMK1, the harvested artery was frozen in Tissue-Tek O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan).

Immunohistochemical Staining of Phosphorylated BMK1

To determine the level of BMK1 activation in the cuffed and sham-operated arteries, immunohistochemistry was performed as described previously (*4*). Phospho-specific BMK1 antibody was used. Immunofluorescence was visualized using a fluorescent microscope (Olympus, Tokyo, Japan).

Statistical Analysis

Values are reported as the means±SD from experiments performed on five different occasions. Two-way ANOVA was used to determine the significance among groups, after which a modified *t*-test with Bonferroni's post hoc test was used for comparisons between individual groups. A value of $p<0.05$ was considered to be statistically significant.

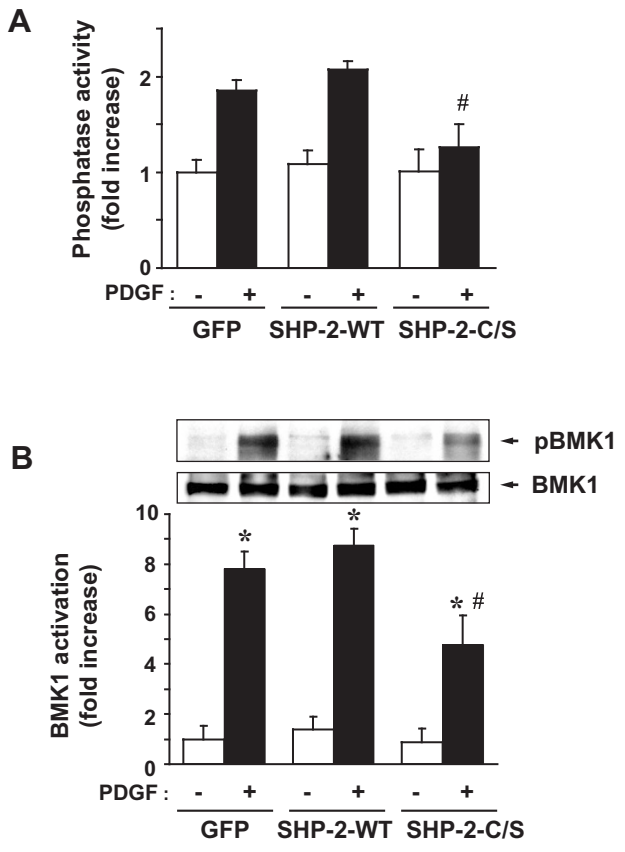

Fig. 3. *Effects of SHP-2-C/S overexpression on PDGF-induced SHP-2 phosphatase activation and BMK1 activation. The GFP, SHP-2-WT, and SHP-2-C/S–overexpressing RASMCs were used. Cells were treated with or without PDGF (10 ng/mL) for 5 min. A: The effects of PDGF on SHP-2 phosphatase activities are shown. Values were normalized by arbitrarily setting the fold increases in the absorbance of control cells (GFP without PDGF) to 1.0 (values are the means±SD, $n=5$; $^{\#}p<0.05$ vs. GFP with PDGF). B: The results of PDGF-induced BMK1 phosphorylation are shown. Values were normalized by arbitrarily setting the densitometry of control cells (GFP without PDGF) to 1.0 (values are the mean±SD, $n=5$). $^{*}p<0.01$ vs. GFP without PDGF; $^{\#}p<0.05$ vs. GFP with PDGF.*

Results

PDGF Stimulates BMK1 Activation and Gab1–SHP-2 Association in RASMCs

To evaluate the magnitude of PDGF-stimulated BMK1 activation in VSMCs, growth-arrested RASMCs were treated with 10 ng/mL PDGF for the indicated period of time up to 60 min. BMK1 was rapidly phosphorylated by PDGF, reaching a maximal response (21.7-fold increase) within 5 to 10 min

compared with the untreated control cells at 0 min (Fig. 1A). PDGF-induced BMK1 activation was increased in a concentration-dependent manner (0.3–30 ng/mL) (Fig. 1B). The magnitude of tyrosine phosphorylation of Gab1 and SHP-2 was also examined as described in the Methods section. As shown in Fig. 2A, Gab1 and SHP-2 were rapidly phosphorylated by PDGF stimulation and reached a maximal response within 5 min. At this peak period, these phosphorylation events were increased in a concentration-dependant manner (Fig. 2B). Moreover, the association between Gab1 and phosphorylated SHP-2 was increased by PDGF stimulation in a time- and concentration-dependent manner (Fig. 2C, D), consistent with the results of PDGF-stimulated tyrosine phosphorylation of Gab1 and SHP-2 (Fig. 2A, B). These results show that PDGF phosphorylated BMK1, Gab1, and SHP-2, and stimulated Gab1–SHP-2 interaction in RASMCs.

PDGF-Increased SHP-2 Tyrosine Phosphatase Activity Is Required for BMK1 Activation in RASMCs

Since it has been reported that Gab1–SHP-2 interaction leads to SHP-2 activation (*35*), we examined the PDGF-induced phosphatase activity of SHP-2. As shown in Fig. 3A, cells transfected with the control vector, GFP or SHP-2-WT exhibited an increase of phosphatase activity by PDGF stimulation. In SHP-2-C/S overexpressing cells, PDGF-stimulated phosphatase activity was suppressed (Fig. 3A). SHP-2-C/S overexpression also inhibited PDGF-stimulated BMK1 activation (Fig. 3B). From these results, it is suggested that BMK1 activation by PDGF requires SHP-2 phosphatase activity.

Inhibition of BMK1 Activation Suppresses PDGF-Induced RASMC Migration

Since BMK1 is considered to be phosphorylated and activated by MEK5α with high specificity (*36*), we used an adenoviral vector carrying DN-MEK5α for the inhibition of PDGF-induced BMK1 activation. In the cells infected with CA-MEK5α or DN-MEK5α, MEK5α protein expression was 3–4 times higher than in the lacZ-expressing control cells (Fig. 4A). Overexpression of CA-MEK5α enhanced both basal and PDGF-stimulated BMK1 activation, and DN-MEK5α suppressed PDGF-induced BMK1 activation to the control level (Fig. 4B). We then proceeded to examine BMK1 involvement in PDGF-induced RASMC migration under the condition of adenovirus infection. First, we confirmed that PDGF stimulation for 6 h increased RASMC invasive migration in a concentration-dependent manner by a modified Boyden chamber assay (Fig. 5A). Examination by a wound healing assay also showed concentration-dependent increases in RASMC transverse migration stimulated by PDGF for 15 h (data not shown). As shown in Fig. 5B, RASMC transverse displacement was clearly enhanced in CA-MEK5–expressing

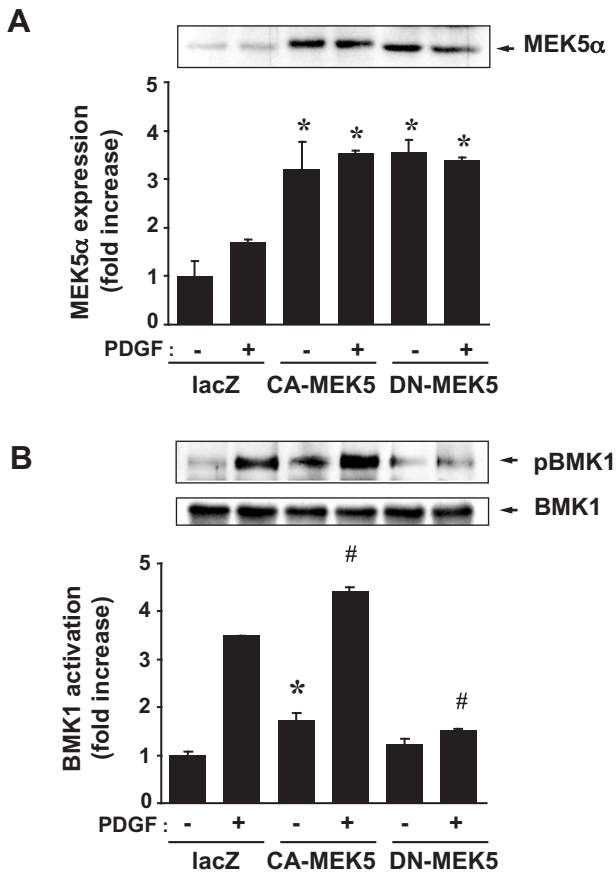

Fig. 4. *BMK1 activation is enhanced by infection with adenoviral vector of constitutively active (CA)-MEK5α, and inhibited with dominant-negative (DN)-MEK5α. Cells grown to 90% confluence were infected with lacZ, CA-MEK5α or DN-MEK5α adenoviral vector for 48 h before the treatment with PDGF (10 ng/mL) for 5 min. The results of MEK5α protein expression (A) and BMK1 phosphorylation (B) are shown. Values were normalized by arbitrarily setting the densitometry of control cells (lacZ infection and without PDGF stimulation) to 1.0 (values are the means ± SD, n = 5). *p < 0.01 vs. lacZ without PDGF stimulation; #p < 0.01 vs. lacZ with PDGF stimulation.*

cells compared to control lacZ cells. In contrast, DN-MEK5–expressing cells showed only a slight migration after 15-h of PDGF stimulation. Consistent with these results, the RASMC invasive migration (as evaluated by a modified Boyden chamber assay) induced by 6-h of PDGF stimulation revealed that the inhibition of BMK1 activation by DN-MEK5α significantly suppressed PDGF-stimulated RASMC migration in comparison to that of lacZ-infected control cells (Fig. 5C). These results indicate that PDGF-stimulated BMK1 activation is involved in RASMC migration.

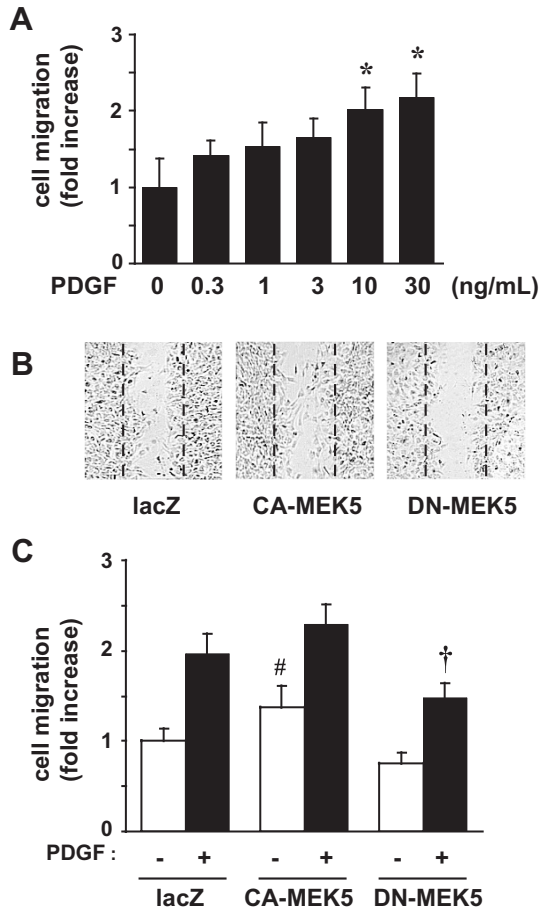

Fig. 5. *PDGF-induced RASMC migration was enhanced by CA-MEK5α infection and PDGF-induced RASMC migration was attenuated by DN-MEK5α infection. A: Concentration-dependant increase of PDGF-induced RASMC migration. Cells were treated with the indicated concentration of PDGF for 6 h. A modified Boyden chamber assay was then performed as described in the Methods section. Values were normalized by arbitrarily setting the fold increases in the migration of control cells (without PDGF treatment) to 1.0 (values are the means ±SD, n = 5, *p < 0.05). B: The results of wound healing assay. The lacZ, CA-MEK5α, or DN-MEK5α–overexpressing RASMCs were used. Adenovirus-infected cells were treated with or without PDGF (10 ng/mL) for 15 h. Representative microscopic photographs are shown. C: The results of modified Boyden chamber assay. The lacZ, CA-MEK5α, or DN-MEK5α–overexpressing RASMCs were used. Adenovirus-infected cells were treated with or without PDGF (10 ng/mL) for 6 h. Values were normalized by arbitrarily setting the fold increases in the migration of control cells to 1.0 (values are the means ±SD, n = 5). $^{\#}p < 0.05$ vs. lacZ without PDGF; $^{\dagger}p < 0.05$ vs. lacZ with PDGF.*

BMK1 Is Activated at the Site of Cuff Injury in the Murine Femoral Artery

To investigate the involvement of BMK1 activation in VSMC migration *in vivo*, we performed a cuff placement experiment around the mouse femoral artery, as described in the Methods section. Cuff placement around arteries is used as a model of inflammatory vascular injury leading to vascular remodeling and the neointimal formation caused by periadventitial injury (*37*). This intervention can induce reproducible site-controlled neointimal formation and stenosis as a result of chronic inflammation (*37*). After 4-week cuff placement, remarkable cellular infiltration was observed around the injured artery (data not shown). Histologic examination with Elastica van Gieson staining of the injured femoral artery revealed the neointima formation and slightly thickened tunica media, compared to the sham-operated control artery (Fig. 6A). Immunostaining for α-smooth muscle actin demonstrated a thickened tunica media in the cuff-injured artery (Fig. 6D). As shown in Fig. 6B, immunofluorescence staining with phosphorylated BMK1 revealed that BMK1 activation was clearly increased in the cuffed arterial wall, compared to the sham-operated control artery. The immunostaining results also showed that BMK1 phosphorylation was increased in the cuff-injured artery compared to the sham-operated artery (Fig. 6C). Moreover, from the results of immunostaining for phosphorylated BMK1 and α-smooth muscle actin in the serial sections, it was confirmed that BMK1 was activated, particularly in the tunica media of cuff-injured arteries (Fig. 6C, D). These observations suggest that BMK1 activation is implicated in VSMC migration *in vivo*, and is related to vascular remodeling.

Discussion

In the present study, we demonstrated for the first time that PDGF-induced BMK1 activation mediates RASMC migration *via* the activation of SHP-2. Moreover, our observations of cuff-injured arteries indicated the possibility that BMK1 activation resulted in VSMC migration and the subsequent vascular remodeling.

Previously, many studies had reported that PDGF-induced VSMC migration appears to be mediated by various candidate molecules, such as PI3K, c-Jun NH_2-terminal kinase, p38, and ERK1/2 (*19*, *20*). The involvement of ERK1/2 activation in VSMC migration has been shown by several studies using the MEK1/2 inhibitors PD98059 or U0126 (*38–40*). However, it has proven difficult to distinguish the contribution of the BMK1 pathway from the involvement of ERK1/2 activation by experiments using such inhibitors, because PD98059 and U0126 inhibit not only ERK1/2 activation but also BMK1 activation (*30*). Therefore, in the present study, we used DN-MEK5α to examine the contribution of BMK1 to VSMC migration. MEK5 was originally cloned as the splice variants MEK5α and MEK5β, which possess inverse

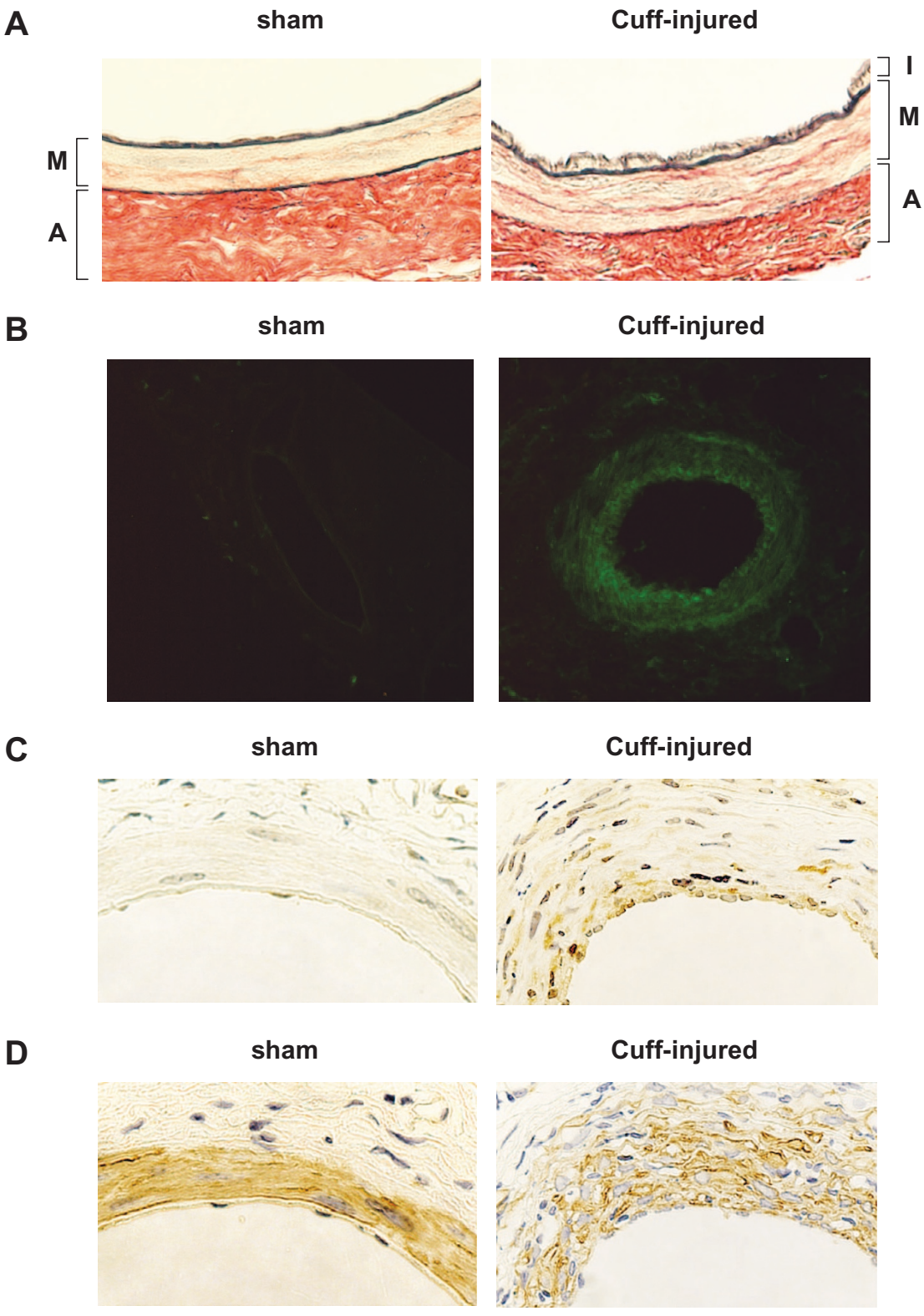

Fig. 6. *Big mitogen-activated protein kinase 1 (BMK1) is activated in the cuff-injured murine femoral artery. Representative cross-sections of sham-operated (left) and cuffed (right) murine femoral arteries are shown. A: Elastica van Gieson staining. I, M, and A represent intima, tunica media, and adventitia, respectively. B: Immunofluorescence staining (IF) for phospho-BMK1. IF was performed and fluorescein isothiocyanate (FITC) signals were visualized as described in Methods. And the representative serial sctions of immunostaining for phopho-BMK1 (C) or α-smooth muscle actin (D) are shown.*

functions (*41*). MEK5α activates BMK1 with high specificity, but MEK5β functions as a naturally dominant negative variant. We used mutated MEK5α, in which Ser311 and Thr315 were replaced by Ala and Val, respectively. As shown in Fig. 3, infection of adenoviral DN-MEK5α inhibited BMK1 activation to the control level. This experiment proved that the inhibition of BMK1 activation partially suppresses the VSMC migration induced by PDGF stimulation. On the other hand, Zhan *et al*. have used dominant-negative ERK1/2 and confirmed the involvement of ERK1/2 activation in PDGF-induced VSMC migration (*20*). From these and our own findings, it is suggested that PDGF-induced VSMC migration requires both BMK1 and ERK1/2 activation. There was a slight difference in the results regarding invasive motility and those for transverse motility, especially in CA-MEK5α–overexpressing cells, as shown in Fig. 5B and C. We consider this to be due to the different durations of PDGF stimulation. The wound healing assay was performed using 15 h of PDGF stimulation, and the influence of cell proliferation could not be excluded. As we have reported previously, BMK1 activation plays a crucial role in not only migration but also proliferation (*4*, *42*). The proliferative reaction resulting from BMK1 activation might affect the enhancement of RASMC migration in CA-MEK5α–expressing cells. In contrast, it is considered that the influence of cell proliferation does not affect the results of the assay using the modified Boyden chamber method.

We also clarified the PDGF-induced intracellular signaling pathway leading to BMK1 activation in RASMCs. Since SHP-2 is reported to be activated by PDGF stimulation and to be recruited to Gab1, we confirmed the phosphorylation of SHP-2 and the binding of SHP-2 to Gab1 as shown in Fig. 2. SHP-2 phosphorylation peaked at 5 min and was sustained to 20 min (Fig. 2A). However, phosphorylation of Gab1-associated SHP-2 was diminished within 20 min (Fig. 2C). This tendency was consistent with the time-course of Gab1 phosphorylation (Fig. 2A). From these results, it was thought that Gab1–SHP-2 association required Gab1 phosphorylation. Therefore, we also explored the SHP-2 involvement in PDGF-induced BMK1 activation. As shown in Fig. 3, PDGF-induced SHP-2 phosphatase activity was required for activation of the MEK5-BMK1 pathway. Consequently, it is indicated that Gab1–SHP-2 interaction and SHP-2 phosphatase activity are required for VSMC migration in response to PDGF stimulation. Supporting these findings, Ronnstrand *et al*. have demonstrated that the inhibition of the association between the PDGF receptor and SHP-2 diminishes ERK2 activation and the aortic endothelial cell migration induced by PDGF (*22*). Recently, it has been reported that the SHP-2 protein level is increased in the rat injured carotid artery compared to control, especially in the media and neointima (*43*). Kallin *et al*. have also reported that embryonic fibroblasts from a Gab1−/− mouse exhibit decreased chemotactic response to PDGF (*44*). These findings suggest that the Gab1–SHP-2–MEK5–BMK1 pathway is a crucial target for the control and regulation of PDGF-induced VSMC migration.

In addition to these findings in *in vitro* studies, we showed for the first time that BMK1 activation was increased in the cuff-injured mouse femoral artery damaged by chronic inflammation. BMK1 activation was obvious especially in the area of the tunica media (Fig. 6). These results imply that BMK1 activation is involved in the VSMC migration in inflammatory vascular remodeling *in vivo*. Since we have previously reported that BMK1 activation causes VSMC proliferation (*42*), it seems likely that VSMC proliferation and migration occur simultaneously and cooperate to form the neointima and thicken the tunica media during atherogenesis (*13*).

In contrast to our hypothesis, several recent studies have proposed a protective role for BMK1 activation in cardiovascular injury (*11*, *45*). With regard to such a model, it seems undeniable that the activation of BMK1 in the cuff-injured artery was the result of a compensatory response to the injury. However, it has also been reported that BMK1 activation is involved in cardiovascular diseases, as shown by studies using gene-targeted mice lacking BMK1 (*2*). Wang *et al*. have reported that BMK1 activation is involved in the hypertrophic remodeling of the heart as one of the compensatory pathways against pressure overload (*46*). Cameron *et al*. demonstrated that BMK1 activation attenuates the cardiac injury which occurs after myocardial ischemia and reperfusion (*47*). Additionally, we have recently reported that cardiac BMK1 is significantly activated in angiotensin II–stimulated mice exhibiting cardiac hypertrophy and fibrosis (*5*). All of these reports support the suggestion that BMK1 activation might be one of the signs of the progression of cardiac diseases. To uncover the pathophysiological significance in greater detail, further studies will certainly be needed, but the present findings do strongly suggest that BMK1 activation plays a significant role in the progression of atherosclerosis and other cardiovascular diseases.

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