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Dose-dependent Biphasic Activity of tRNA Synthetase-associating Factor, p43, in Angiogenesis*

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Mammalian aminoacyl tRNA synthetases form a macromolecular protein complex with three non-enzymatic cofactors. Among these factors, p43 is also secreted to work as a cytokine on endothelial as well as immune cells. Here we investigated the activity of p43 in angiogenesis and determined the related mediators. It promoted the migration of endothelial cells at low dose but induced their apoptosis at high dose. p43 at low concentration activated extracellular signal-regulating kinase, which resulted in the induction and activation of matrix metalloproteinase 9. In contrast, p43 at high concentration activated Jun N-terminal kinase, which mediated apoptosis of endothelial cells. These results suggest that p43 is a novel cytokine playing a dose-dependent biphasic role in angiogenesis.

Aminoacyl-tRNA synthetases (ARSs)[1] are essential enzymes catalyzing the first step of protein synthesis. Several mammalian tRNA synthetases form a macromolecular protein complex with three auxiliary factors, p43, p38, and p18 (1–3). However, the structure and function of this complex have not been fully understood. Although the component enzymes make specific protein-protein interactions via their non-catalytic peptide appendices (4–6) as well as catalytic core domain (7), the assembly and stability of the whole complex are mainly contributed to by one of the three cofactors, p38 (8), which is in contact with many components of the complex (9, 10). Because components dissociated from the multi-ARS complex were subjected to degradation process, at least one function of the complex formation appears to be the maintenance of the cellular stability of the complex-forming enzymes and cofactors (8).

The structure and activity of another complex-associating factor, p43, have been most extensively studied. It was proposed to be located in the center of the complex (11) and binds arginyl-tRNA synthetase (12) as well as tRNA (13) to facilitate the catalysis of the enzyme. Surprisingly, p43 itself is also secreted to work on immune cells and triggers pro-inflammatory response (14, 15). In addition, it showed a potential to interact with the α subunit of ATP synthase (16), which was previously shown to mediate anti-angiogenic activity of angiostatin (17, 18). p43 is also proteolytically cleaved under apoptotic conditions (14, 19). In addition, the C-terminal domain shares homology with the equivalent part of mammalian tyrosyl-tRNA synthetase that is processed to function as two distinct cytokines (20). Another class I tRNA synthetase, tryptophanyl-tRNA synthetase, also showed potent angiostatic activity (21). All of these previous reports led us to expect that the secreted p43 may play an important role in angiogenesis as well as in the inflammation process. Here we investigated the activity of p43 in angiogenesis using various *in vitro* and *in vivo* models and found that p43 shows dose-dependent biphasic activity in angiogenesis.

EXPERIMENTAL PROCEDURES

Cell Culture and Materials—Bovine aorta endothelial cells (BAECs) were isolated from descending thoracic aortas and grown in Dulbecco's modified Eagle's medium containing 20% fetal bovine serum. The primary cells used in this study were between passages 5 and 10. Ac-YVAD-*p*NA, Ac-DEVD-*p*NA, and z-DEVD-fmk were purchased from Calbiochem, and PD98059, SB203580, and SB202190 were purchased from BIOMOL. The antibodies specific to three mitogen-activated protein kinases, ERK1/2, p38 MAPK, and JNK, were obtained from New England BioLabs. JNK dominant negative mutant (JNK-DN) and Jun-binding domain (JBD) of Jun-interacting protein were kind gifts from Dr. E. J. Choi (Korea University, Korea). A Transwell chamber for the endothelial cell migration assay and VEGF were purchased from Corning and R&D systems, respectively.

Purification of p43 and Its Domains—p43 (312 aa) and its N (146 aa)- and C (166 aa)-terminal domains were expressed as His tag fusion protein in *Escherichia coli* BL21(DE3) and purified by nickel affinity and Mono S ion-exchange chromatography (14). To remove lipopolysaccharide (LPS), the protein solution was dialyzed in pyrogen-free buffer (10 mM potassium phosphate buffer, pH 6.0, 100 mM NaCl). After dialysis, the p43 solution was loaded to polymyxin resin (Bio-Rad) pre-equilibrated with the same buffer, incubated for 20 min, and eluted. To further remove residual LPS, the protein solution was dialyzed against PBS containing 20% glycerol and filtered with Posidyne membrane (Pall Gelman laboratory). The concentration of the LPS in p43 was below 20 pg/ml as determined by using the Limulus Amebocyte lysate QCL-1000 kit (BioWhittaker).

Angiogenesis Assays—The activity of p43 in angiogenesis was determined using various *in vitro* and *in vivo* assays. For chorioallantoic membrane (CAM) assay, fertilized chick eggs were incubated in the humidified egg breeder at 37 °C. On the third day of incubation, about 2 ml of egg albumin was removed by an 18-gauge hypodermic needle to detach the developing CAM from the shell. After the incubation for an

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[1] The abbreviations used are: ARS, aminoacyl-tRNA synthetase; RT, reverse transcription; BAEC, bovine aorta endothelial cells; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; DN, dominant negative; JBD, Jun-binding domain; VEGF, vascular epidermal growth factor; aa, amino acid(s); LPS, lipopolysaccharide; CAM, chorioallantoic membrane; DMEM, Dulbecco's modified Eagle's medium; *p*NA, *p*-nitroanilide; MMP, matrix metalloproteinase; GST, glutathione *S*-transferase; EMAP II, endothelial monocyte activating polypeptide II.

additional 6 days, Thermanox coverslips (Nunc) loaded with 0, 0.1, or 1 μg of p43 were placed on the CAM surface, and the remodeling of vascularization was observed after 3 days. The total length of blood vessels within the area of the coverslips was determined by Image-Pro Plus (Media Cybernetics). For tube formation assay, BAECs (5×10^5 cells) were cultivated on Matrigel in the presence of 0, 1, or 100 nM p43 at 37 °C for 18 h. The changes of cell morphology were then captured by phase-contrast microscopy. For the *in vitro* cell migration assay, the cultivated BAECs were wounded with a razor blade and incubated in the media containing 0, 1, or 100 nM p43. The cells were allowed to migrate for 16 h, then fixed with absolute methanol and stained with Giemsa. The BAEC migration assays were performed by using a Transwell chamber (24-well chamber) with polycarbonate membranes (8.0-μm pore size, Costar) as described with slight modifications (22). The wells were coated with 0.5 mg/ml gelatin (Sigma) in phosphate-buffered saline and allowed to air-dry. BAECs were suspended in serum-free DMEM and added to the upper chamber at $2–5 \times 10^4$ cells per well. A chemotactic stimulus, VEGF (0.7 nM), or one of the indicated concentrations of p43 was placed in the lower chamber, and the cells were allowed to migrate for 7 h at 37 °C in a 5% CO_2 incubator. After incubation, non-migrant cells were removed from the upper face of the membrane with a cotton swab. The migrant cells (those attached to lower face) were fixed in 100% methanol and visualized by the hematoxylin (Sigma) staining. The migrant cells were counted in high power fields.

Apoptosis Assays—The *in situ* apoptosis detection was performed by using the Apoptag™ fluorescein kit (Oncor) according to the manufacturer's protocol. Apoptotic nuclei were visualized by using a confocal laser scanning microscope (Bio-Rad MRC 1024). To determine the degradation of chromosomal DNA into the nucleosome-sized fragments, a 500-μl aliquot of the lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2×10^5 cells) and incubated at 37 °C overnight. DNA was obtained by consecutive 1.5 M NaCl and ethanol precipitation, treated with RNase (200 μg/ml), separated in a 1.8% agarose gel, and visualized under UV light. For the caspase assay, BAECs (2×10^6 cells) were treated with or without p43 (100 nM) for 16 h and then lysed with 300 μl of the chilled cell lysis buffer (20 mM HEPES, pH 7.5, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride). The cell lysates were centrifuged at $15,000 \times g$ for 5 min at 4 °C, and the supernatant fractions were used to measure the activities of caspase-1 and -3. The protein extracts (40 μg) of the cell lysates were incubated for 2 h at 30 °C in the assay buffer (20 mM HEPES, pH 7.5, 2 mM dithiothreitol, and 10% glycerol) containing 100 μM caspase-3 substrate, Ac-DEVD-*p*-nitroanilide, or the caspase-1 substrate, Ac-YVAD-*p*-nitroanilide. The amount of *p*-nitroaniline released by the caspase activation was quantitated by the optical density at 405 nm.

Determination of MAPK Activation and in Vitro JNK Kinase Assay—BAECs treated with different concentrations of p43 were washed twice with cold phosphate-buffered saline, lysed with the lysis buffer (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 12 mM β-glycerophosphate, 1 mM dithiothreitol, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 0.1 mM phenylmethylsulfonyl fluoride) containing protease inhibitor mixture (Roche Molecular Biochemicals). The proteins in the lysates were resolved by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore). Antigens were visualized by sequential treatment with specific antibodies, horseradish peroxidase-conjugated secondary antibodies, and an enhanced chemiluminescence substrate kit. JNK immunocomplex *in vitro* kinase assay was performed as described previously (23).

Zymographic Assay—BAECs were seeded onto six-well plates in DMEM containing 20% fetal bovine serum, and cultured to 70–80% confluency. The cells were then washed two times with DMEM with 2% fetal bovine serum and cultured for additional 2 h, and then p43 was added at the indicated concentrations. After 24 h, the media were collected and mixed with 5× FOD buffer (4% SDS, 20% glycerol, 0.01% bromphenol blue, and 125 mM Tris-HCl, pH 6.8). The samples were subjected to 7.5% SDS-PAGE with the gel containing 1 mg/ml gelatin (Sigma). After electrophoresis, the gel was washed two times with 2.5% Triton X-100, briefly with distilled water, and incubated with the reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, 150 mM NaCl, 1% Triton X-100, and 0.002% sodium azide) overnight at 37 °C. The gel was stained with 0.2% Coomassie Brilliant Blue R-250 and destained with 35% methanol.

RT-PCR—Reverse transcription reaction was performed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) following the protocol provided by the manufacturer. The primer sequences for MMP9 cDNA were 5′-GGCAGCTGGCAGAGGAATACCTGT-3′ (forward) and 5′-GGTGGCGCACCAGCGGTAGCCGTC-3′ (reverse). The primer sequences for glyceraldehyde-3-phosphate dehydrogenase cDNA were 5′-TCATTGACCTCAACTACATG-3′ (forward) and 5′-CCAAAGTTGTCATGGATGAC-3′ (reverse). The reaction mixture was first denatured at 95 °C for 5 min, and the PCR condition was 94 °C/1 min, 55 °C/1 min, and 72 °C/1min for 25 cycles, followed by 72 °C for 5 min.

DNA Transfection for Apoptosis—For gene transfection, BAECs were grown overnight in six-well plates and washed in Hanks' buffered salt solution prior to transfection using the method of adenovirus conjugated to polylysine as described previously (24). Empty vector (pcDNA3.1) or the vector expressing JNK-DN or JBD (2 μg) were transfected into BAECs along with 2 μg of the vector expressing enhanced green fluorescent protein, and expressed for 24 h. The transfected cells were treated with p43 (20 nM) for 24 h, and then cell death was determined by counting the apoptotic cells using fluorescence microscopy. The percentage of apoptotic cells was determined by dividing the number of green cells with apoptotic morphology with the total number of green cells. Empty vector was used as the control and gave about 15% or less apoptotic cells.

RESULTS

Dose-sensitive Induction of the Endothelial Cell Migration by p43—The activity of p43 in angiogenesis was determined by several different experiments. In the chorioallantoic membrane assay, the coverslips loaded with the different amounts of p43 were placed on the surface of the membrane. In eight out of ten tested eggs, blood vessels were attracted to the area to which the low dose of p43 (0.1 μg) was spotted, whereas this effect was not observed at the high dose (1 μg) (Fig. 1*A*). The total length of the blood vessel within the area of the coverslip was ~2.2-fold increased with 0.1 μg of p43 but about 0.3-fold decreased with 1 μg of p43 compared with the control. The effect of p43 on tube formation was tested on Matrigel. BAECs were cultivated on Matrigel containing different amounts of p43. The stimulation of tube formation was observed at 1 nM but not at 100 nM p43 (Fig. 1*B*). Third, the activity of p43 was also tested by wound migration assay. In this assay, the cultivated BAECs were scraped with a razor blade and then allowed to migrate in the presence of different concentrations of p43. The cell migration was enhanced at 1 nM but not at 100 nM p43 (Fig. 1*C*). Finally, the chemotactic activity of p43 on BAECs was tested using the Transwell migration system. Different amounts of p43 were added to the lower chamber, and the cells migrating from the upper to lower chamber were counted. The migrated cells were stained with hematoxylin, and the cell counting was performed in high power fields. The cell migration was increased to about 4-fold at 1 nM p43, but the effect of p43 was decreased at the concentrations higher than 1 nM (Fig. 1*D*). Thus, all of these experimental results suggest that p43 may induce the endothelial cell migration but the effect is dose-sensitive.

p43 Induces Apoptosis of the Endothelial Cells at High Concentration—As shown above, the stimulatory effect of p43 on the endothelial cell migration was abolished as its concentration was increased. We have already shown that the endothelial cell proliferation was blocked at high concentrations of p43 (16). Here we investigated whether p43 can induce the death of the endothelial cells at high concentration using BAECs. The effect of p43 on the death of BAECs was monitored by the cell morphology and other typical markers for apoptosis. The number of apoptotic cells was dramatically increased from 10 nM p43 (Fig. 2*A*). The endothelial cell death was further confirmed at 100 nM p43 by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining, DNA laddering, and the activation of caspase-3 (Fig. 2, *B*, *C*, and *D*, respectively). All of the results indicate that p43 may induce apoptosis of the endothelial cells at high concentration.

MAPKs Are Differentially Activated by p43—We have previously shown that p43 activates three major mitogen-activated protein kinases (MAPKs) in monocytes (14, 15). Thus, we tested whether these kinases are also affected by p43 in the

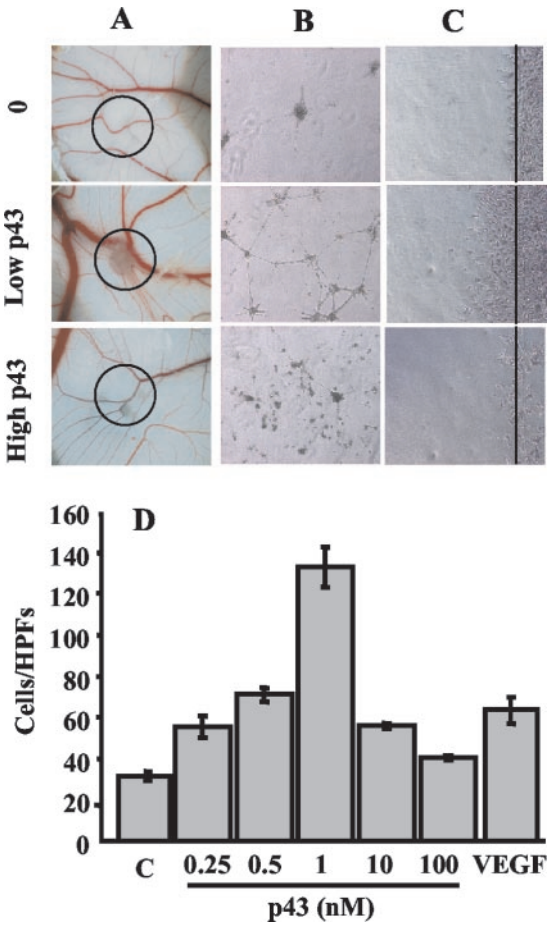

FIG. 1. **p43 induces the endothelial cell migration at low dose.** *A*, the coverslips containing 0, 0.1 (low), and 1 μg (high) of p43 were loaded on the chorioallantoic membrane (*circles*) of the fertilized eggs, and the p43-induced remodeling of vascularization was monitored. *B*, the tube formation of BAECs was observed by phase-contrast microscopy on the Matrigels containing 0, 1 (low), and 100 nM (high) p43. *C*, the BAECs on the culture dishes were scraped with a razor blade and allowed to migrate in the media containing 0, 1 (low), and 100 nM (high) p43. The *lines* stand for the boundary of the wounds introduced by the razor blade. *D*, the effect of p43 on the endothelial cell migration was assayed as described under "Experimental Procedures" using a Transwell chamber with gelatin-coated polycarbonate membrane. BAECs were suspended in the upper chamber, and the indicated concentrations of p43 were filled in the lower chamber. VEGF (0.7 nM) was used as a positive control. The cells migrating to the lower chambers were stained with hematoxylin and counted in high power fields. The data are the averages of the three independent experiments.

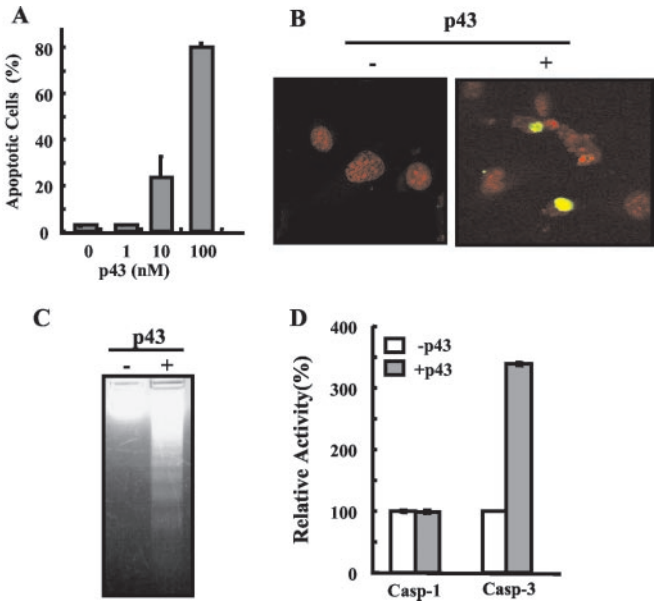

FIG. 2. **p43 induces apoptosis of the endothelial cells at high dose.** *A*, BAECs were treated with the indicated concentrations of p43 for 24 h, and the apoptotic cells were counted by morphological characteristics. *B*, BAECs were treated with 0 (−) and 100 nM (+) p43 and followed by the *in situ* apoptosis staining (*green*). The nuclei were stained with propidium iodide (*red*). *C*, DNA laddering of BAECs treated with 0 (−) and 100 nM (+) p43. After the p43 treatment for 24 h, the nucleosomal fragmentation of the cellular DNA was analyzed by 1.8% agarose gel electrophoresis. *D*, the activities of caspase-1 and -3 were measured from BAECs treated with 0 or 100 nM p43 for 16 h as described under "Experimental Procedures."

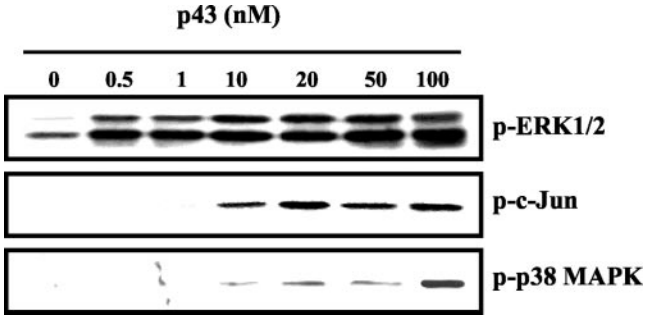

FIG. 3. **Three MAPKs are differentially activated by p43.** The effect of p43 on the activities of three MAPKs (ERK1/2, JNK, and p38 MAPK) was investigated in BAECs. BAECs were treated with the indicated amounts of p43 for 1 h, and the activity of each MAPK was determined as described in previously (14). "*p-*" stands for the phosphorylated form of each protein.

endothelial cells. The activity changes of these proteins were monitored after BAECs were treated with different concentrations of p43. Although all of the three kinases were activated by p43, they responded to different concentrations of p43 (Fig. 3). Although ERK was activated from 0.5 nM p43, the activation of JNK was apparent from 10 nM. The activity of p38 MAPK was increased only at 100 nM p43. Based on this result, we expected that ERK and JNK could be involved in the p43-induced migration and apoptosis of the endothelial cells, respectively.

ERK Is Responsible for the p43-dependent Endothelial Cell Migration and the Activation of MMP9—Because the endothelial cell migration and the activation of ERK occurred at similar concentration of p43 (about 1 nM), we investigated whether ERK mediates the induction of the endothelial cell migration by p43. The activities of ERK and p38 MAPK were suppressed by the treatment of their specific inhibitors, PD98059 and SB203580, respectively. BAECs were incubated with each of these inhibitors in the upper chamber of the Transwell membrane system, and the cell migration was induced with 1 nM p43 in the lower chamber. The cell migration was specifically inhibited by the treatment of PD98059, suggesting that ERK is responsible for the p43-induced cell migration (Fig. 4*A*).

Matrix metalloproteinases (MMPs) secreted by endothelial cells are considered to play a key role in the processes of the matrix remodeling and endothelial cell migration during angiogenesis (25, 26). Particularly the gelatinases, MMP2 and MMP9, capable of degrading native collagen type IV that is the major constituent of basement membranes, are involved in the vascular cell migration and invasion (27, 28). Because we used the gelatin-coated membrane for the Transwell cell migration assay (Fig. 1*D*), we tested whether these two proteinases are involved in the p43-induced cell migration. The activities of these two enzymes were determined by their ability to digest gelatin in the gel matrix as described under "Experimental Procedures." The activity of MMP9 was dramatically enhanced at 1 nM p43 and decreased at the higher concentrations,

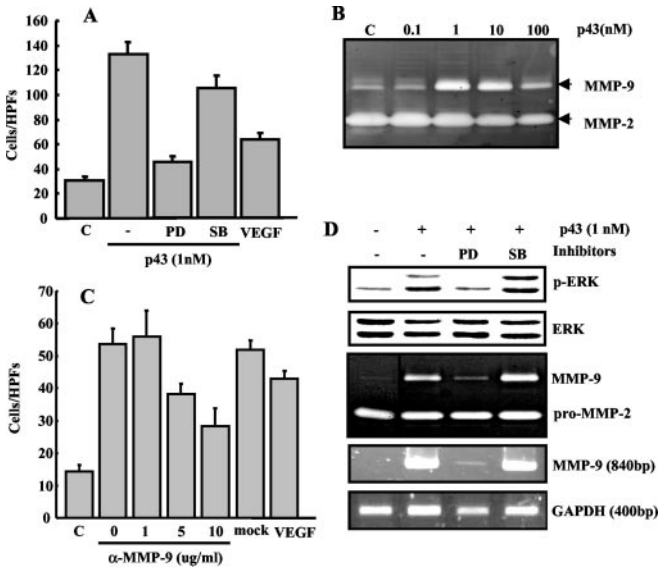

FIG. 4. **ERK is responsible for the p43-induced endothelial cell migration.** *A*, BAECs were suspended with 20 μM PD98059 (*PD*) or 10 μM of SB203580 (*SB*) in the upper chamber, and 1 nM p43 was added to the lower chamber. The cell migration was monitored as described under "Experimental Procedures." VEGF (0.7 nM) was used as positive control. *B*, BAECs were treated with the indicated concentrations of p43, and the activities of MMP2 and -9 were determined by zymographic assay as described under "Experimental Procedures." *C*, the indicated amounts of anti-MMP9 antibody were mixed with BAECS in the upper chamber in Transwell, whereas 1 nM p43 was added to the lower chamber. Mock IgG was added to the concentration of 5 μg/ml. The values are the averages of the two independent experiments. *D*, BAECs were pretreated with 20 μM PD98059 or 10 μM SB203580 for 1 h and treated with 1 nM p43. For the assays of the ERK1/2 phosphorylation, the level of the MMP9 transcript and the activity of MMP9, BAECs were cultured for 1, 12, and 24 h, respectively. RT-PCR and zymography were used to determine the expression and activation of MMP9, respectively. *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) shows that the equal amounts of total RNA were used for RT-PCR.

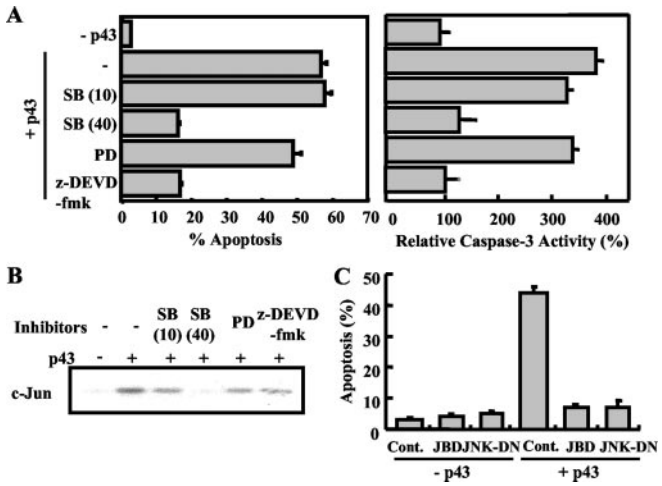

FIG. 5. **JNK mediates for the p43-induced apoptosis.** *A*, BAECs were pretreated with 10 and 40 μM SB202190, 20 μM PD98059, and z-DEVD-fmk for 1 h to inhibit p38 MAPK, JNK, ERK1/2, and caspase-3, respectively, and then treated with 20 nM p43. The number of the apoptotic cells and caspase-3 activity was measured 16 h after the p43 treatment as described under "Experimental Procedures." *B*, BAECs were pretreated with different inhibitors and treated with 20 nM p43. 1 h after the p43 treatment, the JNK activity was measured as described under "Experimental Procedures." *C*, JBD (Jun-binding domain) of Jun-interacting protein and JNK-DN were expressed in BAECs to specifically block the activation of JNK, and the effect of p43 (20 nM) on the cell death was compared. The values for the apoptosis are the averages of the three independent experiments.

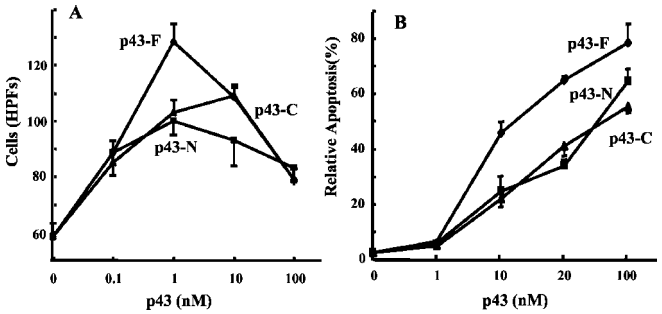

FIG. 6. **Comparison of the full-length p43 and its N- and C-terminal domains for endothelial cell migration and death.** The three different polypeptides of p43 were compared for their activities for inducing endothelial cell migration using a Transwell chamber (*A*) and cell death (*B*) as described under "Experimental Procedures." *p43-F*, *-N*, and *-C* stand for 1–312, 1–146, and 147–312 polypeptides of human p43.

whereas MMP2 gradually decreased as the concentration of p43 was increased (Fig. 4*B*).

To see whether the p43-induced MMP9 is responsible for the cell migration, BAECs were treated with different amounts of anti-MMP9 antibody (26), and the cell migration was induced by 1 nM p43. The cell migration was blocked by the treatment of anti-MMP antibody in a dose-dependent manner while mock antibody did not give a significant effect (Fig. 4*C*), indicating that MMP9 is directly involved in the p43-induced cell migration. To determine the functional linkage of MMP9 and ERK in the p43-induced cell migration, the activity of ERK and the expression and activation of MMP9 were determined in BAECs treated with 1 nM p43. As expected, the activation of ERK by p43 was blocked with PD98059 and but not with SB203580 (Fig. 4*D*), consistent with the effect of PD98059 on the p43-induced cell migration (Fig. 4*A*). The expression and activity of MMP9 were monitored by RT-PCR and zymography, respectively. p43 enhanced both of the expression and activity of MMP9, and the stimulatory effect was also blocked by PD98059 (Fig. 4*D*). Thus, the p43-induced endothelial cell migration is mediated by MMP9 that is induced and activated by ERK.

JNK Mediates the p43-induced Endothelial Cell Death—p43 induced apoptosis of endothelial cells and activated JNK from 10 nM (Figs. 2 and 3). Here we investigated whether JNK is responsible for the p43-induced apoptosis. To address this question, we used SB202190 that inhibits p38 MAPK at 10 μM and blocks both p38 MAPK and JNK at 40 μM (29, 30). BAECs were pretreated with each concentration (10 or 40 μM) of SB202190, PD98059, and the caspase-3 inhibitor, z-DEVD-fmk, and subsequently with 20 nM p43. The induction of apoptosis was determined by cell morphology and the activation of caspase-3. Although the p43-induced apoptosis was not affected by the treatment of SB202190 (10 μM) or PD98059, it was inhibited by the pre-treatment of SB202190 (40 μM) or z-DEVD-fmk (Fig. 5*A*). The activity of JNK was determined by the phosphorylation of GST-Jun as the reaction substrate. The phosphorylation of c-Jun was completely inhibited only with SB202190 (40 μM) among different inhibitors (Fig. 5*B*). These results suggest that JNK, but not p38 MAPK and ERK, should be involved in the p43-induced apoptosis.

To confirm the involvement of JNK in the p43-induced apoptosis more specifically, we used JNK-binding domain (JBD) of JNK-interacting protein 1 or JNK dominant negative form of JNK (JNK-DN), which can block the activity of JNK (31, 32). JNK-DN and JBD were expressed in BAECs by adenoviral transfection as determined by immunoblotting (data not shown). Whereas about 45% of BAECs were turned to apoptotic cells by p43, the effect of p43 was blocked by the

expression of JBD or JNK-DN (Fig. 5*C*). These results clearly indicate that JNK mediates the p43-induced apoptosis of the endothelial cells.

Deletion Mapping of p43 for Endothelial Cell Migration and Apoptosis—We have previously shown that the various truncated p43 fragments retained the activity inducing tumor necrosis factor and interleukin-8 (14). To determine whether the biphasic activity of p43 on endothelial cells can be separated, depending on the peptide region, we have prepared the 146-aa N-terminal and 166-aa C-terminal domains of p43 and compared them with the full-length p43 in the induction of endothelial cell migration and death. In the endothelial cell migration, all of the three polypeptides showed a dose-dependent curve (Fig. 6*A*). However, the maximum effect on the cell migration was shown at 10 nM of the C-terminal domain of p43 but at 1 nM of the full-length p43 and its N-terminal domain. All of the three polypeptides showed the activity inducing endothelial cell death in dose-dependent manner (Fig. 6*B*). In both cases, the full-length p43 showed the highest activity, although all of the three polypeptides showed the similar pattern of the activity. These results suggest that the activities for the endothelial cell migration and apoptosis are not separable by the different domains of p43, but its whole structure is involved for the full activity.

DISCUSSION

p43 is cleaved upon apoptosis to generate its C-terminal domain, which was previously called endothelial monocyte activating polypeptide II (EMAP II) (13, 33). For this reason, p43 has been considered as the precursor for the cytokine, EMAP II. However, we have recently shown that p43, and not EMAP II, is secreted to work as a pro-inflammatory cytokine (14). Nonetheless, EMAP II itself showed a potent anti-angiogenic activity inducing apoptosis of endothelial cells (34, 35). In this work, we found that p43 can induce the migration as well as death of the endothelial cells in a dose-dependent manner (Figs. 1 and 2). Interestingly, both of the N- and the C-terminal EMAP II domains also showed dose-dependent bell-shaped activity in the induction of endothelial cell migration as the full-length p43, although their activities were lower than the full-length p43 (Fig. 6*A*). Thus, the two distinct activities on endothelial cells do not seem to be determined by different structural units but rather dispersed throughout the polypeptide of p43. The p43-dependent signal pathway leads to the activation ERK and MMP9 that is responsible for the endothelial cell migration (Figs. 2 and 4). This result is consistent with the previous reports that the ERK activation is required to induce MMP9 in vascular smooth muscle cells and endothelial cells (36, 37). However, p43 showed little, or very weak if any, effect on the endothelial cell proliferation at concentrations lower than 10 nM (data not shown). Instead, the proliferation of the endothelial cells is inhibited by p43 at higher concentrations (Ref. 16).[2] Thus, the pro-angiogenic activity of p43 appears to result mainly from its capability of inducing migration of the endothelial cells.

The MMP9 activity was significantly decreased at 100 nM p43 (Fig. 4*B*), whereas ERK still remained active (Fig. 3). In addition, MMP9 was still weakly induced and activated when the ERK activity was completely inhibited by the treatment of 20 μM PD98059 (Fig. 4*D*). Likewise, the endothelial cell migration was not completely blocked when the ERK activity was blocked with 20 μM PD98059 or with anti-MMP9 antibody (Fig. 4, *A* and *C*). All of these results implicate that the other signal pathways independent of ERK should be also involved in the endothelial cell migration induced by p43.

p43 also caused apoptosis of the endothelial cells (Fig. 2) via JNK (Fig. 5). It is known that JNK is involved in apoptosis induced by various stimuli such as tumor necrosis factor, ceramide, irradiation, or heat shock (31, 38, 39). The involvement of JNK in apoptosis of the endothelial cells has been also reported previously (40). Although the treatment of SB202190 (40 μM) completely blocked the JNK activity (Fig. 5*B*), apoptosis of the endothelial cells still occurred at a little higher rate than the p43-untreated cells (Fig. 5*A*). Thus, JNK does not appear to be the only mediator for the JNK-induced apoptosis of the endothelial cells.

Angiogenesis is a complex biological process that is determined by the combined effect of multiple factors with different activities. For this reason, the effect of a specific protein factor on angiogenesis may be determined by the balance with other factors near the responding endothelial cells. The biphasic mode of activity in a single protein appears to give an additional complexity in the regulation of angiogenic process. The biphasic activity has been also reported in other signaling molecules such as transforming growth factor-β1 (41), thrombospondin-1 (42), and estrogen (43). Although the detailed mechanism to control their activities may vary, the dual mode of the activity appears to be required for the fine control of angiogenesis.

Although we have previously shown that the N-terminal domain of p43 is responsible for its secretion (14), it does not have any clear sequence motif for secretion. Interestingly, the same domain in p43 is also involved in its association with the multi-ARS complex (12). Also, p43, if not associated with the complex, appears to be unstable in the cell (8). Thus, the activity and cellular turnover of p43 appears to be under complex control. It would be interesting to see whether p43 is secreted independently of the multi-ARS complex or is first held within the multi-ARS complex and then secreted upon appropriate condition or signal.

[2] S. G. Park, Y.-S. Kang, Y. H. Ahn, S. H. Lee, K.-R. Kim, K.-W. Kim, G. Y. Koh, Y.-G. Ko, and S. Kim, unpublished data.

REFERENCES

1. Kisselev, L. L., and Wolfson, A. D. (1994) *Prog. Nucleic Acids Res. Mol. Biol.* **48,** 83–142
2. Mirande, M. (1991) *Prog. Nucleic Acids Res. Mol. Biol.* **40,** 95–142
3. Yang, D. C. H. (1996) *Curr. Top. Cell Regul.* **34,** 101–136
4. Rho, S. B., Lee, K. H., Kim, J. W., Shiba, K., Jo, Y. J., and Kim, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 10128–10133
5. Rho, S. B., Lee, J. S., Jeong, E. J., Kim, K. S., Kim, Y. G., and Kim, S. (1998) *J. Biol. Chem.* **273,** 11267–11273
6. Rho, S. B., Kim, M. J., Lee, J. S., Seol, W., Motegi, H., Kim, S., and Shiba, K. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4488–4493
7. Kim, T., Park, S. G., Kim, J. E., Seol, W., Ko, Y. G., and Kim, S. (2000) *J. Biol. Chem.* **275,** 21768–21772
8. Kim, J. Y., Kang, Y. S., Lee, J. W., Kim, H. J., Ahn, Y. H., Park, H., Ko, Y. G., and Kim, S. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 7912–7916
9. Quevillon, S., Robinson, J.-C., Berthonneau, E., Siatecka, M., and Mirande, M. (1999) *J. Mol. Biol.* **285,** 183–195
10. Robinson, J. C., Kerjan, P., and Mirande, M. (2000) *J. Mol. Biol.* **304,** 989–994
11. Norcum, M. T., and Warrington, J. A. (2000) *J. Biol. Chem.* **275,** 17921–17924
12. Park, S. G., Jung, K. H., Lee, J. S., Jo, Y. J., Motegi, H., Kim, S., and Shiba, K. (1999) *J. Biol. Chem.* **274,** 16673–16676
13. Shalak, V., Kaminska, M., Mitnacht-Kraus, R., Vandenabeele, P., Clauss, M., and Mirande, M. (2001) *J. Biol. Chem.* **276,** 23769–23776
14. Ko, Y.-G., Park, H., Kim, T., Lee, J.-W., Park, S. G., Seol, W., Kim, J. E., Lee, W.-H., Kim, S.-H., Park, J. E., and Kim, S. (2001) *J. Biol. Chem.* **276,** 23028–23033
15. Park, H., Park, S. G., Lee, J.-W., Kim, T., Kim, G., Ko, Y.-G., and Kim, S. (2002) *J. Leukoc. Biol.* **71,** 223–230
16. Chang, S. Y., Park, S. G., Kim, S., and Kang, C. Y. (2002) *J. Biol. Chem.* **277,** 8388–8394
17. Moser, T. L., Stack, M. S., Asplin, I., Enghild, J. J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H. W., and Pizzo, S. V. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 2811–2816
18. Moser, T. L., Kenan, D. J., Ashley, T. A., Roy, J. A., Goodman, M. D., Misra, U. K., Cheek, D. J., and Pizzo, S. V. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 6656–6661
19. Knies, U. E., Behrensdorf, H. A., Mitchell, C. A., Deutsch, U., Risau, W., Drexler, H. C. A., and Cluass, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12322–12327
20. Wakasugi, K., and Schimmel, P. (1999) *Science* **284,** 147–151

21. Wakasugi, K., Slike, B. M., Hood, J., Otani, A., Ewalt, K. L., Friedlander, M., Cheresh, D. A., and Schimmel, P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 173–177
22. Wakasugi, K., and Schimmel, P. (1999) *J. Biol. Chem.* **274,** 23155–23159
23. Ko, Y. G., Kang, Y. S., Park, H., Seol, W., Kim, J., Kim, T., Park, H. S., Choi, E. J., and Kim, S. (2001) *J. Biol. Chem.* **276,** 39103–39106
24. Jo, H., Sipos, K., Go, Y. M., Law, R., Rong, J., and McDonald, J. M. (1997) *J. Biol. Chem.* **272,** 1395–1401
25. Fisher, C., Gilbertson-Beadling, S., Powers, E. A., Petzold, G., Poorman, R., and Mitchell, M. A. (1994) *Dev. Biol.* **162,** 499–510
26. Schnaper, H. W., Grant, D. S., Stetler-Stevenson, W. G., Fridman, R., D'Orazi, G., Murphy, A. N., Bird, R. E., Hoythya, M., Fuerst, T. R., and French, D. L. (1993) *J. Cell. Physiol.* **156,** 235–246
27. Kraling, B. M., Wiederschain, D. G., Boehm, T., Rehn, M., Mulliken, J. B., and Moses, M. A. (1999) *J. Cell Sci.* **112,** 1599–1609
28. Puyraimond, A., Weitzman, J. B., Babiole, E., and Menashi, S. (1999) *J. Cell Sci.* **112,** 1283–1290
29. Jacinto, E., Werlen, G., and Karin, M. (1998) *Immunity* **8,** 31–41
30. Porter, C. M., Havens, M. A., and Clipstone, N. A. (2000) *J. Biol. Chem.* **275,** 3543–3551
31. Verheij, M., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Birrer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. N. (1996) *Nature* **380,** 75–79
32. Harding, T. C., Xue, L., Bienemann, A., Haywood, D., Dickens, M., Tolkovsky, A. M., and Uney, J. B. (2001) *J. Biol. Chem.* **276,** 4531–4534
33. Quevillon, S., Agou, F., Robinson, J.-C., and Mirande, M. (1997) *J. Biol. Chem.* **272,** 32573–32579
34. Berger, A. C., Alexander, H. R., Tang, G., Wu, P. S., Hewitt, S. M., Turner, E., Kruger, E., Figg, W. D., Grove, A., Kohn, E., Stern, D., and Libutti, S. K. (2000) *Microvasc. Res.* **60,** 70–80
35. Schwarz, M. A., Kandel, J., Brett, J., Li, J., Hayward, J., Schwarz, R. E., Chappey, O., Wautier, J. L., Chabot, J., Lo Gerfo, P., and Stern, D. (1999) *J. Exp. Med.* **190,** 341–354
36. Genersch, E., Hayess, K., Neuenfeld, Y., and Haller, H. (2000) *J. Cell Sci.* **113,** 4319–4330
37. Khan, K. M., Falcone, D. J., and Kraemer, R. (2002) *J. Biol. Chem.* **277,** 2353–2359
38. Davis, R. J. (2000) *Cell* **103,** 239–252
39. Tournier, C., Hess, P., Yang, D. D., Xu, J., Turner, T. K., Nimnual, A., Bar-Sagi, D., Jones, S. N., Flavell, R. A., and Davis, R. J. (2000) *Science* **288,** 870–874
40. Ho, F. M., Liu, S. H., Liau, C. S., Huang, P. J., and Lin-Shiau, S. Y. (2000) *Circulation* **101,** 2618–2624
41. Pepper, M. S., Vassalli, J. D., Orci, L., and Montesano, R. (1993) *Exp. Cell Res.* **204,** 356–363
42. Pazouki, S., Pendleton, N., Heerkens, E., Smither, R. L., Moore, J. V., and Schor, A. M. (1996) *Biochem. Soc. Trans.* **24,** 368S
43. Banerjee, S. K., Campbell, D. R., Weston, A. P., and Banerjee, D. K. (1997) *Mol. Cell Biochem.* **177,** 97–105