The migration of endothelial cells in response to various stimulating factors plays an essential role in angiogenesis. The p38 MAPK pathway has been implicated to play an important role in endothelial cell migration because inhibiting p38 MAPK activity down-regulates vascular endothelial growth factor (VEGF)-stimulated migration. Currently, the signaling components in the p38 MAPK activation pathway and especially the mechanisms responsible for p38 MAPK-regulated endothelial cell migration are not well understood. In the present study, we found that p38 MAPK activity is required for endothelial cell migration stimulated by both VEGF and nongrowth factor stimuli, sphingosine 1-phosphate and soluble vascular cell adhesion molecule. By using dominant negative forms of signaling components in the p38 MAPK pathway, we identified that a regulatory pathway consisting of MKK3-p38α/γ-MAPK-activated protein kinase 2 participated in VEGF-stimulated migration. In further studies, we showed that a minimum of a 10-h treatment with SB203580 (specific p38 MAPK inhibitor) was needed to block VEGF-stimulated migration, suggesting an indirect role of p38 MAPK in this cellular event. Most interestingly, the occurrence of SB203580-induced migratory inhibition coincided with a reduction of urokinase plasminogen activator (uPA) expression. Furthermore, agents disrupting uPA and uPA receptor interaction abrogated VEGF-stimulated cell migration. These results suggest a possible association between cell migration and uPA expression. Indeed, VEGF-stimulated migration was not compromised by SB203580 in endothelial cells expressing the uPA transgene; however, VEGF-stimulated migration was inhibited by agents disrupting uPA-uPA receptor interaction. These results thus suggest that the p38 MAPK pathway participates in endothelial cell migration by regulating uPA expression.

The formation of new blood vessels, known as angiogenesis, is necessary for the growth and metastasis of many tumors (1, 2). Angiogenesis involves the activation, proliferation, migration, and reorganization of endothelial cells (1). The migration process is promoted by angiogenic stimulating factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor, sphingosine 1-phosphate (S-1-P), and soluble vascular cell adhesion molecule (VCAM) (3–6). Therapeutic approaches aimed at intercepting angiogenic factor-mediated signaling pathways show promising potential for the inhibition of tumor growth and metastasis (2, 7, 8).

The p38 mitogen-activated protein kinase (MAPK) family contains four members, namely p38α, -β, -γ, and -δ MAPKs (9). p38 MAPKs are activated by upstream MKK3 and MKK6 (9, 10). The effects of p38 MAPKs are mediated by various p38 MAPK substrates including MAPK-activated protein kinase 2 (MK2), MK3, and p38 MAPK-activated kinase (PRAK) (9, 10). In addition to the more defined role in inflammation and cell stress responses (11), p38 MAPK has also been implicated in the cytoskeleton reorganization and the cellular migration of various cell types (12–18). The use of specific inhibitors has demonstrated the importance of p38 MAPK in endothelial cell migration stimulated by angiogenic factors such as VEGF (19). However, the signaling components and the mechanisms of the p38 MAPK pathway involved in endothelial cell migration are not well defined.

Urokinase plasminogen activator (uPA) is a serine protease and, when bound to its receptor, uPAR, initiates the activation of metalloproteinases as well as the conversion of plasminogen to plasmin (20, 21). In addition, it also stimulates the migration of various cell types including smooth muscle and epithelial and endothelial cells (22–26). Blocking uPA or uPAR function with antagonists or down-regulating their expression can significantly impair cell migration (27), suggesting the importance of uPA/uPAR in cell migration. In fact, phosphatidylserinol 3-kinase and protein kinase C have been shown to regulate the motility of breast cancer cells by promoting uPA secretion (28, 29). We and others have demonstrated previously that uPA expression is regulated by the p38 MAPK pathway (30, 31), and this raises the likelihood that p38 MAPK participates in cell migration by regulating uPA expression.

The goal of the present study was to define the mechanisms responsible for p38 MAPK-regulated endothelial cell migration. We showed that p38 MAPK activity is required for VEGF-, S-1-P, and soluble VCAM-stimulated human endothelial cell migration. By using dominant negative forms of the signaling

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‡ To whom correspondence should be addressed: Dept. of Immunology, IMM-19, The Scripps Research Inst., 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-9211; Fax: 858-784-8472; E-mail: shuang@scripps.edu.

1 The abbreviations used are: VEGF, vascular endothelial growth factor; S-1-P, sphingosine 1-phosphate; VCAM, vascular cell adhesion molecule; MAPK, mitogen-activated protein kinase; MKK, MAPK kinase; MKK-activated protein kinase; PRAK, p38-activated kinase; uPA, urokinase plasminogen activator; uPAR, uPA receptor; HUVEC, human umbilical vascular endothelial cell; mAb, monoclonal antibody; RIPA, radioimmunoprecipitation assay; Ad, adenovirus.
components in the p38 MAPK signaling pathway, we identified that only MKK3, p38α/γ MAPK, and MK2 are involved in VEGF-stimulated endothelial cell migration. Furthermore, we found that p38 MAPK-dependent protein expression rather than direct p38 MAPK signaling is essential for VEGF-stimulated cell migration. Because uPA and uPAR are important for endothelial cell migration and their expressions are regulated by the p38 MAPK pathway in various cell types, we examined the involvement of uPA and uPAR in VEGF-stimulated endothelial cell migration. Our results indicate that p38 MAPK regulates uPA expression and that the expression of uPA and the ability of VEGF to stimulate cell migration are closely associated. By using endothelial cells with uPA transgene expression, we demonstrated that forced uPA expression was capable of rescuing VEGF-stimulated cell migration in p38 MAPK-inhibited conditions, which suggests that uPA may be the sole factor responsible for the role of p38 MAPK in VEGF-stimulated endothelial cell migration. Finally, we provide evidence that p38 MAPK-uPA regulated VEGF-stimulated cell migration by facilitating actin reorganization and focal adhesion assembly.

**EXPERIMENTAL PROCEDURES**

**Reagents and Cells—**VEGF and soluble VCAM were obtained from R&D Systems (Minneapolis, MN). S-1-P was purchased from BioMol (Plymouth Meeting, PA). SB203580 and SB202474 were obtained from Calbiochem. Human umbilical embryonic cells (HUVECs) were purchased from Cascade Biologics (Portland, OR) and were maintained in medium containing 20 ng/ml polyamines overnight at 4 °C.

**p38 MAPK, uPA, and Endothelial Cell Migration**

**RESULTS**

**p38 MAPK Activity Is Required for Stimulated Endothelial Cell Migration—**Various agents including VEGF, S-1-P, and soluble VCAM are capable of stimulating endothelial cell migration (3–6). Although it is known that VEGF-stimulated endothelial cell migration requires p38 MAPK activity (19), it has not been clearly shown whether p38 MAPK activity is required for endothelial cell migration stimulated by non-growth factor stimuli such as G-protein-coupled receptors and integrin ligand-soluble VCAM. To investigate this, we treated HUVECs with increasing concentrations of each stimulant, and we measured their ability to induce cell migration. All three stimulants enhanced HUVEC migration in dose-dependent manner with optimal concentrations at 10 ng/ml, 75 nM, and 15 ng/ml, respectively (Fig. 1A). Subsequently, we treated HUVECs with increasing concentrations of SB203580, a highly specific inhibitor for p38 MAPK, for 24 h and then examined the ability of VEGF, S-1-P, or soluble VCAM to stimulate cell migration. SB203580 at 10 μM completely blocked VEGF-, S-1-P, or soluble VCAM-stimulated HUVEC migration (Fig. 1B), whereas the basal level of cell migration was not significantly altered by the addition of SB203580 (data not shown). In control experiments, we treated the cells with the same concentration of SB202474 (a nonfunctional SB203580 structural analog) and did not detect any inhibitory effects on HUVEC migration (Fig. 1B). To rule out the possibility that SB203580 was toxic to HUVECs, we examined the cell viability of both untreated and SB203580-treated cells, and we found that SB203580 caused negligible toxicity on cell viability as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (data not shown). These results indicate that the p38 MAPK activity is required for endothelial cell migration induced by various stimuli but is not required for basal cell migration.

MKK3/p38α/γ-MK2 Pathway Is Involved in VEGF-stimulated Endothelial Cell Migration—The p38 MAPK pathway consists of a 3-tiered signaling cascade with multiple players at
each level, e.g. MKK3 and -6 at the MAPK kinase level, p38α, -β, -γ, and -δ at the MAPK level, and MK2, -3, and PRAK at the level of p38 MAPK downstream substrates (9, 10). To identify the specific kinases involved in VEGF-stimulated endothelial cell migration, we expressed dominant negative forms of these proteins in HUVECs with the aid of recombinant adenovirus. Cells were detached 36 h post-infection and then analyzed for VEGF-stimulated cell migration. The dominant negative forms of the molecules showed no significant effect on cell migration in the unstimulated cells (Fig. 2A). This is consistent with the results that SB203580 did not significantly affect basal HUVEC migration. However, dominant negative forms of MKK3, p38α, p38γ, and MK2, but not dominant negative forms of MKK6, p38β, p38δ, MK3, and PRAK, blocked VEGF-stimulated cell migration (Fig. 2A). Co-expressing dominant negative p38α and p38γ completely abrogated VEGF-stimulated migration (Fig. 2A). These results suggest that a pathway consisting of MKK3-p38α/γ-MK2 is involved in VEGF-stimulated endothelial cell migration.

To determine further the importance of MK2 in VEGF-stimulated endothelial cell migration, we retrovirally introduced constitutively active MK2 or PRAK into HUVECs. Mock control cells and HUVECs expressing constitutively active MK2 or PRAK displayed a similar extent of basal and VEGF-stimulated cell migration (Fig. 2B), suggesting that active MK2 or PRAK did not confer cells with greater basal motility. The addition of SB203580 failed to inhibit VEGF-stimulated cell migration in HUVECs expressing constitutively active MK2 (Fig. 2B). In contrast, SB203580 blocked VEGF-stimulated cell migration in both mock control and constitutively active PRAK-expressing HUVECs (Fig. 2B). These results suggest that the activity of MK2 alone is essential and sufficient for mediating VEGF-stimulated endothelial cell migration.

p38 MAPK Pathway-dependent Gene Expression Rather Than Direct p38 MAPK Signaling Is Responsible for VEGF-stimulated Endothelial Cell Migration—To define the mechanism responsible for p38 MAPK-regulated HUVEC migration, we initially conducted a time course measurement of SB203580 inhibition of p38 MAPK activity. The p38 activity was determined by measuring the phosphorylation extent of
MK2 (a direct p38 MAPK substrate). Because MK2 is activated through phosphorylation at Thr-222 and Thr-334 (35), we used an antibody that recognizes phospho-MK2 (Thr-334) to detect MK2 activity. HUVECs were treated with 10 μM SB203580 and then harvested at various time points indicated in Fig. 3A. An 80% reduction of phosphorylated MK2 was observed in the 1st h, and the activity was diminished 1 h later (Fig. 3A). In the next experiments, we attempted to correlate the data by analyzing HUVEC migration after a similar time course of SB203580 treatment. Endothelial cells were treated with 10 μM SB203580, and at various time points cells were collected for VEGF-stimulated migration assays (Fig. 3B). The earliest time we could detect a significant SB203580-induced migratory inhibition occurred at 6 h, and a minimum of 10 h was necessary to completely inhibit VEGF-stimulated cell migration. The time disparity between the rapid inhibition of MK2 phosphorylation (within 2 h) and delayed onset of migration inhibition (after 6 h) would argue that a p38 MAPK-dependent regulation of gene expression is responsible for VEGF-stimulated cell migration rather than a direct p38 signaling event.

The p38 MAPK pathway has been shown to regulate the expression of various proteins (9). We thus determined whether p38 MAPK-dependent protein expression is required for VEGF-stimulated endothelial cell migration. HUVECs were treated with SB203580 for 24 h, washed thoroughly to remove SB203580, and cultured in complete medium in the absence or presence of 2 μg/ml actinomycin (RNA synthesis inhibitor) or 20 μg/ml cycloheximide (protein synthesis inhibitor) for 4 h. Cells were lysed and lysates immunoprecipitated with anti-MK2 polyclonal antibody. Immunoblotting with the immunoprecipitates using anti-phospho-MK2 (Thr-334) polyclonal antibody showed similar levels of MK2 phosphorylation in cells treated or untreated with either inhibitor (Fig. 4A), demonstrating that the p38 MAPK activity was not compromised in actinomycin- or cycloheximide-treated cells compared with the untreated cells. However, the ability of VEGF to stimulate cell migration was completely lost in actinomycin- or cycloheximide-treated cells (Fig. 4B). These results suggest that p38 MAPK pathway-dependent protein expression is required for VEGF-stimulated endothelial cell migration.

**uPA Expression Is Regulated by p38 MAPK Pathway and Is Required for Cell Migration**—Our previous studies (30, 33) have shown that p38 MAPK regulates uPA and uPAR expression in invasive breast cancer cells. Other studies further demonstrated that uPA and uPAR are involved in migration of various cell types including endothelial cells (26, 36). We thus hypothesized that the p38 MAPK pathway may participate in endothelial cell migration by regulating uPA/uPAR expression. To test this hypothesis, we first examined the effect of inhibiting p38 MAPK activity on uPA/uPAR expression in endothelial cells. HUVECs were treated with an increasing dose of SB203580, and then assayed for VEGF-stimulated chemotaxis using Transwells as described under “Experimental Procedures.” Data are means ± S.E. of triplicates. n = 3, * p < 0.005 versus control in the absence of VEGF. ** p < 0.05 versus control in the absence of VEGF.
SB203580 for 24 h and then lysed, and the cell lysates were subjected to immunoprecipitation with anti-MK2 polyclonal antibody. Immunoprecipitates were subjected to immunoblotting to detect phosphorylated MK2 using anti-phospho-MK2 (Thr-334) polyclonal antibody. The membrane was stripped and reprobed with anti-MK2 polyclonal antibody to measure the endogenous level of MK2. A, cells were lysed and lysates subjected to immunoprecipitation with anti-MK2 polyclonal antibody. Immunoprecipitates were subjected to immunoblotting to detect uPA using anti-uPA mAb and anti-uPAR polyclonal antibody. The membrane was stripped and reprobed with anti-actin polyclonal antibody to ensure equal protein loading. B, HUVECs were infected with control Ad and Ad containing dominant negative forms of MKK3, MKK6, p38α, p38β, p38γ, p38δ, MK2, and PRAK for 36 h. Cells were then lysed, and lysates were subjected to immunoblotting to detect uPA with anti-uPA mAb. The membranes were stripped and reprobed with anti-actin polyclonal antibody to ensure equal protein loading. C, HUVECs were treated with 10 μM SB203580 for varying times and then lysed, and lysates were subjected to immunoblotting to detect uPA with anti-uPA mAb. The membrane was stripped and reprobed with anti-actin polyclonal antibody to ensure equal protein loading.

SB203580 for 24 h and then lysed, and the cell lysates were subjected to immunoblotting to detect uPA and uPAR expression. Although both uPA and uPAR were readily detected, the expression of uPA was significantly down-regulated by SB203580 in a dose-dependent manner (Fig. 5A). Conversely, the level of uPAR expression was unaltered by the presence of SB203580 (Fig. 5A). To identify the specific signaling components in the p38 MAPK pathway controlling uPA expression, we introduced dominant negative forms of these proteins in HUVECs using recombinant adenovirus. Cells were lysed 36 h post-infection and then analyzed for cell-associated uPA expression. The dominant negative forms of MKK3, p38α, p38γ, and MK2, but not dominant negative forms of MKK6, p38β, p38δ, and PRAK, significantly down-regulated uPA expression (Fig. 5B). These results suggest that the same signaling molecules including MKK3, p38α/γ, and MK2 are involved in both endogenous uPA expression and VEGF-stimulated endothelial cell migration.

As 10 μM SB203580 could sufficiently inhibit uPA expression, we used this concentration to examine a time course of uPA inhibition. The earliest apparent reduction in uPA expression was observed 6 h after the addition of SB203580 (Fig. 5C). A significant reduction in uPA expression (>90%) was detected after 8–10 h SB203580 treatment (Fig. 5C). This pattern of uPA expression reduction coincided with the timing observed in the SB203580-mediated inhibition of VEGF-stimulated cell migration (Fig. 3B), suggesting a possible association between uPA expression and VEGF-stimulated cell migration.

In order to examine the role of uPA expression in VEGF-stimulated cell migration, we introduced a panel of mAbs targeting uPA and uPAR, and we analyzed their effects on HUVEC migration. Addition of either anti-uPA mAb 3471 or anti-uPAR mAb 3936, which both function to prevent uPA binding to uPAR, caused significant inhibition of VEGF-stimulated HUVEC migration (Fig. 6); anti-uPA mAb 394, which neutralizes uPA protease activity but does not affect binding of uPA to uPAR, had no effect on basal or VEGF-stimulated cell migration (Fig. 6). Furthermore, the amino-terminal fragment of uPA, which blocks uPA binding to uPAR, also significantly inhibited VEGF-stimulated cell migration (Fig. 6). These results suggest that a signaling event transmitted through uPA-
absence of VEGF.

endothelial cell migration. We treated the uPA transgene-expressing HUVECs with anti-uPA mAb 3471, 394, or anti-uPAR mAb 3936 or 10 ng/ml amino-terminal fragment (ATF) for 2 h and then used in VEGF-stimulated cell migration assays as described under “Experimental Procedures.” Data are means ± S.E. of triplicates. n = 3. *, p < 0.001 versus control in the absence of VEGF; #, p < 0.05 versus control in the absence of VEGF.

uPAR interaction rather than the uPA protease activity is required for VEGF-stimulated endothelial cell migration.

uPA Transgene Expression Rescues VEGF-stimulated Cell Migration in SB203580-treated Endothelial Cells—To investigate further the importance of uPA in VEGF-stimulated endothelial cell migration, we examined whether the expression of uPA transgene was capable of rescuing VEGF-stimulated migration of p38 MAPK-inhibited endothelial cells. Because the p38 MAPK can regulate uPA expression by affecting its mRNA stability via the 3′-untranslated region of its mRNA (31, 33), we subcloned only the complete uPA-coding sequence (lacking both 5′- and 3′-untranslated region) into pBabe/puro retroviral vector. The pBabe/puro plasmid contains its own promoter (long terminal repeat promoter) and polyadenylation signal for RNA expression and processing, so the uPA transgene mRNA expression driven by this retroviral vector should not be affected by p38 MAPK. HUVECs were transduced with this uPA transgene retroviral vector, and a stable line was generated after puromycin selection. We detected uPA overexpression in uPA transgene-expressing HUVECs, and after 24 h of SB203580 treatment, a marginal decrease in uPA levels was observed, presumably because of the down-regulation of endogenous uPA (Fig. 7A). Subsequently, we treated this line with 10 μM SB203580 and measured cell migration. Whereas the VEGF-stimulated cell migration was completely blocked by SB203580 in mock control cells, it was not affected in uPA transgene-expressing HUVECs (Fig. 7B). To confirm that the rescued VEGF-stimulated cell migration in uPA transgene-expressing HUVECs was because of uPA transgene expression, we treated the uPA transgene-expressing HUVECs with anti-uPA mAb 3471, anti-uPAR mAb 3936, or control mouse IgG for 2 h prior to the migration assay. Both anti-uPA and uPAR mAb, but not mouse IgG, inhibited over 80% of VEGF-stimulated cell migration in uPA transgene-expressing HUVECs (Fig. 7B). Thus the SB203580-resistant phenotype of uPA transgene-expressing HUVECs was indeed due to the uPA transgene expression. These results suggest that uPA may be the sole factor responsible for p38 MAPK-regulated endothelial cell migration.

uPA Is Required for VEGF-induced Actin Reorganization and Focal Adhesion Assembly in Endothelial Cells—Cell migration requires the coordinated regulation of actin reorganization and focal adhesion assembly (37, 38). We thus investigated whether p38 MAPK and uPA are involved in these two events. Mock control and uPA-expressing HUVECs were treated with 10 μM SB203580 or left untreated for 24 h and were then induced with 10 ng/ml VEGF for 1 h followed by immunostaining for microscopic analyses. In untreated cells, F-actin was mainly observed in the inner surface of the plasma membrane, and paxillin staining was seen in a diffused pattern throughout the cells with no obvious focal adhesion formation (Fig. 8, A and B). Exposure to VEGF elicited dramatic actin reorganization, resulting in stress fiber formation in both control and uPA transgene-expressing cells (Fig. 8, A and B). Additionally, we observed paxillin staining in the ends of stress fibers in VEGF-stimulated cells (Fig. 8, A and B), suggesting the assembly of focal adhesions in these cells. SB203580 treatment inhibited VEGF-induced stress fiber formation and focal adhesion assembly in mock control HUVECs (Fig. 8A). However, VEGF-induced actin reorganization and focal adhesion assembly was not altered by SB203580 in uPA transgene-expressing HUVECs (Fig. 8B). To determine whether the functionality of uPA is required for VEGF-induced actin reorganization and focal adhesion assembly, both mock control and uPA transgene-expressing cells were incubated with anti-uPA mAb 3471 for 2 h prior to VEGF stimulation. Preincubation with this function-blocking mAb inhibited VEGF-induced actin reorganization and focal adhesion assembly in both control and uPA-expressing HUVECs (Fig. 8, A and B). Taken together, these results suggest that a p38 MAPK-uPA pathway regulates VEGF-stimulated endothelial cell migration by facilitating actin reorganization and focal adhesion assembly.

DISCUSSION

Important aspects of angiogenesis include endothelial cell migration, proliferation, differentiation, and protease production (1). Endothelial cell migration stimulated by angiogenic factors including VEGF, S-1-P, and soluble VCAM have been shown to play crucial roles in the angiogenesis process (3–6). Several recent studies (39) have demonstrated that the specific p38 MAPK inhibitor SB203580 can efficiently block VEGF-stimulated endothelial cell migration. In this report, we investigated whether the activity of p38 MAPK was also required for endothelial cell migration stimulated by nongrowth factor stimulants such as S-1-P (G protein-coupled receptor ligand) and soluble VCAM (integrin ligand). By using a specific p38 MAPK inhibitor SB203580 and its nonfunctional structural analog control SB202474, we found that p38 MAPK activity is required for both VEGF- and nongrowth factor (S-1-P and soluble VCAM)-stimulated endothelial cells migration (Fig. 1). As shown previously, VEGF-stimulated endothelial cell migration is mediated through VEGF receptor 2 and requires Src family kinase activity (19, 39); S-1-P-stimulated endothelial cell migration is mediated through S1P1 and S1P2 receptors and depends on the G protein G12α-linked Rho kinase pathway (40, 41); and soluble VCAM-stimulated cell migration is sensitive to neutralizing anti-α5 integrin and can be blocked by focal adhesion kinase inhibitor (6). Our data demonstrate that p38 MAPK activity is required for endothelial cell migration mediated through diverse signaling pathways (growth factor receptor-, G-protein-coupled receptor-, and integrin-mediated) and thus suggest that the p38 MAPK pathway plays a general role in endothelial cell chemotaxis.

There are multiple potential signaling components at each level of the p38 MAPK signaling pathway (9, 10). Both MKK3 and MKK6 can serve as specific upstream activator kinases for p38 MAPK (9); the p38 MAPK family includes four isoforms (α, β, γ, and δ); and up to six kinases (MK2, MK3, PRAK, MKN1, MSK1, and MSK2) are activated by p38 MAPK (9, 10). Stress stimulants such as sorbitol and arsenide usually lead to the activation of all these signaling components (9, 42). However, signaling components in p38 MAPK pathway can also be differentially activated. For example, transforming growth fac-
tor-β selectively activates MKK3-p38α/p38β but MKK6 and p38β in murine mesangial cells (43, 44). γ-Irradiation preferentially activates the MKK6-p38γ pathway in human osteosarcoma cells (45). Moreover, these signaling components have also been shown to play distinct roles in many cellular events. The activation of p38γ increases AP-1 transcriptional activity, whereas p38γ and p38δ inhibits AP-1 activity in human breast cancer cells (46). MKK6, rather than MKK3, is the dominant activator of p38 MAPK in epithelial cells exposed to tumor necrosis factor-α and interleukin-1 and macrophages stimulated by bacterial lipopolysaccharide (47–49). MK2, rather than PRAK, stimulates phosphorylation-dependent interaction between tuberin and 14-3-3 protein in transfected 293 cells (50). Similarly, we have shown previously (33) that destabilization of the AU-rich element containing uPA is only reverted by constitutively active MK2 but not PRAK. In this report, we showed that dominant negative forms of MKK3, p38α, p38γ, and MK2, but not dominant negative forms of other identified signaling components, can block VEGF-stimulated endothelial cell migration (Fig. 2), suggesting that a pathway consisting of MKK3-p38α/γ-MK2 is specifically involved in VEGF-stimulated endothelial cell migration. Our study thus provides additional evidence for distinct roles of each signaling component in the p38 MAPK pathway-mediated cellular events.

Recent studies (12, 15–18, 51) have demonstrated the importance of the p38 MAPK pathway in cellular migration of various cell types including endothelial cells. It has been suggested that p38 MAPK regulates cell migration by facilitating cytoskeleton reorganization (52–54). However, the involvement of p38 MAPK in cytoskeletal changes and cell migration is not well understood. We showed that a minimum 10-h exposure to SB203580 was required to abrogate VEGF-stimulated cell migration, whereas p38 MAPK activity was diminished within 2 h of SB203580 treatment (Fig. 3), suggesting that the direct p38 MAPK signaling is unlikely to be responsible for VEGF-stimulated endothelial cell migration. By using actinomycin and cycloheximide, we found that p38 MAPK-dependent de novo protein synthesis is necessary for VEGF-stimulated endothelial cell migration (Fig. 4). In search for protein molecules essential for VEGF-stimulated endothelial cell migration, we turned our attention to uPA and uPAR because both proteins have been shown previously to be important for endothelial cell migration (26, 36), and their expression is regulated by the p38 MAPK pathway in various cell types (30, 31). In our studies, we showed that both SB203580 and dominant negative forms of signaling components in the p38 MAPK pathway significantly down-regulated uPA expression in endothelial cells (Fig. 5), and moreover, we detected a correlation between the occurrence of SB203580-induced inhibition in VEGF-stimulated endothelial cell migration and SB203580-induced reduction of uPA expression (Fig. 3 and Fig. 5). The ability of SB203580 to inhibit VEGF-stimulated cell migration is lost in HUVECs expressing constitutively active MK2 and uPA transgenes (Fig. 7). Recent studies (55, 56) have reported that MK2-deficient murine embryonic fibroblasts and neutrophils are defective in platelet-derived growth factor- and formyl-methionyl-leucyl-phenylalanine-stimulated cell migration, respectively. We consider the possibility that the abnormal migration phenotype of MK2-deficient cells may be caused by the lack of uPA expression in these cells.

Early studies by Gonias et al. (57) have demonstrated that binding of uPA to uPAR activates Erk1 and -2 and that this induced Erk activity is required for uPA-induced MCF-7 breast cancer cell migration. They further showed that a signaling
The pathway including FAK-Src-Shc is responsible for uPA-induced Erk activation and cell migration (24). uPA/uPAR expression has been found to correlate very well with the migratory ability of various cell types (36, 58, 59). Blocking uPA/uPAR expression or intercepting uPA-uPAR interaction has been found to inhibit migration in colonic epithelial and glioblastoma cells (60, 61). These findings corroborate well with our results indicating that disrupting uPA and uPAR interaction abrogated VEGF-stimulated endothelial cell migration (Fig. 6). Recent studies showed that phosphatidylinositol 3-kinase, NF-H9260, and protein kinase C regulate motility of breast cancer cells by promoting uPA secretion (28, 29), and this is in excellent agreement with our finding that the p38 MAPK pathway regulates stimulated endothelial cell migration by maintaining uPA expression.

Coordinated regulation of cytoskeleton reorganization and focal adhesion are essential for cell migration (37, 38). VEGF elicited actin reorganization and assembly of paxillin in the focal adhesion plaque (Fig. 8). Either blocking p38 MAPK activity or intercepting uPA-uPAR interaction abrogated VEGF-induced actin reorganization and focal adhesion assembly in mock control HUVECs (Fig. 8A). However, the ability of SB203580, rather than uPA mAb, to inhibit VEGF-induced actin reorganization and focal adhesion assembly is lost (Fig. 8B). These findings further support our hypothesis that the p38 MAPK pathway regulates stimulated endothelial cell migration by maintaining uPA expression and also suggests that uPA-uPAR interaction may promote endothelial cell migration by facilitating cytoskeleton reorganization and focal adhesion assembly.

In conclusion, our data suggest that uPA is a key factor responsible for p38 MAPK-regulated endothelial cell migration. This knowledge may be useful in the design of new anti-angiogenesis approaches aimed at blocking uPA-uPAR interaction.

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