Multiple signaling pathways are involved in endothelin-1-induced brain endothelial cell migration

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Milan, Johanna, Christiana Charalambous, Rashid Elhag, Thomas C. Chen, Wei Li, Shengxi Guan, Florence M. Hofman, and Raphael Zidovetzki. Multiple signaling pathways are involved in endothelin-1-induced brain endothelial cell migration. Am J Physiol Cell Physiol 291: C155–C164, 2006.—We have observed that the vasoactive peptide endothelin-1 is a potent inducer of migration of primary human brain-derived microvascular endothelial cells. By blocking signal transduction pathways with specific inhibitors, and using dominant negative mutant infections, we have demonstrated that multiple pathways are involved in endothelin-1-induced migration. Absolutely required for migration are protein tyrosine kinase Src, Ras, protein kinase C (PKC), phosphatidylinositol 3-kinase, ERK, and JNK; partial requirements were exhibited by cAMP-activated protein kinase and p38 kinase. Partial elucidation of the signal transduction sequences showed that the MAPKs ERK, JNK, and p38 are positioned downstream of both PKC and cAMP-activated protein kinase in the signal transduction scheme. The results show that human brain endothelial cell migration has distinct characteristics, different from cells derived from other vascular beds, or from other species, often used as model systems. Furthermore, the results indicate that endothelin-1, secreted by many tumors, is an important contributor to tumor-produced proangiogenic microenvironment. This growth factor has been associated with increased microvessel density in tumors and is responsible for endothelial cell proliferation, migration, invasion, and tubule formation. Because many signal transduction pathways investigated in this study are potential or current targets for anti-angiogenesis therapy, these results are of critical importance for designing physiological angiogenic protocols.

signal transduction; angiogenesis; microvessels; vasoactive peptides

ANGIOGENESIS, the growth of new blood vessels from preexisting vessels, is a complex process initiated by vascular destabilization followed by endothelial cell (EC) migration, proliferation, and eventually tubule formation (24, 28). Angiogenesis is essential for cancer progression (11, 24), as demonstrated by the correlation between microvessel density and poor clinical outcome (40). Therefore, understanding the mechanisms of angiogenesis in cancer is likely to lead to more effective cancer therapy (11, 25). EC migration, a critical component of angiogenesis, is regulated by growth factors (11, 56). Endothelin-1 (ET-1) is a growth factor known to induce EC migration (60, 72). This polypeptide is a potent vasoconstrictor, normally produced by many cell types, including EC, macrophages, and smooth muscle cells (45). ET-1 is produced in a variety of tumors, and the role of ET-1 in cancer-related angiogenesis is increasingly recognized (5, 6, 20, 57, 58, 62, 69). ET-1 activity is mediated by ETA and ETB receptors expressed on endothelial and a variety of other cell types (78). Our group has previously shown that both receptors are expressed on human brain endothelial cells (BEC) (93). Both ETA and ETB receptors contain seven hydrophobic transmembrane domains common to the superfamily of G protein-coupled receptors (GPCR) and, depending on cell type, can activate each of the three classes of GTP-binding proteins, Gα, Gβ, and Gγ, resulting in activation of multiple intracellular signal transduction pathways (84).

To efficiently block ET-1-induced migration for antiangiogenesis cancer therapy, the signal transduction pathways involved in this process must be clarified. Most studies published on ET-1-induced migration were not performed on EC, and even fewer on human BEC (16, 80, 93); therefore, the precise sequence of intracellular signaling events involved in ET-1-induced migration of brain microvessel EC is not known. Studies using various cell types have demonstrated that ET-1 can activate such enzymes as protein kinase C (PKC) (17, 72, 93, 94), cAMP-dependent protein kinase (PKA) (78, 94), phosphatidylinositol 3-kinase (PI3-K) (8, 26, 83), Ras (26, 83), and non-receptor protein tyrosine kinases, probably of the Src family (8, 19), leading to the activation of each of the three main MAPK modules (ERK, p38, JNK) (8, 17, 19, 23, 26, 83). Moreover, each of these enzymes, depending on the stimulus and cell type, may or may not participate in processes leading to cell migration. In the present work, we show that ET-1 induces migration of primary cultures of human BEC. Furthermore, a partial dissection of the signal transduction pathways involved in this process demonstrates the participation of both ET-1 receptor types and of multiple signal transduction enzymes, including Src, Ras, PKC, PKA, ERK, p38, and JNK.

MATERIALS AND METHODS

Reagents. The following reagents were purchased: ET-1 (Peninsula Laboratories, Belmont, CA); ETA receptor antagonist BQ-610 and ETB receptor antagonist BQ-788 (Alexis Biochemicals, San Diego, CA); Src family tyrosine kinase inhibitors PP1 and PP2 (Biomol Research Laboratories, Plymouth Meeting, PA); Ras inhibitor FTI276, PKC inhibitor bisindolylmaleimide-GF109203X (GF), JNK inhibitor SP600125, and PI3-K inhibitor LY-294002 (Calbiochem, La Jolla, CA); PKC inhibitors bisindolylmaleimide-GF109203X (GF), JNK inhibitor SP600125, and PI3-K inhibitor LY-294002 (Calbiochem, La Jolla, CA); and ETA receptor antagonist BQ-610 and ETB receptor antagonist BQ-788 (Alexis Biochemicals, San Diego, CA); PKC inhibitors bisindolylmaleimide-GF109203X (GF), JNK inhibitor SP600125, and PI3-K inhibitor LY-294002 (Calbiochem, La Jolla, CA).
C156
ENDOTHELIN INDUCES HUMAN BRAIN ENDOTHELIAL CELL MIGRATION

Jolla, CA); P3-K inhibitor wortmannin; PKA inhibitor H-89, adenyllyl cyclase activator forskolin, p38 MAPK inhibitor SB 202190, and phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO); and MEK1/2 inhibitor U0126 and p44/42 MAPK assay kit (Cell Signaling Technology, Beverly, MA).

Cell culture. Human BEC were isolated from ~1–3 g of human normal brain tissue as previously described (29). Cells were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 100 ng/ml endothelial cell growth supplement Endogro (Upstate Biotechnology, Lake Placid, NY), 2 mM l-glutamine, 10 mM HEPES, 24 mM sodium bicarbonate, 300 units of heparin USP, 1% penicillin/streptomycin, and 10% fetal calf serum (FCS). As confirmed by immunocytochemical staining for typical phenotypic markers such as von Willebrand factor for EC, glial fibrillary acid protein for astrocytes, and CD11b for macrophages, the purity of BEC was >98%. Cell viability was >95% as assessed using a Trypan blue exclusion assay. Twenty-four hours before initiation of each experiment, culture medium was replaced with Endogro-free medium containing 1% FCS. At the termination of each experiment, all groups were examined for viability with Trypan blue; cells were routinely >95% viable. In addition, after treatment with inhibitors, cells were examined for viability using the MMT assay; there was no difference between treated and untreated cells. Thus, after 6 h of incubation with the various inhibitors, there was no significant change in cytotoxicity compared with untreated cells, demonstrating that the concentrations of inhibitors employed for the duration of these experiments were not toxic. Only BEC in passage 4 or 5 were used for all experiments presented.

Boyden chamber cell migration assay. Boyden chamber cell migration assay was performed using Transwell chambers with 8-μm pore size filter membranes (BD Bioscience, Bedford, MA). Briefly, the filter membranes were coated with gelatin by immersing the filter membranes into 0.5% gelatin for at least 2 h. The filter membranes were then allowed to air dry by inverting the membranes for 4–12 h. The chambers were then inserted into 24-well culture plates. Subconfluent BCE cultures were harvested by exposure to trypsin-EDTA solution for 30–60 s. A single-cell suspension of EC (5 × 10^4 cells/well) in RPMI medium supplemented with 1% FCS was prepared. One hundred microliters of this suspension were placed into the upper section of the Boyden chamber. Cells were allowed to attach for at least 12 h at 37°C in 5% CO2. After the addition of 600 μl of RPMI medium supplemented with 1% FCS to the bottom of each well, various stimuli and inhibitors or activators were added to the lower compartments of each well. After pretreatment with inhibitors or activators for 30 min (BQ-610, BQ-788), 1 h (P1, P2, P2, wortmannin, LY-294002, GF, H-89, forskolin, U0126, SB 202190, and SP600125), or 24 h (FTI276) and subsequent treatment with ET-1 for 6 h, the filter membranes were recovered and nonmigrated cells attached on the upper surface of the filter membrane were removed with a cotton swab. The longer incubation with FTI276 was required because this farnesyl transferase inhibitor interferes with an essential posttranslational modification required for the translocation of Ras to the plasma membrane and thus has to be administered at an early stage of Ras processing. The remaining cells on the lower surface of the filter membrane, those that migrated through the pores, were subjected to a three-step staining procedure comparable to the Wright-Giemsa method. Briefly, with the use of the Hema 3 stain set, cells were fixed with methanol, stained in Solution I, and stained in Solution II (Fisher Scientific, Middletown, VA). Migration activity was quantified by blind counting of the stained migrated cells in at least 5 fields per chamber with ×20 objective or at least 10 fields per chamber with ×40 objective. Treatment conditions were examined in triplicate in at least three separate experiments using cells derived from different donor cases. In a separate set of experiments, we verified that the DMSO vehicle used for preparing stock solutions does not affect BEC migration or modify ET-1-induced BEC migration at 0.5%, the highest concentration used (data not shown).

RESULTS

ET-1 induces BEC migration in a concentration-dependent manner. To determine whether BEC respond to ET-1 by increased migration, we incubated primary cultures of EC on the surface of gelatin-coated porous filters; different concentrations of ET-1 were added to the lower chamber. Because our group has previously shown that ET-1 induces production of IL-8, a known chemotaxis factor (29), it was important to separate direct effects of ET-1 on BEC migration from the secondary effects via IL-8. Our group’s previous data demonstrated that ET-1 is secreted in detectable quantities only after 12 h following the BEC stimulation by ET-1, as measured using the ELISA technique, and no IL-8 was detected at 6 h (29). Therefore, to exclude the effects of IL-8, we measured the ET-1-induced migration at 6 h. After 6 h, the number of BEC that migrated through the 8-μm pores was counted. All groups were plated in triplicate. The data are expressed as the multiple increase above cell numbers in control groups. The results demonstrate that BEC migration was increased more than twofold at optimal dose, and this increase in migration was dependent on ET-1 concentration (Fig. 1). At 10 nM ET-1, there was a detectable, significant increase in BEC migration. At 30 nM, there was a sharp increase in migration, with an additional, modest increase at 100 nM. On the basis of these data, all subsequent experiments were performed using 100 nM ET-1.
Both ETA and ETB receptors are required for ET-1-induced BEC migration. To determine the signal transduction pathways utilized by ET-1 in inducing BEC migration, we first elucidated the identities of the endothelin receptors involved in this process. Previous studies from this laboratory showed that human BEC express both ET-1 receptors, ETA and ETB (93). Therefore, we tested the role of each receptor using the specific inhibitors BQ-610 (1 μM) and BQ-788 (1 μM), which block ETA and ETB receptor activity, respectively. BEC were plated on the porous filter as described; cells were treated with the receptor antagonists for 30 min before stimulation with ET-1. Figure 2 shows that whereas ET-1 induces a significant increase in migration, each inhibitor blocks the effects of ET-1. Therefore, each receptor can regulate ET-1-induced BEC migration.

Src is required for ET-1-induced BEC migration. Src family members of non-receptor protein tyrosine kinases are involved in ET-1 stimulation (19, 76, 84) and function in mediating migration of EC from different sources, including human microvascular intestinal EC (81). We therefore examined the role of Src in ET-1-induced BEC migration. Src family tyrosine kinase inhibitor PP1 or PP2 was added to BEC cultures at 10 μM for 1 h before ET-1 stimulation. The effects of PP1 and PP2 on ET-1-induced BEC migration are shown in Fig. 3. Pretreatment of cells with the Src inhibitor completely blocked ET-1-induced migration.

Ras is required for ET-1-induced BEC migration. Ras proteins, critical components of signaling pathways and essential for growth factor-induced EC migration (7), are commonly positioned downstream of Src in the scheme of GPCR-induced signal transduction. Therefore, as part of the pathway analysis, we analyzed the role of Ras in ET-1 stimulation. Ras activity was evaluated using FTI276, a farnesyl transferase inhibitor that interferes with an essential posttranslational modification required for the translocation of Ras to the plasma membrane. BEC were incubated with FTI276 at 5 μM for 24 h and then stimulated with ET-1. Figure 4 demonstrates that blocking Ras activity completely abolished ET-1-induced BEC migration.

PI3-K is required for ET-1-induced BEC migration. PI3-K plays a critical role in cell migration and in other processes in angiogenesis (39). Wortmannin and LY-294002, specific inhibitors of PI3-K in vitro and in vivo, were used to test the function of this enzyme in ET-1-induced migration. Our results show that preincubation of BEC with wortmannin (100 nM) or LY-294002 (10 μM) abolished ET-1-induced BEC migration (Fig. 5) and demonstrate the requirement of this enzyme for the migration process.

PKC is required for the ET-1-induced BEC migration. The mammalian PKC family of serine/threonine kinases constitutes a major pathway to activate MAPKs. Previous work from this laboratory has shown that ET-1 activates PKC in BEC (94). Our present results show that inhibition of PKC activity by GF (1 μM) blocked BEC migration (Fig. 6). Furthermore, the role of PKC in BEC migration was confirmed by activating BEC with 3 nM of the PKC activator PMA (Fig. 6).

Partial requirement for PKA in ET-1-induced BEC migration. cAMP/PKA-associated signal transduction pathway has been shown to be involved in EC angiogenesis (21). Previous studies from this laboratory have demonstrated that ET-1 stimulation (19, 76, 84) and function in mediating migration of EC from different sources, including human microvascular intestinal EC (81). We therefore examined the role of Src in ET-1-induced BEC migration. Src family tyrosine kinase inhibitor PP1 or PP2 was added to BEC cultures at 10 μM for 1 h before ET-1 stimulation. The effects of PP1 and PP2 on ET-1-induced BEC migration are shown in Fig. 3. Pretreatment of cells with the Src inhibitor completely blocked ET-1-induced migration.
activates PKA in human BEC (94). To determine whether PKA activity is involved in ET-1-induced BEC migration, we used the PKA blocker H-89 at a concentration of 0.5 μM. Results show that inhibiting PKA activity with H-89 decreased BEC migration by 62 ± 14% (Fig. 7). Consistent with these results, stimulation of cAMP production by the adenylyl cyclase activator forskolin (10 μM) stimulated BEC migration similarly to the effect of ET-1 (Fig. 7).

Involvement of MAPKs in ET-1-induced BEC migration. MAPKs compose a network of signal transduction cascades containing at least five modules, including three main modules (ERK, p38, JNK) (18). MAPKs are activated after the engagement of a variety of cell surface receptors via dual tyrosine and threonine phosphorylation (18). MAPKs predominantly phosphorylate transcription factors, which places MAPKs in the downstream position in the signal transduction sequence (18). Because ERK plays a key role in angiogenesis (61), the function of ERK in ET-1-induced BEC migration was explored. The data obtained using the ERK cascade inhibitor U0126 (10 μM) demonstrate that ERK was involved in ET-1-stimulated BEC migration (Fig. 8).

In some cell types it has been shown that p38 MAPK is involved in ET-1 activation (17). To test this in our system, we used SB 202190 (3 μM), which has been shown to inhibit p38α, β, and γ, but not δ, isoforms (33). The results show that this inhibitor completely blocked ET-1-induced BEC migration (Fig. 8). JNK also has been shown to be activated by ET-1 in various cell populations (23). Therefore, BEC were treated with the novel specific JNK inhibitor SP600125 at 10 μM for 1 h before exposure to ET-1. The results show that JNK was required for ET-1-induced BEC migration (Fig. 8).

Dominant negative infections. Even the best signal transduction inhibitors commonly have nonspecific effects inhibiting cellular processes in addition to the intended targets. To verify participation of signal transduction enzymes in ET-1-induced BEC migration, we verified the inhibitor results by utilizing DN mutants of the three MAPK cascades, ERK, p38, and JNK. Figure 9 shows that infection with control green fluorescent protein (GFP) had no effect on BEC migration and did not affect ET-1-stimulated migration. Similarly, no effect on control or ET-1-stimulated BEC migration was exhibited by infection with WT JNKK1 (Fig. 9). Infection of BEC with DN
JNKK1, the inhibitor of JNK pathway, completely blocked ET-1-induced migration (Fig. 9). As an additional control, we tested the effects of DN mutants on TNF-α-induced migration. TNF-α stimulated BEC migration to an extent similar to that of ET-1 (Fig. 9); however, inhibition of JNK cascade by DN JNKK1 reduced TNF-α-induced migration by only 58% (relative to GFP-transfected) showing that complete inhibition of migration with blocked JNK cascade is specific to ET-1 stimulation. The difference between the ET-1- and TNF-α-associated signaling also was apparent in the case of p38 cascade: inhibition of this cascade with DN p38 resulted in major attenuation of ET-1-induced migration (73 ± 9%, Fig. 10), whereas it had no effect on TNF-α-induced migration (Fig. 10). Blocking ERK cascade with DN MEK1 completely abrogated both ET-1- and TNF-α-induced migration (Fig. 11). Thus these data confirm the inhibitor studies with ERK and JNK inhibitors. In contrast, the complete inhibition of migration by p38 inhibitor SB 202190, compared with only a partial effect of the corresponding DN p38α mutant, is likely due to the lack of specificity of the chemical inhibitor, because DN p38α exerts its inhibitory action by competitive binding to the upstream MKK3/6 enzymes that are common upstream activators of the same p38 isoforms α, β, and γ that are inhibited by SB 202190.

We further investigated the relative positions of PKA or PKC and the three MAPKs in the signal transduction pathway. Direct activation of BEC PKC by PMA increased BEC migration (Fig. 12A). Inhibition of each of the MAPKs by the corresponding inhibitor blocked BEC migration, showing that the MAPKs are downstream of PKC in the migration-inducing signal transduction process. Similar results were obtained in case of direct activation of adenyl cyclase by forskolin, with a resulting increase of cAMP and activation of PKA (Fig. 12B), showing that the MAPKs are positioned downstream of PKA in the signal transduction pathway.

DISCUSSION

In this study, we extended our investigation into the mechanisms of the biological effects of ET-1 on BEC (14, 29, 93, 94) to characterize ET-1-induced migration of these cells. ET-1 exerts a plethora of biological effects utilizing multiple intracellular signal transduction pathways, including activation of GTP-binding proteins, phospholipases, protein kinases and phosphatases, and various transcription factors (78). Perhaps the only consensus that can be deduced from the existing literature is that the effects of ET-1 are cell type dependent. The complexity of the system is increased when realizing that
the pathways involved in inducing angiogenesis in general, and cellular migration in particular, are also stimulus and cell type dependent. Thus, because both migration and ET-1 effects are little studied in human BEC, the present work provides specific information regarding the mechanism of BEC migration in response to ET-1.

We have demonstrated that ET-1 is a potent stimulus for BEC migration. These results disagree with the findings of Wren et al. (88), who reported lack of effect of ET-1 on human umbilical vein endothelial cell (HUVEC) migration. The reason for this discrepancy is not clear and is unlikely to be due to different cell types, because other studies, also using HUVEC as well as bovine or rat microvascular EC, reported ET-1-induced migration (54, 60).

Our results showing that both ETA and ETB receptors are required for ET-1-induced BEC migration are seemingly at variance with previous reports on EC from different origins, where ETB was found to exclusively mediate this process (54, 60). This discrepancy may be due to differences in ET receptor type expression in different cell types. Both Noiri et al. (60) and Morbidelli et al. (54) used HUVEC, which express ETB but not ETA receptors (2, 93), whereas we and others analyzed human and rat brain EC, which express both receptor types (64, 93). In addition to HUVEC, Morbidelli et al. (54) also tested bovine adrenal capillary EC, and Noiri et al. (60) studied rat renal microvascular EC, in which the expressions of ETA receptors are unknown. Therefore, because of a variety of possible species- and organ-specific differences among these EC and the human BEC used in this study, direct comparisons are difficult. Our present results using either receptor antagonist to block BEC migration confirm that ETA receptors on BEC not only are expressed but are fully functional. Furthermore, these data demonstrate that HUVEC are not representative of all EC activity.

Both ET receptors belong to the G protein-coupled family of receptors and activate multiple G proteins and associated signal transduction pathways (76, 84), depending on cell type. Accordingly, we proceeded to test the role of these enzymes in ET-1-induced BEC migration.

Src is a family of non-receptor protein tyrosine kinases, containing at least eight members, with three members (Src, Fyn, and Yes) ubiquitously expressed (1). Our results showing that inhibition of Src by PP1 or PP2 completely blocks ET-1-induced BEC migration agree with previously published results of investigators using EC from different sources and stimulated by various stimuli (77, 81) but not with other results using EC (51, 85), underscoring the stimulus and source dependence of the response. PP1 has been used to block anchorage-dependent growth and tumorigenicity in nude mice of NIH/3T3 fibroblasts expressing the RET/PTC3 oncogene (10). In view of the current use of Src inhibitors for in vivo therapy (30), our results provide additional experimental foundation for their potential efficacy in specific cases.

Generally, signal transduction pathways initiated by GPCR and leading to ERK activation involve Ras, with p38 and JNK activated via different mechanisms (66). However, it also is possible that Ras is involved in activation of p38 and JNK (66). ET-1 activation of Ras was previously demonstrated in several studies.
cell types (26, 73, 83). Ras also was shown to be involved in migration in bovine aortic EC wound healing (77). However, in other cases, Ras inhibition did not block EC migration (51); thus involvement of Ras in migration is stimulus dependent. The present results are the first to show that in human BEC, ET-1-induced migration is completely blocked by a Ras inhibitor. Ras inhibitors, including FTI276, were used in animal models (42), and it has been suggested that Ras can be a target for antiangiogenic therapy (3, 66). Indeed, our results show that Ras inhibition can be potentially beneficial when the antiangiogenesis target has a microvascular origin, similar to BEC used in the present study.

There is no consensus regarding the involvement of PI3-K in EC migration. A crucial role of PI3-K in cell migration has been reported by a number of studies, including those on EC (39, 50, 51, 75, 81). However, others have demonstrated that EC migration is PI3-K independent (77). Most studies report activation of PI3-K by ET-1 in various cell types (8, 26, 83), including EC (49). However, insulin-stimulated PI3-K activity in vascular smooth muscle cells was attenuated by ET-1 (34). The role of PI3-K in the function of human BEC is unknown. Our current results demonstrate that PI3-K is required for ET-1-induced migration of BEC, thereby suggesting another potential site for therapeutic intervention by inhibition of this cancer-associated enzyme (13, 71). Indeed, the PI3-K inhibitors wortmannin and LY-294002 used in this study have been shown to inhibit cancer cell xenograft growths in mouse models (59, 87).

The involvement of PKC in migration and angiogenesis is cell type and stimulus dependent. Thus PKC is required for glucose-induced, but not insulin-induced, EC line migration (75). Moreover, PKC activation inhibits migration of smooth muscle cells (32). Our results demonstrate that ET-1-induced BEC migration is completely dependent on PKC activity. Consistent with the role of PKC, we have shown that direct activation of PKC by PMA strongly induces BEC migration. Furthermore, we have shown that the PMA-induced BEC migration is blocked by the ERK inhibitor U0126, the p38 inhibitor SB 202190, or the JNK inhibitor SP600125, demonstrating that these MAPKs are located downstream from PKC in the signal transduction pathway and that each MAPK is required for induction of the PMA-stimulated BEC migration. Our results obtained using DN p38 infection, which only partially inhibited ET-1-induced migration, question the absolute dependence of the migration on this enzyme (see below). Our results placing PKC upstream of ERK agree with those of previous studies that used different EC (83).

Our present results show that ET-1-mediated migration is partially PKA dependent. There is no consensus in the literature on activation of PKA by ET-1; as was the case for all other enzymes monitored in this study, the PKA involvement was both stimulus and cell type dependent. ET-1 has been reported to increase cAMP levels (80, 94), decrease cAMP (53), or have little effect (90). Moreover, PKA activation may not be relevant for all biological functions. Our group has previously shown that ET-1 activates PKA in human BEC (94); however, the specific PKA inhibitor H-89 did not affect ET-1-induced IL-8 production (93). A preponderance of evidence obtained using other cell types points to inhibition of migration by cAMP elevation (92), but cAMP-induced migration also has been observed (21, 79).

We further investigated the role of elevated cAMP in BEC migration. Consistent with the role of PKA in inducing BEC migration, we have shown that direct activation of adenyl cyclases with forskolin and the consequent increase in intracellular cAMP results in increased BEC migration. Activation of BEC migration with forskolin was completely inhibited by the ERK inhibitor U0126, the p38 inhibitor SB 202190, or the JNK inhibitor SP600125, demonstrating that PKA does act via three modules of MAPK cascade in activating the migration. Consistent with our results, it has been observed that forskolin increases ERK phosphorylation in HUVEC (9). However, inhibition of ERK by cAMP has been observed in other systems (74, 89), and it has even been shown that elevation of cAMP can either inhibit or enhance ERK activation on the same cells, depending on the stimulus (15). It has been suggested that the variable effect of PKA activation on ERK may depend on the relative expressions of Raf-1 and B-Raf (15). According to this hypothesis, in cells in which cAMP activates MAPK, this occurs through PKA-induced activation of the Ras-related small G protein Rap1, which is both a selective activator of B-Raf and an inhibitor of Raf-1 (86). Thus, in cells with relatively high B-Raf expression, activation of Rap1 by cAMP results in activation of the MAPK pathway, whereas in cells with little or no B-Raf, cAMP inhibits the MAPK cascade (15). This scheme may be relevant for the present case, because ET-1 has been shown to activate both Ras/Raf and Rap1/B-Raf systems (73).

The three main MAPK modules (ERK, p38, JNK) activated after the engagement of cell surface receptors are located downstream of Ras and Ras-activated proteins in the signal transduction cascade (18, 66) and are considered separately. ET-1 activates ERK in a variety of cell types (8, 19, 23, 26, 83). However, in hepatic stellate cells, ET-1 inhibits ERK and JNK (52). Although a key role of ERK in angiogenesis is supported by a number of studies (61), there is no consensus in the literature on the requirement for ERK activation in EC migration. ERK has variably been shown to be required (39, 75) or not required (50, 75, 81) in EC migration, depending on the stimulus, origin, and species of the EC. There is no information available on the role of MAPKs in ET-1-induced migration of human BEC. We have shown that the inhibitor of the ERK pathway, U0126, completely blocks ET-1-induced BEC migration. These results were confirmed by infecting BEC with DN MEK1, which also resulted in a complete inhibition of ET-1-induced BEC migration. U0126 also has shown promise as an anticancer drug by inhibiting growth of breast cancer cells (44).

p38 MAPK also was shown to be activated by ET-1 (17, 48). Migration of EC in different systems may (70) or may not (50) be dependent on p38 activity. The proangiogenic effect of inhibiting p38 was reported by Issbrucker et al. (31). In addition, activation of p38 was a pathway of attenuation of EC migration induced by thrombospondin-1 (35). However, a study with genetic knockout of p38 demonstrated an important role for p38 in developmental angiogenesis (55). A possible role of p38 as potential therapy in several disease models was reviewed by Lee et al. (43). The specificity of this signaling pathway for ET-1-induced migration was illustrated by the lack of effect of DN p38 on migration stimulated by TNF-α (Fig. 10). As discussed above, complete inhibition of BEC migration...
for the contraction (95), whereas both receptor types are required for BEC migration (present study). In a description of migration of fibrosarcoma cells, Jo et al. (37) suggested a model in which activation and/or the constitutive activity of multiple independent signaling pathways may be essential to stimulate cell migration in response to urokinase-type plasminogen activator. Antagonizing any one of the pathways may be sufficient to eliminate the response completely. A variation of this model also can be applicable in the present case. Multiple signaling pathways, including Src, PI3-K, and ERK, were also shown to be activated in heparin-affin regulatory peptide-induced migration of HUVEC (65).

In addition to clarifying the details of the signal transduction pathways utilized by ET-1 in inducing BEC migration, an important general finding of the present study is that each of the multiple signal transduction pathways tested is completely or partially required for this process. Although this makes the system very complex for detailed investigation, it also can provide an opportunity for a therapeutic intervention at various regulation sites. Furthermore, in view of variations in angiogenic response as a function of stimulus, species, and vascular bed involved, it is critical for developing novel therapeutic regimens to consider the molecular processes actually taking place in relevant systems, i.e., human BEC, rather than in such common model systems as HUVEC or transformed EC lines, where the mechanisms of angiogenesis are likely to differ in critical details.

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