

c-Jun N-terminal kinase is involved in motility of endothelial cell

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Abbreviations: JNK, c-Jun N-terminal kinase; ERK, extracellular-signal regulated kinase; FGF, fibroblast growth factor; DAR, dominant active Ras; DAS, dominant active SEK1; MMP, matrix metalloprotease; uPA, urokinase plasminogen activator

Abstract

Cell motility is essential for a wide range of cellular activities including angiogenesis as well as metastasis of tumor cells. Ras has been implicated in cell migration and invasion, and functions at upstream of mitogen-activated protein kinase (MAPK) families, which include extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK. In the present study, we examined the role of JNK in endothelial cell motility using stable transfectant (DAR-ECV) of ECV304 endothelial cells expressing previously established oncogenic H-Ras (I⁶¹). DAR-ECV cells showed an enhanced angiogenic potential and motility (~2-fold) compared to ECV304 cells. Western blot analysis revealed constitutive activation of JNK in DAR-ECV cells. Pretreatment of JNK specific inhibitors, curcumin and all trans-retinoic acid, decreased the basal motility of DAR-ECV cells in a dose-dependent manner. These inhibitors also suppressed the motility stimulated by known JNK agonists such as TNF α and anisomycin. To further confirm the role of JNK, ECV304 cells expressing dominant active SEK1 (DAS-ECV) were generated. Basal non-stimulated levels of the cellular migration were greater in DAS-ECV clones than those in control ECV304 cells. These results suggest that Ras-SEK1-JNK pathway regulates motility of endothelial cells during angiogenesis.

Keywords: Endothelial cell, Motility, Angiogenesis, Ras, JNK

Introduction

Cell migration is a critical process for a wide variety of biological phenomena (Diaz-Flores *et al.*, 1994). Co-ordinated migration of various cell types is recurrently required for embryogenesis. Leukocyte migration plays a key role in the inflammation and the defensive immune response to infection (Muller *et al.*, 1999). In metastasis, tumor cells escape from the original tumor mass and migrate to a distant site. Proliferation and migration of vascular smooth muscle cells are an initial step for neointimal hyperplasia in atherosclerosis (Xi *et al.*, 1999). Migration of vascular endothelial cells is essential for angiogenesis and wound healing (Diaz-Flores *et al.*, 1994). For an efficient cellular migration, a series of distinctive events, including morphological polarization, membrane extension, formation of stable contacts between the cell and extracellular matrix (ECM), cytoskeletal contraction, cell body translocation and release of cell-substratum adhesion at the rear of the cell take place in sequence (Pepper *et al.*, 1994). These require an integration of co-ordinated regulation in the cytoskeleton and cell adhesion. Thus, a complex network of signal transduction molecules is involved in a migratory process.

Motility of endothelial cells (EC) is regulated by a number of extracellular stimuli. Growth factors such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) are strong promoters of EC motility as well as angiogenesis (Biro *et al.*, 1994; Anand-Apte *et al.*, 1997). Thus signaling pathways of these growth factors have been subjects of intensive study. Among the intracellular signaling proteins mediating cell migration by these growth factors Ras is of particular interest. Ras is well known for its regulatory role in the diverse biological responses including proliferation and differentiation of mammalian cells (Kang *et al.*, 1999; Olson *et al.*, 2000). Evidence indicates that Ras is also an important regulator of EC motility. Microinjection of oncogenic H-Ras proteins into endothelial cells stimulated random motility (Fox *et al.*, 1994). Ras activity was further shown to be continuously required throughout the motile process, namely initiation of endothelial cell movement as well as the continued movement.

Multiple downstream pathways of Ras have been discovered in vertebrates as well as in lower eucaryotes

(Olson *et al.*, 2000). Regarding the migration of endothelial cells, mitogen-activated protein kinase (MAPK) cascade has been studied most thoroughly (Klemke *et al.*, 1997), because it is a common central pathway mediating Ras activity. It has been reported that rapid phosphorylation of cPLA2 induced by activated extracellular-signal regulated kinase (ERK) MAPK is closely associated with the cell motility. Thus, Ras-MAPK-cPLA2 pathway may be a major route for the basic FGF (bFGF)-induced cellular motility. On the other hand, VEGF-induced motility was dependent on p38 MAPK (Rousseau *et al.*, 1997). Integrin is closely linked to cell adhesion (Cary *et al.*, 1999). It has been shown that Ras regulates both expression of integrins and their activities. The Rho GTPases have been identified as regulators of cytoskeletal reorganization in addition to their effects on cell growth (Kjoller *et al.*, 1999). Members of Rho family proteins sometimes act downstream of Ras. For Ras-induced cell transformation Rho GTPase activities are essential. Ras also activates Rac, a member of Rho GTPases, which is required for the formation of lamellipodium and membrane ruffles (Leng *et al.*, 1999; Ridley *et al.*, 1999).

c-Jun N-terminal kinase (JNK), a member of MAPK, is activated by a number of stresses (Davis, 1999). Thus, it was identified as stress-activated protein kinase (SAPK). However, its activity relates to a wider spectrum of cellular activities than initially thought. ERK and p38 MAPK have been shown to be involved in the EC motility as downstreams of FGF and VEGF (Tanaka *et al.*, 1999), respectively. Evidence indicates that JNK functions downstream of Rho GTPases, which is a key regulator of cytoskeletal rearrangement in several cell types (Atfi *et al.*, 1997). Furthermore, MEK kinase 1 (MEKK1) that regulates JNK pathway is involved in the control of cell motility (Xia *et al.*, 2000; Yujiri *et al.*, 2000). Therefore, we assume that JNK may participate in the EC motility. In the present study, we demonstrate that EC motility is dependent on activation of JNK.

Materials and Methods

Materials

Fetal bovine serum (FBS) and M199 were from Gibco BRL (Rockville, MD, USA). Protein G Sepharose was from Amersham-Pharmacia Biotech (Piscataway, NJ, USA). Phospho-specific JNK antibody and JNK antibody were obtained from New England Biolabs (Beverly, MA, USA). Curcumin and all trans-retinoic acid were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were obtained from Pierce (Rockford, IL, USA). The ECL western blotting detection kit was from Amersham-Pharmacia Biotech.

Cell culture

ECV304 cells were cultured in M199, supplemented with 10% (v/v) fetal bovine serum and maintained at 37°C in an atmosphere of 5% CO₂/95% air. ECV304 cells were kindly provided by Dr. D. T. Fearon (Wellcome Trust Immunology Unit, University of Cambridge School). Other transfected cells were cultured in M199 containing 10% FBS and 200 µg/ml G418.

Transfection

ECV304 cells were plated on 100-mm culture dish at a density of 1×10⁶ cells. Cells were transfected with pcDNASEK(K/R) plasmid expressing constitutive SEK1 using the calcium-phosphate transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For isolation of stable transfectants, cells were cultured with medium containing 1 mg/ml G418 at 37°C, 5% CO₂ for 10-14 days, and drug resistant colonies were isolated. The transfected cells were analyzed on western blot analysis with anti-phosphorylated JNK antibody.

In vitro angiogenesis on Matrigel

Four well plates (NUNC, Denmark) were coated with 300 µl of 10 mg/mL Matrigel (Collaborative Biomedical Product, Becton Dickinson Labware, Bedford, MA, USA) per well, which was allowed to polymerize for 30 min at 37°C. Into each culture well was added 500 µl M199 containing 10% FBS with 1×10⁵ cells of ECV304 or DAR-ECV cells and the plates were incubated at 37°C under 5% CO₂ with frequent observation through inverted microscope. Pictures of angiogenesis were taken at 1.5 h, 5 h, 8 h, 12 h, and 20 h by a camera (Olympus IMT) with a 10X objective lens on an inverted stage phase-contrast microscope.

Zymography

Gelatin and casein zymography were employed to measure the activation of matrix metalloprotease (MMP) and urokinase plasminogen activator (uPA), respectively. Culture supernatants (20 µl) were mixed with the equal volume of non-reducing Laemmli buffer (2X) and loaded on precast 10% SDS-polyacrylamide gels containing 1 mg/ml of gelatin or casein. After electrophoresis, the gels were washed twice with 2.5% Triton X-100 for 10 min to remove SDS and three times for 10 min with H₂O to remove Triton X-100. For the enzyme reactions the gels were incubated in the developing buffer (50 mM Tris HCl, 0.2 M NaCl, 20 mM CaCl₂, pH 7.5, 0.02% Brij 35) at 37°C for 20 h. The gels were stained with 0.5% (w/v) Coomassie brilliant blue R-250 in 45% (v/v) methanol-10% acetic acid, and then destained in the 45% (v/v) methanol-10% acetic acid.

Migration assay

Cell migration was analyzed using Transwell (Nunc, Naperville, IL, USA) containing polycarbonate membranes. Membranes were coated with 1% gelatin/well, and rinsed once with PBS. Cells were starved for 20 h with M199 containing 0.2% FBS. Cells (1×10^5) were added to the chamber of a Transwell with M199 containing 0.2% FBS and then allowed to migrate for 3 h at 37°C. The cells were fixed with 2.5% glutaraldehyde for 10 min. Non-migrating cells on the upper

side of the membrane were removed with a cotton swab, and the invading cells that attached to the bottom membrane were stained with 0.1% crystal violet. The number of invading cells per membrane was counted on six random fields using 20X objective lens (Olympus, PM-10AK3). For wound migration assay, confluent endothelial cells were starved in serum free M199 for 24 h and wounded with a razor blade pressed gently through the cell sheet into the plastic well to mark the

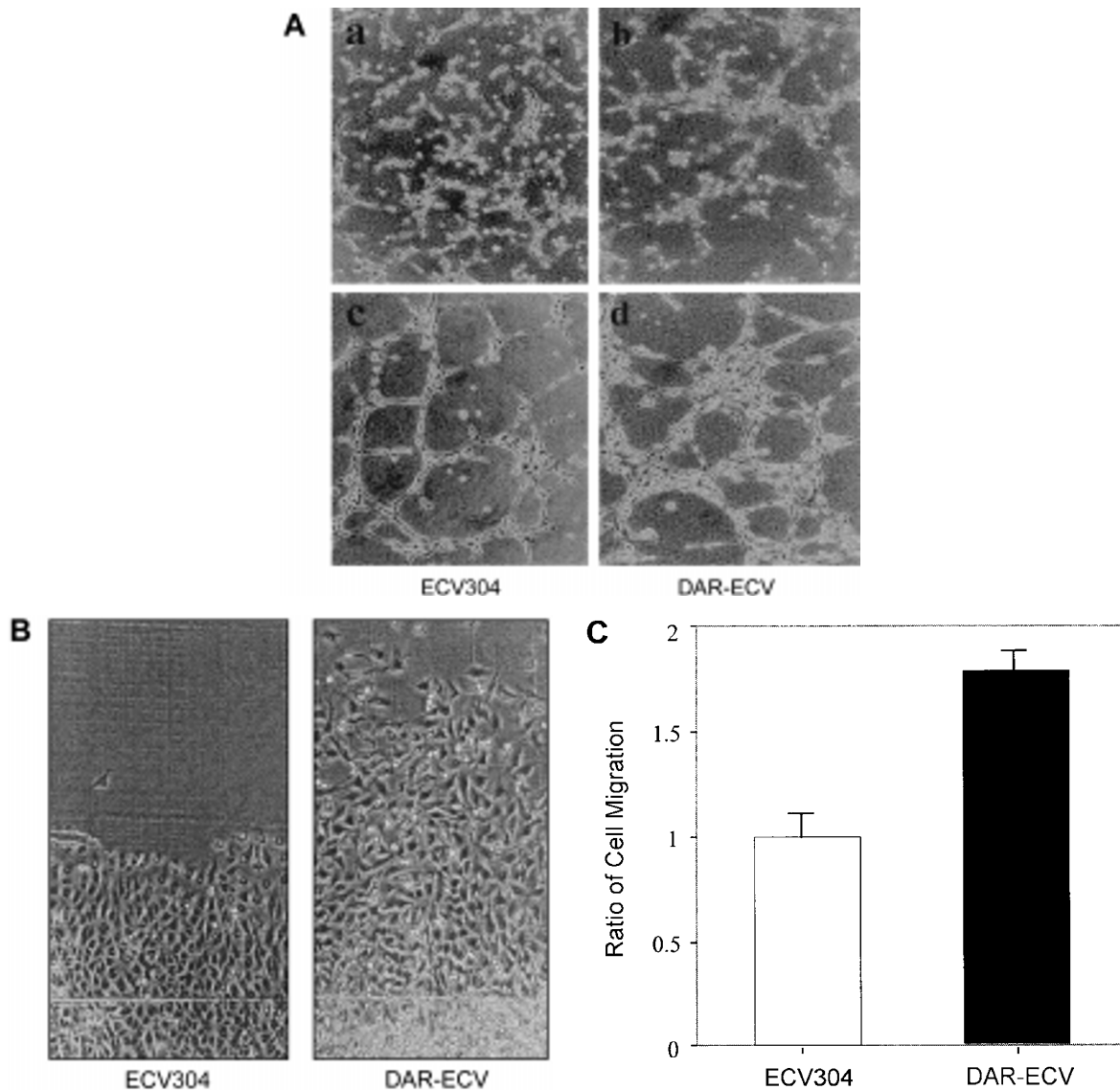


Figure 1. Comparison of angiogenic and migratory potential of ECV304 and DAR-ECV cells. A, Matrigel was dispensed into 4 well plates and solidified for 20 min at 37°C. The cells in M199 containing 10% FBS were dropped on Matrigel at a density of 1×10^5 cells per well, and were placed in 5% CO₂ at 37°C. Pictures were taken under phase-contrast microscope (X100) at the same time points after seeding onto Matrigel, (a) and (b) at 1.5 h and (c) and (d) at 7 h. (a) and (c), ECV304 cells; (b) and (d), DAR-ECV cells. B, Cells were starved for 20 h with M199 containing 0.2% FBS. They were resuspended in 0.5 ml M199 plus 0.2% FBS, wounded with a razor blade, and then allowed to migrate for 18 h and pictures were taken. C, Cells were starved for 20 h in M199 containing 0.2% FBS. They were resuspended in 0.5 ml M199 plus 0.2% FBS, transferred to the upper chamber of Transwell (2×10^5 cells/well), and then allowed to migrate for 2 h at 37°C. The number of migratory cells was counted on ten random fields using 40X objective lens. Migration data are presented as the relative ratio of the number of migratory cells.

origin, and then drawn through the monolayer to remove cells on one side. The cultures were washed with PBS and further incubated in complete M199 media for 18 h. Pictures were taken with a 4X objective lens (Olympus, PM-10AK3) on an inverted stage phase-contrast microscope (Olympus, IMT-2).

Western blot analysis

The proteins were separated on a 10% SDS-PAGE gel and transferred to PVDF membrane at 40 V for 2 h. Membranes were blocked for 30 min at room temperature with TTBS (20 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20) including 3% non-fat dried milk. The blots were then incubated with primary antibody for 1 h at room temperature. Bound primary antibodies were visualized by the ECL detection system, using horseradish peroxidase-conjugated secondary antibody. When necessary, the membranes were stripped in a buffer containing 62.5 mM Tris, pH 6.7, 100 mM α -mercapto-

ethanol, 2% SDS, for 30 min at 37°C.

Results

Active Ras increases the motility of ECV304 endothelial cells

To examine the role of JNK in endothelial cell (EC) motility, the active H-ras transfectants (DAR-ECV) were used as a model system, because Ras is known to activate MAPK families. DAR-ECV cells without endothelial cell specific cobblestone appearance, instead showed fibroblast-like transformed morphology (Shin *et al.*, 1999). To determine whether oncogenic H-Ras further affects angiogenesis, DAR-ECV cells were cultured on Matrigel. Active movement with a number of small processes was observed in DAR-ECV cells during the earlier stage of 1.5 h (Figure 1A-b), while sprouting after initial attachment was less active in ECV304 cells

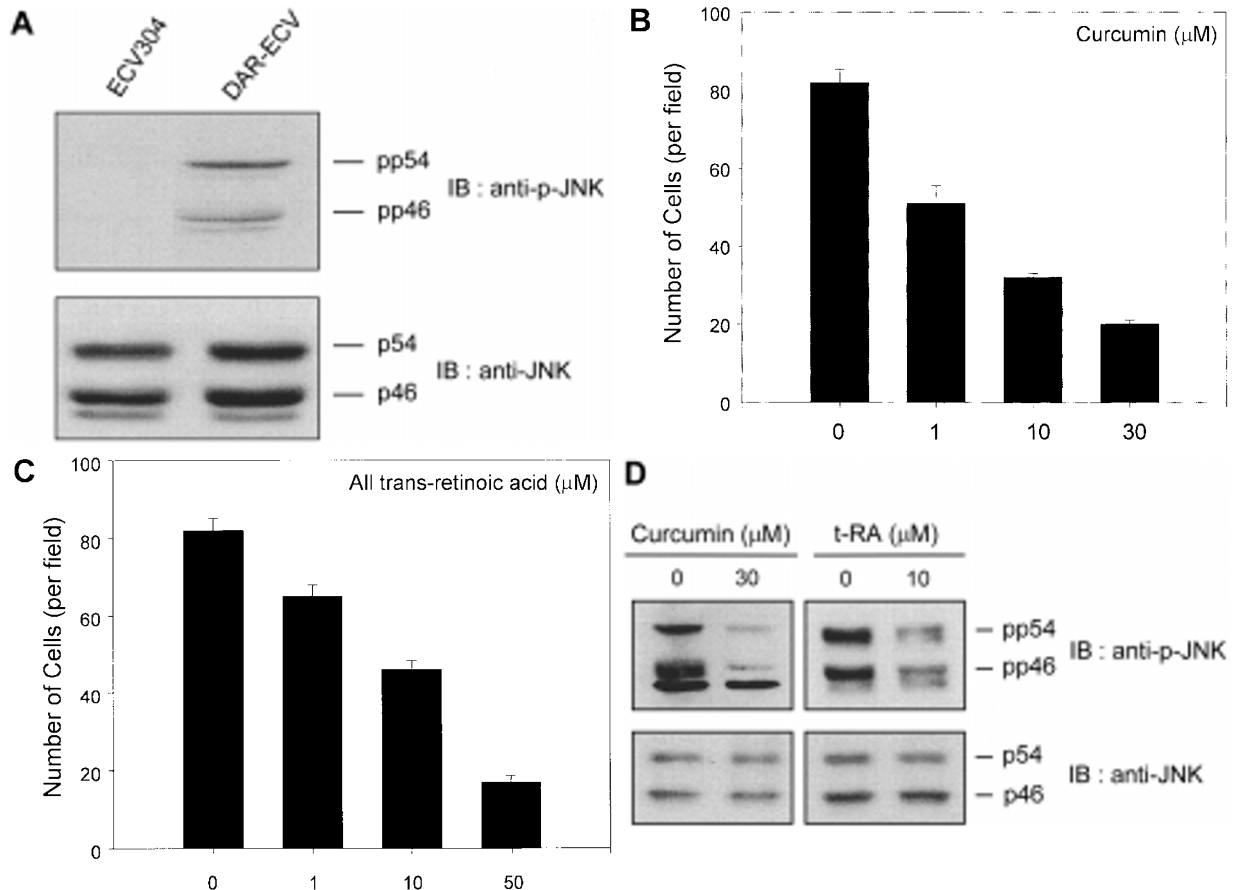


Figure 2. Effect of JNK inhibitors on basal migration of DAR-ECV cells. A, Cell lysates (50 μg) from ECV304 cells and DAR-ECV cells were resolved on a 10% SDS-polyacrylamide gel, and electrottransferred to a nitrocellulose membrane. The membrane was immunoblotted with anti-phosphorylated JNK antibody (upper panel), and reprobred with anti-total JNK antibody (lower panel). Cells were starved for 20 h in M199 plus 0.2% FBS, and pretreated for 1 h with curcumin (B) and all-trans retinoic acid (C) at the indicated concentrations. Migration analysis was performed as described in Figure 1C. Data are expressed as the relative number of cells. D, DAR-ECV cells were treated for 1 h with curcumin (0, or 30 μM), or all-trans retinoic acid (0, or 10 μM), and stimulated with 10 $\mu\text{g}/\text{ml}$ anisomycin at 37°C. The lysates (50 μg) were then resolved on a 10% SDS-polyacrylamide gel, electrottransferred to a nitrocellulose membrane, and immunoblotted with anti-phosphorylated JNK antibody (upper panel) or anti-total JNK antibody (lower panel).

(Figure 1A-a). At 5 h cord formation was almost complete in DAR-ECV cells (Figure 1A-d), although ECV304 networks still showed slender cords (Figure 1A-c). Since DAR-ECV cells exhibited transformed phenotypes, we assumed that they could not make any capillary tubes, a hallmark of endothelial differentiation. Contrary to our expectation, they showed larger capillary networks (not shown) and faster cell motility, ~2-fold greater than parental ECV304 cells (Figure 1B, C).

JNK activity relates to migration of endothelial cells

To determine whether JNK is involved in migration of DAR-ECV cells, we examined JNK activation in DAR-ECV cells. Western blot analysis with anti-phosphorylated JNK antibody revealed significant activation of JNK (Figure 2A). To determine whether elevated JNK activity may have a correlation with EC motility, migration of DAR-ECV cells was assessed in the presence of JNK inhibitors. It has been reported that curcumin and all-trans-retinoic acid have specific inhibitory effect on JNK. Pretreatment with curcumin caused a decrease in the motility of DAR-ECV cells in a dose dependent manner (Figure 2B). In this range curcumin did not show any cytotoxic effect (data not shown). All-trans-retinoic acid also inhibited the migration of DAR-ECV cells in a dose dependent manner (Figure 2C). JNK phosphorylation was inhibited completely by 30 μ M curcumin, but partially by 10 μ M all-trans-retinoic acid (Figure 2D). These results indicate that JNK activity is involved in an enhanced basal migration shown in DAR-ECV cells.

We next determined whether JNK agonists such as TNF α and anisomycin stimulate migration of endothelial cells. ECV304 cells were serum starved for 24 h, transferred to a Transwell, and then stimulated with TNF α or anisomycin. Both TNF α and anisomycin enhanced the migration of ECV-304 cells 85% and 75% more than unstimulated control cells, respectively (Figure 3A). These agonists also stimulated JNK activity as determined by western blot analysis (Figure 3B), raising a possibility that JNK activation might have a correlation with the enhanced migration of endothelial cell. TNF α -induced migration of ECV304 cells was inhibited by pretreatment of curcumin or all-trans-retinoic acid in a concentration dependent manner (Figure 3C).

To further confirm the role of JNK in EC motility, a stable cell line (DAS-ECV) expressing constitutively active SEK1 (MKK4) which functions upstream of JNK was established. Representative DAS-ECV clones with different levels of JNK activation were selected for comparison of migratory ability. Morphologically these clones did not show any transformed features. As illustrated in Figure 4A, three clones showed relatively higher levels of JNK phosphorylation. To measure the basal levels of active SEK1-induced motility no agonist was added in the Transwell, except minimum FBS (0.5%) to maintain

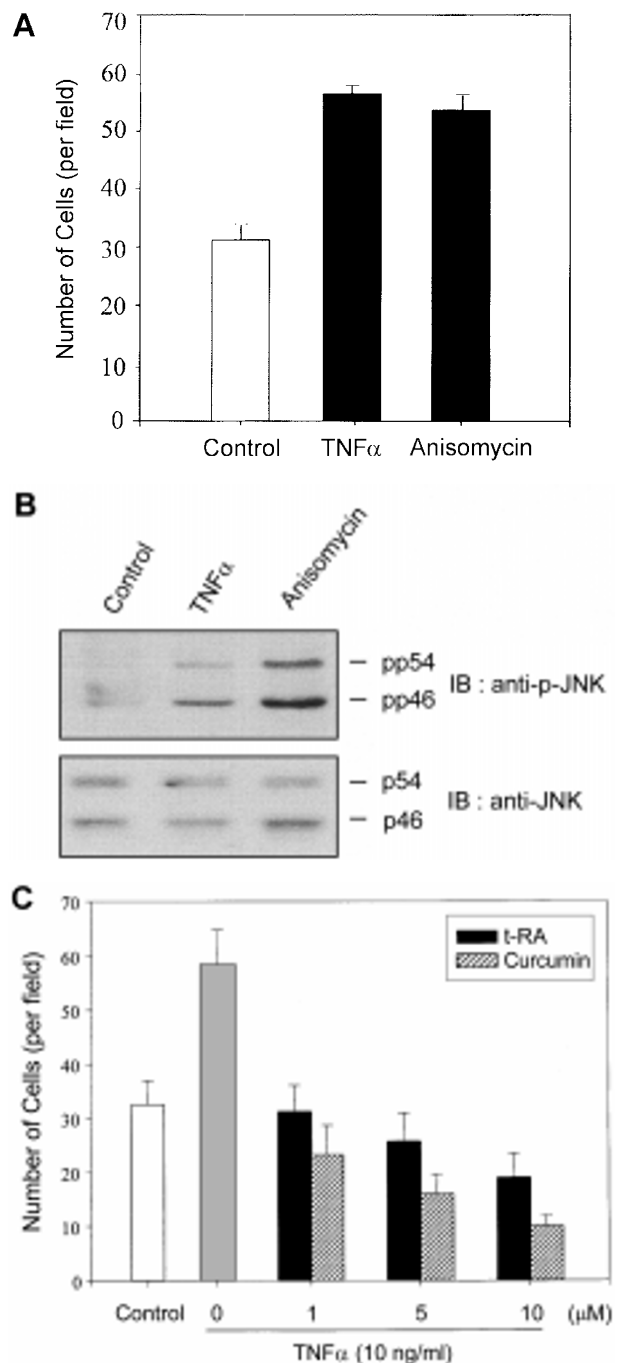


Figure 3. Effect of JNK inhibitors on agonist-induced migration of ECV304 cells. A, Cells were starved for 20 h with M199 plus 0.2% FBS, and treated for 1 h with TNF α (100 ng/ml) or anisomycin (10 μ g/ml). Migration was analyzed by the same procedure as described in Figure 1C. Data are expressed as the number of migratory cells (mean \pm SD). B, Cells were processed as described above. The lysates (50 μ g) were fractionated and immunoblotted with anti-phosphorylated JNK antibody (upper panel) or anti-total JNK antibody (lower panel). C, Cells were starved for 20 h with M199 plus 0.2% FBS, pretreated with curcumin or all-trans-retinoic acid (t-RA) at the indicated concentrations for 1 h and stimulated with 100 ng/ml TNF α . Migration was analyzed by the same procedure as described in Figure 1C. Migration data are expressed as the number of migratory cells (mean \pm SD).

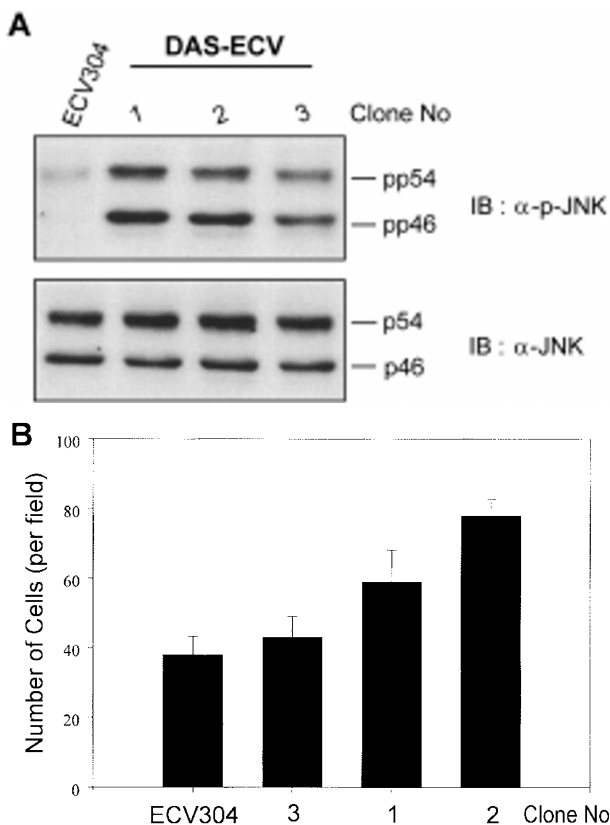


Figure 4. Effect of active SEK1 on basal migration of ECV304 cells. A, DAS-ECV cells expressing active SEK1 were screened by western blot analysis. Cells were grown in M199 plus 0.2% FBS for 24 h and lysed. The lysates (50 μ g) were then resolved on a 10% SDS-polyacrylamide gel, electrottransferred to a nitrocellulose membrane, and immunoblotted with anti-phosphorylated JNK antibody (upper panel) or anti-total JNK antibody (lower panel). B, Migration of the representative three clones was analyzed under the same condition as described in Figure 1C. Migration data are expressed as the number of migratory cells (mean \pm SD).

the cellular viability. All these clones showed an enhanced migratory potential as compared to control cells (Figure 4B). Phosphorylation (activation) levels of JNK in these clones seemed to correlate with motility, but not exactly, suggesting that other factors than JNK might be involved in the motility of these cells.

MMP-2 and uPA are activated downstream of SEK1

Matrix metalloprotease (MMP) has been reported to act downstream of MAPK. Thus, we examined whether MMP is activated in DAS-ECV cells when cultured in dish. For zymography cells were starved for 24 h, then grown in M199 medium. Secreted MMP activity in culture medium was measured using gelatin as a substrate. As shown in Figure 5A, MMP-2 and MMP-9 were constitutively active in both ECV304 and DAS-ECV cells. However, MMP-2 but not MMP-9 was upregulated in DAS-ECV cells. Consistent with this, a similar finding was observed from immunofluorescence microscopy in which stronger

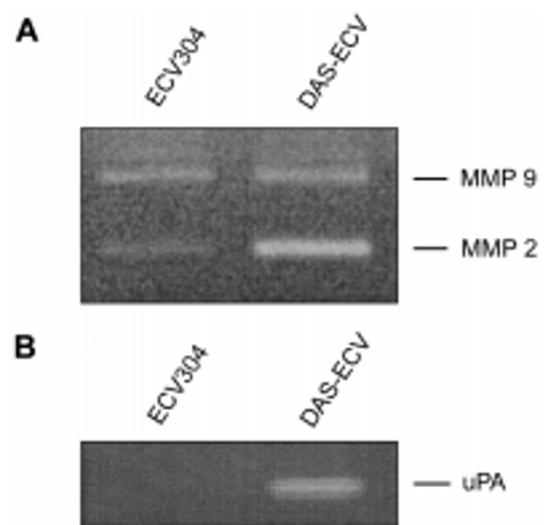


Figure 5. Analysis of MMP and uPA activity in DAS-ECV cells. Gelatin (A) and casein (B) zymography were performed as described in Materials and Methods. Culture supernatants were separated on a SDS-polyacrylamide gel containing 1 mg/ml gelatin or casein under non-reducing condition. For the enzyme reactions gels were incubated in the developing buffer, stained with Coomassie brilliant blue, and then destained in the 45% (v/v) methanol-10% (v/v) acetic acid.

cytoplasmic staining of MMP-2 was shown in DAS-ECV cells as compared to ECV304 cells (data not shown). These results suggest that MMP-2 may act downstream of JNK.

JNK as a downstream of oncogenic Ras switches a protease phenotype from cathepsin L-dependent one to urokinase plasminogen activator (uPA)-dependent one in fibroblast cells (Janulis *et al.*, 1999). We examined whether a similar change would occur in endothelial cells. No band was seen in ECV304 cells, while a band migrating at ~50 kd was clearly seen in DAS-ECV cells, indicating that basal uPA activity was markedly enhanced (Figure 5B). Our observation that SEK1 acts upstream of uPA in endothelial cells is consistent with a previous report (Janulis *et al.*, 1999).

Discussion

In this study, the role of JNK in migration of endothelial cells was examined. Our results showed that JNK inhibitors, curcumin and all trans-retinoic acid, prevented basal and agonist-induced migration. Stimulation of JNK by constitutively active Ras and SEK1 (MKK4) increased unstimulated basal migration. Furthermore, uPA and MMP-2 but not MMP-9 may be major mediators of EC migration. These results implicate that JNK might be involved in both agonist-induced and basal migration of endothelial cells through activation of proteases.

In parallel with the importance of Ras in tumorigenesis

it was suggested that Ras might play a pivotal role in angiogenesis. Despite the enormous results on tumorigenesis its biological function associated with angiogenesis has not been clarified yet. In this study we have provided evidence that Ras is one of critical regulators in angiogenesis using constitutively active H-ras. Constitutively active (oncogenic) H-Ras promoted *in vitro* angiogenesis (Shin *et al.*, 1999), while constitutively negative H-Ras blocked it at the initial stage (data not shown). Our observation was rather surprising, since it was speculated that the Ras transformed ECV304 cells may lose their capacity to differentiate and build a mature capillary network. These results suggest that the cellular transformation and angiogenesis may be independently regulated by H-Ras.

Introduction of oncogenic H-Ras proteins evoked active random movement of endothelial cells (Fox *et al.*, 1994). DAR-ECV cells, which express oncogenic H-Ras stably, showed drastic morphological changes, acquiring the features of transformed cells. In addition, they exhibited not only numerous small spikes (filopodia) following attachment in Matrigel culture but also large and irregular capillary networks that could not be observed during angiogenesis of HUVEC and ECV304 cells. These results strongly suggest that cytoskeletal rearrangement occurred in DAR-ECV cells. Members of Rho GTPase family are key regulatory molecules for the reorganization of actin cytoskeleton (Ridley *et al.*, 1999). Activation of Rho GTPases induces a set of dynamic changes in actin-based cytoskeleton (Nobes *et al.*, 1999): stress fiber by RhoA, lamellipodia and membrane ruffle by Rac, and filopodia by Cdc42. These actin cytoskeletons are closely associated with integrin-based adhesion complexes such as focal adhesion and focal complex (Defilippi *et al.*, 1999). Thus, it is likely that Rho GTPases are involved in Ras-mediated cytoskeletal changes in DAR-ECV cells. Rho GTPases have been reported to regulate the JNK cascade in a PAK-dependent and -independent manner (Teramoto *et al.*, 1996; Rudel *et al.*, 1998). However, DAS-ECV cells did not show any significant cytoskeletal changes, suggesting that SEK1-JNK pathway does not mediate Ras- and Rho-mediated cytoskeletal changes.

Given that JNK does not affect the cytoskeletal rearrangement in DAS-ECV cells, what is the potential target of JNK? Proteases are required to migrate and invade through the Matrigel. Evidence indicates that expression of uPA and MMPs is dependent on co-ordinated activation of MAPK families (Westermarck *et al.*, 1999). JNK has been shown to activate uPA activity in fibroblast cells (Janulis *et al.*, 1999). However, its activity is not obligatory for Ras transformation and invasion. MMP-1, -9 and -13 are also regulated by JNK pathway (Gum *et al.*, 1997; Westermarck *et al.*, 1998; Mengshol *et al.*, 2000). Consistent with this, our results showed that migration of DAR-ECV and DAS-ECV cells was

dependent on JNK and secretion of MMP-2 and uPA was dependent on JNK.

In conclusion, JNK is involved in basal and agonist-induced migration *via* activation of MMP-2 and uPA in ECV304 cells. Further studies to identify downstream mediators of JNK will provide a greater insight into the role of Ras-JNK cascade in cell migration.

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