THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 36, Issue of September 7, pp. 33762–33772, 2001
Printed in U.S.A.

Transendothelial Migration of Colon Carcinoma Cells Requires Expression of E-selectin by Endothelial Cells and Activation of Stress-activated Protein Kinase-2 (SAPK2/p38) in the Tumor Cells*

Received for publication, September 19, 2000, and in revised form, May 9, 2001
Published, JBC Papers in Press, July 11, 2001, DOI 10.1074/jbc.M008564200

Julie Laferrière‡§, François Houle‡, Mohiuddin M. Taher¶, Kristoffer Valerie¶, and Jacques Huot‡‖

From ‡Le Centre de Recherche en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec, Québec G1R-2J6, Canada and the ¶Department of Radiation Oncology, Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298-0058

Adhesion and migration of tumor cells on and through the vascular endothelium are critical steps of the metastatic invasion. We investigated the roles of E-selectin and of stress-activated protein kinase-2 (SAPK2/p38) in modulating endothelial adhesion and transendothelial migration of HT-29 colon carcinoma cells. Tumor necrosis factor α (TNFα) strongly increased the expression of E-selectin in human umbilical vein endothelial cells (HUVEC). This effect was independent of the activation of SAPK2/p38 induced by TNFα. Adhesion of HT-29 cells on a monolayer of HUVEC pretreated with TNFα was dependent on E-selectin expression but was independent of SAPK2/p38 activity of both HUVEC and tumor cells. The adhesion of HT-29 cells to E-selectin-expressing HUVEC led to the activation of SAPK2/p38 in the tumor cells as reflected by the increased phosphorylation of the actin-polymerizing factor HSP27 by mitogen-activated protein kinase 2/3, a direct target of SAPK2/p38. Moreover, a recombinant E-selectin/Fc chimera quickly increased the activation of SAPK2/p38 in HT-29 cells. Blocking the increased activity of SAPK2/p38 of HT-29 cells by SB203580 or by expressing a dominant negative form of SAPK2/p38 inhibited their transendothelial migration. Similarly, HeLa cells stably expressing a kinase-inactive mutant of SAPK2/p38 showed a decreased capacity to cross a layer of HUVEC. Overall, our results suggest that the regulation of transendothelial migration of tumor cells involves two essential steps as follows: adhesion to the endothelium through adhesion molecules, such as E-selectin, and increased motogenic potential through adhesion-mediated activation of the SAPK2/p38 pathway.

Circulating tumor cells attach to adhesive endothelial molecules, and these interactions are pivotal during the metastatic process. E-selectin, whose expression is induced by cytokines and growth factors released by tumor cells, promotes the endothelial adhesion of tumor cells from various origins, and this correlates with metastatic dissemination of tumor cells, *e.g.* to liver, lung, and bones (1–4). The ability of colon tumor cell clones to bind E-selectin on endothelial cells is even directly proportional to their metastatic potential (5). Moreover, inhibiting the expression of E-selectin with drugs such as cimetidine prevents metastasis (6). Metastatic colonization also correlates with the expression of other types of endothelial adhesion molecules such as P-selectin and ICAM[1] (7–12). Furthermore, the metastatic potential is associated with the circulating levels of soluble endothelial adhesion molecules shed by activated endothelial cells of cancer patients (13–17). The increased metastatic potential associated with adhesion of tumor cells to the endothelium might result from two distinctive processes as follows: local intravascular proliferation of the attached tumor cells or extravasation of these cells following their transendothelial migration into the sub-vascular tissues (18, 19). In both cases, the underlying biochemical mechanisms remain ill-defined.

Stress-activated protein kinase-2 (SAPK2/p38), a member of the MAP kinase cascade family, transduces the signals generated by stress and growth factors (20–22). Like other MAP kinase signaling pathways, the SAPK2/p38 pathway consists of the MAP kinase module, the MAP kinase itself (SAPK2/p38), the MAP kinase kinases (*e.g.* MKK3, MKK4, and MKK6), and the MAP kinase kinase kinases (*e.g.* ASK1 and TAK1) (23, 24). Activation of SAPK2/p38 is involved in the synthesis of pro-inflammatory cytokines and activates a number of transcription factors such as MEF2C, ELK-1, and ATF2 (25–28). It also regulates the activation of cytoplasmic kinases such as MAPKAP kinases 2/3 (29–33) which leads to phosphorylation of the actin-polymerizing factor HSP27. In endothelial cells, a cell type that expresses high levels of HSP27, SAPK2/p38-mediated phosphorylation of HSP27 triggers actin polymerization and reorganization into stress fibers in response to oxidative stress and VEGF (21, 22, 34). In the case of VEGF, activation of SAPK2/p38, downstream of VEGFR2, is accompanied by an HSP90-dependent tyrosine phosphorylation of FAK, a key protein kinase involved in the assembly of focal adhesions. SAPK2/38-mediated actin polymerization with FAK-dependent assembly of focal ad-

* This work was supported by Canadian Institutes of Health Research Grant MT15402, the Cancer Research Society Inc., and United States Public Health Grant PHS CA53199. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Holds studentships from FRSQ/FCAR-CRSNG and The Cancer Research Society Inc.

‖ To whom correspondence should be addressed: Centre de Recherche en Cancérologie de l'Université Laval, L'Hôtel-Dieu de Québec, 11 Côte du Palais, Québec, G1R 2J6, Canada. Tel.: 418-691-5553; Fax: 418-691-5439; E-mail: Jacques.Huot@phc.ulaval.ca.

[1] The abbreviations used are: ICAM, intercellular adhesion molecule; ATF2, activating transcription factor-2; FAK, focal adhesion kinase; HSP, heat shock protein; HUVEC, human umbilical vein endothelial cells; JNK, Jun-N-terminal kinase; MAP kinase, mitogen-activated protein kinase; MAPKAP K2, MAP kinase-activated protein kinase 2; SAPK, stress-activated protein kinase; TNFα, tumor necrosis factor α; VEGF, vascular endothelial growth factor; GST, glutathione *S*-transferase; MOPS, 4-morpholinopropanesulfonic acid; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

hesions allow the actin reorganization required for cell migration in various cellular systems (20, 22, 35–38).

In the present study, we show that E-selectin mediates adhesion of colon carcinoma HT-29 cells to endothelial cells. This contributes to activate the SAPK2/p38 pathway in the tumor cells and enhances their motogenic potential and transendothelial migration.

EXPERIMENTAL PROCEDURES

Materials—[γ-^{32}P]ATP (3000 Ci/mmol) and Na^{51}Cr (200–500 mCi/mg) were purchased from DuPont and Amersham Pharmacia Biotech, respectively. TNFα and SB203580 were purchased from Calbiochem. Calcein-AM was obtained from Molecular Probes (Eugene, OR); cycloheximide was from Sigma, and Tfx-50 was from Promega (Madison WI). Recombinant HSP27 was purified from *Escherichia coli* transformed with a plasmid containing the coding sequence for Chinese hamster HSP27 (39). Myc-tagged human HSP27 and LT-tagged-MAPKAP K2 plasmids were obtained from Dr. Jacques Landry (Laval University). pCMV-flag-p38 Ala, Gly, Phe was a gift from Dr. Roger Davis (University of Massachusetts). Recombinant human E-selectin/Fc chimera was obtained from R & D Systems (Minneapolis, MN). pEGFP-C1 was purchased from CLONTECH (Palo Alto, CA). Chemicals for electrophoresis were obtained from Bio-Rad and Fisher.

Antibodies—Anti-MAPKAP kinase 2/3 is a polyclonal antibody raised in the rabbit after injecting a glutathione *S*-transferase (GST) fusion protein containing the 223 C-terminal amino acids of Chinese hamster MAPKAP kinase-2 (33). Anti-E-selectin (Brig-E4 and BBA26) antibodies are mouse monoclonal antibodies that were purchased from R & D Systems and Chemicon (Temicula, CA), respectively. Anti-human TNFα neutralizing antibody was purchased from R & D Systems. Mouse IgG1κ was obtained from Sigma. Myc was detected with the monoclonal antibody 9E10 (40). The phospho-p38/SAPK2 antibody is a rabbit polyclonal antibody purchased from New England Biolabs (Beverly, MA).

Cells—Human umbilical vein endothelial cells (HUVEC) were isolated by collagenase digestion of umbilical veins from undamaged sections of fresh cords (34). Briefly, the umbilical vein was cannulated, washed with Earle's balanced salt solution, and perfused for 10 min with collagenase (1 mg/ml) in Earle's balanced salt solution at 37 °C. After perfusion, the detached cells were collected, and the vein was washed with medium 199 and the wash-off pooled with the perfusate. The cells were washed by centrifugation and plated on gelatin-coated 75-cm^2 culture dishes in medium 199 containing 20% heat-inactivated fetal bovine serum (FBS), endothelial cell growth supplement (60 μg/ml), glutamine, heparin, and antibiotics. Replicated cultures were obtained by trypsinization and were used at passages $\leq$5. The identity of HUVEC as endothelial cells was confirmed by their polygonal morphology and by detecting their immunoreactivity for factor VIII-related antigens. HT-29 human colon carcinoma cells were obtained from ATCC (Manassas, VA). They were cultivated in McCoy 5A medium supplemented with 10% fetal bovine serum. HL-60 cells, obtained from ATCC, were cultivated in RPMI 1640 medium supplemented with 20% heat-inactivated FBS. HeLa cells stably transfected with a plasmid containing a kinase-inactive mutant of SAPK2/p38α (p38(AGF)/HeLa) and the parental HIVCat/HeLa cells (HeLa) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and appropriate selection drugs (G418 and hygromycin B) (41, 42). Cultures were kept at 37 °C in a humidified atmosphere containing 5% CO_2.

Transfection—HT-29 cells were plated 24 h before lipofection (1.3×10^6 cells/25-cm^2 flasks or 1×10^6/60-mm Petri dishes) and incubated for 2 h in the absence of serum with 6.3 or 8.15 μg/dish of plasmids (pCMV-flag-p38 AGF, Myc-tagged human HSP27, LT-tagged-MAPKAP kinase 2, pEGFP-C1) and Tfx-50™ at a ratio of 3:1. The incubation medium was then changed with fresh medium, and treatments were applied 24 h post-transfection.

Immunoprecipitation—After treatments, cells were scraped and extracted in lysis buffer containing 20 mM MOPS, pH 7.0, 10% glycerol, 80 mM β-glycerophosphate, 5 mM EGTA, 0.5 mM EDTA, 1 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 50 mM NaF, 1% Triton X-100, 1 mM benzamidine, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The extracts were vortexed and centrifuged at 17,000 $\times$ *g* for 12 min at 4 °C. The clarified supernatants were stored at −80 °C. The further steps were carried out at 4 °C. The clarified supernatant was diluted 4 times in buffer I (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 1% Triton, 1 mM PMSF). Undiluted antibodies were added in limiting concentrations, and the mixtures were incubated for 1 h. Ten μl of protein A-Sepharose (Amersham Pharmacia Biotech) 50% v/v in buffer I were added, and the mixtures were incubated for 30 min. Samples were centrifuged for 15 s and washed 3 times with 300 μl of buffer I. Immunoprecipitates were directly used for the kinase assays.

Kinase Assays—SAPK2/p38 activation was measured by assessing the activity of its substrate MAPKAP K2. The activity of immunoprecipitated MAPKAP K2 was measured using recombinant HSP27 (34). The assays were carried out in 20 μl of kinase buffer K: 100 μM ATP, 3 μCi of [γ^{32}P]ATP (3000 Ci/mmol), 40 mM *p*-nitrophenyl phosphate, 20 mM MOPS, pH 7, 10% glycerol, 15 mM $MgCl_2$, 0.05% Triton X-100, 1 mM dithiothreitol, 1 mM leupeptin, and 0.1 mM PMSF. The kinase activity was assayed for 30 min at 30 °C and was stopped by the addition of 10 μl of SDS-PAGE loading buffer. In the case of SAPK1/JNK activity, the cell extract was adsorbed on GST-Jun beads, and the kinase was tested using the same GST-N-terminal Jun as substrate (34). Briefly, the GST-Jun fusion proteins bound to glutathione-Sepharose beads were incubated for 30 min at 4 °C with the extracts in buffer I. The beads were then pelleted, washed with I buffer, and incubated for 30 min at 30 °C with 3 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in kinase buffer K containing 10 mM $MgCl_2$. The phosphorylated GST-Jun was boiled in SDS sample buffer to stop the reaction. The activity of the various kinases was quantified by measuring the incorporation of radioactivity into the specific substrate after SDS-PAGE. Kinase activities were evaluated by measuring incorporation of the radioactivity into the specific substrates after resolution by SDS-PAGE and quantification using liquid scintillation counting or by PhosphorImager (Molecular Dynamics). In certain experiments, SAPK2/p38 activity was evaluated by Western blotting using an antibody that recognizes the phosphorylated form of SAPK2/p38 (New England Biolabs).

Phosphorylation of HSP27—HT-29 cells co-transfected with Myc-tagged HSP27, and LT-tagged MAPKAP K2 plasmids were trypsinized, put in suspension, and then left to adhere to plastic only (Petri dish) to control HUVEC or to HUVEC-expressing E-selectin following exposure to TNFα in the presence or not of a neutralizing anti-TNFα antibody. After 30 min, adhering cells were extracted in IEF buffer, and proteins were fractionated by IEF and transferred onto nitrocellulose as described previously (34). After blotting Myc-tagged HSP27 isoforms A–D were revealed with the monoclonal anti-Myc antibody 9E10 and an ECL detection kit (Amersham Pharmacia Biotech). The proportion of each of the isoforms has been quantified after normalization for the same amount of HSP27/sample.

Adhesion Assays—HUVEC were plated on gelatin-coated slides and left to grow to confluence for 24–48 h. HT-29 cells, HL-60 cells, and HeLa cells were labeled for 30 min at 37 °C with calcein. Labeled cells were left to adhere to the endothelial layer for 30 min at 37 °C. The endothelial layer was washed twice with phosphate-buffered saline, and the attached cells were quantified by measuring the fluorescence emission using a fluorometer.

Transendothelial Cell Migration Assay—Cell migration was assayed using a modified Boyden chamber assay. HUVEC (150,000) were grown to confluence (48 h) on a 5.0-μm pore size gelatinized polycarbonate membrane separating the two compartments of a 6.5-mm migration chamber (Transwell Costar). HUVEC were treated or not with 10 ng/ml TNFα for 90 min. Thereafter, culture media were changed for fresh media, and cells were incubated for an additional 2.5 h. Tumor cells in suspension were labeled for 1.5 h with 100 μCi of ^{51}Cr/10^6 cells and then added in migration buffer (medium 199, 10 mM HEPES, pH 7.4, 1.0 mM $MgCl_2$, 0.5% bovine serum albumin) on the monolayer of HUVEC, previously washed with the same buffer. After 4.5 h, cells on the upper face of the membrane were scraped using a cotton swab. The number of tumor cells that have migrated to the lower face of the filter was counted by detaching the membrane and counting the radioactivity.

In some experiments, HT-29 cells were not radiolabeled. After migration, cells on the upper face of the membrane were scraped using a cotton swab, and cells on the lower face were fixed with 3.7% formaldehyde and stained with Mayer's hematoxylin solution. The number of cells on the lower face of the filter was counted in five fields under $\times$ 100 magnification. HT-29 cell number has been determined after correction for the background of HUVEC (<10% of total number of counted cells).

Confocal Fluorescence Microscopy—Confocal microscopy was used for immunofluorescent visualization of F-actin, E-selectin, and ICAM (33). The cells were plated on gelatin-coated LabTek dishes. After treatment, they were fixed with 3.7% formaldehyde and permeabilized with 0.1% saponin in phosphate-buffered saline, pH 7.5. F-actin was detected using fluorescein isothiocyanate-conjugated phalloidin (33.3 μg/ml) diluted 1:50 in phosphate buffer. Brig-E4 monoclonal antibody was used to detect E-selectin. The antigen-antibody complexes were detected with biotin-labeled anti-mouse IgG and were revealed with Texas Red-

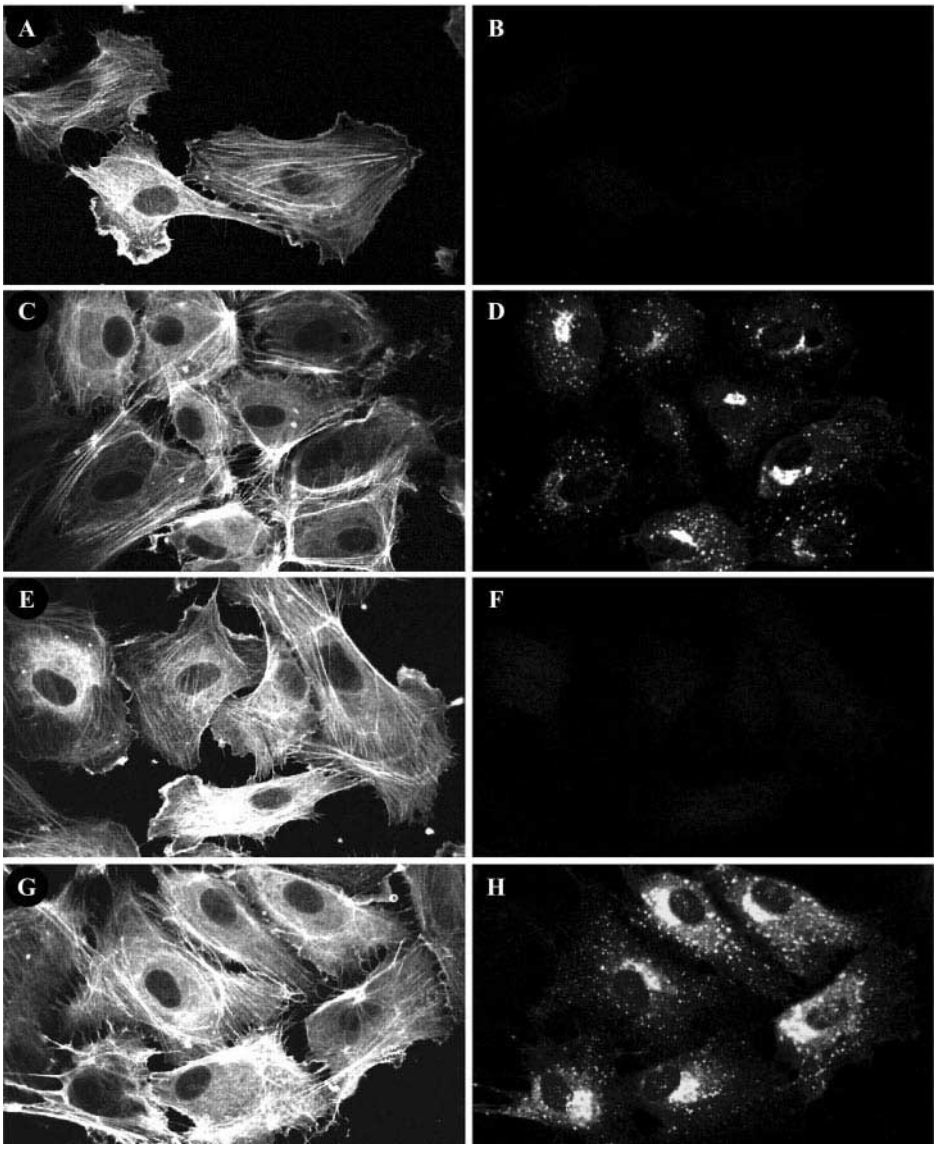

FIG. 1. **TNFα-induced *de novo* expression of E-selectin is not mediated by SAPK2/p38.** HUVEC plated on gelatin-coated slides were left untreated (*A* and *B*) or were pretreated for 60 min with 100 μM cycloheximide (*E* and *F*) or with 5 μM SB203580 (*G* and *H*). Then TNFα (10 ng/ml) was added for 90 min to *C–H*. Thereafter, culture media were changed for fresh media alone (*A–D*) or fresh media containing 100 μM cycloheximide (*E* and *F*) or 5 μM SB203580 (*G* and *H*), and cells were incubated for an additional 2.5 h. After treatments, cells were processed for actin staining using fluorescein isothiocyanate-conjugated phalloidin (*A, C, E,* and *G*) or E-selectin detection with monoclonal antibody Brig-E4 complexed with a biotin-labeled anti-mouse IgG and revealed with Texas Red-conjugated streptavidin (*B, D, F,* and *H*). Cells were then examined by confocal microscopy.

conjugated streptavidin. The cells were examined as reported previously by confocal microscopy with a Bio-Rad MRC-1024 imaging system mounted on a Nikon Diaphot-TDM equipped with a × 60 objective lens with a 1.4 numerical aperture (34).

Statistical Analysis—Data are mean ± S.D. Statistical analysis was done by using the appropriate Student *t* test. $p < 0.05$ was considered as significant.

RESULTS

E-Selectin-dependent Adhesion of Tumor Cells to Endothelial Cells Is Independent of SAPK2/p38 Activity—In primary cultures of HUVEC, TNFα induced a strong activation of the expression of endothelial adhesion proteins that include E-selectin and ICAM (Fig. 1, *A—D,* and data not shown). This induction was maximal after 4 h and required *de novo* protein synthesis being inhibited by cycloheximide (Fig. 1, *E* and *F*). As illustrated in Fig. 2, *A–C*, the expression of E-selectin correlated with an increased adhesion of both colon carcinoma HT-29 cells and HL-60 leukemia cells to a monolayer of HUVEC. After 30 min, the number of HT-29 cells that adhered to HUVEC-expressing E-selectin, following activation with TNFα, was 5-fold higher than when adhering to inactivated HUVEC. Similarly, HT-29 cells quickly adhered to immobilized recombinant human E-selectin/Fc chimera (data not shown). An anti-E-selectin neutralizing antibody, but not a matched isotype antibody, decreased the adhesion of both cancer cell types to the activated endothelium (Fig. 2, *A–C*). Cycloheximide also inhibited the adhesion of HT-29 cells, which is consistent with the fact that adhesion required *de novo* E-selectin synthesis (Fig. 2*A*). These results indicate that E-selectin expression is a major determinant in the adhesion of tumor cells to HUVEC.

TNFα also induced in HUVEC, a marked time- and dose-dependent stimulation of SAPK2/p38 that is characterized by an increased activity of MAPKAP K2/3, a direct physiological target of SAPK2/p38 (21). Maximal stimulation was obtained after a 10-min exposure to concentrations of TNFα equal to or higher than 5 ng/ml (Fig. 3, *A* and *B*). The pyridinylimidazole derivative SB203580, in concentrations of 1–5 μM, completely inhibited the TNFα-induced increase in SAPK2/p38 activity as reflected by the inhibition of MAPKAP K2/3 activation in cells exposed to TNFα (Fig. 3*C*). In contrast, SB203580 had no effect on the activity of SAPK1/JNK that was co-activated with SAPK2/p38 in the presence of TNFα (Fig. 3*D*). Inhibiting the TNFα-induced increase in SAPK2/p38 activity by SB203580 did not impair the expression of E-selectin, which suggested that activation of SAPK2/p38 was not required for the expression of this adhesion molecule (Fig. 1, *G* and *H*). Accordingly, blocking the SAPK2/p38 activity of HUVEC with SB203580 did not inhibit the adhesion of HT-29 cells to HUVEC (Fig. 2*A*).

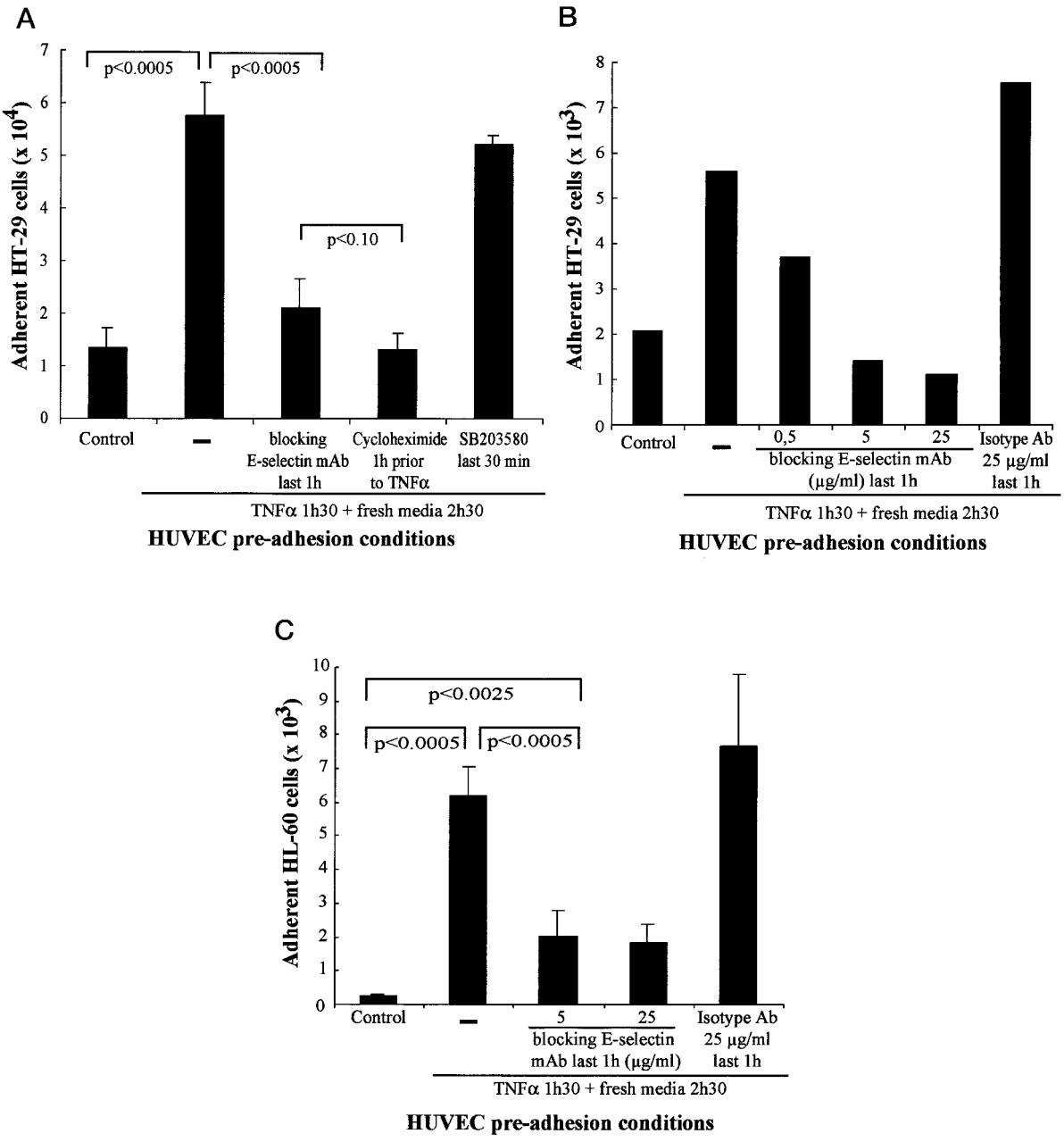

FIG. 2. **E-selectin-dependent adhesion of tumor cells to endothelial cells does not require activation of SAPK2/p38.** *A* and *B*, HUVEC plated on gelatin-coated slides were left untreated or were treated for 90 min with 10 ng/ml TNFα. Thereafter, culture media were changed for fresh media, and cells were incubated for an additional 2.5 h in the presence or absence of increasing concentrations of an anti-E-selectin neutralizing antibody (BBA26, last 60 min), of a matched isotype antibody (last 60 min), of 5 μM SB203580 (last 30 min), or of 100 μM cycloheximide (1 h prior to TNFα treatment). HT-29 cells labeled with calcein-AM were then added to the endothelial layer and left to adhere for 30 min at 37 °C. After washing, fluorescence was quantified to measure the number of adherent cells. *C*, HUVEC plated on gelatin-coated slides were left untreated or were treated for 90 min with 10 ng/ml TNFα. Thereafter, culture media were changed for fresh media, and cells were incubated for an additional 2.5 h in the presence or absence of an anti-E-selectin neutralizing antibody (last 60 min) or with a matched isotype antibody (last 60 min). HL-60 cells labeled with calcein-AM were then added on the HUVEC layer and left to adhere for 30 min at 37 °C. After washing, fluorescence was quantified to determine the number of adherent HL-60 cells. Data points represent the mean + S.D. p was determined by the Student's t test. *, $p < 0.0125$; †, $p < 0.0005$. *mAb*, monoclonal antibody.

Overall, these results indicate that activation of SAPK2/p38 is not necessary for the expression of E-selectin by endothelial cells nor for the adhesion of tumor cells to endothelial cells.

E-Selectin Expression by Endothelial Cells and Activation of SAPK2/p38 in the Tumor Cells Are Both Required for the Transendothelial Migration of Tumor Cells—E-selectin-dependent adhesion of leukocytes to the endothelium is a prerequisite to their transendothelial migration during the inflammatory process (43). We thus verified whether E-selectin-mediated adhesion was required for the migration of tumor cells across an endothelial layer separating the upper and lower compartments of a Boyden-modified chamber. HT-29 cells have by themselves a very low motogenic potential, being unable to traverse a polycarbonate membrane, even following the addition of FBS in the lower chamber (data not shown). However, HT-29 cells migrated across an endothelial layer of HUVEC, and this migration was enhanced by pretreating HUVEC with TNFα (Fig. 4*A*). This increase in cell migration was reduced down to control levels by pretreating HUVEC with the anti-E-selectin antibody indicating the requirement of E-selectin expression and E-selectin-dependent adhesion for HT-29 cell migration across an endothelial cell layer (Fig. 4*A*).

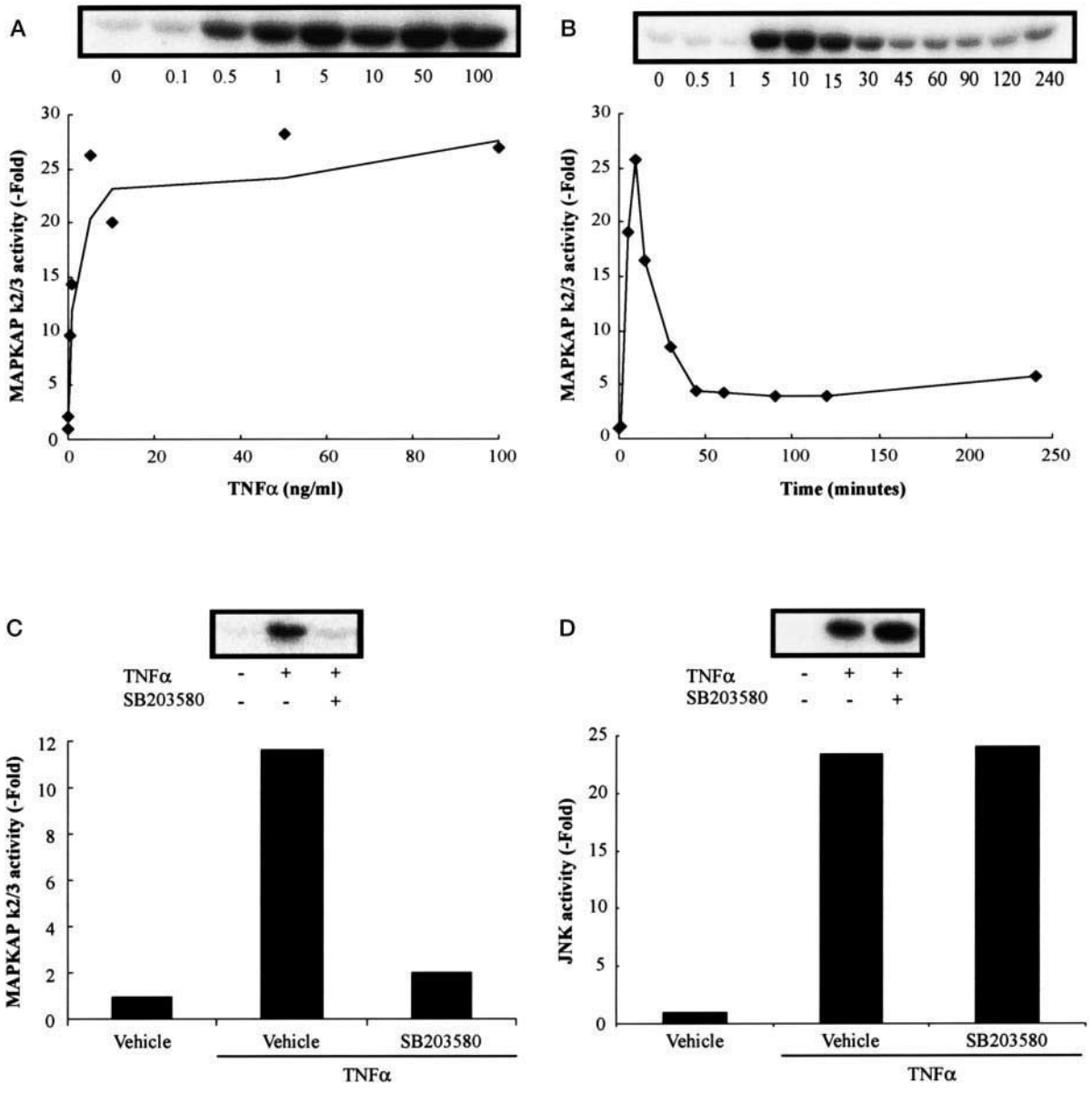

FIG. 3. **Dose- and time-dependent activation of SAPK2/p38 induced by TNFα in HUVEC.** HUVEC were treated for 15 min with increasing concentrations of TNFα (*A*) or for various periods with 10 ng/ml TNFα (*B*). *C*, HUVEC were pretreated for 1 h with vehicle (Me_2SO 0.25%) or with 5 μM SB203580 before administration of 10 ng/ml TNFα for 15 min. After treatments, samples were extracted, and SAPK2/p38 activity was evaluated in immunocomplex assays by measuring the activity of MAPKAP K2, using a specific anti-MAPKAP K2 antibody and rHSP27 as substrate. Results are expressed as the ratio of kinase activity of stimulated cells over the activity of unstimulated cells. Representative results from three experiments are shown. *D*, HUVEC were pretreated for 1 h with vehicle (Me_2SO 0.25%) or with 5 μM SB203580 before administration of 25 ng/ml TNFα for 15 min. After treatments, samples were extracted and were adsorbed on GST-c-Jun beads, and the kinase activity was tested using the same GST-N-terminal Jun as substrate. Results are expressed as the ratio of kinase activity of stimulated cells over the activity of unstimulated cells.

We recently reported that activation of SAPK2/p38, by leading to the phosphorylation of the actin-polymerizing factor HSP27, is importantly involved in transducing the motogenic signal elicited by VEGF in endothelial cells (20, 22). Moreover, SAPK2/p38 was highly reactive in HT-29 cells being activated by cytokines, such as TNFα, that are associated with the neoplastic process. SB203580 inhibited this increased SAPK2/p38 activity in response to TNFα (Fig. 5). From these observations, we hypothesized that E-selectin-mediated adhesion could activate the SAPK2/p38-HSP27 pathway in the tumor cells and that this could trigger their transendothelial migration.

We then examined whether E-selectin-mediated adhesion could activate the SAPK2/p38/HSP27 pathway. HT-29 cells were transiently transfected with Myc-tagged human HSP27 and LT-tagged MAPKAP K2 and then were put in suspension and added to plastic only (Petri dish), to control HUVEC or to HUVEC-expressing E-selectin following activation with TNFα. Thirty minutes after adhesion, cell extracts were prepared from adhering cells, and phosphorylation of HSP27 was evaluated by IEF electrophoresis to separate the four major isoforms of HSP27, A—D, that represent unphosphorylated, monophosphorylated, biphosphorylated, and triphosphorylated variants of the protein. Results showed that adhesion of HT-29 cells to HUVEC-expressing E-selectin was associated with a 3.5-fold increase in the proportion of phosphorylated C form in comparison with the proportion of C form found in HT-29 cells that have adhered to plastic or to untreated HUVEC (Fig. 6, *A* and *D*). This was associated with a proportionally significant decrease in the amount of the unphosphorylated A form in the HT-29 cells adhering to E-selectin-expressing HUVEC (Fig.

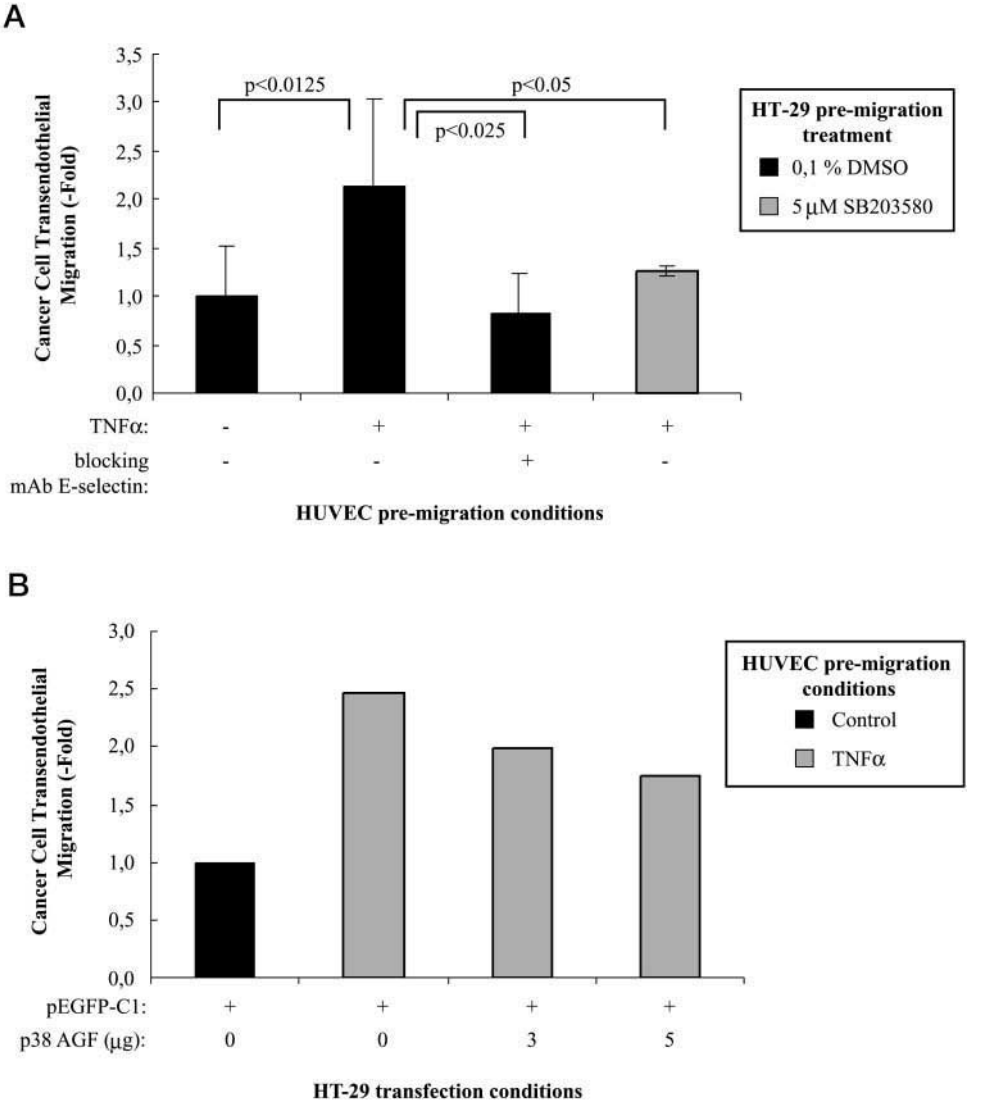

FIG. 4. **Transendothelial migration of HT-29 tumor cells requires E-selectin expression by endothelial cells and increased SAPK2/p38 activity in the tumor cells.** HUVEC were grown to confluency for 48 h on a 5-μm pore size polycarbonate membrane in Boyden-modified chambers. HUVEC were treated or not with 10 ng/ml TNFα for 90 min. Thereafter, culture media were changed for fresh media and cells incubated for an additional 2.5 h. *A,* HUVEC were treated or not for the last 60 min with an anti-E-selectin neutralizing antibody. HT-29 cells pretreated for 30 min with 5 μM SB203580 or with the vehicle (Me_2SO (*DMSO*) 0.1%) were then added on the endothelial layer and left to migrate for 4.5-h at 37 °C. *B,* HT-29 cells were transiently transfected with the indicated amount of pCMV-flag-p38 AGF cDNA for 24 h before being harvested and added to the layer of HUVEC and then left to migrate for 4.5-h. Results are expressed as the ratio of the number of HT-29 cells that have crossed the activated endothelial layer over the number of HT-29 cells that have crossed the unstimulated endothelial layer. *A*, data points represent the mean ± S.D. p was determined by the Student's t test, $n = 3–6$ from two different experiments. *B,* data points represent the means from duplicates, and results are representative of two different experiments.

6*B*). Phosphorylated B form was present in any of the adhering conditions, but its proportion did not vary (Fig. 6*C*). Expression of E-selectin in HUVEC has been induced by pretreating the cells for 90 min with 10 ng/ml TNFα followed by a medium change and a further 2.5-h incubation in fresh medium. Hence, it is possible that a fraction of TNFα exogenously added to HUVEC to trigger synthesis of E-selectin remained bound to HUVEC or in solution in the fresh culture medium at the time of adding HT-29 cells to activated HUVEC. Since TNFα activated SAPK2/p38 in HT-29 cells (Fig. 5), we thus considered the eventuality that residual TNFα contributed to increase the phosphorylation of HSP27 in the adherent HT-29 cells. To exclude this possibility, enzyme-linked immunosorbent assays (Quantikine from R & D Systems) were performed to detect TNFα bound to HUVEC as well as remaining in the fresh culture medium. We found that only trace amounts of TNFα (4.2 pg/5 $\times$ 10^5 cells) were associated with HUVEC, whereas 0.25 ng/ml were found in the fresh culture medium. In both cases, these concentrations were below the minimal concentration of TNFα (0.5 μg/ml) that was required to activate SAPK2/p38 in HT-29 cells. We thus concluded that was unlikely that residual TNFα was involved in activating SAPK2/p38 in HT-29 cells adhering to HUVEC. Accordingly, addition of a neutralizing anti-TNFα, in concentration (0.5 μg/ml) that totally inhibited the activation of SAPK2/p38 by 1 ng/ml TNFα, did not impair the increased phosphorylation of HSP27 in HT-29 adhering to HUVEC (Fig. 6, *E* and *F*). These results support the hypothesis that the E-selectin-dependent adhesion of HT-29 tumor cells to endothelial cells activates the SAPK2/p38-HSP27 pathway in the tumor cells. In fact, the activity of SAPK2/p38 of HT-29 cells was quickly increased by adhesion of the cells to immobilized recombinant human E-selectin/Fc chimera (data not shown). Reciprocally, addition of recombinant human E-selectin/Fc chimera, in concentrations (1 μg/ml) that

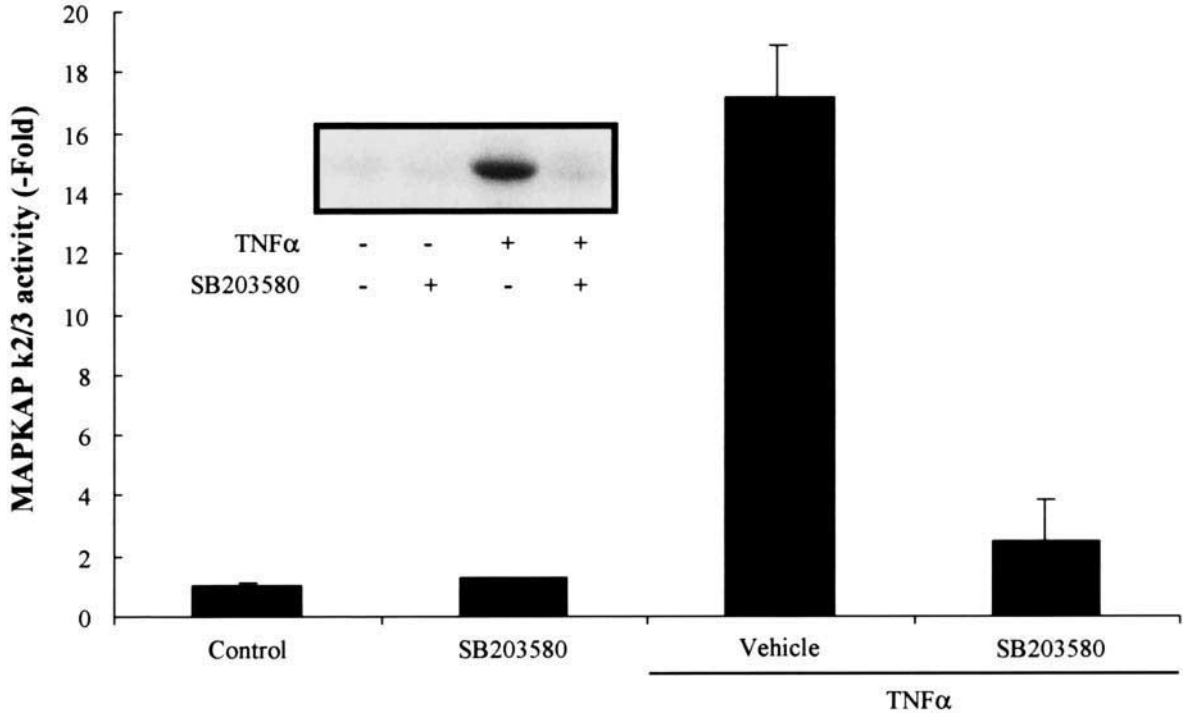

FIG. 5. **Inducibility of the SAPK2/p38 pathway in HT-29 tumor cells.** HT-29 cells were pretreated for 30 min with the vehicle (Me_2SO) or with 5 μM SB203580 before the administration of 20 ng/ml TNFα for 15 min. After treatments, samples were extracted, and SAPK2/p38 activity was evaluated in immunocomplex assays by measuring the activity of MAPKAP K2 using a specific anti-MAPKAP K2 antibody and rHSP27 as substrate. Results are expressed as the ratio of kinase activity of stimulated cells over the activity of unstimulated cells. $n = 2$. Representative results from 2 experiments are shown.

increased adhesion of HT-29 by 10-fold in comparison to bovine serum albumin controls, activated in these cells the SAPK2/38 in a time-dependent manner with a peak of activation of 6.5-fold after 5 min (Fig. 7 and data not shown). Together, these findings indicate that E-selectin did not only mediate the adhesion of HT-29 cells to HUVEC but that it could also act as agonistic ligand that activated the SAPK2/p38-HSP27 motogenic pathway in the tumor cells.

Impairing E-selectin-mediated activation of SAPK2p38 and HSP27 phosphorylation of HT-29 cells with SB203580 (Fig. 4*A* and data not shown) or by expressing a dominant negative form of SAPK2/p38 inhibited their migration across activated HUVEC (Fig. 4*B*). This supports the hypothesis that E-selectin-mediated activation of the SAPK2/p38-HSP27 pathway in HT-29 cells is determinantly involved in triggering the transendothelial migration of these cells. The role of SAPK2/p38 as a motogenic pathway in tumor cells was further supported by the finding that HeLa cells stably expressing a kinase-inactive mutant form of SAPK2/p38 had a lower capacity than the parental cells to migrate across a monolayer of HUVEC (Fig. 8, *A* and *B*). Intriguingly, the adhesion of both types of HeLa cells was not increased when added to HUVEC stimulated with TNFα (Fig. 8*C*). These findings confirmed the results of Fig. 1*A* that SAPK2/p38 was not involved in mediating adhesion of cancer cells to HUVEC, and they indicated that, conversely to HT-29 cells, adhesion of HeLa cells to HUVEC did not require the expression of E-selectin or of other adhesion molecules that depend on TNFα exposure. This raises the interesting possibility that activation of SAPK2/p38 of the cancer cells may be a common motogenic event that involves different types of adhesive interactions that may differ from tumor cells to tumor cells and from endothelial cells from different origins.

DISCUSSION

Adhesion of circulating tumor cells to vascular endothelium and their subsequent transendothelial migration are two important steps associated with extravasation of tumor cells and metastatic spreading. Here, we obtained results that suggest that E-selectin adhesion of tumor cells to endothelial cells contributes to activate the motogenic SAPK2/p38 pathway in the tumor cells, which triggers their transendothelial migration.

E-selectin is not expressed in unstimulated endothelial cells. However, its expression is quickly and transiently turned on following activation of endothelial cells with TNFα. The induction of E-selectin expression results from the transcriptional activation of the E-selectin gene. Three pathways converge on the activation of the E-selectin gene promoter following stimulation of endothelial cells with TNFα, the NF-κB pathway, and the SAPK1/JNK and SAPK2/p38 MAP kinase pathways. Both the SAPK1/JNK and SAPK2/p38 pathways mediate increases in E-selectin gene promoter activity through activation of the transcription factors ATF2 and c-Jun (44). Activation of the NF-κB and SAPK1/JNK pathways are required for full activation of the E-selectin gene (44). In contrast, the SAPK2/p38-mediated activation of the E-selectin gene is ancillary and dispensable for full expression of the protein since we found that inhibiting SAPK2/p38 with SB203580 did not inhibit the expression of E-selectin. This suggests that activation of ATF2 and c-Jun by JNK can rescue the inhibition that results from exposure of cells to SB203580.

The best characterized physiological role for selectins is their involvement in the adhesion of leukocytes to activated endothelial cells during the inflammatory process (45). This adhesion is the first step that underlies the transendothelial migration of leukocytes to the inflammatory sites and to the subsequent destruction of the invading pathogens. Numerous studies have also implicated endothelial adhesion molecules and especially E-selectin in adhesion of carcinoma cells to vascular endothelial cells (2). The necessity of E-selectin expression for the adhesion of tumor cells from solid (HT-29) and hematological tumors (HL-60) is supported by our observation

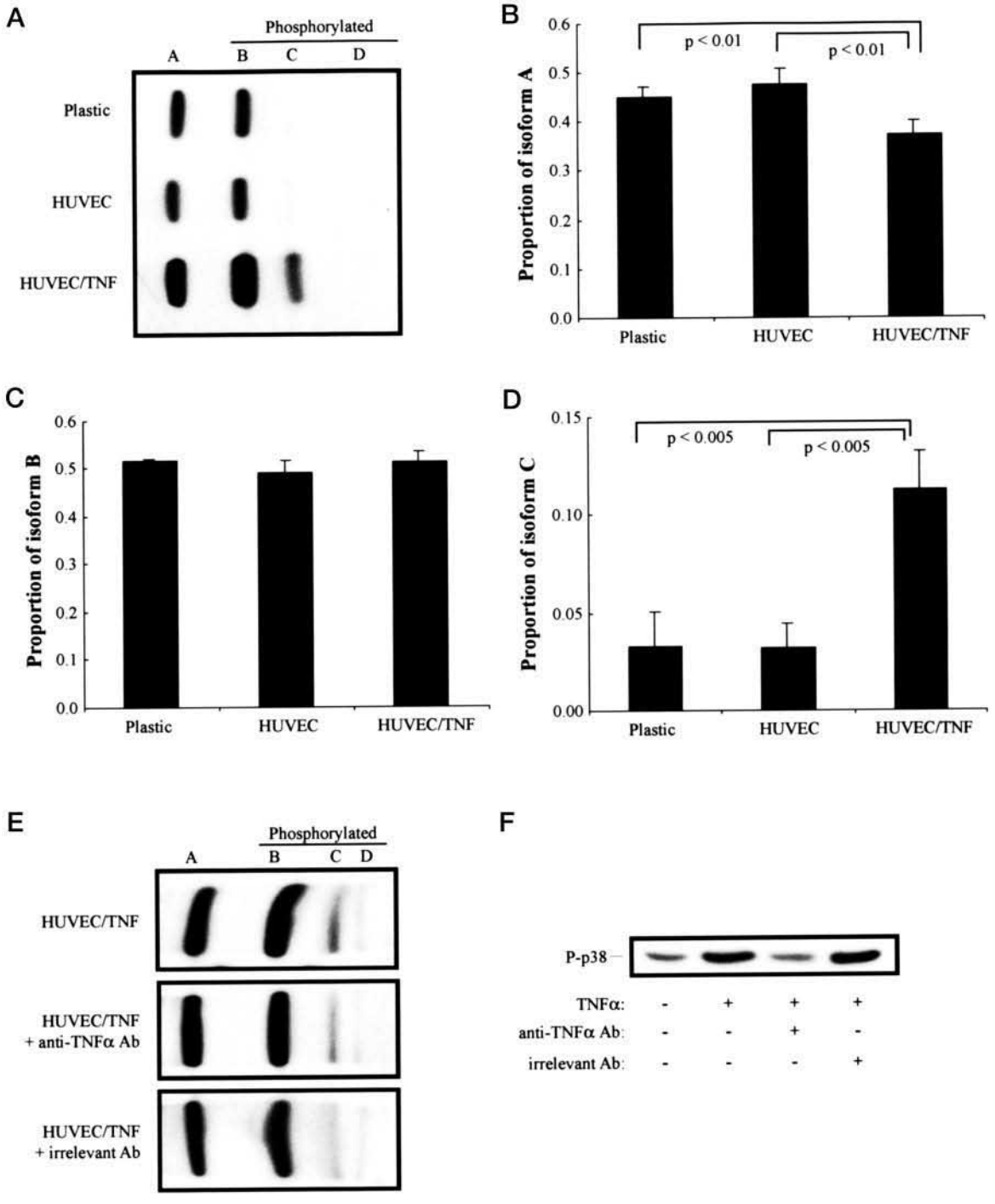

FIG. 6. **E-selectin-mediated adhesion of HT-29 tumor cells to endothelial cells activates the phosphorylation of HSP27 in the tumor cells.** HUVEC were grown to confluency and treated or not with 10 ng/ml TNFα for 90 min. Thereafter, culture media were changed for fresh media, and HUVEC were incubated for an additional 2.5-h. HT-29 cells, transiently transfected with Myc-tagged human HSP27 and LT-tagged MAPKAP K2, were put in suspension in HUVEC media, and then were added to free Petri dishes only (*Plastic*), to a layer of inactivated HUVEC (*HUVEC*), or to a layer of TNFα-activated HUVEC (*HUVEC/TNF*). HT-29 cells were left to adhere for 30 min. Then adherent cells were lysed in IEF buffer, and proteins were fractionated by IEF. The proteins were then transferred on nitrocellulose membrane, and Myc-tagged HSP27 was revealed by Western blotting using the anti-Myc monoclonal antibody 9E10. Representative IEF blots of triplicate samples are shown in *A. B–D* show the quantitative variation in the proportion of the A (unphosphorylated), B (monophosphorylated), and C (biphosphorylated) isoforms of HSP27, respectively. The proportion of each isoforms has been quantified after normalization for the same amount of HSP27/sample. *E,* HUVEC were grown to confluency and treated with 10 ng/ml TNFα for 90 min. Thereafter, culture media were changed for fresh media and HUVEC were incubated for an additional 1.5 h. Then 0.5 μg/ml of an anti-TNFα neutralizing antibody (goat IgG) or 0.5 μg/ml of an irrelevant antibody (goat IgG anti-rabbit) was added for 1 h prior to the addition of transfected HT-29 cells. The extracts were then processed for HSP27 phosphorylation as in *A. F,* HUVEC were pretreated for 1 h with 0.5 μg/ml of an anti-TNFα neutralizing antibody (goat IgG) or with 0.5 μg/ml of an irrelevant antibody (goat IgG anti-rabbit) before the addition of 1.0 ng/ml of TNFα for 10 min. SAPK2/p38 activity was determined by Western blotting using a phospho-p38 antibody (*PY-p38*). Data points represent the mean ± S.D. *p* values were determined by the Student's *t* test.

that pretreating endothelial cells with an anti-E-selectin neutralizing antibody, but not a matched isotype antibody, inhibited in a dose-dependent manner the adhesion of both tumor cell lines to HUVEC. The binding of tumor cells to endothelial cells is clinically significant, being associated with metastasis. Notably, the ability of colon tumor cell clones to bind E-selectin

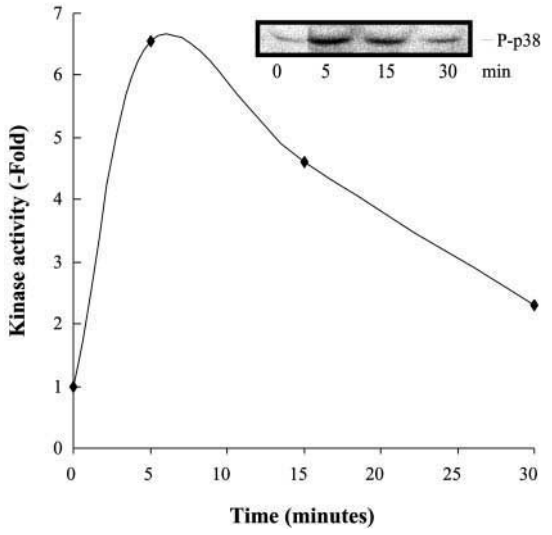

FIG. 7. **Time-dependent activation of SAPK2/p38 by E-selectin.** HT-29 cells were grown for 24 h and then treated for various periods with 1 μg/ml recombinant human E-selectin/Fc chimera. Thereafter, SAPK2/p38 activity was determined by Western blotting using a phospho-p38 antibody (*PY-p38*).

expressed by activated endothelial cells is directly proportional to their metastatic potential (5). Moreover, drugs like cimetidine, which inhibit the expression of E-selectin, prevent metastasis (6).

Two mechanisms may underlie the metastatic development in response to adhesion of tumor cells to the endothelium as follows: intravascular proliferation of attached tumor cells or extravasation of these cells (18, 19). In the latter case, this implicates numerous factors that may work separately or in combination. This implies among others that circulating tumor cells have a higher intrinsic motogenic potential, that they respond to circulating motogenic signals, or that contact of tumor cells with endothelial cells activates the motogenic potential of the tumor cells. A major conclusion of our study is to provide evidence that E-selectin-mediated adhesion of HT-29 tumor cells to HUVEC increased the activity of the motogenic SAPK2/p38 pathway of the tumor cells enabling their transendothelial migration. Two lines of evidence support this conclusion. First, addition of HT-29 cells to HUVEC-expressing E-selectin led to an increased phosphorylation of HSP27, as indicated by the significantly enhanced amount of HSP27-phosphorylated C form in the HT-29 cells that adhered to TNFα-treated HUVEC in comparison to those that adhered

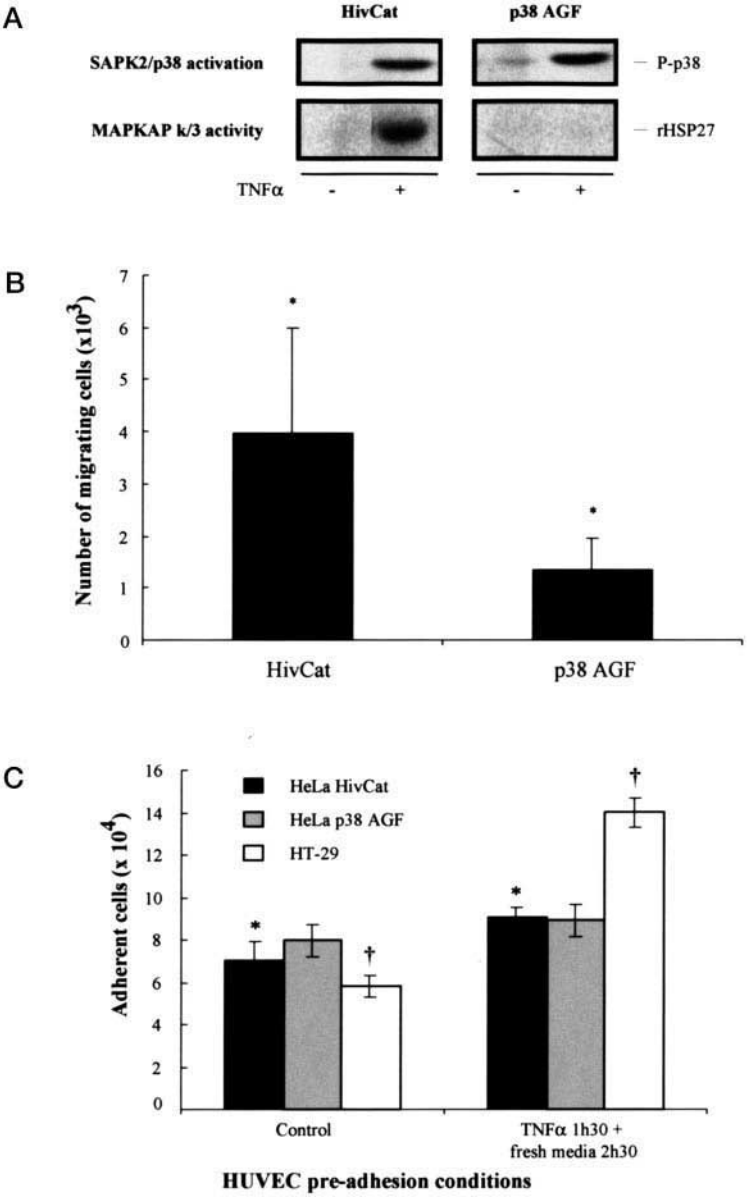

FIG. 8. **Migration of HeLa cells through but not adhesion on endothelial cells depends on SAPK2/p38 activity.** *A,* parental HeLa (HIVCat/HeLa) cells and HeLa cells expressing kinase-inactive mutant of SAPK2/p38 (p38 (AGF)/HeLa) were treated or not for with TNFα, as indicated. Thereafter, SAPK2/p38 activity was determined by Western blotting using a phospho-p38 antibody (*PY-p38*) or by measuring the activity of its substrate MAPKAP K2 in immunocomplexes using a specific anti-MAPKAP K2 antibody and rHSP27 as substrate. *B,* HUVEC were grown to confluency for 48 h on a 5-μm pore size polycarbonate membrane in Boyden-modified chambers. Parental HeLa (HIVCat/HeLa) cells and HeLa cells expressing kinase-inactive mutant of SAPK2/p38 (p38 (AGF)/HeLa) were then added on the endothelial layer and left to migrate for 4.5 h at 37 °C. Results are expressed as the number of HeLa cells that have crossed the endothelial layer. *C,* HUVEC plated on gelatin-coated slides were left untreated or were treated for 90 min with 10 ng/ml TNFα. Thereafter, culture media were changed for fresh media, and cells were incubated for an additional 2.5 h. HT-29 and parental HeLa (HIVCat/HeLa) cells or HeLa cells expressing a kinase-inactive mutant of SAPK2/p38 (p38 AGF/HeLa) were labeled with calcein-AM and added to a monolayer of unstimulated or TNFα-stimulated HUVEC. Cells were left for adhesion during 30 min at 37 °C, washed twice, and then fluorescence was quantified. The number of HT-29 cells and HeLa cells were determined using standard curves. Data points represent the mean ± S.D. p was determined by the Student's t test. *, $p < 0.0125$; †, $p < 0.0005$.

only to plastic or to untreated HUVEC. HSP27 is an actin-polymerizing factor whose phosphorylation downstream of the SAPK2/p38 pathway (34) contributes with FAK phosphorylation to induce the actin reorganization that is required for cell migration (20, 46–48). Second, inhibiting SAPK2/p38 activity and phosphorylation of HSP27 of HT-29 cells with SB203580 or with an inactive kinase mutant of SAPK2/p38 resulted in an inhibition of the transendothelial migration of the tumor cells.

The finding that recombinant human E-selectin/Fc chimera activates SAPK2/p38 indicates that E-selectin acts as an agonist that binds to counter-receptors at the surface of tumor cells to initiate a cascade of events leading to SAPK2/p38 activation. The tumor cells binding to E-selectin involves oligosaccharides such as sialyl Lewis a and x presented by counter-receptors for E-selectin (49). Binding of E-selectin to these receptors initiates signaling events involving tyrosine phosphorylation of various proteins (50). One such potential E-selectin receptor on HT-29 cells might be E-selectin ligand-1, a member of the fibroblast growth factor tyrosine kinase receptor family that is expressed by various tumor cell lines including myeloid cells. SAPK2/p38 is strongly activated by VEGF binding to VEGFR2, another tyrosine kinase receptor (20, 22). E-selectin ligand-1 is thus possibly implicated as a counter-receptor responsible for binding of E-selectin and for transmitting the signal that triggers activation of SAPK2/p38. The capacity of selectins to activate SAPK2/p38 has recently been reported in a study that showed that clustering of L-selectin in neutrophils activates SAPK2/p38, which triggers neutrophil degranulation (51). It remains possible that a secondary adhesion molecule could contribute with E-selectin to trigger adhesion-mediated signaling to SAPK2/p38. In this context, the role of ICAM that is co-expressed with E-selectin in endothelial cells activated by TNFα remains to be investigated. Integrins are importantly involved in transducing signals initiated by cell-cell adhesion (52). For example, tumor cell-bound α_4 integrin strengthens adhesion of tumor cells to the endothelium and promotes transendothelial migration (53). Moreover, activation of SAPK2/p38 by adhesion of osteosarcoma cells onto collagen is mediated by $\alpha_2\beta_1$ integrin (54). Thus, integrins may act jointly with selectins to regulate the SAPK2/p38-mediated motogenic signal elicited in tumor cells when they adhere to endothelial cells.

Interestingly, adhesion of HeLa cells to HUVEC is not markedly increased following treatment of endothelial cells with TNFα suggesting that E-selectin does not have a major role in the process. Nevertheless, transendothelial migration of HeLa cells also required SAPK2/p38 activity since HeLa cells stably expressing a kinase-inactive mutant of SAPK2/p38 showed a decreased capacity to cross the endothelial layer compared with the parental cells. These observations suggest the following: first, activation of SAPK2/p38 might be a common mechanism that triggers transendothelial migration of tumor cells following their adhesion to the endothelium, and second, different endothelial adhesive molecules may contribute to activate this pathway. Endothelial adhesive molecules differ between endothelial cells from different origins, and the specificity of the cancer cell-endothelial cell interactions may well constitute the basis for the organ specificity of metastatic colonization. Notably, hepatic colonization by metastatic cells requires the expression of E-selectin by liver sinusoidal endothelial cells, whereas pulmonary metastasis rather requires the expression of the lung endothelial cell adhesion molecule, LuECAM (3, 55).

In summary, we have shown here that transendothelial migration of tumor cells requires the expression of endothelial adhesion molecules such as E-selectin, which are necessary to enable tumor cells to adhere to the endothelium adhesion and which contribute to activate the motogenic pathway SAPK2/p38-HSP27 in the tumor cells. This might represent a pivotal and insidious paracrine mechanism of metastatic spreading since tumor cells may activate the expression of E-selectin (3).

Acknowledgments—We thank Dr. Jacques Landry, Steve Charette, and Herman Lambert for providing Myc-tagged HSP27 construct and Dr. Roger J. Davis for giving pCMV-flag-p38 (Ala, Gly, Phe).

REFERENCES

1. Mannori, G., Santoro, D., Carter, L., Corless, C., Nelson, R. M., and Bevilacqua, M. P. (1997) *Am. J. Pathol.* **151,** 233–243
2. Brodt, P., Fallavollita, L., Bresalier, R. S., Meterissian, S., Norton, C. R., and Wolitzky, B. A. (1997) *Int. J. Cancer* **71,** 612–619
3. Khatib, A. M., Kontogiannea, M., Fallavollita, L., Jamison, B., Meterissian, S., and Brodt, P. (1999) *Cancer Res.* **59,** 1356–1361
4. Zhang, G. J., and Adachi, I. (1999) *Int. J. Oncol.* **14,** 71–77
5. Sawada, R., Tsuboi, S., and Fukuda, M. (1994) *J. Biol. Chem.* **269,** 1425–1431
6. Kobayashi, K., Matsumoto, S., Morishima, T., Kawabe, T., and Okamoto, T. (2000) *Cancer Res.* **60,** 3978–3984
7. Aigner, S., Ramos, C. L., Hafezi-Moghadam, A., Lawrence, M. B., Friederichs, J., Altevogt, P., and Ley, K. (1998) *FASEB J.* **12,** 1241–1251
8. Haraldsen, G., Kvale, D., Lien, B., Farstad, I. N., and Brandtzaeg, P. (1996) *J. Immunol.* **156,** 2558–2565
9. Sun, J. J., Zhou, X. D., Liu, Y. K., Tang, Z. Y., Feng, J. X., Zhou, G., Xue, Q., and Chen, J. (1999) *J. Cancer Res. Clin. Oncol.* **125,** 28–34
10. Chirivi, R. G., Nicoletti, M. I., Remuzzi, A., and Giavazzi, R. (1994) *Cell Adhes. Commun.* **2,** 219–224
11. Johnson, J. P. (1992) *Int. J. Clin. & Lab. Res.* **22,** 69–72
12. Krause, T., and Turner, G. A. (1999) *Clin. Exp. Metastasis* **17,** 183–192
13. Hebbar, M., Revillion, F., Louchez, M. M., Fournier, C., Bonneterre, J., and Peyrat, J. P. (1999) *Clin. Cancer Res.* **5,** 1427–1433
14. Benekli, M., Gullu, I. H., Tekuzman, G., Savas, M. C., Hayran, M., Hascelik, G., and Firat, D. (1998) *Br. J. Cancer* **78,** 267–271
15. Matsuura, N., Narita, T., Mitsuoka, C., Kimura, N., Kannagi, R., Imai, T., Funahashi, H., and Takagi, H. (1997) *Jpn. J. Clin. Oncol.* **27,** 135–139
16. Gruss, H. J., Dolken, G., Brach, M. A., Mertelsmann, R., and Herrmann, F. (1993) *Leukemia* (*Baltimore*) **7,** 1245–1249
17. Banks, R. E., Gearing, A. J., Hemingway, I. K., Norfolk, D. R., Perren, T. J., and Selby, P. J. (1993) *Br. J. Cancer* **68,** 122–124
18. Al-Mehdi, A. B., Tozawa, K., Fisher, A. B., Shientag, L., Lee, A., and Muschel, R. J. (2000) *Nat. Med.* **6,** 100–102
19. St-Pierre, Y., Aoudjit, F., Lalancette, M., and Potworowski, E. F. (1999) *Leuk. & Lymphoma* **34,** 53–61
20. Rousseau, S., Houle, F., Kotanides, H., Witte, L., Waltenberger, J., Landry, J., and Huot, J. (2000) *J. Biol. Chem.* **275,** 10661–10672
21. Huot, J., Houle, F., Rousseau, S., Deschesnes, R. G., Shah, G. M., and Landry, J. (1998) *J. Cell Biol.* **143,** 1361–1373
22. Rousseau, S., Houle, F., Landry, J., and Huot, J. (1997) *Oncogene* **15,** 2169–2177
23. Landry, J., and Huot, J. (1999) *Biochem. Soc. Symp.* **64,** 79–89
24. Tibbles, L. A., and Woodgett, J. R. (1999) *Cell. Mol. Life Sci.* **55,** 1230–1254
25. Treisman, R. (1996) *Curr. Opin. Cell Biol.* **8,** 205–215
26. Lee, J. C., Laydon, J. T., McConnell, P. C., Gallagher, T. F., Kumar, S., Green, D., McNulty, D., Blumenthal, M. J., Heys, J. R., Lanvatter, S. W., Strickler, J. E., McLaughlin, M. M., Siemens, I., Fisher, S., Livi, G. P., White, J. R., Adams, J. L., and Young, P. R. (1994) *Nature* **372,** 739–746
27. Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997) *Nature* **386,** 296–299
28. Karin, M. (1995) *J. Biol. Chem.* **270,** 16483–16486
29. Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., Hunt, T., and Nebreda, A. R. (1994) *Cell* **78,** 1027–1037
30. Freshney, N. W., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., and Saklatvala, J. (1994) *Cell* **78,** 1039–1049
31. Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen, P., Gallagher, T. F., Young, P. R., and Lee, J. C. (1995) *FEBS Lett.* **364,** 229–233
32. McLaughlin, M. M., Kumar, S., McDonnell, P. C., Van Horn, S., Lee, J. C., Livi, G. P., and Young, P. R. (1996) *J. Biol. Chem.* **271,** 8488–8492
33. Huot, J., Lambert, H., Lavoie, J. N., Guimond, A., Houle, F., and Landry, J. (1995) *Eur. J. Biochem.* **227,** 416–427
34. Huot, J., Houle, F., Marceau, F., and Landry, J. (1997) *Circ. Res.* **80,** 383–392
35. Jaakkola, P., Kontusaari, S., Kauppi, T., Maata, A., and Jalkanen, M. (1998) *FASEB J.* **12,** 959–969
36. Heuertz, R. M., Tricomi, S. M., Ezekiel, U. R., and Webster, R. O. (1999) *J. Biol. Chem.* **274,** 17968–17974
37. Ogura, M., and Kitamura, M. (1998) *J. Immunol.* **161,** 3569–3574
38. Matsumoto, T., Yokote, K., Tamura, K., Takemoto, M., Ueno, H., Saito, Y., and Mori, S. (1999) *J. Biol. Chem.* **274,** 13954–13960
39. Landry, J., Lambert, H., Zhou, M., Lavoie, J. N., Hickey, E., Weber, L. A., and Anderson, C. W. (1992) *J. Biol. Chem.* **267,** 794–803
40. Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J., and Landry, J. (1997) *J. Cell Sci.* **110,** 357–368
41. Taher, M. M., Baumgardner, T., Dent, P., and Valerie, K. (1999) *Biochemistry* **38,** 13055–13062
42. Taher, M. M., Oakley, J. D., Hershey, C., and Valerie, K. (2000) *Biochemistry* **39,** 1709–1715
43. McEver, R. P. (1991) *Thromb. Haemostasis* **65,** 223–228
44. Read, M. A., Whitley, M. Z., Gupta, S., Pierce, J. W., Best, J., Davis, R. J., and

Collins, T. (1997) *J. Biol. Chem.* **272,** 2753–2761
45. Aplin, A. E., Howe, A., Alahari, S. K., and Juliano, R. L. (1998) *Pharmacol. Rev.* **50,** 197–263
46. Piotrowicz, R. S., Hickey, E., and Levin, E. G. (1998) *FASEB J.* **12,** 1481–1490
47. Richardson, A., Malik, R. K., Hildebrand, J. D., and Parsons, J. T. (1997) *Mol. Cell. Biol.* **17,** 6906–6914
48. Romer, L. H., McLean, N., Turner, C. E., and Burridge, K. (1994) *Mol. Biol. Cell* **5,** 349–361
49. Fukuda, M., Hiraoka, N., and Yeh, J. C. (1999) *J. Cell Biol.* **147,** 467–470
50. Soltesz, S. A., Powers, E. A., Geng, J. G., and Fisher, C. (1997) *Int. J. Cancer* **71,** 645–653
51. Smolen, J. E., Petersen, T. K., Koch, C., O'Keefe, S. J., Hanlon, W. A., Seo, S., Pearson, D., Fossett, M. C., and Simon, S. I. (2000) *J. Biol. Chem.* **275,** 15876–15884
52. Giancotti, F. G. (2000) *Nat. Cell Biol.* **2,** E13–E14
53. Holzmann, B., Gosslar, U., and Bittner, M. (1998) *Curr. Top. Microbiol. Immunol.* **231,** 125–141
54. Ivaska, J., Reunanen, H., Westermarck, J., Koivisto, L., Kahari, V. M., and Heino, J. (1999) *J. Cell Biol.* **147,** 401–416
55. Zetter, B. R. (1993) *Semin. Cancer Biol.* **4,** 219–229