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Intracellular Signal-transducing Elements Involved in Transendothelial Migration of Lymphoma Cells

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To investigate the molecular mechanisms underlying transendothelial migration of tumor cells, an essential process for their hematogenous dissemination, we developed an *in vitro* model system that allows the separate monitoring of cell adhesion and transmigration processes. This system uses a human pre-B lymphoma cell line, Nalm-6, and a cultured mouse endothelial cell line, KOP2.16. Nalm-6 cells rapidly adhered to KOP2.16 and subsequently transmigrated underneath them. Using this model, we examined the effects on transendothelial migration, of various reagents which specifically interfere with the function of intracellular signal transduction molecules. Treatment of Nalm-6 cells with wortmannin (WMN), herbimycin A, pertussis toxin, or C3 exoenzyme of *Clostridium botulinum*, which specifically inhibit PI3 kinase and/or myosin light chain kinase, herbimycin-sensitive tyrosine kinases, heterotrimeric G proteins, and the small G proteins *rho*/*rac*, respectively, reduced transmigration in a dose-dependent manner. Pretreatment of KOP2.16 endothelial cells with WMN also reduced transmigration in a dose-dependent manner. Binding of Nalm-6 to KOP2.16 was not affected, even when Nalm-6 or KOP2.16 cells were pretreated with these inhibitors, indicating that the reduction of transmigration was not due to a reduction of Nalm-6 binding to KOP2.16. These results also indicate that the signal transduction pathway(s) involved in transmigration can be dissociated from that of adhesion. Our results support the notion that endothelial cells are not a passive barrier in lymphoma extravasation, but that they assist lymphoma cell extravasation.

Key words: Extravasation — Transendothelial migration — Hematogenous dissemination — Tumor — Metastasis

An understanding of the molecular mechanisms of tumor metastasis is essential for the development of new diagnostic and therapeutic tools against malignant tumors. While the process of tumor metastasis involves sequential and complicated steps, attachment of tumor cells to cellular and extracellular matrix determinants on host endothelial cells and their extravasation through endothelial junctions are thought to be particularly important in determining the tissue specificity of metastasis observed in certain tumors. To mimic these steps under experimental conditions, a number of methods have been established during the past two decades, including tumor cell adhesion assays, colony scattering assays and Matrigel invasion assays.[1–5] Recent studies using these methods have demonstrated that interactions between tumor cells and endothelial cells are mediated by a variety of adhesion molecules, such as selectins, integrins, cadherins, members of the immunoglobulin superfamily, and CD44.[6–13] Binding of these adhesion molecules on the tumor cell surface to their counterpart receptors on endothelial cells is required for tumor cell extravasation into surrounding tissues. Although much progress has been made recently in demonstrating the functional roles of cell surface molecules and signal transducers in cell adhesion and motility, relatively little is understood about details of post-adhesive, intracellular events leading to tumor cell extravasation.

In this study, we present a model system for biochemical analysis of signal transduction pathways leading to tumor cell extravasation using a human pre-B lymphoma cell line, Nalm-6,[14] and a mouse endothelial cell line, KOP2.16,[15] which was previously established in our laboratory. The KOP2.16 cell line allows reproducible, quantitative assessment of cell extravasation, since it permits lymphoid cells, including normal lymphocytes, and some tumor cells to transmigrate readily. To elucidate the post-

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adhesive intracellular events in both tumor cells and endothelial cells in the metastatic process, we examined the effects of pharmacological agents, known to interfere with the function of specific intracellular molecules, on Nalm-6 transmigration through KOP2.16. Our observations may help to define the molecular mechanisms of transendothelial lymphoma migration and aid in designing strategies to prevent hematogenous dissemination of malignant tumor cells.

MATERIALS AND METHODS

Cell culture The cultured mouse endothelial cell line, KOP2.16,[15] was maintained in DMEM (Gibco, New York, NY) containing 20% fetal calf serum (FCS) (Lot No.; FBK09, Mitsubishi Kasei Co., Tokyo), 10 m*M* Hepes, 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 10^{-4} *M* 2-mercaptoethanol, 1% (v/v) 100X non-essential amino acids (Flow Labs, Irvine, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (complete medium). The human pre-B lymphoma cell line, Nalm-6,[14] was cultured in complete RPMI 1640 medium (Flow Labs) containing 10% FCS.

Reagents Herbimycin A (HbA) and pertussis toxin (PTX) were purchased from Seikagaku Kogyo (Tokyo). Wortmannin (WMN)[16, 17] and C3 exoenzyme (C3)[18, 19] were prepared as described previously. BCECF-AM [2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester] was purchased from Molecular Probes Inc. (Eugene, OR).

Transmigration assay Transmigration of Nalm-6 cells beneath the KOP2.16 monolayer was assessed microscopically. KOP2.16 cells were plated at a confluent density (2×10^5/well) in flat-bottomed wells of Costar 24-well plates. Nalm-6 cells suspended in DMEM containing 20% FCS were added to each well (7×10^5/well). After 90 min incubation at 37°C, Nalm-6 cells bound on the surface of the KOP2.16 monolayer were removed by vigorous washing. In experiments where signal transduction inhibitors were used, Nalm-6 or KOP2.16 cells were pretreated with the indicated reagent or an appropriate concentration of solvent used to dissolve the reagent for indicated time periods at 37°C. Nalm-6 cells which transmigrated underneath the KOP2.16 monolayer were easily distinguishable from those bound on the surface of KOP2.16 cells due to their flattened and phase-dense appearance. Therefore, to quantify the process of transmigration, four randomly selected fields were photographed under a phase-contrast microscope, and the number of phase-dense Nalm-6 cells underneath the monolayer was counted.

Cell adhesion assay Adhesion of Nalm-6 cells to the KOP2.16 monolayer was quantified using BCECF-labeled cells as described previously.[15] Briefly, KOP2.16 cells were plated at a confluent density (2×10^4 cells/well) in flat-bottomed wells of 96-well microtiter plates. Nalm-6 cells were suspended in serum-free DMEM containing 5 μ*M* BCECF-AM. After incubation for 45 min at 37°C, Nalm-6 cells were washed and resuspended in culture medium. BCECF-labeled lymphoma cells (5×10^5/well) were then added to the KOP2.16 monolayer in quadruplicate. After 15 min incubation, wells were filled up with medium, sealed with a film, and then converted. Nonadherent cells were removed by aspirating the culture medium. Adherent cells were solubilized with 0.1% NP-40 in phosphate-buffered saline, and the fluorescence intensity of each well was measured with a Fluoroscan II (Flow Labs, McLean, VA).

RESULTS

Establishment of an *in vitro* model system for investigation of lymphoma infiltration When the human pre-B lymphoma cell line, Nalm-6, was added to the KOP2.16 endothelial monolayer (Fig. 1A), Nalm-6 cells avidly bound to the apical surface of KOP2.16 cells (Fig. 1B). Subsequently, they rapidly transmigrated and located themselves underneath the KOP2.16 cells, showing a flattened, phase-dense morphology (Fig. 1C). A significant transmigration was observed within 30 min of addition of Nalm-6 cells and reached a plateau by 90 min (Fig. 1D). Using this *in vitro* assay system, we quantitatively assessed the lymphoma binding to, and transmigration through, the endothelial monolayer.

Effects of various pharmacological agents on lymphoma infiltration In order to understand the molecular mechanisms involved in lymphoma infiltration, lymphoma cells and endothelial cells were treated with various reagents known to be specific inhibitors of various signal transduction pathways. When Nalm-6 cells were pretreated with 10 n*M* WMN, a specific inhibitor of myosin light chain kinase (MLCK) and/or phosphatidylinositol 3 (PI3) kinases,[16, 17] transmigration was significantly inhibited (Fig. 2A). Inhibition was observed in a dose-dependent manner, and was almost complete at 10 μ*M*. Transmigration was also inhibited by pretreatment with PTX, which inhibits heterotrimeric G protein-mediated signaling (Fig. 2B).[20] Significant inhibition by PTX was observed at 1 ng/ml, and was complete at 100 ng/ml (Fig. 2B).

The inhibitory effect on transmigration was also observed when Nalm-6 cells were pretreated with HbA, a protein tyrosine kinase inhibitor,[21] and C3 enzyme, an inhibitor for the small G proteins, *rho* and *rac*.[18, 19] HbA was inhibitory at concentrations between 1 and 10 μ*M* (Fig 2A). C3 was relatively less effective compared with WMN, PTX and HbA, although this may be due to its inefficient penetration into cells.[22] The inhibitory effects of these reagents were not due to their cytotoxic activity,

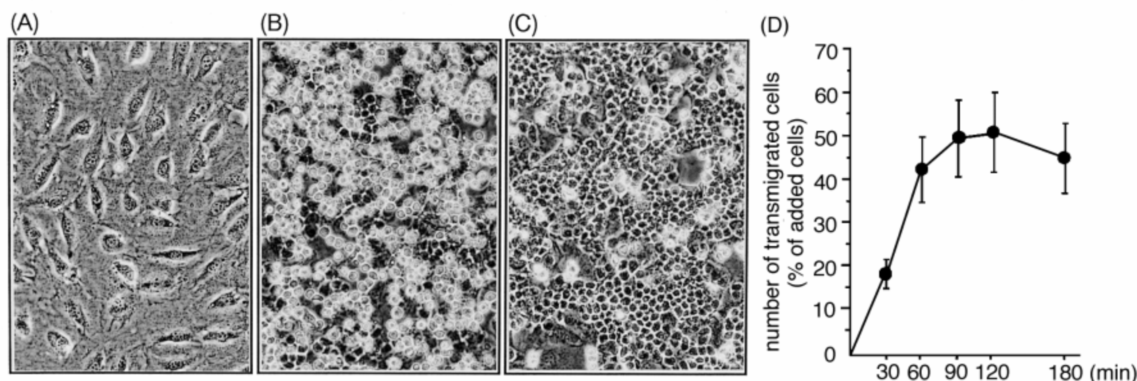

Fig. 1. Interaction between Nalm-6 cells and KOP2.16 endothelial cells. (A) Morphology of KOP2.16. (B) Nalm-6 cells bound on the surface of KOP2.16 cells. Nalm-6 cells were added to the KOP2.16 monolayer, and incubated for 15 min. Non-adherent cells were removed by washing. Small, round and brightened cells are Nalm-6 cells adhering to the KOP2.16 surface. (C) Nalm-6 cells transmigrated underneath the KOP2.16 monolayer. After 90 min incubation of KOP2.16 cells with Nalm-6 cells, Nalm-6 cells bound to KOP2.16 cells were removed by vigorous washing. In contrast to bound cells, shown in (B), transmigrated cells showed dark, phase-dense, and flattened morphology. (D) Time course of Nalm-6 transmigration. Nalm-6 cells (7×10^5/well) were incubated on the KOP2.16 monolayer for the indicated time periods at 37°C followed by washing to remove adherent cells on the surface. Transmigrated cells were counted as described in "Materials and Methods." The number of transmigrated cells reached a plateau after 90 min.

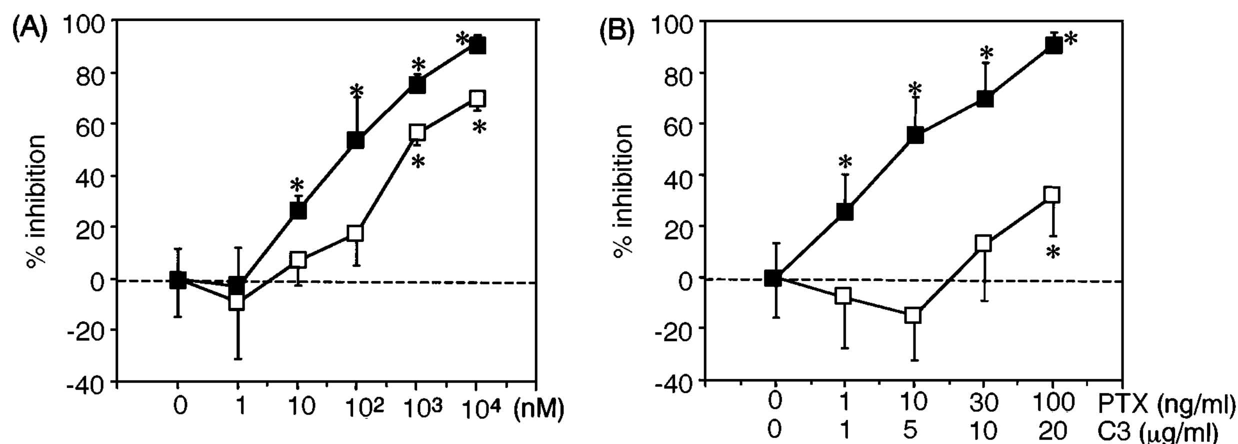

Fig. 2. Dose-dependent inhibition of transmigration by pretreatment of Nalm-6 cells with inhibitors. Nalm-6 cells were pretreated with the indicated concentrations of (A) WMN (closed squares) or HbA (open squares) for 30 min, (B) PTX (closed squares) for 2 h or C3 (open squares) for 72 h, or an equivalent volume of diluent alone. These cells were washed and added to the KOP2.16 monolayer. Transmigrated cells were counted after 90 min of incubation. Data are mean±SD percent inhibition of migration relative to the number of migrated cells treated with the diluent alone (0 n*M* or 0 μg/ml; dashed line). * P<0.05, compared with control.

because they had no effect on cell viability at any of the concentrations used (data not shown). These results suggest that MLCK, PI3 kinases, heterotrimeric G proteins, and tyrosine kinases are involved in the regulation of transendothelial migration of Nalm-6 lymphoma cells.

To investigate whether intracellular signaling events in endothelial cells also participate in lymphoma transmigration, we treated KOP2.16 endothelial cells with various reagents and then performed the transmigration assay. Transmigration was efficiently inhibited by pretreatment of the endothelial monolayer with WMN (Fig. 3). The inhibition was statistically significant in the dose range of 10 n*M* to 10 μM, similar to that seen with pretreatment of Nalm-6, although the extent of inhibition was smaller. No

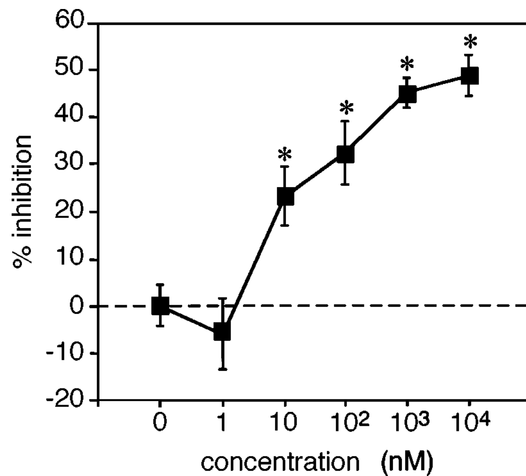

Fig. 3. Dose-dependent inhibition of transmigration by pretreatment of KOP2.16 with WMN. The KOP2.16 monolayer was pretreated with the indicated concentration of WMN or diluent for 30 min, and washed with warmed culture medium. Nalm-6 cells were then added to the KOP2.16 monolayer. Transmigrated cells were counted after a 90 min incubation. Data are mean±SD percent inhibition of migration as compared with the number of migrated cells treated with the diluent alone (0 n*M*; dashed horizontal line). * $P<0.05$, compared with control.

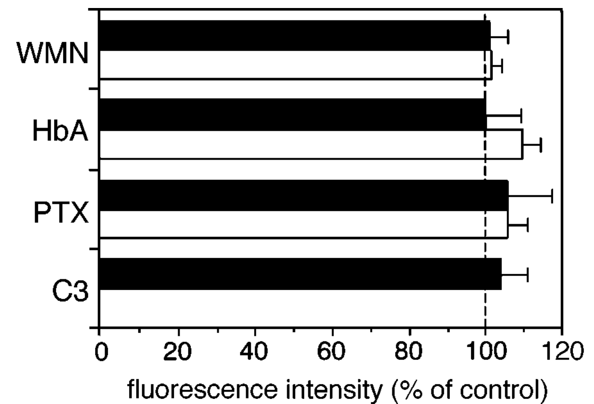

Fig. 4. Effects of inhibitors on Nalm-6 binding to KOP2.16. Nalm-6 cells (solid columns) or the KOP2.16 monolayer (open columns) were pretreated with inhibitors. Inhibitors used were WMN (5 μM), HbA (2 μM), PTX (100 ng/ml), and C3 (20 μg/ml), respectively. Adhesion was measured as described in "Materials and Methods." Treatment of KOP2.16 with C3 resulted in rounding of the cell morphology, so Nalm-6 binding could not be assessed. Data are mean±SD percent of fluorescence intensity relative to the binding of cells pretreated with diluent alone.

difference was observed in cell viability or morphology between untreated and WMN-treated KOP2.16 cells. Endothelial treatment with HbA or PTX at concentrations found to be inhibitory when Nalm-6 cells were pretreated, did not affect the transmigration (data not shown). The role of *rho*/*rac* proteins in endothelial cells could not be estimated because significant morphological changes of the KOP2.16 endothelial cells were induced during treatment with C3 exoenzyme (data not shown).

Effects of various pharmacological agents on lymphoma adhesion to endothelial cells To examine whether the inhibitory effects of these reagents were due to decreased binding of Nalm-6 to KOP2.16 cells, the cell binding assay was performed before and after treatment of either of the cell types with the reagents. As shown in Fig. 4, none of these reagents inhibited Nalm-6 binding to the KOP2.16 monolayer surface, indicating that inhibition of Nalm-6 transmigration was not due to inhibition of the binding of Nalm-6 cells to the KOP2.16 endothelial surface.

DISCUSSION

Tumor metastasis is a complex, multistep process that includes dissociation of tumor cells from the primary site, extravasation, degradation and penetration of the extracellular matrix, uncontrolled cell proliferation and angiogenesis in target organs. In this study, we focused on the signal transduction pathways employed in both endothelial cells and tumor cells during tumor cell extravasation. To identify signal transduction molecules involved in transendothelial migration of tumor cells, we used an *in vitro* cell monolayer transendothelial migration assay, and made the following observations. Firstly, PTX-sensitive G proteins in Nalm-6 lymphoma cells are essential for transmigration. Secondly, lymphoma cell MLCK, PI3 kinases, and small G proteins, *rho* and/or *rac*, also contribute to signal transduction pathways involved in the transmigration of lymphoma cells. Thirdly, endothelial MLCK and PI3 kinases also play an important role in transmigration of lymphoma cells across the endothelial cell barrier. Since none of the reagents caused any detectable changes in cell viability and adhesion of lymphoma cells to the endothelial surface at any concentration used, we postulate that inhibition of transmigration is due to inhibition of post-adhesive, intracellular events.

WMN binds specifically to MLCK and PI3 kinases in an irreversible manner and inhibits the kinase activities of both enzymes with estimated IC_{50} values of 3.0 μM and 0.2 μM, respectively.[16, 17] WMN markedly reduced Nalm-6 transmigration in our experiments. We observed a dose-dependent inhibition of transmigration after WMN treatment of Nalm-6 cells at concentrations ranging from 10 n*M* to 10 μM, indicating that both PI3 kinases and MLCK are involved in this process. Although a number of studies

have examined the biochemical nature and signaling ability of these kinases, the roles of these kinases in tumor cells are not well understood and appear to be complex.[23] MLCK phosphorylates the myosin light chain (MLC); MLC phosphorylation correlates with ATPase activity of myosin.[24, 25] PI3 kinases phosphorylate the D3 position of the inositol ring of phosphatidylinositol, and products of the kinases are implicated in the regulation of various biological functions including DNA synthesis, prevention of apoptosis, and vesicle trafficking.[23] In addition to these functions, PI3 kinases have been implicated in regulating the actin cytoskeleton.[26] Phosphatidylinositol 4,5-diphosphate, one of the products of PI3 kinases, can promote the growth of actin filaments by binding to actin-capping proteins, and releasing these proteins from F-actin ends.[27, 28] Further, there is growing evidence that PI3 kinases are required for activation of integrins and cell adhesion through regulation of the actin cytoskeleton.[29–31] Preliminary results from our laboratory have shown that transmigration of Nalm-6 is completely inhibited by anti-VLA $\alpha4$ and anti-VLA $\alpha5$ antibodies, suggesting that VLA-4 and VLA-5 integrins mediate Nalm-6 transendothelial migration (unpublished data). It has been suggested that VLA-4 can transduce a signal to activate the phosphoinositide pathway.[32] Although the biochemical mechanisms of inhibition of transmigration by WMN are obscure at present, we speculate that inactivation of MLCK and PI3 kinases with WMN leads to inhibition of integrin function through functional impairment of the actin cytoskeleton, resulting in inhibition of transendothelial migration.

That treatment of endothelial cells with WMN also significantly reduced tumor cell extravasation deserves emphasis, as this result indicates that endothelial cells are *active* and not *passive* participants in transendothelial migration of tumor cells. There is growing evidence that some of the endothelial adhesion molecules, such as ICAM-1 and VCAM-1, can transduce signals.[32, 33] It has been reported that VCAM-1 can transduce a signal that involves activation of the phosphoinositide pathway and the mobilization of Ca^{2+} in cultured endothelial cells.[32] Since KOP2.16 cells express VCAM-1 on the surface,[15] ligation of endothelial VCAM-1 with VLA-4 expressed on Nalm-6 may be partly responsible for activation of the WMN-sensitive pathway. Precisely how MLCK and/or PI3 kinases of endothelial cells mediate lymphoma transmigration is not clear, but it is tempting to speculate that both kinases may be important in regulation of the actin cytoskeleton. The endothelial cytoskeleton could be involved in the regulation of functions of cell surface molecules associated with the actin cytoskeleton, and/or of endothelial retraction which might be essential for passage of leukocytes through endothelial cell-cell junctions. This possibility is supported by previously reported evidence that endothelial cells retract in the presence of endothelial-cell-retraction factor derived from tumor cells.[34] Cellular retraction, which results in opening of the intraendothelial cell junctions, is required for subsequent movement of tumor cells across the endothelial monolayer.[34] The functional role of the endothelial cytoskeleton in tumor extravasation requires further exploration.

PTX also inhibited lymphoma cell transmigration. PTX ADP ribosylates the α subunit of certain classes of heterotrimeric GTP-binding proteins.[20] This results in the functional uncoupling of G proteins from their cognate surface receptors, thus blocking signal transduction. In previous studies, PTX has been shown to inhibit migration of lymphocytes,[35] neutrophils,[36, 37] and thymocytes,[38] indicating that PTX-sensitive G proteins are required for leukocyte migration. This is also relevant to invasion and metastasis of lymphoma cells. PTX inhibits liver metastasis formation by lymphoma cells *in vivo*,[39] and also inhibits invasion of T lymphomas to the fibroblast monolayer.[40] Although our observation suggests that PTX-sensitive G proteins are involved in transmigration of lymphoma cells, the classes of cell surface receptors on Nalm-6 lymphoma cells that are functionally linked to G proteins and contribute to transmigration are currently unknown. Additional studies using *in vitro* transmigration assays are required to identify the receptors and downstream effectors involved in this event.

Treatment of Nalm-6 with HbA markedly reduced the number of transmigrated cells, whereas treatment of endothelial cells had no effect (data not shown), suggesting that endothelial protein kinases are not essential in Nalm-6 transmigration. The target proteins whose phosphorylation by HbA-sensitive protein kinases contributes to transmigration, were not examined in the present study, and the identification of HbA-sensitive protein kinases and their target proteins will require further studies.

Various *in vitro* attempts have been made to investigate molecular mechanisms underlying tumor cell invasion. For example, assays using fibroblast and mesothelial monolayers have been reported.[5, 34, 40–42] Transendothelial chemotaxis assay, in which HUVEC was used in combination with chemokines, has been found to be useful for studying the transendothelial migration process.[43] Here we have described an experimental model for lymphoma invasion using a cultured mouse endothelial cell monolayer. We used the endothelial cell line, KOP2.16,[15] which we considered to be a suitable model for investigating molecular aspects of transendothelial migration of tumor cells for the following reasons. Firstly, the KOP2.16 cells permit lymphoma cells to migrate rapidly across the endothelial barrier without addition of any exogenous chemotactic factor. Secondly, since the number of transmigrated lymphoma cells is dependent on the time of incubation and the number of lymphoma cells added to the assay, a reproducible, quantitative assessment of the

transmigration can be made. Thirdly, adhesion and transmigration events can be examined separately in this system, and as shown in the present study, under appropriate conditions signal transduction pathways involved in transmigration can be dissociated from those involved in adhesion. Finally, our *in vitro* model system may be useful as a convenient assay to screen substances that modify tumor cell extravasation.

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