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Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices

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We previously described an inverse correlation between galectin-9 (Gal-9) expression and metastasis in patients with malignant melanoma and breast cancer. This study verified the ability of Gal-9 to inhibit lung metastasis in experimental mouse models using highly metastatic B16F10 melanoma and Colon26 colon cancer cells. B16F10 cells transfected with a secreted form of Gal-9 lost their metastatic potential. Intravenous Gal-9 administration reduced the number of metastases of both B16F10 and Colon26 cells in the lung, indicating that secreted Gal-9 suppresses metastasis. Analysis of adhesive molecule expression revealed that B16F10 cells highly express CD44, integrin α1, α4, αV, and β1, and that Colon26 cells express CD44, integrin α2, α5, αV, and β1, suggesting that Gal-9 may inhibit the adhesion of tumor cells to vascular endothelium and the extracellular matrix (ECM) by binding to such adhesive molecules. Indeed, Gal-9 suppressed the binding of hyaluronic acid to CD44 on both B16F10 and Colon26 cells, and also suppressed the binding of vascular cell adhesion molecule-1 to very late antigen-4 on B16F10 cells. Furthermore, Gal-9 inhibited the binding of tumor cells to ECM components, resulting in the suppression of tumor cell migration. The present results suggest that Gal-9 suppresses both attachment and invasion of tumor cells by inhibiting the binding of adhesive molecules on tumor cells to ligands on vascular endothelium and ECM.

Keywords: CD44/ECM/Galectin-9/metastasis/VLA-4

Introduction

Invasion and metastasis are biological hallmarks of malignant tumors (Hanahan and Weinberg 2000). They appear to be the major causes of malignant tumor-related morbidity and mortality (Wittekind and Neid 2005). Thus, suppression of metastasis and invasion has become major goals for overcoming malignant tumor-related mortality, and extensive studies have been conducted to clarify the mechanisms underlying invasion and metastasis. Furthermore, adhesive molecules, such as the integrin family, CD44, and others, are widely known to be critical players in the metastasis of malignant tumors (Dedhar 1990; Jothy 2003; Eccles et al. 2005).

Galectins are animal lectins that exhibit selective affinity for β-galactosides (Kilpatrick 2002) and bind to a variety of β-galactoside-containing glycoproteins and glycolipids both on the cell surface and in extracellular matrices (ECM) (Hsu and Liu 2004). By binding to these glycoconjugates, galectins deliver signals intracellularly as well as mediate cell–cell and cell–ECM adhesion (Hughes 2001). Galectins thus modulate cell adhesion and migration by affecting the process of tumor cell metastasis (Danguy et al. 2002). We have previously described that high galectin-9 (Gal-9) expression in the cytoplasm of tumor cells is closely associated with reduced metastasis and low recurrence in patients with malignant melanoma (Kageshita et al. 2002) and breast cancer (Irie et al. 2005), raising a hypothesis that Gal-9 is involved in the suppression of tumor cell metastasis. It has been shown that Gal-9 is localized in the cytoplasm and on the cell surface of tumor cells (Kashio et al. 2003). Moreover, Gal-9 seems to be released from tumor cells (Keryer-Bibens et al. 2006). However, it remains unclear which form of Gal-9 is critically involved in the process of tumor cell metastasis.

The purpose of the present experiments is to show that secreted Gal-9 suppresses tumor cell metastasis. Gal-9 accomplishes this by blocking the interaction between adhesive molecules on tumor cells and ligands on both endothelium and ECM.

Results

Effects of Gal-9 transfection on the metastatic potential of B16F10 cells

In our preliminary experiments, we found that the level of cytoplasmic Gal-9 and the amount of Gal-9 secreted in the culture supernatants of B16F10 cells (a highly metastatic cell line) (<3 pg/mL) were significantly less than those of B16F1 cells (a less metastatic cell line) (404 ± 75 pg/mL). We transfected two types of mouse Gal-9 genes, a secreted form of mouse Gal-9 (sec mGal-9) and a non-secreted form of mouse Gal-9 (non-sec mGal-9), into B16F10 cells. Both sets of transfectants expressed more evident Gal-9 mRNA compared to mock-transfected cells (Figure 1A). However, only sec mGal-9-transfected cells secreted high levels of Gal-9 protein during the culture compared to mock-transfected cells (Figure 1B). We further found

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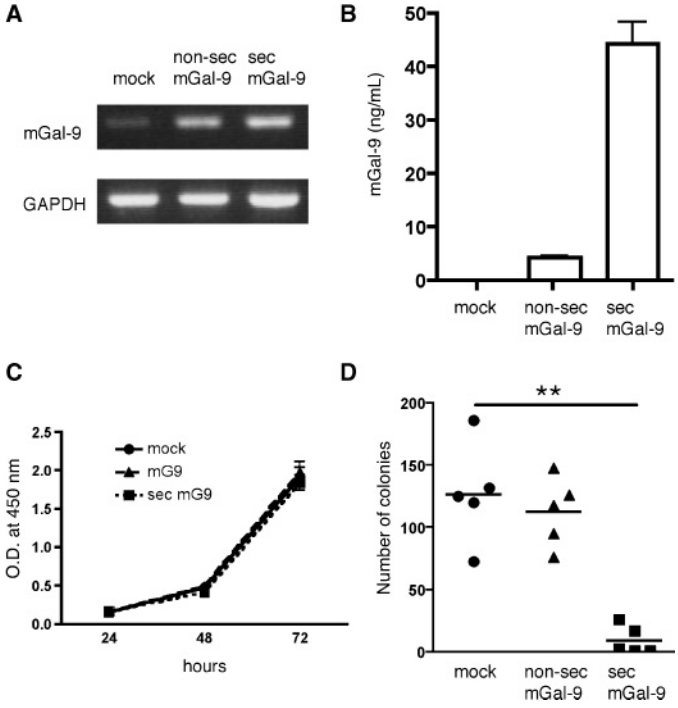

Fig. 1. Transfection of B16F10 cells with mock, non-secreted form of mouse Gal-9 (non-sec mGal-9), and secreted form of mouse Gal-9 (sec mGal-9). (**A**) RT-PCR analysis of mouse Gal-9 gene was performed on mock-, non-sec mGal-9-, and sec mGal-9-transfected cells. GAPDH was used as a control. (**B**) Amounts of mouse Gal-9 in the culture medium of mock-, non-sec mGal-9-, or sec mGal-9-transfected cells. The cells (4×10^4 cells/well) were cultured for 3 days in a fresh medium, and the levels of mouse Gal-9 in the culture fluids were assessed by ELISA. (**C**) Proliferation of mock-, non-sec mGal-9-, and sec mGal-9-transfected cells was determined by WST-1 assay at 24 h, 48 h, and 72 h. (**D**) Transfected cells (2×10^5/mouse) were injected i.v. into C57BL/6 mice. Twelve days later, metastatic colonies in the lung were counted. $^{**}P < 0.01$, Mann–Whitney *U* test (mock versus sec mGal-9).

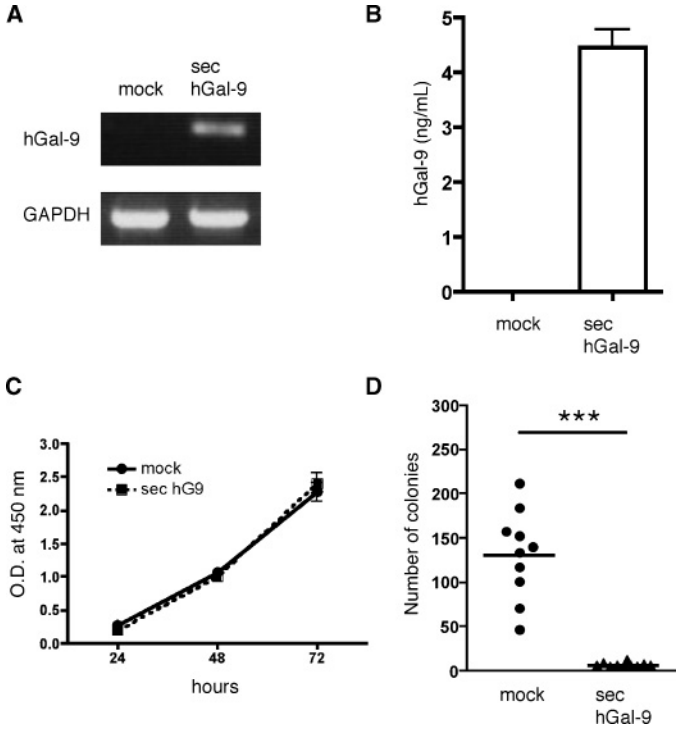

Fig. 2. Transfection of B16F10 with mock and secreting form of stable human Gal-9 (sec hGal-9). (**A**) RT-PCR analysis of human Gal-9 gene was performed on mock- and sec hGal-9-transfected cells. GAPDH was used as a control. (**B**) Amounts of human Gal-9 in the culture medium of mock- or sec hGal-9-transfected cells. The cells (4×10^4 cells/well) were cultured for 3 days in a fresh medium, and the levels of human Gal-9 in the culture fluids were assessed by ELISA. (**C**). Proliferation of mock- and sec hGal-9-transfected cells was determined using WST-1 assay on 24 h, 48 h, and 72 h. (**D**) Transfected cells (2×10^5/mouse) were i.v. injected via the tail vein of C57BL/6 mice. Twelve days later, the lung colonies were counted. $^{***}P < 0.0001$, Mann–Whitney *U* test (mock versus sec hGal-9).

that Gal-9 transfection had no influence on the proliferation of B16F10 cells (Figure 1C).

We inoculated intravenously (i.v.) sec mGal-9-, non-sec mGal-9-, or mock-transfected cells into C57BL/6 mice in order to evaluate the metastatic potential of the transfected cells. A large number of metastatic foci (112.6 ± 12.5 and 127.2 ± 18) were detected in the lungs of all mice inoculated with non-sec mGal-9- and mock-transfected cells, whereas few metastatic foci were detected in lungs of mice that received sec mGal-9-transfected cells (9.6 ± 5.1) (Figure 1D), suggesting the influence of secreted Gal-9 in the suppression of tumor cell metastasis.

To assess the effects of human Gal-9 on pulmonary metastasis, we also used B16F10 cells transfected with a secreted form of stable human Gal-9 (sec hGal-9). Similar to sec mGal-9-transfected B16F10 cells, sec hGal-9-transfected B16 F10 cells expressed high levels of Gal-9 mRNA (Figure 2A), secreted Gal-9 in the culture supernatant (Figure 2B), and there was little influence on the proliferation of B16F10 cells (Figure 2C). Moreover, we found that sec hGal-9-transfected B16F10 cells almost completely lost their metastatic potential (hGal-9, 7 ± 0.8, $n = 10$; mock, 131 ± 15.8, $n = 10$; $P < 0.0001$) (Figure 2D). Based on these data, we decided to use the stable form of human Gal-9 for further experiments.

Suppression of pulmonary metastasis by administration of Gal-9

Experiments were performed to further clarify whether administration of Gal-9 suppresses tumor cell metastasis in vivo. Daily i.v. injection of Gal-9 after i.v. inoculation of B16F10 cells (a mesenchymal malignant melanoma) in C57BL/6 mice significantly reduced the number of pulmonary metastatic foci in the lung (Gal-9, 43.4 ± 7.6, $n = 9$; PBS, 77.9 ± 12.9, $n = 10$; $P < 0.05$) (Figure 3A).

Since the metastatic potential of malignant tumor cells can vary according to the tumor cell lines (Algarra et al. 1999), we did further experiments to generalize Gal-9-mediated suppression of metastasis using Colon26 cells, a murine colon adenocarcinoma, in BALB/c mice. Similar to B16F10 cell metastasis in C57BL/6 mice, daily Gal-9 injection significantly reduced the number of metastatic foci in lung (Figure 3B, Gal-9, 25.7 ± 3.8, $n = 7$; PBS, 51.6 ± 5.6, $n = 7$; $P < 0.05$). These results suggested that Gal-9 is involved in the suppression of metastasis of malignant tumor cells, and these data extend to two heterogenous malignant cell lines (melanoma and adenocarcinoma cell lines).

Effects of Gal-9 on the proliferation and cell death of B16F10 and Colon26 cells

The possibility that Gal-9-mediated inhibition of tumor metastasis is the result of affecting proliferation and/or cell death

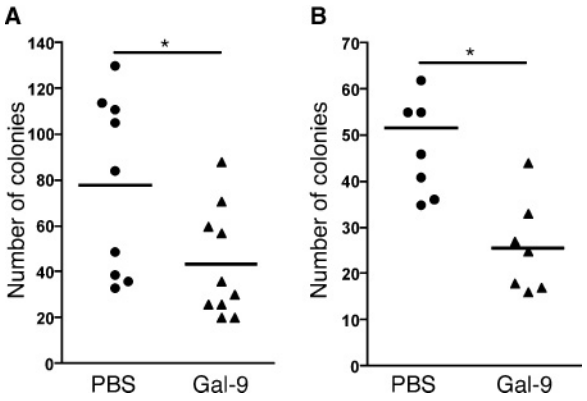

Fig. 3. Effects of Gal-9 on lung metastasis. B16F10 cells (**A**) or Colon26 cells (**B**) were i.v. inoculated into C57BL/6 mice ($n = 9$ or 10) or Balb/c mice ($n = 7$), respectively. One hour later, C57BL/6 mice or Balb/c mice were treated daily with i.v. Gal-9 (30 μg/mouse) from day 0 to day 11 or from day 0 to day 22, respectively. One day after the treatment was stopped, the number of colonies in the lung was counted. $^*P < 0.05$ (PBS-treated versus Gal-9-treated).

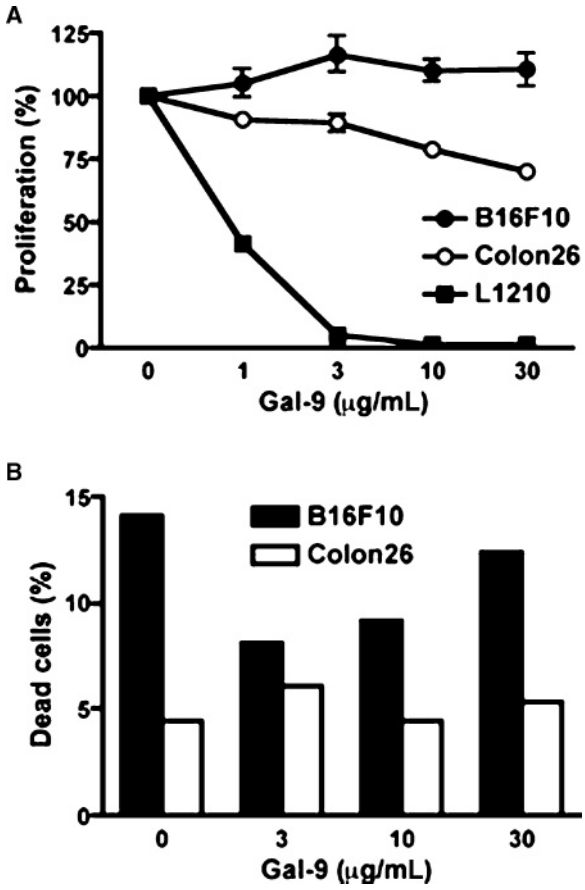

Fig. 4. Effect of Gal-9 on the proliferation and cell death of B16F10 and Colon26 cells. (**A**) B16F10 and Colon26 cells (3×10^3/well) were incubated for 24 h, and Gal-9 was added and cultured for further 24 h. Cells were incubated with WST-1 for 2– 4 h at 37°C. The number of surviving cells was quantified by measuring absorbance at 450 nm. As a control, an L1210 mouse leukemia cell line was used. Data represented are the means ± SD of two experiments in triplicate. (**B**) B16F10 and Colon26 cells (1×10^4/ well) were cultured in 96-well plates with various concentrations of Gal-9 (0–30 μg/mL) for 24 h, and dead cells were measured by a flow cytometric 7-AAD assay. Cell death, indicated by 7AAD positivity, is graphed. Representative data of three experiments are included.

cannot be excluded because Gal-9 induces cell death in certain type of cells (Kageshita et al. 2002; Irie et al. 2005). Thus, experiments were first done to clarify whether Gal-9 affects the proliferation of B16F10 and Colon26 cells using WST-1 assays. Gal-9 dramatically suppressed the proliferation of L1210 mouse leukemia cell line cells. Almost complete suppression was induced by as little as 3 μg/mL Gal-9 (Figure 4A). In contrast, Gal-9 did not affect the proliferation of B16F10 cells at any concentration tested, and the proliferation of Colon26 cells was only slightly suppressed by Gal-9 in a dose-dependent manner (Figure 4A).

Next, we did experiments to clarify whether Gal-9 induces cell death in those cell line cells. Gal-9 did not induce cell death of B16F10 cells and Colon26 cells at concentrations as high as 30 μg/mL (Figure 4B). From these results, it is clear that the effects of Gal-9 on proliferation and cell death, including apoptosis are, at most, only minimally involved in the Gal-9-mediated inhibition of metastasis.

Expression of adhesive molecules on B16F10 and Colon26 cells

It is well known that adhesive molecules play a crucial role in malignant tumor cell metastasis (Nguyen 2004). Therefore, experiments were done to measure the expression of adhesive molecules, such as CD44, sialyl Lewis X, and integrins on the cell surface of B16F10 and Colon26 cells. B16F10 cells expressed high levels of CD44 and integrins α1, α4, αV, and β1 chain. They also expressed low levels of integrins α5 and α6, but not integrins α2, α3, β2 chains (Figure 5A), and sialyl Lewis X (B16F10 cells do not adhere to E-selectin). Colon26 cells expressed high levels of CD44, integrins α2, α5, αV, and β1 chains, and low level of integrin α6 chain, but not the integrins α1, α3, α4, and β2 chains, differing from B16F10 cells (Figure 5B). These results indicate that adhesive molecules expressed on the respective tumor cell lines differ somewhat although Gal-9 similarly suppresses the metastasis of these tumor cell lines. Based on these results, we hypothesize that Gal-9 suppresses metastasis by blocking binding between adhesive molecules expressed on the respective tumor cells and ligands on endothelial cells or in ECM.

Blockade of the binding of tumor cells to hyaluronic acid (HA)

Since B16F10 and Colon26 cells express high levels of CD44 and interaction between CD44 and HA is critical for the adhesion of tumor cells to endothelium (Mummert et al. 2003), experiments were first done to clarify whether Gal-9 inhibits the binding of CD44-expressing tumor cells to HA. The binding of B16F10 cells to HA was significantly inhibited by Gal-9 in a dose-dependent manner and the intensity of inhibition was comparable to anti-CD44 antibody (70 μg/mL) (Figure 6). It was further found that such suppression was abrogated in the presence of a representative β-galactoside, lactose (Figure 6), but not sucrose (data not shown). The binding of Colon26 cells to HA was also inhibited by Gal-9 (Figure 6), suggesting that Gal-9 regulates the adhesion of these two tumor cells to vascular endothelium and components of ECM by inhibiting the binding between CD44 and HA through its lectin nature.

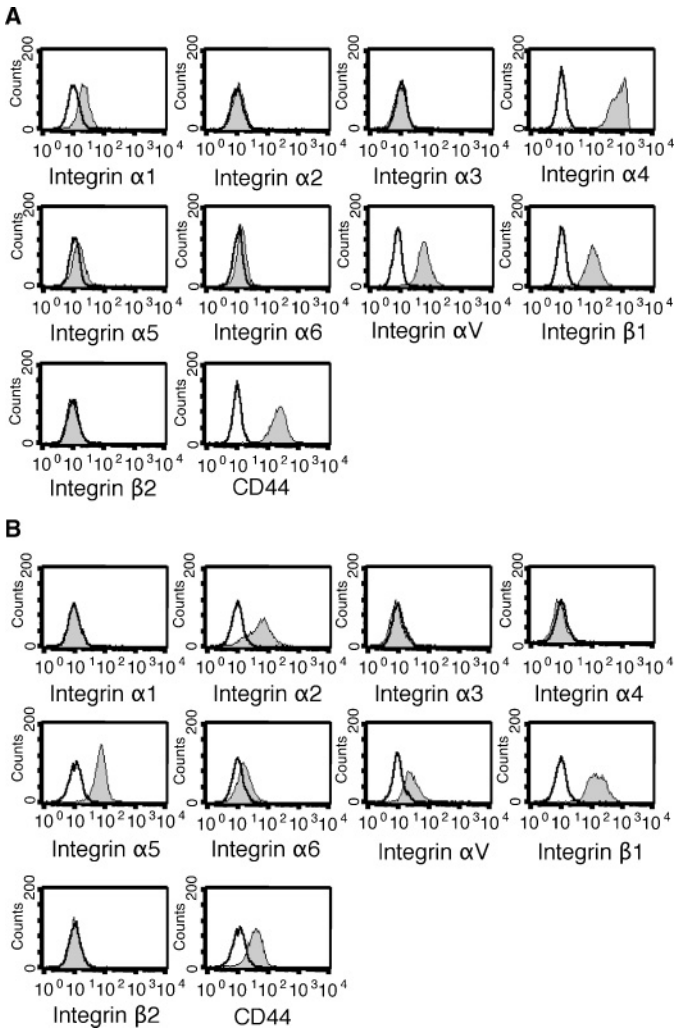

Fig. 5. Expression profiles of adhesive molecules on B16F10 and Colon26 cells. B16F10 (**A**) and Colon26 cells (**B**) were stained with anti-integrin α1, α2, α3, α4, α5, α6, αV, β1, β2, CD44 (shaded histograms) or isotype control IgG (open histograms) and analyzed by flow cytometry. Data are representative of three independent experiments.

Blockade of binding between very late antigen-4 and vascular cell adhesion molecule-1

B16F10 cells, but not Colon26 cells, express high levels of integrin α4β1 (very late antigen-4, VLA-4) on their surface (Figure 5A). Since interaction of VLA-4 with vascular cell adhesion molecule-1 (VCAM-1) is supposed to be critical for tumor cell metastasis (Qian et al. 1994), we did experiments to evaluate whether Gal-9 directly inhibits the binding of VCAM-1 to B16F10 cells in vitro. When B16F10 cells were exposed to soluble VCAM-1/Fc chimera or E-selectin/Fc chimera (as a negative control) in the presence and absence of Gal-9, Gal-9 inhibited the binding of VCAM-1/Fc to B16F10 cells through VLA-4 binding (Figure 7A). In contrast to B16 F10 cells, Colon26 cells did not bind VCAM-1/Fc chimera (data not shown), confirming that Colon26 cells do not express VLA-4 on their surface (Figure 5B).

In order to clarify whether Gal-9 suppresses the binding of B16F10 cells to VCAM-1, B16F10 cells were exposed to

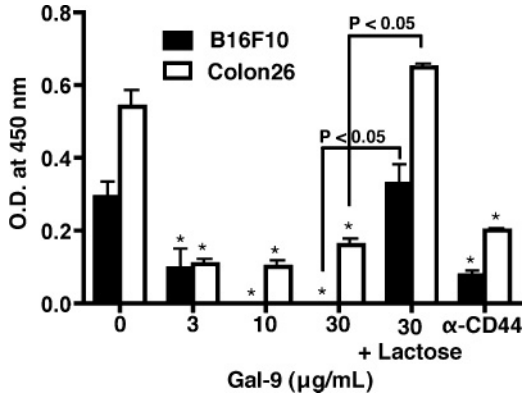

Fig. 6. Inhibition of B16F10 and Colon26 cell adhesion to hyaluronic acid (HA). B16F10 and Colon26 cells (4×10^4 cells/well) were suspended in RPMI containing 0.02% BSA with Gal-9 (0, 3, 10, 30 μg/mL, and 30 μg/mL Gal-9 + lactose) or anti-CD44 antibody (α-CD44, 70 μg/mL), and then incubated for 60 min on a HA-coated plate. After gentle washing with warm PBS, the number of adherent cells was evaluated by WST-1 assay. Data are the means ± SD of two experiments performed in triplicate. $^*P < 0.05$ (compared with PBS treatment)

Gal-9 or blocking antibodies against VLA-4 on plates coated with VCAM-1/Fc chimera. We found that Gal-9 suppressed the binding of B16F10 cells to VCAM-1/Fc-coated plates in a dose-dependent manner (Figure 7B, $P < 0.05$). A representative β-galactoside (lactose) that binds to Gal-9 decreased the inhibitory effects of Gal-9 ($P < 0.05$), suggesting that Gal-9-induced suppression is ascribed by its lectin nature.

Gal-9 inhibits the adhesion of tumor cells to ECM

We next assessed the binding of B16F10 and Colon26 cells to ECM components (collagen types 1 and 4, fibronectin, laminin, and vitronectin). Gal-9 significantly inhibited the adhesion of both tumor cell line cells to ECM components in a dose-dependent manner, although the intensity of suppression on the binding of those tumor cells differed according to the components (Figure 8A), suggesting that Gal-9 also inhibits the adhesion of tumor cells to ECM.

In order to confirm the above findings, inhibition of tumor cell migration by Gal-9 in vitro was evaluated by a chemotactic assay using membranes coated with Matrigel containing ECM components. Migration of B16F10 cells was significantly suppressed by Gal-9 in a dose-dependent manner (Figure 8B), and Gal-9-mediated inhibition was abrogated by lactose but not sucrose (Figure 8B), indicating that Gal-9 inhibits the invasion of tumor cells in ECM by suppressing the adhesion of tumor cells to ECM components.

Discussion

In the present experiments, we have shown that transfection of B16F10 cells with sec Gal-9 or administration of recombinant stable human Gal-9 significantly suppresses lung metastasis of melanoma (B16F10) cells and colon adenocarcinoma (Colon26) cells. We have described that transfection of B16F10 cells with sec Gal-9 almost completely abrogates the metastatic potential of B16F10 cells, while not significantly affecting the proliferation of those cells (Figures 1 and 2). These results suggest that

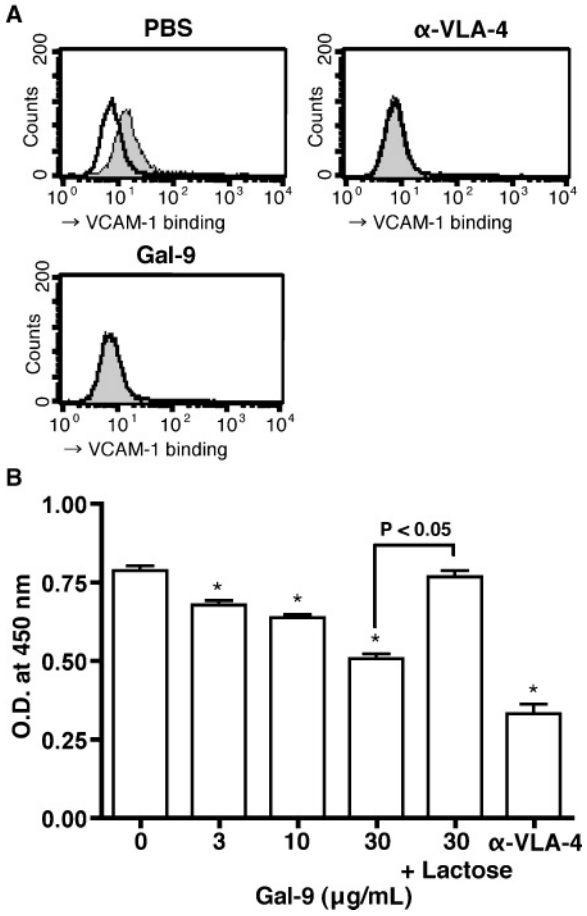

Fig. 7. Suppression of B16F10 cell adhesion to VCAM-1 by Gal-9. (**A**) B16F10 cells were pretreated with Gal-9 (30 μg/mL), PBS or anti-VLA-4 antibody (10 μg/mL). Two micrograms per milliliter VCAM-1/Fc (shaded histograms) or E-selectin/Fc (open histograms, as a control) were added, fixed, and stained with a FITC-mouse anti-human IgG. Data are representative of three independent experiments. (**B**) B16F10 cells (4×10^4 cells/well) were suspended in RPMI containing 0.02% BSA with Gal-9 (0, 3, 10, 30 μg/mL, and 30 μg/mL Gal-9 + lactose) or anti-VLA-4 antibody (α-VLA4, 30 μg/mL) and then incubated for 60 min on a VCAM-1/Fc-coated plate. After gentle washing with warm PBS, the number of adherent cells was evaluated by WST-1 assay. Data are representative of two independent experiments performed in triplicate, presented are the means ± SD. $^*P < 0.05$ (compared with PBS treatment)

mechanisms other than cell death participate in Gal-9-mediated suppression of lung metastasis, and that exogenous Gal-9 plays a crucial role in the suppression of metastasis.

The fact that B16F10 cells transfected with non-sec mGal-9 metastasize similar to mock-transfected B16F10 cells may be inconsistent with our previous findings that high Gal-9 expression in breast cancer cells and melanoma cells correlated with better prognosis (Kageshita et al. 2002; Irie et al. 2005). Possible explanations can be proposed to account for the inconsistency. The first is that Gal-9-positive tumor cells in the patients can secrete Gal-9 unlike to established tumor cells that do not release Gal-9. The second is that Gal-9 released from other cells infiltrating in the lesions plays a prognostic role. In any case, evaluating not only Gal-9 expression in the cytoplasm of cancer cells but also Gal-9 secreted in the lesion may be important for a more precise prediction of metastasis in cancer patients.

It has been described that Gal-1 secreted by tumors facilitates tumor immune escape by killing cytolytic T cells targeting the tumor (Rubinstein et al. 2004). As previously shown, Gal-9 induces cell death in activated Th1 cells, human melanoma cell line cells, and synoviocytes from patients with rheumatoid arthritis (Kageshita et al. 2002; Kashio et al. 2003; Zhu et al. 2005; Seki et al. 2007; Seki et al. 2008), suggesting that the ability of Gal-9 to induce cell death mediates the suppression of metastasis. However, we have found that Gal-9 does not induce evident cell death in B16F10 cells, although it induces cell death in certain types of cell lines such as L1210 mouse leukemia cells (Figure 4) and MethA fibrosarcoma cells (data not shown). Indeed, Gal-9 exhibits a variety of other biological functions, including induction of maturation and activation of innate immune cells (Dai et al. 2005; Anderson et al. 2007; Seki et al. 2008), and proliferation of osteoblasts (Tanikawa et al. 2008). We suggest that mechanisms other than cell death are responsible for Gal-9-mediated suppression of metastasis although the possibility that cell death signals are partly involved cannot be completely excluded.

Galectins recognize integrins in a carbohydrate-dependent manner. For instance, Gal-1 binds to α5β1 (Fischer et al. 2005), Gal-3 binds to α1β1 (Ochieng et al. 1998), and Gal-8 binds to α3β1 and α6β1 (Hadari et al. 2000) as well as αM (Nishi et al. 2003). It has been also shown that pulmonary metastasis of B16F10 cells is inhibited by antibodies against integrin α5 and CD44 (Qian et al. 2005), and peptide antagonists such as a synthetic peptide, Pep-1, that blocks the function of HA (Mummert et al. 2003) and bifunctional peptides, RGD-4C-GG-D (KLAKLAK)2, that bind selectively to αVβ3 integrin receptors (Smolarczyk et al. 2006). These results suggest that Gal-9 inhibits the adhesion and invasion of cancer cells to vascular endothelium and ECM components through binding with integrins expressed on the surface of cancer cells. In contrast, Gal-8, belonging to the tandem repeat-type galectins, binds to integrins and induces apoptosis by inhibiting cell adhesion (Hadari et al. 2000). Gal-9 does not induce apoptosis in B16F10 cells, as described above. Such a discrepancy may be ascribed to the difference in the sugar binding affinity between Gal-8 and Gal-9 (Hirabayashi et al. 2002).

Cell–cell and cell–ECM interactions are involved in the establishment of metastasis (Saiki 1997; Ellenrieder et al. 1999; Pasco et al. 2004; Zhou et al. 2006). The process of metastasis involves five critical steps: detachment of cancer cells from primary sites, movement (invasion) of cancer cells in ECM, attachment of cancer cells to vascular endothelial cells at distal sites, invasion of cancer cells through vascular endothelium, and tumor growth with neovascularization, which are essential for accomplishing malignant tumor metastasis (Hanahan and Weinberg 2000). From the present results, it is conceivable that Gal-9 suppresses multiple steps of metastasis by inhibiting the binding of adhesive molecules on tumor cells to the ligands on vascular endothelium. HA is one of the representative ligands of CD44 (Miyake et al. 1990; Liu et al. 1998), and interaction between CD44 and HA promotes the metastasis of tumor cells (Lesley et al. 1997). Thus, it has been described that CD44 on tumor cells and T cells binds to HA of both ECM components and endothelium to promote metastasis of tumor cells and infiltration of T cells into inflammatory sites, respectively (Sleeman et al. 1996; DeGrendele et al. 1997). Moreover, inhibitory molecules for CD44, such as anti-CD44 antibody and

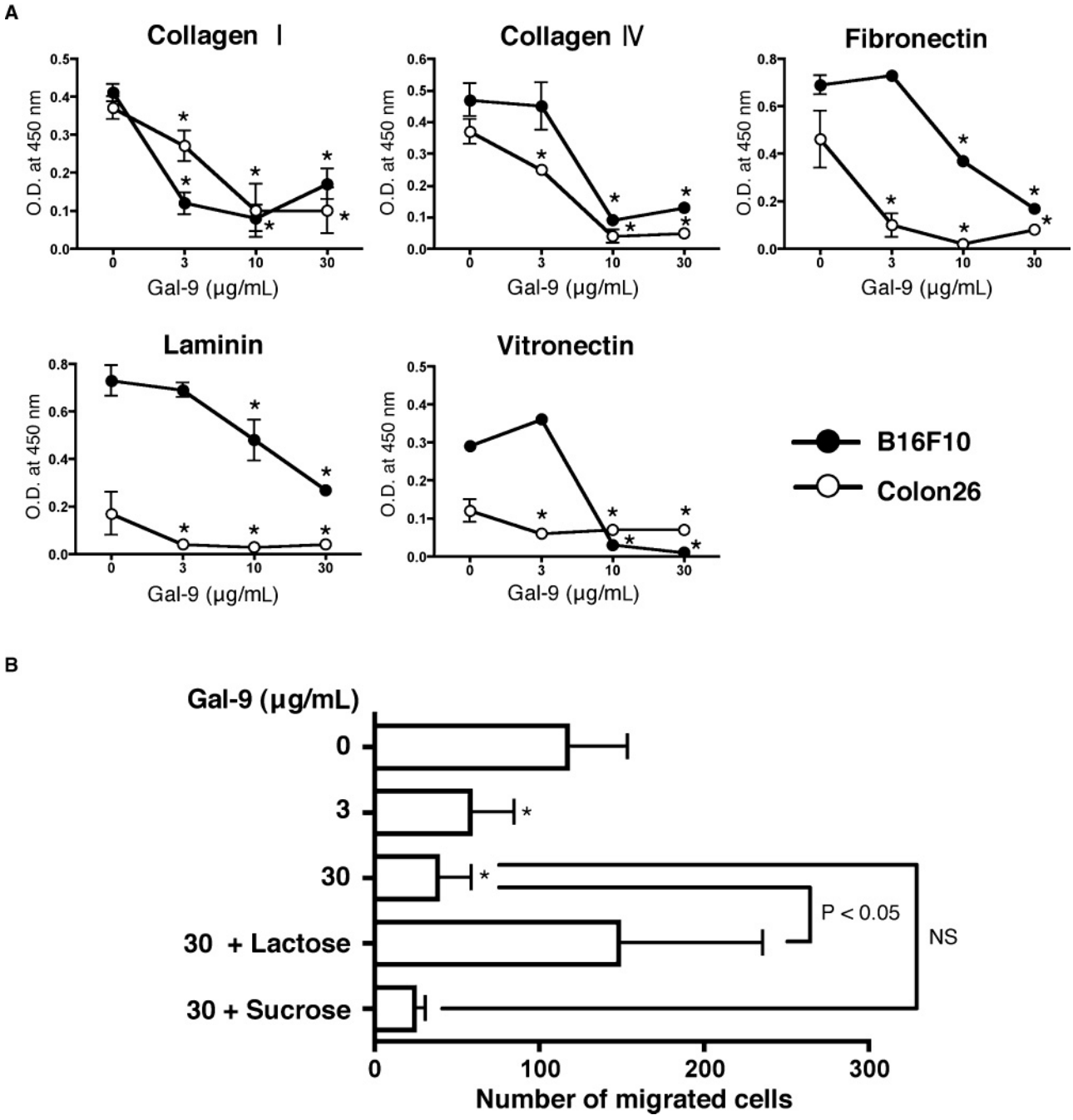

Fig. 8. Inhibition of B16F10 and Colon26 cell adhesion to ECM by Gal-9. (**A**) B16F10 and Colon26 cells were suspended in RPMI containing 0.02% BSA with Gal-9 (0, 3, 10, and 30 μg/mL), transferred to the wells (4×10^4 cells/well) coated with ECM components (collagen types 1 and 4, fibronectin, laminin, and vitronectin), and then incubated for 60 min. After gentle washing with warm PBS, the number of adherent cells was evaluated by WST-1 assay. Data are representative of three independent experiments performed in triplicate, and presented are the means ± SD. $^*P < 0.05$ (PBS-treated versus Gal-9-treated). (**B**) Inhibition of B16F10 cell migration in vitro. Polycarbonate filters were coated with 1 μg Matrigel. B16F10 cells (1×10^5/well) were suspended in RPMI containing 0.5% FBS with Gal-9 (0, 3, 30 μg/mL, 30 μg/mL Gal-9 + lactose, and 30 μg/mL Gal-9 + sucrose), transferred into the upper wells of 48-mutiwell chemotaxis chambers, and the lower wells were filled with RPMI containing 10% FBS. After incubation for 24 h at 37°C in 5% CO_2, the filters were removed and stained with a Diff–Quik staining solution. The number of migrated cells was counted. Data are presented as the means ± SD and are representative of three independent experiments in triplicate. $^*P < 0.05$ (PBS-treated versus Gal-9-treated).

CD44/Fc fusion proteins suppress the growth, invasion, and metastasis of tumor cells (Guo et al. 1994; Zawadzki et al. 1998). Our previous study indicates that Gal-9 directly binds the adhesion molecule CD44, inhibits interactions with HA, and suppresses a murine model of allergic asthma (Katoh et al. 2007). Based on the findings that B16F10 and Colon26 cells express abundant CD44 molecules on their surface, and that Gal-9 suppresses the binding of these CD44-expressing cells to HA in a carbohydrate-dependent manner, Gal-9 may suppress the binding of tumor cells to endothelium by inhibiting the

interaction between CD44 and HA, resulting in the suppression of lung metastasis.

In addition to the interaction of CD44 with HA, interaction between VLA-4 on tumor cells and VCAM-1 on endothelium has also been thought to be a critical factor for the adhesion of tumor cells to endothelium (Zetter 1993; Tomita and Saito 1995; Sanz-Rodriguez and Teixido 2001). Indeed, the binding of α4β1 integrin (VLA-4), but not β2 on B16F10 cells and VCAM-1 on endothelial cells, plays a critical role in metastasis (Qian et al. 1994). We also have found that Gal-9 significantly suppresses the binding between VLA-4 and VCAM-1 in vitro (Figure 7). Since B16F10 cells express abundant VLA-4 on their surface in addition to CD44, it is reasonable that Gal-9 inhibits both interactions (CD44/HA and VLA-4/VCAM-1) and suppresses B16F10 cell metastasis.

The adhesive interaction of metastatic tumor cells and components of ECM appears to be obligatory for successful target organ colonization (Engbring and Kleinman 2003). Gal-1 and Gal-3 promote the adhesion of ovarian and prostate cancer cells to ECM (Clausse et al. 1999; Ellerhorst et al. 1999; Inufusa et al. 2001; O′Driscoll et al. 2002). Moreover, highly metastatic human breast carcinoma cells express higher levels of Gal-3 and have increased adhesion to endothelial cells in vitro compared to their nonmetastatic counterparts (Khaldoyanidi et al. 2003). Similar to Gal-1, Gal-3, and Gal-8 (Andre et al. 1999; Hadari et al. 2000; Baptiste et al. 2007), we have found that Gal-9 also suppresses the adhesion of tumor cells to components of ECM, such as collagen types 1 and 4, fibronectin, laminin, and vitronectin, and that Gal-9 significantly suppresses the migration of tumor cells in vitro (Figure 8). We have previously shown that Gal-9 promotes the homotypic cell adhesion of human melanoma lines and breast carcinoma lines (Kageshita et al. 2002; Irie et al. 2005). We have, however, found that 3 and 10 μg/mL Gal-9 induces little aggregation though 30 μg/mL Gal-9 induces only a little aggregation (data not shown), suggesting that Gal-9-induced suppression of adhesion and migration may not be the results of cell aggregation induced by Gal-9. Thus, it is likely that Gal-3 promotes invasion and metastasis of tumor cells, but Gal-9 suppresses both the detachment of tumor cells from primary sites and the cell adhesion to ECM necessary for cellular movement.

The present results suggest that Gal-9 suppresses both attachment and invasion of two different tumor cells in two different strains of mice (B16F10 in C57BL/6 mice and Colon26 in Balb/c mice) by inhibiting the binding of adhesive molecules on tumors to ligands of both vascular endothelium and ECM, respectively, resulting in the suppression of metastasis.

Materials and methods

Mice, cell lines, and reagents

Female C57BL/6 mice (6–8 weeks old) and BALB/c mice (6–8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). All animal experiments were approved by the Experimental Animal Center of Kagawa University. B16F10 cells and Colon26 cells were provided by the Cell Resource Center for Biomedical Research, Tohoku University and cultured in a RPMI-1640 solution (Sigma, St. Louis, MO) supplemented with 10% (v/v) heat-inactivated FBS (JRH Biosciences, Lenexa, KS), 10 mM HEPES (Sigma), antibiotic antimycotic solution (Sigma), and 2-mercaptoethanol (Gibco, Grand Island, NY). Recombinant human stable Gal-9 was prepared by deleting the linker peptide region as previously described and used instead of full-length Gal-9 throughout the present experiments (Nishi et al. 2005).

Flow cytometry

For flow cytometric analysis and integrin binding experiments, anti-integrin α1 (clone: Ha31/8, BD Biosciences Pharmingen, San Diego, CA), FITC-anti-integrin α2 (clone: Hma2, eBioscience, San Diego, CA), anti-integrin α3 (C-18) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-integrin α4 (clone: PS2, a gift from Dr. Kensuke Miyake, University of Tokyo), anti-integrin α5 (clone: 5H10-27, Fitzgerald, Concord, MA), anti-integrin α6 (clone: NKI-GoH3, Chemicon, Temecula, CA), PE-anti-integrin αV (clone: RMV-7, eBioscience), FITC-anti-integrin β1 (clone: HMb1-1, Santa Cruz Biotechnology), FITC-anti-integrin β2 (clone: M18/2, eBioscience), anti-CD44 (clone: KM201) (Zheng et al. 1995), isotype control FITC-hamster IgG (clone: eBio299Arm, eBioscience), isotype control hamster IgG2λ1 (clone: ant-KLH, BD Biosciences Pharmingen), isotype control mouse IgM (clone: 11E10, eBioscience), isotype control FITC-rat IgG2a (Immunotech, Marseille, France), isotype control goat IgG (Santa Cruz Biotechnology), isotype control rat IgG1 (clone: 1H5, MBL, Nagoya, Japan), secondary FITC-rabbit anti-goat IgG (H+L) (American Qualex, San Clemente CA), and secondary FITC-anti-rat IgG (eBioscience) were used.

Recombinant plasmids

The expression plasmid for human stable Gal-9 (pET-stable Gal-9) was described previously (Nishi et al. 2005). The plasmid was digested with *Nde*I and *Xba*I, and then inserted with *Sfi*I-adaptor (5′-ctagagttggcctg-3′,5′-tacaggccaact-3′) at the 5′ end of a human stable Gal-9 open reading frame. The resulting plasmid was digested with *Sfi*I, treated with T4 DNA polymerase followed by *Hind*III digestion to excise a DNA fragment containing human stable Gal-9 cDNA. The DNA fragment was inserted into the *Sfi*I–*Hind*III site of pSecTag2/HygroA (Invitrogen Life technologies, Carlsbad, CA) to generate an expression plasmid for a secretary form of stable Gal-9 (pSecTag2/HygroA-signal-human stable Gal-9). This plasmid encodes a full-length human stable Gal-9 protein (296 amino acids) and the Ig-kappa signal sequence (METDTLLLWVLLLWVPGSTGD) added at the N-terminus. To construct an expression plasmid for a secretary form of mouse Gal-9, a wild-type mouse Gal-9 (M type; 322 amino acids) cDNA was used (Wada and Kanwar 1997). The Ig kappa-mouse Gal-9 cDNA fragment from pSecTag2/HygroB-mouse Gal-9 was inserted into the *Nde*I–*Sma*I site of a pCXN2linker vector to generate an expression plasmid.

Transfection

B16F10 cells were transfected with the vectors encoding sec mGal-9, sec hGal-9, non-sec mGal-9, or a mock vector using a Lipofectamine 2000 reagent (Invitrogen Life Technologies). Transfected cells were selected in a medium containing 100 μg/mL G418 (for transfected mouse vectors) (Gibco) and 100 μg/mL Hygromycin B (for transfected human vectors) (Calbiochem, La Jolla, CA), and subcultured with the conditioned RPMI 1640 solution.

RT-PCR assay

Total RNA from B16F10 cells was extracted with TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Using a Gene Amp RNA PCR kit (Perkin Elmer, Foster City, CA), total RNA (0.5 μg) was reverse-transcripted for 15 min at 42°C with reverse transcriptase. For amplification, cDNA was mixed with the following primer sequences synthesized at Sigma and reacted with ampliTaq DNA polymerase. The PCR program consisted of 30 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 1 min. Reverse transcription and PCR were conducted using an iCycler (Bio-Rad, Hercules, CA). The PCR products were run on a 2% agarose gel. Mouse Gal-9: 5′-ggtcagagttcaaggtgatggtga-3′ and 5′-gcctgatatcatgatggacttgga-3′. Human Gal-9: 5′-cgtcaatggctctgtgcagctgtc-3′ and 5′-agatccacactgagaagctctggc-3′. GAPDH: 5′-gccatcaatgccccttcattcattgac-3′ and 5′-acggaaggccatgccagtgagctt-3′.

ELISA analysis

Plates were coated with an anti-mouse Gal-9 monoclonal antibody (3 μg/mL, clone 52A1) or anti-human Gal-9 monoclonal antibody (5 μg/mL, clone: 9S2-3) in phosphate buffered saline (PBS) overnight at 4°C. The plates were washed twice in PBS-T (PBS containing 0.05% Tween20) and then blocked by 20 mg/mL BSA in PBS-T at room temperature for 1 h. The culture supernatants of transfected B16F10 cells (4×10^4 cells/well, 3 days' culture in the fresh RPMI 1640 solution) were incubated in the plates for 1 h at 37°C. Wells were washed and an anti-mouse Gal-9 polyclonal antibody (3 μg/mL) or anti-human Gal-9 polyclonal antibody (5 μg/mL) was incubated for 1 h at 37°C. Then, horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Biosciences, Buckinghamshire, UK) was incubated for 1 h 37°C. The TMB solution (KPL, Gaithersburg, MD) was incubated in the dark for 30 min. The colorimetric reaction was stopped by the addition of 1 M phosphoric acid. Finally, the plates were measured at OD 450 nm with a microtiter plate reader (Multiskan JX; Thermo Lab Systems, Helsinki, Finland).

Experimental lung metastasis models

B16F10 melanoma cells (2×10^5 cells/mouse) were injected via the tail vein of C57BL/6 mice, and mice were treated daily with i.v. Gal-9 (30 μg/mouse) or PBS 1 h after the tumor inoculation for 11 days. Transfected cells (2×10^5 cells/mouse) were injected via the tail vein of C57BL/6 mice. Twelve days later, the animals were killed by an overdose of pentobarbital sodium, and the number of lung colonies was counted.

Colon26 cells (4×10^4 cells/mouse) were also injected via the tail vein of BALB/c mice, and mice were daily treated with i.v. Gal-9 (30 μg/mouse) or PBS 1 h after the tumor inoculation for 23 days. Twenty-four days later, the animals were sacrificed by an overdose of pentobarbital sodium, and the number of lung colonies was enumerated.

Effect of Gal-9 on cell proliferation and cell death

Transfected cells (3×10^3/100 μL/well) were cultured in triplicate for 24 h, 48 h, and 72 h at 37°C in 96-well plates. B16F10 and Colon26 cells (3×10^3/100 μL/well) were seeded into 96-well plates. After 24 h, various concentrations of Gal-9 were added and cultured for a further 24 h. At the end of the culture, 10 μL of WST-1 reagent (Roche Diagnostics, Lewes, UK) was added, and cell cultures were continued for another 2–4 h at 37°C. The absorbance of each sample was measured at 450 nm using a microtiter plate reader. As a control cell line, L1210 mouse leukemia cell line was used.

For cytotoxicity, B16F10 and Colon26 cells (1×10^4/well) were cultured in a 96-well plate (Sumilon, Tokyo, Japan) with various concentrations of Gal-9 (0, 3, 10, and 30 μg/mL) for 24 h. The cells were incubated with 7-amino-actinomycin D (7-AAD, BD Biosciences Pharmingen) for 15 min at room temperature in the dark. The cells were analyzed with a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA), and 7-AAD-positive cells were defined as dead cells.

Flow cytometric analysis of cell surface adhesive molecules

Cells were first incubated with each antibody (5 μg/mL) for 30 min at 4°C in a FACS buffer (1% BSA and 0.1% NaN_3 in PBS), washed twice with the FACS buffer, and then incubated with a secondary antibody (1:100) for 30 min at 4°C unless using directly conjugated FITC or PE antibodies. The stained cells were acquired with a BD FACSCalibur flow cytometer and analyzed with CellQuest software (BD Biosciences).

Soluble VCAM-1 binding assay

VCAM-1 binding was carried out as previously described (Cardones et al. 2003). Briefly, B16F10 cells were exposed to Gal-9 (30 μg/mL), PBS, or anti-VLA-4 antibody (10 μg/mL) in 10% FCS-containing RPMI 1640 solution for 30 min at 37°C. VCAM-1/Fc (2 μg/mL, R&D Systems, Minneapolis, MN) or E-selectin/Fc (as a control) was then added to the cell suspension for 45 s, and the cells were fixed for 10 min with 4% paraformaldehyde at room temperature. After washing with the FACS buffer, the cells were incubated with a FITC-mouse anti-human IgG antibody (Zymed, San Francisco, CA) at 4°C for 30 min, and analyzed by a FACSCalibur.

Cell adhesion to ECM components

Plates coated with a component of ECM (collagen types 1 and 4, fibronectin, laminin, and vitronectin) were purchased from Chemicon. VCAM-1- or HA-coated plates (Sigma) were prepared by incubating with VCAM-1/Fc (5 μg/mL) or HA (100 μg/mL) overnight at 4°C. The plates were then blocked with 1% BSA/RPMI for 60 min at room temperature. B16F10 and Colon26 cells (4×10^4 cells/well) were incubated with Gal-9 (0–30 μg/mL) in 96-well plates coated with the above adhesion molecules in the 0.02% BSA/serum-free RPMI 1640 solution. After incubation for 60 min at 37°C, the plates were washed twice with warm PBS to remove nonadherent cells and the adherent cells were incubated with WST-1 for 120 min at 37°C. The number of adherent cells was quantified by measuring absorbance at 450 nm with a plate reader.

Cell migration

In vitro cell migration was assessed with a 48-multiwell chemotaxis chamber assay (Neuro Probe, Cabin John, MD). In brief, 12 μm pore-size polyvinylpyrolidone-free polycarbonate filters (Poretics Corporation, Livermore, CA) were coated with 1 μg of Matrigel (BD Biosciences). The lower wells were filled with RPMI containing 10% FBS. B16F10 cells (1×10^5 cells/well in RPMI with 0.5% FBS) were added to each of the upper

wells with Gal-9 (0–30 μg/mL, with or without lactose and sucrose). After incubation for 24 h at 37°C in 5% CO_2, the filters were removed and stained with a Diff–Quik staining solution (Sysmex, Kobe, Japan). The number of migrated cells to the lower surface of the membrane was counted.

Statistical analysis

A comparison between two groups was done by the Mann–Whitney *U* test. All statistical analyses were done with Prizm 4 software (GraphPad Software, San Diego, CA).

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Conflict of interest statement

The authors declare that there are no financial or other interests regards to this manuscript that might be constructed as a conflict of interest.

Abbreviations

7-AAD, 7-amino-actinomycin D; ECM, extracellular matrix; Gal-9, galectin-9; HA, hyaluronic acid; i.v., inoculated intravenously; PBS, phosphate buffered saline; sec hGal-9, secreted form of stable human Gal-9; sec mGal-9, secreted form of mouse Gal-9, non-sec mGal-9, non-secreted form of mouse Gal-9; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

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