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Association of $\alpha_v\beta_3$ integrin expression with the metastatic potential and migratory and chemotactic ability of human osteosarcoma cells

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Abstract

Introduction. Expression of adhesion molecules such as $\alpha_v\beta_3$ integrin has been associated with the metastatic potential of tumor cells. The purpose of this study was to determine whether $\alpha_v\beta_3$ expression correlated with the metastatic potential of human osteosarcoma cells. *Materials and methods*. We developed a series of sublines (LM2–LM7) from human osteosarcoma SAOS parental cells, with progressively increasing potential to form lung metastases in nude mice after intravenous injection. SAOS parental and LM2 cells were poorly metastatic, but LM7 cells resulted in visible metastatic lung nodules by 6–8 weeks. We quantified $\alpha_v\beta_3$ integrin expression using flow cytometry. *Results*. $\alpha_v\beta_3$ expression correlated with the metastatic potential of the cells, with LM7 cells showing the highest expression. LM7 cell adhesion to vitronectin decreased after treatment with echistatin, a RGD-containing peptide antagonist of $\alpha_v\beta_3$. LM7 cells demonstrated higher chemotactic activity than SAOS cells to a homogenate made from lung tissue. This chemotactic activity was also inhibited by echistatin. These data indicated that $\alpha_v\beta_3$ was critical for the migration of LM7 cells to the lung homogenate. Chemotaxis to a liver homogenate was the same for LM7 and SAOS cells. Migration of LM7 cells through lung endothelial cells was higher than that through liver endothelial cells, and echistatin again inhibited this migration. *Conclusions*. $\alpha_v\beta_3$ integrin expression may play a role in the metastatic potential of osteosarcoma cells by enhancing the ability of the cells to migrate specifically to the lung. $\alpha_v\beta_3$ integrin may therefore be a potential new target for osteosarcoma.

Abbreviations: BSA – bovine serum albumin; EC – endothelial cell; FBS – fetal bovine serum; OS – osteosarcoma; PBS – phosphate-buffered saline; VN – vitronectin

Introduction

Osteosarcoma (OS), the most common primary malignant bone tumor, continues to be a challenge to treatment options. Despite aggressive chemotherapy and surgical resection of the primary tumor, 30–40% of patients with newly diagnosed OS develop pulmonary metastases [1]. New strategies are therefore needed to prevent and eradicate macro- and micrometastases to the lung and to improve the disease-free survival rate. Insight into the mechanisms by which OS cells metastasize may contribute to the development of more effective and specific therapies.

During the metastatic process, tumor cells leave the primary tumor, travel through the bloodstream, and colonize target organs. Tumor cells must arrest at the vessel wall, penetrate the endothelium and basal membrane, and migrate invasively toward their target tissue [2]. Many of these steps involve integrins, a class of molecules that mediate adhesion to cell molecules and cell-surface ligands. All integrins are structurally similar heterodimers composed of α- and β-subunit transmembrane glycoproteins that are noncovalently linked [3]. Integrins play pivotal roles in diverse cellular processes, such as cell migration, proliferation, and attachment [4]. In several types of cancer, including melanoma, breast cancer, prostate cancer, colon cancer, and glioma [2, 5–9], tumor cells

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express $\alpha_v\beta_3$ integrin; such expression has been shown to be associated with progression and metastasis in melanoma, breast cancer, and prostate cancer [2, 5–9]. We had previously developed an experimental OS lung metastasis mouse model and isolated cell sublines that have increasing metastatic potential [10]. In this study, to understand the specific contribution of $\alpha_v\beta_3$ integrin expression to tumor cell functions that support OS metastasis in the model, we compared the $\alpha_v\beta_3$ integrin expression and migratory activities of the OS sublines with different metastatic potentials. We demonstrated increased $\alpha_v\beta_3$ expression and higher chemotactic activity to lung homogenates in cells with higher metastatic potential. $\alpha_v\beta_3$ expression was found to be critical for OS cell migration through lung endothelial cells.

Materials and methods

Reagents and antibodies

Eagle's minimal essential medium (EMEM), Dulbecco's modified Eagle medium (DMEM), Hanks' balanced salt solution without Ca^{2+} or Mg^{2+} (HBSS), nonessential amino acids, sodium pyruvate, minimal essential medium vitamins, L-glutamine, and 2.5% trypsin were purchased from Whittaker Bioproducts (Walkersville, MD). Fetal bovine serum (FBS) was purchased from Intergen. (Purchase, NY), CM-Dil was purchased from Molecular Probes (Eugene, OR), and vitronectin was purchased from Promega (Madison, WI). Echistatin was purchased from Bachem California Inc. (Torrance, CA). Mouse anti-human $\alpha_v\beta_3$ integrin monoclonal antibody LM609 was purchased from Chemicon International (Temecula, CA).

Cell lines

The human OS cell line SAOS-2 was obtained from the American Type Culture Collection (Manassas, VA). The LM2, LM3, LM4, LM5, LM6, and LM7 sublines were derived from SAOS-2 cells by initial selection of cells on 0.9% agarose followed by intravenous injection into nude mice and then by repeated intravenous recycling through the lungs of nude mice [10]. Cells were maintained in EMEM supplemented with nonessential amino acids, sodium pyruvate, L-glutamine, and 10% FBS (heating at 56 °C for 30 min). Murine lung and liver endothelial cells (EC) were established as previously described [11]. ECs were maintained in DMEM supplemented with nonessential amino acids, sodium pyruvate, L-glutamine, and 10% inactivated FBS. These cells were verified to be free of mycoplasmas and pathogenic murine viruses (mycoplasm plus PCR Primer kit, Stratagen Inc., La Jolla, CA, Whittaker). For *in vivo* injection, OS cells in the midlog growth phase were trypsinized with 0.25% trypsin/0.02% EDTA (wt/vol), and then resuspended in supplemented medium. The cells were washed and resuspended in HBSS at a concentration of 5×10^6 cells/ml, and then 1×10^6 cells in 0.2 ml were injected into the lateral tail vein of the nude mice. At designated times (10, 12 or 17 weeks), the mice were sacrificed. The lungs were fixed in Bouin's solution, and metastases were counted with the aid of a dissecting microscope.

Mouse model

Male specific-pathogen-free athymic nude (nu/nu) mice, 4–6 weeks old, were purchased from Charles River Breeding Laboratories (Wilmington, MA). The mice were maintained in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, the Department of Health and Human Services, and the National Institute of Health. Mice were housed 5 to a cage and kept in a laminar-flow cabinet under specific pathogen-free conditions for 2 weeks before their use. Intravenous injections of 10^6 LM6 cells resulted in development of microscopic lung metastases by 5 weeks and visible lung metastatic nodules by 8 weeks [10]. LM7 cells, developed by an additional cycling of LM6 cells, caused visible lung metastases 6 weeks after the injection.

In vitro *growth and proliferation assay*

Parental SAOS cells and the LM cell sublines in the midlog phase of growth were harvested by trypsinization, washed, resuspended in supplemented EMEM, and adjusted to contain 3×10^4 cells/ml. Aliquots containing 3×10^3 cells were seeded into each well of a 96-well plate (Becton Dickinson Labware, Franklin Lakes, NJ) and then incubated at 37 °C in a humidified 5% CO_2 incubator for 24, 48, 72, or 96 h. During the final 24 h of incubation, the cells were labeled with 0.2 μCi/well of [^{3}H] thymidine (ICN Biomedicals Inc., MP Biochemicals, Irvine, CA). The cells were then washed twice with HBSS and lysed using 0.1 ml of 0.1 N potassium hydroxide. Incorporation of radioactivity was quantified with a plate harvester (Hamden, CT) and a Beta Plate Counter (Wallac OY, Turku, Finland). The doubling time was calculated with the following formula: time $\times$ log 2/log (n/n_0), in which n_0 is the counts per minute of cells incubated for 24 h and n is the counts per min of cells incubated for 48, 72 or 96 h.

Flow cytometric analysis

$\alpha_v\beta_3$ Integrin expression in the OS cell lines was analyzed by flow cytometry using the monoclonal

Table 1. Metastatic characteristics of the SAOS parental and LM sublines.

Cell line	Doubling time (h)[a]	Lung metastases[b]			
		Time (weeks)	Incidence[c]	Median no. (range)	Diameter (mm)
SAOS parental	45.7 ± 3.3	17	0	0	0
LM2	43.6 ± 4.2	17	0	0	0
LM3	44.1 ± 2.6	17	2/5	0 (0–1)	0.5–1.0
LM4	40.0 ± 0.9	17	3/4	9 (0–100)	0.5–2.0
LM5	37.2 ± 3.8	17	4/4	88 (7–>200)	0.5–5.0
LM6	34.9 ± 1.4	12	9/9	92 (30–>200)	0.5–5.6
LM7	26.8 ± 1.3	10	12/12	100 (30–>200)	0.5–7.0

[a]Data are given as hours ± SD. SAOS parental or the indicated LM cells (3×10^3 cells) were plated and incubated at 37 °C for 24, 48, 72, or 96 h. The cells were labeled with [^{3}H]thymidine during the last 24 h of incubation. Doubling time was calculated as described in 'Materials and methods' and is expressed as the mean ± SD of three independent experiments.
[b]SAOS parental or the indicated LM cells (1×10^6 cells) were injected into the lateral tail vein of mice. Mice were sacrificed at 10, 12, or 17 weeks. The lungs were removed and fixed, and tumor nodules were counted and measured.
[c]Number of tumor-positive mice/number of inoculated mice.

antibody LM609 [5]. Cells were harvested by trypsin/EDTA treatment, washed with PBS, and incubated with PE-conjugated mouse anti-human integrin $\alpha_v\beta_3$ antibody (1 : 200) for 30 min on ice. After another washing with PBS, the cells were subjected to flow cytometric analysis.

Adhesion assays

Adhesion assays were performed as previously described [12]. The 96-well plates were precoated with 2 μg/ml of vitronectin in PBS, followed by blocking of nonspecific sites with 0.2% bovine serum albumin in PBS for 2 h at 37 °C. LM7 cells were seeded onto the precoated 96-well plates, incubated at 37 °C for 2 h, washed with PBS, stained with crystal violet, and then quantified by measuring absorbance at 540 nm using a plate reader.

Chemotaxis assay

A chemotaxis assay was performed using transwell chambers (Corning, Acton, MA) [13]. LM7 or SAOS parental cells (4×10^4) were resuspended in serum-free EMEM and seeded into the upper compartment of the chamber. LM7-conditioned medium and a homogenate made from liver or lung tissue were transferred by pipette into the bottom compartment of the chamber. The two compartments were separated by a polycarbonate filter having 8.0-μm pores. The cells were allowed to migrate at 37 °C in humidified air with 5% CO_2 for 4 h. Nonmigrated cells were then removed with cotton swabs, and the membranes were removed, briefly fixed in methanol, and then stained with xanthene and thiazine dye using a Diff-Quick system (StatLab, Lewisville, TX). Migrated cells on the bottom of the filter were quantified by counting five fields per membrane using 10× objective.

Transendothelial migration assays

The in vitro transendothelial migration assay was performed as previously described [14]. Endothelial cells from the lung and liver (3×10^3/0.2 ml DMEM) were seeded into the upper compartment of a transwell chamber. DMEM with 10% inactivated FBS was transferred by pipette into the bottom wells of the chamber. The two compartments were separated by an 8.0-μm pore polycarbonate filter. LM7 cells (4×10^4 in 40 μl EMEM), prelabeled with CM-Dil (2.5 μg/ml for 10 min at 37 °C and then for 15 min at 4 °C), were added to the monolayer of endothelial cells 48 h later. After 4 h the nonmigrated cells were removed with cotton swabs. Migrated cells on the bottom of the filter were quantified as described above. For inhibition experiments, 1 μl echistatin (stock solution 1 μg/μl water) was added to the monolayers 1 h after the addition of LM7 cells.

Statistical analyses

Data were analyzed by the Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

$\alpha_v\beta_3$ *Integrin expression in the SAOS sublines*

The metastatic characteristics of the SAOS parental and LM sublines are described in Table 1. As shown in Figure 1, the expression of $\alpha_v\beta_3$ integrin increased progressively from 28% in SAOS parental cells to 43%, 72%, and 86% in the LM3, LM5, and LM7 cell lines, respectively. To study the $\alpha_v\beta_3$ integrin-mediated signaling pathway, we disrupted its cellular functions using echistatin, a selective $\alpha_v\beta_3$, integrin RGD-containing antagonist. LM7 adhesion to vitronectin decreased from 100% to 7% after treatment

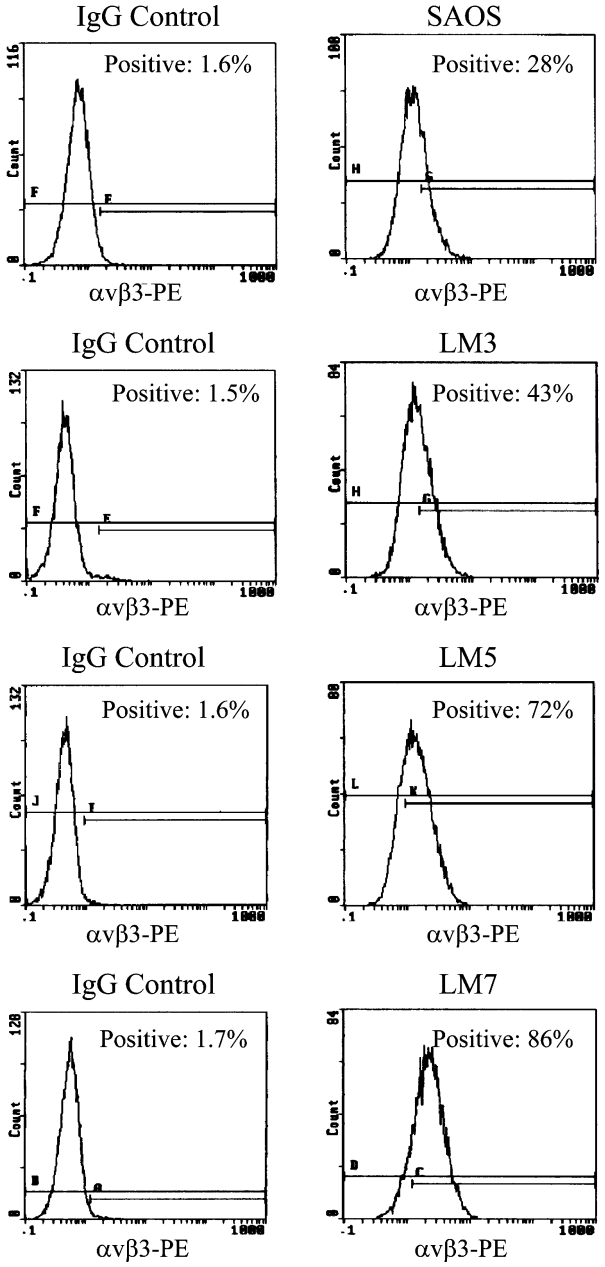

Figure 1. Expression of $\alpha_v\beta_3$ integrin in SAOS parental cells and the LM3, LM5 and LM7 sublines. $\alpha_v\beta_3$ Integrin expression was quantified by flow cytometry using the monoclonal antibody LM609. Increased expression of $\alpha_v\beta_3$ integrin was associated with increased metastatic potential.

with echistatin, demonstrating the importance of $\alpha_v\beta_3$ integrin to this process (Figure 2).

Effect of echistatin on the migration of LM7 cells

To examine whether $\alpha_v\beta_3$ integrin plays a role in the migration of OS cells, we determined the effect of echistatin on the ability of LM7 cells to migrate to a chemotactic stimulus. LM7 cells were plated in chambers using LM7-conditioned medium as the chemoattractant. To minimize the effect of echistatin on detachment, echistatin was added 1 h after the cells had adhered. Echistatin significantly reduced the migration of LM7 (Figure 3). Echistatin had no effect on cell viability or proliferation as assessed by trypan blue and MTT, respectively (Data not shown).

Because OS metastasizes almost exclusively to the lung, we next determined whether OS cells preferentially migrated to a homogenate made from lung tissue as opposed to one made from liver tissue. As shown in Figures 4A–C, the migration of LM7 cells was significantly increased when lung homogenates as opposed to liver homogenates were used as the chemotactic stimulus. Treatment of the cells with echistatin reduced this migration (Figures 4A and D). In contrast, SAOS parental cells, which are poorly metastatic to the lung and express little $\alpha_v\beta_3$ integrin showed no migration preference to the lung homogenate (Figures 4A, E and F).

Inhibition of LM7 cell transendothelial migration by echistatin

The ability of LM7 cells to migrate through endothelial cells was quantified using an *in vitro* transendothelial migration assay. Endothelial cells from either lung or liver were used for the endothelial monolayers. LM7 cells were prelabeled with CM-Dil before seeding on to the endothelial cell monolayer in order to distinguish them from the endothelial cells. As shown in Figure 5, the migration of LM7 cells through the lung endothelial monolayer was significantly higher than through the liver endothelial monolayer. Treatment with echistatin, once again, significantly decreased this migration through the lung endothelial monolayer, however treatment with echistatin did not significantly decrease this migration through the liver endothelial monolayer (Figure 5).

SAOS, LM3, LM5, and LM7 cell transendothelial migration

The ability of SAOS, LM3, LM5, and LM7 cells to migrate through lung ECs was quantified using an *in vitro* transendothelial migration assay. Lung ECs were used for the endothelial monolayers. SAOS, LM3, LM5, and LM7 cells were prelabeled with CM-Dil before seeding on to the endothelial cell monolayer in order to distinguish them from the endothelial cells. As shown in Figure 6, the migration of the highly metastatic LM7 cells was significantly higher than that of the poorly metastatic LM3 cells or the non-metastatic SAOS cells. Migration of LM3 cells was also significantly higher than that of SAOS (Figure 6).

Discussion

The data from this study demonstrate that $\alpha_v\beta_3$ integrin expression correlated with the metastatic potential of human OS cells and participates in their migratory and chemotactic ability. Echistatin, an

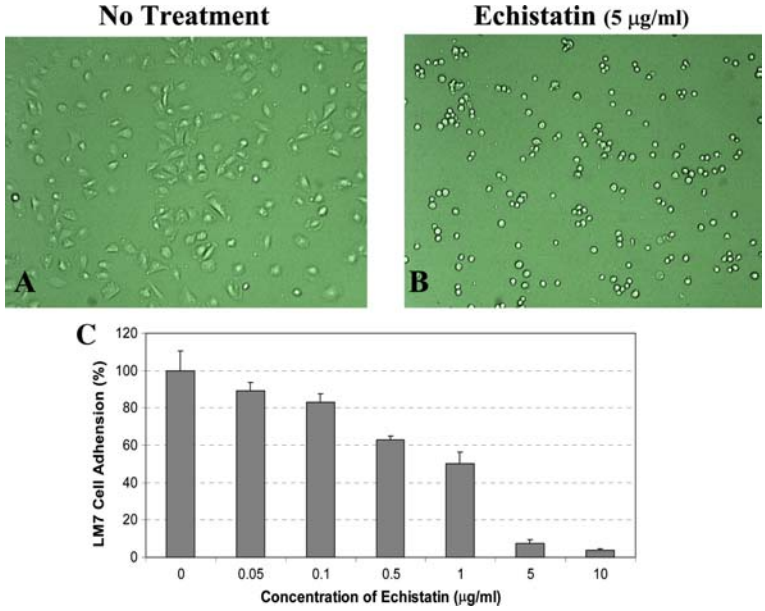

Figure 2. Effect of echistatin on LM7 cell adhesion. LM7 cells adhered to vitronectin-coated plates (A) which was disrupted by echistatin (B). This adhesion was decreased in a dose-dependent manner (C). Error bars indicate standard deviation.

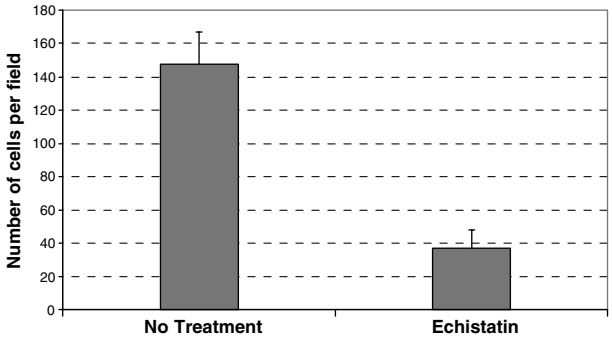

Figure 3. Effect of echistatin on LM7 cell migration. LM7 cells were seeded into the upper compartments of transwell chambers. LM7-conditioned medium served as the chemoattractant. Migration of LM7 cells was decreased after treatment with echistatin. Error bars indicate standard deviation.

RGD-containing peptide antagonist of $\alpha_v\beta_3$ integrin, inhibited both the migration of LM7 cells and their adhesion to vitronectin.

The process of tumor metastasis consists of a complex cascade of adhesive interactions between tumor cells and host cells [15–17]. Human melanoma cells metastasize early in the course of the disease, primarily using the bloodstream to colonize preferred target organs such as lung, bone, liver, and brain [18]. Metastasis depends on specific adhesive, invasive, and migratory properties of the tumor cells. Expression of the $\alpha_v\beta_3$ integrin has been shown to be associated with the invasiveness of a subset of tumor cells that eventually leave the primary tumor and cause secondary growth [2]. Certain tumors metastasize to specific organs independent of vascular anatomy, rate of blood flow, and number of tumor cells delivered to each organ [19]. Because OS almost exclusively metastasizes to the lung, we reasoned that these cells may preferentially migrate to the lung as opposed to other organs. Indeed, we demonstrated preferential chemotaxis to a homogenate made from lung tissue vs. one made from liver tissue. We also demonstrated the ability of LM7 cells to migrate through the lung endothelium. $\alpha_v\beta_3$ Integrin was critical to this process because treatment of the cells with echistatin significantly decreased migration. Our finding that migration through the lung endothelium was much higher than that through the liver endothelium was intriguing: this preferential migration may play a role in the pattern of metastasis seen in OS.

The $\alpha_v\beta_3$ integrin supports a number of adhesive and invasive tumor cell functions that are thought to be critical during tumor progression [15, 20, 21]. The specific ability of $\alpha_v\beta_3$ integrin to support OS cell migration and the finding of its increased expression in cells with a higher metastatic potential suggest that $\alpha_v\beta_3$ integrin expression may contribute to the metastatic process of OS cells. Understanding the mechanisms involved in the metastatic spread may ultimately reveal novel therapeutic targets for treating OS. $\alpha_v\beta_3$ Integrin may therefore be one of these potential new targets for therapy.

Acknowledgements

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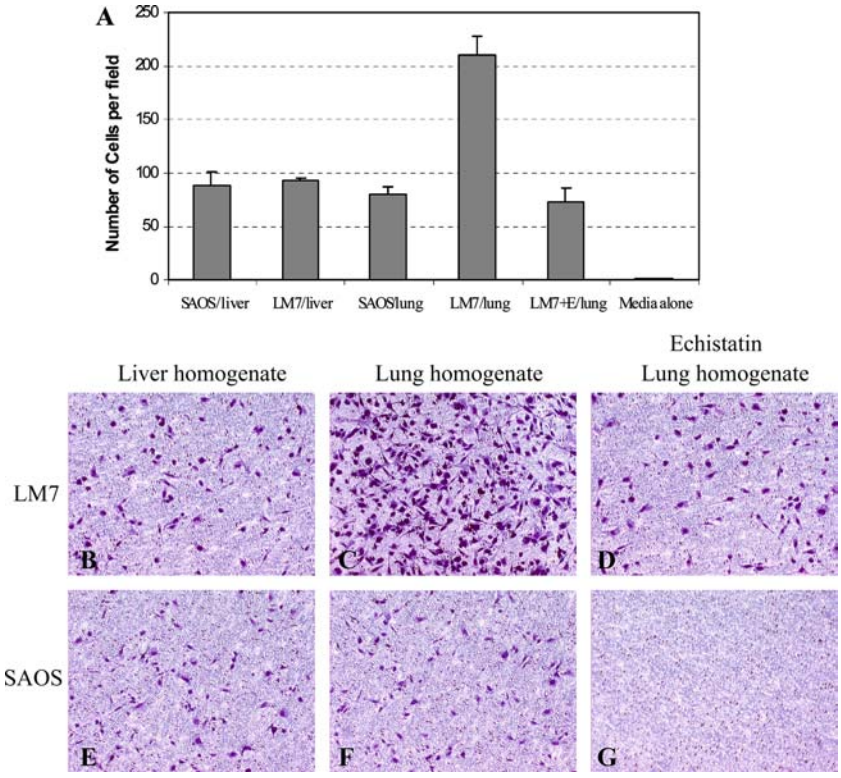

Figure 4. Migration of SAOS parental and LM7 cells to liver and lung homogenates (A). SAOS parental and LM7 cells were seeded into the upper compartments of transwell chambers; homogenates made from liver tissue (B and E) or lung tissue (C, D and F) and medium (G) were transferred by pipette into the bottom wells. Results presented are from one of three representative experiments. LM7 cell migration to lung homogenates vs LM7 cell migration to liver homogenates, $P < 0.01$; migration of LM7 vs SAOS cells lung homogenates, $P < 0.01$; migration of untreated LM7 cells vs LM7 cells treated with echistatin to lung homogenates, $P < 0.01$.

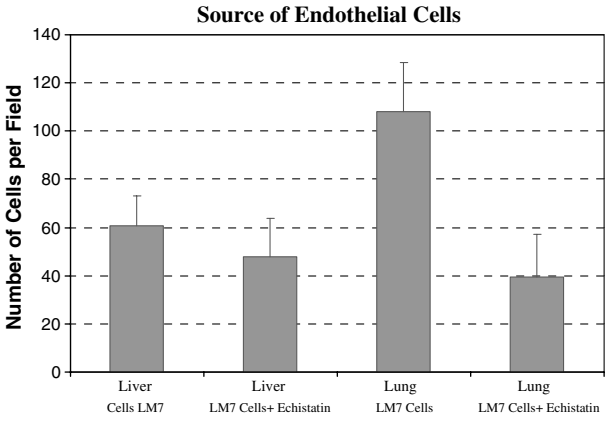

Figure 5. Echistatin inhibits the transendothelial migration of LM7 cells through lung but not liver ECs. Migration of LM7 cells through lung or liver ECs was quantified using transwell chambers as described in Materials and methods. Migration through lung ECs was significantly higher than through liver ECs ($P < 0.01$). Echistatin inhibited the migration through lung ECs ($P < 0.001$) but had no effect on the migration through liver ECs ($P > 0.05$).

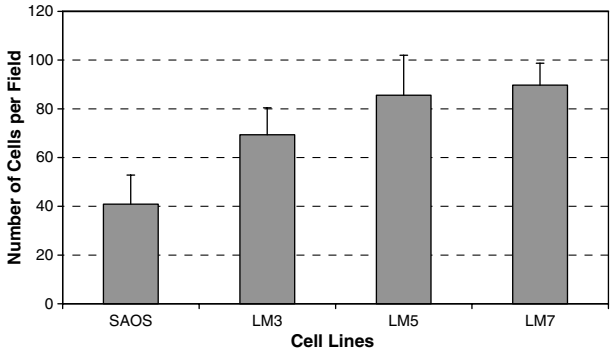

Figure 6. Transendothelial migration through lung ECs correlates with metastatic potential. Transendothelial migration through lung ECs was quantified as described in Figure 5. Migration of LM7 cells was significantly higher than LM3 cells ($P < 0.001$) and SAOS cells ($P < 0.001$). Migration of LM3 cells was significantly higher than SAOS cells ($P < 0.001$).

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