ANTICANCER RESEARCH *40*: 6171-6178 (2020)
doi:10.21873/anticanres.14637

A Potential Role of Adhesion Molecules on Lung Metastasis Enhanced by Local Inflammation

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Abstract. *Background/Aim: Local and systemic inflammations are associated with negative long-term outcomes; however, their precise mechanism of action remains unclear. We previously demonstrated that hepatocyte growth factor (HGF)/c-Met signaling contributed to the enhancement of liver metastasis associated with peritonitis model. The aim of this study is to investigate the effect of local inflammation on the development of lung metastasis. Materials and Methods: NL-17 cells were injected into BALB/c mice via the tail vein to produce a high potential model for lung metastasis. After injection of NL-17 cells, lipopolysaccharide (LPS) and live Pseudomonas aeruginosa, and phosphate-buffered saline were administered intratracheally to induce acute lung injury (ALI) and pneumonia, respectively. Results: In both ALI and pneumonia mice, lung metastasis was significantly promoted compared to control mice. Concentrations of Interleukin-6, tumor necrosis factor-α, and HGF in the bronchoalveolar lavage fluid were significantly higher in ALI and pneumonia mice than in control mice. Neither administration of recombinant mouse HGF nor c-Met knockdown in NL-17 cells influenced the magnitude of lung metastasis. Yet stimulation with LPS increased the expression of α2 integrin, vascular cell-adhesion protein-1, and intercellular adhesion molecule-1 (ICAM-1) in the lung. Invasive activity of NL-17 cells was significantly up-regulated by LPS, but was suppressed by anti-ICAM-1 antibody. While LPS-stimulated NL-17 cells showed significantly promoted lung metastasis, E-selectin expression in the lungs of mice with ALI or pneumonia was significantly enhanced compared with control mice. Conclusion: Up-regulation of adhesion molecules, but not HGF/c-Met signaling, may contribute to the lung metastasis enhanced by local infection/inflammation.*

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Key Words: Lung metastasis, acute lung injury (ALI), pneumonia, intercellular adhesion molecule-1 (ICAM-1), E-selectin.

Since DerHagopian *et al*. introduced the concept of inflammatory oncotaxis in 1978, in which persistent inflammation could stimulate dormant tumor cells to promote organ metastasis (1), many researchers have reported that postoperative complications may affect recurrence and cause poor long-term survival in various malignancies, such as head and neck, colorectal, esophageal, and gastric cancers (2-6). Although researchers have proposed a hypothesis that systemic infection may suppress host tumor immunity and promote tumor growth, the precise mechanism on how systemic infection leads to poor cancer survival still remains unclear. We previously reported that intra-abdominal infection induced by cecal ligation and puncture promoted liver metastasis and that it could be a good animal model for the analysis of inflammation-enhanced cancer metastasis (7, 8). Based on those reports, we have proposed that suppression of the number and activity of intrahepatic natural killer cells plays an important role as one of key mechanisms underpinning this phenomenon. In addition, we recently revealed the HGF/c-Met signaling pathway should contribute to the enhanced liver metastasis associated with intra-abdominal infection (9).

High frequency malignant tumor metastatic sites include liver, lungs, bones, and peritoneum, and dominant sites of metastasis and/or recurrence differ depending on the types of tumors. This is understood to be due to the difference in the expression of adhesion molecules, the production of growth factors, and the affinity of tumor cells to metastatic organs (10). Thus, when considering the possibility of tumor metastasis, a detailed examination of each metastatic organ is required (11).

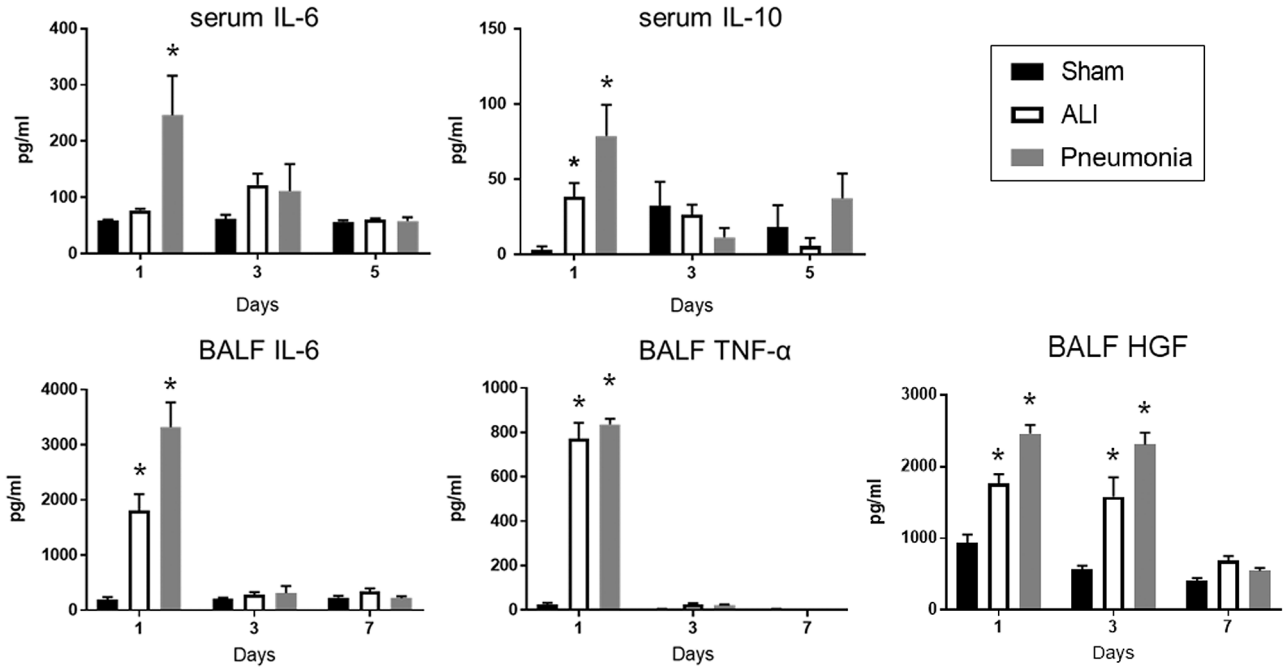

Figure 1. *Wet lung weight (A) and area of resected lungs (B) of metastasis model mice on Day 14 after treatment. ALI: Acute lung injury. (n=5/group) *p<0.05 vs. control.*

In this study, we established a murine lung metastasis model and investigated the effect of local inflammation on metastatic development.

Materials and Methods

Mice and cell line. Female (8-10 weeks) BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan) and given *ad-libitum* access to food and water. NL-17 cells, a murine colon cancer cell line derived from colon 26 cells which have a high potential to induce liver metastasis, were kindly provided from the Division of Molecular Pharmacology, Cancer Chemotherapy Center (Japanese Foundation for Cancer Research, Tokyo, Japan). The cells were maintained in Roswell Park Memorial Institute 1640 (RPMI-1640) medium containing 5% heat-inactivated fetal bovine serum and antibiotics in a humidified atmosphere of 5% CO_2 at 37˚C.

Animal models. The NL-17 cells were suspended in Hanks' Balanced Salt Solution (HBSS) and adjusted to the concentration of 5×10^6 cells/ml. Under general anesthesia, mice were injected intravenously with 5×10^5 NL-17 cells in 100 μl HBSS *via* the tail vein using a 29-G needle syringe. Immediately after injection of tumor cells, the mice were intratracheally administered with lipopolysaccharide (LPS) derived from *Escherichia coli* (O111: B4; Sigma, St Louis, MO, USA) suspended in phosphate-buffered saline (PBS) at a concentration of 20 μg/50 μl to induce acute lung injury (ALI). Similarly, they were intratracheally administered with live Pseudomonas aeruginosa bacteria (1.5×10^6 colony forming unit/50 μl PBS) to induce pneumonia. As a control, mice were administered with 50 μl of PBS intratracheally. The survival rate on Day 14 after the above treatment was 100% in each model. The extent of lung metastasis was evaluated by measuring the lung weight because there was no difference in the lung weight on Day 14 among the three models unless tumor cells were inoculated (data not shown).

To investigate serum cytokine concentrations, blood samples were obtained from mice on days 1, 3, and 5 after treatment. Bronchoalveolar lavage fluid (BALF) was also collected on days 1, 3, and 7 after treatment. Briefly, after cervical dislocation, a 24-G elastic needle was inserted into the tracheal lumen for irrigation. BALF was performed with 2.5 ml of PBS four times (total volume=10 ml).

A photocopy of the resected lungs with a ruler was scanned and saved as a TIFF file. The volume of resected lung was determined using Image J software for Windows (available at: https://imagej.nih.gov/ij/download.html).

To evaluate the direct effect of hepatocyte growth factor (HGF) on lung metastasis, mice were intraperitoneally administered with 5 μg/100 μl of recombinant mouse HGF (R&D systems, Inc., Minneapolis, MN, USA) or 100 μl of PBS as a control, and the lung weight was monitored for 10 days after injection.

Cytokine measurement. Cytokine concentrations in the sera and BALF were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' protocols. Interleukin-6 (IL-6), IL-10, and tumor necrosis factor-α (TNF-α) were measured using BD OptEIA™ ELISA Set (Becton, Dickinson and Company, NJ, USA), while HGF was measured using DuoSet® ELISA Mouse HGF (R&D Systems Inc., Minneapolis, MN, USA).

Measurement of E-selectin in the lung tissue. After homogenization of the lung tissue in 1 ml of PBS with a 200-G stainless steel mesh

filtration, concentrations of E-selectin and total protein in each tissue sample were measured with commercially available ELISA kits (E-selectin, R&D Systems Inc.; total protein, Bio-Rad Laboratories, Richmond, CA, USA) according to the manufacturers' protocols. Pulmonary tissue E-selectin concentrations were corrected with the corresponding protein concentration and expressed as relative values (pg/mg protein).

Establishment of c-Met knockdown cells. c-Met-knockdown cells were established using the RNA interference technology, as previously described (12). Briefly, NL-17 cells were infected with adenovirus containing siRNA encoding murine c-Met (clone 178M) with a multiplicity of infection of 100 and incubated for 72 h in a humidified atmosphere of 5% CO_2 at 37˚C. c-Met knockdown was confirmed by measuring the expression of c-Met by Western blot. NL-17 cells infected with non-specific adenovirus (clone GL-2, which encodes siRNA against firefly luciferase) under the same condition were used for control.

Western blotting. Cell extracts boiled under a reduced condition were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred to polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Waltham, MA, the USA). Membranes were incubated with antibodies against Met (SP260: sc-162, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or β-actin (AC-15: ab6276, Abcam, Cambridge, MA, USA) followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology). After incubation with enhanced chemiluminescence reagent (Amersham Biosciences, Buckinghamshire, UK), chemiluminescence signals were photographed and quantitated *via* image analysis.

Flow cytometric analysis of adhesion molecules expression. NL-17 cells (5×10^5 cells) were stimulated with LPS (final concentration=100 ng/ml), and the expression of several adhesion molecules was evaluated using the FACScan™ system (BD Japan, Tokyo). Primary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) used in this study were as follows: phycoerythrin (PE)-conjugated β1 integrin, fluorescein isothiocyanate (FITC)-conjugated β2 integrin, PE-conjugated β3 integrin, PE-conjugated β4 integrin, PE-conjugated β5 integrin, PE-conjugated α2 integrin, PE-conjugated α4 integrin, FITC-conjugated α5 integrin, PE-conjugated α6 integrin, PE-conjugated αV integrin, PE-conjugated E-selectin, PE-conjugated intercellular adhesion molecule-1 (ICAM-1), and PE-conjugated vascular cell adhesion molecule-1 (VCAM-1).

Invasion assay. Invasive activity of NL-17 cells was evaluated by the modified Boyden method with a blind-well chamber (Corning Inc., NY, USA). Briefly, 200 μl of the test sample, *i.e.* either 100 ng/ml of LPS, 100 ng/ml of LPS + 40 mg/ml of anti-ICAM-1 antibody (Thermo Fisher Scientific, Waltham, MA, USA), or 100 ng/ml of LPS + 40 mg/ml of isotype immunoglobulin (Ig) G antibody (Thermo Fisher Scientific, Waltham, MA, USA) was placed in the lower chamber of the wells, and then 1×10^5 NL-17 cells were placed in the upper chamber which was separated from the lower chamber by a 3-mm-pore polycarbonate membrane (Nuclepore, NY, USA). The assembled chamber was incubated for 12 hours at 37˚C under 5% CO_2. The membrane was then fixed in methanol and the cells were stained with hematoxylin and eosin. Invasive activity was determined by counting the number of cells that had invaded onto the lower surface of the membrane filter in three separate high-power (×200) fields (HPF).

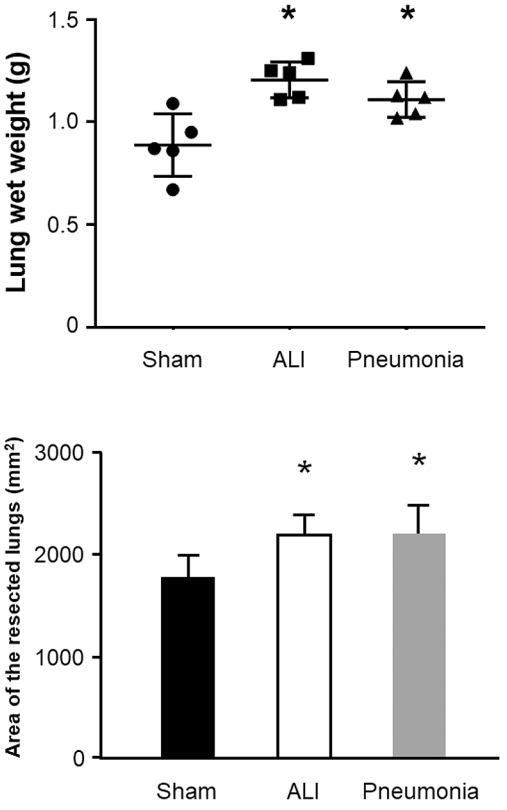

Figure 2. *Cytokine levels in sera and BALF obtained from lung metastasis model mice with ALI and pneumonia. ALI: Acute lung injury, BALF: bronchoalveolar lavage fluid, IL: interleukin, TNF: tumor necrosis factor, HGF: hepatocyte growth factor. (n=5/group) *p<0.05 vs. control.*

Ethics. All animal protocols were based on the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines, and were approved by the Institutional Review Board for the Care of Animal Subjects of National Defense Medical College (Approval number: 15010).

Statistical analysis. JMP Pro 14.0.0 software (SAS Institute, Cary, NC, USA) was used for statistical analyses. Data were expressed as mean±standard error. Statistical analyses were performed using the Mann–Whitney *U* or Chi-squared test with Fisher's exact test, as appropriate. Survival rates were calculated using the Kaplan–Meier method, and differences in survival rates were determined using the log-rank test. The *p*-Values of <0.05 were considered as statistically significant.

Results

As shown in Figure 1, lung metastasis evaluated by measuring lung weight and area of the resected lung on Day 14 was significantly increased in both ALI and pneumonia groups compared to the control group.

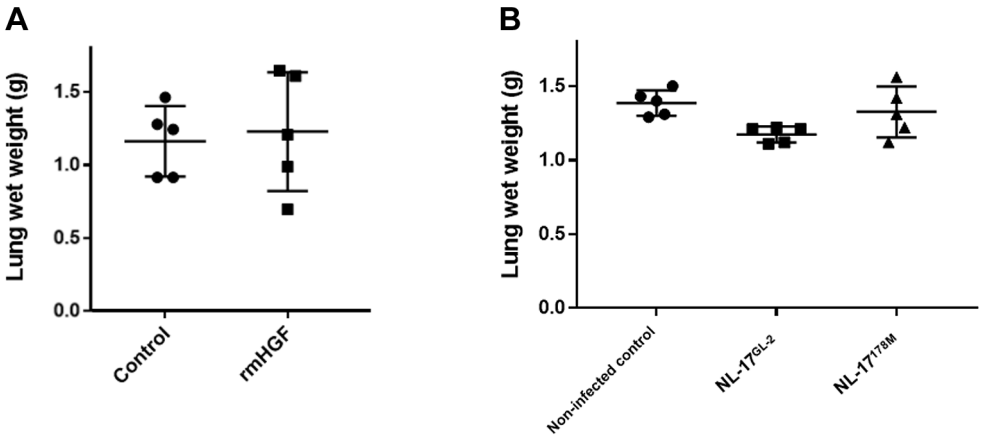

Figure 3. *Wet lung weight on Day 14 after rmHGF or c-Met knockdown treatment. (A) rmHGF: recombinant mouse hepatocyte growth factor, (n=5/group) (B) NL-17GL-2: NL-17 cells infected with control virus, NL-17178M: NL-17 cells infected with c-Met knockdown virus (n=5/group). rmHGF: Recombinant mouse hepatocyte growth factor.*

To see the effect of ALI/pneumonia on the inflammatory stress in the lung metastasis model mice, changes in serum and BALF cytokines were measured (Figure 2). Serum IL-6 concentration in the serum of pneumonia group on Day 1 was significantly higher than that of control group; however, such an increase of serum IL-6 was not observed in the ALI group. Also, there was no statistical difference in the serum IL-6 levels among the three groups on Days 3 and 5. Serum IL-10 concentrations in both ALI and pneumonia groups on Day 1 showed significantly higher values compared to the control group. Again no difference in serum IL-10 levels was observed among the three groups on Days 3 and 5 (Figure 2, upper panels). Cytokine concentrations in BALF showed much higher values than that in serum presumably because the stimulants were administered through the tracheal canal. IL-6 and TNF-α concentrations in BALF on Day 1 were significantly higher in the ALI and pneumonia groups than in the control group, but there was no statistical difference in the levels of those two cytokines among the three groups on Days 3 and 5. Serum HGF concentrations were below the sensitivity threshold for detection in all groups throughout the experimental period. HGF concentrations in BALF in the ALI and pneumonia groups were significantly higher than those in the control group on Days 1 and 3 (Figure 2, lower panels).

To assess a direct effect of HGF on lung metastasis, recombinant mouse HGF (rmHGF) was intraperitoneally administered to the tumor metastasis model mice and lung wet weight was measured (Figure 3A). However, there was no significant difference in lung wet weight between the mice administered with rmHGF and those with normal saline (control group). Similarly, there was no statistical difference in lung metastasis, *i.e.* lung wet weight among the three groups in which NL-17 cells had been pretreated with saline (non-infected control group), control virus ($NL\text{-}17^{GL\text{-}2}$) targeting firefly luciferase, and c-Met knockdown virus ($NL\text{-}17^{178M}$), respectively.

Next, the expression of adhesion molecules on the cell surface of NL-17 cells after LPS stimulation was evaluated using flow cytometry (Figure 4A). There was no significant enhancement in the expression of α5, α6, αV, β1, or β4 integrins; however, α2 integrin, VCAM-1, and ICAM-1 were up-regulated by LPS stimulation. Particularly, the percentage of ICAM-1-positive NL-17 cells significantly increased after LPS stimulation showing a peak value at 6 hours (Figure 4B). The percentage of ICAM-1-positive cells at 24 h was still higher than the cells that had not been subjected to LPS stimulation.

Invasive activity of NL-17 cells was significantly up-regulated by LPS stimulation as shown in the trans-well invasion assay (Figure 5), and this invasiveness was significantly suppressed by the administration of anti-ICAM-1 antibody.

Lung metastasis of LPS-stimulated NL17 cells assessed by lung weight at Day 14 was significantly promoted compared with non-stimulated cells (Figure 6A). Concentration of E-selectin in the lung of metastasis model mice with ALI and pneumonia showed significantly higher concentration compared with that in the sham stimulation mice (Figure 6B).

Discussion

In this study, we demonstrated that lung metastasis was enhanced by local inflammation and infection which was associated with up-regulation of adhesion molecules activated by local inflammatory reactions, but not through the HGF/c-Met signaling which had been reported to be enhanced in liver metastasis with intra-abdominal infection (9).

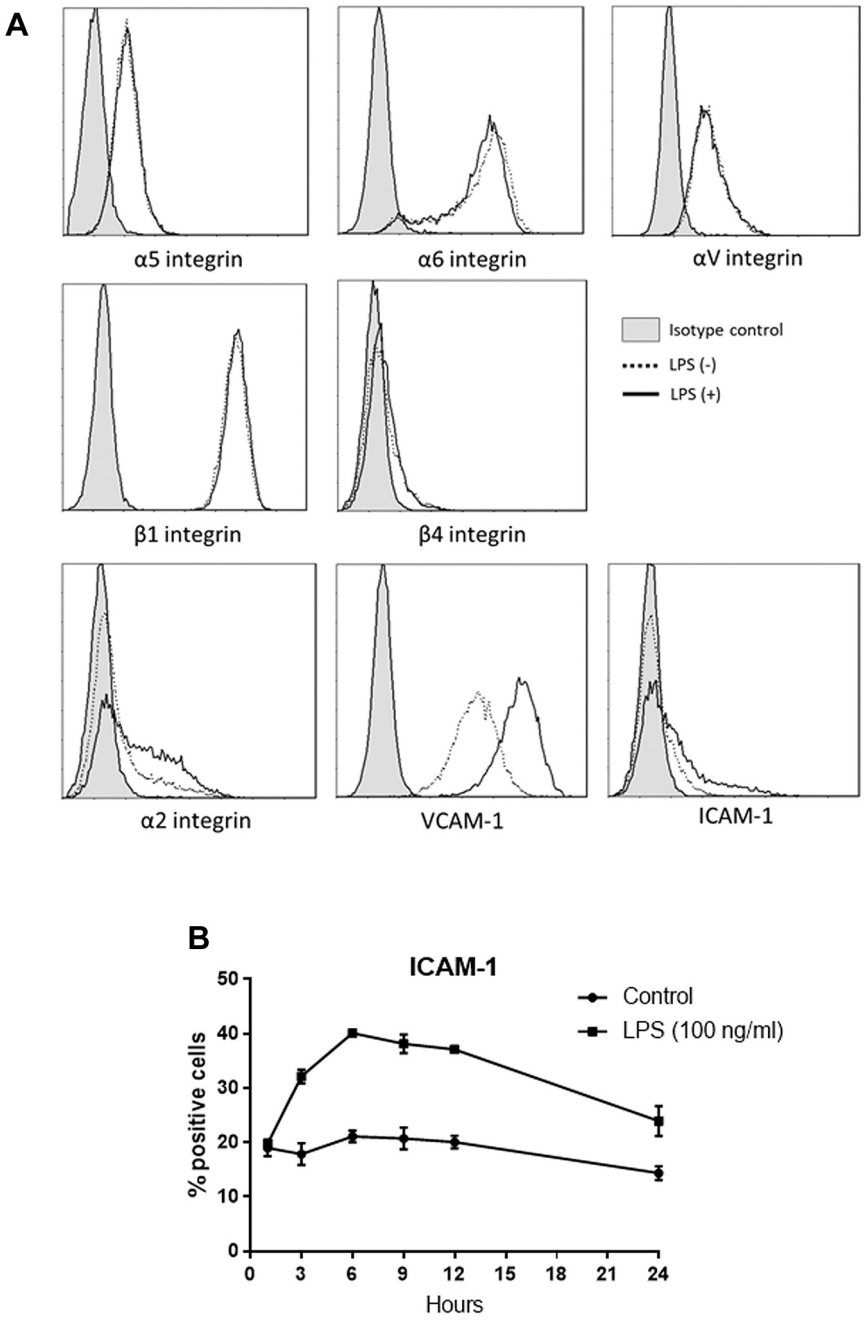

Figure 4. *Expression of adhesion molecules in NL-17 cells after LPS stimulation. (A) Representative flow cytometric histograms of various adhesion molecules on NL-17 cells from one of at least three experiments with similar results. (B) Changes in ICAM-1 expression after LPS stimulation. (n=5/group). Gray filled: Isotype control, dashed lines: NL-17 cells without LPS stimulation, bold lines: NL-17 cells after LPS stimulation. *p<0.05 vs. control. LPS: Lipopolysaccharide, VCAM-1: vascular cell-adhesion molecule-1, ICAM-1: intracellular adhesion molecule-1.*

Malignant tumor metastasis is achieved by complex multistep processes that involve tumor dissemination through the bloodstream beyond its organ of origin, resulting in colonization of other organs and aggressive tumor growth at metastatic sites (13). Thus, the mechanism by which local or systemic infection promotes metastasis of malignant tumors should encompass these steps. Because HGF concentration in BALF was significantly higher in the metastasis model mice with ALI or pneumonia compared to the control group on Day 1, we first hypothesized that the HGF/c-Met signaling

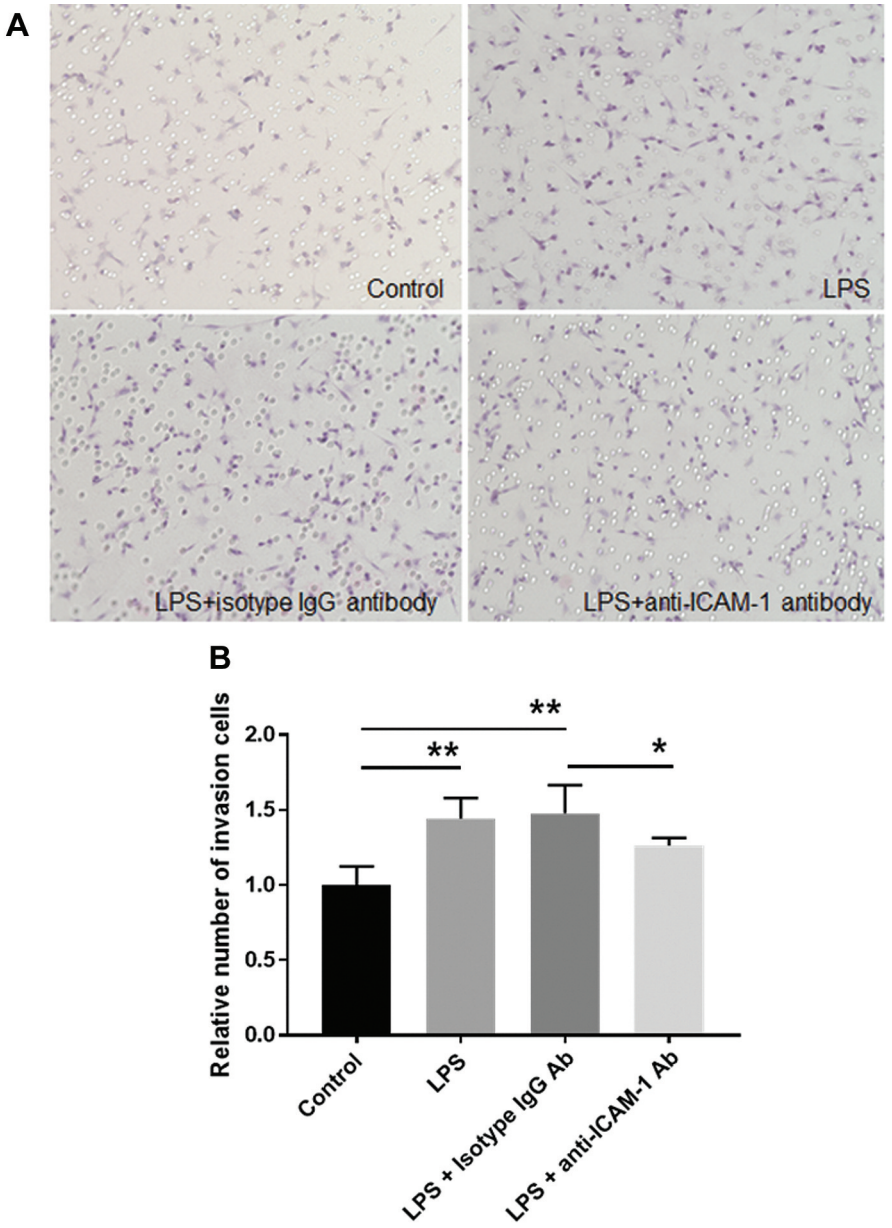

Figure 5. *Trans-well invasion assay of NL-17 cells. (A) Representative photographs of the lower filter surface from one of the four experiments with similar results. The invasive response was determined by counting the number of cells that had invaded onto the lower filter surface in three high-power (×200) fields. (B) Relative number of invading cells. Anti-ICAM-1 Ab effectively inhibited invasive capacity of NL-17 cells (n=4/group). LPS: Lipopolysaccharide, Ab: antibody, ICAM-1: intracellular adhesion molecule-1. *p<0.05 vs. LPS + isotype IgG antibody. **p<0.05 vs. control.*

pathway might promote lung metastasis during local and systemic inflammation processes, as seen in the liver metastasis model that was enhanced by intraabdominal infection (8). However, an increase in lung metastasis was not observed in the septic model (data not shown). In this study, there was no direct effect of rmHGF on lung metastasis, and also any difference in the extent of lung metastasis was not identified depending on the expression of c-Met in NL-17 cells. Thus, processes different from liver metastasis seemed to contribute to the development of lung metastasis.

Several Ig superfamily molecules, such as integrins and selectins, are components of cell-adhesion molecules and involved in cell–matrix and cell–cell adhesion (14). These molecules are also expressed in some tumor cells and are

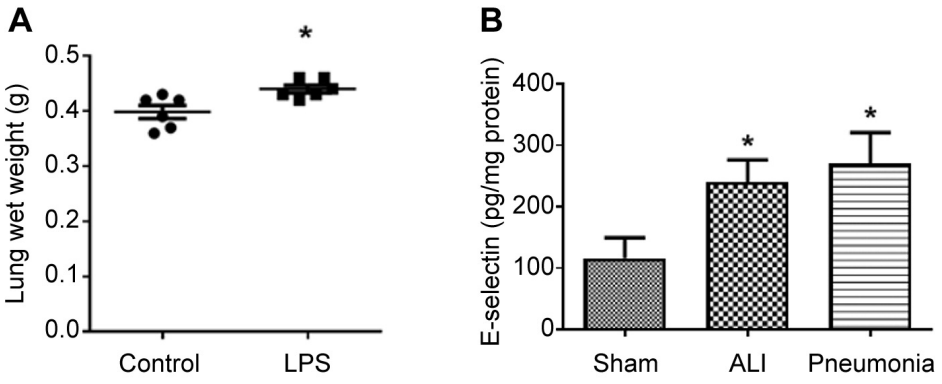

Figure 6. *Effect of LPS stimulation on lung metastatic activity of NL-17 cells. (A) Wet lung weight in lung metastasis model mice using LPS-stimulated NL-17 cells. LPS: lipopolysaccharide, *p<0.05 vs. control. (n=6/group) (B) E-selectin concentrations in the lungs with ALI or pneumonia. (n=6/group) ALI: acute lung injury, *p<0.05 vs. control.*

involved in cell adhesion, proliferation, migration, and invasion of tumor cells, leading to tumor cell metastasis (15, 16). However, their involvement in tumor growth and metastasis during infection in cancer-bearing hosts has not been sufficiently clarified. In this study, we demonstrated that NL-17 cells ubiquitously expressed several adhesion molecules, and LPS stimulation increased expression of α2 integrin, VCAM-1, and ICAM-1 on NL-17 cells. We also showed that invasion enhanced by LPS stimulation was partially suppressed by the administration of anti-mouse ICAM-1 antibody, suggesting that enhanced expression of adhesion molecules was involved in tumor metastasis. In addition, LPS-stimulated NL-17 cells showed promoted lung metastasis compared to non-stimulated NL-17 cells, and E-selectin concentrations in lung tissue were elevated in mice with ALI or pneumonia. E-selectin expression on vascular surfaces is induced in response to inflammatory cytokines and is elevated in tumor-associated vessels (17). Therefore, enhanced expression of adhesion molecules in both tumor cells and at metastatic sites may be involved in the mechanism of lung metastasis in ALI and pneumonia models.

In conclusion, we showed that local inflammation and lung infection such as ALI and pneumonia up-regulated cell-adhesion molecules, such as ICAM-1 and E-selectin, which might contribute to lung metastasis enhanced by local inflammation. In contrast, direct effect of HGF/c-Met on lung metastasis was unclear. Our results suggest that tumor cell metastasis may be promoted by different mechanisms in different organs, such as in the liver and lungs.

Conflicts of Interest

The Authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

Authors' Contributions

Hiroyuki Horiguchi, Hironori Tsujimoto, Nariyoshi Shinomiya, Shinsuke Nomura, and Hiromi Miyazaki contributed to the design and experiment of this study. Takao Yamori contributed to the cell culture and in vitro study. Hironori Tsujimoto, Daizoh Saitoh, Yoji Kishi, and Hideki Ueno contributed to writing the draft and supervising this study. All authors have approved the manuscript and agree with its submission.

Acknowledgements

Part of this work has been supported by the Grant-in-Aid for Scientific Research, Japan Society for the Promotion of Science, (Grant/Award Number: 18H02685).

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Received August 26, 2020
Revised September 8, 2020
Accepted September 11, 2020