Involvement of Very Late Activation Antigen 4 (VLA-4) and Vascular Cell Adhesion Molecule 1 (VCAM-1) in Tumor Necrosis Factor \( \alpha \) Enhancement of Experimental Metastasis

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ABSTRACT

In this study, we examined the effect of tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) on pulmonary metastasis of murine melanoma B16-BL6 by focusing on the intercellular adhesion molecules involved in the metastatic process. TNF-\( \alpha \) administration before B16-BL6 inoculation significantly enhanced the experimental pulmonary metastasis. The enhancement was seen when TNF-\( \alpha \) was administered 4 h, but not 24 h, before B16-BL6 inoculation. Administration of 50–5000 units of TNF-\( \alpha \) increased the number of metastatic lung colonies in a dose-dependent manner. Flow cytometric analysis demonstrated a high expression of very late activation antigen 4 (VLA-4) on the surface of B16-BL6 cells. Immunoperoxidase staining demonstrated that a ligand for VLA-4, vascular cell adhesion molecule 1, was expressed on lung vascular endothelium 4 h after administration of TNF-\( \alpha \). Pretreatment of B16-BL6 cells with an anti-VLA-4 monoclonal antibody abolished the TNF-\( \alpha \)-enhanced pulmonary lung colonies. Administration of an anti-vascular cell adhesion molecule 1 monoclonal antibody also abolished the enhancement. These results indicate that the interaction between VLA-4 on tumor cells and vascular cell adhesion molecule 1 on activated endothelial cells is critically involved in TNF-\( \alpha \) enhancement of metastasis.

INTRODUCTION

Tumor cell surface adhesion molecules, including integrins and selectin ligands, have recently been thought to be implicated in tumor metastasis and invasion (1, 2). It has been reported that activation of endothelial cells by inflammatory cytokines increased the adhesion of tumor cells in vitro (3–6). An inducible endothelial cell surface glycoprotein, INCAM-110, that was identified later as VCAM-1, mediated adhesion of tumor cells to IL-1/2 or TNF-activated endothelial cells in vitro (7, 8). IL-1, TNF, and \( \gamma \)-interferon have been also reported to enhance the metastasis of human melanoma cells in nude mice (9). However, the involvement of VLA-4/VCAM-1 interaction in this enhanced metastasis has not been directly demonstrated in vivo. To investigate the relevance of these observations in vivo, we evaluated the effect of TNF on pulmonary metastasis of murine melanoma B16-BL6 and the involvement of the VLA-4/VCAM-1 interaction in the enhanced metastasis.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice were obtained from Nippon SLC (Shizuoka, Japan). In accordance with the institutional guidelines, mice did not suffer unnecessary discomfort, pain, or injury, and received proper care and maintenance.

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2 The abbreviations used are: IL-1, interleukin 1; TNF, tumor necrosis factor; VLA-4, very late activation antigen 4; VCAM-1, vascular cell adhesion molecule 1; PBS, phosphate-buffered saline; mAb, monoclonal antibody; ICAM-1, intercellular adhesion molecule 1.

Cells. Mouse melanoma B16-BL6 established by Poste et al. (10) were maintained in RPMI 1640 medium (Nikkei Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (JR Scientific, Inc., Woodland, CA). B16-BL6 cells were harvested by brief exposure to 0.25% trypsin-0.02% EDTA solution, washed with PBS once, and resuspended in PBS at 1 × 10^7 cells/ml for inoculation.

Experimental Pulmonary Metastasis. Single cell suspension of B16-BL6 (>95% viable as estimated by trypsin blue exclusion, 1 × 10^7 in 0.1 ml of PBS) was inoculated into the lateral tail vein of mice. The mice were sacrificed and their lungs were removed on days 11 to 13. The number of colonies on the surface of the lungs was counted. Differences in the number of lung colonies were analyzed by the Student t test.

TNF-\( \alpha \) Administration. Recombinant human TNF-\( \alpha \) (specific activity, 2.2 × 10^6 units/mg) was a generous gift from Asahi Chemical Industry Co. Ltd. (Tokyo, Japan) (11). Fifty, 500, or 5000 units/mouse of TNF-\( \alpha \) in 0.1 ml of PBS was administered in the lateral tail vein of mice 4 h before B16-BL6 cell inoculation. Eleven days later, the number of colonies on the surface of the lungs was counted. B16-BL6 cells were inoculated 0–48 h after TNF-\( \alpha \) (500 units/mouse) administration. Eleven days later, the number of colonies on the surface of the lungs was counted.

Antibodies. Monoclonal rat IgG2b antibody to murine VLA-4 \( \alpha \) chain, PS/2 (12), was purified from ascites by protein G affinity chromatography. This mAb did not affect the growth of B16-BL6 cells in vitro when added to the culture even up to 100 \( \mu \)g/ml (data not shown). B16-BL6 cells (1 × 10^6) were incubated with 1 \( \mu \)g of PS/2 for 30 min at 4°C. After one washing with PBS, these cells were inoculated into the lateral tail vein of mice that were inoculated with 5000 units of TNF-\( \alpha \) 4 h earlier. The number of lung colonies on day 13 was counted. RMV-7 (monoclonal rat IgG2b antibody to murine vitronectin receptor \( \alpha \) chain) (13) and K-9 (monoclonal rat IgG2a, antibody to murine H-2K\( \alpha \), established by H. Ishikawa, Keio University, Tokyo, Japan) were used as control antibodies. The same numbers of B16-BL6 cells were preincubated with 1 \( \mu \)g of RMV-7 or K-9 before inoculation. Monoclonal rat IgG1 antibody to murine VCAM-1, M/K-2, was also described previously (14, 15). Monoclonal rat IgG2a antibody to murine ICAM-1, KAT-1, was recently established by K. Kato (Juntendo University) and used as a control antibody. One hundred \( \mu \)g of M/K-2 or KAT-1 mAb was administered simultaneously with 5000 units of TNF-\( \alpha \) in the lateral tail vein of mice. Four h later, B16-BL6 were inoculated into the lateral tail vein of mice. The numbers of lung colonies on day 13 were counted.

Flow Cytometry. B16-BL6 cells (1 × 10^6) were incubated with 1 \( \mu \)g of PS/2 or anti-mouse \( \beta \)2 (LST272, Phar Minger, San Diego, CA) mAb for 1 h at 4°C. Cells were washed with PBS twice, then the cells were treated with fluorescein isothiocyanate-labeled goat anti-murine immunoglobulin antibody (Caltag, San Francisco, CA) for 1 h at 4°C. After washing with PBS twice, the cells were analyzed on a fluorescence-activated cell sorter (FACSscan; Becton Dickinson, Sunnyvale, CA).

Immunoperoxidase Staining. Lungs were removed from the mice 4 or 24 h after TNF-\( \alpha \) (5000 units) administration. The lungs were frozen immediately in liquid nitrogen and kept at −80°C until cryostat sectioning. Cryostat sections were fixed with 4% paraformaldehyde for 12 h at room temperature. After washing with PBS, these sections were incubated with M/K-2 mAb or a negative control rat IgG for 1 h at room temperature, washed with PBS twice, and were reacted with biotin-labeled goat anti-murine immunoglobulin antibody (Vector, Burlingame, CA) for 1 h at room temperature. After washing with PBS, the sections were reacted with avidin-biotin-peroxidase complex (Nichirei, Inc., Tokyo, Japan), washed, and developed with diaminobenzidine and \( \text{H}_2\text{O}_2 \).
**RESULTS**

**Effect of TNF-α on Pulmonary Metastasis of B16-BL6.** Administration of TNF-α 4 h before tumor inoculation increased the number of metastatic lung colonies in a dose-dependent manner (Fig. 1). The mean number of metastatic colonies in untreated mice was 33.4 ± 24.4 (SD). The mean numbers of metastatic colonies in the mice administered 50 or 500 units of TNF-α were 77.8 ± 33.4 and 101 ± 34.3, respectively. When 5000 units of TNF-α were administered, the colonies strikingly increased by about 10-fold (318 ± 66.7). In the following studies, 5000 units of TNF-α were administered to mice. We next examined the time kinetics of the TNF-α-enhanced pulmonary lung colonies. As indicated in Fig. 2, the enhancing effect of TNF-α was transient. The mean numbers of metastatic colonies in the mice treated with 5000 units of TNF-α 0, 4, 12, 24, or 48 h before B16-BL6 inoculation were 95.2 ± 14.2, 177.2 ± 25.5, 94.6 ± 17.7, 42.8 ± 4.4, and 16.2 ± 9.5, respectively. This result demonstrated that TNF-α enhanced pulmonary metastasis of B16-BL6 most strikingly when administered 4 h before tumor inoculation, but the enhancing effect disappeared at 24 h after administration as compared with the untreated mice (41.6 ± 9.9).

**Expression of VLA-4 and VCAM-1.** Flow cytometric analysis with an anti-VLA-4 mAb, PS/2, demonstrated that B16-BL6 cells expressed a high level of VLA-4 on their surface (Fig. 3). In contrast, an anti-β7 mAb did not react with B16-BL6 cells, indicating that these cells express the classical VLA-4 (α4β1) but not the α4β7 complex (data not shown). On the other hand, immunohistochemical staining with an anti-VCAM-1 mAb, M/K-2, indicated that the VCAM-1 expression was barely detected on normal lung vascular endothelium, but was highly induced at 4 h after administration of 5000 units of TNF-α. Thereafter, the VCAM-1 expression decreased at 24 h after TNF-α administration (Fig. 4).

**Inhibitory Effect of Anti-VLA-4 mAb on TNF-α-enhanced Pulmonary Metastasis.** We next examined the involvement of VLA-4 in the TNF-α-enhanced pulmonary lung colonies by preincubating B16-BL6 cells with a saturating amount of PS/2 mAb, which has been shown to efficiently inhibit the binding of VLA-4 to VCAM-1 (12) before inoculation (Fig. 5A). An anti-vitronectin receptor α chain mAb (RMV-7) and an anti-H-2Kb mAb (K-9), which bind to B16-BL6 to an extent similar to that of PS/2 (data not shown), were used as nonspecific control antibodies. Preincubation of B16-BL6 cells with PS/2 mAb completely abrogated the TNF-α-enhanced pulmonary metastasis (mean colony number, 110.0 ± 27.7 and 21.2 ± 16.9 for Ab(—) and PS/2, respectively). In contrast, the preincubation with RMV-7 or K-9 mAb did not significantly affect the enhanced metastasis (mean colony number, 81.0 ± 15.9 and 132.0 ± 43.2 for RMV-7 and K-9, respectively).

**Inhibitory Effect of Anti-VCAM-1 mAb on TNF-α-enhanced Pulmonary Metastasis.** We also examined the involvement of VCAM-1 that was expressed on the lung vascular endothelium after TNF-α administration, by administering M/K-2 mAb before i.v. inoculation of B16-BL6 (Fig. 5B). The TNF-α-enhanced metastasis was significantly reduced by M/K-2 but not by a control anti-ICAM-1 mAb (KAT-1), which also binds to the TNF-α-activated lung endothelium (mean colony number, 52.8 ± 25.7, 20.0 ± 11.9 and 101.4 ± 49.1 for Ab(—), M/K-2, and KAT-1, respectively).

**DISCUSSION**

Clinical treatment of malignant tumors is very difficult because they frequently form metastasis, the major cause of morbidity and mortality in cancer patients (16) and, therefore, it is very important to analyze the mechanisms of metastasis. The bloodstream is the most common pathway for metastasis formation. Hematogenous metastasis requires the arrest and extravasation of circulating tumor cells. An important step in this metastatic process is the arrest of tumor cells on vascular endothelium of the target organ. For many years vascular...
endothelium was viewed as a passive barrier between the circulation and the extravascular tissue. However, recently, it has become evident that endothelial cells play an active role in many biological processes such as angiogenesis, coagulation, leukocyte migration, and metastasis. According to the recent knowledge about invasion and metastasis, tumor cell surface adhesion molecules, including integrins and selectin ligands, have been implicated in the arrest of tumor cells on vascular endothelium. Tumor cells use a variety of surface adhesion molecules to attach to and invade basement membranes (1, 2, 17, 18). In addition, adhesive interactions between tumor cells and endothelial cells are mediated through a variety of cell surface proteins. Rice and Bevilacqua (7) reported that an inducible endothelial cell surface glycoprotein (INCAM-110), which has been identified as VCAM-1, mediates the adhesion of melanoma cells to IL-1- or TNF-α-activated endothelial cells in vitro. Taichman et al. (8) reported that the adhesion of human melanoma, osteosarcoma, and kidney carcinoma cell lines to activated endothelium is mediated by VLA-4 and VCAM-1 in vitro. Martin-Padura et al. (19) and Kawaguchi et al. (20) also reported that VLA-4 may play a key role in metastasis of some tumor cells. On the other hand, Giavazzi et al. (9) reported that the number of melanoma lung colonies is increased in IL-1-treated nude mice. Orosz et al. (21) reported that a single i.p. injection of recombinant human TNF or recombinant mouse TNF into mice 5 h before i.v. inoculation of methylcholanthrene-induced fibrosarcoma cells induced a significant enhancement of pulmonary metastasis. However, these studies have not addressed the molecular mechanisms relevant to the enhancement, and the role of VLA-4 in hematogenous metastasis has not been confirmed directly in vivo. To investigate the relevance in vivo, we examined the involvement of VLA-4/VCAM-1 interaction in experimental pulmonary metastasis of B16-BL6 cells, that highly expressed VLA-4 (Fig. 3), in TNF-α-treated mouse. The administration of TNF-α greatly enhanced the pulmonary lung colonies (Fig. 1). The enhancement was most striking when B16-BL6 cells were inoculated at 4 h after TNF-α administration, but disappeared at 24 h after TNF-α administration. This kinetics of enhanced metastasis is in good correlation with that of the VCAM-1 induction on lung vascular endothelium after TNF-α administration (Fig. 4). Immunoperoxidase staining showed high expression of VCAM-1 on the lung vascular endothelium at 4 h after TNF-α administration, but the expression decreased at 24 h later. These results suggested that the enhancement of pulmonary lung colonies by administration of TNF-α was caused by the VLA-4/VCAM-1 interaction. To prove this, we tested whether anti-VLA-4 or anti-VCAM-1 mAb could prevent the TNF-α enhancement of pulmonary lung colonies. The TNF-α-enhanced metastasis was inhibited completely by the pretreatment of B16-BL6 with an anti-VLA-4 mAb and partially by the administration of an anti-VCAM-1 mAb (Figs. 5A and 5B). These results clearly implicated the intercellular interaction between VLA-4 on B16-BL6 cells and VCAM-1 on TNF-α-activated lung vascular endothelium in the TNF-α enhancement of pulmonary lung colonies. The partial
inhibitory effect of anti-VCAM-1 mAb as compared with anti-VLA-4 mAb was also observed in other cell-cell interaction systems and seems to be due to the fact that most VCAM-1 has two VLA-4-binding domains, only one of which was blocked by M/β2 (22). Alternatively, fibronectin, another ligand for VLA-4, might be involved in the enhanced metastasis, since PS/2 inhibits VLA-4 binding to both VCAM-1 and fibronectin.

The present study indicated that TNF-α enhances pulmonary metastasis of B16-BL6 melanoma cells in vivo via the VLA-4/VCAM-1 interaction. This suggests that a high serum concentration of TNF-α, which is associated with cachexia, sepsis, surgical stress, or therapeutic administration of recombinant TNF-α, raises the risk of metastasis of certain tumors by up-regulating the expression of endothelial ligands for tumor cell surface adhesion molecules. Therefore, the treatment that interferes with such interactions would be effective to reduce the risk of metastasis.

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REFERENCES


Fig. 5. Inhibitory effect of anti-VLA-4 and VCAM-1 mAb on TNF-α-enhanced pulmonary metastasis. A, B16-BL6 cells (1 × 10⁵), which were preincubated with PS/2 (anti-VLA-4), RMV-7 (anti-vitronectin receptor), or K-9 (anti-H-2Kt) mAb, were inoculated into the mice given 5000 units of TNF-α or PBS 4 h earlier, and the lung colonies were counted 13 days later. The data indicated mean ± SD of five mice for each treatment. *, P < 0.01; **, P < 0.05. Similar results were obtained in three independent experiments.


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