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ORIGINAL ARTICLE

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Involvement of tumor necrosis factor α and very late activation antigen 4/vascular cell adhesion molecule 1 interaction in surgical-stress-enhanced experimental metastasis

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Abstract We examined the influence of surgical stress on hematogenous metastasis of malignant tumor cells. The study was performed by focusing on the involvement of inflammatory cytokines in the serum, raised acutely after surgery, and endothelial adhesion molecules in the metastatic process. Surgical stress, given to C57BL/6 mice before B16-BL6 melanoma inoculation, significantly enhanced the pulmonary metastasis. This enhancement was seen when the surgery lasted for more than 2 h. After the 2-h surgery, the enhancement of pulmonary metastasis was seen most remarkably when B16-BL6 was inoculated 24 h after surgery. The serum level of tumor necrosis factor α (TNFα) in the mice that underwent the 2-h surgery peaked 12 h after the surgery. In contrast, serum interferon γ was not detectable. Administration of an anti-TNFα mAb before the surgery inhibited the enhanced metastasis by inhibiting the increased expression of vascular cell adhesion molecule 1 (VCAM-1) on lung vascular endothelium after the surgery. Pretreatment of B16-BL6 cells with an anti-very late activation antigen 4 (anti-VLA-4) mAb completely inhibited the enhanced metastasis after surgery. Administration of an anti-VCAM-1 mAb before surgery also inhibited the enhancement. These results indicate that serum TNFα, raised by surgical stress, is critically involved in the enhanced pulmonary metastasis of mouse melanoma by inducing VCAM-1 expression on lung vascular endothelium.

Key words Surgical stress · Pulmonary metastasis · TNF-α · VLA-4 · VCAM-1

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Introduction

Many physicians have the impression that metastasis of malignant tumors to distant organs is often accelerated after surgery [1]. Recently, tumor cell-surface-adhesion molecules, including integrins and selectin ligands, have been be implicated in tumor metastasis and invasion [2, 3]. We previously demonstrated that inflammatory cytokines, such as tumor necrosis factor α (TNFα), up-regulate the expression of adhesion molecules on lung vascular endothelium and enhance lung metastasis of mouse melanoma [4]. On the other hand, an increased serum level of some inflammatory cytokines after surgery has been reported by several groups [5–7]. In this context, we evaluated the influence of surgical stress on tumor metastasis by characterizing cytokines and adhesion molecules involved in the enhanced metastasis. The clinical implication of the present findings is discussed.

Materials and methods

Animals

Female C57BL/6 mice, 6–8 weeks old, were obtained from Nippon SLC (Shizuoka, Japan). In accordance with the institutional guidelines, the mice did not suffer unnecessary discomfort, pain or injury, and received proper care and maintenance.

Tumor cells

Mouse melanoma B16-BL6 cells established by Poste [8] were maintained in RPMI-1640 medium (Nikkei Bio Medical Laboratory, Tokyo, Japan) supplemented with 10% fetal bovine serum (JR Scientific Inc., Woodland, Calif.). B16-BL6 cells were harvested by brief exposure to 0.25% trypsin/0.02% EDTA solution, washed with phosphate-buffered saline (PBS) once, and resuspended in PBS at 5×10^5 cells/ml for inoculation.

Surgery

Anesthesia was achieved by intraperitoneal injection of Nembutal (Dinabot Co. Ltd., Tokyo, Japan) at 60 mg/kg. The mice underwent

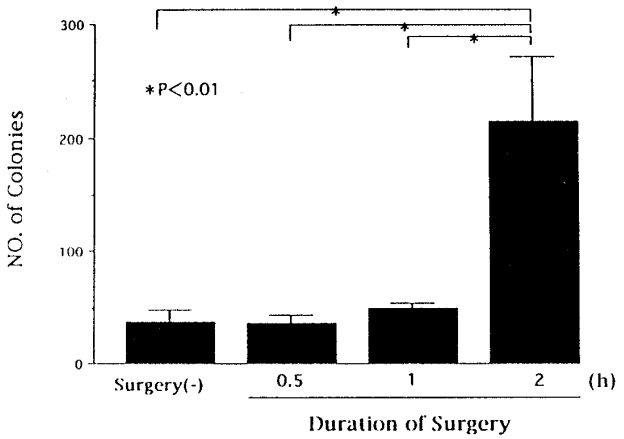

Fig. 1 Relation between duration of surgery and pulmonary metastasis. Surgery was performed on the mice for the indicated lengths of time, and 5×10^4 B16-BL6 cells were injected into the lateral tail veins. The mice were sacrificed and their lungs were removed on days 13–15. The number of colonies on the surface of the lungs was counted. The data indicate the mean $\pm$SD of seven mice for each treatment. Surgery for 2 h significantly increased the number of metastatic lung colonies (*, $P<0.01$)

laparotomy. Their bowels were drawn extraperitoneally and then covered by gauze wetted with saline. After various lengths of time, the bowels were settled back to the abdomen carefully and the wound was closed by auto clip(C-A 7631 autoclip 9 mm, Becton Dickinson).

Control mice

Mice anesthetized by intraperitoneal injection of Nembutal but without surgery were chosen as the control group.

Experimental pulmonary metastasis

B16-BL6 cells (5×10^4) suspended in 0.2 ml PBS were injected into the lateral tail vein 24 h after surgery unless otherwise indicated. The tumor cells were injected in the same way into the control mice. The mice were sacrificed and their lungs were removed on days 13–15. The number of colonies on the surface of the lung was visually counted.

Determination of serum levels of TNFα and interferon γ (IFNγ)

After the 2-h surgery, peripheral blood was periodically collected by cardiac puncture under ether anesthesia. The blood was centrifuged at 2000 rpm for 5 min, and the serum was collected. The serum level of TNFα was determined by enzyme-linked immunosorbent assay (ELISA) (Factor-Test TMmTNF-α, Genzyme, Cambridge, Mass.). The serum level of IFNγ was also determined by ELISA (Factor-Test TMmIFN-γ, Genzyme).

Treatment with antibodies

Monoclonal rat IgG2b antibody to murine very late activation antigen 4 (VLA-4) α chain, PS/2 [9], 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 μg/ml (data not shown). B16-BL6 cell (1×10^5) were incubated with 1 μg PS/2 for 30 min at 4 °C. After one washing with PBS, these cells were injected into the lateral tail vein of the mice that had undergone the 2-h surgery 24 h before. RMV-7 (monoclonal rat IgG2b antibody to murine vitronectin receptor α chain) [10] was used as a control antibody. The treated tumor cells were also injected into the lateral tail vein of the control mice. Monoclonal rat IgG1 antibody to murine vascular cell adhesion molecule 1 (VCAM-1), M/K-2 [11, 12], was kindly provided by Dr. K. Miyake (Saga Medical School). Monoclonal rat IgG1 antibody to murine TNFα (MP6-XT3) was obtained from PharMingen Inc. (San Diego, Calif.). Normal rat Ig (Organo Teknika, Calif.) was used as a control. A 500-μg dose of M/K-2, MP6-XT3, or normal rat Ig was administered to the mice in the lateral tail vein 1 h before the 2-h surgery, and B16-BL6 cells were inoculated 24 h after the surgery.

Immunoperoxidase staining

The lungs were removed 24 h after the 2-h surgery from the mice that were untreated or had received anti-TNFα mAb or normal rat Ig. The lungs were frozen immediately in optical cutting temperature compound and kept at −80 °C. Cryostat sections were fixed with 4% paraformardehyde for 10 min at 4 °C, then incubated with 2% bovine serum albumin for 30 min at room temperature. After washing with PBS, these sections were incubated with biotin-labeled M/K-2 mAb for 2 h at room temperature. After washing with PBS, the sections were reacted with avidin-biotin-peroxidase complex (Nichirei, Inc., Tokyo), washed, and developed with dimethylaminobenzidine and H_2O_2.

Statistical analysis

Differences in the number of lung colonies were analyzed by Student's *t*-test.

Results

Influence of surgical stress on pulmonary metastasis of B16-BL6

To evaluate the influence of surgical stress on metastasis, we counted the number of metastatic colonies on the surface of the lungs on days 13–15 after inoculating B16-BL6 cells into the mice that had undergone surgery for 0.5, 1, or 2 h (Fig. 1). The mean number of metastatic colonies in untreated mice was 37.1 ± 11.3. The mean numbers of metastatic colonies in the mice that had undergone 0.5, 1, or 2 h of surgery were 36.3 ± 7.4, 49.3 ± 4.9, and 213.6 ± 57.5 respectively. The 2-h surgery greatly increased the number of metastatic lung colonies. On the basis of this result, mice underwent the 2-h surgery in subsequent studies.

We next examined the timing of B16-BL6 inoculation after the surgery (Fig. 2). The mean number of metastatic colonies in untreated mice was 18.0 ± 4.1. The mean numbers of metastatic colonies in the lung, when tumor cells were inoculated 1, 4, 12, 24, or 48 h after the surgery, were 31.7 ± 6.6, 32.0 ± 8.9, 75.3 ± 8.7, 117.5 ± 22.0, and 36.8 ± 10.9 respectively. The enhanced pulmonary metastasis caused by surgical stress was found most strikingly when the tumor cells were inoculated 24 h after the surgery, but was no longer observed at 48 h. This indicates the transient nature of the mechanism responsible for the surgical-stress-enhanced metastasis. On the basis of this result, tumor cells were inoculated 24 h after the surgery in the subsequent studies.

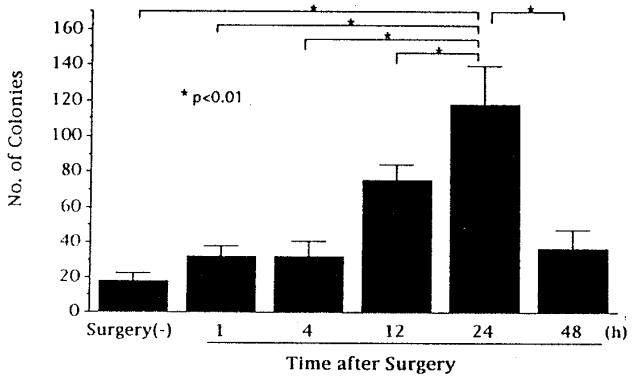

Fig. 2 Relation between time after surgery and pulmonary metastasis. The number of metastatic colonies in the lung was counted on day 14 after tumor cell inoculation 1, 4, 12, 24, or 48 h after 2-h surgery. The data indicate the mean ±SD of seven mice for each treatment. This result demonstrates that enhanced pulmonary metastasis was found most strikingly when the tumor cells were inoculated 24 h after the surgery (*, $P < 0.01$)

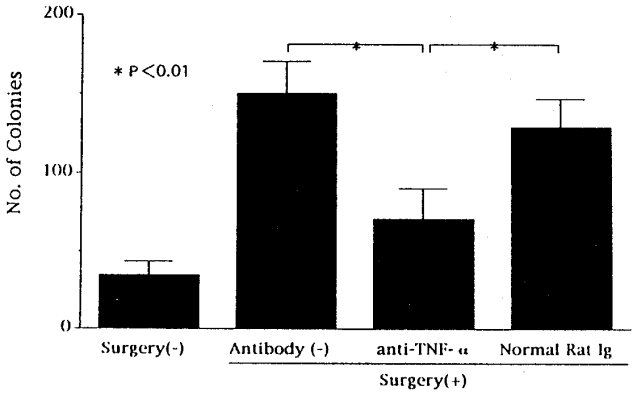

Fig. 4 Inhibitory effect of anti-TNFα mAb on pulmonary metastasis. A 500-μg dose of anti-TNFα mAb or normal rat Ig was administered 1 h before surgery, and B16-BL6 cells were inoculated 24 h after 2-h surgery. The data indicate the mean ±SD of seven mice for each treatment. Anti-TNFα mAb, but not normal rat Ig, significantly decreased the colonies (*, $P < 0.01$)

Serum levels of TNFα and IFNγ after surgery

We next examined inflammatory cytokines in the serum after the surgery, which might be responsible for the transient nature of the enhancement. The serum level of TNFα began to rise at 3 h and peaked 12 h after the 2-h surgery. On the other hand, IFNγ was not detectable in the serum during these postoperative periods (Fig. 3).

Inhibitory effect of anti-TNFα mAb on pulmonary metastasis enhanced by surgery

We next examined whether the transiently increased TNFα in the serum is responsible for the enhanced metastasis. A neutralizing anti-TNFα mAb was administered 1 h before the surgery. The mean number of metastatic colonies in the non-operated mice was 20.1±12.5, that in the operated mice without antibody was 149.6±20.5, that in the operated mice treated with anti-TNFα mAb was 71.2±19.5, and that in the operated mice treated with normal rat Ig was 129.0±18.2 (Fig. 4). Apparently, the enhanced metastasis was inhibited by the anti-TNFα mAb administration. This indicates that serum TNFα, raised by surgical stress, is responsible for the enhanced metastasis.

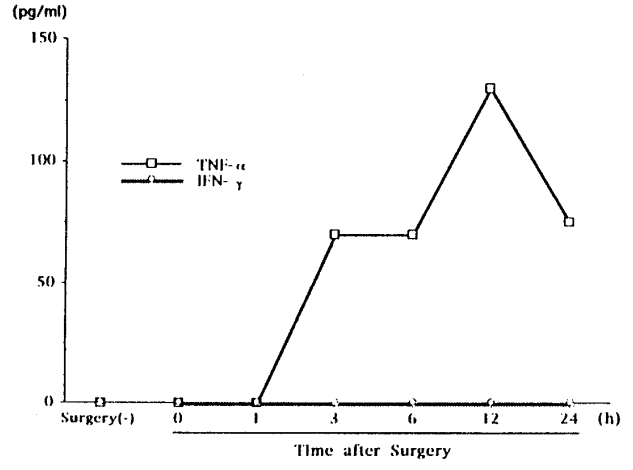

Fig. 3 Serum levels of tumor necrosis factor α (*TNF*α) and interferon γ (*IFN*γ) after surgery. The concentration of TNFα and IFNγ in the serum was periodically measured by ELISA. The TNF level began to elevate after 3 h and then peaked at 12 h. On the other hand, IFNγ was not detectable at any point

Inhibitory effects of anti-VLA-4 and anti-VCAM-1 mAb on pulmonary metastasis enhanced by surgery

We previously demonstrated that administration of recombinant TNFα enhanced the pulmonary metastasis of B16-BL6 by up-regulating the expression of VCAM-1 on the vascular endothelium that binds to VLA-4 on B16-BL6 [4]. We therefore examined the involvement of VLA-4 and VCAM-1 in surgical-stress-enhanced metastasis.

Preincubation of B16-BL6 cells with an anti-VLA-4 mAb (PS/2) completely suppressed the increase of metastatic pulmonary colonies after surgery (the mean numbers of the metastatic colonies were 85.4±34.5 and 5.2±2.7 for Ab^- and PS/2 respectively). In contrast, the preincubation with an anti-vitronectin receptor mAb (RMV-7) did not significantly reduce the colonies (the mean number was 91.2±12.9) (Fig. 5). This indicates a critical role of VLA-4 on B16-BL6 in the surgical-stress-enhanced metastasis.

We also examined the contribution of VCAM-1, which is a ligand for VLA-4. Administration of an anti-VCAM-1 mAb (M/K-2), but not of a control rat Ig, inhibited the enhanced metastasis (the mean number of metastatic colonies of the non-operated mice was 20.1±12.5, that of the operated mice without antibody treatment was 149.6±20.5, that of the operated mice treated with M/K-2 was 61.6±10.9, and that of the operated mice treated with normal rat Ig was 129.0±18.2) (Fig. 6). This indicates a

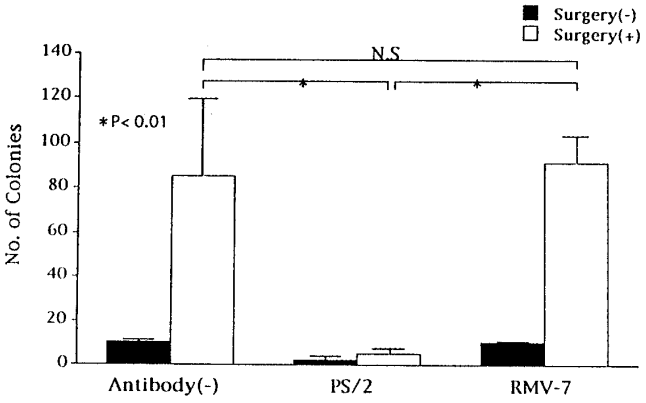

Fig. 5 Inhibitory effect of anti-VLA-4 mAb on pulmonary metastasis. B16-BL6 cells (1×10^5) were preincubated with 1 μg PS/2 (anti-VLA-4 mAb) or RMV-7 [anti-(vitronectin receptor mAb)] for 30 min at 4 °C. After one wash with PBS, these cells were injected into the lateral tail vein of mice that had undergone 2-h surgery 24 h earlier. The number of lung colonies was counted on days 13–15. The data indicate the mean ±SD of six mice for each treatment. Preincubation of B16-BL6 cells with PS/2 completely suppressed the increased metastasis after surgery (*, $P<0.01$)

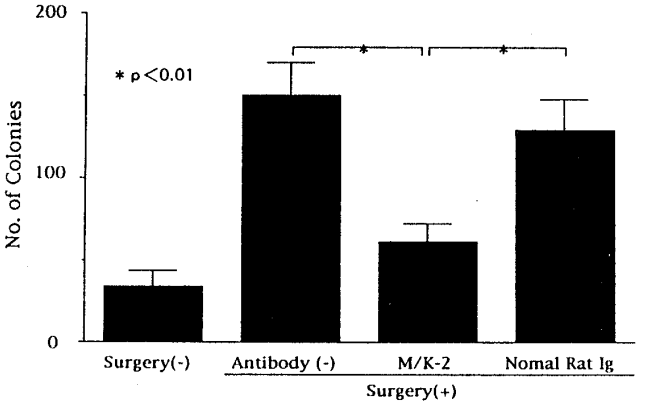

Fig. 6 Inhibitory effect of anti-VCAM-1 mAb on pulmonary metastasis. A 500-μg dose of anti-VCAM-1 mAb (M/K-2) or normal rat Ig was administered before the surgery, and B16-BL6 cells were inoculated 24 h after 2-h surgery. The data indicate the mean ±SD of seven mice for each treatment. The enhanced pulmonary metastasis was significantly inhibited by M/K-2 but not by a control rat Ig (*, $P<0.01$)

contribution of VCAM-1 to the enhanced metastasis, probably as the ligand for VLA-4 on B16-BL6.

Expression of VCAM-1 on the lung vascular endothelium

We finally examined whether the surgical-stress-induced serum TNFα is responsible for VCAM-1 up-regulation on the lung vascular endothelium, thereby enhancing the pulmonary metastasis of B16-BL6 expressing VLA-4. Immunohistochemical staining with an anti-VCAM-1 mAb, M/K-2, showed that the VCAM-1 expression was barely detectable on normal lung vascular endothelium (Fig. 7A), but was strongly induced 24 h after the 2-h surgery (Fig. 7B). In contrast, the expression of VCAM-1 was greatly decreased by the administration of anti-TNFα mAb before the surgery (Fig. 7C), but not by administration of normal rat Ig (Fig. 7D). This indicates that TNFα was predominantly responsible for the VCAM-1 induction on the vascular endothelium after surgical stress, which led to enhanced metastasis of VLA-4-expressing tumor.

Discussion

Surgical operations, that remove or reduce the tumor mass and examine its character or expansion in the body, are very important for treating malignant neoplasms. However, many surgeons have the impression that metastasis to distant organs is enhanced after surgery [1], but the molecular mechanisms for this have not been clarified. Of greater clinical importance is the fact that much of the mortality of malignant neoplasms is attributed to their ability to develop secondary growth in organs at a distance from the primary tumor mass [13]. Therefore, elucidation of the mechanisms for enhanced metastasis after surgery is crucial for improving the treatment of malignant tumors.

In order to establish an animal model system, we used experimental lung metastasis of B16-BL6 melanoma cells injected i.v. into C57BL/6 mice. The mice underwent surgical stress by laparotomy for various periods before inoculation of the tumor cells. The number of metastatic lung colonies was significantly increased after the 2-h surgery, although no significant increase was found after 0.5-h or 1-h surgery. This result indicates that longer surgery, which would cause more surgical stress, does enhance metastasis. We also examined the kinetics of the enhanced metastasis after the surgery. The enhancement was transient with a peak 24 h after the surgery. This suggested that some transient factor induced by surgical stress was responsible for the enhanced metastasis.

It has been reported that serum levels of inflammatory cytokines, including interleukin-1 (IL-1) and TNFα, were raised in the acute phase after surgery [5–7]. TNF, which is released by macrophages and mast cells, is a mediator of pathophysiological manifestations of sepsis, inflammation, and multiple organ failure. IL-1 is produced by macrophages in response to endotoxin, silica, and TNF. TNF stimulates IL-1 production by endothelial cells, and IL-1 can stimulate its own production. TNF and IL-1 have synergistic effects on inflammatory reactions [14]. TNF and IL-1 have been also known to stimulate endothelial cells and up-regulate the expression of various adhesion molecules on them. Moreover, we and others have demonstrated that administration of TNF and IL-1 enhances the experimental metastasis. In our experimental system, the serum TNFα level was indeed raised transiently with a peak 12 h after the surgery. A critical contribution of this increased TNF to metastasis enhanced by surgical stress was revealed by the inhibitory effect of anti-TNFα mAb administration. As TNF and IL-1 have been known to up-regulate the expression of various adhesion molecules on

the endothelial cells synergistically, administration of anti-TNFα plus anti-IL-1 antibodies would possibly be very effective in inhibiting the increase in experimental metastasis. On the other hand, it has been reported that not only TNF and IL-1 but also other mediators, such as IL-6, IL-8 and granulocyte-colony-stimulating factor etc., were induced by surgical stress, partially through increased TNF and IL-1 levels, and these cytokines have also been thought to enhance metastasis after surgery. In our study, the administration of anti-TNFα reduced but did not eliminate the sugrical-stress-induced increase in experimental metastasis. The anti-TNFα antibody was suspected to be only partially effective in reducing blood levels of TNFα, and suppressing the increase in metastasis that was induced by increasing serum TNFα.

We previously demonstrated that i.v. administration of recombinant TNFα enhanced the lung metastasis of B16-BL6 by up-regulating the expression of VCAM-1 on vascular endothelium which binds to VLA-4 on the tumor cells [4]. We then examinined the involvement of VLA-4 and VCAM-1 in surgical-stress-enhanced metastasis. The treatment with either anti-VLA-4 or anti-VCAM-1 mAb inhiited the enhanced metastasis, indicating the critical involvement of VLA-4/VCAM-1 in this system also. Immunohistological staining of the lung tissue indicated that the VCAM-1 expression was up-regulated 24 h after the surgery, and that this was blocked by the anti-TNFα mAb

Fig. 7A–D Immunohistochemical staining of lung vascular endothelium with anti-VCAM-1 mAb. VCAM-1 expression was barely detectable on normal lung vascular endothelium (**A**), but was strongly detected 24 h after 2-h surgery (**B**). The expression of VCAM-1 was decreased by the treatment with anti-TNFα mAb (**C**), but not by normal rat Ig (**D**)

administration. Taken together, these results indicated that TNFα, induced by surgical stress, up-regulated the VCAM-1 expression on lung vascular endothelium, thereby enhanding the metastasis of B16-BL6 expressing VLA-4.

In our previous study, huge amounts of TNFα were required to increase the experimental metastatic colonies. The kinetics indicated that TNFα increased the pulmonary metastasis most efficiently when administered 4 h before B16 cell inoculation. The increase continued for 12 h and had disappeared 24 h after administration. We also demonstrated that VCAM-1 expression on lung vascular endothelium was strongly induced at 4 h and had decreased 24 h after administration of TNFα [4]. In this study, the elevation of serum TNFα began after 3 h, peaked at 12 h and had declined 24 h after surgery (Fig. 3). After serum TNFα had decreased to an ineffective level and lost the ability to induce VCAM-1 expression, the expression of VCAM-1 was suspected to return to normal. It was considered that the appearance and then the loss of a surgically induced increase in experimental metastasis is consistent with an

increase in and then a return to normal expression of VCAM-1. The result that the enhanced pulmonary metastasis was found most strikingly at 24 h and had decreased 48 h after surgery (Fig. 2) was in good correlation with this hypothesis.

TNFα has also been known to up-regulate some adhesion molecules other than VCAM-1, including E-selectin and ICAM-1. In our preliminary results, administration of TNFα as well as surgical stress enhanced lung metastasis of a mouse colon cancer cell line (colon 26), which may be mediated by E-selectin up-regulation (unpublished data). Similarly, it has been reported that TNFα administration enhanced lung metastasis of a mouse fibrosarcoma but no relevant adhesion molecules have been characterized [15]. These results suggest that circulating inflammatory cytokines induced by surgical stress may be rather generally involved in enhanced hematogenous metastasis by up-regulating vascular endothelial adhesion molecules that bind to tumor surface ligands. Therefore, application of some antagonist to TNF (such as anti-TNF mAb and soluble TNF receptor) or adhesion molecules (such as blocking mAb and soluble ligands) would be effective in preventing the risk of hematogenous metastasis, especially during and shortly after surgery.

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