laboratory and animal investigations

Vascular Endothelial Growth Factor Increased by Pulmonary Surgery Accelerates the Growth of Micrometastases in Metastatic Lung Cancer*

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Background: The use of surgery for metastatic lung cancer has been established recently and the indications have been extended to multiple and bilateral lung metastases. However, in some patients, secondary lung metastasis appears soon after the first pulmonary surgery, making curative treatment very difficult. Postoperative weakness of tumor angiogenesis suppression mechanisms seems to play an important role in the recurrence of lung metastases. To verify this hypothesis, we performed a clinical and an experimental study.

Results and conclusion: The clinical study revealed that serum vascular endothelial growth factor (VEGF), also known as vascular permeability factor, increased after pulmonary surgery. The experimental study showed that VEGF played an important role in the rapid growth of dormant micrometastases of the lung. These results suggested that the postoperative increase of VEGF disrupted angiogenesis suppression and induced the growth of dormant micrometastases early in the postoperative period. It was also demonstrated that this effect of VEGF on micrometastases was abolished by AGM-1470, an angiogenesis inhibitor. In conclusion, postoperative treatment with AGM-1470 might inhibit the early recurrence of malignant tumors.

(CHEST 1998; 114:1668–1675)

Key words: angiogenesis inhibitor; lung metastasis; metastatic lung cancer; vascular endothelial growth factor; vascular permeability factor

Abbreviations: ELISA = enzyme-linked immunosorbent assay; mRNA = messenger RNA; VEGF = vascular endothelial growth factor; VPF = vascular permeability factor

The use of surgery for metastatic tumors in the lung was first advocated in 1965 by Thomford et al. in their analysis of 205 clinical cases. Later, surgery was extended to multiple and bilateral lung metastases. However, in some patients, secondary lung metastases appear soon after the first pulmonary surgery and are difficult to manage. In our observation of 120 patients who underwent surgery for lung metastases, secondary lung metastases developed in 12 patients (10%) within 6 months after the first operation, and their prognosis was significantly worse. Figure 1 shows the Kaplan-Meier survival plot of these 120 patients divided into two groups, those who developed additional metastases during the first 6 months (lower curve) and those who did not (upper curve).

We hypothesized that postoperative weakening of tumor angiogenesis suppression mechanisms may play an important role in the recurrence of lung metastases.
We planned clinical and experimental studies to clarify the role of vascular endothelial growth factor (VEGF), also known as vascular permeability factor, in the recurrence of lung metastases. These studies revealed that serum VEGF levels increased after pulmonary surgery and that VEGF may play an important role in the rapid growth of dormant lung micrometastases.

**Materials and Methods**

**Patients**

Lung resection via thoracotomy was carried out in 13 patients with benign and malignant diseases (emphysema, 1; granuloma, 2; primary lung carcinoma, 10) in our hospital from June 25, 1996 to January 31, 1997. Ten patients were men and three were women; their average age was 58.8 ± 16.8 years. Partial lung resection was performed in seven cases and lobectomy in six cases. The average operating time was 256.8 ± 126.8 min.

**Serum VEGF Levels in Surgical Patients**

Peripheral venous blood was obtained from all patients before surgery, soon after surgery, and 6, 12, 18, 24, and 48 h afterwards. Blood samples were centrifuged immediately and serum was stored at −84°C. The serum VEGF concentration was determined using a commercially available enzyme-linked immunosorbent assay kit (R & D Systems, Inc; Minneapolis, MN).

**Cell Culture**

Lewis lung carcinoma cells were obtained from the Cancer Cell Repository of Tohoku University. Cells were cultured in RPMI-1640 medium supplemented with penicillin (100 u/mL), streptomycin (100 u/mL), and 10% bovine calf serum. Immediately before injection into mice, tumor cells were suspended in RPMI at 1.0 × 10⁶ cells/mL.

**Animals**

Eight-week-old female BDF-1 mice (Charles River Japan Inc; Yokohama, Japan), were fed a commercially available pellet diet (MF; Oriental Yeast Co Ltd; Tokyo, Japan) and were given tap water ad libitum.

**VEGF**

The 165-amino acid homodimeric species of recombinant human VEGF (rhVEGF₁₆₅) was purified by Pepro Tech EC Ltd (London, England). Lyophilized VEGF was reconstituted in distilled water at a concentration of 100 ng/μL and stored at −20°C. Then 10 ng, 200 ng, and 1,000 ng of VEGF diluted into 10 μL of saline was injected intraperitoneally into mice.

**Angiogenesis Inhibitor**

AGM-1470, a fumagillin derivative angiogenesis inhibitor, was synthesized by Takeda Chemical Industries Ltd. (Tokyo, Japan) and stored at −20°C. It was diluted in a 10% solution of 100% ethanol and stored at 4°C. Then 9.7 mL of 0.9% sodium chloride was added to 0.3 mL of this ethanol solution just before administration by subcutaneous injection at a dose of 30 mg/kg/d.

**Experimental Design**

Hematogenous lung metastases were established by injection of 1.0 × 10⁷ Lewis lung carcinoma cells into the tail veins of mice (day 0). To assess the effect of VEGF on the growth of micrometastases, VEGF was injected intraperitoneally on day 11 in four groups of mice. Group A received 10 ng of VEGF, group...
B received 200 ng of VEGF, and group C received 1 μg of VEGF. Group D received 200 ng of VEGF followed by 30 mg/kg of AGM-1470 injected subcutaneously every 4 days after VEGF administration. The control animals received no treatment after injection of Lewis lung carcinoma cells. Lung metastases of > 2.0 mm in diameter were counted macroscopically on day 20.

**Microvessel Density in Tumors**

To evaluate the pathologic role of angiogenesis, we immunostained metastatic lesions with factor VIII–related antigen. Rabbit anti-human factor VIII (Dako Corp; Carpinteria, CA) was used for this assay. Tissues were fixed in 10% neutral buffered phosphate formalin and then embedded in paraffin. Immunocytochemistry was done using the avidin-biotin peroxidase complex method and peroxidase activity was visualized with dianinobenzidine. Microvessel density was expressed as the average microvessel count per 1 mm².

**Data Analysis**

All data were expressed as the mean ± SE. Statistical significance was evaluated using analysis of variance and a p value of < 0.05 was considered to denote statistical significance.

**RESULTS**

**Increase of VEGF Under Surgical Stress in Clinical Cases**

Increased VEGF₁₆₅ levels were detected in the postoperative period. The maximum VEGF concentration was higher after surgery than before surgery in all except one subject (Fig 2, A). The peak value was at 12 h after surgery and it was significantly higher than the values before surgery (p < 0.05) or just after surgery (p < 0.005)(Fig 2, B).

**Effects of VEGF and Angiogenesis Inhibitor on Experimental Micrometastases**

Dormant micrometastases were detected in the lungs on day 11 (Fig 3) and lung metastases were observed in all animals on day 20 (Fig 4). None of the treatments affected food or water intake or weight gain.

The number of metastases > 2.0 mm was significantly increased in groups B and C compared with the control group (p < 0.05). AGM-1470 completely suppressed the effect of VEGF on the growth of lung metastases (Fig 5).

Immunohistochemical staining of metastatic lesions clearly showed progressive infiltration of microvessels into the metastases > 2.0 mm in diameter (Fig 6, A). In contrast, microvessels were rare inside the metastases ≤ 2.0 mm in diameter (Fig 6, B). In all groups, the microvessel density was significantly higher in tumors > 2.0 mm than in tumors ≤ 2.0 mm (p < 0.05 in groups A, B, and D; p < 0.01 in group C). However, there was no significant effect of either VEGF or AGM-1470 on microvessel density (Fig 7).

**DISCUSSION**

The development of metastases involves a long series of sequential interrelated steps, and tumor cell proliferation in the target organ is the final one.⁵ At this stage, angiogenesis is required for expansion of the metastatic colony.⁶ Extensive vascularization has certainly occurred if a tumor mass exceeds 2 mm in diameter.⁷ In cancer patients, dormant micrometas-
tases are often asymptomatic and clinically undetectable because of balanced proliferation and apoptosis in the presence of angiogenesis suppression.\(^8\)

This theory is applicable to surgical cases of lung metastases. Even if the lung parenchyma has been invaded by tumor in various places, most lesions can be controlled by angiogenesis suppression. Only a few tumor cells escape this suppression and start proliferating. As a result, in some patients with lung metastases, clinically detectable lesions are only a part of the tumor colonies scattered in the lung parenchyma. Usually some dormant micrometastases, in addition to the resected lesions, are found in the lung parenchyma at the time of surgery. If surgical stress induced by lung resection disrupts angiogenesis suppression, dormant micrometastases will start growing.

VEGF, or vascular permeability factor, is a specific growth factor for vascular endothelial cells.\(^9\)-\(^12\) Alternative splicing of the VEGF gene transcript yields four isoforms of 121, 165, 189, and 206 amino acids, and the 165-amino acid isoform is the most abundant in vivo.\(^13\) Secretory forms of VEGF are produced by
vascular cells, including endothelial cells, smooth muscle cells, mesangial cells, and pericytes. Two kinds of VEGF receptors, fms-like tyrosine kinase 1 (flt-1) and kinase insert domain-containing receptor (kdr), are expressed in endothelial cells. VEGF participates in the process of angiogenesis via autocrine and paracrine pathways. Angiogenesis is involved in many physiologic and pathologic responses, such as wound healing, collateral blood flow in ischemic tissues, and retinopathy. VEGF is also released by a variety of tumor cells and it is a tumor angiogenesis factor in vivo.

Inefficient vascular supply and the resultant reduction in tissue oxygen tension often induces neovascularization and the release of VEGF. VEGF messenger RNA (mRNA) levels dramatically in-

Figure 6. Staining of endothelial cells with Factor VIII–related antigen. A. In tumors that were > 2.0 mm in diameter, dense infiltration of microvessels was observed. B. Few microvessels were detected in the metastases ≤ 2.0 mm. Scale bars = 50 μm.
We can secrete VEGF which consequently increases lung vessels by perfusion of isolated skin explants in vitro. Furthermore, ex vivo perfusion of isolated rat lungs under hypoxic conditions caused an increase of VEGF mRNA in lung tissue and an increase of VEGF receptor levels. VEGF is regarded as the principal angiogenic factor under ischemic and hypoxic conditions.

In our study, serum VEGF increased in the postoperative period, as a physiologic response to hypoxia. Surgical stress tends to expose peripheral tissues to hypoxia. Thoracotomy involves a series of procedures that result in hypoxia of the lung tissues, which consequently secrete VEGF. The increase of serum VEGF detected in our study corresponded to an overflow of VEGF from ischemic lung tissues, so we can assume that at the local level the secretion of VEGF was much higher.

Hematogenous lung metastases were established by IV injection of Lewis lung carcinoma cells into mice. In previously reported experimental models of lung metastases, tumor cells were injected subcutaneously and growth of the metastases was accelerated by resection of the primary tumor. We injected cells intravenously to avoid any influence of the primary tumor on the development of the lung lesions. Dormant micrometastases were detected in the lungs on day 11 after the cells were injected, so VEGF was injected on that day to assess its effect on micrometastases. To evaluate the effect of VEGF, the number of tumors that exceeded 2 mm in diameter was counted on day 20 because tumor masses need extensive vascularization to exceed this diameter.

Measurement of microvessel density showed a relationship between angiogenesis and tumor growth. In all groups, the microvessel density of tumors > 2.0 mm in size was higher than that of tumors ≤ 2.0 mm. These results show that small micrometastases remained in the prevascular phase and larger metastases had been infiltrated by microvessels. Therefore, tumors > 2.0 mm in size correspond to lesions infiltrated by microvessels.

In our experimental study, VEGF was the trigger that changed the prevascular phase of metastases into the proliferating phase, and VEGF was related more to an increase in vascular permeability for tumor cells than to an increase in the density of vessels. In groups B and C, more micrometastases started proliferation because of the higher activity of injected VEGF. In the control group, on the other hand, autogenous VEGF and other angiogenic factors hardly stimulated micrometastases. Therefore,

![Diagram](http://journal.publications.chestnet.org/pdaccess.ashx?url=/data/journals/chest/21893/ on 04/06/2017)
the number of tumors > 2.0 mm in size reflected the activity of VEGF as a trigger of angiogenesis.

These results further demonstrated that VEGF plays an important role in the rapid growth of dormant micrometastases as a result of its high angiogenic activity. The postoperative increase of VEGF seems to disrupt angiogenesis suppression and induce the growth of dormant micrometastases early in the postoperative period.

AGM-1470 is a synthetic analogue of fumagillin and is a potent angiogenesis inhibitor. AGM-1470 inhibits endothelial cell proliferation in vitro and also suppresses angiogenesis in vivo. It has been suggested that the target of AGM-1470 is located relatively late in the G1 phase and includes an upstream regulatory point for the expression of mRNA for a subset of cdk/cyclin subunits in endothelial cells. Thanks to its inhibitory effect on angiogenesis, it suppresses the growth of a variety of different tumors. Our study showed that the stimulatory effect of VEGF on micrometastases was abolished by AGM-1470. Therefore, administration of AGM-1470 in the postoperative period might inhibit the early recurrence of malignant tumors induced by increased levels of VEGF. However, examination of its effect on wound healing and other processes is needed before the clinical trials of AGM-1470 can proceed.

REFERENCES