

Vascular endothelial growth factor-A and changes in a tumor-bearing mouse model with Lewis lung cancer

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Abstract. Vascular endothelial growth factor-A (VEGF-A) affects tumor growth and metastasis through stimulation of angiogenesis. The purpose of this study was to describe features of Lewis lung cancer (LLC) in mice and compare the serum VEGF-A levels with those of normal control mice. Two groups of mice were compared: one was subcutaneously injected with LLC cells (n=16) and the other served as the normal control (n=6). The serum VEGF-A levels were measured by ELISA prior to inoculation, and at 7, 21 and 35 days post-inoculation. The tumor weight and the metastatic condition were evaluated on day 35. Changes in body weight and serum VEGF-A concentration over a period of time were compared between the groups using generalized estimating equations. The relationship between the primary tumor and the metastatic condition was analyzed using the Spearman's rank correlation test. The survival rate was 56.3% on day 35 post-tumor inoculation. No difference was found between the groups with regard to gastrocnemius muscle weight on day 35 post-inoculation [0.1315 ± 0.0066 g vs. 0.1308 ± 0.0069 g (normal control)]. In tumor-bearing mice, the weight gain at sacrifice was less (0.24 ± 0.45 vs. 1.93 ± 0.47 g, $P=0.01$), the final mean tumor volume and weight were 4264.69 ± 1038.32 mm³ and 3.70 ± 0.83 g, the number of nodules in the lungs and livers was 6.33 (range 0-20) and 2.22 (range 0-11), respectively, and the serum VEGF-A levels were significantly higher than those of control mice. In conclusion, lower body weight gain, metastasis in the liver and lungs, and elevated VEGF-A levels are features of LLC in mice.

Introduction

Lung cancer is a common cause of cancer mortality (1,2). Lung cancer has been associated with a 32% decrease in pre-illness weight and poor survival of patients (3). Thus, establishing tumor-bearing models in order to prevent lung cancer and slow the course of the disease is crucial for cancer research.

Animal models have been developed to study the pathophysiology of this disease and the effects of therapy. Lewis lung carcinoma (LLC) was isolated from the epidermoid carcinoma of the lung in mice. It is regarded as an essential tumor model in studies of metastasis (4,5), vessel formation (6), and effects of therapy (7). A tumor-bearing model of mice with LLC exhibits a greater metastatic potential than a tumor-bearing model alone (8,9). LLC cells have been inoculated into various sites, including the tail vein (5), muscle (10), subcutaneous tissue (9,11), lungs (9), intrathorax and intrabronchus (12). LLC-bearing mice have been used in experimental models of lung tumor (13), brain metastasis (14) and cachexia (15). In the study by Li *et al* mice were implanted with LLC cells in various sites, and the results were compared. These authors found that intrabronchial implantation yielded slow-growing tumors, but no distant metastasis. Additionally, mice with intrathoracic implantation succumbed more rapidly than those with intrabronchial implantation. Intrathoracic and intrabronchial implantation were not considered favorable tumorigenic and metastatic models (12). Recently, Liu *et al* compared intrapulmonary and subcutaneous models of lung cancer using LLC cells and found a higher rate of tumor formation, stronger transfer characteristics and a shorter survival time in the intrapulmonary than in the subcutaneous model (9). However, the lack of distant metastasis suggests that intrapulmonary inoculation is unsuitable for use in metastatic study. In the orthotopic model, it is not possible to evaluate the time-related change in tumor growth until the point of sacrifice. Harlos *et al* reported the subcutaneous and intramuscular models of lung cancer. Their results showed that there is no difference in tumor growth in mice receiving the subcutaneous and intramuscular inoculation (16). However, they observed that intramuscular inoculation with cancer cells may damage skeletal muscle and decrease activity during tumor formation in mice. It may also affect the outcome measurement of muscle

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mass in cancer cachexia. To assess the effect of tumor growth and cancer cachexia, the subcutaneous inoculation model was considered appropriate.

Tumor growth and metastasis require angiogenesis, which is regulated by various factors, including the vascular endothelial growth factor (VEGF) (17). VEGF overexpression is an indicator of poor prognosis in patients with non-small and small cell lung cancer (18). Shimanuki *et al* measured the serum VEGF levels preoperatively in patients with non-small cell lung cancer and revealed a correlation with microvessel density in the resected tumor. Their results showed that the average overall survival and disease-free survival time were significantly longer when the VEGF levels were lower (19). VEGF also plays a significant role in the rapid growth of micro-metastasis in the lungs (5). To understand the tumor growth, biology of metastases and cancer cachexia, an animal model with a subcutaneous inoculation of LLC cells was used in the present study. The aim of this study was to describe the features of Lewis lung cancer (LLC) in mice and compare the serum VEGF-A levels with that of normal control mice.

Materials and methods

Animals, cell culture and study guidelines. A total of 22 8-week-old, healthy C57BL/6 male mice were purchased from the National Animal Center (Taipei, Taiwan). The animals were housed in cages under a 12-h light/dark cycle at the Laboratory Animal Center of the National Taiwan University (Taipei, Taiwan) and were provided with food and water *ad libitum*. LLC cells were cultured in tissue culture flasks containing DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 mg/dl streptomycin, and 100 U/ml penicillin, and were maintained at 37°C in a humidified atmosphere containing 5% CO₂ (7).

The mice were given one week to acclimatize to their environment before any treatment was administered, and then divided into two groups. Sixteen mice were inoculated subcutaneously with 5×10⁵ LLC cells in 0.1 ml PBS and 0.1 ml Matrigel in the back. The remaining 6 mice were used as normal controls and were injected with PBS and Matrigel devoid of tumor cells.

Blood samples (150 µl) were drawn prior to inoculation, and on days 7, 21 and 35 post-inoculation to measure the serum VEGF-A levels. Body weight was measured 3 times a week. After 5 weeks, the surviving mice were sacrificed by CO₂ asphyxiation. This study was approved by the National Taiwan University College of Medicine and the College of Public Health Institutional Animal Care and Use Committee (IACUC).

Assessment of tumor volume and metastasis. The tumor volume [$V = L \text{ (longest diameter)} \times W^2 \text{ (shortest diameter)} \times 0.5$] was measured 3 times a week. Following sacrifice, the tumors were excised and weighed. The lungs and liver were harvested and the surface nodules were counted to evaluate the metastatic spread of the tumor. The tissues were fixed in 4% paraformaldehyde and stained with hematoxylin and eosin (20,21).

Enzyme-linked immunosorbent assay (ELISA). Blood samples (150 µl) were drawn from mice by submandibular venipuncture, collected using Eppendorf tubes, clotted overnight at 4°C, and centrifuged for 20 min at 2,000 × g. The serum was

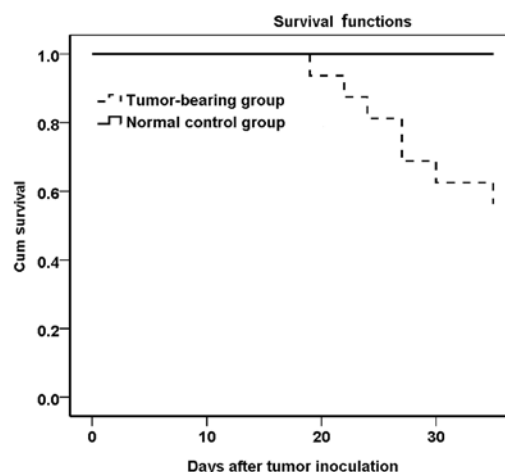


Figure 1. Comparison of survival rate in the tumor-bearing and normal control groups using Kaplan-Meier analysis with the log rank test, $P=0.069$.

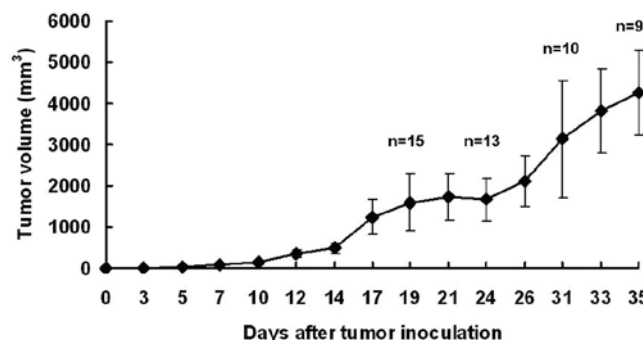


Figure 2. Tumor volume in the tumor-bearing group. Values are the means ± SEM of the survival tumor-bearing mice.

collected and stored at -80°C. The levels of serum VEGF-A were determined using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions (22).

Statistical analysis. The statistical software package SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to perform data analyses. Data analysis included the calculation of descriptive statistics (means ± SEM). The Kaplan-Meier analysis was performed for survival analysis. The significance of differences between groups was assessed using the Mann-Whitney U test. Generalized estimating equations (GEE) were also used to test the significance of differences in tumor effects on body weight and serum VEGF-A concentration at each measurement point. The α -value was set at 0.05.

Results

Survival rate. The survival rate was 56.3% (9/16) in the tumor-bearing group on day 35 post-tumor inoculation (Fig. 1).

Body weight. The mean body weight was not significantly different between the tumor-bearing ($n=16$) and normal control ($n=6$) groups prior to tumor inoculation (23.88 ± 0.36 and 23.41 ± 0.51 g, respectively). GEE analysis revealed a

Table I. Characteristics in tumor-bearing and normal control groups.

	Tumor-bearing (n=9)	Normal control (n=6)
Body weight ^a (g)	27.54 (0.81)	25.34 (0.88) ^d
Tumor weight (g)	3.70 (0.83)	NA
Carcass ^b (g)	23.84 (0.51)	25.34 (0.88)
Body weight gain ^c (g)	0.24 (0.45)	1.93 (0.47) ^d
Average gastrocnemius weight (g)	0.1315 (0.0066)	0.1308 (0.0069)
Lung weight (g)	0.1568 (0.0076)	0.1326 (0.0126)
Liver weight (g)	1.4779 (0.0466)	1.1640 (0.0485) ^d
Nodules in liver	2.22 (1.20)	NA
Nodules in lung	6.33 (2.51)	NA

Data are shown as the means \pm SEM. ^aBody weight on day 35 post-tumor inoculation; ^bBody weight on day 35 post-tumor inoculation minus tumor weight; ^cCarcass minus body weight prior to tumor inoculation. ^dSignificant difference between the two groups using the Mann-Whitney U test ($P<0.05$). NA, not applicable.

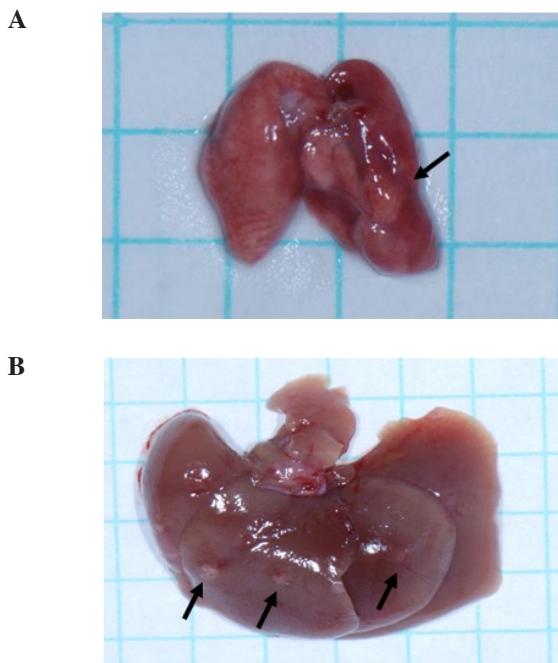


Figure 3. Tumor metastasis. Metastasis in the (A) lungs and (B) liver. The arrow indicates a nodule.

significant time effect ($P<0.01$) and a group-time interaction effect ($P<0.01$) on body weight, but no group effect was found ($P=0.368$). The total weight gain was higher in the tumor-bearing mice than in the control mice [3.94 ± 0.74 (n=9) vs. 1.93 ± 0.47 g (n=6), $P=0.03$] on day 35 post-inoculation; however, following tumor resection, total weight gain was found to be lower (0.24 ± 0.45 vs. 1.93 ± 0.47 g, $P=0.01$) (Table I). Seven mice in the tumor-bearing group died prior to the end of the experiment (35 days post-tumor inoculation) with a mean body weight of 1.05 ± 0.08 times the mean body weight of the normal control group. In two mice, the body weight decreased prior to their death.

Tumor growth and metastasis. Primary tumor formation was detected 5-7 days after tumor inoculation, and all mice in

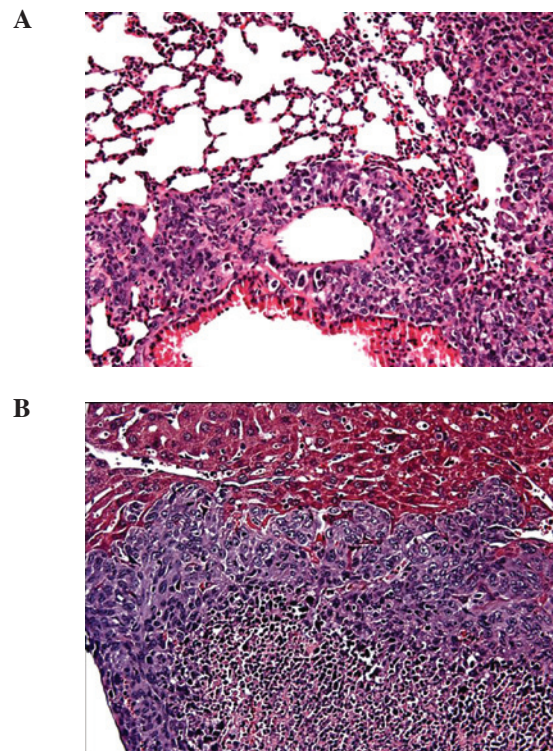


Figure 4. Histological features of LLC in the lung and liver. Photomicrographs of H&E-stained sections in the (A) lungs and (B) liver. The lung and liver were collected five weeks after tumor inoculation for histological analysis. Magnification, $\times 200$.

the tumor-bearing group developed tumors. At the end of the experiment, the tumor-bearing mice had a mean tumor volume of 4264.69 ± 1038.32 mm³ (Fig. 2). After exposing the skin, the primary tumor was large, vascularized, soft and roughly spherical in shape. The mean tumor weight was 3.70 ± 0.83 g.

A number of tumor nodules were found in the lungs and liver (Fig. 3). The mean number of nodules in the lungs and liver was 6.33 (range 0-20) and 2.22 (range 0-11), respectively (Table I). The liver weight was higher in the tumor-bearing group than in the control group ($P<0.01$) and correlated with

Table II. Serum VEGF-A expression according to generalized estimating equation analyses.

	Before inoculation	7 days post-inoculation	21 days post-inoculation	35 days post-inoculation
Tumor-bearing	188.50±37.30	104.56±7.29	123.34±23.61	111.69±6.93
Normal control	130.37±20.75	88.15±8.41	89.32±3.82	92.06±14.87

Data are shown as the means ± SEM. A significant group effect ($P=0.009$) was found, but no time effect ($P=0.623$) and group-time interaction ($P=0.779$) based on the generalized estimating equation.

the primary tumor weight ($r=0.667$, $P<0.05$). The lung weight was higher in the tumor-bearing group than in the control group, but no significant difference was found ($P=0.077$). The histological analysis revealed metastasis in the liver and lungs (Fig. 4).

Muscle wasting. No significant differences were found in the average gastrocnemius muscle weight of the two hindlimbs [0.1315 ± 0.0066 g ($n=9$) for the tumor-bearing group vs. 0.1308 ± 0.0069 g ($n=5$) for the normal control group], indicating the absence of cancer-induced wasting of the gastrocnemius muscle.

Serum VEGF-A. GEE equations revealed a significant group effect ($P=0.009$), but no time effect ($P=0.623$) or group-time interaction ($P=0.779$). The serum levels of VEGF-A were significantly higher in the tumor-bearing group (Table II).

Discussion

This study demonstrated that the tumor formation rate was 100% and that the primary tumor grew significantly following the subcutaneous inoculation of LLC cells. The tumor-bearing mice developed significant metastasis in the liver and lungs, and an increased liver weight was evident. The tumor-bearing mice gained less body weight following tumor resection and the serum VEGF-A levels were increased.

The lungs and liver are the most common sites for the occurrence of metastasis (23). In the present study, metastasis to the liver and lungs was considerable and the liver weight in tumor-bearing mice was higher than that of the control mice. These results are supported by the study of Argilés *et al* (10). The different metastatic specificities may be associated with different extracellular matrix molecules (24). The lung weight in tumor-bearing mice was not significantly different from that in the control mice; this was also found in a study by O'Reilly *et al* (4).

In tumor-bearing mice, metastasis in the liver and lungs and the liver weight were found to increase significantly. At the same time, body weight gain following tumor resection was lower than that observed in the control group, which demonstrated that cancer cachexia was present. The body weight loss in tumor-bearing mice compared with the control mice in this study may be due to loss of body fat. Our hypothesis is supported by the findings of Argilés *et al* that inoculation of LLC cells results in decreased adipose tissue mass (10).

Argilés *et al* revealed marked skeletal muscle wasting following the injection of LLC cells into hindlimb muscle

(10,25). The skeletal muscle wasting in the intramuscular implantation was due to increasing protein degradation (25) and apoptosis (26). However, the gastrocnemius muscle weight did not decrease in the present study. In addition to the different sites of inoculation. As shown in a previous study, resistance exercise attenuates extensor digitorum longus muscle wasting following inoculation in mice bearing colon-26 adenocarcinoma (27). We suggest that the lower extremity muscles received more loading with the subcutaneous inoculation on the back, which may account for the different findings in our study. However, identifying the main factor involved in skeletal muscle wasting in cancer cachexia, and whether the mechanism of muscle wasting changes or not in different sites of inoculation is crucial.

VEGF increases endothelial cell permeability and enhances endothelial cell migration and proliferation (28), rendering it crucial for tumor growth and angiogenesis (29). VEGF-A binds the two receptor tyrosine kinases, (RTK) VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR), and transduces signals for angiogenesis. Maniwa *et al* reported that micro-metastasis was enhanced following the intraperitoneal injection of VEGF, demonstrating the significance of VEGF in micrometastatic development (5). Another study reported that anti-VEGF monoclonal antibody therapy significantly attenuated micrometastatic tumor growth (30). These results confirm the significance of VEGF in tumor metastasis. To study the effects VEGF have on cancer and therapy, we established a tumor-bearing model and examined the VEGF concentration with LLC. Similar to patients with hematological malignancies (31), the tumor-bearing mice in our study had significantly higher serum VEGF-A levels than their healthy counterparts. Vicioso *et al* demonstrated a positive correlation between the serum VEGF levels and tumor size in primary breast cancer (32). However, the serum VEGF165 levels did not correlate with tumor size in HCC (33). Similarly, the serum VEGF-A levels did not correlate with tumor volume or tumor weight on day 35 post-tumor cell inoculation in our study. VEGF may be secreted from the tumor, platelets and muscle contraction (19,34). Poon *et al* demonstrated that the serum VEGF165 levels/platelet count ratio correlated significantly with tumor cytosolic VEGF165 (33). Although the platelet count and the amount of VEGF in the tumor was not measured, we assume that the tumor is the main source of VEGF. Furthermore, O'Reilly reported that metastatic growth was suppressed by a circulating angiostatin (angiogenesis inhibitor) when a primary tumor was present (4). Other angiogenic or anti-angiogenic factors should also be considered in future studies.

In conclusion, our tumor-bearing mice had obvious metastases in the liver and lungs, a lower body-weight gain and higher VEGF-A levels as compared to the control mice. This animal model may be employed to study cancer pathophysiology, metastasis and the effects of intervention.

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