Angiopoietin-3 Inhibits Pulmonary Metastasis by Inhibiting Tumor Angiogenesis

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ABSTRACT

Angiopoietins (Ang-1, Ang-2, and Ang-3) are the ligands of Tie-2 receptor tyrosine kinase. The essential roles of Ang-1 and Tie-2 in embryonic angiogenesis have been established, and studies have demonstrated the involvement of Ang-1 and Ang-2 in tumor angiogenesis. However, the role of Ang-3 in tumor angiogenesis and metastasis and the mechanism underlying its function are totally unknown. We have shown recently that Ang-3 is tethered on cell surface via heparan sulfate proteoglycans. In our current study, we have demonstrated that overexpression of Ang-3 inhibits pulmonary metastasis of Lewis lung carcinoma and TA3 mammary carcinoma (TA3) cells by inhibiting tumor angiogenesis and promoting apoptosis of the tumor cells. In addition, we have demonstrated that the binding of Ang-3 to the cell surface is required for the effective inhibition of Ang-3 on tumor metastasis and that Ang-3 inhibits endothelial cell proliferation and survival and blocks Ang-1- and vascular endothelial growth factor-induced activation of extracellular signal-regulated kinase 1/2 and Akt kinases, which likely underlie the Ang-3-mediated inhibition on tumor angiogenesis and metastasis.

INTRODUCTION

Angiogenesis plays important roles in tumor growth and metastasis (1–4), and numerous molecules are involved in this process (5–7). Angiopoietins are among these molecules and are the ligands of Tie-2 receptor kinase, which is primarily expressed by endothelial cells (8–10). Three Tie-2 ligands have been identified: angiopoietin-1, angiopoietin-2, and angiopoietin-3 (Ang-1, Ang-2, and Ang-3; refs. 11–13). Angiopoietin has a similar protein structure, which consists of a signal peptide, an NH2-terminal coiled-coil domain, a linker peptide and a signal peptide, an NH2-terminal coiled-coil domain, a linker peptide and a fibrinogen-like domain, which is responsible for dimerization/multimerization of angiopoietin, whereas the fibrinogen homology domain binds to Tie-2 receptor (12–14).

Ang-1 activates Tie-2 receptor by inducing tyrosine phosphorylation of Tie-2 and promotes recruitment of periendothelial cells. Ang-2 and Ang-3 are believed to compete with Ang-1 for binding of Tie-2; however, they do not induce Tie-2 phosphorylation (12, 13). Thus, Ang-2 and Ang-3 are considered as naturally occurring antagonists of Tie-2 receptor. Tie-2 has been shown to play important role in tumor angiogenesis (15, 16), and recent data demonstrated that Ang-1 and Ang-2 are expressed by tumor cells and involved in tumor angiogenesis (17–21). However, the role of Ang-3 in tumor angiogenesis and metastasis has not been established.

In our current study, we investigated how Ang-3 affects tumor angiogenesis and metastasis and the mechanism underlying its function. We demonstrated that overexpression of Ang-3 inhibits pulmonary metastasis of Lewis lung carcinoma (LLC) and TA3 mammary carcinoma (TA3) cells by inhibiting tumor angiogenesis and progression of pulmonary micrometastases to life-threatening macrometastases. In addition, we demonstrated that cell surface tethering of Ang-3 is required for effective inhibition of Ang-3 on tumor metastasis and that Ang-3 inhibits endothelial cell proliferation and survival and blocks Ang-1- and vascular endothelial growth factor (VEGF)-induced activation of extracellular signal-regulated kinase 1/2 (Erk1/2) and Akt kinases, which likely underlie the Ang-3-mediated inhibition on tumor angiogenesis and metastasis.

MATERIALS AND METHODS

Cells and Reagents. Human umbilical vein endothelial cells (HUVECs), human dermal microvascular endothelial cells, and human umbilical artery smooth muscle cells were obtained from Cambrex (Walkersville, MD). MS1 endothelial cells were obtained from American Type Culture Collection (Manassas, VA). Anti-CDF4 (American Type Culture Collection), anti-v5 epitope (Invitrogen, Carlsbad, CA), anti-Tie2, anti-Ang-1, -Ang-2, and -Ang-3 (Santa Cruz Biotechnology, Santa Cruz, CA); anti-von Willebrand factor, anti-smooth muscle actin (Dako, Carpinteria, CA), anti-Erk1/2, anti- phospho-Erk1/2 (Santa Cruz Biotechnology), anti-Akt, and anti- phospho-Akt (Cell Signaling, Beverly, MA) antibodies, and Apoptag kit (Chemicon, Temecula, CA) were used in the experiments.

Reverse Transcription-PCR and Expression Constructions. Reverse transcription-PCR was performed as previously described (22) using the primer pairs corresponding to the 5′- and 3′-end of 24 nucleotides of the coding sequence of mouse Ang-3 or β-actin under the accession numbers AF113707 and X03672, respectively. Full-length Ang-1, Ang-2, and Ang-3 cDNAs were obtained by PCR using mouse placenta cDNAs as templates and Pfu DNA polymerase (Strategene, La Jolla, CA) and the pairs of primers corresponding to the 5′- and 3′-end of 24 nucleotides of the coding sequence of each molecule (the accession numbers U83509, AF004326, and AF113707). The stop codons were omitted from the reverse primers to fuse angiopoietins to the COOH-terminal v5 epitope tag existed in the expression vector (pEF6/v5-HisTOPO; Invitrogen).

Transfection. Lipofectamine (Invitrogen) was used to transfect a subline of LLC and a clonal TA3 cell with the empty expression vector alone or with the expression constructs containing cDNA inserts encoding mouse Ang-3 or Ang-2/Ang-3 hybrid. The transfected LLC and TA3 cells were selected for their resistance to blasticidin, and the expression level of v5-epitope-tagged Ang-3 or Ang2/Ang3 by the transfected cells was measured by Western blotting with anti-v5 monoclonal antibody.

Erk and Akt Phosphorylation. HUVECs were cultured until subconfluent and switched to serum-free medium for overnight. Different amounts of purified Ang-1, Ang-3, VEGF, and basic fibroblast growth factor (bFGF) in different combinations were applied to the serum-starved HUVECs for 25 minutes or 24 hours as detailed in the figure legend (Fig. 6). The cells were then lysed, and equal amounts of the proteins were analyzed by Western blotting with anti-phospho-Erk1/2 or anti-phospho-Akt antibody to detect phospho-Erk1/2 and phospho-Akt or with anti-Erk or anti-Akt antibody to detect total amount of Erk and Akt, respectively.

Cell Proliferation and Apoptosis Assay. Cell proliferation assay was performed by seeding HUVECs, human dermal microvascular endothelial cells, and umbilical artery smooth muscle cells at 5 × 104 cells/well of 96-well plates in triplicate. After 1 day, the cells were switched to serum-free medium and cultured for additional 8 hours. Fresh serum-free medium or 2% fetal bovine serum medium alone, serum-free medium containing bFGF or VEGF (15 ng/mL), or 200 ng/mL purified angiopoietins or 2% fetal bovine serum containing 200 ng/mL of angiopoietins were added to these cells. The serum starved HUVECs were applied with serum-free medium alone, serum-free medium containing 10 ng/mL bFGF or VEGF or 300
ng/mL Ang-3 alone or 10 ng/mL bFGF or VEGF plus 300 ng/mL Ang-3. These cells were cultured for an additional 48 hours. Ten microliters of WST1 reagent (Roche, Indianapolis, IN) were applied into each well, and color was allowed to develop for 2 hours and measured at wavelength of 450 nm.

Apoptosis assay was performed by seeding 5-bromo-2′-deoxyuridine-labeled HUVECs into 24-well plates in triplicate (5 × 10⁴ cells/well) and cultured in EGM-2 complete medium (Cambrex) for 4 hours, switched to serum-free medium for 8 hours, and replaced with fresh serum-free medium alone or serum-free medium containing 15 ng/mL bFGF or 200 ng/mL purified angiopoietins, and cultured for an additional 24 hours. The floating and adherent cells were collected and extent of apoptosis was determined by the cellular DNA fragmentation Elisa kit (Roche).

Pulmonary Metastasis Experiments. In the spontaneous pulmonary metastasis experiments, 1 × 10⁶ viable LLC transfectants were injected s.c. into the left flanks of each syngeneic C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). Five independent clonal LLC transfectants expressing Ang-3 or Ang-2/Ang-3 or transfected with the empty expression vector were used. For each type of the experiment, six mice were injected with each clonal transfectant, and two independent experiments were performed. These s.c. tumors were removed surgically ~3 weeks after the tumor implantation when size of the tumors reaches ~1.5–2.0 cm in the longest diameter. Two types of the experiments were performed using these mice. In one set of the experiments, the experimental mice were observed daily after surgery, and duration of mouse survival was recorded. The survival rate of these mice was calculated as the following: survival rate (%) = (number of mice are still alive/total number of the experimental mice) × 100%. The mice that are free of symptom 60 days after the surgery were sacrificed, and their lungs were examined. In the second set of experiments, 3 weeks after surgical removal of the s.c. tumors, pulmonary metastatic burden was assessed by counting surface pulmonary tumors under dissection microscope and by measuring weight of the mouse lungs. Gross pictures of the mouse lungs were taken and the digital images were analyzed by Image-Pro Plus software 4.5. (MediaCybernetics, Silver Spring, MD). One-hundred randomly selected pulmonary metastases were measured to obtain average diameter of these metastases.

To determine average number and diameter of the micrometastases developed in the lungs of the experimental mice received LLCAng-3 cells, 10 randomly selected lungs were fixed and cut into 15-μm sections. These sections were stained with H&E, and micrometastases in these sections were counted under a microscope. Average diameter of these micrometastases was determined by measuring the diameters of 100 randomly selected micrometastases using Image-Pro Plus software.

Experimental pulmonary metastasis was carried out as detailed previously (18) using five independent clonal TA3Ang-3 or TA3wt cells. The survival rate and pulmonary metastatic burden of these experimental mice were determined as described above.

Histology and Immunohistochemistry. Histology was performed as described previously (18). The sections were reacted with anti-von Willebrand factor and anti-smooth muscle actin (Dako) antibody to assess tumor angiogenesis, with anti-CD44 antibody (monoclonal antibody IM7.8) to highlight the localization of CD44-positive micrometastases or with Apoptag kit to detect apoptotic cells in situ. Total number of tumor cells and number of apoptotic cells in five randomly selected ×400 microscopic fields within the pulmonary metastases or micrometastases were counted using Image-Pro Plus software, and >2000 cells were counted for each type of transfectants. The apoptosis rate was calculated as the following: apoptosis rate = (number of apoptotic tumor cells per microscopic field/total number of tumor cells per microscopic field) × 100%.

To determine blood vessel density, 10 randomly selected ×100 microscopic fields that contain pulmonary macro- or micrometastases were photographed. The numbers of von Willebrand factor-positive blood vessels within the pulmonary metastases or micrometastases were counted, and area values of these pulmonary tumors were measured using Image-Pro Plus software. The blood vessel density was expressed as average number of blood vessels per mm² of pulmonary tumors.

RESULTS

Ang-3 Inhibits Pulmonary Metastasis of LLC and TA3 Cells. To determine which cell types express Ang-3, we performed RT-PCR using RNAs derived from several different cell lines. Our result showed that Ang-3 is expressed by mesenchymal cells, including vascular smooth muscle cells and by CMT-93 colon carcinoma cells, but not by LLC and TA3 cells (Fig. 1A). Unlike LLC and TA3 cells, which form tumors in their corresponding syngeneic mice, CMT-93 cells are not tumorigenic in their syngeneic mice (data not shown).

Angiogenesis is essential for tumor metastasis, and the factors produced by tumor cells and their surrounding stromal cells play important roles in regulating tumor angiogenesis (1–3, 23). To determine how Ang-3 affects tumor angiogenesis and metastasis, we used two in vivo models: a breast carcinoma (TA3) pulmonary metastasis model and a spontaneous pulmonary metastasis model using a subline of LLC cell, which forms pulmonary metastases after removal of s.c. tumors. Using this LLC subline, we established LLC transfectants expressing v-5 epitope-tagged Ang-3 (LLCAng-3; Fig. 1B, Lanes 1–5) or transfected with the empty expression (LLCw). V5 epitope is a 14 amino acid epitope derived from P and V proteins of the paramyxovirus SV5 (24).

We have shown that Ang-3 is tethered on cell surface through
heparan sulfate proteoglycans (HSPGs; Fig. 1B). 1 To determine whether binding of Ang-3 to cell surface affects the role of Ang-3 in pulmonary metastasis of LLC cells, we established an Ang-3 mutant that is incapable of binding to cell surface. The domain that tethers Ang-3 on cell surface was located in the coiled-coil region of Ang-3,1 and it has been shown that bioactivity of Ang-3 is determined by the fibrinogen homology domain of Ang-3, and the hybrid between the coiled-coil domain of Ang-2 and the fibrinogen homology domain of Ang-3 behaves like wild-type Ang-3 and evokes the same response from Tie-2 receptor (13). On the basis of these data, we generated a hybrid construct between the coiled-coil domain of Ang-2 and the fibrinogen homology domain of Ang-3 (Ang-2/Ang-3). Western blot result showed that Ang-2/Ang-3 hybrid is secreted with very limited binding to the cell surface (Fig. 1B, Lanes 6–10).

Five independent clonal LLC transfectants, which express a similar level of Ang-3 or Ang-2/Ang-3 (Fig. 1B) or transfected with the empty expression vector, were used in the spontaneous pulmonary metastasis experiments. Our results showed that expression of Ang-3 effectively inhibits pulmonary metastasis of LLCAng-3 cells and significantly extended the survival time of the mice implanted with LLCAng-3 cells compared with that of the mice received LLCwt cells (Fig. 2, A and B). In addition, we demonstrated that expression of Ang-2/Ang-3 neither effectively block pulmonary metastasis of LLC cells nor significantly extend the survival time of the experimental mice (Fig. 2, A and B), suggesting tethering Ang-3 to the cell surface is required for its effective inhibition of pulmonary metastasis.

The metastatic burden was expressed by average weight of the mouse lungs (Fig. 2B) and average number and size of macro- and micrometastatic lesions per mouse lung (Table 1). We demonstrated that comparing to average weight of healthy lungs, metastatic tumor burden derived from LLCwt or LLCAng-2/Ang-3 cells caused >160 or 120% increase of lung weight, respectively, whereas there is only a ~29% increase of lung weight in the mice received LLCAng-3 cells. In addition, there are ~30 or 24 visible metastatic lesions per lung

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Fig. 2. Ang-3 inhibits spontaneous pulmonary metastasis of LLC cells. A, survival rate of the experimental mice after surgical removal of the s.c. tumors. Total of 30 mice were used for each type transfectant. B, pulmonary metastasis burden is expressed as the weight of the experimental mouse lungs 3 weeks after removal the s.c. tumors. C, histological and immunological analysis of the lung sections. Immediately after removal of the s.c. tumors, the CD44-positive micrometastases derived from LLCwt (C-b) and LLCAng-3 (C-c) cells are localized around the preexisted pulmonary vessels (arrows). C-a, evenly distributed CD44-positive macrophages in normal lung parenchyma. H&E staining of normal lung section (C-d) and the sections derived from the experimental mice 3 weeks after removal of the s.c. tumors derived from LLCwt (C-e) or LLCAng-3 (C-f) cells are shown. Bar: 200 μm.
with average diameter of 4.44 or 2.53 mm derived from LLC wt or LLCAng-2/Ang-3 cells, respectively (Table 1). On the contrary, only 3.3 visible metastatic nodules per lung with average diameter of 1.927 mm were detected in the mice received LLCAng-3 cells; however, 126 micrometastatic lesions (0.288 mm in diameter) per lung were detected in the lung sections (Table 1). These results suggest that expression of Ang-3 inhibits progression of pulmonary micrometastases to macrometastases.

H&E staining indicated that at the early stage of pulmonary metastasis, many of the micrometastases are attached to the preexisted pulmonary blood vessels (data not shown). Because LLC cells express high level of CD44 (25), a principal cell surface receptor for hyaluronan, anti-CD44 antibody was used to highlight these micrometastases (Fig. 2C-b and c). In healthy lungs, evenly distributed macrophages are the major CD44-positive cells (ref. 26; Fig. 2C-a). We showed that the CD44-positive micrometastases derived from LLCAng-3 (Fig. 2C-c) LLCAng-2/Ang-3 (data not shown), and LLC wt (Fig. 2C-b) cells frequently aggregate around the preexisted pulmonary blood vessels (arrows in Fig. 2C-b and c). Three weeks after removal of the s.c. tumors, the micrometastases derived from LLCwt or LLCAng-2/Ang-3 cells were able to grow and form visible metastatic lesions (Fig. 2C-e and data not shown), whereas expression of Ang-3 inhibited progression of the micrometastases, which are still small and attached to pulmonary blood vessels (Fig. 2C-f, arrows).

Table 1  Ang-3 inhibits pulmonary metastasis of LLC cells by repressing tumor angiogenesis

<table>
<thead>
<tr>
<th>Transfectants</th>
<th>LLCwt</th>
<th>LLCAng-3</th>
<th>LLCAng-2/Ang-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of macrometastasis/lung</td>
<td>30.1 ± 9.78</td>
<td>3.3 ± 2.98</td>
<td>23.6 ± 6.00</td>
</tr>
<tr>
<td>Diameter of macrometastasis (mm)</td>
<td>4.44 ± 1.69</td>
<td>1.927 ± 1.08</td>
<td>2.53 ± 1.37</td>
</tr>
<tr>
<td>No. of micrometastasis/lung</td>
<td>ND*</td>
<td>125.6 ± 43.99</td>
<td>ND*</td>
</tr>
<tr>
<td>Diameter of micrometastasis (mm)</td>
<td>ND*</td>
<td>0.288 ± 0.131</td>
<td>ND*</td>
</tr>
<tr>
<td>No. of blood vessels/mm² of the pulmonary tumors</td>
<td>16.7 ± 2.02</td>
<td>3.91 ± 2.59</td>
<td>14.38 ± 3.52</td>
</tr>
<tr>
<td>Apoptosis rate of the tumor cells in vivo (%)</td>
<td>9.49 ± 5.69</td>
<td>70.27 ± 5.35</td>
<td>13.78 ± 3.94</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.
* Massive invasion and fusion of pulmonary metastases in lung parenchyma make it impossible to count micrometastases which may be present in the lungs of the mice that received LLCwt and LLCAng-2/Ang-3 cells.

Fig. 3. Ang-3 inhibits pulmonary metastasis of TA3 cells by inhibiting tumor angiogenesis. A, representative gross pictures of the mouse lungs 3 weeks after i.v. injection of TA3wt (A-a–c) or TA3Ang-3 (A-d–f) cells. Bar: 3 mm. B, survival rate of the experimental mice. A total of 30 mice was used for each type of transfectants. C, pulmonary metastatic burden is expressed by weight of the lungs derived from experimental mice three weeks after the i.v. injection. D, histological and immunological analysis. The representative lung sections were stained with H&E (D-a and b), anti-von Willebrand factor antibody (D-c and d), or Apoptag (D, e and f). These sections were derived from the mice received TA3wt (D-a, c, and e) or TA3Ang-3 (D-b, d, and f) cells. Bar: 200 µm in a–d and 50 µm in e and f. E and F, the quantitative data that reveals the effect of Ang-3 on tumor angiogenesis and tumor cell apoptosis.
implying that adequate angiogenesis was not established in these lesions.

To determine whether the inhibitory effect of Ang-3 on tumor metastasis is limited to LLC cells, we investigated the effect of Ang-3 on pulmonary metastasis of TAs mammary carcinoma cells. To eliminate heterogeneity in TA3 cells, we first transfected wild-type TA3 cells with empty expression vector containing a G418-resistant gene. A clonal TA3 cell that undergoes aggressive pulmonary metastasis after i.v. injection was selected (data not shown) to be transfected with the expression constructs. Five independent clonal TA3 transfectants transfected with the empty expression vector containing blasticidin-resistant gene (TA3wt) or expressing a similar level of Ang-3 (TA3Ang-3) were identified and used in the pulmonary metastasis experiments. We showed that massive pulmonary metastases were formed 3 weeks after i.v. injection of TA3wt cells and expression of Ang-3 inhibits pulmonary metastasis (Fig. 3). The metastatic tumors derived from TA3wt cells are invasive and fused together, which made it difficult to determine accurate number of the metastatic lesions. Thus, metastatic burden was quantified by average weight of the lungs. Our results showed that expression of Ang-3 dramatically reduce metastatic burden (Fig. 3C) and significantly extended the survival time of the mice (Fig. 3B).

**Overexpression of Ang-3 Inhibits Angiogenesis in the Pulmonary Micrometastases.** To investigate whether adequate angiogenesis was not established in the pulmonary micrometastases derived from LLCAng-3 and TA3Ang-3 cells, the lung sections were analyzed for the presence of blood vessels using anti-von Willebrand factor (vWF) antibody to reveal blood vessels (A, D, and G), with anti-smooth muscle actin antibody to show smooth muscle cells around blood vessels and bronchi (B, E, and H) and with Apoptag to detect apoptotic cells in situ (C, F, and I). Bar: 200 μm.

![Fig. 4. Ang-3 blocks tumor angiogenesis and promotes tumor cell apoptosis in the pulmonary micrometastases derived from LLCAng-3 cells. The lung sections derived from a normal mouse (A–C) or from the experimental mice 3 weeks after removal of the s.c. tumors derived from LLCwt (D–F) or LLCAng-3 (G–I) cells. These sections were reacted with anti-von Willebrand factor (vWF) antibody to reveal blood vessels (A, D, and G), with anti-smooth muscle actin antibody to show smooth muscle cells around blood vessels and bronchi (B, E, and H) and with Apoptag to detect apoptotic cells in situ (C, F, and I). Bar: 200 μm.](https://www.cancerres.aacrjournals.org/content/64/13/5123/F6.large.jpg)
micrometastases/small metastases derived from TA3Ang-3 or LL-CAng-3 cells (Figs. 3, D-f and F, and 4f; Table 1). Taken together, these results indicated that Ang-3 inhibits pulmonary metastasis of LLC and TA3 cells by inhibiting tumor angiogenesis and promoting apoptosis of the tumor cells.

Ang-3 Inhibits Endothelial Cell Proliferation and Survival by Blocking the Activation of Erk and Akt Kinases Induced by Ang-1 and VEGF. To elucidate the cellular mechanism underlying the antiangiogenic activity of Ang-3, we investigated the effect of Ang-3 on endothelial cell proliferation and survival using purified...
ANG-3. Purified ANG-3 is capable of binding to heparin and to the cell surface (data not shown). We demonstrated that unlike ANG-1, which has a very weak effect on endothelial cell proliferation (Fig. 5, A and B) and prevents endothelial cell apoptosis (Fig. 5D), ANG-3 inhibits endothelial cell proliferation in serum-free and low serum conditions and inhibits endothelial cell proliferation induced by VEGF but not that induced by bFGF (Fig. 5A–C). In addition, ANG-3 promotes apoptosis of the serum-starved endothelial cells (Fig. 5D).

To further determine the molecular mechanism underlying the effects of ANG-3 on endothelial cells, we investigated how ANG-3 affects the Erk and Akt signaling pathways (27–29), which play important roles in cell proliferation and survival. We showed that similar to VEGF_165, ANG-1 induces phosphorylation of Erk1/2 in the serum starved HUVECs, whereas ANG-3 blocks phosphorylation of Erk1/2 induced by ANG-1 or VEGF_165 but not that induced by bFGF (Fig. 6A–C). ANG-1 induces a sustained Erk1/2 phosphorylation that lasts much longer than that induced by VEGF_165 and ANG-1 and VEGF_165 or ANG-1 and bFGF displayed a synergistic stimulatory effect on Erk1/2 activation (Fig. 6, B and C, and data not shown). Furthermore, we showed that activation of Akt induced by ANG-1 or VEGF_165 is inhibited by ANG-3 (Fig. 6D, Lanes 5 and 7). These results suggest that ANG-3 inhibits endothelial cell proliferation and survival by blocking the signals derived from ANG-1 and VEGF_165, which likely underlie the inhibitory activity of ANG-3 on tumor angiogenesis and metastasis.

**DISCUSSION**

It is well established that angiogenesis is regulated by pro- and antiangiogenic factors, and adequate angiogenesis is essential for successful tumor metastasis. Different factors produced by tumor cells and surrounding stromal cells play important roles in regulating tumor angiogenesis by activating or blocking different pathways (3, 30). To use antiangiogenesis approach successfully as an anticancer therapy, it is essential to identify these essential pro- and antiangiogenic factors and understand the mechanisms underlying their functions. In our current study, we provided results that outlined how and why ANG-3 affects pulmonary metastasis of carcinoma cells. Pulmonary metastasis is pathologically relevant to lung and breast cancers. Thus, the results obtained herein are relevant to the pathological situation and may provide a potential reagent for cancer therapy in the future.

We have shown that ANG-3 inhibits pulmonary metastasis by inhibiting tumor angiogenesis and promoting tumor cell apoptosis. Pulmonary metastasis starts with growth of a small number of metastatic tumor cells inside of lung parenchyma to form micrometastases. We have shown that these micrometastases are often formed around preexistent pulmonary blood vessels, which provide necessary nutrition and oxygen to the micrometastases to support their initial growth without evoking hypoxia, and the new blood vessels are sequentially induced via actions of the angiogenic factors in the microenvironment. This situation is described as vessel co-option (30). As shown in our current study, angiopoietin-Tie-2 pathway likely plays a predominant and essential role in this type of angiogenesis.

**Tethering ANG-3 on Cell Surface Is Required for Its Antiangiogenic and Anti metastasis Activity.** Studies have shown that HSPGs can positively or negatively modulate bioactivities of many growth factors (31–33). Although it is well understood how HSPGs affect activity of some growth factors such as that of bFGF, it remains unclear how HSPGs regulate activity of other factors, including angiogenic activity of VEGF. In the current study, we have demonstrated that binding of ANG-3 to cell surface is required for the antiangiogenic and antimetastasis activity of ANG-3, which provides first clear example that HSPGs play an essential role in regulating activity of an antiangiogenic factor.

HSPGs may enhance the antiangiogenic activity of ANG-3 by the following mechanisms. Unlike growth/angiogenic factors, which often have short half-lives and exist in low concentrations, HSPGs are relative stable and abundant (33). Our result showed that ANG-3 protein is cleaved proteolytically (data not shown). Tethering ANG-3 on cell surface via HSPGs likely promotes ANG-3 from proteolytic cleavage, which extends its half-life. In addition, binding to HSPGs can concentrate ANG-3 on cell surface, which may be critical for effectively blocking the proangiogenic activity of ANG-1 and repressing tumor angiogenesis. Finally, ANG-3 and HSPG complexes may generate unique signals that are different from the ones derived from soluble ANG-3 and HSPGs separately.

**ANG-3 and ANG-1 Play Opposite Roles in Regulating Endothelial Cell Behavior and Angiogenesis.** We have shown that ANG-1 and ANG-3 displayed opposite effects on endothelial cell proliferation and survival and on activation of Erk1/2 and Akt kinases. ANG-3 likely blocks Ang-1-induced activation of Erk1/2 and Akt kinases by competing with ANG-1 for the binding of Tie-2 and therefore exerts an antagonistic effect. As a HSPG binding factor, ANG-3 likely blocks activation of Erk1/2 and Akt kinases induced by VEGF through indirect modulation of the binding of VEGF to HSPGs. Additional study is required to confirm this hypothesis.

Loss of endothelial layer health and integrity is the root of many vascular diseases (34–36). Our results suggested that ANG-1 and ANG-3 represent two important factors produced by peri-endothelial cells that may play antagonistic roles in maintaining health of endothelial cells in adult tissues, and the balanced activity of ANG-1 and ANG-3 may be important for initiation of angiogenesis during embryogenesis and tissue repair and, imbalanced up-regulation of ANG-3 activity and/or down-regulation of ANG-1 activity inhibits tumor angiogenesis and may contribute to vascular diseases such as ischemic heart and limbs, atherosclerosis, and restenosis.

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