Expression of Angiopoietin-1 in Human Glioblastomas Regulates Tumor-Induced Angiogenesis In Vivo and In Vitro Studies

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Abstract—To define a role for the angiopoietin/Tie2 system in astrocytoma angiogenesis, we examined the expression of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2) in these tumors by immunohistochemistry and in situ hybridization. Furthermore, we studied in vitro the effects elicited by glioblastoma cell–secreted Ang1 or by recombinant Ang1 on functions of endothelial cells (ECs). Our observations of astrocytomas show that a stage-specific induction of angiopoietins occurs and is correlated with angiogenic phases of different intensity. Ang1 expression was found in a few astrocytes scattered in the tumor at all stages of astrocytoma progression. In blood vessels, Ang1 mRNA increased progressively in high-grade glioblastomas, in which the number of vessels was higher than in low-grade tumors. Ang2 was detected in tumor cells and in ECs in high-grade astrocytomas, whereas its expression was negligible in low-grade tumors. Coculture of glioblastoma cell lines producing Ang1 with endothelium demonstrated a key role of this ligand in the control of EC network organization. We found that recombinant Ang1 in vitro induces EC spreading and reorganization of the cell monolayer into cordlike structures. These results suggest that Ang1 directly acts on ECs by modulating cell-cell and cell-matrix associations and promoting the differentiation phase of angiogenesis. (Arterioscler Thromb Vasc Biol. 2001;21:536-541.)

Key Words: angiopoietins • glioblastomas • angiogenesis

Astrocytic tumors can be separated by histological grading into low- and high-grade astrocytomas (anaplastic astrocytoma and glioblastoma multiforme), on the basis of increasing malignant phenotype.1 Their progression is accompanied by an increased tissue vascularization with aspects of prominent proliferation that is peculiar to high-grade astrocytomas.2

The establishment and the remodeling of new blood vessels in normal tissues and in tumors, including those of the central nervous system, require paracrine signals.3–5 Angiopoietins (Ang1 and Ang2) constitute a family of endothelial growth factors that are ligands for the tyrosine kinase receptor Tie2, which is expressed in endothelial cells (ECs) and upregulated in tumor microvessels.6–9 In vitro, Ang1 promotes EC sprouting, survival, and migration,10–12 whereas Ang2 blocks the activation of Tie2 induced by Ang1.7 Disruption of the function of either Tie2 or Ang1 in mice resulted in lethal defects in the developing vasculature, such as simplification of the vascular branching pattern and failure to recruit accessory cells.13,14 Consistent with its action as an Ang1/Tie2 inhibitor, overexpression of Ang2 in mice results in defects similar to those observed in Ang1 or Tie2 knockouts.7 These studies suggest that angiopoietins play their role during vascular remodeling and maturation. Furthermore, they contribute to the vessel integrity through the establishment of appropriate cell-cell and cell-matrix connections.6,7

To clarify the role of angiopoietins in the vascularization of human astrocytomas, we have analyzed their expression in a series of specimens of different histological grading and in glioblastoma cell lines. Furthermore, the functional role of Ang1 in vascular formation has been investigated in ECs cocultured with glioblastoma cells and by the use of the recombinant molecule.

Methods

Tumor Samples and Cell Cultures

Three low-grade astrocytomas, 4 anaplastic astrocytomas, and 3 glioblastomas multiformes diagnosed according to World Health Organization classification1 and 3 normal brain specimens were studied.

Human umbilical vein ECs (HUVECs) and human microvascular ECs (Dr E. Ades, Biological Products Branch, Atlanta, Ga) were cultured as previously described.15 Glioblastoma cell lines ADF (Dr G. Donelli, Istituto Superiore di Sanità, Rome, Italy), DF and LI (Dr G. Zupi, Istituto Regina Elena, Rome, Italy), U87 (American Tissue
Culture Collection), and U373 (Dr A. Colombatti, Centro di Riferimento Oncologico, Aviano, Italy) were maintained in RPMI 1640 (Sigma Chemical Co) supplemented with 10% FCS (Sigma) and antibiotics.

Immunohistochemistry and Quantitative Analysis
Deparaffinized sections were saturated in blocking solution (DAO AS) for 30 minutes and then incubated overnight at 4°C with (1) goat polyclonal antibodies against the C-terminus peptide of human Ang1 and Ang2 (1:200, Santa Cruz Biotechnology Inc), (2) mAb against human CD31 (1:40, DAKO), and (3) mAb against glial fibrillary acidic protein (GFAP, 1:100, Calbiochem). Specificity of anti-Ang1 and anti-Ang2 was tested by incubating antibodies with the respective immunizing peptides before immunostaining. Sections were incubated with secondary antibodies for 1 hour (biotinylated rabbit anti-mouse IgG [1:200] or anti-goat IgG [1:400], DAKO). For negative controls, the primary antibody was deleted. Staining was developed by the streptavidin-peroxidase-complex (DAKO). Immunoreactivity of antibody against Ang1 was amplified by the Tyramide Signal Amplification (TSA) method (NEN Life Sciences).

For microvessel quantification, please see http://atvb.ahajournals.org.

In Situ Hybridization and Reverse Transcriptase–Polymerase Chain Reaction
The full human Ang1 and Ang2 cDNA and a 1940-bp Tie2 cDNA fragment (positions 88 to 2022) subcloned into pBluescript SK were used to generate RNA probes by in vitro transcription with the use of T7 and T3 polymerases in the presence of digoxigenin-labeled UTP (Boehringer-Mannheim). RNA probes were reduced to 400- to 500-bp fragments with 0.1 mol/L NaOH. Frozen sections were postfixed in 4% paraformaldehyde for 10 minutes, washed in PBS, and acetylated with 0.1 mol/L triethanolamine and 0.25% acetic anhydride for 10 minutes. After dehydration, the sections were incubated in chloroform and then rehydrated in 100% and 95% ethanol. Hybridization was performed as described previously.15 Color detection was amplified by the TSA method.

For a description of primers and methods used for reverse transcriptase–polymerase chain reaction, please see http://atvb.ahajournals.org.

Coculture Assay
Matrigel (growth factor free, 300 μL, Becton Dickinson) was added to a Transwell chamber insert (12-mm diameter, 0.45 μm pores, Becton-Dickinson) and allowed to gel. HUVECs (70,000 cells well) were plated on the gel, and the insert was transferred into the culture plate, where glialblastoma cells were grown to confluence. For the control condition, HUVECs were cocultured with medium alone. The polyclonal anti-Ang1 antibody or nonsense mouse IgG (1:50, Sigma) was added to the Matrigel before its solidification and to the medium. After 6 hours, the cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.05 mol/L sodium cacodylate, pH 7.4.

Recombinant Ang1 and Immunoprecipitation
Ang1 was expressed with the baculovirus system.17 The full human Ang1 cDNA was subcloned into the BarnHI-Kpn1 site of the baculovirus vector pBlueBac 4.5 (Invitrogen). The vector was cotransfected with the linearized baculoviral DNA into Sf9 insect cells, and the recombinant virus was plaque-purified and amplified. Characterization of Ang1 in conditioned media was performed by immunoblotting.

For immunoprecipitation and immunoblotting, please see http://atvb.ahajournals.org.

Cell Behavior in Tridimensional and Bidimensional Culture Conditions
HUVECs (30,000 or 70,000 cells well) were cultured for 6 hours on 300 μL Matrigel in M199 and 0.5% FCS and stimulated for 6 hours with 80 ng/mL Ang1 or 10 ng/mL vascular endothelial growth factor (VEGF)-A16 (Sigma) or mock supernatant from insect cells. In selected experiments, Ang1 was neutralized by overnight incubation at 4°C with anti-Ang1 antibody or with goat IgG (1:50). Alternatively, HUVECs were starved for 5 hours in M199, 2% BSA, and 2% FCS and plated in gelatin-coated 12-well tissue culture plates. Cells (20,000) in 10 μL of starving medium were seeded in the center of every well; the cells remain confluent at the well center and have enough space to spread and to migrate toward the margins. Cells were stimulated for 6 hours with Ang1 (80, 250, and 500 ng/mL), VEGF-A16 (10 mg/mL), or mock supernatant in 0.5% FCS. Cells were stained as described.15

Results
Expression of Ang1 and Ang2 in Astrocytomas
Ang1 and Ang2 expression was analyzed by immunohistochemistry in specimens ranging from low- to high-grade astrocytomas.

Low-grade astrocytomas contained a higher number of astrocytes than did normal brain. The vasculature closely resembled that of a normal brain, as indicated by CD31 staining (please see online Figure I at http://atvb.ahajournals.org); blood vessels were of small caliber without signs of neovascularization and endothelial hyperplasia. Their number was similar to that in normal brain (please see online Table I at http://atvb.ahajournals.org). Necrotic areas were absent. In serial sections, Ang1 was detected in ECs lining the vessels, whereas it was nearly absent in the vessels of normal brain. It was also detected in a few tumor cells scattered in the tissue as well as in a few neuronal cells of the normal brain. In both tissues, Ang1-positive cells ranged from 4% to 10%. Ang2 expression was negligible in normal brain and in large areas of low-grade astrocytomas. Nevertheless, restricted areas of these tumors contained CD31-positive ECs and GFAP-positive astrocytes with a weak positivity for Ang2. The number of Ang2-positive astrocytes ranged from 4% to 11%. In these areas, an extracellular signal was observed, probably because of diffusion of the protein in intercellular spaces.

In anaplastic astrocytomas, cellularity and nuclear polymorphism were increased. Astrocytes still expressed GFAP, mitoses were more frequent, and small necroses appeared. CD31 staining showed endothelial hyperplasia with an abundance of branched blood vessels positive for Ang1 (please see online Figure II and Table I at http://atvb.ahajournals.org). Ang1 expression in astrocytes was comparable to that observed in low-grade astrocytomas. In this tumor, Ang2-expressing astrocytes were not confined to selected areas, as found in low-grade astrocytomas, but they were diffused in the tissue, and their number was up to 12% to 20%. Ang2 was also detected in CD31-positive ECs of numerous vessels.

In glioblastoma multiforme, there was a marked cellular density and nuclear polymorphism. Astrocytes had different sizes, round or elongated shapes, and variable GFAP positivity. Mitosis and necrosis were abundant, as was the vessel number (please see online Table I). Newly formed vessels were common (Figure 1A, 1B, and 1C), together with glomeruloid proliferation formed by endothelial hyperplastic cells (Figure 1E). Immunohistochemistry of these specimens showed that nearly all vessels expressed Ang1 and Ang2, which appeared to be more abundant than the expression in vessels of low-grade astrocytomas, most probably a result of EC hyperplasia (Figure 1B and 1C).

Ang1 and Ang2 mRNA expression was also studied by in situ hybridization in glioblastoma multiforme. Figure 1E and 1G (left) shows Ang1 and Ang2 mRNA, respectively, in ECs...
of blood vessels. Vessels also expressed Tie2 mRNA (Figure 1I) as well as the protein (not shown). Tissues incubated with Ang1 and Ang2 sense RNA probes did not show positive signals (Figure 1F and 1H). The number of tumor cells expressing Ang1 was ~10% (not shown) without focal upregulation in selected areas. In contrast, Ang2 was focally expressed in many tumor cells (range 15% to 25%), as detected by immunostaining (Figure 1D) and by in situ hybridization (Figure 1G, right).

Expression of Ang1 by Glioblastoma Cells Promotes Angiogenesis of Cocultured ECs

The in vivo analysis suggests that Ang1 is involved in the early stage of vascular activation during glioblastoma progression. To characterize the effects of Ang1 expressed and released by tumor cells on EC morphogenesis, a coculture system was used.

First, endothelial and glioblastoma cell lines were investigated for the expression of Ang1 and Ang2 mRNA by using reverse transcriptase–polymerase chain reaction. Two glioblastoma cell lines (ADF and U87) expressed both angiopoietins, as demonstrated by the presence of 2 products of 771 and 545 bp, which correspond to the expected sizes of Ang1 and Ang2 (please see online Figure III at http://atvb.ahajournals.org). DF glioblastoma cells expressed only Ang2, whereas LI and U373 cells did not express either. HUVECs and human microvascular ECs expressed Ang1 and Ang2 transcripts. All the cell lines tested expressed 3 isoforms of VEGF-A, namely, VEGF-A$_{121}$ (452 bp), VEGF-A$_{165}$ (584 bp), and VEGF-A$_{189}$ (656 bp).

In coculture experiments, glioblastoma cells were seeded in the lower chamber of a Transwell; in the upper chamber, HUVECs were plated over a layer of Matrigel. Here, ECs form vascular tubes connecting cellular nodes, a phenomenon known as in vitro angiogenesis. This assay allows for in vitro conditions that more closely mimic the in vivo environment permissive for cell differentiation into capillaries. HUVECs cultured on Matrigel alone in low serum became elongated and aligned with each other, and they formed a network of thin cord of interconnecting cells. Nevertheless, they failed to establish cell-cell contacts to neighboring cells, giving origin to fragmented tubes. Moreover, many cells exhibited a small round shape and did not spread (Figure 2A). HUVECs cocultured with the DF, U373, or LI cell lines, which do not express Ang1, showed a pattern similar to the control pattern (data not shown). By contrast, in the presence of ADF (Figure 2B) or U87 (not shown) glioblastoma cells, which express VEGF-A and Ang1, ECs migrated throughout the Matrigel surface to form a better organized meshwork of anastomosing, not fragmented, cordlike structures compared with the control. The pattern observed could be induced by a
A variety of soluble molecules produced by glioblastoma cells. Therefore, the defined role played by Ang1 in EC differentiation during the coculture experiments was characterized with a neutralizing anti-Ang1 antibody. This antibody inhibits Ang1-mediated Tie2 phosphorylation (please see online Figure IV at http://atvb.ahajournals.org), and when it was added to the coculture system, it produced the appearance of a large number of HUVECs with a cobblestone-like morphology (Figure 2C, arrows). Cells failed to align and elongate, giving origin to less extended cordlike structures with nests composed of an increased number of cells. Nonimmune goat IgG or anti-Ang1 antibody preadsorbed to the immunizing peptide did not affect EC organization (not shown). These data suggest that Ang1 influences reciprocal interactions between EC and favors their transition from cobblestone-like morphology to capillary-like structures.

**Ang1 Modifies Cell Behavior in Tridimensional and Bidimensional Cultures**

To test the hypothesis that Ang1 produced by glioblastoma affects in vitro angiogenesis, the recombinant molecule was used to evaluate its effect on EC behavior in tridimensional Matrigel. Recombinant Ang1 was functionally active, as assessed by its effect on Tie2 phosphorylation in HUVECs (please see online Figure IV at http://atvb.ahajournals.org). Cell lysates from stimulated and unstimulated HUVECs were immunoprecipitated with an anti-Tie2 polyclonal antibody, and proteins were separated by SDS-PAGE and blotted with a mAb anti-phosphotyrosine. Ang1 induced Tie2 phosphorylation, and the effect was neutralized by its preincubation with a specific blocking antibody.

The Ang1 effect on HUVEC organization in Matrigel was evaluated by plating ECs at low density (30,000 cells) in low serum. In this condition, they spontaneously formed a network of cordlike structures (Figure 2D), and the addition of Ang1 or VEGF-A165 had no effect on EC differentiation (data not shown). At higher density (70,000 cells), HUVECs did not spontaneously form the network of tubes but remained confluent (Figure 2E). The addition of Ang1 determined the formation of a well-organized network of cordlike structure (Figure 2F). This effect was abolished by preincubation of Ang1 with a neutralizing antibody (Figure 2G) but not with nonimmune goat IgG (not shown). The addition of VEGF-A165 had no effect on endothelial organization, producing a pattern similar to that of the control condition (Figure 2H).
A step of the complex effects of Ang1 in inducing in vitro angiogenesis seems to be the loss of monolayer structure (see Figure 2E versus 2F) that requires cell separation. Actually, the loss of cell-cell contact is necessary in the beginning of in vitro and in vivo angiogenesis. To demonstrate that cell-cell detachment belongs to the mechanism of in vitro angiogenesis induced by Ang1, HUVECs were plated at confluence on gelatin-coated plates (Figure 3A). After 5 hours, Ang1-stimulated cells appeared detached from each other and assumed a spread and elongated shape (Figure 3B). In contrast, cells stimulated with VEGF-A165 were similar to the control condition (Figure 3C).

Discussion

In the present study, we have combined an in vivo and an in vitro approach to study the role of angiopoietins in neoplastic angiogenesis during astrocytoma progression.

We have first analyzed in vivo the expression of Ang1 and Ang2 in human astrocytomas of different pathological grades. Ang1 expression in normal tissue was very low and involved few neuronal cells and sporadic vessels. In tumor cells, Ang1 was detected in a few astrocytes of low-grade astrocytomas and remained constant during the tumor progression. On the contrary, the intensity of Ang1 staining and the number of positive vessels were increased during progression from low- to high-grade astrocytomas.

Ang2 expression was activated later than Ang1 expression. Whereas in normal brain and in low-grade astrocytomas, the signal was absent, in anaplastic astrocytomas and glioblastoma multiforme, Ang2 was expressed in tumor and vascular ECs. Ang2 expression in tumor cells was different from that of Ang1 in that it involved a higher number of astrocytes grouped in areas with high vessel density. Like Ang1, the intensity of Ang2 staining in ECs and the number of positive vessels were increased.

Our observations are consistent with the role assigned to angiopoietins in vascular remodeling. Ang1 expression in most adult quiescent tissue and, as shown in the present study, in all phases of glioblastoma progression suggests that this molecule provides a stabilizing signal maintaining the integrity of interaction between the newly formed endothelium and the underlying matrix. Precisely regulated activation of Ang2 expression, by hypoxia or other inducers, in adult tissue undergoing vascular remodeling and in high-grade astrocytomas would negate the stabilizing signal and allow localized disruption of the vessel wall, rendering ECs more accessible to angiogenic inducers. In glioblastoma, this angiogenic signal most likely is provided by VEGF-A. The coexpression in vascular vessels of high levels of both angiopoietins during astrocytoma progression could explain the abnormal architecture of vasculature in these tumors. Transgenic overexpression of Ang1 or Ang2 results in the formation of more numerous and larger vessels or in the establishment of a discontinued vascular network, respectively. Notably, human ECs can express both molecules in vitro (see online Figure III) as well as in vivo.

Our results disagree with those published by others who studied in the same model the expression of Ang1 and Ang2 by in situ hybridization. They found expression of Ang2 but not of Ang1 in the tumor vessels. This discrepancy may be based on technical differences. In our experience, only the application of the TSA method in immunohistochemistry and in situ hybridization has allowed the detection of Ang1 protein and mRNA. Furthermore, in previous experiments, 570- and 640-bp probes for Ang1 and Ang2, respectively, have been used. In the present study, Ang1 and Ang2 full-length probes were used, a condition that could differentiate the sensitivity of the assay. However, it would be intriguing to speculate that a complex pattern of transcription and translation of angiopoietin genes occurs in glioblastomas, thus explaining the observed discrepancies in a small series of patients.

In Ang1 knockout mice, poor association between ECs and periendothelial cells as well as a lack of EC spreading and flattening are responsible for immature vessels. It has been demonstrated that Ang1 promotes sprouting, cell survival, and migration in ECs. Therefore, it can be speculated that Ang1 not only plays a role in the maintaining of interactions between ECs and support cells but also seems to have a direct effect on EC behavior during vascular remodeling.

To define the role of Ang1 secreted by tumor cells in neoplastic angiogenesis, we have identified a number of Ang1-expressing glioblastoma cell lines and have set up an in vitro coculture system with ECs. In the presence of Ang1-expressing glioblastoma cells, ECs appeared markedly flattened.
and migrated throughout the Matrigel surface, forming a well-organized network of anastomosing, not fragmented, cordlike structures. The formation of such tubular structures is a very complex process that involves the combined effects on cell-cell and cell-matrix adhesion, proteolytic remodeling of the matrix and migration. The use of a blocking anti-Ang1 antibody predominantly affected cell-cell adhesion and migration. Actually, ECs remained grouped, failing to align and elongate and giving origin to less extended cordlike structure and nests constituted by an increased number of cells.

In the Matrigel system, the use of recombinant Ang1 confirms this scenario. The formation of capillary-like structures by ECs plated at high concentration was reduced in the absence of Ang1. As seen in the coculture experiments, the addition of Ang1 favors EC separation and reorganization of the cell monolayer into cordlike structures. This is also supported by the observation that in a bidimensional culture, Ang1 induces the spreading of the EC monolayer.

These data extend the biological role of Ang1 in regulating the reciprocal interaction between ECs not only in stabilized vessels but also at the beginning of an angiogenic event, favoring the loss of cell-cell contacts and the EC movement toward each other, which are required for fusion into capillary structures. These effects are contradictory and suggest that the molecular events driving them are extremely complex and not completely clarified. Along this line, it has been demonstrated that the association of VEGF receptor 2 with αβ\textsubscript{3} integrin or with vascular-endothelial cadherin differentiates the biological response of ECs to the ligand, with the former being the association favorable for migration and the latter being favorable for survival.\textsuperscript{28,29} Indeed, the cooperation with other membrane proteins may discriminate the biological responses (ie, stabilization of vessel wall versus loss of cell-cell contacts) triggered by the Ang1/Tie2 pathway.

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