Angiopoietin-2 induces human glioma invasion through the activation of matrix metalloprotease-2

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A hallmark of highly malignant human gliomas is their infiltration of the brain. We analyzed a large number of primary human glioma biopsies and found high levels of expression of an angiogenic regulator, angiopoietin-2 (Ang2), in the invasive areas, but not in the central regions, of those tumors. In the invasive regions where Ang2 was overexpressed, increased levels of matrix metalloprotease-2 (MMP-2) were also apparent. Consonant with these features, intracranial xenografts of glioma cells engineered to express Ang2 were highly invasive into adjacent brain parenchyma compared with isogenic control tumors. In regions of the Ang2-expressing tumors that were actively invading the brain, high levels of expression of MMP-2 and increased angiogenesis were also evident. A link between these two features was apparent, because stable expression of Ang2 by U87MG cells or treatment of several glioma cell lines with recombinant Ang2 *in vitro* caused activation of MMP-2 and acquisition of increased invasiveness. Conversely, MMP inhibitors suppressed Ang2-stimulated activation of MMP-2 and Ang2-induced cell invasion. These results suggest that Ang2 plays a critical role in inducing tumor cell infiltration, and that this invasive phenotype is caused by activation of MMP-2.

glioblastoma

One of the major pathophysiological features of malignant human gliomas is their ability to diffusely invade into surrounding brain tissues. The rapid dissemination of single tumor cells throughout the brain renders these tumors incurable by surgical removal even when combined with adjuvant radiation and either chemotherapies or immunotherapies (1) and also underlies their great propensity for recurrence. Invasion of glioma cells involves the attachment of invading tumor cells to extracellular matrix (ECM), disruption of ECM components, and subsequent cell penetration into adjacent brain structures. This is accomplished in part by tumor-secreted matrix metalloproteases (MMPs) that degrade the ECM at tumor-invasive fronts to overcome ECM barrier (2). MMPs are a family of 22 ECM-modifying enzymes (3). Up-regulation of MMP-2, a member of the MMP family, has been found in glioma cell lines and in high-grade glioma specimens (4), and its activation has been linked to enhanced glioma invasion in several *in vitro* and *in vivo* model systems (2, 5). Although acquisition of this invasive phenotype by tumor cells is a turning point during glioma progression, and this transition involves gene products such as MMP-2, the mechanisms of initiation and maintenance of glioma invasiveness remain unknown.

Angiopoietin-2 (Ang2), a naturally occurring antagonist of Ang1, plays important roles in angiogenesis and tumor progression. Both Ang2 and Ang1 act as ligands of the endothelial cell (EC)-specific tyrosine kinase receptor, Tie2. Through binding to Tie2, Ang1 promotes interactions between ECs and peri-ECs to stabilize the established vasculature. Ang2 modulates Ang1-mediated vessel stabilization by competitively inhibiting the binding of Ang1 to Tie2 (6). Accumulated evidence also indicates that production of Ang2 is implicated in tumor progression. In human glioma, colon, gastric, or breast cancer tissues, in addition to expression of Ang2 in ECs, up-regulated Ang2 protein was found in tumor cells (7–11). Overexpression of Ang2 by colon or gastric cancer cells enhanced tumor angiogenesis and growth in mice (7, 9). Correlation of Ang2 expression with tumor invasiveness in primary tumor specimens or increased metastases of Ang2 stably transfected gastric tumors in mice suggested the involvement of Ang2 in tumor invasion or metastases (8–11). However, whether Ang2 can promote tumor progression by directly stimulating Tie2-deficient tumor cells has not been described.

Here, we report that Ang2 induces human glioma cell invasion. In invasive areas of primary human glioma specimens, up-regulated expression of Ang2 was detected in tumor cells. Correspondingly higher levels of MMP-2 expression were present in Ang2-expressing tumor cells in these gliomas. These features and their potential interactions were modeled by using intracranial xenografts in mouse brains where overexpression of Ang2 induced glioma invasion. In these invasive tumors, there was increased expression of MMP-2 at the invasive front. *In vitro* invasion analyses showed that Ang2 promoted glioma cell invasion and stimulated activation of MMP-2. Moreover, inhibition of MMP-2 by MMP inhibitors impeded Ang2-induced cell invasion. These findings implicate Ang2 action on tumor cells through activation of MMP-2 in glioma invasion and suggest another function for Ang2 in addition to its primary role in vascular and tissue development.

Materials and Methods

Cell Lines and Reagents. Human U87MG, U373MG, and T98G glioma cells and human umbilical vascular EC (HUVEC) cells were from American Type Culture Collection, and their culture was described previously (12). The following reagents were used for this study: human U251MG glioma cells (from C. Gladson, University of Alabama); rabbit polyclonal anti-Myc-tag antibody (1 μg/ml, Medical and Biological Laboratories, Nagoya, Japan); goat polyclonal anti-Ang2 antibody (C-19, 1:50, Santa Cruz Biotechnology); mouse monoclonal antiphosphotyrosine antibody (4G10, 1 μg/ml, Upstate Biotechnology, Lake Placid, NY); rat monoclonal anti-mouse CD31 antibody (1:1,000, BD-PharMingen); rabbit polyclonal anti-MMP-2 antibody (AB809, 1:200, Chemicon); rabbit polyclonal anti-von Willebrand factor-antibody (1:1,000, DAKO); mouse monoclonal anti-Tie2 antibody (1 μg/ml, C. Counter, Duke University); recombinant human Ang2 protein (1 μg/ml) and rabbit polyclonal anti-Ang2 antibody (AF623, 0.2 μg/ml, R& D Systems); rabbit polyclonal antisecreted protein acidic and rich in cysteine (SPARC) antibody (1:200, R. Brekken, University of

Abbreviations: ECM, extracellular matrix; Ang, angiopoietin; MMP, matrix metalloprotease; EC, endothelial cell; HUVEC, human umbilical vascular EC; CM, conditioned media; IHC, immunohistochemistry/immunohistochemical; VN, vitronectin; SPARC, secreted protein acidic and rich in cysteine; MVD, microvascular density; WHO, World Health Organization.

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Texas, Southwestern Medical Center, Dallas); vitronectin (VN), fibronectin, and laminin (400 ng/ml, Invitrogen); Matrigel (0.78 μg/ml, Becton Dickinson Biosciences); MMP inhibitors (50 μM, MMP inhibitor II or III, GM6001, Calbiochem); and Marimastat (20 μM, W. R. Bishop, Schering-Plough). Other reagents were from Invitrogen, Sigma, or Fisher Scientific.

Immunohistochemical (IHC) Analyses of Primary Human Glioma Specimens. Of the 79 human glioma specimens investigated, there were 32 glioblastoma multiforme [World Health Organization (WHO) grade IV], 12 anaplastic astrocytoma (WHO grade III), 3 anaplastic oligodendroglioma (WHO grade III), 5 anaplastic oligoastrocytoma (WHO grade III), 16 diffuse astrocytoma (WHO grade II), 6 oligodendroglioma (WHO grade II), five pilocytic astrocytoma (WHO grade I), and four normal human brain specimens. All surgical specimens were obtained during the past 6 yr at the Department of Neurosurgery, Saitama Medical School, Saitama, Japan. The glioma grades and presence of invasive areas within paraffin sections stained by hematoxylin/eosin were independently verified by neuropathologists from Saitama Medical School and the University of Pittsburgh. IHC analyses were performed as described (12).

Generation of Glioma Cells That Stably Express Angs. U87MG cells were stably transfected with cDNAs for Ang1 or Ang2 in a pSecTagB/Myc-His(+) expression vector (Invitrogen). The clones that expressed Ang1 or Ang2 were characterized as described (12). To assess the effect of tyrosine phosphorylation of Tie2 receptor in HUVEC by U87MG-derived Ang1 or Ang2, HUVEC cells were treated with conditioned media (CM) containing Ang1 or Ang2 or a mixture of these CM at 37°C, 5% CO_2 for 30 min. Cell lysates containing 30 μg of protein were analyzed by Western blotting by using an antiphosphotyrosine antibody (4G10). The membrane was then stripped, reprobed with an anti-Tie2 antibody, and developed. Quantification of Tie2 phosphorylation was done by importing the scanned images into the IMAGE PRO PLUS program and analyzed (Version 4.1, Media Cybernetics, Silver Spring, MD).

Analyses of Tie2 Expression in Various Cells by Using RT-PCR. cDNA templates for PCR analyses were synthesized from human dermal vascular EC or various glioma cell lines (12). PCR was performed by using 5′-ATCCCATTTGCAAAGCTTCTGGCTGGC-3′ (sense primer) and 5′-TGTGAAGCGTCTCACAGGTCCAGGATG-3′ (antisense primer). PCR reactions were performed at: 95°C, 3 min; 35 cycles of 95°C, 1 min; and 60°C, 30 s; 72°C, 1 min; and 72°C, 10 min. The amplified Tie2 cDNA fragment was analyzed by 4% agarose gel electrophoresis.

Tumorigenicity, Glioma Invasion, Mouse Brain Tissue Processing, and IHC. U87MG (5×10^5) or U87MG Ang2-expressing cell clones were stereotactically implanted into individual nude mouse brains with five mice per group. When mice developed neurological symptoms due to disturbance of their central nervous systems, they were killed and their brains were removed, processed, and analyzed (12). The distance of invading glioma cells from tumor masses was assessed by capturing serial images of hematoxylin/eosin-stained sections by using a Olympus BX51 (Melville, NY) microscope equipped with a digital camera and calculated by the fact that under a ×100 magnification, one frame is equal to 1 mm long. To quantify microvascular density (MVD) or the degree of staining by antibodies, five to seven serial-cut sections were stained with the anti-CD31 or anti-Ang2 or anti-MMP-2 antibodies. Captured images (10 or more random areas per slide) were imported into the IMAGE PRO PLUS program. The mean values of MVD or relative intensity of the antibody staining from serial brain sections (five or more individual mouse per group) in each group were used. MVD were expressed as the ratio of positively stained areas to the total area of the image (object areas/mm^2). The degree of the antibody staining was shown as folds of increase to that in U87MG/LacZ tumors.

Gelatin Zymography Analyses (13). Serum-free CM collected after 24 or 48 hr of cell culture was analyzed for the proteolytic activities of MMP-2 toward gelatin. CM containing 20 μg of total protein was separated at 4°C in a 7.5% SDS polyacrylamide gel containing 0.2% gelatin. The gel was washed in a substrate buffer containing 2% of Triton X-100, rinsed, and developed in a buffer containing 1% of Triton X-100 and 5 mM of $CaCl_2$ at 37°C for 16 h. The gel was then stained, destained, and photographed. Inhibition of MMP activities by synthetic MMP inhibitors, MMP inhibitor II (50 μM), III (50 μM), GM6001 (50 μM), or Marimastat (20 μM) was done by including each inhibitor separately in the cell cultures.

***In Vitro* Invasion Assays (14, 15).** Transwell inserts for 24-well plates (Costar) were coated with prediluted Matrigel (0.78 μg/ml) or VN (400 ng/ml). One milliliter of serum-free medium was added into the bottom wells of the plate. Five hundred microliters of cell suspension of the various types of cells (1×10^6 cells/ml) were added to triplicate inserts. The cells were allowed to invade through the matrices at 37°C for 48 h. The filters were then fixed and stained. Nonmigrating cells on the upper surface were removed, and the membranes were cut. In some experiments, an anti-Ang2 antibody (10 μg/ml), MMP inhibitors (50 μM), or recombinant Ang2 (1.0 μg/ml) was included in the upper wells. The number of invading cells was quantified by counting them in 10 random high-powered fields (×200 of total magnification) per filter.

Results

Up-Regulation of Ang2 and MMP-2 in Invading Glioma Cells and Neovessels in the Invasive Areas of Human Primary Glioma Specimens. To determine whether Ang2 and MMP-2 were associated with glioma progression, we performed IHC analyses by using an anti-Ang2 antibody (7, 8, 10, 11) on a collection of human glioma specimens. Among 79 glioma specimens analyzed, 20 samples of various WHO glioma grades had clearly identifiable borders between tumor mass and "normal" brain tissues where glioma cell invasion had occurred. Fig. 1 shows a glioblastoma multiforme (WHO grade IV) tissue where the glioma cells invaded into the adjacent brain parenchyma (Fig. 1 *b*, arrows, and *c*). In the central region of the glioma mass, Ang2 protein was either not detected (Fig. 1 *a* and *d*) or was expressed at low levels (data not shown). In contrast, in the border areas of all 20 specimens, strong immunostaining by the anti-Ang2 antibody was apparent in the invading glioma cells and the activated neural cells as well as neovessels (Fig. 1 *e*, *f*, *m*, and *n*). Expression of Ang2 in the invading glioma cells and neovessels was found in all invasive areas of the glioma specimens of various tumor grades (WHO grades II–IV) analyzed (Fig. 1 and data not shown), suggesting that the expression of Ang2 detected in cells was phenotypically consistent with the invasive phenotype of human gliomas. Expression of Ang2 in neovessels in tumor borders and glioma invasion regions suggests that EC-expressing Ang2 stimulates angiogenesis in these areas (Fig. 1 *m* and *n*).

Next, we sought to determine the expression of MMP-2 in various areas of the glioma specimens. Similar to the pattern observed for Ang2, no MMP-2 protein was found in the central regions of the glioma tissue (Fig. 1*h*), whereas expression of MMP-2 was clearly detected in the border (Fig. 1*i*) and the invasive areas (Fig. 1*j*) of the tumor. A high level of expression of MMP-2 was seen in the tumor cells and the activated neural cells (arrows in Fig. 1 *i* and *j*) as well as some vessels (arrowheads in Fig. 1 *b*, *c*, *e*, *f*, *i*, *j*, *m*, and *n*), in which Ang2 was overexpressed. Thus, coexpression of Ang2 and MMP-2 in the invasive areas in human glioma tissues suggests that both molecules are associated with glioma invasion.

Expression of Angs and Tie2 in Human U87MG Glioma Cells. To test the roles of Ang2 in glioma progression, a glioma model was developed

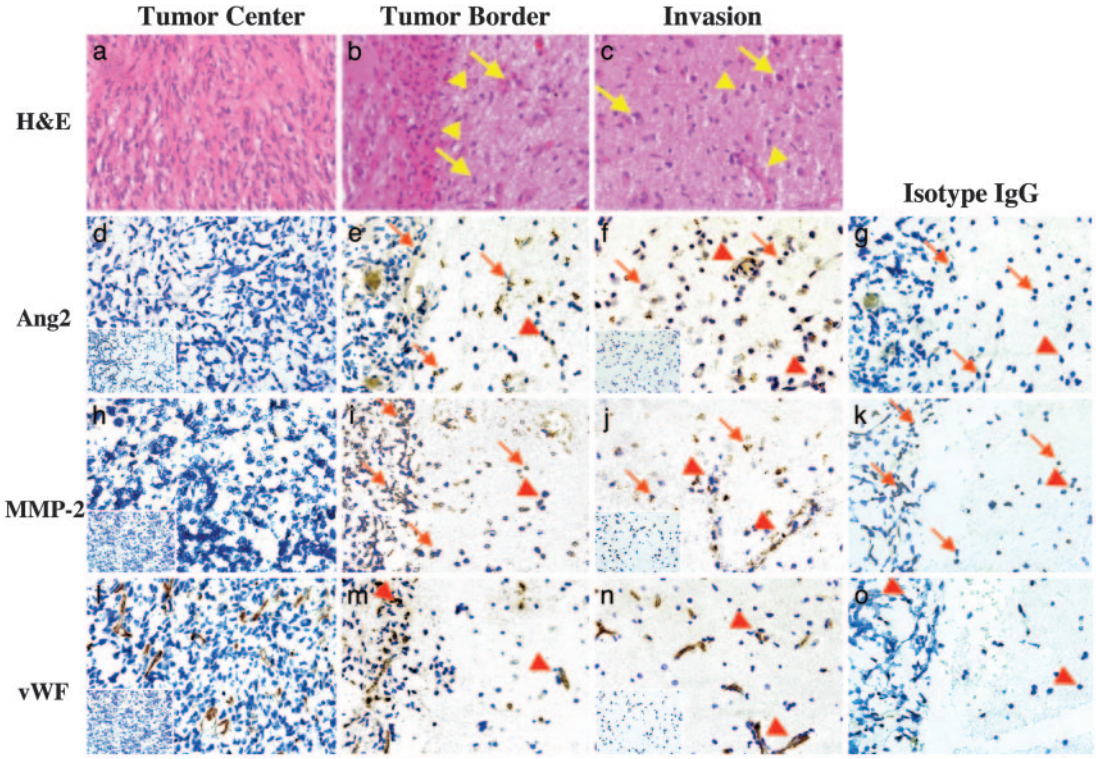

Fig. 1. Cooverexpression of Ang2 and MMP-2 in invasive areas of primary human glioma tissues. IHC on serial sections of a glioblastoma multiforme tissue using hematoxylin/eosin (*a–c*), a goat polyclonal anti-Ang2 antibody (*d–f*), a rabbit polyclonal anti-MMP-2 antibody (*h–j*), a rabbit polyclonal anti-von Willebrand factor antibody (*l–n*), and their isotype-matched IgG controls (*g*, *k*, and *o*; identical areas shown in *e*, *I*, and *m*; *Insets* in *d*, *f*, *h*, *j*, *l*, and *o*; identical areas in each panel). (*a*, *d*, *h*, and *l*) Central region in the tumor mass. (*b*, *e*, *g*, *i*, *k*, *m*, and *o*) Tumor borders. Arrowheads in *b* indicate the border between tumor mass (far left, high cellular density) and ''normal'' brain tissues (low cellular density). (*c*, *f*, *j*, and *n*) An area 0.25 mm away from the tumor edge shown in *b*, *e*, *i*, and *m*. Arrowheads in *c–o* indicate tumor vessels. Arrows in *c–g* and *h–k* are glioma cells or activated neural cells stained by the anti-Ang2 and the anti-MMP-2 antibodies, respectively. A total of 20 individual primary tumor specimens (WHO grade II–IV) that contain invasive areas were analyzed. The experiments were repeated two additional times with similar results. Original magnification, ×400.

by stably transfecting U87MG cells with Ang2 or Ang1 cDNA in a pSecTag expression vector. Five independent clones of each class that expressed exogenous Ang2 or Ang1 proteins were characterized. As shown in Fig. 2*A*, after 48 h of cell culture, U87MG cell clones in each class secreted Ang1 (72 kDa, Fig. 2*A Upper*) or Ang2 (72 kDa, Fig. 2*A Lower*) at various levels into their CM. The U87MG cell-derived Ang proteins were biologically active because the secreted Ang1 strongly stimulated tyrosine phosphorylation of Tie2 in HUVEC cells (a 5.5-fold increase when compared with untreated cells), whereas the U87MG cell-derived Ang2 competitively inhibited Ang1–stimulated tyrosine phosphorylation of Tie2 in ECs (a 2.3-fold increase when compared with the control, Fig. 2*B*).

The cognate receptor for Ang2, Tie2, is expressed in ECs. To examine whether it is also present in U87MG glioma cells, RT-PCR analyses were performed to amplify a specific cDNA fragment of Tie2 in various types of cells. As shown in Fig. 2*C*, the expression of the Tie2 receptor was found in human dermal vascular EC, but not in U87MG, five U87MG Ang2-expressing cell clones, or other glioma cell lines that were examined (data not shown).

Overexpression of Ang2 by U87MG Gliomas Caused Aggressive Tumor Invasion in Mouse Brains. To determine whether overexpression of Ang2 by U87MG cells would stimulate glioma progression *in vivo*, we stereotactically implanted U87MG/LacZ (isogenic control) or five individual Ang2-expressing (U87MG/Ang2) cell clones separately into the brains of mice. In general, U87MG/LacZ cells formed oval-shaped intracranial tumors with sharp edges that expanded as spheroids (Fig. 3 *a* and *c*). Similarly, mice that received 5×10^5 U87MG/LacZ cells developed noninvasive tumors with a volume of 56 mm^3 or larger in 40.2 ± 2.2 days (12). However, mice that received a single U87MG/Ang2 cell clone or a mixture of two cell clones developed highly invasive gliomas (Fig. 3 *b* and *d*). Among 75 mice that received various U87MG/Ang2 cell clones (five mice per group in six separate experiments), 61 (81.3%) showed a morphologically invasive phenotype. Microscopically, the Ang2-expressing gliomas displayed interspersed fibroblast-like structures. The borders of these tumors assumed zigzag shapes and formed spike-like structures that invaded into the normal brain structures (Fig. 3 *b* and *d*). Some Ang2-expressing gliomas displayed a palisading pattern of tumor cells (data not shown), this being one of the distinct morphological features in human gliomas (1) and rarely encountered in xenografted models. Glioma cells migrated well beyond the initial tumor masses or became groups of individual tumor clusters that localized at 2.5–4.3 mm from the tumor mass in various invasive tumors (Fig. 3 *b* and *d*). Thus, overexpression of Ang2 by U87MG gliomas caused aggressive glioma invasion in brains with phenotypic characteristics reminiscent of the clinical appearance of diffusely invasive human gliomas (1).

Overexpression of MMP-2 at Tumor Invasive Fronts of U87MG/Ang2 Gliomas. We next determined whether the invasive phenotype displayed by U87MG/Ang2 gliomas corresponded with the overexpression of Ayg2. With an anti-human Ang2 antibody or an anti-c-Myc tag antibody (data not shown), high levels of Ang2 expression were detected in the U87MG/Ang2 gliomas (Fig. 3*f*) but not in the U87MG/LacZ tumors (Fig. 3*e*). We then determined whether the regional cooverexpression of Ang2 and MMP2 that was found in invasive areas of human gliomas (Fig. 1) was also evident in the U87MG/Ang2 tumors. The expression of MMP-2

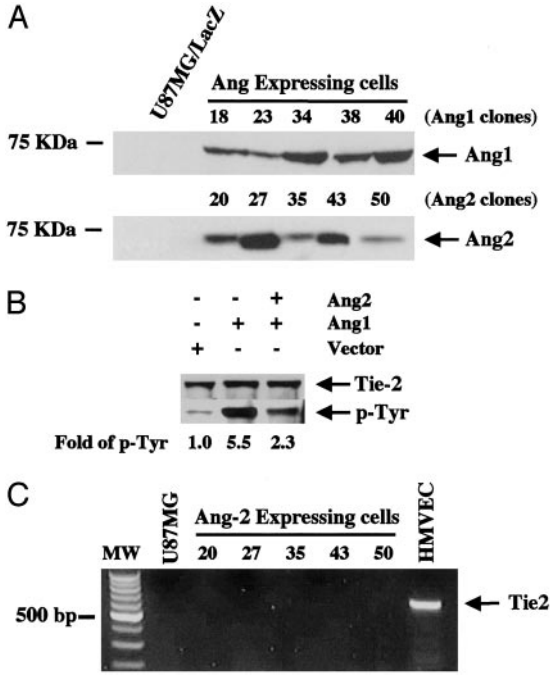

Fig. 2. Expression of Ang2 and Tie2 in U87MG glioma cells. (*A*) Overexpression of Ang1 or Ang2 in U87MG cells. CM from U87MG/LacZ or five Ang1- or Ang2-expressing cell clones were analyzed by Western blot by using an anti-c-Myc antibody. Lanes labeled 18, 23, 34, 38, and 40, Ang1-expressing cell clones. Lanes labeled 20, 27, 35, 43, and 50, Ang2-expressing cell clones. Ang1 or Ang2 proteins derived from U87MG cells ran at 72 kDa. (*B*) Tyrosine phosphorylation of Tie2 in HUVEC cells. (*Upper*) Tie2 proteins. (*Lower*) Phosphorylated tyrosine in the Tie2 protein (*p*-Tyr). The purified recombinant Ang2 proteins were also able to inhibit Ang1-stimulated Tie2 tyrosine phosphorylation in HUVEC cells (data not shown). (*C*) Lack of expression of Tie2 in parental U87MG and U87MG/Ang2 cell clones. Synthesized first-strand cDNA from total cellular RNA were used for PCR reactions. The length of synthesized cDNA fragment of Tie2 is 512 bp. MW, DNA molecular weight marker. Lanes labeled 20, 27, 35, 43, and 50, Ang2-expressing cell clones. HMVEC, human dermal vascular EC. The experiments were performed three individual times with similar results.

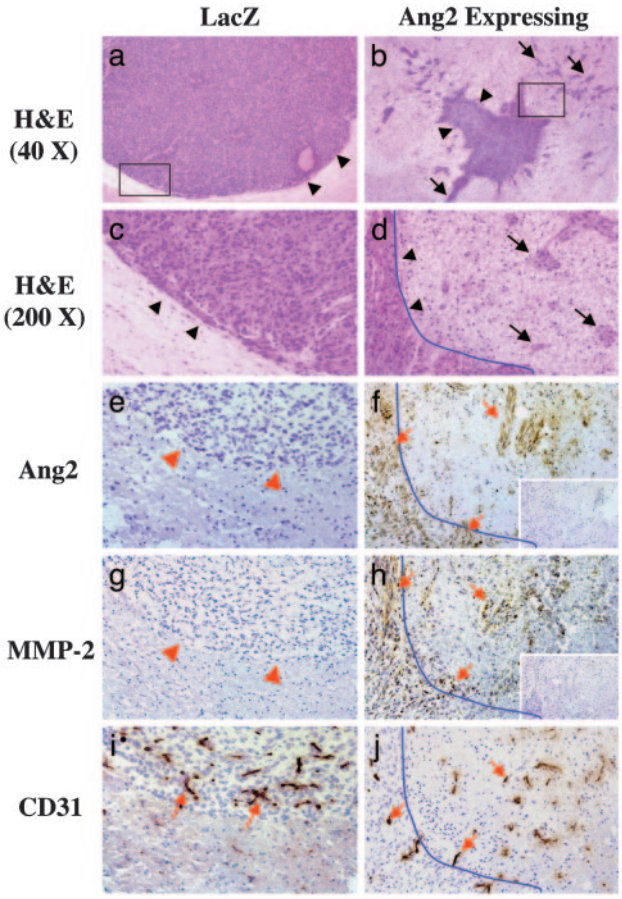

Fig. 3. Overexpression of Ang2 by U87MG cells induces glioma invasion in brains. IHC on various U87MG gliomas established in mouse brains. (*a–d*) Hematoxylin/eosin staining. (*a* and *c*) (Enlarged area indicated by a rectangle in *a*), glioma established by control U87MG/LacZ cells. (*b* and *d*) (Enlarged area indicated by a rectangle in *b*), invasive glioma formed by U87MG/Ang2 cells. Arrowheads in *a* and *b* indicate tumor mass. Arrowheads in *c* indicate the clean edge of U87MG/LacZ tumor spheroid. Arrows in *b* and *d* show invasive spikes as well as disseminated tumor clusters of U87MG/Ang2 gliomas. (*e–j*) IHC on serial sections of U87MG/LacZ or U87MG/Ang2 gliomas using an anti-Ang2 antibody (*e* and *f*), an anti-MMP-2 antibody (*g* and *h*), and an anti-CD31 antibody (*i* and *j*). Arrowheads in *e* and *g* indicate the clean edges of U87MG/LacZ tumor spheroid. A blue line in *d*, *f*, *h*, and *j* marks the tumor edge. Arrows in *f* and *h* show the invasive tumor-spike and disseminated tumor clusters that expressed Ang2 (*f*) or MMP-2 (*h*). Arrows in *i* and *j* indicate stained tumor vessels. *Insets* in *f* and *h*, negative controls (see Fig. 1). Ten or more individual samples in each class were analyzed. The experiments were repeated two additional times with similar results. (Original magnifications: *a* and *b*, ×40; *c–j*, ×200.)

was up-regulated in the Ang2-expressing gliomas (a 2- to 5-fold increase when compared with that in U87MG/LacZ tumors, Fig. 3*h*), whereas no expression of the MMP-2 proteins was detected in the control tumors (Fig. 3*g*). Because MMP-2 is an ECM protein, the protein stains detected in the invasive Ang2 tumors were relatively diffusive. The highest expressions of Ang2 or MMP-2 (a 4- to 5-fold increase when compared with that in U87MG/LacZ tumors) were found at the invasive fronts as well as in the disseminated tumor clusters of the U87MG/Ang2 gliomas (arrows in Fig. 3 *f* and *h*).

Overexpression of Ang2 Stimulated Vessel Growth at the Invasive Fronts of the U87MG/Ang2 Gliomas. To determine whether tumor angiogenesis was correlated with Ang2-induced glioma invasion, we assessed vessel growth in the various U87MG gliomas. In contrast to well neovascularized control U87MG/LacZ gliomas within the tumor mass (arrows in Fig. 3*i*, MVD: 299.0 ± 63.1 object areas/mm^2, $n = 5$), increased angiogenesis was most evident in the peripheral areas of the U87MG/Ang2 gliomas. Stimulated vessels surrounded the invasive forks as well as disseminated tumor cell clusters (arrows in Fig. 3*j*, MVD: 1,646.7 ± 485.6 object areas/mm^2, $n = 5$). Thus, overexpression of Ang2 stimulated host vessel growth in front of invading or disseminated tumor clusters.

Ang2 Stimulated Human Glioma Cell Invasion *in Vitro*. The aggressive phenotype of U87MG/Ang2 glioma cell invasion into adjacent brain parenchyma prompted us to determine whether Ang2 is capable of directly stimulating the invasiveness of Tie2-deficient tumor cells. We performed an *in vitro* assay that assesses the invasiveness of various glioma cells through membranes coated with Matrigel or VN (14, 15). As shown in Fig. 4*A*, U87MG/Ang2 cells had a 4-fold increase in invasion compared with that of parental U87MG cells. When an anti-Ang2 antibody (10 μg/ml) was included in the invasion assays, the cell invasion stimulated by Ang2 overexpression was inhibited by >50%. To further establish that the induced invasiveness of U87MG/Ang2 cells was the result of the effect of Ang2 on U87MG cells and not from other effects caused by Ang2 overexpression, we purified recombinant Ang2 proteins from CM collected from the U87MG/Ang2 cells (data not shown). We then determined whether those recombinant Ang2 proteins were capable of promoting U87MG cell invasion *in vitro*. Exposure of the U87MG cells to recombinant Ang2 resulted in a concentra-

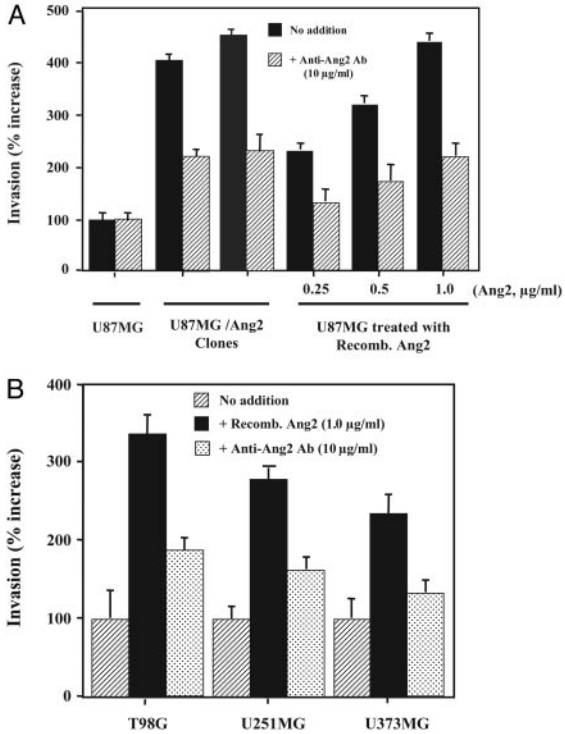

Fig. 4. Ang2 stimulates glioma cell invasion *in vitro*. (*A*) U87MG/Ang2 cell clones or parental U87MG cells that were treated with recombinant Ang2 protein at various concentrations have elicited capacity of cellular invasion through the Matrigel-coated membranes. (*B*) Recombinant Ang2 (1 μg/ml) stimulates T98MG, U251MG, and U373MG glioma cell invasion. In both sets of experiments, a goat anti-Ang2 antibody (10 μg/ml) was included in some assays. The data are shown as means; bars, $\pm$SD. The assay was performed in triplicate three independent times with similar results.

tion-dependent stimulation. Treatment by 1.0 μg/ml of the Ang2 protein caused a 4-fold increase in the number of U87MG cells invading through the Matrigel- (Fig. 4*A*) or VN- (data not shown) coated membranes compared with that of untreated controls. A similar effect was achieved by using a commercially available recombinant Ang2 protein (data not shown). Importantly, the recombinant Ang2-stimulated cell invasion was inhibited by the anti-Ang2-antibody (10 μg/ml). Furthermore, invasion by T98G, U251MG, or U373MG glioma cells was also strongly promoted by exposure to the recombinant Ang2 protein (1.0 μg/ml), and such effects were ablated by the anti-Ang2 antibody (Fig. 4*B*). Thus, Ang2 is capable of stimulating Tie2-deficeint glioma cell invasion both *in vivo* (Fig. 3) and *in vitro* (Fig. 4)

Ang2 Stimulated Activation of MMP-2 in Human Glioma Cells. We next determined whether Ang2 expression or exposure to the recombinant Ang2 protein could stimulate activation of MMP-2 in human glioma cells. To promote tumor invasion, pro-MMP-2, which is a secreted enzyme, has to be converted to fully activated MMP-2 through proteolysis (3). Therefore, we determined whether Ang2 caused MMP-2 to be activated in the U87MG cells by using an *in situ* zymography assay (13) with CM collected from parental U87MG or Ang2-expressing cells. As shown in Fig. 5*A*, increased activities of the intermediate (64 kDa) and active forms of MMP-2 (62 kDa) were found in the CM of the Ang2-expressing cells. When parental U87MG cells were stimulated with the purified recombinant Ang2 or a commercially available Ang2 (data not shown), a concentration-dependent increase in the activation of MMP-2 was found in the Ang2-treated U87MG cells (Fig. 5*A*). We then tested whether Ang2 could activate MMP-2 in other glioma cells by

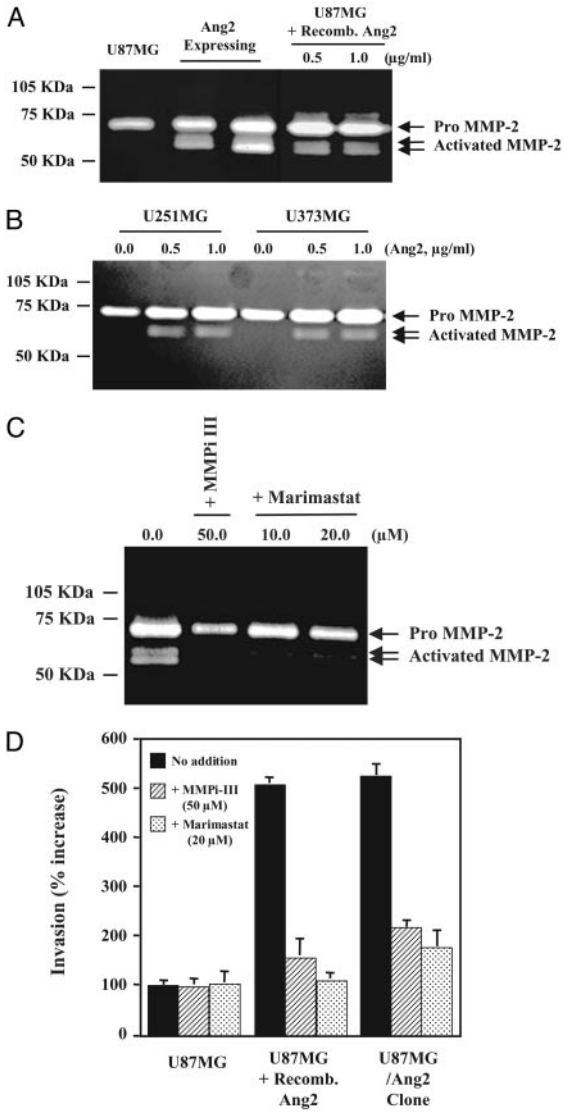

Fig. 5. Ang2 stimulates glioma cell invasion through the activation of MMP-2 in glioma cells *in vitro*. (*A–C*) Zymography analyses of MMP-2 activity in CM collected from various cells. (*D*) *In vitro* cell invasion through Matrigel. (*A*) Overexpression of Ang2 in U87MG cells or treatments on parental U87MG by recombinant Ang2 protein stimulate the activation of MMP-2. (*B*) Recombinant Ang2 stimulates the activation of MMP-2 in U251MG or U373MG glioma cells. (*C*) MMP inhibitors, MMP inhibitor III (50 μM), or Marimastat (10 or 20 μM) inhibit the activation of MMP-2 in U87MG/Ang2 cells. The Pro-MMP-2 ran at 72 kDa, the intermediate form of MMP-2 ran at 64 kDa, and the mature form of MMP-2 ran at 62 kDa. (*D*) MMP inhibitors, MMP inhibitor III (50 μM), or Marimastat (20 μM) inhibit Ang2-stimulated glioma cell invasion. The experiments were performed three independent times with similar results.

stimulating U251MG or U373MG glioma cells with the recombinant Ang2. As shown in Fig. 5*B*, the enzymatic activities of MMP-2 in both types of cells were increased by such exposure.

Ang2 Promotion of *in Vitro* Human Glioma Cell Invasion Is Mediated Through Activation of MMP-2. Having demonstrated that Ang2 stimulated the activation of MMP-2 in glioma cells, we next examined whether MMP-2 activation by Ang2 modulates glioma cell invasion. We first determined whether the three MMP inhibitors, Marimastat, MMP inhibitor III, or GM6001 (16), were capable of inhibiting Ang2-stimulated MMP-2 activities in the glioma cells. We added the MMP inhibitors separately into cell cultures of U87MG/Ang2 or parental U87MG cells that had been

stimulated by the recombinant Ang2. Exposure to 50 μM of MMP inhibitor III, 20 μM of Marimastat (Fig. 5*C*), or 50 μM of GM6001 (data not shown) effectively inhibited the Ang2-stimulated enzymatic activities of MMP-2 in U87MG/Ang2 or the Ang2-stimulated U87MG glioma cells.

Finally, we determined whether Ang2-stimulated *in vitro* cell invasiveness was mediated by modulation of the activation of MMP-2 in glioma cells. We performed the cell invasion assays by using U87MG/Ang2 or parental U87MG cells treated with recombinant Ang2, in the presence or absence of MMP inhibitor III (50 μM) or Marimastat (20 μM). Both MMP inhibitors prevented glioma cell invasion stimulated by Ang2 either by overexpression of Ang2 or by exposure to the recombinant Ang2 (Fig. 5*D*). Thus, Ang2 promotes human glioma cell invasion through the activation of MMP-2 in glioma cells both *in vivo* (Fig. 3) and *in vitro* (Figs. 4 and 5).

Discussion

Although the diverse roles of Ang2 in tumor progression have been studied extensively, investigations of its functions have been primarily focused on its action on angiogenesis through a Tie2-dependent pathway in ECs (6). Increased expression of Ang2 in human tumor tissues or enhanced tumor growth by overexpressing Ang2 in xenografted tumors has been attributed to the effects of Ang2 on ECs (7–11). For example, a recent study that nicely describes a role of Ang2 in gastric cancer progression ascribes the effect of Ang2 on tumor metastases and dissemination to the stimulation of Ang2 on ECs (9). In this report, we present evidence that Ang2 can also directly induce glioma cell invasion in the absence of detectable Tie2 expression. Ectopic expression of Ang2 by U87MG glioma cells conferred an aggressively invasive phenotype to otherwise spheroid-shaped U87MG gliomas. The *in vivo* induction of U87MG glioma invasion by Ang2 was accompanied by increased vessel growth only at the invasive fronts (tumor-adjacent brain parenchyma) of the invading U87MG/Ang2 tumors. Ang2 was also capable of directly inducing invasive behaviors by Tie2-deificent glioma cells *in vitro* where it increased their abilities to invade through Matrigel or VN. These activities were apparent whether the tumor cells expressed the Ang2 or were exposed exogenously to it. Thus, Ang2 is capable of affecting glioma invasion both through its direct activity on Tie2-deficient tumor cells as well as its well documented activity through the Tie2 receptor on EC.

It has been reported that increased expression of MMP-2, MMP-9, and membrane type-1 MMP (MT1-MMP) is correlated with the invasive phenotype of glioma and other types of tumors (2, 17). MT1-MMP is involved in the activation of MMP-2 (17). Although inhibition of MMP-2 expression suppresses the invasiveness of tumor cells in several model systems (14, 18, 19), which molecules induce MMP-2 activation during tumor development has not been defined (17). Our results demonstrate that Ang2 can function as such an inducer and thereby affect glioma cell invasiveness. Interestingly, we also found that MT1-MMP was coexpressed with Ang2 and MMP-2 in the invasive regions in clinical glioma specimens and the Ang2-expressing gliomas (data not shown), suggesting a role for it in Ang2-induced glioma invasion. In contrast, we found diffuse expression of MMP-9 in the invasive Ang2-expressing gliomas, suggesting that it was produced by tumor stroma (20). The responsiveness of the glioma cells to Ang2 even though they lack detectable Tie2 expression raises the question of the details of Ang2 signaling in this case. However, it has been recently reported that Ang2 interacts with certain cell surface integrins to support adhesion for fibroblasts and EC *in vitro* through a Tie2-independent pathway (21). Currently, we are investigating the involvement of integrins and other molecules in modulating the activation of MMP-2 during the Ang2-induced glioma invasion.

In addition to MMPs, compelling evidence shows that integrins (22), urokinase-like plasminogen activator receptor (23), osteopontin, tenasin (24), and SPARC (25) are also involved in glioma invasion. SPARC is an ECM-binding protein that is up-regulated in human primary gliomas. Overexpression of SPARC by U87MG cells induced glioma cell invasion *in vitro* as well as *in vivo* (26). Our studies demonstrate that Ang2 has a similar capacity of inducing glioma cell invasion. Analyses of parental U87MG, LacZ-, or Ang2-expressing cells or various xenografted gliomas derived from these cells showed similar levels of SPARC expression (data not shown). We thus postulate that both SPARC and Ang2 are able to induce glioma invasion with similar phenotypes in brain, and that these two molecules may act independently or synergistically during the glioma progression.

In summary, our data show that in addition to modulating vessel growth, Ang2 is capable of causing MMP-2 activation and thereby inducing glioma cell invasion clearly *in vitro* and may also be of relevance to the *in vivo* setting. We have established a system that should prove useful in deciphering the pathophysiological mechanisms underlying the invasive behaviors of glioma cells *in vivo* and *in vitro*. Defining the pathways by which Ang2 induces tumor invasion could provide critical information with regard to the potential of Ang2 and its effectors as new therapeutic targets in glioma treatment.

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