(ADR) which alone has a cytotoxic effect to glioma cells[22] and which is improved in combination with hyperthermia, even in ADR-resistant cells[10]. Despite its great anti-neoplastic potential the clinical application of ADR is limited since the blood–brain barrier (BBB) restricts the transport of the water-soluble drug to the tumour. It was further reported that hyperthermia could increase the permeability of the BBB[18,23]. Thus, the combination of ADR and hyperthermia should have a greater efficacy with a glioma than single treatment modalities. Based on experience on interstitial hyperthermic therapy for malignant human gliomas[24–28], the therapeutic effect of IA chemotherapy with ADR in combination with interstitial hyperthermia was studied using an established tumour model for rat brain.

2. Materials and methods

2.1. *Brain tumour model*

The malignant C_6 glioma growing in the brain of male Wistar rats (body weight 250–300 g) was used for these studies. Animals were housed in animal care facilities and received a standard diet and acidified water *ad libitum*. Tumours were inoculated by injection of $\sim 10^6$ C_6 glioma cells/10 µl through a borehole into the right part of the brain of anaesthetized rats. The site of inoculation was at a depth of 5 mm beneath the skull surface, 3 mm lateral to the midline, and 2 mm posterior to the coronal suture. C_6 glioma cells (Japanese Cancer Research Resources Bank) for injection were maintained in a monolayer culture in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated foetal bovine serum.

2.2. *Hyperthermia and treatment procedure*

Treatment of tumours in animals anaesthetized with 2–2.5% halothane was performed at day 10 or 12 after tumour inoculation when tumours had grown to a size of 5 and 6 mm in diameter, respectively. Local tumour heating was carried out interstitially using a golden needle antenna (0.9 mm in diameter) designed at the institute and operated at a radiofrequency of 13.56 MHz[25–27]. The antenna was inserted into the centre of the tumour through the same borehole used for tumour inoculation. A thermocouple was placed at a distance of 2.5 or 3.0 mm from the antenna (for 10- or 12-day old tumours, respectively) to maintain the temperature at the tumour margin at 40°C. This was achieved by a temperature control unit of the RF-generator. Previous studies on the temperature distribution in rat gliomas have shown that during the RF heating of the tumour, the temperature of the surrounding normal brain tissue (3 mm apart) remained below 39°C during the time of heating[44]. ADR treatment was defined to be when the pre-set temperature of 40°C was reached. This was achieved $\sim$ 5 min after the start of heating and was maintained for 15 min, the time of ADR infusion. Adriamycin (ADR, Kyowa Hakko Kogyo Comp, Japan) at a dosage of 1 mg/kg body weight was infused within 15 min into the rat via the tail vein (i.v.) or via the carotid artery (i.a.) using an infusion pump (Microsyringe pump, type KDS-200, Neuroscience Comp.). The intracarotid infusion of the rat has been described previously[29]. Briefly, a polyethylene catheter (PE-10) was inserted into the external carotid artery in retrograde manner up to the bifurcation of the common carotid artery ipsilateral to the brain tumour. After the treatment, the catheter was removed and the external carotid artery was ligated.

2.3. *Biological parameters*

The effectiveness of the various treatments was investigated by determining the following biological parameters: ADR concentration in the tumour, median survival rate of the animals, and tumour necrosis.

2.4. *Tumour ADR uptake*

Adriamycin uptake was determined in 12-day old tumours treated with ADR administered intravenously (group A), intra-arterially (group B), intravenously in combination with hyperthermia (group C), and intra-arterially in combination with hyperthermia (group D). In each group, eight tumour-bearing rats had been treated. Immediately after the treatments, rats were decapitated and their brain tumours were removed. The concentration of ADR in the tumour was determined using high performance liquid chromatography (HPLC) with a fluorescence detector. Differences in ADR content of the groups were statistically analysed with the Fisher PLSD test.

2.5. *Survival rate*

The survival rate of the animals after the various treatments was studied by means of Kaplan-Meier plots. Animals 10 days after tumour inoculation were separated into six groups consisting of eight rats each: group A (ADR administered i.v.), group B (ADR i.a.), group C (ADR i.v. and hyperthermia), group D (ADR i.a. and hyperthermia), group E (hyperthermia alone), and group F. (untreated control). Differences in the survival rates among the groups were analysed using the Wilcoxon test.

2.6. *Histopathological studies*

It has been described that heat-induced morphological changes in normal brain and brain tumour are after several days more distinctly expressed rather than immediately after treatment[25,26,30–32]. Therefore, tumour and brain tissue were studied 7 days after treatment with intra-arterial ADR infusion and interstitial hyperthermia. Five brain tumours of groups D (ADR i.a. and hyperthermia) and F (untreated control) were histologically investigated. The rat brain with the tumour was totally removed, and after a horizontal cut slices of ~2 mm of the equatorial plane of the tumour were taken for routine paraffin histology. Tissue specimens of 3 μm were stained with haematoxylin and eosin (H&E).

3. Results

3.1. *ADR concentration in tumour*

Figure 1 shows the uptake of ADR in C_6 gliomas after the various treatments. An intravenous or intra-arterial infusion of ADR (groups A and B) led nearly to the same concentrations in the tumours. Differences in ADR concentrations were observed in combination with hyperthermia (groups C and D). Whereas only a little increase in the ADR concentration after i.v. and additional heating was found, a considerable increase after intra-arterial ADR infusion and hyperthermia was obtained. The ADR concentration in the tumour increased from 186 ± 41 ng/g after i.a. administration alone to 1058 ± 236 ng/g in combination with interstitial hyperthermia. This increase of ADR uptake is a factor of ~5, which is highly significant ($p < 0.05$) in comparison to all the other groups (A, B and C). Thus,

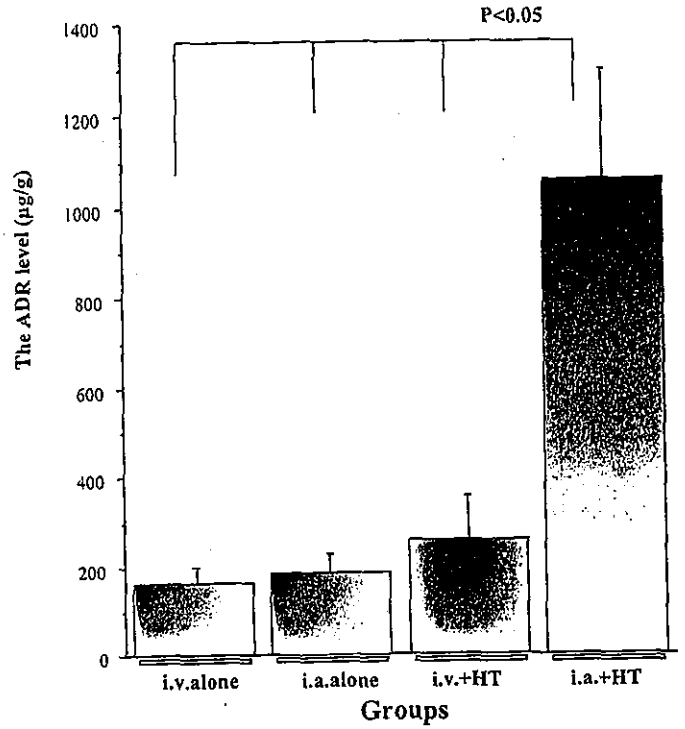


Figure 1. Concentrations of adriamycin (ADR) in rat gliomas C_6 measured by HPLC immediately after intravenous (i.v.) and intra-arterial (i.a.) administration alone or in combination with interstitial hyperthermia (HT). Each bar represents the mean value ± SD. The ADR level after treatment with i.a. ADR and hyperthermia was significantly higher compared to the other treatments ($p < 0.05$).

one potential advantage of this combined treatment is that it selectively enhances the uptake of the drug in the tumour.

3.2. *Survival time*

Figure 2 shows the Kaplan-Meier plots of the survival of rats after the various treatments. The shortest overall survival was obtained for those animals whose gliomas had not been treated (group F) or had been treated with intravenous or intra-arterial ADR only (group A and B). Interstitial hyperthermia alone or in combination with ADR administration intravenously both showed slightly longer survival times which were not significantly different in comparison to each other (group C and E). The longest survival time (46 days) was achieved when ADR was administered intra-arterially and combined with hyperthermia (group D). The difference of the median survival time was significant ($p < 0.05$) in comparison to the other treated groups. Single drug administration did not prolong the survival time regardless of the method of infusion (i.v. or i.a.). Interstitial hyperthermia alone of the C_6 glioma did not result in a greater survival time. Table 1 summarizes the median survival times for the treatments applied.

3.3. *Histopathological studies*

Figure 3(*a*) shows the light microscopic picture of a C_6 glioma in the rat brain 7 days after inoculation. The section through an equatorial plane of the brain demonstrates a massive tumour in the right half of the brain which is invasively growing into the brain tissue. After a single treatment with intra-arterial ADR infusion and

Table 1. Mean survival times of Wistar rats with malignant gliomas C_6 after various treatments with adriamycin (ADR) in combination with interstitial hyperthermia (HT).

Treatment	Number of tumors	Mean survival times (days)
Control	8	6.4 ± 0.8
ADR i.v.	8	7.1 ± 1.2
ADR i.a.	8	7.8 ± 0.9
HT only	8	7.9 ± 1.7
ADR i.v. + HT	8	10.9 ± 1.9
ADR i.a. + HT	8	24.1 ± 4.8

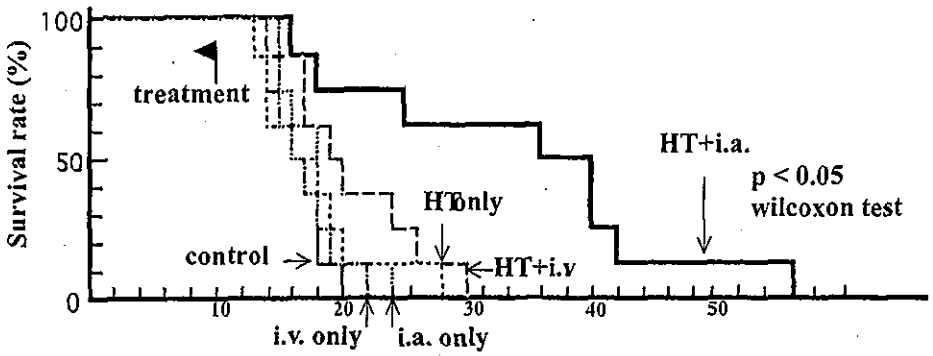


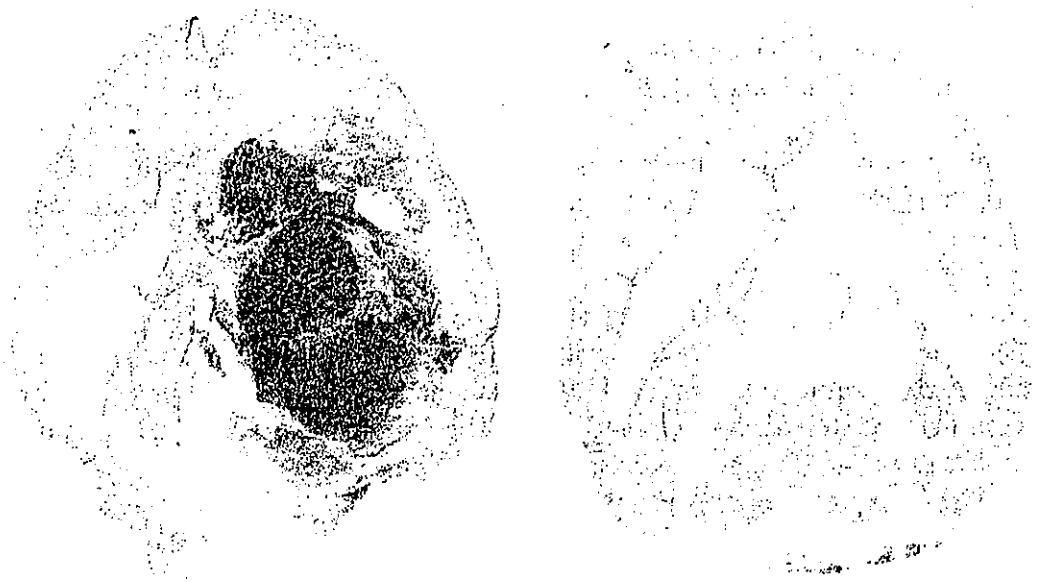
Figure 2. Survival curves (Kaplan-Meier plots) of Wistar rats after single treatment of gliomas C_6 tumours with adriamycin, administered intravenously (i.v.), intra-arterially (i.a.) alone, or in combination with interstitial hyperthermia (HT), with hyperthermia alone, and without any treatment (control). Rats were treated 10 days after tumour inoculation. A significant prolongation of the survival time was achieved by treating the tumour with i.a. ADR and hyperthermia.

interstitial hyperthermia (figure 3(*b*)), the tumour tissue from the centre to the tumour margin becomes completely necrotic. The surrounding normal brain tissue shows no signs of tissue damage and still retains its normal morphology.

4. Discussion

4.1. *Effects of the combination therapy of rat gliomas using intra-arterial chemotherapy and local tumour heating*

It is generally supposed that brain tumours respond poorly to chemotherapy because the blood–brain barrier (BBB) and the blood–tumor barrier (BTB) both restrict an adequate delivery of antineoplastic drugs to tumours[33–35]. This is supported by the studies on lipid-soluble drugs like nitrosoureas which are most effective[36–38]. Intra-arterial chemotherapy of brain tumours has been considered favourable for drug delivery. In this way, large amounts of drugs via blood vessels can be transferred to the tumour and their uptake in the tumour tissue after the first circulation can be increased. Drugs for brain tumours has been administered by intracarotid infusion of mannitol to overcome the BBB and BTB of animals[5] and humans[6,7]. Hyperosmotic barrier opening drugs resulted in an acceptable response of human malignant brain tumours[9]. However, the therapeutic effect of this therapy has certain limitations since these drugs are also potentially cytotoxic to the normal brain[39,40]. Therefore, the aim of an intra-arterial chemotherapy should be to selectively achieve a high drug concentration in the tumour without any side effects



(*a*) (*b*)

Figure 3. Light microscopic appearance of the glioma C_6 in the rat brain. (*a*) Untreated tumour, day 17 after tumour inoculation. Tumour is characterized by growing invasively in the brain. (*b*) Tumour 7 days (day 17 after tumour inoculation) after single treatment with interstitial hyperthermia and adriamycin administered intra-arterially. The tumour tissue shows complete necrosis, the surrounding normal brain tissue still retains its normal structures.

in the normal tissue. To reach this goal, several unique techniques and new drugs have been investigated up to now, such as RMP-7 (Bradykinin Analogue)[2], calcium antagonists[3], angiotensin II[8], and theophylline[4]. The previous studies are positive. Another possibility to enhance the efficacy of a drug in a tumour is the combination of IA-chemotherapy with interstitial hyperthermia.

Two important factors that affect intra-arterial drug delivery to malignant brain tumours are the blocking by the BBB and the regulation of the cerebral blood flow (CBF). It is well known that hyperthermia increases the transport of drugs to the tumour and increases the permeability of the BBB[42,23]. Ohmoto *et al.*[43] reported that the CBF was considerably enhanced to ~ 130–200% in the temperature range of 41–43°C as compared to normal CBF. Moreover, several investigators reported that hyperthermia enhances the cytotoxic effect of several chemotherapeutic drugs including ADR[10,13–15,17–19,22].

The results and findings of this study have clearly demonstrated that a combination therapy of a rat glioma with interstitial hyperthermia and intra-arterial ADR administration significantly (1) increased the drug concentration in the tumour, (2) enhanced significantly the survival time of the animals, and (3) induced tumour necrosis without damaging the surrounding normal brain tissue. Moreover, side effects like disorder in the mobility, visual faculty and body weight of the animals were not observed during the survival study. The most surprising point was that a remarkable higher uptake of the water-soluble adriamycin (ADR) was achieved in the tumour by additional local heating (figure 1). This may be based on the enhancement of tumour blood flow by hyperthermia. Since the tumour is not heated homogeneously by the RF-antenna, tumour blood flow will depend on the local tumour temperatures as well as the damage of the tumour cells and the changes in the

tumour micromilieu[41]. Therefore, the higher therapeutic effect of the combined treatment (figure 2, ADR i.a. + HT) can be explained in part by the effect of the drug itself, by the enhanced cytotoxicity of hyperthermia, and by the hyperthermic toxicity.

Consequently, a combined treatment of brain tumours with interstitial hyperthermia resulted in a higher therapeutic potential in comparison to chemotherapy alone.

4.2. *Clinical implication and future prospects*

The interstitial hyperthermia device developed at the institute is suitable for heating brain tumours locally. The RF antenna can be inserted into the brain tumours using a stereotactic apparatus[25,26]. The antenna can adequately and safely heat the tumour with no side effects, even in the case of deep-seated tumours[39]. In most cases, complete tumour response after hyperthermia treatment was diagnosed by contrast enhanced CT or MRI. In some cases, however, where the tumour was located at an eloquent area and/or was of rather irregular shape, complete tumour necrosis could not always be achieved. The treatment of brain tumours with interstitial hyperthermia and intra-arterial chemotherapy is performed under light local anaesthesia and can be applied even to older patients who cannot undergo surgical operation because of the high risks. The present study indicates that other antitumour agents could also be applied in combination with local hyperthermia for the treatment of malignant brain tumours, regardless of the BBB problem. Based on the advantages of this combination therapy, a clinical study on brain tumours in the hospital has just been started.

5. Conclusion

These results of the study on a rat brain tumour model indicate that a combined treatment of localized interstitial hyperthermia and intra-arterial chemotherapy with adriamycin was significantly more effective than single treatments of hyperthermia or chemotherapy. A combination of hyperthermia and intravenous administration of adriamycin was therapeutically less effective. Tumour heating in the brain with the RF-antenna was reliable and adequate with regard to the temperature (40°C) at the tumour rim. Thus, this method can be recommended for a successful treatment of malignant brain tumours.

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References

1. Walker RW, Dantis E, Shapiro WR. Treatment of recurrent glioma with carboplatin. *Proc Am Soc Clin Oncol* 1987; 6: 72.
2. Matsukado K, Nomura T, Ikezaki K, Fukui M. Selective increase in blood-tumor barrier permeability by calcium antagonists in transplanted rat brain tumors. *Acta Neurochir* 1994; 60 (Suppl): 403–5.

3. Matsukado K, Inamura T, Nakano S, Fukui M, Bartus RT, Black KL. Enhanced tumor uptake of carboplatin and survival in glioma-bearing rats by intracarotid infusion of bradykinin analog, RMP-7. *Neurosurgery* 1996; 39: 125–34.
4. Natori Y, Baba T, Moriguchi M, Tkeshita I, Fukui M. Effects of theophylline on the selective increases in intratumoral blood flow induced by intracarotid infusion of adenosine and adenosine triphosphate in C6 glioma-transplanted rat brains. *Surg Neurol* 1992; 37: 8–14.
5. Neuwelt EA, Barnett PA, McCormick Cl, Frenkel EP, Minna JD. Osmotic blood–brain barrier modification: monoclonal antibody, albumin, and methotrexate delivery to cerebrospinal fluid and brain. *Neurosurgery* 1985; 17: 419–23.
6. Neuwelt EA, Hill SA, Frenkel EP. Osmotic blood–brain barrier modification and combination chemotherapy: concurrent tumor regression in areas of barrier opening and progression in brain regions distant to barrier opening. *Neurosurgery* 1984; 15: 362–6.
7. Neuwelt EA, Rapoport SI. Modification of the blood–brain barrier in the chemotherapy of malignant brain tumors. *Fed Proc* 1984; 43: 214–9.
8. Ogasawara H, Uozumi T, Kiya K, Kurisu K, Mikami T, Hotta T, *et al.* Analysis and distribution of etoposide in rat brain tumor model: intracarotid versus intracarotid with angiotensin 2-induced hypertension. *Cancer Invest* 1993; 11: 299–305.
9. Willams PC, Henner WD, Roman-Goldstein S, Dahlborg SA, Brummett RE, Tableman M, *et al.* Toxicity and efficacy of carboplatin and etoposide in conjunction with disruption of the blood–brain tumor barrier in the treatment of intracranial neoplasms. *Neurosurgery* 1995; 37: 17–28.
10. Asami J, Kawasaki S, Nishikawa K, Kkuroda M, Hiraki Y. Effects of hyperthermia and cepharanthin on Adriamycin accumulation with changes in extracellular pH. *Int J Hyperthermia* 1995; 11: 27–35.
11. Colombo R, Lec A, Da Pozzo LF, Freschi M, Gallus G, Rigatti P. A new approach using local combined microwave hyperthermia and chemotherapy in superficial transitional bladder carcinoma treatment. *J Urol* 1995; 153: 959–63.
12. Di Filippo F, Carlini S. Local hyperthermia and systemic chemotherapy for treatment of recurrent melanoma. *World J Surg* 1995; 19: 359–62.
13. Kawai H, Minamiya Y, Kitamura M, Matsuzaki I, Hashimoto M, Suzuki H, *et al.*: Direct measurement of doxorubicin concentration in the intact, living single cancer cell during hyperthermia. *Cancer* 1997; 79: 214–9.
14. Leibovici J, Klorin G, Huszar M, Hoenig S, Klein O, Michowitz M, *et al.* Sensitivity to thermochemotherapy of AKR lymphoma and B16 melanoma variants of malignancy. *Clin Exp Metastasis* 1990; 8: 33–46.
15. Los G, van Vugt MJ, Pinedo HM. Response of peritoneal solid tumours after intraperitoneal chemohyperthermia treatment with cisplatin or carboplatin. *Br J Cancer* 1994; 69: 235–41.
16. Lui RY, Matsumoto K, Kunishio K, Mizumatsu S, Tamiya T, Furuta T, *et al.* Proliferrative potential and apotosis in rat glioma cell lines after hyperthermia. *Neurologia Medico-chirurgica (Tokyo)* 1998; 38: 196–202.
17. Miyahara T, Ueda K, Akaboshi M, Shimada Y, Imamura M, Utsumi H. Hyperthermic enhancement of cytotoxicity and increased uptake of cis-diamminedichloroplatinum(II) in cultured human esophageal cancer cells. *Jpn J Cancer Res* 1993; 84: 336–40.
18. Sakaguchi Y, Maehara Y, Inutsuka S, Takahashi I, Yoshida M, Emi Y, *et al.* Laser flow cytometric studies on the intracellular accumulation of anthracyclines when combined with heat. *Cancer Chemother Pharmacol* 1994; 33: 371-7.
19. Siegal A, Hoenig S, Liebovici J. Combined effect *in vitro* of chemotherapy with agents acting on the cell membrane of lewis lung carcinoma. *Chemotherapy* 1990; 36: 230–9.
20. Sugimachi K, Kuwano H, Ide H, Toge T, Saku M, Oshimi Y. Chemotherapy combined with or without hyperthermia for patients with oesophageal carcinoma: a prospective randomized trial. *Int J Hyperthermia* 1994; 10: 485–93.
21. Tsukiyama I, Ogino T. Hyperthermia for bone and soft tissue sarcoma: relationship between computerized tomographic and histological findings. *Radiat Med* 1994; 12: 231–6.
22. Watanabe M, Tanaka R, Hondo H, Kuroki M. Effect of antineoplastic agents and hyperthermia on cytotoxicity toward chronically hypoxic glioma cells. *Int J Hyperthermia* 1992; 8: 131–8.

23. Vaupel PW. Effects of physiological parameters on tissue response to hyperthermia: new experimental facts and their relevance to clinical problems. In Gerner EW, Cetas TC, eds, *Hyperthermic Oncology* 1992. Arizona Board of Regents, 1993; 2: 17–23.
24. Hoopes PJ. The effects of heat on the nervous system. In Gutin PH, Leibel SA, Sheline GE, eds, *Radiation Injury to the Nervous System*. New York: Raven Press, 1992; 407–30.
25. Takahashi H, Tanaka R, Hondo H *et al.* Clinical experiences of RF hyperthermia for malignant brain tumors. *Neurosurgeons* 1993; 12: 245–55 (in Japanese).
26. Takahashi H, Tanaka R, Watanabe M, Kakinuma K, Suda T, Takahashi S, *et al.* Clinical result of RF interstitial hyperthermia for malignant brain tumors. Japanese J of Hyperthermic Oncology 1995(Jpn);11:61-67.
27. Tanaka R, Kim CH, Yamada N, Saito Y. Radiofrequency hyperthermia for malignant brain tumors. *Neurosurgery* 1987; 21: 478–83.
28. Uzuka T, Tanaka R, Takahashi H, Kakinuma K, Matsuda J, Kato K. Planning of hyperthermic treatment for malignant glioma using computer simulation. *Int J Hyperthermia* 2001; 17: 114–22.
29. Shem BC, Krossnes BK. Enhancement of ACNU treatment of the BT_4An rat glioma by local brain hyperthermia and intra arterial drug administration. *Euro J Cancer* 1995; 11: 1860–74.
30. Takahashi S, Tanaka R, Watanabe M, Takahashi H, Kakinuma K, Suda T, *et al.* Effects of whole-body hyperthermia on the canine central nervous system. *Int J Hyperthermia* 1999; 15: 203–16.
31. Kakinuma K, Tanaka R, Takahashi H. Targeting chemotherapy for malignant brain tumour using thermosensitive liposome and localized hyperthermia. *J Neurosurg* 1996; 84: 180–4.
32. Lyons BE, Obana WG, Borcich JK, Kleinman R, Singh D, Britt RH. Chronic histological effects of ultrasonic hyperthermia on normal feline brain tissue. *Radiat Res* 1986; 106: 234–51.
33. Fenstermacher JD, Cowles AL. Theoretic limitations of intracarotid infusion in brain tumor chemotherapy. *Cancer Treat Rep* 1977: 61: 519–26.
34. Groothuis DR, Fisher JR, Lapin G, Bigner, DD, Vick NA. Permeability of different experimental brain tumor models to horseradish peroxidase. *J Neuropathol Exp Neurol* 1982; 41: 164–85.
35. Neuwelt EA, Barnett PA, Bigner DD, Frenkel EP. Effects of adrenal cortical steroids and osmotic blood–brain barrier opening on methotrexate delivery to gliomas in the rodent: The factor of the blood-brain barrier. *Proc Natl Acad Sci USA* 1982; 79: 4420–3.
36. Levin VA, Kabra P. Effectiveness of the nitrosoureas as a function of their lipid solubility in the chemotherapy of experimental rat brain tumors. *Cancer Chemother Rep* 1974; 58: 787–92.
37. Levin VA, Shapiro WR, Clancy TP, Oliverio VT. The uptake distribution, and antitumor activity of 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea in the murine glioma. *Cancer Res* 1970; 30: 2451–5.
38. Tator CH, Day A, Ng R, Liberman L. Chemotherapy of an experimental glioma with nitrosoureas. *Cancer Res* 1977; 37: 476–81.
39. Hiesiger EM, Voorhies RM, Basler GA, Lipschutz LE, Posner JB, Shapiro WR. Opening the blood–brain and blood–tumor barriers in experimental rat brain tumors: the effect of intracarotid hyperosmolar mannitol on capillary permeability and blood flow. *Ann Neurol* 1986; 19: 50–9.
40. Inoue T, Fukui M, Nishino S, Kitamura K, Nagara H. Hyperosmotic blood–brain barrier disruption in brains of rats with an intracerebrally transplanted RG-C6 tumor. *J Neurosurg* 1987; 66: 256–63.
41. Hall EJ. *Radiology to the Radiologist*. Philadelphia: J.B. Lipincott Comp, 1988; 293–329.
42. Lin JC, Lin MF. Microwave hyperthermia-induced blood-brain barrier alternations. *Radiat Res* 1982; 89: 77–87.
43. Ohmoto R, Fujisawa H, Ishikawa T, Koizumi H, Matsuda T, Ito H. Sequential changes in cerebral blood flow, early neuropathological consequences and blood-barrier disruption following radiofrequency-induced localized hyperthermia in the rat. *Int J Hyperthermia* 1996; 12: 321–34.
44. Kakinuma K, Tanaka R, Kato M. Hyperthermia enhancement by thermosensitive liposome as a drug delivery system for malignant glioma chemotherapy. *Drug Del Sys* 1998; 13: 389–99.

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Review Article

New generation, conditionally replicating herpes simplex virus G47Δ as a potential backbone vector for expressing foreign proteins

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CONTENTS

Abstract

The use of replication-competent viruses that can selectively replicate, spread *in situ* and lyse malignant cells is an attractive strategy for treating cancer. G207 is a multimutated, conditionally replicating HSV-1 vector, and recent clinical trials in recurrent malignant glioma patients showed its safety when inoculated into human brain tumors. Whereas G207 has been shown to be effective in various types of cancer in animal models, its replication capability in tumor cells is considerably attenuated compared with wild-type HSV-1. In order to improve the antitumor efficacy, we have recently constructed a new generation vector, G47Δ, by creating an additional deletion in the *α47* gene of G207. G47Δ showed enhanced tumor cell killing and increased major histocompatibility complex class I expression in infected human cells. This paper reviews the circumstances that led to the development of G47Δ and the features of the vector that may be suitable as a backbone for expressing foreign molecules.

Introduction

The use of replication-competent viruses that can selectively replicate, spread *in situ*, and lyse malignant cells is an attractive strategy for treating cancer (1). It has been more than a decade, since genetically engineered herpes simplex virus type 1 (HSV-1) has been introduced as a suitable means for oncolytic virus therapy (2). However, development of replication-competent HSV-1 vectors adequate for clinical application has not been an easy task. The pathogenicity of the vector needs to be attenuated enough not to cause any damage to normal human tissue, while retaining its replication capability in tumor cells. G207 is a multimutated, conditionally-replicating HSV-1 vector created with an emphasis on safety (3), and recent clinical trials in recurrent malignant glioma patients showed that G207 can in fact be safely inoculated into human brains (4). Whereas G207 has shown efficacy in various types of cancer in animal models (5), its replication capability in tumor cells is also considerably attenuated compared with wild-type HSV-1. In order to improve the antitumor efficacy, we have recently constructed a new generation vector, G47Δ, by creating an additional deletion in the *α47* gene of G207 (6). G47Δ showed enhanced tumor cell killing and increased major histocompatibility complex (MHC) class I expression in infected human cells. In this paper, we review the circumstances that led to the development of G47Δ and the features of the vector that may be suitable as a backbone for expressing foreign molecules.

HSV-1 as a therapeutic bioreagent

Conditionally-replicating HSV-1 is an effective means for treating cancer (1). Oncolytic HSV-1 therapy uses the natural characteristics of the virus to kill host cells in the course of viral replication. Mutations by genetic engineering in the genes associated with virulence and/or viral

DNA synthesis can limit the virus to replicate only in transformed cells. Recent studies have demonstrated that oncolytic activities by HSV-1 vectors can also induce antitumor immune responses (7-9). HSV-1 has many advantages as an oncolytic vector (1,10): (i) It infects a variety of tumor cell types. (ii) Total cell killing can be achieved by a relatively low multiplicity of infection (MOI). (iii) Circulating anti-HSV-1 antibody does not affect the cell-to-cell spread of the virus, therefore a vector can be administered repeatedly in immunocompetent hosts. (iv) The large genome (~152 kb) is well characterized and contains many nonessential genes that can be mutated or replaced with large-sized transgenes (11), which is in contrast with adenovirus whose genome size (35 kb) limits the size of transgenes. (v) Antiherpes drugs, such as aciclovir and ganciclovir, are available and can be used for optional termination of the therapy (12, 13). (vi) Unlike retrovirus, HSV-1 DNA remains episomal and is not integrated into the cellular genome. (vii) There are mice and nonhuman primates that are susceptible to HSV-1 and can be used for preclinical evaluation for safety and efficacy.

Since genetically engineered HSV-1 was first described in 1991 as a tool for treating malignant glioma (2), several replication-competent HSV-1 vectors have now been used in patients. Besides G207, a *γ34.5*-deleted HSV-1, strain 1716, has been tested for safety in patients with malignant glioma (4, 14) and also in melanoma patients (15). A phase I trial is under way with an attenuated HSV-1, NV1020, originally designed for HSV-1 vaccination, for patients with liver metastasis of colon cancer in which the virus is administered into the hepatic artery (16). So far, all these trials have demonstrated the feasibility of using replication-competent HSV-1 vectors for treating cancer patients.

G207: a multimutated, replication-competent HSV-1 vector

G207 was constructed from recombinant HSV-1 (R3616) that originates in HSV-1 laboratory strain F and has deletions in both copies of the *γ34.5* gene, the major determinant of HSV-1 neurovirulence (3). G207 also has an insertion of the *E. coli LacZ* gene in the infected-cell protein 6 (ICP6) coding region (UL39), inactivating ribonucleotide reductase, a key enzyme for viral DNA synthesis in nondividing cells but not in dividing cells. The double mutation confers favorable properties on G207 for cancer therapy: (i) It replicates selectively in cancer cells and causes no damage to normal tissues. (ii) The chance of reverting to wild-type is minimal. (iii) The reporter gene *LacZ* allows easy histochemical detection of the replicating virus. (iv) G207 is hypersensitive to ganciclovir/aciclovir.

G207 was first shown to inhibit the tumor growth and/or prolong the survival in athymic mice harboring malignant glioma in the brain or under the skin when inoculated intraneoplastically (3). Since then, G207 has shown efficacy in a variety of immunoincompetent and -competent animal tumor models (5). Those tumors include malignant meningioma (17), breast cancer (18), colorectal cancer (19), prostate adenocarcinoma (20), head and neck cancer (21), bladder cancer (22), gastric cancer (23), ovarian cancer (24), neuroblastoma (25), hepatic cancer (26), gallbladder cancer (26) and malignant melanoma (6).

G207 is significantly less toxic than wild-type HSV when injected into the brain and prostate of mice and nonhuman primates (3, 27-29). G207 was the first replication-competent HSV-1 vector used in the US in humans and was tested for safety in patients with recurrent malignant glioma. The dose-escalation study starting at 1×10^5 plaque forming units (pfu) demonstrated that intracerebral inoculation of G207 was safe at doses up to 3×10^9 pfu (4). The results warrant further clinical trials for brain tumors as well as other types of cancer. Phase Ib/II studies for malignant glioma are under way.

Antitumor immunity induction by G207

The tumor cell killing effect of G207 depends on the extent that tumor cells can support G207 replication. Thus there is a wide range of variation among different cell lines (5). The antitumor effect also depends on the extent of antitumor immunity induction in the course of G207 oncolytic activity (7-9). One way to enhance the antitumor efficacy of G207 while retaining the safety features is to harness this action of inducing antitumor immune responses. A potential contradicting consequence is that enhancing immune responses could inhibit viral replication within the tumor, leading to a reduced cytopathic effect. With adenovirus vectors, it has been shown that antiviral immune responses act adversely to the therapeutic efficacy (30, 31). Innate immunity may also limit the therapeutic efficacy of HSV-1 vectors (32).

In several immunocompetent animal tumor models, G207 has been shown to induce specific and systemic antitumor immunity (7, 9, 26). Intraneoplastic inoculation of G207 into a s.c. N18 neuroblastoma in syngeneic A/J mice inhibited the growth of remote tumors in the brain as well as in the periphery via systemic antitumor immune responses (7). The therapy also conferred tumor-specific protective immunity with long-term memory, which was demonstrated by cured animals protected from a s.c. rechallenge with N18 cells but not Sal/N cells (7). Antitumor immunity was associated with an elevation of specific cytolytic T lymphocyte (CTL) activity against tumor cells (7). A similar antitumor effect of G207 on remote tumors via systemic immune responses was observed in other bilateral s.c. tumor models such as CT26 colorectal carcinoma in BALB/c mice (9), M3 melanoma in DBA/2 mice (9) and KIGB-5 gallbladder carcinoma in Syrian hamsters (26). In the CT26 model, intraneoplastic G207 inoculation induced $CD8^+$ T cells that recognized a dominant tumor antigen in an MHC class I-restricted fashion (9).

Defective HSV-1 vectors expressing immunostimulatory molecules

Defective HSV-1 vectors have been used to introduce immunostimulatory genes into tumor cells both *ex vivo* and *in situ* (33). A replication-incompetent HSV-1 vector expressing interleukin (IL)-2 was effective in inducing antitumor immune responses against head and neck metastases of renal carcinoma in animal models (34). An HSV-1 amplicon vector expressing murine granulocyte-macrophage colony stimulating factor (GM-CSF) or IL-2 was used in combination with i.p. injections of interferon-γ (IFN-γ) in an animal hepatoma model. The combination therapy was more effective than any treatment alone. Complete elimination of the tumor was observed in 4 of 12 animals receiving GM-CSF/IFN-γ and 8 of 11 animals given IL-2/IFN-γ (35). An HSV-1 amplicon vector expressing IL-2 also showed a significant suppression of the growth of lung squamous cell carcinoma implanted s.c. in animals (36, 37). Treatment with the IL-2 vector caused a retardation of the growth of tumors remote from vector inoculation sites and led to a significant improvement in animal survival. In a bilateral s.c. tumor model of murine melanoma, intraneoplastic inoculation of a defective HSV-1 vector encoding murine GM-CSF significantly inhibited the growth of both the inoculated and noninoculated contralateral tumors (38).

An HSV-1 amplicon can be combined with oncolytic HSV-1 vectors by using an oncolytic HSV-1 vector as a helper virus when generating the defective HSV-1 vector. Several amplicon vectors expressing immunostimulatory molecules have been used in combination with G207 (39-41). When a mixture of G207 and a defective vector expressing IL-12, for example, is inoculated into the tumor, tumor cells infected with G207 allow the virus to replicate and are ultimately destroyed, further spreading progeny G207 to surrounding tumor cells. On the other hand, tumor cells infected with the defective vector produce IL-12 and recruit immune cells, which augment the antitumor immune response elicited by the oncolytic activity of G207. Intraneoplastic inoculation of the IL-12 defective vector with G207 significantly augmented the antitumor immune response compared with G207 alone in a s.c. CT26 tumor model (40). Increases in tumor-specific CTL activity and production of IFN-β by splenocytes were observed (40). A defective HSV vector (dvB7Ig) expressing a soluble form of B7-1, one of the most potent costimulatory molecules (42), was also used in combination with G207 (41). Soluble B7-1 was designed as a fusion protein of the extracellular domain of B7-1 and the Fc portion of IgG, so that it is secreted by tumor cells rather than expressed on the cell surface. The *in vivo* efficacy was tested in the poorly-immunogenic murine neuroblastoma, Neuro2a, in A/J mice. Intraneoplastic inoculation of dvB7Ig/G207 at a low titer successfully inhibited the growth of established s.c. tumors despite the fact that the expression of B7-1-Ig was detected in only 1% or less of tumor cells at the inoculation site; treatment also prolonged the survival of mice bearing intracerebral tumors. Inoculation of dvB7Ig/G207 induced a significant influx of $CD4^+$ and $CD8^+$ T cells into the tumor. *In vivo* depletion of immune cell subsets further revealed that the antitumor effect required $CD8^+$ T cells but not $CD4^+$ T cells. Treatment with dvB7Ig/G207 conferred tumor-specific protective immunity in cured animals.

Replication-competent HSV-1 vectors for amplified gene delivery

The use of replication-competent vectors for transgene expression has multiple attractive advantages over defective vectors: i) continuous generation of a high-titer, homogenous vector stock is possible which allows manufacturing of a large amount with a better quality control; ii) amplified gene delivery can be obtained *in vivo;* and iii) transgene expression may lower administering doses required therefore decreasing toxicity. On the other hand, potential demerits of using replication-competent vectors for expressing foreign proteins are that the transgene expression may: i) be shorter in duration than defective vectors due to destruction of the host cell by viral replication; ii) increase the toxicity of the vector; and iii) interfere with viral replication.

Recently, several replication-competent HSV-1 vectors have been created that contain transgenes for immunostimulatory molecules. *γ34.5*-Deficient HSV-1 containing the murine IL-4 gene displayed significantly higher antitumor activity and prolonged survival of mice with intracranial tumors as compared to its parent virus or to the virus expressing IL-10 (43). Recombinant HSV-1 expressing IL-12 (M002 and NV1042) showed improved *in vivo* efficacy against murine neuroblastoma (44), murine squamous cell carcinoma (45) and murine colorectal tumor (46). Immunohistochemical analyses of tumors treated with these HSV-1 mutants revealed a significant influx of $CD4^+$, $CD8^+$ T cells and macrophages. The replication-competent HSV-1 vector expressing IL-12 (NV1042) was more effective than the vector expressing GM-CSF in the same backbone (NV1034) in mice with s.c. squamous cell carcinoma (45). The mice cured by NV1042 had a higher rate of rejecting rechallenged tumor cells than those cured by NV1034 (45).

G47Δ

Another way to improve the antitumor action of G207 while retaining its safety is to construct a new HSV-1 vector by adding further mutations to the genome of G207. The *α47* gene product (ICP47) of HSV-1 inhibits the transporter associated with antigen presentation (TAP) that mediates antigen presentation in the context of MHC class I by translocating peptides across the endoplasmic reticulum (47-50). Upon infection, ICP47 therefore causes downregulation of MHC class I expression on the cell surface, allowing the HSV-1-infected host cells to escape the host immune surveillance (51). An *α47*-deleted

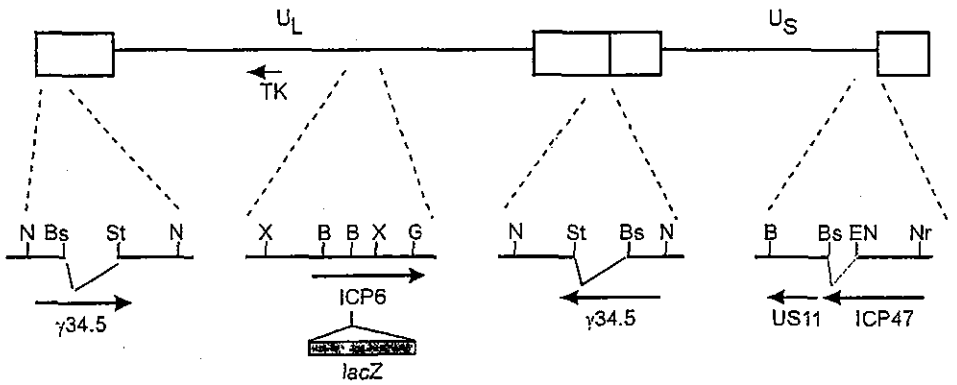

Fig. 1. Structure of G47Δ. The boxes (top line) represent inverted repeat sequences flanking the long (U_L) and short (U_S) unique sequences of HSV-1 DNA. The *E. coli LacZ* coding sequence is inserted into the indicated BamHI site in the ICP6 coding region. There is a 1.0-kb deletion in both copies of the *γ34.5* gene located in the expanded domain of the long inverted repeat sequences. G47Δ was created from G207 by further deleting 312 bp between the indicated BstEII and EcoNI sites in the ICP47 locus. The deletion also places the *US11* gene under control of the ICP47 immediate-early promoter. Thick arrows indicate transcribed region. (N, NcoI; Bs, BstEII; St, StuI; X, XhoI; B, BamHI; G, BglII; EN, EcoNI; Nr, NruI)

HSV-1 vector did not downregulate MHC class I expression in human melanoma cells (52). Also, *α47*-deleted replication-competent HSV-1 was less virulent than the parent wild-type virus in the brains of HSV-1-sensitive A/J mice, and the attenuation of neurovirulence was dependent on $CD8^+$ T cells (53). In order to confer an enhanced MHC class I presentation, we have constructed a new generation, replication-competent HSV-1 vector, G47Δ, by further creating a 312 bp deletion within the *α47* gene of G207 (Fig. 1). G47Δ-infected human melanoma cells which showed the greatest enhancement of MHC class I expression among cells tested, caused a significantly better stimulation of tumor infiltrating lymphocytes (TIL) as compared to G207-infected cells, resulting in 41% more IFN-γ secretion (6). The results showed that higher MHC class I expression in cells infected with G47Δ can indeed enhance the antitumor T cell stimulation.

Because of the overlap of the *US11* promoter region and the *α47* gene, the 312 bp deletion in G47Δ also results in deletion of the *US11* promoter region, placing the late *US11* gene under control of the immediate-early *α47* promoter (6). The altered expression of the *US11* gene functions as a second-site suppressor of the *γ34.5* mutation (54-56) and recovers the impaired growth properties of *γ34.5*-deficient HSV-1 mutants by precluding the shutoff of protein synthesis. G47Δ gave higher virus yields than G207 in all cell lines tested, resulting in an approximately 4- to 1000-fold increase in titer (6). The improved replication capability of G47Δ also led to an enhanced cytopathic effect in a variety of tumor cells tested, including glioma, neuroblastoma and prostate cancer (6; Y. Ino, H. Fukuhara, T. Todo, unpublished data).

The enhanced tumor cell killing of G47Δ shown *in vitro* was reflected in the improved antitumor efficacy *in vivo*. In athymic mice with s.c. U87MG human glioma, intraneoplastic inoculation of G47Δ caused a significantly greater reduction in tumor growth, leading to higher numbers of "cures" as compared G207-treated animals. In the same model, tumors were harvested periodically to measure the recovery of infectious viruses, and the peak recovery of G47Δ was 20-fold higher than the peak obtained for G207 (57). G47Δ was also significantly more efficacious than G207 in immunocompetent A/J mice with s.c., poorly immunogenic Neuro2a tumors, prolonging the survival of animals compared with mock or G207 (6). Moreover, in A/J mice bearing intracerebral Neuro2a tumors, intraneoplastic inoculations of G47Δ at a low dose caused moderate extension of survival compared with G207-treated animals (58). Most importantly, G47Δ was as safe as G207 when inoculated into the brains of A/J mice at 2×10^6 pfu (6).

Thus, G47Δ has attractive features for human cancer therapy, including potent stimulation of antitumor immune cells, high yields of virus, improved oncolytic activity, and safety in an HSV-1-sensitive rodent model. The preserving of the host TAP function also make G47Δ useful as a backbone vector for expressing foreign antigens in the context of vaccination. While the use of replication-competent HSV-1 vectors for expressing immunostimulatory cytokines has proven useful (44-46), the backbone vectors used by others to test the strategy were likely less attenuated compared with G207, leaving concerns for clinical applications. The safety and preclusion of the shutoff of protein synthesis make G47Δ an ideal replication-competent vector to express any foreign protein molecules. We are currently generating multiple replication-competent vectors with different transgenes using G47Δ as the backbone.

Conclusions

The development of G47Δ was built upon accumulated experience of over a decade by us as well as by

others with so-called first- and second-generation replication-competent HSV-1 vectors. This new generation HSV-1 vector is particularly attractive for clinical application because it may be the safest oncolytic HSV-1 vector described to date while retaining its ability to replicate in tumor cells which leads to enhanced antitumor activity. The capability of obtaining high yields of G47Δ is also beneficial from a manufacturing standpoint. Furthermore, G47Δ is an ideal vector for expressing foreign proteins, especially immunostimulatory molecules, due to the preservation of the host TAP function, leading to enhanced MHC class I expression. We believe G47Δ will contribute to the progress of oncolytic virus therapy.

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References

1. Martuza, R.L. *Conditionally replicating herpes vectors for cancer therapy.* J Clin Invest 2000, 105: 841-6.

2. Martuza, R.L., Malick, A., Markert, J.M., Ruffner, K.L., Coen, D.M. *Experimental therapy of human glioma by means of a genetically engineered virus mutant.* Science 1991, 252: 854-6.

3. Mineta, T., Rabkin, S.D., Yazaki, T., Hunter, W.D., Martuza, R.L. *Attenuated multi-mutated herpes simplex virus-1 for the treatment of malignant gliomas.* Nat Med 1995, 1: 938-43.

4. Markert, J.M., Medlock, M.D., Rabkin, S.D. et al. *Conditionally replicating herpes simplex virus mutant, G207 for the treatment of malignant glioma: Results of a phase I trial.* Gene Ther 2000, 7: 867-74.

5. Todo, T., Ebright, M.I., Fong, Y., Rabkin, S.D. *Oncolytic herpes simplex virus (G207): From basic to clinical.* In: Tumor-Suppressing Viruses, Genes, and Drugs - Innovative Cancer Therapy Approaches. H. Maruta (Ed.). Academic Press: San Diego 2002, 45-75.

6. Todo, T., Martuza, R.L., Rabkin, S.D., Johnson, P.A. *Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing.* Proc Natl Acad Sci USA 2001, 98: 6396-401.

7. Todo, T., Rabkin, S.D., Sundaresan, P. et al. *Systemic antitumor immunity in experimental brain tumor therapy using a multimutated, replication-competent herpes simplex virus.* Hum Gene Ther 1999, 10: 2741-55.

8. Todo, T., Rabkin, S.D., Chahlavi, A., Martuza, R.L. *Corticosteroid administration does not affect viral oncolytic activity, but inhibits antitumor immunity in replication-competent herpes simplex virus tumor therapy.* Hum Gene Ther 1999, 10: 2869-78.

9. Toda, M., Rabkin, S.D., Kojima, H., Martuza, R.L. *Herpes simplex virus as an in situ cancer vaccine for the induction of specific anti-tumor immunity.* Hum Gene Ther 1999, 10: 385-93.

10. Mullen, J.T., Tanabe, K.K. *Viral oncolysis.* Oncologist 2002, 7: 106-19.

11. Roizman, B. *The function of herpes simplex virus genes: A primer for genetic engineering of novel vectors.* Proc Natl Acad Sci USA 1996, 93: 11307-12.

12. Coen, D.M., Goldstein, D.J., Weller, S.K. *Herpes simplex virus ribonucleotide reductase mutants are hypersensitive to acyclovir.* Antimicrob Agents Chemother 1989, 33: 1395-9.

13. Balfour, H.H. Jr. *Antiviral drugs.* N Engl J Med 1999, 340: 1255-68.

14. Rampling, R., Cruickshank, G., Papanastassiou, V. et al. *Toxicity evaluation of replication-competent herpes simplex virus (ICP 34.5 null mutant 1716) in patients with recurrent malignant glioma.* Gene Ther 2000, 7: 859-66.

15. MacKie, R.M., Stewart, B., Brown, S.M. *Intralesional injection of herpes simplex virus 1716 in metastatic melanoma.* Lancet 2001, 357: 525-6.

16. Mayer-Kuckuk, P., Banerjee, D., Kemeny, N., Fong, Y., Bertino, J.R. *Molecular therapies for colorectal cancer metastatic to the liver.* Mol Ther 2002, 5: 492-500.

17. Yazaki, T., Manz, H.J., Rabkin, S.D., Martuza, R.L. *Treatment of human malignant meningiomas by G207, a replication-competent multimutated herpes simplex virus 1.* Cancer Res 1995, 55: 4752-6.

18. Toda, M., Rabkin, S.D., Martuza, R.L. *Treatment of human breast cancer in a brain metastatic model by G207, a replication-competent multimutated herpes simplex virus 1.* Hum Gene Ther 1998, 9: 2177-85.

19. Kooby, D.A., Carew, J.F., Halterman, M.W. et al. *Oncolytic viral therapy for human colorectal cancer and liver metastases using a multi-mutated herpes simplex virus type-1 (G207).* FASEB J 1999, 13: 1325-34.

20. Walker, J.R., McGeagh, K.G., Sundaresan, P., Jorgensen, T.J., Rabkin, S.D., Martuza, R.L. *Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector G207.* Hum Gene Ther 1999, 10: 2237-43.

21. Chahlavi, A., Todo, T., Martuza, R.L., Rabkin, S.D. *Replication-competent herpes simplex virus vector G207 and cisplatin combination therapy for head and neck squamous cell carcinoma.* Neoplasia 1999, 1: 162-9.

22. Oyama, M., Yazaki, T., Ohigashi, T. et al. *Application of conditionally replicating herpes vector for gene therapy treatment of urologic neoplasms.* Mol Urol 2000, 4: 83-7.

23. Bennett, J.J., Kooby, D.A., Delman, K. et al. *Antitumor efficacy of regional oncolytic viral therapy for peritoneally disseminated cancer.* J Mol Med 2000, 78: 166-74.

24. Coukos, G., Makrigiannakis, A., Kang, E.H. et al. *Use of carrier cells to deliver a replication-selective herpes simplex virus-1 mutant for the intraperitoneal therapy of epithelial ovarian cancer.* Clin Cancer Res 1999, 5: 1523-37.

25. Mashour, G.A., Moulding, H.D., Chahlavi, A. et al. *Therapeutic efficacy of G207 in a novel peripheral nerve sheath tumor model.* Exp Neurol 2001, 169: 64-71.

26. Nakano, K., Todo, T., Chijiiwa, K., Tanaka, M. *Therapeutic efficacy of G207, a conditionally replicating herpes simplex virus type 1 mutant, for gallbladder carcinoma in immunocompetent hamsters.* Mol Ther 2001, 3: 431-7.

27. Hunter, W.D., Martuza, R.L., Feigenbaum, F. et al. *Attenuated, replication-competent herpes simplex virus type 1 mutant G207: Safety evaluation of intracerebral injection in nonhuman primates.* J Virol 1999, 73: 6319-26.

28. Sundaresan, P., Hunter, W.D., Martuza, R.L., Rabkin, S.D. *Attenuated, replication-competent herpes simplex virus type 1 mutant G207: Safety evaluation in mice.* J Virol 2000, 74: 3832-41.

29. Todo, T., Feigenbaum, F., Rabkin, S.D. et al. *Viral shedding and biodistribution of G207, a multimutated, conditionally replicating herpes simplex virus type 1, after intracerebral inoculation in aotus.* Mol Ther 2000, 2: 588-95.

30. Yang, Y., Nunes, F.A., Berencsi, K., Furth, E.E., Gonczol, E., Wilson, J.M. *Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy.* Proc Natl Acad Sci USA 1994, 91: 4407-11.

31. Yang, Y., Li, Q., Ertl, H.C., Wilson, J.M. *Cellular and humoral immune responses to viral antigens create barriers to lung-directed gene therapy with recombinant adenoviruses.* J Virol 1995, 69: 2004-15.

32. Ikeda, K., Ichikawa, T., Wakimoto, H. et al. *Oncolytic virus therapy of multiple tumors in the brain requires suppression of innate and elicited antiviral responses.* Nat Med 1999, 5: 881-7.

33. Tuting, T., Storkus, W.J., Lotze, M.T. *Gene-based strategies for the immunotherapy of cancer.* J Mol Med 1997, 75: 478-91.

34. Mizuno, H., Yanoma, S., Nishimura, G. et al. *Therapeutic efficiency of IL-2 gene transduced tumor vaccine for head and neck carcinoma.* Cancer Lett 2000, 152: 175-85.

35. Karpoff, H.M., D'Angelica, M., Blair, S., Brownlee, M.D., Federoff, H., Fong, Y. *Prevention of hepatic tumor metastases in rats with herpes viral vaccines and gamma-interferon.* J Clin Invest 1997, 99: 799-804.

36. Carew, J.F., Federoff, H., Halterman, M. et al. *Efficient gene transfer to human squamous cell carcinomas by the herpes simplex virus type 1 amplicon vector.* Am J Surg 1998, 176: 404-8.

37. D'Angelica, M., Karpoff, H., Halterman, M. et al. *In vivo interleukin-2 gene therapy of established tumors with herpes simplex amplicon vectors.* Cancer Immunol Immunother 1999, 47: 265-71.

38. Toda, M., Martuza, R.L., Rabkin, S.D. *Tumor growth inhibition by intratumoral inoculation of defective herpes simplex virus vectors expressing granulocyte-macrophage colony-stimulating factor.* Mol Ther 2000, 2: 324-9.

39. Carew, J.F., Kooby, D.A., Halterman, M.W., Kim, S.H., Federoff, H.J., Fong, Y. *A novel approach to cancer therapy using an oncolytic herpes virus to package amplicons containing cytokine genes.* Mol Ther 2001, 4: 250-6.

40. Toda, M., Martuza, R.L., Kojima, H., Rabkin, S.D. *In situ cancer vaccination: An IL-12 defective vector/replication-competent herpes simplex virus combination induces local and systemic antitumor activity.* J Immunol 1998, 160: 4457-64.

41. Todo, T., Martuza, R.L., Dallman, M.J., Rabkin, S.D. *In situ expression of soluble B7-1 in the context of oncolytic herpes simplex virus induces potent antitumor immunity.* Cancer Res 2001, 61: 153-61.

42. Galea-Lauri, J., Farzaneh, F., Gaken, J. *Novel costimulators in the immune gene therapy of cancer.* Cancer Gene Ther 1996, 3: 202-14.

43. Andreansky, S., He, B., van Cott, J. et al. *Treatment of intracranial gliomas in immunocompetent mice using herpes simplex viruses that express murine interleukins.* Gene Ther 1998, 5: 121-30.

44. Parker, J.N., Gillespie, G.Y., Love, C.E., Randall, S., Whitley, R.J., Markert, J.M. *Engineered herpes simplex virus expressing IL-12 in the treatment of experimental murine brain tumors.* Proc Natl Acad Sci USA 2000, 97: 2208-13.

45. Wong, R.J., Patel, S.G., Kim, S. et al. *Cytokine gene transfer enhances herpes oncolytic therapy in murine squamous cell carcinoma.* Hum Gene Ther 2001, 12: 253-65.

46. Bennett, J.J., Malhotra, S., Wong, R.J. et al. *Interleukin 12 secretion enhances antitumor efficacy of oncolytic herpes simplex viral therapy for colorectal cancer.* Ann Surg 2001, 233: 819-26.

47. York, I.A., Roop, C., Andrews, D.W., Riddell, S.R., Graham, F.L., Johnson, D.C. *A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8⁺ T lymphocytes.* Cell 1994, 77: 525-35.

48. Grandea, A.G., 3rd, Androlewicz, M.J., Athwal, R.S., Geraghty, D.E., Spies, T. *Dependence of peptide binding by MHC class I molecules on their interaction with TAP.* Science 1995, 270: 105-8.

49. Hill, A., Jugovic, P., York, I. et al. *Herpes simplex virus turns off the TAP to evade host immunity.* Nature 1995, 375: 411-5.

50. Fruh, K., Ahn, K., Djaballah, H. et al. *A viral inhibitor of peptide transporters for antigen presentation.* Nature 1995, 375: 415-8.

51. Hill, A.B., Barnett, B.C., McMichael, A.J., McGeoch, D.J. *HLA class I molecules are not transported to the cell surface in cells infected with herpes simplex virus types 1 and 2.* J Immunol 1994, 152: 2736-41.

52. Krisky, D.M., Marconi, P.C., Oligino, T.J. et al. *Development of herpes simplex virus replication-defective multigene vectors for combination gene therapy applications.* Gene Ther 1998, 5: 1517-30.

53. Goldsmith, K., Chen, W., Johnson, D.C., Hendricks, R.L. *Infected cell protein (ICP)47 enhances herpes simplex virus neurovirulence by blocking the CD8⁺ T cell response.* J Exp Med 1998, 187: 341-8.

54. Mohr, I., Gluzman, Y. *A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function.* EMBO J 1996, 15: 4759-66.

55. He, B., Chou, J., Brandimarti, R., Mohr, I., Gluzman, Y., Roizman, B. *Suppression of the phenotype of $\gamma_1$34.5- herpes simplex virus 1: Failure of activated RNA-dependent protein kinase to shut off protein synthesis is associated with a deletion in the domain of the α47 gene.* J Virol 1997, 71: 6049-54.

56. Cassady, K.A., Gross, M., Roizman, B. *The second-site mutation in the herpes simplex virus recombinants lacking the $\gamma_1$34.5 genes precludes shutoff of protein synthesis by blocking the phosphorylation of eIF-2α.* J Virol 1998, 72: 7005-11.

57. Ino, Y., Martuza, R.L., Rabkin, S.D., Todo, T. *Improved efficacy of replication competent oncolytic HSV-1 vector G47Δ in brain tumors.* 5th Annual Meeting of the American Society of Gene Therapy (June 5-9, Boston) 2002, Abst 457.

58. Ino, Y., Martuza, R.L., Rabkin, S.D., Todo, T. *Oncolytic virus therapy for brain tumors using a third-generation, conditionally-replicating HSV-1 vector G47Δ.* 93rd Annual Meeting of American Association for Cancer Research (April 6-10, San Francisco) 2002, Abst 5442.

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

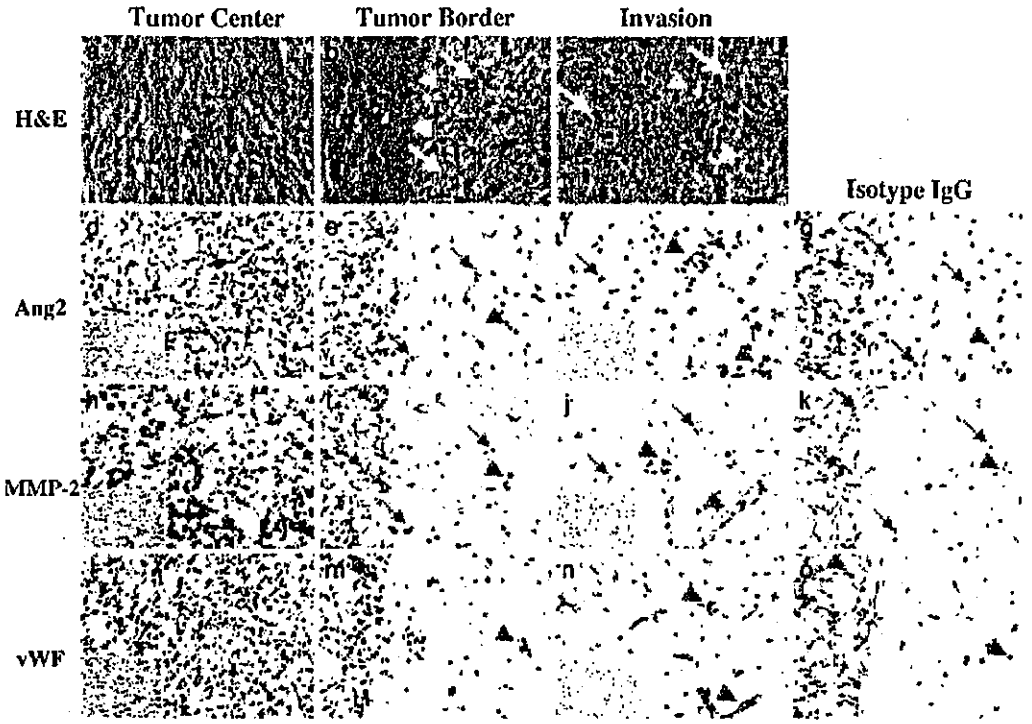

Fig. 1. Cooverexpression of Ang2 and MMP-2 in invasive areas of primary human glioma tissues. IHC on serial sections of a glioblastoma multiforme tissue using hematoxylin/eosin (*a–c*), a goat polyclonal anti-Ang2 antibody (*d–f*), a rabbit polyclonal anti-MMP-2 antibody (*h–j*), a rabbit polyclonal 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

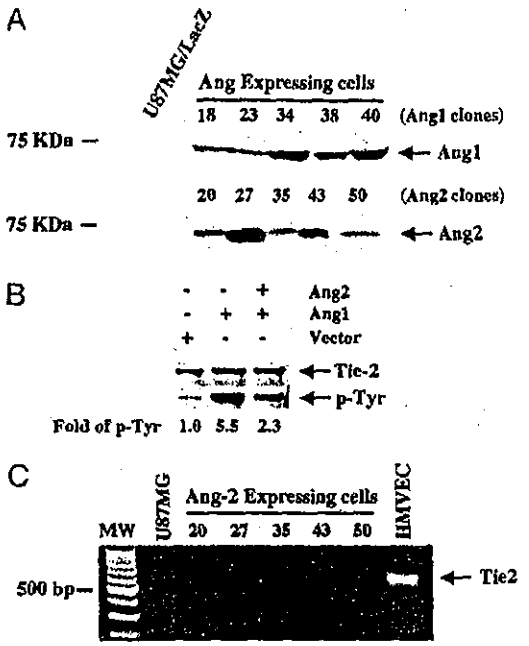

Fig. 2. Expression of Ang2 and Tie2 in U87MG glioma cells. (*A*) Overexpression of Ang1 or Ang2 in U87MG cells. CM from U87MG/LacZ or five Ang1- or Ang2-expressing cell clones were analyzed by Western blot by using an anti-c-Myc antibody. Lanes labeled 18, 23, 34, 38, and 40, Ang1-expressing cell clones. 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.

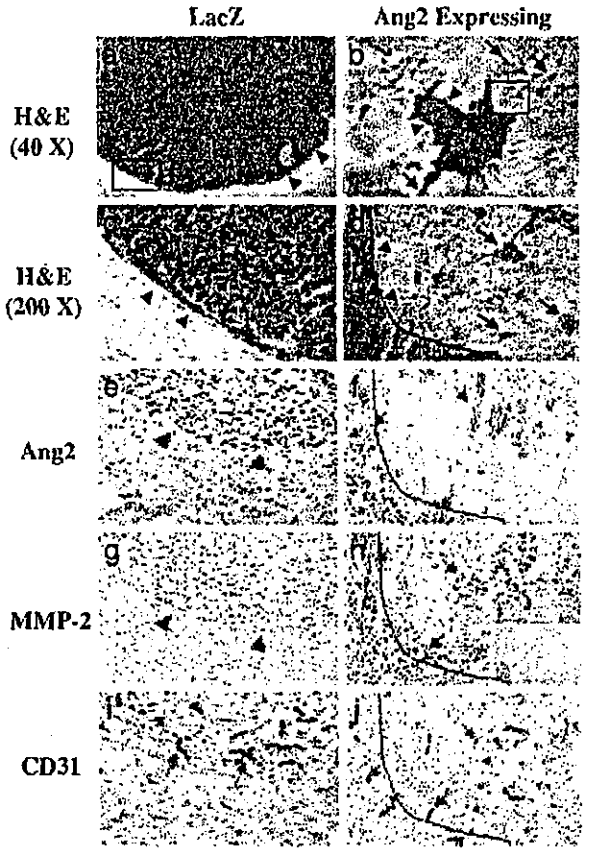

Fig. 3. Overexpression of Ang2 by U87MG cells induces glioma invasion in brains. IHC on various U87MG gliomas established in mouse brains. (*a–d*) Hematoxylin/eosin staining. (a and c) (Enlarged area indicated by a rectangle in *a*), glioma established by control U87MG/LacZ cells. (*b* and *d*) (Enlarged 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-

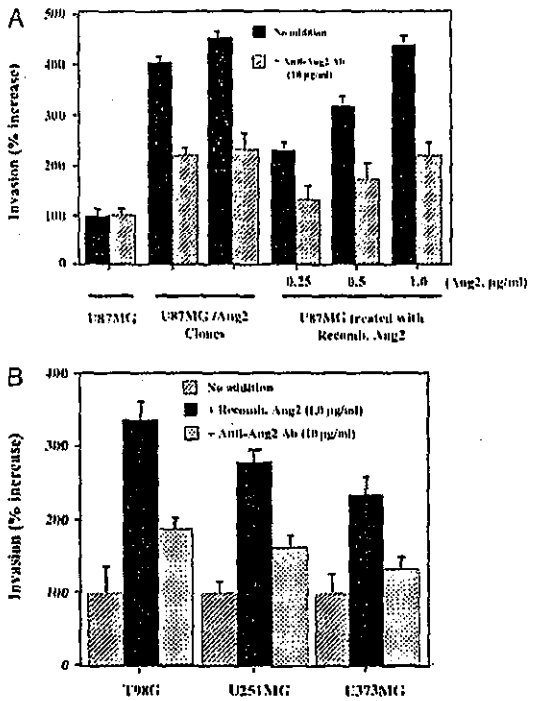

Fig. 4. Ang2 stimulates glioma cell invasion *in vitro*. (*A*) U87MG/Ang2 cell clones or parental U87MG cells that were treated with recombinant Ang2 protein at various concentrations have elicited capacity of cellular invasion through the Matrigel-coated membranes. (*B*) Recombinant Ang2 (1 μg/ml) stimulates 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, ±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

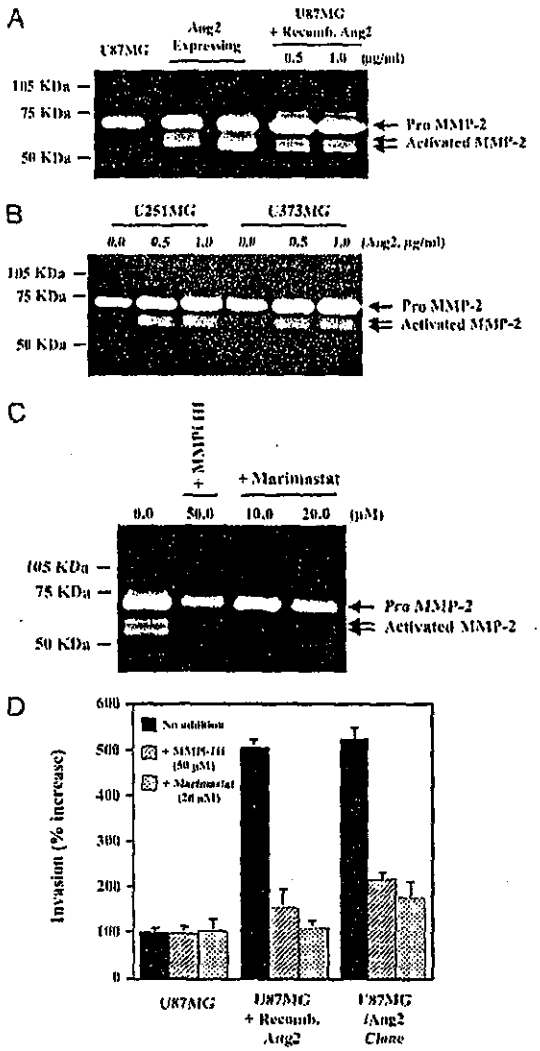

Fig. 5. Ang2 stimulates glioma cell invasion through the activation of MMP-2 in glioma cells in vitro. (A–C) Zymography analyses of MMP-2 activity in CM collected from various cells. (D) In vitro cell invasion through Matrigel. (A) Overexpression of Ang2 in U87MG cells or treatments on parental U87MG 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