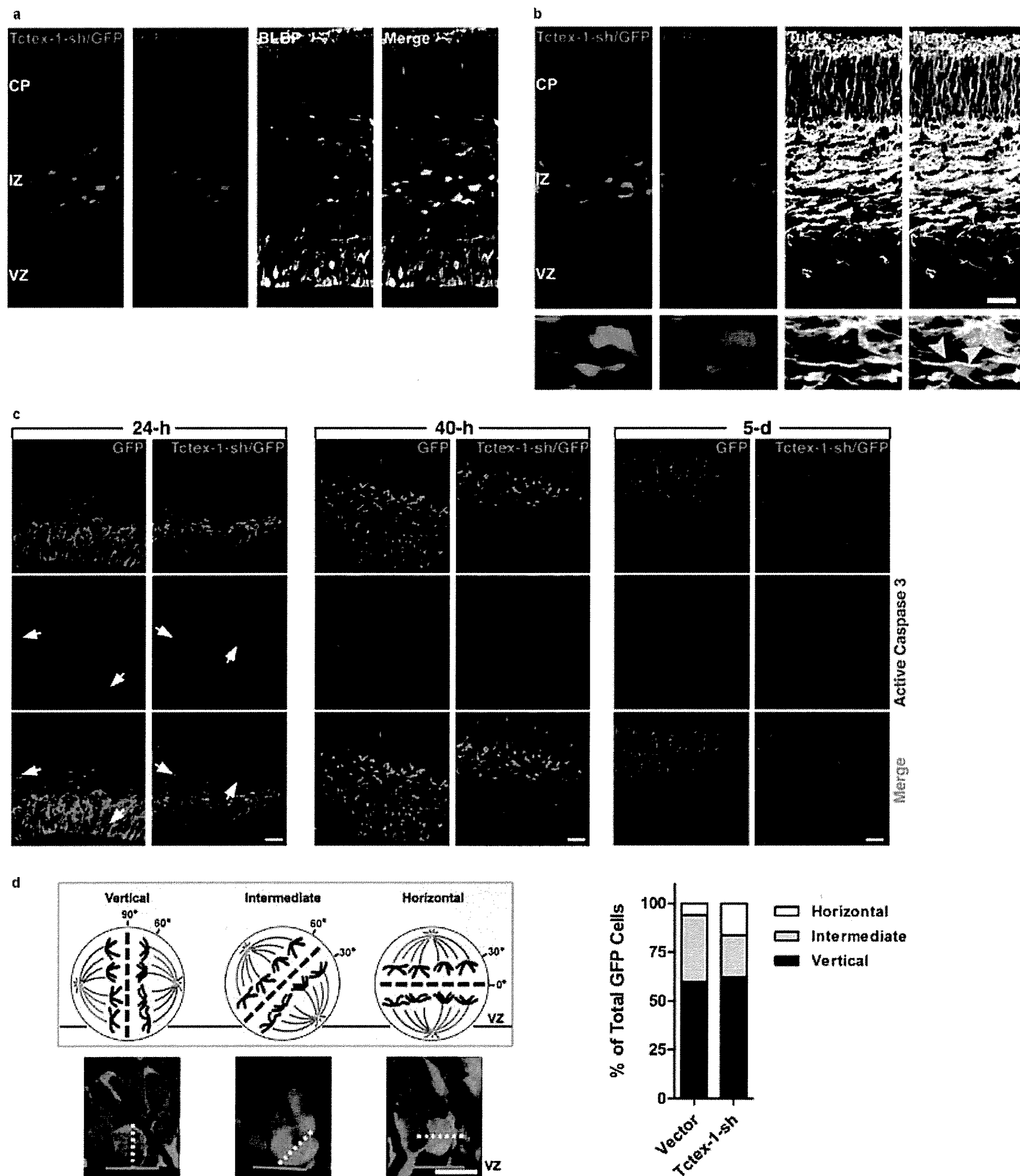


Figure S5 (**a, b**) 3-mm-thick confocal images of E15.5 brain that was co-electroporated with Tctex-1-sh/GFP and HcRed and immunolabeled for BLBP (a) or Tuj1 (b). Almost all transfected cells were double-positive for both GFP and HcRed. Very few transfected cells were positive for BLBP, whereas a large majority of them exhibited Tuj1. (**c**) Cortical slices, harvested from brain 24 hr, 40 hr or 5 days after electroporation, were labeled for active caspase 3. Arrows pointed to the apoptotic nuclei. Non-specific labeling of blood vessels was also seen in the 40-hr and 5-day samples. Bars= 50 mm (24-hr, 40-hr brains); 100 mm (5-day brain). (d) Schematic drawings (top, left) and representative images (bottom, left) of three alternative orientations of cleavage planes in relation to the ventricular surfaces. Bar=20mm. Based on the alignment of chromosome, the fractions of GFP-labeled dividing cells displayed the vertical cleavage plane (60°-90°), intermediate (30°-60°), and horizontal (0°-30°) were quantified (n=67, 7 brains for GFP transfection, and n=37, 9 brains for Tctex-1-sh/GFP transfection) (right).

© 2011 Macmillan Publishers Limited. All rights reserved.

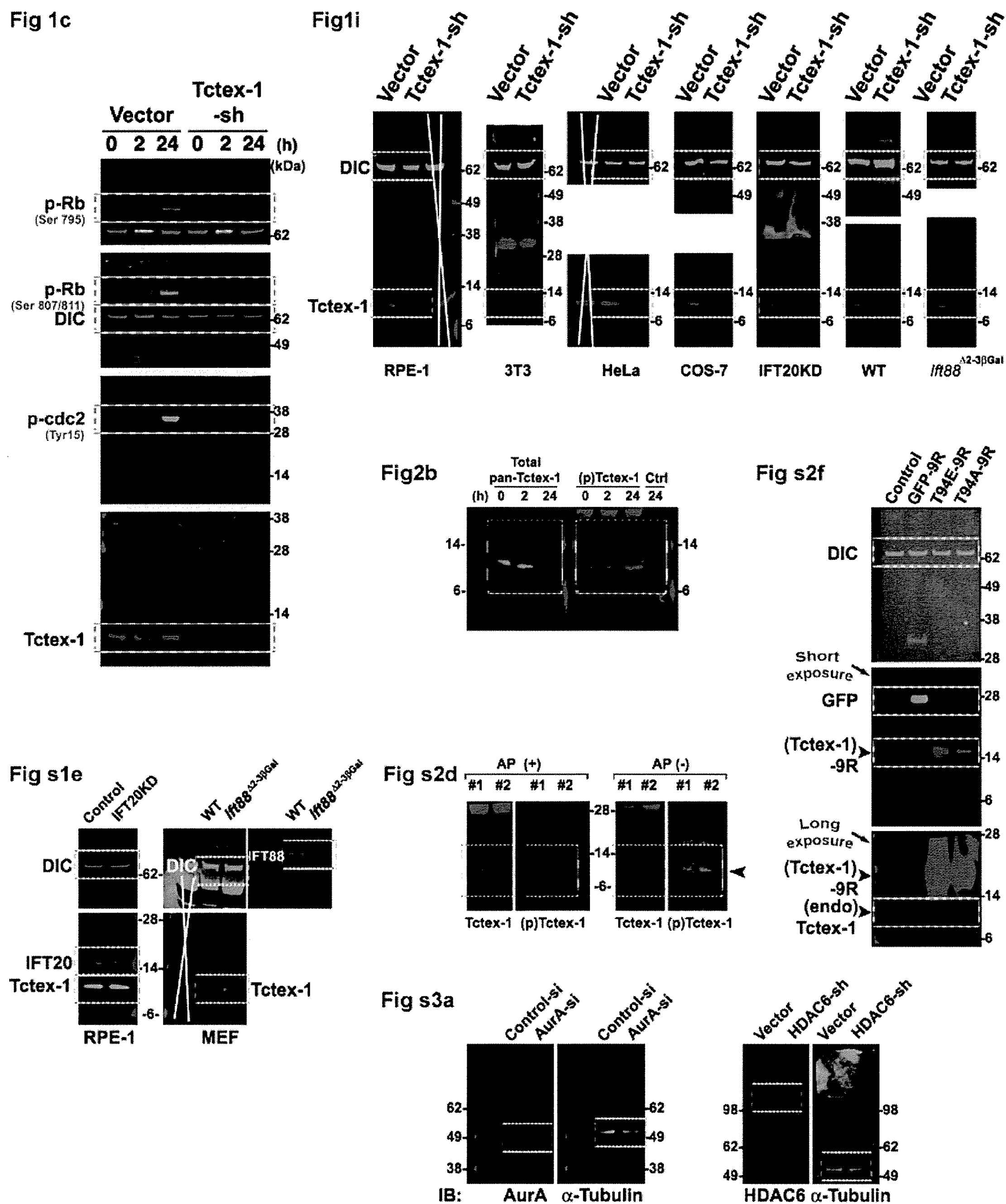


Figure S6 Full scans of Western blots.

© 2011 Macmillan Publishers Limited. All rights reserved.

Protein transduction into the mouse otocyst using arginine-rich cell-penetrating peptides

Toru Miwa[a], Ryosei Minoda[a], Taku Kaitsuka[b], Momoko Ise[a], Kazuhito Tomizawa[b] and Eiji Yumoto[a]

The mouse otocyst, an anlage of the inner ear, is an attractive experimental target for developing treatment modalities for congenital inner ear diseases and for studying inner ear development. Poly-arginine (6–12 residues) is a cell-penetrating peptide and can be used to deliver cargo into cells. Here, we achieved transutero delivery of enhanced green fluorescent protein (EGFP) fused to a nine-arginine peptide into mouse embryonic otocysts. The EGFP signal was detected both in the lining cells of the otocysts and in their vicinity at 18 h post injection. Mice injected with EGFP fused to a nine-arginine peptide had normal auditory and vestibular functions. These data suggest that protein transduction using poly-arginine may be a useful alternative strategy to commonly used gene delivery methods for delivering therapeutically relevant molecules to the developing inner ear. *NeuroReport* **22:994–999 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

NeuroReport 2011, 22:994–999

Keywords: cell-penetrating peptides, otocyst, poly-arginine, protein transduction

[a]Department of Otolaryngology-Head and Neck Surgery, Kumamoto University, Graduate School of Medicine and [b]Department of Molecular Physiology, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

Correspondence to Dr Ryosei Minoda, MD, PhD, Kumamoto University, Graduate School of Medicine, 1-1-1 Honjo Kumamoto, 860-0811, Japan
Tel: +81 96 373 5255; fax: +81 96 373 5256;
e-mail: minoda@gpo.kumamoto-u.ac.jp

Ryosei Minoda and Taku Kaitsuka contributed equally to this study.

Received 27 September 2011 **accepted** 29 September 2011

Introduction

Manipulation of the developing inner ear is an attractive experimental strategy for investigating treatment modalities for inner ear diseases and for studying the course of the inner ear development. The otocyst, which is formed by invagination of the otic placode on each side of the head at the level of the hindbrain at embryonic day 9.5 (E9.5) in mice, may be the best target for this because it is a closed and isolated epithelial vesicle and the origin of the anatomical structure of the adult inner ear [1].

Various approaches such as the use of viral vectors, electroporation, microinjection and liposome encapsulation have been used to introduce target genes and molecules into otocysts to manipulate the cells. However, there have been few reports on direct manipulation of otocysts in mammals *in vivo* because mammal otocyst inoculation requires an intricate surgical technique; the otocysts cannot be directly visualized, and it is necessary to manipulate the embryos by the uterine wall. Previous studies have demonstrated successful otocystic inoculation of exogenous genes in mice using viral vectors [1,2] and electroporation [3], but these strategies have several limitations, including low transfection efficiency, restrictions based on cell type, cellular toxicity, and the requirement for optimization for each tissue [1,2].

The HIV-type 1 TAT protein can enter cells when added to culture media [4,5]. The protein transduction domain of the TAT protein, which contains a high proportion of arginine and lysine residues, has been identified as being responsible for its ability to penetrate the plasma membrane [6]. Short peptide sequences such as the protein transduction domain of TAT are referred to as cell-penetrating peptides (CPPs). The CPPs comprise a class of short cationic peptides with the ability to traverse the cell membrane of many different types of mammalian cells [7,8]. A wide variety of macromolecules have been attached to these peptides and subsequently internalized. The cargo molecules that penetrate the cells maintain their biological activities [7,8]. The major application of CPPs is the transduction of proteins and peptides. Another application is CPP-mediated oligonucleotide delivery for the purpose of RNA-based gene silencing, which interrupts or suppresses gene expression [9].

Among the different CPPs, the arginine-rich CPPs have been the most widely studied [5,7,10–12]. Simple poly-arginine peptides with an optimal length of 9–11 residues induce significantly higher cell penetration rates than TAT [13–15]. Here, we have shown that a peptide comprising nine arginine residues (9R) effectively delivered enhanced green fluorescent protein (EGFP) into the developing inner ear when inoculated into mouse otocysts, without causing any deterioration of auditory or vestibular function.

Materials and methods

Recombinant enhanced green fluorescent protein fused to a nine-arginine peptide and enhanced green fluorescent proteins

Expression and purification of EGFP and EGFP conjugated to a nine-arginine peptide (EGFP-9R) were

0959-4965 © 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/WNR.0b013e32834da8f8

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

performed as described previously [16]. In brief, the constructed plasmids were transfected into BL21-DE3 *Escherichia coli* cells, and protein expression was induced with 0.1 mM of isopropyl 1-thio-β-D-galactopyranoside. The expressed proteins were purified using a nickel-nitrilotriacetic acid-agarose column (Invitrogen, Carlsbad, California, USA). After dialysis against phosphate-buffered saline (PBS), the proteins were stored at −80°C.

Animals

Timed-pregnant CD-1 mice were purchased from Charles River Laboratory (Kanagawa, Japan). On arrival, the mice were housed conventionally. Noon on the day on which a vaginal plug was detected was designated as E0.5 of development. All animal experiments were approved by the Committee on the Use and Care of Animals at Kumamoto University and were performed using accepted veterinary standards.

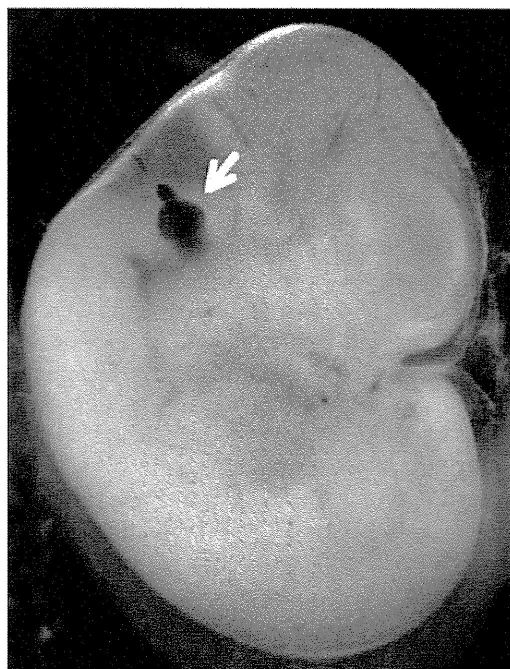
Otocystic inoculation of recombinant proteins in E11.5 mouse embryos

Pregnant mice at 11.5 days postcoitum were deeply anesthetized with intraperitoneal administration of 4 mg/kg of xylazine (Bayer, Shawnee Mission, Kansas, USA) and 120 mg/kg of ketamine–HCl (Daiichisankyo, Tokyo, Japan) in 0.9% NaCl. The abdominal wall was sterilized with 70% alcohol and 10% povidone-iodine. The uterus was exposed by low midline laparotomy. It was then placed on a transparent surgical stage and illuminated from beneath with a fiber optic beam to visualize the rostral and caudal branches of the primary head vein, between which the otocyst resides. EGFP-9R or EGFP, which contained 0.1% fast green (Sigma, St. Louis, Missouri, USA) as a tracking dye, was injected by oral pressure into the lumen of the left-side otocyst (Fig. 1, arrow) using a heat-pulled glass micropipette connected to a Silastic tube with an outer diameter of 5 mm. During surgery, the locations of the embryos that had undergone inoculation were recorded so that they could be identified later. The abdominal skin was closed with a stapler (MikRon Precision, Inc., Gardena, California, USA), and 30 mg/kg of chloramphenicol (Daiichisankyo) were administered intraperitoneally.

Immunohistochemistry

Pregnant mice whose embryos had undergone otocystic inoculation were euthanized under deep anesthesia at 3, 6, 12, 18, and 24 h after inoculation. The embryos were harvested and fixed in 4% paraformaldehyde in PBS for 24 h at 4°C. The tissues were aligned for sectioning, frozen in a dry ice/alcohol mixture, and stored at −80°C until sectioning. For cryostat sections, the entire head was embedded in OCT compound (Sakura Finetek Japan Co. Ltd., Tokyo, Japan) and serially sectioned to a thickness of 10 μm. The cryostat sections were blocked in blocking solution (PBS containing 5% goat serum) and then incubated with rabbit polyclonal anti-Jagged-1 antibody (Abcam Inc., Cambridge, UK), diluted 1 : 500 in PBS, for 1 h at room temperature. After three washes with PBS (5 min per wash), the tissues were reacted with Cy5-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific, Waltham, Massachusetts, USA), diluted 1 : 1000 in PBS, for 1 h at room temperature. Thereafter, Hoechst 33258 dye (Molecular Probes, Eugene, Oregon, USA) was applied for 30 s for nuclear staining, and the specimens were mounted on glass slides with Fluoromount (Diagnostic BioSystems, Pleasanton, California, USA).

Fig. 1



An otocyst was inoculated with enhanced green fluorescent protein fused to a nine-arginine peptide and 0.1% fast green. After otocystic inoculation, the embryo was removed by opening the uterine wall. The arrow indicates the otocyst.

The samples were examined under a BX51 fluorescence microscope (Olympus, Tokyo, Japan). Images were captured with a DP72 camera.

For surface morphological observation of the cochlea, postnatal day 30 (P30) mice were fixed by cardiac perfusion with paraformaldehyde/PBS under deep anesthesia, and the bony capsule and lateral wall of the cochlea were removed. Texas Red-X phalloidin (Molecular Probes) was diluted 1 : 500 in PBS and applied for 30 min at room temperature. The dissected organs of Corti were mounted on glass slides with Fluoromount.

Auditory function analysis

To check for any harmful effects of EGFP-9R inoculation, we assessed the hearing threshold of each ear of the five

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

EGFP-9R-inoculated pups, using the threshold of the noninoculated side (right side) as a control. The animals were anesthetized with intraperitoneal administration of 4 mg/kg of xylazine (Bayer) and 120 mg/kg of ketamine–HCl (Daiichisankyo) in 0.9% NaCl. Electrodes were placed beneath the pinna of the test ear and at the vertex, just below the surface of the skin [3]. The ground electrode was placed under the contralateral ear. The auditory brainstem response was recorded at 4, 12, and 20 kHz (15-ms duration, 1-ms rise/fall time, tone burst). Presentation levels of the stimuli varied in five steps near the thresholds, and a total of 1024 sweeps were averaged for each recording. The threshold was defined as the lowest level at which auditory brainstem response waves could be clearly detected by visual inspection.

Vestibular function analysis

Vestibular function was evaluated at P30 in mice that had undergone inoculation of EGFP-9R into the otocyst. Circling behavior and head tilt were assessed as described previously [17].

Results

Mouse embryo otocysts inoculated with enhanced green fluorescent protein fused to a nine-arginine peptide

In the embryos that had undergone otocystic inoculation of EGFP-9R, EGFP was detected in the lining cells in the otocysts and in their vicinity in a diffused manner (Fig. 2). The EGFP signal overlapped with that for Jagged-1, a marker of the prosensory domain of the otocyst [18], suggesting that EGFP-9R was delivered into the prosensory domain of otocysts (Fig. 2). To investigate how rapidly EGFP-9R was delivered and how long it remained in the otocyst, we examined time-dependent changes in the EGFP signal in embryonic otocysts after inoculation. EGFP was weakly detected in two of five embryos 3 h after inoculation, and a strong signal was observed in the otocysts of all embryos 6 and 12 h after injection (Figs 3, 4). At 18 h, the signal was faint in the embryonic otocysts (Figs 3, 4). EGFP was not detected in the control mouse embryo otocysts inoculated with recombinant EGFP without 9R (data not shown).

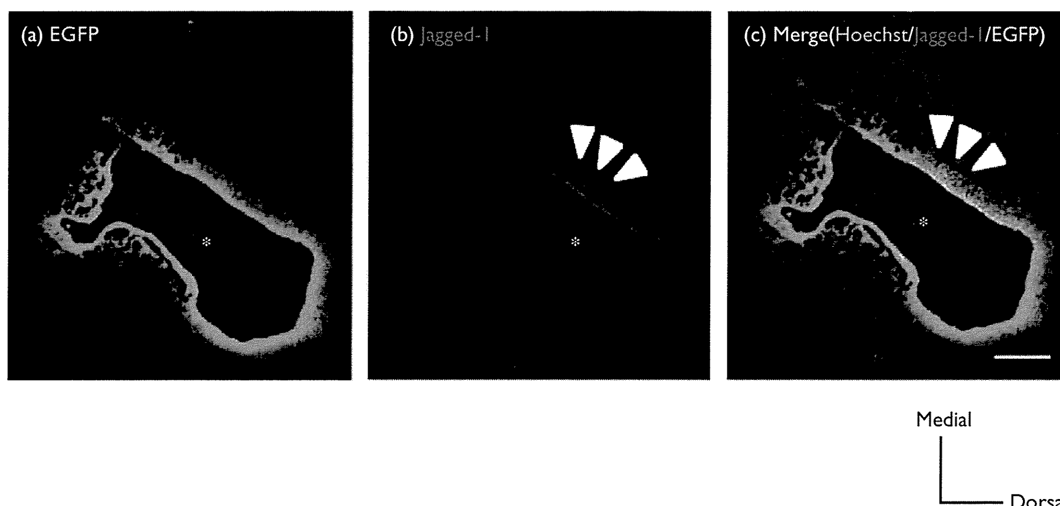
Effect of enhanced green fluorescent protein fused to a nine-arginine peptide inoculation on auditory function

We next examined whether inoculation of EGFP-9R into the otocysts of mouse embryos affected auditory function. There were no statistically significant differences between the thresholds of the inoculated side (left, $n = 5$) and those of the non-inoculated side (right, $n = 5$; $P > 0.05$, Mann–Whitney U-test; Fig. 5). The means ± SDs of the hearing thresholds were 24.6 ± 7.16, 10.0 ± 6.63, and 17.8 ± 4.09 dB SPL at 4, 12, and 20 kHz, respectively, in the inoculated sides (left), and 21.0 ± 15.6, 9.20 ± 2.59, and 18.4 ± 9.21 dB sound pressure level at 4, 12, and 20 kHz, respectively, in the noninoculated sides.

Surface morphology of the cochleae after otocystic inoculation of enhanced green fluorescent protein fused to a nine-arginine peptide

We also assessed the surface morphology of the cochleae in five pups at P30. There was no hair cell loss or disturbance of hair cell alignment in the inoculated or noninoculated sides (Fig. 6).

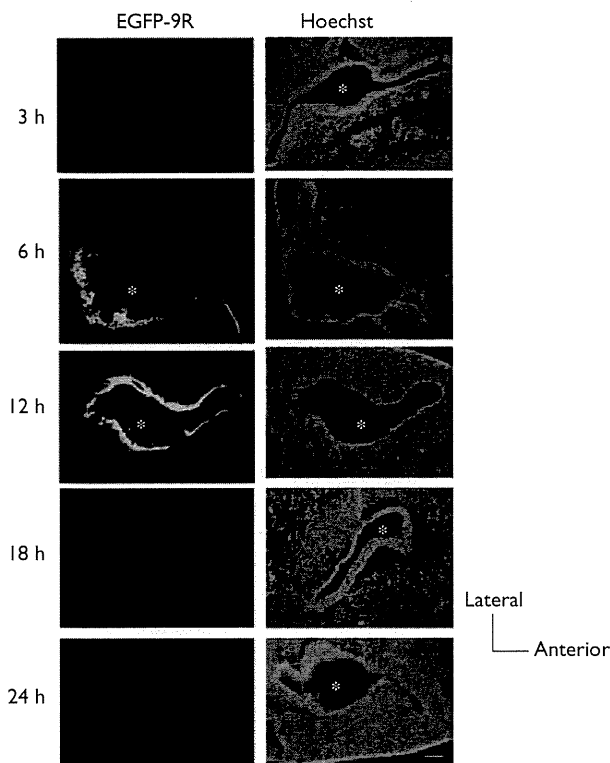
Fig. 2



High-magnification images of double fluorescence labeling with enhanced green fluorescent protein fused to a nine-arginine peptide (EGFP-9R; green) and Hoechst stain (blue). (a) The images show the otocyst at 12 h after EGFP-9R inoculation. EGFP fluorescence was detected in the lining cells of the otocyst and their vicinity in a diffuse manner. (b) Immunolabeled jagged-1 was detected at the ventromedial side of the lining cells of otocysts (arrowheads). (c) Merged image of Jagged-1, EGFP, and Hoechst signals. Asterisks indicate otocysts. The scale bar indicates 100 μm.

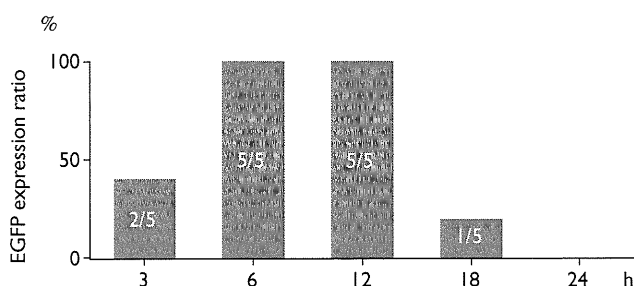
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 3



Sequential images of enhanced green fluorescent protein fused to a nine-arginine peptide (EGFP-9R)-inoculated otocysts fluorescently labeled with EGFP-9R (green) and Hoechst stain (blue). EGFP expression was clearly detected at 6 and 12 h and had disappeared at 18 and 24 h. The scale bar indicates 100 μm.

Fig. 4



Enhanced green fluorescent protein (EGFP) fused to a nine-arginine peptide expression ratio. The numbers in the bars indicate number of otocysts with EGFP expression/number of EGFP fused to a nine-arginine peptide-inoculated otocysts. The ratio reached 100% at 6–12 h and fell to 0% by 24 h after inoculation.

Vestibular function after otocystic inoculation of enhanced green fluorescent protein fused to a nine-arginine peptide

No animals inoculated with EGFR-9R showed circling behavior or head tilt at P30 (data not shown).

Discussion

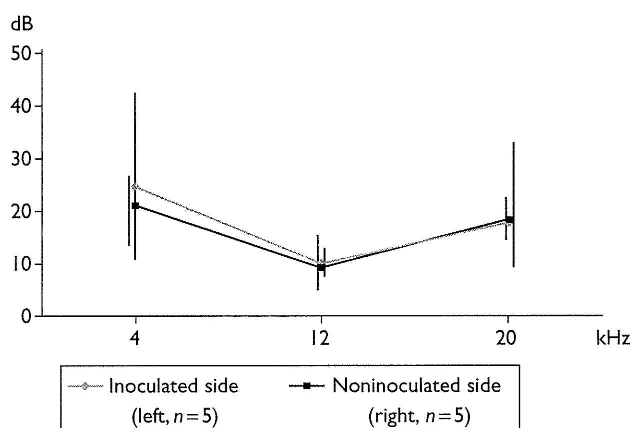
In the present study, we showed that inoculation of EGFP-9R into mouse otocysts induced highly effective transduction of protein to the lining cells of the otocysts and adjacent cells. The detection of an EGFP signal in these cells indicates that the EGFP was delivered into the cells and retained its function. Moreover, mice that underwent EGFP-9R inoculation did not show postnatal deterioration in auditory or vestibular function.

Previous studies have reported the use of viral vectors [1,2] or electroporation [3] to successfully transfect genes into mouse otocystic cells. Adeno-associated viral vectors induce gene transfection into otocystic cells more effectively than adenoviral vectors. Nevertheless, the usefulness of the viral vectors is limited by their low transfection efficiency, restrictions based on cell type, and cellular toxicity. Electroporation-mediated gene transfer is effective for transfecting genes into a wide range of cell types, including the lining cells and its vicinity of the otocysts; however, the electroporation-mediated gene transfer has several drawbacks. The electroporation-mediated gene transfer requires two steps: inoculation of a plasmid and electroporation. The longer exposure time of the embryos that is required for these two-step manipulations increases the risk of embryo survival after the manipulation. In addition, the nonoptimized electroporation conditions may cause less effective gene transfection or cell toxicity. In contrast, the protein transduction does not require multiple step manipulations, and in-vivo protein transduction using octa-arginine or nona-arginine gave high transfection efficiency, with no cell specificity and minimal cytotoxicity [16]. Taken together, the 9R-mediated protein transduction into otocysts is a simple method compared with the electroporation-mediated gene transfer, and may be an effective and safe method of delivering target proteins into and around the lining cells of otocysts.

CPP-mediated otocystic transduction has several drawbacks. These include a short half-life and non-specific targeting of the transduced proteins by CPPs. The present study showed that EGFP-9R levels in otocysts were not maintained for 24 h. The short half-life may limit its usefulness. Repeated application of the proteins may resolve this problem. However, it is technically difficult to repeatedly inoculate a substance into the developing inner ear. Moreover, repeated application may disturb the normal development of the inner ear. We have previously shown that most peptides and proteins delivered into cells by CPPs are degraded in lysosomes [19,20]. It is important to optimize the method for long-term stabilization of transduced proteins. We recently showed that cell-penetrating D-isomer peptides of the C-terminus of p53 were resistant to degradation and had long-term inhibitory effects on tumor growth [21]. A single application of the peptides

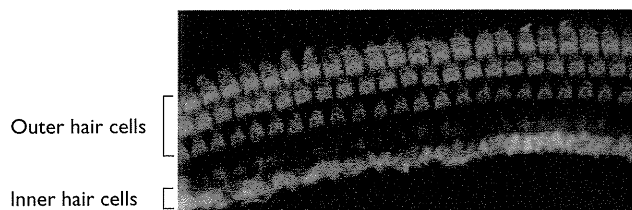
Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 5



Auditory thresholds in P30 mice. There was no statistically significant difference in auditory threshold between the enhanced green fluorescent protein fused to a nine-arginine peptide (EGFP-9R)-inoculated (left) and noninoculated sides (right; $P > 0.05$).

Fig. 6



Surface morphology of an inoculated-side cochlea of a P30 mouse stained with Texas Red-X phalloidin. There was no hair cell loss or disturbance of hair cell alignment.

inhibited the growth of bladder cancer and increased long-term disease-free survival *in vivo* [21]. Thus, the transduction of D-isomer proteins fused to CPPs may be useful for protein transduction into embryonic otocysts. In addition, the non-specific nature of CPP-mediated protein delivery may become a disadvantage. Site-targeted or tissue-targeted delivery would be preferable. Laakkonen *et al.* [22] reported that LyP-1, a kind of CPP, induces strong accumulation in primary MDAMB-435 breast cancer xenografts and their metastases after intravenous peptide injections. Intracellular localization of cargos may also be controlled by the addition of a nuclear localization signal. Kemp *et al.* [23] reported that it resulted in the exclusive delivery of a peptide into the nuclear compartment of the cells. Thus, the attachment of such homing peptides or other targeting motifs to CPPs may increase retention in a specific tissue or cell type [24].

Conclusion

EGFP-9R was effectively delivered into the otocysts of mouse embryos and remained for 12–18 h after otocystic inoculation. The embryos that underwent EGFP-9R inoculation developed normally, and their auditory and vestibular functions were normal after birth. CPP-mediated protein transduction may be a useful alternative to commonly used gene delivery methods for the delivery of therapeutically relevant molecules. Short half-life and nonspecific transduction may be drawbacks of this method. Nevertheless, recent studies provide some clues to overcoming these problems.

Acknowledgements

The authors thank members of the Molecular Physiology and Gene Technology Center at Kumamoto University for their important contributions to the experiments. This work was supported in part by the Advanced Education Program for Integrated Clinical, Basic and Social Medicine, Graduate School of Medical Sciences, Kumamoto University (Program for Enhancing Systematic Education in Graduate Schools, Mext, Japan).

Conflicts of interest and source of funding

There are no conflicts of interest.

References

1 Bedrosian JC, Gratton MA, Brigande JV, Tang W, Landau J, Benette J. In-vivo delivery of recombinant viruses to the fetal murine cochlea: transduction characteristics and long-term effects on auditory function. *Mol Ther* 2006; **14**:328–335.
2 Minoda R, Kawamoto K, Raphael Y. Trans-utero gene transfer into the mouse otocyst. *Assoc Res Otolaryngol Abs* 2003; **26**:162–163.
3 Gubbels SP, Woessner DW, Mitchell JC, Ricci AJ, Brigande JV. Functional auditory hair cells produced in the mammalian cochlea by in utero gene transfer. *Nature* 2008; **455**:537–541.
4 Green M, Loewenstein PM. Autonomous functional domains of chemically synthesized human immunodeficiency virus Tat trans-activator protein. *Cell* 1998; **55**:1179–1188.
5 Frankel AD, Pabo CO. Cellular uptake of the tat protein from human immunodeficiency virus. *Cell* 1988; **55**:1189–1193.
6 Nagahara H, Vocero-Akbani AM, Snyder EL, Ho A, Latham DG, Lissy NA, *et al.* Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p-27Kip1 induces cell migration. *Nat Med* 1998; **4**:1449–1452.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

7 Schmidt N, Mishra A, Lai GH, Wong GCL. Arginine-rich cell-penetrating peptides. *FEBS Lett* 2010; **584**:1806–1813.

8 Schwarze SR, Ho A, Vocero-Akbani AM, Dowdy SF. In-vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* 1999; **285**:1569–1572.

9 Eguchi A, Meade BR, Chang YC, Fredrickson CT, Willert K, Nitin P, *et al.* Efficient siRNA delivery into primary cells by a peptide transduction domain-dsRNA binding domain fusion protein. *Nat Biotechnol* 2009; **27**:567–571.

10 Takenobu T, Tomizawa K, Matsushita M, Li ST, Moriwaki A, Lu YF, *et al.* Development of p53 protein transduction therapy using membrane-permeable peptides and the application to oral cancer cells. *Mol Cancer Ther* 2002; **1**:1043–1049.

11 Futaki S. Membrane-permeable arginine-rich peptides and the translocation mechanisms. *Adv Drug Deliv Rev* 2005; **5**:547–558.

12 Futaki S. Arginine-rich peptides: potential for intracellular delivery of macromolecules and the mystery of the translocation mechanisms. *Int J Pharmaceut* 2002; **245**:1–7.

13 Wender PA, Mitchell DJ, Pattabiraman K, Pelkey ET, Steinman L, Rothbard JB. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *PNAS* 2000; **9**:13003–13008.

14 Rothbard JB, Garlington S, Lin Q, Kirschberq T, Kreider E, McGrane PL, *et al.* Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat Med* 2000; **6**:1253–1257.

15 Lindsay MA. Peptide-mediated cell delivery: application in protein target validation. *Curr Opin Pharmacol* 2002; **2**:587–594.

16 Matsushita M, Tomizawa K, Moriwaki A, Li ST, Terada H, Matsui H. High-efficiency protein transduction system demonstrating the role of PKA in long-lasting long-term potentiation. *J Neuroscience* 2001; **16**:6000–6007.

17 Kawamoto K, Oh SH, Kanzaki S, Brown N, Raphael Y. The functional and structural outcome of inner ear gene transfer via the vestibular and cochlear fluids in mice. *Mol Ther* 2001; **4**:575–585.

18 Hartman BH, Reh TA, Bermingham-McDonogh O. Notch signaling specifies prosensory domains via lateral induction in the developing mammalian inner ear. *Proc Natl Acad Sci USA* 2010; **107**:15792–15797.

19 Matsushita M, Noguchi H, Lu YF, Tomizawa K, Michiue H, Li ST, *et al.* Photo-acceleration of protein release from endosome in the protein transduction system. *FEBS Lett* 2004; **572**:221–226.

20 Michiue H, Tomizawa K, Wei FY, Matsushita M, Lu YF, Ichikawa T, *et al.* The NH2 terminus of influenza virus hemagglutinin-2 subunit peptides enhances the antitumor potency of polyarginine-mediated p53 protein transduction. *J Biol Chem* 2005; **280**:8285–8289.

21 Araki D, Takayama K, Inoue M, Watanabe T, Kumon H, Futaki S, *et al.* Cell-penetrating D-isomer peptides of p53 C-terminus: long-term inhibitory effect on the growth of bladder cancer. *Urology* 2010; **75**:813–819.

22 Laakkonen P, Akerman ME, Biliran H, Yang M, Ferrer F, Karpanen T, *et al.* Antitumor activity of a homing peptide that targets tumor lymphatics and tumor cells. *Proc Natl Acad Sci USA* 2004; **101**:9381–9386.

23 Kemp BE, Cheng HC, Walsh DA. Peptide inhibitors of cAMP-dependent protein kinase. *Methods Enzymol* 1988; **159**:173–183.

24 Järver P, Mäger I, Langel Ü. In-vivo biodistribution and efficacy of peptide mediated delivery. *Trends Pharmacol Sci* 2010; **31**:528–535.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Brain Tumor Pathol (2011) 28:13–24
DOI 10.1007/s10014-010-0007-z

REVIEW ARTICLE

Angiogenesis and invasion in glioma

**Manabu Onishi · Tomotsugu Ichikawa ·
Kazuhiko Kurozumi · Isao Date**

Received: 20 July 2010 / Accepted: 22 September 2010 / Published online: 8 January 2011
© The Japan Society of Brain Tumor Pathology 2010

Abstract Despite advances in surgical and medical therapy, glioblastoma consistently remains a fatal disease. Over the last 20 years, no significant increase in survival has been achieved for patients with this disease. The formation of abnormal tumor vasculature and glioma cell invasion along white matter tracts are believed to be the major factors responsible for the resistance of these tumors to treatment. Therefore, investigation of angiogenesis and invasion in glioblastoma is essential for the development of a curative therapy. In our report, we first reviewed certain histopathological studies that focus on angiogenesis and invasion of human malignant gliomas. Second, we considered several animal models of glioma available for studying angiogenesis and invasion, including our novel animal models. Third, we focused on the molecular aspects of glioma angiogenesis and invasion, and the key mediators of these processes. Finally, we discussed the recent and ongoing clinical trials targeting tumor angiogenesis and invasion in glioma patients. A better understanding of the mechanism of glioma angiogenesis and invasion will lead to the development of new treatment methods.

Keywords Glioma · Invasion · Angiogenesis · Hypoxia · Molecular targeted therapy

M. Onishi · T. Ichikawa (✉) · K. Kurozumi · I. Date
Department of Neurological Surgery,
Okayama University Graduate School of Medicine,
Dentistry and Pharmaceutical Sciences,
2-5-1, Shikata-cho, Kita-ku, Okayama 700-8558, Japan
e-mail: tomoichi@cc.okayama-u.ac.jp

M. Onishi
e-mail: onishi_manabu@yahoo.co.jp

Introduction

Despite advances in surgical and medical therapy, glioblastoma consistently remains a fatal disease. The pathophysiological processes of angiogenesis and tumor cell invasion play pivotal roles in glioma development and growth already in the earliest phase [1]. The formation of abnormal tumor vasculature and glioma cell invasion along white matter tracts are believed to be the major reasons for the resistance of these tumors to treatment. Recent insight into the fundamental processes governing glioma angiogenesis and invasion have provided renewed hope for developing novel strategies aimed at reducing morbidity due to this fatal disease. However, glioma angiogenesis and invasion are challenging to study in experimental settings because most of the animal models fail to mimic the unique angiogenesis and invasiveness of human glioma cells.

In this review, we first discuss the histopathological features of angiogenesis and invasion in human malignant glioma. Second, we considered several animal glioma models available for studying angiogenesis and invasion. We then focus our attention on the molecular biology of glioma angiogenesis and invasion, examining the key mediators of these processes. Lastly, we briefly discuss the recent and ongoing clinical trials targeting mediators of angiogenesis or invasion in glioma patients.

Histopathological studies of glioma angiogenesis and invasion

Glial tumors are believed to arise from neuroepithelial tissue and can be classified into astrocytic, oligodendroglial, and oligoastrocytic tumors. According to the World Health Organization (WHO), astrocytomas are classified

into four grades (I–IV) based on the histopathological features that correlate with prognosis [2]. Grade I tumors (pilocytic astrocytomas) are rare and biologically benign. Grade II tumors display nuclear atypia, and the median survival of patients with this tumor type is approximately 10 years. The median survival of patients with tumors classified as grade III (anaplastic astrocytoma) is approximately 2 years. Grade IV tumors, also called glioblastoma multiforme (GBM), have a very poor prognosis, and the median survival of patients with GBM is approximately 14.6 months [3]. Glioblastoma, the most malignant tumor among the astrocytic gliomas, is the most common primary brain tumor in adults.

Malignant glioma (WHO grade III and IV) occurs most often in the subcortical white matter of the cerebral hemispheres. Many reports have described the histopathological features of malignant gliomas. Marked proliferation, angiogenesis, and invasion have been recognized as hallmarks of this disease. Proliferation activity is usually prominent, with detectable mitoses present in nearly every case. Vascular proliferation is seen throughout the lesion, often around necrotic foci and in the peripheral infiltration zone. Glioblastomas are among the most vascularized human tumors [4]. Tumor infiltration often extends into the adjacent cortex and through the corpus callosum into the contralateral hemisphere.

Histopathological analysis of angiogenesis

Neovascularization in and around the malignant glioma is well recognized. Glioblastomas, one of the most well-studied tumor types, with regard to angiogenesis, are known to have blood vessels of increased diameter with high permeability, thickened basement membranes, and highly proliferative endothelial cells [5]. The presence of microvascular proliferation with the formation of glomerular capillary loops in garland-like formation [6] is a histopathological hallmark of glioblastoma. Increased neoplastic proliferation of glial cells running parallel to endothelial vascular proliferation is one of the malignancy evaluation criteria [7]. Vascular density in glioblastoma is markedly higher than that in tumors of lower histological grade [8]. An increase in vascularization significantly worsens prognosis [7]. Necrotic foci surrounded by pseudopalisading cells are a configuration relatively unique to glioblastoma. Recently, Rong et al. [9] demonstrated that pseudopalisading cells are present in severely hypoxic states and they overexpress hypoxia-inducible factor (HIF-1) and secreting proangiogenic factors, such as VEGF and IL-8. Thus, microvascular hyperplasia in glioblastoma, which provides a new vasculature and promotes peripheral tumor extension, is closely linked to the appearance of pseudopalisades.

Histopathological analysis of invasion

Although infiltrative spread is a common feature of all diffuse astrocytic tumors, glioblastoma is particularly infamous for its rapid invasion into neighboring brain structures [10]. Supratentorial bilateral extension occurs because of rapid growth along myelinated structures, in particular across the corpus callosum and along the fornices toward the temporal lobes. Extension within and along perivascular spaces is another typical mode of infiltration [11].

Magnetic resonance imaging (MRI) is frequently used to evaluate tumor vasculature and invasion [12]. In human glioma patients, only the central leaky part of high-grade gliomas can be visualized by gadolinium contrast enhancement, whereas the other outer rims of the tumor and infiltrating cells in the normal surrounding brain tissue, which are protected by normal vasculature, are not visible. The diffuse invasive area around the enhanced mass of a malignant glioma is depicted as high-intensity signals on T2-weighted images [13]. Although MRI is a powerful tool for high resolution in vivo imaging, sub-millimeter lesions are not always detected by MRI.

While histopathological studies have given insight into tumor invasion, there are limitations to this methodology. Several problems have been encountered in investigating glioma invasion, the most critical being the lack of glioma-specific antibodies for immunohistochemical analysis. Exact localization of invading glioma cells within seemingly normal brain parenchyma is crucial for the precise evaluation of invasion patterns. Recently, MAP2e, a splice variant of MAP2 (microtubule-associated protein), was reported as a candidate glioma-specific antigen. Most cells in CNS tumors, particularly oligodendrogliomas and glioblastomas, stain positively for MAP2e. Immunohistochemistry targeting MAP2e can therefore localize invasive glioma cells [14].

We examined samples of human glioblastomas by dual immunohistochemical staining with glioma- and endothelial-specific antibodies (MAP2e and vWf; von Willebrand factor, respectively), and examined the relationship between angiogenesis and invasion (Fig. 1). Our findings confirmed that there are at least two angiogenic and invasive glioma phenotypes. Clusters of glioma cells were seen around newly developed vessels in the normal parenchyma adjacent to the tumor margins. Single cell infiltrations into normal brain parenchyma independent of vasculature were also seen. These different invasive and angiogenic phenotypes are called angiogenesis-dependent and angiogenesis-independent invasions [15]. Malignant astrocytoma and glioblastoma consist of a mixture of subclones with both angiogenesis-dependent and angiogenesis-independent invasion phenotypes present in various proportions.

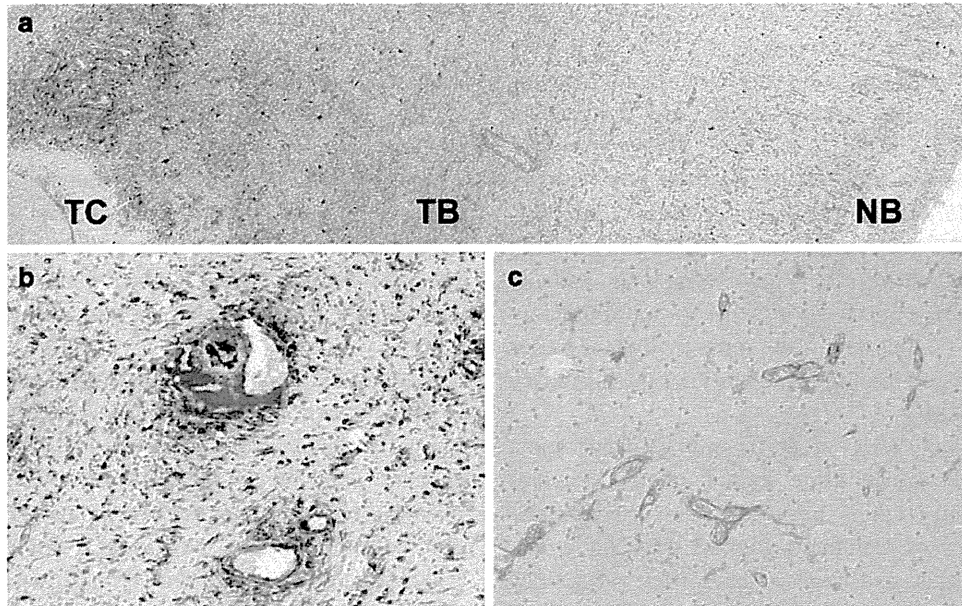


Fig. 1 MAP2e and vWf immunohistochemical staining of a human glioblastoma sample showing two distinct invasive and angiogenic phenotypes. Macroscopic appearance of glioblastoma sample (**a**). All tumor cells were positive for MAP2e and represented the diffuse infiltration from the tumor core (TC) to surrounding normal brain (NB) and thereby the absence of a border between tumor and normal brain (**a**). The center of the tumor showed high tumor cell density, endothelial proliferation, necrosis, and pseudopalisading of tumor cells around the necrotic focus. At the tumor border (TB), clusters of MAP2e-positive tumor cells were observed around dilated vessels (**b**). In normal brain (NB) tissues where no dilated vessels were observed, single MAP2e-positive tumor cells were diffusely distributed (**c**). MAP2e: DAB, vWf: DAB-Ni, counterstain: hematoxylin. *TC* tumor core, *TB* tumor border, *NB* normal brain

Moreover, gliomatosis cerebri is an extreme example of a tumor that consists solely of subclones with an angiogenesis-independent invasion phenotype.

Animal models for studying glioma invasion and angiogenesis

To study the histopathological and molecular bases of malignant glioma, several animal models of glioma have been established by intracerebral inoculation of highly proliferative glioma cells in culture. Glioma invasion and angiogenesis are challenging areas to study because most traditional animal models do not recapitulate the unique features of invasiveness and angiogenesis of human glioma cells [16]. Typically, such transplantable tumors in mice or rats form solid nodules at the injection site, which compress rather than invade the surrounding brain regions. Recently, several new glioma models have been developed, which replicate the invasive behavior and show neovascularization. In particular, some sophisticated animal models exist in which human glioma xenografts can show various degrees of true invasion. These models include (1) direct implantation of patient surgical specimens into the brains of nude mice [17], (2) transplantation of patient surgical material s.c. in nude mice followed by dissociation and orthotopic reinjection of these xenotransplants [18], (3) engraftment of glioblastoma-derived spheroids after short-term culture into rat brain [19], and (4) engraftment of glioblastoma stem cell-enriched cultures into mouse brain [20, 21]. Although these models are reproducible, they require some special procedures to develop the brain tumors, such as in vivo preparation of xenografts or in vitro spheroid formation before implantation.

We have developed a pair of glioma cell lines that show different invasive and angiogenic behaviors in the brains of immunocompromised animals (S. Inoue, T. Ichikawa, T. Maruo et al. Novel animal glioma models that separately exhibit two different invasive and angiogenic phenotypes of human glioblastomas; submitted). Histopathologically, one shows angiogenesis-dependent invasion with cluster formation around neovascular vessels in the tumor margin, and the other shows single cell infiltration into the normal brain parenchyma without angiogenesis (Fig. 2). These animal glioma models separately mimic the two phenotypes of human glioblastoma invasion: angiogenesis-dependent and angiogenesis-independent invasions. Our models can be readily established without any complicated cell preparation, and they give steady reproducibility of tumor development with the same phenotypic growth as that observed in human glioma. These novel models would be particularly beneficial for analyzing the molecular mechanisms of invasion and angiogenesis in glioma and investigation of new therapies for glioma.

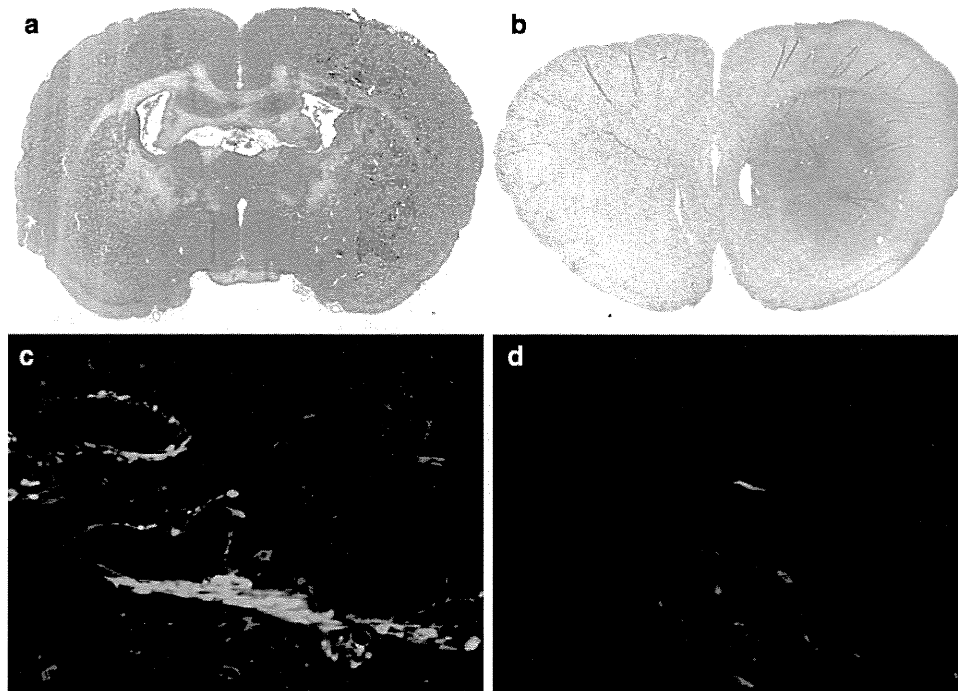


Fig. 2 Two animal brain tumor models showing distinct phenotypes of invasion. J3T-1 brain tumor established in athymic rat brain (**a**). J3T-1 cells formed well-demarcated and highly angiogenic tumors in the rat brain. Clusters of tumor cells were seen around newly developed vessels in adjacent normal brain. J3T-1G cells expressing green fluorescent protein (GFP) coopting around dilated vessels (*red*) (**c**). J3T-2 brain tumor established in athymic rat brain (**b**). J3T-2 cells formed poorly demarcated tumors with tumor cells gradually dispersed from the tumor center to the normal brain parenchyma. J3T-2G single cells diffusely invaded normal brain tissue (**d**). **a**, **b** H&E staining. **c**, **d** Tumor cell (GFP, *green*), vascular stain (RECA-1, *red*), nuclear stain (DAPI, *blue*)

Molecular biology of angiogenesis in glioma

Angiogenesis is a key event in the progression of malignant gliomas. The presence of microvascular proliferation leads to the histological diagnosis of GBM [22]. Among all solid tumors, GBM has been reported to be the most angiogenic by displaying the highest degree of vascular proliferation and endothelial cell hyperplasia [23]. Such intense vascularization might be responsible for the peritumoral edema, one of the pathological features of GBM [24, 25].

Angiogenesis is the formation of new blood vessels by rerouting or remodeling of existing vessels, and it is believed to be the primary method of vessel formation in gliomas. Angiogenesis requires three distinct steps: (1) blood vessel breakdown, (2) degradation of the vessel basement membrane and surrounding extracellular matrix (ECM), and (3) migration of endothelial cells and the formation of new blood vessels (Fig. 3).

Blood vessel breakdown

The first step in forming new blood vessels from existing vessels is the dissolution of aspects of native vessels. Glioma cells first accumulate around the existing cerebral blood vessels and lift off the astrocytic foot processes, which leads to the disruption of the normal contact between endothelial cells and the basement membrane [26]. The affected endothelial cells express angiopoietin-2 (Ang-2), resulting in destabilization of the vessel wall and decreased pericyte coverage [26–28]. Ang-1 and -2 are important endothelial growth factors that signal via the Tie2 receptor tyrosine kinase (RTK) expressed on endothelial cells. In the normal brain, Ang-1 binds to Tie2 to induce association between pericytes and endothelial cells, resulting in stabilization of the vasculature [29, 30]. On the other hand, Ang-2 may act as an antagonist to Tie2 phosphorylation, leading to destabilization of blood vessels. Therefore, Ang-2 represents a checkpoint for Ang-1/Tie2-mediated angiogenesis [26, 31].

Degradation of the vessel basement membrane and surrounding ECM

Degradation of the vessel basement membrane and surrounding ECM, which also facilitates the invasion of endothelial cells, is an integral part of the ongoing angiogenic process [32]. The matrix metalloproteinase (MMP) family enzymes that degrade components of ECM consist of four groups according to their substrates: collagenases, gelatinases, stromelysins, and membrane-associated MMPs.

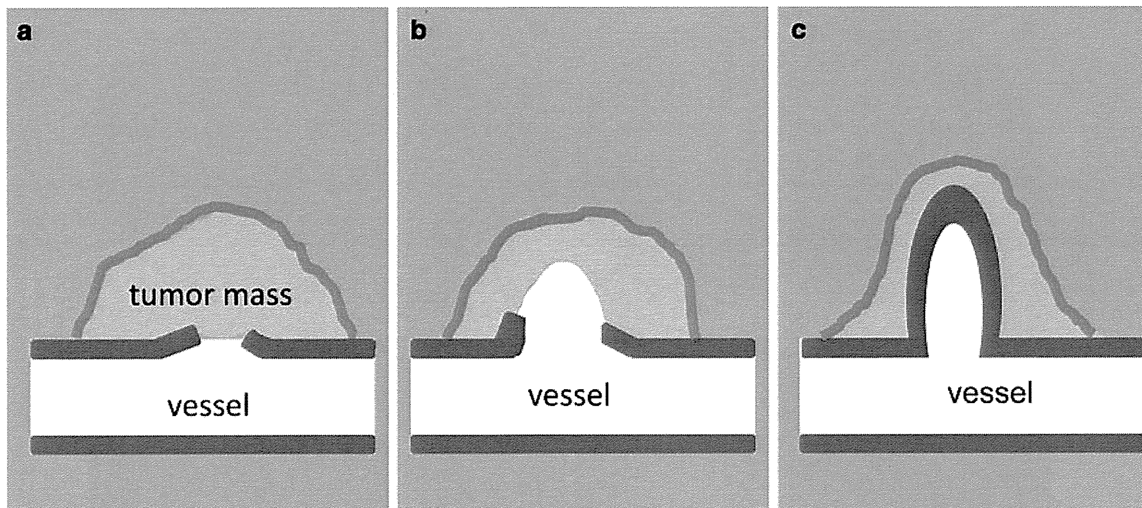


Fig. 3 Schematic representation of the mechanism of angiogenesis in glioma. Angiogenesis requires three distinct steps: (**a**) blood vessel breakdown, (**b**) degradation of the vessel basement membrane and surrounding ECM, and (**c**) migration of endothelial cells and formation of new blood vessels

Gelatinases-A (MMP-2) and gelatinases-B (MMP-9) are highly expressed in astrocytomas, and their expression levels, especially those of MMP-9, correlate with the histological grade of tumor. Both MMP-2 and MMP-9 have been detected in blood vessels as well as in tumor cells [33, 34]. MMP-2 and MMP-9 expression is strongly induced by hypoxia, and these two molecules appear to have a synergistic effect on basement membrane degradation [35].

Migration of endothelial cells and formation of new blood vessels

After regression of existing vessels and breakdown of the basement membrane, endothelial cells proliferate and migrate toward the tumor cells expressing pro-angiogenic compounds. Integrin $\alpha v\beta 3$ and $\alpha 5\beta 1$ are upregulated in endothelial cells during angiogenesis, enhancing endothelial cell adhesion and migration [36, 37]. In addition to migration of endothelial cells, migration of pericytes is an important part of tumor vessel formation. Platelet-derived growth factor (PDGF) secretion by activated endothelial cells recruits pericytes to the site of newly sprouting vessels and aids in establishing a new basement membrane [38, 39].

Hypoxia and angiogenesis

Vascular homeostasis is governed by a balance between pro-angiogenic and anti-angiogenic stimuli [40]. Angiogenesis is activated in growing gliomas when the pro-angiogenic stimuli outweigh the anti-angiogenic stimuli. The most potent activator of angiogenic mechanisms in brain tumors is tissue hypoxia. One well-studied pathway is the HIF-1/VEGF-A pathway, which leads to endothelial cell proliferation and migration [41]. The transcription factor HIF-1, which is composed of two subunits, HIF-1β, and HIF-1α, is the classic and best-characterized hypoxia-regulated molecule. Interestingly, HIF-1β remains unchanged under hypoxic conditions. However, at the posttranscriptional level, HIF-1α protein is upregulated under hypoxic (1–2% O_2) conditions [42]. Under these conditions, HIF-1 activates DNA promoter regions known as hypoxia response elements (HREs). HREs induce transcription of >100 genes that help the cell cope with low O_2 conditions [43, 44]. VEGF, which regulates tumor edema and blood vessel formation, is an example of a gene regulated by an HIF-1 though an HRE. Specifically, VEGF-A is known to be upregulated in glioblastoma and is produced by multiple cell types, including tumor, stromal, and inflammatory cells [45]. VEGF-A binds to two RTKs (receptor; tyrosine kinases), VEGFR-1 (Flt-1) and VEGFR-2 (KDR, Flk-1) [46]. VEGF-A regulates endothelial cell survival, proliferation, permeability, and migration primarily via VEGF-receptor 2 (VEGFR2) [47]. VEGF promotes endothelial proliferation via activation of the MAPK pathway [22]. VEGF also enhances vascular permeability through the MAPK signaling cascade by rearranging cadherin/catenin complexes and loosening adhering junctions between endothelial cells [48, 49]. VEGF stimulates endothelial production of urokinase-type plasminogen activator (uPA) [50], which induces conversion of plasminogen to plasmin, causing the breakdown of ECM components and leading to ECM remodeling [22]. The end result of VEGF signaling in tumors is the production of immature, highly permeable blood vessels with subsequent poor maintenance of BBB and parenchymal edema [40, 51].

Molecular biology of glioma invasion

One of the insidious features of gliomas is the potential of single cells to invade normal brain tissue, blurring tumor margins, and establishing numerous microtumors at a distance from the primary tumor. The detailed mechanisms of glioma invasion are only beginning to be understood.

Invasion of tumor cells into normal tissue is thought to be a multifactorial process, consisting of cell interactions with ECM and with adjacent cells, as well as accompanying biochemical processes supportive of active cell movement. It is well known that cell motility is not a de novo feature that emerges coincident to carcinogenesis. Several cell types exhibit active movement during various stages of embryonal development, during wound healing, and in the course of immune responses. This motile behavior is regulated in a very rigid manner, suggesting that the reappearance of a motile phenotype in cancer cells results from the loss or cessation of normal inhibitory controls [52, 53]. Many have proposed that the highly infiltrative nature of human gliomas recapitulates the migratory behavior of glial progenitors [14, 54, 55].

Tumor cell invasion requires four distinct steps: (1) detachment of invading cells from the primary tumor mass, (2) adhesion to ECM, (3) degradation of ECM, and (4) cell motility and contractility (Fig. 4).

Detachment of invading cells from the primary tumor mass

The detachment of invading glioma cells from the primary tumor mass involves several events. The first event entails destabilization and disorganization of cadherin-mediated junctions that hold the primary mass together. Cadherins (E-, P-, and N-cadherin) form adherent junctions, which are calcium-dependent, transmembrane, cell–cell adhesion complexes. Due to their contribution to processes such as morphological differentiation and contact inhibition of growth and motility, cadherins may function as suppressors of tumor growth and invasion [53]. During carcinoma progression, decreased cadherin function is correlated with de-differentiation, metastasis, and poor prognosis [56].

The second event is a decline in the expression of connexin 43, which leads to a reduction in gap junction formation. Cell–cell communication is important in growth control and differentiation, and it is partly achieved using gap junctions and via second messengers [57]. Decreased gap junction formation may result in fewer inhibitory signals, facilitating uncontrolled cell division and de-differentiation [58]. Connexin 43 is the most abundant gap junction protein in CNS and is expressed primarily in astrocytes [59]. McDonough and coworkers [60] have reported that reduced gap junction formation is correlated with increased motility of glioma cells in vitro. Increased malignancy of glioma specimens correlates with reduced in situ gap junction formation as well as reduced connexin 43 expression [61]. These observations suggest that decreased expression of connexin 43 is important for increased growth and invasion of gliomas [53].

The third event is cleavage of CD44, which anchors the primary mass to ECM, by the metalloproteinase ADAM. CD44 is a transmembrane glycoprotein belonging to the immunoglobulin receptor superfamily, which interacts with hyaluronic acid as its ligand. Hyaluronic acid comprises a substantial fraction of brain ECM and is implicated in a wide variety of physiological and pathological

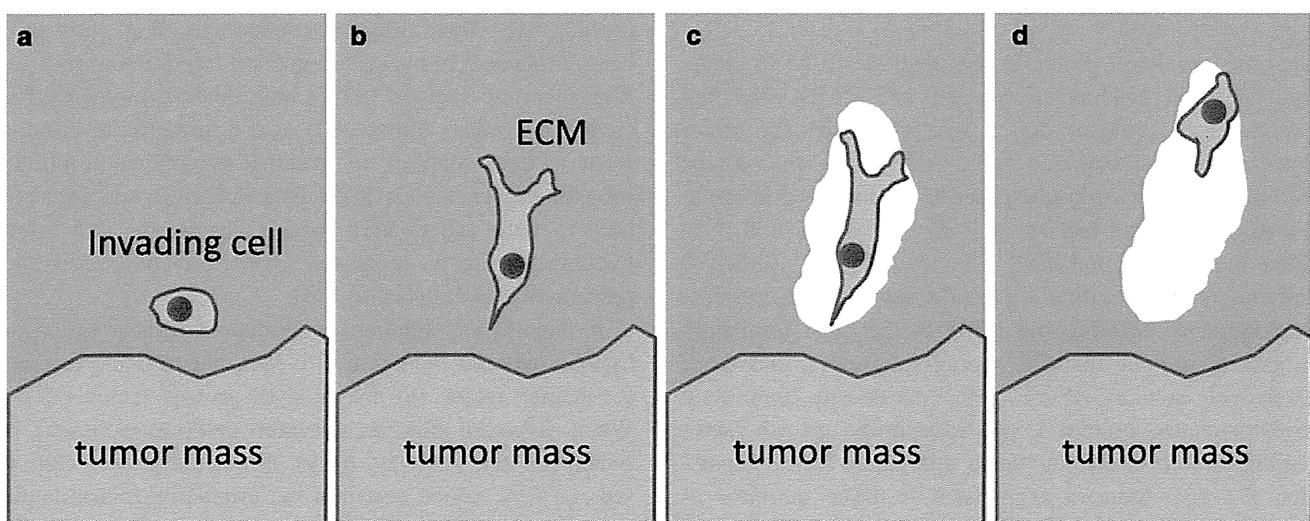


Fig. 4 Schematic representation of the mechanism of glioma invasion. Invasion requires four distinct steps: (**a**) detachment of invading cells from the primary tumor mass, (**b**) adhesion to ECM, (**c**) degradation of ECM, and (**d**) cell motility and contractility

processes [62]. Monoclonal antibodies directed against CD44 decrease intracerebral invasion of glioma cells in vivo and through matrigel matrices in vitro [63]. CD44 can be cleaved by ADAM 10 and 17, and both the extracellular and intracellular cleaved components of CD44 promote cell migration [64].

Adhesion to ECM

The most common molecules that allow glioma cells to adhere to ECM are the integrins. Integrins are transmembrane glycoproteins composed of two subunits (α and β). They interact with two large groups of ligands: a variety of ECM proteins, such as fibronectin, vitronectin, fibrinogen, and cell surface molecules, that are members of the immunoglobulin supergene family, such as intracellular adhesion molecules (ICAM-1, ICAM-2) and vascular cell adhesion molecule (VCAM-1). In particular, the integrin $\alpha v\beta 3$, which binds to fibronectin, vitronectin, and tenascin-C in ECM, is thought to play a central role in glioma invasion [65]. Increased expression of integrin $\alpha v\beta 3$ leads to increased motility in human glioma cells with a concomitant decrease in apoptosis sensitivity. Conversely, inhibition of integrin $\alpha v\beta 3$ decreases glioma cell motility [66]. Several factors expressed in glioma cells have been found to regulate integrin expression. Particularly, uPA secreted by glioma cells has been shown to upregulate integrin $\alpha v\beta 3$ expression by autocrine mechanism [67]. Glioma expression of focal adhesion kinase, a nonreceptor cytoplasmic tyrosine kinase, has been shown to increase phosphorylation of the enhancer of filamentation 1, which in turn stimulates PDGF-mediated stimulation of glioma integrin adhesion to ECM [68].

Degradation of ECM

The most common proteases that degrade ECM to create the space for invading glioma cells are MMPs. The first experimental evidence for a role of MMPs in tumor development was obtained in the early 1980s when a type IV collagenase was demonstrated to be involved in melanoma invasion and metastasis [69]. Wild-Bode et al. [70] found that MMP-2 and MMP-3 levels and MMP-2/MMP-9 activity correlated with glioma cell migration and invasion. Regulation of MMP-2 and MMP-9, the most frequently expressed glioma invasion-mediating factors, has been extensively studied. NF-κB, uPA, low-density lipoprotein receptor-related protein 1, and insulin-like growth factor binding protein-2 are known to upregulate MMP expression [71–73]. Induced expression of tissue inhibitor of metalloproteinases-3 (TIMP-3), a putative inhibitor of MMP activity, has been shown to suppress infiltration and also to induce apoptosis in cancer cell lines [74]. Similarly, several MMP inhibitors have been observed to effectively downregulate glioma invasion in vitro [75, 76].

Cell motility and contractility

Cell motility requires cytoplasmic contractile force. Glioma cells migrate similarly to nontransformed neural progenitor cells, extending a prominent leading cytoplasmic process followed by a burst of forward movement in the cell body. As is the case with neural progenitor cells, glioma motility and contractility require A and B isoforms of myosin II. Myosin II is the major source of cytoplasmic contractile force. Myosin II allows glioma cells to squeeze through pores smaller than their nuclear diameter, which is important because the brain has particularly narrow extracellular spaces [77]. The activity of myosin II is controlled by phosphorylation of a serine residue on its regulatory light chain (RLC). Phosphorylation of RLC by myosin light chain kinase (MLCK) activates myosin II. This action is opposed by dephosphorylation of RLC, which is mediated by a specific MLC (myosin light chain) phosphatase. Thus, the degree of activity of myosin II in a cell is controlled by the relative balance of activities of MLCK and MLC phosphatase. Small GTPases, such as RhoA, Rac, cdc42, as well as RLC-interacting protein, are also involved in this process in glioma cells [78, 79].

Hypoxia and glioma invasion

In studies of human GBM surgical specimens, pseudopalisading cells found in the necrotic areas are known to be hypoxic, as demonstrated by their dramatic upregulation of HIF-1, a nuclear transcription factor that orchestrates the adaptive response of the cells to lower oxygen [9, 80, 81]. Therefore, the pseudopalisading cells have been implicated in hypoxia-regulated migration away from these necrotic regions [82]. Limitations in oxygen diffusion would cause tumor cells at the furthermost distance from arterial supplies to became hypoxic and migrate towards viable vessels [82]. This is further supported by both in vitro and in vivo models, suggesting that tumor hypoxia results in increased GBM cell migration and, presumably, also invasion [83].

A significant number of human GBMs respond to hypoxia with an induction of c-Met, which is the receptor for scatter factor (SF)/hepatocyte growth factor (HGF). When hypoxia stimulates tumor cells to activate the c-met/SF/HGF pathway, c-met stimulates glioma cells to secrete uPA, which converts the circulating plasminogen into plasmin. Then plasmin degrades a variety of ECM proteins and activates several MMPs, which help glioma cells to invade the surrounding tissue [84, 85].

Molecular targeted therapy for glioma angiogenesis and invasion

Despite the genetic heterogeneity of malignant gliomas, common aberrations exist in the signaling elements of their angiogenesis and invasion pathways. New treatments have emerged to target molecules in these signaling pathways with the goal of clinical efficacy and minimal toxicity. Here, we review several clinical trials of new therapies that target the molecular mediators of glioma angiogenesis and invasion.

Bevacizumab

Bevacizumab, a humanized neutralizing monoclonal antibody against VEGF, has demonstrated encouraging radiographic responses in patients with recurrent malignant gliomas in combination with irinotecan [86]. Although a significant antitumor effect was observed in this phase II trial with a 63% radiographic response, the 6-month progression-free survival, which is a more positive primary endpoint for phase II trials in malignant gliomas, was 32% for GBM [87]. Recent data concerning the clinical use of anti-angiogenic drugs for recurrent malignant gliomas have been disappointing [88]. Some patients treated with bevacizumab have shown a reduction in the Gd-enhanced tumor size, but significant increases in the volume of infiltrative tumor relative to the enhanced tumor were also observed. Indeed, an experimental study showed that anti-angiogenic therapy of murine gliomas with an antibody against VEGF-R2 caused small satellite tumors to arise near the primary mass, centered around core vessels, akin to the perivascular invasion found more recently in the VEGF knockout glioma cell lines described previously [89]. Further research will be needed to identify mediators of this invasion and to determine whether the invasion seen after bevacizumab treatment of human glioblastomas is the perivascular invasion seen in the murine cell lines or the parenchymal type of invasion along white matter typically seen in glioblastomas [1]. One recent clinical trial suggested that the presence of tumor hypoxia markers predicts radiographic response and survival of patients treated with bevacizumab and irinotecan [90].

It has been proposed that hypoxia caused by vessel regression during the course of anti-angiogenic therapy leads to upregulation of proangiogenic factors and recruitment of bone marrow-derived cells (BMDCs) that have the capacity to increase tumor growth by means of new blood vessel growth [91]. Glioma cells evade antiangiogenic therapies by upregulating alternative proangiogenic signal circuits, including those utilizing fibroblast growth factor, ephrin A1, and angiopoietin 1. Another adaptive measure is the hypoxia-regulated recruitment of vascular progenitor cells and proangiogenic monocytes from the bone marrow to tumors [91].

Cilengitide

Integrins, cell adhesion molecules, play an important role in both glioma cell migration and angiogenesis. Cilengitide, an intravenous inhibitor of $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, demonstrated efficacy against an animal malignant glioma model [92]. Blocking $\alpha v\beta 3$ integrin inhibits blood vessel formation in vivo [93]. Cilengitide induces apoptosis in U87 glioma cells by preventing adherence to vitronectin and tenascin, matrix protein mediators of brain tumor invasion, and growth [94]. A phase I clinical trial of cilengitide in recurrent malignant gliomas by the New Approaches to Brain Tumor Therapy (NABTT) has been completed with no dose-limiting toxicities [95]. The most notable trial to date was a randomized phase II study of cilengitide, which was associated with a median survival of 10 months in recurrent glioma patients [96]. The North American Brain Tumor Consortium (NABTC) study was designed to determine whether cilengitide effectively penetrates into GBM in human patients. This study confirmed that cilengitide is effectively delivered into primary human GBM tumors with good retention. Recently, phase II studies of cilengitide in newly diagnosed GBM patients were presented. Patients with lowered MGMT expression based on gene promoter methylation appeared to benefit more with the addition of cilengitide [97]. The effect of combination therapy, such as cilengitide with XRT or with another chemotherapeutic agent, is likely to be cumulative.

Marimastat

Increased MMP levels are associated with glioma invasion and angiogenesis. Marimastat (MT), an orally active drug, can reduce MMP levels in patients with gliomas [98]. One phase II study evaluated MT combined with temozolomide (TMZ) in patients with recurrent malignant glioma [99]. For all patients, the progression-free survival (PFS) at 6 months was 39%. Median PFS was 17 weeks, median overall survival was 45 weeks, and 12-month PFS was 16%. A good outcome was documented, but joint and tendon pain was reported in 47% of patients. Eleven percent of patients were removed from the study because of intolerable joint pain. Further investigation of therapy-induced joint pain is needed.

Thalidomide

Thalidomide is an immunomodulatory agent that inhibits angiogenesis [100]. While thalidomide's anti-angiogenic mechanism is not understood, it has been suggested to

interfere with the expression of integrin receptors $\alpha v\beta 3$ and $\alpha v\beta 5$ and also to inhibit VEGF [101]. While thalidomide proved to be unsuccessful when used as a monotherapy in GBM, the measured response rate was 6%, and the 12-month overall survival was 22% [102].

Sorafenib

Sorafenib is a multikinase inhibitor, and inhibits Raf, VEGF, PDGFR-b, and c-Kit. Sorafenib suppresses angiogenesis via inhibition of VEGFR and PDGFR activities in endothelial cells [103]. Sorafenib-treated mice showed significant inhibition of glioblastoma cell proliferation, increased apoptosis and autophagy, and suppression of angiogenesis in vivo [104]. Phase II trials of sorafenib in patients with malignant gliomas are ongoing [22].

Imatinib

Imatinib is a kinase inhibitor of PDGFR, c-kit, and bcr-abl. PDGF and its cognate receptors, PDGFR, are important in growth and angiogenesis of glioma [105]. The in vitro effects of imatinib on glioma cell proliferation have been investigated. Imatinib at low concentrations can act as a cytostatic agent, whereas at high concentrations it mainly behaves as a cytotoxic agent [106]. Imatinib monotherapy has failed to show any significant clinical benefits. There are several potential reasons for the disappointing results with imatinib monotherapy in malignant gliomas. The penetration of the drug across BBB is likely to be limited by P-glycoprotein and other efflux pumps, thus reducing tumor concentrations of the drug. A second reason for the limited activity of imatinib may be that inhibition of PDGFR alone is insufficient to prevent growth of malignant gliomas [107].

Tenascin-C immunotherapy

Tenascin-C has been identified in hyperplastic vessels and is found to promote migration of endothelial cells in astrocytic tumors [108, 109]. Therefore, targeting tenascin with an antibody to suppress angiogenesis seems biologically reasonable. A tenascin-specific antibody radiolabeled with I-131 was tested in patients with high-grade gliomas [110]. The phase II studies with I-131 in malignant glioma were reported to yield a slight increase in survival time [111].

Conclusion

The mechanisms of glioma angiogenesis and invasion are only just beginning to be investigated, and many aspects are yet to be clarified. A better understanding of the molecular components responsible for glioma angiogenesis and invasion will hopefully lead to the development of new treatment methods. We hope that our novel glioma model described in this review can contribute in this aspect.

References

1. Bello L, Giussani C, Carrabba G et al (2004) Angiogenesis and invasion in gliomas. Cancer Treat Res 117:263–284
2. Kleihues P, Louis DN, Scheithauer BW et al (2002) The WHO classification of tumors of the nervous system. J Neuropathol Exp Neurol 61:215–225 (discussion 26–29)
3. Stupp R, Mason WP, van den Bent MJ et al (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. N Engl J Med 352:987–996
4. Plate KH, Risau W (1995) Angiogenesis in malignant gliomas. Glia 15:339–347
5. Lopes MB (2003) Angiogenesis in brain tumors. Microsc Res Tech 60:225–230
6. Reifenberger G, Collins VP (2004) Pathology and molecular genetics of astrocytic gliomas. J Mol Med 82:656–670
7. Lebelt A, Dzieciol J, Guzinska-Ustymowicz K et al (2008) Angiogenesis in gliomas. Folia Histochem Cytobiol 46:69–72
8. Wesseling P, Ruiter DJ, Burger PC (1997) Angiogenesis in brain tumors; pathobiological and clinical aspects. J Neurooncol 32:253–265
9. Rong Y, Durden DL, Van Meir EG et al (2006) 'Pseudopalisading' necrosis in glioblastoma: a familiar morphologic feature that links vascular pathology, hypoxia, and angiogenesis. J Neuropathol Exp Neurol 65:529–539
10. Cingolani A, De Luca A, Larocca LM et al (1998) Minimally invasive diagnosis of acquired immunodeficiency syndrome-related primary central nervous system lymphoma. J Natl Cancer Inst 90:364–369
11. Burger PC, Heinz ER, Shibata T et al (1988) Topographic anatomy and CT correlations in the untreated glioblastoma multiforme. J Neurosurg 68:698–704
12. Koutcher JA, Hu X, Xu S et al (2002) MRI of mouse models for gliomas shows similarities to humans and can be used to identify mice for preclinical trials. Neoplasia 4:480–485
13. Tovi M, Hartman M, Lilja A et al (1994) MR imaging in cerebral gliomas tissue component analysis in correlation with histopathology of whole-brain specimens. Acta Radiol 35:495–505
14. Suzuki SO, Kitai R, Llena J et al (2002) MAP-2e, a novel MAP-2 isoform, is expressed in gliomas and delineates tumor architecture and patterns of infiltration. J Neuropathol Exp Neurol 61:403–412
15. Sakariassen PO, Prestegarden L, Wang J et al (2006) Angiogenesis-independent tumor growth mediated by stem-like cancer cells. Proc Natl Acad Sci USA 103:16466–16471
16. Tate MC, Aghi MK (2009) Biology of angiogenesis and invasion in glioma. Neurotherapeutics 6:447–457
17. Horten BC, Basler GA, Shapiro WR (1981) Xenograft of human malignant glial tumors into brains of nude mice. A histopathological study. J Neuropathol Exp Neurol 40:493–511
18. Giannini C, Sarkaria JN, Saito A et al (2005) Patient tumor EGFR and PDGFRA gene amplifications retained in an invasive intracranial xenograft model of glioblastoma multiforme. Neuro Oncol 7:164–176

19. Engebraaten O, Fodstad O (1999) Site-specific experimental metastasis patterns of two human breast cancer cell lines in nude rats. Int J Cancer 82:219–225
20. Galli R, Binda E, Orfanelli U et al (2004) Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. Cancer Res 64:7011–7021
21. Gunther HS, Schmidt NO, Phillips HS et al (2008) Glioblastoma-derived stem cell-enriched cultures form distinct subgroups according to molecular and phenotypic criteria. Oncogene 27:2897–2909
22. Wong ML, Prawira A, Kaye AH et al (2009) Tumour angiogenesis: its mechanism and therapeutic implications in malignant gliomas. J Clin Neurosci 16:1119–1130
23. Brem S, Cotran R, Folkman J (1972) Tumor angiogenesis: a quantitative method for histologic grading. J Natl Cancer Inst 48:347–356
24. Zagzag D, Goldenberg M, Brem S (1989) Angiogenesis and blood–brain barrier breakdown modulate CT contrast enhancement: an experimental study in a rabbit brain-tumor model. Am J Roentgenol 153:141–146
25. Del Maestro RF, Megyesi JF, Farrell CL (1990) Mechanisms of tumor-associated edema: a review. Can J Neurol Sci 17:177–183
26. Zagzag D, Amirnovin R, Greco MA et al (2000) Vascular apoptosis and involution in gliomas precede neovascularization: a novel concept for glioma growth and angiogenesis. Lab Invest 80:837–849
27. Holash J, Maisonpierre PC, Compton D et al (1999) Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. Science 284:1994–1998
28. Zagzag D, Hooper A, Friedlander DR et al (1999) In situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis. Exp Neurol 159:391–400
29. Bergers G, Song S (2005) The role of pericytes in blood-vessel formation and maintenance. Neuro Oncol 7:452–464
30. Reiss Y, Machein MR, Plate KH (2005) The role of angiopoietins during angiogenesis in gliomas. Brain Pathol 15:311–317
31. Maisonpierre PC, Suri C, Jones PF et al (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 277:55–60
32. Rooprai HK, McCormick D (1997) Proteases and their inhibitors in human brain tumours: a review. Anticancer Res 17:4151–4162
33. Rao JS, Yamamoto M, Mohaman S et al (1996) Expression and localization of 92 kDa type IV collagenase/gelatinase B (MMP-9) in human gliomas. Clin Exp Metastasis 14:12–18
34. Forsyth PA, Wong H, Laing TD et al (1999) Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. Br J Cancer 79:1828–1835
35. Lakka SS, Gondi CS, Rao JS (2005) Proteases and glioma angiogenesis. Brain Pathol 15:327–341
36. Brooks PC, Clark RA, Cheresh DA (1994) Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science 264:569–571
37. Kim S, Bell K, Mousa SA et al (2000) Regulation of angiogenesis in vivo by ligation of integrin alpha5beta1 with the central cell-binding domain of fibronectin. Am J Pathol 156:1345–1362
38. Lindahl P, Johansson BR, Leveen P et al (1997) Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science 277:242–245
39. Ferrara N, Kerbel RS (2005) Angiogenesis as a therapeutic target. Nature 438:967–974
40. Jain RK, di Tomaso E, Duda DG et al (2007) Angiogenesis in brain tumours. Nat Rev Neurosci 8:610–622
41. Safran M, Kaelin WG Jr (2003) HIF hydroxylation and the mammalian oxygen-sensing pathway. J Clin Invest 111:779–783
42. Jiang BH, Rue E, Wang GL et al (1996) Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. J Biol Chem 271:17771–17778
43. Wenger RH, Stiehl DP, Camenisch G (2005) Integration of oxygen signaling at the consensus HRE. Sci STKE 2005:re12
44. Brahimi-Horn C, Berra E, Pouyssegur J (2001) Hypoxia: the tumor's gateway to progression along the angiogenic pathway. Trends Cell Biol 11:S32–S36
45. Hanahan D, Folkman J (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 86:353–364
46. Ferrara N (2004) Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 25:581–611
47. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257
48. Esser S, Lampugnani MG, Corada M et al (1998) Vascular endothelial growth factor induces VE-cadherin tyrosine phosphorylation in endothelial cells. J Cell Sci 111(Pt 13):1853–1865
49. Kevil CG, Payne DK, Mire E et al (1998) Vascular permeability factor/vascular endothelial cell growth factor-mediated permeability occurs through disorganization of endothelial junctional proteins. J Biol Chem 273:15099–15103
50. Mandriota SJ, Seghezzi G, Vassalli JD et al (1995) Vascular endothelial growth factor increases urokinase receptor expression in vascular endothelial cells. J Biol Chem 270:9709–9716
51. Jain RK (2003) Molecular regulation of vessel maturation. Nat Med 9:685–693
52. Graham CH, Connelly I, MacDougall JR et al (1994) Resistance of malignant trophoblast cells to both the anti-proliferative and anti-invasive effects of transforming growth factor-beta. Exp Cell Res 214:93–99
53. Demuth T, Berens ME (2004) Molecular mechanisms of glioma cell migration and invasion. J Neurooncol 70:217–228
54. Dirks PB (2001) Glioma migration: clues from the biology of neural progenitor cells and embryonic CNS cell migration. J Neurooncol 53:203–212
55. Farin PW, Crosier AE, Farin CE (2001) Influence of in vitro systems on embryo survival and fetal development in cattle. Theriogenology 55:151–170
56. Bremnes RM, Veve R, Hirsch FR et al (2002) The E-cadherin cell–cell adhesion complex and lung cancer invasion, metastasis, and prognosis. Lung Cancer 36:115–124
57. Goodenough DA, Goliger JA, Paul DL (1996) Connexins, connexons, and intercellular communication. Annu Rev Biochem 65:475–502
58. Ruch RJ (1994) The role of gap junctional intercellular communication in neoplasia. Ann Clin Lab Sci 24:216–231
59. Dermietzel R, Spray DC (1993) Gap junctions in the brain: where, what type, how many and why? Trends Neurosci 16:186–192
60. McDonough WS, Johansson A, Joffee H et al (1999) Gap junction intercellular communication in gliomas is inversely related to cell motility. Int J Dev Neurosci 17:601–611
61. Soroceanu L, Manning TJ Jr, Sontheimer H (2001) Reduced expression of connexin-43 and functional gap junction coupling in human gliomas. Glia 33:107–117
62. Nagano O, Saya H (2004) Mechanism and biological significance of CD44 cleavage. Cancer Sci 95:930–935
63. Gunia S, Hussein S, Radu DL et al (1999) CD44s-targeted treatment with monoclonal antibody blocks intracerebral invasion and growth of 9L gliosarcoma. Clin Exp Metastasis 17:221–230

64. Okamoto I, Kawano Y, Matsumoto M et al (1999) Regulated CD44 cleavage under the control of protein kinase C, calcium influx, and the Rho family of small G proteins. J Biol Chem 274:25525–25534
65. Leavesley DI, Ferguson GD, Wayner EA et al (1992) Requirement of the integrin beta 3 subunit for carcinoma cell spreading or migration on vitronectin and fibrinogen. J Cell Biol 117:1101–1107
66. Platten M, Wick W, Wild-Bode C et al (2000) Transforming growth factors beta(1) (TGF-beta(1)) and TGF-beta(2) promote glioma cell migration via up-regulation of alpha(V)beta(3) integrin expression. Biochem Biophys Res Commun 268:607–611
67. Adachi Y, Lakka SS, Chandrasekar N et al (2001) Down-regulation of integrin alpha(v)beta(3) expression and integrin-mediated signaling in glioma cells by adenovirus-mediated transfer of antisense urokinase-type plasminogen activator receptor (uPAR) and sense p16 genes. J Biol Chem 276:47171–47177
68. Natarajan M, Stewart JE, Golemis EA et al (2006) HEF1 is a necessary and specific downstream effector of FAK that promotes the migration of glioblastoma cells. Oncogene 25:1721–1732
69. Liotta LA, Tryggvason K, Garbisa S et al (1980) Metastatic potential correlates with enzymatic degradation of basement membrane collagen. Nature 284:67–68
70. Wild-Bode C, Weller M, Wick W (2001) Molecular determinants of glioma cell migration and invasion. J Neurosurg 94:978–984
71. Li L, Gondi CS, Dinh DH et al (2007) Transfection with anti-p65 intrabody suppresses invasion and angiogenesis in glioma cells by blocking nuclear factor-kappaB transcriptional activity. Clin Cancer Res 13:2178–2190
72. Song H, Li Y, Lee J et al (2009) Low-density lipoprotein receptor-related protein 1 promotes cancer cell migration and invasion by inducing the expression of matrix metalloproteinases 2 and 9. Cancer Res 69:879–886
73. Wang H, Shen W, Huang H et al (2003) Insulin-like growth factor binding protein 2 enhances glioblastoma invasion by activating invasion-enhancing genes. Cancer Res 63:4315–4321
74. Baker AH, George SJ, Zaltsman AB et al (1999) Inhibition of invasion and induction of apoptotic cell death of cancer cell lines by overexpression of TIMP-3. Br J Cancer 79:1347–1355
75. Tonn JC, Kerkau S, Hanke A et al (1999) Effect of synthetic matrix-metalloproteinase inhibitors on invasive capacity and proliferation of human malignant gliomas in vitro. Int J Cancer 80:764–772
76. Tonn JC, Goldbrunner R (2003) Mechanisms of glioma cell invasion. Acta Neurochir Suppl 88:163–167
77. Beadle C, Assanah MC, Monzo P et al (2008) The role of myosin II in glioma invasion of the brain. Mol Biol Cell 19:3357–3368
78. Salhia B, Rutten F, Nakada M et al (2005) Inhibition of Rho-kinase affects astrocytoma morphology, motility, and invasion through activation of Rac1. Cancer Res 65:8792–8800
79. Bornhauser BC, Lindholm D (2005) MSAP enhances migration of C6 glioma cells through phosphorylation of the myosin regulatory light chain. Cell Mol Life Sci 62:1260–1266
80. Brat DJ, Mapstone TB (2003) Malignant glioma physiology: cellular response to hypoxia and its role in tumor progression. Ann Intern Med 138:659–668
81. Zagzag D, Zhong H, Scalzitti JM et al (2000) Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. Cancer 88:2606–2618
82. Brat DJ, Castellano-Sanchez AA, Hunter SB et al (2004) Pseudopalisades in glioblastoma are hypoxic, express extracellular matrix proteases, and are formed by an actively migrating cell population. Cancer Res 64:920–927
83. Elstner A, Holtkamp N, von Deimling A (2007) Involvement of Hif-1 in desferrioxamine-induced invasion of glioblastoma cells. Clin Exp Metastasis 24:57–66
84. Martens T, Schmidt NO, Eckerich C et al (2006) A novel one-armed anti-c-Met antibody inhibits glioblastoma growth in vivo. Clin Cancer Res 12:6144–6152
85. Eckerich C, Zapf S, Fillbrandt R et al (2007) Hypoxia can induce c-Met expression in glioma cells and enhance SF/HGF-induced cell migration. Int J Cancer 121:276–283
86. Sathornsumetee S, Reardon DA, Desjardins A et al (2007) Molecularly targeted therapy for malignant glioma. Cancer 110:13–24
87. Vredenburgh JJ, Desjardins A, Herndon JE 2nd et al (2007) Phase II trial of bevacizumab and irinotecan in recurrent malignant glioma. Clin Cancer Res 13:1253–1259
88. Norden AD, Young GS, Setayesh K et al (2008) Bevacizumab for recurrent malignant gliomas: efficacy, toxicity, and patterns of recurrence. Neurology 70:779–787
89. Lamszus K, Kunkel P, Westphal M (2003) Invasion as limitation to anti-angiogenic glioma therapy. Acta Neurochir Suppl 88:169–177
90. Sathornsumetee S, Cao Y, Marcello JE et al (2008) Tumor angiogenic and hypoxic profiles predict radiographic response and survival in malignant astrocytoma patients treated with bevacizumab and irinotecan. J Clin Oncol 26:271–278
91. Bergers G, Hanahan D (2008) Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 8:592–603
92. MacDonald TJ, Taga T, Shimada H et al (2001) Preferential susceptibility of brain tumors to the antiangiogenic effects of an alpha(v) integrin antagonist. Neurosurgery 48:151–157
93. Fu Y, Ponce ML, Thill M, Yuan P et al (2007) Angiogenesis inhibition and choroidal neovascularization suppression by sustained delivery of an integrin antagonist, EMD478761. Invest Ophthalmol Vis Sci 48:5184–5190
94. Taga T, Suzuki A, Gonzalez-Gomez I et al (2002) alpha v-Integrin antagonist EMD 121974 induces apoptosis in brain tumor cells growing on vitronectin and tenascin. Int J Cancer 98:690–697
95. Eskens FA, Dumez H, Hoekstra R et al (2003) Phase I and pharmacokinetic study of continuous twice weekly intravenous administration of cilengitide (EMD 121974), a novel inhibitor of the integrins alphavbeta3 and alphavbeta5 in patients with advanced solid tumours. Eur J Cancer 39:917–926
96. Reardon DA, Fink KL, Mikkelsen T et al (2008) Randomized phase II study of cilengitide, an integrin-targeting arginine-glycine-aspartic acid peptide, in recurrent glioblastoma multiforme. J Clin Oncol 26:5610–5617
97. Reardon DA, Nabors LB, Stupp R et al (2008) Cilengitide: an integrin-targeting arginine–glycine–aspartic acid peptide with promising activity for glioblastoma multiforme. Exp Opin Invest Drugs 17:1225–1235
98. Levin VA, Phuphanich S, Yung WK et al (2006) Randomized, double-blind, placebo-controlled trial of marimastat in glioblastoma multiforme patients following surgery and irradiation. J Neurooncol 78:295–302
99. Groves MD, Puduvalli VK, Hess KR et al (2002) Phase II trial of temozolomide plus the matrix metalloproteinase inhibitor, marimastat, in recurrent and progressive glioblastoma multiforme. J Clin Oncol 20:1383–1388
100. D'Amato RJ, Loughnan MS, Flynn E et al (1994) Thalidomide is an inhibitor of angiogenesis. Proc Natl Acad Sci USA 91:4082–4085
101. Hansen JM, Harris C (2004) A novel hypothesis for thalidomide-induced limb teratogenesis: redox misregulation of the NF-kappaB pathway. Antioxid Redox Signal 6:1–14

102. Fine HA, Figg WD, Jaeckle K et al (2000) Phase II trial of the antiangiogenic agent thalidomide in patients with recurrent high-grade gliomas. J Clin Oncol 18:708–715
103. Adnane L, Trail PA, Taylor I, Wilhelm SM (2006) Sorafenib (BAY 43-9006, Nexavar), a dual-action inhibitor that targets RAF/MEK/ERK pathway in tumor cells and tyrosine kinases VEGFR/PDGFR in tumor vasculature. Methods Enzymol 407:597–612
104. Siegelin MD, Raskett CM, Gilbert CA, Ross AH, Altieri DC (2010) Sorafenib exerts anti-glioma activity in vitro and in vivo. Neurosci Lett 478:165–170
105. Kilic T, Alberta JA, Zdunek PR et al (2000) Intracranial inhibition of platelet-derived growth factor-mediated glioblastoma cell growth by an orally active kinase inhibitor of the 2-phenylaminopyrimidine class. Cancer Res 60:5143–5150
106. Ranza E, Mazzini G, Facoetti A, Nano R (2010) In vitro effects of the tyrosine kinase inhibitor imatinib on glioblastoma cell proliferation. J Neurooncol 96:349–357
107. Wen PY, Yung WK, Lamborn KR et al (2006) Phase I/II study of imatinib mesylate for recurrent malignant gliomas: North American Brain Tumor Consortium Study 99-08. Clin Cancer Res 12:4899–4907
108. Zagzag D, Shiff B, Jallo GI et al (2002) Tenascin-C promotes microvascular cell migration and phosphorylation of focal adhesion kinase. Cancer Res 62:2660–2668
109. Zagzag D, Friedlander DR, Dosik J et al (1996) Tenascin-C expression by angiogenic vessels in human astrocytomas and by human brain endothelial cells in vitro. Cancer Res 56:182–189
110. Bigner DD, Brown M, Coleman RE et al (1995) Phase I studies of treatment of malignant gliomas and neoplastic meningitis with 131I-radiolabeled monoclonal antibodies anti-tenascin 81C6 and anti-chondroitin proteoglycan sulfate Me1-14 F (ab′)2—a preliminary report. J Neurooncol 24:109–122
111. Reardon DA, Akabani G, Coleman RE et al (2002) Phase II trial of murine (131)I-labeled antitenascin monoclonal antibody 81C6 administered into surgically created resection cavities of patients with newly diagnosed malignant gliomas. J Clin Oncol 20:1389–1397