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IN FOCUS

The role of thrombin in gliomas

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Summary. *Background:* In a previous study we found that intracerebral infusion of argatroban, a specific thrombin inhibitor, reduces brain edema and neurologic deficits in a C6 glioma model. *Objectives:* To examine the role of thrombin in gliomas and whether systemic argatroban administration can reduce glioma mass and neurologic deficits and extend survival time in C6 and F98 gliomas. *Methods:* The presence of thrombin in human glioblastoma samples and rat C6 glioma cells (*in vitro* and *in vivo*) was assessed using immunohistochemistry. The effect of thrombin on C6 cell proliferation *in vitro* was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. The role of thrombin *in vivo* was assessed in rat C6 and F98 glioma cell models using argatroban, a thrombin inhibitor. The effects of argatroban on tumor mass, neurologic deficits and survival time were investigated. *Results:* Thrombin immunoreactivity was found in cultured rat C6 glioma cells and human glioblastomas. Thrombin induced C6 cell proliferation *in vitro*. In C6 glioma, argatroban reduced glioma mass ($P < 0.05$) and neurologic deficits ($P < 0.05$) at day 9. In F98 glioma, argatroban prolonged survival time ($P < 0.05$). *Conclusion:* These results suggest that thrombin plays an important role in glioma growth. Thrombin may be a new therapeutic target for gliomas.

Keywords: argatroban, glioma, thrombin.

Introduction

It has long been known that thrombin has a pivotal role in the coagulation cascade. However, the discovery and cloning of a series of thrombin receptors [1], has suggested that thrombin has a wide range of potential actions. Several pieces of evidence led us to examine whether thrombin might have a major role in malignant gliomas. (A) In contrast to normal brain, the vasculature of these tumors is typically highly permeable with the potential for prothrombin entry from blood into tumor. (B) Brain edema in and around gliomas contributes to the high mortality (within months) of patients with malignant gliomas by causing herniation-related death. Our previous studies indicate that thrombin plays an important role in edema formation after intracerebral hemorrhage [2,3]. (C) Thrombin is a potent mitogen, which enhances the synthesis and secretion of nerve growth factor in glial cells and stimulates astrocyte and tumor cell proliferation [4,5]. (D) Angiogenesis is essential for rapid glioma growth because of the need for oxygen and metabolites. Although many factors regulate angiogenesis, thrombin may play an important role [6].

In a preliminary study, we found that intracerebral infusion of argatroban, a specific thrombin inhibitor, reduces brain edema and neurologic deficits in a rat C6 glioma model [7]. The current experiments expand on this initial finding and specifically determine whether thrombin is expressed in human glioblastomas and in rat glioma cells *in vivo* and *in vitro*, to examine whether thrombin can modulate C6 cell proliferation *in vitro* and whether thrombin inhibition affects tumor mass, tumor-induced behavioral deficits and survival time in rat C6 and F98 glioma models.

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Materials and methods

This study was in six parts. The first part determined whether rat C6 and F98 glioma cells express thrombin in culture using immunohistochemistry. The second part examined whether human glioblastoma cells also express thrombin, again using immunohistochemistry. The third part examined whether thrombin can modulate C6 cell proliferation *in vitro*. The fourth part tested whether thrombin can increase vascular endothelial growth factor (VEGF) levels *in vitro*. The fifth part examined whether systemic use of argatroban reduces tumor mass and neurologic deficits in C6 glioma model. The last part examined whether argatroban could prolong survival time in F98 glioma model. Argatroban is a small molecular weight (MW 508.7), direct thrombin inhibitor that inhibits both free and fibrin-bound thrombin [8].

Cell culture

Rat C6 (passage number 36–42) and F98 (passage numbers 9–11) glioma cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). C6 glioma cells were grown at 37 °C in air with 5% CO_2 in Ham's F-10 medium with 2.5% fetal bovine serum and 15% horse serum. F98 glioma cells were obtained from ATCC and grown in DMEM (ATCC) medium with 10% fetal bovine serum at 37 °C in air with 5% CO_2. Cells were maintained in a monolayer culture.

Human glioma specimens

Surgical glioblastoma specimens ($n = 10$) were obtained from the Department of Neurosurgery at the University of Michigan. The protocol was approved by the Institutional Review Boards at the University of Michigan. The specimens were frozen in liquid nitrogen and stored at −80 °C. Frozen sections (18 μm thick) were taken on a cryostat for immunohistochemistry.

Cell proliferation in vitro

C6 glioma cells were cultured in 96-well plates at a cell density of 5×10^3 cells per well. Cells were incubated in serum-free medium for 24 h and then different doses of rat thrombin (0, 0.1, 0.25, 0.5, 1.0 and 2.5 U mL^{-1}; Sigma, St Louis, MO, USA) with or without argatroban (10 μM; GlaxoSmithKline, Research Triangle, NC, USA) were added to the cell culture. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay [9] was performed 48 h later. Metabolically active cells can change yellow tetrazolium salt MTT to indigo-blue formazan. For the assay, MTT (5 mg mL^{-1}, 25 μL per 200 μL medium) was filtered, added to the wells and incubated at 37 °C for 4 h. Medium was removed gently using a 21-gauge needle and 200 μL dimethyl sulfoxide was added. After 10 min, the optical density was measured on a microplate reader at 540 nm with a reference wavelength of 630 nm. All *in vitro* studies were repeated three times.

Animal preparation and the C6 and F98 glioma models

The protocols in this study were approved by the University of Michigan Committee on the Use and Care of Animals. Adult male Fischer 344 rats (200–225 g; Charles River Laboratories, Portage, MI, USA) were anesthetized with pentobarbital (50 mg kg^{-1}, i.p.). Aseptic precautions were utilized in all surgical procedures. After anesthesia was achieved, a polyethylene catheter (PE-50) was inserted into the right femoral artery in order to monitor arterial blood pressure and to obtain blood for analysis of blood gases, blood pH, hematocrit, and blood glucose concentration. They were then placed in a Kopf stereotaxic frame. Body temperature was maintained at 37 ± 1 °C using a rectal temperature probe and a feedback regulator. A small burr hole was drilled in the skull overlying the right caudate nucleus. The tip of a 26-gauge stainless steel cannula was then lowered into the right caudate (0.2 mm anterior, 5.5 mm ventral, 3.5 mm lateral to the bregma) and 1×10^6 C6 glioma cells were infused [10]. Sham animal had only a needle insertion.

Alzet osmotic minipump implantation and argatroban treatment

To examine whether thrombin contributed to brain edema formation, glioma growth, glioma-related neurologic deficits and survival time, animals were treated with argatroban, a thrombin inhibitor. At the time of C6 or F98 glioma cell implantation, rats were implanted intraperitoneally with an osmotic minipump (Alzet, Cupertino, CA, USA). The pump was preloaded with argatroban (60 mg mL^{-1} and delivers 5 μL h^{-1}) or vehicle.

Immunohistochemistry

Immunohistochemistry was performed as previously described [11]. Briefly, rats ($n = 3$) were reanesthetized and were perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The brains were removed and kept in 4% paraformaldehyde for 12 h, then immersed in 25% sucrose for 3–4 days at 4 °C. After embedding in the mixture of 25% sucrose and OCT, 18-μm sections were taken on a cryostat. The avidin–biotin complex technique was used in the staining and hematoxylin was used for counter staining. The primary antibody was sheep anti-human thrombin IgG (1:400 dilution; Affinity Biologicals Inc., Ancaster, Canada). The sheep anti-human thrombin IgG is raised against inactivated human alpha-thrombin and reacts with human, rat and mouse thrombin. Normal sheep IgG was used as a negative control.

Enzyme-linked immunosorbent assay (ELISA)

C6 glioma cells were cultured in 96-well plates at a cell density of 5×10^4 cells per well. Cells were incubated in serum-free medium for 24 h and then different doses of thrombin (0, 0.25 and 0.5 U mL^{-1}; Sigma) were added to the cell culture. VEGF levels in the supernatant were measured at 24 h after thrombin stimulation with an ELISA kit (R & D systems). Data were expressed as pg mL^{-1} supernatant.

Reverse transcription-polymerase chain reaction (RT-PCR)

At 9 days after C6 cell implantation or sham operation, animals were reanesthetized with pentobarbital (60 mg kg^{-1}, i.p.) and killed by decapitation. C6 and F98 glioma cells were also grown *in vitro*. The C6 gliomas and glioma cells were sampled for RT-PCR [12]. Total RNA was extracted with Trizol reagent (Gibco BRL, Grand Island, NY, USA), 1-μg RNA was digested with deoxyribonuclease (DNaseI, Amplification Grade, Gibco BRL). cDNA was synthesized by RT using the digested 1 μg RNA (11 μL) with 14 μL of reaction buffer (Perkin-Elmer, Foster City, CA, USA) containing

dNTP (dATP, dCTP, dGTP and dTTP), 25 mM MgCl2, 10x PCR buffer II, Random Hexamer Primer, RNase inhibitor and MuLV reverse transcriptase. The reaction was performed at 42 °C for 30 min and terminated at 99 °C for 5 min. Diethyl pyrocarbonate water (75 μL) was added to dilute the cDNA to 100 μL and stored at −20 °C until use.

Polymerase chain reaction was performed with 15 μL of the reverse transcriptase reaction mixture (Perkin-Elmer) containing 25 mM MgCl2, dNTP, 10x PCR buffer II and AmpliTaq DNA Polymerase in a final volume of 50 μL. The rat prothrombin primers (NIH GenBank database) corresponded to nucleotides 1674–1692 (sense primer, 5′-TCCTCGCTTGGTGTCATTC-3′) and nucleotides 1349–1367 (antisense primer, 5′-CAAGCACTCCAGAACCAGA-3′). Rat GAPDH primers (5′-CTCAGTGTAGCCCAGGATGC-3′, 5′-ACCACCATGGAGAAGGCTGG-3′) were used to amplify GAPDH mRNA, a housekeeping gene used as a control. Amplification was performed in a DNA thermal cycler (MJ Research, Watertown, MO, USA). Samples were subjected to 30 cycles (94 °C, 30 s; 55 °C, 30 s; and 72 °C, 1 min). PCR production was analyzed by the use of electrophoresis on a 1% agarose gel. Gels were visualized with ethidium bromide staining and ultraviolet transillumination. Photographs were taken with black and white film (Polaroid Corp., Waltham, MA, USA) and analyzed using NIH image 1.62.

Tumor mass

We used the weight difference between the ipsilateral (tumor side) and contralateral hemisphere to estimate the tumor mass.

Behavioral tests

Animals were placed in a cylindrical enclosure to record preferential use of the non-impaired forelimb for weight shifting movements during spontaneous vertical exploration. The percentage independent use of the non-impaired forelimb (ipsilateral to the tumor), the percentage independent use of the contralateral forelimb, and the percentage use of both forelimbs together in rapid succession for stepping movements along the walls of the cylinder were assessed. A single score was then used to reflect forelimb use asymmetry: percentage ipsilateral limb use minus percentage contralateral limb use (low score = better function). In addition, a vibrissae-stimulated forelimb placing test (10 trials per side for each rat) was used to examine sensorimotor/proprioceptive capacity (high score = better function) [13]. All behavior was scored by experimenters who were blind to both neurologic and treatment conditions. These tests are highly correlated with extent of striatal injury without being influenced by repeated testing.

Statistics

The results are reported as mean ± S.D. Data were analyzed by analysis of variance (ANOVA) followed by Scheffe's post hoc test, Student's *t*-test, Mann–Whitney *U*-rank test or Trend Peto–Peto–Wilcoxon test. Differences were considered significant at the $P < 0.05$ level.

Results

Thrombin immunoreactivities were detected in cultured C6 and F98 glioma cells *in vitro* (Fig. 1A,B). Similarly, thrombin was detected in human glioblastomas. Figure 1C shows a representative example of the 10 glioblastomas sampled.

To examine whether thrombin would affect C6 glioma cell proliferation *in vitro*, cells were exposed to thrombin at a concentration range of 0–2.5 U mL^{-1} (higher concentrations are cytotoxic [14]) and proliferation assessed with an MTT assay. Thrombin induced proliferation at 0.1 and 0.25 U mL^{-1}, but not at higher concentrations (Fig. 2A). The thrombin-induced proliferation was blocked by addition of argatroban, a thrombin inhibitor (Fig. 2B).

Thrombin can stimulate VEGF production *in vitro* ($P < 0.05$). The VEGF levels in the supernatant of C6 cell culture were 101 ± 16, 151 ± 21 and 136 ± 21 pg mL^{-1} at 24 h after 0, 0.25 and 0.5 U mL^{-1} thrombin stimulation, respectively.

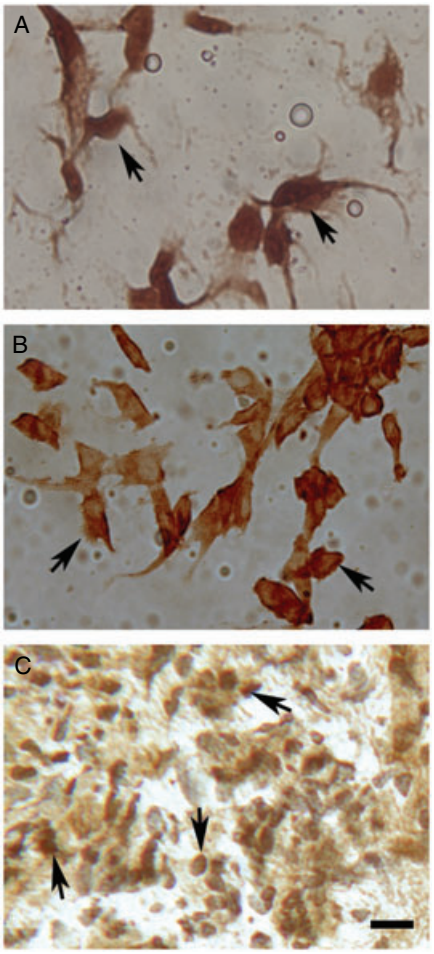

Fig. 1. The presence of thrombin immunoreactivity in cultured C6 (A) and F98 (B) glioma cells and a human glioblastoma tissue sample (C). Arrows indicate thrombin-positive cells. Scale bar = 20 μm.

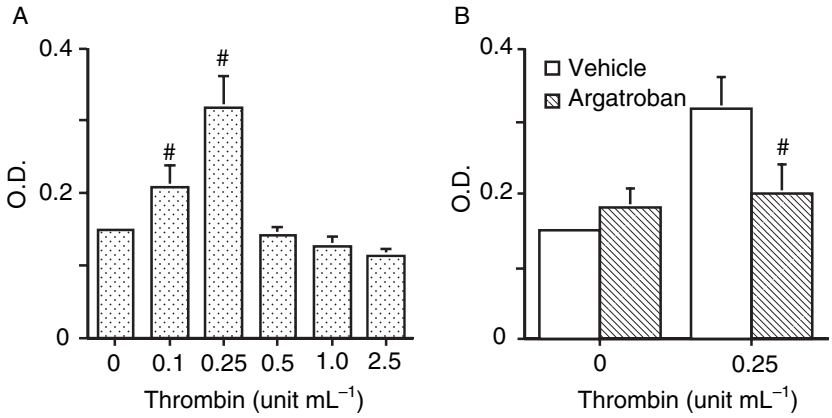

Fig. 2. Thrombin stimulation of C6 cell proliferation as assessed by MTT assay. (A) C6 glioma cells were exposed to different doses of thrombin (0, 0.1, 0.25, 0.5, 1.0 and 2.5 U mL^{-1}) and the MTT assay was performed 48 h later. Values are mean ± SD ($n = 9$). indicates a significant difference from control (no thrombin) at the $P < 0.01$ level. (B) C6 glioma cells were treated with either vehicle, argatroban (10 μM), thrombin (0.25 U mL^{-1}) or thrombin (0.25 U mL^{-1}) plus argatroban (10 μM), cell growth was assayed by MTT 48 h later. Values are mean ± SD ($n = 9$). # indicates a significant difference between the vehicle and argatroban-treated groups at the $P < 0.01$ level.

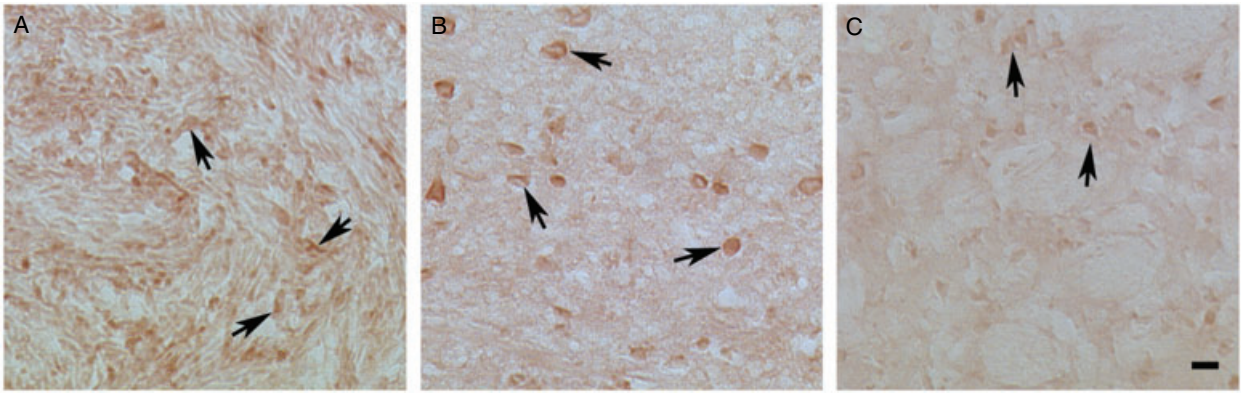

Fig. 3. Immunohistochemistry for thrombin in C6 gliomas. (A) Center of the C6 cell glioma, (B) the edge of the glioma and (C) the contralateral hemisphere. Arrows indicate thrombin-positive cells. Scale bar = 20 μm.

In this C6 glioma model, thrombin was detected in the tumor using an antibody to thrombin (Fig. 3A,B). Thrombin immunoreactivity in glioma was much greater than that found in the contralateral hemisphere (Fig. 3C). Thrombin-positive cells were localized in the tumor center and the tumor edge. In the glioma center, most of the tumor cells are thrombin positive. In addition, prothrombin mRNA was detected in C6 and F98 glioma cells in culture (Fig. 4A). Prothrombin mRNA levels increased in the C6 glioma (1732 ± 93 pixels vs. 970 ± 133 pixels in the sham, $P < 0.05$, Fig. 4B).

We used the weight difference between the ipsilateral (tumor side) and the contralateral hemisphere to estimate the tumor mass. Argatroban (0.3 mg h^{-1} per rat) reduced C6 glioma mass at day 9 (43 ± 31 mg vs. 80 ± 35 mg in the vehicle group, $n = 8$, $P < 0.05$). Argatroban treatment also improved C6 glioma-related neurologic deficits including forelimb use asymmetry and forelimb placing (Fig. 5).

Rats were examined every day and started to die from 10 days after F98 glioma cell implantation. Argatroban prolonged survival time in F98 glioma model (18 ± 3 days vs. 14 ± 4 days in the vehicle group, $n = 20$, $P < 0.05$, Trend Peto–Peto–Wilcoxon test, Fig. 6).

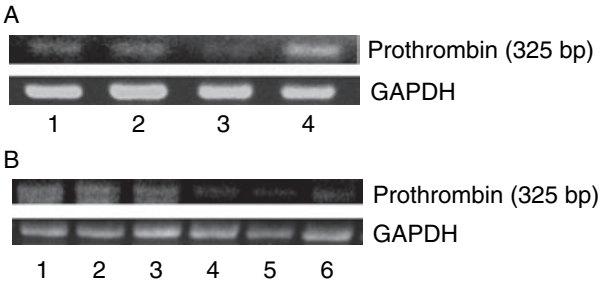

Fig. 4. (A) Prothrombin mRNA levels measured by RT-PCR in C6 (lanes 1 and 2) and F98 glioma cells in culture. (B) Prothrombin mRNA levels in three gliomas 9 days after intracerebral infusion of C6 glioma cells (lanes 1–3) and in the ipsilateral basal ganglia 9 days after sham operation (lanes 4–6).

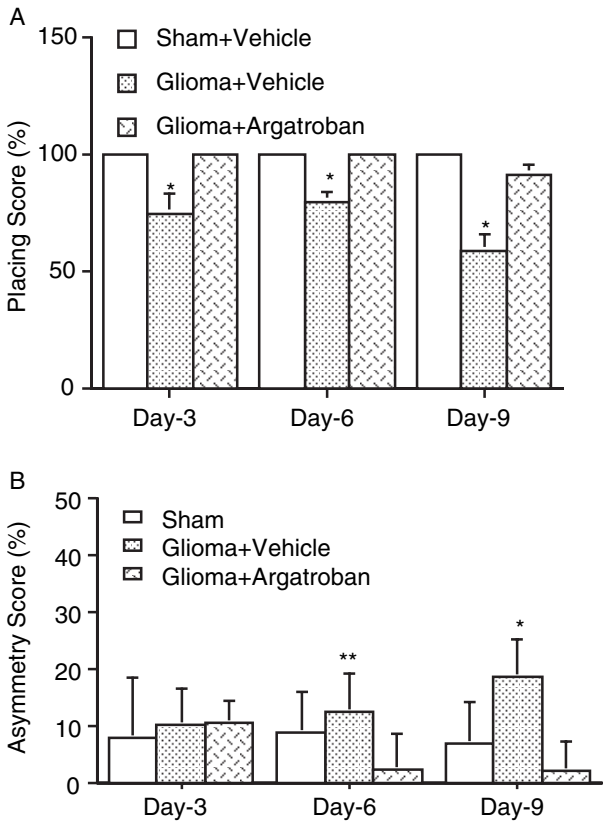

Fig. 5. The effects of argatroban on neurologic deficits in the C6 glioma model. Forelimb placing score (A) and forelimb use asymmetry (B) in rats after intracerebral implantation of C6 glioma cells. The rats were treated with argatroban or vehicle. Values are mean ± SD, $n = 9$–10, $^{*}P < 0.05$ vs. other groups, $^{**}P < 0.05$ vs. glioma + argatroban. Mann–Whitney *U*-rank test. For the forelimb placing test, a score of 100% indicates no deficit. For the forelimb asymmetry test, a score of 0% indicates no deficit.

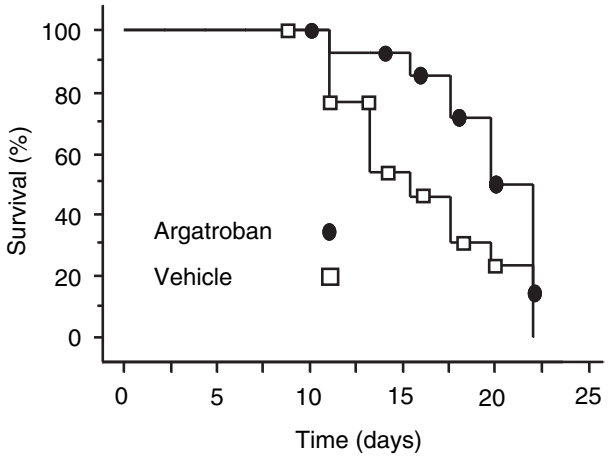

Fig. 6. The effect of argatroban on rat survival time after F98 glioma implantation.

Discussion

This study demonstrates that thrombin is expressed in rat C6, F98 glioma cells and human glioblastomas, that thrombin can induce proliferation of C6 glioma cells *in vitro* and that systemic use of argatroban, a thrombin inhibitor, can reduce tumor mass and behavioral deficits in a C6 glioma model in the rat. Argatroban also improved F98 glioma-induced behavioral deficits and prolonged survival time in that glioma model. Together, this suggests that thrombin could be a therapeutic target in gliomas. This adds to a growing body of evidence that thrombin can have profound non-hemostatic effects in the brain that are important in brain injury and disease [1,15].

The presence of thrombin in the C6 glioma model *in vivo* and in the human glioblastoma samples could be derived from prothrombin entering the tumor from the systemic circulation via a disrupted blood–tumor barrier, or it may be derived from the tumor itself or a combination of both. As yet it is impossible to determine the significance of these potential sources. However, the fact that prothrombin mRNA and thrombin were detected in C6 and F98 cells in culture, and prothrombin mRNA levels were increased in C6 gliomas indicate that tumor cell-derived thrombin is at least a component. This suggestion is supported by evidence that the brain, including astrocytes, produces prothrombin mRNA [16]. It should be noted, however, that non-cancerous cells within the tumor might contribute to the increase of prothrombin mRNA levels in the C6 glioma *in vivo*. Thus, macrophages in gliomas commonly produce prothrombin and express the clotting factors necessary for thrombin production [17]. Clotting factors other than prothrombin have also been identified in brain parenchyma [15].

The current study demonstrates that thrombin can increase C6 cell proliferation *in vitro*. There is other evidence supporting that thrombin can regulate cell proliferation. Thrombin enhances the synthesis and secretion of nerve growth factor in glial cells [4] and stimulates astrocyte proliferation [5]. In addition, thrombin may act as a growth factor for tumor cells [18] and induces proliferative response in T-47D mammary tumor cells [19]. In T98G and TM-1 human glioma cells, thrombin also induced proliferation. This mitogenic effect was abolished by hirudin, a thrombin inhibitor [20]. In our study, the effect might have been blocked by argatroban, another thrombin inhibitor.

Although the primary role of thrombin in hemostasis is through cleaving fibrinogen to fibrin, other important cellular activities of thrombin may be related to thrombin receptor activation. Three protease-activated receptors (PARs), PAR-1, PAR-3 and PAR-4, have been identified as thrombin receptors [1]. Thrombin receptors are activated by proteolytic cleavage rather than by ligand binding and thrombin receptor-activated peptides are able to mimic many cellular activities of thrombin. Recent studies indicate that PARs mediate some of the pathophysiological effects of thrombin. For example, PAR-1 activation by thrombin receptor activating peptide results in angiogenesis [6]. Thrombin receptors have been found in many types of tumor cells [21] including rat C6 cells ([22] and Y. Hua, R.F. Keep, G. Xi, unpublished data). Whether the proliferative effects of thrombin on C6 cells are PAR mediated deserves further investigation. It is also possible that the absence of a thrombin-induced proliferation at high concentrations might

reflect the activation of different types of PAR receptor. There has been debate about whether or not thrombin in gliomas is enzymatically active based on the cleavage of fibrinogen [23]. Our findings on the effects of argatroban *in vivo* and *in vitro* suggest that gliomas have functionally active thrombin but do not address the issue of whether this involves thrombin receptor-mediated or fibrinogen cleavage mechanisms.

Evidence for a potentially important role for thrombin in gliomas was also found *in vivo*. Argatroban reduced tumor mass, neurologic deficits and prolonged survival time. Argatroban was chosen as the thrombin inhibitor in this study. It is a small molecule (MW 508.7) and a direct thrombin inhibitor binding to the catalytic site of the thrombin molecule. Argatroban is an effective inhibitor of thrombin both bound to fibrin and thrombin free in solution [8,24]. The effect of thrombin on tumor mass may result from a direct effect on tumor cell proliferation, as described *in vitro*. As our measure of tumor mass includes edema, the effect of argatroban on mass may also reflect reduced edema formation. Another possibility is thrombin may increase tumor mass by affecting blood vessel proliferation as thrombin is a potent promoter of angiogenesis [25].

Other experimental data suggest a role for coagulation and fibrinolysis systems in tumor development, progression and metastasis [23,26]. Clinical data also suggest that targeting the coagulation system might influence the course of malignant disease. The anticoagulant drug warfarin prolongs the length of survival of patients with small cell lung cancer. Heparin has a benefit in patients with several types of cancer [27]. Our results suggest that drugs directly targeting thrombin may have similar actions and that warfarin and heparin may be having their effects by reducing thrombin production or activity.

In this study, argatroban reduced neurologic deficits in the glioma model. Traditional preclinical investigations of brain tumor therapies have focused primarily on inhibiting tumor cell proliferation or survival with little regard to behavioral assessment. However, in clinical trials brain function has been an essential index of treatment benefit. Moreover, it seems clear from the literature on brain injury and recovery of function that mechanisms of neural plasticity needed for optimal behavioral outcome in a brain compromised by tumor growth might be vulnerable to some tumor treatments, including irradiation, pro-apoptotic drugs, or agents that adversely affect angiogenesis, mitosis or neurotrophic factor activity [7,28,29]. A model that includes sensitive functional outcome measures would appear to represent a significant advantage in brain tumor research.

The current study provides evidence that thrombin could be a therapeutic target for malignant gliomas. It does not address the question of whether thrombin plays a role in other forms of brain tumor, i.e. is it specific for gliomas or does thrombin stimulate tumor growth in, for example, low-grade astrocytomas? But, in relation to this point, there is evidence that the effect of thrombin is not confined to gliomas from examination of tumors outside of the brain [30]. In particular, the effects of thrombin on angiogenesis suggest that it may affect growth in a variety of solid tumors which depend on the formation of new blood vessels [6,26,30].

In summary, our results have shown that anti-thrombin treatment with argatroban reduces tumor mass, improves neurologic scores and prolongs survival time. Clarification of the role of thrombin in glioma proliferation, angiogenesis and edema formation should help to develop new therapeutic strategies for glioma treatment.

Conflict of interest disclosure

This study was supported by the Mitsubishi Pharma Corporation.

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