A thrombin inhibitor reduces brain edema, glioma mass and neurological deficits in a rat glioma model*

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Summary

Although thrombin is a critical enzyme in the coagulation cascade, it has become apparent that it has many other effects. Thus, it may induce brain edema formation, angiogenesis and cell proliferation. Because of the importance of these three factors in the extremely poor prognosis of glioma patients, the present study examined the role of thrombin in that disease state. We found that thrombin activity is increased in a rat glioma model and thrombin-positive cells were present in the tumor. Anti-thrombin treatment with argatroban reduced brain edema, tumor growth, and tumor-related neurological deficits. Our results suggest that thrombin is a new target for glioma treatment.

Keywords: Glioma; brain edema; thrombin; argatroban; neurological deficits; rats.

Introduction

Rapidly growing brain tumors, such as malignant gliomas, result in a high mortality within months. Brain edema contributes to the high mortality in glioma patients by causing herniation-related death. Our previous studies indicate that thrombin plays an important role in edema formation after intracerebral hemorrhage [6, 18]. Angiogenesis is essential for rapid glioma growth because of the need for oxygen and metabolites. Although many factors regulate angiogenesis, thrombin may play a key role [8]. In addition, 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 [1, 11]. Because of these multiple potential effects of thrombin in gliomas, the present study investigated whether a thrombin inhibitor, argatroban, can reduce edema formation, glioma mass and tumor-induced neurological deficits in a rat C6 glioma model.

Materials and methods

Animal groups

Rat C6 glioma cells (6 × 10^4) were infused into the right caudate of adult male Fischer 344 rat. Two sets of experiments were performed. In the first set, rats were killed at day 12 for edema, glioma mass and thrombin activity measurements. Brain thrombin activity was measured using the thrombin-specific chromogenic substrate S2238. To further confirm the specificity of thrombin activity measurement, a specific thrombin inhibitor, PPACK was used to inhibit thrombin activity. In the second set, rats were implanted with an osmotic minipump to deliver a thrombin inhibitor (argatroban, 1 µg/µl, 0.5 µl/h) or saline into the right caudate at the time of C6 cell implantation. After 9 days, the rat brains were removed for determination of brain water content and glioma mass. Over the period of the experiment, the animals underwent behavioral testing [4, 15].

Glioma model

The rats were anesthetized with pentobarbital (40 mg/kg, i.p.). They then were placed in a Kopf stereotaxic frame. Body temperature was maintained at 37.5°C using a rectal temperature probe and a feed-back regulator. A small burr hole was drilled in the skull overlying the right caudate nucleus. The tip of a 28 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 6 × 10^5 C6 glioma cells were infused.

Alzet osmotic mini-pump implantation

At the time of C6 glioma cell implantation (or saline injection), rats were implanted with an osmotic minipump to deliver a thrombin inhibitor (argatroban) or saline into the right basal ganglia. Utilizing the same burr hole as used for C6 cell infusion, the tip of a 28-gauge stainless steel cannula was lowered into the caudate. The cannula was fixed to the skull with dental acrylic and screws. An osmotic pump (Alzet) was connected to the cannula and implanted in the

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back of the neck via PE-60 tubing. The pumps were preloaded with or without drug and delivered 0.5 μl/h. The skin incisions were then closed and the animal was allowed to recover.

Behavioral tests

Animals were placed in a cylindrical enclosure to record preferential use of the non-impairred forelimb for weight shifting movements during spontaneous vertical exploration. The % independent use of the non-impairred forelimb (ipsilateral to the tumor), the % independent use of the contralateral forelimb, and the % 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: % ipsilateral limb use minus % 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/propriceptive capacity (high score = better function). These tests are highly correlated with extent of striatal injury without being influenced by repeated testing. All behavior was scored by experimenters who were blind to both neurological and treatment conditions.

Brain water content measurement

Rats were sacrificed by decapitation under deep pentobarbital anesthesia. The brains were removed immediately and cut into three parts: front part (frontal pole to 3 mm back), the middle part (3–7 mm from frontal pole) and the rear part (7 mm to occipital pole). The brain samples were then divided into cortex or basal ganglia (ipsilateral or contralateral). Tissue samples were weighed on an electronic analytical balance to obtain the wet weight (WW). The tissue was then dried in a gravity oven at 100°C for more than 24 hours to determine the dry weight (DW). Tissue water contents (%) are calculated as ((WW – DW)/WW) × 100.

Tumor mass

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

Thrombin activity measurement

For thrombin activity measurements, rat brains were perfused transcardially with saline and sampled as described in the water content section. Brain samples were homogenized and thrombin activities were measured using the thrombin-specific chromogenic substrate S2238 (Chromogenix). The final concentration of S2238 was 0.3 mmol/L in PBS and the absorption at 405 nm of the supernatant was measured after one hour. A calibration curve of thrombin activity was made for the thrombin activity quantitation [16]. Measurements were made in the presence and absence of phenyl-propyl-arginyl-chloromethyl ketone (PPACK), a thrombin inhibitor, to ensure that the measurements were a reflection of thrombin activity.

Immunohistochemistry

Immunohistochemical studies were performed as in our previous report [17]. The primary antibody was sheep anti-human thrombin. Normal rabbit IgG was used as negative control.

Statistics

Data were analyzed by Student t test or Mann-Whitney U nonparametric test. Differences were considered significant at the p < 0.05 level.

Results

Intra-caudate implantation of C6 glioma cells caused brain edema and neurological deficits in the rat. Thrombin activity was increased in the glioma (340 ± 12 vs. 200 ± 40 mU/gram brain tissue in the contralateral side, p < 0.05). Immunohistochemistry also demonstrated the presence of thrombin-positive cells in and around the glioma.

Treatment with argatroban reduced brain edema (81.6 ± 0.5% vs. 82.7 ± 0.3% in control, n = 5, p < 0.05, Fig. 1), glioma mass (44 ± 21 mg vs. 106 ± 57 mg in the control, n = 4, p < 0.05) and neurological deficits (forelimb placing score: 85 ± 7% vs. 9 ± 4% in control, n = 10, p < 0.05, Fig. 2; Forelimb use asymmetry: −10 ± 3% vs. 25 ± 12% in control, n = 10, p < 0.05, Fig. 3) in rats at day 9.

Discussion

Our present findings demonstrate that thrombin (by activity assay and immunohistochemistry) is increased in the glioma. Anti-thrombin treatment reduces peri-gliomal edema formation, glioma mass and glioma-induced neurological deficits indicating that thrombin plays an important role in glioma growth.

As early as 1865, Trousseau found a connection between malignant cell growth and the coagulation. Much experimental data suggest a role for the coagu-
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A thrombin inhibitor reduces brain edema, glioma mass and neurological deficits in a rat glioma model. The coagulation cascade (thrombin) may also be a therapeutic target in brain gliomas.

We chose this injection model for this glioma study because of several advantages. First, C6 glioma cells were injected into the caudate. This takes advantage of the fact that a number of behavioral tests have been devised to assess caudate damage induced by ischemia, hemorrhage or neurotoxins. Second, this model is stable and reproducible in terms of edema, tumor mass and neurological deficit measurements. Third, the properties of C6 glioma cell line can be retained for several years [5]. C6 gliomas have a disrupted blood-brain barrier suggesting that increased thrombin activity within the tumor might be due to an influx of prothrombin from blood. However, production of prothrombin by the tumor or adjacent cells cannot yet be excluded.

Thrombin plays a major role in edema formation in the brain after intracerebral hemorrhage [6, 18]. Our current results suggest it may have a similar edemogenic role in tumors, although it is difficult to dissociate effects on tumor mass and edema. Thrombin is produced immediately in the brain after intracerebral hemorrhage or blood-brain barrier breakdown following many kinds of brain injury [3]. Direct infusion of large doses of thrombin into brain causes inflammatory cell infiltration, mesenchymal cell proliferation and brain edema formation [6, 13, 18]. Thrombin-induced brain edema results from a direct opening of the blood-brain barrier or a direct neurotoxicity [7].

The effect of thrombin on tumor mass may result from a direct effect on tumor cell proliferation. Thrombin enhances the synthesis and secretion of nerve growth factor in glial cells [11] and stimulates astrocyte proliferation [1]. In addition, thrombin may act as a growth factor for tumor cells [2] and induces proliferative response in T-47D mammary tumor cells [10]. In T98G and TM-1 human glioma cells, thrombin also induced proliferation. This mitogenic effect was abolished by hirudin, a specific thrombin inhibitor [14]. Thrombin is also a potent promoter of angiogenesis. Thrombin activates an angiogenic cascade through, at least in part, modulating hypoxia inducible factor-1, matrix metalloproteases, vascular endothelial growth factor (VEGF) and the receptors of VEGF [8, 9, 12, 21, 22].

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, the literature on brain injury and functional recovery indicates that mechanisms of neural plasticity needed for optimal behavioral outcome in tumor patients might be vulnerable to some tumor treatments, including irradiation, pro-apoptotic drugs, or agents that adversely affect angiogenesis, mitosis or neurotrophic factor activity. A model that includes sensitive functional outcome measures would appear to represent a significant advantage in brain tumor research.

In summary, thrombin is present in the glioma and administration of a thrombin inhibitor (argatroban) results in less brain edema, glioma mass and neurological deficits. These results suggest that thrombin plays an important role in glioma growth. Modulating thrombin activity, either using thrombin inhibitors or upregulating endogenous thrombin inhibitors in the brain, may produce novel strategies for glioma treatment.

References


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