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Thrombin-induced Migration and Matrix Metalloproteinase-9 Expression Are Regulated by MAPK and PI3K Pathways in C6 Glioma Cells

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Glioblastoma multiforme is one of the most common and aggressive tumors in central nervous system. It often possesses characteristic necrotic lesions with hemorrhages, which increase the chances of exposure to thrombin. Thrombin has been known as a regulator of MMP-9 expression and cancer cell migration. However, the effects of thrombin on glioma cells have not been clearly understood. In the present study, influences of thrombin on glioma cell migration were examined using Boyden chamber migration assay and thrombin-induced changes in MMP-9 expression were measured using zymography, semi-quantitative RT-PCR, and Western blotting. Furthermore, underlying signaling pathways by which thrombin induces MMP-9 expression were examined. Thrombin-induced migration and MMP-9 expression were significantly potentiated in the presence of wortmannin, a PI3K inhibitor, whereas MAPK inhibitors suppressed thrombin-induced migration and MMP-9 expression in C6 glioma cells. The present data strongly demonstrate that MAPK and PI3K pathways evidently regulate thrombin-induced migration and MMP-9 expression of C6 glioma cells. Therefore, the control of these pathways might be a beneficial therapeutic strategy for treatment of invasive glioblastoma multiforme.

Key Words: Thrombin, MMP-9, C6 glioma cells, MAPK, PI3K

INTRODUCTION

Gliomas constitute nearly 60% of primary brain tumors and usually infiltrate normal brain parenchyma. Gliomas are categorized as their histologic features, and the major type of gliomas are astrocytomas [1]. Glioblastoma multiforme (GBM) is the most common type of astrocytomas arising in adults and clinically very aggressive [2]. These tumors typically show necrosis and hemorrhage. Patients diagnosed as GBM survive less than 1 year due to diffusely infiltrating nature of GBM. Glioma cells migrate away from the primary tumor site so that surgical resection of a tumor mass is not effective for remission [3].

Matrix metalloproteinases (MMPs) are critically involved in many physiological and pathological processes such as tissue remodeling and cancer progression [4]. Especially, MMP-2 and MMP-9 (known as gelatinases) have been reported to play crucial roles in tumor angiogenesis and metastasis. It has been also reported that enhanced gelatinase expression in various types of cancers corresponds to increased invasion and metastasis [5].

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Thrombin, a trypsin-like serine protease, is the most copious enzyme participating in the coagulation cascade. In addition to its hemostatic roles, thrombin modulates a diversity of pathophysiological functions such as inflammation, cell migration and proliferation, apoptosis and so on. There are many evidences that thrombin regulates MMP-9 expression [6-8] and cancer cell migration [9-11]. Nevertheless, the effect of thrombin on glioma cell migration is not fully understood even though thrombin released by hemorrhagic site of GBM may have a important role in invasion. In the present study, we focused on demonstrating the effects of thrombin on the migration of C6 rat glioma cells, which are morphologically similar to GBM [12], and possible underlying signaling pathways that thrombin induces MMP-9-mediated migration.

METHODS

Reagents

Thrombin and wortmannin were obtained from Sigma-Aldrich (St. Louis, MO, USA) and Calbiochem (Darmstadt, Germany), respectively. SB203580, SP600125, U0126 and MMP-9 inhibitor, batimastat, were purchased from Tocris

ABBREVIATIONS: MMP-9, matrix metalloproteinase-9; PI3K, phosphatidylinositol 3-kinase; MAPK, mitogen-activated protein kinase.

Bioscience (Ellisville, MO, USA).

Cell culture and thrombin treatment

The C6 rat glioma cell line was cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY, USA) in the presence of 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc.), 100 U/ml of penicillin, 100 mg/ml of streptomycin and 2 mM of glutamine (GIBCO BRL, Grand Island, NY, USA). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. All inhibitors were pretreated in serum-deprived culture medium for 1 hr followed by thrombin treatment as indicated.

Cell migration assay

Cell migration assay was performed by BD Falcon™ Cell Culture Inserts (BD Bioscience, Bedford, MA; pore size, 8-μm) in 24-well culture plates. Cell culture inserts were coated with fibronectin (Sigma, St. Louis, MO, USA; 3 μg/ml) both upper and lower surfaces. Approximately 0.7×10^5 cells in 400 μl of serum-free medium were seeded in the upper chamber and 400 μl of the same medium was placed in the lower chamber. When seeded, cells were respectively pretreated with appropriate concentration of inhibitors; SB203580, SP600125, U0126, and wortmannin. After 1 hr of pretreatment, thrombin was added to the lower chamber and the plates were incubated for 6 hr at 37°C in 5% CO_2 incubator. Subsequently, cells were fixed in 3.7% formaldehyde solution for 10 min and permeabilized by 0.2% Triton X-100 for 10 min. Then, cells on the lower surface were stained with 0.2% crystal violet and rinsed with PBS several times. Non-migrating cells in the upper chamber were attentively removed by cotton swabs. The migrating cells were counted by light microscopy.

Western blot analysis

After removed the culture media, the C6 rat glioma cells were harvested and lysed with PRO-PREP™ Protein Extraction Solution (iNtRON Biotechnology, Inc.). The obtained protein samples were quantified by the BCA protein assay and the same amounts of total cellular proteins were resolved on 10% SDS-polyacrylamide gels. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were incubated in blocking solution (5% skim milk in TBST) for 1 hr at room temperature and then probed overnight with suitable antibodies at 4°C; anti-MMP-9 (Affinity BioReagents, Golden, CO, USA; 1 : 1,000), p-Akt, Akt (Cell Signaling Technology, Inc.; 1 : 1,000), or β-actin (Sigma-Aldrich, St. Louis, MO, USA; 1 : 5,000). After three washes with TBST, the membranes were incubated with either horseradish peroxidase (HRP)-conjugated goat anti-rabbit or donkey anti-mouse secondary antibody (Jackson ImmunoResearch) for 2 hr at room temperature. The blots were detected by enhanced chemiluminescence using Kodak® BioMax™ XAR Film.

RNA isolation and semi-quantitative RT-PCR analysis

Isolation of total cellular RNA from C6 glioma cells was performed using the Total RNA Extraction Kit (iNtRON Biotechnology, Inc.) according to the manufacturer's instructions. Total RNA was quantified by NanoDrop spectrophotometer (Thermo Fischer Scientific Inc., Wilmington, DE, USA) and 2 μg of total RNA was immediately reverse-transcribed by Omniscript® RT Kit (QIAGEN GmbH, Hilden, Germany) with oligo-(dT) 15 primers (Promega, Madison, WI, USA). The cDNA was amplified by polymerase chain reaction (PCR) with appropriate primers (MMP-9 forward, TCCAGTAGACAATCCTTGCA and reverse, CTCCGTGATTCGAGAACTTC; GAPDH forward, GACAACTTTGGCATCGTGGA and reverse, ATGCAGGGATGATGTTCTGG). 30 cycles of PCR was performed by n*Taq* polymerase under the following conditions: denaturing at 94°C for 30 sec, annealing at 62°C for 45 sec, and elongation at 72°C for 30 sec. The PCR products (MMP-9, 110 bp; GAPDH, 133 bp) were electrophoresed on a 2% agarose gel containing ethidium bromide and visualized by UV transillumination.

Gelatin zymography

The gelatin zymography was carried out in 8% polyacrylamide gels containing 1 mg/ml of gelatin. C6 glioma cells were plated in 60 mm culture dishes and incubated overnight in serum- free medium. Cell culture supernatants were collected and concentrated by Amicon® Ultra Centrifugal Filter (Millipore, Bedford, MA) subsequent to 24 hr-thrombin treatment. These samples were mixed with non-reducing 5x loading buffer without heating and electrophoresed on the gel as mentioned above. After PAGE, gels were thoroughly washed with enzyme renaturing buffer (2.5% Triton X-100 solution) at room temperature for 4 times every 15 min. The gels were then transferred into incubation buffer (50 mM Tris-HCl, 0.15 M NaCl, 10 mM $CaCl_2$, and 0.02% NaN_3) and placed in a 37°C shaking incubator for 24 hr followed by Coomassie brilliant blue R-250 staining and destaining procedures.

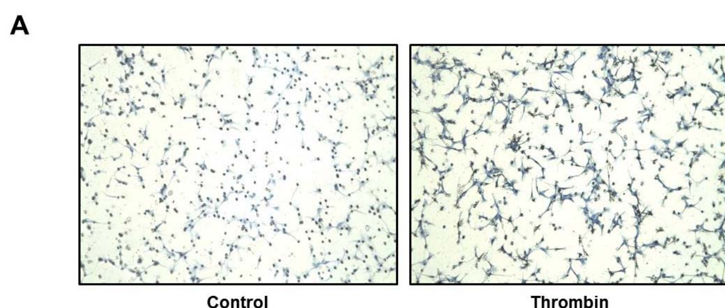
Statistical analysis

All data are expressed as the mean±standard deviation (SD). Statistical significance was analyzed by two-tailed Student's t-test. Data with values of $p<0.05$ were considered as statistically significant. Single (* and #) and double (** and ##) marks represent statistical significance in $p<0.05$ and $p<0.01$, respectively.

RESULTS

Thrombin induced the migration of C6 glioma cells

In the previous studies, it has been demonstrated that thrombin induces cell motility in many cell lines [9,13,14]. To investigate the effect of thrombin on the migration of C6 glioma cells, we used the Boyden chamber migration assay. Approximately 0.7×10^5 cells were plated in the upper chamber of cell culture inserts and incubated with or without 25 U/ml of thrombin. After 6 hr of incubation, the cells migrating through the membrane toward thrombin containing serum-free media were stained with crystal violet and the number of migrated cells was counted (Fig. 1). The number of migrated cells was significantly increased in the presence of thrombin compared to control, suggesting that thrombin has a chemotactic effect on C6 glioma cells.



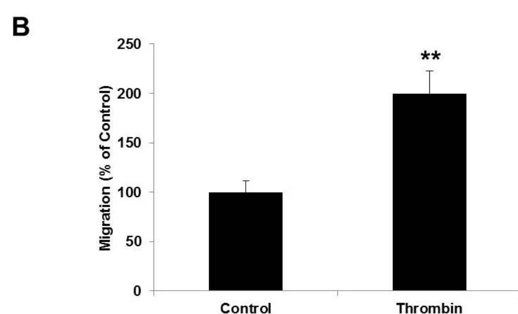
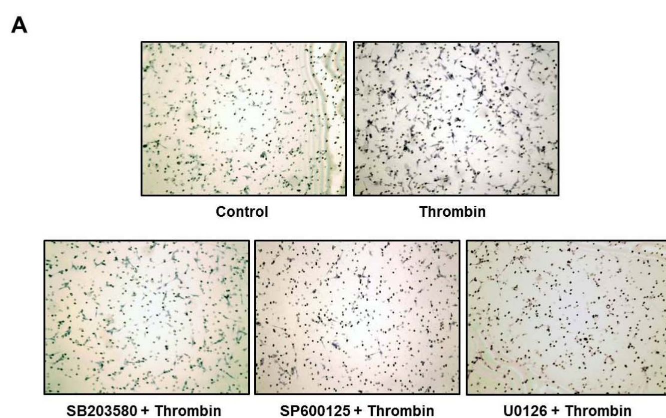


Fig. 1. Thrombin stimulates C6 glioma cell migration. (A) Representative images of C6 cell migration assay. Thrombin (25 U/ml) treatment for 6 hr exhibited approximately 2 fold increase in number of migrating cells. Migrating cells and unmigrating cells were separated by polyethylene terephthalate (PET) membranes. Microscopy images were detected the migrating cells on the lower surface of the membrane. (B) Quantitative analysis of migration assay. The cell migration was quantified by counting the cells that migrating through the membranes. Cell counting data were expressed as a percentage in comparison with control group. The experiments were conducted in triplicate and data are shown as the means±SD. $^{**}p<0.01$ indicates statistically significant difference with control.



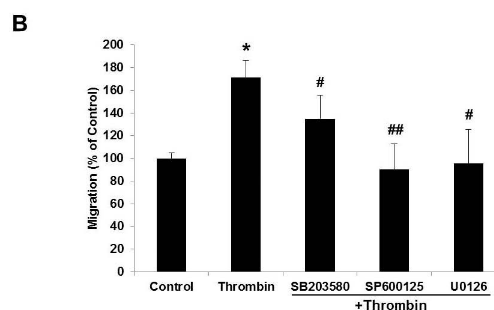


Fig. 2. MAPK inhibitors (MAPKIs) inhibit thrombin-induced C6 glioma cell migration. (A) Representative images of the MAPKI effects on thrombin-induced migration. SB203580, SP600125, and U0126 were pretreated for 1 hr at the concentrations of 20, 20, and 10 μM, respectively, followed by thrombin treatment for 6 hr. (B) Quantitative analysis of migration assay. The cell migration was quantified by counting the cells that migrating through the membranes. All data were obtained from three independent experiments and presented as the means±SD. $^{*}p<0.05$ indicates statistically significant difference with control. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ indicate statistically signiticant difference with thrombin.

MAPK inhibitors had an inhibitory effect on thrombin-induced migration of C6 glioma cells

Involvement of MAPK pathway in the regulation of cancer cell migration has been investigated by many studies [15-17]. In the present study, to determine the underlying mechanism for chemotactic action of thrombin, C6 cells were treated with some MAPK pathway inhibitors: SB203580 (20 μM), a specific inhibitor of p38-MAPK; SP600125 (20 μM), a selective inhibitor of c-Jun N-terminal kinase (JNK); U0126 (10 μM), a selective inhibitor of MEK/ERK. All inhibitors were pretreated for 1 hr in serum free media with indicated concentrations and thrombin was subsequently treated for 6 hr. As shown in Fig. 2, thrombin-induced migrating cells were reduced with pretreatment of the MAPK inhibitors. Inhibition of p38 with SB203580 showed significant inhibition of C6 migration (Fig. 2). Especially, inhibition of JNK and ERK with SP600125 and U0126, respectively, exerted such a strong inhibition that the number of migrated cells was reduced almost to control level (Fig. 2). These results suggest that MAPK pathway may regulate thrombin-induced migration of C6 glioma cells.

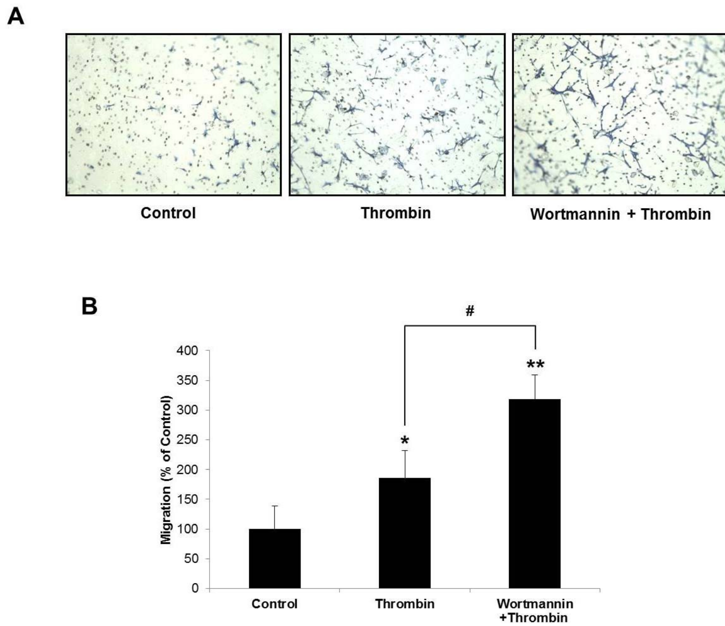


Fig. 3. Thrombin-induced chemotactic migration was markedly increased by PI3K inhibitor, wortmannin. (A) Potentiating effects were observed in the group that treated thrombin for 6 hr subsequent to preincubated with wortmannin for 1 hr. (B) Quantitative analysis of migration assay. The cell migration was quantified by counting the cells that migrating through the membranes. All data were obtained from three independent experiments and presented as the means±SD. $*p<0.05$ and $**p<0.01$ indicate statisticodly significant difference with control. $^{\#}p<0.05$ indicates statistically significant difference between thrombin and wortmannin thrombin.

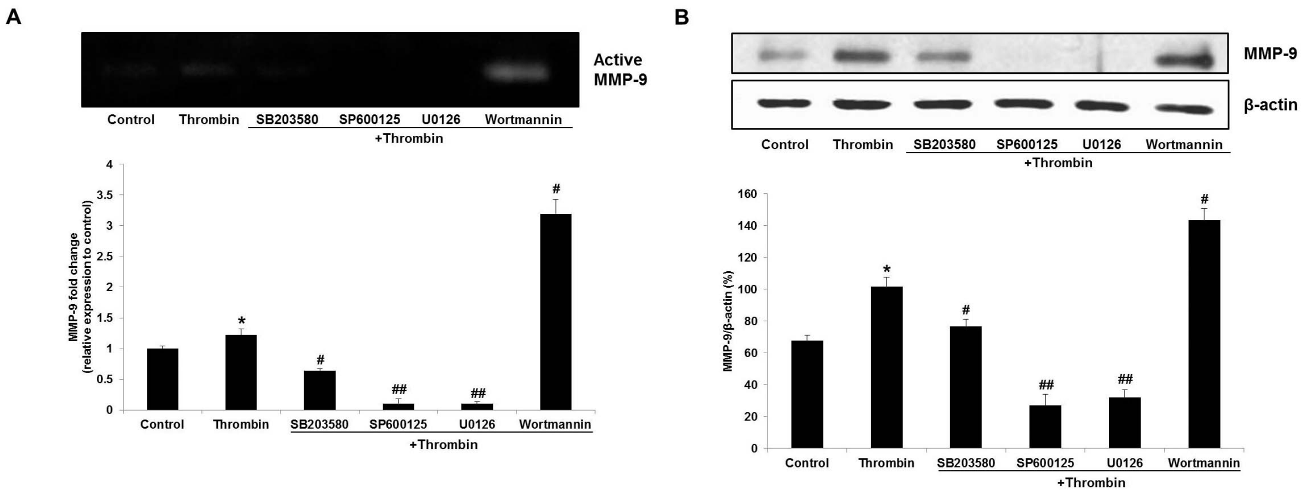


Fig. 4. Effects of thrombin and MAPK or PI3K inhibitors treatment on MMP-9 expression in protein level. All inhibitors were pretreated for 1 hr at the indicated concentrations followed by 24 hr of thrombin (5 U/ml) treatment: SB203580 and SP600125, 20 μM; U0126 and wortmannin, 10 μM. C6 cells were incubated with inhibitors in the serum free media. (A) MMP-9 activities were analyzed by zymography. Thrombin-induced MMP-9 expression was blocked by MAPK inhibitors and increased by PI3K inhibitor. (B) The cellular level of MMP-9 was examined by western blot analysis. β-actin was used as an internal control. $*p<0.05$ indicates statistically signiticant difference with control. $^{\#}p<0.05$ and $^{\#\#}p<0.01$ indicate statistically signiticant difference with thrombin.

Thrombin-induced C6 cell migration was significantly increased with wortmannin, a PI3K inhibitor

The previous studies have been shown that PI3K pathway is also related to the cancer cell motility [18,19]. On this account, we investigated the effect of the PI3K pathway on thrombin-induced C6 cell migration with a PI3K inhibitor wortmannin, an antifungal antibiotic. 100 nM of wortmannin was used for migration assay and other experimental procedures were the same as mentioned above. Wortmannin significantly potentiated the migratory activity induced by thrombin (Fig. 3). This result may suggest that the PI3K pathway negatively regulates thrombin-induced C6 cell migration.

MMP-9 expression was correlated with C6 cell migration propensity

To elucidate whether the thrombin-induced migration and MMP-9 expression are directly related, we used an MMP-9 inhibitor (MMPI), batimastat, for migration assay.

The suppressive effect of MMPI is represented in Fig. 6. It clearly showed that MMP-9 is directly involved in cell migration stimulated by thrombin.

To determine whether MMP-9 is regulated by MAPK and PI3K pathways in the thrombin-induced migration of C6 glioma cells, we examined the changes of MMP-9 expression in multiple stages such as mRNA, protein, and extracellular levels. C6 cells were pre-incubated with the MAPK or PI3K inhibitors for 1 hr and thrombin was subsequently treated for 24 hr in serum-free media. After incubation, the conditioned media were collected and concentrated for zymography and the cells were harvested and lysed for western blot analysis. Gelatinolytic activities of MMP-9 were visualized by zymography. As shown in Fig. 4A, thrombin induces MMP-9 secretion and it is suppressed by MAPK inhibitors whereas thrombin-induced MMP-9 secretion was potently increased by wortmannin (Fig. 4A). These results closely correspond to migration assay data. Western blot analysis also presented the same tendency to zymographic data. To further confirm, we measured MMP-9 expression in mRNA level using semi-quantitative RT-PCR assay. After pretreated the inhibitors for 1 hr, C6 cells were incubated with thrombin for 4 hr. MMP-9 mRNA was expressed in the same manner as MMP-9 protein expression (Fig. 5). These data strongly support that thrombin-mediated MMP-9 expression is regulated by MAPK and PI3K pathways.

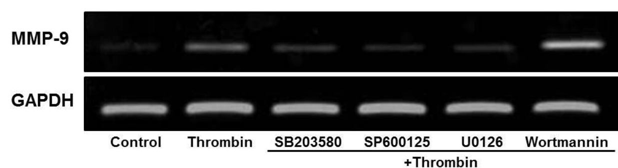


Fig. 5. Effects of thrombin and MAPK or PI3K inhibitors treatment on MMP-9 expression in mRNA level. C6 cells were pretreated with inhibitors for 1 hr, followed by thrombin (5 U/ml) treatment for 4 hr. Subsequently, RNA samples were isolated and subjected to semi-quantitative RT-PCR for MMP-9 with a housekeeping gene, GAPDH, as an internal control. MMP-9 mRNA expression patterns were similar to western blot and zymographic data.

DISCUSSION

Glioblastoma multiforme (GBM) is one of the most aggressive CNS tumors with very poor prognosis because of its highly-infiltrative nature [20]. GBM has a histologically characteristic feature which forms a serpentine pattern of necrosis and vascular or endothelial cell proliferation. These necrotic lesions often contain hemorrhagic components [2]. These conditions lead glioma cells to have more chances of exposure to thrombin. Previous studies have been revealed that thrombin is involved in cancer cell migration and invasion [9,10,21]. In the present study, we found that thrombin enhances migration of C6 rat glioma cells by Boyden chamber migration assay.

To determine the mechanism of the thrombin effect, we considered MAPK and PI3K pathways as pivotal factors based on the former studies [22,23]. Thrombin-induced migratory ability was significantly blocked by MAPK inhibitors. Wortmannin, a specific inhibitor of the PI3K pathway, on the other hand, surprisingly reinforced the thrombin-mediated cell migration. We used 100 nM and 1 μM of wortmannin for migration assay, but 1 μM was less potent than 100 nM of wortmannin in inducing migratory ability (data not shown). These results might be caused by excessive PI3K pathway inhibition in 1 μM of wortmannin treatment. Because PI3K pathway is critical in cell survival, its inordinate suppression subjects to cellular damage. The C6 cells treated with 1 μM of wortmannin presented slight morphological changes and with 10 μM showed more severe changes in migration assay.

As MMPs are key components of cell migration and invasion [5,24], we investigated the relationship between C6 cell migration and MMP-9. Thrombin-induced migration was effectively suppressed by an MMP-9 inhibitor, batimastat. It supports that MMP-9 is directly involved in cell migration stimulated by thrombin. However, batimastat alone could not totally block the thrombin-induced migration. This implies that there are more factors responsible

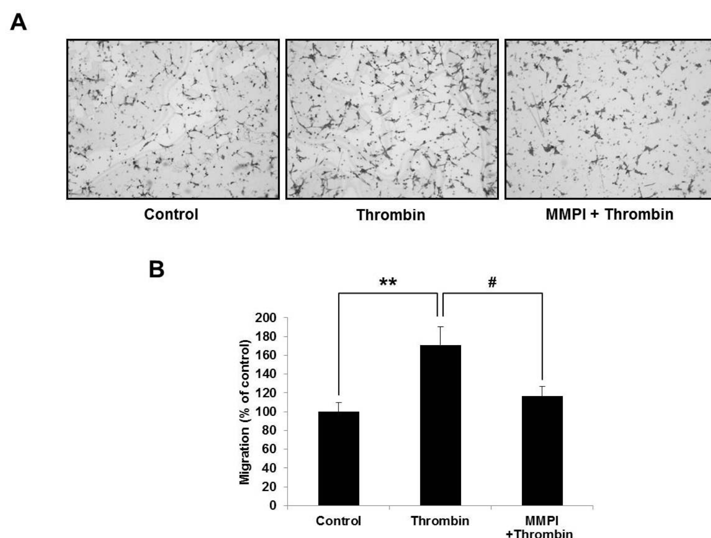


Fig. 6. Thrombin-induced migration was directly blocked by batimastat, an MMP-9 inhibitor (MMPI). (A) Representative images of the effect of MMPI on thrombin-induced C6 cell migration. 0.5 μM of batimastat was pretreated for 1 hr and thrombin was subsequently treated for 6 hr. (B) Quantitative analysis of migration assay. The cell migration was quantified by counting the cells that migrating through the membranes. All data were obtained from three independent experiments and presented as the means±SD. **$p < 0.01$ and $^{\#}p < 0.05$ indicate statistically signficant difference between control and thrombin, and thrombin and MMPI thrombin, respectively.

for thrombin-induced cell migration other than MMP-9. Thrombin induced MMP-9 expression in protein and mRNA level, parallel to increase in C6 cell migration. Induced MMP-9 expression was significantly attenuated by MAPK inhibitors and augmented by the PI3K inhibitor. Our results indicate that these pathways regulate thrombin-mediated MMP-9 expression and it affects migratory activity of C6 cells. That is, the activator of PI3K pathway, such as the platelet-derived growth factor (PDGF), or MAPK inhibitors might inhibit the thrombin-induced MMP-9 expression and migration. A previous study actually demonstrated that PDGF inhibits MMP-9 activity in C6 cells [25]. Wortmannin, for itself, did not increase both MMP-9 expression and cell migration (Data not shown).

In conclusion, the present study suggests that thrombin mediates C6 glioma cell migration by increasing expression of MMP-9. In addition, MAPK and PI3K pathways might be involved in thrombin-induced cell migration and MMP-9 expression. The migratory ability of cells is an important factor for cancer metastasis. Together with the previous studies, this study suggests that inhibition of MAPK pathway or potentiation of PI3K pathway could be a valuable therapeutic target for the treatment of metastatic glioblastoma.

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