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Rosiglitazone reduces cell invasiveness by inducing MKP-1 in human U87MG glioma cells

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ABSTRACT

We sought to investigate the molecular mechanisms by which rosiglitazone (RGZ) inhibits cell invasion in human glioma cells. In this study, we found that RGZ attenuated MMP-2 protein levels, MMP-2 gelatinolytic activity, and cell invasiveness through a PPAR-γ independent pathway. RGZ increased mitogen activated protein kinase phosphatase-1 (MKP-1) expression. The addition of triptolide (a diterpenoid triepoxide, which blocked MKP-1 induction) abolished the inhibitory effects by RGZ. Furthermore, we demonstrated that the knock down of MKP-1 by MKP-1 specific small interference RNA reversed the reduction of MMP-2 secretion, and of cell invasiveness by RGZ. In contrast, the stable expression of MKP-1 in glioma cell lines decreased MMP-2 activity and cell invasiveness. These results suggest that RGZ may mediate the inhibitory effects through MKP-1 induction. Thus, MKP-1 could be a potential target in glioma therapy.

1. Introduction

Human malignant glioma cells are characterized by invasive growth. Patients diagnosed with gliomas have a low survival rate. Until now, available therapeutic strategies are still largely unsuccessful, and have shown only a minimal impact on the patients' survival rate [1,2]. Proteolytic enzymes are involved in a diverse range of physiological and pathological processes. Matrix metalloproteinases (MMPs) are a family of extracellular endopeptidases requiring metal ions for their enzymatic activities degrade extracellular matrix (ECM) proteins such as gelatins, and collagens [3]. MMP-2 (or gelatinase-A) is a member of the MMPs family. MMP-2 is constitutively expressed in higher-grade astrocytomas but not in the lower-grade gliomas. Over-expression of MMP-2 has been implicated in increasing cell invasive capacity *in vitro* [3,4]. MMP-2 gene expression and activity are regulated by mitogen activated protein kinases (MAPKs) (p38, extracellular signal-regulated kinase ERK, and c-Jun N-terminal kinase JNK) in many cells [5–7]. Reduction of MAPK activities results in suppression of MMP-2 expression [8].

Thiazolidinediones (TZDs) are peroxisome proliferators activated receptor-γ (PPAR-γ) agonists, which have been shown to improve insulin sensitivity *in vivo* and are being used as new class of antidiabetic drugs [9]. TZDs may affect cell viability, apoptosis and expression of cell cycle-related proteins in cancer cell lines derived from human gliomas [10–12]. Recently, TZDs have been shown to suppress MMP-2 activity by a PPARγ-independent pathway in pancreatic cancer cells [13,14]. MAPKs-related signal transduction pathways have been implicated in these TZD-induced effects [15]. Thus, TZDs may regulate cell responses through MAPK signaling pathways.

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Abbreviations: MMP-2, matrix metalloproteinase-2; RGZ, rosiglitazone; MKP-1, mitogen activated protein kinase phosphatase-1; ECM, extracellular matrix; Trp, triptolide.

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MAP kinase phosphatase-1 (MKP-1) is a member of the dual-specificity MKPs family [16,17]. MKP-1 plays a role in the negative regulation of cellular responses mediated by ERK, JNK, and p38 kinases [18,19]. There are 10 MKPs that act as negative regulators of MAPK activity [18,20]. Abnormal MAPK signaling leads to development and progression of human cancer; the inactivation of the aberrant MAPKs activation by MKP-1 may be considered as a therapeutic strategy in cancer therapy [21,22].

Here, we characterized the role of MMP-2 in the invasiveness of human gliomas. We demonstrated that RGZ reduced MMP-2 activity and decreased glioma cell invasiveness via a mechanism dependent upon MKP-1 induction. Ectopic expression of MKP-1 in U87MG or GBM 8401 cells reduced MMP-2 activity and decreased cell invasiveness, suggesting that MKP-1 may play a pivotal role in inhibiting MMP-2 induction and tumor invasiveness. Our results suggest that MKP-1 can be considered as a possible therapeutic target controls the cell invasiveness of human gliomas.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), glutamine, gentamycin, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). All antibodies specific for MMP-2, MKP-1, phospho-p38, phospho-ERK, phospho-JNK, and α-tubulin and MKP-1 siRNA were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). *N*-acetyl-L-cysteine (*l*-NAC) was purchased from Calbiochem-Novabiochem (San Diego, CA). Horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody was purchased from Bio-Rad (Hercules, CA).

2.2. Culture of human glioma cells and preparation of cell lysates

Human U87MG cells (ATCC No. HTB-14) and cell lines derived from human glioma biopsies were cultured in DMEM supplemented with 10% heat-inactivated FCS, and penicillin (100 U/ml)/streptomycin (100 μg/ml). After reaching confluence, cells were treated with various concentrations of indicated agents and incubated for the indicated time in a 5% CO_2 humidified incubator at 37 °C. Cells were scraped off using a rubber policeman and add lysis buffer added to obtain cell lysates for subsequent experiments.

2.3. Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Proteins from cell lysates were separated by SDS–PAGE and transferred onto PVDF membranes for immunoblotting. Each membrane was blocked with blocking solution containing 3% bovine serum albumin in PBS containing 0.1% Tween 20 for 1 h at room temperature followed by primary and secondary antibody incubations. Immunodetection was carried out using LumiGLO chemiluminescence kit (Amersham, UK).

2.4. MTT assay

Cell viability was evaluated by using a [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide] (MTT) assay. Briefly, human glioma cells were seeded at an initial density of 2×10^4 cells/ml in a 24-well plate and incubated at 37 °C for 24 h. Cells were then incubated with serum-free medium containing various concentrations of rosiglitazone or triptolide for 24 h. After treatment, MTT was added to each well at a final concentration of 500 μg/mL, and the cells were incubated for 1.5 h at 37 °C. The medium was then removed, and the cell layer was dissolved with dimethylsulfoxide (DMSO). The formazen reaction product was quantified spectrophotometrically at 570 nm using a Synergy HT plate reader (BioTek Instruments, Vermont, USA). The results are expressed as the percentage of absorbance measured in control cultures after subtracting the background absorbance from all values.

2.5. Gelatin zymography

MMP-2 activities were determined by gelatin zymography. Equal numbers of cells (1×10^6) were seeded onto 100-mm dishes and cultured with serum-free medium for 24 h. Equal amount of media were loaded onto gelatin gels and analyzed by zymography. MMP-2 activities were determined by gelatin zymography. Gradient SDS–PAGE (10%) contained 0.1% gelatin. After the addition of 2X sample buffer, cell media were directly loaded on to gels for electrophoresis. After electrophoresis, sample were renatured by exchanging SDS with 2.5% Triton X-100. The gel was incubated at 37 °C in developing buffer containing 50 mM Tris–HCl, (pH 7.6) and10 mM $CaCl_2$. Gel was then stained with 0.25% Coomassie blue R250, 40% methanol, and 10% acetic acid at room temperature and destained with 40% methanol, 10% acetic acid until the bands of lysis became clear. The MMP-2-relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system (BioSpectrumAC Imaging System Vision with LS software, UVP Inc., Upland, California, USA).

2.6. Expression constructs

Human dominant negative MKP-1 cDNA was PCR-amplified with primers containing *BamH*I and *Eco*RI linkers and was inserted into pcDNA3.1 vector (Promega). U87MG cells were seeded at 5×10^5 cells in 6-cm plates and allowed to adhere overnight pcDNA3.1/MKP-1 and pcDNA3.1 plasmids were transfected into glioma cells using Lipofectamine (Invitrogen). G418 was used to select stable expression clones.

2.7. Transfection of MKP-1 specific siRNA

MKP-1 gene expression was knocked down with MKP-1 specific small interference RNA. Glioma cells seeded at 5×10^5 cells per 6-cm plate and allowed to adhere overnight, were transfected with MKP-1-specific small interference RNA (siRNA) (Santa Cruz, CA) using Lipofectamine. After 24 h, transfected cells were split equally into 6-cm plates and allowed to adhere overnight. The transfected cells were treated with the RGZ (30 μM) for 24 h. Experi-

mental cell lysates were collected in parallel for Western analysis.

2.8. In vitro invasion assay

Cell invasiveness capacity was determined by the Boyden-chamber assay method using the BD BioCoat Matrigel Invasion Chambers (BD Biosciences, San Jose, CA). Human glioma cell were suspended in serum-free DMEM and placed in the upper compartment of each chamber (1×10^5 cells/well), while the lower compartments were filled with DMEM containing 10% FCS. Non-invading cells were gently removed after 24 h. Cells on the bottom side of the filter were fixed, stained, and counted under a light microscope

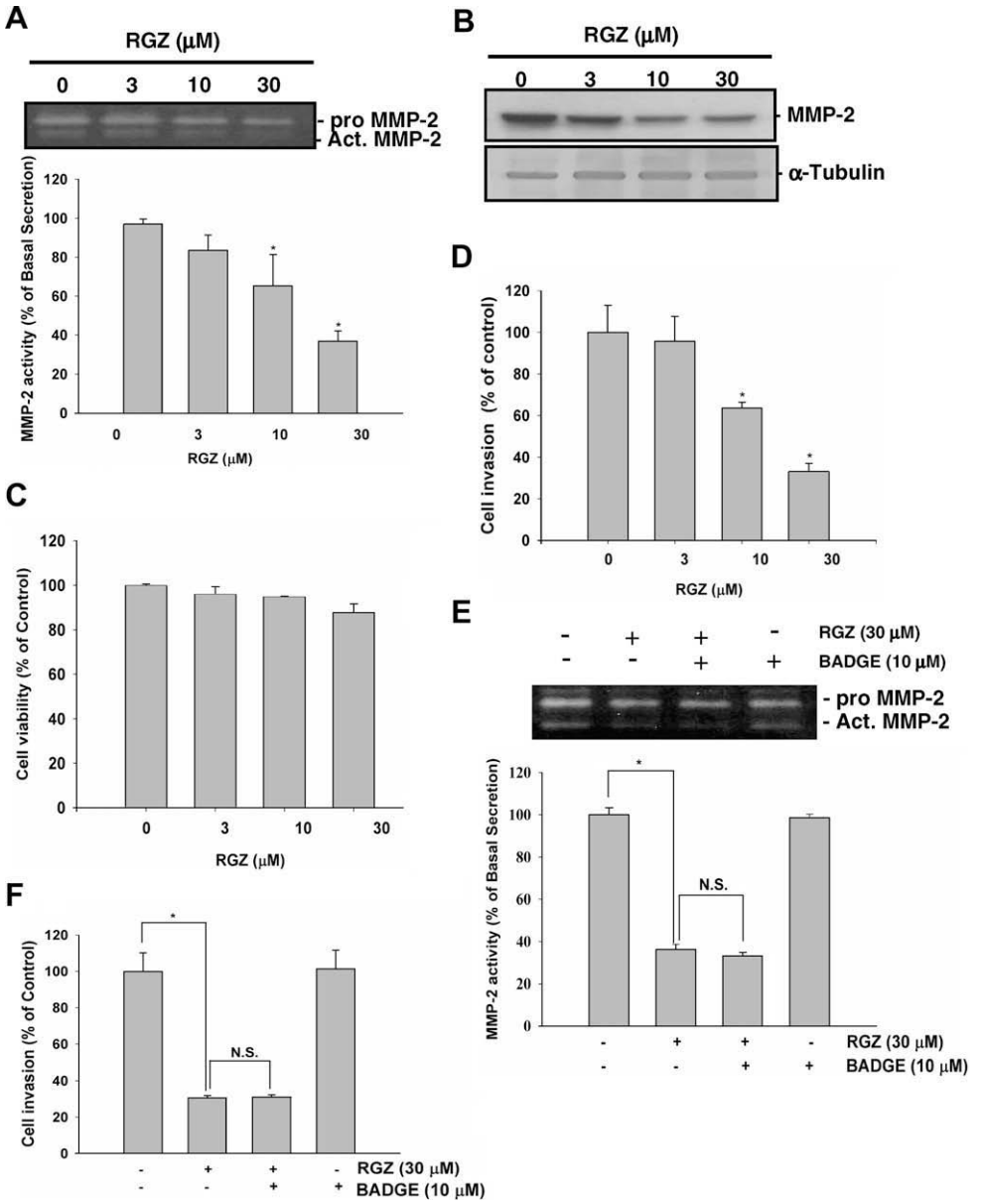

Fig. 1. Rosiglitazone reduces the gelatinolytic activity and the expression of matrix metalloproteinase-2 (MMP-2), and decreases cell invasiveness in U87MG cells. U87MG Cell were treated with different concentrations of RGZ (0–30 μM). (A) Cultured media were collected and their gelatinolytic activity were measured by zymography. (B) Total cell lysates were subjected to Western blot analysis probed with anti-MMP-2 or anti-α-tubulin antibodies. (C) Cell viability was tested by MTT assay after a 24 h incubation. (D) U87MG cells (1×10^5 cells) were incubated in serum-free DMEM medium and placed in the upper part of transwell chamber seperated by a Matrigel coated filter. After 24 h, cells on the bottom side of the filter were fixed, stained, and counted. Data represent the means ± SEM of three independent experiments. (E) U87MG cells were treated with RGZ (30 μM) in the absence or presence of bisphenol A diglycidyl ether, (BADGE; 10 μM) in serum-free medium for 24 h. Media were then collected for zymographic assay. (F) U87MG cells (1×10^5 cells) were incubated with RGZ (30 μM) in the absence or presence of BADGE (10 μM) in serum-free medium and placed in the upper part of transwell chamber. Cell invasiveness was assessed as described above. Data represent the means ± SD of three independent experiments. Statistically significant ($^*p < 0.05$, as compared with the basal secretion; N.S., no significant difference) was determined by Student's *t*-test.

(200× magnification). All experiments were repeated three times.

2.9. Statistical analysis

All data are expressed as the means ± SD for the number of experiments. Statistical significance ($p < 0.05$) between experimental groups was tested by a single factor ANOVA for multiple groups or unpaired student's t test for two groups.

3. Results

3.1. Effects of RGZ on MMP-2 and cell invasiveness

MMP-2 expression has been linked to the invasiveness of human gliomas. We first investigated whether RGZ reduces MMP-2 activity in human glioma cells. Treatment of U87MG cells with RGZ (0–30 μM) reduced MMP-2 gelatinolytic activity in a concentration-dependent manner using zymography (Fig. 1A). Treatment of U87MG cells with different concentrations of RGZ reduced MMP-2 protein expression in a concentration-dependent manner (Fig. 1B). The viability of glioma cells was examined using the MTT assay. Treatment of cells with RGZ (0–30 μM) did not alter the viable cell numbers (Fig. 1C). We next used a Matrigel-coated filter chamber to investigate the effects of RGZ on glioma invasiveness. As shown in Fig. 1D, RGZ inhibited invasiveness of U87MG cells. We further examined whether the incubation of cells with a PPAR-γ inhibitor, BADGE, would reverse the inhibition by RGZ. As depicted in Fig. 1E, pretreatment of U87MG cells with BADGE did not reverse the inhibition of MMP-2 gelatinolytic activity by RGZ. Consistently, BADGE did not reverse the RGZ-induced invasiveness of U87MG cells (Fig. 1F). These findings support the notion that RGZ reduces MMP-2 activity and cell invasiveness might through a PPAR-γ-independent pathway.

3.2. RGZ induces MKP-1 protein expression in human glioma cells

Several studies have demonstrated that MAPKs regulate MMP-2 activation [6,7]. MKP-1 is one of the key regulators of MAPK signal transduction pathways. MKP-1 serves as a negative regulator of different MAPK pathways and regulates a variety of cellular functions *in vivo* and *in vitro* [19], but little is known about its biological roles in tumor invasion. In human umbilical vascular epithelial cells, RGZ has been shown to inhibit activation of p44/p42 ERK and p38 mitogen activated protein kinase (p38MAPK) [23]. In agreement, treatment of U87MG cells with RGZ reduced activity all three members of the MAP kinase family (Fig. 2A). Because MAPKs may be dephosphorylated and inactivated by MKP-1, we hypothesized that RGZ

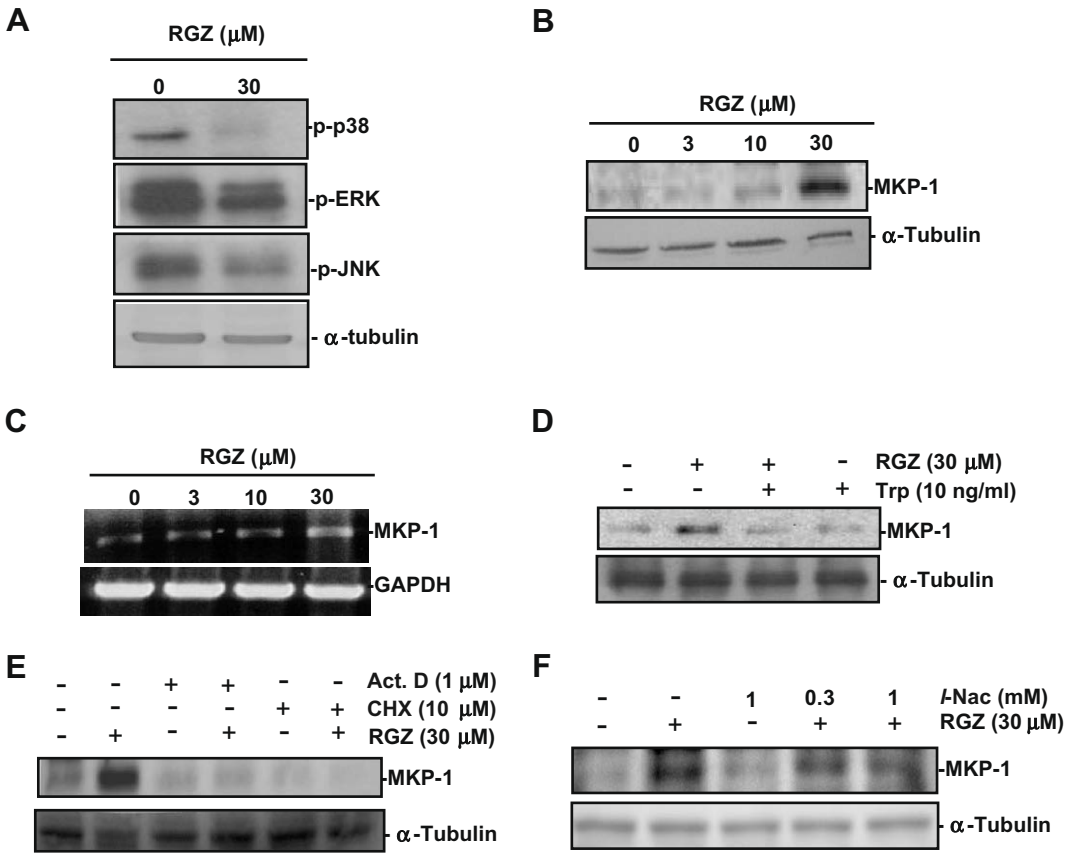

Fig. 2. Rosiglitazone induces de novo MAP kinase phosphatase-1 expression via a ROS-dependent pathway. (A) U87MG cells were treated with RGZ (0–30 μM) for 24 h, and cell lysates were immunoblotted with antibodies specific for phospho-p38 MAPK, phospho-ERK, phospho-JNK, or α-tubulin. In (B), U87MG cells were treated with RGZ (0–30 μM) for 24 h, and MKP-1 protein level was measured by Western blot analysis. In (C), U87MG cells were treated with RGZ (0–30 μM) for 24 h, and RNA were extracted for RT-PCR analysis. (D) U87MG cells were pretreated with triptolide (10 ng/ml) for 30 min, cells were then treated with RGZ (30 μM) for 24 h. MKP-1 expression was analyzed by Western blotting. (E) U87MG cells were pretreated with actinomycin D (Act. D) (1 μM) or cycloheximide (CHX) (10 μM) for 30 min before being treated with RGZ (30 μM) for 24 h. MKP-1 protein was detected by Western blot analysis using anti-MKP-1 antibodies. (F) U87MG cells were treated with different concentrations of an ROS scavenger N-acetyl-L-cysteine (l-NAC) (0.3–1 mM) for 30 min prior to the addition of RGZ (30 μM) and incubated for 24 h. MKP-1 protein expression was detected by Western blot analysis. The protein levels of α-tubulin serve as equal loading control for all western blots analysis.

may attenuate MAPKs activities via MKP-1 induction. To address this issue, we first investigated whether RGZ-induced MKP-1 in U87MG cells. Incubation of U87MG cells with different concentrations of RGZ increased MKP-1 protein expression (Fig. 2B). Treatment of U87MG cells with RGZ (0–30 μM) induced MKP-1 mRNA expression (Fig. 2C). These data suggest that RGZ might MKP-1 induce MKP-1 expression at the transcription and translation levels. Triptolide, an oxygenated diterpene derived from a Chinese herb that potently blocks MKP-1 induction, reduced RGZ-induced MKP-1 protein expression in U87MG cells (Fig. 2D). Treatment of glioma cells with actinomycin D Act. D (an inhibitor of gene transcription) or Cycloheximide CHX (an inhibitor of protein translation) prior to the addition of RGZ decreased MKP-1 protein levels (Fig. 2E), suggesting that RGZ-induced MKP-1 expression requires *de novo* gene expression and protein synthesis. Treatment of cells with *l*-NAC, which increased the cellular levels of reduced glutathione, decreased MKP-1 protein induction by RGZ (Fig. 2F). These data suggest that intracellular ROS are involved in MKP-1 induction.

3.3. RGZ reduces MMP-2 expression and cell invasiveness via a MKP-1 dependent mechanism

To further link MKP-1 to RGZ-inhibition of MMP-2 activity, U87MG cells were pretreated with triptolide to reduce MKP-1 expression prior to the addition of RGZ. As shown in Fig. 3A, treatment of cells with triptolide prevented the decrease in MMP-2 activity caused by RGZ in U87MG cells. In addition, RGZ-inhibition of MMP-2 expression was reversed by treatment of cells with triptolide (Fig. 3B). Triptolide also reversed RGZ-mediated cell invasiveness of U87MG cells (Fig. 3C). Treatment of cells with triptolide (0–10 ng/ml) did not alter the viable cell numbers in three glioma cell lines (Fig. 3D). We next used zymography to investigate the effects of triptolide on MMP-2 secretion in glioma cell lines. As depicted in Fig. 3E, triptolide did not alter MMP-2 activity in the cell lines. These findings support the notion that RGZ reduces MMP-2 activity and cell invasiveness through the MKP-1-dependent pathway. To further address the role of MKP-1 in the inhibition of MMP-2 production and in cell invasiveness, a small interference RNA (siRNA) specific for MKP-1 was used to knock down MKP-1 expression in U87MG and GBM cells. Knockdown of MKP-1 expression was demonstrated by suppression of MKP-1 protein expression in U87MG and GBM8401 cells (Fig. 4B). MKP-1 knockdown reversed the inhibitory effects of RGZ on MMP-2 secretion and protein expression in U87MG cells (Fig. 4A, B). Additionally, knockdown of MKP-1 reversed the inhibition of cell invasiveness by RGZ in U87MG and GBM8401 cells. These results suggest that MKP-1 may play a pivotal role in mediating the inhibition of glioma invasiveness by RGZ.

3.4. MKP-1 regulates cell invasiveness in U87MG cells

To confirm that the induction of MKP-1 is indeed linked to the inhibition of MMP-2, U87MG cells were stably expressed with an empty vector (pcDNA3.1) or pcDNA3.1/MKP-1 encoding wild-type MKP-1. Ectopic expression of MKP-1 reduced MMP-2 activity in U87MG and GBM8401

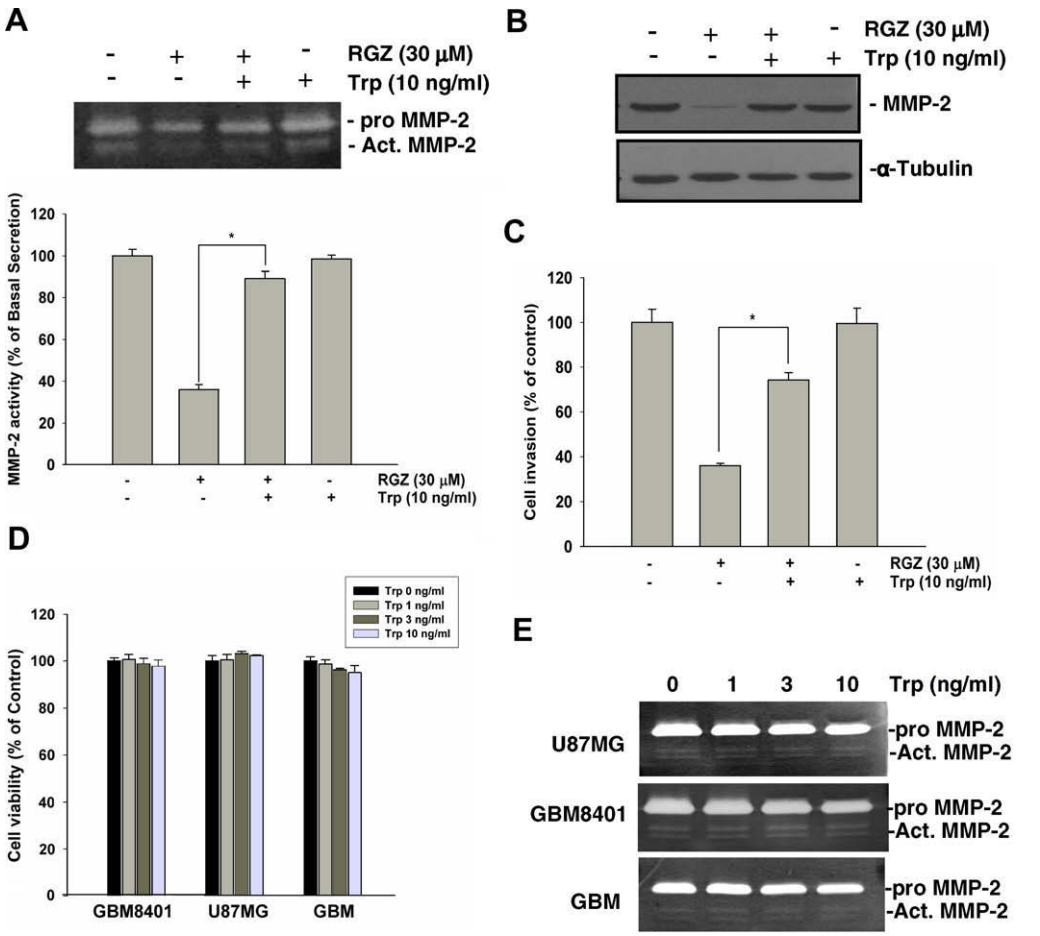

Fig. 3. Triptolide reversed rosiglitazone-inhibited gelatinolytic activity and expression of MMP-2 and glioma cell invasiveness. U87MG cells were pretreated with triptolide (10 ng/ml) 30 min prior to the addition of RGZ (30 μM) in serum-free medium for 24 h. (A) Media were then collected for the zymographic assay. (B) Cell lysates were analyzed for MMP-2 expression by Western blotting. (C) U87MG cells were pre-incubated with triptolide (10 ng/ml) for 30 min and then treated with or without RGZ (30 μM) for 24 h. Data represent the means ± SD of three independent experiments (* $p < 0.05$, as compared with the RGZ+Trp). Glioma cell lines (U87MG, GBM8401, GBM) were treated with triptolide (0–10 ng/ml) for 24 h. (D) Cell viability was analyzed by the MTT assay. (E) Media were then collected for a zymographic assay for matrix metalloproteinase-2 (MMP-2) activity.

cells (Fig. 5A). MKP-1 reduced the phosphorylations of p38, ERK, and JNK, inhibited MMP-2 expression (Fig. 5B), and inhibited invasiveness of U87MG and GBM8401 cells (Fig. 5C).

4. Discussion

The prognosis for patients with gliomas remains dismal because current therapies fail to control gliomas from invading contiguous normal brain tissues. It has been shown that MMP-2 plays a pivotal role in invasion and development of human malignant gliomas. In the present study, we demonstrate that treatment with RGZ reduced MMP-2 expression activity, and cell invasiveness in human U87 glioma cells. We present evidences that RGZ increased MKP-1 protein levels and MKP-1 inhibition by triptolide or MKP-1 knockdown by siRNA reversed the inhibition of MMP-2 by RGZ. Although RGZ has been shown to inhibit the growth and invasiveness of many cancer cell lines, this study is the first report emphasizing the role of MKP-1 in the inhibitory effect of RGZ on cell invasion by human gliomas. Our results suggest that induction of MKP-1 may be considered as a potential therapeutic strategy in the treatment of human malignant gliomas.

Activation of PPAR-γ by conjugating linoleic acid inhibits the cell proliferation rate, induces apoptosis, and decreases cell migration and invasiveness in a human glioblastoma cell line [24]. Many thiazolidinedione derivatives (TZDs) are PPAR-γ ligands. However, TZDs can exert their effects through a signal transduction pathway independent of PPAR-γ activation. For example, Galli et al. [13] demonstrated that a similar dose-dependent inhibition of cell invasiveness was found in both PPAR-γ-expressing and non-expressing pancreatic cell lines. Our results are in line with those of Galli et al. [8,13], because in the inhibition of cell invasion, MMP-2 expression and MKP-1 induction by RGZ were not affected by BADGE, the PPAR-γ antagonist. In the present study, we found that RGZ-induced MKP-1 might be involved in regulating glioma cell fate, and that RGZ-induced MKP-1 expression was dependent upon ROS induction. These results are in line with a previous report showing RGZ-induced protein-tyrosine phosphatase (PTP)-1B expression in glioma cells via a PPAR-γ-independent pathway [25]. Our data also agree with many other reports showing troglitazone or pioglitazone, thiazolidinedione (TZD) derivatives, reduced glioma cell growth and invasion *in vivo* [26]. TZDs might be a promising drug for malignant gliomas because they facilitate the phosphatase cascade, thereby suppress MMP-2 activity and cell invasiveness in malignant glioma cells.

It is well established that activation of MAPK pathways is important for MMP-2 expression. Our results agree with

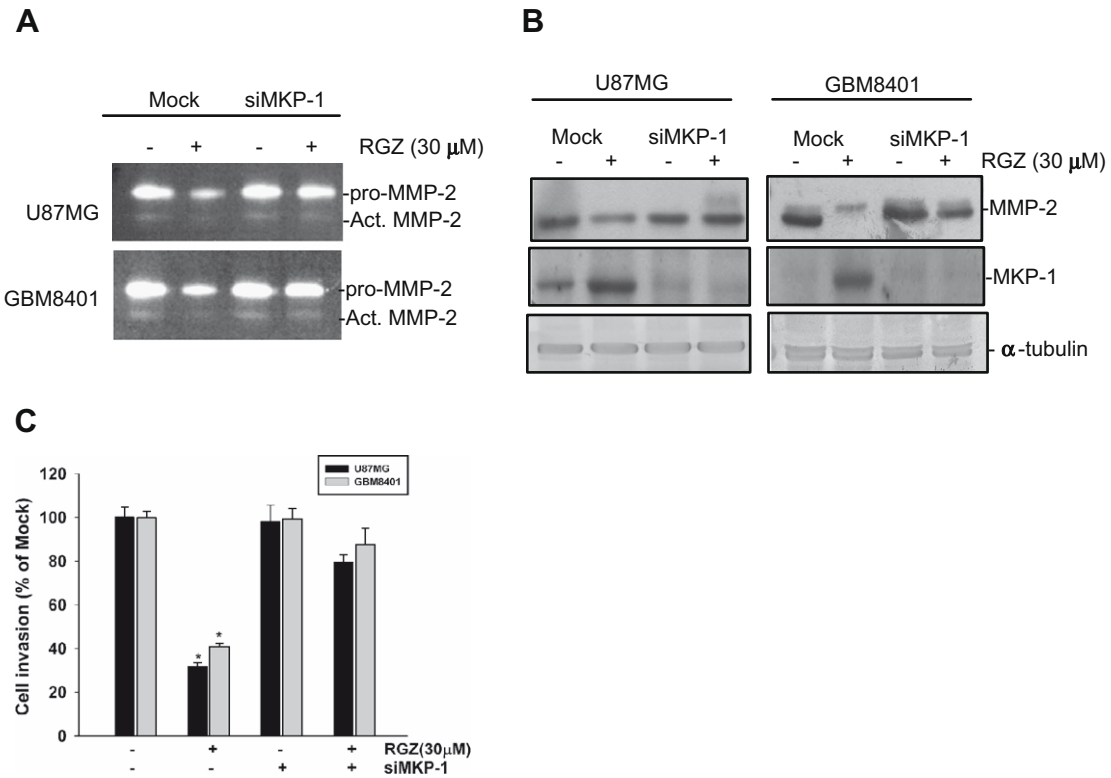

Fig. 4. Rosiglitazone inhibits MMP-2 gelatinolytic activity, and glioma cell invasiveness via MKP-1. U87MG and GBM8401 cells were transfected with either mock or MKP-1 specific siRNA and incubated in serum-free medium for 24 h, prior to the addition of RGZ (30 μM). (A) Media were collected for the zymographic assay. (B) MKP-1 and MMP-2 expressions were analyzed by Western blotting. (C) For the *in vitro* invasion assay, equal numbers of glioma cells were seeded in the upper part of a transwell chamber with or without the RGZ. After 24 h, cells on the bottom side of the filter were fixed, stained, and counted. Data represent the mean ± SD of three independent experiments.

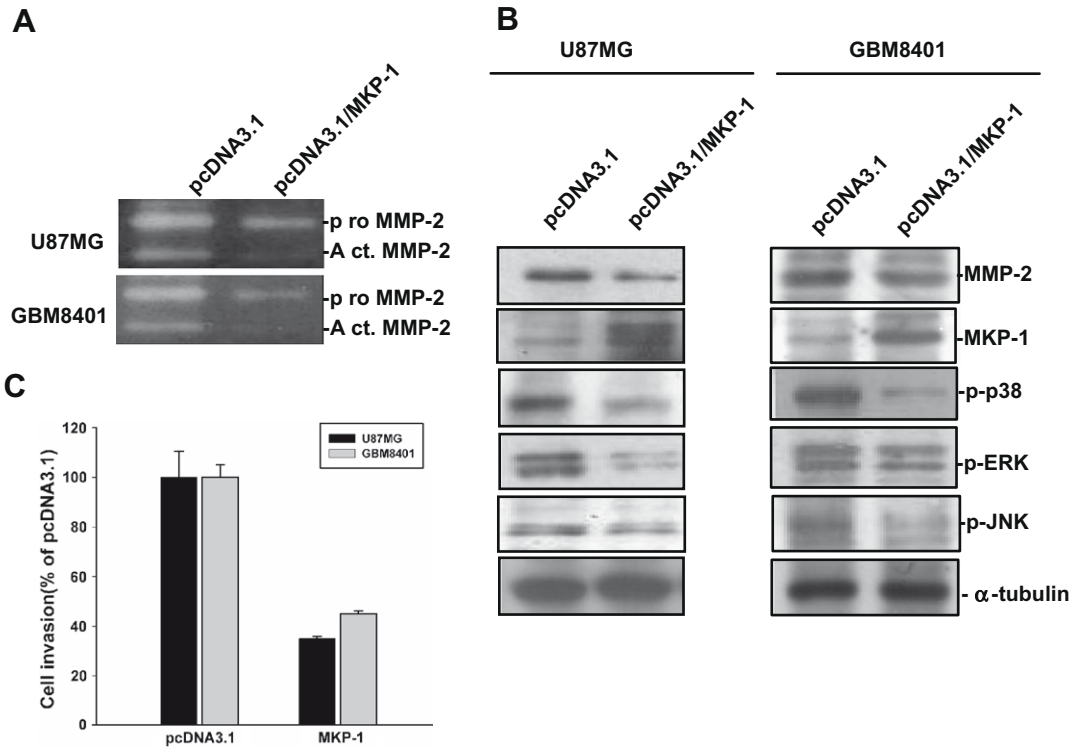

Fig. 5. Ectopic expression of MKP-1 reduces MMP-2 activity and cell invasiveness in U87MG and GBM8401 cells. U87MG or GBM8401 cells were stably expressed with either control vector (pcDNA3.1) or wild-type MKP-1 (pcDNA3.1/MKP-1). (A) Media were collected for MMP-2 zymography. (B) Cell lysates were immunoblotted with antibodies specific for MMP-2, MKP-1, phospho-p38, phospho-ERK, phosphor-JNK, or α-tubulin. (C) Cell invasiveness was analyzed by an *in vitro* Matrigel invasion assay. Data are presented as the means ± SD of three independent experiments performed in triplicate. ($^{*}p < 0.05$, as compared with the control).

recent reports showing that the inhibition of p38 by genistein blocked MMP-2 induction and cell invasion [7], and that the ERK1/2 pathway is involved in MMP-2 expression [8]. We previously found that inhibition of MAPKs by pharmacological inhibitors suppresses MMP-2 secretion and cell invasiveness in glioma cells [22]. RGZ has been shown to inhibit cell invasiveness [13], and tumor metastasis [27] through a PPAR-γ-independent pathway. Because RGZ inhibits MAPK activities [28], it is highly possible that RGZ exerts these effects by inhibiting MAPKs activity. This assumption is supported by the observation that RGZ induces MKP-1 expression in human glioma cells. Taken together, our data suggest that RGZ may induce MKP-1 expression, which in turn inhibit MAPK activativities and MMP-2 secretion in U87MG cells.

Conflicts of interest statement

The authors declare that we do not have financial and personal relationships with other people or organisations that could inappropriately influence (bias) our work.

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