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Dexamethasone reduced invasiveness of human malignant glioblastoma cells through a MAPK phosphatase-1 (MKP-1) dependent mechanism

Yu-Min Lin [a,c], Hsun-Jin Jan [d], Chin-Cheng Lee [b], Hsiao-Yi Tao [d], Yu-Lueng Shih [b], Hen-Wei Wei [f], Horng-Mo Lee [e,*]

[a] Department of Internal Medicine, National Defense Medical Center, Shin Kong Memorial Hospital, Taipei 111, Taiwan
[b] Department of Pathology, National Defense Medical Center, Shin Kong Memorial Hospital, Taipei 111, Taiwan
[c] Graduate Institute of Medical Sciences, National Defense Medical Center, Shin Kong Memorial Hospital, Taipei 111, Taiwan
[d] Graduate Institute of Medical Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan
[e] School of Medical Laboratory Sciences and Biotechnology, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan
[f] Department of Animal Science and Technology, National Taiwan University, Taipei 106, Taiwan

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ABSTRACT

Dexamethasone has been shown to inhibit tumor invasiveness. In the present study, the effects of dexamethasone on matrix metalloproteinases-2 (MMP-2) secretion, cell invasiveness, and intravasation in human U87MG glioma cells were examined. Dexamethasone decreased MMP-2 secretion and cell invasiveness in human glioma cells. Incubation of cells with dexamethasone increased mitogen activated protein kinase phosphatase-1 (MKP-1) expression. Ectopic expression of MKP-1 decreased cell invasiveness *in vitro* and intravasation *in vivo*. Because expression of inducible nitric oxide synthase (iNOS) has been implicated in the progression of malignant gliomas, we next investigated the possible roles of NO^- in MMP-2 secretion and cell invasiveness in human U87MG glioma cells. Treatment of glioma cells with nitric oxide donor, sodium nitroprusside (SNP), increased MMP-2 secretion and the capacity of cell invasion in U87MG cells. Addition of dexamethasone or ectopic expression of wild-type MKP-1 suppressed the SNP-stimulated MMP-2 activation and glioma cell invasiveness in U87MG cells. Taken together, these results suggest that dexamethasone may suppress MMP-2 secretion and cell invasion through MKP-1 induction in human glioma cells.

1. Introduction

Gliomas are the most frequent and malignant among human intracranial tumors (Ohgaki, 2005). Despite radical surgery, the prognosis with radiation therapy and conventional chemotherapy remains low due to invasion of surrounding brain tissues (Demuth and Berens, 2004). Tumor cell hyperproliferation and invasiveness are key features of gliomas. Cell invasion is a complex process consisting of proteolysis of extracellular matrix components and adhesion of tumor cells to normal brain elements. Matrix metalloproteinases (MMPs) have been implicated as important factors in the control of the invasive capability of gliomas. MMPs play roles in pathological conditions involving untimely and accelerated turnover of extracellular matrix, including inflammation, angiogenesis, and metastasis (Nakano et al., 1995; Price et al., 2001; Rao, 2003). As MMPs are important regulators of tumor invasiveness, MMPs and their regulatory pathways have been considered as possible therapeutic approach for cancer therapy. Among MMPs, attention has been focused on gelatinases (MMP-2 and MMP-9) in human gliomas. A strong correlation between glioma invasion and a high expression level of MMP-2 has been demonstrated in cultured glioma cells (Nakano et al., 1995; Rao et al., 2003).

Mitogen-activated protein kinases (MAPKs), including extracellular signal-regulating kinase (ERK), p38 MAPK, and c-Jun N-terminal protein kinase (JNK) play important roles in cell proliferation, apoptosis, and many other nuclear events. MAPKs pathways have been implicated in tumorigenesis in a variety of cells and targeting malignant glioma MAPKs signaling may improve clinical outcomes (Kapoor and O'Rourke, 2003; Kondo et al., 2004; McLendon et al., 2007; Mischel and Cloughesy, 2003; Wong et al., 2007). MAPKs have been shown to regulate MMP-2 activity in tumor cells (Galli et al., 2005; Park et al., 2002; Puli et al., 2006). MAPKs are negatively regulated by mitogen activated protein kinase phosphatase-1 (MKP-1) (also known as CL100, 3CH134, Erp, and hVH-1), a dual-specificity phosphatase, which inactivates MAPKs by dephosphorylation of both threonine and tyrosine residues within the activation motif (Keyse, 2008). MKP-1 has been shown to inhibit a number of cellular responses mediated by ERK, JNK, and p38 MAPK (Duff et al., 1995;

* Corresponding author. Tel.: +886 2 2736 1661x3316; fax: +886 2 2732 4510.
E-mail address: leehorng@tmu.edu.tw (H.-M. Lee).

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Engelbrecht et al., 2003; Gupta et al., 1996; Keyse and Emslie, 1992; Lai et al., 1996; Liu et al., 1995; Wadgaonkar et al., 2004).

Nitric oxide synthases have been linked to MMP-2 activation and implicated in the pathophysiology of high-grade astrocytic tumors (Yamaguchi et al., 2002; Badn et al., 2007). Among the 3 NOS isoforms, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS), enhanced iNOS expression was observed in 54% of oligodendrogliomas and in 29% of anaplastic oligodendrogliomas (Broholm et al., 2001). The enhanced iNOS expression in astroglial tumors suggests that iNOS may play a role in the progression from low-grade oligodendrogliomas to more-anaplastic types. Although inhibition or knockout of iNOS dramatically reduces the tumorgenicity of gliomas (Badn et al., 2007; Yamaguchi et al., 2002), little is known about the relationships between the nitric oxide production and the invasiveness of glioma cells.

Dexamethasone is a member of glucocorticoid family, which is broadly used as an anti-inflammatory agent and for management of cerebral edema in patients with brain tumors. Dexamethasone exerts its anti-inflammatory effects at least partly through induction of MKP-1 (Van Molle and Libert, 2005). MKP-1 is also one of the genes that are associated with the anti-tumor activity of glucocorticoids (Vedoy and Sogayar, 2002). Although the underlying mechanism of glucocorticoid on malignant glioma cell invasiveness is still unknown, it is possible that dexamethasone may regulate cell invasiveness through MKP-1 expression. In the present study, we tested the hypothesis of whether dexamethasone inhibits MMP-2 activity and invasiveness of human malignant glioma cells through a MKP-1-dependent mechanism. This study also addressed whether the effects of NO^- on MMP-2 secretion and cell invasiveness can be regulated by dexamethasone through MKP-1 induction. We demonstrated that dexamethasone induced MKP-1 expression in U87MG cells. Expression of dominant negative MKP-1 suppressed the inhibitory effects of dexamethasone on MMP-2 secretion, cell invasion and intravasation, suggesting dexamethasone may inhibit glioma cell invasiveness through induction of MKP-1. NO^- overproduction has been implicated in the malignancy of human gliomas. Because NO^- induced MMP-2 secretion and cell invasion were suppressed by dexamethasone or expression of wild-type MKP-1, possibly dexamethasone exerts its anti-invasive effect through suppression of iNOS expression. These data suggest that dexamethasone may exert therapeutic effect through MKP-1 induction, which regulates the invasiveness of gliomas.

2. Materials and methods

2.1. Chemicals

Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), glutamine, gentamycin, penicillin, and streptomycin were purchased from Life Technologies (Gaithersburg, MD). Antibodies specific for iNOS, phospho-p38 MAPK, phospho-ERK, phospho-JNK, MKP-1 and α-tubulin were purchased from Santa Cruz Biochemicals (Santa Cruz, CA). Antibody specific for MMP-2 was purchased from Sigma Chemical Co. (St. Louis, MO, USA). SB 203580, SP 600125, and PD 98059 were purchased from Calbiochem-Novabiochem (San Diego, CA). A horseradish peroxidase-conjugated anti-rabbit Immunoglobulin G antibody was purchased from Bio-Rad (Hercules, CA). Protease inhibitor cocktail tablets were purchased from Boehringer Mannheim (Mannheim, Germany).

2.2. Plasmids and stable clones

Human MKP-1 cDNA was PCR-amplified with primers containing EcoRI and BamHI linkers and was inserted into the polycloning site of pcDNA3.1. The dominant negative MKP-1CS was inserted in pcDNA3.1. U87MG cells were seeded at 5×10^5 cells in 6-cm plates and allowed to adhere overnight. The next day, pcDNA3.1/MKP-1, pcDNA3.1/MKP-1CS, and pcDNA3.1 vectors were transfected into U87MG cells using lipofectamine. After 24 h, cell-conditioned medium was changed to select G418-resistant clones. Experimental cell lysates were collected in parallel for Western analysis.

2.3. Preparation of cell lysates and Western blot analysis

Human U87MG (American Type Culture Collection no. HTB-14) and other primary human glioma cells (from Shin Kong Memorial Hospital) were cultured in DMEM supplemented with 13.1 mM $NaHCO_3$, 13 mM glucose, 2 mM glutamine, 10% heat-inactivated FCS, and penicillin (100 U/ml)/streptomycin (100 mg/ml). Cells were attached to a Petri dish after 24 h of incubation. After reaching confluence, cells were treated with various concentrations of dexamethasone for the indicated time intervals and incubated in a humidified incubator at 37 °C. After incubation, cells were lysed by adding lysis buffer containing 10 mM Tris–HCl (pH 7.5), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM DTT, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktails (with final concentrations of 0.2 mM PMSF, 0.1% aprotinin, and 50 μg/ml leupeptin). For Western blot analysis, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted. Blots were subsequently incubated with 5% non-fat milk in PBS for 1 h to block non-specific binding and then overnight with antibodies against MKP-1, MMP-2, or MAPKs (ERK 1/2, JNK 1/2, and p38). Blots were then incubated with horseradish peroxidase goat anti-rabbit or anti-mouse immunoglobulin G for 1 h. All incubations were carried out at 37 °C and intensive PBS washing was performed. After the final PBS washing, the signal was developed using an ECL (enhanced chemiluminescence) plus detection kit (Amersham Life Sciences, Piscataway, NJ), and the relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system (BioSpectrum AC Imaging System Vision Work LS software).

2.4. MTT assay

Cell viability, an indicator of cytotoxicity, was evaluated using an [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenytetrazolium bromide] (MTT) assay. Glioma cells grown on 150-mm plates were washed twice with phosphate-buffered saline (PBS) and resuspended in DMEM. The suspended cells were plated on 24-well plates (2×10^5 cells/well) and treated with the indicated reagent(s) for 24 h. MTT was added to the medium (1 mg/ml), and cells were incubated at 37 °C for 2 h. Then dimethylsulfoxide (DMSO) (100 μl) was applied to the medium to dissolve the formazan crystal derived from mitochondrial cleavage of the tetrazolium ring of MTT. The absorbency at 570 nm in each well was measured on a micro-enzyme-linked immunosorbent assay (ELISA) plate reader. None of the reagents used in this study interfered with the MTT values.

2.5. Gelatinolytic zymography

Gelatinolytic zymography was used to detect the activity of MMP-2 in the cultured media. Briefly, the collected medium was loaded to onto a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel co-polymerized with 0.1% gelatin and subjected to electrophoresis. After electrophoresis, the gel was washed twice with a 2.5% Triton X-100 solution and incubated in incubation buffer (0.05M Tris–HCl buffer (pH 8.0), 5 mM $CaCl_2$, and 5 μM $ZnCl_2$), at 37 °C overnight. The gel was stained with PhastGel Blue R at room temperature for 2h. Gelatinases in the media were detected as unstained gelatin-degraded zones on the gel. The MMP-2-relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system (BioSpectrum AC Imaging System Vision with LS software).

2.6. In vitro Matrigel invasion assay

Matrigel invasion assays were performed with a cell invasion assay kit (Chemicon). Briefly, cells were seeded in the upper part at a density of 1×10^5 cells/well in 300 μl of serum-free medium and pretreated with or without the indicated concentrations of various agents. After 30 min, cells were incubated with dexamethasone. Lower compartments were filled with DMEM that contained 10% FCS. After incubation 12 h in culture incubator, non-invading cells were gently removed using a cotton-tipped swab while invading cells on the lower surface of the membrane were stained. For quantitative assessment, the stained cells were dissolved in DMSO (100–200 μl/well) and the dye/solute mixtures were transferred to a 96-well plate for colorimetric reading of OD at 560 nm.

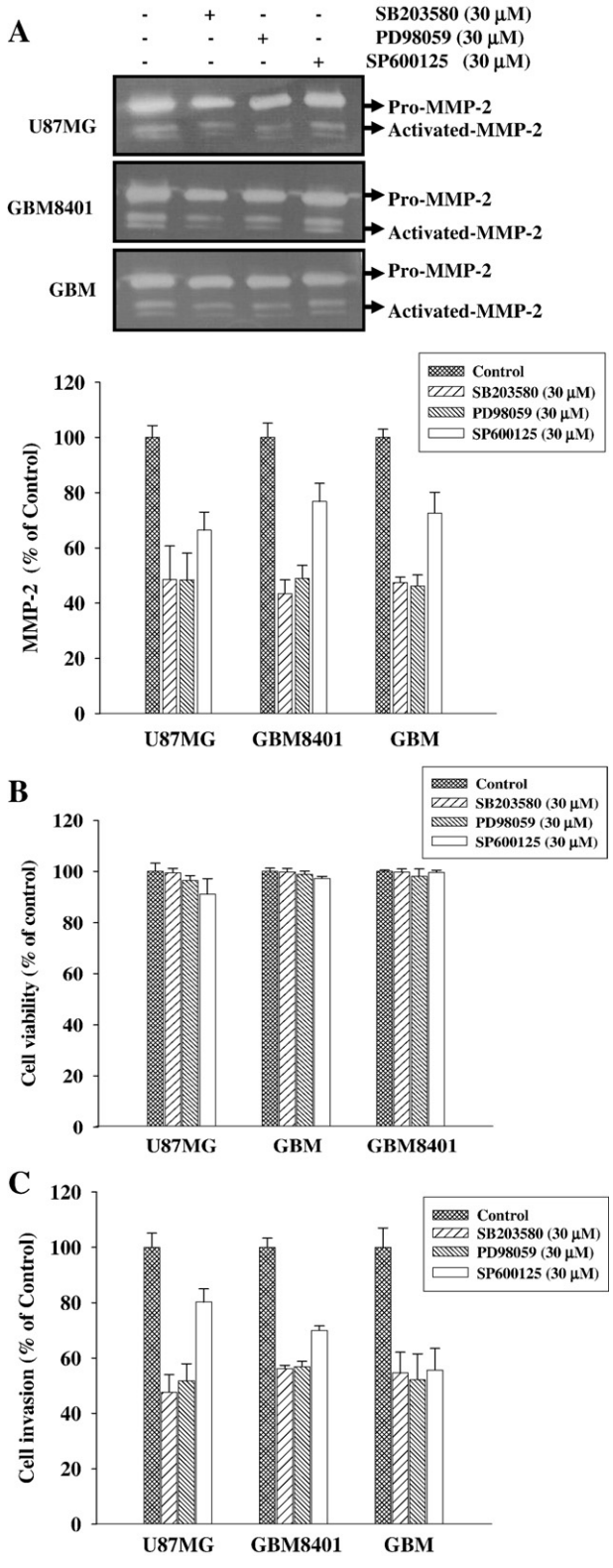

Fig. 1. Effects of MAPK inhibitors on matrix metalloproteinase-2 (MMP-2) secretion and invasiveness in human glioma cell lines derived from human gliomas (GBM, GBM8401) and U87MG cells were treated with SB203580 (30 μM), PD98059 (30 μM), or SP600125 (30 μM) for 24 h. In (A), the gelatinolytic activities of MMP-2 in cultured media were analyzed by zymography. The MMP-2-relative photographic density (in lower of panel A) was quantitated by scanning the photographic negatives on a gel analysis system. Mean photographic density ± S.E.M. of three independent experiments is shown. In (B), cell viability was determined by the addition of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) to the cultures at the end of incubation periods. The reaction was stopped after incubating for another 2 h, and the changes in absorbance quantified using a plate reader at 590 nm. In (C), *in vitro* invasion assay, glioma cell lines were treated with MAPKs inhibitors for 24 h, and cells on the bottom side of the filter were quantitated as described in the "Materials and methods". Data are given as the means ± S.E.M. of three independent experiments performed in triplicate.

2.7. In vivo chicken embryo chorioallantoic membrane intravasation assay

The assay of the chicken embryo chorioallantoic membrane (CAM) was conducted according to the description by Kim *et al.* (Kim et al. (1998) except for a few modifications. Fertilized eggs were incubated in a rotary incubator at 38 °C with 60% humidity. On day 10, a small hole was drilled in the top of an egg above the area of greatest vasculature, causing the CAM to detach from the shell membrane. Glioma cells (10^5) in a volume of 50 μl of cell culture media were inoculated onto the dropped CAM with a pipette tip (five eggs for each tumor type). The holes were sealed with tape, and the eggs were returned to the incubator. Four days after inoculation (day 14) eggs were carefully opened. The CAMs lining the cavity of the eggshell were removed, snap-frozen, and used for extraction of genomic DNA. The frozen CAMs were crushed to powder, suspended in digestion buffer (100 mM NaCl, 10 mM Tris–Cl, (pH 8.0), 25 mM EDTA, (pH 8.0), 0.5% SDS, and 0.1 mg/ml proteinase K), and incubated at 50 °C for 18 h. The samples were extracted using a genomic isolation kit (from Sigma). The PCR, which produced an *Alu* band of 224 bp, was performed according to Kim *et al.* (Kim et al. (1998). The PCR products were resolved on a 2% agarose gel containing 1 μg/ml ethidium bromide. The primers for human *Alu* were forward, 5′-ACGCCTGTAATCCCAGCACTT-3′ and reverse, 5′-TCGCCCAGGCTGGAGTGCA-3′.

2.8. Immunohistochemistry

Formalin-fixed, paraffin embedded tissue sections were used to examine for the expression of the iNOS protein. Briefly, 4–6μm thick sections were cut using microtome and applied to PLL coated slides. Sections were dewaxed in xylene for 10min, rehydrated in alcohol solution and placed in 5% hydrogen peroxide in absolute alcohol for 10min to block endogenous peroxidase activity. To unmask antigen by heat treatment, the slides were placed in a container and covered with 10 mM sodium citrate buffer, pH 6 and heated at 95 °C for 5 min. Topped off with fresh buffer and heated at 95 °C for 5 min and allowed slides to cool in buffer for 20 min. Slides were washed in deionized water three times for 2min each on stir plate. All subsequent steps were carried out at room temperature in a humidified chamber. Tissue sections were not allowed to dry out at any time during the procedure. The primary antibody was diluted (1:50) and added in sufficient volume to cover this tissue, incubated for 2h. Slides were rinsed with PBS, washed in PBS twice for 2min each on stir plate. The sections were incubated for 30min in

biotinylated secondary antibody at a dilution of 1:200. Rinse with PBS, then wash with PBS twice for 2min each on stir plate. Tissue sections were then incubated with HRP-streptavidin complex (Dako) for 30min before washing again with PBS. The immuno-staining was visualized by developing in diaminobenzidine before counter stained with Mayer's haematoxylline.

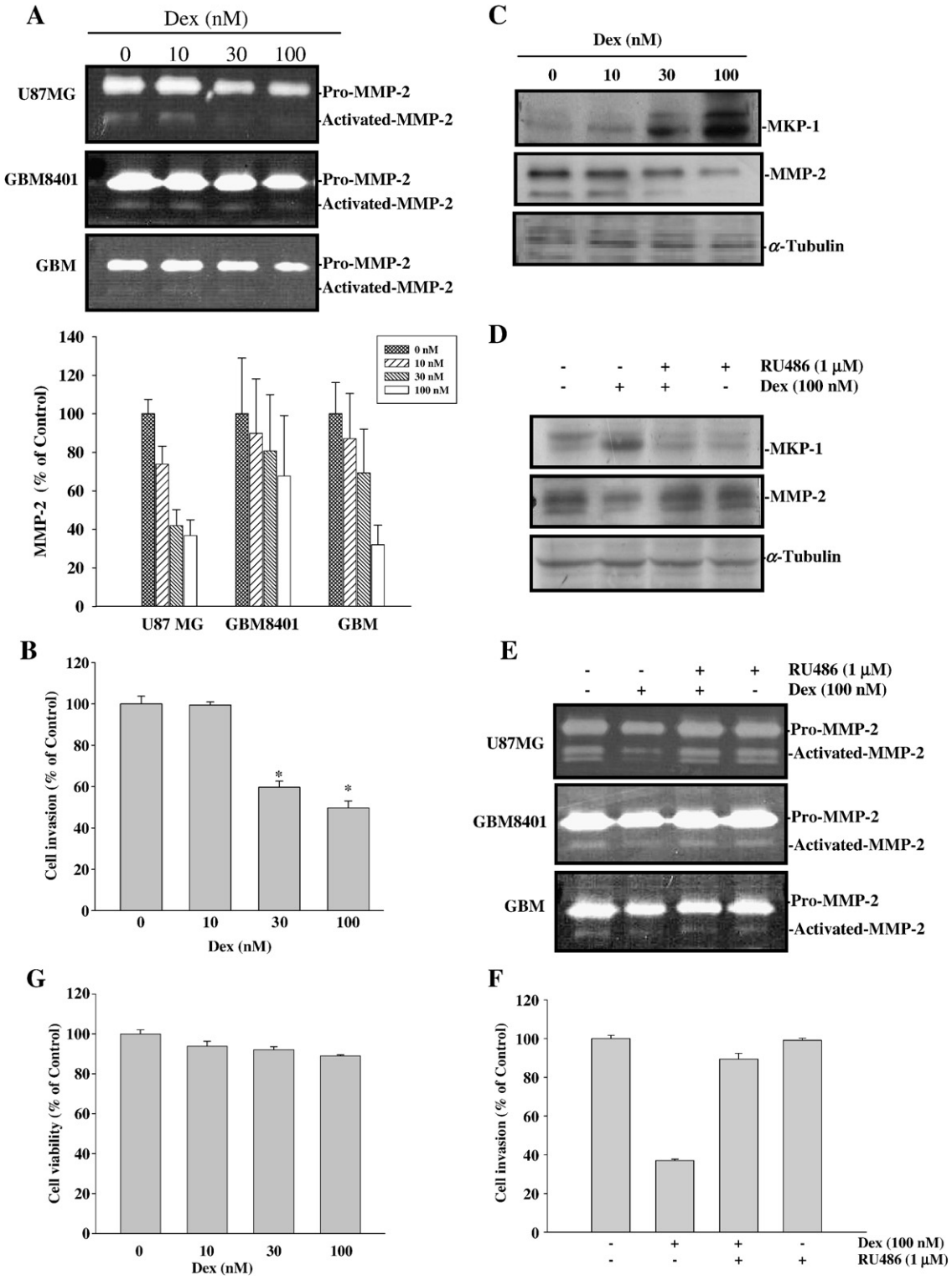

Fig. 2. Effects of dexamethasone (Dex) on MMP-2 secretion and cell invasiveness in human glioma cell lines. GBM, GBM8401 and U87MG cells were treated with different concentrations of dexamethasone for 24 h. In (A), media were collected and MMP-2 activities analyzed by zymography. In the lower panel of (A), the MMP-2-relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system. Mean photographic density ± S.E.M. of three independent experiments is shown (lower panel). In (B), *in vitro* Matrigel invasion assay was performed as described in the "Materials and methods". In (C), U87MG cells were treated with different concentrations of dexamethasone, and total cell lysates were subjected to immunoblot analysis probing with anti-MKP-1, anti-MMP-2 or α-tubulin antibodies. In (D), (E), and (F), U87MG cells were pretreated with the glucocorticoid receptor antagonist, RU486 (1 μM) for 30 min before incubation with dexamethasone (100 nM) for 24 h. Total cell lysates were subjected to immunoblot analysis probing with anti-MKP-1, anti-MMP-2 or α-tubulin antibodies (D). Cultured media were collected and their MMP-2 activities analyzed by zymography (E). Cell invasiveness was analyzed by an *in vitro* Matrigel invasion assay (F). In (G), cell viability was evaluated using the MTT assay as described above. Data are given as the means ± S.E.M. of three independent experiments performed in triplicate. *$P<0.05$.

2.9. Statistical analysis

All data are expressed as the mean ± S.E.M. Statistical analysis was performed using one-way ANOvA and student *t*-test. A difference between groups of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Roles of MAPKs in regulating MMP-2 secretion and cell invasiveness in human glioma cell lines

Because MAPK inhibitors have been shown to mediate MMP-2 expression, we first evaluated whether MAPKs regulate MMP-2 secretion and cell invasiveness in human glioma cell lines. Cell lines derived from human glioma biopsies of World Health Organization (WHO) grades IV (GBM and GBM8401) and U87MG cells (from ATCC) were incubated in serum-free medium for 24h, and cultured media were collected and assayed for MMP-2 gelatinolytic activity. Fig. 1A shows all 3 cell lines constitutively secreted MMP-2, and this effect was inhibited by pretreatment of cells with SB203580 (a p38 MAPK-specific inhibitor) or PD98059 (a MEK-specific inhibitor), and SP600125 (a JNK inhibitor) in human glioma cell lines. Incubation of cells with highest concentrations of these MAPK inhibitors did not cause significant cytotoxicity (Fig. 1B). The inhibition of MMP-2 secretion was associated with decreased cell invasiveness in human glioma cells (Fig. 1C). These data provide evidences that MAPKs may regulate MMP-2 secretion and cell invasiveness in human glioma cells.

3.2. Dexamethasone regulates MMP-2 secretion and invasiveness in glioma cells through a MKP-1 dependent mechanism

Because glucocorticoids have been shown to suppress MAPK activities, we investigated whether dexamethasone inhibits MMP-2 secretion in human glioma cells. Fig. 2A shows that MMP-2 gelatinolytic activity in cultured media was suppressed in a concentration-dependent manner by treating cells with dexamethasone. We next examined whether dexamethasone attenuated glioma cell invasiveness *in vitro* by Matrigel invasion assay. As shown in Fig. 2B, treatment of U87MG cells with dexamethasone (10–100nM) reduced glioma invasiveness. Glucocorticoids have been shown to inhibit MAPKs through MKP-1 induction in many cells. We next tested whether dexamethasone induced MKP-1 protein expression in human glioma cells. Dexamethasone induced MKP-1 protein expression in a concentration-dependent manner (Fig. 2C). Notably, the increase of MKP-1 protein levels was inversely correlated to the decrease of MMP-2 protein levels in glioma cells (Fig. 2C). These data provide strong evidence that dexamethasone might regulate MMP-2 through MKP-1 induction. To elucidate whether dexamethasone is mediated through glucocorticoid receptor, cells were incubated with RU486, a glucocorticoid receptor antagonist, prior the addition of dexamethasone. RU486 blocked the MKP-1 protein expression (Fig. 2D) and reversed the inhibition of MMP-2 secretion (Fig. 2E) and cell invasion (Fig. 2F) in human glioma cells. These data suggest that dexamethasone might reduce MMP-2 secretion and cell invasiveness through glucocorticoid receptor. Because dexamethasone (10–100nM) did not alter the cell viability (Fig. 2G), these data suggest that dexamethasone-induced MKP-1 expression might play a pivotal role in regulating MMP-2 secretion and cell invasiveness in human glioma cells.

3.3. MKP-1 plays a pivotal role in regulating MMP-2 secretion and cell invasiveness in glioma cells

To address these further, U87MG cells were stable transfected with pcDNA3.1, pcDNA3.1/MKP-1, or pcDNA3.1/MKP-1CS, encoding an empty vector, a wild-type MKP-1, or a catalytically inactive mutant of MKP-1 (MKP-1CS), respectively. The expression of wild-type MKP-1 inhibited MAPKs phosphorylation (p38, ERK, and JNK) and MMP-2 expression in U87MG cells. Opposite effects were observed in cells bearing the catalytically inactive mutant of MKP-1 (Fig. 3A). While the expression of catalytically inactive MKP-1 did not inhibit MMP-2

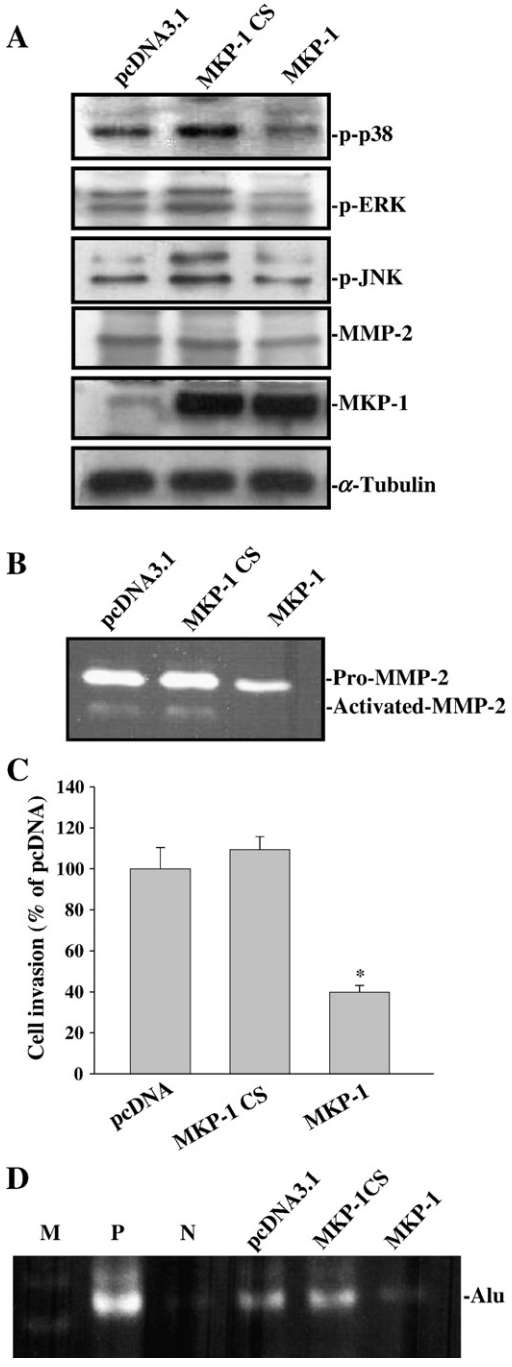

Fig. 3. Roles of MKP-1 in MMP-2 secretion and cell invasiveness in U87MG cells. U87MG cells were stably expressed with either the control vector (pcDNA3.1), dominant negative mutant of MKP-1 (pcDNA3.1/MKP-1CS), or wild-type MKP-1 (pDNA3.1/MKP-1). In (A), cell lysates were immunoblotted with antibodies specific for phosphorylated p38MAPK, ERK, JNK, or MMP-2, MKP-1, α-tubulin. In (B), cultured media were collected for MMP-2 zymography. In (C), cell invasiveness was analyzed by an *in vitro* Matrigel invasion assay. Data are given as the mean±S.E.M. of three independent experiments performed in triplicate. *$P<0.05$. In (D), for *in vivo* intravasation assay, cells (1×10^5) were inoculated onto the dropped chorioallantoic membrane (CAM) with a pipette tip. After inoculation, the CAMs lining the cavity of the eggshell were removed, snap-frozen, and used for extraction of genomic DNA. PCR amplification of the human *Alu* sequence is shown. P, positive control; N, negative control. The experiment was repeated three times, all producing similar results.

secretion, stable expression of MKP-1 (pcDNA/MKP-1) reduced MMP-2 gelatinolytic activity in cultured media (Fig. 3B). Consistently, while the catalytically inactive mutant of MKP-1 did not affect cell invasiveness *in vitro*, over expression of MKP-1 significantly decreased the cell invasiveness ($P < 0.001$) (Fig. 3C). To confirm that MKP-1 may regulate glioma cell invasiveness *in vivo*, a cell intravasation assay using the chicken embryal chorioallantoic membrane (CAM) was used. Chicken embryos incubated at day 10 were inoculated with 1×10^5 cells and then incubated for additional 4 days. The intravasation of tumor cells was demonstrated by PCR for the human *Alu* sequence of the lower CAM. As shown in Fig. 3D, cells that overexpressed MKP-1 failed to intravasate into the CAM. However, the human *Alu* sequence was readily detected by PCR in the CAM of embryos that were inoculated with control U87MG cells or U87MG cells that expressed a dominant negative MKP-1 construct.

3.4. Roles of nitric oxide in regulating MMP-2 secretion and cell invasiveness

Overexpression of inducible nitric oxide synthase (iNOS) has been implicated in the malignancy of human gliomas. To determine whether iNOS overexpression and NO overproduction play important roles in the invasiveness of the malignant glioma tumors, we first evaluated the localization and expression levels of iNOS in tissue sections of typical high-grade astrocytomas and benign brain tumor (Fig. 4A). The immuno-staining of iNOS was seen in astrocytoma cells and endothelial cells of intratumoral vessels, but not in a benign brain tumor. We next examine whether NO^- production is linked to MMP-2 activity in the gliomas. Human U87MG glioma cells were treated with a NO^- donor, sodium nitroprusside (SNP), and the activity of MMP-2 in media examined. SNP increased the gelatinolytic activity in cultured media (Fig. 4B) and MMP-2 expression in U87MG cells (Fig. 4C). These results suggested that NO^- is a positive regulator of MMP-2 secretion. Furthermore, SNP increases cell invasiveness in the U87MG glioma cells (Fig. 4D).

3.5. Dexamethasone inhibits NO^- induced MMP-2 secretion and cell invasiveness

To determine whether dexamethasone inhibited SNP-induced MMP-2 secretion, cells were pretreated with dexamethasone for 30min before SNP were added. Treatment of glioma cells with

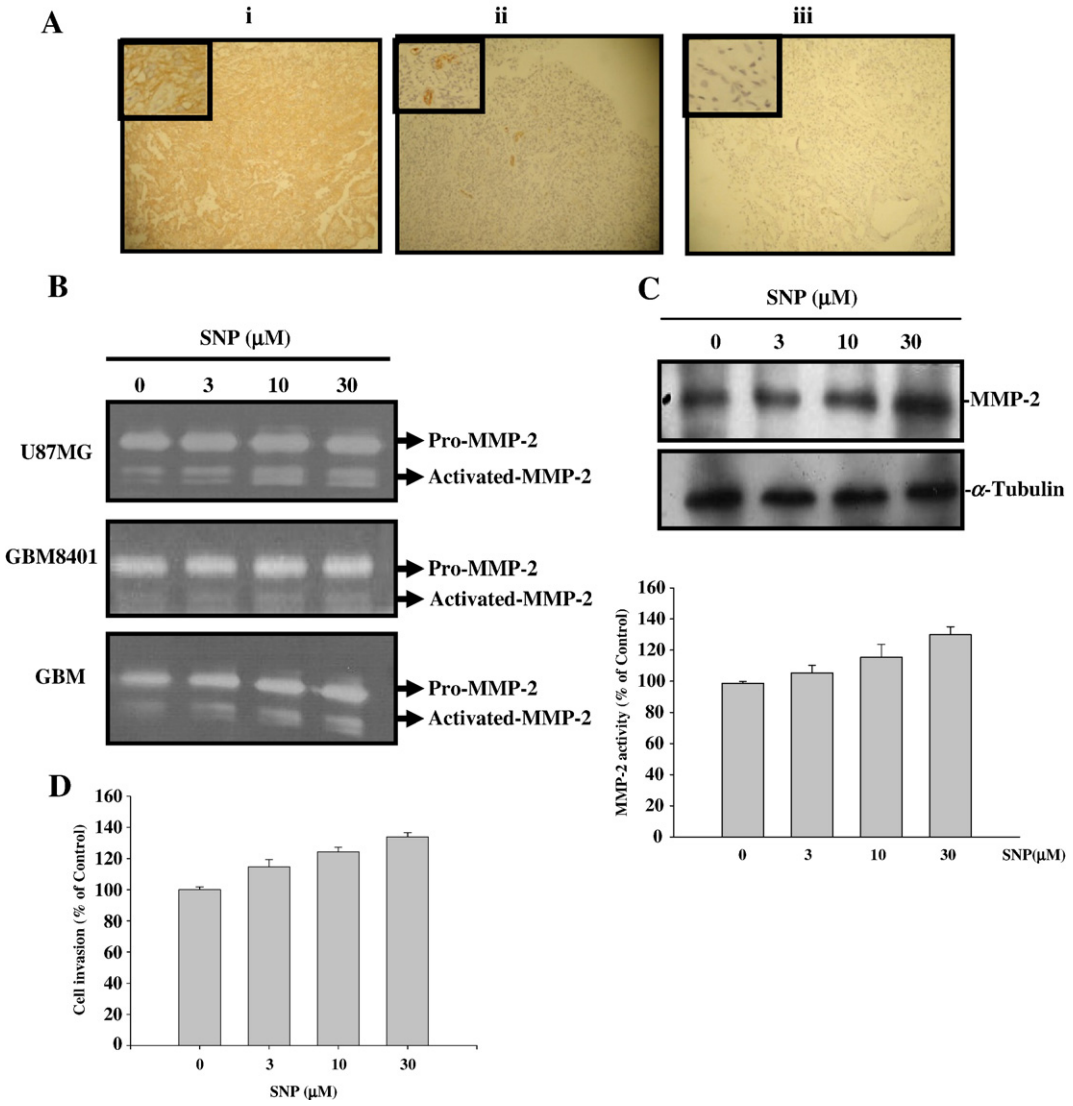

Fig. 4. NO^- regulated matrix metalloproteinase-2 (MMP-2) activity in glioma cells. In (A), immunohistochemistry of iNOS in a high-grade astrocytomas (i, ii), and meningioma (iii). Strong positive staining was noted in the high-grade category (original magnification, ×100). In (B), (C), and (D), U87MG cells were treated with the NO^- donor, sodium nitroprusside (SNP), for 24 h. In (B), cultured media were collected and MMP-2 activities were detected by zymography. In (C), cell lysates were immunoblotted with antibodies specific for MMP-2 or α-tubulin. In (D), U87MG cells were treated with differential concentrations of SNP for 24 h, and cell invasiveness was analyzed by an *in vitro* Matrigel invasion assay.

dexamethasone attenuated SNP-induced MMP-2 secretion (Fig. 5A) and cell invasiveness in U87MG cells (Fig. 5B). To confirm that these effects were mediated through MKP-1, U87MG cells were transfected with wild-type MKP-1 (pcDNA/MKP-1) or empty vector (pcDNA3.1) and the MAPK phosphorylation, MMP-2 expression and cell invasiveness were compared. Fig. 5C shows overexpression of MKP-1 reduces SNP-stimulated MAPKs phosphorylation and MMP-2 expression in U87MG cells. The ectopic expression of MKP-1 also reduced MMP-2 secretion in the media (Fig. 5D) and cell invasiveness in U87MG cells *in vitro* (Fig. 5E). These data suggested that MKP-1 may also play a role in regulating of NO^- induced glioma cell invasion.

4. Discussion

In the present study, we demonstrated that human glioma cells constitutively secreted MMP-2 in the media, and this effect was

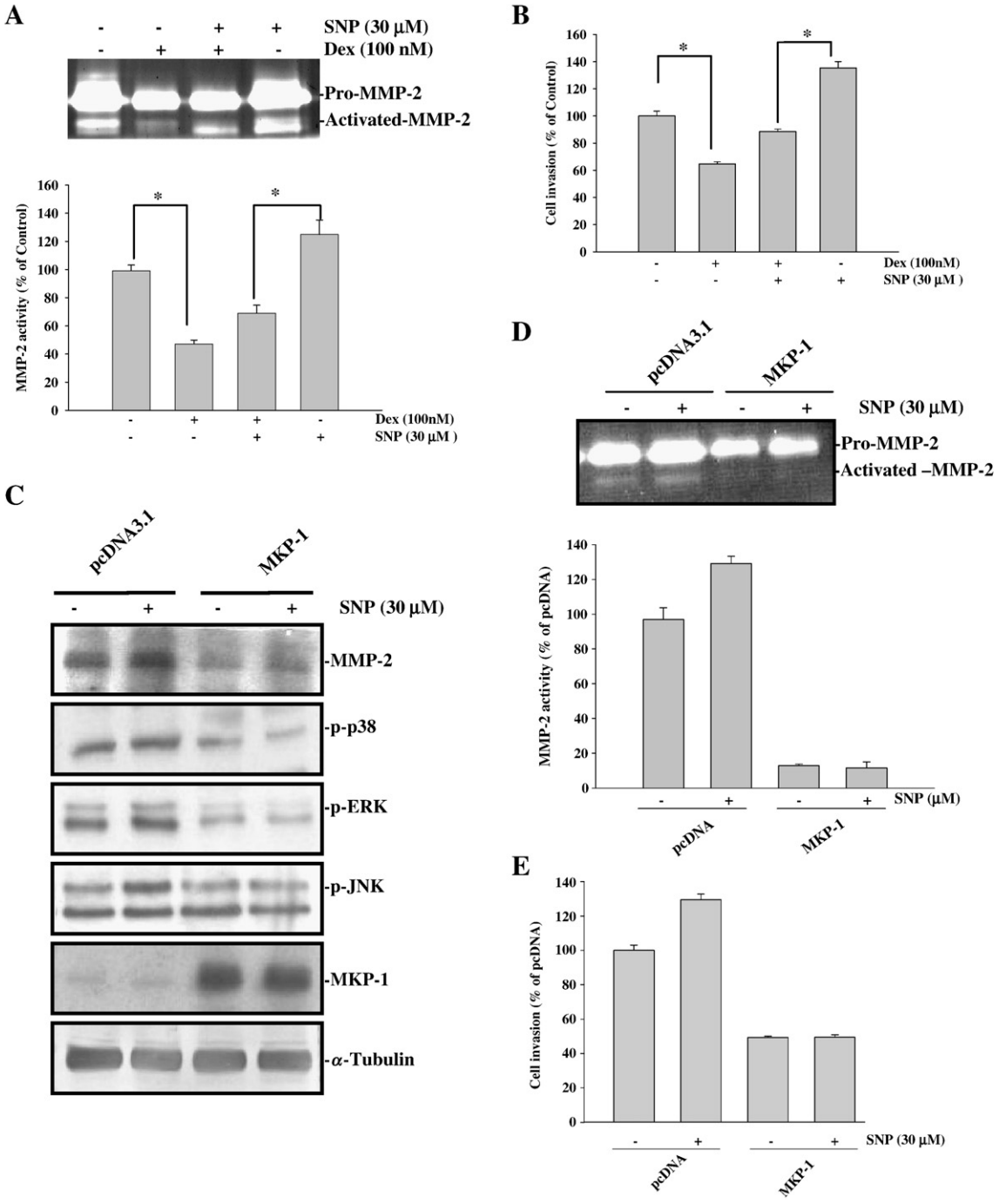

Fig. 5. Effects of dexamethasone (Dex) on SNP-induced MMP-2 secretion and cell invasion. In (A), U87MG cells were pretreated with dexamethasone (100 nM) for 30 min before incubation with SNP (30 μM) for 24 h. Media were collected for gelatinolytic zymography. In the lower panel, the MMP-2-relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system. Mean photographic density ± S.E.M. of three independent experiments is shown. *$P<0.05$. In (B), cell invasiveness was analyzed by an *in vitro* Matrigel invasion assay. Data are given as the mean ± S.E.M. of three independent experiments performed in triplicate. *$P<0.05$. In (C), U87MG cells were either expressed with control vector pcDNA3.1 or pDNA3.1/MKP-1, and cells were treated with SNP (30 μM) for 24 h. Total lysates were prepared and subjected to Western analysis probing with anti-MMP-2, MKP-1, or α-tubulin. In (D), cultured media were collected for MMP-2 zymography. In the lower panel, the activated MMP-2 relative photographic density was quantitated by scanning the photographic negatives on a gel analysis system. Mean photographic density ± S.E.M. of three independent experiments is shown. In (E), cell invasiveness was analyzed by an *in vitro* Matrigel invasion assay. Data are given as the means ± S.E.M. of three independent experiments performed in triplicate.

inhibited by pharmacological inhibitors specific for ERK, p38MAPK and JNK. We present evidences that dexamethasone may suppress MMP-2 secretion and cell invasiveness through glucocorticoid receptor-dependent MKP-1 induction. This is supported by the facts that ectopic expression of MKP-1 suppresses gelatinolytic activities in cultured media, the MMP-2 protein expression and cell invasiveness in U87MG cells. The *in vitro* effect was further confirmed by an *in vivo* chicken embryo CAM model. Additionally, we show that increase of iNOS expression may play a role in regulating MMP-2 secretion and cell invasiveness in human glioma cells. We demonstrated that treatment of glioma cells with the NO^- donor, SNP, increase MMP-2 secretion and cell invasiveness in human glioma cells, and these effects were blocked by overexpression of MKP-1 in U87MG cells. These findings suggest that the induction of MKP-1 may serve as a therapeutic target in human malignant gliomas.

Steroidal anti-inflammatory drugs such as dexamethasone are frequently used to reduce brain edema and may exert beneficial effects on decreasing cell invasiveness in glioma (Vedoy and Sogayar, 2002). Consistently, we demonstrated that dexamethasone decreases MMP-2 secretion and cell invasiveness in human glioma cells. MMP-2 secretion is regulated by many different factors. Among these factors, MAPK pathways have been implicated in regulating MMP-2 expression in cancer cells (Mook et al., 2004) including human glioma cells (Park et al., 2002). MAPKs may regulate MMP-2 expression by controlling the transcription initiation of MMP-2 genes. There are many *cis*-acting regulatory elements including cAMP-response element-binding protein (CREB), p53, Ets-1, CCAAT/enhancer-binding protein (C/EBP), Sp1 and AP-1 sites in the upstream control region of MMP-2 gene (Mook et al., 2004). In the present study, we show that dexamethasone may suppress MMP-2 secretion via MKP-1 induction. MKP-1 is a negative regulator of extracellular signal-regulated kinase (ERK), JNK, and p38 kinases (Duff et al., 1995; Engelbrecht et al., 2003; Gupta et al., 1996; Keyse and Emslie, 1992; Lai et al., 1996; Liu et al., 1995; Wadgaonkar et al., 2004). The role of MKP-1 was further confirmed by the facts that the over expression of MKP-1 in human glioma cells suppresses MMP-2 activity and cell invasiveness in U87MG cells. Because intracellular MAPK signal transduction networks are interconnected, inhibition of one of the MAPK signal transduction pathways may not be enough to suppress the MAPKs-mediated effects. Induction of MKP-1 regulates ERK, JNK, and p38 MAPK pathways by triggering a phosphatase cascade, which may be more promising therapeutic strategies than using individual MAPK inhibitors to treat malignant gliomas.

The role of nitric oxide (NO^-) in tumor biology remains controversial and poorly understood. While many studies have demonstrated that NO^- is detrimental in tumor cells, and consequently, their metastatic ability, evidence indicates that NO^- is correlated with angiogenesis and tumor invasion (Ekmekcioglu et al., 2005). The expression level of iNOS has been correlated with angiogenesis and prognostic significance with glioma grade (Hara and Okayasu, 2004). In the present study, we show that iNOS is strongly expressed in high-grade glioma cells but not in a benign brain tumor. These data suggest that iNOS may play a role in glioma progression. We demonstrated that treatment of glioma cells with SNP, an NO^- donor, increased MMP-2 secretion, suggesting that NO^- is a positive regulator of MMP-2 in malignant gliomas. Our results are in line with those reported by Chen et al. (2005), in which they demonstrated that the gelatinolytic activity was dramatically reduced in the liver of iNOS (−/−) mice. These results were also consistent with that of Pfeilschifter et al. (2001) showing MMP-2 is under the control of nitric oxide.

In conclusion, our results suggest that dexamethasone may suppress MMP-2 secretion and cell invasion in U87MG cells through MKP-1 induction. Because NO^- induces MMP-2 secretion and cell invasion, our data support the notion that iNOS up-regulation and nitric oxide overproduction contribute to cell invasion. Taken together, these data suggest that dexamethasone may exert the anti-invasive effect by inhibiting nitric oxide-induced MMP-2 secretion and thus invasion in human gliomas.

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