

S-Adenosylhomocysteine Promotes the Invasion of C6 Glioma Cells via Increased Secretion of Matrix Metalloproteinase-2 in Murine Microglial BV2 Cells

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S-Adenosylhomocysteine (SAH) is a risk factor for many diseases, including tumor progression and neurodegenerative disease. In this study, we examined the hypothesis that SAH may indirectly enhance the invasion of C6 glioma cells by induction of matrix metalloproteinase-2 (MMP-2) secreted from the murine microglia BV2 cells. We obtained conditioned medium (CM) by incubating BV2 cells with SAH (1–50nM) for 24 h. We found that the SAH-containing CM (SAH-BV2-CM) strongly enhanced the invasiveness of C6 glioma cells and that this effect increased with increasing concentrations of SAH in the SAH-BV2-CM. The effect of CM could be attributed to its MMP-2 activity, as a result of increased protein and messenger RNA expression of MMP-2 in BV2 cells induced by SAH. In BV2 cells treated with SAH, the binding abilities of nuclear factor-kappa B (NF- κ B) and stimulatory protein-1 (Sp1) to the MMP-2 promoter were increased, whereas the level of NF- κ B inhibitor was decreased. In addition, SAH significantly increased the phosphorylation of extracellular signal-regulated kinase (ERK) and phosphatidylinositol-3-kinase/serine/threonine protein kinase (or protein kinase B) (PI3K/Akt) proteins but did not affect that of c-Jun NH2-terminal kinase or p38. Pretreatment of BV2 cells with an inhibitor specific for ERK (U0126) markedly abated the expression of ERK and MMP-2. Furthermore, SAH significantly and dose dependently decreased tissue inhibitor of metalloproteinase-2 (TIMP-2) in BV2 cells. Thus, SAH may induce the invasiveness of C6 glioma cells by decreased TIMP-2 expression and increased MMP-2 expression in BV2 cells. The latter effect is likely mediated through the ERK and PI3K/Akt pathways, with increased binding activities of NF- κ B and Sp1 to the MMP-2 gene promoter.

Key Words: S-Adenosylhomocysteine; invasion; MMP-2.

Glioma represents the most frequent type of human brain tumor, and its strong invasiveness is a major clinical problem. Microglia, the immunocompetent cells of the brain, contribute

significantly to the tumor and are potent interaction partners of the glioma cells. Activated microglia release many factors, including cytokines and proteases, which directly or indirectly influence tumor progression (Watters *et al.*, 2005). Recent studies demonstrated a strong ability of microglial cells to enhance tumor invasion in cell cocultures and brain organotypic slice cultures (Markovic *et al.*, 2005).

Matrix degradation by metalloproteinases (MMPs) is a key feature in mediating the malignant characteristics of gliomas (Rao, 2003). In human brain tissues, the activity of MMP-2 and MMP-9, especially MMP-2, has been associated with the malignancy of brain tumors (Yamamoto *et al.*, 1996). MMP-2 activity is regulated at a posttranslational level through interaction with tissue inhibitor of metalloproteinase-2 (TIMP-2; Zhang *et al.*, 2004), and signal-dependent activation and repression of gene transcription is the principal level of control for several MMP genes (Yan and Boyd, 2007). The mitogen-activated protein kinases (MAPK) play an important regulatory role in cell growth, differentiation, apoptosis, and metastasis (Chan-Hui and Weaver, 1998). In addition, phosphatidylinositol-3-kinase/serine/threonine protein kinase (or protein kinase B) (PI3K/Akt) signal transduction pathway is involved in the development, progression, and metastasis of various tumors (Liu *et al.*, 2009).

S-Adenosylhomocysteine (SAH), which forms homocysteine (Hcy) reversibly by SAH hydrolase, is a key intermediate in the metabolism of the amino acid methionine (Finkelstein, 2000). SAH can accumulate in liver, pancreas, kidney, brain, and intestine (Hoffman *et al.*, 1980) and is highly correlated with Hcy-related disease (Kerins *et al.*, 2001). Importantly, SAH has been suggested to be a pivotal biomarker of cardiovascular (Kerins *et al.*, 2001; Wagner and Koury, 2007), chronic renal (Fu *et al.*, 2000; Valli *et al.*, 2008), Alzheimer's (Kennedy *et al.*, 2004), and vascular diseases. However, very little is known whether SAH and Hcy are involved in cancer cell invasion. We previously showed that the invasive ability of different hepatoma cells correlates positively with intracellular levels of SAH (Yang and Hu,

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2006). Interestingly, Hcy has been shown to activate MMP-9 in ischemic stroke, and this important event leads to loss of brain endothelial integrity and macromolecular leakage (Lominadze *et al.*, 2006; Rosell *et al.*, 2006). It is unclear whether SAH may behave similarly Hcy in activating MMPs in disease states such as cancer cell invasion.

It has recently been shown that microglia stimulate the invasiveness of glioma cells by increasing the activity of MMP-2 (Markovic *et al.*, 2005). Glioma cells can form intracranial tumors that characteristically invade surrounding brain, both as mass and as signal cells (Bernstein *et al.*, 1990; Goldberg *et al.*, 1991). In the present study, we hypothesized that SAH may enhance the invasion of C6 glioma cells via the induction of MMP-2 secretion by BV2 cells, a murine microglial cell line, as MMPs have been suggested to contribute to neuropathological diseases, including glioma, stroke, and Alzheimer's disease (Yong *et al.*, 2001). We first incubated BV2 cells with SAH for 24 h to obtain the conditioned medium (CM), which was then used to induce the invasion of C6 glioma cells.

MATERIALS AND METHODS

Chemicals. All chemicals used are of reagent grade. SAH was from Sigma Chemical Co. (St Louis, MO). Dulbecco's minimal essential medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin, streptomycin, sodium pyruvate, nonessential amino acid, and Giemsa stain were from Gibco/BRL (Bethesda, MD) and transwell chambers from Costar (Cambridge, MA). TIMP-2 protein and electrophoretic mobility shift assay (EMSA) kit were purchased from Amersham (Piscataway, NJ) and Pierce Biotechnology (Rockford, IL), respectively. The antibody against Akt, MAPK/extracellular signal-regulated kinase (ERK) 1/2, c-Jun NH2-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK proteins and phosphorylated proteins were purchased from Cell Signaling Technology (Beverly, MA). Anti-MMP-2, anti-ERK2, anti-PI3K, and anti-I κ B- α mouse monoclonal antibody and anti-mouse goat anti-rabbit horseradish peroxidase antibody were purchased from US Biological Co. (Swampscott, MA) and Santa Cruz Biotechnology Co. (Santa Cruz, CA), respectively.

Preparation of CM and culture of C6 glioma. The murine microglial BV2 cells were cultured with SAH (1–50nM) for 24 h, after which the medium was collected and centrifuged at 13,000 \times g. The supernatant was filtered to remove cellular material, and the resulting medium was called conditioned medium (SAH-BV2-CM). SAH-BV2-CM was stored at -20°C before being applied to C6 glioma cells. BV2 and C6 cells were grown in DMEM medium containing 10% (vol/vol) FBS, 0.37% (wt/vol) NaHCO_3 , penicillin (100 U/ml), and streptomycin (100 U/ml) in a humidified incubator under 5% CO_2 and 95% air at 37°C .

Cell invasion assay. Tumor cell invasion was assayed in transwell chambers (Costar) according to the method reported by Repesh (1989), with some modifications. Briefly, transwell chambers (Costar) with 6.5-mm polycarbonate filters of 8- μm pore size were used, and each filter was coated with 100 μl of 1:20 diluted Matrigel in cold DMEM to form a thin continuous film on the top of the filter. The number of C6 glioma cells was adjusted to $5 \times 10^5/\text{ml}$, and a 100- μl aliquot containing 5×10^4 cells was added to each of triplicate wells in DMEM containing 10% FBS. After incubation for 12 h, the cells invading the lower side of the filter were stained with Giemsa and then the number was counted under a microscope. For each replicate, the tumor cells in 10 randomly selected fields were determined and the counts averaged.

Gelatin zymography. MMP-2 activity was assayed using gelatin zymography according to the method reported by Hwang *et al.* (2006), with some

modifications. For the assay of gelatin zymography, the culture medium was electrophoresed in a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel containing 0.1% (wt/vol) gelatin. The gel (MMP-gel) was washed for 30 min at room temperature in a solution containing 2.5% (vol/vol) Triton X-100 with two changes and subsequently transferred to a reaction buffer for enzymatic reaction containing 1% NaN_3 , 10mM CaCl_2 , and 40mM Tris-HCl (pH 8.0) at 37°C with shaking overnight (for 12 and 15 h). Finally, the MMP-gel was stained for 30 min with 0.25% (wt/vol) Coomassie blue in 10% acetic acid (vol/vol) and 50% methanol (vol/vol) and destained in 10% acetic acid (vol/vol) and 50% methanol (vol/vol). The relative MMP-2 activities were quantitated by Matrox Inspector 2.1 software.

Western blot. MMP-2, I κ B- α , ERK2, PI3K, Akt, and TIMP-2 protein levels were assayed by Western blot as described previously (Sampieri *et al.*, 2008). Total cellular proteins were prepared using lysis buffer (Ripa buffer from Sigma Chemical Co.) containing 20% SDS and 1mM PMSF. The lysate was sonicated for 30 s on ice, followed by centrifugation for 30 min at 4°C . Protein levels were determined by Bio-Rad assay (Bio-Rad, Hercules, CA). To each lane of a 12% SDS-PAGE gel, 50 μg protein was added, and the primary and secondary antibodies of MMP-2, I κ B- α , ERK2, PI3K, Akt, and TIMP-2 were diluted 1:1000 for Western blotting. The relative MMP-2, I κ B- α , ERK2, PI3K, Akt, and TIMP-2 levels were quantitated by Matrox Inspector 2.1 software.

Reverse transcription-PCR (RNA isolation and sequencing). Total cellular RNA was isolated from cell culture (RNAzol-kit) and reverse transcribed into complementary DNA (cDNA; MMLV Reverse Transcriptase; Gibco/BRL) using oligo (dT)₁₅ as primers and then co-amplified with four primer bases on MMP-2 and β -actin (internal control) sequences. The primers for amplifying MMP-2 cDNA were as follows: 5'-CACAGCCAACACTACGATGACG-3', located in the 5'-untranslated region, and 5'-CTCCTGAATGCCCTTGATGT-3', located in the 3' untranslated region. The primers for amplifying β -actin cDNA were as follows: 5'-GTGGGGCGCCCCAGG-CACCA-3' and 5'-CTCCTTAATGTACGCACGATTTC-3'. PCR amplification was carried out in a thermal cycler, as follows: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 90 s (31 cycles), followed by a final incubation at 72°C for 7 min. The sizes of the amplification products of MMP-2 and β -actin were 825 and 541 base pairs (bp), respectively. The PCR products were subjected to 1% agarose gel electrophoresis and stained with ethidium bromide. The relative MMP-2 levels were quantitated by Matrox Inspector 2.1 software.

Preparation of nuclear extracts and EMSA. Nuclear protein extracts (5 μg) of BV2 cells incubated with SAH were prepared according to the modified method of a previous study (Chen *et al.*, 1996). Binding activities of transcription factors including nuclear factor-kappa B (NF- κ B), stimulatory protein-1 (Sp1), and activator protein-1 (AP-1) were analyzed by gel mobility shift assays (EMSA) using Lightshift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL), as described previously (Shukla *et al.*, 2005), using double-stranded oligonucleotides based on sequences of NF- κ B (5'-AGTTGAGGGGACTTCCAGGC-3') and Sp1 (5'-ATTCGATCGGGGCGGGGCGAG-3') sites in the MMP-2 promoter or a consensus AP-1 (5'-CGCTTGATGACTCAGCCGGAA-3') site. The relative NF- κ B and Sp1 levels were quantitated by Matrox Inspector 2.1 software.

Chromatin immunoprecipitation assay. Cells were fixed with 1% formaldehyde at 37°C for 10 min, washed twice with ice-cold PBS with protease inhibitors (1mM PMSF, 1 $\mu\text{g}/\text{ml}$ aprotinin, and 1 $\mu\text{g}/\text{ml}$ pepstatin A), and then scraped and pelleted by centrifugation at 4°C . After resuspending in a lysis buffer (1% SDS, 10mM EDTA, and 50mM Tris-HCl, pH 8.1), the cells were incubated for 10 min on ice and sonicated to shear DNA. After sonication, the lysate was centrifuged for 10 min at 16,000 \times g at 4°C . Then, the supernatant was diluted in chromatin immunoprecipitation (ChIP) dilution buffer (0.01% SDS, 1% Triton X-100, 2mM EDTA, 16.7mM Tris-HCl [pH 8.1], 167mM NaCl, and protease inhibitors) to a final volume of 2 ml. Twenty microliters (1%) of the ChIP dilution solution was kept as input control.

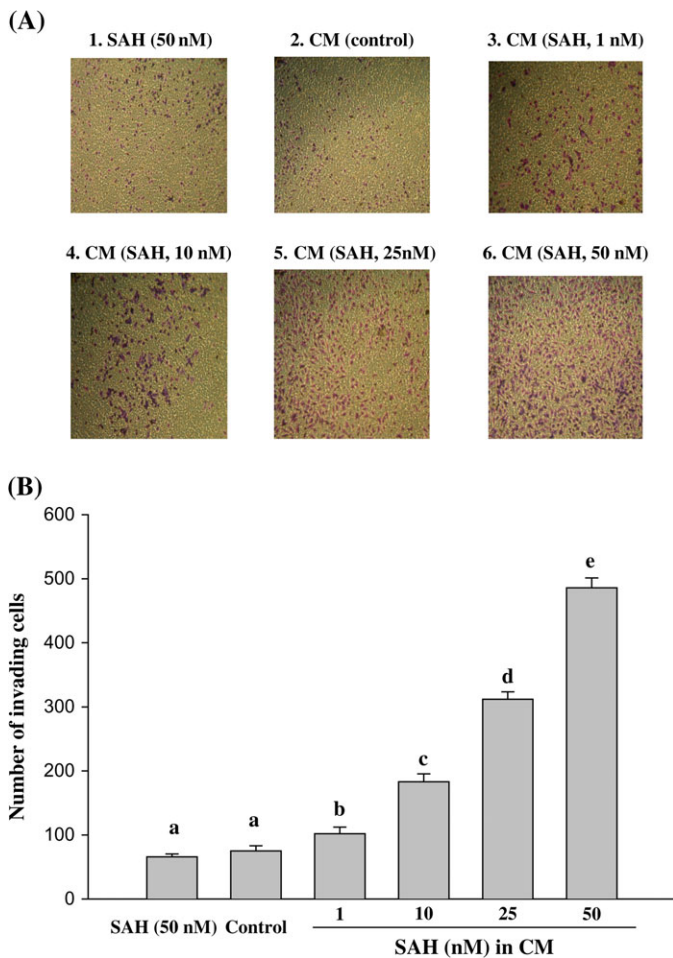


FIG. 1. (A) A representative micrograph of the invasion of C6 glioma cells induced by the CM obtained from incubation of BV2 cells with SAH (1–50nM) for 24 h. Panel 1, SAH (50nM): C6 glioma cells directly incubated with 50nM SAH rather than with CM; panel 2, CM (control): C6 glioma cells incubated with control medium (i.e., CM prepared in the absence of SAH); panels 3–6: CM (SAH 1, 10, 25, and 50nM) represent C6 glioma cells incubated with CM prepared with indicated concentrations of SAH. (B) Number of invading C6 glioma cells measured using the transwell chambers (Costar). Values (means \pm SD, $n = 3$) not sharing an alphabetic letter differ significantly ($p < 0.05$).

Primary antibodies were added and incubated overnight at 4°C with rotation. The immunocomplex was collected by protein A/G agarose beads and washed with a low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 200mM Tris-HCl [pH 8.1], and 150mM NaCl), then with a high-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 200mM Tris-HCl [pH 8.1], and 500mM NaCl), with LiCl washing buffer (0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, and 10mM Tris-HCl, pH 8.1), and finally with 1 \times TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8.0). Then, the immunocomplex was eluted by the elution buffer (1% SDS, 0.1M NaHCO₃, and 200mM NaCl) and the cross-links were reversed by heating at 65°C for 6 h. After the reaction, the samples were adjusted to 10mM EDTA, 20mM Tris-HCl (pH 6.5), and 40 μ g/ml proteinase K and incubated at 45°C for 1 h. DNA was recovered and subjected to PCR amplification of the rat MMP-2 promoter, which contains the SP1 site at the –1433/–1342 bp region compliance with the human promoter. The sequences for the primers were as follows: forward 5'-ATCACTGGCTCTCAACTTGG-3' and reverse 5'-TTAGCTGCAAG-GAGTCTCTT-3'. The predicted size for the PCR product was 250 bp and

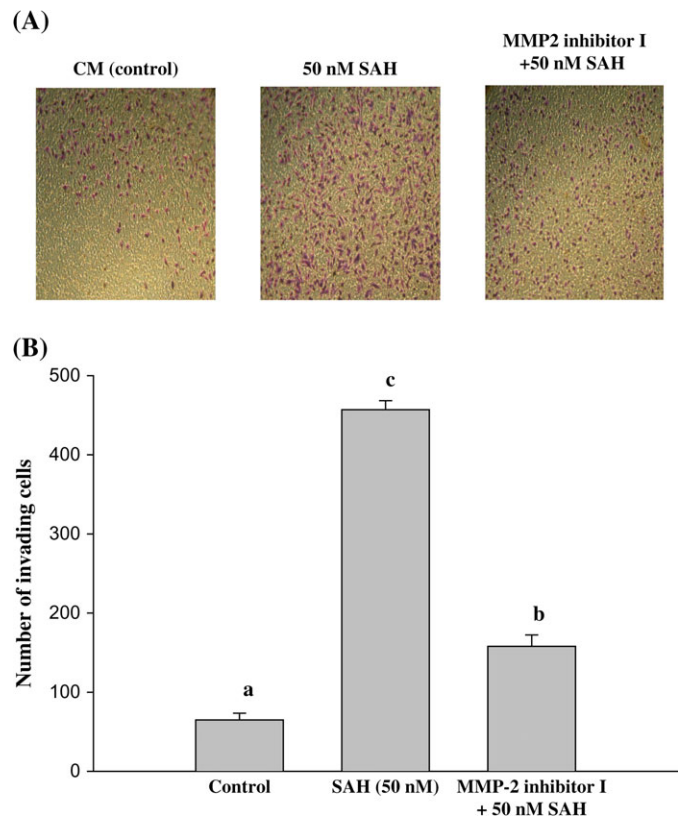


FIG. 2. (A) A representative micrograph of the invading C6 glioma cells induced by the control CM or the medium obtained from the incubation of BV2 cells with 50nM SAH. A third group of cells were preincubated with MMP-2 inhibitor I (30 μ M) for 30 min before incubation with 50nM SAH for 24 h. (B) Number of invading C6 glioma cells measured using the transwell chambers (Costar). Values (means \pm SD, $n = 3$) not sharing an alphabetic letter differ significantly ($p < 0.05$).

analyzed by agarose gel electrophoresis, followed by quantification using Matrox Inspector 2.1 software.

Statistical analysis. Values are expressed as means \pm SD and analyzed using one-way ANOVA, followed by Duncan's multiple range test for comparisons of group means. The statistical analysis was carried out using SPSS for Windows, version 10 (SPSS, Inc., Chicago, IL); $p < 0.05$ was considered statistically significant.

RESULTS

SAH-BV2-CM Enhanced the Invasiveness of C6 Glioma Cells

We first incubated C6 glioma cells directly with SAH (1–50nM) for 24 h and found that SAH added at a concentration up to 50nM did not affect the invasiveness of C6 glioma cells (Fig. 1A, panel 1). To investigate whether SAH may affect C6 glioma cell invasiveness indirectly through the secretion of BV2 cells, we incubated C6 glioma cells with the CM of BV2 cells after incubation with SAH for 24 h (SAH-BV2-CM). We found that SAH-BV2-CM increased C6 glioma cell invasiveness in a manner that was proportional to the concentration of

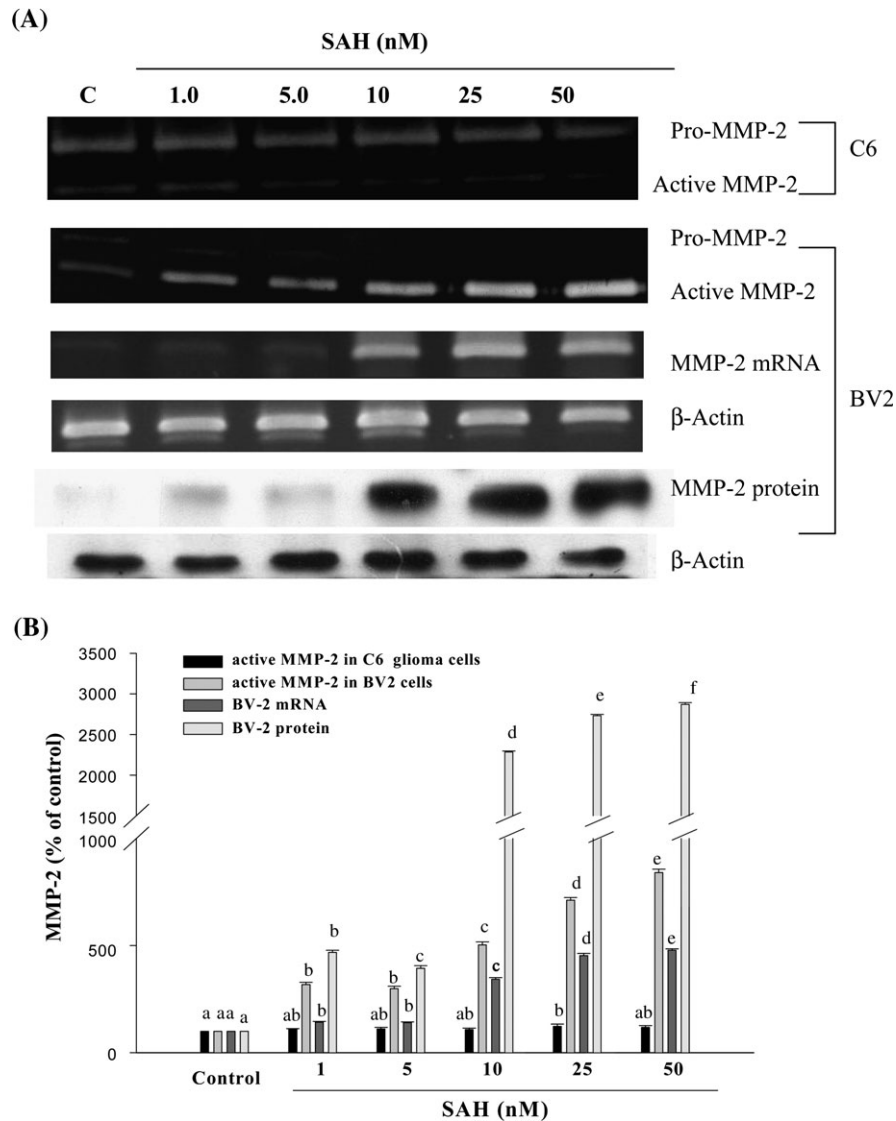


FIG. 3. Effects of SAH on MMP-2 activity and MMP-2 protein and mRNA expression in BV2 cells and C6 glioma cells (C6). (A) MMP-2 activity (gelatin zymography), protein expression (Western blotting), and mRNA expression (RT-PCR). (B) Densitometric analysis of A. Values (means ± SD, n = 3) of individual items (columns) not sharing an alphabetic letter are significantly different (p < 0.05).

SAH (Fig. 1A, panels 2–6). The number of invading C6 glioma cells treated with SAH-BV2-CM obtained from 50nM SAH (330 cells/transwell) was six times that of the control group (55 cells/transwell; Fig. 1B).

We then used an MMP-2 inhibitor to confirm that the MMP-2 from BV2 cells is involved in the invasion of C6 glioma cells. The BV2 cells were preincubated with MMP-2 inhibitor I (30µM in control CM) for 30 min, followed by incubation with SAH (50nM in BV2-CM) for 24 h. We found that the MMP-2 inhibitor significantly decreased the invasiveness of C6 glioma cells (Fig. 2A). The number of invading C6 glioma cells treated with 50nM SAH (480 cells/transwell) was three times that pretreated with MMP-2 inhibitor I (160 cells/transwell; Fig. 2B). However, the number of invading cells in the MMP-2 inhibitor I

pretreated group was still significantly higher than that of the control, although the concentration ratio of MMP-2 inhibitor I to SAH was exceedingly high (30µM/50nM = 600).

SAH Enhanced MMP-2 Expression at Both the Protein and the Messenger RNA Levels in BV2 Cells

The gelatin zymographic assay was used to determine the activity of MMP-2 in both C6 glioma cells and BV2 cells (Fig. 3A). We showed that C6 glioma cells expressed mostly pro-MMP-2 protein (72 kDa) and little active MMP-2 (62 kDa) and that SAH did not significantly affect the level of active MMP-2 in C6 glioma cells (Fig. 2B). By contrast, SAH significantly and dose dependently increased the active MMP-2 in BV2 cells, with an 8.5-fold increase at 50nM SAH (Fig. 3B). We then

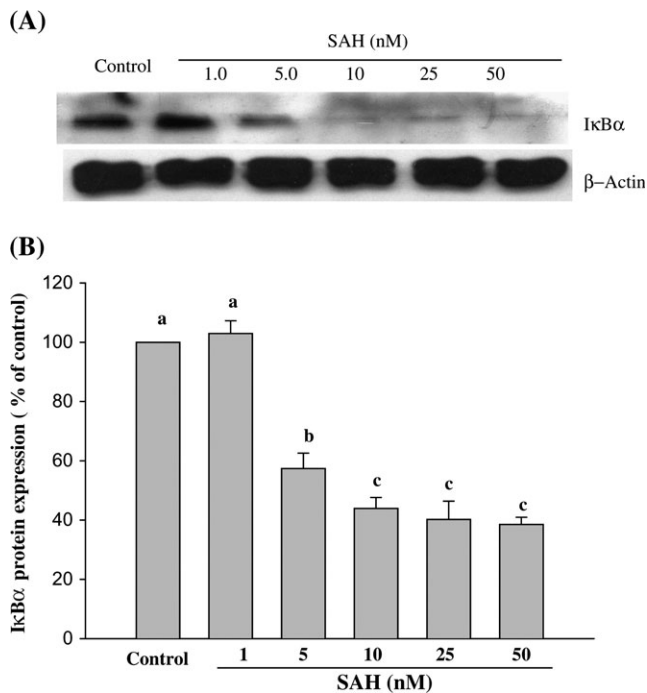


FIG. 4. Levels of IκB-α protein in BV2 cells (1×10^5 cells/ml) incubated with SAH for 24 h. (A) Western blots of IκB-α and β-actin. (B) Densitometric analysis of A. Values (means \pm SD, $n = 3$) not sharing an alphabetic letter are significantly different ($p < 0.05$).

analyzed the protein and messenger RNA (mRNA) expression of MMP-2 in BV2 cells (Fig. 3A) and found that SAH significantly and dose dependently increased the expressions of MMP-2 mRNA and protein. The increase in protein expression was particularly strong when the SAH concentration reached 10nM (Fig. 3B).

SAH Inhibited IκB-α Protein Levels and Enhanced the Binding Activities of NF-κB and Sp1 in BV2 Cells

To examine the mechanisms for the effect of SAH on MMP-2 in BV2 cells, we determined the protein levels of IκB-α and the binding activities of NF-κB and Sp1 in BV2 cells. We found that the expression of IκB-α protein in BV2 cells was significantly inhibited by SAH in a dose-dependent manner after incubation for 24 h (Fig. 4A). An half maximal inhibitory concentration (IC_{50}) of approximately 7.8nM SAH was obtained for the inhibition of IκB-α protein levels by SAH (Fig. 4B).

The binding activities of NF-κB and Sp1 were increased in BV2 cells incubated with SAH for 24 h, and the effect of SAH was concentration dependent (Fig. 5A). The levels of NF-κB and Sp1 in BV2 cells incubated with 50nM SAH were approximately 208 and 724%, respectively, of the control level (Fig. 5B). By contrast, SAH had little or no effect on AP-1-binding activity (data not shown).

We then used ChIP assay to specifically target the Sp1-binding region of MMP-2 promoter in BV2 cells. As shown in

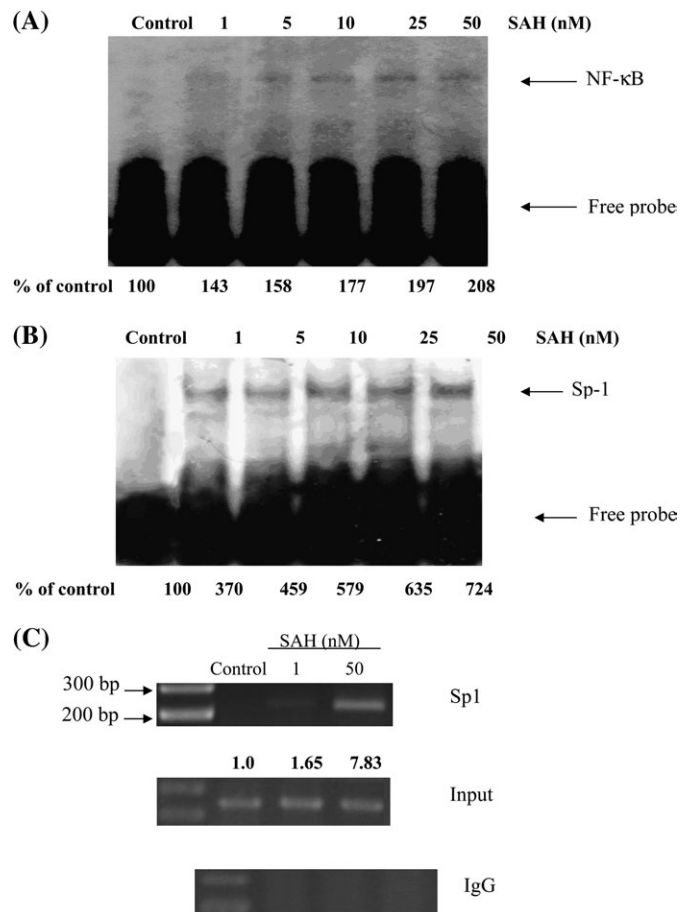


FIG. 5. Induction of SAH on nuclear-binding activities of NF-κB (A) and Sp1 (B). Nuclear extracts were prepared from BV2 cells treated with SAH for 24 h, and the binding activities were analyzed using gel mobility shift assay. (C) The ChIP assay of the targeted binding of Sp1 to the MMP-2 promoter-binding site. The ChIP dilution solution (20 μ l) was kept as input control, and IgG served as the negative control.

Figure 5C, incubation of BV2 cells with 50nM SAH for 24 h resulted in a 7.83-fold increase in Sp1 binding to Sp1 sites.

SAH Enhanced ERK and PI3K/Akt Signaling Pathway in BV2 Cells

To further elucidate the signaling pathways for the effect of SAH in BV2 cells, we determined MAPK and PI3K/Akt signaling proteins and their phosphorylated protein levels. The Western blot analysis showed that SAH markedly and concentration dependently increased the levels of PI3K, p-Akt, and p-ERK at 6 h after incubation (Fig. 6A), with 2.0-, 4.5-, and 7-fold increases respectively (Fig. 6B), at 50nM SAH. These effects of SAH weakened markedly at 24 h after incubation (data not shown). However, SAH had little or no effect on the levels of total Akt and ERK (Fig. 6A, only the Western blots are shown). We further showed that SAH affected neither the protein expression of JNK and p38 nor the phosphorylated forms of these proteins (data not shown). Thus,

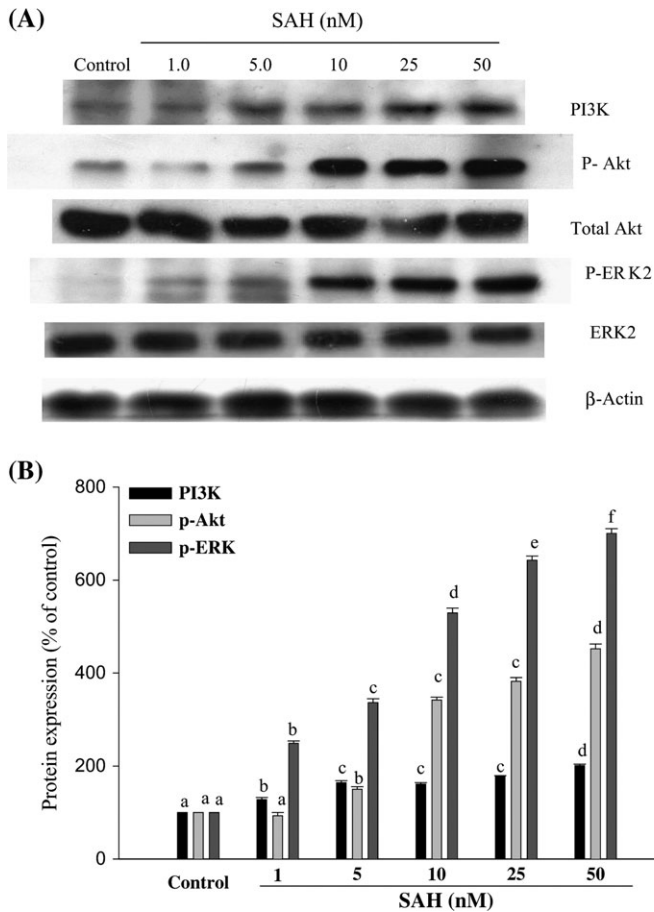


FIG. 6. Levels of PI3K, Akt, and ERK proteins and the phosphorylated forms (p-Akt and p-ERK) in BV2 cells (1×10^5 cells/ml) incubated with SAH for 6 h. (A) Western blots of ERK, p-ERK, PI3K, and p-PI3K proteins. (B) Densitometric analysis of A. Values (means \pm SD, $n = 3$) of the individual protein (PI3K, p-Akt, or p-ERK) not sharing an alphabetic letter are significantly different ($p < 0.05$).

among the three major mammalian MAPKs, only ERK was affected by SAH (via enhancing the phosphorylation of ERK).

We then used an ERK inhibitor (U0126) to confirm that the ERK signaling pathway is involved in the enhancement of MMP-2 in BV2 cells by SAH. We determined the protein expression of p-ERK and MMP-2 in BV2 cells preincubated with U0126 for 30 min, followed by incubation with SAH (50nM) for 6 h (for p-ERK expression) or 24 h (for MMP-2 expression; Fig. 7A). As shown in Figure 6B, the level of ERK in the 50nM SAH + U0126 group (230% of control) was substantially lower than that of the 50nM SAH group (650% of control). Similarly, the level of MMP-2 in the 50nM SAH + U0126 group (105% of control) was markedly lower than that of the 50nM SAH treatment (530% of control; Fig. 7B).

SAH Inhibited TIMP-2 Protein Levels in BV2 Cells

To determine whether SAH may also affect the level of TIMP-2 in BV2 cells, we used Western blot analysis to

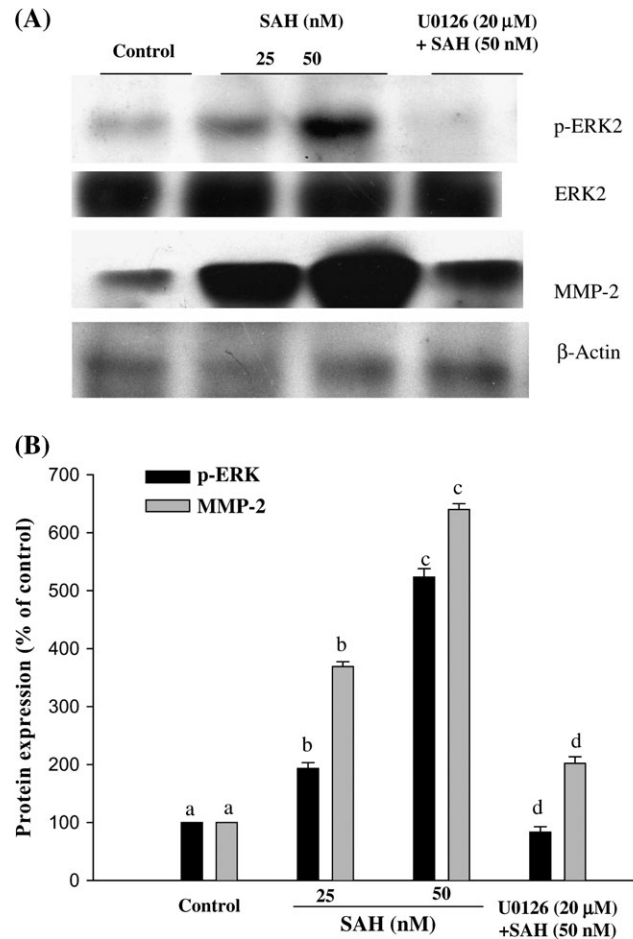


FIG. 7. Effect of ERK inhibitor (U0126) on the activities of MMP-2 and ERK in BV2 cells. The cells were seeded in a six-well plate and preincubated with U0126 (20 μ M) for 30 min, followed by incubation with SAH (50nM) for either 6 h (ERK) or 24h (MMP-2). (A) Western blots of ERK and MMP-2. (B) Densitometric analysis of A. Values (means \pm SD, $n = 3$) of individual protein (ERK or MMP-2) not sharing an alphabetic letter are significantly different ($p < 0.05$).

measure the level of TIMP-2 in BV2 cells incubated with SAH (1–50nM) for 24 h (Fig. 8). We found that SAH markedly decreased the level of TIMP-2 protein in BV2 cells (Fig. 8A), and the effect of SAH was concentration dependent, with an IC_{50} of approximately 7.5nM (Fig. 8B).

DISCUSSION

In the present study, we examined our hypothesis that SAH may induce the invasiveness of C6 glioma cells via increased secretion of MMP-2 in BV2 cells. We showed that the invasiveness of C6 glioma cells incubated with the CM obtained from the incubation of BV2 cells with SAH (SAH-BV2-CM) increased strongly and proportionally to increased SAH concentration (1–50nM). By contrast, direct incubation of C6 glioma cells with SAH (50nM) did not affect the

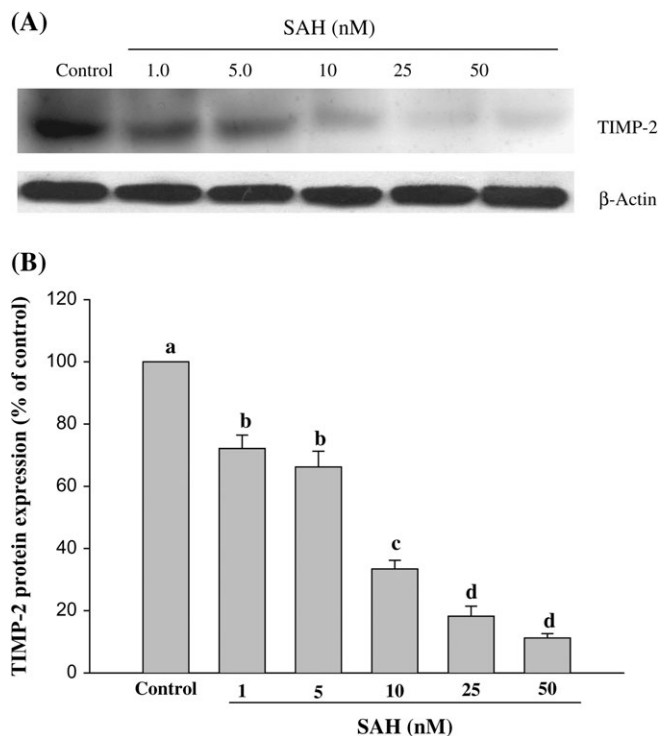


FIG. 8. Levels of TIMP-2 in BV2 cells (1×10^5 cells/ml) incubated with SAH for 24 h. (A) Western blots of TIMP-2. (B) Densitometric analysis of A. Values (means \pm SD, $n = 3$) not sharing an alphabetic letter are significantly different ($p < 0.05$).

invasiveness of C6 glioma cells. The concentrations of SAH (1–50nM) used in the present study are comparable with those of human plasma levels (~ 0.02 – $0.04\mu\text{M}$). In fact, plasma SAH concentrations can increase markedly in Hcy-related diseases (James *et al.*, 2002; Yi *et al.*, 2000).

Matrix degradation by MMPs is a key feature in mediating the malignant characteristics of gliomas (Rao, 2003). Microglia may secrete many factors including cytokines and proteases that directly or indirectly influence tumor progression (Watters *et al.*, 2005). Moreover, microglia have been shown to stimulate the invasiveness of glioma cell lines by increasing the activity of MMP-2 (Markovic *et al.*, 2005). In the present study, we showed that SAH slightly inhibited MMP-2 activity in C6 glioma cells, whereas it strongly and dose dependently upregulated the expression of MMP-2 mRNA and protein in BV2 cells. It should be noted that very little MMP-9 activity was detected in BV2 cells and that the effect of SAH was not evident. The elevated active MMP-2 enzyme in BV2 cells, which was clearly demonstrated by gelatin zymographic assay, was then released into the medium and, in turn, enhanced the invasiveness of C6 glioma cells. The present findings confirmed that C6 glioma cells express relatively large amounts of soluble pro-MMP-2 (Fig. 3, lane 1), especially at the invading front (Guo *et al.*, 2005; Tews, 2000). However, these cells are not able to efficiently convert the pro-MMP-2 into the

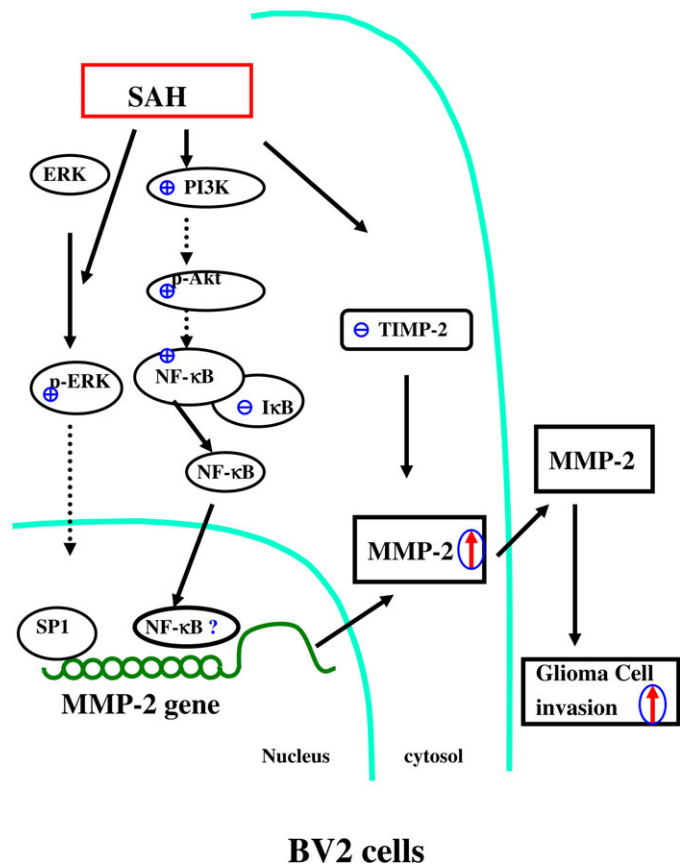


FIG. 9. Proposed mechanisms by which SAH increases the expression and activity of MMP-2 in BV2 cells: promotion and inhibition. The question mark alongside of NF- κ B denotes uncertainty of its binding to the MMP-2 promoter-binding site.

active form (Fig. 3, lane 1), as a result of insufficient expression of membrane-type matrix MMP-1 (Deryugina *et al.*, 1997). Thus, it is clear that the MMP-2 required for C6 glioma cell invasion is essentially contributed from BV2 cells upon activation by SAH. However, it cannot be excluded that BV2 cells may secrete some unknown factors that are involved in C6 cell invasion. This is implicated by our results that pretreatment of BV2 cells with a high concentration of MMP-2 inhibitor I ($30\mu\text{M}$) followed by incubation with SAH (50nM) markedly, but did not completely, inhibit C6 glioma cell invasiveness.

We also have studied the possible mechanism underlying the enhancing effect of SAH on MMP-2 expression in BV2 cells (Fig. 9). The 5' flanking region of the MMP-2 gene contains several function regulatory motifs that interact with well-characterized transcription factors, including NF- κ B, AP-1, and Sp1 (Sato *et al.*, 1993; Takahra *et al.*, 2004). NF- κ B, which is normally bound to its inhibitor I κ B, is located in the cytoplasm, but when dissociated from I κ B, it moves into the nucleus and promotes cancer cell proliferation, angiogenesis, and metastasis. One of the main upstream regulators of Sp1 is

the ERKs, and this signaling pathway also plays an important role in enhancing cell migration and invasion. In the present study, we showed that SAH inhibited the expression of I κ B and enhanced the phosphorylation of Akt (through increased expression of PI3K) and ERK in BV2 cells. These coordinated actions of SAH resulted in increased NF- κ B and Sp1 nuclear-binding activity and led to increased levels of MMP-2 in BV2 cells.

The EMSA provided evidence for nonspecific nuclear-binding activity of NF- κ B and Sp1 to the MMP-2 promoter in BV2 cells. We then further demonstrated the specific binding of Sp1 to the MMP-2 promoter-binding site in BV2 cells by ChIP assay. However, we did not directly demonstrate the actual binding of NF- κ B to the MMP-2 promoter-binding site in BV2 cells. This is because the MMP-2 promoter does not contain binding sites for NF- κ B, although it has been reported that MMP-2 activation occurs in tumor cells through an AP-1- or NF- κ B-dependent pathway (Bergman *et al.*, 2003; Philip and Kundu, 2003). Interestingly, several genes, such as those for granulocyte-macrophage colony-stimulating factor (Schreck and Baeuerle, 1990) and tumor necrosis factor- α (Collart *et al.*, 1990), have been shown to be regulated through NF- κ B-like-binding sites. It is presently unclear whether there is an NF- κ B-like-binding site on the promoter of MMP-2.

By contrast, we observed that SAH had little or no effect on AP-1 nuclear-binding activity and that SAH affected neither the expression of JNK and p38 nor the phosphorylation of these proteins (data not shown). Thus, among the three major mammalian MAPKs (ERK, JNK, and p38), only the ERK signaling pathway is affected by SAH in BV2 cells. The involvement of ERK pathway was confirmed by our finding that pretreatment of BV2 cells with the ERK inhibitor U0126 markedly decreased SAH-induced expression of both p-ERK and MMP-2 proteins (Fig. 6). Indeed, the importance of ERK and PI3K/Akt in mediating the invasiveness of bladder, renal, and colon cancer cells has been demonstrated (Kotelevets *et al.*, 1998; Theodorescu *et al.*, 1998). Interestingly, Moshal *et al.* (2006) have demonstrated that homocysteine-mediated MMP-9 induction is regulated by the ERK1/2 signaling pathways in cultured human heart microvascular endothelial cells. It remains to be investigated as to whether the effects of homocysteine and SAH are related to each other.

Another important mechanism for the regulation of the activity of MMPs is through binding of MMPs to a family of endogenous homologous proteins known as tissue inhibitors of metalloproteinases (TIMPs; Brew *et al.*, 2000). It has been reported that MMP-2 activity can be inhibited by forming a 1:1 complex with TIMP-2 (Boone *et al.*, 1990; Greene *et al.*, 1996). In the present study, we demonstrated that TIMP-2 expression in BV2 cells was significantly and dose dependently decreased by SAH. Thus, SAH can increase the activity of MMP-2 in BV2 cells both by inducing the expression of

MMP-2 and by reducing the expression of TIMP-2 in BV2 cells. Previous studies have demonstrated that microglial cells express only TIMP-2 mRNA (Crocker *et al.*, 2006) but not TIMP-1 and TIMP-3.

In summary, we demonstrate in this cell culture study that SAH is able to induce the invasion of C6 glioma cells through increased MMP-2 secretion from BV2 cells. The mechanism by which SAH increases MMP-2 activity is likely mediated by the PI3K/Akt and ERK signaling pathways, with increased binding activities of NF- κ B and Sp1 to the 5' flanking region of the MMP-2 gene.

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REFERENCES

- Bergman, M. R., Cheng, S., Honbo, N., Piacentini, L., Karliner, J. S., and Lovett, D. H. (2003). A functional activating protein 1 (AP-1) site regulates matrix metalloproteinase 2 (MMP-2) transcription by cardiac cells through interactions with JunB-Fra1 and JunB-FosB heterodimers. *Biochem. J* **369**, 485–496.
- Bernstein, J. J., Goldberg, W. J., Laws, E. R., Jr, Conger, D., Morreale, V., and Wood, L. R. (1990). C6 glioma cell invasion and migration of rat brain after neural homografting: ultrastructure. *Neurosurgery* **26**, 622–628.
- Bonne, T. C., Johnson, M. J., De Clerck, Y. A., and Langley, K. E. (1990). cDNA cloning and expression of a metalloproteinase inhibitor related to tissue inhibitor of metalloproteinase. *Proc. Natl. Acad. Sci. U.S.A* **87**, 2800–2804.
- Brew, K., Dinakarpanian, D., and Nagase, H. (2000). Tissue inhibitors of metalloproteinase: Evolution, structure and function. *Biochim. Biophys. Acta* **1477**, 267–283.
- Chan-Hui, P., and Weaver, R. (1998). Human mitogen-activated protein kinase kinase mediates the stress-induced activation of mitogen-activated protein kinase cascades. *Biochem. J* **336**, 599–609.
- Chen, Y. C., Kuo, T. C., Lin-Shiau, S. Y., and Lin, J. K. (1996). Induction of HSP70 gene expression by modulation of Ca²⁺ ion and cellular p53 protein by curcumin in colorectal carcinoma cells. *Mol. Carcinog* **17**, 224–234.
- Collart, M. A., Baeuerle, P., and Vasalli, P. (1990). Regulation of tumor necrosis factor alpha transcription in macrophages: Involvement of four kappa B-like motifs and of constitutive and inducible forms of NF-kappa B. *Mol. Cell. Biol.* **19**, 1498–1505.
- Crocker, S. J., Milner, R., Pham-Mitchell, N., and Campbell, I. L. (2006). Cell and agonist-specific regulation of genes for matrix metalloproteinases and their tissue inhibitors by primary glial cells. *J. Neurochem.* **98**, 812–823.
- Deryugina, E. I., Bourdon, M. A., Luo, G. X., Reisfeld, R. A., and Strongin, A. (1997). Matrix metalloproteinase-2 activation modulates glioma cell migration. *J. Cell Sci.* **110**, 2473–2482.
- Finkelstein, J. D. (2000). Pathways and regulation of homocysteine metabolism in mammals. *Semin. Thromb. Hemost.* **26**, 219–225.
- Fu, W., Dudman, N. P. B., Perry, M. A., Young, K., and Wang, X. I. (2000). Interrelation between plasma homocysteine and intracellular S-adenosylhomocysteine. *Biochem. Biophys. Res. Commun* **271**, 47–53.
- Goldberg, W. J., Law, E. R., Jr, and Bernstein, J. J. (1991). Individual C6 glioma cells migrate in adult rat brain after neural homografting. *Int. J. Dev. Neurosci* **9**, 427–437.

- Greene, J., Wang, M., Liu, Y. E., Raymond, L. A., Rosen, C., and Shi, Y. E. (1996). Molecular cloning and characterization of human tissue inhibitor of metalloproteinase 4. *J. Biol. Chem.* **271**, 30375–30380.
- Guo, P., Imanishi, Y., and Cackowski, F. C. (2005). Up-regulation of angiopoietin-2, matrix metalloproteinase-2, membrane type 1 metalloproteinase, and laminin 5 gamma 2 correlates with the invasiveness of human glioma. *Am. J. Pathol.* **196**, 701–711.
- Hoffman, D. R., Marion, D. W., Cornatzer, W. E., and Duerre, J. A. (1980). S-adenosylmethionine and S-adenosylhomocysteine metabolism in isolated liver. *J. Biol. Chem.* **22**, 10822–10827.
- Hwang, H. J., Park, H. J., Chung, H. J., Min, H. Y., Park, E. J., Hong, J. Y., and Lee, S. K. (2006). Inhibitory effects of caffeic acid phenethyl ester on cancer cell metastasis mediated by the down-regulation of matrix metalloproteinase expression in human HT1080 fibrosarcoma cells. *J. Nutr. Biochem.* **17**, 356–362.
- James, S. J., Melnyk, S., Pogribny, M., Pogribny, I. P., and Caudill, M. A. (2002). Elevation in S-adenosylhomocysteine and DNA hypomethylation: Potential epigenetic mechanism for homocysteine-related pathology. *J. Nutr.* **132**, 2361S–2366S.
- Kennedy, B. P., Bottiglieri, T., Hansen, A., and Masliah, E. (2004). Elevated S-adenosylhomocysteine in Alzheimer's brain: Influence on methyltransferase and cognitive function. *J. Neural. Transm.* **111**, 547–567.
- Kerins, D. M., Koury, M. J., Capdevila, A., Rana, S., and Wagner, C. (2001). Plasma S-adenosylhomocysteine is a more sensitive indicator of cardiovascular disease than plasma homocysteine. *Am. J. Clin. Nutr.* **74**, 723–729.
- Kotelevets, L., Noë, V., Bruyneel, E., Myssiakine, E., Chastre, E., Mareel, M., and Gaspach, C. (1998). Inhibition by platelet-activating factor of Src- and hepatocyte growth factor-dependent invasiveness of intestinal and kidney epithelial cells. Phosphatidylinositol 3'-kinase is a critical mediator of tumor invasion. *J. Biol. Chem.* **273**, 14138–14145.
- Liu, P., Cheng, H., Roberts, T. M., and Zhao, J. J. (2009). Targeting the phosphoinositide 3-kinase pathway in cancer. *Nat. Rev. Drug Discov.* **8**, 627–644.
- Lominadze, D., Roberts, A., Tyagi, N., and Tyagi, S. (2006). Homocysteine causes cerebrovascular leakage in mice. *Am. J. Physiol. Heart Circ. Physiol.* **290**, H1206–H1213.
- Markovic, D. S., Glass, R., Synowitz, M., Rooijen, N., and Kettenmann, H. (2005). Microglia stimulate the invasiveness of glioma cells by increasing the activity of metalloproteinase-2. *J. Neuropathol. Exp. Neurol.* **64**, 754–762.
- Moshal, K. S., Sen, U., Tyagi, N., Henderson, B., Steed, M., Ovechkin, A. V., and Tyagi, S. C. (2006). Regulation of homocysteine-induced MMP-9 by ERK1/2 pathways. *Am. J. Physiol. Cell Physiol.* **290**, 883–891.
- Philip, S., and Kundu, G. C. (2003). Osteopontin induces nuclear factor kappa B-mediated promatrix metalloproteinase-2 activation through I kappa B alpha/IKK signaling pathways, and curcumin (diferulolylmethane) down-regulates these pathways. *J. Biol. Chem.* **278**, 14487–14497.
- Rao, J. S. (2003). Molecular mechanisms of glioma invasiveness: The role of proteases. *Nat. Rev. Cancer* **3**, 489–501.
- Reפש, L. A. (1989). A new in vitro assay for quantitating tumor cell invasion. *Invasion Metastasis* **9**, 192–208.
- Rosell, A., Ortega-Aznar, A., Alvarez-Sabin, J., Ribo, M., Molina, C. A., Lo, E. H., and Montaner, J. (2006). Increased brain expression of matrix metalloproteinase-9 after ischemic and hemorrhagic human stroke. *Stroke* **37**, 1399–1406.
- Sampieri, C. L., Nuttall, R. K., Young, D. A., Goldspink, D., Clark, I. M., and Edward, D. R. (2008). Activation of p38 and JNK MAPK pathways abrogates requirement for new protein synthesis for phorbol ester mediated induction of select MMP and TIMP genes. *Matrix Biol.* **27**, 128–138.
- Sato, H., and Seiki, M. (1993). Regulatory mechanism of 92 kDa type IV collagenase gene expression which is associated with invasiveness of tumor cells. *Oncogene* **8**, 395–405.
- Schreck, R., and Baeuerle, P. A. (1990). NF-kappa B as inducible transcriptional activator of the granulocyte-macrophage colony-stimulating factor gene. *Mol. Cell. Biol.* **10**, 1281–1286.
- Shukla, S., MacLennan, G. T., Marengo, S. R., Resnick, M. I., and Gupta, S. (2005). Constitutive activation of P13 K-Akt and NF-kappaB during prostate cancer progression in autochthonous transgenic mouse model. *Prostate* **64**, 224–239.
- Takahra, T., Smart, D. E., Oakley, F., and Mann, D. A. (2004). Induction of myofibroblast MMP-9 transcription in three-dimensional collagen I gel cultures: Regulation by NF-kappaB, AP-1 and Sp1. *Int. J. Biochem. Cell Biol.* **36**, 353–363.
- Tews, D. S. (2000). Adhesive and invasive features in gliomas. *Pathol. Res. Pract.* **196**, 701–711.
- Theodorescu, D., Laderoute, K. R., and Gulding, K. M. (1998). Epidermal growth factor receptor-regulated human bladder cancer motility is in part a phosphatidylinositol 3-kinase-mediated process. *Cell Growth Differ.* **9**, 919–928.
- Valli, A., Carrero, J. J., Qureshi, A. R., Garibotto, G., Barany, P., Axelsson, J., Lindholm, B., Stenvinkel, P., Anderstam, B., and Suliman, M. E. (2008). Elevated serum levels of S-adenosylhomocysteine, but not homocysteine, are associated with cardiovascular disease in stage 5 chronic kidney disease patients. *Clin. Chim. Acta* **395**, 106–110.
- Wagner, C., and Koury, M. J. (2007). S-adenosylhomocysteine: A better indicator of vascular disease than homocysteine? *Am. J. Clin. Nutr.* **86**, 1581–1585.
- Watters, J. J., Schartner, J. M., and Badie, B. (2005). Microglia function in brain tumors. *J. Neurosci. Res.* **81**, 447–455.
- Yamamoto, M., Mohanam, S., Sawaya, R., Fuller, G. N., Seiki, M., Sato, H., Gokaslan, Z. L., Liotta, L. A., Nicolson, G. L., and Rao, J. S. (1996). Differential expression of membrane-type matrix metalloproteinase and its correlation with gelatinase A activation in human malignant brain tumors in vivo and in vitro. *Cancer Res.* **56**, 384–392.
- Yan, C., and Boyd, D. D. (2007). Regulation of matrix metalloproteinase gene expression. *J. Cell Physiol.* **211**, 19–26.
- Yang, T. H., and Hu, M. L. (2006). Intracellular levels of S-Adenosylhomocysteine but not homocysteine are highly correlated to the expression of nm23-H1 and the level of 5-methyldeoxycytidine in human hepatoma cells with different invasion activities. *Nutr. Cancer* **55**, 224–231.
- Yi, P., Melnyk, S., Pogribna, M., Pogribny, I. P., Hinet, R. J., and James, S. J. (2000). Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J. Biol. Chem.* **275**, 29318–323.
- Yong, V. W., Power, C., Forsyth, P., and Edwards, D. (2001). Metalloproteinases in biology and pathology of the nervous system. *Nat. Rev. Neurosci.* **2**, 502–511.
- Zhang, Z. H., Liu, X. G., Chen, G. P., Zhang, X. R., and Guo, M. Y. (2004). Significance of MMP-2 and TIMP-2 mRNA expressions on glomerular cells in the development of glomerulosclerosis. *Chin. Med. Sci. J.* **9**, 84–88.