available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/biochempharm

Repression of matrix metalloproteinase gene expression by ginsenoside Rh2 in human astroglioma cells

So-Young Kim [a], Dong-Hyun Kim [b], Sang-Jun Han [b], Jin-Won Hyun [c], Hee-Sun Kim [a,*]

[a] Department of Neuroscience and Medical Research Institute, College of Medicine, Ewha Womans University, Mok-6-dong 911-1, Yangchun-Ku, Seoul 158-710, Republic of Korea
[b] Department of Microbial Chemistry, College of Pharmacy, Kyung Hee University, Seoul, Republic of Korea
[c] Department of Biochemistry, College of Medicine, Cheju National University, Jeju, Republic of Korea

ARTICLE INFO

Article history:
Received 25 June 2007
Accepted 14 August 2007

Keywords:
Rh2
Glioma invasion
MMP
NF-κB
AP-1
MAP kinases

ABSTRACT

Matrix metalloproteinases (MMPs) play an important role in glioma infiltration, facilitating cell migration and tumor invasion through their ability to degrade the extracellular matrix. Therefore, the inhibition of MMPs has been suggested to be a promising therapeutic strategy for brain tumors. This study examined the effect of ginsenoside Rh2 on the expression of MMPs in human astroglioma cells. Rh2 inhibited the PMA-induced mRNA expression of MMP-1, -3, -9, and -14, suggesting that Rh2 has a broad-spectrum inhibitory effect on MMPs. The molecular mechanism underlying MMP-9 inhibition was further investigated because MMP-9 plays a major role in the invasiveness of glioma. It was found that Rh2 inhibited the secretion and protein expression of MMP-9 induced by PMA in human astroglioma cells. The Rh2-mediated inhibition of MMP-9 gene expression appears to occur through NF-κB and AP-1 because their DNA binding and transcriptional activities were suppressed by the agent. Furthermore, Rh2 significantly repressed the PMA-mediated activation of p38 MAPK, ERK and JNK, which are upstream modulators of NF-κB and AP-1. Finally, Rh2 inhibited the in vitro invasiveness of glioma cells, which might be attributed to the broad-spectrum inhibition of MMPs by Rh2. Overall, the strong inhibition of MMP expression by Rh2 might provide a potential therapeutic modality for brain tumors.

1. Introduction

Malignant gliomas are characterized by rapid cell proliferation, a high level of invasiveness into the surrounding brain and extensive vascularization through angiogenesis [1]. This diffusely infiltrative nature of malignant glioma is one of the major obstacles to successful curative treatments, leading to a short survival time, generally less than 1 year [2]. Progress in the treatment of gliomas now depends on better understanding of the biology of these tumors. Recent studies into the biology of gliomas have found that matrix metalloproteinases (MMPs) and the related signal transduction pathways play a role in tumor initiation and maintenance [2,3]. In many reports, various MMPs have been implicated as markers of tumor progression in gliomas, due to the positive correlation between increasing expres-

* *Corresponding author*. Tel.: +82 2 2650 5823; fax: +82 2 2653 8891.
E-mail address: hskimp@ewha.ac.kr (H.-S. Kim).

Abbreviations: MMP, matrix metalloproteinase; ECM, extracellular matrix; PMA, phorbol myristate acetate; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor-κB; AP, activator protein; EMSA, electrophoretic mobility shift assay.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.08.015

sion levels and the histological grade of the malignancy [2,4,5].

The matrix metalloproteinases (MMPs) are a family of structurally related zinc-dependent endopeptidases that are capable of degrading almost all the extracellular matrix (ECM) components [4,6]. There is evidence suggesting that MMPs contribute to glioma cell invasion to the surrounding normal tissues through degradation of the cell surface ECM [2,4,6]. Studies assessing the invasiveness of glioma cells in vitro have demonstrated a strong correlation between glioma invasion and high levels of MMP-2 or -9 expression [7–9]. Although many studies focused on the inhibition of gelatinases such as MMP-2 and MMP-9, which play a major role in glioma invasion and migration, a better solution would be to determine a way to control the various MMPs simultaneously. This is because several studies have reported that various MMPs (i.e. MMP-1, -3, -9, -14, etc.) are involved in glioma cell invasion and angiogenesis [10–12].

Ginsenosides, which are glycosides containing an aglycone (protopanaxadiol or protopanaxatriol) with a dammarane skeleton, are the major effective components of ginseng and have been shown to have a wide variety of biological activities including immunomodulatory effects, anti-inflammatory and anti-tumor activity [13–15]. Rh2 is produced by the bacterial transformation of ginsenoside Rg3, and belongs to the protopanaxadiol family. Rh2 has attracted considerable attention owing to its potential tumor-inhibitory activity [13,16]. It inhibits cell growth in MCF-7 human breast cancer cells and SK-HEP-1 hepatoma cells [17,18] and can also induce apoptosis in various cell lines including C6 rat glioma, SK-N-BE(2)C human neuroblastoma, and A375-S2 human malignant melanoma cells [19–21]. In addition, Rg3, the proform of Rh2, and other ginsenosides have been reported to inhibit the formation of PMA-induced mouse skin tumors and/or COX-2 expression in mouse skin and cultured mammary epithelial cells [22,23].

However, there are no reports on the effects of Rh2 on glioma invasion or the detailed molecular mechanism. Therefore, this study examined the effects of Rh2 on the expression of MMPs, which play a key role in glioma invasion. Rh2 showed a broad-spectrum inhibitory effect against MMP-1, -3, -9, and -14 at both the mRNA and promoter levels. The effects of Rh2 on the PMA-induced activation of NF-κB, AP-1, and the upstream signaling MAP kinases were examined to further analyze the molecular mechanism for the inhibition of MMP-9 by Rh2. The results also showed that Rh2 remarkably inhibited the invasiveness of glioma cells, which appears to be related to the broad-spectrum inhibition of MMPs by Rh2.

2. Materials and methods

2.1. Reagents

The red ginseng extract was prepared and its main constituent ginsenoside Rg3 isolated based on the previously described procedure [24]. To isolate the main metabolite of ginsenoside Rg3, it (0.2 g) was anaerobically incubated with human fecal suspension (1 g) in the anaerobic medium (0.5 L) according to the previous method [25]. The reaction mixture was extracted with *n*-BuOH (1 L) twice, and evaporated (dried extract, 0.15 g) followed by being defatted with *n*-hexane. The deffated *n*-BuOH extract was subjected to a silica gel column chromatography (2 cm × 20 cm) and eluted with a stepwise gradient of CH_2Cl_2:methanol and then underwent further chromatography on silica gel columns, employing the same eluent systems, to give a ginsenoside Rh2 (35 mg). The extracted ginsenoside Rh2 was identified by comparison to an authentic standard by nuclear magnetic resonance spectrometry (Bruker AM-500, 500 MHz) and FAB mass spectrometry, and their structures are shown in Fig. 1.

All reagents used for cell culture containing penicillin/streptomycin, trypsin, and DMEM were purchased from Gibco BRL (Grand Island, NY). TRI reagent and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma Chemical Co. (St. Louis, MO). Restriction enzymes, Taq polymerase, and antibodies against phospho-/total form of p38 MAPK, ERK1/2, and SAPK/JNK were purchased form Cell Signaling Technology (Beverley, MA). All other chemicals were obtained from Sigma–Aldrich, unless stated otherwise.

2.2. Cell culture and transient transfection assays

Human astroglioma U87MG and U373MG cells (American Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Hyclone), streptomycin, and penicillin. The CRT-MG human astroglioma cell line (a generous gift of Dr. Etty N. Benveniste, University of Alabama, Birmingham, USA) was grown in RPMI 1640 medium supplemented with 10 mM HEPES (pH 7.2), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. Transfection was performed by a standard calcium phosphate method. Cells (2×10^5 in 60 mm-diameter dishes) were transfected with 4 μg of the reporter construct, 1 μg of pRSV β-gal and pUC19 plasmid to a total of 10 μg of DNA. The MMP reporter plasmids (hMMP1-luc, hMMP3-luc, hMMP9-luc) were constructed by our lab as described previously [10], and NF-κB and AP-1 reporter plasmids were purchased from Clontech (Mountain View, CA). Plasmids used

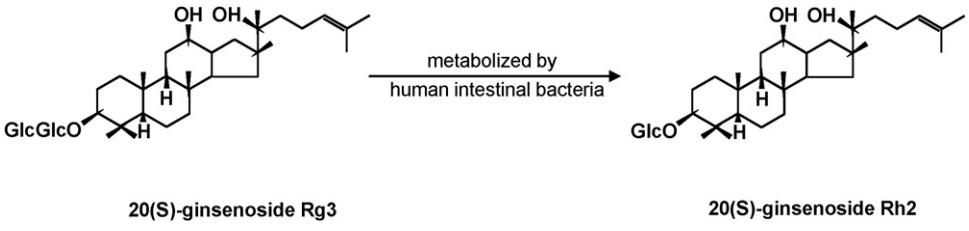

Fig. 1 – Structure of ginsenoside Rg3 and its metabolic compound Rh2.

for transient transfection assays were prepared by using Qiagen (Santa Clarita, CA) columns. After 48 h, cells were harvested and luciferase assays were performed as previously described [26]. To correct for differences in transfection efficiencies among different DNA precipitates, luciferase activity was normalized to that of β-galactosidase activity determined by ONPG assay. All transfection assays were performed at least three times in duplicate.

2.3. RT-PCR

Total cellular RNA was extracted from appropriately treated U87MG cells with TRI reagent according to the manufacturer's protocol. Total RNA (2 μg) was reverse transcribed for 1 h at 37 °C in a reaction mixture containing 5 U RNase inhibitor (Invitrogen), 0.5 mM dNTP (Böeringer Mannheim, Indianapolis, IN), 2 μM random hexamer (Promega, Medison, WI), 1× RT buffer and 5 U reverse transcriptase (Qiagen). PCR was performed using primers for human MMP-1, -2, -3, -9, -14 and GAPDH as below. Analysis of the resulting PCR products on 1% agarose gels showed single-band amplification products with expected sizes.

	Forward primer (5′ → 3′)	Reverse primer (5′ → 3′)	Size (bp)
MMP-1	ATATCGGGGCTTTGATGTACC	AGCTGTAGATGTCCTTGGGGT	408
MMP-2	GAAGTATGGGAACGCCGATGG	TTGTCGCGGTCGTAGTCCTCA	311
MMP-3	GATATAAATGGCATTCAGTCCCTC	TCCTTGCTAGTAACTTCATATGCG	287
MMP-9	ATGT ACCCTATGTACCGCTTCACT	CAGAGAAGAAGAAAAGCTTCTTGG	496
MMP-14	CCGAAGCCTGGCTACAGCAAT	ATGTGGCATA CTCGCCCACCT	317
GAPDH	GGTCGGTGTGAACGGATTTGGCCG	GGTTCACACCCATCACAAACATGG	395

2.4. Gelatin zymography

The gelatinolytic activity of MMP-9 secreted in conditioned media was assayed by means of gelatin-substrate gel electrophoresis to identify the levels of metalloproteinase activity. Conditioned media were harvested and the protein concentration measured, followed by concentrating by precipitation with 2-volumes of absolute ethanol. Twenty micrograms of protein was resuspended with 2× sample buffer without reducing agent (0.5 M Tris–HCl, pH 6.8, 10% SDS, 0.1% bromophenol blue, 10% glycerol) and then subjected to 7.5% SDS-PAGE gel containing 0.1% (w/v) gelatin without prior boiling. After electrophoresis at 4 °C, the gel was washed with 2.5% Triton X-100 (v/v) for 30 min at RT to remove SDS and allow protein to renature and subsequently incubated in substrate buffer (50 mM Tris–HCl, pH 7.5, 1 mM $ZnCl_2$, 5 mM $CaCl_2$) at 37 °C for 48–72 h. The gel was then stained with 0.5% (w/v) Coomassie blue in 10% acetic acid (v/v) and 30% methanol (v/v) and then destained with the same solution without Coomassie blue. Proteolytic activities were detected by clear bands indicating the lysis of the substrate. Quantification of MMP-9 band density was carried out using the image analysis program luminescent image analyzer Las-3000 (Fujifilm, Japan).

2.5. Western blot analysis

Cells were appropriately treated and total cell lysates were prepared as described previously [27]. The proteins (20–100 μg) were heated with 4× SDS sample buffer and separated by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes (Amersham, Piscataway, NJ). The membranes were blocked with 5% bovine serum albumin in 10 mM Tris–HCl containing 150 mM NaCl and 0.5% Tween-20 (TBST) and then incubated with primary antibodies (1:1000) that recognize the phospho- or the total forms of MAP kinases. After thoroughly washing with TBST, horseradish peroxidase-conjugated secondary antibodies (Amersham, Piscataway, NJ; 1:2000 dilution in TBST) were applied and the blots were developed using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ).

2.6. Electrophoretic mobility shift assay (EMSA)

U87MG glioma cells were treated with PMA in the absence or presence of Rh2, and nuclear extracts were prepared as described [27]. Nuclear proteins (5 μg) were incubated with ^{32}P-labeled NF-κB or AP-1 probe on ice for 30 min, and the DNA–protein complex was separated on a 5% acrylamide gel. The oligonucleotide sequences of the NF-κB and AP-1 probes were those from the human MMP-9 promoter: for NF-κB, 5′-CCCCAGTGGAATTCCCCAGCCTTG-3′ (sense), 5′-GCAAGGCTGGGGAA TTCCACTGGG-3′ (antisense), for proximal AP-1, 5′-CTGACCCCTGAGTCAGCAC TTGC-3′ (sense), 5′-CAA-GTGCTGACTCAGGGGTCAGG-3′ (antisense), for upstream AP-1, 5′-GAGGAAGCTGAGTCAAAGAAGGC-3′ (sense), 5′-TCTCCTT-CGACTCA GTTCTTCC-3′ (antisense). The sense and antisense oligomers were annealed and double strands were isolated with PAGE gel and used for labeling. For supershift assay, antibodies against the p65 or p50 subunits of NF-κB (Santa Cruz Biotechnology, Santa Cruz, CA) were coincubated with the nuclear extract mix for 30 min at 4 °C before adding the radiolabeled probe.

2.7. Invasion assay

Invasion assays were carried out using modified Boyden chambers consisting of Transwell (Corning Costar, Cambridge, MA) membrane filter (6.5-mm diameter, 8-μm pore size). The upper surfaces of the transwell membranes were coated with 1 mg/ml Matrigel matrix (Becton–Dickinson Labware, Franklin Lakes, NJ). Cells were plated on the Matrigel-coated transwell with or without Rh2 in the presence of PMA. The medium in the lower chambers also contained 0.1 mg/ml bovine serum albumin. The inserts were incubated at 37 °C for 24 h. Non-invading cells were removed by wiping the upper side of the membrane, and the invaded cells were fixed with methanol and stained with hematoxylin. Random fields were counted under a light microscope.

2.8. Statistical analysis

Data are expressed as means ± standard error (S.E). and analyzed for statistical significance using analysis of variance (ANOVA), followed by Scheffe's test for multiple comparisons. A P value <0.05 was considered significant.

3. Results

3.1. Ginsenoside Rh2 inhibits the transcription of the MMP-1, -3, -9, and -14 genes in human astroglioma cells

RT-PCR was carried out to examine the MMPs expressed from U87MG human astroglioma cells. As shown in Fig. 2, the expression of MMP-1, -3, -9, and -14 mRNA in U87MG cells was induced significantly by a strong tumor promoter, PMA (50 ng/ml), while MMP-2 expression was constitutive. Pretreatment with Rh2 significantly suppressed the PMA-induced expression of MMP-1, -3, -9, and -14 in a dose-dependent manner (Fig. 2A). Moreover, Rh2 repressed the promoter activities of MMP-1, -3, and -9 induced by PMA (Fig. 2B). However, the expression of MMP-2 was not significantly altered by Rh2. Similar results were obtained in other human glioma cells such as U373MG and CRT-MG cells (data not shown). Therefore, these results suggest that Rh2 is a potent broad-spectrum inhibitor of the MMPs expressed in human astroglioma cells. The molecular mechanism underlying MMP-9 inhibition by Rh2 was further investigated because MMP-9 is a key enzyme among the MMPs implicated in the invasiveness of glioma.

3.2. Rh2 suppresses MMP-9 secretion in human glioma cells

Gelatin zymography was carried out to determine the effect of Rh2 on the PMA-induced MMP-9 secretion in human astroglioma cells. The gelatinolytic activity at 92 kDa, which corresponded to the molecular mass of pro-MMP-9, was detected in the conditioned medium from PMA-stimulated astroglioma cells. The most remarkable induction of MMP-9 gelatinolytic activity was observed with 50 ng/ml of PMA for 48 h (data not shown). As shown in Fig. 3, Rh2 markedly suppressed the PMA-induced secretion of pro-MMP-9 in a dose-dependent manner. The inhibitory effect of Rh2 on the

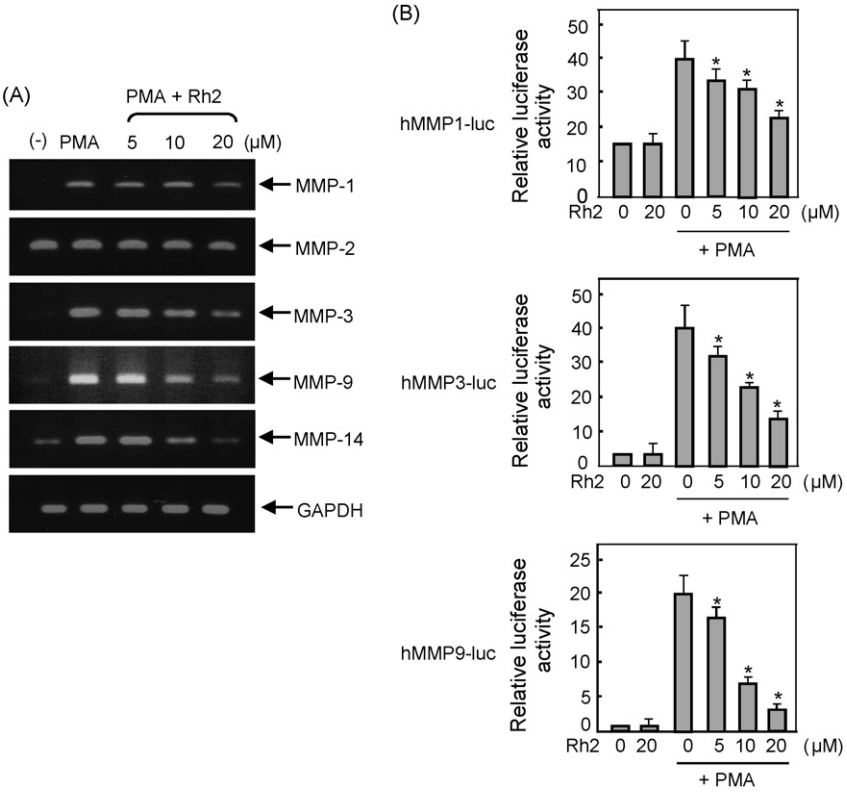

Fig. 2 – The effects of Rh2 on MMPs expressed from human astroglioma U87MG cells. (A) RT-PCR was performed to detect MMPs expressed from U87MG cells in the absence or presence of PMA (50 ng/ml). The mRNA expressions of MMP-1, -3, -9, -14 were dramatically induced by PMA, whereas the expression of MMP-2 was constitutive. The elevated levels of MMPs were significantly downregulated by pretreatment of Rh2. The MMP mRNA expression was normalized to GAPDH mRNA. The data are representative of three independent experiments. (B) Inhibitory effects of ginsenoside Rh2 on promoter activities of MMP-1, -3, and -9 in U87MG cells. U87MG glioma cells were transfected with three kinds of reporter plasmids each containing one of the MMP-1, -3, and -9 promoters and treated with 50 ng/ml of PMA in the presence of indicated doses of Rh2 for 18 h before harvest. Luciferase assay using the cell lysates revealed that Rh2 significantly decreased the PMA-induced promoter activities of MMP-1, -3, and -9 in a dose-dependent manner. Values correspond to the mean ± S.E. of three independent experiments. *P < 0.05, significantly different from luciferase activities in cells treated with PMA alone.

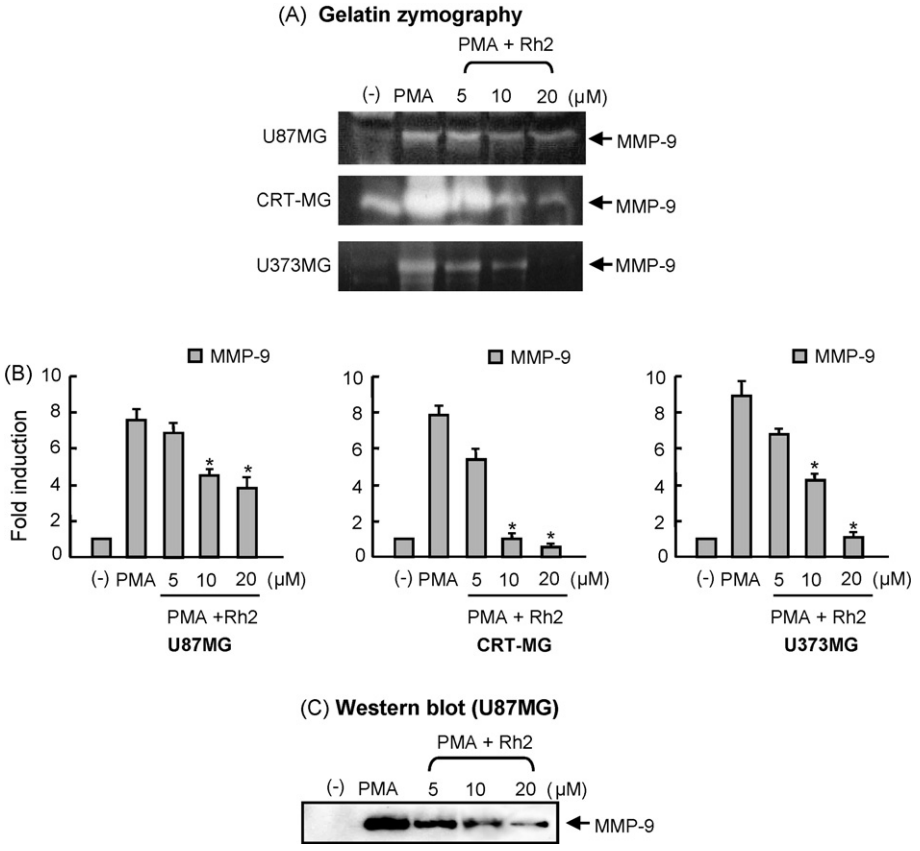

Fig. 3 – Rh2 repressed the secretion and protein expression of MMP-9. (A) Three kinds of human glioma cell were treated with different concentrations of Rh2 in the absence or presence of PMA (50 ng/ml) in serum-free media. Conditioned media were collected after 48 h, and gelatin zymography was performed. The data are representative of three independent experiments. (B) Quantification of zymography data. Levels of the secreted MMPs were normalized with respect to the amount of protein and expressed as relative fold changes in comparison to control samples, which were arbitrary set to 1.0. Values correspond to the mean ± S.E. of three independent experiments. *P < 0.05, significantly different from the PMA-treated sample. (C) Western blot analysis using the conditioned medium of U87MG cells used for zymography also shows that Rh2 decreased the protein amount of MMP-9 in a dose-dependent manner.

secretion of MMP-9 was observed in three human astroglioma cell lines (U87MG, U373MG and CRT-MG) (Fig. 3A). The concentration of Rh2 (up to 20 μM) used in these experiments did not affect the cell-viability in the MTT assay (data not shown). The band density of MMP-9 in the zymogram was normalized to the amount of protein and the results are shown in Fig. 3B. Western blot analysis was performed using the conditioned medium used for zymography in order to determine if the decrease in MMP-9 secretion was the result of a decrease in the amount of protein. As shown in Fig. 3C, Rh2 inhibited the PMA-induced expression of the MMP-9 protein in a dose-dependent manner. Therefore, the decreased secretion of MMP-9 correlates with the reduced level of MMP-9 protein synthesis after the Rh2 treatment.

3.3. *Rh2 inhibits the DNA binding and transcriptional activities of NF-κB and AP-1*

The effects of Rh2 on NF-κB and AP-1 were examined to determine the downregulation mechanism of MMP-9 by Rh2. The NF-κB and AP-1 sites resided on the MMP-9 promoter (Fig. 4A), and have been reported to play an important role in controlling the basal and cytokine-induced MMP-9 expression in various cancer cell lines. In order to determine the effect of Rh2 on the NF-κB DNA-binding activity, the nuclear extracts were prepared from the cells treated with PMA in the presence or absence of Rh2 (20 μM), and were incubated with the ^{32}P-labeled NF-κB probe. As shown in Fig. 4B, Rh2 reduced the NF-κB binding activity. An antibody supershift assay using the NF-κB specific antibody showed that the DNA–protein complex contains the p50 and p65 subunits of NF-κB (Fig. 4C). Moreover, Rh2 inhibited the NF-κB-mediated transcriptional activity, as shown by the NF-κB reporter gene assay (Fig. 4D). Next, the effects of Rh2 on both AP-1 DNA binding and AP-1-dependent transcriptional activity were examined. As shown in Fig. 5, Rh2 significantly inhibited the nuclear protein binding to the proximal and upstream AP-1 sites that had been induced by PMA. Competition assay using a molar excess of cold oligonucleotides of their own or consensus AP-1 revealed the DNA–protein complex to be specific to AP-1 (Fig. 5B). In addition, the transient transfection assay showed that Rh2 inhibited the AP-1-mediated transcriptional activity in U87MG

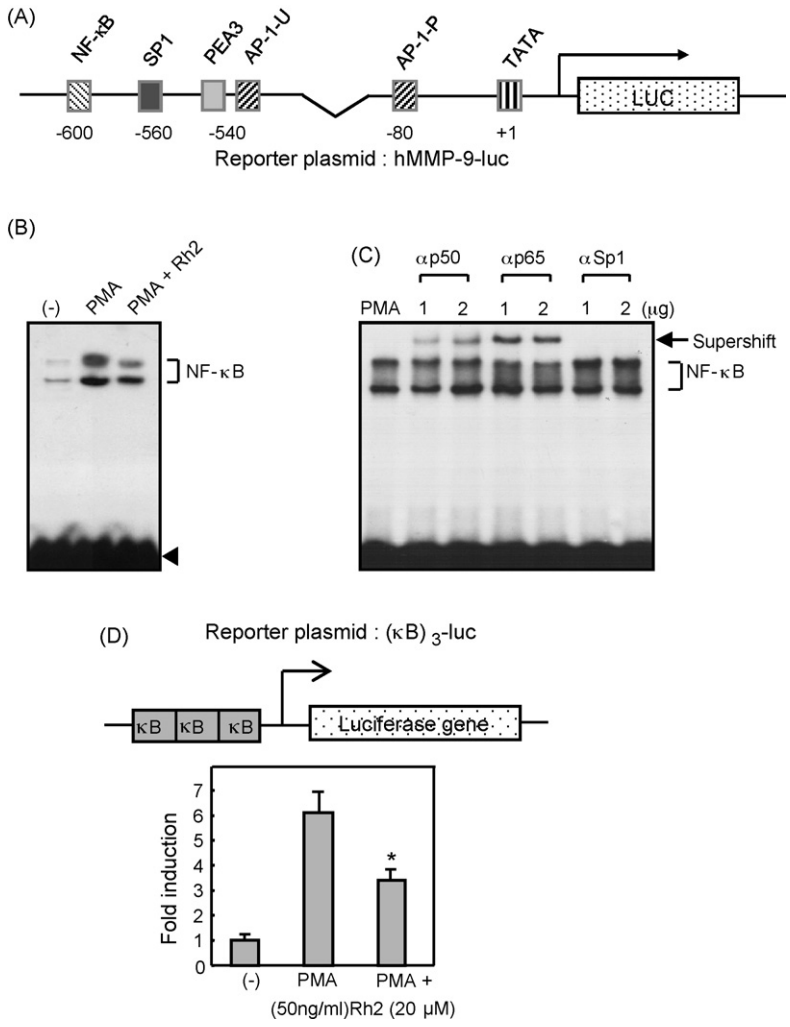

Fig. 4 – Effects of Rh2 on NF-κB activities in U87MG cells. (A) Structure of MMP-9 reporter plasmid (hMMP9-luc). Transcription factor binding sites are indicated. (B) EMSA for NF-κB DNA-binding activity. Nuclear extracts were prepared from U87MG cells after treatment with Rh2 (20 μM) for 6 h in the absence or presence of PMA (50 ng/ml). Bracket indicates a DNA–protein complex of NF-κB. (C) Supershift assay indicated that the NF-κB complex is composed of p50 and p65 subunits because the complex was supershifted by antibodies against the p50 or p65 subunit (αp50, αp65), but not by Sp1 antibody (αSp1). (D) Effect of Rh2 on NF-κB reporter gene activity. NF-κB reporter plasmid was transfected into U87MG cells, and PMA with or without Rh2 (20 μM) was added 6 h before harvest. Luciferase assay using cell lysates revealed that Rh2 inhibited the NF-κB-mediated transcriptional activity induced by PMA. Values correspond to the mean ± S.E. of three independent experiments. *P < 0.05, significantly different from the PMA-treated sample.

glioma cells (Fig. 5C). Therefore, Rh2 suppresses MMP-9 gene expression by inhibiting the activity of NF-κB and AP-1.

3.4. *Rh2 suppresses the phosphorylation of three types of MAP kinases*

It was previously reported that MAP kinases play a role as upstream modulators in MMP-9 gene expression as well as in NF-κB and AP-1 activity [27,28]. Therefore, the effects of Rh2 on the MAP kinase activities in U87MG glioma cells were examined. As shown in Fig. 6, Rh2 significantly suppressed the phosphorylation of the three types of MAP kinases (ERK, JNK, p38 MAPK) induced by PMA. The degree of inhibition by 20 μM Rh2 was approximately 80% for each MAP kinase (Fig. 6B).

3.5. *The effect of Rh2 on in vitro invasion of glioma cells*

Matrigel invasion chambers were used to examine the effect of Rh2 on the invasiveness of U87MG glioma cells. As shown in Fig. 7, a treatment with 20 μM Rh2 for 24 h inhibited the cell invasiveness by more than 90%, without affecting the cell viability. To determine whether the inhibitory effect is directly correlated with the repression of MMP activities by Rh2, we examined the effect of various MMP inhibitors on PMA-induced invasion of U87MG glioma cells: MMP inhibitors significantly suppressed invasiveness (data not shown). Therefore, the strong inhibitory effect of Rh2 on an in vitro invasion appears to be related to its broad-spectrum inhibition of MMP expression.

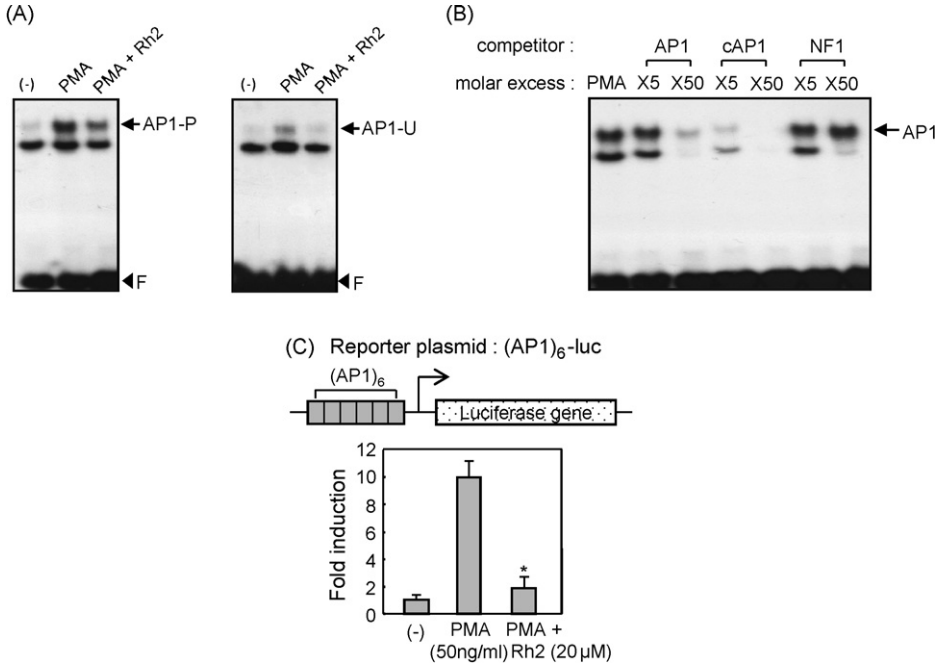

Fig. 5 – Effect of Rh2 on the DNA-binding and transcriptional activities of AP-1 in the proximal and upstream region of MMP-9 promoter. (A) EMSA for AP-1 DNA-binding activity. Oligonucleotides containing the upstream or proximal AP-1 sequence were used as a probe and incubated with the same nuclear extracts used for NF-κB EMSA. The arrow indicates a DNA–protein complex of AP-1. AP1-P means AP-1 protein bound to AP-1 proximal sequence, and AP1-U to upstream sequence, respectively. (B) Competition assay revealed that the complex is AP-1-specific since it was diminished by a molar excess of cold oligonucleotide of its own or consensus AP-1 (cAP-1) but not by other nonspecific oligonucleotide (NF1). (C) Rh2 (20 μM) repressed AP-1-mediated transcription. AP-1 reporter plasmid containing six copies of the AP-1-binding sequence fused to the luciferase gene was transfected into U87MG cells, and luciferase activity was determined. Values correspond to the mean ± S.E. of three independent experiments. *P < 0.05, significantly different from the PMA-treated sample.</ce:figure

4. Discussion

Malignant human gliomas are pathophysiologically characterized by their insidious infiltration to the brain. The invasion of tumor cells into the surrounding normal brain is the primary cause of local recurrence and treatment failure of malignant gliomas [1,2]. The invasion of glioma cells into the adjacent brain structures occurs through the activation of multigenic programs, including matrix metalloproteinses (MMP) such as MMP-2, -9, which degrade the extracellular matrix to overcome the extracellular matrix barrier at the invasive fronts of tumors [4–6]. Many studies have shown that the up-regulation of MMPs correlates with the invasiveness of human gliomas, and MMPs offer an interesting target for examining the mechanism of glioma invasion [4,29,30].

The effect of Rh2 on the expression of MMPs induced by a strong tumor promoter PMA in human astroglioma cells was first examined to determine if ginsenoside Rh2 has potential for controlling MMP and resultant glioma invasion. PMA remarkably induced the mRNA expression of MMP-1, -3, -9, and -14, which play key roles in brain tumor progression in vivo. A pretreatment with Rh2 inhibited the expression of those MMPs. In contrast, the expression of MMP-2 expression was constitutive and was not affected by either PMA or Rh2. Glioma invasion is known to occur through the complex activities of various MMP molecules. It was reported that the MMP-1 (collagenase-1) protein level increases with tumor grade and is related to increased glioma invasiveness [9,31]. MMP-3 has also been detected in invasive astrocytoma cells, and is up-regulated in primary adult and child astrocytomas [32,33]. In addition, MMP-14 (MT1-MMP) appears to play an important role in remodeling of the ECM because this membrane-bound protease can stimulate proMMP-2 activation [34]. The up-regulation of VEGF by MMP-14 has also been reported during the progression of human gliomas [35]. Moreover, among the MMPs, MMP-9 is a well-characterized enzyme that has been implicated in the glioma invasiveness. The MMP-9 level was found to increase during the growth of glioblastoma cells, which had been intracerebrally implanted in nude mice [36]. In addition, the antisense MMP-9 vector or adenovirus expressing antisense-MMP-9 inhibited the PMA-induced migration and invasion of glioblastoma cells [37,38]. Therefore, the strong inhibition of glioma invasion by Rh2 might be due to the broad-spectrum inhibition of these series of MMPs.

A subsequent study was carried out to unravel the inhibitory mechanism of MMP-9 by Rh2 because MMP-9 is a key enzyme among the MMPs involved in glioma invasion. It was demonstrated that Rh2 represses the NF-κB, AP-1 and MAPK activities in PMA-stimulated glioma cells. Previously, it was reported that the inhibition of three types of MAP kinases results in the suppression of the MMP-9 promoter activity as

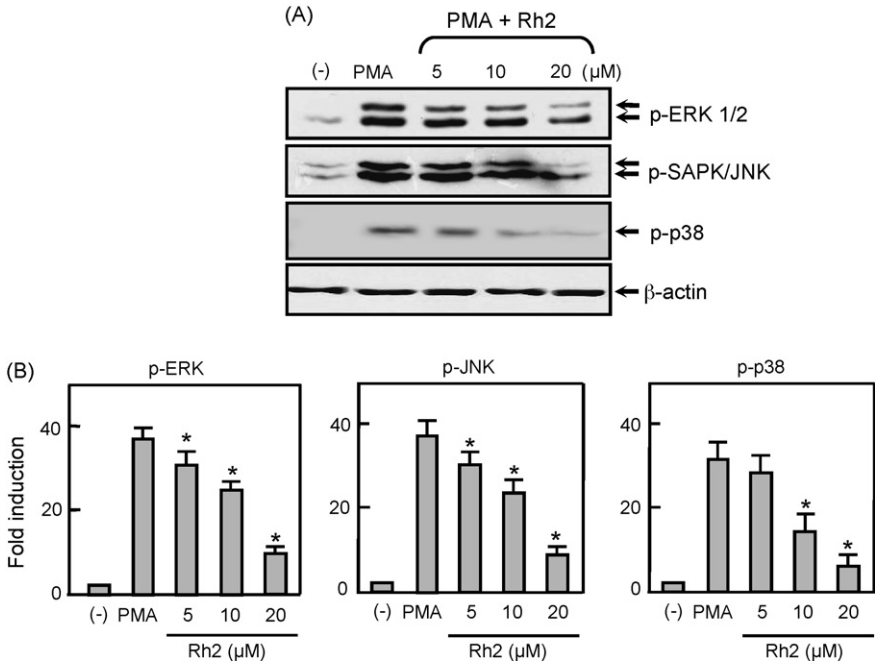

Fig. 6 – Effect of Rh2 on MAP kinase activities. (A) Cell extracts were prepared from U87MG cells treated with PMA for 30 min in the absence or presence of Rh2 and subjected to immunoblot analysis using antibodies against phospho- or the total form of 3 MAPKs (p38 MAPK, ERK1/2, JNK). Arrows indicate bands recognized by each antibody. As to ERK and JNK, two different isoforms were identified: pERK (p44 and p42) and pJNK (p54 and p46). (B) Quantification of Western blot data. Levels of the active forms of MAPKs were normalized with respect to β-actin and expressed as relative fold changes in comparison to control samples, which were arbitrarily set to 1.0. Values correspond to the mean ± S.E. of three independent experiments. *P < 0.05, significantly different from the PMA-treated sample.

well as the activities of NF-κB and AP-1 [27,28]. Furthermore, three types of MAP kinase inhibitors significantly repressed the in vitro invasiveness of glioma cells [28]. Therefore, these results suggest that Rh2 represses the expression of MMP-9, at least through the inhibition of NF-κB, AP-1 and MAPK activities, and finally inhibit glioma invasion. Our previous data showed that the extent of the involvement of each MAP kinase in MMP gene regulation differs according to the MMP subtypes [10]. Therefore, the inhibition of MAP kinase activity by Rh2 might also contribute to the inhibition of other MMPs besides MMP-9 (i.e. MMP-1, MMP-3).

Several studies have reported the neuroprotective properties of Rh2 and its proform Rg3 [39–43]. Ginsenoside Rg3 has been found to inhibit the ligand-gated ion channels such as NMDA receptors, 5-HT receptors and nicotinic acetylcholine receptors, which mediate neurotoxicity [40,41]. Ginsenoside

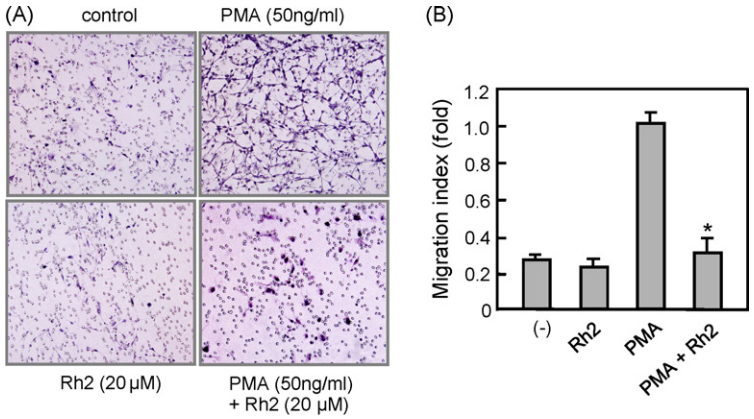

Fig. 7 – Cell invasion assay in U87MG cells. (A) Cell invasion through Matrigel basement membrane was analyzed using a modified Boyden chamber method, as described in Section 2. Cells that invaded to the lower surface of the membrane were fixed and stained. (B) Mean cell counts from at least 10 fields and data represent the mean ± S.E. of at least three independent experiments.

Rh2 has been also reported to reduce the level of ischemic brain injury in rats [42] and ameliorate neuronal cell death associated with hypoxia or glutamate toxicity [43]. These results suggest that Rg3 and Rh2 exert their effect on various CNS disorders. Generally, ginsenosides are barely absorbed as intact forms from the intestine to the blood. They are transformed by intestinal microflora and the transformants should be absorbed into the blood [44]. As a main metabolite of Rg3 by human intestinal bacteria, Rh2 is likely to have more potent biological activity than ginsenoside Rg3. In support of this, Rh2 inhibited the expression of MMP-9 more prominently than Rg3 (unpublished data).

In conclusion, this study reports for the first time the inhibitory effect of ginsenoside Rh2 on glioma invasion and MMP expression. Considering that glioma invasion progresses through the multiple interplay of many MMP molecules, the broad-spectrum inhibition of Rh2 against MMP expression might have therapeutic potential for controlling the growth and invasiveness of brain tumors.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KFR-2005-015-E00174), and in part by Neurobiology Research Program (M10412000042-04N1200-04210) of the Ministry of Science and Technology, Republic of Korea.

REFERENCES

[1] Kargiotis O, Rao JS, Kyritsis AP. Mechanisms of angiogenesis in gliomas. J Neurooncol 2006;78:281–93.
[2] Rao JS. Molecular mechanisms of glioma invasiveness: the role of proteases. Nat Rev Cancer 2003;3:489–501.
[3] Salhia B, Tran NL, Symons M, Winkles JA, Rutka JT, Berens ME. Molecular pathways triggering glioma cell invasion. Expert Rev Mol Diagn 2006;6:613–26.
[4] Stamenkovic I. Matrix metalloproteinases in tumor invasion and metastasis. Semin Cancer Biol 2000;10:415–33.
[5] Lakka SS, Gondi CS, Rao JS. Proteases and glioma angiogenesis. Brain Pathol 2005;15:327–41.
[6] VanMeter TE, Rooprai HK, Kibble MM, Fillmore HL, Broaddus WC, Pilkington GJ. The role of matrix metalloproteinase genes in glioma invasion: co-dependent and interactive proteolysis. J Neurooncol 2001;53:213–35.
[7] Zhao JX, Yang LP, Wang YF, Qin LP, Liu DQ, Bai CX, et al. Gelatinolytic activity of matrix metalloproteinase-2 and matrix metalloproteinase-9 in rat brain after implantation of 9L rat glioma cells. Eur J Neurol 2007;14:510–6.
[8] Hu B, Jarzynka MJ, Guo P, Imanishi Y, Schlaepfer DD, Cheng SY. Angiopoietin 2 induces glioma cell invasion by stimulating matrix metalloprotease 2 expression through the alphavbeta1 integrin and focal adhesion kinase signaling pathway. Cancer Res 2006;66:775–83.
[9] Gondi CS, Lakka SS, Dinh DH, Olivero WC, Gujrati M, Rao JS. Downregulation of uPA, uPAR and MMP-9 using small, interfering, hairpin RNA (siRNA) inhibits glioma cell invasion, angiogenesis and tumor growth. Neuron Glia Biol 2004;1:165–76.
[10] Kim SY, Jung SH, Kim HS. Curcumin is a potent broad spectrum inhibitor of matrix metalloproteinase gene expression in human astroglioma cells. Biochem Biophys Res Commun 2005;337:510–6.
[11] Laurent M, Martinerie C, Thibout H, Hoffman MP, Verrecchia F, Le Bouc Y, et al. NOVH increases MMP3 expression and cell migration in glioblastoma cells via a PDGFR-alpha-dependent mechanism. FASEB J 2003;17:1919–21.
[12] Sounni NE, Devy L, Hajitou A, Frankenne F, Munaut C, Gilles C, et al. MT1-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression. FASEB J 2002;16:555–64.
[13] Shibata S. Chemistry and cancer preventing activities of ginseng saponins and some related triterpenoid compounds. J Korean Med Sci 2001;16:S28–37.
[14] Park EK, Choo MK, Kim EJ, Han MJ, Kim DH. Antiallergic activity of ginsenoside Rh2. Biol Pharm Bull 2003;26:1581–4.
[15] Kenarova B, Neychev H, Hadjiivanova C, Petkov VD. Immunomodulating activity of ginsenoside Rg1 from Panax ginseng. Jpn J Pharmacol 1990;54:447–54.
[16] Yun TK. Experimental and epidemiological evidence on non-organ specific cancer preventive effect of Korean ginseng and identification of active compounds. Mutat Res 2003;523/524:63–74.
[17] Oh M, Choi YH, Choi S, Chung H, Kim K, Kim SI, et al. Anti-proliferating effects of ginsenoside Rh2 on MCF-7 human breast cancer cells. Int J Oncol 1999;14:869–75.
[18] Oh JI, Chun KH, Joo SH, Oh YT, Lee SK. Caspase-3-dependent protein kinase C delta activity is required for the progression of Ginsenoside-Rh2-induced apoptosis in SK-HEP-1 cells. Cancer Lett 2005;230:228–38.
[19] Kim HE, Oh JH, Lee SK, Oh YJ. Ginsenoside RH-2 induces apoptotic cell death in rat C6 glioma via a reactive oxygen- and caspase-dependent but Bcl-X(L)-independent pathway. Life Sci 1999;65:33–40.
[20] Kim YS, Jin SH. Ginsenoside Rh2 induces apoptosis via activation of caspase-1 and -3 and up-regulation of Bax in human neuroblastoma. Arch Pharm Res 2004;27:834–9.
[21] Fei XF, Wang BX, Tashiro S, Li TJ, Ma JS, Ikejima T. Apoptotic effects of ginsenoside Rh2 on human malignant melanoma A375-S2 cells. Acta Pharmacol Sin 2002;23:315–22.
[22] Surh YJ, Lee JY, Choi KJ, Ko SR. Effects of selected ginsenosides on phorbol ester-induced expression of cyclooxygenase-2 and activation of NF-kappaB and ERK1/2 in mouse skin. Ann NY Acad Sci 2002;973:396–401.
[23] Park SA, Kim EH, Na HK, Surh YJ. KG-135 inhibits COX-2 expression by blocking the activation of JNK and AP-1 in phorbol ester-stimulated human breast epithelial cells. Ann NY Acad Sci 2007;1095:545–53.
[24] Bae EA, Hyun YJ, Choo MK, Oh JK, Ryu JH, Kim DH. Protective effect of fermented red ginseng on a transient focal ischemic rats. Arch Pharm Res 2004;27:1136–40.
[25] Bae EA, Han MJ, Choo MK, Park SY, Kim DH. Metabolism of 20(S)- and 20(R)-ginsenoside Rg3 by human intestinal bacteria and its relation to in vitro biological activities. Biol Pharm Bull 2002;25:58–63.
[26] Kim HS, Goncalves NM, Henson JW. Glial cell-specific regulation of the JC virus early promoter by large T antigen. J Virol 2000;74:755–63.
[27] Woo MS, Jung SH, Kim SY, Hyun JW, Ko KH, Kim WK, et al. Curcumin suppresses phorbol ester-induced matrix metalloproteinase-9 expression by inhibiting the PKC to MAPK signaling pathways in human astroglioma cells. Biochem Biophys Res Commun 2005;335:1017–25.
[28] Jung SH, Woo MS, Kim SY, Kim WK, Hyun JW, Kim EJ, et al. Ginseng saponin metabolite suppresses phorbol ester-

induced matrix metalloproteinase-9 expression through inhibition of activator protein-1 and mitogen-activated protein kinase signaling pathways in human astroglioma cells. Int J Cancer 2006;118:490–7.
[29] Chintala SK, Tonn JC, Rao JS. Matrix metalloproteinases and their biological function in human gliomas. Int J Dev Neurosci 1999;17:495–502.
[30] Nabeshima K, Inoue T, Shimao Y, Sameshima T. Matrix metalloproteinases in tumor invasion: role for cell migration. Pathol Int 2002;52:255–64.
[31] Lorenzl S, Albers DS, Chirichigno JW, Augood SJ, Beal MF. Elevated levels of matrix metalloproteinases-9 and -1 and of tissue inhibitors of MMPs, TIMP-1 and TIMP-2 in postmortem brain tissue of progressive supranuclear palsy. J Neurol Sci 2004;218:39–45.
[32] Mercapide J, Lopez De Cicco R, Castresana JS, Klein-Szanto AJ. Stromelysin-1/matrix metalloproteinase-3 (MMP-3) expression accounts for invasive properties of human astrocytoma cell lines. Int J Cancer 2003;106:676–82.
[33] Bodey B, Bodey Jr B, Siegel SE, Kaiser HE. Matrix metalloproteinase expression in childhood astrocytomas. Anticancer Res 2000;20:3287–92.
[34] Deryugina EI, Ratnikov B, Monosov E, Postnova TI, DiScipio R, Smith JW, et al. MT1-MMP initiates activation of pro-MMP-2 and integrin alphavbeta3 promotes maturation of MMP-2 in breast carcinoma cells. Exp Cell Res 2001;263:209–23.
[35] Munaut C, Noel A, Hougrand O, Foidart JM, Boniver J, Deprez M. Vascular endothelial growth factor expression correlates with matrix metalloproteinases MT1-MMP, MMP-2 and MMP-9 in human glioblastomas. Int J Cancer 2003;106:848–55.
[36] Sawaya R, Go Y, Kyritisis AP, Uhm J, Venkaiah B, Mohanam S, et al. Elevated levels of Mr 92,000 type IV collagenase during tumor growth in vivo. Biochem Biophys Res Commun 1998;251:632–6.
[37] Kondraganti S, Mohanam S, Chintala SK, Kin Y, Jasti SL, Nirmala C, et al. Selective suppression of matrix metalloproteinase-9 in human glioblastoma cells by antisense gene transfer impairs glioblastoma cell invasion. Cancer Res 2000;60:6851–5.
[38] Lakka SS, Rajan M, Gondi C, Yanamandra N, Chandrasekar N, Jasti SL, et al. Adenovirus-mediated expression of antisense MMP-9 in glioma cells inhibits tumor growth and invasion. Oncogene 2002;21:8011–9.
[39] Lim JH, Wen TC, Matsuda S, Tanaka J, Maeda N, Peng H, et al. Protection of ischemic hippocampal neurons by ginsenoside Rb1, a main ingredient of ginseng root. Neurosci Res 1997;28:191–200.
[40] Lee E, Kim S, Chung KC, Choo MK, Kim DH, Nam G, et al. 20(S)-ginsenoside Rh2, a newly identified active ingredient of ginseng, inhibits NMDA receptors in cultured rat hippocampal neurons. Eur J Pharmacol 2006;536:69–77.
[41] Lee BH, Jeong SM, Ha TS, Park CS, Lee JH, Kim JH, et al. Ginsenosides regulate ligand-gated ion channels from the outside. Mol Cells 2004;18:115–21.
[42] Park EK, Choo MK, Oh JK, Ryu JH, Kim DH. Ginsenoside Rh2 reduces ischemic brain injury in rats. Biol Pharm Bull 2004;27:433–6.
[43] Jang S, Ryu JH, Kim DH, Oh S. Changes of [3H]MK-801, [3H]muscimol and [3H]flunitrazepam binding in rat brain by the prolonged ventricular infusion of transformed ginsenosides. Neurochem Res 2004;29:2257–66.
[44] Bae EA, Han MJ, Kim EJ, Kim DH. Transformation of ginseng saponins to ginsenoside Rh2 by acids and human intestinal bacteria and biological activities of their transformants. Arch Pharm Res 2004;27:61–7.