HET

Human and Experimental Toxicology
30(5) 406–415
© The Author(s) 2010
Reprints and permission:
sagepub.co.uk/journalsPermissions.nav
DOI: 10.1177/0960327110372405
het.sagepub.com
SAGE

Gypenosides inhibits migration and invasion of human oral cancer SAS cells through the inhibition of matrix metalloproteinase-2 -9 and urokinase-plasminogen by ERK1/2 and NF-kappa B signaling pathways

Kung-Wen Lu[1], Jung-Chou Chen[1], Tung-Yuan Lai[2,3], Jai-Sing Yang[4], Shu-Wen Weng[1], Yi-Shih Ma[1], Pei-Jung Lu[5], Jing-Ru Weng[6], Fu-Shin Chueh[7], W Gibson Wood[8] and Jing-Gung Chung[6,9]

Abstract

Gypenosides (Gyp), found in *Gynostemma pentaphyllum* Makino, has been used as a folk medicine in the Chinese population for centuries and is known to have diverse pharmacologic effects, including anti-proliferative and anti-cancer actions. However, the effects of Gyp on prevention from invasion and migration of oral cancer cells are still unsatisfactory. The purpose of this study was to investigate effects of Gyp treatment on migration and invasion of SAS human oral cancer cells. SAS cells were cultured in the presence of 90 and 180 μg/mL Gyp for 24 and 48 hours. Gyp induced cytotoxic effects and inhibited SAS cells migration and invasion in dose- and time-dependent response. Wound-healing assay and boyden chamber assay were carried out to investigate Gyp-inhibited migration and invasion of SAS cells. Gyp decreased the abundance of several proteins, including nuclear factor-kappa B (NF-κB), cyclooxygenase-2 (COX-2), extracellular signal-regulated kinase 1/2 (ERK1/2), matrix metalloproteinase-9, -2 (MMP-9, -2), sevenless homolog (SOS), Ras, urokinase-type plasminogen activator (uPA), focal adhesion kinase (FAK) and RAC-alpha serine/threonine-protein kinase (Akt), in a time-dependent manner. In addition, Gyp decreased mRNA levels of MMP-2, MMP-7, MMP-9 but did not affect FAK and Rho A mRNA levels in SAS cells. These results provide evidences for the role of Gyp as a potent anti-metastatic agent, which can markedly inhibit the metastatic and invasive capacity of oral cancer cells. The inhibition of NF-κB and MMP-2, -7 and -9 signaling may be one of the mechanisms that is present in Gyp-inhibited cancer cell invasion and migration.

[1] School of Chinese Medicine, China Medical University, Taichung, Taiwan
[2] School of Post-baccalaureate Chinese Medicine, China Medical University, Taichung, Taiwan
[3] Department of Chinese Internal Medicine, China Medical University Hospital, Taichung, Taiwan
[4] Department of Pharmacology, China Medical University, Taichung, Taiwan
[5] Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan
[6] Department of Biological Science and Technology, China Medical University, Taichung, Taiwan
[7] Department of Health and Nutrition Biotechnology, Asia University, Wufeng, Taichung, Taiwan
[8] Department of Pharmacology, School of Medicine, Geriatric Research, Education and Clinical Center, VA Medical Center, University of Minnesota, Minneapolis, Minnesota, USA
[9] Department of Biotechnology, Asia University, Wufeng, Taichung, Taiwan

Corresponding author:
Tung-Yuan Lai, School of Post-baccalaureate Chinese Medicine, China Medical University, No 91, Hsueh-Shih Road, Taichung 40402, Taiwan
Email: tylai@mail.cmu.edu.tw

Keywords
gypenosides, cell migration, cell invasion, matrix metalloproteinase-2 -9, urokinase-plasminogen, SAS cells

Introduction

Over 300,000 cases of oral and oropharyngeal cancers are diagnosed annually worldwide.[1,2] To date, efficacy of different treatments (surgery, radiotherapy and chemotherapy) for oral and oropharyngeal cancers have not been satisfactory. Many components from natural plants and their derivatives have been shown to inhibit cancer, such as paclitaxel which is derived from *Taxus brevifolia,*[1,3] and it has been used clinically for lung cancer for over 20 years.[3] *Gynostemma pentaphyllum* Makino (family Cucurbitaceae) has been used as a folk medicine in the Chinese population for centuries. Gypenosides (Gyp) are the major components in *Gynostemma pentaphyllum* Makino,[4] and it has a variety of pharmacological effects including anti-inflammatory, anti-oxidative,[5] anti-hyperlipidemic, anti-cardiovascular[6-8] and anti-cancer.[9-12] Gyp induced apoptosis in human hepatoma cells via the mitochondria-dependent pathway.[9] In our laboratory, we found that Gyp affected *N*-acetyltransferase activity and gene expression in human cervical cancer cells.[13] We had reported that Gyp induced apoptosis in human colon cancer colo 205 cells through the mitochondria-dependent pathway and activation of caspase-3.[14] Recently, we reported that Gyp induced apoptosis in human tongue cancer SCC-4 cells through the endoplasmic reticulum stress and mitochondria-dependent pathways.[1]

There is no information on effects of Gyp on migration and invasion of human oral cancer SAS cells. Therefore, in the present study, we selected human oral cancer SAS cells for examining the effects of Gyp on migration and invasion of SAS cells. Gyp inhibited the migration and invasion of SAS cells by inhibition of matrix metalloproteinase (MMP)-2, -7 and -9 by nuclear factor-kappa B (NF-κB) signaling pathways.

Materials and methods

Chemicals, reagents and cell culture

Gyp was kindly provided by Dr Jung-Chou Chen (School of Chinese Medicine, China Medical University). Dimethyl sulfoxide (DMSO), potassium phosphates and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Dulbecco's modified Eagle's medium (DMEM), L-glutamine, fetal bovine serum (FBS) and penicillin–streptomycin, trypsin-EDTA were obtained from Invitrogen (Carlsbad, California, USA). The primary antibodies (anti-NF-κB, -COX-2, -ERK1/2, - MMP-9, -MMP-2, -SOS, -Ras, -uPA, -FAK, -PI3K, -Akt) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, California, USA). All materials used for gel electrophoresis were obtained from Bio-Rad Laboratories, Inc. (Hercules, California, USA). The SAS cell line (human oral squamous cell carcinoma) was obtained from Dr Pei-Jung Lu (Graduate Institute of Clinical Medicine, National Cheng Kung University, Tainan, Taiwan). The cells were cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 100 Units/mL penicillin and 100 μg/mL streptomycin in 75 cm^2 tissue culture flasks at 37°C under a humidified 5% CO_2 and 95% air atmosphere as we have previously reported.[1]

Viability of SAS cells

Cells (2×10^5 cells/well) were plated in 12-well plates and incubated at 37°C for 24 hours before each well were co-treated with 0, 90 and 180 μg/mL Gyp for 24 hours. DMSO was used for the control regimen. All cells were harvested and stained with Propidium iodide (PI) (5 μg/mL) and then were analyzed by flow cytometry (Becton-Dickinson, San Jose, California, USA) as previously described.[15,16]

Wound healing assay

Cells were cultured in 6-well plates for 24 hours and then were wounded by scratching with a sterile pipette tip (to remove the cells but both sides still have original cells) and incubated with DMEM containing no FBS, and then the cells were treated with Gyp (90 and 180 μg/mL) or without (control) for 0, 24 and 48 hours. The migrated cells in each well were photographed under phase-contrast microscopy (×200) as previously described.[17]

Invasion and migration determinations

Cell invasion was determined by using Matrigel (BD Biosciences, Franklin Lakes, New Jersey,

USA)-coated transwell cell culture chambers (8 μm pore size; Millipore, Billerica, Massachusetts, USA) as previously described.[18] SAS cells were kept for 24 hours in serum-free medium and then were trypsinized and resuspended in serum-free DMEM and placed in the upper chamber of the transwell insert (5×10^4 cells/well) and incubated with 0.5% DMSO or Gyp (90 and 180 μg/mL), and DMEM containing 10% FBS was added to the lower chamber. The plates were incubated in a humidified atmosphere with 95% air and 5% CO_2 at 37°C for 24 or 48 hours, non-invasive cells in the upper chamber were removed by wiping with a cotton swab and invasive cells were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. Cells in the lower surface of the filter that penetrated through the Matrigel were counted under a light microscope at ×200.[17] Cell migration was determined as described for cell invasion assay except that the filter membrane was not coated with Matrigel. Cells located on the underside of the filter were counted under a light microscope at ×200.[17,19]

Western blotting analysis

SAS cells were cultured on 6-well culture plates and grown for 24 hours. Gyp was added to cells at a final concentration of 180 μg/mL, while DMSO (solvent) alone was added to control cells. Cells from each treatment were incubated in DMEM with 0.5% FBS at 37°C for 0, 6, 12, 24, 48 and 72 hours. The cells in each treatment were harvested by centrifugation and resuspended in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing 2 mM EDTA and 0.1% Triton X-100. The collected cells were sonicated and centrifugated at 13,000*g* for 10 min at 4°C to remove cell debris and the supernatant collected for determination of total protein concentration by using a Bio-Rad protein assay kit (Hercules), with bovine serum albumin (BSA) as the standard. Sodium dodecyl sulfate (SDS) gel electrophoresis and Western blotting were performed as described previously[17] for determining the effects of Gyp on protein levels of NF-κB, cyclooxygenase-2 (COX-2), extracellular signal-regulated kinase 1/2 (ERK1/2), MMP-9, MMP-2, sevenless homolog (SOS), Ras, urokinase-type plasminogen activator (uPA), focal adhesion kinase (FAK), PI3K and alpha serine/threonine-protein kinase (Akt). Due to the observation that Gyp treatment altered total protein levels, β-actin, which was not altered by Gyp, was used to normalize changes in specific protein levels.

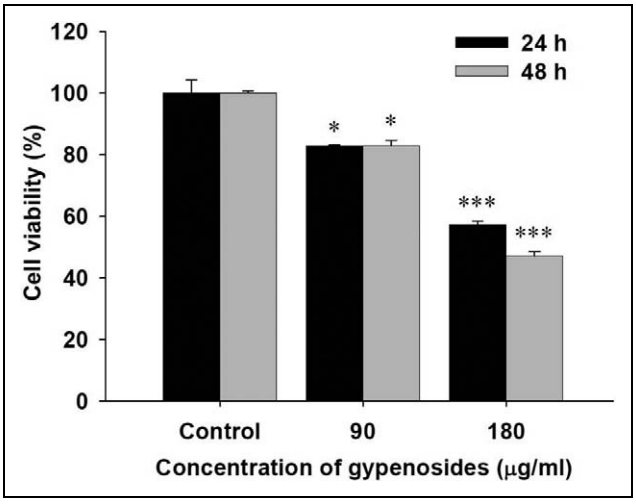

Figure 1. Gypenosides (Gyp) affected the viability of SAS cells. The SAS cells (2×10^5 cells/well) were placed in 12-well plates and incubated at 37°C for 24 hours before they were co-treated with various doses of Gyp for 24 hours. DMSO (dimethyl sulfoxide; solvent) was used for the control regimen. Cells were stained with PI and analyzed by flow cytometry as described in materials and methods section. Each point is mean ± SD of three experiments. *$p < 0.05$ and ***$p < 0.001$ vs control.

Real-time polymerase chain reaction (PCR)

Cells were cultured in 6-well culture plates and 180 μg/mL Gyp was added to cells for 24 or 48 hours. Cells were collected and total RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, California, USA) as previously described.[17,20] RNA was reverse-transcribed for 30 min at 42°C with High Capacity cDNA Reverse Transcription Kit according to the standard protocol of the supplier (Applied Biosystems, Foster City, California, USA). Quantitative PCR was performed by the following conditions: 2 min at 50°C, 10 min at 95°C and 40 cycles of 15 sec at 95°C, 1 min at 60°C using 1 μL of the cDNA reverse-transcribed as described above, 2X SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward (F) and reverse (R) primers, including MMP-2: F-CCCCAGACAGGTGATCTT GAC, R-GCTTGCGAGGGAAGAAGTTG; MMP7: F-GGATGGTAGCAGTCTAGGGATTAACT, R-AG GTTGGATA CCACTGCATTAGG; MMP9: F-CG CTGGGCTTAGATCATTCC, R-GTGCCGGATGCC ATTCAC; FAK: F-TGAA TGGAACCTCGCAGTCA, R-TCCGCATGCCTTGCTTTT; Rho A: F- TCAAGC CGGAGGTCAACAAC, R-ACGAGCTGCCCATAGC AGAA; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): F-ACACCCACTCCTCCACCTTT, R-TA

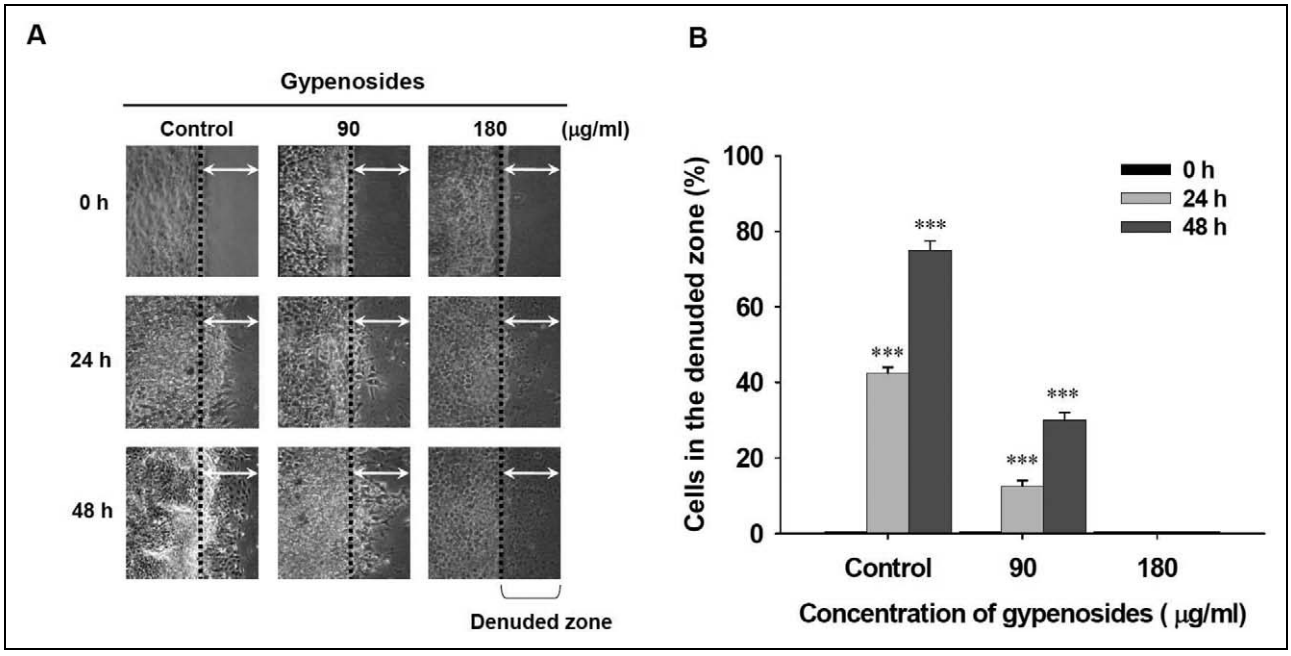

Figure 2. Gypenosides (Gyp) inhibited the migration of SAS cells in vitro. Cells in 6-well plates were wounded by scratching with a pipette tip and the cells were incubated with Dulbecco's modified Eagle's medium (DMEM) containing no fetal bovine serum (FBS) and incubation with or without Gyp for 24 and 48 hours. The cells were photographed under phase-contrast microscopy (×200; A) performed by counting cells (B). ***$p < 0.001$ vs control.

GCCAAATTCGTTGTCATACC. Each assay was run on an Applied Biosystems 7300 Real-Time PCR system in triplicates and expression fold-changes were derived using the comparative C_T method.[17,20]

Statistical analysis

All results were expressed triplicate as mean ± SD. Differences between the Gyp-treated and control groups were analyzed by Student's *t* test, with values of *$p < 0.05$ and ***$p < 0.001$ considered significant.

Results

Gyp affected the viability of human oral cancer SAS cells

Cells were incubated with 90 and 180 μg/mL of Gyp for 24 and 48 hours, and cell viability was determined. Figure 1 shows that there were fewer viable cells as Gyp concentration increased when compared to control groups. These effects were time-dependent.

Gyp inhibited the migration of SAS cells in vitro

Inhibition of cell migration by Gyp was determined using a wound-healing assay and the results are shown in Figure 2A and B. The higher Gyp concentration (180 μg/mL) and longer period of time (48 hours) incubation time produced greater inhibition as compared with lower concentrations and shorter incubation times. Percentage of inhibition ratio was 30%–48% for the 24-hour treatment, 30%–58% for the 48-hour treatment. Gyp significantly inhibited SAS cells motility.

Gyp suppressed the invasion and migration of SAS cells

One of the major characteristic of metastasis is the migratory and invasive ability of tumor cells. Effects of Gyp inhibition of invasion and migration were examined by Boyden Chamber assay and these results are shown in Figure 3. Gyp inhibited cell migration with increasing concentration in a dose-and time-dependent manner (Figure 3A and B). At 90 μg/mL of Gyp, the cell migration was reduced to 32% and 46% for 24- and 48-hour treatment, respectively, and at 180 μg/mL of Gyp, the cell migration was reduced to 63% and 78% for 24- and 48-hour treatment, respectively. Gyp also induced a dose-dependent decrease in invasion (Figure 3C and D). At 90 μg/mL of Gyp, cell invasion was reduced to 28% and 65% for 24-hour and 48-hour treatment, respectively, and at 180 μg/mL of Gyp, the invasion was reduced to 51% and 80% for 24-hour and 48-hours treatment,

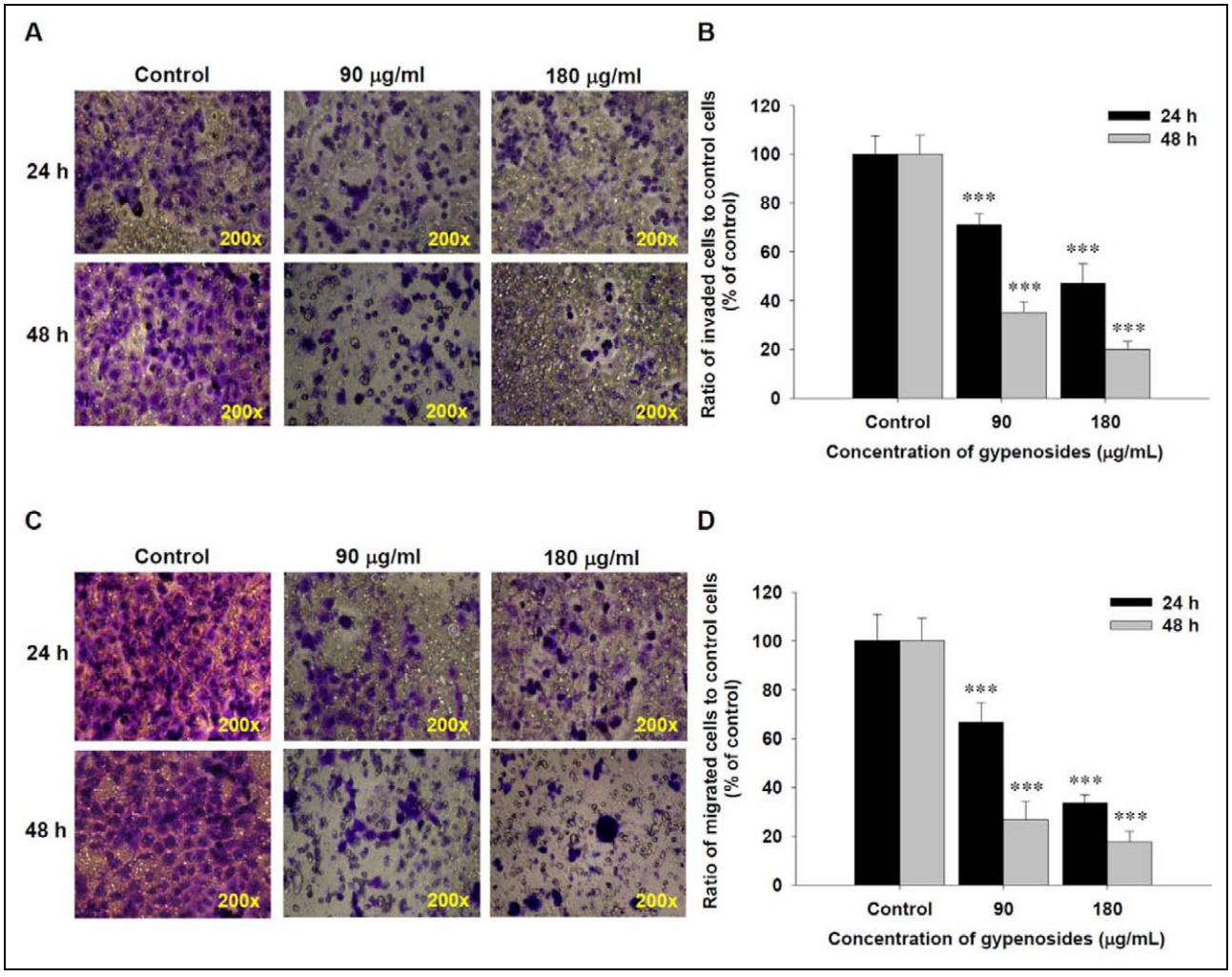

Figure 3. Gypenosides (Gyp) suppressed the migration and invasion of SAS cells in vitro. Cells that penetrated through without or with the Matrigel to the lower surface of the filter were stained with crystal violet and photographed under a light microscope at ×200 (A and C). Quantification of cells in the lower chambers was performed by counting cells at ×200 (B and D). Columns repeat the mean from three independent experiments. ***$p < 0.001$, significant difference between Gyp-treated groups and the control as analyzed by Student's *t* test.

respectively. These results demonstrated that Gyp significantly inhibited the migration and invasion of SAS cells.

Gyp decreased the levels of migration- and invasion-associated proteins in SAS cells

Levels of invasion- and migration-associated proteins in SAS cells after treatment with Gyp for different time periods were determined by Western blotting. The results indicate that the levels of NF-κB, COX-2, ERK1/2, MMP-2 and MMP-9 (Figure 4A) and SOS, Ras, uPA, FAK and Akt (Figure 4B) were lower in Gyp-treated cells than control cells. However, PI3K (Figure 4B) was lower than that of control for up to 12 hours and then started to increase to that of control levels for up to 48 hours.

Gyp reduced MMP-2, MMP-7 and MMP-9 mRNA expressions in SAS cells

In order to further investigate whether Gyp affected migration- and invasion-associated gene expression in SAS cells, the cells were treated with Gyp (180 μg/mL) for 0, 24 or 48 hours. Total RNA was isolated from Gyp-treated and control groups, and gene expressions were examined by real-time PCR. The results are shown in Figure 5A and B which indicate that the expression levels of MMP-2, MMP-7 and MMP-9 (Figure 5A) were inhibited during Gyp treatment for 48 hours and

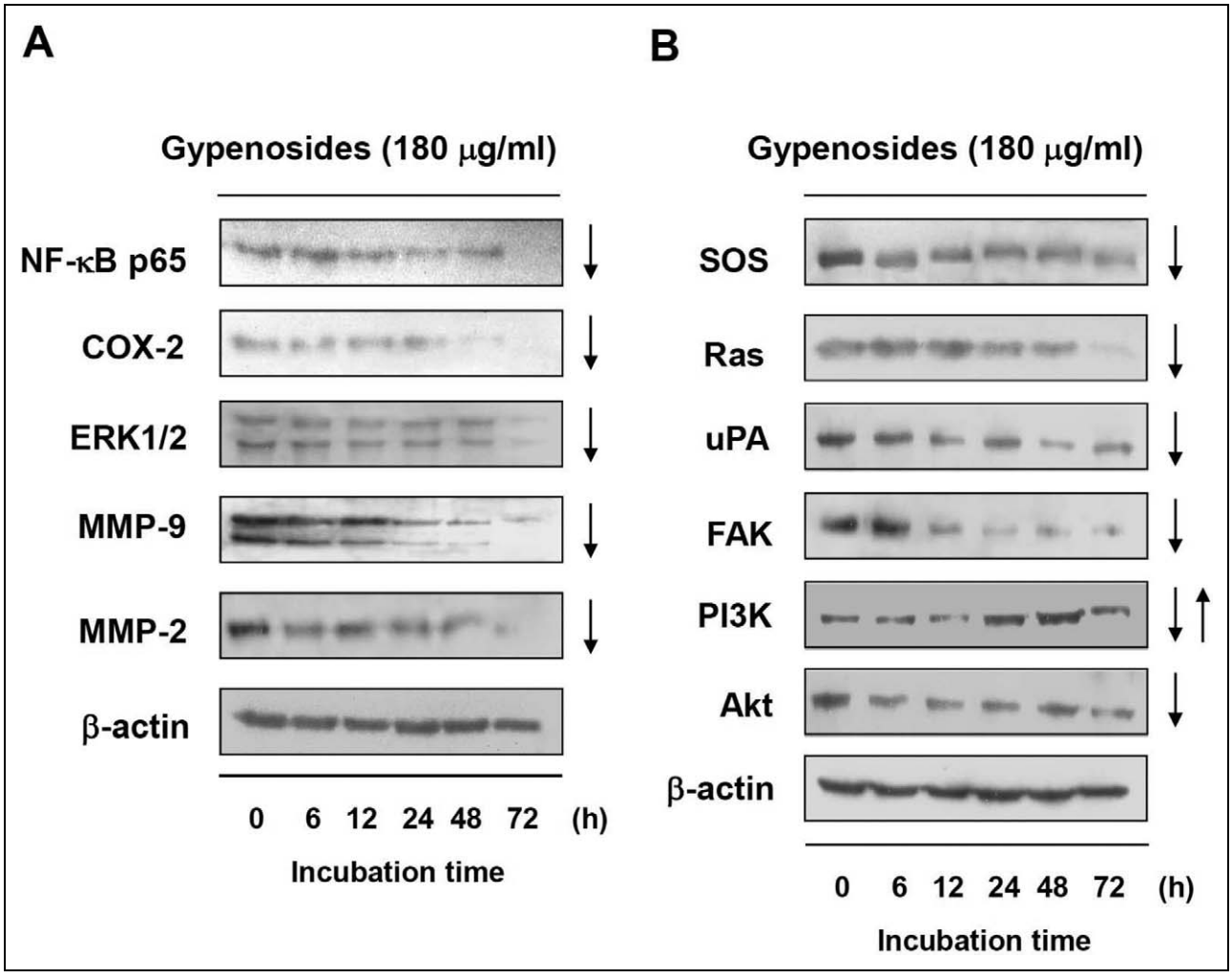

Figure 4. Representative Western blotting analyses demonstrated the changes in the levels of associated proteins in migration and invasion of SAS cells after exposure to gypenosides (Gyp). The SAS cells (5×10^6 cells/well) were treated with Gyp at 180 μg/mL for different periods of time (0, 6, 12, 24, 48 and 72 hours) and then cells were collected and the total protein extracts were prepared and determined as described in materials and methods section. The levels of nuclear factor-kappa B (NF-κB), cyclooxygenase-2 (COX-2), extracellular signal-regulated kinase 1/2 (ERK1/2), matrix metalloproteinase (MMP)-9 and MMP-2 (panel A), sevenless homolog (SOS), Ras, urokinase-type plasminogen activator (uPA), focal adhesion kinase (FAK), PI3K and threonine-protein kinase (Akt; panel B) expressions were estimated by Western blotting as described in materials and methods.

FAK and Rho A (Figure 5B) were not significantly reduced after exposure to Gyp for 24 hours.

Discussion

Oral squamous cell carcinoma (OSCC) can be aggressive if not detected early. Approximately 50% of OSCC patients have nodal metastases and the 5-year survival rate is less than 50%.[21] Metastasis is one of the major challenges for a successful cancer treatment. In the present study, we show that Gyp significantly inhibited the invasion and migration of SAS cells, which is one kind of OSCC cell line. Furthermore, our findings showed that Gyp could decrease protein levels of tumor metastasis-related proteins such as MMP-2, -9 and uPA (Figure 4A and B). These findings are the first reported to address the inhibitory effects of Gyp on oral cancer invasiveness and migration via decreasing the production of tumor-associated metastatic-related proteins in SAS cells.

Tumor invasion requires degradation of basement membranes, proteolysis of extracellular matrix (ECM), pseudopodial extension and cell migration.[22] The basement membrane is the first barrier of the ECM

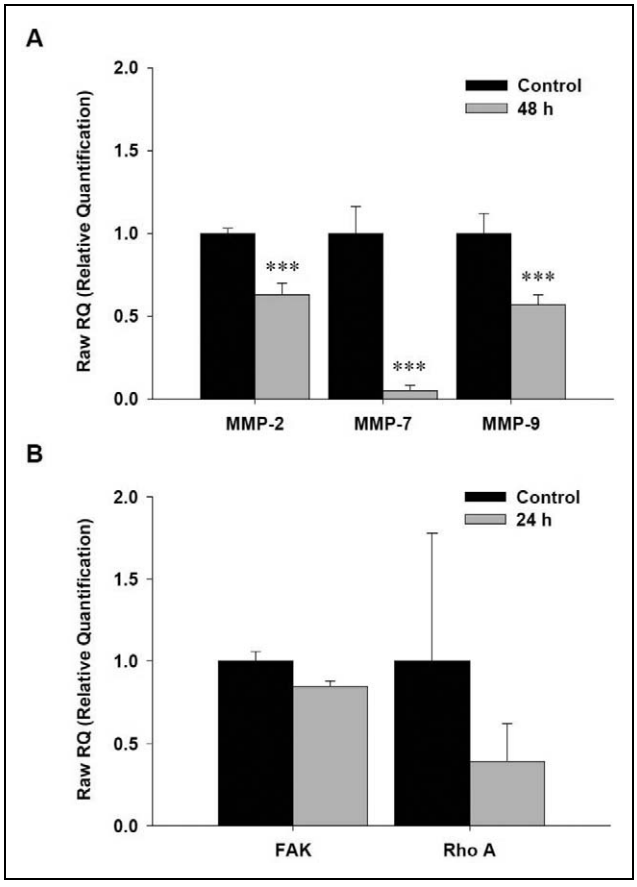

Figure 5. Effects of gypenosides (Gyp) on matrix metalloproteinase (MMP)-2, MMP-7, MMP-9, FAK and Rho A mRNA expressions in SAS cells. The total RNA was extracted from SAS cells after Gyp (180 μg/ml) treatment for 0, 24 and 48 hours, and RNA samples were reverse-transcribed cDNA then for real-time PCR as described in materials and methods section. The ratios of MMP-2, -7 and -9 (A), focal adhesion kinase (FAK) and Rho A (B) mRNA/GAPDH are presented in panels A and B. Data represents mean ± SD of three experiments. ***Indicate $p < 0.001$ vs control.

against cancer invasion. A number of proteolytic enzymes, including MMPs and serine proteinases, contribute to the degradation of underlying basement membrane. MMPs play a role in the progression of oral cancer[23] and they are secreted by invasive cancer cells, Therefore, MMPs play an important role in cancer cell invasion and metastasis.[24,25] In addition to MMPs, the serine proteinase, uPA and cathepsins are involved during metastasis and angiogenesis, which degrades basement membranes and activates pro-MMPs.[26] Inhibition of the invasion-mediated by MMP-2 and uPA can reduce metastasis, suggesting the possible usefulness of specifically selected MMP inhibitors would be worthy of investigation also as chemopreventive agents in patients at high risk of developing oral cancer. Other reports have shown that MMP-2 was associated with tongue cancer[27] and MMP-9 associated with nasopharyngeal carcinoma[28] and with the recurrence rate of head and neck SCC.[29] Thus, elevated expression of MMP appears to be related to the invasion, aggressiveness and overall survival of patients with head and neck cancers.

The 52-kDa uPA plays a major role in the decomposition of basement membranes, and expression of uPA is increased in solid tumors. Our results showed that Gyp decreased the uPA protein levels in SAS cells. It was also reported that the activation of the uPA/uPAR/plasmin proteolytic network occurred with tumor invasion and dissemination of various malignancies.[30,31] The presence of uPA in tumors can be useful as a potential prognostic factor and a prognostic enzyme-linked immunosorbent assay (ELISA) for uPA for solid cancers.[32] Moreover, the levels of uPA and uPAR expression have served as prognostic markers in various malignancies.[33] Thus, we examined the expression of MMPs, FAK and Rho A in SAS cells and observed that real-time PCR revealed a marked decrease in the level of MMP-2, -7 and -9 mRNA after Gyp treatment for 24 hours. These results indicated that Gyp inhibited the expression of MMP-2, -7 and -9 in oral cavity carcinoma cells (SAS). Our results showed that Gyp suppressed the levels of ERK1/2, NF-κB, uPA, Ras, SOS, COX-2, Akt, FAK, MMP-2 and -9 protein levels in SAS cells. It has been reported that MMP-9 and uPA proteins require NF-κB[34,35] and activator protein-1 (AP-1) for production. Other investigators also showed that the activation of ERK will stimulate two cis-acting regulatory elements such as the binding sites of AP-1 and NF-κB, which play an important role in controlling *MMP-9* gene expression.[36]

Gyp also inhibited protein levels of FAK, Ras and ERK1/2 in SAS cells. Clinical evidence indicates that high FAK expression relates to tumor progression and reduced FAK expression reduced expression of MMPs.[37] FAK is a positive regulator of nonmalignant cell migration and cell survival[38] and increased FAK expression has been detected in several types of human cancers.[37] FAK is required for tumor development induced by some oncogenic proteins, which activate a positive FAK-c-Src feedback loop with ERK and Akt. Ras has been demonstrated to be either mutated or activated in many types of human cancer, and it is

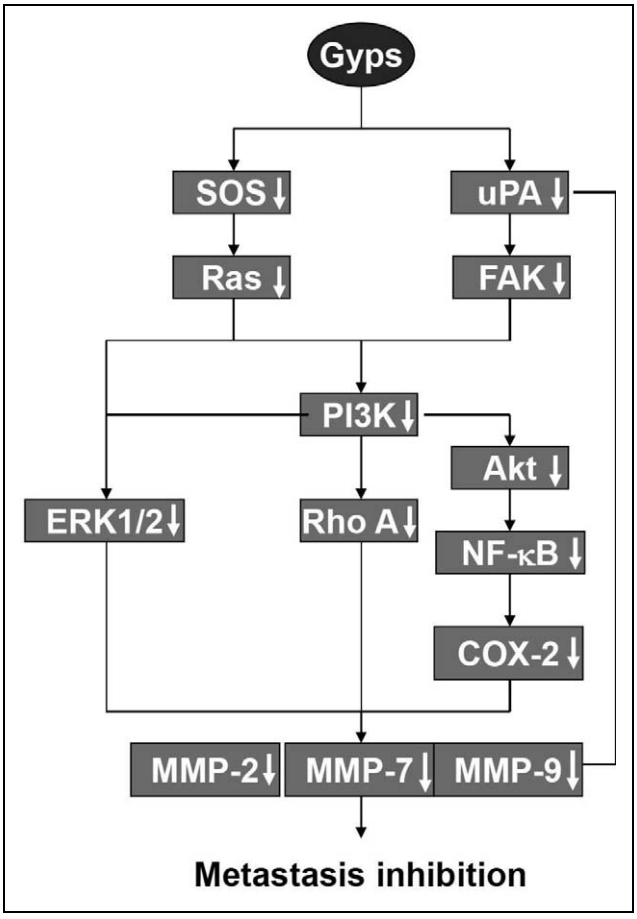

Figure 6. The possible signaling pathways for gypenosides (Gyp) inhibited cell invasion and migration in human oral cancer SAS cells.

recognized as a driving force for tumor development in mouse models.[39] Aberrant activation of Ras protein has been found in the malignant phenotype of cancer cells that are associated with cellular proliferation, transformation, invasion and metastasis.[40] Other reports also showed that elevated Ras correlated with ERK activation that has been detected in human glioblastoma multiforme (GBM) specimens.[41] We demonstrated that Gyp inhibited MMP-2 and -9, which are associated with ERK1/2, Ras, FAK and Rho A in SAS cells.

In conclusion, our observations show that Gyp exerted an inhibitory effect on several essential steps of metastasis, including cell invasion and migration, through NF-κB and ERK signaling pathways, resulting in the inhibition of MMP-2 and -9 as summarized in Figure 6. Gyp and other flavones could possibly regulate the activities of invasion-associated proteinases and their natural inhibitors. Our results indicate that Gyp may be a powerful candidate to be used as a preventive agent against oral cancer metastasis.

Acknowledgement

Tung-Yuan Lai and Jing-Gung Chung contributed equally to this work.

Funding

This work was supported by grant CMU97-094 from China Medical University, Taichung, Taiwan.

References

1. Chen JC, Lu KW, Tsai ML, et al. Gypenosides induced G0/G1 arrest via CHk2 and apoptosis through endoplasmic reticulum stress and mitochondria-dependent pathways in human tongue cancer SCC-4 cells. *Oral Oncol* 2009; 45: 273–283.
2. Pintos J, Black MJ, Sadeghi N, et al. Human papillomavirus infection and oral cancer: a case-control study in Montreal, Canada. *Oral Oncol* 2008; 44: 242–250.
3. Shingyoji M, Ando S, Nishimura H, et al. VEGF in patients with non-small cell lung cancer during combination chemotherapy of carboplatin and paclitaxel. *Anticancer Res* 2009; 29: 2635–2639.
4. Cui J, Eneroth P, Bruhn JG. Gynostemma pentaphyllum: identification of major sapogenins and differentiation from Panax species. *Eur J Pharm Sci* 1999; 8: 187–191.
5. Shang L, Liu J, Zhu Q, et al. Gypenosides protect primary cultures of rat cortical cells against oxidative neurotoxicity. *Brain Res* 2006; 1102: 163–174.
6. Megalli S, Davies NM, Roufogalis BD. Anti-hyperlipidemic and hypoglycemic effects of Gynostemma pentaphyllum in the Zucker fatty rat. *J Pharm Pharm Sci* 2006; 9: 281–291.
7. Circosta C, De Pasquale R, Occhiuto F. Cardiovascular effects of the aqueous extract of Gynostemma pentaphyllum Makino. *Phytomedicine* 2005; 12: 638–643.
8. Aktan F, Henness S, Roufogalis BD, et al. Gypenosides derived from *Gynostemma pentaphyllum* suppress NO synthesis in murine macrophages by inhibiting iNOS enzymatic activity and attenuating NF-kappa B-mediated iNOS protein expression. *Nitric Oxide* 2003; 8: 235–242.
9. Wang QF, Chen JC, Hsieh SJ, et al. Regulation of Bcl-2 family molecules and activation of caspase cascade involved in gypenosides-induced apoptosis in human hepatoma cells. *Cancer Lett* 2002; 183: 169–178.
10. Hu L, Chen Z, Xie Y. New triterpenoid saponins from *Gynostemma pentaphyllum*. *J Nat Prod* 1996; 59: 1143–1145.
11. Wang C, Wang X, Li Y, et al. A preliminary observation of preventive and blocking effect of Gynostemma pentaphyllum (Thunb) Makino on esophageal cancer

in rats. *Hua Xi Yi Ke Da Xue Xue Bao* 1995; 26: 430–432.
12. Zhou Z, Wang Y, Zhou Y. The effect of *Gynostemma pentaphyllum* mak (GP) on carcinogenesis of the golden hamster cheek pouch induced by DMBA. *Zhonghua Kou Qiang Yi Xue Za Zhi* 1996; 31: 267–270.
13. Chiu TH, Chen JC, Chung JG. N-acetyltransferase is involved in gypenosides-induced *N*-acetylation of 2-aminofluorene and DNA adduct formation in human cervix epidermoid carcinoma cells (Ca Ski). *In Vivo* 2003; 17: 281–288.
14. Chen JC, Lu KW, Lee JH, et al. Gypenosides induced apoptosis in human colon cancer cells through the mitochondria-dependent pathways and activation of caspase-3. *Anticancer Res* 2006; 26: 4313–4326.
15. Lai WW, Yang JS, Lai KC, et al. Rhein induced apoptosis through the endoplasmic reticulum stress, caspase- and mitochondria-dependent pathways in SCC-4 human tongue squamous cancer cells. *In Vivo* 2009; 23: 309–316.
16. Lin SY, Lai WW, Ho CC, et al. Emodin induces apoptosis of human tongue squamous cancer SCC-4 cells through reactive oxygen species and mitochondria-dependent pathways. *Anticancer Res* 2009; 29: 327–335.
17. Ho YT, Yang JS, Li TC, et al. Berberine suppresses in vitro migration and invasion of human SCC-4 tongue squamous cancer cells through the inhibitions of FAK, IKK, NF-kappa-B, u-PA and MMP-2 and -9. *Cancer Lett* 2009; 279: 155–162.
18. Fishman DA, Liu Y, Ellerbroek SM, et al. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and MMP-dependent invasion in ovarian cancer cells. *Cancer Res* 2001; 61: 3194–3199.
19. Hsu SC, Kuo CL, Lin JP, et al. Crude extracts of Euchresta formosana radix inhibit invasion and migration of human hepatocellular carcinoma cells. *Anticancer Res* 2007; 27: 2377–2384.
20. Lin SS, Lai KC, Hsu SC, et al. Curcumin inhibits the migration and invasion of human A549 lung cancer cells through the inhibition of matrix metalloproteinase-2 and -9 and vascular endothelial growth factor (VEGF). *Cancer Lett* 2009; 285: 127-133.
21. Zhang X, Liu Y, Gilcrease MZ, et al. A lymph node metastatic mouse model reveals alterations of metastasis-related gene expression in metastatic human oral carcinoma sublines selected from a poorly metastatic parental cell line. *Cancer* 2002; 95: 1663–1672.
22. Woodhouse EC, Chuaqui RF, Liotta LA. General mechanisms of metastasis. *Cancer* 1997; 80: 1529–1537.
23. Thomas GT, Lewis MP, Speight PM. Matrix metalloproteinases and oral cancer. *Oral Oncol* 1999; 35: 227–233.
24. Stacker SA, Achen MG, Jussila L, et al. Lymphangiogenesis and cancer metastasis. *Nat Rev Cancer* 2002; 2: 573–583.
25. Tryggvason K, Hoyhtya M, Salo T. Proteolytic degradation of extracellular matrix in tumor invasion. *Biochim Biophys Acta* 1987; 907: 191–217.
26. Pepper MS. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. *Arterioscler Thromb Vasc Biol* 2001; 21: 1104–1117.
27. Yoshizaki T, Maruyama Y, Sato H, et al. Expression of tissue inhibitor of matrix metalloproteinase-2 correlates with activation of matrix metalloproteinase-2 and predicts poor prognosis in tongue squamous cell carcinoma. *Int J Cancer* 2001; 95: 44–50.
28. Jeon YK, Lee BY, Kim JE, et al. Molecular characterization of Epstein-Barr virus and oncoprotein expression in nasopharyngeal carcinoma in Korea. *Head Neck* 2004; 26: 573–583.
29. Pacheco MM, Kowalski LP, Nishimoto IN, et al. Differential expression of c-jun and c-fos mRNAs in squamous cell carcinoma of the head and neck: associations with uPA, gelatinase B, and matrilysin mRNAs. *Head Neck* 2002; 24: 24–32.
30. Andreasen PA, Kjoller L, Christensen L, et al. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int J Cancer* 1997; 72: 1–22.
31. Kwaan HC. The plasminogen-plasmin system in malignancy. *Cancer Metastasis Rev* 1992; 11: 291–311 .
32. Blasi F. The urokinase receptor. A cell surface, regulated chemokine. *APMIS* 1999; 107: 96–101.
33. Duffy MJ, Maguire TM, McDermott EW, et al. Urokinase plasminogen activator: a prognostic marker in multiple types of cancer. *J Surg Oncol* 1999; 71: 130–135.
34. Farina AR, Tacconelli A, Vacca A, et al. Transcriptional up-regulation of matrix metalloproteinase-9 expression during spontaneous epithelial to neuroblast phenotype conversion by SK-N-SH neuroblastoma cells, involved in enhanced invasivity, depends upon GT-box and nuclear factor kappaB elements. *Cell Growth Differ* 1999; 10: 353–367.
35. Bond M, Fabunmi RP, Baker AH, et al. Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappa B. *FEBS Lett* 1998; 435: 29–34.

36. Rhee JW, Lee KW, Sohn WJ, et al. Regulation of matrix metalloproteinase-9 gene expression and cell migration by NF-kappa B in response to CpG-oligodeoxynucleotides in RAW 264.7 cells. *Mol Immunol* 2007; 44: 1393–400.
37. McLean GW, Carragher NO, Avizienyte E, et al. The role of focal-adhesion kinase in cancer – a new therapeutic opportunity. *Nat Rev Cancer* 2005; 5: 505–515.
38. Parsons JT. Focal adhesion kinase: the first ten years. *J Cell Sci* 2003; 116: 1409–1416.
39. Chin L, Tam A, Pomerantz J, et al. Essential role for oncogenic Ras in tumour maintenance. *Nature* 1999; 400: 468–472.
40. Campbell PM, Der CJ. Oncogenic Ras and its role in tumor cell invasion and metastasis. *Semin Cancer Biol* 2004; 14: 105–114.
41. Feldkamp MM, Lala P, Lau N, et al. Expression of activated epidermal growth factor receptors, Ras-guanosine triphosphate, and mitogen-activated protein kinase in human glioblastoma multiforme specimens. *Neurosurgery* 1999; 45: 1442–1453.