Emodin, aloe-emodin and rhein inhibit migration and invasion in human tongue cancer SCC-4 cells through the inhibition of gene expression of matrix metalloproteinase-9

YA-YIN CHEN[1,4], SU-YIN CHIANG[1], JAUNG-GENG LIN[1], YI-SHIH MA[1,6], CHING-LUNG LIAO[1], SHU-WEN WENG[1], TUNG-YUAN LAI[3,5] and JING-GUNG CHUNG[2]

[1]School of Chinese Medicine, [2]Department of Biological Science and Technology, [3]School of Post-Baccalaureate Chinese Medicine, China Medical University, Taichung 404; [4]Department of Chinese Medicine and [5]Chinese Internal Medicine, China Medical University Hospital, Taichung 404; [6]Department of Chinese Medicine, Chang-Hua Hospital, Department of Health, Executive Yuan, Changhua, Taiwan, R.O.C.

Received September 28, 2009; Accepted December 17, 2009

DOI: 10.3892/ijo_00000593

Abstract. Emodin, aloe-emodin and rhein are major compounds in rhubarb (*Rheum palmatum* L.), used in Chinese herbal medicine, and found to have antitumor properties including cell cycle arrest and apoptosis in many human cancer cells. Our previous studies also showed that emodin, aloe-emodin and rhein induced apoptosis in human tongue cancer SCC-4 cells. However, the detail regarding emodin, aloe-emodin and rhein affecting migration and invasion in SCC-4 cells are not clear. In the present study, we investigated whether or not emodin, aloe-emodin and rhein inhibited migration and invasion of SCC-4 cells. Herein, we demonstrate that emodin, aloe-emodin and rhein inhibit the protein levels and activities of matrix metalloproteinase-2 (MMP-2) but did not affect gene expression of MMP-2, however, they inhibited the gene expression of MMP-9 and all also inhibited the migration and invasion of human tongue cancer SCC-4 cells. MMP-9 (gelatinase-B) plays an important role and is the most associated with tumor migration, invasion and metastasis in various human cancers. Results from zymography and Western blotting showed that emodin, aloe-emodin and rhein treatment decrease the levels of MMP-2, urokinase plasminogen activator (u-PA) in a concentration-dependent manner. The order of inhibition of associated protein levels and gene expression of migration and invasion in SCC-4 cells are emodin >aloe-emodin >rhein. Our results provide new insight into the mechanisms by which emodin, aloe-emodin and rhein inhibit tongue cancers. In conclusion, these findings suggest that molecular targeting of MMP-9 mRNA expression by emodin, aloe-emodin and rhein might be a useful strategy for chemo-prevention and/or chemotherapeutics of tongue cancers.

Correspondence to: Dr Jing-Gung Chung, Department of Biological Science and Technology, China Medical University, No. 91, Hsueh-Shih Road, Taichung 404, Taiwan, R.O.C.
E-mail: jgchung@mail.cmu.edu.tw

Key words: emodin, aloe-emodin, rhein, migration, invasion, matrix metalloproteinases, human tongue cancer SCC-4 cells

Introduction

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), aloe-emodin [1,8-dihydroxy-3-(hydroxymethyl)-anthraqui-none] and rhein (4,5-dihydroxyanthraquinone-2-carboxylic acid), are the major compounds in the root of rhubarb (*Rheum palmatum* L.) (1-3). Emodin possesses anticancer, antibacterial, diuretic, and vasorelaxant effects (4-6), aloe-emodin has a specific anti-neuroectodermal tumor activity (7) and rhein inhibits the growth of tumor cells in rat liver (8), human glioma (9), and Ehrlich ascites tumor (10) *in vivo*. Recently, in our laboratory we have found that emodin (11), aloe-emodin (12) and rhein (13) induced cell cycle arrest and apoptosis in human tongue cancer SCC-4 cells. Although various bioactivity studies of emodin, aloe-emodin and rhein have been carried out, studies regarding the molecular mechanisms by which emodin, aloe-emodin and rhein act on the expression of metalloproteinase (MMP)-9 and the invasiveness of SCC-4 are still undefined.

Invasion and metastasis are fundamental properties of various malignant cancer cells. Matrix metalloproteinases (MMPs), are a family of zinc-dependent endopeptidases, are deeply involved in the invasion and metastasis of various tumor cells (14-16). About 24 kinds of MMPs have been identified, and MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are most associated with tumor migration, invasion and metastasis for various human cancers (1,2,4). MMP-2 is often overexpressed in highly metastatic tumors, whereas MMP-9 can be stimulated by an inflammatory cytokine (17), a growth factor (18,19), or an oncogene (18,19) through activation of different intracellular-signaling pathways. It was reported that MMP-2 and MMP-9 are capable of degrading most extracellular matrix

(ECM) components that form the basal membrane (20). Numerous reports have been demonstrated that inhibition of MMP expressions or enzyme activities may be considered to be early targets for preventing cancer metastasis (21,22). Therefore, agents possessing the ability to suppress the expression of MMP-2 or -9 are worthy of development for anti-hepatoma cancer invasion and metastasis.

Materials and methods

Chemicals and reagents. Aloe-emodin, emodin, rhein, dimethyl sulfoxide (DMSO), propidium iodide (PI), Tris-HCl, Triton X-100 and trypan blue were obtained from Sigma Chemical Co. RPMI-1640, fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA, USA). High Capacity cDNA Reverse Transcription kit and 2X SYBR Green PCR Master mix was obtained from Applied Biosystems (Carlsbad, CA, USA).

Human tongue cancer cells. Human tongue cancer cell line (SCC-4) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan) and were cultured at 37˚C under a humidified 5% CO_2 and 95% air at one atmosphere with RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin (100 U/ml penicillin and 100 μg/ml streptomycin) and 2 mM L-glutamine. The medium was changed every 2 days (11,23,24).

Assessment of viability of SCC-4 cells after exposure to emodin, aloe-emodin and rhein. The SCC-4 cells ($2x10^5$ cells/well) were cultured in 12-well plates and incubated at 37˚C for 24 h then were treated individually with 0, 20, 30 and 40 μM emodin, 0, 25, 50 and 100 μM aloe-emodin and 0, 25, 50 and 100 μM rhein for 24 h. DMSO (solvent) was used for the control regimen. Cells from each treatment were stained with PI (5 μg/ml) and analyzed by flow cytometry (Becton-Dickinson, San Jose, CA, USA) as previously described (13,23).

Emodin, aloe-emodin and rhein inhibit the invasion and migration of SCC-4 cells. The SCC-4 cells ($1x10^5$ cells/well) were co-treated with or without emodin, aloe-emodin and rhein then the cell invasion was measured using Matrigel-coated transwell cell culture chambers (8 μm pore size; Costar, Acton, MA, USA) as described previously (25). SCC-4 cells ($1x10^5$ cells/well) were placed in serum-free RPMI-1640 medium for 24 h then were resuspended in serum-free RPMI-1640 medium and placed in the upper chamber of the Matrigel-coated (0.8 μg/μl, 37˚C, 2 h; BD Biosciences, San Diego, CA, USA) transwell insert ($5x10^4$ cells/well) and with 0.5% DMSO or emodin (0, 15 or 30 μM), aloe-emodin (0, 25 or 50 μM) and rhein (0, 25 or 50 μM). However, the RPMI-1640 medium containing 10% FBS was placed in the lower chamber. Cells were placed in an incubator for 24 or 48 h at 37˚C in humidified atmosphere with 95% air and 5% CO_2. Cells were allowed to invade in 100 μl serum-free RPMI-1640 for 24 or 48 h through a polycarbonate membrane. Invasive cells in membrane were fixed with 4% formaldehyde in PBS and stained with 2% crystal violet in 2% ethanol. In the upper chamber, the non-invasive cells were removed by wiping the upper side of the membrane with a cotton swab, cells located on the underside of the filter were counted under a light microscope at x200 (26,27). The procedures of migration assay of SCC-4 cells are the same as in invasion assay of SCC-4 cells as described above except the filter membrane was not coated with Matrigel. Cells located on the underside of the filter were counted under a light microscope at x200 (26,27). Per treatment and control were performed with three chambers and the values obtained were calculated by averaging the total number of cells from three filters (experiments).

Emodin, aloe-emodin and rhein affect the associated protein levels of invasion and migration from SCC-4 cells. The SCC-4 cells ($2x10^6$ cells/well) were placed in 6-well tissue culture plates and grown for 24 h. Emodin, aloe-emodin and rhein was individually added to each cell at a final concentration of 0, 20, 25, 30 or 50 μM, while DMSO (solvent) alone was added to the wells as control cells. Cells from each treatment including control were incubated with emodin, aloe-emodin and rhein in 90% RPMI-1640 medium with 0.5% FBS at 37˚C for 0, and 24 h then were harvested and resuspended in ice-cold 50 mM potassium phosphate buffer at pH 7.4 containing 0.1% Triton X-100 and 2 mM EDTA. The collected cells were sonicated and centrifuged at 4˚C for 10 min at 13,000 x g to remove cell debris and the supernatant was collected to determine the total protein concentration by using a Bio-Rad protein assay kit (Hercules, CA, USA) with bovine serum albumin (BSA) as the standard as described previously (26,27). SDS gel electrophoresis and Western blotting were performed to determine the effects of emodin, aloe-emodin and rhein on protein levels of MMP-2, MMP-9, u-PA, TIMP-1, FAK, NF-κB p65, p-AKT, p-P38, p-JNK and p-ERK as described previously (28,29).

Emodin, aloe-emodin and rhein affect the activities of MMP-2 from SCC-4 cells. The activity of MMP-2 of SCC-4 cells after exposure to emodin, aloe-emodin and rhein were examined by Gelatin zymography. The SCC-4 cells ($2x10^6$ cells/well) were cultured in 6-well tissue culture plates in serum-free RPMI-1640 medium in the presence of emodin (20 and 30 μM), aloe-emodin (25 and 50 μM) and rhein (25 and 50 μM) for 24 h, the conditioned medium was then collected as samples. The unboiled medium from each treatment were separated by electrophoresis on 10% SDS-PAGE containing 0.1% gelatin then the gels were soaked in 2.5% Triton X-100 in dH_2O twice for total 60 min at room temperature, then incubated in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.02% NaN_3 and 1% Triton X-100, pH 8.0) at 37˚C for 18 h. Bands corresponding to activity of MMP-2 were visualized by negative staining using 0.3% Coomassie blue in 50% methanol and 10% acetic acid (28) and the fold-change between control and treatment was quantified.

Emodin, aloe-emodin and rhein affect the gene expression of MMP-2, MMP-7 and MMP-9 in SCC-4 cells. The gene expression of SCC-4 after exposure to emodin, aloe-emodin and rhein was performed by real-time PCR. The SCC-4 cells ($2x10^6$ cells/well) were cultured in 6-well plates for 24 h.

Table I. The DNA sequence was evaluated using the primer express software.

Primer Name	Primer sequence
Homo MMP2-F	CCCCAGACAGGTGATCTTGAC
Homo MMP2-R	GCTTGCGAGGGAAGAAGTTG
Homo MMP7-F	GGATGGTAGCAGTCTAGGGAT TAACT
Homo MMP7-R	AGGTTGGATACATCACTGCA TTAGG
Homo MMP9-F	CGCTGGGCTTAGATCATTCC
Homo MMP9-R	AGGTTGGATACATCACTGCA TTAGG

Emodin, aloe-emodin and rhein was individually added to cells for a final concentration of 20 and 30, 25 and 50, 25 and 50 μM, respectively for 24 h, then total RNA was extracted using the Qiagen RNeasy Mini kit as described previously (31,32). The RNA samples from each treatment were individually reverse-transcribed at 42˚C with high capacity cDNA Reverse Transcription kit for 30 min according to the protocol of the supplier (Applied Biosystems). Quantitative PCR conditions were: 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 and reverse primers as shown in Table I. Applied Biosystems 7300 real-time PCR system was used for each assay in triplicate and expression fold-changes were derived using the comparative C_T method (29,30).

Statistical analysis. Student's t-test was used to analyze differences between emodin, aloe-emodin and rhein-treated and untreated (control) groups.

Results

Emodin, aloe-emodin and rhein decreased the percentage of human tongue cancer (SCC-4) cells. SCC-4 cells were individually exposed to different concentrations of emodin, aloe-emodin and rhein for 24 and 48 h, and cells were collected for PI staining to determine the percentage of viability. The results are present in Fig. 1. There were fewer viable cells as concentration increased when compared to control groups. The cytotoxic effects were in the order emodin >aloe-emodin >rhein. These effects are dose- and time-dependent.

Emodin, aloe-emodin and rhein inhibit migration of SCC-4 cells. SCC-4 cells were individually exposed to different concentrations of emodin, aloe-emodin and rhein for 24 and 48 h, and cells were determined as percentage of inhibition of migration and results are presented in Fig. 2, which indicates

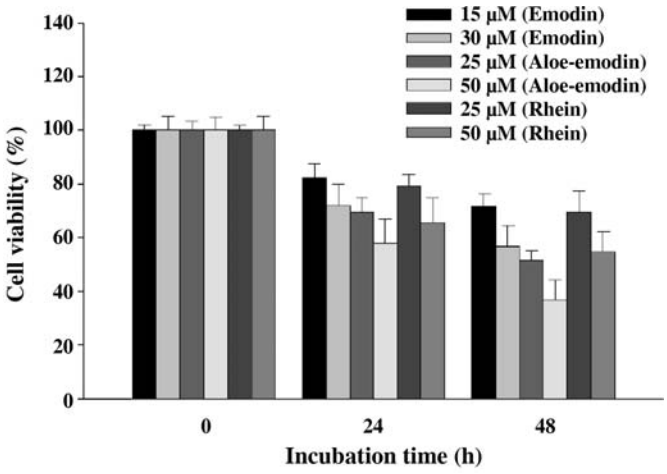

Figure 1. Emodin, aloe-emodin and rhein decrease the percentage of viable SCC-4 cells. The SCC-4 cells ($2x10^5$ cells/well) were placed in 12-well plates and incubated at 37˚C for 24 h then were co-treated with various doses of emodin, aloe-emodin and rhein for 24 and 48 h. DMSO (solvent) was used for the control regimen. Cells were harvested and stained with PI then analyzed by flow cytometry as described in Materials and methods. Each point is mean ± SD of three experiments. $^{*}p<0.05$; $^{**}p<0.001$.

that emodin, aloe-emodin and rhein had a significant inhibitory effect on cell migration at concentrations between 15-30 μM for emodin, 25-50 μM for aloe-emodin and rhein. Data in Fig. 2 indicate that the inhibition was at 45-58 and 54-79%, 44-74 and 52-82%, 46-75 and 56-86% when cells were incubated with emodin, aloe-emodin and rhein for 24- and 48-h treatment, respectively.

Emodin, aloe-emodin and rhein inhibit invasion of SCC-4 cells. SCC-4 cells were individually exposed to different concentrations of emodin, aloe-emodin and rhein for 24 and 48 h, and cells were determined as percentage of inhibition of invasion. The results are presented in Fig. 3, which indicates that emodin, aloe-emodin and rhein had a significant inhibitory effect on cell invasion at concentrations between 15-30 μM for emodin, 25-50 μM for aloe-emodin and rhein. Data in Fig. 3 indicate that the inhibition was at 36-54% and 52-74%, 22-38% and 35-66%, 19-52% and 28-56% when cells were incubated with emodin, aloe-emodin and rhein for 24- and 48-h treatment, respectively.

Emodin, aloe-emodin and rhein affect associated protein levels of migration and invasion of SCC-4 cells. SCC-4 cells were individually exposed to different concentrations of emodin, aloe-emodin and rhein for 24 h, cells were harvested from each treatment then total levels were determined as described for Western blotting. The results are shown in Fig. 4 indicating that emodin, aloe-emodin and rhein decreased the levels of MMP-2, u-PA, FAK, NF-κB p65, p-AKT, p-P38, p-JNK and p-ERK and increased the level of TIMP-1 but had no effect the the level of MMP-9.

Emodin, aloe-emodin and rhein inhibit the activities of MMP-2 from SCC-4 cells. The effects of emodin, aloe-emodin and rhein on MMP-2 activities were examined by Gelatin

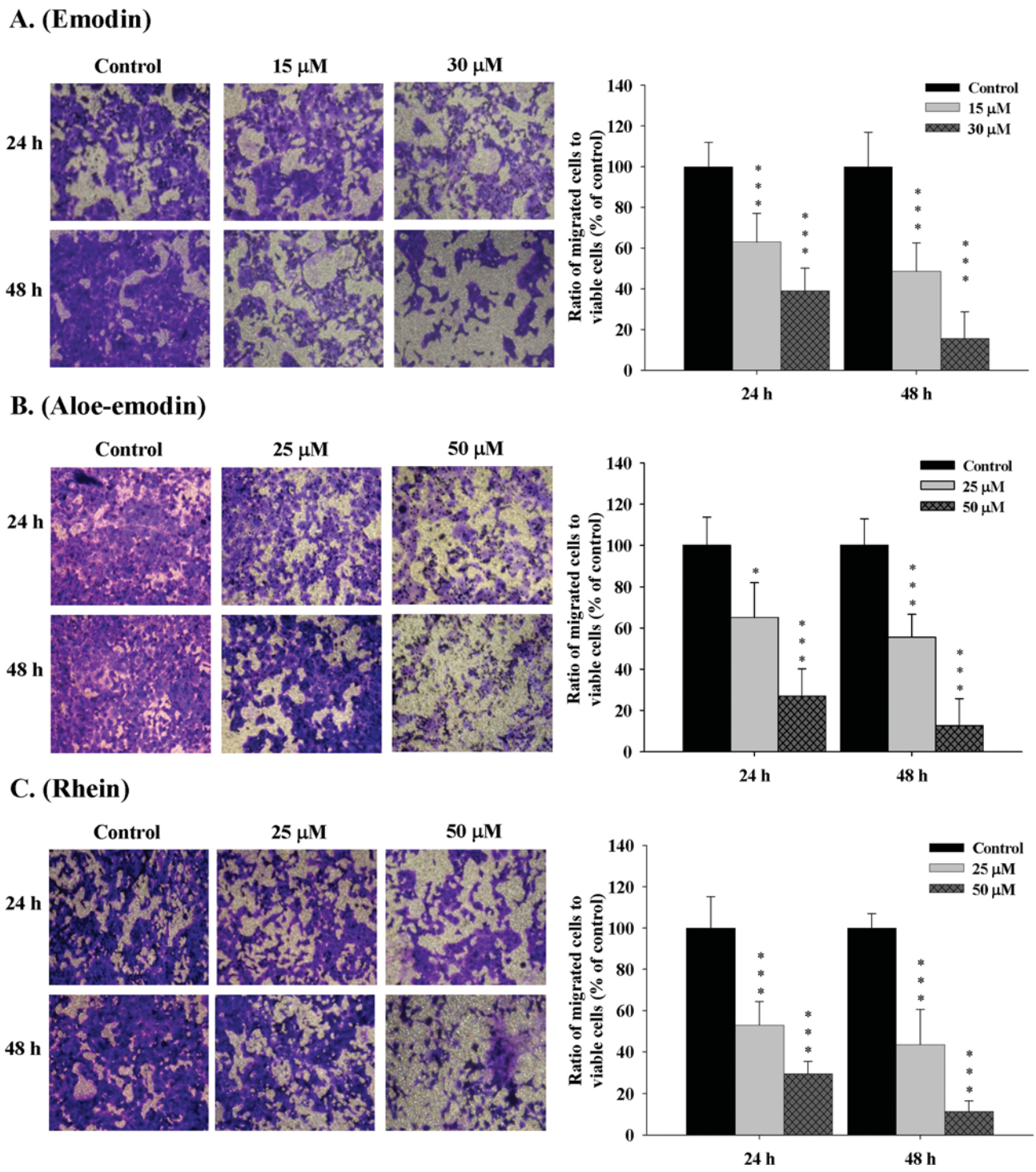

Figure 2. Emodin, aloe-emodin and rhein inhibited migration of SCC-4 cells. The SCC-4 cells ($2x10^5$ cells/well; 12-well plates) were incubated individually with various concentrations of emodin, aloe-emodin and rhein for 24 and 48 h and migrate cells were determined as described in Materials and methods. (A) Representative picture of migrated cells of emodin treatment and the percentage of inhibition. (B) Representative picture of migrated cells of aloe-emodin treatment and the percentage of inhibition. (C) Representative picture of migrated cells of rhein treatment and the percentage of inhibition. Each point is mean ± SD of three experiments. $^{*}p<0.05$; $^{**}p<0.001$.

zymography for SCC-4 cells at 24 h. As shown in Fig. 5, the results indicate that emodin, aloe-emodin and rhein inhibited MMP-2 activities. This was also confirmed by Western blotting as shown in Fig. 4B, and the results indicated that emodin, aloe-emodin and rhein inhibited the protein levels of MMP-2.

Emodin, aloe-emodin and rhein inhibit MMP-9 mRNA expression in SCC-4 cells. In order to investigate whether emodin, aloe-emodin and rhein affect migration- and invasion-associated gene expression in SCC-4 cells, cells were individually treated with emodin, aloe-emodin and rhein (25 μM) for 0, 24 and 48 h. Total RNA was isolated from control and emodin, aloe-emodin and rhein treatment, gene

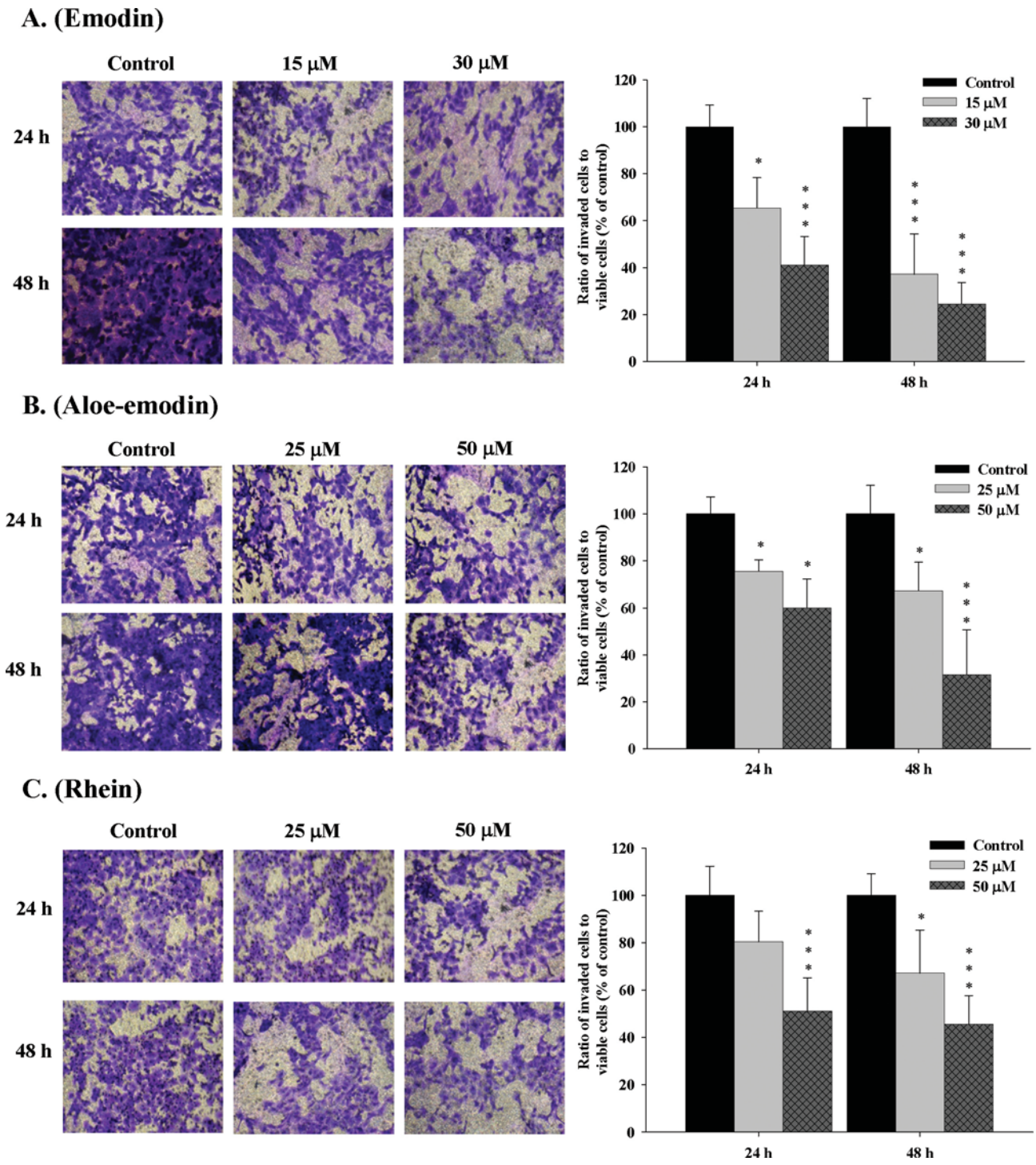

Figure 3. Emodin, aloe-emodin and rhein inhibited invasion of SCC-4 cells. The SCC-4 cells ($2x10^5$ cells/well; 12-well plates) were incubated individually with various concentrations of emodin, aloe-emodin and rhein for 24 and 48 h and invasive cells were determined as described in Materials and methods. (A) Representative picture of invaded cells of emodin treatment and the percentage of inhibition. (B) Representative picture of invaded cells of aloe-emodin treatment and the percentage of inhibition. (C) Representative picture of invaded cells of rhein treatment and the percentage of inhibition. Each point is mean ± SD of three experiments. $^{*}p<0.05$; $^{**}p<0.001$.

expressions were examined by real-time PCR. The results shown in Fig. 6 indicate that the expression levels of MMP-9 mRNA were decreased and these effects were time-dependent (Fig. 6) but they did not affect MMP-2 and MMP-9 in SCC-4 cells.

Discussion

In the oral cavity, oral squamous cell carcinoma (OSCC) is the most malignant neoplasm and can be aggressive, 50% of oral tongue SCC patients were diagnosis with nodal metastases and the 5-year survival rate is less than 50% for

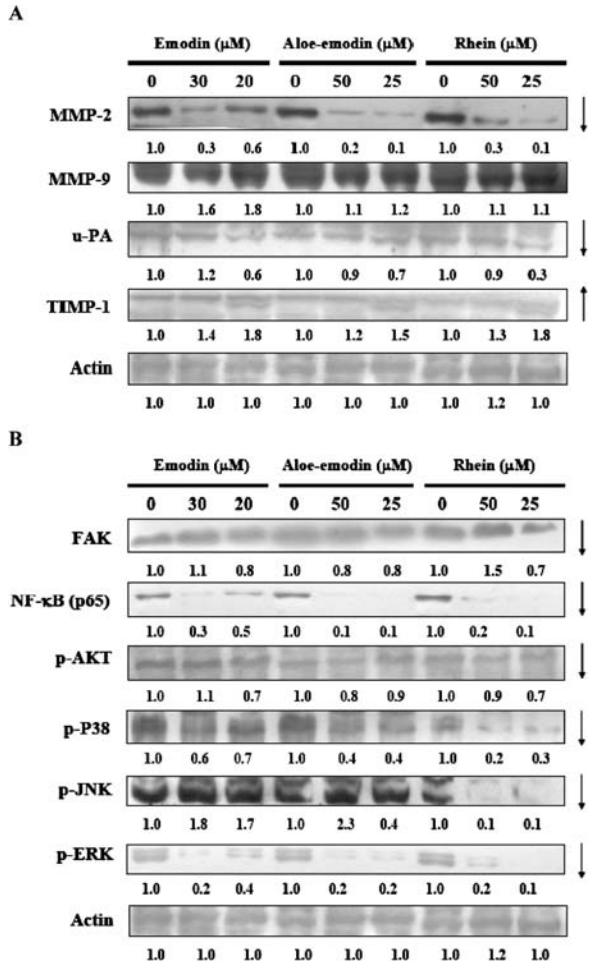

Figure 4. Emodin, aloe-emodin and rhein affect the proteins levels of associated proteins for invasion and migration in SCC-4 cells. The SCC-4 cells (1x10^6 cells/well; 12-well plates) were incubated individually with various concentrations of emodin, aloe-emodin and rhein for 6, 12, 24 and 48 h then the total proteins were collected and the protein levels of (A) MMP-2, MMP-9, u-PA and TIMP-1 and (B) FAK, NF-κB p65, p-AKT, p-P38, p-JNK and p-ERK) were measured by SDS-PAGE and Western blotting as described in Materials and methods.

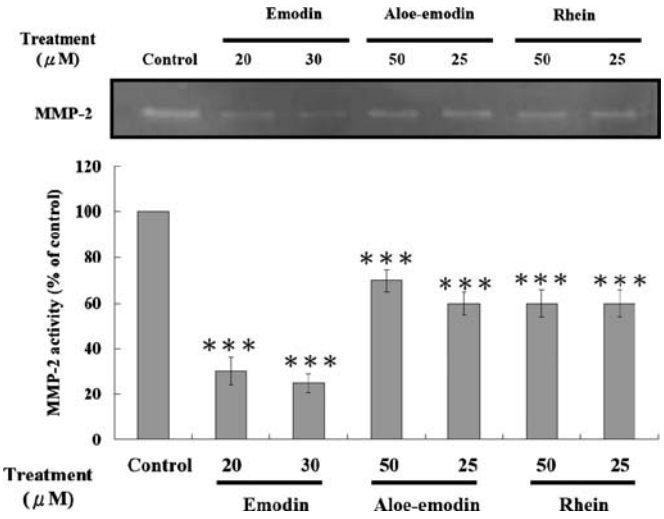

Figure 5. Emodin, aloe-emodin and rhein inhibit the activity of MMP-2 in SCC-4 cells. SCC-4 cells (5x10^4 cells/well) were incubated with various doses of emodin, aloe-emodin and rhein for 24 h. Cells were harvested and separated by gelatin zymography as described in Materials and methods. The ratio of MMP-2 activities was quantified.

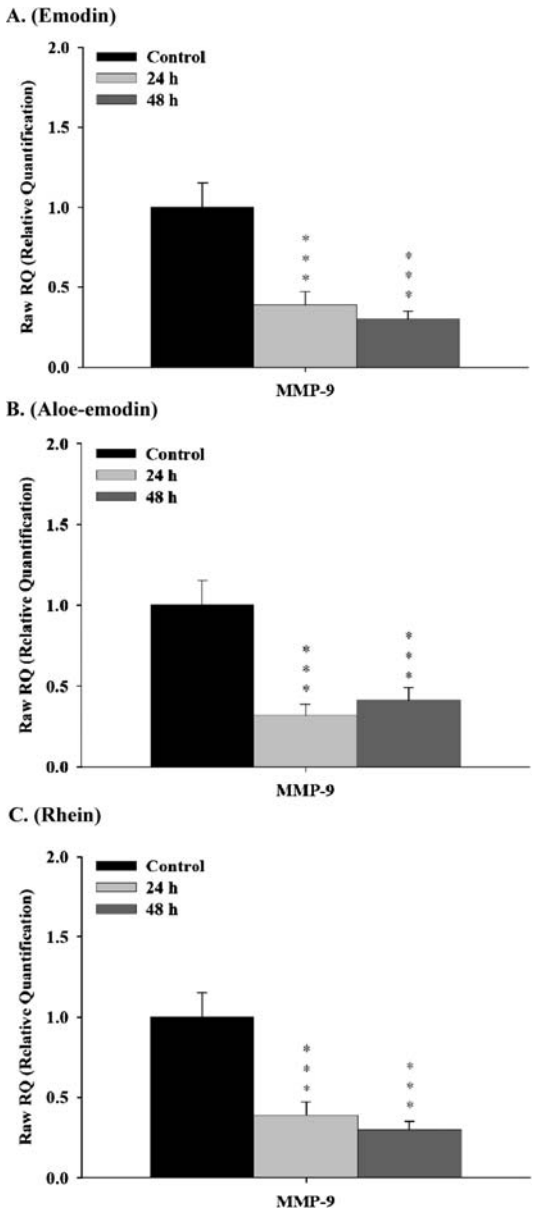

Figure 6. Emodin, aloe-emodin and rhein inhibit MMP-9 mRNA expression in SCC-4 cells. The SCC-4 cells (5x10^5 cells/well) were incubated with 30 μM of emodin, aloe-emodin and rhein for 24 and 48 h. The total RNA was extracted from each treatment of SCC-4 cells and RNA samples were reverse-transcribed for real-time PCR as described in Materials and methods. The ratios of MMP-2 mRNA/GAPDH are presented in the panel. Data are the mean ± SD of three experiments. *P<0.05, ***P<0.001.

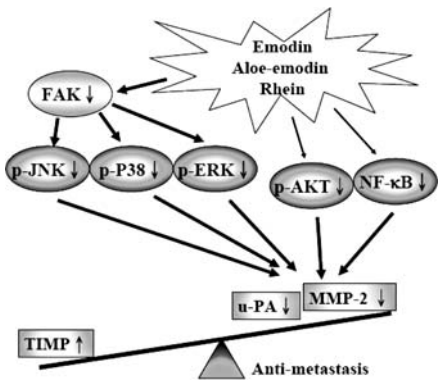

Figure 7. The proposed inhibition mechanisms of emodin, aloe-emodin and rhein on migration and invasion of SCC-4 cells.

patients who have lymph node metastasis (19). Therefore, metastasis is one of the important factors associated with success or failure in cancer treatment. Many reports have shown that emodin, aloe-emodin and rhein induced cytotoxic effects leading to a decrease in the percentage of viable cancer cells through cell cycle arrest and induction apoptosis. However, there is no available information to show the effect of emodin, aloe-emodin and rhein on the migration and invasion human tongur cancer SCC-4 cells. Herein, we revealed that emodin, aloe-emodin and rhein could significantly inhibit the invasive and migration ability of SCC-4 cells *in vitro*. The inhibition ratio among the three compounds is emodin >aloe-emodin >rhein. Furthermore, our findings showed that emodin, aloe-emodin and rhein could decrease protein levels of tumor metastasis-related proteins such as MMP-2 and u-PA. This is the first report related to the inhibitory effect of emodin, aloe-emodin and rhein on oral cancer invasiveness via decreased production of tumor metastasis-related proteins.

Many studies have shown that emodin, aloe-emodin and rhein possess potent antiproliferative effect and induction of apoptosis in various cancer cells also have been reported (4-13). Furthermore, our primary studies have shown that emodin, aloe-emodin and rhein induced apoptosis in SCC-4 cells (11-13). In the present study, similar results were also shown that emodin, aloe-emodin and rhein significantly decreased the percentage of viable SCC-4 cells (Fig. 1). These findings suggest that emodin, aloe-emodin and rhein may be powerful candidates to develop as preventive agents for oral cancer metastasis. However, the detailed mechanisms of inhibition of oral cancer cell invasion by emodin, aloe-emodin and rhein are still not completely clear, therefore, further studies are required.

It was reported that many MMPs play a role in the progression of oral cancer (31) and play important roles in cancer cell invasion and metastasis (32,33). The tumour cells showed only low levels of invasion in the absence of either u-PA or MMPs (34). Tumor-secreted MMPs destroy extracellular matrix components in tissue surrounding a tumor, enter and survive in the circulation, lymphatics or peritoneal spaces and can arrest in a distant target organ.

In the present study, we show that emodin, aloe-emodin and rhein could inhibit protein levels of tumor metastasis-related proteins such as MMP-2 (Fig. 4) but all examined compounds did not significantly inhibit MMP-9. However, results from real-time PCR (Fig. 6) indicated that all the examined compounds inhibited the mRNA expression of MMP-9. Thus, the possible usefulness of specifically selected MMP inhibitor, would be worthy of investigation also as chemopreventive agents in patients at high risk of developing oral cancer.

Our results (Fig. 4) from Western blotting also show that emodin, aloe-emodin and rhein inhibited the levels of NF-κB in SCC-4 cells. It was reported that the transcription of MMP gene is regulated by upstream regulatory elements, including NF-κB and AP-1 binding sites (35,36). There is substantial evidence showing that one or more of these binding sites have been implicated in mediating the effects of a diverse set of agents. It was reported that the suppression of the NF-κB, c-Fos and c-Jun activities, or blockage of the factors binding to their regulatory elements may potentially block tumor invasion (37,38).

These results demonstrated that emodin, aloe-emodin and rhein may be important in the inhibition of migration and invasion of SCC-4 cells *in vitro* through multiple mechanisms: inhibition of migration, matrix degradation and metastasis. Based on these results, we propose a schematic presentation of possible mechanisms for the effect of emodin, aloe-emodin and rhein on migration and invasion of SCC-49 cells (Fig. 7). Emodin, aloe-emodin and rhein individual treatment inhibited the cell migration/invasion of SCC-4 cells via levels of MMP-2, u-PA, FAK, NF-κB p65, p-AKT, p-P38, p-JNK and p-ERK and increased the level of TIMP-1, followed by the inhibition of MMP-2 production and the inhibition of mRNA expression of MMP-9, as well as the increase of TIMP-1. However, the cross-talk between these two pathways is unclear. Our findings are expected to provide clues for the development of novel therapeutic strategies to inhibit the migration and invasion of SCC-4 cells by emodin, aloe-emodin and rhein.

Acknowledgements

This study was supported by Grants CMU96-086 and CMU96-087 from China Medical University, Taichung, Taiwan.

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