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Suppression on Metastasis by Rhubarb Through Modulation on MMP-2 and u-PA in Human A549 Lung Adenocarcinoma: an Ex-vivo Approach

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Abbreviations: *b.i.d.*, *bis in die* (= twice a day); MMP2, matrix metalloproteinase-2; MMP9, matrix metalloproteinase-9; MT1-MMP, membrane type I matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; uPA, urokinase-type plasminogen activator; PAI1, plasminogen activator inhibitor1; TCM, traditional Chinese medicine

Key Words: Traditional Chinese medicine, Rhubarb, Metabolic event, Metastasis, MMP-2, u-PA

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ABSTRACT

In Chinese society, rhubarb is used as a central ingredient in the prescription for treatment of cancer patients. Several lines of evidences have demonstrated that rhubarb extracts can inhibit tumor cell proliferation and metastasis. However, most of these bioactivity studies were done *in vitro* and focused exclusively on the parental form of the components. This does not take into account the achievable physiological *in vivo* concentrations and the metabolic fates of these components. To mimic the functionality of rhubarb in clinical, our laboratory has established an experimental model allowing us to address the anti-metastatic activity of rhubarb under physiological conditions in which rats were orally administered rhubarb decoction and then serum metabolites were prepared and characterized to assay for their anti-proliferative and anti-tumor metastasis capability *in vitro*. Our results demonstrated that the cell mobility was strongly inhibited and the enzymatic activity of MMP-2 was decreased following culture with the rhubarb serum metabolite in human lung adenocarcinoma A549 cells. Further experiments demonstrated the down regulation of MMP-2 enzymatic activity might be through both transcriptional and post-translational mechanisms. Taken together, our results provide possible rhubarb anti-tumor metastatic mechanisms and the methodology to identify new metabolically active compounds for cancer treatment.

1. INTRODUCTION

The incidence and mortality of lung cancer has continued to rise worldwide. Clinically, approximately 40% of lung cancer patients are diagnosed as non-small-cell adenocarcinoma, which quickly develops both radio- and chemo-resistance, and often presents at stages too late for surgical intervention. Together, the above mentioned factors resulting low overall survival at 5 years of less than15% [1]. Furthermore, often local invasion or migration of the cancer cells to distant organs occurs by the time of diagnosis and consequently, most deaths are not caused by the primary tumor itself, but rather due to metastases of the tumor into distant vital organ [2].

Since tumor cells have to produce and/or recruit proteases to degrade the surrounding extracellular matrix to facilitate cell migration and invasion, the search for effective treatments to inhibit metastasis-promoting proteases is of great interest in cancer research [3,4]. The matrix metalloproteinases (MMPs) are a family of zinc-dependent proteinases involved in the degradation of the extracellular matrix [5,6]. Among them, the enzymatic activities of MMP-2 and -9 have been reported to strongly associated with tumor metastasis, angiogenesis and prognostic stage in various cancers[7].

Extensive preclinical data have shown that administration of MMP inhibitors to different animal models results in reduction of primary tumor growth in terms of number as well as size of metastatic lesions. However, the first and second generation of MMP inhibitors did not perform adequately in clinical trials due to the poor selectivity. Therefore, identifying highly selective MMP inhibitors to prevent cancer metastasis with minimal adverse affects is crucial in designing new MMP-inhibitors. Current, some new generation anti-tumor MMP-inhibitors in development now take inhibitor specificity into

consideration. [8,9].

Recently, naturally occurring substances, including plant foods and traditional Chinese medicine (TCM), have been considered as fruitful anticancer candidates[10,11]. Continued efforts of our team search for potential anti-metastatic agents using an *ex-vivo* screening programs were established. In this procedure, numerous herbs and prescriptions, which were indicated for preventing or treating cancers in TCM practice were tested. Among them, rhubarb was one of the most promising anti-metastatic agents without observable cell cytotoxicity (Data not shown). Rhubarb, called Dahunag in Chinese, is the dried root and rhizome of *Rheum palmatum* L. Under botanical classification, rhubarb is considered a vegetable and its consumption in food, primarily drinks and meat stews, have been employed for a long time in China to treat many diseases including constipation, jaundice, gastrointestinal hemorrhage, and ulcers [12]. Rhubarb contains many anthraquinones and their glycosides, including aloe-emodin, rhein, emodin, and chrysophanol, which are thought to be the major active components for inhibiting tumor growth [13-17].

Despite previous promising findings, several lines of evidence have demonstrated that the *in vitro* results did not completely translate into the *in vivo* or clinical studies. One explanation of these findings may be that most *in vitro* studies ignored the achievable active drug concentration *in vivo* or the lack of consideration for the metabolic process. As a result, high concentration of parent form compounds was used for its bioactivity test. [18-21]. In fact, our previous pharmacokinetics result revealed that most anthraquinones were quickly metabolized to glucuronides or sulfates after oral administration of rhubarb decoction in rats [22]. These findings indicate that the

in vitro bioactivities of parent form of anthraquinons might not appropriately predict the *in vivo* effects. Therefore, we propose that the conjugated metabolites of anthraquinones may play a more prominent role *in vivo* than their parent forms. To mimic the *in vivo* conditions, the serum metabolites of rhubarb were prepared and characterized after feeding rats with rhubarb decoction orally based on our previous study [22] and used for *in vitro* experiments to study the molecular mechanism of blockage on tumor metastasis. Our experiments demonstrate that rhubarb suppresses *in vivo* lung metastasis through a regulatory mechanism involving MMP-2 and u-PA using an *ex-vivo* approach.

2. MATERIALS AND METHODS

2.1. Reagents. Monoclonal antibodies to MMP-2, MMP-9, TIMP-2 and uPA, as well as horseradish peroxidase-conjugated secondary antibody were purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). Cell culture reagents were obtained from Hyclone (South Logan, UT). Acrylamide, bisacryamide, ammonium persulfate, and N,N, N', N'-tetramethylethylene diamine were from Bio-Rad (Richmond, CA). Western blot chemilluminescent reagent was purchased from Millipore (Boston, MA). All of the other chemicals were from USB (Darmstadt, Germany) or Sigma Chemical (St. Louis, MO), and were standard analytic grade or higher.

2.2. Cell Lines. Human lung adenocarcinoma epithelial cell line (A549) was purchased from American Type Culture Collection (ATCC; Rockville, MD). Gastric carcinoma epithelial cell line (MKN45) was obtained from Japanese Collection of

Research Bioresources (JCRB). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL of penicillin, and 100 mg/mL streptomycin sulfate at 37 ^{0}C in a humidified atmosphere of 5% CO_2/ 95% air.

2.3. Animals. Four to six-weeks old male Sprague-Dawley rats and NOD.CB17-Prkdc^{scid} mice were obtained from the animal center (National Taiwan University, Taiwan) and maintained under specific pathogen-free conditions throughout the study. The animal studies strictly adhered to "The Guidebook for the Care and Use of Laboratory Animals (2002)" (Published by the Chinese Society for the Laboratory Animal Science, Taiwan, R.O.C.). The animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of China Medical University, Taichung, Taiwan. The permission number of animal protocol is 97-80-N.

2.4. Preparation of Rhubarb Decoction. The rhizomes of *Rheum palmatum* was purchased from a Chinese drug store in Taichung city, Taiwan. The origin of the crude drug was identified by microscopic examination. The rhubarb specimen was deposited in the Institute of Chinese Pharmaceutical Sciences, China Medical University. The decoction preparation followed the traditional method, briefly, 300 mL of water was added to the dried crude drug of rhubarb (15 g) and heating was carried out on a gas stove. After boiling, gentle heating continued until the volume reduced to less than 30 mL. The mixture was filtered to remove the large insoluble particles while hot and sufficient water was added to make 30 mL and frozen at −30°C until later use. The final concentration of rhubarb decoction was equivalent to 0.5 g/mL of crude drug [22].

2.5. Preparation and Characterization of Rhubarb Metabolites in Rat Serum.

The serum metabolites were prepared from rats and characterized according to previous pharmacokinetic study [22]. Briefly, after overnight fasting, male Sprague-Dawley rats were fed orally with 10 mL/5.0 g/kg of rhubarb decoction via gastric gavages. Half hour later, rats were boosted with another dose of decoction. Twenty minutes after second booster, 10 mL blood was collected by immediate cardiac puncture. Serums was collected and serum proteins were removed by mixing with four times volumes methanol following centrifugation [22]. The supernatant was lyophilized then reconstituted with PBS. Blank serum from untreated rat was processed in the same manner and was used as control treatment for *in vitro* experiments.

2.6. Microculture Tetrazolium Assay. The colorimetric assay for cellular growth and survival was described by Hansen *et al.* with modification [23]. Briefly, logarithmic growth phase cells were seeded in a 96-well microplate for overnight culture. Therefore, test samples, including rhubarb serum metabolite or control serum, were added to the above culture in total volume of 150 μL. Forty-eight hours later, 15μL of MTT solution (5 mg/mL) was added to each well, and then cells were incubated at 37℃ for 4 hours. Then, 75μL lysis buffer (20% SDS-50% *N, N*-Dimethyl formamide) was added to each well, and the culture plate was incubated at 37℃ for overnight to dissolve the dark blue crystals. The absorption of formazan solution at 570 nm was measured using a microplate reader.

2.7. Analysis of MMP-2 and MMP-9 Activities by Gelatin Zymography. Gelatinase activities of MMP-2 and MMP-9 were examined by gelatin zymography as previous described by Rao et al. [24]. Briefly, one million tumor cells were seeded on 24 well plates for overnight. Serum contained medium was removed and replenished with

culture medium devoid of FBS in the presence or absence of the serum metabolites. The conditional medium was collected after 48 hrs and mixed with equal volume loading buffer (125 mM Tris–HCl, pH 6.8, 3% SDS, 40% glycerol, 0.02% bromophenol blue) without boiling. To measure the MMP-2/MMP-9 activity, the secretary MMPs were separated by 8 % SDS-PAGE containing 0.1% gelatin under the non-reducing condition. The gelatinolytic bands were documented by BioDoc-it Image System (UVP, USA).

2.8. Analysis of Urokinase Type Plasminogen Activator (uPA) Activities by Casein-Plasminogen Zymography. Activities of uPA was examined by casein-plasminogen zymography as previously described [25]. Briefly, 2% casein (w/v) and 20 μg/mL plasminogen were added to 8% SDS–PAGE, and then performed as described in the gelatin zymography. After electrophoresis, the gels were rinsed twice with 2.5% Triton X-100 and MMPs/uPA activities were measured by incubated the gel overnight at 37 °C in the developing buffer (40 mM Tris–HCl, pH 8.0; 10 mM $CaCl_2$ and 0.01% NaN_3) for 12 h at 37^0C. The gels were stained with 0.25% CoomassieBlueR-250 and destain till the clear bands were visualized. The gelatinolytic bands were documented by BioDoc-it Image System (UVP, USA).

2.9. Wound Healing Assay. To assess the alterations of cell motility and migration ability after the treatment, wound-healing method describe previously by Ongusaha *et al.* were used [26]. Briefly, one million cells were seeded into a 6-well plate 18 hrs before the experiment. An artificial “wound” was carefully created at 0h, using a pipette tip to scratch on the monolayer cell. Floating and loosely attached cells were carefully removed by PBS wash and tested compounds were added at pre-determined concentration. Microphotographs were taken every 6 hours and the area of each wounding area was

calculated by Image J software. All experiments were conducted in triplicate. Plates were marked for consistent photo documentation.

2.10. Reverse Transcription – Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from cells using a TRIzol^{R} reagent according to the manufacture instruction. Four µg of total RNA was reverse transcripted by SuperScript III using oligo dT as primer following the manufacture suggestion. The mRNA expression level of each protein was measured by PCR with the following primer sets: MMP-2 (forward: 5'-gCCgTCgCCCATCATCAAgT-3'; reverse: 5'ggAAggCACgAgCAAAggCA3'); uPA (forward: TTGCGGCCATCTACAGGAG; reverse: ACTGGGGATCGTTATACATC) TIMP-2 (forward: TTTATCTACACGGCCCCCTCCTCAG; reverse: ACGGGTCCTCGATGTCAAGAAACTC) and PAI-1(forward: GGATCCAGCCACTGGAAAGGCAACATG; reverse: GGATCCGTGCCGGACCACAAAGAGGAA). The final products were resolved on a 2 % agarose gel and stained with ethidium bromide then visualized under UV Transilluminator and documented by BioDoc-it Image System (UVP, USA).

2.11. Preparation of Cell Lysates and Western Blot Analysis. Cells were initially seeded at 1×10^{5} cells in 6 well plate. After treatment for the indicated time with various concentrations of serum metabolites, cells were washed twice with PBS, gently scraped from the dishes, then centrifuged, then lysed in ice-cold lysis buffer (100 mM Tris [pH 7.4], 1 % NP40, 0.01% SDS, 1 mM PMSF, 10 µg/mL pepstatin, and 30 µg/mL leupeptin), and cleared by centrifugation. Protein concentrations of lysates were determined using the BCA Protein Assay Reagent (PIERCE Biotechnology, Rockford, IL). Equal amount of protein from each sample were separated on SDS-PAGE gels and transferred to PVDF-

membrane. After soaking in a blocking solution TBS with 0.05% Tween 20, and 5% skim milk, the blot was incubated with the primary antibody and antibody binding was detected using the appropriate secondary antibody coupled with horseradish peroxidase according to the instructions of manufacturer. Enhanced chemiluminescence was used to detect the relevant proteins following protocols suggested by the manufacturer and then images were taken on LAS-4000 (Fuji Film, Japan).

2.12. Experimental Murine Lung Metastasis Studies . A549 cells (2×10^6) suspended in HBSS were injected into NOD.CB17-Prkdc^{scid} mice via the tail vein. One week later the mice were randomly divided into two groups (N=5/group) which were fed orally twice a day with or without rhubarb decoction (0.75g/kg). The mouse was euthanized on day 42. Lungs were removed, weighed and fixed in Bouin's solution for 24 h, and the numbers of metastatic lesions were counted macroscopically.

2.13. Statistical Analysis. All assays were carried out in triplicate. Data was expressed as a mean with standard deviation (SD). Student's *t*-test was used to compare the mean of each group with that of the control group. A p value <0.05 was considered statistically significant.

3. RESULTS

3.1. Rhubarb Serum Metabolite Does Not Possess Direct Cytotoxicity Against A549 and MKN45 *in Vitro*. Several reports have shown that the parent form of anthraquinones in the rhubarb can inhibit the tumor growth in the *in vitro* experiments [13-17]. However, our previous studies have shown that anthraquinones were quickly metabolized and most of their parent forms were below the detection level in serum

samples of rhubarb-treated rats [22]. Therefore, the serum concentration of anthraquinones should not reach the cytotoxicity level as shown in previous reports [13-17]. In order to clarify whether the cellular cytotoxicity contributed to *in vivo* anti-metastatic effect of rhubarb, we treated cancer cells with rhubarb serum metabolite as well as control serum, and then determined cell viability. As shown in **Figure 1**, the results demonstrated that the serum metabolite of rhubarb did not possess any noticeable cytotoxicity against MKN45 and A549. The cell viability of both cell lines in experimental groups at 48h are almost identical to that of control cultures.

3.2. Rhubarb Serum Metabolite Suppresses the Functional Activity and Expression Level of MMP-2 in A549 Cells. Because *in vitro* growth inhibition assays showed that rhubarb serum metabolites did not possess cytotoxicity against A549 and MKN45 cell proliferation at pharmacological concentration, we therefore, hypothesized that the blockage of long distance metastasis by rhubarb might be due to the inhibition of cell invasion and/or migration ability. To understand the non-cytotoxic anti-metastatic mechanism of rhubarb against highly metastatic lung cancer cell line A549 *in vivo*, several metastatic related target proteins were chosen for further studies. Recently, several lines of evidence have shown that matrix metalloproteases (MMPs) play an important role in cancer with respect to invasion, metastasis and angiogenesis [27]. Among them, the MMP-2 and MMP-9 have been reported to be mostly associated with tumor metastasis [28]. Thus the enzymatic activity of both MMP-2 and MMP-9 were measured in the cancer cells in the presence or absence of rhubarb serum metabolites. As shown in **Figure 2A**, rhubarb serum metabolite significantly inhibits MMP-2 functionality in A549 cells in a concentration dependent manner. The addition of rhubarb

serum metabolite causes a 22% and 92% reduction in MMP-2 activity when cells were co-cultured with half or full-concentration serum, respectability. However, the impact of rhubarb serum metabolite on MMP-9 activity was inconclusive due to extremely low level of MMP-9 expression in A549 cells. (**Figure 2A**)

In order to further explore the causes of protease activity reduction by rhubarb treatment, the protein expression level was determined by Western blot analysis. Similar to MMP-2 activity, rhubarb serum metabolite provoked a decrease in MMP-2 protein expression, including the pro- and active- forms of MMP-2, in a time-dependent manner (**Figure 2B**). These results suggest that rhubarb serum metabolite inhibited the MMP-2 activity might arise from reducing the production of pro-MMP-2 and the activation of MMP-2 protease.

3.3. Rhubarb Serum Metabolite Inhibits MMP-2 transcription through NF-κB/ c-Jun Pathway in A549 Cells. Since rhubarb serum metabolite treatment induces decrease of pro-MMP-2 protein in A549 cells, we therefore, proposed that transcriptional regulation may be involved in downregulating MMP-2. As shown in **Figure 3A**, the level of MMP-2 mRNA was reduced by rhubarb serum metabolite treatment. Several studies have indicated that transcription factors, including NF-κB, AP-1, c-Fos and c-Jun, are involved in the transcriptional regulation of MMP-2 [29-33]. To assess the underlying mechanism, A549 cells were treated with rhubarb serum metabolite at 1× serum concentration for the indicated time intervals, and the phosphorylation status of the selected transcription factors were analyzed by Western blotting. As shown in **Figure 3B**, the phosphorylation status of NF-κB and c-Jun were gradually diminished in a time-dependent manner upon treatment of rhubarb serum metabolite. This result suggests that

down-regulation of MMP-2 activity by rhubarb serum metabolite, at least in part, contributes by inhibiting of NF-κB/ c-Jun pathway.

3.4. Rhubarb Serum Metabolite Reduces MMP-2 Functionality through uPA Modulation. Several lines of evidence have demonstrated that MMP-2 activation may be through plasminogen activator/plasmin system in which pro-urokinase plasminogen activator (pro-uPA) binds to its receptor, uPA receptor (uPAR) [34], and this binding results in uPA activation, accelerating the conversion of plasminogen to plasmin on the cell surface, thereby cleaving and activating MMP-2 to enhance degradation of ECM components [35,36]. In this pathway, plasminogen activator inhibitor-1 (PAI-1) controls the rate of plasmin generation by forming irreversible inhibitory complexes with u-PA [37]. To further explore whether the suppression of MMP-2 activity by rhubarb serum metabolite might involves this process, the expression level of uPA, PAI-1, and TIMP-2 mRNA was investigated by RT-PCR. As shown in **Figure 4A**, there was no notable change in mRNA levels of PAI-1 and TIMP-2 in A549 cells by rhubarb serum metabolite treatment under all treatment conditions. However, a significant reduction in the uPA mRNA levels was noticed in a time-dependent manner (**Figure 4A and 4B**). Consistently, the protein level and functional activity of uPA was also decreased with rhubarb serum metabolite treatment in A549 cells (**Figure 4C and 4D**). Since uPA is an upstream activating enzyme of MMP-2, this result suggests that rhubarb serum metabolite reduces MMP-2 expression through post-translational modification by inhibiting uPA expression.

3.5. Rhubarb Serum metabolite Treatment Blocks Cell Motility. In addition to inhibiting MMP-2 activity, a variety of strategies have also been targeted to prevent occurrences of metastasis. Therefore we want to evaluate the inhibitory role of rhubarb

serum metabolite in the migratory ability of tumor cells. As shown in **Figure 5A**, treatment with control serum induces tumor cells to close the wound area in 72h. In contrast, rhubarb serum metabolite treatment inhibits wound healing of tumor cells in a time-dependent manner (**Figure 5B**), indicating that rhubarb serum metabolite appears to inhibit the migration ability of tumor cells.

3.6. Rhubarb Inhibits Experimental Lung Metastasis of Human A549 Adenocarcinoma *in Vivo*.

In order to confirm that rhubarb could block the metastatic ability of A549 in experimental lung metastatic mouse model, 0.75 g/kg of rhubarb decoction was orally fed to mice twice a day in A549 cancer cells bearing mice. This experimental setup mimics clinical administration of rhubarb decoction and maximum non-toxic tolerance dose according to previous studies [38]. As shown in Table 1, mice were scarified and the number of metastatic nodules in the lungs was examined in the indicated time. In the control groups, we found 205 ± 5 (n=5) metastatic nodules. Notably, the corresponding nodule numbers in mice dosed with 0.75 g/kg of rhubarb decoction were significantly decreased to 44.2 ± 35 (n=5). This result indicates that rhubarb is a potent anti-metastatic substance.

4. DISCUSSION

Herbal medicines, including medicinal foods, are used frequently as supplemental therapy for many chronic diseases including cancer. However, the mechanisms by which herbal medicine could improve clinical status of cancer patients is under-explored. Currently, several lines of evidence demonstrated that the rhubarb anthraquinones might contribute to anti-cancer capacity. For examples, emodin inhibits EGF-induced cell

migration in various cancer cells through PI3K pathway [39]; rhein suppresses the IL-1α-induced production of pro-MMP-1, -3, -9, and -13 in rabbit chondrocytes [40]; and aloe-emodin inhibits tumor metastasis through PKC, p38, and ERK pathway [41,42].

Our previous pharmacokinetic study showed that most anthraquinones were rapidly and extensively metabolize to glucuronides or sulfated forms after oral administration of rhubarb decoction in rats [22]. Therefore, previous *in vitro* bioactivity studies focusing on the parent form of anthraquinons might not appropriately predict the *in vivo* effects of anthraquinones. Thus, detailed knowledge concerning the metabolic fates and the resulting plasma levels of anthraquinons following ingestion of rhubarb are crucial for understanding their bioactivity. In order to further elucidate the mechanisms of rhubarb tumor metastatic suppression, we used an *ex vivo* system where several tumor cell lines were treated with serum metabolite of rhubarb treated rats. This then allowed us to exam the suppressive actions of rhubarb metabolite in an *in vitro* assay under physiological active metabolites and concentration.

The prevention of tumor metastasis is well recognized through diverse mechanisms, including tumor cell death induction. In present study, we first revealed the absence of cytotoxic effects of rhubarb serum metabolite on A549 cells (**Figure 1**), suggesting that induction of cell death might not be responsible for the prevention of tumor cell colony formation on the lung surface.

Furthermore, many studies have reported that proteinases related cell matrix degradation are required for tumor cell metastasis, and that an enhanced production of MMPs and u-PAs are correlated with invasion, migration, and angiogenesis of tumors. The impacts of rhubarb-serum metabolite on several proteases involved in ECM

degradation were investigated, and our results demonstrated that rhubarb serum metabolite could suppress protease activity of MMP-2 (**Figure 2A**). In addition, the expression level of MMP-2 protein was also dramatically inhibited by the treatment of rhubarb serum metabolite on A549 cells (**Figure 2B**). It is interesting to find that the addition of rhubarb serum metabolite causes a 92% reduction in MMP-2 activity (**Figure 2A**), but only a 50% reduction in the protein level of MMP-2 (pro-form) and 23% (active form) (**Figure 2B**) as compared to control serum in the same treatment duration of 48 h. Therefore, we propose the regulation of MMP-2 expression by rhubarb serum metabolite in A549 cells might be through both transcriptional as well as post-translational modification mechanisms.

Indeed, the mRNA level of MMP-2 was down-regulated by the treatment of rhubarb-serum metabolite in a time-dependent manner (**Figure 3A**). The transcription of MMP-2 gene is regulated by upstream regulatory factors including NF-κB, c-Jun, and AP-1, [31-33,43,44]. We analyzed the activity status of c-Jun and NF-κB in rhubarb serum metabolite treated A549 cells by Western blot analysis, the data showed that a notably reduction of c-Jun (p48) and NF-κB (p65) activation in the presence of rhubarb metabolite (**Figure 4B**). On the basis of these data, it appears that the inhibitory effect of rhubarb serum metabolite on c-Jun and NF-κB might be able to explain the suppression of MMP-2 gene expression, and detail mechanism and related regulation are under investigation in our laboratory.

In addition to transcription regulation, the plasminogen activation system may also control MMP-2 functionality. The plasminogen activation system consists of uPA, uPAR, PAI-1 and TIMP-2, and plays a key role in invasion and metastasis in many cancer types

including lung cancer [45,46]. Extracellular uPA facilitates matrix degradation by converting plasminogen to the active serine protease plasmin, which degrades fibronectin, laminin, collagen IV, and other noncollagenous extracellular matrix proteins. In this study, we demonstrated that the rhubarb serum metabolite could significantly inhibited the functional activity, protein level and mRNA level of uPA in A549 cells in a time dependent manner while the expression levels of TIMP-2 & PAI-1 were not affected (**Figure 4**). In addition to TIMP-2 and PAI-1, previous reports have also demonstrated that membrane type I MMP (MT1-MMP) serves as the first physiological activator of pro-MMP-2 [47-49]. Therefore, we current investigate if MT1-MMP involves in post-translational modification process of MMP-2 in rhubarb serum metabolite-treated cells as well. Moreover, rhubarb serum metabolite could also block cancer cell motility. This result confirms that rhubarb is a potential inhibitor in invasion and metastasis. The identification of the active components in the rhubarb metabolite and their effect on different signaling pathways is actively investigated in our laboratory.

Our animal experiments further demonstrated a significant reduction in lung metastatic colonies in rhubarb-treated mice, suggesting that rhubarb contain enriched active components that block cancer metastasis (**Table 1**).

Taken together, we have provided a novel *ex-vivo* approach to mimic clinical condition by using serum metabolite of rhubarb to investigate the anti-metastatic activity and mechanism in an *in vitro* bioactivity assay. Using this strategy, we have identified that rhubarb is a potent anti-metastatic herb that functions through the down-regulation of the transcriptional and post-translational modification processes of MMP-2.

5. ACKNOWLEDGEMENTS

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Table 1. Antimetastatic effect of rhubarb in the *in vivo* experimental metastasis model[a]

Treatment	Body weight (g)		Lung weight (mg) in Day 42	Lung metastasis			
	Day 0[b]	Day 42		Incidence	Number of metastatic nodules	Medium	Range
Control	26.1 ± 1.2	20.9 ± 3.3	233.6 ± 62.1	5/5	205.2 ± 4.8	208	All > 200
0.75 g/kg	26.1 ± 1.5	20.5 ± 2.7	211.4 ± 74.6	5/5	44.2 ± 35.0[c]	30	22 ~ 105

[a] A549 cells (2×10^6) were inoculated *i.v.* into SCID mice. Seven days later, groups of mice were orally administered rhubarb decoction *b.i.d.* All mice were killed on day 42, and formation of lung metastases was evaluated.

[b] Data expressed as mean ± S.D.

[c] $P < 0.01$

FIGURE LEGEND

Figure 1. Effect of rhubarb serum metabolite on the viability of A549 and MKN45 cells. Rats were orally administered rhubarb decoction and then serum metabolite was isolated and lyophilized. The lyophilized serum samples were reconstituted with PBS in 10-fold serum concentration, then those serum samples were added to culture medium in a final concentration equal to 1x serum. The A549 (lung carcinoma) and MKN45 (gastric carcinoma) cells were treated with those serum samples at 37℃ for 48 hours and then subjected to MTT assay to investigate the percent of cell viability. Each data point represents the mean ± S.D. of three independent experiments.

Figure 2. **Effect of rhubarb serum metabolite on the functional activity and expression level of MMP-2 and MMP-9 in A549 Cells.** (A) Rhubarb serum metabolite represses protease activity of MMP-2. Serum metabolite was prepared as mention in the previous figure legend then added to culturing medium to a final concentration equal to 0.5 or 1x serum. Cells were treated for 48h. Gelatinolytic activities of MMP-2 and MMP-9 in the conditioned medium were determined by gelatin zymography. The band intensity of active MMP-2 was quantified by densitometry. The data represent the mean ± S.D. of three independent experiments. (B) Time effect of rhubarb serum metabolite on regulation of MMP-2 protein expression. The A549 cells were harvested after rhubarb serum metabolite treatment at the indicated time points, and cell extract was prepared and loaded on SDS-PAGE. After SDS-PAGE separation, proteins were transferred on the PVDF-membrane, and expression level of MMP-2 was analyzed by Western blot analysis.

β-actin has been used as internal loading control.

Figure 3. Rhubarb serum metabolite affects MMP-2 transcript and phosphorylation status of NF-κB and c-Jun. (A) RT-PCR analysis of MMP-2 mRNA levels in A549 cells after rhubarb serum metabolite treatment. Cells were exposed to rhubarb serum metabolite and control serum at 1x serum concentration. MMP-2 mRNA levels were evaluated at the indicated times. Total RNA isolated from each group was subjected to semi-quantitative RT-PCR as described in methods. The band intensity of the MMP-2 mRNA was quantified by densitometry. The data represents the mean ± S.D. of three independent experiments. Human GAPDH was used as an internal control. (B) Phosphorylation status of NF-κB and c-Jun in A549 cells after rhubarb serum metabolite treatment. Cells were exposed to rhubarb serum metabolite at 1x serum concentration for the indicated times, and cell extract was prepared and loaded on SDS-PAGE. After SDS-PAGE separation, proteins were transferred on the PVDF-membrane, and phosphorylation status of NF-κB and c-Jun protein were analyzed by Western blot analysis.

Figure 4. **Rhubarb serum metabolite treatment inhibits uPA level and activity in A549 Cells.** (A) Cells were treated with the rhubarb serum metabolite and control serum at 1x serum concentration at 37^0C for the indicated times. Total RNA isolated from each group was subjected to semi-quantitative RT-PCR for the expression level of uPA, PAI-1, TIMP-2 mRNA as described in methods. GAPDH was used as an internal control. (B) The band intensities of the uPA mRNA were quantitated by densitometry. The data

represents the mean ± S.D. of three independent experiments. (C) Time effect of rhubarb serum metabolite on regulation of uPA protein expression. A549 cells were harvested after 1x rhubarb serum metabolite treated for the indicated times, and cell extract was prepared and loaded on SDS-PAGE. After electrophoresis, proteins were transferred on thePVDF-membrane, and expression level of uPA was analyzed by Western blot analysis. β-actin was used as an internal control. (D) Rhubarb serum metabolite represses protease activity of uPA. A549 cells were harvested after rhubarb serum metabolite at 0.5X and 1x serum concentration treated for 48 hours. Proteolytic activity of uPA in the conditioned medium was determined by casein zymography. The band intensity of uPA was quantified by densitometry. The data represent the mean ± S.D. of three independent experiments.

Figure 5. **Rhubarb serum metabolite treatment reduces the migration ability of MKN45 cells.** (A) *In vitro* cell motility of the rhubarb serum metabolite-treated cells. Confluent cell cultures were treated with rhubarb serum metabolite and control serum and wounded by scratching with plastic micropipette tips (top). Cells were photographed at 72 h after wounding by phase contrast microscopy (bottom) (×100). (B) The percentage of area wound healing of cells was quantified, and values represent mean ± S.D. from biological triplicate experiments. * $p < 0.05$.

Figure 1

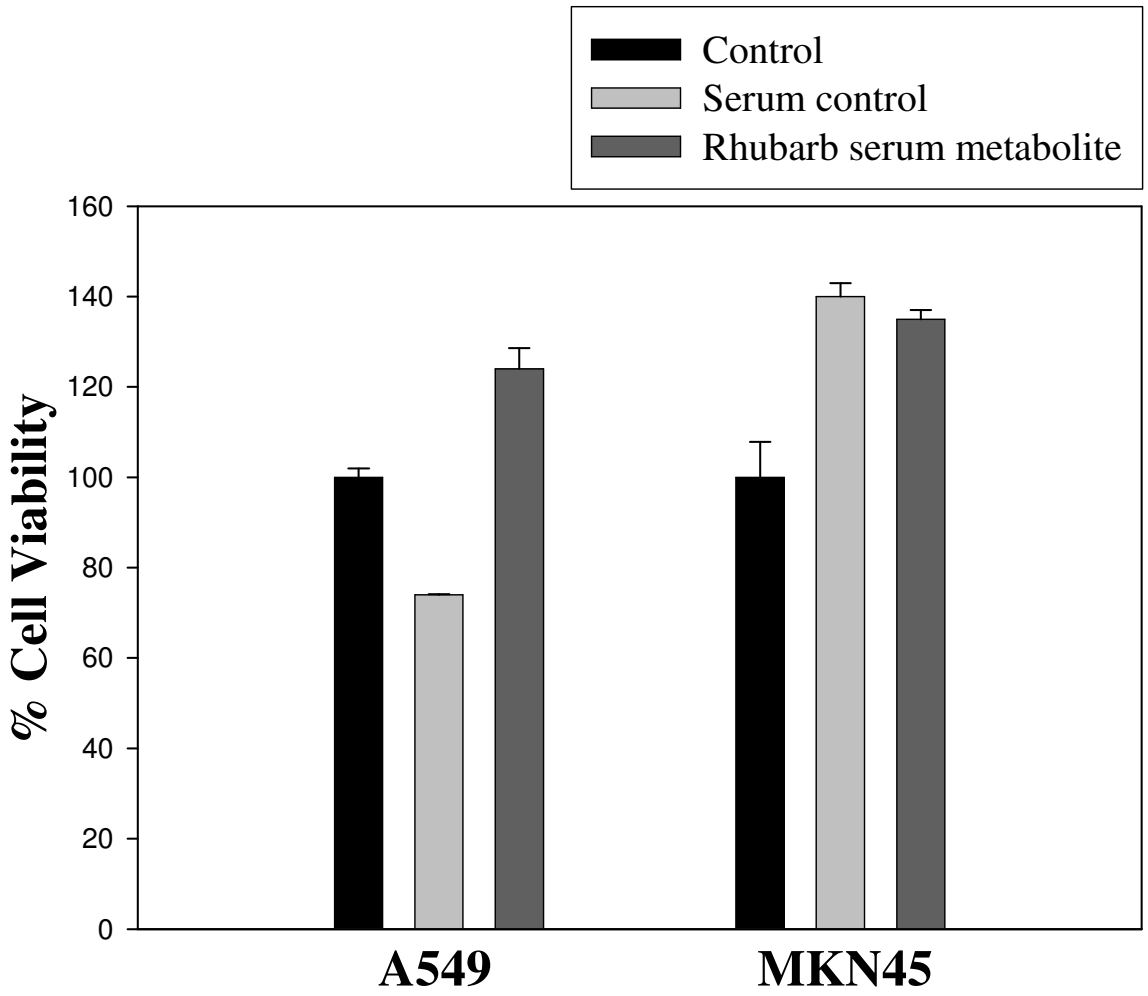

Control
Serum control
Rhubarb serum metabolite
% Cell Viability
160
140
120
100
80
60
40
20
0
A549
MKN45

Figure 2

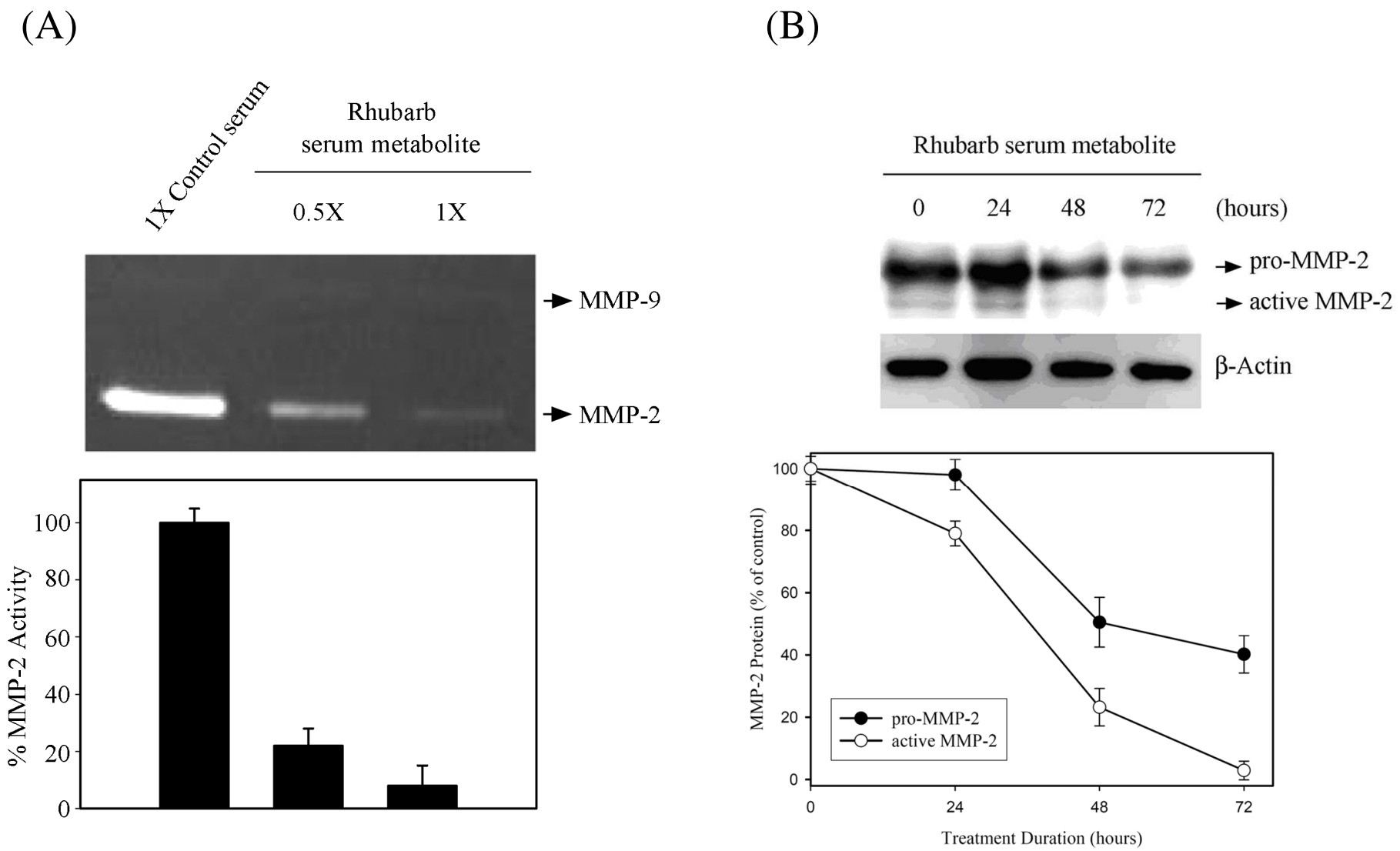
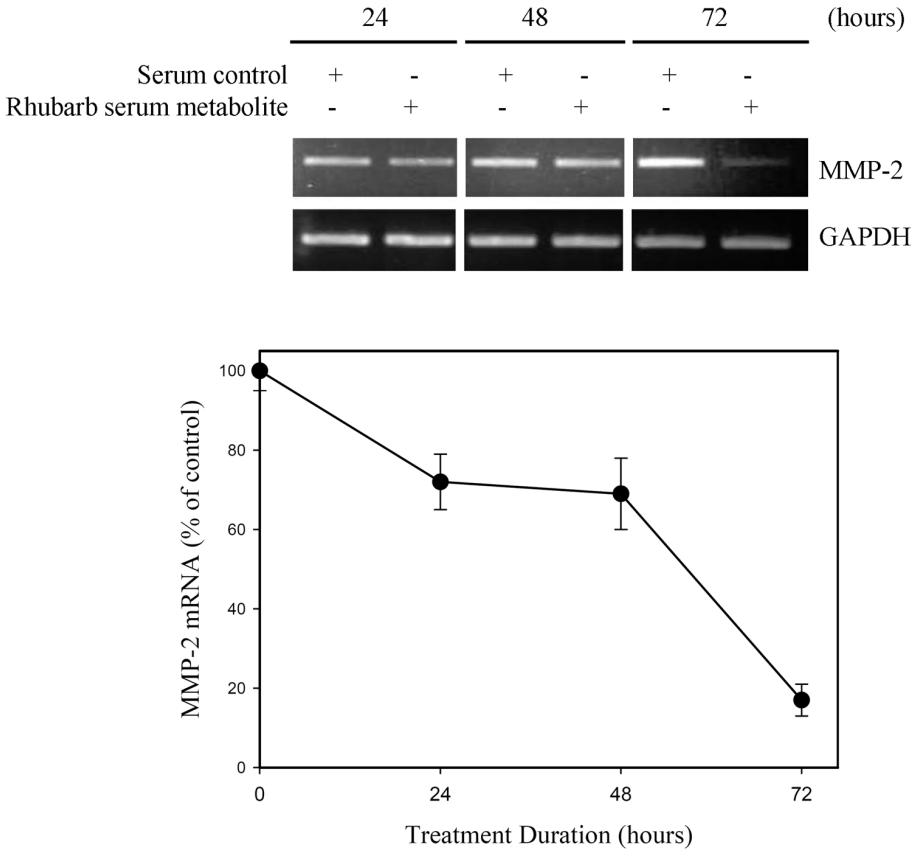

(A)
1X Control serum
Rhubarb serum metabolite
0.5X
1X
MMP-9
MMP-2
% MMP-2 Activity
100
80
60
40
20
0
(B)
Rhubarb serum metabolite
0
24
48
72
(hours)
pro-MMP-2
active MMP-2
β-Actin
MMP-2 Protein (% of control)
pro-MMP-2
active MMP-2
Treatment Duration (hours)

Figure 3

(A)


24
48
72
(hours)
Serum control + - + - + -
Rhubarb serum metabolite - + - + - +
MMP-2
GAPDH
MMP-2 mRNA (% of control)
100
80
60
40
20
0
0
24
48
72
Treatment Duration (hours)

(B)

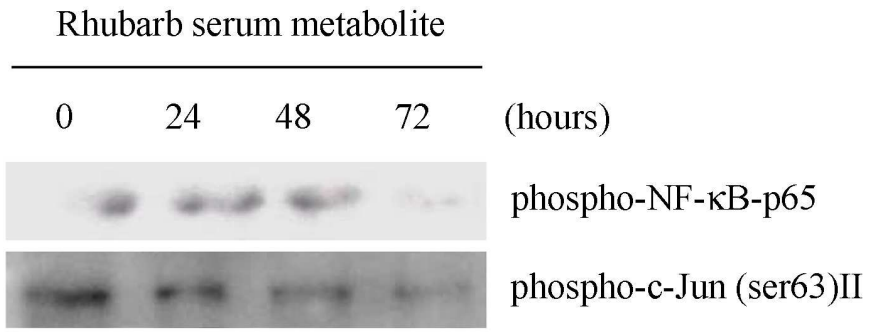
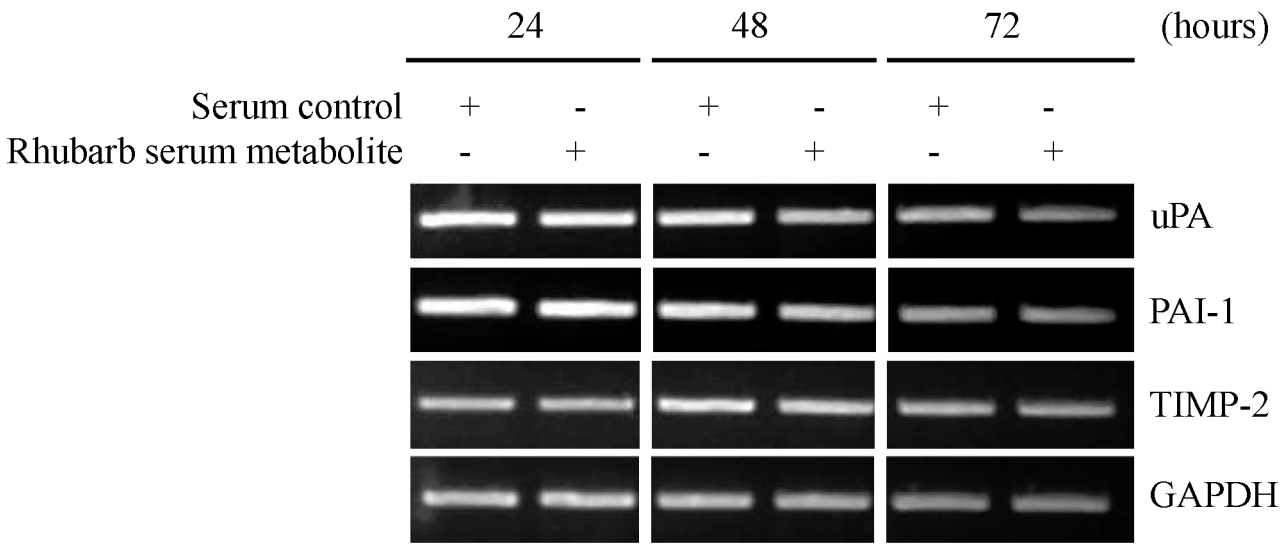

Rhubarb serum metabolite
0
24
48
72
(hours)
phospho-NF-κB-p65
phospho-c-Jun (ser63)II

Figure 4

(A)


24
48
72
(hours)
Serum control + - + - + -
Rhubarb serum metabolite - + - + - +
uPA
PAI-1
TIMP-2
GAPDH

(B)

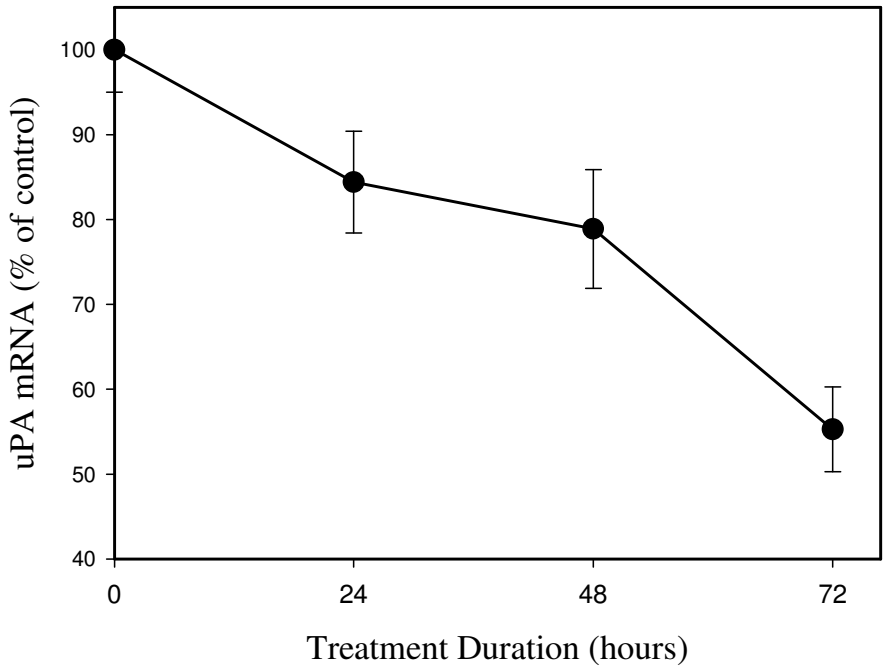

uPA mRNA (% of control)
100
90
80
70
60
50
40
0
24
48
72
Treatment Duration (hours)

Figure 4

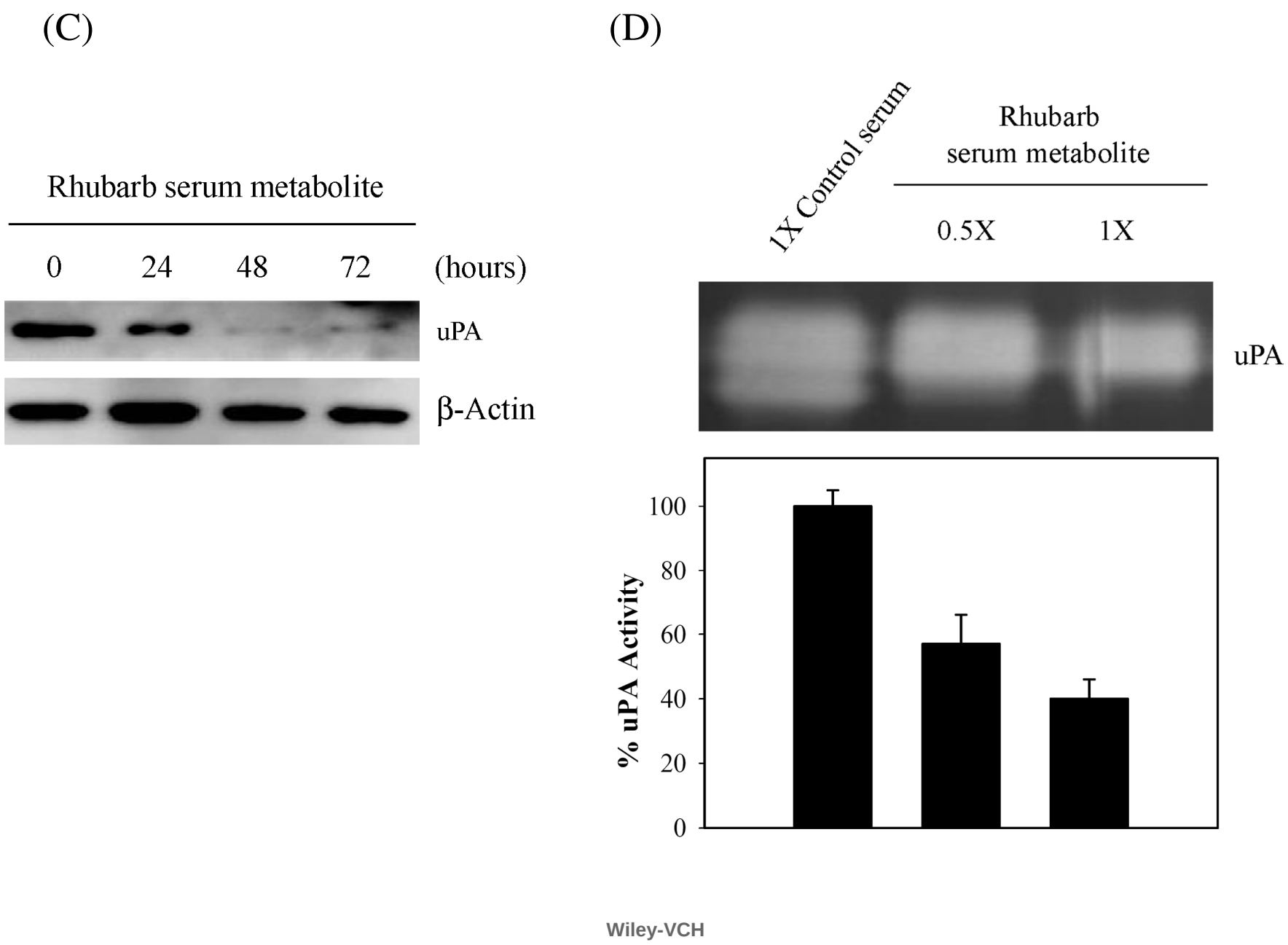
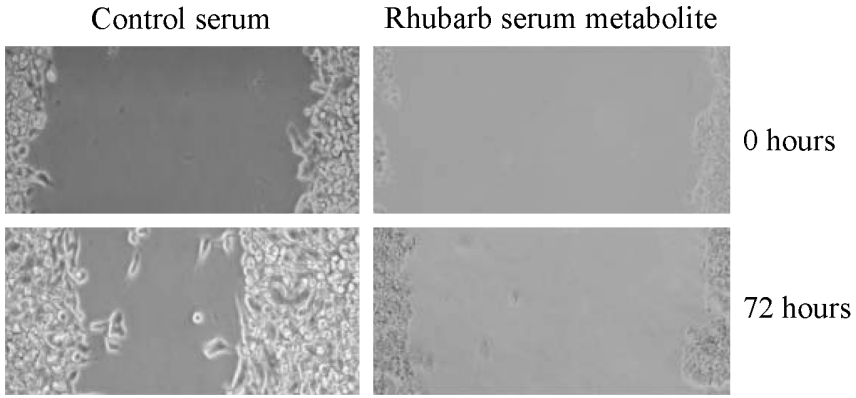
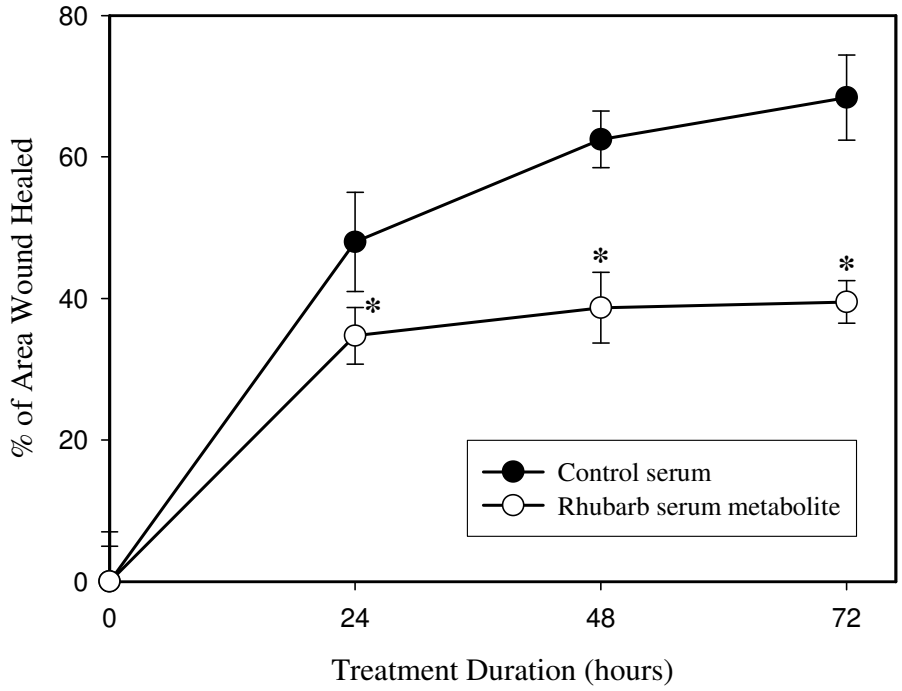

(C)
Rhubarb serum metabolite
0
24
48
72
(hours)
uPA
β-Actin
(D)
1X Control serum
Rhubarb
serum metabolite
0.5X
1X
uPA
% uPA Activity
100
80
60
40
20
0

Figure 5

(A)


Control serum
Rhubarb serum metabolite
0 hours
72 hours

(B)


% of Area Wound Healed
Treatment Duration (hours)
0
20
40
60
80
0
24
48
72
Control serum
Rhubarb serum metabolite
*
*
*