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Effect of matrine on HeLa cell adhesion and migration

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Abstract

This study sought to explore the role of matrine in the metastasis of cancer cells and to gain insight into the possible mechanism of matrine's ability to inhibit cancer metastasis. Accordingly, changes in vasodilator-stimulated phosphoprotein (VASP) phosphorylation and in cAMP-dependent protein kinase (PKA) activity during cell detachment and reattachment were first investigated. After administration of matrine (50 μg/ml), the decrease in VASP phosphorylation paralleled the decrease in PKA activity. Matrine was found to significantly inhibit HeLa cell adhesion to collagen I. To determine the effect of matrine on the migration of HeLa cells, we analysed the migratory behaviour of HeLa cells in a two and three-dimensional cell migration assay. In a two-dimensional cell migration assay, the average cell migration velocity was significantly reduced by matrine compared with the control. Moreover, in a three-dimensional cell migration assay performed with the Transwell system, HeLa cells treated with matrine (50 μg/ml) were found to migrate less than the control cells. These data suggest that the inhibitory effect of matrine may be produced by decreased phosphorylation of VASP due to inhibition of the activity of PKA during HeLa cell adhesion and migration.

Keywords: Matrine; Cervical cancer; VASP (Vasodilator-stimulated phosphoprotein); Cell adhesion; Cell migration

1. Introduction

Use of the Pap smear for cervical cancer screening has led to a decreased incidence of this malignancy in Western countries. However, carcinoma of the uterine cervix continues to be the leading cause of death from cancer among women in most developing countries. Distant metastasis is a major cause of death in patients with cervical cancer. The ability of cancer cells to invade and metastasize represents the final and most difficult-to-treat stage of cervical cancer, and it is this change that most often leads to the patient's death. Therefore, strategies to control the metastasis of cancer cells have received much attention in cervical cancer therapy.

However, cancer metastasis is a long series of sequential, interrelated, complex processes between cancer cells and host cells (Brooks, 1996). The metastatic cells first must break loose from neighbouring cells. Then, with the help of metalloproteinases (MMPs), cancer cells must weave their way through the extracellular matrix and capsule enclosing the tumour, then make their way into the blood or lymphatic vessels. Finally, they must migrate through the bloodstream to a distant site, exit the bloodstream, and establish new colonies (Folkman, 1996; Jiang et al., 1995). It is easy to conclude that cell–matrix, cell–cell adhesion, and cell migration have an important role in cancer metastasis.

Cell migration requires a coordinated series of events involving cell adhesion to the extracellular matrix, the assembly, disassembly or reorganization of the actin cytoskeleton, and membrane protrusion and retraction in space and time to generate productive and net forward movement. It is now widely accepted that cell migration consists of four distinct processes: actin-driven extension of protrusions such as lamellipodia and filopodia, stabilization of these structures by attachment to the extracellular matrix *via* the formation of focal adhesions at the front, translocation of the cell body, and

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detachment of the rear part of the cell upon disassembly of focal adhesions at this site (Lehmann, 2001; Pollard and Borisy, 2003; Raftopoulou and Hall, 2004).

Matrine, one of the major alkaloid components found in *Sophora* roots, has long been regarded as an anticancer herb in China. Matrine, first isolated and identified in 1958, is obtained primarily from *Sophora japonica* (kushen), but also from *Sophora subprostrata* (shandougen) and from the above-ground portion of *Sophora alopecuroides* (Lai et al., 2003; Zhang et al., 2001; Liu et al., 2000). The intensive investigation of the pharmacologic and clinical applications of these alkaloids remains a focus of Chinese medical research. The main clinical applications are treatment of cancer, viral hepatitis, cardiac diseases (such as viral myocarditis), and skin diseases (such as psoriasis and eczema). Injection of *Sophora* extract, containing mainly alkaloids, was used as an adjuvant to standard medical therapy in 200 cases of lung cancer, with reportedly good results. Xu Xiangru and Jiang Jikai reported that the alkaloids could inhibit the growth of tumour cells directly and could also affect immune functions (Xu and Jiang, 1998). In clinical work, they described the use of *Sophora* alkaloids for treating radiation or chemotherapy-induced leukopenia and for treating certain cancers, notably uterine cervical cancer and leukemia. In addition, matrine is also considered an important component of the treatment of oesophageal and laryngeal cancer. In a recent pharmacologic study, it was reported that matrine facilitated the differentiation of leukemia cells into mature and normal white blood cells. An *in vitro* study showed that by inhibition of adhesion of endothelial cells to metastatic lung cancer cell lines, expression of adhesion molecules, and permeability of endothelial cells, matrine inhibits the metastasis of cancer cells. Moreover, matrine induces differentiation by inhibiting cell proliferation and significantly inhibiting the invasion and metastatic potential of SMMC-7721 (Wang et al., 2003).

To gain insight into the role of matrine in the metastasis of cancer cells and to explore the possible mechanism of the inhibiting effect of matrine on cancer metastasis, we studied the effect of matrine on vasodilator-stimulated phosphoprotein (VASP) phosphorylation and cAMP-dependent protein kinase (PKA) activity during HeLa cell detachment and reattachment, the role of matrine in HeLa cell adhesion and migration, and the effect of matrine on the phosphorylation of VASP during HeLa cell adhesion and migration. We demonstrated that VASP phosphorylation *via* PKA is dynamically regulated during the process of HeLa cell adhesion and detachment. We further showed that matrine can significantly suppress the adhesion and

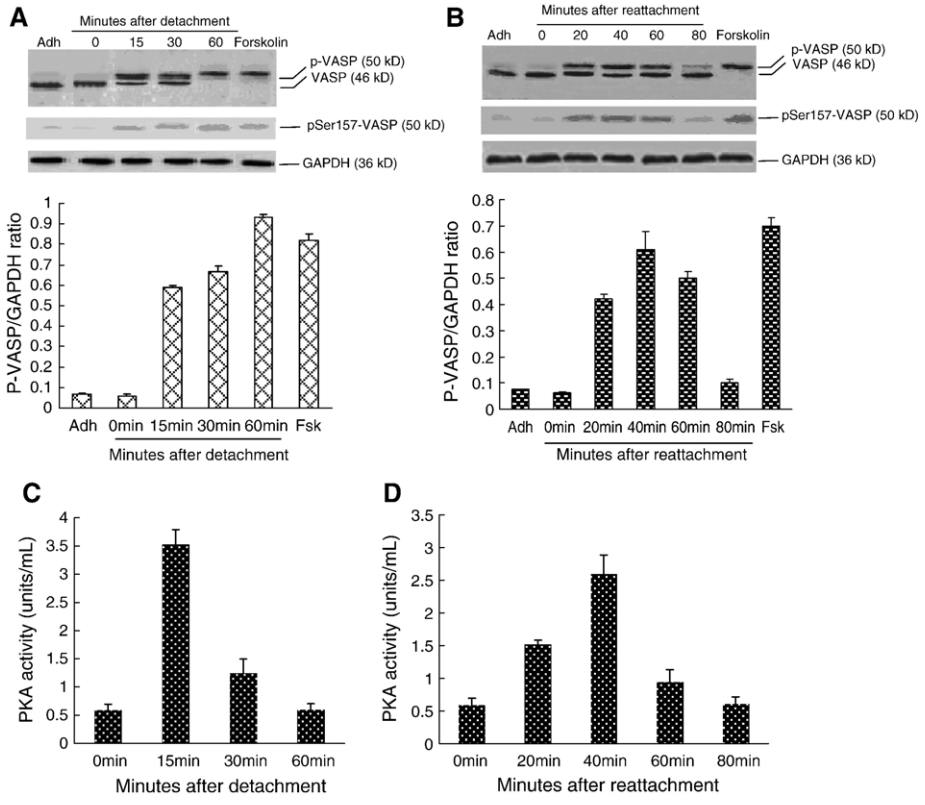

Fig. 1. Changes in VASP phosphorylation and PKA activity during HeLa cell detachment and reattachment. (A) and (B) HeLa cells were detached from the cell culture flask and incubated in suspension and then allowed to reattach to collagen I-coated plates for the indicated number of minutes and then harvested for immunoblot analysis with antibodies against total VASP and a phospho-specific antibody(pSer-157). Lysates from stably adherent (Adh) cells and cells treated with forskolin were included as references for unphosphorylated and fully phosphorylated VASP, respectively. 0 min after detachment and 0 min after reattachment indicate cells in suspension for 0 min. (C) and (D) HeLa cells were detached and held in suspension or allowed to reattach to collagen I for the indicated number of minutes and then harvested for PKA activity assay. The average and standard error from three separate experiments are shown.

migration of HeLa cells and decrease the level of VASP phosphorylation.

2. Materials and methods

2.1. Materials

HeLa cells were preserved by the Department of Pathophysiology, Medical College of Wuhan University. RPMI-1640 medium was from Hyclone Company. Matrine injection and docetaxel for injection were purchased from Guangzhou Mingxing Pharmaceutical Co., Ltd. and Qilu Pharmaceutical Co., Ltd. Pep Tag®. Non-Radioactive cAMP-dependent protein kinase assay kit was from Promega Company (USA). The monoclonal mouse anti-VASP antibody (IE273) was obtained from ImmunoGlobe Company. Phospho-VASP (Ser157) antibody was from Cell Signal Technology. The DAB Kit was purchased from Beijing Zhongshan Biotechnology Co., Ltd. The 24-well Transwell system was from Costar Corporation.

2.2. Cell culture

Human HeLa cells were maintained in RPMI-1640 medium with 10% calf serum at 37 °C in a humidified incubator supplemented with 5% carbon dioxide. For all experiments, HeLa cells were grown to near-confluence, then serum-starved overnight (12–16 h) in serum-free RPMI-1640 medium. Forskolin was dissolved in Me_2SO and added to the cells (at 25 μM) for 20 min. Detachment and reattachment on collagen-coated plates was performed as described (Howe and Juliano, 2000). Cells were detached with trypsin-EDTA and collected into RPMI-1640 containing 1% bovine serum albumin (BSA) and 1 mg/ml soybean trypsin inhibitor. After centrifugation for 10 min at 1000 ×*g*, the cells were washed with phosphate-buffered saline (PBS) once and resuspended in RPMI-BSA. They were then incubated in suspension with gentle rotation for the indicated period of time. For reattachment, 10-cm Petri dishes were coated with 20 μg/ml type-I collagen (Sigma) for 2 h at room temperature, then washed with PBS, and blocked for 30 min with RPMI-1640+2% BSA.

2.3. Cell adhesion assay

Ninety-six-well plates were incubated with 50 μl collagen I (20 μg/ml) at 37 °C for 1 h, then washed with PBS for twice and blocked with RPMI-1640+2% BSA for 30 min at 37 °C. HeLa cells were grown to 80–90% confluence and then harvested with 0.25% trypsin-EDTA and resuspended to a density of 8×10^5 cells/ml in serum-free RPMI-1640 medium. The cells were then treated with matrine in different concentrations (0, 5, 50, 100, 250, 500 μg/ml) for 24 h at 37 °C in a humidified incubator supplemented with 5% carbon dioxide. After digestion with trypsin-EDTA, 100 μl of cell suspension (8×10^5 cells/ml) was added to each well and cells were allowed to attach for 1 h at 37 °C. After the cells were washed gently with PBS twice to remove the non-adherent cells, 50 μl 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium-bromide (MTT) was added to each well for 4 h, then MTT was removed and 200 μl dimethyl sulfoxide (DMSO) was added to each well. The optical density (OD) of each well was measured with a microplate reader at 570 nm 15 min later. The experiments were performed three times. The control cells were not treated with matrine. The positive control cells were treated with docetaxel

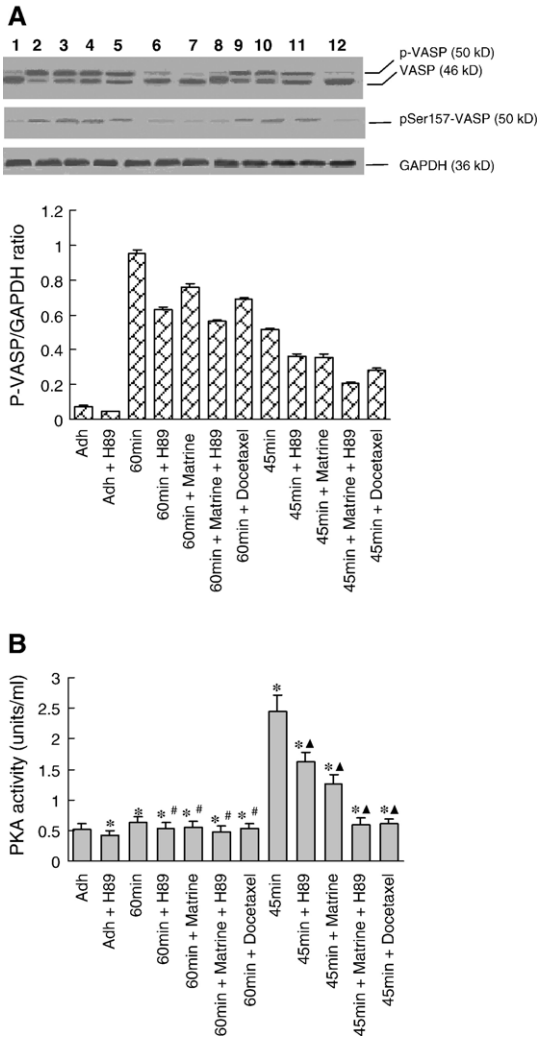

Fig. 2. Effect of matrine on VASP phosphorylation and PKA activity during HeLa cell detachment and reattachment. (A) and (B) HeLa cells treated with serum-free RPMI-1640 medium or matrine (50 μg/ml) or docetaxel (0.05 μg/ml) for 24 h at 37 °C were detached from the cell culture flask and incubated in suspension for 60 min (60 min, 60 min+H89, 60 min+Matrine, 60 min+Matrine+H89, 60 min+Docetaxel, respectively, lanes 2, 9, 3, 10,4) and allowed to reattach to collagen I-coated plates for 45 min (45 min, 45 min+H89, 45 min+Matrine, 45 min+Matrine+H89, 45 min+Docetaxel, respectively, lanes 5, 11, 6, 12, 7), and then were harvested for immunoblot analysis with total VASP antibody and a phospho-specific antibody(pSer-157) and PKA activity assay, respectively. HeLa cells treated with docetaxel (0.05 μg/ml) were regarded as a positive control. The average and standard error of values from three separate experiments are shown. Statistical analyses were performed using the *t*-test and one-way ANOVA. * ($P<0.05$) indicates a significant difference compared with the adherent (Adh, lanes 1 or Adh+H89, lanes 8) HeLa cell group. # ($P<0.05$) indicates a significant difference compared with the time point of 60 min after cell detachment. ▲ ($P<0.05$) indicates a significant difference compared with the time point of 45 min after cell reattachment.

(0.05 μg/ml). The cell adhesion ratio was calculated using the following formula:

The cell adhesion ratio
$= OD_{570\ \text{treated group}} / OD_{570\ \text{untreated group}}$

2.4. Two-dimensional cell migration assay

A two-dimensional cell migration assay using a wound-healing model was performed as previously described (Santos et al., 1997). After being serum-starved for 12 h in serum-free RPMI1640 medium, a confluent monolayer of HeLa cells was treated with matrine (50 μg/ml) and docetaxel (0.05 μg/ml) in 96-well plates for 24 h. The monolayer was then scraped with a sterile 200 μl pipette tip into a 96-well-plate and washed with PBS. After this, time-lapse images were captured using an inverted phase-contrast microscope at 100× magnification for 24 h. The control cells were not treated with matrine. The positive control cells were treated with docetaxel (0.05 μg/ml). Cell migration was evaluated by calculating the average cell migration velocity using the following formula:

The average cell migration velocity
= migration distance/ migration time

2.5. Three-dimensional cell migration assay

A three-dimensional cell migration assay was performed with the Transwell system, which allows cells to migrate through an 8-μm pore size polycarbonate membrane. Serum-free RPMI-1640 medium was first added to the 24-well plate well (the lower chamber of Transwell), and then to the Transwell insert (the upper chamber of Transwell), which then was equilibrated overnight to allow cell attachment at 37 °C in a incubator supplemented with 5% CO_2. Cells were trypsinized, washed, and resuspended in

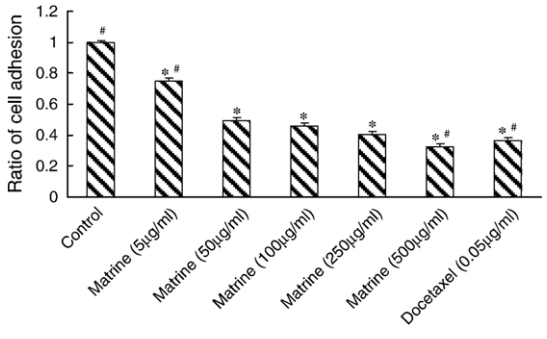

Fig. 3. Effect of matrine on the adhesion of HeLa cells. HeLa cells treated with serum-free RPMI-1640 medium or matrine (0, 5, 50, 100, 250, 500 μg/ml) or docetaxel (0.05 μg/ml) for 24 h at 37 °C were seeded in clear-bottom 96-well plates at a density of 8×10^5 cells/ml and allowed to attach 1 h at 37 °C. After the cells were gently washed with PBS twice to remove the non-adherent cells, the HeLa cell adhesion ratio was quantified by the MTT assay. The positive controls were HeLa cells treated with docetaxel (0.05 μg/ml). The average and standard error of values from three separate experiments are shown. Statistical analyses were performed using the *t*-test and one-way ANOVA. * ($P<0.01$) indicates a significant difference compared with the control group. # ($P<0.01$) indicates a significant difference compared with the matrine (50 μg/ml) group.

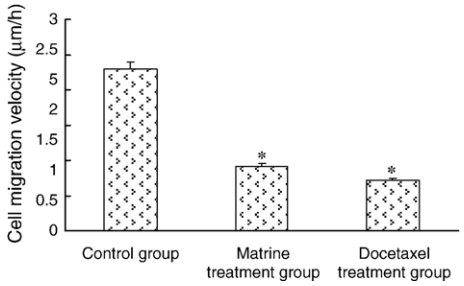

Fig. 4. Effect of matrine on the migration of HeLa cells in a two-dimensional cell migration assay. HeLa cells treated with serum-free RPMI-1640 medium, matrine (50 μg/ml) or docetaxel (0.05 μg/ml) were seeded in clear-bottom 96-well plates at high density and allowed to form monolayers overnight. Then, the cells were damaged with a pipette tip. After wound healing for 24 h, the cell migration velocity was computed as the ratio of the migration distance to the migration time (Materials and methods). HeLa cells treated with docetaxel (0.05 μg/ml) were regarded as a positive control. Data are expressed as mean cell migration velocity ± S.E.M. At least 10 migrating cells were counted to calculate the velocity. The average and standard error of values from three separate experiments are shown. Statistical analyses were performed using the *t*-test and one-way ANOVA. * ($P<0.01$) indicates a significant difference compared with the control group.

RPMI-1640 medium containing 10% calf serum (1×10^6 cells/ml). This suspension (100 μl) was added to the upper chamber of the Transwell. The lower chamber was filled with 600 μl RPMI-1640 medium with 20% calf serum. HeLa cells adhered to the Transwell filters during a 2 h incubation at 37 °C in the presence of 5% CO_2. Then the RPMI-1640 medium containing 10% calf serum in the upper chamber was replaced with serum-free RPMI-1640 medium or matrine (50 μg/ml) or docetaxel (0.05 μg/ml). After incubation for 12 h at 37 °C in the presence of 5% CO_2, the cells were fixed for 30 min in 4% formaldehyde and stained for 15 min with crystal violet. The filters were then rinsed thoroughly in distilled water and checked by brightfield microscopy to ensure that the cells were adherent and had migrated. The non-migrating cells were then carefully removed from the upper surface (inside) of the Transwell with a wet cotton swab. To quantify cell motility, cells that had migrated to the bottom surface of the filter were counted. Nine evenly spaced fields of cells were counted in each well, using an inverted phase-contrast microscope at 200× magnification. The control cells were not treated with matrine. The positive control cells were treated with docetaxel (0.05 μg/ml).

2.6. PKA activity assay

PKA activity was measured exactly according to the protocol of Pep Tag® Non-Radioactive cAMP-dependent protein kinase assay kit. Cells in suspension or on collagen I-coated plates were rapidly washed twice with ice-cold PBS, then resuspended or covered in PKA extraction buffer on ice for 15 min. The lysates were scraped into tubes (for adherent cells) and clarified by centrifugation at 14,000 ×*g* for 10 min at 4 °C. Supernatants were assayed for PKA activity.

2.7. SDS-PAGE and Western blotting

All cells were washed twice with ice-cold PBS and then lysed in lysis buffer (1% Triton X-100, 20 mM Tris–Cl, pH7.5,

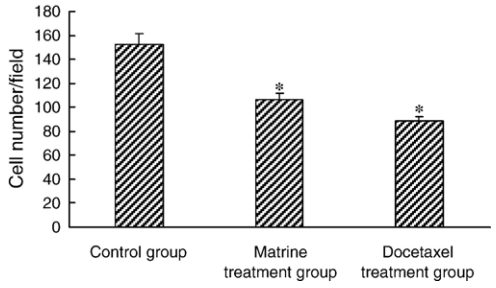

Fig. 5. Effect of matrine on the migration of HeLa cells in a three-dimensional cell migration assay with the Transwell system. HeLa cells treated with serum-free RPMI-1640 medium, matrine (50 μg/ml) or docetaxel (0.05 μg/ml) were seeded into the upper chamber of Transwell at a density of 1×10^6 cells/ml and allowed to migrate through the pores for 12 h. Then, the number of cells per field that had migrated to the bottom surface of the filter were counted. The positive controls were HeLa cells treated with docetaxel (0.05 μg/ml). The average and standard error from three separate experiments are shown. Statistical analyses were performed using the *t*-test and one-way ANOVA. * ($P<0.05$) indicates a significant difference compared with the control group.

150 mM NaCl), using 50 μl lysis buffer per well. After 15 min on ice, lysates were scraped into microcentrifuge tubes and centrifuged at 14,000 ×*g* for 15 min at 4 °C. The protein concentration of the supernatant lysate was determined by Coomassie Brilliant Blue assay. For direct immunoblotting, aliquots of lysate were mixed with 5×sample buffer containing 2-mercaptoethanol and boiled for 5 min before loading on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel with a 5% (v/v) stacking gel and a 12% (v/v) gradient gel. Following SDS-PAGE separation, proteins were transferred to a nitrocellulose membrane overnight at 40 mA. After blocking with 5% (w/v) BSA in Tris-buffered saline (TBS) at 37 °C for 2 h, membrane strips were incubated with a 1:1500 dilution of anti-VASP mAb at 37 °C for 2 h. After extensive washing, membrane strips were incubated with a 1:2000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG at 37 °C for 2 h and then extensively washed again. Detection was performed with DAB (3, 3-diaminobenzidine). The immune enzymic reaction was stopped by rinsing in distilled water.

2.8. Statistical analysis

All values are expressed as means±standard error of the mean (S.E.M.). The difference of means between multiple groups was assessed using analysis of variance (ANOVA). Statistical significance was established between two groups using the *t*-test. A probability value of $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Changes in VASP phosphorylation and PKA activity during HeLa cell detachment and reattachment

To investigate whether VASP phosphorylation is affected by matrine, we first studied the changes in VASP phosphorylation during cell detachment and reattachment. HeLa cells were left untreated, detached and incubated in suspension for 0 min, 15 min, 30 min, and 60 min. Cell extracts were then subjected to immunoblotting analysis with anti-VASP antibodies (IE273). Phosphorylation of VASP at Ser-157, the preferred PKA site, but not at Ser-235 or Thr-274, causes a significant electrophoretic mobility shift of VASP on SDS-PAGE gels, and this shift is routinely used as a reliable marker of Ser-157 phosphorylation (Butt et al., 1994; Smolenski et al., 1998; Harbeck et al., 2000). In our research, VASP was predominant in the faster migrating, unphosphorylated form in untreated, stably adherent cells (Fig. 1). Detachment of cells induced gradual phosphorylation of VASP at Ser-157 to almost the same extent as treatment with forskolin (a positive control, Fig. 1). This was confirmed by immunoblotting with a phospho-specific antibody that recognizes VASP only when it is phosphorylated near the Pro-rich region (pSer-157). Conversion of VASP to its phosphorylated form was more than 60% complete within 15 min after HeLa cell detachment and more than 95% complete after 60 min. This detachment-induced phosphorylation of VASP at Ser-157 was

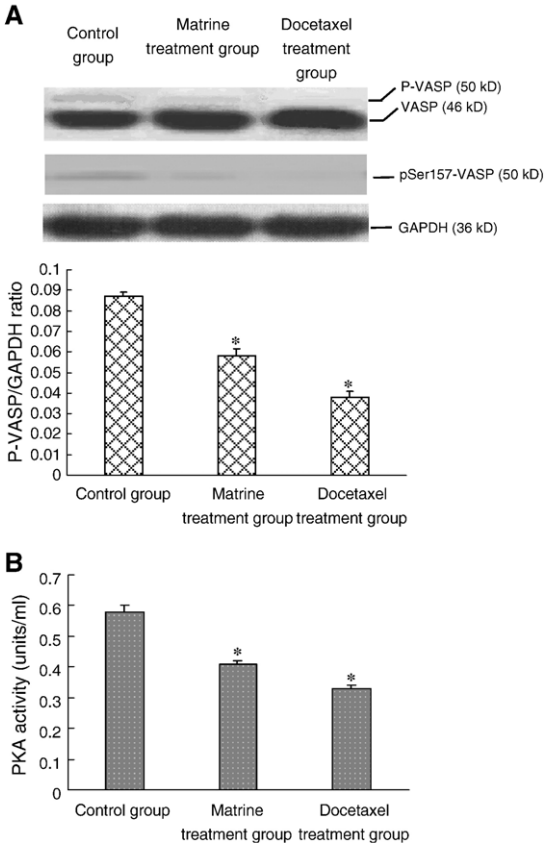

Fig. 6. Effect of matrine on the phosphorylation of VASP and on PKA activity during HeLa cell migration. (A) and (B) HeLa cells treated with serum-free RPMI-1640 medium, matrine (50 μg/ml) or docetaxel (0.05 μg/ml) for 24 h were harvested for immunoblot analysis with antibodies against VASP and PKA activity assay respectively. The positive controls were HeLa cells treated with docetaxel (0.05 μg/ml). The average and standard error of values from three separate experiments are shown. Statistical analyses were performed using the *t*-test and one-way ANOVA. * ($P<0.05$) indicates a significant difference compared with the control group.

also observed in an endothelial cell line (ECV304) and a gastric carcinoma cell line (SGC7901) (data not shown). During cell detachment, PKA activity in HeLa cells was high in cells soon after detachment but decreased as cells remained in suspension and showed a transient upregulation, peaking at 15 min (Fig. 1). These data demonstrate that cell detachment induces the phosphorylation of VASP, which may depend on intracellular PKA activity.

To determine whether the detachment-induced phosphorylation of VASP was reversible, HeLa cells were detached with trypsin-EDTA and then allowed to reattach on collagen-coated plates for 0 min, 20 min, 40 min, 60 min and 80 min and harvested for immunoblot analysis. VASP were rapidly phosphorylated following adhesion to collagen I as judged by the increased electrophoretic mobility of the proteins (Fig. 1), and showed an intermediate level of phosphorylation during cell spreading. At the same time, after adhesion to collagen I, PKA activity decreased initially but increased during cell spreading and returned to a low level at 80 min. This time course closely paralleled that of VASP phosphorylation at Ser-157 (Fig. 1). The changes in PKA activity correlated with the changes in VASP phosphorylation at Ser-157 during cell reattachment and cell spreading.

3.2. Effect of matrine on VASP phosphorylation and PKA activity during HeLa cell detachment and reattachment

To explore the effect of matrine on VASP phosphorylation and PKA activity during HeLa cell detachment and reattachment, HeLa cells were treated with matrine and docetaxel 60 min after cell detachment and 45 min after cell reattachment, based on the above experimental results. Sixty minutes after cell detachment and 45 min after cell reattachment, phosphorylation of VASP at Ser-157 was decreased compared with that at the corresponding time points when HeLa cells were not treated with matrine and docetaxel. Matrine did not cause a significant decrease in phosphorylation of VASP compared with docetaxel. PKA activity also decreased 60 min after cell detachment and 45 min after cell reattachment after incubation with matrine and docetaxel for 12 h. The decrease in PKA activity paralleled that in VASP phosphorylation (Fig. 2). VASP phosphorylation and PKA activity also decreased after administration of H89, a specific inhibitor of PKA. It must be pointed out that VASP phosphorylation and PKA activity decreased further when HeLa cells were treated with matrine. These data suggest that matrine may decrease the phosphorylation of VASP by inhibiting PKA activity during the detachment and reattachment of HeLa cells.

3.3. Effect of matrine on HeLa cell adhesion

To determine the effect of matrine on the adhesion of HeLa cells, we analysed the adhesion behaviour of HeLa cells in a cell adhesion assay using 96-well plates coated with collagen I. The HeLa cell adhesion ratio was quantified with the MTT assay. After HeLa cells were treated with matrine at 5, 50, 100, 250 and 500 μg/ml for 24 h, the cell adhesion ratio was reduced to 74.68%, 48.97%, 45.66%, 40.68% and 32.61%, respectively, compared with that of the control cells. But when HeLa cells were treated with docetaxel (a positive control) (0.05 μg/ml), the cell adhesion ratio was reduced to 36.22% (Fig. 3). These data indicate that matrine can inhibit HeLa cell adhesion to collagen I, although docetaxel has a stronger inhibitory effect on HeLa cell adhesion to collagen I.

3.4. Effect of matrine on HeLa cell migration

To determine the effect of matrine on the migration of HeLa cells, we analysed the migratory behaviour of HeLa cells in a two and three-dimensional cell migration assay. In a two-dimensional cell migration assay, a wound-healing model was used to assess the migratory behaviour of HeLa cells (Fig. 4). In the control group, observable wound healing was found 24 h after the cell monolayer was damaged with a pipette tip. Significant cell migration could be seen at 24 h at the wound margin. But wound healing was not clearly seen after cells were treated with matrine and docetaxel for 24 h. Cell migration was markedly inhibited at 24 h at the wound margin. The average cell migration velocity was significantly reduced compared with that of the control group. In a three-dimensional cell migration assay with the Transwell system, cells treated with matrine (50 μg/ml) or docetaxel (0.05 μg/ml) for 12 h were found to migrate less than the control cells (Fig. 5). These results indicate that matrine suppresses HeLa cell migration, possibly *via* PKA.

3.5. Effect of matrine on the phosphorylation of VASP and PKA activity during HeLa cell migration

To explore the possible mechanism of the inhibiting effect of matrine on HeLa cell migration, we investigated the effect of matrine on VASP phosphorylation and PKA activity. In a two-dimensional cell migration assay, the phosphorylation of VASP decreased compared with that of cells not treated with matrine (50 μg/ml) or docetaxel (0.05 μg/ml), which demonstrates that matrine may inhibit VASP phosphorylation during HeLa cell migration. PKA activity also decreased after incubation with matrine and docetaxel for 24 h. The decrease in PKA activity paralleled that in VASP phosphorylation (Fig. 6). These results suggest that matrine may decrease the phosphorylation of VASP by inhibiting the activity of PKA during HeLa cell migration.

4. Discussion

The mammalian vasodilator-stimulated phosphoprotein (VASP), which was first described in human platelets, is the patriarch of the Ena/VASP family of cytoskeletal regulatory proteins that, in mammals, includes the mammalian homologue of the *Drosophila* Ena protein (Mena) and the Ena/VASP-like protein EVL (Reinhard et al., 2001; Krause et al., 2003). All of these proteins share a tripartite domain structure comprising an amino-terminal Ena/VASP homology (EVH)-1 domain, a carboxyl-terminal EVH-2 domain, and a more variable central proline-rich region (PRR). Previous studies performed with different systems have suggested that this family of proteins has a universal role in the control of cell motility and intracellular

actin dynamics. VASP is predominantly localized in stress fibres, cell–matrix and cell–cell adherent junctions, and highly dynamic membrane areas (Reinhard et al., 1992). VASP is a substrate of both cGMP- and cAMP-dependent protein kinases (Halbrugge et al., 1990; Walter et al., 1993). Three common cGMP-dependent protein kinase/cAMP-dependent protein kinase phosphorylation sites have been biochemically identified in human VASP (Ser-157, Ser-239, and Thr-278) (Butt et al., 1994; Smolenski et al., 1998), two of which are also conserved in Mena (mammalian Enabled) and one in Evl (Ena-VASP-like), two other family members (Gertler et al., 1996). Although each phosphorylation site can be regulated by both PKA and the cGMP-dependent protein kinase, Ser-157 is the preferred target for PKA (Butt et al., 1994; Smolenski et al., 1998). Phosphorylation of VASP has been shown to be crucial for regulating its function (Krause et al., 2003; Howe et al., 2005), particularly its role in regulating cell migration (Loureiro et al., 2002; Lebrand et al., 2004). Previous studies have suggested that VASP may have a role in trophoblast invasion, angiogenesis, cell proliferation, and tumorigenesis (Kayisli et al., 2002a, b; Chen et al., 2004; Liu et al., 1999). Dertsiz et al. have shown that VASP expression is higher in adenocarcinomas than in normal bronchial epithelial cells and pneumocytes. They also found a gradual increase in VASP expression in adenocarcinoma tissue in parallel to an increase in tumour stage, which suggests that VASP may be involved in the invasive behaviour of lung adenocarcinomas, possibly by regulating intracellular F-actin formation, focal adhesion, and cell migration. So, phosphorylation of VASP may play an important role in cancer invasion and metastasis (Dertsiz et al., 2005).

Matrine, one of the major alkaloid components found in *Sophora* roots, has long been regarded as an anticancer herb in China (Lai et al., 2003; Zhang et al., 2001; Liu et al., 2000). In this study, we showed that the phosphorylation of VASP at Ser-157 gradually increased and that PKA activity was transiently upregulated during cell detachment. This is consistent with the report of Howe et al. (2002). The disparity in the observed decrease in PKA activity with prolonged incubation in suspension and the sustained phosphorylation of VASP at Ser-157 remains to be explained. Perhaps it is involved in the PKC signal pathway during cell detachment (Wentworth et al., 2006; Chitaley et al., 2004). VASP was rapidly phosphorylated following adhesion to collagen I, as judged by the increased electrophoretic mobility of the proteins, and then showed an intermediate level of phosphorylation during cell spreading. At the same time, following adhesion to collagen I, PKA activity decreased initially but increased during cell spreading and returned to a low level at 90 min, a time course that closely paralleled that of VASP phosphorylation at Ser-157. After administration of matrine, VASP phosphorylation decreased during HeLa cell detachment and reattachment. PKA activity also decreased, paralleling to that in VASP phosphorylation. These data suggest that matrine may decrease the phosphorylation of VASP by inhibiting PKA activity during the detachment and reattachment of HeLa cells.

In addition, we also studied the effect of matrine on HeLa cell adhesion and migration. In cell adhesion assays, matrine significantly reduced the ratio of cell adhesion to collagen I, and cell adhesion was markedly inhibited compared with that of the control cells. The two-dimensional cell migration assay showed that the average cell migration velocity was significantly reduced compared with that of the control group, which demonstrates that matrine may inhibit the average migration velocity of HeLa cells. Thus matrine may suppress HeLa cell migration. Likewise, the three-dimensional cell migration assay also showed that cells treated with matrine for 12 h were found to migrate less than the control cells. The result indicates that matrine suppressed HeLa cell migration. Matrine also has an inhibitory effect on the phosphorylation of VASP and on PKA activity during HeLa cell migration. We found that the phosphorylation of VASP was decreased compared with that of the control cells. At the same time, PKA activity was also decreased after administration of matrine for 24 h. The decrease in PKA activity paralleled that in VASP phosphorylation. These results suggest that matrine may decrease the phosphorylation of VASP by inhibiting the activity of PKA during HeLa cell migration.

In conclusion, we found that matrine can inhibit HeLa cell adhesion and migration. The inhibitory effect of matrine is possibly produced by a decreased phosphorylation of VASP due to inhibition of the activity of PKA during HeLa cell adhesion and migration. In addition, our results also show that matrine may decrease the phosphorylation of VASP by inhibiting PKA activity during detachment and reattachment of HeLa cells. The exact molecular mechanism of the inhibiting effect of matrine on cell adhesion, migration, and cancer metastasis needs further study.

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References

Brooks, P.C., 1996. Cell adhesion molecules in angiogenesis. Can. Met. Rev. 15, 187–194.

Butt, E., Abel, K., Krieger, M., Palm, D., Hoppe, V., Hoppe, J., Walter, U., 1994. cAMP- and cGMP-dependent protein kinase phosphorylation sites of the focal adhesion vasodilator-stimulated phosphoprotein (VASP) in vitro and in intact human platelets. J. Biol. Chem. 269, 14509–14517.

Chen, L., Daum, G., Chitaley, K., Coats, S.A., Bowen-Pope, D.F., Eigenthaler, M., Thumati, N.R., Walter, U., Clowes, A.W., 2004. Vasodilator-stimulated phosphoprotein regulates proliferation and growth inhibition by nitric oxide in vascular smooth muscle cells. Arterioscler. Thromb. Vasc. Biol. 24, 1403–1408.

Chitaley, K., Chen, L., Galler, A., Walter, U., Daum, G., Clowes, A.W., 2004. Vasodilator-stimulated phosphoprotein is a substrate for protein kinase C. FEBS Lett. 556, 211–215.

Dertsiz, L., Ozbilim, G., Kayisli, Y., Gokhan, G.A., Demircan, A., Kayisli, U.A., 2005. Differential expression of VASP in normal lung tissue and lung adenocarcinomas. Thorax 60, 576–581.

Folkman, J., 1996. Fighting cancer by attacking its blood supply. Sci. Am. 275, 150–154.

Gertler, F.B., Niebuhr, K., Reinhard, M., Wehland, J., Soriano, P., 1996. Mena, a relative of VASP and *Drosophila* Enabled, is implicated in the control of microfilament dynamics. Cell 87, 227–239.

Halbrugge, M., Friedrich, C., Eigenthaler, M., Schanzenbacher, P., Walter, U., 1990. Stoichiometric and reversible phosphorylation of a 46-kDa protein in human platelets in response to cGMP- and cAMP-elevating vasodilators. J. Biol. Chem. 265, 3088–3093.

Harbeck, B., Huttelmaier, S., Schluter, K., Jockusch, B.M., Illenberger, S., 2000. Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. J. Biol. Chem. 275, 30817–30825.

Howe, A.K., Juliano, R.L., 2000. Regulation of anchorage-dependent signal transduction by protein kinase A and p21-activated kinase. Nat. Cell Biol. 2, 593–600.

Howe, A.K., Hogan, B.P., Juliano, R.L., 2002. Regulation of vasodilator-stimulated phosphoprotein phosphorylation and interaction with Abl by protein kinase A and cell adhesion. J. Biol. Chem. 11 (277), 38121–38126.

Howe, A.K., Baldor, L.C., Hogan, B.P., 2005. Spatial regulation of the cAMP-dependent protein kinase during chemotactic cell migration. Proc. Natl. Acad. Sci. U. S. A. 102, 14320–14325.

Jiang, W.G., Hiscox, S., Singhrao, S.K., Puntis, M.C., Nakamura, T., Mansel, R.E., Hallett, M.B., 1995. Induction of tyrosine phosphorylation and translocation of ezrin by hepatocytegrowth factor/scatter factor. Biochem. Biophys. Res. Commun. 217, 1062–1069.

Kayisli, U.A., Demir, R., Erguler, G., Arici, A., 2002a. Vasodilator-stimulated phosphoprotein expression and its cytokine-mediated regulation in vasculogenesis during human placental development. Mol. Hum. Reprod. 8, 1023–1030.

Kayisli, U.A., Selam, B., Demir, R., Arici, A., 2002b. Expression of vasodilator-stimulated phosphoprotein in human placenta: possible implications in trophoblast invasion. Mol. Hum. Reprod. 8, 88–94.

Krause, M., Dent, E.W., Bear, J.E., Loureiro, J.J., Gertler, F.B., 2003. Ena/VASP proteins: regulators of the actin cytoskeleton and cell migration. Annu. Rev. Cell Dev. Biol. 19, 541–564.

Lai, J.P., He, X.W., Jiang, Y., Chen, F., 2003. Preparative separation and determination of matrine from the Chinese medicinal plant *Sophora flavescens* Ait by molecularly imprinted solid-phase extraction. Anal. Bioanal. Chem. 375, 264–269.

Lebrand, C., Dent, E.W., Strasser, G.A., Lanier, L.M., Krause, M., Svitkina, T.M., Borisy, G.G., Gertler, F.B., 2004. Critical role of Ena/VASP proteins for filopodia formation in neurons and in function downstream of netrin-1. Neuron 42, 37–49.

Lehmann, R., 2001. Cell migration in invertebrates: clues from border and distal tip cells. Curr. Opin. Genet. Dev. 11, 457–463.

Liu, K., Li, L., Nisson, P.E., Gruber, C., Jessee, J., Cohen, S.N., 1999. Reversible tumorigenesis induced by deficiency of vasodilator-stimulated phosphoprotein. Mol. Cell. Biol. 19, 3696–3703.

Liu, J., Shen, H.M., Ong, C.N., 2000. Salvia miltiorrhiza inhibits cell growth and induces apoptosis in human hepatoma Hep G(2) cells. Cancer Lett. 153, 85–93.

Loureiro, J.J., Rubinson, D.A., Bear, J.E., Baltus, G.A., Kwiatkowski, A.V., Gertler, F.B., 2002. Critical roles of phosphorylation and actin binding motifs, but not the central proline-rich region, for Ena/vasodilator-stimulated phosphoprotein (VASP) function during cell migration. Mol. Biol. Cell 13, 2533–2546.

Pollard, T.D., Borisy, G.G., 2003. Cellular motility driven by assembly and disassembly of actin filaments. Cell 112, 453–465.

Raftopoulou, M., Hall, A., 2004. Cell migration: Rho GTPases lead the way. Dev. Biol. 265, 23–32.

Reinhard, M., Halbrugge, M., Scheer, U., Wiegand, C., Jockusch, B.M., Walter, U., 1992. The 46/50 kDa phosphoprotein VASP purified from human platelets is a novel protein associated with actin filaments and focal contacts. EMBO J. 11, 2063–2070.

Reinhard, M., Jarchau, T., Walter, U., 2001. Actin-based motility: stop and go with Ena/VASP proteins. Trends Biochem. Sci. 26, 243–249.

Santos, M.F., McCormack, S.A., Guo, Z., Okolicany, J., Zheng, Y., Johnson, L.R., Tigyi, G., 1997. Rho proteins play a critical role in cell migration during the early phase of mucosal restitution. J. Clin. Invest. 100, 216–225.

Smolenski, A., Bachmann, C., Reinhard, K., Honig-Liedl, P., Jarchau, T., Hoschuetzky, H., Walter, U., 1998. Analysis and regulation of vasodilator-stimulated phosphoprotein serine 239 phosphorylation in vitro and in intact cells using a phosphospecific monoclonal antibody. J. Biol. Chem. 273, 20029–20035.

Walter, U., Eigenthaler, M., Geiger, J., Reinhard, M., 1993. Role of cyclic nucleotide-dependent protein kinases and their common substrate VASP in the regulation of human platelets. Adv. Exp. Med. Biol. 344, 237–249.

Wang, Y., Peng, C., Zhang, G., Liu, Y., Li, H., Shan, J., 2003. Study on invasion and metastasis related factors in differentiation of SMMC-7721 cells induced by matrine. Zhong Yao Cai 26, 566–569.

Wentworth, J.K., Pula, G., Poole, A.W., 2006. Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated on Ser157 by protein kinase C-dependent and -independent mechanisms in thrombin-stimulated human platelets. Biochem. J. 393, 555–564.

Xu, X.R., Jiang, J.K., 1998. Recent progress in anticancer bioactivity study of *Sophora flavescens* and its alkaloids. Chinese J. Integrated Trad. Chinese Western Med. 4, 235–239.

Zhang, L.P., Jiang, J.K., Tam, J.W., Zhang, Y., Liu, X.S., Xu, X.R., Liu, B.Z., He, Y.J., 2001. Effects of matrine on proliferation and differentiation in K2562 cells. Leuk. Res. 25, 793–800.