Ginsenoside Rg1 attenuates invasion and migration by inhibiting transforming growth factor-β1-induced epithelial to mesenchymal transition in HepG2 cells

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Abstract. Ginseng has become one of the most commonly used alternative herbal medicines and its active component, ginsenoside Rg1, possesses known pharmacological effects, including anticancer properties. However, the effects of ginsenoside Rg1 on metastasis have not been investigated. The present study demonstrated that ginsenoside Rg1 was able to suppress transforming growth factor-β1 (TGF-β1)-induced invasion and migration in HepG2 liver cancer cells. This suppression was associated with the inhibition of TGF-β1-induced epithelial to mesenchymal transition (EMT). Furthermore, TGF-β1 induced HepG2 cells to undergo EMT and significantly promoted cell invasion and migration. When cells were pretreated with ginsenoside Rg1 for 24 h and subsequently exposed to TGF-β1 for 24 h, the results demonstrated that ginsenoside Rg1 inhibited the initiation of TGF-β1-induced EMT. In addition, HepG2 cells exhibited a mesenchymal phenotype when exposed to TGF-β1, but when exposed to ginsenoside Rg1 this effect was reversed and the cells exhibited a classical epithelial morphology. Ginsenoside Rg1 also increased the expression of the epithelial phenotype marker E-cadherin and repressed the expression of the mesenchymal phenotype marker vimentin. In conclusion, the results of the present study suggest that ginsenoside Rg1 may suppress liver cancer invasion and migration *in vitro* through inhibiting TGF-β1-induced EMT.

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Introduction

Hepatocellular carcinoma is a major health problem worldwide and the third largest cause of cancer-associated mortality in the world annually (1). Metastasis remains a continuing problem for the management of liver cancer. The multi-step process of metastasis includes local invasion, intravasation to the lymph and blood systems, survival in the bloodstream, extravasation from the microvessels and colonization at a secondary site (2,3). There is evidence to suggest that epithelial to mesenchymal transition (EMT) contributes to cancer progression, invasion and migration in various types of cancer (4,5). EMT is a cellular process during which epithelial polarized cells become motile mesenchymal-appearing cells (6). The hallmarks of EMT include loss of cell-cell adhesion, actin cytoskeleton reorganization and acquisition of increased migratory characteristics. EMT is characterized by the downregulation of epithelial differentiation markers, including E-cadherin and the upregulation of mesenchymal markers, including vimentin.

EMT can be initiated by external signals, including fibroblast growth factor, epidermal growth factor (EGF) and transforming growth factor (TGF)-β1 (7,8). TGF-β1 is the multifunctional cytokine implicated in different biological processes by inducing EMT, such as during wound healing, fibrotic diseases, embryonic development and cancer pathogenesis (9). Commonly, tumor cells lose the ability to inhibit the growth activity of TGF-β1.

There is increasing interest in the role of Traditional Chinese Medicine in maintaining health and treating disease. Ginsenoside Rg1, is one of most active and abundant components in ginseng, which has pharmacological effects in the central nervous system, cardiovascular system and immune system and also exerts anticancer properties (10-18). However, the effect of ginsenoside Rg1 on cancer metastasis has not been investigated. In the present study, the effects of ginsenoside Rg1 on TGF-β1-induced invasion and migration in liver cancer were demonstrated and a potential mechanism for these effects was examined. It was hypothesized that TGF-β1 induces HepG2 cells to undergo EMT and promotes cell invasion and migration. Ginsenoside Rg1 may suppress

liver cancer invasion and migration through inhibiting TGF-β1-induced EMT.

Materials and methods

Materials. Ginsenoside Rg1 was obtained from Shanghai International Port (Group) Co., Ltd. (Shanghai, China) Sulforhodamine B (SRB), trichloroacetic acid (TCA), acetic acid, anti-β-actin and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Monoclonal rabbit antibodies for E-cadherin (1:1,000; #3195) and vimentin (1:1,000; #5741) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Secondary mono-clonal rabbit antibodies (1:5,000; PA1-14444) for western blotting were obtained from Amersham Biosciences Corp. (Piscataway, NJ, USA). All other reagents were obtained from Sigma-Aldrich unless stated otherwise.

SRB assay. Cytotoxicity was determined using an SRB assay. Cells were seeded into 96-well plates and exposed to different concentrations of ginsenoside Rg1 (50, 100, 200 and 400 μM). After 48 h of incubation, the cells were fixed with TCA for 1 h at 4˚C, air-dried and then stained with 0.4% SRB solution for 30 min at room temperature. Following staining, the SRB solution was removed and cells were washed five times with 1% acetic acid. Subsequently, 10 mM Tris base solution (pH 10.5) was added to dissolve the protein-bound dye and plates were incubated on a plate shaker for 10 min. The OD_{570} nm was determined using a 96-well plate reader (MRX; Dynex Technologies, Chantilly, VA, USA).

Wound-healing assay. Cells were cultured in a 6-well plate and incubated until they reached 80% confluence. Cell mono-layers were carefully wounded by scratching with a 200 μl sterile plastic pipette tip. Subsequently, cells were washed twice with phosphate-buffered saline and then replaced with fresh medium without serum. For each scratch, images were captured at 0 and 24 h using an inverted microscope (Nikon Eclipse TS100 1064; Nikon, Tokyo, Japan) in the same field.

Matrigel invasion assay. Invasion of HepG2 cells was performed in a 24-well transwell unit (8 μM pore size) and was coated with 1 mg/ml Matrigel matrix as described previ-ously (19). Briefly, cells were placed on the Matrigel-coated transwell (the upper compartment of the invasion chamber), and cells were exposed to TGF-β1 (1.25-5.00 ng/ml) for 24 h, or cells were pretreated with ginsenoside Rg1 (50-200 μM) for 48 h in the presence or absence of 5 ng/ml TGF-β1 for 24 h. The cells were then resuspended in 200 μl serum-free medium and placed in the upper chambers at 5×10^4 cells. Conditioned medium (600 μl) was added to the lower compartment of the invasion chamber. Following incubation at 37˚C for 48 h, cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Random fields were counted by light microscopy (MF53; Olympus Corporation, Tokyo, Japan).

Western blotting. Cells were harvested and lysed for total cellular protein extraction and then centrifuged at 2,250 x g for 30 min at 4˚C. A DC protein assay kit was used to determine the protein concentrations (Bio-Rad, Hercules, CA, USA). Total protein (25 μg) was separated using 8-12% sodium dodecyl sulfate-polyacrylamide gels and subsequently trans-ferred onto a polyvinylidene difluoride membrane. Following blocking with skimmed milk, the membranes were incubated with various primary antibodies, E-cadherin (1:1,000), vimentin (1:1,000) and β-actin (1:10,000), at 4˚C overnight, respectively. Immunopositive bands were visualized using the Amersham ECL™ plus western blotting detection kit (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis. Statistical analysis between groups was performed using an unpaired Student's t-test with Sigmaplot 10.0 software (Jandel Scientific, San Rafael, CA, USA). Data are presented as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

TGF-β1 induces HepG2 cells to undergo EMT. Initially, the optimum concentrations required for TGF-β1 to initiate EMT in HepG2 cells were ascertained. Cells were treated with 1.25-5 ng/ml TGF-β1 for 24 h. Fig. 1 shows that the cells acquired a spindle-like fibroblastic phenotype and reduced their cell-cell contact when exposed to higher doses of TGF-β1 (5 ng/ml). Since EMT is closely associ-ated with the loss of E-cadherin expression and acquisition of vimentin expression, the expression of E-cadherin and vimentin by western blotting was also assessed. The results demonstrated that TGF-β1 decreased E-cadherin expression in a dose-dependent manner, while it significantly induced the expression of vimentin. The maximal effects of TGF-β1 in initiating EMT in HepG2 cells were achieved at a concen-tration of 5 ng/ml, therefore this concentration was used in subsequent experiments.

TGF-β1 promotes the invasion and migration of HepG2 cells. Previous studies have demonstrated that EMT is involved in cancer invasion and metastasis, which are key processes in cancer progression and metastasis (20,21). Thus, in the present study, the effect of TGF-β1 on invasion and migration in HepG2 cells were assessed using transwell and wound-healing assays. Cells were exposed to different concentrations of TGF-β1 (1.25-5 ng/ml) for 24 h. The results demonstrated that TGF-β1 significantly promoted HepG2 cell invasion and migration compared with the untreated cells (Fig. 2A, B and C).

Ginsenoside Rg1 inhibits TGF-β1-induced cell invasion and migration. In order to observe the effect of ginsenoside Rg1 on cell viability in HepG2 cells, cells were treated with increasing concentrations of ginsenoside Rg1 (50-400 μM) for 48 h and then assessed using an SRB assay. Ginsenoside Rg1 had no effect on cell viability up to a concentration of 400 μM. Thus, the non-cytotoxic concentrations of ginsen-oside Rg1, between 50 and 200 μM were used in subsequent experiments (Fig. 3A).

To investigate the effects of ginsenoside Rg1 on TGF-β1-induced cell invasion and migration, transwell

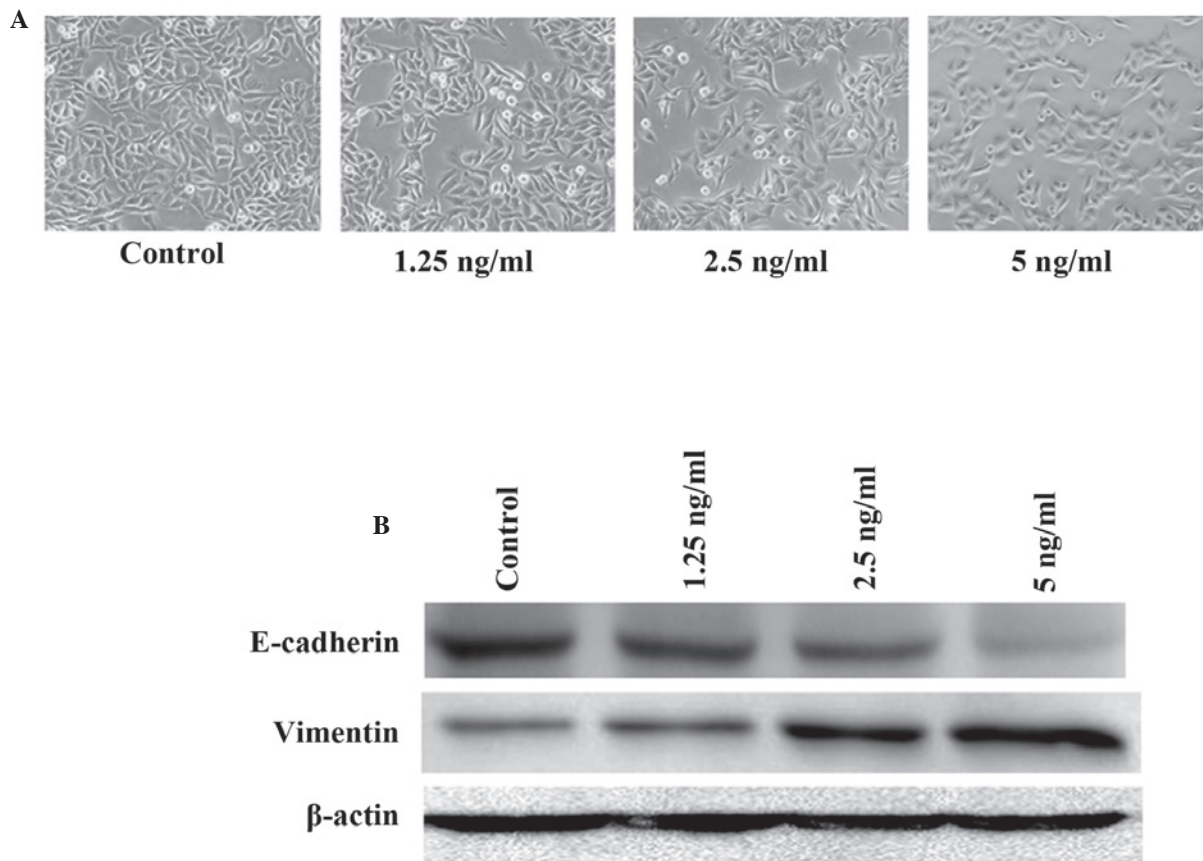Figure 1. TGF-β1 induces HepG2 cells to undergo epithelial to mesenchymal transition. HepG2 cells were exposed to different concentrations of TGF-β1 (1.25-5 ng/ml) for 24 h. (A) Images showing the morphological alterations of HepG2 cells following exposure to TGF-β1. (B) Cellular protein levels of E-cadherin and vimentin were measured using western blotting. TGF-β1, transforming growth factor-β1.

and wound-healing assays were performed. Cells were treated with ginsenoside Rg1 (50-200 μM) for 48 h with or without 5 ng/ml TGF-β1 for 24 h. TGF-β1 significantly increased invasion and migration of HepG2 cells compared with TGF-β1-untreated control cells, while ginsenoside Rg1 reversed this effect (Fig. 3B, C and D). These results suggest that ginsenoside Rg1 may constitute an effective inhibitor of invasion and migration in HepG2 cells.

Ginsenoside Rg1 regulates morphological alterations and EMT marker expression during TGF-β1-induced EMT. To further clarify whether suppression of TGF-β1-induced invasion and migration by ginsenoside Rg1 resulted from regulation of EMT, the expression of EMT marker proteins vimentin and E-cadherin and alterations in cell morphology were examined. Fig. 4A shows that HepG2 cells exhibited a mesenchymal phenotype when exposed to TGF-β1 (5 ng/ml), but cells pretreated with ginsenoside Rg1 exhibited a classical epithelial morphology. The present study also analyzed the expression of the epithelial phenotype marker E-cadherin and the mesenchymal phenotype marker vimentin using western blotting. The results in Fig. 4B demonstrate that TGF-β1 (5 ng/ml) significantly decreased the expression of E-cadherin, but induced the expression of vimentin compared with TGF-β1-untreated control cells, while ginsenoside Rg1 reversed these effects. These results suggest that ginsenoside Rg1 may prevent invasion and migration via inhibition of TGF-β1-induced EMT in HepG2 cells.

Discussion

In the present study, the results demonstrated that ginsenoside Rg1 significantly suppressed TGF-β1-induced invasion and migration. It is likely that the suppressive effects of ginsenoside Rg1 on invasion and metastasis are mediated through the inhibition of TGF-β1-induced EMT. This indicated that ginsenoside Rg1 may be a potential inhibitor in preventing the invasion and migration of human liver cancer.

Tumor metastasis is a multi-stage process beginning with tumor cell migration and invasion. These proceses require EMT. During EMT, well-polarized and adhesive epithelial cells lose polarity and intercellular adhesion, which is mediated by cadherins. These cells then acquire a highly motile fibroblastoid or mesenchymal phenotype (22,23). TGF-β1 is major inducer of EMT and several studies have demonstrated that TGF-β1 alone or in combination with other growth factors, including EGF and hepatocyte growth factor is critical in mediating EMT in various types of malignant tumor (24,25). Consistent with these studies, the present study found that TGF-β1 induces HepG2 cells to undergo EMT and the cells acquire a spindle-like fibroblastic phenotype and reduce their cell-cell contact. TGF-β1 (5 ng/ml) also significantly upregulated the expression of the mesenchymal marker vimentin, downregulated the expression of the epithelial marker E-cadherin and promoted invasion and migration of HepG2 cells.
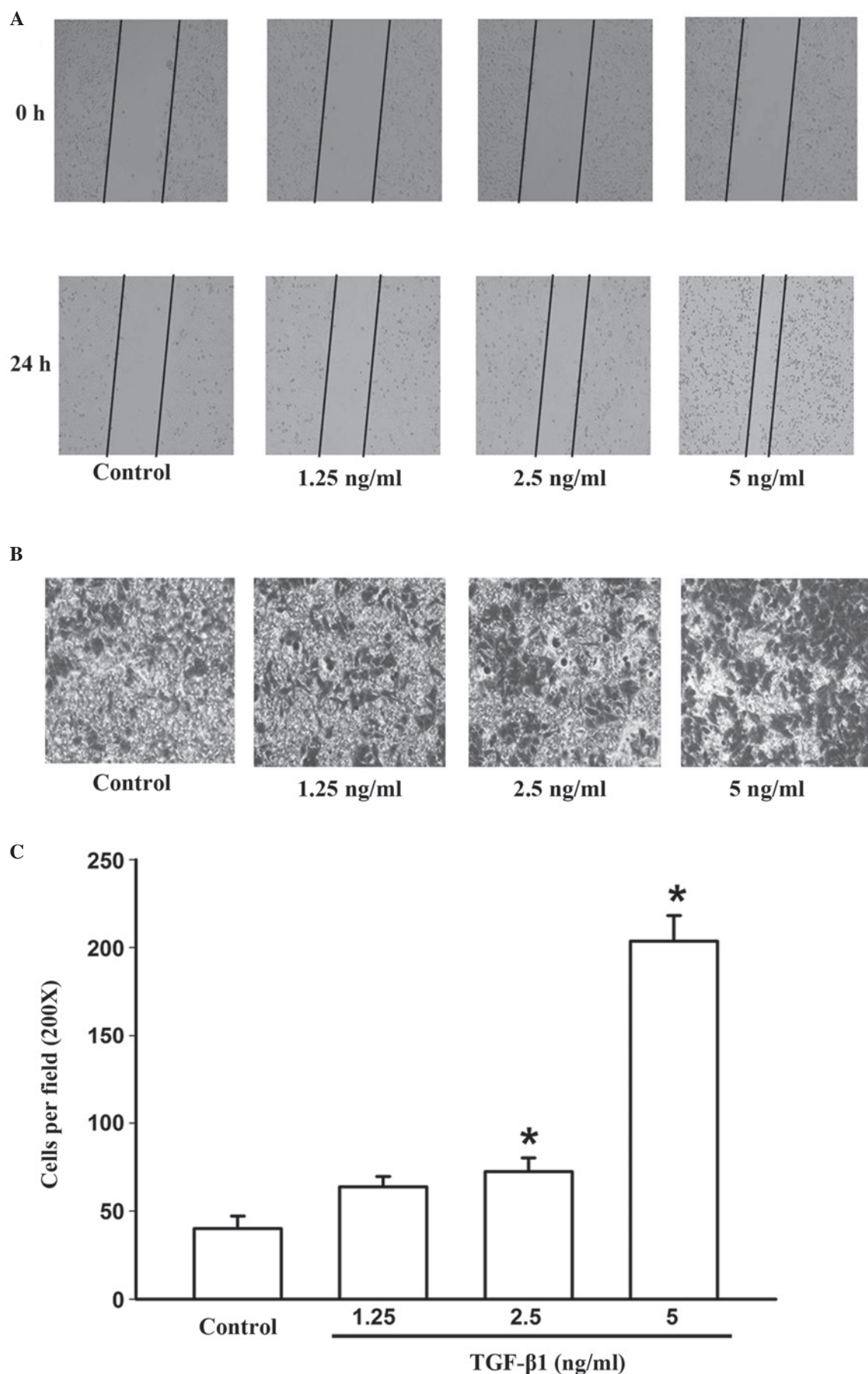
Figure 2. TGF-β1 promotes the invasion and migration of HepG2 cells. HepG2 cells were exposed to different concentrations of TGF-β1 (1.25-5 ng/ml) for 24 h. (A) Migration ability of HepG2 cells was analyzed using a wound-healing assay. Cells were scratched with a pipette tip and then exposed to TGF-β1 (1.25-5 ng/ml) for 24 h. (B and C) Invasive ability of HepG2 cells was assessed using a Matrigel invasion assay. Results are presented as the mean ± standard error of the mean of three independent experiments. *P<0.05 vs. control. TGF-β1, transforming growth factor-β1.
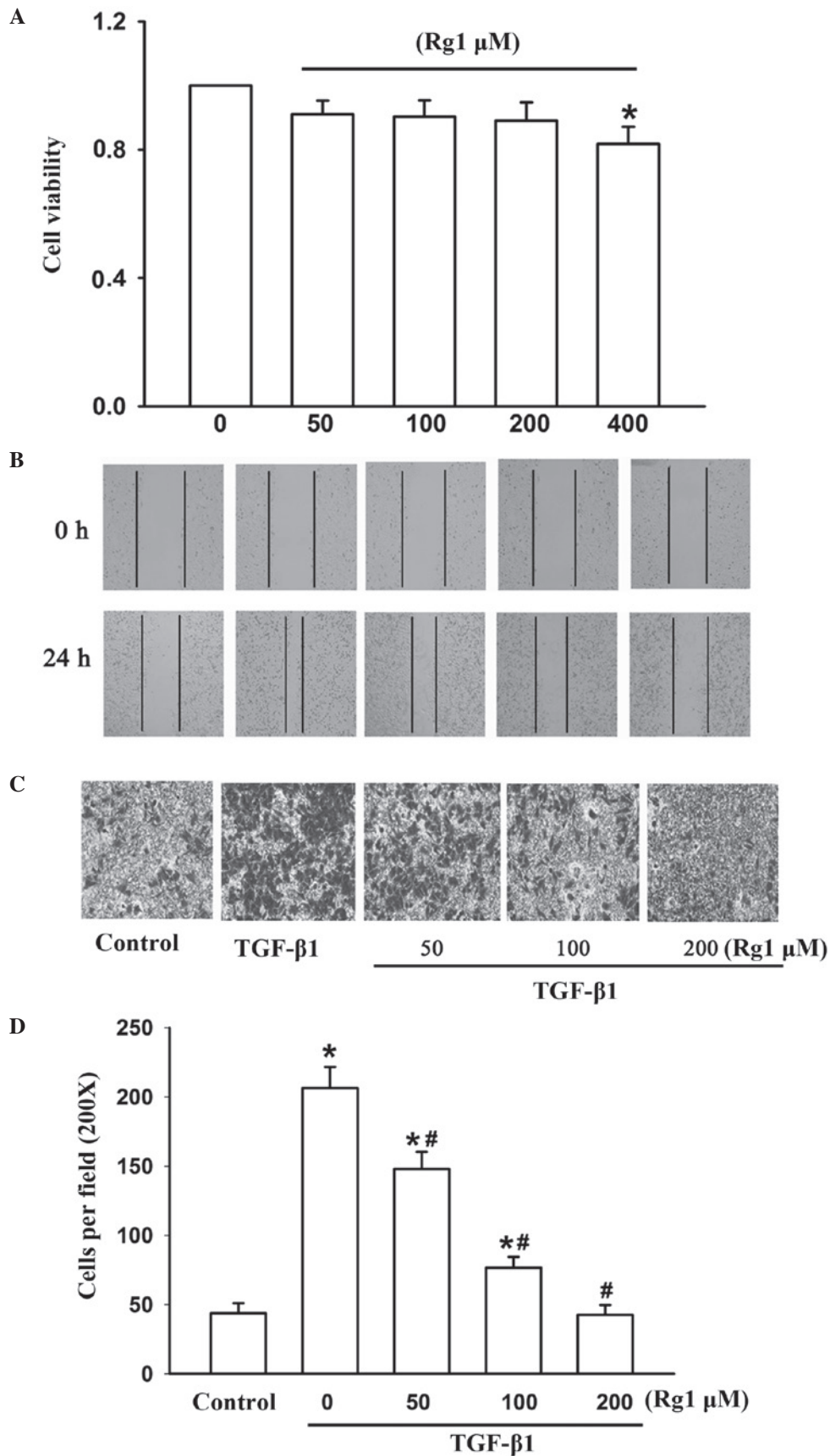
Figure 3. Rg1 inhibits TGF-β1-induced cell invasion and migration. (A) Cells were treated with increasing concentrations of Rg1 (50-400 μM) for 48 h and cell viability was assessed using a sulforhodamine B assay. Results are presented as the mean ± standard error of the mean of three independent experiments; *P<0.05 vs. untreated cells. (B) HepG2 cells were scratched with a pipette tip and then exposed to 50-200 μM Rg1 for 48 h in the presence of absence of 5 ng/ml TGF-β1 for 24 h. Results are representative of three independent experiments. (C and D) Invasive ability of HepG2 cells was assessed by Matrigel invasion assay. Cells were treated with 50-200 μM Rg1 for 48 h in the presence or absence of 5 ng/ml TGF-β1 for 24 h. Results are presented as the mean ± standard error of the mean of four independent experiments. *P<0.05 vs. control; #P<0.05 vs. TGF-β1 group. TGF-β1, transforming growth factor-β1; Rg1, ginsenoside Rg1.
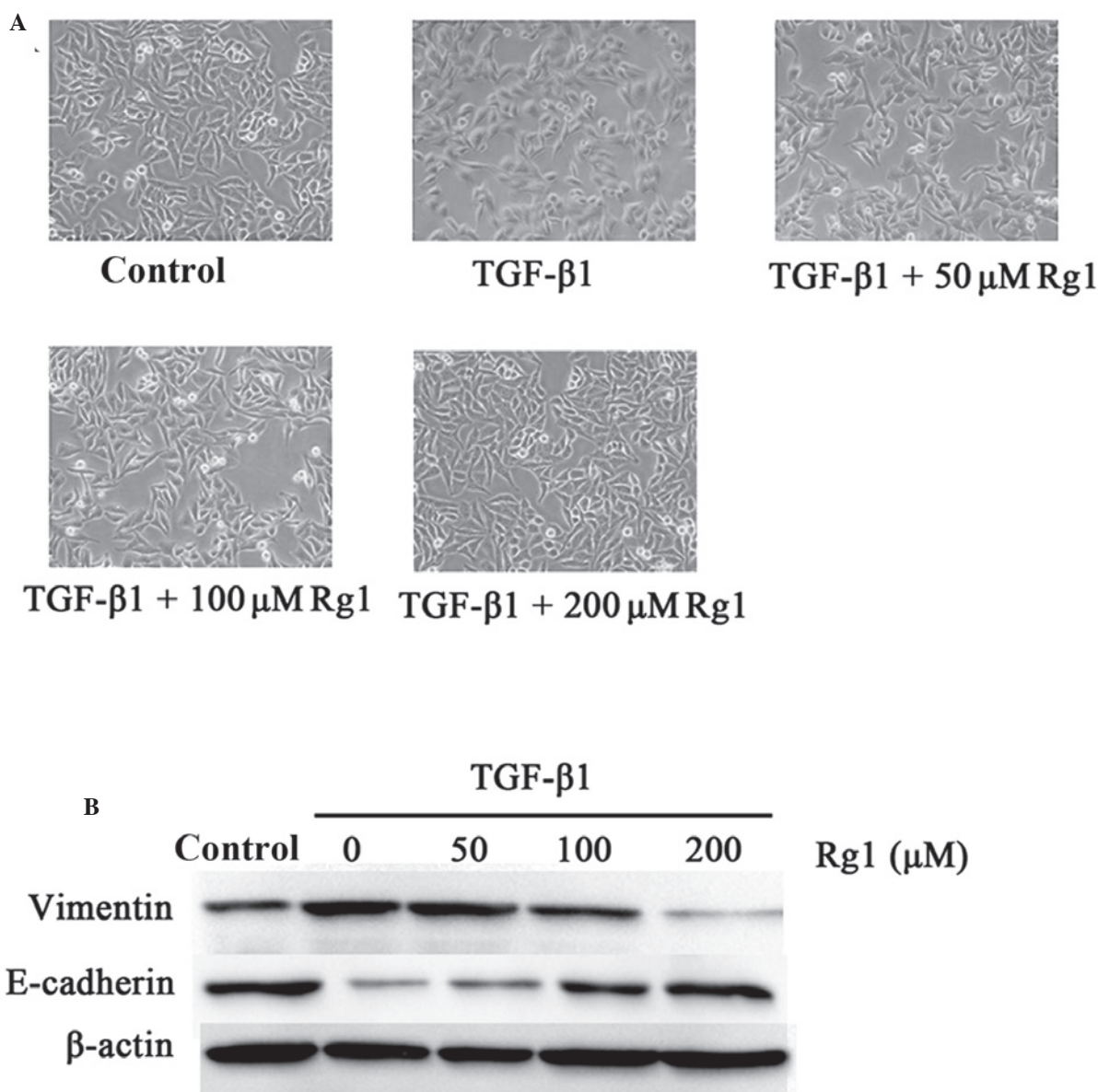
Figure 4. Rg1 regulates TGF-β1-induced morphological alterations and epithelial to mesenchymal transition marker expression in HepG2 cells. Cells were treated with Rg1 (50-200 μM) for 48 h in the presence or absence of 5 ng/ml TGF-β1 for 24 h. (A) Representative images of TGF-β1-induced morphological alterations in HepG2 cells. (B) Cellular protein levels of E-cadherin and vimentin were measured using western blotting. TGF-β1, transforming growth factor-β1; Rg1, ginsenoside Rg1.

Previous studies have demonstrated that ginsenoside Rg1 may exert anti-cancer properties (13,18,26,27), however, this is the first study, to the best of our knowledge, to demonstrate that ginsenoside Rg1 may be a potential inhibitor of invasion and migration. The results of the transwell and wound-healing assays demonstrated that ginsenoside Rg1 inhibited TGF-β1-induced cell invasion and migration in HepG2 cells. To further investigate how ginsenoside Rg1 suppresses TGF-β1-induced cell invasion and migration, the effect of ginsenoside Rg1 on TGF-β1-induced EMT was assessed. The results demonstrated that HepG2 cells exhibit a mesenchymal phenotype when exposed to TGF-β1, but when exposed to ginsenoside Rg1 this effect was reversed and the cells exhibited a classical epithelial morphology. Ginsenoside Rg1 also increased the expression of the epithelial phenotype marker E-cadherin and repressed the expression of the mesenchymal phenotype marker vimentin.

These results suggest that ginsenoside Rg1 may prevent invasion and migration via inhibition of TGF-β1-induced EMT in HepG2 cells.

In conclusion, the present study demonstrated that ginsenoside Rg1 was able to inhibit liver cancer cell invasion and migration *in vitro* by inhibiting TGF-β1-induced EMT. This indicated that ginsenoside Rg1 may serve as a potential inhibitor in preventing the invasion and migration of human liver cancer.

Acknowledgements

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