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Scutellarin promotes *in vitro* angiogenesis in human umbilical vein endothelial cells

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ABSTRACT

Angiogenesis is critical to a wide range of physiological and pathological processes. Scutellarin, a major flavonoid of a Chinese herbal medicine *Erigeron breviscapus* (Vant.) Hand. Mazz. has been shown to offer beneficial effects on cardiovascular and cerebrovascular functions. However, scutellarin's effects on angiogenesis and underlying mechanisms are not fully elucidated. Here, we studied angiogenic effects of scutellarin on human umbilical vein endothelial cells (HUVECs) *in vitro*. Scutellarin was found by MTT assay to induce proliferation of HUVECs. In scutellarin-treated HUVECs, a dramatic increase in migration was measured by wound healing assay; Transwell chamber assay found significantly more invading cells in scutellarin-treated groups. Scutellarin also promoted capillary-like tube formation in HUVECs on Matrigel, and significantly upregulated platelet endothelial cell adhesion molecule-1 at both mRNA and protein levels. Scutellarin's angiogenic mechanism was investigated *in vitro* by measuring expression of angiogenic factors associated with cell migration and invasion. Scutellarin strongly induced MMP-2 activation and mRNA expression in cultured HUVECs in a concentration-dependent manner. Taken together, these results suggest that scutellarin promotes angiogenesis and may form a basis for angiogenic therapy.

1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing endothelium [1], is critical to a variety of processes, both physiologic (embryonic development and wound healing) and pathologic (malignancy and chronic inflammation) [2,3]. It is a complex biologic function regulated by angiogenic factors [4] and comprising several steps, including sequential basement membrane degradation, endothelial cell migration and proliferation, tube formation, inhibition of endothelial proliferation, and the stabilization of new vessels. Modulation of any of these steps would affect new vessel formation.

Supplying oxygen, nutrients and various growth factors to sites of tissue repair or regeneration requires angiogenesis, as does removal of waste products [2,5]. Improved angiogenic processes can repair ischemia-associated tissue damage, such as arteriosclerosis, myocardial infarction and limb ischemia [6,7], and can thus significantly aid therapeutic neovascularization. Although many angiogenic factors have strong stimulating effects on angiogenesis, the biological activity of protein-type growth factors cannot last long *in vivo* because of their poor stability [8]. If administered systemically in large doses, angiogenic factors can cause harmful side effects.

Scutellarin, a known flavone glycoside, is the primary active component of the traditional Chinese herbal medicine *Erigeron breviscapus* (Vant.) Hand. Mazz. Scutellarin has been extensively used in clinics to treat cardiovascular diseases and cerebrovascular injury, such as angina pectoris, myocardial infarction, stroke and cerebral thrombotic diseases, which are associated with vascular endothelial cell dysfunction. It has been shown that scutellarin exhibits a variety of pharmacological actions, including anti-oxidative [9], anti-inflammatory [10], vasodilator [11] as well as cardiovascular and cerebrovascular ischemia protective effects [12,13], indicating beneficial vascular effects of scutellarin. Therefore, it is speculated that scutellarin may be able to stimulate angiogenesis, which could be beneficial in the treatment of ischemic disease, wound healing and tissue regeneration. Scutellarin has been recently demonstrated to promote functional neovascularization in the polyglycolic acid scaffold *in vivo* [14]. However, there is no sufficient evidence proving the effects of scutellarin on angiogenesis, especially at the cellular and molecular levels. Therefore, the

Abbreviations: ECM, extracellular matrix; ECGM, endothelial cell growth medium; HUVECs, human umbilical vein endothelial cells; MMP-2, matrix metalloproteinase-2; PECAM-1, platelet endothelial cell adhesion molecule-1; PKC, protein kinase C; TIMP-2, tissue inhibitor of metalloproteinase-2; VEGF, vascular endothelial growth factor.

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purpose of the present study was to elucidate the direct angiogenic actions of scutellarin on human umbilical vein endothelial cells (HUVECs) *in vitro*.

Our results showed that scutellarin markedly induced endothelial cell proliferation, migration, invasion, and capillary-like tube formation, suggesting a potential for increasing angiogenesis. The data from this study provide an experimental evidence that scutellarin should be considered as a potential therapeutic angiogenesis drug.

2. Materials and methods

2.1. Chemicals and reagents

Scutellarin was purchased from Bei-Dou-Xing Pharmaceutical Co. Ltd. (Tianjin, China), and recombinant human vascular endothelial growth factor (VEGF) from PeproTech Inc. Matrigel was obtained from BD Biosciences. Rabbit anti-platelet endothelial cell adhesion molecule-1 (PECAM-1) and rabbit anti-β-actin antibodies were provided by Santa Cruz Biotechnology, Inc. All other chemicals and reagents were supplied by Sigma–Aldrich, Inc., unless indicated otherwise.

2.2. Cell culture

Primary HUVECs and their appropriate medium were purchased from ScienCell Research Laboratories. Tissue culture flasks were pre-coated with poly-L-lysine. Cells were cultured in endothelial cell growth medium (ECGM) and maintained at 37 °C with 5% CO_2, according to the supplier's instructions. The ECGM consists of endothelial cell medium supplemented with 5% fetal bovine serum, endothelial cell growth supplement and penicillin/streptomycin solution. Tightly confluent monolayers of HUVECs from 2nd to 5th passage were used in all the experiments.

2.3. Cell proliferation assay

HUVECs were seeded onto 96-well plates (2×10^4 cells/well) and cultured in ECGM for 24 h. Their medium was changed to medium containing 10 ng/mL VEGF (positive control) or scutellarin at various concentrations (1, 5 or 10 μM). After incubating 48 h, cell proliferation was determined with MTT assay. Briefly, 50 μL MTT solution (0.5 mg/mL) was added to each well and incubated at 37 °C for 4 h to allow the formation of blue formazan crystals. Residual MTT was carefully removed, and crystals dissolved by incubation with dimethyl sulfoxide (150 μL). Plates were shaken for 10 min; absorbance at 490 nm was measured using a microplate reader.

2.4. Wound healing assay

Migration of HUVECs was performed by a wound healing assay. HUVECs were cultured on 24-well plates (2×10^5 cells/well) in ECGM for 36 h. When confluent, the endothelial monolayers were scratched horizontally with a yellow pipette tip to obtain a monolayer culture with space without cells. Media and dislodged cells were aspirated and endothelial cell medium was then added to the plate along with 10 ng/mL VEGF or scutellarin at various concentrations (1, 5 or 10 μM). Three randomly selected fields along the scraped line were photographed on each well using a phase contrast inverted microscope. After incubation at 37 °C for 8 h, cell migration was observed; images were taken by the same method.

2.5. Chemoinvasion assay

Cell invasion assay was performed using Transwell chambers with 6.5 mm diameter polycarbonate membrane (8-μm-sized pores). Upper surfaces of Transwell inserts were coated with Matrigel. The bottom chamber of the apparatus contained 600 μL of endothelial cell medium supplemented with 10 ng/mL VEGF or scutellarin at various concentrations (1, 5 or 10 μM). The HUVECs (100 μL) were added to the upper chamber (2×10^4 cells/well) and incubated in endothelial cell medium. After 24 h incubation at 37 °C, non-invasive cells on the upper membrane surfaces were removed by wiping with cotton swabs. Invading cells were fixed with methanol and stained with Giemsa. Cell invasion was quantified by counting cells on the lower surface using phase contrast microscopy.

2.6. Tube formation assay

Scutellarin's effect on HUVEC differentiation was examined by *in vitro* tube formation on Matrigel. Growth factor-reduced Matrigel was thawed on ice overnight, spread evenly over each well (100 μL) of 48-well plates and polymerized for 30 min at 37 °C. HUVECs (2×10^4 cells/well) were plated onto the Matrigel layer and cultured in endothelial cell medium containing 10 ng/mL VEGF or supplemented with scutellarin at various concentrations (1, 5 or 10 μM). After 24 h of incubation at 37 °C, cell morphological changes were observed and captured with a phase contrast inverted microscope.

2.7. Western blot analysis

Cells were lysed in RIPA buffer (Beyotime Biotechnology, China). Protein concentrations were determined using a BCA Protein Assay Kit (Beyotime Biotechnology, China). Cell lysates (50 μg protein) were separated by 10% SDS–PAGE and electrophoretically transferred onto a polyvinylidene fluoride membrane. After blocking in TBS-T containing 5% nonfat dry milk, the membranes were incubated overnight at 4 °C with primary antibodies against target proteins. After washing twice, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies, and protein levels were detected by an enzymatic chemiluminescence kit (Pierce).

2.8. Gelatin zymography

Conditioned media were collected from HUVECs after 24 h culture in endothelial cell medium, containing 10 ng/mL VEGF or supplemented with scutellarin at various concentrations (1, 5 or 10 μM). Following one freeze and thaw cycle, 20 μL conditioned media of each sample was separated on 10% polyacrylamide gel containing 0.1% gelatin under nonreducing conditions. After electrophoresis, gels were washed twice for 15 min in 2.5% Triton X-100, then incubated for 12 h in developing buffer (0.5 M Tris–HCl [pH 7.5], 10 mM $CaCl_2$). Each gel was stained with 0.5% Coomassie brilliant blue R-250 for 2 h and destained in 50% methanol–10% acetic acid.

2.9. RT-PCR analysis

Total RNAs were extracted from HUVECs with Trizol reagent (Invitrogen). One microgram of total RNA was reverse transcribed into cDNA and amplified using an RT-PCR system (Promega). The primer sequences and product sizes are listed in Table 1. PCR products were separated on a 1% agarose gel. GAPDH was used as an internal control.

2.10. Statistical analysis

All experiments were performed at least three times. Data are presented as mean values ± SD. Statistical differences were evaluated by one-way ANOVA, with $P < 0.05$ considered significant.

Table 1
Primers used for RT-PCR analysis.

Sequence name	Primers	Tm (°C)	Size (bp)
MMP-2-F	5′-GTGGATGATGCCTTTGCTCG-3′	62	272
MMP-2-R	5′-CCATCGGCGTTCCCATACTT-3′	62	
TIMP-2-F	5′-CCGCTCAAATACCTTCACAA-3′	58	222
TIMP-2-R	5′-ATTACGGCAGCAAGTCCAAT-3′	58	
PECAM-1-F	5′-AGGTGGCTCACGCCTGTAAT-3′	62	203
PECAM-1-R	5′-AACCTCCGTTTCCTGGGTTC-3′	62	
GAPDH-F	5′-AACGGATTTGGTCGTATTGG-3′	58	209
GAPDH-R	5′-TGGAAGATGGTGATGGGATT-3′	58	

3. Results

3.1. Effects of scutellarin on proliferation of HUVECs

An MTT assay was used to assess the effect of scutellarin on endothelial cell proliferation. Cells were cultured and treated for 48 h with 10 ng/mL VEGF or different concentrations of scutellarin (1, 5 or 10 μM). As a positive control, VEGF significantly increased HUVEC proliferation. Compared with control, scutellarin strongly induced HUVEC proliferation in a dose-dependent manner. The effect of scutellarin at 10 μM on HUVEC proliferation is comparable to that of VEGF at 10 ng/mL (Fig. 1A).

3.2. Effects of scutellarin on migration of HUVECs

Migration of endothelial cells is a critical initiating event in the formation of new blood vessels and the repair of injured vessels. Scutellarin's effect on HUVEC migration was examined with a wound healing assay. Monolayer cultures with spaces without cells were incubated with 10 ng/mL VEGF or different concentrations of scutellarin, and cell migration over the following 8 h was observed. Fig. 1B shows that there was little migration measured in the control group, whereas in medium supplemented with VEGF, HUVEC migration was significantly enhanced. When compared to controls, endothelial cells treated with scutellarin migrated into the denuded area, covered the exposed surface, and reduced the uncovered area, showing a dramatic increasement in migration in a dose-dependent manner.

3.3. Effects of scutellarin on invasion of HUVECs

The angiogenesis process requires migrating endothelial cells to break and transverse through their basement membranes to form new blood vessels. We thus observed scutellarin's effects on HUVEC invasion using Transwell chambers. HUVECs were placed on the polycarbonate membranes of Matrigel-coated Transwell inserts and allowed to invade in the presence of 10 ng/mL VEGF or different concentrations of scutellarin. The VEGF-treated cells, serving as positive controls, indicated enhancement of invasion. Compared with the control group, scutellarin significantly increased HUVEC invasion in a dose-dependent manner, with maximum effects at 10 μM (Fig. 1C).

3.4. Effects of scutellarin on tube formation of HUVECs

To investigate scutellarin's effects on HUVEC tube formation, we used a common method for gauging *in vitro* angiogenesis, the

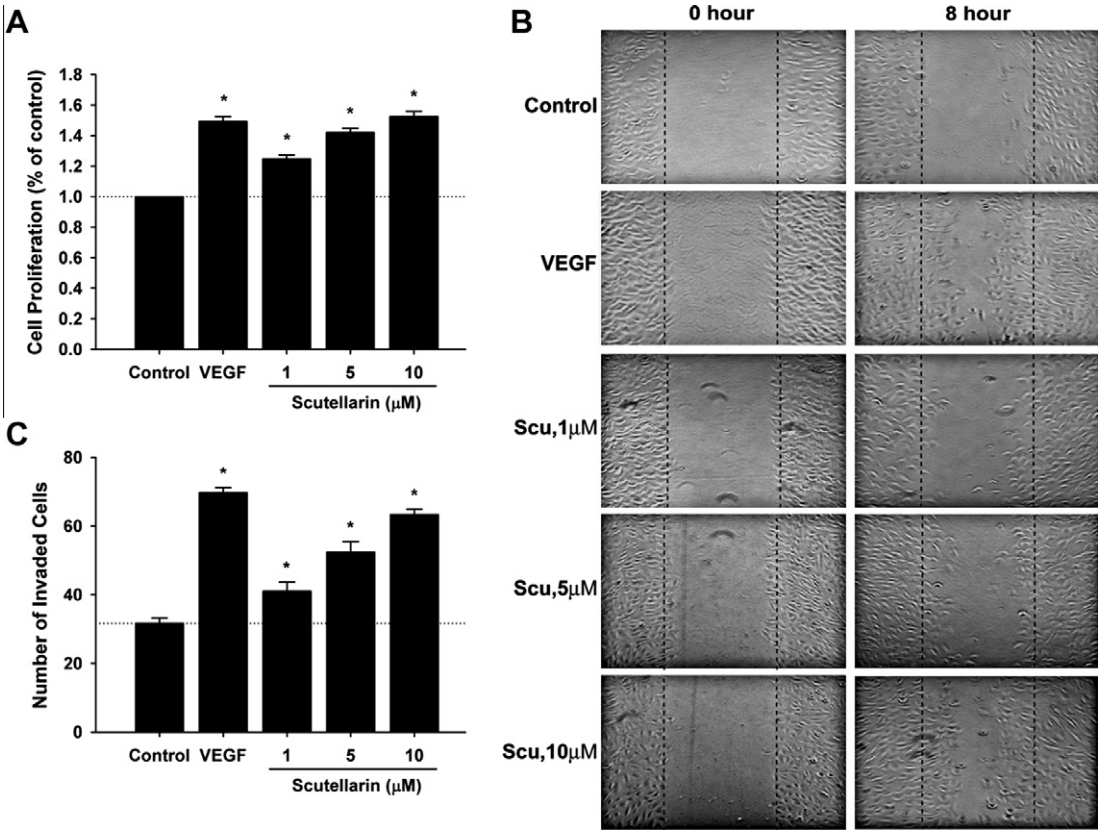

Fig. 1. Effects of scutellarin on HUVEC proliferation, migration, and invasion. (A) Cells were exposed to 10 ng/mL VEGF (positive control) or scutellarin at different concentrations (1, 5 or 10 μM) for 48 h. Cell proliferation was assessed by MTT assay. (B) A confluent HUVEC monolayer was wounded with a yellow pipette tip and then incubated with 10 ng/mL VEGF (positive control) or scutellarin at different concentrations (1, 5 or 10 μM). After 8 h, closure of the wound was visualized under a phase contrast inverted microscope. (C) Cells were treated with graded concentrations (1, 5 or 10 μM) of scutellarin using Transwell chambers for 24 h. Cells cultured in 10 ng/mL VEGF served as positive controls. After washing, fixation and staining, invading cells were counted by a phase contrast inverted microscope. "Scu" means scutellarin. Data shown in graphs are expressed as mean values ± SD of three individual experiments. $^{*}P < 0.05$ compared with control.

capillary-like tube formation assay on Matrigel. After seeding on Matrigel, HUVECs spread and aligned with each other to develop hollow, tube-like structures. When HUVECs were cultured in endothelial cell medium, there was little tube formation. In treatment groups, however, VEGF and scutellarin induced morphogenetic changes. Treatment with different scutellarin concentrations significantly stimulated the capillary network formation of endothelial cells, including increasing areas covered by HUVECs and lengths of the network compared to controls (Fig. 2). Scutellarin also enhanced tube formation in a dose-dependent manner, with near maximal activity at 10 μM.

3.5. Effects of scutellarin on the expressions of PECAM-1 in HUVECs

The effect of scutellarin was further analyzed by studying the expression of PECAM-1, a biochemical marker of angiogenesis. As with the positive control, PECAM-1 levels steadily increased with the concentration of scutellarin, and were significantly higher in cells treated with scutellarin than the control group (Fig. 3A). Analysis with RT-PCR further confirmed that PECAM-1 mRNA expression was also upregulated in cells exposed to scutellarin, in a dose-dependent manner (Fig. 3B). Increased PECAM-1 expression was also consistent with the morphological changes.

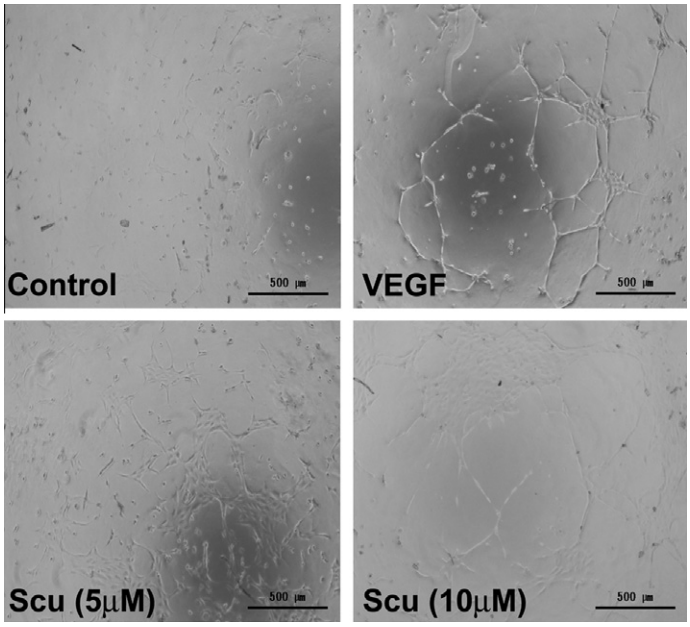

Fig. 2. Effects of scutellarin on HUVEC tube formation. Cells were cultured on a layer of Matrigel and incubated with medium containing 5, 10 μM scutellarin or 10 ng/mL VEGF (positive control). After 24 h, capillary-like tube formation was visualized using an inverted phase contrast microscope. "Scu" means scutellarin.

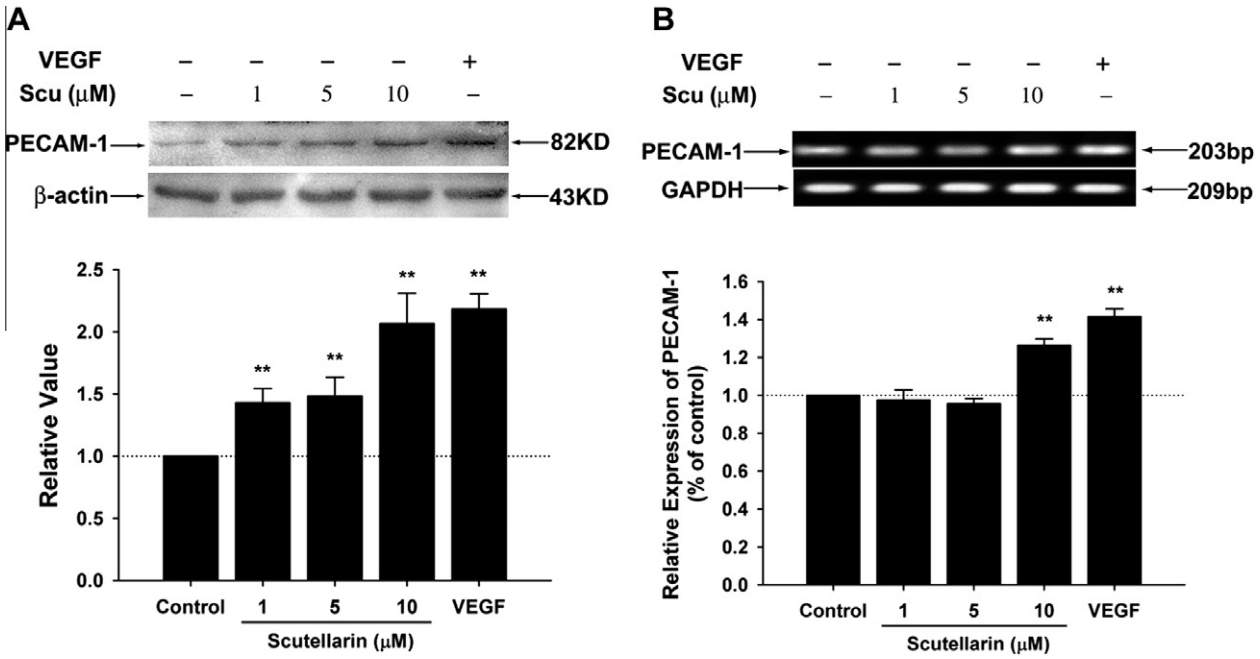

Fig. 3. Expression levels of PECAM-1 in scutellarin-treated HUVECs. Cells were treated with 1, 5 or 10 μM scutellarin or 10 ng/mL VEGF (positive control) for 24 h. (A) Western blot was used to analyze PECAM-1 protein expression in the cell lysate. (B) The mRNA expression of PECAM-1 was examined by RT-PCR. β-Actin and GAPDH were used as an internal controls for western blot and RT-PCR, respectively. The bands were quantitated by densitometry. "Scu" means scutellarin. Data shown in graphs are expressed as mean values ± SD of three individual experiments. $^{**}P < 0.01$ compared with control.

3.6. Effects of scutellarin on the expressions of MMP-2 and TIMP-2 in HUVECs

We next evaluated the angiogenic mechanism of scutellarin *in vitro* by measuring expression of well-characterized angiogenic factors, especially those associated with cell migration and invasion. RT-PCR was used to evaluate expression levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) mRNA after VEGF or scutellarin treatment in HUVECs. As shown in Fig. 4A, we found that, compared to controls, scutellarin-exposed HUVECs produced MMP-2 mRNA in a dose-dependent manner. In contrast, TIMP-2 mRNA levels decreased in cells treated with only 1 μM scutellarin, but increased in cells treated with scutellarin at 5 and 10 μM; however, this increase was not statistically significant compared to controls. Scutellarin-induced upregulation of MMP-2 protein was also confirmed by gelatin zymography (Fig. 4B).

4. Discussion

Angiogenesis plays an important role in physiologic and pathologic processes and is involved in such diseases as ischemic heart disease, diabetes, chronic inflammation and cancer [15]. All forms of angiogenesis are thought to share certain basic features, including degradation of the basement membrane, migration and proliferation of endothelial cells, and lumen formation. Although scutellarin's role in vascular function has been widely reported [11–14], its effect on endothelial cells has not been elucidated. Here, we attempted to determine whether scutellarin directly affected human endothelial cells. We found that scutellarin significantly induced *in vitro* angiogenic processes, including proliferation, migration, invasion, and tube formation, in primary cultured HUVECs. Scutellarin's angiogenic activities at 10 μM were comparable to those of 10 ng/mL VEGF. Furthermore, PECAM-1 is a known marker for angiogenesis [16]. We found that PECAM-1 expression increased significantly in cells treated with scutellarin compared with controls. These results provide strong evidence that scutellarin has powerful angiogenic activity via stimulation of various angiogenic steps, suggesting an application as an angiogenesis inducer for several angiogenesis-related human diseases.

The degradation of subendothelial basement membrane and surrounding extracellular matrix (ECM) is an initial step in the angiogenic process [17]. Following matrix breakdown, endothelial cells migrate and proliferate to form new vessels. Therefore, proteolytic enzyme activity for degrading ECM is essential in angiogenesis. The MMPs are a family of Zn^{2+}-dependent endopeptidases that digest a variety of ECM components [18]. Accumulating evidence suggests that MMPs play an important role in angiogenesis [19,20], particularly in endothelial cell migration, capillary basement membrane breakdown, and pericellular fibrinolysis [21]. MMP-2 is the most widely distributed of MMPs and is expressed constitutively by a number of cells, including endothelial cells. It

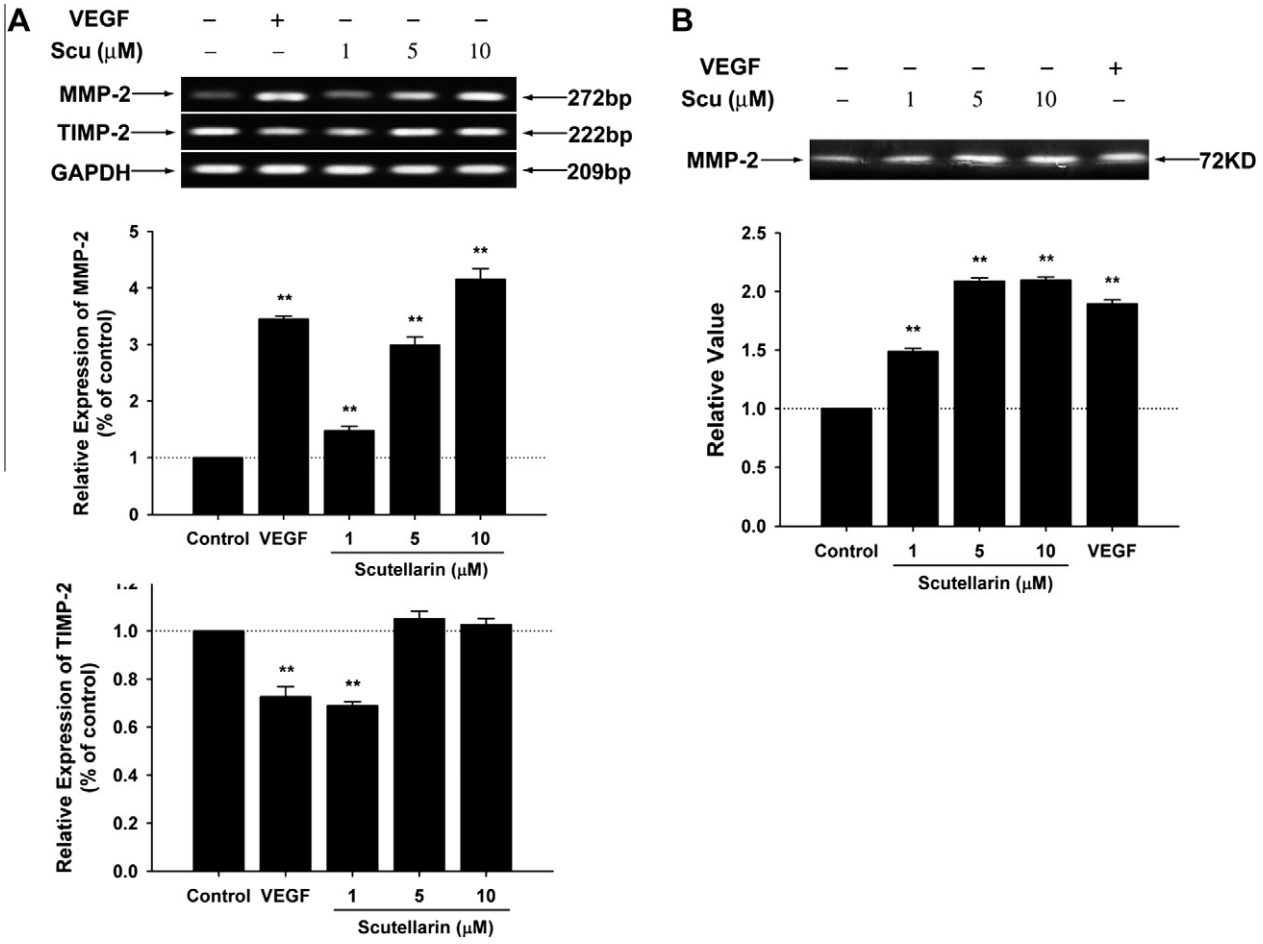

Fig. 4. Expression levels of MMP-2 and TIMP-2 in scutellarin-treated HUVECs. Cells were incubated in medium containing different concentrations (1, 5 or 10 μM) of scutellarin or 10 ng/mL VEGF (positive control) for 24 h. (A) For mRNA levels, total RNAs were extracted and subjected to RT-PCR for MMP-2 and TIMP-2, with GAPDH as an internal control. (B) For protein levels, the culture medium was harvested and subjected to gelatin zymography to analyze MMP-2 activity. The bands were quantitated by densitometry. "Scu" means scutellarin. Data shown in graphs are expressed as mean values ± SD of three individual experiments. $^{**}P < 0.01$ compared with control.

participates in the breakdown of collagen type IV, a major component of subendothelial basement membranes [22]. Therefore, MMP-2 production by endothelial or surrounding cells may be vital for the formation of new functional blood vessels. Given these important roles of MMPs in angiogenesis, we measured the expression of MMP-2 and TIMP-2 (the specific physiological MMP-2 inhibitor) in HUVECs. In this study, we found that scutellarin-treated HUVECs significantly increased MMP-2 expression at both mRNA and protein levels in a dose-dependent manner. On the other hand, TIMP-2 mRNA expression initially decreased with scutellarin treatment at a 1-μM dose, and then increased along with the scutellarin concentration. It may be related to the involvement of TIMP-2 at low levels in regulating MMP-2 activation by forming a membrane complex with MT1-MMP [23,24]. Taken together, these data could help us to understand the molecular mechanism of scutellarin-induced angiogenesis.

Previous studies have suggested that scutellarin has an inhibitory effect on protein kinase C (PKC). Because emerging evidence indicates that PKC is involved in mediating VEGF-induced angiogenesis [25,26], the inhibition of PKC would be expected to attenuate *in vitro* angiogenesis, which contradicts our results. The PKC family of serine/threonine kinases comprises at least 12 isozymes grouped into three classes: conventional PKC, novel PKC, and atypical PKC. Different PKC isoforms may have unique and even opposing functions during angiogenesis [27]. Overexpression of PKCδ, which is generally considered a growth inhibitory or pro-apoptotic PKC, in rat epididymal fat pad endothelial cells inhibited endothelial differentiation in a matrigel assay [28], whereas inhibition/down-regulation of PKCα in HUVEC inhibited vessel formation *in vitro* and myocardial neovascularization *in vivo* [29]. Scutellarin might also affect angiogenesis by activating additional signal pathways, such as nitric oxide synthase and its isoforms [13] or the estrogen receptor pathway [30], which are unrelated to its inhibition of PKC activity.

In conclusion, this study showed scutellarin to directly induce *in vitro* angiogenesis, which is closely correlated with upregulated MMP-2 expression. Although further study is needed to shed light on pathways involved in scutellarin's effect on HUVEC angiogenesis, scutellarin could be the basis for potent therapies for several angiogenesis-related human diseases.

Acknowledgments

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