Kallistatin Suppresses Cell Proliferation and Invasion and Promotes Apoptosis in Cervical Cancer Through Blocking NF-κB Signaling

Tao Wang, Fan Shi, JiQuan Wang, Zi Liu, and Jin Su

Department of Radiation Oncology, The First Affiliated Hospital of Xi'an Jiaotong University, Xi'an, P.R. China

Kallistatin has been recognized as an endogenous angiogenesis inhibitor and exerts pleiotropic effects in inhibiting tumor growth, migration, apoptosis, and inflammation. The purpose of the present study was to investigate the potential role and mechanisms of kallistatin in cervical cancer. We demonstrated that kallistatin effectively inhibited cell proliferation and enhanced apoptosis in a dose-dependent manner. Additionally, kallistatin suppressed migration and invasion activities and markedly reduced the expression of matrix-degrading metalloproteinases, progelatinase (MMP-2), MMP-9, and urokinase-type PA (uPA). Kallistatin reversed the epithelial–mesenchymal transition (EMT) and caused the upregulation of epithelial markers such as E-cadherin and inhibited mesenchymal markers such as N-cadherin and vimentin. Moreover, kallistatin led to a marked decrease in the expression of vascular endothelial growth factor (VEGF) and HIF-1α. In a xenograft mouse model, kallistatin treatment reduced tumor growth. Importantly, kallistatin strikingly impeded NF-κB activation by suppressing IκBα degradation and the level of phosphorylation of p65. Interestingly, similar to kallistatin, treatment with PDTC (an inhibitor of NF-κB) also attenuated cell invasion and migration. Taken together, these findings suggest that kallistatin suppresses cervical cancer cell proliferation, migration, and EMT and promotes cell apoptosis by blocking the NF-κB signaling pathway, suggesting that kallistatin may be a novel therapeutic target for cervical cancer treatment.

Key words: Cervical cancer; Kallistatin; Migration; Apoptosis; NF-κB signaling

INTRODUCTION

Cervical cancer, a potentially preventable disease with a high incidence, remains the second most common malignancy in women worldwide. Although advanced surgical techniques and chemoradiotherapy can improve the treatment rate of cervical cancer, the mortality rate remains high because of tumor recurrence and drug resistance. Therefore, efforts to identify additional novel molecular markers for the detection and diagnosis of cervical cancer are of great clinical importance.

Tumor malignancy consists of a series of complicated processes including proliferation, invasion, migration, and angiogenesis. During tumor progression, tumor cells acquire the expression of mesenchymal markers, such as vimentin, N-cadherin, and fibronectin, and the loss of epithelial markers such as E-cadherin, resulting in epithelial–mesenchymal transition (EMT), subsequent tumor metastasis, and proliferation at distant sites[1]. Angiogenesis is critical to the growth and metastasis of solid tumors. As such, targeting tumor neovascularization is a favorable strategy for cancer therapy. A number of angiogenesis inhibitors, including endostatin, have been identified and are being investigated in preclinical experiments. Several inhibitors have also been applied in clinical trials[2,3].

Kallistatin, detected in human plasma, was first discovered and identified as a tissue kallikrein-binding protein (KBP) and a unique serine proteinase inhibitor, and has emerged as a novel inhibitor of angiogenesis[4]. Plasma levels of kallistatin are known to be reduced in patients with sepsis, liver disease[5], and obesity[6], as well as in various cancers[7]. Kallistatin has a variety of biological effects in pathological and physiological responses, such as blood pressure regulation, antiangiogenesis, anti-inflammation, as well as antitumor and antioxidant effects[8–11]. Kallistatin suppresses tumor growth and angiogenesis in nude mice by antagonizing VEGF-mediated cell proliferation, migration, and invasion of cultured endothelial cells[10]. However, the role of kallistatin in human cervical carcinoma and the underlying mechanisms of such effects remain unclear.

In the present study, we investigated the potential role and the underlying mechanisms of kallistatin on cervical cancer. The results show that kallistatin inhibited tumor growth and migration and enhanced cell apoptosis

Address correspondence to Tao Wang, Department of Radiation Oncology, The First Affiliated Hospital of Xi'an Jiaotong University, No. 277 Yan Tower West Road, Xi'an 710061, P.R. China. Tel: +86-029-85324019; E-mail: twangrad@163.com

in vitro and in vivo, which suggest that kallistatin could be a suitable candidate for the regulation of tumor progress. These experiments demonstrate that kallistatin possesses strong antiproliferation and may be useful as an anticancer drug.

MATERIALS AND METHODS

Purification and Characterization of Recombinant Human Kallistatin

Recombinant human kallistatin was secreted into the serum-free medium of cultured human embryonic kidney cells (HEK293T). The culture medium was concentrated by ammonium sulfate precipitation followed by nickel affinity chromatography, as previously described[12]. The purity and identity of human kallistatin were verified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using a specific monoclonal antibody[5,12].

Cell Culture

The human cervical cancer cell lines HeLa and SiHa were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA), and human umbilical vein endothelial cells (HUVECs) were obtained from the Bioresource Collection and Research Center (Hsinchu City, P.R. China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (HyClone, Logan, UT, USA) at 37°C in a humidified 5% CO_2 atmosphere.

Cell Viability Assessed by the MTT Assay

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2, 5-dephenyltetrazoliumbromide (MTT; Sigma-Aldrich) assay according to the manufacturer's protocol. Briefly, cells were plated into 96-well microplates and treated with various concentrations of kallistatin (0.5, 1, and 2 μM). At the end of the incubation, the cells were exposed to MTT (0.5 mg/ml) at 37°C for 4 h. The absorbance value (A) at 570 nm was read using a Benchmark Microplate Reader (Bio-Rad Laboratories, Hercules, CA, USA). Three independent experiments were performed to generate an average value as the final result for analysis.

Apoptosis Assay

Apoptosis was determined using flow cytometry with a commercial Annexin-V–FITC Apoptosis Kit (Cell Signaling Technology, Boston, MA, USA) according to the manufacturer's protocol. Briefly, after being treated under different conditions, the cells were washed with ice-cold PBS and collected in a trypsin–EDTA solution (Invitrogen, Carlsbad, CA, USA). After centrifugation to remove the trypsin–EDTA, the cells were resuspended in binding buffer containing annexin V–FITC and propidium iodide (PI), and then incubated for 15 min at room temperature in the dark prior to analysis on a Becton Dickinson LSR II flow cytometer (BD Bioscience, San Jose, CA, USA).

Wound Healing Assay

Cells were seeded into 12-well plates and allowed to reach 100% confluence. The cell monolayer was scratched with a 200-μl pipette tip to yield scratches of a constant width. Cells were then incubated with the indicated treatments, and cells invading the wound line were photographed under a microscope. Three independent experiments were performed.

Boyden Chamber Invasion Assay

Filters (8 μm) coated with Matrigel (20 μg/filter) were placed in Boyden chambers (Neuro Probe, Cabin John, MD, USA). The cells suspended in DMEM containing 0.1% BSA and treated with kallistatin were placed in the top chamber. Conditioned medium from NIH/3T3 mouse fibroblasts was used as a chemoattractant and placed in the bottom compartment. Following 24 h of incubation at 37°C, noninvading cells were scraped off, and cells that had migrated to the lower surface of the filter inserts were fixed using 100% methanol for 10 min and stained with hematoxylin. Results were expressed as the percentage of migrated cells compared with control.

Quantitative Real-Time PCR (qRT-PCR) Analysis

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) following the manufacturer's protocol. cDNA was synthesized with the High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's instructions. Real-time PCR was performed with FastStart Universal SYBR Green Master kit (Roche Diagnostics, Indianapolis, IN, USA) and analyzed with an Applied Biosystems 7900 Real-Time PCR System. Fold changes in expression were calculated. The mRNA expression levels were calculated and expressed as $2^{-\Delta\Delta CT}$.

Western Blot Analysis

Protein contents of cleared lysates were determined with a BCA Protein Quantitative Analysis Kit (Shenergy Biocolors Technologies, Shanghai, P.R. China), and equal amounts of protein were loaded into each lane of a 12% SDS-PAGE gel for protein separation. The protein bands were then transferred to polyvinylidene difluoride (PVDF) membrane (GE Healthcare, Piscataway, NJ, USA). The membranes were blocked with 7% milk in TBST (20 mM Tris, 500 mM NaCl, and 0.1% Tween 20) for 1 h. After washing with TBST twice, membranes were incubated with primary antibody overnight at 4°C. The

membranes were washed twice with TBST and incubated with a horseradish peroxidase-conjugated secondary goat anti-mouse IgG (GenScript, P.R. China) for 2 h at room temperature. The signal was developed using enhanced chemiluminescence (Super ECL Plus; Applygene Technologies, Nanjing, P.R. China) to expose a photographic film according to the manufacturer's instructions. The experiment was repeated three times. The densitometry of bands was quantified with ImageJ2 software.

ELISA for VEGF

The treatments were assessed using a VEGF ELISA kit according to the manufacturer's protocol. The cultivated cells were incubated in six-well plates for 24 h. The VEGF concentrations were measured using an ELISA kit (Elabscience, P.R. China) after the culture medium had been collected and centrifuged to remove cell debris.

Tumor Xenografts in Nude Mice

Nude mice (BALB/c nu/nu, females; 4–5 weeks old) were purchased from the Laboratory Animal Center of Xi'an Jiaotong University and housed under specific pathogen-free conditions. The experimental protocols were approved by the Animal Research Committee of The First Affiliated Hospital of Xi'an Jiaotong University. HeLa cells (5×10^6) suspended in PBS were injected subcutaneously into the right front axilla of the mice. When the tumors reached approximately 180 mm^3 in volume, the mice were treated with intraperitoneal (IP) injection without or with kallistatin every 2 days. Tumors were measured every 2 days using calipers, and the tumor volume was calculated according to the formula $V = 0.5 \times A^2 \times B$, where A represents the smallest superficial diameter, and B represents the largest superficial diameter. On day 32 after injection, the mice were sacrificed, and the tumors were excised and weighed. All procedures were performed in accordance with the guidelines of the Chinese Association for Laboratory Animal Science.

Statistical Analysis

All data are presented as mean±SD for experiments performed at least three times. Statistical analysis was performed using GraphPad Prism 5 software (La Jolla, CA, USA) and SPSS 13.0 software (Chicago, IL, USA). The statistical significance of differences was determined by Student's two-tailed *t*-test for two groups and one-way ANOVA for multiple groups. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

Kallistatin Restrains Cell Viability and Promotes Apoptosis in Cervical Cancer Cells

To determine the effect of kallistatin on the viability of cervical cancer cells, HeLa and SiHa cells were treated with various concentrations of kallistatin, and cell viability was evaluated by MTT assay. The results show that kallistatin strongly suppressed the viability of the two cervical cancer cell lines in a dose- and time-dependent manner (Fig. 1A and B). For further confirmation of apoptosis by kallistatin, we performed annexin V–FITC staining of kallistatin-treated HeLa and SiHa cells. Kallistatin markedly increased the percentage of apoptotic cells in the two cervical cancer cell lines after 48 h of treatment (Fig. 1C and D). Collectively, these data demonstrate that kallistatin has the ability to suppress cell proliferation and promote apoptosis in cervical cancer cells.

Kallistatin Impedes Cervical Cancer Cell Migration, Invasion, and EMT

We further examined whether kallistatin affects cell migration and invasion in cervical cancer cells using the wound healing assay and invasion assay. Kallistatin was able to significantly inhibit invasion of HeLa and SiHa cells in a dose-dependent manner (Fig. 2A and B). In addition, kallistatin exhibited markedly reduced migration of HeLa and SiHa cells in a concentration-dependent manner (Fig. 2C and D). We further examined the effect of kallistatin on major regulators and markers of EMT. Kallistatin treatment significantly elicited the upregulation of epithelial markers such as E-cadherin and also decreased the expression of mesenchymal markers such as N-cadherin and vimentin (Fig. 2E and F). In combination, these data indicate that kallistatin notably suppresses cell migration, invasion, and EMT in cervical cancer cells.

Kallistatin Exerts an Inhibitory Effect on uPA, MMP-2, and MMP-9

Several proteins that perform primary functions in the invasion and migration of cervical cancer include uPA, MMP-2, and MMP-9. We conducted qPCR and Western blot analysis to determine whether the expression levels of uPA, MMP-2, and MMP-9 were influenced by kallistatin in HeLa cells. The results show that the mRNA and protein levels of uPA, MMP-2, and MMP-9 were markedly decreased after kallistatin treatment (Fig. 3A and B). The above results suggest that kallistatin inhibits the expression and activity of uPA, MMP-2, and MMP-9 and thereby prevents aggressive tumor invasion.

The Effect of Kallistatin on VEGF and HIF-1α in HUVECs

We further examined whether kallistatin was capable of inhibiting tumor-induced proliferation and invasion of HUVECs induced by HeLa. HUVEC proliferation was evaluated by the MTT assay. The results show that HeLa cells promoted viability, but kallistatin gradually reduced the viability of HUVECs in a dose-dependent manner
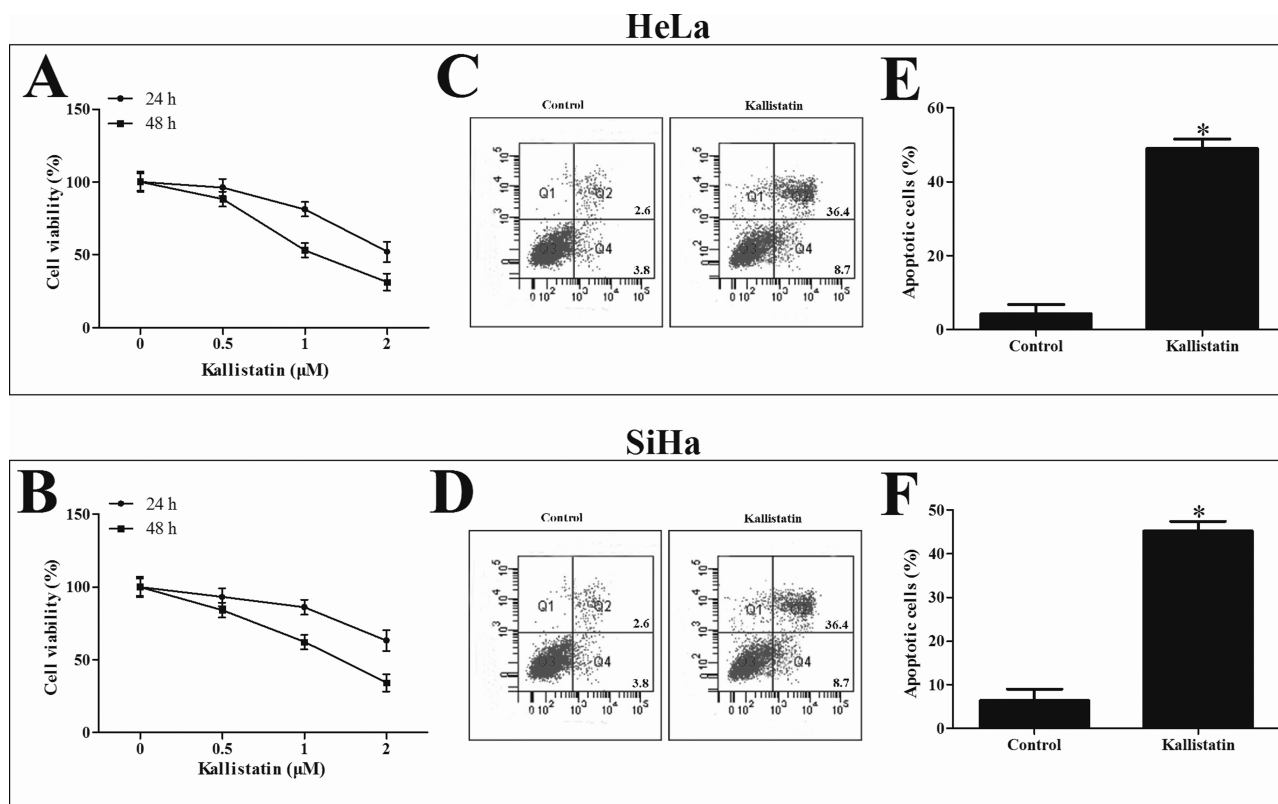
HeLa

SiHa

Figure 1. The effect of kallistatin on cell viability and apoptosis in human cervical cancer cells. HeLa (A) and SiHa (B) cells were treated with various concentrations of kallistatin for 24 and 48 h. Cell viability was assessed with the MTT assay. (C, D) Apoptotic cells were analyzed by flow cytometry with annexin V–FITC/PI staining. (E, F) Quantitative analyses of apoptotic populations following kallistatin treatment. Data are presented as the mean±SD of at least three independent experiments. *$p < 0.05$ versus Control.

(Fig. 4A). The Matrigel invasion assay was performed to assess whether kallistatin affects HUVECs invasion. HeLa cells significantly stimulated HUVEC migration, and kallistatin obviously suppressed the invasion of HUVECs in a concentration-dependent manner (Fig. 4B). VEGF is the most potent angiogenic factor and is associated with tumor-induced angiogenesis. To determine the effects of kallistatin on VEGF secretion, the VEGF protein level in the HUVECs treated with HeLa was measured by ELISA. These results indicate that kallistatin reduced VEGF secretion in a dose-dependent manner (Fig. 4C). Western blot was further performed to confirm the effects of kallistatin on VEGF expression. VEGF protein expression was significantly decreased with the increasing concentrations of kallistatin in HUVECs (Fig. 4D). HIF-1α is an important modulator for VEGF transcription and significantly increased VEGF expression. The effects of kallistatin on the expression HIF-1α were inspected by Western blot. The results show that a dose-dependent decrease in HIF-1α expression was observed (Fig. 4D). The abovementioned results demonstrate that kallistatin inhibits endothelial cell viability and invasion, and negatively regulated the expression of VEGF and HIF-1α.

Kallistatin Dampens the NF-κB Pathway

NF-κB is known to regulate a variety of cell functions including proliferation, apoptosis, and migration. We further investigated the effects of kallistatin on the NF-κB pathway. The degradation of IκBα was effectively attenuated by kallistatin (Fig. 5A and B). Kallistatin also suppressed the phosphorylation of p65 but not the expression of p65. These observations suggest that kallistatin may dispute NF-κB activation in HeLa cells via the suppression of IκBα degradation and impairs nuclear translocation of NF-κB. To further investigate the roles of NF-κB pathways in the inhibitory effect of kallistatin on HeLa cell migration and invasion, the effects of PDTC (an inhibitor of NF-κB) on the invasion and migration were observed. The results are similar to the effect of kallistatin on cell invasion and migration (Fig. 5C and D). Exposure of the cells to 200 μmol/L PDTC also suppressed cell invasion and migration.

Kallistatin Suppresses Tumor Progression In Vivo

To further investigate the antitumor effect of kallistatin in vivo, we established a HeLa cell xenograft model in nude mice. The results show that kallistatin dramatically
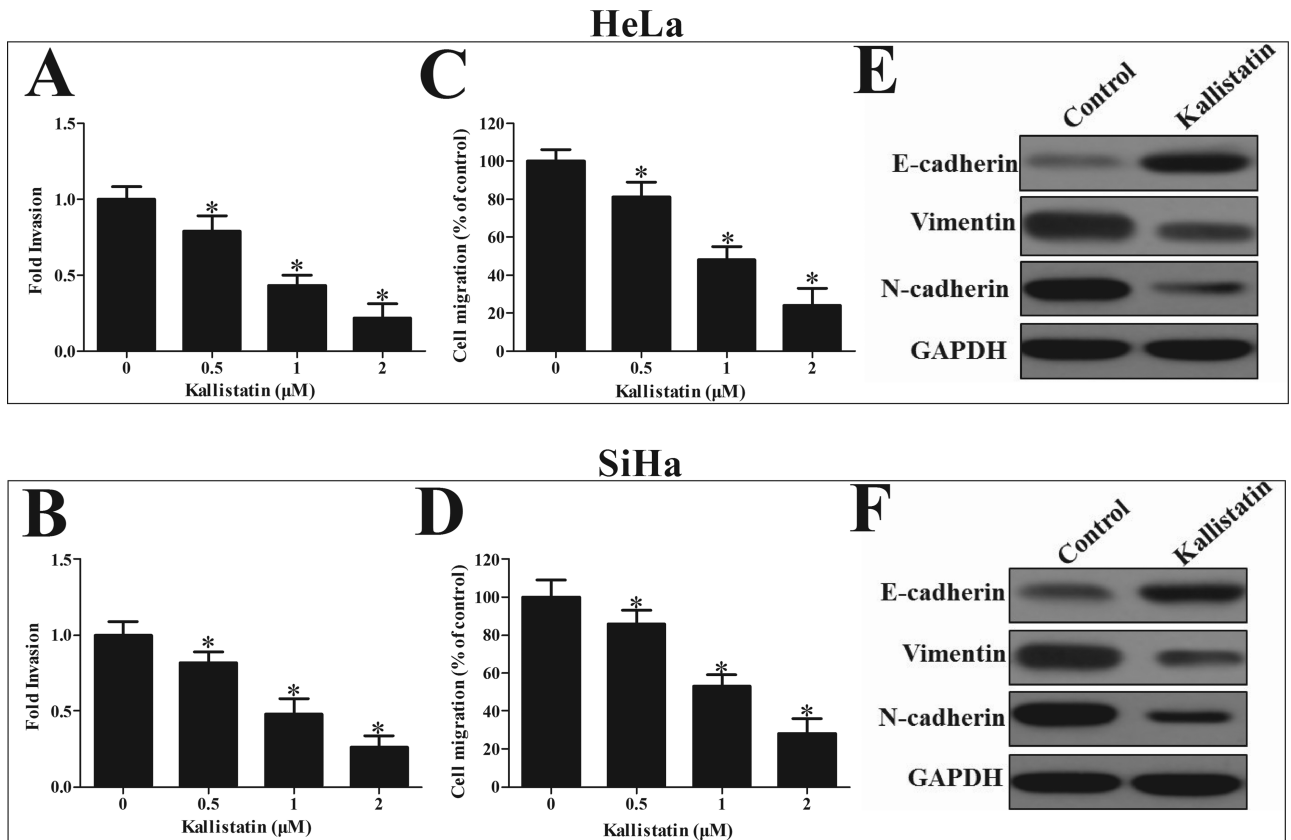
HeLa

SiHa

Figure 2. The effects of kallistatin on cell invasion, migration, and EMT in human cervical cancer cell lines. HeLa (A) and SiHa (B) cells were analyzed for cell migration by the wound healing assay. HeLa (C) and SiHa (D) cells were treated with various concentrations of kallistatin for 24 h, followed by an invasion assay. Western blot analysis was used to detect the expression of E-cadherin, vimentin, and N-cadherin in HeLa (E) and SiHa (F) cells. $*p < 0.05$ versus Control.

decreased tumor volume (Fig. 6A). Consistent with this profound effect on tumor volume, a significant reduction in the weight of tumors was observed in the group treated with kallistatin (Fig. 6B). These data indicate that kallistatin markedly inhibits tumorigenicity in nude mice.

DISCUSSION

In the present study, we demonstrated the potential therapeutic efficacy of kallistatin in inhibiting cervical carcinogenesis in vitro and in vivo by hampering the NF-κB pathway. Kallistatin, a plasma protein, has been
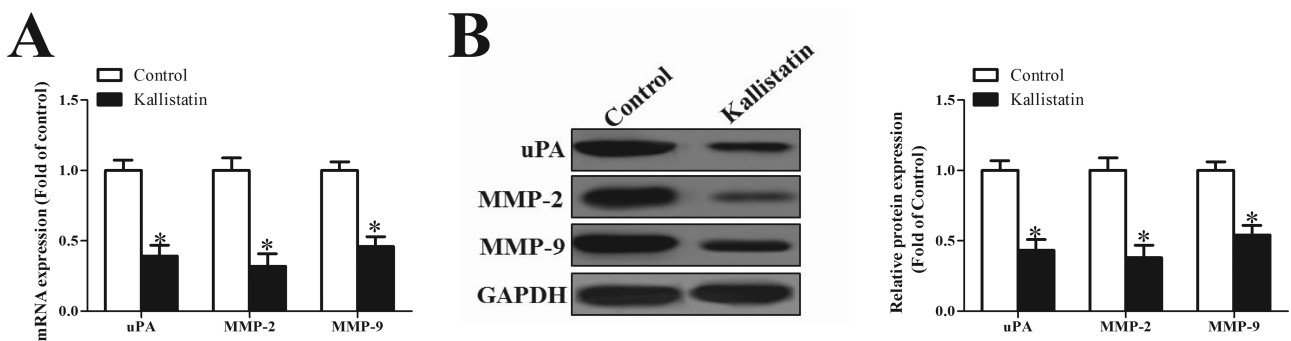

Figure 3. The effects of kallistatin on the proteinase and transcription activities of uPA, MMP-2, and MMP-9. (A) The mRNA levels of uPA, MMP-2, and MMP-9 were determined by RT-PCR. (B) Protein levels of uPA, MMP-2, and MMP-9 were inspected by Western blot analysis. $*p < 0.05$ versus Control.
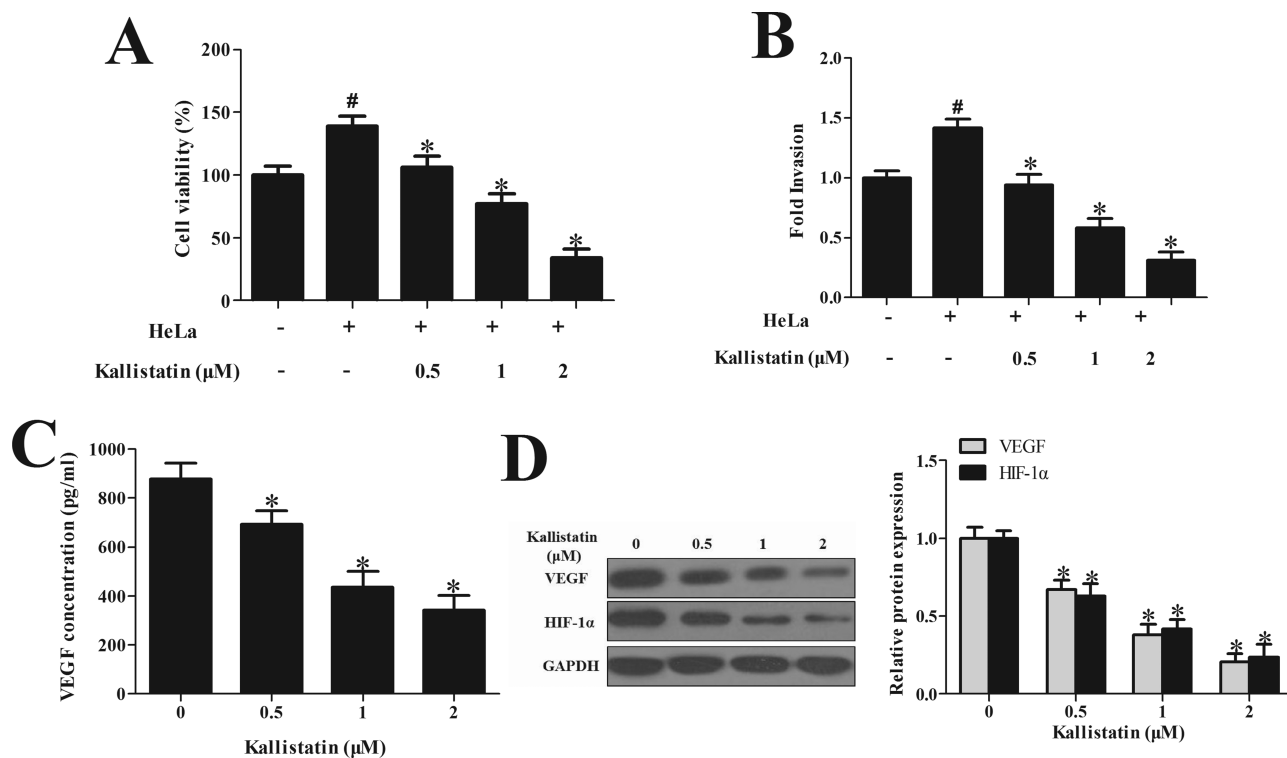
Figure 4. HUVECs were treated with kallistatin at the indicated concentration. HUVEC viability (A) and cell invasion (B) were assessed. (C) The concentration of VEGF was determined using ELISA. (D) The protein expression of VEGF and HIF-1α was detected by Western blotting. *$p < 0.05$ versus Control.

demonstrated to exert multiple biological functions. The pleiotropic effects of kallistatin include inhibiting inflammation, angiogenesis, oxidative stress, apoptosis, tumor growth, and cellular invasion in animal models and cultured cells. It is present in many human tissues, including the eye, kidney, liver, heart, arteries and veins, atheromas, blood cells, and body fluids[5]. In addition, tissue kallikrein is highly expressed in adenocarcinomas[13] and can efficiently activate two matrix-degrading metalloproteinases, progelatinase (MMP-2) and MMP-9[14]. Like plasmin, tissue kallikrein may have a role in degrading the extracellular matrix to promote tumor invasion. In support of this notion, a previous study has shown that recombinant wild-type kallistatin, a specific tissue kallikrein inhibitor, significantly attenuated the invasiveness of human breast tumor cells[15]. Another recent study has identified that kallistatin inhibits tumor angiogenesis via inhibition of the NF-κB signaling pathway[16]. However, the role of kallistatin in cell proliferation and migration of cervical cancer remains to be elucidated.

Tumorigenesis occurs as a result of excessive proliferation combined with reduced apoptosis. Recently, it has been shown that kallistatin markedly inhibits angiogenesis by suppressing VEGF- and bFGF-induced proliferation, migration, and adhesion of endothelial cells[10]. In the

current study, we elucidated the function of kallistatin in the tumorigenesis of cervical cancers. The MTT assay and flow cytometry results showed that kallistatin remarkably attenuated cell viability in a dose-dependent manner and promoted apoptosis in both HeLa and SiHa cells. Considering the biological nature of these cells, we also found a similar pattern in our constructed nude mouse model, as reflected by a reduction in tumor volume and weight following kallistatin treatment. Neoplastic metastasis is a major cause of cancer-mediated death. EMT, a process by which epithelial cells lose their polarity and acquire a migratory mesenchymal phenotype, is a crucial process in the induction of tumor invasion and metastasis. Studies have simultaneously demonstrated that kallistatin also reverses EMT by regulating the expression of E-cadherin, N-cadherin, and vimentin.

The metastasis of tumor cells is dependent on the degradation of the components of the extracellular matrix by MMPs, particularly MMP-2 and MMP-9[17]. These enzymes directly participate in angiogenesis and metastasis and have an effect on clinical outcome and prognosis[18]. It has been reported that MMP-2 and MMP-9 secreted from HeLa cells are involved in the proteolytic events required for tumor migration, metastasis, and angiogenesis[19,20]. In addition, the invasive ability of tumor cells consists of
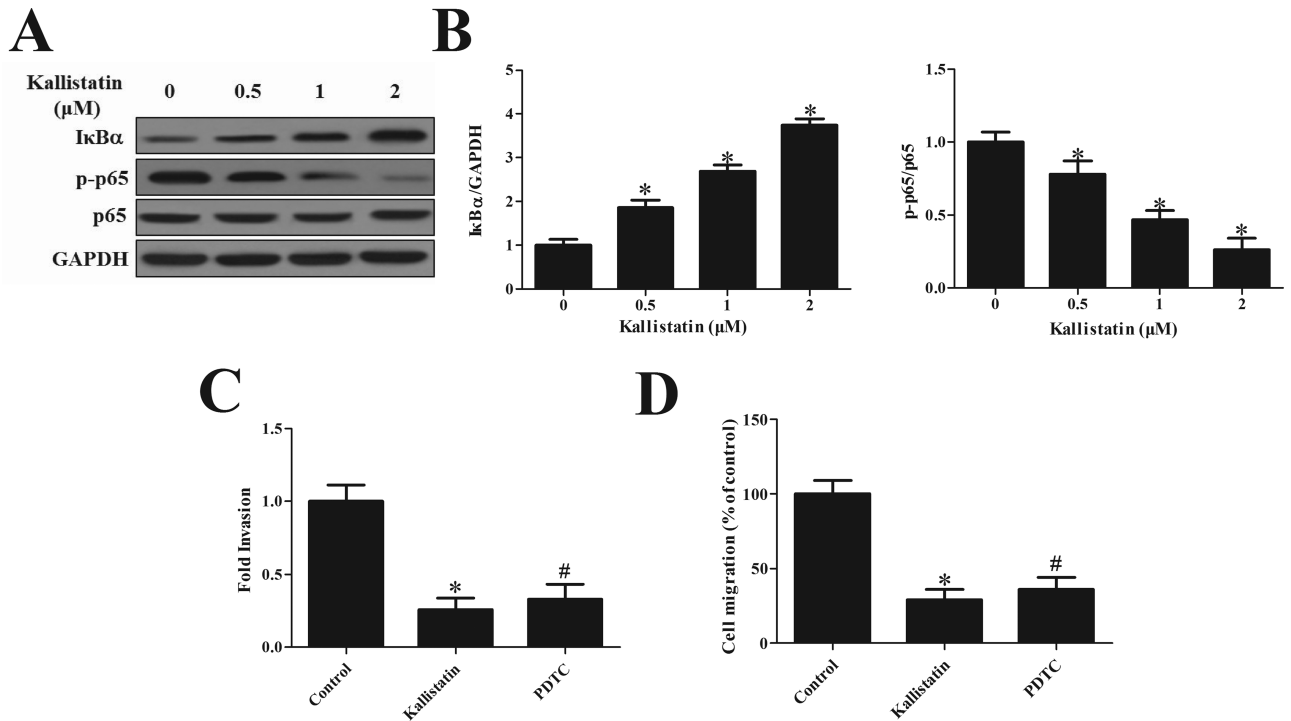
Figure 5. Effect of kallistatin on the NF-κB pathway. (A) Western blot analysis of IκBα and p-p65 expression. (B) Quantification of band intensity in (A). HeLa cells were exposed to 200 μmol/L PDTC. (C) Cell invasion ability was assessed by Matrigel invasion assay. (D) Cell migration was detected using wound healing assay. Data are presented as mean±SD. *$p<0.05$ versus Control; #$p<0.05$ versus Control.

various aspects such as the urokinase-type PA (uPA) system, which consists of uPA and its specific cell surface receptor uPAR. The uPA level is upregulated in pancreatic cancer cells. In addition, suppression of the uPA–uPAR system results in the downregulation of angiogenin and a decrease in angiogenic potential in vitro and in vivo[21]. Here we demonstrate that kallistatin repressed the expression of MMP-2, MMP-9, and uPA at the mRNA and protein levels in HeLa cells. In conclusion, these data

support the view that kallistatin suppresses migration by regulating the activity of MMP-2, MMP-9, and uPA.

Angiogenesis is a critical role in tumor progression, invasion, and metastasis; therefore, it represents a rational target for therapeutic intervention[22]. Compelling evidence has indicated that VEGF is involved in angiogenesis and tumor growth; this biomarker has also been implicated in tumor progression[23]. HIF-1α is a transcription factor known to be associated with most hypoxic
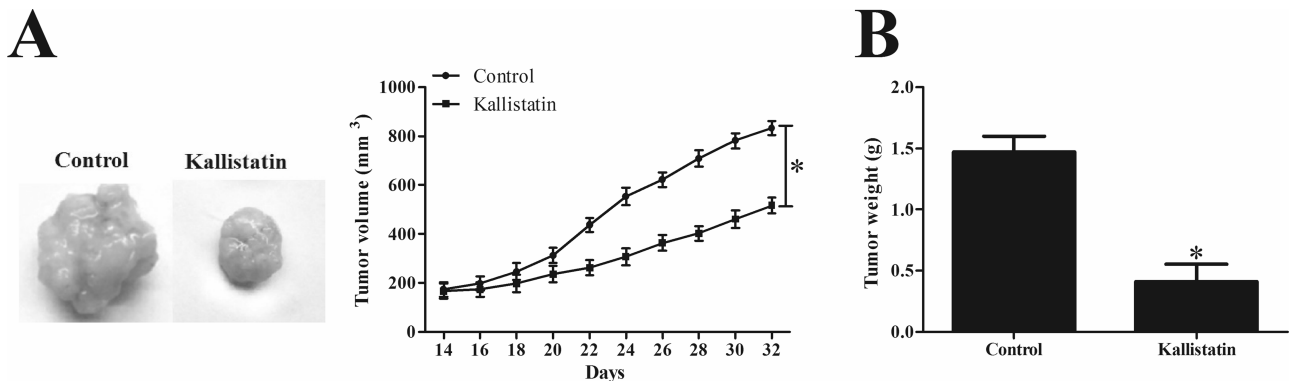

Figure 6. Kallistatin hindered tumor progression in vivo. (A) Tumor growth curves after injection in nude mice. Tumor volumes (mm^3) were measured every 2 days. (B) Analysis of tumor weight after 32 days of treatment. $n=7$. *$p<0.05$ versus Control.

solid tumors. This factor is consistently overexpressed in various human cancers and promotes tumorigenesis through angiogenesis. The present study elucidates that kallistatin suppresses the capacity of HeLa to induce cell viability and invasion, suggesting that kallistatin could influence the crosstalk between vascular endothelial cells and HeLa cancer cells. Subsequent analysis revealed that kallistatin prevented the secretion of VEGF and down-regulated the expression of HIF-1α in HeLa cells. Taken together, these results demonstrate that kallistatin effectively inhibits tumor growth of HeLa cells, suggesting that kallistatin has therapeutic potential to inhibit migration in vivo and in vitro.

NF-κB is a family of important transcriptional factors known to regulate a wide range of biological effects, including proliferation, cellular invasion, apoptosis, and angiogenesis, via its downstream target genes[24]. Earlier reports have indicated that NF-κB plays a significant role in the expression of MMP-2 and uPA[25]. Additionally, NF-κB regulates the expression of many genes whose products are involved in tumor growth[26]. Recently, it has been shown that kallistatin inhibits vascular inflammation by antagonizing TNF-α-induced NF-κB activation[27]. In the current study, the impact of kallistatin on NF-κB activity was examined, and the results show that kallistatin suppressed IκBα degradation and reduced the level of p65 phosphorylation. To further define the point in the NF-κB pathway at which kallistatin regulates the invasion and migration of cervical cancer cells, we treated HeLa cells with an NF-κB inhibitor (PDTC). We demonstrated that PDTC dramatically repressed cell invasion and migration, and these effects are similar to those of kallistatin, indicating involvement of the NF-κB pathway in the inhibitory effect of kallistatin on cell invasive ability.

In conclusion, this study demonstrates that kallistatin inhibits tumor growth and migration and promotes cell apoptosis, associated with blocking the NF-κB pathway. Targeting kallistatin could be a useful and new therapeutic strategy to control the progression in tumor treatment. Further preclinical and clinical trials are required to research the full potential of this important protein.

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