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Short communication

Saxatilin Suppresses Tumor-induced Angiogenesis by Regulating VEGF Expression in NCI-H460 Human Lung Cancer Cells

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Tumor growth and metastasis are dependent on angiogenesis, and endothelial cell invasion and migration are apparent means of regulating tumor progression. We report here that saxatilin, a snake venom-derived disintegrin, suppresses the angiogenesis-inducing properties of NCI-H460 human lung cancer cells. Culture supernatants of NCI-H460 cells are able to induce human umbilical vascular endothelial cell (HUVEC) invasion and tube formation. However, treatment of the cancer cells with saxatilin resulted in reduced angiogenic activity of the culture supernatant. This suppressed angiogenic property was found to be associated with the level of vascular endothelial growth factor (VEGF) in the culture supernatant. Further experimental evidence indicated that saxatilin inhibits VEGF production in NCI-H460 cells by affecting hypoxia induced factor-1α (HIF-1α) expression via the Akt pathway.

Keywords: Angiogenesis, Disintegrin, HIF-1α, Lung cancer, VEGF

Introduction

Angiogenesis plays a key role in embryonic vascular development, wound healing, and organ regeneration, as well as in pathological processes such as tumor growth (Ferrara, 1997; Risau, 1997). A complex network of growth factors and cytokines regulates angiogenesis. Tumor angiogenesis is induced by the release of angiogenic peptides from tumor cells, macrophages, and the extracellular matrix (ECM) (Risau, 1997). VEGF is a unique, endothelial cell-specific mitogen that promotes many events that are necessary for angiogenesis (Brown *et al*., 1997; Shibuya, 2006). A variety of malignant human tumors, including breast, lung, and prostate carcinomas, are known to secrete VEGF (Brown *et al*., 1993; Ohta *et al*., 1996; Joseph *et al*., 1997).

Integrins mediate interactions between endothelial cells and the ECM and have been implicated in angiogenesis (Stromblad and Cheresh, 1996). For example, integrin αvβ3 is not readily detectable in quiescent vessels but becomes highly expressed in angiogenic vessels (Brooks *et al*., 1994). Both Brooks *et al*. and Yeh *et al*. evidenced the dependence of angiogenesis on vascular cell adhesion events *in vivo* with the observation that both antibody and snake venom proteins that antagonize integrin αvβ3 also blocked angiogenesis in the chick chorioallantoic membrane (CAM) model (Brooks *et al*., 1994; Yeh *et al*., 1998). These facts imply that antagonists specific to a certain integrin may be selective for a given angiogenic disease or process.

In previous studies, we characterized both saxatilin and salmosin, disintegrins that are derived from the venom of the Korean snakes, *G. saxatilis and Gloydius halys brevicaudus*, respectively (Kang *et al*., 1998; Hong *et al*., 2002a; Hong *et al*., 2002b). Saxatilin binds specifically to integrins αIIbβ3 and αvβ3 that are expressed on platelets and other cells, including vascular endothelial cells and certain tumor cells. Saxatilin has also been reported to suppress bFGF-induced human umbilical vein endothelial cell (HUVEC) proliferation and vitronectin-induced smooth muscle cell (SMC) migration at higher concentrations (20-40 mg/ml) as well as to inhibit platelet aggregation with an IC_{50} of 127 nM (Hong *et al*., 2002a). Salmosin, which is structurally homologous to saxatilin, inhibits fibrinogen-dependent platelet aggregation, experimental tumor metastasis, tumor growth, and bFGF-induced angiogenesis without affecting the proliferation of normal endothelial cells (Kang *et al*., 1998; 1999; 2000).

In this study, we examined the anti-angiogenic activity of saxatilin in human lung cancer cells and observed the molecular changes induced by saxatilin in these cells.

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Materials and Methods

Cells and preparation of culture supernatant. HUVECs were isolated from human umbilical cord veins by collagenase treatment as described previously (Jaffe *et al*., 1973) and used during passages 7-9. The cells were grown in M199 medium (GIBCO) supplemented with 20% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml basic fibroblast growth factor, and 5 units/ml heparin at 37°C under humidified air with 5% CO_2. NCI-H460 cells, obtained from the American Type Culture Collection (VA), were maintained in RPMI 1640 medium containing 10% FBS, L-glutamine, and antibiotics. The cells were incubated with or without saxatilin at 37°C for 24 h and the culture supernatant was collected and centrifuged at 2,000 × *g* for 5 min at 4°C. The supernatant was isolated and stored at −80°C until used for further assays.

Chemoinvasion assay. The HUVEC invasion assay was performed *in vitro* using a transwell chamber (Costar) system with 8.0-μm pore polycarbonate filter inserts as described previously (Tan *et al*., 2004). Six hundred microliters of either M199 medium or cancer cell culture supernatant were placed in the lower wells. HUVECs (10^5 cells/100 μl) were loaded into each of the upper wells. After the cells were incubated at 37°C for 24 h, the cells on the lower surface were counted at 40 × magnification in 4 fields. Each sample was assayed in duplicate and the experiment was repeated three times independently.

***In vitro* endothelial capillary tube formation assay.** The tube formation assay was carried out as previously described (Kubota *et al.,* 1988) with slight modifications. Matrigel (250 μl of a 10-mg/ml concentration; BD Biosciences) was placed in a 24-well culture plate and polymerized for 30 min at 37°C. HUVECs (3×10^5 cells/ml) were resuspended in either M199 medium or cancer cell culture supernatants and loaded onto the Matrigel. The cells were incubated for 18 h and fixed in 3.7% formaldehyde. Three random fields of view in two replicate wells for each test condition were visualized under 40 × magnification. Each sample was assayed in duplicate and the experiment was repeated three times independently.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis. Cellular RNA was extracted from NCI-H460 cells that were incubated for the indicated times in 100 nM saxatilin using the RNeasy Protect Kit (Qiagen) according to the manufacturer's instructions. Aliquots (2 μg) of total RNA were converted into cDNA using oligo $(dT)_{15}$ with AMV reverse transcriptase following the instructions of the manufacturer (Promega). The following primers were used for semi-quantitative RT-PCR analysis: VEGF (forward): 5'- GTG TGC CCC TGA TGC GAT GCG -3'; VEGF (reverse): 5'- ACC GCC TCG GCT TGT CAC -3'; actin (forward): 5'- GGG TCA GAA GGA TTC CTA TG -3'; actin (reverse): 5'- CCT TAA TGT CAC GCA CGA TTT -3'. Reactions were performed in an Eppendorf Gradient Mastercycler (Eppendorf) with an initial denaturation at 94°C for 2 min, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 90 s, and an additional 5-min polymerization step at 72°C using Takara Ex *Taq* polymerase (Takara-Bio). Amplified PCR products were resolved by electrophoresis on agarose gels and photographed using Kodak's digital science, 1D image analysis software.

VEGF Enzyme-linked immunosorbant assay (ELISA). VEGF levels in NCI-H460 cell culture supernatants were determined using an ELISA kit (R&D Bioscience) according to the manufacturer's instructions and analyzed using a Labsystems Multiscan reader. The experiment was repeated three times independently.

Immunoblotting studies. NCI-H460 cells were incubated with the indicated concentrations of saxatilin at 37°C for either 4 h or 12 h and the lysates were subjected to immunoblotting analysis as described previously (Ueda *et al*., 2006). The following antibodies were used at a dilution of 1 : 1,000 : anti-PKB/Akt-phospho-Ser473, anti-PTEN-phospho-ser380 (both from Cell Signaling Technology), anti-HIF-1α, and anti-actin (both from CHEMICON). Bands on immunoblots were analyzed using image reader LAS-3000 (version 2.1; FUJIFILM).

Immunofluorescence. NCI-H460 cells were resuspended in RPMI containing 10% FBS, seeded on coverslips, and incubated at 37°C for 18 h. The cells were treated with the indicated concentrations of saxatilin for 12 h and fixed with 4% formaldehyde at room temperature for 20 min. Antibodies against HIF-1α were diluted in PBS containing 0.1% BSA and incubated with the fixed cells at room temperature for 2 h. The coverslips were washed with PBS and then incubated with FITC-conjugated secondary antibody diluted in PBS containing 0.1% BSA at room temperature for 30 min in the dark. Each coverslip was mounted on a drop of VECTASHIELD mounting medium for fluorescence with DAPI (VECTOR Laboratories). Results were analyzed using an Axioplan 2 imaging fluorescent microscope system (ZEISS).

Results and Discussion

Angiogenesis consists of proliferation and alignment of endothelial cells to form tubular structures (Breier and Risau, 1996). The inhibition of tumor angiogenesis is an important strategy for cancer therapy. It is well demonstrated that an increase in tumor growth must be preceded by an increase in the microvasculature that supplies the neoplasm (Folkman, 1995; Risau, 1997). Also, it has been demonstrated that the supernatants from lung cancer cells or HNSCC cells induce angiogenic responses (Petruzelli *et al*., 1993; Chang *et al*., 2003).

In this work, we examined the effects of saxatilin on NCI-H460 human lung carcinoma cells that were associated with angiogenic properties. NCI-H460 cells were incubated in the presence or absence of saxatilin for 24 h under normoxic conditions in RPMI 1640 medium. Culture supernatants from NCI-H460 cells induced HUVEC migration and tube formation *in vitro*. On the other hand, compared with those of untreated cells, the culture supernatants generated from NCI-H460 cells treated with 100 nM saxatilin suffered 50% loss of their ability to induce HUVEC migration. Also, culture supernatants from cancer cells treated with saxatilin showed a decreased ability to induce HUVEC tube formation. However, 100 nM saxatilin in

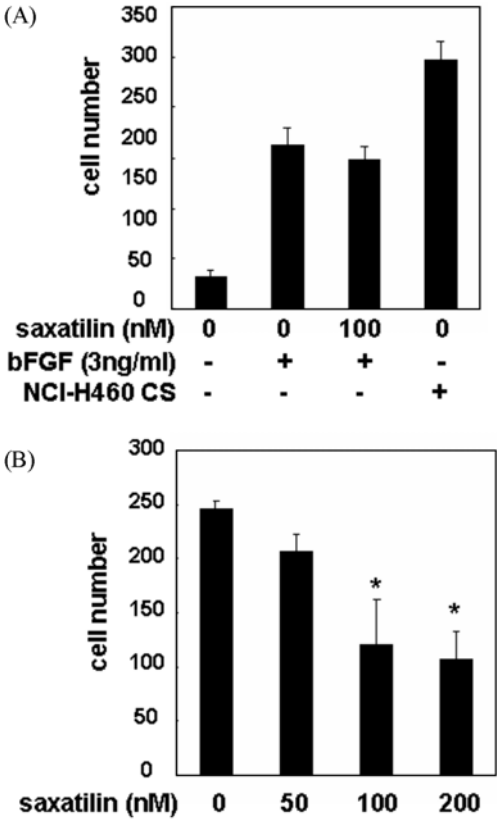

Fig. 1. Chemoinvasion assay of HUVECs stimulated by culture supernatants of NCI-H460 cells. (A) HUVEC chemoinvasion assays were performed using either bFGF or culture supernatants of NCI-H460 cells as the chemoattractant. (B) HUVEC chemoinvasion assays were performed using culture supernatants that were derived from NCI-H460 cells treated with the indicated concentrations of saxatilin. The total number of cells that migrated to the lower surface of the transwell membrane after a 24-h incubation period was counted. Results are expressed as the mean ± SD. (*) $p < 0.05$ vs. cells invaded toward untreated culture supernatants.

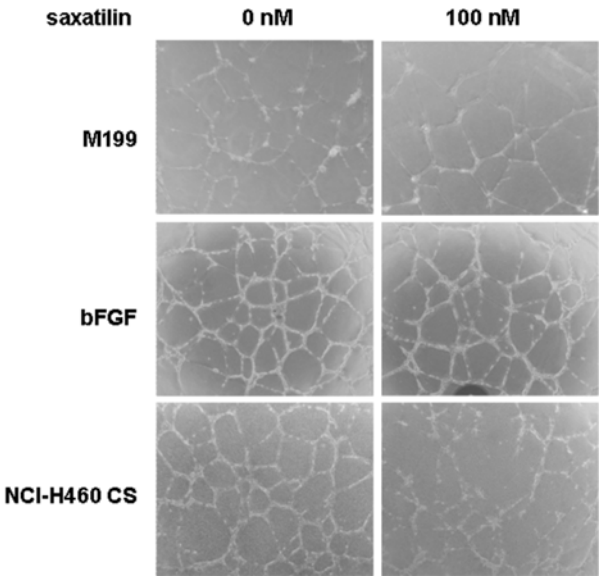

Fig. 2. Tube formation of HUVECs induced by culture supernatants of NCI-H460 cells. HUVEC tube formation assays were performed using culture supernatants of NCI-H460 cells incubated in the presence or absence of saxatilin. Three independent experiments were performed in duplicate and representative photomicrographs (40 × magnification) are shown.

fresh M199 media did not exhibit an inhibitory effect on either HUVEC migration or tube formation (Figs. 1 and 2). These data suggest that saxatilin is a potent anti-angiogenic agent.

To examine whether saxatilin gains its anti-angiogenic activity by suppressing cell growth, we performed 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assays of both HUVECs and NCI-H460 cells in the presence of saxatilin for either 24 h or 72 h and found that saxatilin did not affect cancer cell proliferation at the same concentrations (50-200 nM; data not shown). The results of *in vitro* angiogenesis assays indicated that NCI-H460 cells may release growth factors and that culture supernatants from saxatilin-treated NCI-H460 cells might lack the growth factor(s) required for angiogenic induction.

We, therefore, next examined whether saxatilin down-regulates the expression of VEGF in NCI-H460 cells. After treating cancer cells with saxatilin for the indicated times, total mRNA was prepared and subjected to RT-PCR analyses for both VEGF and actin. As shown in Fig. 3A, saxatilin treatment reduced the expression of VEGF at the transcriptional level. To quantitate VEGF secretion in the culture supernatants of NCI-H460 cells, ELISA was performed after incubating the cells with or without saxatilin for 24 h. VEGF levels were significantly decreased by saxatilin treatment in NCI-H460 cells in a dose-dependent manner (Fig. 3B). There are a number of reported HIF-1α-binding sites in the VEGF promoter region; HIF-1α binding to the promoter stimulates VEGF expression (Shima *et al*., 1996; Forsythe *et al*., 1996). HIF-1α is also known to be constitutively over-expressed in a number of human cancers (Zhong *et al*., 2002; Jubb *et al*., 2004) and its expression is activated by either the PI-3K/Akt, which prevents cell apoptosis, MEK1/ERK signaling pathways or the ILK/Akt/mTOR signaling pathway (Tan *et al*., 2004; Shi *et al*., 2005; Kang *et al*., 2006). Once activated, HIF-1 stimulates the transcription of many growth factors, including VEGF, bFGF-2, FGF-7, TGF-β (Berger *et al*., 2003), and erythropoietin (Grimm and Ratcliffe, 2002), as well as glycolytic enzymes (Gleadle *et al*., 1997), all of which are implicated in angiogenesis and in cell survival and proliferation during hypoxia.

Therefore, we next examined the impact of saxatilin on the activation of Akt and PTEN, which are associated with ILK activity and the expression of HIF-1α (Wu and Dedhar, 2001). Immunoblotting analyses showed that 100 nM saxatilin significantly decreased the phosphorylation of Akt. On the other hand, saxatilin increased the phosphorylation of PTEN

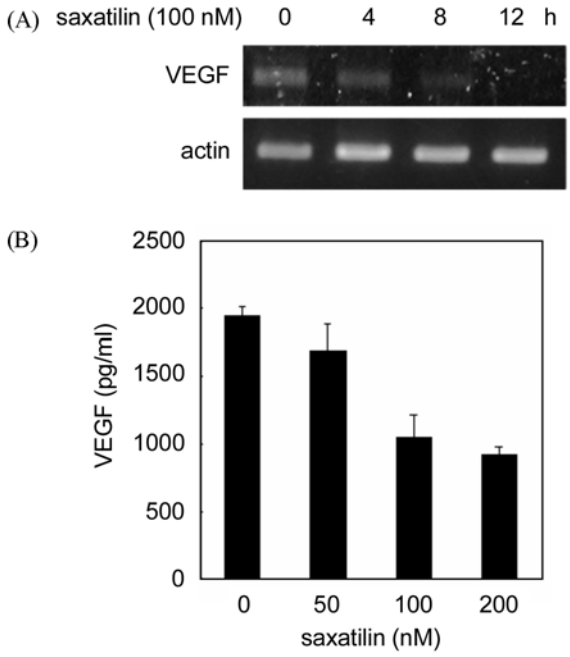

Fig. 3. Inhibition of VEGF expression in NCI-H460 cells by saxatilin. (A) NCI-H460 cells were treated with 100 nM saxatilin for the indicated times at 37°C. RT-PCR analyses for both VEGF and actin were performed. (B) ELISA was performed to quantitate VEGF levels in culture supernatants of NCI-H460 cells treated with the indicated concentrations of saxatilin for 24 h. The experiment was repeated four times. Results are expressed as the mean ± SD.

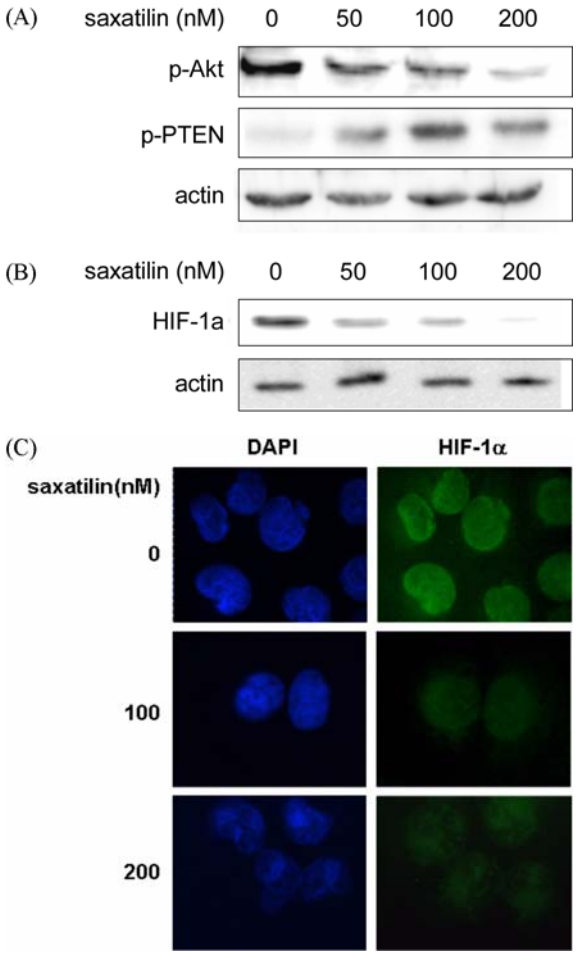

Fig. 4. Inhibition of HIF-1α expression in NCI-H460 cells by saxatilin. (A) NCI-H460 cells were treated with the indicated concentrations of saxatilin for 4 h at 37°C and lysates were subjected to immunoblot analysis with antibodies to phosphor-Akt, phosphor-PTEN, or actin as indicated. (B) NCI-H460 cells were treated with the indicated concentrations of saxatilin for 12 h at 37°C and lysates were subjected to immunoblot analysis with antibodies to either HIF-1α or actin as indicated. Representative data of three separate experiments are shown. (C) NCI-H460 cells were treated with the indicated concentrations of saxatilin for 12 h at 37°C and stained with rabbit antibody against HIF-1α and FITC-conjugated secondary antibody.

at the same concentration (Fig. 4A). In addition, we investigated the nuclear localization as well as the cellular levels of HIF-1α. As shown in Fig. 4B and C, HIF-1α expression was decreased in saxatilin-treated NCI-H460 cells. Saxatilin treatment induced changes in the levels of both VEGF and HIF-1α, consistent with the overall reduction in the angiogenic properties of the cells. Taken together with the findings that the ILK/Akt pathway regulates HIF-1α expression (Tan *et al*., 2004) and that HIF-1α stimulates the transcriptional activity of VEGF (Berger *et al*., 2003), our data suggest that saxatilin inhibits VEGF expression in NCI-H460 cells by transcriptional repression through the ILK/Akt pathway.

In summary, we demonstrate that VEGF expression in lung cancer cells is significantly inhibited by saxatilin in both a dose- and time-dependent manner. Additionally, HUVEC migration and tube formation induced by lung cancer cell culture supernatants were decreased when the cancer cells were treated with saxatilin. These results indicate that saxatilin is a potent inhibitory agent of lung cancer-induced angiogenesis.

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