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Short communication

Saxatilin, a Snake Venom Disintegrin, Suppresses TNF-α-induced Ovarian Cancer Cell Invasion

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Saxatilin is a disintegrin known to inhibit tumor progression *in vivo* and *in vitro*. The role of saxatilin in cancer cell invasion was examined by a modified Boyden chamber assay in MDAH 2774 human ovarian cancer cell line. Saxatilin (50 nM) significantly inhibited cancer cell invasion induced by tumor necrosis factor-α (TNF-α). Saxatilin also reduced MMP-9 mRNA levels in cancer cells in a dose-dependent manner. In addition, TNF-α-induced MMP-9 activity was reduced by the treatment of saxatilin. These results indicate that transcriptional regulation of MMP-9 is an important mechanism for the tumor suppressive effects of saxatilin in MDAH 2774 human ovarian cancer cells.

Keywords: Disintegrin, Invasion, MMP-9, Ovarian cancer, TNF-α

Introduction

Ovarian cancer is thought to metastasize into the peritoneal cavity and other organs at an early stage of the disease (Ellerbroek *et al*., 1999). During metastasis, reorganization of the extracellular matrix (ECM) and angiogenesis are key steps for migration and invasion of cancer cells (Davidson *et al*., 2003). ECM degradation is mainly regulated by gelatinases such as matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), which also have a critical role in angiogenesis and cancer proliferation in ovarian carcinomas (Roomi *et al*., 2006).

MMPs are a large family of proteins regulating cell survival, matrix remodeling, angiogenesis, and cell signaling (Sternlicht *et al*., 2001). MMP-9 (also known as gelatinase B) is an 89 kDa protein which is important for tumor metastasis, and has roles in the immune system and central nervous system (Opdenakker *et al*., 2001). Elevated MMP-9 expression is often correlated with malignancy of ovarian epithelial cancers whose progression is dependent on migration through the surrounding ECM (Davidson *et al*., 1999; Rauvala *et al*., 2005). MMP-9 is regulated by several mechanisms: transcriptional regulation, post-translational activation, and regulation by tissue inhibitors of metalloproteinase (TIMP) (Bjorklund *et al*., 2005). Appropriate manipulation of MMP-9 activity has been suggested as a cancer treatment preventing tumor metastasis and angiogenesis (Troussard *et al*., 2000).

Integrins are heterodimeric receptors critical in both cell-cell interaction and cell adhesion to ECM, and regulate cell survival, growth, migration, and invasion (Hood *et al*., 2002). Integrins form over 25 combinations of 18α and 8β subunits that determine ligand specificity (Bjorklund *et al*., 2005). When integrins bind their ligands, they initiate signal transduction into the cell in response to the extracellular environment. They also take part in signal transduction from in to out by modulating their affinity to ligands such as fibrinogen (Ginsberg *et al*., 2005). Integrins are known to regulate the activity of MMPs during tumor progression by modulating gene expression, activation, and localization (Bjorklund *et al*., 2005). Thus, disintegrin, as an integrin antagonist, is considered to be a potent tumor suppressing agent, along with integrin blocking antibodies and peptide.

Saxatilin is a monomeric disintegrin containing the tripeptide sequence Arg-Gly-Asp, a typical binding motif of adhesive proteins such as glycoprotein IIb-IIIa of platelets and $\alpha_v\beta_3$ integrin of endothelial cells (Hong *et al*., 2002). Besides inhibiting platelet aggregation, saxatilin is known to inhibit angiogenesis and melanoma pulmonary metastasis in a mouse model (Kim *et al*., 2006). We have previously reported that a very low concentration of saxatlin (50 nM) suppresses interleukin-8 (IL-8) gene expression at the transcriptional level (Kim *et al*., 2007). We have postulated that the anti-angiogenic and anti-metastatic activity of saxatilin might be due to the modulation of genes associated with migration and invasion of tumor cells. In this report, we demonstrate that

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saxatilin suppresses MMP-9 gene expression, reducing cell invasion in MDAH 2774 human ovarian cancer cells.

Materials and Methods

Cell culture and reagents. The human ovarian cancer cell line MDAH 2774 was obtained from American Type Culture Collection (ATCC) and grown in DMEM medium with 10% FBS, 25 mM HEPES, 100 U/ml of penicillin, and 100 mg/ml of streptomycin. All culture reagents were purchased from Life Technologies.

RNA isolation and Reverse-transcription PCR analysis. After cells were treated with the indicated concentrations of saxatilin for 4 h, total RNA was extracted with a MicroRNA Isolation Kit (Stratagene) as previously described (Choi *et al.*, 2006). Total RNA was reverse-transcribed in the first-strand synthesis buffer containing oligo(dT) primer, StrataScript^{TM} reverse transcriptase, dNTPs, and RNase inhibitor at 42°C for 1 h. The cDNA was subjected to a standard PCR reaction for 25 cycles using the following primer sets: Actin forward 5'-GGG TCA GAA GGA TTC CTA TG-3', reverse 5'-CCT TAA TGT CAC GCA CGA TTT-3'; MMP-9 forward 5'-CAC TGT CCA CCC CTC AGA GC-3', reverse 5'-GCC ACT TGT CGG CGA TAA GG-3'; bFGF forward 5'-CCC GAC GGC CGA GTT GAC-3', reverse 5'-CAC ATT TAG AAG CCA GTA ATC T-3'. PCR products were resolved on a 1% agarose gel and visualized with UV light after staining with ethidium bromide.

Gelatin zymographic assay. MDAH 2774 cells were incubated with TNF-α (10 ng/ml), VEGF (10 ng/ml), and EGF (10 ng/ml) for 24 h under serum-free conditions in the presence or absence of various concentrations of saxatilin. The cell culture medium was analyzed for proteins with gelatinolytic activity by identification of substrate lysis in 7.5% SDS-polyacrylamide gels containing 2 mg/ml gelatin. Gels were washed with 2.5% Triton X-100 for 1 h and incubated for 16 h at 37°C in 50 mM Tris-HCl, pH 7.5 containing 10 mM $CaCl_2$. Gels were stained with Coomassie Brilliant Blue R-250 (0.2%) in 40% methanol and 10% acetic acid.

MMP activity assay. The gelatinolytic proteinase activity of MMPs in cultured supernatants was determined by enzyme-linked immunosorbent assay (ELISA) after incubating 1×10^6 cells in 1 ml of serum-free medium containing TNF-α (10 ng/ml) in the presence or absence of saxatilin (50 nM) for 24 h. The supernatant was analyzed to estimate net gelatinase activity using MMP Collagenase Activity Assay Kit (CHEMICON) according to the manufacturer's instructions.

Cancer cell invasion assay. The invasion assay was carried out as previously described (Hong *et al.*, 2002) with some modifications. Briefly, polycarbonate filters were coated with 10 mg of Matrigel and placed in a transwell chamber. Serum-free medium was placed in the lower compartment of the chamber with or without 10% fetal bovine serum (FBS). Cell suspensions (3×10^5) in DMEM medium without FBS were loaded into the upper compartment of the chamber in the presence of saxatilin (50 nM) or TNF-α (10 ng/ml). After incubation for 24 h at 37°C, the filters were fixed with methanol and the cells invading through the basement membrane were counted.

Results and Discussion

Neoplastic cells tend to induce endothelial cells to migrate and invade surrounding tissues during tumor metastasis. During metastasis, cancer cells undergo several processes such as proliferation, matrix remodeling, invasion, migration, and differentiation. Integrins are known to play a critical role in these physiological events by regulating cell/cell interactions and cell/ECM adhesion both of which determine the invasiveness of cancer cells (Hood *et al.*, 2002).

There are several reports that integrin signaling is associated with MMP expression or cell migration. Fibronectin induces MMP-9 expression through AP-1 activation in human lung carcinoma cells (Han *et al.*, 2006). Moreover, contortrostatin, a homodimeric disintgrin containing RGD sequences, mimics the intracellular signaling evoked by fibronectin (Schmitmeier *et al.*, 2005). Alternagin-C, a disintegrin-like protein from snake venom, even induces neutrophil chemotaxis with a potency similar to the classic neutrophil chemoattractant N-formyl-methionyl-leucyl-phenylalanine (Mariano-Oliveira *et al.*, 2003). The effects of these disintegrins are distinguishable from traditional disintegrins (such as echistatin), which need micromolar concentrations to exert their physiological effects on endothelial cells (Schmitmeier *et al.*, 2005).

Saxatilin, a disintegrin derived from snake venom, was introduced as an inhibitor of platelet aggregation by antagonizing integrin $\alpha_{IIb}\beta_3$ on platelets and integrin $\alpha_v\beta_3$ on the surface of endothelial cells (Hong *et al.*, 2002). Furthermore, it is reported that saxatilin inhibits tumor metastasis in a mouse model (Kim *et al.*, 2006). Recently, we reported that low concentrations of saxatilin inhibits ovarian cancer cell proliferation by suppressing IL-8 expression, implying that saxatilin can directly influence the expression of genes associated with tumor progression. In this study, saxatilin is shown to have a strong effect on MMP-9 expression and invasiveness in a human ovarian cancer cell line.

A matrigel invasion assay was employed to investigate the role of saxatilin in the invasiveness of the MDAH 2774 ovarian carcinoma. Saxtatlin or TNF-α was present in the upper chamber while FBS was used as chemoattractant in the lower chamber. TNF-α, a regulator of many angiogenic genes including MMP-9 (Thommesen *et al.*, 2005; Cordiali-Fei *et al.*, 2006), induced cell invasion as shown in Fig. 1. This cell invasion was reduced more than 80% in the presence of 50 nM saxatilin. One possibility is that saxatilin inhibited the ovarian cancer cell invasion by regulating integrin-mediated signaling. However, disintegrins are also known to reduce cell migration by physically blocking integrin. We note that half-maximal inhibition of vitronectin-induced smooth muscle cell migration is obtained with 2.6 mM of saxatilin (Hong *et al.*,

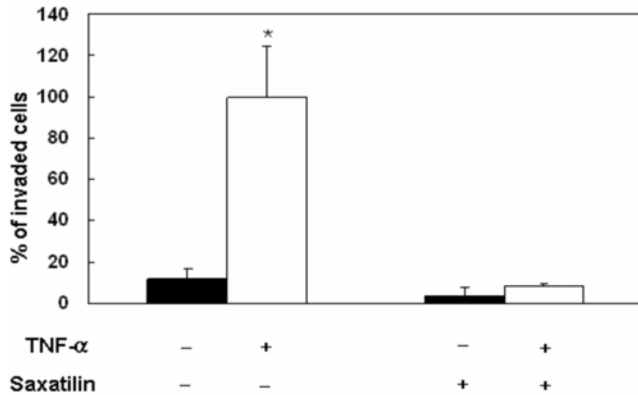

Fig. 1. The effect of saxatilin on ovarian cancer cell invasion. MDAH 2774 human ovarian cancer cell invasion was induced by treatment with TNF-α (10 ng). Cell invasion was suppressed by 50 nM saxatilin. The inhibition assay was performed in a transwell plate as described in Materials and Methods. *$p < 0.01$ compared with saxatilin treated group.

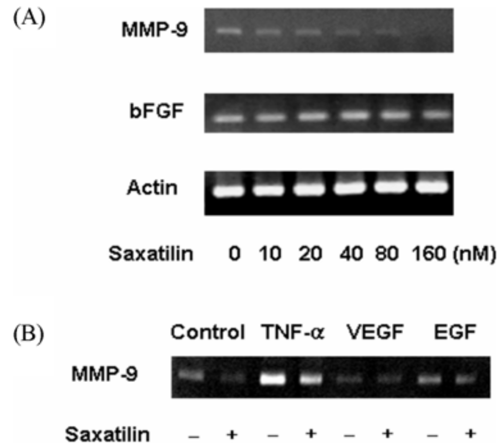

Fig. 2. MMP-9 expression in ovarian cancer cells treated with saxatilin. (A) MDAH 2774 cells were treated with the indicated concentrations of saxatilin for 4 h. The levels of MMP-9 and bFGF mRNA were analyzed by RT-PCR. The level of human actin mRNA was used as a control. (B) Cells were treated with 10 ng of TNF-α, VEGF, and EGF for 24 h in the presence or absence of saxatilin (50 nM). The culture media was then subjected to gelatin zymography.

2002), a concentration over 50 times higher was used in this study. Though the experimental conditions and cells were different, it is reasonable to think that the effect of direct blocking of integrin by saxatilin on the cancer cell invasion is negligible. At nanomolar concentrations, disintegrins like saxatilin show effects distinct from those traditionally observed like integrin blocking. Alternagin-C, jarastatin, and EC3 all can induce neutrophil migration by activating signaling cascades associated with integrins (Mariano-Oliveira *et al*., 2003; Coelho *et al*., 2004). Alternagin-C and EC3 do not contain an RGD motif whereas jarastatin does. Alternagin-C and jarastatin exist as monomers, while EC-3 is a heterodimer. Despite remarkable contrasts in sequence and structure, all three induce integrin signaling which modulates cell survival, cell motility and gene expression in similar ways. Thus, it is thought that saxatilin can play diverse roles in cell movement in various cell types and under a variety of circumstances via integrin-associated signaling and regulation of gene expression.

To identify the role of saxatilin in suppressing cancer progression at the transcriptional level, RT-PCR was performed on MDAH 2774 human ovarian cancer cells after saxatilin treatment. The levels of MMP-9 mRNA decreased after saxatilin treatment in a dose dependent manner, while that of bFGF or actin were unchanged (Fig. 2A). VEGF and EGF are known to be abundant in serum of ovarian cancer patients (Baron *et al*., 1999; Chen *et al*., 1999; Nash *et al*., 1999). When cells were treated with TNF-α, gelatin zymography showed that MMP-9 activity in the cell culture supernatant increased markedly. Cells treated with VEGF or EGF showed little change. However, MMP-9 activities in the cell culture supernatant of cells treated with saxatilin were significantly decreased under all conditions (Fig. 2B).

Next, we examined the effect of saxatilin on MMP-9 expression induced by TNF-α in ovarian cencer cells. MMP-9 activity of the cell culture medium was analyzed in gelatin

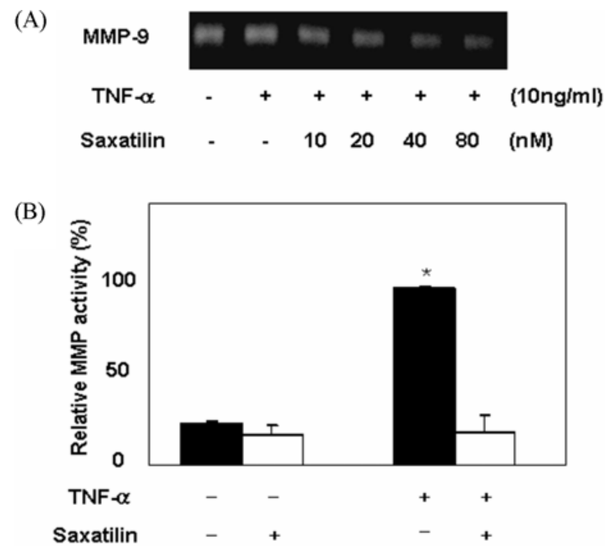

Fig. 3. TNF-α-induced MMP-9 activity was decreased by saxatilin treatment. (A) Ovarian cancer cells were treated with 10 ng TNF-α and saxatilin at the indicated concentrations in serum free medium for 24 h. The culture media was then subjected to zymography. (B) Cells were incubated with 10 ng TNF-α for 24 h in the presence or absence of saxatilin (50 nM). The culture supernatants were analyzed by MMP Collagenase Activity Assay Kit (CHEMICON). Each bar indicates the relative MMP activity in the cultured medium. *$p < 0.01$ compared with saxatilin treated group.

zymography after the cells were treated with TNF-α for 24 h with or without saxatilin pretreatment for 2 h. TNF-α-induced MMP-9 activities were suppressed by saxatilin treatment in a dose-dependent manner in the zymogram (Fig. 3A). The

catalytic activity of MMPs in the culture medium, including MMP-2 and MMP-9, was analyzed by ELISA. In accordance with the above data, gelatinolytic MMP activities were also increased by TNF-α treatment whereas the activity was greatly decreased in the presence of saxatilin (Fig. 3B). Taken together, it is apparent that TNF-α-induced MMP-9 expression was inhibited by saxatilin treatment. However, it is interesting to note that the MMP activity was more dramatically decreased in the activity assay than in the gelatin zymography (compare Fig. 2B and Fig. 3B). Therefore, there may be another inhibitory mechanism of MMP activity besides the inhibition of MMP-9 expression. MMPs are important in diverse pathologies, including tumor progression (Bjorklund *et al*., 2005), thus discovering the mechanism of MMP inhibition by saxatilin could be helpful in treating various diseases.

In conclusion, we have shown that a low concentration of saxatilin decreased cell invasion through the regulation of MMP-9 activity in a human ovarian cancer cell line. These data suggest that saxatilin inhibits tumor progression in a novel manner, the molecular mechanism of which remains to be elucidated.

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