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Disulfiram Suppresses Invasive Ability of Osteosarcoma Cells Via the Inhibition of MMP-2 and MMP-9 Expression

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Cancer cells, characterized by local invasion and distant metastasis, are very much dependant on the extracellular matrix. The expression of matrix metalloproteinases (MMPs) has been implicated in the invasion and metastasis of cancer cells. In this study, we reported the effects of disulfiram, a clinically used anti-alcoholism drug, on tumor invasion suppression, as well as its effects on the activity of MMP-2 and MMP-9 in human osteosarcoma cells (U2OS). Disulfiram has been used for alcohol aversion therapy. However, recent reports have shown that disulfiram may have potential in the treatment of human cancers. Herewith, we showed that the anti-tumor effects of disulfiram, in an invasion assay using U2OS cells and that disulfiram has a type IV collagenase inhibitory activity that inhibits expression of genes and proteins responsible for both cell and non-cell mediated invasion on pathways. In conclusion, disulfiram inhibited expression of MMP-2 and MMP-9 and it regulated the invasion of human osteosarcoma cells. These observations raise the possibility of disulfiram being used clinical for the inhibition of cancer invasion.

Keywords: Disulfiram, Invasion, MMP-2, MMP-9, U2OS

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Introduction

Osteosarcoma is the most common primary malignant tumor of the bone and is known to metastasize. Changes in the cell-extracellular matrix (ECM) interactions, dissociation of intercellular adhesion complexes and degradation of ECM are needed for metastasis of osteosarcoma to occur (Yoon *et al*., 2001; Stewart *et al*., 2004). Degradation and dissociation of the ECM is a special feature of tumor invasion, metastasis and angiogenesis in osteosarcoma.

The degradation of ECM has been linked to MMPs. MMPs are known to be closely associated with tumor growth and metastasis as well (Matsunaga *et al*., 2004). MMPs are a family of zinc endopeptidases consisting of at least 20 different members (Soini *et al*., 2001; Yasuhito *et al*., 2002) and they play an important role in various cellular metabolic processes. They induce a variety of biological effects including growth, morphogenesis, apoptosis, tissue destruction, and cancer formation (Lee *et al*., 2005; Soreide *et al*., 2006). Among the human MMPs reported previously, two gelatinases, gelatinase A (72-kDa type VI collagenase, MMP-2) and gelatinase B (92-kDa type VI collagenase, MMP-9) can degrade type IV collagen and fibronectin, and are closely related to the invasion and metastasis of tumor cells (Liotta *et al*., 1991; Masahiro *et al*., 2002; Tanimura *et al*., 2005).

MMP-2 and MMP-9 expression are known to be regulated by transcription factors, AP-1, NF-κB and Sp-1 (Woo *et al*., 2004). It was recently found that AP-1 regulates downstream targets such as the matrix metalloproteinase-9 promoter in human renal carcinoma cells (Hong *et al*., 2005; Cho *et al*., 2007). Although MMPs are associated with invasion and

metastasis, the mechanisms of MMP-2 and MMP-9 expression in tumor cells remain largely unknown.

Disulfiram (DSF) has been used for over half a century for alcohol aversion therapy (Shen *et al*., 2000). Disulfiram is a chelator of heavy metals and an inhibitor of aldehyde dehydrogenase (Marikovsky *et al*., 2003). Chelation is the process of reversible binding of a ligand to a meal ion, forming a metal-chelator complex. Chelators are used in chemical analysis and are also employed to safely bind poisonous metal agents such as mercury, arsenic or lead, to stabilize them and allow them to be excreted without further interaction within the body. Commonly used synthetic chelators include iron-chelating agents such as EDTA (Li *et al*., 2007), D-penicillamine (DPA) and 2,3 dimercaptosuccinic acid (DMSA) (Dhawan *et al*., 1989). Recent reports indicate that disulfiram and other dithiocarbamates may have a significant potential in the treatment of human cancers (Chen *et al*., 2006). Disulfiram has also been shown to inhibit the activation of the transcription factor/cyclic-AMP-responsive element binding protein, which is implicated in the growth and progression of melanomas (Brar *et al*., 2004). These preceding results reveal that disulfiram may have an important role as an adjuvant in the chemotherapy of human cancers. In this paper, we report the effects of disulfiram on type IV collagenase, MMP-2 and MMP-9 in human osteosarcoma cells and its suppression-related mechanism. These results provide evidence that the invasion phenotype of tumorsis correlated with MMP-2 and MMP-9 activity, suggesting that inhibition of MMP-2 and MMP-9 activity via the chelating agent disulfiram may serve as a way to inhibit human osteosarcoma cell invasion.

Materials and methods

Cell culture and conditioned medium. Cell lines used in the study were maintained in humidity in a 5% CO_2 incubator. U2OS (human osteosarcoma), Caki-1 (renal carcinoma), Caski (cervical carcinoma cell), MDA-MB-231 (human breast cancer) cells were obtained from the American Type Culture Collection. Cells were cultured in DMEM-High glucose (Dulbecco's Modified Eagle's medium) (Gibco-BRL) medium containing 10% fetal bovine serum (Hyclone Laboratories) and 1% ampicillin (Gibco). After a 24 h attachment, the medium was replaced with serum-free medium and various dose of disulfiram (SIGMA) and PMA (phorbol 12-myristate 13-acetat) (SIGMA) were added.

Soft agar colony formation assay. Effects of disulfiram on the soft agar colony formation of U2OS cells were investigated. Single-cell suspensions of U2OS cells were treated with or without different concentrations of disulfiram, and then mixed with agarose in a final concentration of 0.35%. Aliquots of 1.5 ml containing 10^4 cells and 10% FBS were plated in triplicate in 60 mm culture dishes over a base layer of 0.7% agarose and allowed to solidify. Following 14 days of incubation, the colonies were stained with 0.005% crystal violet and those which were >60 μm were counted using a dissecting microscope.

Cell viability assay. Reduction of 3-[4,5-dimethlyhiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Roche Applied Science) assays were performed as described in the supplier's protocol to evaluate the cytotoxicity of disulfiram.

Cell invasion assay. For invasion assay, 5×10^4 cells were seeded in the upper well of each transwell chamber. Before invasion assay, the upper parts of the transwell (Corning Costar) were coated with 30 μl of a 1 : 2 mixture of matrigel : phosphate-buffered saline. Cells were plated on the matrigel-coated transwells in the presence of various concentrations of disulfiram. Culture media was placed in the lower compartment of the cemotaxis chamber as a source of chemoattractants. The inserts were incubated for 24 h at 37°C. Cells that had invaded the lower surface of the membrane were fixed with methanol and stained with hematoxylin and eosin. Using light microscopy, random fields were selected and cells in each field were counted under high power (×200).

Zymographic assay for metalloproteinase. Zymography was used to determine the effect of disulfiram on PMA-induced MMP-9 activity after the cells were treated with disulfiram in the presence of 75 nM PMA. Cell were suspended in medium containing 10% fetal bovine serum and plated at 3×10^5 cells/60 mm dish. The dishes were incubated until the cultures were 80% confluent. The medium was then changed to fresh serum-free medium with or without disulfiram. Supernatant was collected after incubation for 24 h. The cellected cell culture medium was mixed with 4×SDS sample buffer (without β-mercaptoethanol) for electrophoresis. Sample was loaded onto the SDS-PAGE in 10% polyacrylamide gel containing 0.1% gelatin and electrophoresed. After electrophoresis, the gels were washed thrice in 2.5% triton X-100 for 10 min at room temperature, then incubated for 24 h at 37°C in incubation buffer containing 5 mM $CaCl_2$ and 1 μM $ZnCl_2$. Gels were stained with 0.25% Coomassie Brilliant Blue R250 for 1 h and then de-stained for 30 min~1 h at room temperature. Proteolytic activity was visible in the form of clear bands against the blue background of the stained gelatin.

Western Bolt Analysis. Cellular lysates were prepared by suspending 3×10^5 cells/60 mm plates in lysis buffer. The cells were disrupted and extracted at 4°C for 1 h. After 1 h, they were centrifuged and the supernatant containing proteins were collected into new e-tube. Each lane was loaded with samples on 10% SDS-PAGE gel, separated and electro-transferred to immobilon-p membranes (Millipore Co.). Detection of specific proteins was carried out by enhanced chemiluminescence following the manufacture's instructions (Amersham Biosciences). MMP-9 and MMP-2 rabbit polyclonal antibodies were purchased from Chemicon (Chemicon International).

Isolation of RNA and RT-PCR. Total cellular RNA was isolated using a RNA ZolBee (Life Technology). For reverse transcription reaction, cDNA was synthesized from total RNA using moloney murin leukemia virus reverse transcriptase (Promega). PCR primers sequences used were as follows: MMP-2 sense 5'-GGCCCTGTCA CTCCTGAGAT-3' and antisense 5'-GGCATCCAGGTTATCGGG GA-3'; MMP-9 sense 5'-CGGAGCACGGAGACGGGTAT-3' and antisense 5'-TGAAGGGGAAGACGCACAGC-3'. PCR products were resolved electrophoretically on a 1.0% (w/v) agarose gel, and

DNA bands were visualized by staining with ethidium bromide. The expression of measured genes in each sample was normalized to β-actin expression. All samples were analyzed in triplicate.

Luciferase reporter gene assay. MMP-2 wild type (pGL3-MMP-2WT-luc) luciferase promoter constructs and MMP-9 wild type (pGL3-MMP-9WT-luc) luciferase promoter constructs were used in transient transfection assays. U2OS cells were plated at a density of 3×10^5 cells/60 mm dish and grown to 60% confluence in 2 ml of growth medium. The cells were transiently transfected with 5 μg of various plasmid constructs for 6 h using lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were cultured in serum-free medium with 20 μM disulfiram for 24 h. The cells were then washed twice with cold PBS and lysed with reporter lysis buffer (Promega). After centrifugation, cell extract and luciferase assay reagent were mixed at room temperature and placed in a luminometer (Luminometer, Microlumat Plus, Berthold Technologies, Dortmund, Germany) to detect the light produced.

Statistical analysis. All values were expressed as mean ± S.E. Each value is the mean of at least three repetitive experiments in each group. T-test was used to compare the effect of the compounds and their difference from the vehicle-treated control was analyzed for significance.

Results

Disulfiram suppresses MMP-2 and MMP-9 activity. The gelatin zymography assay showed that the secretion of MMP-9 in the conditioned medium of U2OS, Caski and Caki-1 cells were dramatically induced by PMA, and activity of MMP-2 and MMP-9 was decreased by DSF (Fig. 1). As shown in Fig. 1, treatment of U2OS with disulfiram, at doses above 20 μM, strongly suppressed PMA-induced MMP-9 and MMP-2 activity compared to caki-1 and caski cells in a dose-dependent manner.

These results indicate that disulfiram inhibits MMP-2 and MMP-9 activity in U2OS more than the other cell lines tested.

Effect of disulfiram on growth and proliferation of human osteosarcoma cells. It has been reported that the ability of cells to form colonies in a semi-solid medium is generally considered a marker of anchorage independence and is positively correlated with metastatic potential. The soft agar colony formation assay showed that disulfiram significantly reduced the soft agar colony forming capacity of osteosarcoma cells. The colonies that appeared were scanty and were small in size (Fig. 2A-b and B). The results also revealed a dose-dependent effect and at 20 μM, disulfiram inhibited colony formation by 95% (Fig. 2A-b and B). Disulfiram cytotoxicity as a cause of the above results was eliminated when cell viability testing on U2OS cells treated with 10 μM of disulfiram for 24 h showed that the viability decreased by only 10 to 20% (Fig. 2C).

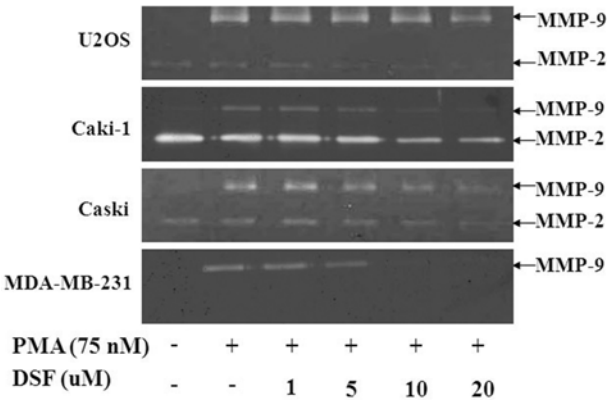

Fig. 1. Effects of disulfiram in various cell lines. Various cell lines were treated with different concentrations of disulfiram in the presence of PMA (75 nM). Conditioned medium was collected from cultures after 24 h and analyzed by gelatin zymography. The data represents the mean ± S.E. of at least three independent experiments. Results were deemed statistically significant using Student's *t*-test.

Inhibitory effect of disulfiram on the invasion of human osteosarcoma cells in an *in vitro* assay. Transwell assay was performed to further evaluate the anti-invasion activity of disulfiram. As shown in Fig. 2D, disulfiram inhibited PMA-dependent invasion in a dose-dependent manner, with in the 10~20 μM disulfiram treated cells reaching the levels similar to the control cells. Our data suggested that disulfiram counteracts the effects of PMA that induces the invasive activity in human osteosarcoma cells.

Effects of disulfiram on the MMP-2 and MMP-9 activities of human osteosarcoma cells. U2OS cells, which release MMP-2 and MMP-9 when cultured in serum-free medium, were treated with various concentrations of PMA for 24 h. The level of MMP-2 expression was not significantly altered by PMA (75 nM), but the expression of MMP-9 was induced, as evidenced by gelatin zymography (Fig. 3 and data not shown). From 1 to 20 μM, disulfiram caused a dose-dependent decreases in PMA-induced MMP-9 activity as well as the MMP-2 activity (Fig. 3). These findings demonstrated that disulfiram inhibits type VI collagenase activity, especially for MMP-2 and MMP-9. Therefore, this inhibition maybe mediated largely through direct drug-protein interaction rather than by the cell-mediated pathway.

Effects of disulfiram on the MMP-2 and MMP-9 protein expression of human osteosarcoma cells. The levels of MMP-2 and MMP-9 proteins in the cells were analyzed in order to explore whether the decreased enzyme activity was due to a diminished amount of MMP-2 and MMP-9 proteins. Our data revealed that the amount of MMP-2 and MMP-9 proteins were decreased in the cells treated with disulfiram (Fig. 4A and B). Similar to the zymographic data, it revealed that the amount of MMP-9 protein decreased in a dose-

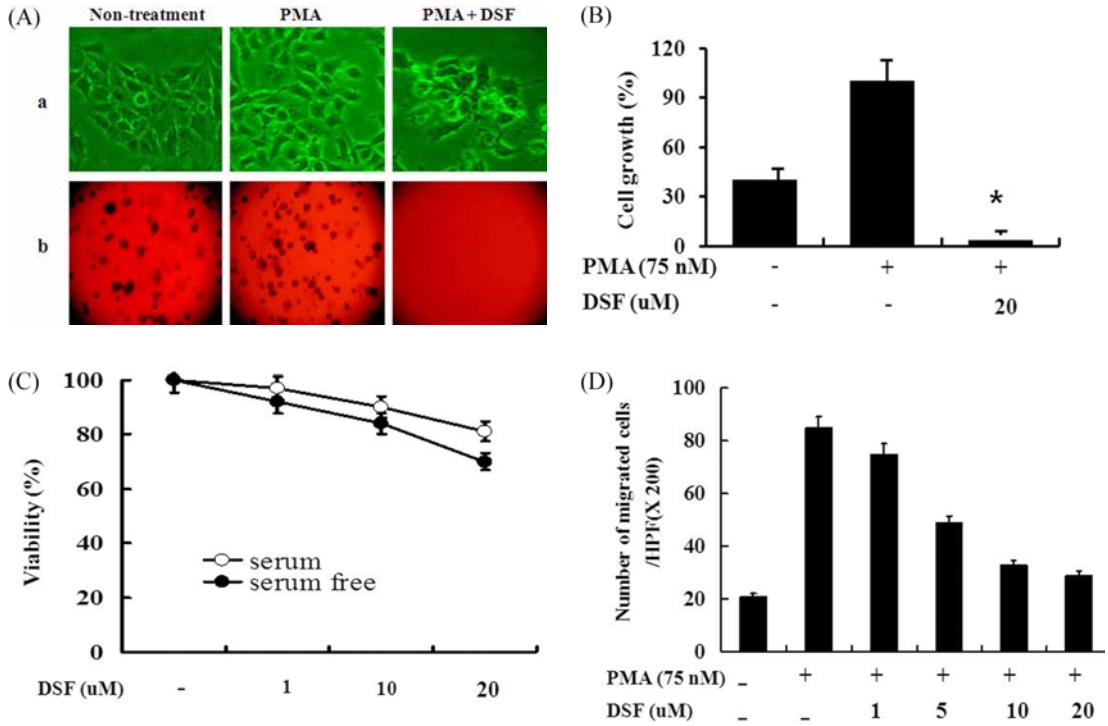

Fig. 2. Effects of disulfiram in U2OS cells. Effects of disulfiram on morphology (A-a), soft agar colony formation assay (A-b) and quantification by colony formation (B) in U2OS cells. (A-a) U2OS cells were treated with PMA (75 nM) and disulfiram (20 uM). Cell is detected under light microscopy. (A-b) U2OS cells were treated with disulfiram in 0.35% agarose containing 10% FBS over 0.7% agarose containing 10% FBS. Cell colonies were counted after 15-days under light microscopy. C. Cytotoxicity of U2OS cells treated with different concentrations of disulfiram. U2OS cell viability was tested by MTT assay after 24 h. D. Effects of disulfiram *in vitro* invasion of U2OS cells. For invasion assays, the upper parts of trans-wells were coated with Matrigel, and U2OS cells with various concentrations of disulfiram and 75 nM of PMA were added. After 24 h, cells on the bottom side of the filter were fixed, stained, and counted. Data represent the mean ± S.E. of at least three independent experiments. Results were deemed statistically significant using Student's *t*-test.

dependant manner as compared to that of MMP-2 protein. The reduced MMP-9 activity was also due to the smaller quantity of proteins produced. Our results suggest that disulfiram inhibits the protein expression and activity of MMP-2 and MMP-9.

Suppression of MMP-2 and MMP-9 expression by disulfiram. RT-PCR and reporter gene analysis were done to determine whether the inhibition of MMP-2 and MMP-9 expression by disulfiram was due to a decreased level of transcription. The treatment of U2OS cells with disulfiram showed a decrease in the levels of PMA-induced MMP-9 and MMP-2 mRNA, whereas TIMP (-1 and TIMP-2 remained largely unchanged (Fig. 4C and D). To test which of these transcription factors may regulate the MMP-2 and MMP-9 genes in U2OS cells, cells were transiently transfected with reporter genes that included the wild type (WT) MMP-2 promoter and WT MMP-9 promoter. Luciferase activity in the cells with the WT MMP-2 and WT MMP-9 construct was significantly reduced when treated with disulfiram (Fig. 5). These results indicate that disulfiram inhibits the expression of MMP-2 and MMP-9 through regulation of transcription levels.

Therefore, the inhibition of MMP-2 and MMP-9 are regulated through both non-cell-mediated and cell-mediated pathways.

Discussion

The metastatic spread of cancer causes 90% of human cancer related deaths, and as a result, remains one of the greatest barrier to curing cancer (Christofori, 2006). Several proteins are altered in cancer cells that promote their invasive or metastatic capabilities. Among these proteins, tumor-associated matrix metalloproteinases (MMPs) are important components in the metastatic process through their capacity to degrade extracellular matrix proteins. Among the previously reported human MMPs, MMP-2 and MMP-9 are considered to be particularly good targets for anticancer drugs because both enzymes degrade gelatins, which are major components of the basement membrane. The expression of both these enzymes correlated with an aggressive, advanced invasive or metastatic tumor phenotype (Sato and Seiki, 1993; Hong *et al*., 2005).

Disulfiram is an inhibitor of aldehyde dehydrogenase and is

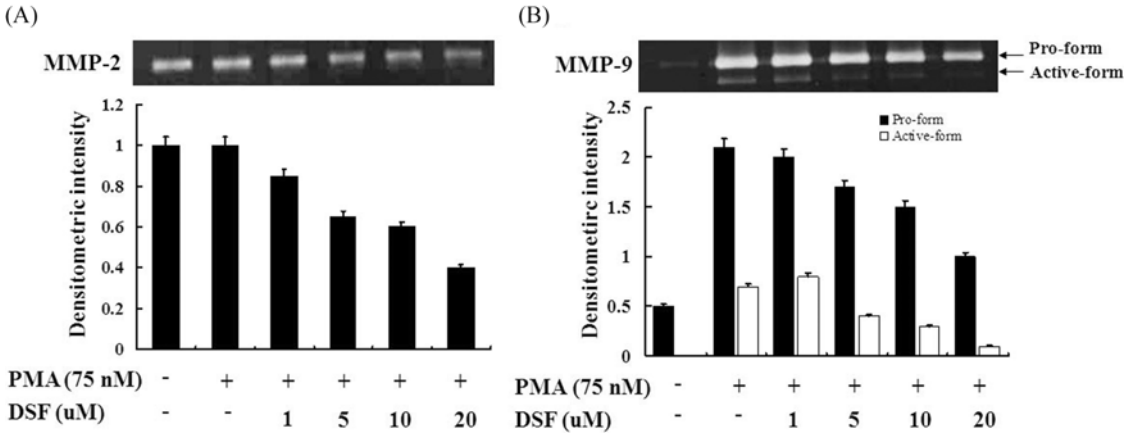

Fig. 3. Effect of disulfiram on MMP-2 activity (A) and PMA-induced MMP-9 activity (B). U2OS cells were treated with various concentrations of disulfiram in the presence of PMA (75 nM). Conditioned medium was collected from cultures after 24 h and analyzed by gelatin zymography. Data represents the mean ± S.E. of at least three independent experiments. Results were deemed statistically significant using Student's *t*-test.

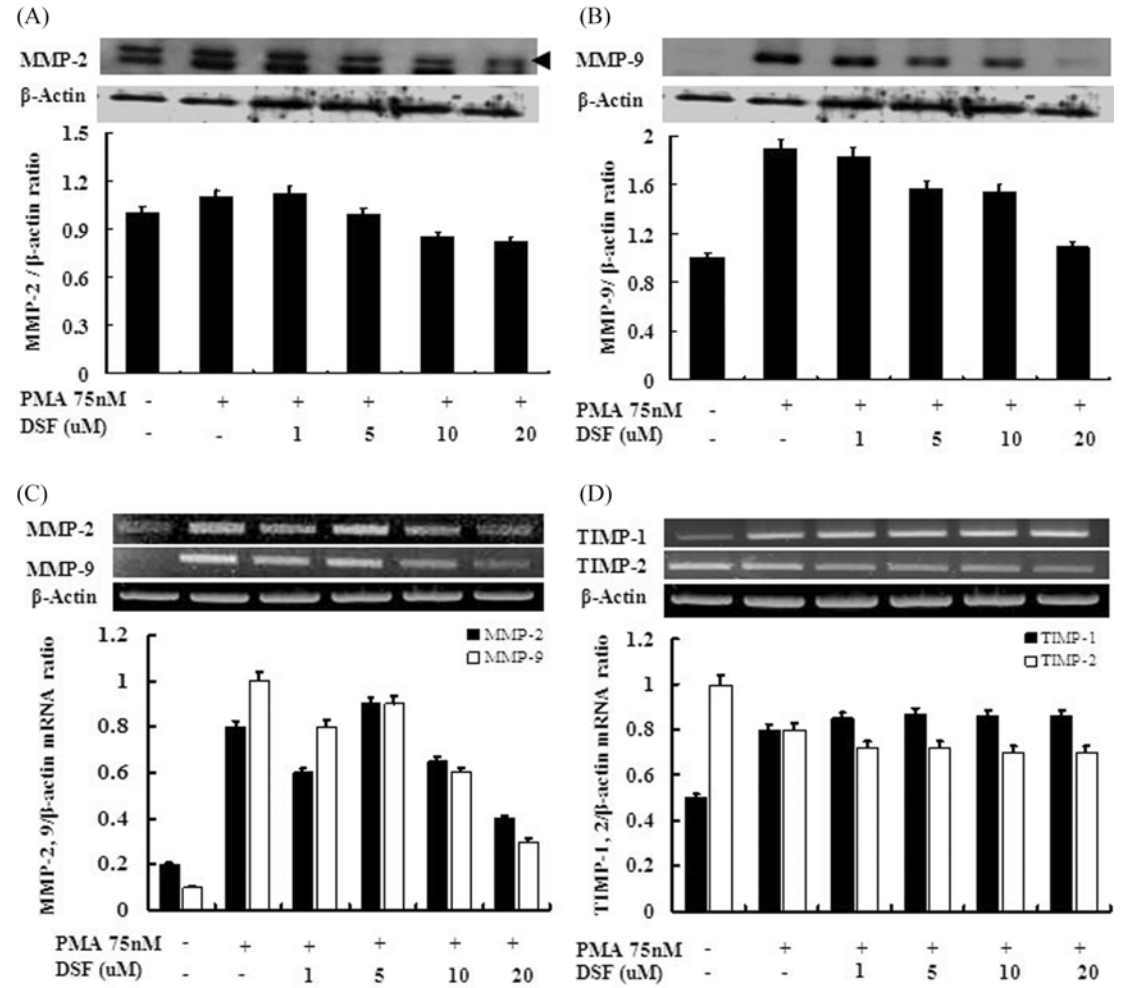

Fig. 4. Effect of disulfiram on MMP-2 and MMP-9 expression and mRNA expression of MMP-2 and MMP-9, TIMP-1 and TIMP-2. A and B, expression of MMP-2 and MMP-9 treated with vehicle and disulfiram in the presence of PMA for 24 h was evaluated by western blot analysis using anti-MMP-2 and anti-MMP-9. C and D, using RT-PCR, the levels of MMP-2, MMP-9, TIMP-1 and TIMP-2 mRNA from cells treated as indicated were determined. Data represent the mean ± S.E. of at least three independent experiments. Results were statistically significant using Student's *t*-test.

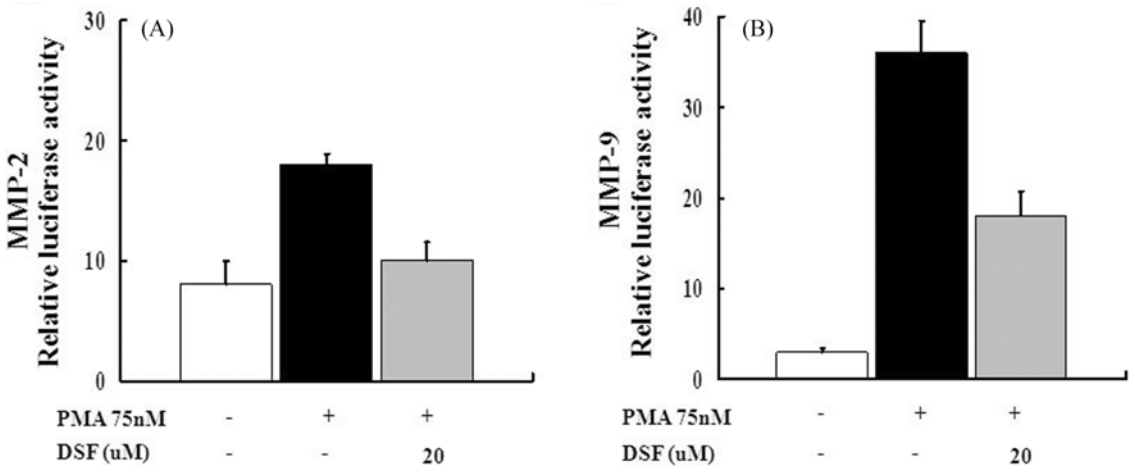

Fig. 5. Effects of disulfiram on the activities that function through MMP-2 (A) and MMP-9 (B) promoter. U2OS cells were transfected with pGL3-MMP-2WT and pGL3-MMP-9WT reporter plasmids, and then cultured with PMA (75 nM) and disulfiram (20 μM). U2OS cells transfected with the indicated reporters were cultured with disulfiram (20 μM) and/or PMA (75 nM) for 24 h, and luciferase activity in cell extracts was then determined. Values represent the mean of at least three independent experiments. Data represent the mean ± S.E. of at least three independent experiments. Results were deemed statistically significant using Student's *t*-test.

used clinically in the treatment of alcoholism (Lipsky *et al*., 2001). Disulfiram has zinc chelating capability and is a zinc ejector (Nash *et al*., 1998). It has been reported to possess antiretroviral activity and can eject intrinsic zinc out of human immunodeficiency virus (HIV) nucleocapsid protein, induce apoptosis, show metal ion-dependent antineoplastic activity and arrests angiogenesis (McDonnel *et al*., 1997). Disulfiram has also been shown to directly affect at least three of above traits-inducing apoptosis, acting as an anti-angiogenesis agent and preventing tissue invasiveness and metastasis (Sauna *et al*., 2005; Chen *et al*., 2006). However, the mechanism for disulfiram's effects remains unclear, and the use of disulfiram has not yet been reported in the treatment for human osteosarcoma.

In this study, we explored whether disulfiram can modulate cancer invasion and metastasis in cultured human osteosarcoma cells through MMPs by analyzing its effect on MMP-2 and MMP-9. Our results revealed that the non-toxic ranges of disulfiram suppressed MMP-2 and MMP-9 activity and expression, producing an almost complete growth inhibition at 10 μM concentration of disulfiram.

Therefore, the inhibitory effect of disulfiram on tumor invasion may be associated with the degradative cascade of the ECM and basement membrane. However, it is not clear whether disulfiram is able to directly inhibit the activity of the active form of MMP. Nonetheless, these results clearly indicate that the anti-invasive effect of disulfiram is associated with inhibition of the enzymatic degradative processes involved in tumor invasion and disulfiram acts as a potent inhibitor of MMP-2 and MMP-9 expression. These results provide evidence suggesting that inhibition of MMPs activity via the chelating agent disulfiram may serve as a therapy to inhibit tumor cell invasion and metastasis. In addition, disulfiram has been shown to directly inhibit the growth of cancer cells both *in vitro* and *in vivo*, while divalent metal ions have been shown to enhance its antineoplastic activity (Sauna *et al*., 2005). Disulfiram inhibited type IV collagen degradation through both the cell-mediated and non-cell-mediated pathways, suggesting that there are at least two possible mechanisms involved in this inhibition process. Our data strongly suggested that disulfiram could directly interact with MMP-2 and MMP-9 proteins through a non-cell-mediated pathway. Furthermore RT-PCR analysis and reporter gene assays revealed that the reduction of the lytic content of MMP-2 and MMP-9 are due to cell-mediated mechanism as well. Disulfiram has the ability to inhibit the invasion of tumor cells by diminishing the efficiency of gene transcription (Fig. 4). Interestingly, our results indicated that TIMP-1 and TIMP-2 mRNA levels were unchanged (Fig. 4C and D). TIMPs play an important role in regulating the activity of the secreted matrix metalloproteases. In addition to their ability to bind at the active site, TIMP-1 and TIMP-2 can form complexes with specific matrix metallproteinases (Stetler-Stevenson *et al*., 1990). In the present study, the expressions of these inhibitors were not related to the regulation of MMP-2 and MMP-9 expressions. As shown in Fig. 5, disulfiram was found to inhibit luciferase activity in reporter gene assay in a dose-dependent manner. These results clearly indicate that the down-regulation of the MMP-2 and MMP-9 genes expression by disulfiram is associated with inhibition of transcription.

MMPs gene expression is primarily regulated at the transcriptional level through AP-1, NF-kB and so forth via mitogen activated protein kinase pathways (Yao *et al*., 2001). Among the MMPs, the MMP-2 gene expression in cancer cells is dependent on several transcription factors, including Sp1, AP-1, NF-κB and p53 (Ma *et al*., 2004; Qin *et al*., 1999).

It has been previously documented that the MMP-9 promoter has several transcription-factor-binding motifs that can affect its transcription, including NF-κB, AP-1, Sp1 (Sato and Seiki, 1993; Huang *et al*., 2005). Disulfiram affected components could downregulate c-Jun/c-Fos and NF-κB expression levels and then decrease the DNA binding activity of AP-1 and NF-κB (Liu *et al*., 1998; Shian *et al*., 2003). MMP-2 and MMP-9 may also influence the expression of genes directly responsible for regulating invasion. Taken together, these phenomena may provide possible cell-mediated signaling pathways as a result of disulfiram interference. Disulfiram's suppression of MMP-2 and MMP-9 expression could conceivably be through these common factors (AP-1, NF-κB). However, its exact mechanism of action still remains elusive. We can therefore theorize that the inhibitory effect of disulfiram on AP-1 and NF-kB activity might explain the resulting suppression of the MMP-2 and MMP-9 genes. In conclusion, we report that disulfiram down-regulates the expression of invasion-associated proteins MMP-2 and MMP-9.

These results provide new insights on the potential use of disulfiram in controlling cancer invasion and metastasis. We demonstrated that disulfiram has anti-invasive and anti-metastatic activity via the inhibition of MMP-2 and MMP-9 activity in human osteosarcoma cells. Thus disulfiram can be a new anti-cancer drug for the management of human osteosarcoma.

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