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Tubulin-binding agents down-regulate matrix metalloproteinase-2 and -9 in human hormone-refractory prostate cancer cells – A critical role of Cdk1 in mitotic entry

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ABSTRACT

Tubulin is an important target for anticancer therapy. Taxanes and *vinca* alkaloids are two groups of tubulin-binding agents in cancer chemotherapy. Besides tubulin binding, these groups of agents can also down-regulate protein levels of matrix metalloproteinase (MMP)-2 and -9, two important cancer-associated zinc-dependent endopeptidases in invasion and metastasis. However, the mechanism of action waits to be explored. In this study, protein levels but not mRNA expressions of MMP-2 and -9 were down-regulated by paclitaxel (a microtubule-stabilization agent), vincristine and evodiamine (two tubulin-depolymerization agents). These agents induced an increase of protein expression of cyclin B1, MPM2 (mitosis-specific phosphoprotein) and polo-like kinase (PLK) 1 phosphorylation. The data showed a negative relationship between the levels of mitotic proteins and MMP-2 and -9 expressions. MG132 (a specific cell-permeable proteasome inhibitor) blocked mitotic entry and arrested cell cycle at G2 phase, preventing down-regulation of MMP-2 and -9. Cell cycle synchronization experiments by thymidine block or nocodazole treatment showed that mitotic exit inhibited the down-regulation of MMP-2 and -9, confirming negative relationship between cell mitosis and protein levels of MMP-2 and -9 expressions. Cyclin-dependent kinase (Cdk) 1 is a key kinase in mitotic entry. Knockdown of Cdk1 almost completely inhibited the down-regulation of MMP-2 and -9 induced by tubulin-binding agents. In conclusion, the data suggest that mitotic entry and Cdk1 plays a central role in down-regulation of MMP-2 and -9 protein expressions. Tubulin-binding agents cause mitotic arrest and Cdk1 activation, which may contribute largely to the down-regulation of both MMP-2 and -9 expressions.

1. Introduction

Matrix metalloproteinases (MMPs) are a large family of proteolytic enzymes, which have been characterized for their ability in degradation of many different components of extracellular matrix and have been extensively studied in tumor invasion and metastasis [1]. MMPs involve in many biological processes which are different from the well-identified role in matrix degradation, including the interaction with specific cell molecules and signaling pathways in maintaining tumor microenvironment and promoting early stages of tumor development and growth [1,2]. MMP-2 and -9, two cancer-associated zinc-dependent endopeptidases, cleave a wide variety of targets (e.g., growth factors and receptors, cytokines and extracellular matrix) and interact with integral membrane proteins (e.g., CD44 and several integrins), regulating cell migration, invasion, inflammation and angiogenesis [3–5]. High levels of MMP-2 and -9 are expressed in cancer tissues and invasive cell lines, and increasing lines of evidence reveal that MMP-2 and -9 are negative prognostic markers for survival in cancer patients, including prostate cancer patients [6–10]. Accordingly, MMP-2 and -9 have been suggested as targets for the development of anticancer drugs [11,12].

Taxanes and *vinca* alkaloids are two of the most clinically important families that target tubulin/microtubule. The binding sites on tubulin and the effect on microtubule assembly/disassembly may be varied among different tubulin-binding agents. The ultimate result is the same in perturbing microtubule dynamics, leading to cell cycle arrest at mitotic phase and subsequent apoptotic cell death [13,14]. Besides the known pharmacodynamic properties, tubulin-binding agents show anti-angiogenic activity through the inhibition of cell proliferation, motility and capillary network formation of endothelial cells

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[15,16], down-regulation of vascular endothelial growth factor (VEGF) and angiopoietin-1 [17,18] and induction of both gene and protein expression of thrombospondin-1 [19].

Down-regulation of VEGF, MMP-2 and MMP-9 expression is responsible to the inhibition of intratumor angiogenesis by tubulin-binding agents [16,20,21]. Stearns and Wang [22] have selected and subcloned bone metastatic and non-metastatic lines from human prostate cancer PC-3 parent cells in SCID mice models and have found that MMP-2 levels are high in subcutaneously grown metastatic clones but low in non-metastatic clones. High MMP-2 production is abolished by the administration of paclitaxel. Moreover, Kato and the colleagues [23] have established paclitaxel-resistant prostatic cancer cells from PC-3 cells and have discovered an elevated invasive activity through increasing secretion of MMP-2 and -9. The data indicate that once paclitaxel loses inhibitory activity on MMP-2 and MMP-9, cancer invasion will be enhanced thereafter. Although it has been suggested that tubulin-binding agents can down-regulate protein levels of MMP-2 and MMP-9, the mechanism of action still waits to be explored. The present study has identified the mechanism suggesting that cyclin-dependent kinase (Cdk)-1 and mitotic entry may play crucial roles in regulating the expression of MMP-2 and -9 in human hormone-refractory prostate cancer (HRPC) cells.

2. Materials and methods

2.1. Materials

RPMI 1640 medium and fetal bovine serum (FBS) were obtained from GIBCO/BRL Life Technologies (Grand Island, NY). Antibodies to MMP-2, MMP-9, cyclin B1 and anti-mouse and anti-rabbit IgGs were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to p-mTOR^{Ser2448} (mammalian target of rapamycin), p-Akt^{Ser473}, p-p38$^{Thr180/Tyr182}$, p-PLK1^{Thr210} (polo like kinase), Cdk-1, p-Cdk1^{Tyr15}, p-Cdk1^{Thr161} and GAPDH were from Cell Signaling Technologies (Boston, MA). Antibody to mitotic protein monoclonal 2 (MPM2) was from BD Biosciences PharMingen (San Diego, CA). Chemical compounds were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell lines and cell culture

HRPC cell lines, PC-3 and DU-145, were from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium with 10% FBS (v/v), penicillin (100 units/ml) and streptomycin (100 μg/ml). Cultures were maintained in a 37 °C incubator with 5% CO_2/95% air.

2.3. Cell cycle synchronization

For thymidine block and mitotic synchronization, cells were treated with 2 mM thymidine and 0.25 mM nocodazole for 24 h, respectively. After phosphate-buffered saline (PBS) washing, the cells were re-plated in drug-free medium. The block was released by incubation in fresh medium/10% FCS (indicated as time zero), and cells were harvested at the indicated times. Cell cycle progression was detected by flow cytometric analysis.

2.4. Flow cytometric assay of DNA content

After the treatment of cells with vehicle (0.1% DMSO) or the indicated agent, the cells were harvested by trypsinization, fixed with 70% (v/v) alcohol at 4 °C for 30 min and washed with PBS. After centrifugation, cells were incubated in 0.1 ml of phosphate–citric acid buffer (0.2 M $NaHPO_4$, 0.1 M citric acid, pH 7.8) for 30 min at room temperature. Then, the cells were centrifuged and resuspended with 0.5 ml PI solution containing Triton X-100 (0.1%, v/v), RNase (100 μg/ml) and PI (80 μg/ml). DNA content was analyzed with FACScan and CellQuest software (Becton Dickinson, Mountain View, CA).

2.5. Western blotting

After the treatment, cells were harvested with trypsinization, centrifuged and lysed in 0.1 ml of lysis buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF and 100 μM sodium orthovanadate. Total protein was quantified, mixed with sample buffer and boiled at 90 °C for 5 min. Equal amount of protein (30 μg) was separated by electrophoresis in 12% SDS-PAGE, transferred to PVDF membranes and detected with specific antibodies. The immunoreactive proteins after incubation with appropriately labeled secondary antibody were detected with an enhanced chemiluminescence detection kit (Amersham, Buckinghamshire, UK).

2.6. Small interfering RNA (siRNA) transfection

For transfection, PC-3 cells were seeded into 60-mm tissue culture dishes with 30% confluence and grown for 24 h to 50–60% confluence. Each dish was washed with serum-free Opti-MEM (Life Technologies, Ground Island, NY), and 2 ml of the same medium was added. Aliquots containing siRNA in serum-free Opti-MEM were transfected into cells using Lipofectamine 2000 (Life Technologies, Ground Island, NY) according to manufacturer's instructions. After incubation for 6 h at 37 °C, cells were washed with medium and incubated in 10% FBS-containing RPMI-1640 medium for 48 h. Then, the cells were treated in the absence or the presence of the indicated agents and the subsequent experiments were performed.

2.7. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA). First-strand cDNA was synthesized from 1 mg of RNA using M-MLV reverse transcriptase (Promega Corporation, Madison, WI). After RT, PCR amplification was performed in a final volume of 25 μl of 5× Taq master mix (Protech, Taiwan) containing 1 μl of cDNA. Primer sequences are: MMP-2: forward, 5′-GTATTTGATGGCATCGCTCA-3′, reverse, 5′-CATTCCCTGCAAAGAACACA-3′; MMP-9: forward, 5′-CGCTACCACCTCGAACTTTG-3′, reverse 5′-GCCATTCACGTCGTCCTTAT-3′. Reaction mixtures were incubated at 95 °C for 5 min to pre-denature, and then amplification was performed in optimized cycles (denaturized for 30 s at 95 °C, renaturated for 20 s and extended for 30 s at 72 °C), followed by 72 °C for 10 min. Each PCR product was separated on a 2% agarose gel and visualized by staining with SYBR Green.

2.8. Detection of the release of MMP-2 and -9

Amount of MMP-2 and -9 in conditioned media was measured in triplicate by commercially available enzyme-linked immunosorbent assay kits (R&D Systems, Inc., Minneapolis, MN) according to manufacturer's protocols.

2.9. Data analysis

The compound was dissolved in DMSO. The final concentration of DMSO was 0.1% in cells. Data are presented as the mean ± SEM for the indicated number of separate experiments. Statistical analysis was performed with one-way analysis of variance (ANOVA). Student's