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Docetaxel-mediated Apoptosis in Myeloid Progenitor TF-1 Cells is Mitigated by Zinc: Potential Implication for Prostate Cancer Therapy

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Abstract

Background—Docetaxel-based combination chemotherapy is approved by the FDA for the treatment of metastatic castration-resistant prostate cancer. Unfortunately, docetaxel's efficacy is significantly limited by its considerable toxicity on hematopoietic progenitor cells, thus necessitating dose reduction or even discontinuation of the chemotherapy. Induction of pre-mitotic arrest protects cells against docetaxel-mediated toxicity and affords therapeutic opportunities.

Methods—Cell cycle progression was examined by propidium iodide staining. Zinc uptake was determined by FluoZin-3 AM staining. Apoptotic DNA fragmentation was detected using APO-BRDU kit.

Results—In the course of our current work, we treated the myeloid progenitor TF-1 cells and the castration-resistant PC-3 and DU-145 prostate cancer cells with physiologically relevant concentrations of zinc. In doing so, we were able to prevent docetaxel-mediated mitotic arrest in zinc accumulating myeloid progenitor TF-1 cells but not in castration-resistant PC-3 and DU-145 prostate cancer cells. Moreover, pre-treatment with zinc abolished docetaxel-induced apoptosis in TF-1 cells, whereas such treatment had no effect on apoptosis in PC-3 and DU-145 prostate cancer cells.

Conclusions—Our results suggest that zinc can protect myeloid progenitor cells against docetaxel-induced toxicity without compromising the drug's anti-tumor activity.

Keywords

prostate; cancer; zinc; docetaxel; apoptosis

Introduction

Prostate cancer is the most commonly diagnosed malignancy in men and the second most common cause of male cancer deaths in the United States. Docetaxel-based combination chemotherapy is the mainline therapy approved by the FDA for treatment of castration-resistant prostate cancer (CRPC), conferring a median survival of 18.9 months [1]. Docetaxel is a member of the taxane family and is a semi-synthetic analogue of paclitaxel (Taxol). Three weekly intravenous infusions of docetaxel (75 mg/m² every 3 weeks) are now considered standard of care for patients with metastatic CRPC. Unfortunately, docetaxel's administration is significantly limited by its considerable toxicities. Rapidly

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dividing cells such as the bone marrow, hair follicles, epithelium of the gastrointestinal tract, and the skin and the nailbed are markedly affected along with the tumor cells, resulting in myelosuppression, hair loss, diarrhea/nausea, skin peeling/blistering and loss of the nail plate [2]. Adverse effects, especially significant myelosuppression, can lead to substantial morbidity, may cause early mortality, and can prevent continuation of docetaxel.

Docetaxel acts by stabilizing microtubules through reversible binding to polymeric tubulin, preventing its disassembly. In cells treated with docetaxel, centrosome organization is disrupted resulting in cell cycle arrest at the metaphase/anaphase transition of mitosis [3]. Inhibition of mitotic progression and mitotic arrest correlate with the cytotoxicity of microtubule-active drugs [4–7]. As such, recent studies demonstrate that low concentrations of doxorubicin can cause pre-mitotic arrest, protecting colon [8], promyelocytic leukemia [6] breast and ovarian [9] cancer cells against the cytotoxic effects of microtubule-active drugs such as docetaxel. Similar results have been noted in experiments with normal primary lymphocytes, as Demidenko et al. reported that no mitotic or apoptotic cells were detected in PHA-stimulated doxorubicin-treated lymphocytes following treatment with docetaxel [6].

The process whereby a normal prostate epithelial cell transforms into a cancerous cell involves the loss of the cell's ability to accumulate intracellular zinc [10,11]. This loss of zinc accumulation is one of the most consistent and persistent characteristics of prostatic malignancy. Moreover, decreased zinc concentrations have been reported in the serum of patients with metastatic prostate carcinoma [12]. Clinically, cancer cells that maintain higher levels of intracellular zinc have been shown to have significantly slower growth rates [13,14]. Our experiments demonstrate that the administration of physiologic levels of zinc prevents docetaxel-mediated mitotic arrest, thereby protecting zinc accumulating myeloid progenitor TF-1 cells from docetaxel-mediated toxicity. Such treatment has no effect on apoptosis in castration-resistant PC-3 and DU-145 prostate cancer cells where zinc metabolism is already disrupted. These results suggest that the exogenous administration of zinc could be protective of normal hematopoietic cells against docetaxel-mediated toxicity, thereby obviating the need for chemotherapeutic dose reduction or discontinuation.

Materials and Methods

Cell culture and reagents

Cell lines were obtained from ATCC (Rockville, MD) and maintained in complete cell culture medium (RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% FCS (Hyclone, Logan, UT), gentamicin (50 mg/L), sodium pyruvate (1 mM) and non-essential amino acids (0.1 mM)). During the experiments, the cells were incubated with indicated concentrations of zinc in the form of ZnSO_4 in RPMI 1640 medium for 3 hours. After this incubation, RPMI 1640 medium was replaced with complete cell culture medium for indicated times as described in Figure Legends. Propidium Iodide (PI), methylseleninic acid (MSA), and pyrithione were obtained from Sigma Corporation (St. Louis, MO). TRAIL was obtained from Biomol (Plymouth Meeting, PA, USA). FluoZin-3 AM was obtained from Molecular Probes (Eugene, OR).

Cell cycle analysis

Cells were harvested, washed with PBS, fixed in 70 % ethanol at -20°C for 30 minutes followed by incubation with RNase A (100 $\mu\text{g/ml}$) and PI (50 $\mu\text{g/ml}$) at 37°C for 1 hour. Analysis was performed using FACScan (Becton Dickinson, Franklin Lakes, NJ) using acquisition and analysis software (Cell Quest, Becton Dickinson).

FluoZin-3 staining

Cells were incubated with 1.5 µg/ml of zinc in the form of ZnSO₄ in RPMI 1640 medium for 1 hour, washed twice with PBS, loaded with 5 µM FluoZin-3 AM at room temperature for 30 min and analyzed by flow cytometry. Analysis was performed using FACSscan (Becton Dickinson). Individual fluorescent populations were determined through the use of acquisition and analysis software (Cell Quest, Becton Dickinson).

Measurement of apoptosis

DNA fragmentation was detected using APO-BRDU kit (The Phoenix Flow Systems, Inc., San Diego, CA).

Results and Discussion

The development and progression of prostate cancer involves the loss of the prostate cell's ability to accumulate intracellular zinc. The discrepancy in cellular zinc levels is well preserved in long-term cultured malignant prostate cancer cell lines [15]. We examined cellular zinc uptake in myeloid progenitor TF-1 cells and castration-resistant PC-3 and DU-145 prostate cancer cells. Figure 1 demonstrates that zinc accumulation is significantly lower in advanced prostate cancer cells when compared with TF-1 cells, as measured by the cell-permeable zinc-specific fluorescent indicator, Fluo-Zin-3.

Recent studies reveal that increased intracellular zinc uptake inhibits proliferation at multiple checkpoints in hematopoietic cells [16]. Therefore, we set out to examine the effect of physiologically relevant levels of zinc (0.5–1.5 µg/ml [17]) on docetaxel-mediated mitotic arrest in zinc accumulating TF-1 cells and zinc-refractory PC-3 and DU-145 cells. Figure 2 demonstrates that co-administration of zinc and docetaxel had no significant effect on docetaxel-mediated mitotic arrest in PC-3 and DU-145 cells. In contrast, the combination of docetaxel and zinc at physiologically relevant level of 1.5 µg/ml effectively prevented mitotic arrest in TF-1 cells. Interestingly, treatment with zinc alone had no significant effect on cell cycle distribution in either TF-1 or CRPC cells. Our data are in contrast with those reported by Liang et al. [18] who found that treatment with zinc resulted in the increase of LNCaP and PC-3 cell populations in G2/M phase. The modulation of cellular growth by zinc could be a consequence of its inhibitory effect of terminal oxidation [15], truncation of the Krebs cycle [19], and decreased energy production [20]. In addition, zinc modulates the expression and/or activation of genes functionally relevant to various phases of cell cycle progression, i.e., metallothioneins [21], Hsp70 [22], p21(Cip/WAF1) [18] and NF-κB [23]. Down-regulation of NF-κB activity could lead to the inhibition of cell growth regulation mediated by selective alterations in G1 cell cycle regulatory proteins [24] whereas over-expression of Hsp70 was shown to arrest cells at S phase [25]. Interestingly, recent studies demonstrated that p21 might inhibit cell cycle progression at both G1/S and G2/M transitions [26]. Thus, increased intracellular zinc uptake could potentially inhibit cell cycle progression at multiple checkpoints and vary depending on the cell line assayed. Regardless, zinc appears to be an important factor in the regulation of cell cycle progression, the impact of which requires further investigation.

Myelosuppression is one of the major dose-limiting toxicity in patients treated with docetaxel. Given that mitotic arrest correlates with the cytotoxicity of microtubule-active drugs [4–7], we examined whether zinc could protect bone marrow cells from docetaxel-mediated apoptosis. The cytotoxic effect of docetaxel in combination with zinc was evaluated in TF-1, PC-3 and DU-145 cells using a DNA fragmentation assay. Treatment with docetaxel alone induced significant levels of apoptosis in both bone marrow and prostate cancer cells. Concomitant treatment with docetaxel and zinc resulted in

dramatically reduced levels of DNA fragmentation in TF-1 cells only, whereas addition of zinc had no significant effect on docetaxel-mediated cell death in PC-3 and DU-145 cells (Fig. 3A).

Zinc can directly inhibit the activity of various caspase family members, specifically caspases -3, -6, -7 and -8 [27,28]. Thus, the effect of zinc on docetaxel-induced apoptosis could be mediated at least in part via suppression of caspase enzymatic activities. To assess the potential involvement of caspase family members in this process, we tested whether zinc could prevent DNA fragmentation in TF-1 cells simultaneously treated with TRAIL and methylseleninic acid (MSA). We have demonstrated previously that apoptosis in prostate cancer cells treated with the combination of TRAIL and MSA is mediated via a caspase-dependent process [29]. Figure 3B demonstrates that zinc failed to block TRAIL/MSA-mediated apoptosis in TF-1 cells. The pan-caspase inhibitor Z-VAD.fmk was used as a control and blocked TRAIL/MSA-mediated apoptosis to background level. These experiments appear to demonstrate that zinc inhibits docetaxel-mediated apoptosis via a caspase-independent process.

To validate the regulatory role of intracellular zinc levels on docetaxel-mediated cytotoxicity, we examined the effect of concomitant administration of zinc and the zinc ionophore pyrithione using zinc non-accumulating PC-3 prostate cancer cells. Intracellular zinc accumulation was significantly increased in PC-3 cells cultured in the presence of both zinc and pyrithione (Fig. 4A). In addition, increased zinc uptake in PC-3 cells treated concomitantly with zinc and pyrithione had a functional impact on the docetaxel-mediated mitotic arrest. Figure 4B demonstrates that simultaneous treatment with zinc, pyrithione, and docetaxel significantly reduces the number of PC-3 cells undergoing mitotic arrest. Moreover, increased zinc uptake also coincided with a dramatic reduction of apoptotic-mediated cell death in docetaxel-treated PC-3 cells (Fig. 4C). Although reports from several laboratories have revealed that zinc administration can induce apoptosis in a variety of different cell lines [18,30,31], we did not encounter similar findings in our set of experiments. In these prior studies [30,31], it is important to note that super-physiological levels of zinc were used, which most likely does not replicate *in situ* conditions. In contrast, the report by Liang et al. [18] demonstrates a pro-apoptotic effect at physiological levels of zinc (0.5–1.0 $\mu\text{g/ml}$) on LNCaP and PC-3 prostate cancer cells. These results are puzzling since the growth of native PC-3 prostate cancer cells *in vivo* was not affected by plasma zinc levels of 0.6–1.2 $\mu\text{g/ml}$ [32]. Nevertheless, the findings by Liang et al. warrant consideration and could possibly be explained by the fact that these experiments were performed in serum-free medium [18].

Furthermore, we saw no effect on the amount of apoptosis induced even when we treated cells with zinc in the presence of the zinc ionophore pyrithione. Although one would expect a greater effect on cells with increased zinc accumulation, we believe that this lack of effect was due to the relatively transient exposure of cells to pyrithione. As described in the Materials and Methods section, we co-incubated zinc and pyrithione for only 3 hours and then removed them prior to treating the cells with docetaxel. This short incubation probably did not allow for the full zinc-mediated cellular effects to occur.

Despite the relatively indolent nature of prostate cancer, approximately 15–20% of men still die from castration-resistant prostate cancer. Docetaxel has emerged as a frontline treatment for the subset of patient, but its use and effectiveness have been limited by its considerable toxicities. Manipulation of zinc metabolism both in malignant prostate cells and normal rapidly dividing cells represents a novel strategy to enhance docetaxel's efficacy and broaden its use to all men with CRPC.

Conclusions

In summary, docetaxel is now an established first line treatment for men with CRPC, producing objective responses and prolonging survival. Similar to other chemotherapeutic agents, docetaxel affects both benign and malignant rapidly dividing cells. Its effects on bone marrow progenitors can be dose-limiting and restrict patients with advanced prostate cancer from receiving potentially curative therapy. In this manuscript we show that bone marrow cells, but not CRPC cells, can be shielded from the cytotoxic effects of docetaxel by co-administration of parenteral zinc. We demonstrate that zinc prevents docetaxel-mediated mitotic arrest and thereby protects TF-1 cells from the cytotoxic action of docetaxel. In turn, because CRPC cells fail to accumulate zinc, docetaxel's cytotoxicity is not blunted under zinc-rich conditions. Importantly, it has been observed that serum zinc levels are significantly reduced in patients with metastatic prostate carcinoma compared with healthy individuals [12]. Thus, daily zinc supplementation in patients with CRPC would not endanger these patients from developing zinc toxicity due to ingestion of supraphysiologic doses of zinc. In essence, zinc supplementation would merely restore these patients' serum zinc levels to a normal concentration, thereby protecting these individuals from the dose-limiting toxicities of docetaxel. We believe these findings present exciting and novel therapeutic opportunities.

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Figure 1.

Accumulation of zinc in the myeloid progenitor TF-1 cells and the castration-resistant PC-3 and DU-145 prostate cancer cells. Cells were pre-incubated with or without zinc followed by staining with FluoZin-3 AM as described in Materials and Methods. X axis represents fluorescence intensity, Y axis represents cell number. The numbers represent the percentage of cells in populations positively stained with FluoZin-3.

Figure 2.

Cell cycle analysis in TF-1, PC-3 and DU-145 cells. Cells were pre-incubated with or without zinc as described in Materials and Methods followed by the addition of docetaxel (50nM) for 18 hours. Cells were stained with PI and analyzed by flow cytometry. Numbers represent percentage of cells in G1, S and G2/M phases of cell cycle respectively.

Figure 3.

(A) Physiologically relevant concentrations of zinc prevent docetaxel-mediated apoptosis in zinc-accumulating myeloid progenitor TF-1 cells but not in PC-3 and DU-145 prostate cancer cells. Cells were pre-incubated with zinc as described in Materials and Methods followed by treatment with docetaxel (50nM) for 48 hours. The percentage of apoptotic cells was determined by TUNEL assay followed by flow cytometry analysis. X axis represents DNA content; Y axis represents staining intensity. (B) Zinc doesn't protect TF-1 cells from TRAIL/MSA-mediated apoptosis. TF-1 cells were pre-incubated with zinc as described in panel A, followed by treatment with TRAIL (200 ng/ml) and MSA (5 μ M) for 18 hours. The percentage of apoptotic cells was determined by TUNEL assay followed by flow cytometry analysis.

Figure 4.

Pyrithione-mediated zinc uptake modulates the effect of docetaxel on cell cycle progression and apoptosis in PC-3 cells. (A) Pyrithione facilitates zinc accumulation in PC-3 cells. Cells were pre-incubated with zinc (0.5 $\mu\text{g/ml}$) in the form of ZnSO_4 with or without pyrithione (3 μM) for 1 hour followed by staining with FluoZin-3 AM as described in Materials and Methods. (B) PC-3 cells were pre-incubated with or without zinc (0.5 $\mu\text{g/ml}$) and pyrithione (3 μM) for 3 hours followed by the addition of docetaxel (50nM) for 18 hours. Numbers represent percentage of cells in G1, S and G2/M phases of cell cycle respectively. (C) Increased zinc uptake protects PC-3 cells from docetaxel-mediated apoptosis. Cells were treated as described in panel B for 48 hours. The percentage of apoptotic cells was determined by TUNEL assay followed by flow cytometry analysis.