Zinc sensitizes prostate cancer cells to sorafenib and regulates the expression of Livin

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In prostate carcinogenesis, normal zinc-accumulating epithelial cells are transformed into malignant cells that do not accumulate zinc. Increased levels of zinc have been shown to induce apoptosis through a caspase-dependent mechanism with down-regulated anti-apoptotic proteins in prostate cancer cells. Our previous study showed that, as a member of the inhibitor of apoptosis proteins (IAPs) family, Livin could play an important role in the initiation of human prostate cancer and promote cell proliferation by altering the G1–S cell cycle transition. In the present study, we measured the apoptosis sensitivity of prostate cancer cells to zinc and sorafenib and found that zinc sensitized prostate cancer cells to sorafenib-induced apoptosis. Surprisingly, we also found that, unlike its counterparts Survivin and cIAP2, Livin was not decreased all the time; instead, it was compensatively increased in zinc-mediated apoptosis at 48 h in prostate cancer cells. Our results offer potential treatment combinations that may augment the effect of sorafenib, and also reveal, for the first time, that increased Livin expression may play a role in the early cell death response of prostate cancer cells to zinc.

Keywords zinc; prostate cancer; sorafenib; Livin

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Introduction

Zinc is an essential trace element that serves as an important factor in various physiological and biochemical circumstances. In humans, normal prostate epithelial cells accumulate the highest levels of zinc in any soft tissue of the body. This unique capability is retained in benign prostatic hyperplasia [1]. However, prostate carcinogenesis involves the transformation of zinc-accumulating normal epithelial cells into malignant cells, which do not accumulate zinc. The loss of the unique ability of the prostate to retain normal intracellular levels of zinc may be an important factor in the development and progression of prostate cancer. Until now, the exact mechanism underlying the reduced levels of zinc in prostate cancer has not been determined. Some studies reported that the cellular accumulation of zinc has an inhibitory effect on the proliferation of prostate cancer cells by inducing apoptosis [2,3].

Apoptotic pathways, which should be tightly controlled, are so perturbed by carcinogenic factors that the cell’s fate is altered. In recent years, the complex networks of apoptotic and anti-apoptotic proteins that govern apoptosis have been identified as biomarkers of disease or potential therapeutic targets against cancer. Thus, they are receiving increasing attention from academics and clinicians. Among these proteins, inhibitor of apoptosis proteins (IAPs), which could inhibit the downstream components of the caspase activation pathways, play important roles in regulating the process of apoptosis in many species [4,5]. Our previous study showed that, as a member of the IAP family, Livin might play an important role in the initiation of human prostate cancer and promote cell proliferation by regulating the G1–S cell cycle transition [6]. Another previous report by our group showed that Livin directly regulates prostate cancer cell invasion by impacting the nuclear factor-kappaB (NF-κB) signaling pathway and the expression of FN and CXCR4, resulting in the inhibition of FAK, Src, and α5 and β3 integrins [7]. The interpretation of these data suggested that Livin may be a potential therapeutic target to regulate the development and progression of prostate cancer.

It has been reported that zinc may induce apoptosis through a caspase-dependent mechanism with down-regulated IAPs (such as Survivin or cIAP2) in androgen-independent prostate cancer cells [8,9]. However, as the family member of IAPs, Livin has not been reported in this process. Because IAP family members are structurally similar and some of them can act cooperatively via particular pathways to regulate apoptosis and proliferation [10,11], we speculated that Livin might have the same effect on zinc-mediated apoptosis in prostate cancer cells.

In this study, we detected the apoptosis sensitivity of prostate cancer cells to zinc and sorafenib and investigated...
the expression of Livin in the zinc-induced apoptosis in prostate cancer cells.

Materials and Methods

Cell line and cell culture
The human prostate cancer cell line PC-3 was obtained from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were incubated in RPMI-1640 (Gibco, Carlsbad, USA) with 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin under a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Cell viability assay
Cell proliferation and cell viability were evaluated in 96-well microplates using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cells (2 x 10^3) were plated onto 96-well plates. After adherence, the cells were grown for 6 h in serum-free medium. Serum-starved cells were incubated with various concentrations of either ZnSO₄ (Sigma, St Louis, USA), sorafenib (Bayer HealthCare AG, Leverkusen, Germany), or both for the indicated time periods. The cells were incubated with MTT (5 mg/ml; Invitrogen, Carlsbad, USA) for 4 h at 37°C in a humidified atmosphere of 5% CO₂, and then 200 μl of dimethyl sulfoxide was added for 10 min at room temperature to solubilize the crystals. The absorbance values at a wavelength of 490 nm were measured using a microplate reader (Bio-Rad, Hercules, USA).

Apoptosis assays
To confirm that zinc and sorafenib cause apoptosis in human prostate cancer cells, a Hoechst apoptosis kit (Beyotime Biotechnology, Nantong, China) was used. For the Hoechst staining assay, PC-3 cells (1 x 10⁶) were plated on cover slips in six-well plates. The next day, the cells were treated with phosphate-buffered saline (PBS) or zinc and sorafenib for 24 h. The cells were then fixed in methanol/acetic acid (3:1) for 10 min. After fixation, the cells were washed with PBS and stained with Hoechst 33258 for 15 min, and their nuclear morphology was examined by fluorescence microscopy. The cells with apoptotic morphology, characterized by condensed or fragmented nuclei, were counted [12].

F-actin staining
Cells were seeded in six-well plates containing 19-mm diameter cover slips and incubated at 37°C overnight. After adherence, the cells were grown for 6 h in serum-free medium. Serum-starved cells were treated with drugs for 24 h. To detect F-actin, the cells were fixed for 30 min with 0.2% Triton-X 100 in PBS. The cells were then incubated with phalloidin-TRITC (P1951, Sigma) at a 1:1000 dilution and with 0.2 mg/ml 4',6-diamidino-2-phenylindole (DAPI) for 3 h. The slides were analyzed with an Olympus FV1000 confocal microscope (Tokyo, Japan) using a x40 objective.

Western blot analysis
Cultured cells, washed with cold PBS, were lysed with lysis buffer supplemented with protease inhibitor phenylmethylsulfonyl fluoride. The lysates were vortexed for 15 min at 4°C and centrifuged at 14,000 g for 15 min at 4°C. The supernatants were collected and heated with loading buffer at 95°C for 5 min. Fifteen to 30 mg of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked at 4°C overnight with blocking buffer (5% non-fat milk powder in Tris-buffered saline containing 0.5% Tween-20) and then incubated overnight with rabbit anti-Livin polyclonal antibody ML-IAP (H-90) (sc-30161; Santa Cruz Biotechnology, Santa Cruz, USA) at 1:100 overnight at 4°C. Rabbit anti-β-actin monoclonal antibody (Cell Signaling Technology, Danvers, USA) at 1:100 was used as an internal control. The membranes were incubated with HRP-conjugated goat anti-rabbit/mouse IgG (1:1000; ZSGB-BIO, Beijing, China). The detection was performed using an ECL kit (Amersham Biosciences, Piscataway, USA) according to the manufacturer’s instructions. The band density was measured using the Gel-Pro analyzer 4.0 software (Cybernetics, Rockville, USA) and normalized against the density of the respective housekeeping β-actin.

Statistical analysis
The SPSS13.0 software was used to determine the statistical significance. Each assay was performed at least three times. The data were expressed as the mean ± SD, and Student’s t-test was used to determine the significance of the differences in multiple comparisons. A P value < 0.05, indicated by an asterisk, was considered statistically significant.

Results

Effects of zinc and sorafenib on prostate cancer cell viability
To quantify the zinc- and sorafenib-induced cell death in prostate cancer cells, cytotoxicity was measured using the MTT cell viability assay. When PC-3 cells were exposed to various concentrations of zinc sulfate for 24 and 48 h, their growth was inhibited in a dose-dependent manner up to 250 μM [Fig. 1(A)]. When PC-3 cells were exposed to various concentrations of sorafenib for 24 and 48 h, their
growth was also inhibited in a dose-dependent manner up to 30 \( \mu \text{M} \) [Fig. 1(B)].

Treatment with \(<50 \mu\text{M}\) of zinc sulfate only had a minimal effect on PC-3 cell survival. Therefore, we added 25 and 50 \( \mu\text{M}\) zinc sulfate to various concentrations of sorafenib to examine alterations in cell viability. We found that the combination of zinc sulfate and sorafenib inhibited cell viability to a greater extent than sorafenib alone in PC-3 cells [Fig. 1(C)].

Synergistic effects of zinc and sorafenib on apoptosis in prostate cancer cells

Human prostate cancer cells treated with PBS, zinc, or sorafenib were stained with Hoechst 33258. The cells treated with zinc or sorafenib showed chromatin condensation in their nuclei. PC-3 cells underwent apoptosis in a concentration-dependent manner after treatment with zinc or sorafenib. Almost no apoptotic nuclei were detected in the cells that were treated with PBS. The cells treated with a combination of zinc and sorafenib showed significantly more apoptosis than those treated with either of these reagents alone (Fig. 2).

Effects of zinc and sorafenib on the cytoskeleton in prostate cancer cells

Apoptosis is closely associated with changes in the organization of F-actin that is related to the formation of apoptotic bodies. Rhodamine-conjugated phalloidin was used to localize F-actin. F-actin changed very little in cells exposed to 50 \( \mu\text{M}\) zinc or 5 \( \mu\text{M}\) sorafenib (Fig. 3). However, the combination of 5 \( \mu\text{M}\) sorafenib and 50 \( \mu\text{M}\) zinc changed F-actin extensively. The change observed in the cytoskeletal organization of cells treated with the combination of sorafenib and zinc suggests that zinc could sensitize prostate cancer cells to sorafenib-mediated apoptosis.

Expression of Livin in prostate cancer cells treated with zinc sulfate

Western blot analysis was used to determine the expression levels of Livin in PC-3 cells treated with zinc sulfate. PC-3 cells were incubated with 0, 25, or 50 \( \mu\text{M}\) zinc sulfate for 24 or 48 h. There was a decrease in the expression of Livin protein in cells treated with zinc sulfate at 24 h. However, there was a compensatory increase in the expression of Livin protein in cells treated with 50 \( \mu\text{M}\) zinc sulfate compared with the control at 48 h (Fig. 4). These data indicated that the effect of Livin differs from that of other IAP members on zinc-induced apoptosis in prostate cancer cells [8,9].

Discussion

Recently, some researches have reported that zinc may induce apoptosis through a caspase-dependent mechanism with down-regulated IAPs in androgen-independent prostate cancer cells. Ku et al. [8] reported that reduced Survivin expression might play a role in the early (apoptotic) and overall (clonogenic) cell death response of prostate cancer cells to zinc. Uzzo et al. [9] showed that zinc could reduce the expression of NF-\(\kappa\)B-controlled anti-apoptotic protein c-IAP2 and sensitize prostate cancer cells to tumor necrosis factor-\(\alpha\) and paclitaxel-mediated cell death. It appears that the members of the family of inhibitors of apoptosis proteins (IAPs) may play an important role in zinc-induced apoptosis in prostate cancer cells.

In the present study, we obtained an unanticipated result. Unlike its counterparts Survivin or cIAP2, the anti-apoptotic protein Livin was not decreased all the time; instead, it compensatively increased with a non-lethal dose of zinc-mediated early apoptosis at 48 h in prostate cancer cells.

Livin, which is also referred to as melanoma inhibitor of apoptosis protein (ML-IAP) or kidney inhibitor of apoptosis protein (KIAP), is the most recently identified member of the IAP family. Its anti-apoptotic mechanism is mediated through the inhibition of caspase-3, caspase-7, and caspase-9 and by its E3 ubiquitin-ligase-like activity, which promotes the degradation of Smac/DIABLO [13]. Livin has been shown to be extensively expressed in many types of cancers and either not expressed or expressed at substantially lower levels in their normal tissue counterparts. It seems that the exclusively cancer cell-expressed Livin may be the valuable biomarker of different types of cancer.

Livin and other members of IAP family are homologs with highly conserved sequences, and they are structurally similar. They constitute a family of proteins that possess one to three baculovirus IAP repeats, and several of the proteins also have a gene (RING) finger domain at the C-terminus. Currently, eight IAPs have been identified in mammals, including X-linked inhibitor of apoptosis (XIAP), cellular IAP-1 (cIAP-1), cIAP-2, Livin, IAP-like protein 2 (ILP2), neuronal apoptosis inhibitory protein (NAIP), Survivin, and BRUCE [14–16]. IAPs are a group of anti-apoptotic factors in the apoptotic pathway that render cancer cells insensitive to apoptotic stimulation [17,18].

Tumor cells often express more than one IAP family member simultaneously to enhance their resistance to apoptosis. IAPs could have complex interactions with each other, which may lead to inhibition of an IAP member or cooperative synergistic action to protect cells from apoptosis [19]. In addition to their roles in cytoprotection, it has been demonstrated that IAPs function as broader regulators of cellular homeostasis and play roles in cell division, metabolism, and the activation of multiple intracellular signaling pathways, including NF-\(\kappa\)B, transforming growth factor-\(\beta\), and Janus kinase [14]. The overexpression of IAPs is highly correlated with cancer progression and resistance...
to chemotherapy and is associated with a poor prognosis [20]. Furthermore, Harlin et al. [21] reported that XIAP-deficient mice do not have any obvious defects in their development or in the regulation of apoptosis. A change was observed in cells derived from XIAP-deficient mice; the levels of c-IAP1 and c-IAP2 protein were increased. This phenomenon suggests the existence of a compensatory mechanism that leads to the up-regulation of other IAP family members when XIAP expression is lost. The changes in c-IAP1 and c-IAP2 expression may provide functional compensation for the loss of XIAP during development or in the induction of apoptosis. In addition, our previous study showed that Livin was highly expressed in prostate cancer but it was inhibited post-transcriptionally due to the expression of miR-198 in DU145 cells [22]. These IAP mechanisms may explain why Livin was not decreased but was instead compensatively increased during zinc-mediated apoptosis in prostate cancer cells.

We also found that a non-lethal dose of zinc may sensitize prostate cancer cells to sorafenib-induced apoptosis. Sorafenib is an oral multi-kinase inhibitor that targets the Ras/Raf kinase pathway, the vascular endothelial growth factor, and the platelet-derived growth factor receptor [23]. It has gained Food and Drug Administration approval for renal cell and hepatocellular cancers [24,25] and has shown promising activity in a variety of other cancers [26,27]. Some studies indicated that sorafenib could exert antiproliferative and pro-apoptotic activities in human prostate cancer cells [28–30]. Currently, sorafenib has undergone phase I/II clinical evaluation for the treatment of prostate cancer [31–35]. Nevertheless, there are several side effects, such as hypertension and hypothyroidism, and diverse cutaneous side effects associated with sorafenib therapy. Therefore, a reduction of the dose of sorafenib that continues to maintain its therapeutic effectiveness may be the goal in drug development. Our data indicated that the synergistic effects of zinc and sorafenib in cell apoptosis could support the combination therapy approach and may lead to a therapeutically effective reduced dose of sorafenib.

In summary, our results not only offer treatment combinations that could potentially augment the effect of sorafenib, but they also reveal, for the first time, that
increased Livin expression may have a role in the early cell death response of prostate cancer cells to zinc. However, the roles of other members of the IAP family in zinc-mediated apoptosis require further investigation.

The synergistic effects of zinc and sorafenib may be validated through additional in vitro and in vivo experiments to determine whether this result could provide a route for clinical application.

Figure 3 Localization of F-actin in PC-3 cells The cells were treated with PBS, 50 μM zinc, 5 μM sorafenib, or both 50 μM zinc and 5 μM sorafenib for 24 h. Actin filaments were visualized by phalloidin-TRITC, and nuclei were stained with DAPI. Stress fibers were absent. Slides were analyzed with the Olympus FV1000 confocal microscope using a ×40 objective.

Figure 4 Zinc regulates the expression of Livin in PC-3 cells PC-3 cells treated with zinc sulfate after 24 and 48 h were analyzed by western blotting for Livin expression. The band density was measured using the Gel-Pro analyzer 4.0 software and normalized against the density of its respective housekeeping β-actin. *P < 0.05 compared with the untreated group.
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References


