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Direct Effect of Zinc on Mitochondrial Apoptogenesis in Prostate Cells

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Abstract

BACKGROUND—Prostate epithelial cells uniquely accumulate significantly higher levels of zinc than other mammalian cells. We previously showed that the accumulation of high intracellular zinc levels in specific prostate cells results in the induction of apoptosis and the inhibition of cell growth. The apoptotic effect is due to zinc induction of mitochondrial apoptogenesis. We now report additional studies that corroborate this effect of zinc and provide insight into the mechanism of this unique effect.

METHODS—The effect of exposure to physiological levels of zinc on apoptosis was determined for three human prostate cell lines (PC-3, BPH, and HPR-1). Zinc-induced apoptosis was identified by DNA fragmentation. The direct effect of zinc on isolated mitochondrial preparations from each cell line was determined. The mitochondrial release of cytochrome c was determined by Western blot.

RESULTS—Exposure to zinc induced apoptosis in PC-3 and BPH cells but not in HPR-1 cells. The zinc accumulation in PC-3 (4.3 ± 0.3) and BPH (2.8 ± 0.4) was higher than that in HPR-1 cells (1.8 ± 0.1). The apoptotic effect of zinc on PC-3 cells could be observed as early as 4–6 hr of zinc treatment, and this effect was not reversible. The exposure of isolated mitochondria from PC-3 and BPH cells to zinc resulted in the release of cytochrome c; but zinc had no effect on mitochondria from HPR-1 cells.

CONCLUSIONS—Exposure to zinc induces apoptosis in PC-3 and BPH cells, which accumulate high intracellular levels of zinc, but not in HPR-1 cells, which do not accumulate high levels of zinc. Once initiated, the induction of apoptosis is not reversed by the removal of zinc, i.e., it is an irreversible process. The apoptogenic effect is due to a direct effect of zinc on mitochondria that results in the release of cytochrome c. The cell specificity of zinc induction of apoptogenesis is dependent on the ability of the cells to accumulate high levels of intracellular zinc and on the ability of the mitochondria to respond to the direct effect of zinc.

Keywords

zinc; prostate; mitochondria; apoptosis; PC-3; BPH; HPR-1

INTRODUCTION

Normal human prostate secretory epithelial cells accumulate the highest zinc levels of any cells in the body. In prostate cancer (PCa), the malignant prostate cells have lost this ability; compelling evidence now exists for the implication of zinc in the pathogenesis and progression of prostate malignancy [1–3]. Consequently, zinc plays a critical role in the normal function and pathology of the prostate gland. For detailed reviews of these relationships, see Costello and Franklin [3, 4].

An important question is, “What are the consequences and role of the accumulation of high zinc levels in the prostate secretory epithelial cells that possess the unique capability of transporting and accumulating high levels of zinc (i.e., zinc-accumulating prostate cells)?” We recently demonstrated that zinc accumulation in prostate cells results in increased apoptosis [5, 6] and that this effect was manifested by the zinc induction of mitochondrial apoptogenesis [6]. Exposure of these cells to zinc rapidly induces the translocation of cytochrome c from mitochondria into cytosol, which triggers caspase activation that leads to cascading cytosolic and nuclear apoptotic events. In contrast, exposure of cells to zinc that are incapable of high zinc accumulation does not result in apoptogenesis. This present report confirms and extends our earlier reports and addresses two important issues relating to the mechanism of zinc-induced mitochondrial apoptogenesis: (1) whether or not the effect of zinc is due to a direct effect of zinc on the mitochondria, and (2) if the cell-specific effect of zinc is due to the level of cellular zinc and/or in the capability of mitochondria to respond to accumulated zinc.

MATERIALS AND METHODS**Cell Lines and Cultures**

The present studies were conducted with PC-3 cells, a human malignant prostate cell line obtained from ATCC as we previously described [5]; BPH cells (kindly provided by Dr. S. Haywood, University of California, San Francisco), an epithelial cell line that was derived from human benign prostatic hyperplasia (BPH) tissue; and HPR-1 cells (kindly provided by Dr. C. K. Choo, University of Hong Kong, Hong Kong, China), an epithelial cell line that was derived from normal human prostate tissue. PC-3 cells and BPH cells were cultured in RPMI-1640 medium with 10% (PC-3) and 5% (BPH) fetal bovine serum and 1% penicillin/streptomycin. HPR-1 cells were cultured in serum-free keratinocyte medium with EGF (2.5 µg/500 ml) and bovine pituitary extracts (25 mg/500 ml) (Gibco BRL, Life Technologies, Bethesda, MD) and 1% penicillin/streptomycin.

Mitochondrial Preparation and Detection of Cytochrome c Release

The cells were collected by centrifugation at 250g for 5 min at 4°C. The cells were washed with ice-cold PBS twice and resuspended in 5 volumes of mitochondrial isolation buffer

(MIB) composed of 220 mM mannitol, 68 mM sucrose, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 10 mM HEPES, 0.1% bovine serum albumin (BSA), with added fresh 1 mM DTT and protease inhibitors (pepstatin A, 5 µg/ml; leupeptin, 10 µg/ml; aprotinin, 2 µg/ml), pH 7.4. The cells were homogenized gently on the ice with a glass homogenizer and followed by a centrifugation at 800 ×g for 10 min. The resulting supernatant fluid was centrifuged at 10,000g for 5 min at 4°C. The pellet (mito-chondria) was resuspended in MRB buffer composed of 200 mM mannitol, 50 mM sucrose, 10 mM succinate, 5 mM potassium phosphate, 10 mM HEPES, 0.1% BSA, pH 7.4, and kept on ice. Aliquots of the mitochondrial suspension (200 µg of protein/40-µl reaction) were exposed to zinc for various time periods at 30°C under conditions described in the Results section. At the conclusion of the incubation period, the mitochondria were separated from the reaction by rapid centrifugation at 10,000g for 5 min. The supernatant fluid was assayed for cytochrome c by Western blot. The protein concentration of the mitochondrial preparations was determined by the method of Bradford [7]. Western blot assays were performed with specific anti-cytochrome c and β-actin antibodies (BD Transduction Laboratories, San Diego, CA) under the conditions recommended by the manufacturer.

Detection of Cell Apoptosis

The extraction of DNA and detection of DNA fragmentation were performed as previously described [5]. The morphology of the cells treated with or without zinc in six-well culture plate for designated time periods and the characteristics of apoptotic cells were observed under an inverted microscope (Nikon, Eclipse TE200) and photographed.

Determination of Cellular Zinc

Prostatic cells were grown in 75 cm² flasks until 90% confluence of the culture. The cells were treated with or without zinc (1,000 ng/ml) in fresh serum-free medium for 3 hr. Before harvest, the cells were washed once with 1 × PBS and then washed twice after the collection to remove extracellular zinc. The cells were resuspended in sucrose buffer (250 mM sucrose, 20 mM HEPES, pH 7.4) and homogenized on ice. The nuclei and cell membranes were separated by centrifugation at 800g for 10 min. The supernatants were then centrifuged at 10,000g for 5 min, and these supernatants were used as cytosol samples. The protein concentrations of the samples were measured by Bradford method [7]. Thirty microliters of each sample (200 µg of protein) were placed in a 96-well plate and mixed well with 60 µl of TSQ buffer, which was composed of 1.9 g of sodium acetate, 2.9 g of sodium barbital, 1.5 mg of TSQ (Molecular Probes, Eugene, OR) dissolved in 100 µl of warmed ethanol, then double distilled H₂O added to 100 ml, pH 10. The fluorescence of zinc labeled by TSQ was detected by using a Fluoroskan Ascent, Labsystems, Microplate Reader (Life Sciences International Company, USA) with excitation of 355 and emission of 485 [8, 9].

In this study, all experiments were repeated three or more times to establish the consistency and reproducibility of the results. The data presented are representative of the results of the repeated experiments.

RESULTS

Zinc Induces Apoptosis in Human Prostatic Malignant (PC-3) and Benign Hyperplasia (BPH) Cells, but Not in HPR-1 Cells Derived From Normal Prostatic Epithelial Cells

We previously demonstrated that exposure of LNCaP and PC-3 cells to physiological levels of zinc results in increased apoptosis and decreased cell growth while having no effect on HPV-1 cells, which are derived from human normal prostatic epithelial cells [5, 6]. Correspondingly, LNCaP and PC-3 cells will accumulate high zinc levels; whereas HPV-1 cells do not accumulate high zinc levels (unpublished information). To extend this important relationship, the effect of zinc on the induction of apoptosis was studied in BPH cells, in HPR-1 cells that, like HPV-1 cells, purport to be representative of normal human prostate secretory epithelial cells, and in PC-3 cells for comparison of the effects. Figure 1 reveals that zinc exposure (1 $\mu\text{g/ml}$ for 24 hr) of PC-3 and BPH cells resulted in a marked decrease in the cell density while having no effect on HPR-1 cells. Correspondingly, cell apoptotic morphologic features were observed in both PC-3 and BPH cells treated with zinc but no similar effect was observed in HPR-1 cells. The apoptotic changes observed in PC-3 and BPH cells included the appearance of cell membrane “blebbing,” nuclear chromosome condensation, and eventually the formation of apoptotic bodies. Apoptosis was further confirmed by genomic DNA fragmentation (Fig. 2). Multiple 180-bp DNA fragments, resulting from inter-nucleosomal cleavage, were clearly visible in PC-3 and BPH cells treated with 0.25, 0.50, and 1.0 $\mu\text{g/ml}$ zinc for 24 and 48 hr. To determine that these effects were possibly associated with a cellular accumulation of zinc, the level of increased zinc accumulation in the zinc-treated cells vs. the control cells was determined to be 4.3-fold for PC-3 cells, 2.7-fold for BPH cells, and only 1.8-fold for HPR-1 cells. The values for PC-3 and HPR-1 cells are consistent with the cellular increases as measured by atomic absorption (unpublished information). Thus, zinc induction of apoptosis is a major factor in the inhibitory effect of zinc on prostate cell growth as we reported earlier [5].

A time-course study of zinc-induced apoptosis was further conducted in PC-3 cells (Fig. 3A). The results show that zinc induction of apoptosis in PC-3 cells is time dependent. Apoptotic cells appeared as early as 4 hr after zinc treatment, and apoptosis was clearly evident after 6 hr of zinc treatment. Between 6 and 24 hr after the start of zinc treatment, the increase in apoptotic cells was associated with significant decreased cell density.

In another study (Fig. 3B), we wanted to determine whether zinc-induced apoptogenesis could be reversed by removal of the zinc. PC-3 cells were recovered for 24 hr in normal growth medium after 2, 4, 6, and 24 hr of zinc (1 $\mu\text{g/ml}$) treatment. It is clear that as early as 4–6 hr of exposure of the cells to zinc irreversibly induced apoptosis and subsequent decreased cell density. The longer time of zinc treatment, the more extensive apoptosis appeared. This recovery study of zinc treatment showed that the inhibitory effect of zinc on PC-3 cell growth is not reversible.

Zinc Induction of Cytochrome c Release in Isolated Mitochondrial Preparations

We reported previously that the induction of apoptosis by zinc in PC-3 cells involved the release of cytochrome c from mitochondria into cytosol, which then triggered caspase

activation leading to downstream apoptotic events [6]. However, a key unresolved issue was whether the accumulated cellular zinc acted directly on the mitochondria to release cytochrome c, or if the accumulated zinc triggered some other cellular event that was required to initiate the release of cytochrome c. To address this issue, the direct effect of zinc on isolated mitochondrial preparations was determined (Fig. 4). Figure 4A reveals that exposure of isolated mitochondria from PC-3 cells to either 38 μM or 76 μM zinc (equivalent to 0.01 and 0.02 μmol zinc/mg mitochondrial protein, respectively) significantly increased (approximately 50%) the release of cytochrome c within 10 min of exposure. Further increases (90% and 190% for 38 μM and 76 μM zinc, respectively) of cytochrome c release were evident after 20 min of exposure to zinc. Even after the prolonged period of 60 min of exposure during which the endogenous release of cytochrome c is increased, zinc treatment resulted in the additional (50%) release of cytochrome c. The effects of zinc on cytochrome c release from BPH mitochondria were essentially the same as the effects obtained for PC-3 mitochondria (Fig. 4B). Thus, it is clearly evident that zinc-induction of the release of cytochrome c is due to a direct and rapid effect of zinc on mitochondria. These results extend our earlier report, which showed that zinc induces mitochondrial apoptosis in intact PC-3 cells by means of a stimulatory effect on the release of cytochrome c [6].

It is extremely important to note that (Fig. 4C), in sharp contrast to PC-3 and BPH, neither 38 μM nor 76 μM zinc had an effect on the release of cytochrome c from isolated mitochondria from the HPR-1 cells. This finding is consistent with the absence of zinc induction of apoptosis in these cells under conditions that induce apoptosis in PC-3 and BPH cells (Fig. 1). The results reveal that after zinc treatment the lower cytosol accumulation of zinc by HPR-1 cells as shown in Figure 1 was not the only reason for the absence of an apoptotic effect of zinc. The endogenous level of cytosol zinc is higher in HPR-1 cells than in BPH and PC-3 cells; yet these HPR-1 cells do not exhibit apoptosis (Fig. 1). It is now evident that the mitochondria in these cells, unlike PC-3 and BPH mitochondria, are not responsive to the direct effects of zinc on the release of cytochrome c.

The results presented in Figure 4 indicated that PC-3 and BPH mitochondrial exposure to zinc for 20 min was optimal for the release of cytochrome c. It was then important to conduct a dose-response study to determine the minimal concentration and the range of zinc that could result in the release of cytochrome c (Fig. 5). Studies with BPH mitochondria reveal that a dose-response effect of 20-min exposure to zinc on the release of cytochrome c is evident over the range of 0.002–0.040 μmol zinc/mg protein (equivalent to 7.6–152 μM zinc). Over this range, the cytochrome c released increased from 44% (at 0.002 μmol /mg protein) to approximately 300% (at 0.04 μmol /mg protein) when compared with control mitochondria.

DISCUSSION

These studies extend our previous observations that zinc accumulation in prostate cells induces mitochondrial apoptosis. Most importantly, it is now evident that the induction of mitochondrial apoptosis is due to a direct effect of zinc on the mitochondria. Heretofore, it was thought that the cellular accumulation of zinc could have inducible effects

that were prior to and required for the manifestation of the mitochondrial response. That zinc induces the release of cytochrome c from isolated prostate mitochondria eliminates any required interaction of zinc on non-mitochondrial conditions or components before its mitochondrial effect. The next critical issue to investigate is the mechanism by which zinc alters mitochondrial function that leads to the release of cytochrome c and the onset of apoptogenesis.

It is extremely important to note the difference between the PC-3 and BPH cells vs. the HPR-1 cells. PC-3 cells, BPH cells, and, as previously reported [10], LNCaP cells accumulate high zinc levels when exposed to physiological levels of extracellular zinc. Correspondingly, the accumulation of zinc induces mitochondrial apoptogenesis in these cells. In addition, freshly prepared rat ventral prostate epithelial cells also undergo mitochondrial apoptogenesis when exposed to zinc [6]. Clearly, in zinc-accumulating prostate cells, exposure to zinc results in apoptogenesis. In contrast, the exposure of PZ-HPV cells (purported to be derived from normal peripheral zone) to zinc does not result in the cellular accumulation of high zinc levels and, therefore, does not undergo zinc-induced apoptosis [6]. Similarly, HPR-1 cells do not accumulate high zinc levels and do not exhibit zinc-induced apoptogenesis. Evidently the ability of cells to accumulate high zinc levels is a requirement for the induction of apoptosis by exposure to extracellular zinc (i.e., plasma levels).

However, the present studies reveal another significant relationship. The issue raised is whether the lack of a pro-apoptotic effect of zinc on HPR-1 cells is due to an insufficient level of intracellular zinc and/or due to a lack of responsiveness of the mitochondria to zinc. Under the same experimental conditions that induced the release of cytochrome c from isolated PC-3 and BPH mitochondria, the isolated mitochondria from HPR-1 cells were not responsive to the direct exposure to zinc. It is now evident that the cell-specific effects of zinc induction of mitochondrial apoptogenesis as demonstrated in this and earlier reports [5, 6] are dependent upon two factors: (1) the ability of cells to accumulate sufficiently high levels of mobile reactive zinc necessary to induce mitochondrial release of cytochrome c; and (2) the ability of mitochondria from different cells to respond to the direct effect of zinc. An important issue that has to be pursued is the reason for the different zinc-responsivenesses of mitochondria from different cells. In this regard, we have shown that zinc-altered mitochondrial aconitase equilibrium is unique to prostate mitochondria due to a specific protein not associated with other mitochondria [11].

A direct effect of zinc on the mitochondrial release of cytochrome c was evident at zinc concentrations as low as 7.6 μM (0.004 μmol zinc/mg mitochondrial protein). Although the free zinc concentrations of mammalian cells is unknown, the cytosolic pool of free zinc has been estimated to be 10^{-5} – 10^{-12} M and even as low as 10^{-15} M [12, 13]. The total cellular zinc has been calculated to be in the range of approximately 10^{-3} M. These values are likely higher for prostate cells, because prostate accumulates approximately three to five times more zinc than other tissues. Moreover, cellular reactions of zinc are dependent upon mobile reactive zinc, not solely free zinc, through intermolecular exchange of zinc. This pool of zinc, although its cytosolic concentration is unknown, is magnitudes greater than the free zinc pool. Consequently, the effectiveness of zinc levels in the range of 10^{-6} M zinc as used

in this study likely is representative of in situ cytosolic reactive zinc levels and is, therefore, a physiological effect of zinc in the prostate cells.

The present studies combined with our previous reports demonstrate a consistent and persistent effect of zinc in the induction of mitochondrial apoptogenesis in specific prostate epithelial cells. This effect is observed when the cells are exposed to conditions that are representative of the plasma level of zinc (approximately 2–15 μM). In contrast, most reports with other mammalian cells describe a role of zinc in the inhibition of apoptosis [14, 15]. In the overwhelming majority of those reports, the levels of zinc to which the cells were exposed were in the millimolar range, i.e., unphysiological concentrations to which cells in situ would never be exposed. This, in part, could account for the difference in the zinc effects on apoptosis. This concern is also applicable to the report that zinc induces necrosis in prostate cells. In that study, 200–500 μM zinc (conditions that would never exist in situ) was required to induce necrosis; and the necrotic effect was not evident at lower, but still unphysiologic, levels of zinc [16]. However, similar to our observations with prostate cells, a few reports exist that also demonstrate zinc induction of apoptosis in select mammalian cells [17–19]. In support of our observations, Untergasser et al. recently reported that zinc induces mitochondrial alterations in prostate cells that result in apoptosis [20]. The divergent reported effects of zinc as an inhibitor of apoptosis in some cells or conversely a stimulator of apoptosis in other cells might be due, in part, to the ability or lack thereof of the mitochondria in different cells to respond to the direct apoptogenic effect of zinc as demonstrated in this present study. Consequently, the generalization that zinc is a physiological inhibitor of apoptosis in mammalian cells is not tenable. Recognition of the cell-specific role of zinc in apoptosis and cell growth is essential. Moreover, our earlier report combined with this present report with prostate cells provides the first demonstration in any mammalian cells (to our knowledge) that the zinc-induced apoptotic effect is targeted directly at the induction of mitochondrial apoptogenesis.

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Zinc Accumulation in Cytosol			
	Control	Zinc 1µg/ml	Zinc/Control
PC-3	4.2 ± 0.4	18.1 ± 2.4	4.3 ± 0.3
BPH	7.5 ± 1.1	20.5 ± 0.9	2.8 ± 0.4
HPR-1	9.0 ± 1.3	14.1 ± 1.2	1.8 ± 0.1

Fig. 1.

Accumulation of zinc, which induces apoptosis in human prostatic malignant PC-3 and BPH cells but not in human HPR-1 cells derived from normal prostate epithelial cell. The cells were cultured in six well/plate at a density of approximately 4×10^5 cells/well in the growth medium. Twenty-four hours before the zinc treatment, growth medium was replaced by serum and/or growth hormone-free medium. Zinc (1, 000 ng/ml) was then added for another 24 hr. Triplicate cultures were used for each treatment. Cell morphologic features were observed and photographed under a microscope (Nikon, Eclipse, TE200). Table: The cellular accumulation of zinc was measured by TSQ as described in the Materials and Methods.

Fig. 2. Effect of zinc on DNA fragmentation in PC-3 and BPH cells. Shown is a representative electrophoretic fragmentation pattern of DNA isolated from PC-3 and BPH cells treated with or without zinc (250, 500, and 1,000 ng/ml) for 24 (PC-3 only) and 48 hr. Medium and the zinc treatment were replaced every 24 hr.

Fig. 3.

The inhibitory effect of zinc on PC-3 cell growth is time-dependent (A) and is not reversible (B). PC-3 cells were treated with (right panels) or without (left panels, control) zinc (1,000 ng/ml) for 2–24 hr, as indicated. After each treatment, zinc medium was removed and cells were washed once with serum medium and followed by another 24-hr culture in fresh growth medium (B). The cell morphologic changes were observed and recorded by photograph.

Fig. 4.

Zinc induction of mitochondrial cytochrome c release in prostatic cell-free system. Mitochondria were prepared from PC-3 cells (A), BPH cells (B), HPR-1 cells (C) as described in the Materials and Methods section and were incubated with or without zinc (0.01–0.02 μ M/mg protein) for the time periods indicated. The levels of released cytochrome c determined by Western blot are presented by the relative O.D. from densitometry scan of the cytochrome c (Cyto c) bands corrected by the values of β actin as internal controls.

Fig. 5.

Zinc induction of mitochondrial cytochrome c release in BPH cell-free system is concentration dependent. Mitochondria prepared from BPH cells were incubated with zinc at the concentrations indicated for 20 min at 30°C. The cytochrome c (Cyto c) release was determined as described in Figure 4.