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Abstract

Prostate cancer is an age-related disease that is linked to the inability of prostate cells to accumulate zinc following transformation. It is shown in the present study that the basal percentage of normal prostate cells expressing senescence-associated β-galactosidase (SA-β-gal) is higher than that of the cancer cells. In the presence of high zinc in the cell culture medium, the percentage of normal prostate cells expressing the SA-β-gal increased but not that of the cancer cells. Increased intracellular zinc occurs in the prostate cancer cells treated with supraphysiologic concentration of zinc but it does not induce senescence or decrease the telomerase activities in these cells. Senescence, however, occurred when the prostate cancer cells DNA is damaged by irradiation. These findings suggest that prostate cancer cells are insensitive to the senescence-inducing effects of zinc but the cancer cells retain the capacity to undergo senescence through other pathways.

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Introduction

Prostate cancer is one of the most common malignancy affecting Western males [1]. Genetic predisposition and numerous environmental factors, including diet and hormonal changes, play important roles in the pathogenesis of the disease [2]. Prostate cancer tends to occur more commonly in older men, suggesting that ageing is possibly one of the contributing factors. Abnormal prostatic growth such as benign prostatic hyperplasia (BPH) is also common in older males and this is thought to be due to hormonal changes during aging, which affects the proliferative and survival capacity of prostate cells. Various factors such as reduced apoptosis rate and increased basal proliferative rates of prostate tissue contribute to BPH [3]. More recently, numerous prostate gland epithelial cells expressing the senescence marker, senescence-associated β-galactosidase (SA-β-gal) were found in BPH patients with prostatic enlargement, suggesting that senescence may have a role in the development of prostatic enlargement [4–5]. Even though the association of senescence with the development of prostate cancer is not known, loss of senescence potentials in prostate cells, perhaps, may have a role in prostate tumorigenesis. One characteristic feature of the tissues of the prostate gland unlike other tissues is their ability to accumulate uniquely high concentration of zinc, which is toxic to tissues elsewhere in the body. This characteristic, however, is lost when the prostate cells are transformed [6]. The significance of high zinc concentration in these cells is not known but zinc is an important essential trace element that affects various enzymes and transcription factors important for normal cell proliferation and
differentiation. It modulates DNA replication, protein synthesis and the cellular signaling pathways [7]. It is still unknown how exactly the relationship between prostate cell senescence and zinc accumulation capability could contribute to the development of prostate cancer. The present study, hence, aimed to investigate the effects of zinc supplementation on the induction of senescence in prostate normal and cancer cell cultures.

Materials and methods

Cell culture

Human prostate cancer cells LNCaP.FGC was purchased from American Type Culture Collection (ATCC, USA). Human normal prostate epithelial cells, PNT2, were obtained as gift from Dr. R. Rosli, Universiti Putra Malaysia. All cells were cultured in RPMI 1640 (Flowlab, Australia) supplemented with 10% fetal bovine serum (FBS; Biowhittaker, USA), nonessential amino acid (1 mM), L-glutamine (2 mM) and 10 mM HEPES at 37 °C in humidified atmosphere of 5% CO2.

Zinc studies

Approximately 5 × 10⁴ cells were seeded into 60 mm tissue culture petri dishes (Falcon, USA). The following day, cells were pre-treated with zinc pyrithione, 1-hydroxypyridine-2-thione (250 µM; Sigma, USA) for 30 min at 37 °C to assist in zinc uptake. The treatment medium was then removed and replaced with growth medium containing various concentrations of zinc (3.5, 35, 70 and 175 µM ZnSO4 prepared in serum-free medium) and incubated for 72 h. For controls, the cells were either left untreated or treated with only zinc pyrithione and incubated in parallel with the zinc-treated cells. Zinc concentration in growth medium supplemented with FBS prior to addition of zinc supplements was found to be ~3.4 µM. For long-term zinc treatments, cells were propagated in growth medium supplemented with additional zinc (35 µM) for up to 24 passages (one passage per week). Control cell cultures were supplemented with only the zinc diluent and propagated in parallel with the zinc-supplemented cells. Chelation of zinc from cells was performed by treating cells with N, N, N', N'-tetrakis(2-pyridylmethyl)-ethylenediamine (TPEN; Sigma USA). Briefly, the cell growth medium was replaced with serum-free medium containing TPEN (100 µM). The cell culture was then incubated at 37 °C for 90 min.

SA-β-galactosidase pH 6.0 staining

Cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde prepared in phosphate-buffered saline (PBS). The fixed cells were washed twice in PBS and then stained overnight with 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) staining solution consisting of 1 mg/mL X-gal, 40 mM citric acid/sodium phosphate (pH 6.0), potassium ferricyanide (5 mM), potassium ferrocyanide (5 mM), NaCl (150 mM) and MgCl₂ (2 mM). Cells expressing SA-β-gal at pH 6.0 were stained blue when viewed under a microscope. The percentage of senescent cells was determined by counting the blue-stained cells and dividing it with the total number of cells observed in a microscopic field. The average of at least 10 microscopic fields was used per datum and each experiment was performed in duplicates and repeated twice. Gamma-irradiated (10 Gy) cells incubated in parallel were used as positive control for senescing cells. Cells cultured in growth medium without any treatment and cells treated with only zinc pyrithione were used as negative controls for the treatment.

Intracellular zinc staining

Approximately 5 × 10⁴ LNCaP cells were seeded onto 13 mm in diameter glass cover slips (Menzel-Glaser, Germany). The cover slips were placed in 24 well tissue culture plates. The cells were grown to 70% confluency, washed twice in PBS and fixed with 4% paraformaldehyde on ice for 30 min. After fixation, the cells were washed in PBS, briefly rinsed in water and air dried. The slides were stained with 25 µM Zinquin ethyl ester (Calbiochem, USA) in PBS in a humidified chamber for 30 min at 37 °C. Following the incubation, the slides were washed twice in PBS and briefly dipped into propidium iodide solution (10 µg/ml). The slides were then washed as above, rinsed in water, air dried and observed under a UV-equipped microscope using the DAPI and Texas Red filter (Leica Microsystem, Germany).

Telomerase assay

Telomerase activity was performed using the TRAPEze telomerase detection kit (Chemicon, USA) following the manufacturer’s protocol. Briefly, 1 × 10⁶ cells were trypsinized and washed with PBS. For telomerase extract preparation, the cell lysates were incubated on ice for 30 min with 1 × CHAPS lysis buffer (10 mM Tris–HCl, pH 7.5, 1 mM MgCl₂, 1 mM EGTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 0.5% CHAPS and 10% glycerol), followed by centrifugation at 12,000 g for 20 min at 4 °C. The resulting supernatant was transferred into new tubes and protein concentration was determined using Micro BCA Protein Assay System (Pierce, USA). Prior to telomere repeat amplification, the PCR mixture and 750 ng/µl of telomerase extract were incubated at 30 °C for 30 min followed by PCR cycling at 94 °C for 30 s; 50 °C for 30 s and 72 °C for 1 min for 30 cycles [8]. The amplified DNA fragments were electrophoresed on 12.5% SDS-PAGE gels at 160 V for approximately 76 min. The gels were then stained with ethidium bromide and observed under UV light.
stained with ethidium bromide (10 μg/ml). Band intensity detection was performed using BioNumerics version 2.50 (Applied Maths, Kortrijk, Belgium).

**Statistical analysis**

Statistical analyses were performed using GraphPad Prism Version 4.03 (GraphPad Software Inc., USA). Data were expressed as the mean ± standard error of mean (SEM). Statistical differences were analyzed using one-way ANOVA by comparing all data to those obtained for the cell cultures treated with zinc pyrithione only. A p-value < 0.05 was considered as statistically significant difference.

**Results**

The effects of zinc on the induction of senescence in prostate cell cultures were analyzed using the senescence marker, SA-β-gal following 72 h of exposure. Cells expressing SA-β-gal were enlarged and flattened with heavily granulated cytoplasm as previously described [9,10]. These cells were observed in both the prostate normal, PNT2 (Fig. 1a) and prostate cancer, LNCaP cell cultures (Fig. 1b). The basal level of senescent cells in both the prostate normal and cancer cell cultures were ~27% and 11%, respectively (Fig. 2). The percentage of senescent cell in both the PNT2 prostate normal and LNCaP prostate cancer cell cultures increased two-fold to ~54% and 38%, respectively, when cellular senescent was induced by γ-irradiation (10 Gy). This finding shows that both the PNT2 prostate normal and LNCaP prostate cancer cell retained the capacity to undergo senescence.

The percentage of senescent cells in prostate normal cell cultures (24–27%) treated with zinc (3.5–70 μM) were comparable to the percentage of senescent cells noted in the untreated cell culture (27%) and the zinc pyrithione-treated prostate normal cell cultures, 28% (Fig. 2). Similarly, the percentage of senescent cells in LNCaP cell cultures treated with 3.5–70 μM zinc did not increase and remained comparable to the percentage of senescent cells in the non-treated LNCaP cell culture (11%) or LNCaP cell culture treated with zinc pyrithione only, 12% (Fig. 2). Conversely, when the PNT2 cells were treated with high concentration of zinc (175 μM), the percentage of senescent cells significantly increased (~34%; p < 0.05, ANOVA) in comparison with the control LNCaP cells treated with zinc pyrithione only (~28%). No change in the percentage of senescent cells, however, was noted in LNCaP prostate cancer cell culture treated with 175 μM of zinc (~12%) when compared to the LNCaP cells treated with zinc pyrithione only, 12% (Fig. 2). These

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**Fig. 1.** Senescence-associated β-galactosidase staining of PNT2 prostate normal and LNCaP prostate cancer cells. PNT2 prostate normal cells (a) and LNCaP prostate cancer cells (b) expressing senescence-associated β-galactosidase activity at pH 6.0 (SA-β-gal) are stained blue (black arrow) and cells negative for SA-β-gal staining are indicated by white arrow. Cells were viewed and photographed under 40 × magnification using Nikon D70 camera (Japan).

**Fig. 2.** Percentage of senescent prostate cells following treatment with zinc. PNT2 prostate normal cells and LNCaP prostate cancer cells were treated with various concentrations of zinc (3.5–175.0 μM) following pre-treatment with zinc pyrithione to increase zinc uptake. The cells were stained with senescence-associated β-gal staining solutions at pH 6.0 following 72 h exposure to zinc. Prostate cells subjected to γ-irradiation were used as positive control while the negative control cells were cells that were either left untreated or treated with zinc pyrithione only. Results are expressed as the mean of the percentage (± SEM) of senescent cells generated from counting the blue-stained cells and dividing it by the total number of cells observed per microscopic field for 10 microscopic fields per datum. Statistically significant data, when compared to cell cultures treated with zinc pyrithione only (p < 0.05, ANOVA), are marked with asterisks (*).
observations show that high intracellular zinc induced senescence in the PNT2 prostate normal cells but not the LNCaP prostate cancer cells.

The concentration of intracellular zinc in the zinc-treated cells was determined by staining with Zinquin, a zinc-specific fluorescent probe. Both the PNT2 and LNCaP cells showed the presence of characteristic high-intensity fluorescing vesicular structures of zincosomes in the cytoplasm when stained with Zinquin, the zinc-specific fluorescent probe (Figs. 3a and b). Similar observations were made for LNCaP cells treated with only zinc (35 μM) and zinc (3.5 μM) in the presence of zinc pyrithione (Fig. 3c and d). The zincosomes, however, were absent in the zinc-supplemented cells treated with TPEN, a zinc chelator (Fig. 3e). These cells exhibited a dull homogenous fluorescence, which is not representative of zinc-specific staining. The presence of zincosomes in LNCaP cells treated with zinc suggests the presence of intracellular zinc in these cells.

Prolonged supplementation of the LNCaP cells with 35 μM zinc resulted in senescence in ~11–14% cells. No increase in the percentage was noted from passage 5 up to passage 24, the last time point of the study (Fig. 4). Telomere shortening repeatedly occurs during each cell division and this eventually leads to cellular senescence in normal cells. Cancer cells are known to have overcome senescence control by expressing telomerase activities for the extension of telomeric repeats to compensate for the telomere shortening [11]. The telomerase activities of the LNCaP prostate cancer cells, hence, were determined following prolonged exposure to zinc to investigate the effects of zinc on the cell telomerase activities. The telomerase activities of both the zinc-treated (Figs. 5a and b, lane 4) and non-treated cells (Figs. 5a and b, lane 3), measured using TRAPeze telomerase assay, showed the typical laddering of DNA fragments with six-base increments, similar to the positive control samples (Figs. 5a and b, lane 1). In contrast, both the negative control and heat-inactivated zinc-treated samples showed only the internal control bands (Figs. 5a and b, lane 2; Fig. 5b, lane 5). These observations confirmed the presence of telomerase activity in the prostate cancer cells. A comparison of the intensities of the ladders, however, did not indicate any significant difference in the telomerase levels of both the zinc-treated and control cells (Figs. 5a and b, lanes 3 and 4). These findings suggest that prolonged exposure to zinc does not induce senescence or affect telomerase activity of prostate cancer LNCaP cells.

Discussion

Reproducible senescence is a stage where cells cultured in vitro have achieved their proliferative capacity and arrested at G1 phase of the cell cycle. It results from
gradual shortening of the telomeres at the ends of chromosomes, which then triggers a cascade of events leading to cell cycle arrest [12–13]. The role of zinc in ageing and immunity is well established, where zinc deficiency and impaired cell-mediated immunity in ageing individuals increase susceptibility to infection and other age-related diseases [14]. As zinc is an antioxidant, deregulation of zinc metabolism also results in increased susceptibility to free radical damage and therefore malignant transformation [15]. Moreover, certain molecular and biological changes observed in ageing are associated with pathological features of malignancy [16]. Accumulations of senescing prostate epithelial cells were reported in prostate tissues of BPH patients [4–5] and zinc, which is present in uniquely high levels in the prostate normal cells, is reduced in prostate adenocarcinoma [6]. Taken together, these earlier findings are suggestive of the role of senescence in the development of prostate cancer but the association of senescence with zinc, however, requires further investigations as reports of the role of zinc in prostate senescence are limited.

In the present study, we showed that LNCaP prostate cancer cells are inherently less susceptible to senescence when cultured under similar culture conditions compared to that of the PNT2 prostate normal cells. The LNCaP cells are also not susceptible to zinc-induced senescence as the percentage of senescent cells is less in cell cultures treated with high zinc or prolonged cultivation in growth medium supplemented with supraphysiologic concentration of zinc. This is in contrast to PNT2 cells, which exhibited increased percentage of senescent cells when treated with high zinc. The inability to accumulate intracellular zinc reported for cancerous prostate tissues is not likely to have played any role in its failure to undergo zinc-induced senescence as the LNCaP cells, as shown in the present study, accumulated and retained zinc, especially in cell cultures maintained in growth medium supplemented with high zinc.

Zinc is known to affect various cellular pathways. It affects cell replication, differentiation, division, growth arrest, signal transmission and gene expression [17]. Our results, which showed that zinc induces senescence in PNT2 cells, are important as they relate to the role of zinc in regulating cellular pathways associated with induction of senescence. The inability to respond to the regulatory effects of zinc such as that shown in LNCaP cells in the present study could be amongst the reasons why prostate cells become transformed and cancerous, as the cells are no longer responsive to cellular signals to undergo senescence. Hence, this perhaps explains the high accumulation of zinc in normal prostate as it plays a regulatory role in maintaining healthy prostate tissues.
by tightly regulating cellular senescence. Results from telomerase assay further reinforced the unresponsiveness of LNCaP prostate cancer cells to the senescence-inducing effects of zinc although high zinc was reported to affect telomerase activity in the androgen-independent prostate cancer cell line, DU145 [8].

Findings from the present study highlight the potential ineffectiveness of dietary zinc supplementation or zinc-based therapy once the prostate cells have become transformed or cancerous. High zinc could instead superinduce the cells’ resistance to zinc induction of senescence signals, causing the cells to become recalcitrant to other treatment modalities. However, results from this study shows that although the LNCaP prostate cancer cells do not respond to zinc-induced senescence, the cells could still undergo senescence comparable to the normal prostate cells when the genome is severely damaged by gamma irradiation. These imply that the capacity to undergo senescence is still retained in LNCaP prostate cancer cells and this offers hope that there are potentially other pathways that can be used to induce senescence in prostate cancer cells.

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