ABSTRACT

Objective: To investigate the antiproliferative effects of zinc-citrate compound on hormone refractory prostate cancer (HRPC).

Methods: HRPC cell line (DU145) and normal prostate cell line (RWPE-1) were treated with zinc, citrate and zinc-citrate compound at different time intervals and concentrations to investigate the effect of zinc-citrate compound. Mitochondrial (m)-aconitase activity was determined using aconitase assay. DNA laddering analysis was performed to investigate apoptosis of DU145 cells. Molecular mechanism of apoptosis was investigated by Western blot analysis of P53, P21Waf1, Bcl-2, Bcl-xl and Bax, and also caspase-3 activity analysis.

Results: Treatment with zinc-citrate compound resulted in a time- and dose-dependent decrease in cell number of DU145 cells in comparison with RWPE-1. M-aconitase activity was significantly decreased. DNA laddering analysis indicated apoptosis of DU145 cells. Zinc-citrate compound increased the expression of P21Waf1 and P53, and reduced the expression of Bcl-2 and Bcl-xl proteins but induced the expression of Bax protein. Zinc-citrate compound induced apoptosis of DU145 cells by activation of the caspase-3 pathway.

Conclusion: Zinc-citrate compound can induce apoptotic cell death in DU145, by caspase-3 activation through up-regulation of apoptotic proteins and down-regulation of antiapoptotic proteins.

Key words: Prostatic neoplasm; Zinc; Apoptosis

INTRODUCTION

Prostate cancer is the most frequently diagnosed malignancy in men in Europe and the United States[1]. Despite improvements in early detection and treatment, up to 40% of men develop biochemical recurrence after radical retropubic prostatectomy[2]. Although antiandrogen therapy is initially effective, resistance inevitably occurs within a few years. There are limited options for hormone-refractory prostate cancer (HRPC). Zinc has antioxidant, anti-inflammatory and proapoptotic activities and plays a role in genetic stability and function[3,4]. Higher concentration of zinc is maintained in normal prostatic cells in comparison with other cells and the change of zinc concentration within the prostate is associated with the development of prostate cancer[5].

Citrate, one of the components of the Krebs cycle, has been reported to elevate the solubility of heavy metals by binding to heavy metal ions, and upon binding with zinc it may increase the bioavailability of zinc[6]. In addition, similar to zinc, citrate is accumulated in normal prostatic cells at high concentrations, and the concentration has been found to be markedly decreased in prostate cancer cells[5].

We demonstrated the antiproliferative activity of zinc-citrate compound on the HRPC cell line and investigated its inhibitory mechanism of action.

MATERIALS AND METHODS

Preparation of Zinc-citrate Compound

Zinc-citrate compound was composed of zinc chloride (Sigma Chemical Co., St. Louis, MO, USA) and citric acid anhydrous (Sigma Chemical Co., St. Louis, MO, USA), and boiled for 15 min in a 121°C autoclave.
**Cell Culture**

Normal prostate cell line, RWPE-1 (ATCC, Manassas, VA, USA) and androgen-independent prostate cancer cell line, DU145 (Korean Cell Line Bank, Seoul, Korea) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 300 mg/L L-glutamine, 25 mmol/L Ca²⁺, Mg²⁺-free Hanks' solution containing 25 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 25 mmol/L NaHCO₃.

**Intracellular Zinc Concentration**

Intracellular zinc concentration was measured with Zinc Assay Kit (Sentinel CH, Milan, Italy). RWPE-1 and DU145 cells were treated with zinc-citrate compound or zinc only for 1, 3, and 6 h. Cells were centrifuged at 1,500 r/min for 5 min. Cells (1×10⁶/100 μl in PBS) were sonicated three times for 10 s, and centrifuged at 14,000 r/min. Solution AC was prepared with Reagent C 40 mg and 1 vial of Reagent A; 5 μl of sample was mixed with 100 μl of solution AC and 10 μl of Reagent C and was read in a Bio-Rad Model 3550 Microplate Reader (Richmond, CA, USA).

**Cell Viability Assay**

RWPE-1 and DU145 cells were treated at different time intervals and with varying doses to evaluate the effects of zinc-citrate compound on cell growth and survival. Cells were inoculated into 96-well plates at the density of 1×10³ cells/well and incubated for 12 h. Subsequently, the cells were cultured in the media containing diverse concentrations of zinc or citrate only, or a zinc-citrate compound (0.25/5, 0.5/10, 1/20, 2/40 mmol/L) for 24 h. IC₅₀ (the half maximal inhibitory concentration) was obtained, and the cells were incubated with zinc-citrate compound at IC₅₀ concentration for 6, 12, 24, and 48 h. After the exposure to the zinc-citrate compound, 20 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (1 mg/ml in PBS) was added to each well, and incubated at 37°C for 4 h. The plates were read at 450 nm wavelength using the Bio-Rad Model 3550 Microplate Reader (Richmond, CA, USA). The wells containing only RPMI 1640-FBS and MTT were used as the control group.

**Mitochondrial Aconitase Activity**

Mitochondrial (m)-aconitase activity in cell extract was measured using the Bioxytech aconitase-340 assay (Oxis Research, Foster City, CA, USA). RWPE-1 cells and DU145 cells were treated with the zinc-citrate compound for 1, 2, and 4 h, and observed at 340 nm wavelength for 5 min at 37°C.

**DNA Laddering Analysis**

RWPE-1 cells and DU145 cells (2×10⁶) treated with the zinc-citrate compound (0.4/8 mmol/L) for 6, 12, 24, or 48 h were washed with PBS, and 200 μl binding/lysis buffer was added. The total volume was adjusted to 400 μl, and the solution was mixed. After incubating at 15–25°C for 10 min, 100 μl isopropanol was added, and shaken. To a filter tube, a collection tube was attached and subsequently the samples were added to the filter tube and centrifuged at 800 r/min for 1 min. A total of 500 μl washing buffer was added and centrifuged at 800 r/min for 1 min twice, and centrifuged a third time at 13,000 r/min for 10 s. Elution buffer 100 μl was added, and centrifuged at 800 r/min for 1 min. Finally, electrophoresis was performed on 1% agarose gel at 75 V voltage for 1.5 h, and assessed under ultraviolet light.

**Western Blot Analysis**

DU145 cells treated with the zinc-citrate compound for 6, 12, 24, or 48 h were extracted by centrifuging at 4°C, 2,000 r/min, for 5 min, and lysing by the reaction with 100 μl lysis buffer for 30 min at room temperature. DU145 cell lysate exposed to the zinc-citrate compound (50 μg of protein) was electrophoresed on 10% sodium dodecyl sulfate (SDS)/polyacrylamide gel at 100 V for 2 h, and treated for 1 h using PBS containing 5% non-fat milk and 0.05% Tween-20. P53 and P21waf1 were assessed, and the antiapoptotic protein, Bcl-2 as well as Bcl-xL and the proapoptotic protein, Bax were assayed. The samples reacted with the primary antibody to P53, P21waf1, Bcl-2, Bcl-xL, and Bax (diluted 1:500) at 4°C overnight. Membranes were washed 3 times with PBS containing 0.05% Tween-20, and reacted with anti-mouse or anti-rabbit secondary antibody (Calbiochem, San Diego, CA, USA) diluted 1:5,000 for 1 h at room temperature. Each membrane was washed with PBS containing 0.05% Tween-20 three times. Specific protein bands were examined using the ECL Western blotting system (Amersham, Piscataway, NJ, USA).

**Caspase-3 Activity Assay**

RWPE-1 cells and DU145 cells exposed to the zinc-citrate compound were washed with PBS, and 50 μl lysis buffer was added and kept on ice for 10 min. Cell lysate was centrifuged at 4°C and 12,000 r/min, and the supernatant was transferred to a new tube, and stored on ice. Caspase-3 activity was measured with Caspase-3 colorimetric protease assay kit (BioSource, Camarillo, CA, USA). Lysis buffer 50 μl was adjusted to contain 100 μg of protein. Then, 10 μl 0.1 mol/L dithiothreitol (DTT) was mixed with 1 ml 2× reaction buffer, 50 μl 0.1 mol/L DTT-2× reaction buffer was added to the samples, and 5 μl 4 mmol/L Asp-Glu-Val-Asp p-nitroanilide (DEVD-pNA) was added, then wrapped with aluminum foil, and kept at 37°C for 2 h. Absorbance was measured at 405 nm using an
enzyme-linked immunosorbent assay (ELISA) plate reader.

**Statistical Analysis**

All experiments were performed on three separate cultures. All data were presented as $\bar{x} \pm s$, where $P<0.05$ was considered to be statistically significant. Overall comparisons between groups were performed using SAS 8.0 (SAS Institute, Raleigh, NC, USA). Analysis of variance (ANOVA) with Duncan test was performed to detect differences over the time course and between the groups.

**RESULTS**

**Changes of Intracellular Zinc Concentration of Cell Lines**

The zinc concentration in DU145 cells before the addition of the zinc-citrate compound was markedly low in comparison with RWPE-1 cells ($P<0.0001$). The zinc concentration in DU145 cells was significantly increased after the addition of the zinc-citrate compound ($P<0.0001$). However, the zinc concentration in RWPE-1 cells was not increased ($P=0.1467$). The elevation of the zinc concentration of the zinc-citrate compound group was higher than that of the zinc only group in DU145 cells ($P<0.0001$) (Figure 1).

**Figure 1.** Intracellular zinc concentration. A: Treatment with zinc-citrate compound (0.5/10 mmol/L); B: Treatment with zinc (0.5 mmol/L). The elevation of the zinc concentration of the zinc-citrate compound group was higher than that of the zinc only group in DU145 cells ($P<0.0001$). NT: No treatment.

**Result of Cell Viability Assay**

DU145 cells exposed to the zinc-citrate compound showed a pattern of the decrease in a time- and dose-dependent manner. DU145 cells exposed to the diverse concentrations of the zinc-citrate compound for 24 h showed a clear reduction of cell viability in MTT assay ($P<0.0001$). In DU145 cells, the antiproliferative effect was higher in the zinc-citrate compound group than in the zinc or citrate only group ($P<0.0001$). In contrast with DU145 cells, the antiproliferative effect in RWPE-1 cells was lower in the zinc-citrate compound group than in the zinc or citrate only group ($P<0.0001$) (Figure 2).

The IC50 of zinc-citrate compound against DU145 was estimated to be 0.4 mmol/L. DU145 cells and RWPE-1 cells were exposed to 0.4/0.8 mmol/L of zinc-citrate compound for 48 h, and the reduction of cell viability depending on time in DU145 cells was clearly observed in comparison with RWPE-1 cells ($P<0.0001$). In DU145 cells, the antiproliferative effect was higher in the zinc-citrate compound group than in the zinc or citrate only group ($P<0.0001$). The antiproliferative effect in RWPE-1 cells was lower in the zinc-citrate compound group than in the zinc only group ($P<0.0001$), and a statistical difference from the citrate only group was not shown ($P=0.990$) (Figure 3).

**Figure 2.** Result of cell viability assay. In DU145 cells, the antiproliferative effect was higher in the zinc-citrate compound group than in the zinc or citrate only group ($P<0.0001$).

**Figure 3.** Result of cell viability assay at IC50. In DU145 cells, the antiproliferative effect was higher in the zinc-citrate compound group than in the zinc or citrate only group ($P<0.0001$).
Effect of Zinc-Citrate Compound on M-Aconitase Activity

M-aconitase activity was decreased significantly after the addition of the zinc-citrate compound to DU145 cells ($P<0.0001$) although it was maintained constantly in RWPE-1 cells ($P=0.1579$) (Figure 4).

Apoptotic Effect of Zinc-Citrate Compound on DU-145 cell

The migration of fragmented DNA as a laddering pattern was observed by electrophoresis in DU145 cells although it was not observed in RWPE-1 cells (Figure 5).

Through Western blot method, in DU145 cells, when the zinc-citrate compound was added, the elevation of the expression of P53 and P21waf1 was shown. The expression of the antiapoptotic proteins Bcl-2 and Bcl-xL was reduced, and simultaneously, the expression of the proapoptotic protein Bax was elevated, and the Bcl-2/Bax ratio and the Bcl-xL/Bax ratio were decreased with time (Figure 6).

Figure 4. Result of m-aconitase activity. M-aconitase activity was decreased significantly after the addition of the zinc-citrate compound to DU145 cells ($P<0.0001$) although it was maintained constantly in RWPE-1 cells ($P=0.1579$).

Figure 5. Result of DNA laddering analysis. The migration of fragmented DNA as a laddering pattern was observed by electrophoresis in DU145 cells although it was not observed in RWPE-1 cells.

Figure 6. Western blot results. The expression of the antiapoptotic proteins Bcl-2 and Bcl-xL was reduced, and simultaneously, the expression of the proapoptotic protein Bax was elevated, and the Bcl-2/Bax ratio and the Bcl-xL/Bax ratio were decreased with time.
medium-induced apoptosis, high zinc concentrations induce apoptosis or increase serum-free low zinc concentrations (from 7.5 to 15.0 μmol/L) depending on the zinc concentration used. Although an opposite effect on apoptosis, inhibiting or increasing medium and DEX-induced thymocyte apoptosis. However, Schrantz, et al. [8] reported that low zinc concentrations (from 10 to 50 μmol/L) inhibit both serum-free medium-induced apoptosis, high zinc concentrations (from 75 to 600 μmol/L) inhibit both serum-free medium and DEX-induced thymocyte apoptosis. However, Provinciali, et al. [7] reported that zinc has an opposite effect on apoptosis, inhibiting or increasing it depending on the zinc concentration used. Although low zinc concentrations (from 7.5 to 15.0 μmol/L) induce apoptosis or increase serum-free medium-induced apoptosis, high zinc concentrations (from 75 to 600 μmol/L) inhibit both serum-free medium and DEX-induced thymocyte apoptosis. However, Schrantz, et al. [8] reported that low zinc concentrations (from 10 to 50 μmol/L) inhibit apoptosis whereas higher concentrations of zinc (from 50 to 100 μmol/L) activate a death pathway associated with apoptotic-like features in human Burkitt lymphoma B cell line. The prostatic secretory epithelium accumulates high levels of zinc, approximately 3 to 10-fold higher than other soft tissues. The higher levels of mitochondrial zinc inhibit m-aconitase activity and subsequent citrate oxidation. The inhibition of citrate oxidation eliminates the coupled energy (ATP) production that normally occurs from Krebs cycle oxidation[9]. However, prostate cancer cells characteristically have lost the ability to accumulate zinc and essential energy required for the proliferation of cells which should be supplied by the Krebs cycle. In our experiment, before the addition of the zinc-citrate compound to DU145 cells, the concentration of zinc was noticeably reduced in comparison with RWPE-1 cells, and when the zinc-citrate compound was added to DU145 cells, zinc was accumulated in mitochondria and suppressed m-aconitase activation within 4 hours. Therefore, it is thought that it blocks the oxidation and generation of coupled ATP required for the proliferation of cells, and in doing so suppresses the proliferation of DU145 cells.

Prostate secretory epithelium has the capability of accumulating and secreting extraordinarily high levels of citrate. But citrate level is markedly decreased in prostate cancer[10]. Thus, citrate shows similar characteristics to zinc in normal prostate and prostate cancer. Because of such characteristics that would aid in the prevention and treatment of prostate cancer, interests in zinc are on the rise. Reports have shown that the decrease of blood zinc concentration may be associated with prostate cancer[11], but conversely there is no evidence that the increase of blood zinc concentration is associated with prostate cancer. To provide prostate cancer cells with an environment to accelerate the absorption as well as accumulation of zinc may be a means to prevent the development of prostate cancer. The simplest method may be to elevate blood zinc concentration that would in turn accelerate zinc absorption, plus oral administration may be attempted, however such an approach has several problems. In terms of aging, the bioavailability of zinc decreases gradually, so that even if blood zinc concentration is high, the concentration of zinc in tissues is low[12]. In addition, intracellular absorption of zinc is not carried out by diffusion, but rather carried out by specific zinc transporting mechanisms such as zinc uptake transporter 1 (ZIP1)[13]. In our study, it was administered in the form of zinc-citrate compound, and in DU145 cells, its antiproliferative effects were increased in comparison with cases that used either zinc or citrate only. This may have been due to the fact that when a zinc-citrate compound is administered as opposed to zinc only, the solubility of zinc is greater resulting in the increase of the bioavailability of zinc in cells[9]. On the other hand, in RWPE-1 cells, compared with cells treated with zinc or citrate only, the reduction of antiproliferative effect or the effect of protecting normal cells from apoptosis was observed. For this reason, use of a zinc-citrate compound in contrast to zinc only may have advantages that could ultimately inhibit cancer cells more effectively and protect normal cells from zinc toxicity simultaneously.

Zinc has been reported to play an important role in the activation of caspases. Schrantz, et al. [8] have reported that in human Burkitt lymphoma B cells, zinc interferes in apoptosis by suppressing the activation of caspase-3 but at high concentrations, it rather accelerates it and thus induces apoptosis. In the laddering analysis performed in our study, DNA fragmentation was observed, and thus it was found that the mechanism of cell death caused by the zinc-citrate compound was apoptosis, and depending on the time exposed to the zinc-citrate compound, caspase-3 activity was elevated, and thus it was found that

Figure 7. Caspase-3 activity. In comparison with RWPE-1 cells, in DU145 cells, caspase-3 activity was increased in proportion to the time exposed to the zinc-citrate compound (P<0.0001).

According to caspase-3 activity analysis, in comparison with RWPE-1 cells, in DU145 cells, caspase-3 activity was increased in proportion to the time exposed to the zinc-citrate compound (P<0.0001) (Figure 7).

**DISCUSSION**

Zinc has conflicting reports from stimulation to inhibition of apoptosis, depending upon the cell type or concentration. Provinciali, et al. [7] reported that zinc has an opposite effect on apoptosis, inhibiting or increasing it depending on the zinc concentration used. Although low zinc concentrations (from 7.5 to 15.0 μmol/L) induce apoptosis or increase serum-free medium-induced apoptosis, high zinc concentrations (from 75 to 600 μmol/L) inhibit both serum-free medium and DEX-induced thymocyte apoptosis. However, Schrantz, et al. [8] reported that low zinc concentrations (from 10 to 50 μmol/L) inhibit apoptosis whereas higher concentrations of zinc (from 50 to 100 μmol/L) activate a death pathway associated with apoptotic-like features in human Burkitt lymphoma B cell line.

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apoptosis was ongoing. P53 arrests cell cycle at the G1 stage and induces apoptosis\cite{14}, and P21 intervenes in DNA replication by interacting with proliferating cell nuclear antigen and induces cell arrest between G1 and G2 stages, and is also known to be involved in cellular aging\cite{15}. It has been reported that \textit{p21} gene is controlled primarily by P53 at the transcription level\cite{14}, and in our experiments, it was similarly observed that in DU145 cells treated with the zinc-citrate compound, together with P21\textsuperscript{waf1}, the expression of P53 was increased. This is believed to be due to that P21\textsuperscript{waf1} is an intermediate stage pertinent to the suppression of cell growth through P53.

Bcl-2 family proteins control apoptosis sensitivity by stimulating or suppressing the migration of cytochrome c to the cytoplasm, it is suppressed by the antiapoptotic proteins Bcl-2 and Bcl-xL, and accelerated by the proapoptotic protein Bax\cite{16}. Bax has been shown to suppress the antiapoptosis activity of Bcl-2 by forming a heterodimer with Bcl-2\cite{17}. In this study, after the addition of the zinc-citrate compound to HRPC cell line DU145, the expression of Bcl-2 and Bcl-xL was decreased and the expression of Bax was increased. Bcl-2/Bax ratio and the Bcl-xL/Bax ratio were also decreased. Such results demonstrated that the apoptosis of DU145 cells induced by the zinc-citrate compound may be induced via the reduction of Bcl-2 and Bcl-xL and the increase of the expression of Bax pertinent to the caspase-3 apoptosis pathway.

Zinc-citrate compound suppressed antiapoptotic genes and stimulated apoptotic genes resulting in the induction of apoptosis of HRPC cell line, DU145, possibly through the activation of caspase-3. For the clinical application of the zinc-citrate compound to the prevention and treatment of HRPC, the development of experimental models reflecting the metabolism of zinc-citrate compounds in human prostate cancer is required.

Disclosure of Potential Conflicts of Interest
No Potential conflicts of interest were disclosed.

REFERENCES