Effect of zinc(II) ions on the expression of pro- and anti-apoptotic factors in high-grade prostate carcinoma cells

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Abstract. Several typical characteristics of prostate tissue have been identified including the ability to accumulate zinc(II). However, this feature of prostate cells is lost during carcinogenesis and, thus, prostate cells are unable to accumulate zinc(II) ions in high levels. Therefore, we can expect that zinc(II) ions can significantly contribute to the progression of tumour disease and to the ability of prostate cell lines to metastasize. In this study, we aimed our attention on determining the expression of Bcl-2, c-Fos, c-Jun, Ki-67, NF-κB and p53 genes in two prostate cell lines, as the 22Rv1 cell line, a model of aggressive partially androgen-sensitive prostate cancer and the PNT1A cell line, a normal prostate cell line model. Moreover, we were interested in the mechanisms through which exposure of these cell lines to zinc(II) ions could influence expression of the above-mentioned genes. We found that zinc(II) ions caused elevated expression of Ki-67, a marker of proliferation, extremely low expression of p53, high expression of Bcl-2 and no changes in the expression of p53. Our experimental data show different effect of zinc(II) ions on expression of the above-mentioned regulatory genes, which may give us more information on their impact on cancer development and progression with possible using for cancer therapy.

Introduction

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death in males worldwide (1,2). Due to its high incidence and mortality, early diagnosis, identification of highly aggressive clinically silent forms and understanding of disease pathogenesis with typical metabolic differences in order to develop specifically targeted therapy are needed. To target these issues, biochemistry of normal and tumour prostate cells is investigated. Based on these investigations, several typical characteristics of prostate tissue have been found including the ability to accumulate zinc(II) ions (3-7), which are shown in Fig. 1. The intracellular concentration of zinc(II) ions in prostate tissue exceeds up to ten times the concentrations detected in other cell and tissue types. However, this feature of prostate cell lines is lost during carcinogenesis and, thus, prostate cells are unable to accumulate zinc(II) ions in high levels. Therefore, we can expect that zinc(II) ions can significantly contribute to the progression of tumour disease and to the ability of prostate cell lines to metastasize (8).

As a result of numerous studies on cells as well as on prostate cancer patients, several compounds have been found connected with tumourigenesis in prostate cells including Bcl-2. This intracellular protein belongs to a large group of proteins the Bcl-2 family (9), and acts as an inhibitor of apoptosis. Bcl-2 has been established to block apoptotic death in various cell types such as lymphocytes and motoric neurons. It prevents both apoptosis dependent on caspases and oxidative necrosis. Under normal conditions, Bcl-2 is anchored to the outer mitochondrial membrane and heads out into the cytosol, which gives this protein the opportunity to interact with other proteins. These interactions are important for maintaining mitochondrial membrane integrity and function. By binding to the pro-apoptotic family members, Bcl-2 prevents activation of mitochondrial pathway of apoptosis based on the formation of pores that disrupt the permeability of mitochondrial membrane (10-12). It has been suggested that overexpression of the Bcl-2 oncoprotein in human cancer cells contributes to their resistance to chemotherapy- and radiotherapy-induced apoptosis and is connected with unfavourable prognosis (13). The majority of human prostate tumours overexpress Bcl-2,

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which is responsible for tumour resistance to radiotherapy and chemotherapy (14,15). This event is supported by the fact that Bcl-2 knocked down by antisense oligodeoxynucleotides induces radiosensitization in human PC-3 prostate tumour xenografts (13). Moreover, it has been reported that Bcl-2 expression is associated with tumour progression and unfavourable prognosis in prostate cancer patients (13,16,17) and is associated with the development of androgen-independent prostate cancer (18). Possible associations with other proteins connected with tumour processes such as c-Fos, c-Jun, Ki-67, NF-κB and p53 can be expected. Therefore, we aimed our attention at determining of expression of Bcl-2, c-Fos, c-Jun, Ki-67, NF-κB and p53 genes in two prostate cell lines the 22Rv1 cell line, a model of aggressive partially androgen-sensitive prostate cancer and the PNT1A cell line, a model of healthy cell line. Moreover, we were interested in the issue how exposure of these cell lines to zinc(II) ions could influence expression of the above-mentioned genes. The expression levels were correlated with the results obtained with a fluorescence microscopy of treated cells, and zinc and -SH moieties content.

Materials and methods

Chemical and biochemical reagents. RPMI-1640 medium, Ham's F12 medium, fetal bovine serum (FBS) (mycoplasma-free), penicillin/streptomycin and trypsin were purchased from PAA Laboratories GmbH (Pasching, Austria). PBS was purchased from Invitrogen Corp. (Carlsbad, CA, USA). Ethylenediaminetetraacetic acid (EDTA), zinc(II) sulphate (BioReagent grade, suitable for cell cultures), RIPA buffer and all other chemicals of ACS purity were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), unless noted otherwise.

Cell cultures. Two human prostatic cell lines were used in this study: a) PNT1A human cell line established by immortalisation of normal adult prostatic epithelial cells by transfection with a plasmid containing SV40 genome with a defective replication origin; the primary culture was obtained from the normal prostatic tissue of a 35-year old male at *post mortem*, and b) 22Rv1 human cell line derived from a xenograft that was serially propagated in mice after castration. Both cell lines used in this study were purchased from HPA Culture Collections (Salisbury, UK).

Cell cultivation. PNT1A cells were cultured in RPMI-1640 medium supplemented by 10% FBS. 22Rv1 cells were cultured in RPMI-1640 without phenol red (medium) with 10% FBS. The media were supplemented with penicillin (100 U/ml) and streptomycin (0.1 mg/ml), and the cells were maintained at 37˚C in a humidified (60%) incubator with 5% CO_2 (Sanyo, Japan). The passages of PNT1A and 22Rv1 cell lines ranged from 10 to 35 h.

Zinc(II) treatments of cell cultures. Immediately the cells grew up to 50-60% confluence, the cultivation media were replaced by a fresh medium to synchronize cell growth. Cells were cultivated under these conditions for 24 h, then cells were treated with zinc(II) sulphate (0-100 μM for both cell lines) dissolved in fresh medium for 48 h.

RNA isolation, cDNA preparation. High pure total RNA isolation kit (Roche, Basel, Switzerland) was used for RNA isolation. Briefly, cultivation medium was removed and samples were washed twice with 5 ml of ice-cold PBS. Cells were transferred to clean tubes and centrifuged at 15,000 x g for 5 min at 4˚C. After it, lysis buffer was added and RNA isolation was carried out according to the manufacturer's instructions. Isolated RNA was used for cDNA construction. Total RNA (600 ng) was transcribed using transcriptor first strand cDNA synthesis kit (Roche). Prepared cDNA (20 μl) was diluted with RNase-free water to 100 μl and directly analysed by real-time polymerase chain reaction.

Real-time reverse-transcription polymerase chain reaction (RT-PCR). RT-PCR was performed in triplicates using the TaqMan gene expression assay with 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The amplified DNA was analysed by the comparative Ct method using β-actin as an endogenous control. The primer and probe sets for β-actin (assay ID: Hs00185826_m1), Fos (assay ID: Hs00170630_m1), c-Jun (assay ID: Hs00277190_m1), NF-κB (assay ID: Hs00765730_m1), p53 (assay ID: Hs01034649_m1), Bcl-2 (assay ID: Hs99999018_m1) were selected from TaqMan gene expression assay. Real-time PCR was performed under following amplification conditions: total volume of 20 μl, initial denaturation 95˚C/10 min, then 40 cycles 95˚C/15 sec, 60˚C/1 min.

Cell content quantification. Total cell content was analysed using semi-automated image-based cell analyser (Cedex XS, Innovatis, Roche, Basel, Switzerland) according to the following protocol. Cultivation medium was removed and samples were two times washed with 5 ml of ice-cold PBS to maintain only viable cells. Cells were scraped and transferred to clean tubes. Trypan blue solution (Innovatis) was diluted to 0.2% prior to use and added to samples. Following settings were used in operating software: cell type: standard cells, dilution: none, process type: standard. All samples were measured in duplicates.

Measurements of cell viability - MTT test. MTT assay was used to determine cell viability. The suspension of cells was diluted to the density of 5,000 cells/ml in the cultivation medium. Volume of 200 μl was transferred to 2-11 wells of standard microtiter plates. Medium (200 μl) was added to the first and to the last column (1 and 12, control). Plates were incubated for 2 days at 37˚C to ensure cell growth under the same conditions described in Cell cultivation. Medium was removed from columns 2 to 11. Columns 3-10 were filled with 200 μl of medium containing increasing concentration of zinc(II) (0, 50, 100, 150, 200, 250, 300 and 500 μM). As control, columns 2 and 11 were not filled with medium containing zinc(II) ions. Plates were incubated for 24 h; then, media were removed and replaced by a fresh medium, three times a day. Columns 1 to 11 were filled with 200 μl of medium containing 50 μl of MTT (5 mg/ml in PBS) and incubated in a humidified atmosphere for 4 h at 37˚C, wrapped in aluminium foil. After the incubation, MTT-containing medium was replaced by 200 μl of 99.9% dimethyl sulphoxide (DMSO) to dissolve MTT-formazan crystals. Then, 25 μl of glycine buffer was added to all wells and absorbance was

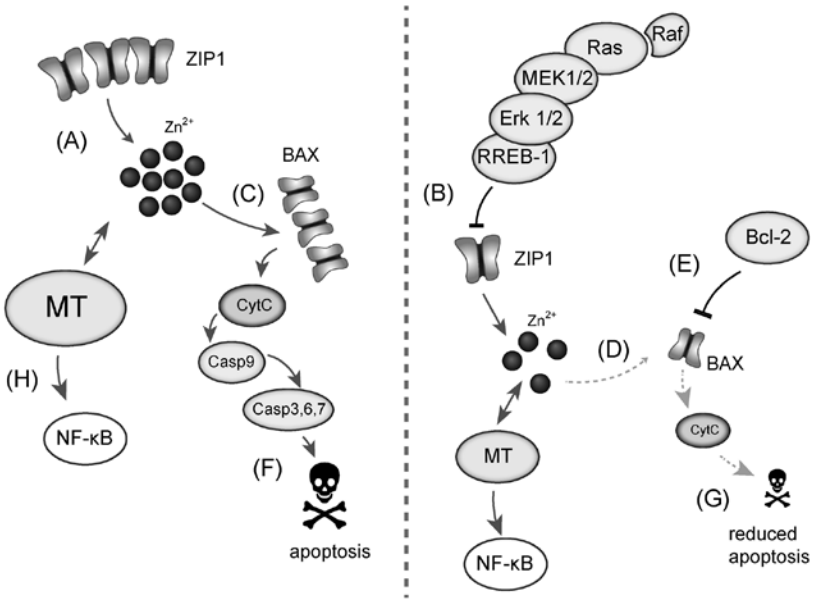

Figure 1. Distinctions in zinc(II) mediated mechanisms in healthy and tumour tissue. (A) In healthy prostate, high level of cellular zinc is present compared to (B) tumour tissue, where zinc-transporter ZIP1 is inhibited by up-regulated Ras-Raf-Mek-Erk cascade. (C) Zinc(II) up-regulates formation of BAX pores on mitochondrial membrane and (F) thus induces cytochrome c (CytC)/caspase(Casp)-mediated apoptosis. On the contrary, in prostate cancer, formation of BAX is inhibited by (E) Bcl-2 and by (D) low intracellular zinc, (G) resulting in lower proapoptic effects. (H) Different zinc(II) levels in healthy and tumour cells interacts with metallotionein (MT), which promotes NF-κB expression differently.

immediately determined at 570 nm (VersaMax microplate reader, Molecular Devices, Sunnyvale, CA, USA).

Cell growth and proliferation assay using impedance measurement with xCELLigence system. The xCELLigence system (Roche Applied Science and ACEA Biosciences, San Diego, CA, USA) consists of four main components: the RTCA analyser, the RTCA DP station, the RTCA computer with integrated software and disposable E-plate 16. Firstly, the optimal seeding concentration for proliferation and cytotoxic assay was determined. After seeding the total number of cells in 200 μl medium to each well in E-plate 16, the attachment, proliferation and spreading of the cells was monitored every 15 min. All experiments were carried out for 250 h. The results are expressed as relative impedance using the manufacturer's software (Roche Applied Science and ACEA Biosciences).

Densitometric and statistical analysis. Software Statistica 10 (StatSoft Inc., Tulsa, OK, USA) was used for statistical analysis. Student's t-test for independent values was used to evaluate differences between two groups. Simple linear correlations were performed to reveal the relationships between variables. Unless noted otherwise, a level of statistical significance was set at $p<0.05$.

Fluorescence microscopy and cell staining. For fluorescence microscopy, cells were cultivated directly on microscope glass slides (75x25 mm, thickness 1 mm, Fischer Scientific, Pardubice, Czech Republic) in Petri dishes in above-described cultivation media (see Cultured cell conditions). Cells were transferred directly onto slides, which were submerged in cultivation media. After treatment, microscope glass slides with monolayer of cells were removed from Petri dishes, rinsed with cultivation medium without zinc(II) supplementation and PBS buffer and directly used for staining and fluorescence microscopy.

For the staining of free thiols, respectively free -SH groups, 5-(bromomethyl)fluorescein (5-BMF, Sigma-Aldrich) was used. This probe reacts more slowly with thiols of peptides, proteins and thiolated nucleic acids in comparison with other fluorescent probes. However, it forms stronger thioether bonds that are expected to remain stable under the conditions required for fluorescence microscopy. Stock solution of 5-BMF (4 mM, anhydrous DMSO) was prepared prior to staining because of 5-BMF stability. Working solution was prepared immediately using stock solution by diluting to final concentration of 20 μM (PBS buffer, pH 7.6). Cells were incubated for 1 h at 37˚C and in dark. Then, cells on microscope glass slide were three times washed by PBS buffer (pH 7.6) and observed using fluorescence microscope (Axioskop 40, Carl Zeiss AG, Oberkochen, Germany) equipped with wideband excitation and set of filters (FITC, DAPI, Carl Zeiss). Photographs were taken using digital camera (Olympus Camedia 750, Olympus, Tokyo, Japan). Program NIS-elements was used for evaluation of intensity of emission, all values were recalculated to control (100%). Ten random fields from each variant and replicate were evaluated.

For free zinc(II) ion staining, fluorescent probe N-(6-methoxy-8-quinolyl)-p-toluene sulphonamide (TSQ, Invitrogen) was used. Working solution (10 μM, phosphate buffer pH 7.6) was prepared by diluting of TSQ stock solution (10 mM, acetone). Cells were carefully rinsed by PBS buffer to remove all cultivation medium containing free zinc(II) ions, subsequently stained by working TSQ solution (30 min, 37˚C, dark), three times washed by PBS buffer (pH 7.6) and observed under a fluorescence microscope (Axioskop 40, Carl Zeiss) equipped by FITC and DAPI filters (Carl Zeiss). Photographs were taken on digital camera (Olympus

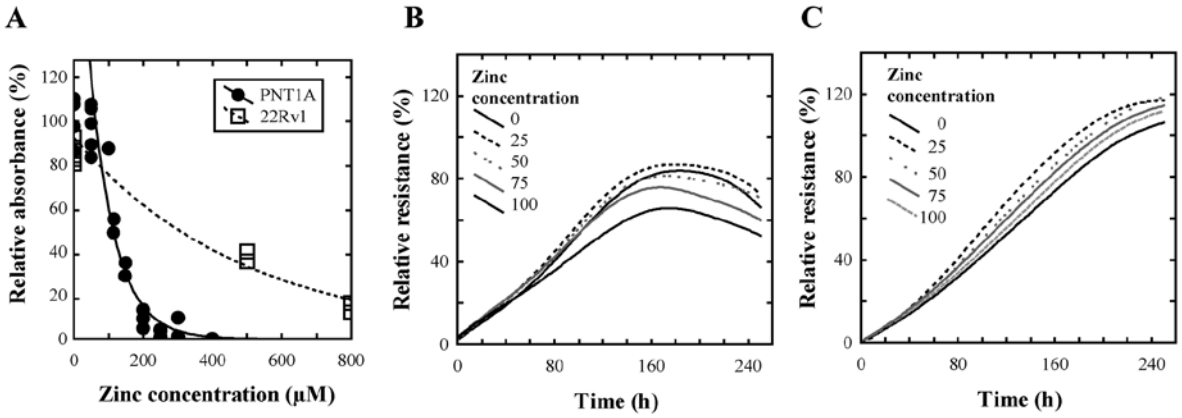

Figure 2. Real-time monitoring of cell adhesion and proliferation using the cytotoxic MTT assay and xCELLigence system. (A) MTT-determined response to zinc(II) treatment. Significantly higher IC_{50} is reached compared to xCELLigence-determined viability in the case of PNT1A and 22Rv1 cells (B vs. C). (B) PNT1A and (C) 22Rv1 cells were seeded at a density of 10,000 cells/well in E-plates 16 (gold working electrodes). After 24 h, the zinc(II) sulphate was added and cell index was monitored (n=2). Other experimental parameters are detailed in Materials and methods.

Camedia 750, Olympus). NIS-element program was used for evaluation of intensity of emission, all values were recalculated to control (=100%). Ten random fields from each variant and replicate were evaluated.

Results

Growth and viability of treated PNT1A and 22Rv1 cells. To select suitable concentration range of zinc(II) ions for treatment of the cell lines, we determined IC_{50} values of zinc(II) for both cell lines using standard MTT cytotoxicity assay with simple linear regression from the descending part of the curve. We obtained IC_{50} corresponding to concentration of 197.9 μM for non-tumour PNT1A and concentration of 369.1 μM for 22Rv1 tumour cells (Fig. 2A). Moreover, we found that PNT1A cells reached stationary phase of growth after 160 h while 22Rv1 cells after 240 h. Thus, we decided to use zinc(II) ion concentrations as follows: 0 (control), 25, 50, 75 and 100 μM for both cell lines for the monitoring of changes in gene expression for 240 h.

Further, we were interested to monitor growth of the cell lines in real time. It is difficult to monitor on-line growth of cell lines during experiment due to the destructive methods for its determination. xCELLigence system offers good possibility to monitor cell growth and proliferation in real time using impedance measurement (19), therefore, we used this system. Primarily, it was necessary to determine optimal cell counts for real time monitoring. We obtained optimal signal with 10,000 cells in each well. This signal corresponded to the cell count. In wells with lower cell count, lower relative impedance signal level was determined and, in some cases, higher relative standard deviation was obtained. Higher cell counts gave also well repeatable results with low relative standard deviation, however, higher count of cells was not optimal from the point of view of 240 h long experiment expecting increasing proliferation and therefore cell count. Therefore, we decided to use 10,000 cells per well to examine the effect of zinc(II) ions on prostatic cells either derived from normal prostate epithelium (PNT1A) or cells derived from primary prostate carcinoma (22Rv1). The IC_{50} values of zinc(II) ions using the xCELLigence system were also determined to evaluate the selected concentration range. These IC_{50} values were as follows: 150.8 μM zinc for PNT1A cells and 445.5 μM zinc for 22Rv1 cells. The changes between these values and values determined using MTT assay can be associated with the fact that both methods are based on measuring of very different physical parameters, which are associated with different physiological phenomena. Based on both measurements of IC_{50} it may be concluded that zinc(II) ions are more toxic to non-tumour cell line at 2.9-fold lower concentration. Based on the obtained results 10,000 cells per well were treated with the above-mentioned concentrations of zinc(II) ions for 240 h. In the beginning of the treatment we observed negligible decrease in relative impedance, i.e. in growth, of PNT1A cells after 15-h long treatment (Fig. 2B) and no changes in cell proliferation of 22Rv1 cells (Fig. 2C). These changes were compared with cell lines treated with 0 μM of zinc(II) ions. Concentrations of 100 μM of zinc(II) ions induced 1.4-and 1.1-fold decrease of relative resistance in PNT1A and 22Rv1 cell lines, respectively, compared to non-treated samples. These results clearly show that the selected concentration range is non-toxic for both cell lines and we can observe the effects of physiological doses of zinc(II) ions on expression of the selected markers as Bcl-2, c-Fos, c-Jun, NF-κB, Ki-67 and p53.

Comparison of the expression of Bcl-2 and other regulatory genes after zinc treatment. Further, we focused on comparison of the base line expression and changes in transcription of Bcl-2, c-Fos, c-Jun, NF-κB, Ki-67 and p53 genes on the RNA level in prostate cell lines treated with zinc(II) ions. Baseline transcription level and zinc(II) ions effect on transcription levels of selected genes was performed by RT-PCR. Fig. 3 shows that 22Rv1 cells demonstrate different expression patterns in monitored genes compared to PNT1A cells. 22Rv1 cell line has 4.5-fold higher level of Bcl-2 anti-apoptotic gene expression (n=5). We found no significant differences ($p>0.05$) in c-Jun gene expression in either cell line. c-Fos gene that together with c-Jun forms important part of AP-1 transcription factor shows 2.5-fold down-regulation in 22Rv1 cells. Ki-67, a nuclear protein that is associated with cellular proliferation, is present in 22Rv1 cell line in 2-fold higher concentration compared to PNT1A.

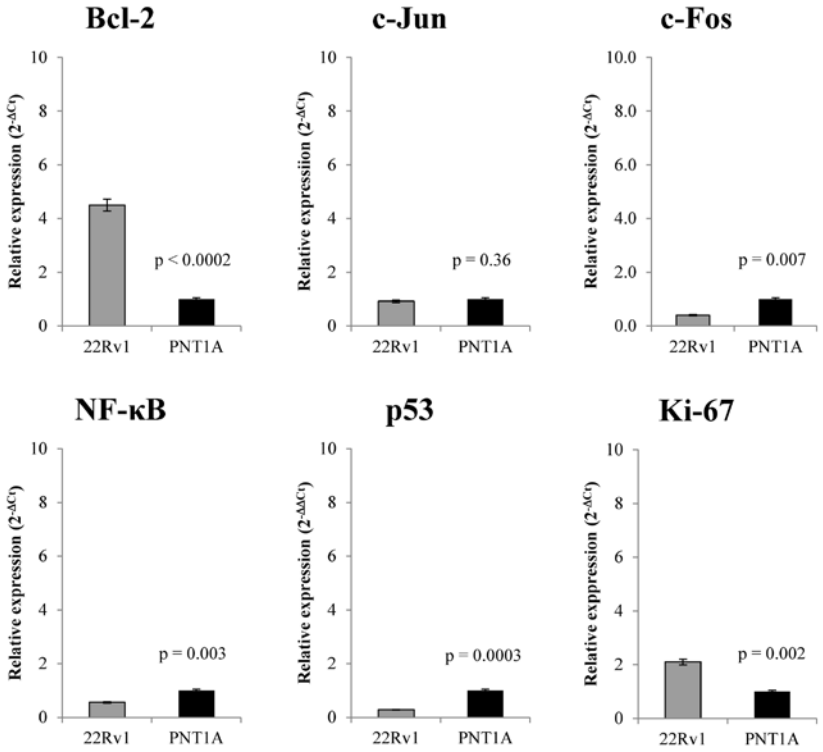

Figure 3. Comparison of the base line expression of c-Fos, c-Jun, NF-κB, Ki-67 and p53 genes on the RNA level. Base line transcription level of selected genes was conducted by RT-PCR. For other experimental parameters see Material and methods.

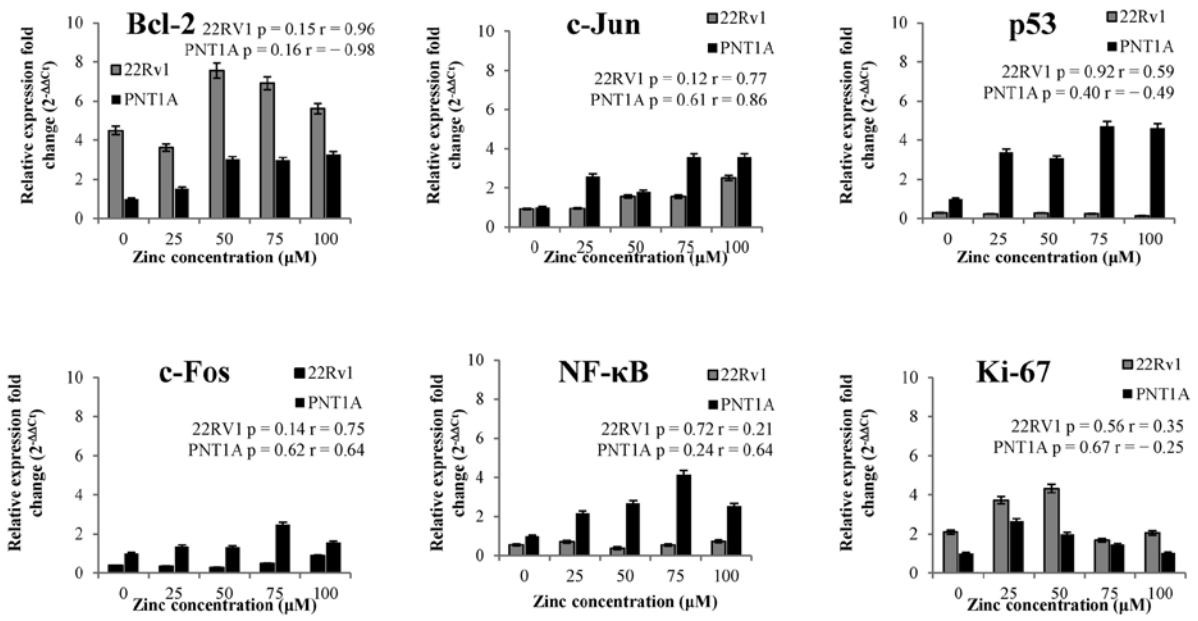

Figure 4. Zinc(II) treatment effect on expression changes of c-Fos, c-Jun, NF-κB, Ki-67 and p53 genes at mRNA level. 22Rv1 cell line shows not only different expression levels of these genes in comparison to healthy PNT1A prostatic cells but also zinc(II) treatment has different effect on their transcriptional levels in both cell lines. For other experimental parameters see Materials and methods and Fig. 2.

Moreover, NF-κB is present in half concentration in 22Rv1cells compared to PNT1A and p53, a key regulator of apoptosis, shows 3.3-fold decreased level compared with PNT1A cell line. After basic characterization of the expression of selected markers in both studied cell lines, we focused on zinc(II) ion treatment effect on the expression changes of these selected regulatory genes (Fig. 4).

We found that zinc(II) ions influence positively expression of Bcl-2 gene in both tested cell lines, however, more in PNT1A cells [≤3.2-fold change in case of 100 μM zinc(II) ions treatment]. In addition, we found no significant difference ($p>0.05$) in p53 expression levels after zinc treatment in 22Rv1 cell line (Fig. 4). On the other hand, zinc(II) ions treatment resulted in the increasing p53 expression from 3- up to 4.7-fold change in

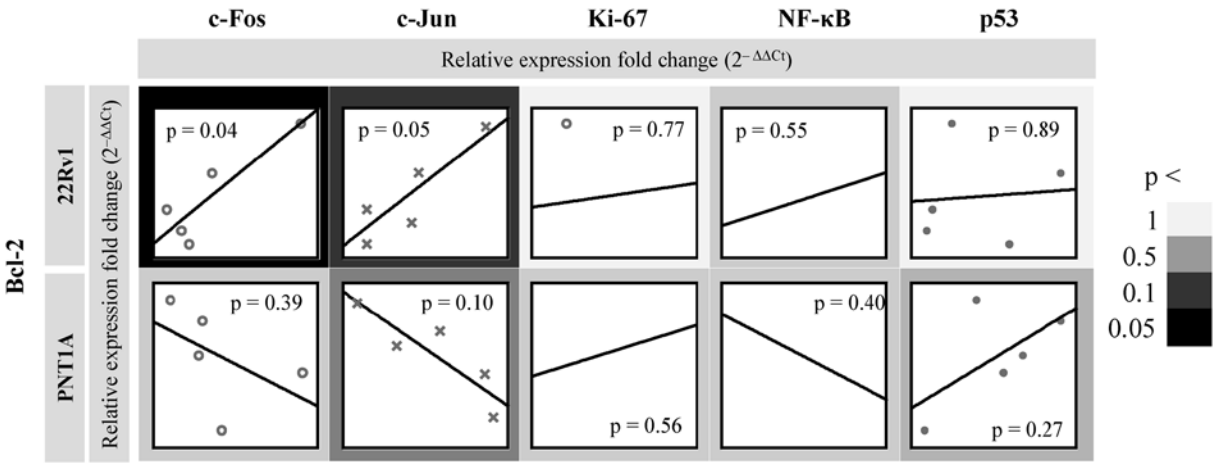

Figure 5. Correlation analysis between Bcl-2 mRNA and other regulatory genes at mRNA level. When exposed to zinc treatment, distinct trends were found between Bcl-2 and c-Fos and c-Jun and no significant correlations between Bcl-2 and Ki-67, NF-κB and p53 in both cell lines. Other experimental parameters are detailed in Materials and methods and in Fig. 2.

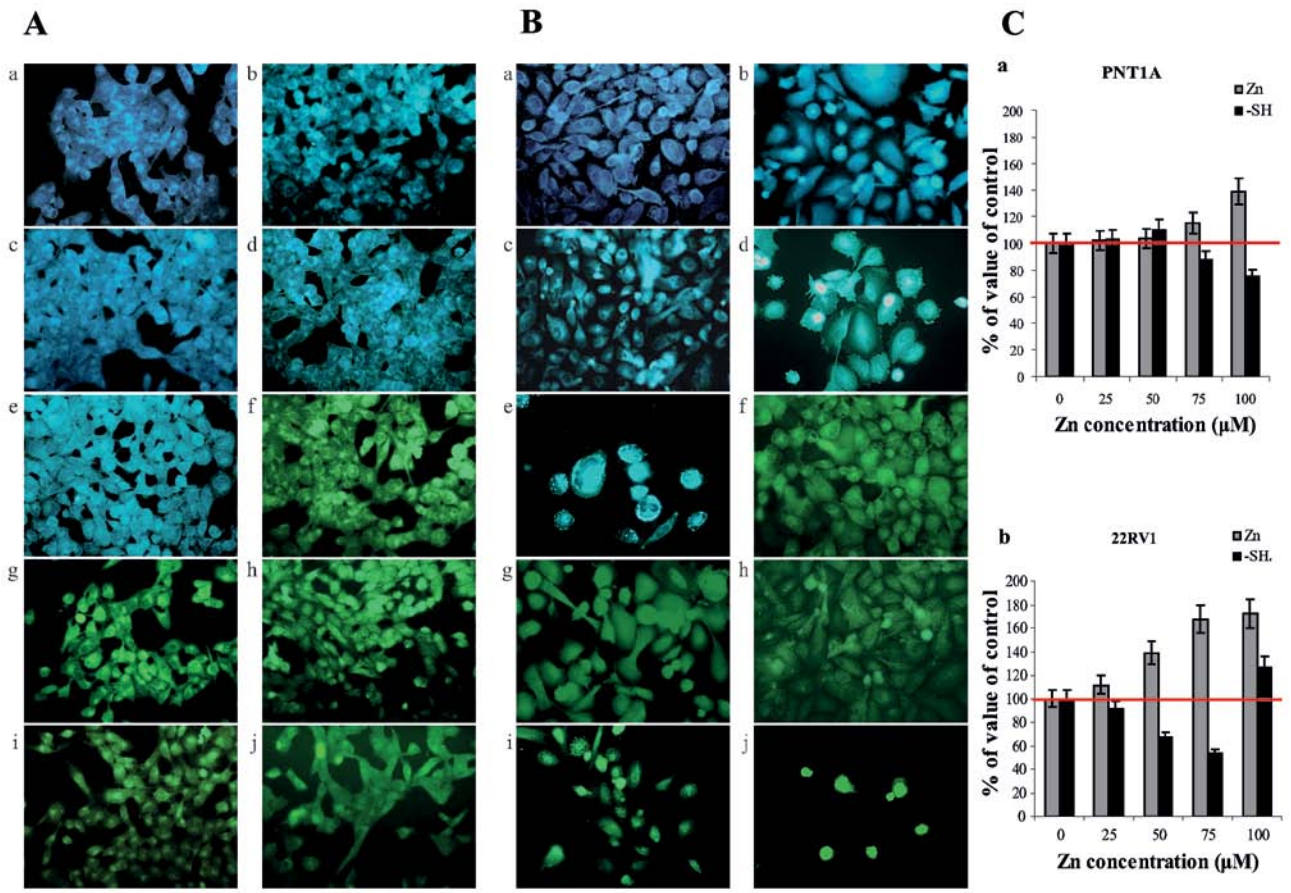

Figure 6. Distribution of free zinc(II) ions and thiols in (A) PNT1A and (B) 22Rv1 cell lines. Cells were treated with 0 (a and f), 25 (b and g), 50 (c and h), 75 (d and i), and 100 μM (e and j) zinc(II) ion concentrations. Distribution of (a-e) free zinc(II) ions and (f-j) free thiol groups. Note presence of bright spots - zincosomes - in 22Rv1 cells treated with the highest zinc(II) ion dose. Evaluation of emission intensity after fluorescence staining using NIE-element program recalculated to control (100%). (C) Content of zinc ions and free thiol moieties in (a) PNT1A and (b) 22Rv1 cell lines. For other experimental parameters see Materials and methods.

PNT1A. Ki-67 gene shows similar pattern in both cell lines after zinc(II) treatment and it is not surprising that 22Rv1 cell line, characterized by higher proliferation rate (Fig. 2A and B), demonstrated higher expression as a gene involved in the proliferation process. Zinc(II) ions had up-regulative effect on c-Jun gene in both cell lines. PNT1A demonstrated higher c-Jun expression in all zinc treatments compared to 22Rv1. In addition, we found no correlation between c-Jun and c-Fos gene expression after zinc treatment, but expression profile of c-Fos showed statistically significant positive correlation (data not shown) with

expression of NF-κB gene after the treatment (compare trends in Fig. 4). Both genes are expressed in higher concentration in PNT1A cell line on the base expression level as well as after zinc treatment.

Correlation analysis of selected genes. Subsequently, we focused on possible dependencies between Bcl-2 mRNA and the mRNA levels of other regulatory genes. After exposure to zinc(II) ions, we observed distinct trends between Bcl-2 and c-Fos and c-Jun and no significant correlations between Bcl-2 and Ki-67, NF-κB and p53 in either cell line (Fig. 5). Bcl-2-p53 showed strong positive correlation at r=0.61. In the case of c-Fos and c-Jun, we found strong positive correlations at r>0.85 in 22Rv1 tumour cell line and strong negative correlations at r<-0.50 in PNT1A non-tumour cell line. In terms of statistical significance, Bcl-2-c-Fos and - c-Jun showed correlations p<0.05 only in 22Rv1 cell line. In contrast, non-tumour cell line PNT1A showed an inverse trend i.e. decrease of Bcl-2 in relation to regulatory genes. In this cell line, negative significant correlation was detected at Bcl-2-c-Jun at p=0.10 with r=-0.79 (Fig. 5).

Determination of free and bound zinc(II) cellular levels after zinc(II) exposure. We characterized cell lines from the point of view of pro- and anti-apoptotic factor expressions, free zinc(II) ions were visualized using fluorescent probe N-(6-methoxy-8-quinolyl)-*p*-toluene sulphonamide, probe specific to these ions. Significant differences in zinc(II) ion localization in both non-tumour PNT1A cells (Fig. 6Aa-e) and tumour 22Rv1 (Fig. 6Ba-e) cells were found. For quantification of changes in free zinc(II) ion levels in cell lines, program NIS-elements for image analysis was used. Detected values (10 fields for each concentration and repetition) were recalculated to control cells (100%) (Fig. 6C). In both PNT1A and 22Rv1 cells, free zinc(II) ions levels were closely connected with zinc(II) ions treatment in concentration-dependent manner. In the case of PNT1A cell line, localization of zinc(II) ions around nuclei and irregularly in nuclei was evident in cells treated with the highest zinc(II) concentration (100 μM). Peripheral parts of cytoplasm demonstrate only weak emission, representing only low free zinc(II) levels in these localizations. In 22Rv1 cells, the intensity of emission of fluorescence product significantly increases with the increased supplementation of cultivation medium by zinc(II) ions (Fig. 6Ba-e). In the lowest Zn(II) supplementation, free zinc(II) ions were localized especially around the nuclei. At the highest Zn(II) supplementation, free zinc(II) ions were localized in nuclei and around the nuclei in the form of spots with high emission. The origin of these spots, which were visible only in the case of 22Rv1 cells in the highest Zn(II) ions supplementation, is probably the zincosomes, compartments of endoplasmic reticulum origin (20).

Determination of free thiols. Compounds rich in -SH moieties including low molecular mass peptides and proteins as reduced glutathione and metallothionein (21-25) are responsible for binging metal ions in intracellular space. Therefore, monitoring of such compounds as well as detection of free zinc(II) ions can answer questions on metabolizing of metal ions in a cell. 5-(bromomethyl)fluorescein, the probe that provides formation of fluorescent product after reaction with -SH groups of thiols, was used for detection and cellular compartmentation of free thiols. In the case of both PNT1A (Fig. 6Af-j) and 22Rv1 cells (Fig. 6Bf-j), amount of free thiols continually decreases with the increasing zinc(II) ions supplementation, however, selection of cells with high content of free thiols was evident. This fact was evident in 22Rv1 cells treated with the highest Zn(II) concentration. In both cell lines, free thiols were localized around the nuclei and in nuclei in cells treated with lower Zn(II) concentrations. On the other hand, highest free thiols levels were detected in the nuclei in the case of tumour 22Rv1 cell line. This fact is evidence on the possible role of free thiols in transcriptional activity, which is regulated not only by free thiols, but also by zinc(II) ions whose concentration was high in nuclei in the same case.

Discussion

Zinc is involved in energetic metabolism (26), proliferation and apoptosis (27-29) in prostate, therefore it is expectable that zinc may play an important role in prostate cancer pathogenesis (4,30,31). Our results revealed that the base line expression of the anti-apoptic gene Bcl-2 is 4.5-fold higher in 22Rv1 than in PNT1A. This result is in accordance with the previously published reports, where elevated Bcl-2 expression in prostate cancer tumours has been reported (14,16). Furthermore, this elevation was associated with the development of androgen-independent prostate cancer (18) and also with radiotherapy and chemotherapy resistance (13,14,32). Interestingly, we observed further increase in Bcl-2 expression in both cell lines after zinc(II) treatment. However, in the case of 22Rv1 this enhancement in expression was higher, up to 7.5-fold, compared to base line expression of Bcl-2 in PNT1A cell line. Although the exact mechanism remains unclear, our findings suggest that higher zinc(II) ion concentrations in prostate may contribute to prostate tumour therapy resistance associated with elevated Bcl-2 expression.

c-Jun forms homodimers and heterodimers with c-Fos and other Jun-related proteins, which together comprise the AP-1 transcription factor that binds TPA response elements (TREs). Therefore, c-Jun mediates transcriptional regulation in response to a variety of stimuli, including cytokines, growth factors and stress (33). Generally, AP-1 controls a number of cellular processes including differentiation, proliferation, and apoptosis (34). Although there is considerable evidence that c-Jun activation can represent a positive step in the events leading towards apoptosis, there are numerous contrary reports. Possible role of c-Jun in inhibition of apoptosis and promoting of proliferation/cell differentiation is reviewed in ref. 35. Growing amount of such evidence implicates c-Jun in the protection of cells from stress-induced apoptosis. It was reported that cells expressing the Ser63Ala, Ser73Ala mutant of c-Jun are not protected against apoptosis triggered by UV irradiation (36). As 22Rv1 cells show higher viability and proliferation (Fig. 2) after zinc treatment, we can conclude that the increasing expression of c-Jun does not have negative impact on 22Rv1 cells.

NF-κB is a transcription factor, which effect cannot be easily evaluated. Its expression strongly correlates with c-Fos transcription in both cell lines used in our experiments. This finding correlates with known fact that NF-κB enhances c-Fos transcription via the direct binding to a response element situ-

ated in the first intron (37). Surprisingly, only PNT1A cells react on zinc(II) treatment by increased expression of NF-κB. In the case of 22Rv1 we observed no significant changes in NF-κB expression. However, this finding is not in accordance with the previously reported studies that show association of progression of prostate cells toward greater tumourigenic potential with the increasing constitutive levels of NF-κB activity (38,39).

NF-κB is generally viewed as anti-apoptotic and oncogenic. However, recent reports suggest that NF-κB may promote apoptosis and is not necessarily an anti-apoptotic factor in some situations (40). For example, Bohuslav *et al* have shown that p53 stimulates the ribosomal S6 kinase, which in turn phosphorylates the p65/RELA subunit (41). This phosphorylation of p65 was found to reduce its affinity to IκBα, thereby preventing IκBα-mediated nuclear export of NF-κB (41). These results indicate a novel non-classical mechanism of NF-κB activation via p53. However, it is worth mentioning at this point that in contrast to these co-operative efforts between p53 and NF-κB, there have been a few reports which indicate an antagonistic relationship. For instance, it was shown that p53 and NF-κB repress each other by competing for a limiting pool of transcriptional co-activator proteins p300 and CREB-binding protein (CBP) (42). In our study, NF-κB expression after zinc(II) treatment correlates with p53 expression in both cell lines, which supports the previous findings. In our experiments, 22Rv1 did not increase expression of p53 after zinc treatment. It has been reported that metals in general are able to induce formation of reactive oxygen species (ROS) and activate p53-dependend apoptotic pathway (43,44). Moreover, it has been published that p53 activation by ROS is in some cases of metal-based drug treatment necessary for induction of apoptosis (45). Based on our results and previously published studies, it can be expected that low expression of p53 and no significant changes in its expression after zinc treatment may prevent cells from undergoing p53-dependent apoptosis e.g., caused by oxidative stress. However, this expectation needs to be experimentally proven.

Ki-67 is a nuclear protein closely associated with ribosomal RNA transcription and may be crucial for cellular proliferation (46). Inactivation of Ki-67 leads to inhibition of ribosomal RNA synthesis (47). Ki-67 has been established as a promising marker of aggressive prostate cancer. There is plenty of evidence on the overexpression of this protein in prostate cancer (48-52). Our results indicate that 22Rv1 cells express at base line two-fold more Ki-67 than PNT1A cells, in accordance with previously published data. Zinc treatment increased Ki-67 significantly in both tumour and non-tumour cell lines.

Collectively, elevated expression of Ki-67, a marker of proliferation, extremely low expression of p53 and also no changes in expression of p53 after zinc treatment, high expression of Bcl-2, especially after zinc treatment and viability and proliferation curves (Fig. 2) indicate that zinc has significant positive effect on 22Rv1 cell line proliferation and viability.

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