Zinc Induces Apoptosis of Human Melanoma Cells, Increasing Reactive Oxygen Species, p53 and FAS Ligand

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Abstract. *The aim of this study was to examine the in vitro effect of zinc on the apoptosis of human melanoma cells, by studying the zinc-dependent modulation of intracellular levels of reactive oxygen species (ROS) and of p53 and FAS ligand proteins. We showed that zinc concentrations ranging from 33.7 μM to 75 μM Zn^{2+} induced apoptosis in the human melanoma cell line WM 266-4. This apoptosis was associated with an increased production of intracellular ROS, and of p53 and FAS ligand protein. Treatment of tumor cells with the antioxidant N-acetylcysteine was able to prevent Zn^{2+}-induced apoptosis, as well as the increase of p53 and FAS ligand protein induced by zinc. Zinc induces apoptosis in melanoma cells by increasing ROS and this effect may be mediated by the ROS-dependent induction of p53 and FAS/FAS ligand.*

Melanoma is the most aggressive form of skin cancer. The poor prognosis in patients with advanced disease largely results from resistance to conventional chemotherapy, namely cytotoxic drugs. The basis for drug resistance in melanoma is, at least partly, related to a dysregulation of apoptosis, although other mechanisms, including drug transport, de-toxification, and enhanced DNA repair, may also play a role.

Evidence suggest an intriguing link between zinc and cancer. Whereas some anticancer actions of zinc might be related to a modulation of immune system effectiveness or to the zinc-dependent regulation of the production of other anticancer substances (1, 2), some evidence supports the possible modulation of apoptosis by zinc. In fact, zinc has been shown to induce apoptosis in glioma (3), bladder (4), prostate (5), as well as breast (6) cancer cells. A zinc-dependent modulation of reactive oxygen species (ROS), which have been implicated as modulators of apoptosis (7, 8), has been reported in both non-tumoral (9-11) and tumoral (6) models, suggesting the influence of zinc on apoptosis through the modulation of the intracellular redox state. Prevention of the zinc-induced increase of intracellular ROS by *N*-acetylcysteine (NAC) in mammary carcinoma cells further emphasizes this possibility (6). Furthermore, zinc has been shown to modulate the tumor suppressor p53, acting either at the level of mRNA (6) or protein (6) expression, or on its functional conformation (12, 13). Finally, zinc has been shown to influence the FAS/FAS ligand (FASL) apoptotic pathway, enhancing FAS-mediated apoptosis of Jurkat cells (14) and increasing FAS/FASL mRNA and protein in mammary cancer cells (6).

In the present study, we examined the effect of zinc on the apoptosis of human melanoma cells, studying the zinc-dependent modulation of intracellular ROS, and p53 and FASL protein.

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Materials and Methods

Tumor cells and cell culture conditions. The human melanoma WM 266-4 tumor cell line (American Type Culture Collection, Rockville, USA) was maintained *in vitro* using sterile, disposable flasks (Nunc, Roskilde, Denmark) at 37˚C in a humidified atmosphere with 5% CO_2, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin (referred to as complete medium, CM; all from Gibco, Life Technologies, Milan, Italy). Established monolayers of sub-confluent and exponentially-growing tumor cells were incubated overnight at 37˚C in 5% CO_2 with $ZnSO_4$ (Carlo Erba, Milan, Italy) at concentrations of 37.5 μM or 75 μM Zn^{2+} in serum-free AIMV medium (Gibco) in 25 cm^2 sterile flasks or in Thermanox Coverslips (13 mm; Nunc) inserted into 24-well plates (Nunc). Controls were incubated in serum-free AIMV medium alone. AIMV is a serum-free culture medium containing a small amount of Zn^{2+} (0.8 μM Zn^{2+}), as we determined by atomic absorption spectrophotometry according to the method of Fernandez and Kahn (15). Control

cultures were maintained in either AIMV medium alone or in the appropriate CM. Untreated and Zn^{2+}-treated tumor cells were fixed in 4% formalin and examined under light microscopy for their morphology. *N*-Acetylcysteine (NAC; Sigma, St. Louis, MO, USA), was added at a concentration of 10 mM to the media of cultured cells 4 h prior administration of Zn^{2+}. Viability of tumor cells was determined by Trypan Blue exclusion.

Quantification of apoptosis by flow cytometry. Apoptosis was measured using flow cytometric detection of changes in cell morphology and by annexin V binding to the cell membrane. Freshly harvested cells were washed in phosphate buffered saline (PBS), resuspended in isotonic solution, and analyzed for their light-scatter properties by flow cytometry. Apoptotic cells were recognized from decreased forward scatter and increased side scatter using a Coulter XL flow cytometer (Coulter, Hialeah, FL, USA). For annexin V binding to the cell membrane, cells were washed twice in PBS after treatment and stained with a fluorescein isothiocyanate (FITC)-labeled recombinant annexin V (Alexis Co., Laufelfingen, Switzerland). The non-vital dye propidium iodide (Sigma) was added immediately before flow cytometric analysis. Simultaneous staining of tumor cells with annexin V (green fluorescence) and with propidium iodide (red fluorescence) allowed the discrimination of intact cells ($FITC^-PI^-$), early apoptotic ($FITC^+PI^-$), and late apoptotic or necrotic cells ($FITC^+PI^+$). Fluorescence was determined using an XL flow cytometer (Coulter, Hialeah, FL, USA).

Flow cytometric analysis of rates of intracellular oxidation. Hydroethidine (HE) was obtained from Molecular Probes (Eugene, OR, USA), and stock solutions were prepared and stored at −20˚C. As previously reported (16), cells were incubated with HE (160 μM) after zinc treatment and samples were analyzed using an XL flow cytometer. Dead cells and debris were excluded from the analysis by electronic gating of forward and side scatter measurements.

Flow cytometric measurement of p53 and FASL proteins. To evaluate the p53 protein expression through flow cytometry, control and zinc-treated tumor cells were washed with a solution of PBS, 5% FCS, and 0.04% NaN_3 and fixed with 2% formalin. Fixed cells were resuspended in 1 ml of 0.2% Tween 20 (Sigma) in PBS at room temperature and incubated for 15 min at 37˚C. Cells were washed again with PBS with 5% FCS, and 0.04% NaN3 and incubated with a monoclonal antibody to p53 (Ab-1; Oncogene Science, Cambridge, MA, USA) for 30 min at 4˚C. After washing, cells were incubated with an FITC-conjugated anti-mouse IgG_{2a} (Pharmingen, San Diego, CA, USA) for 30 min at 4˚C. The cell pellet was washed with PBS with 0.1% NaN_3 and resuspended in ISOTON® II diluent (Beckman Coulter, Brea, California, United States). Mouse IgG_{2a} (Pharmingen, San Diego, CA) was used as isotype control. To evaluate FasL, cells were incubated with a FITC-labeled rat monoclonal antibody to CD95 Ligand (CD95L) which recognizes both mouse and human FASL (Alexis Corporation, Nottingham, UK) for 30 min at 4˚C. The cell pellet was washed with PBS with 0.1% NaN3 and resuspended in Isoton II. Rat IgG2a (Pharmingen) was used as isotype control. Cell fluorescence was analyzed via an XL flow cytometer.

Statistical analysis. Statistical significance of differences was determined by Student's *t*-test. Difference between means were considered significant at $p<0.05$. Data analysis was performed with SigmaStat software version 1.03 (Jandel Scientific, Germany).

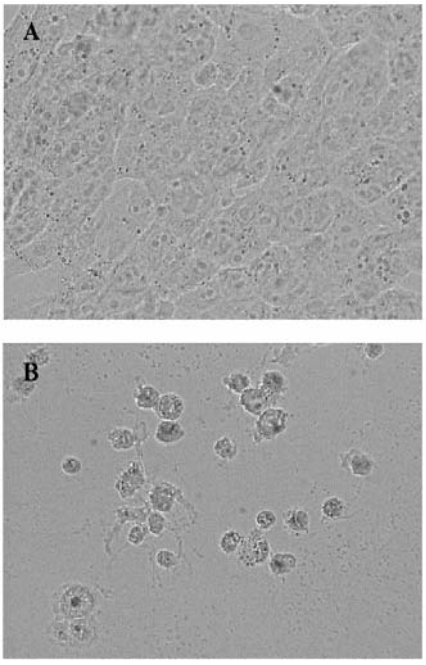

Figure 1. *Effect of Zn^{2+} on the morphology of human melanoma cells. WM 266-4 melanoma cells were incubated overnight in medium alone or in medium supplemented with 37.5 μM Zn^{2+}. A: WM 266-4 cells incubated overnight in medium alone form a confluent adherent monolayer with sparse cytoplasm and heterogeneous nuclear chromatin; B: WM 266-4 cells treated with 37.5 μM Zn^{2+} are detached and exhibit the characteristic signs of apoptosis, the cytoplasm is shrunken and the chromatin is pyknotic.*

Results

Effect of Zn^{2+} on the apoptosis of WM 266-4 melanoma cells. Melanoma cells were cultured overnight in the presence of different Zn^{2+} concentrations and analyzed for the number of apoptotic cells. WM 266-4 cells were grown in a serum-free medium (AIMV medium) to avoid the presence of zinc, which is present in high amounts in FCS. Neither the number nor the viability of tumor cells were significantly different after incubation overnight, or for five days in AIMV medium in comparison with FCS-supplemented cultures.

Figure 1 shows the morphology of WM 266-4 cells after incubation in medium alone and in medium supplemented with 37.5 μM Zn^{2+}. As shown, untreated melanoma cells appeared as a confluent adherent monolayer, had a sparse cytoplasm and heterogeneous nuclear chromatin (Figure 1A). Overnight incubation with 37.5 μM Zn^{2+} caused the

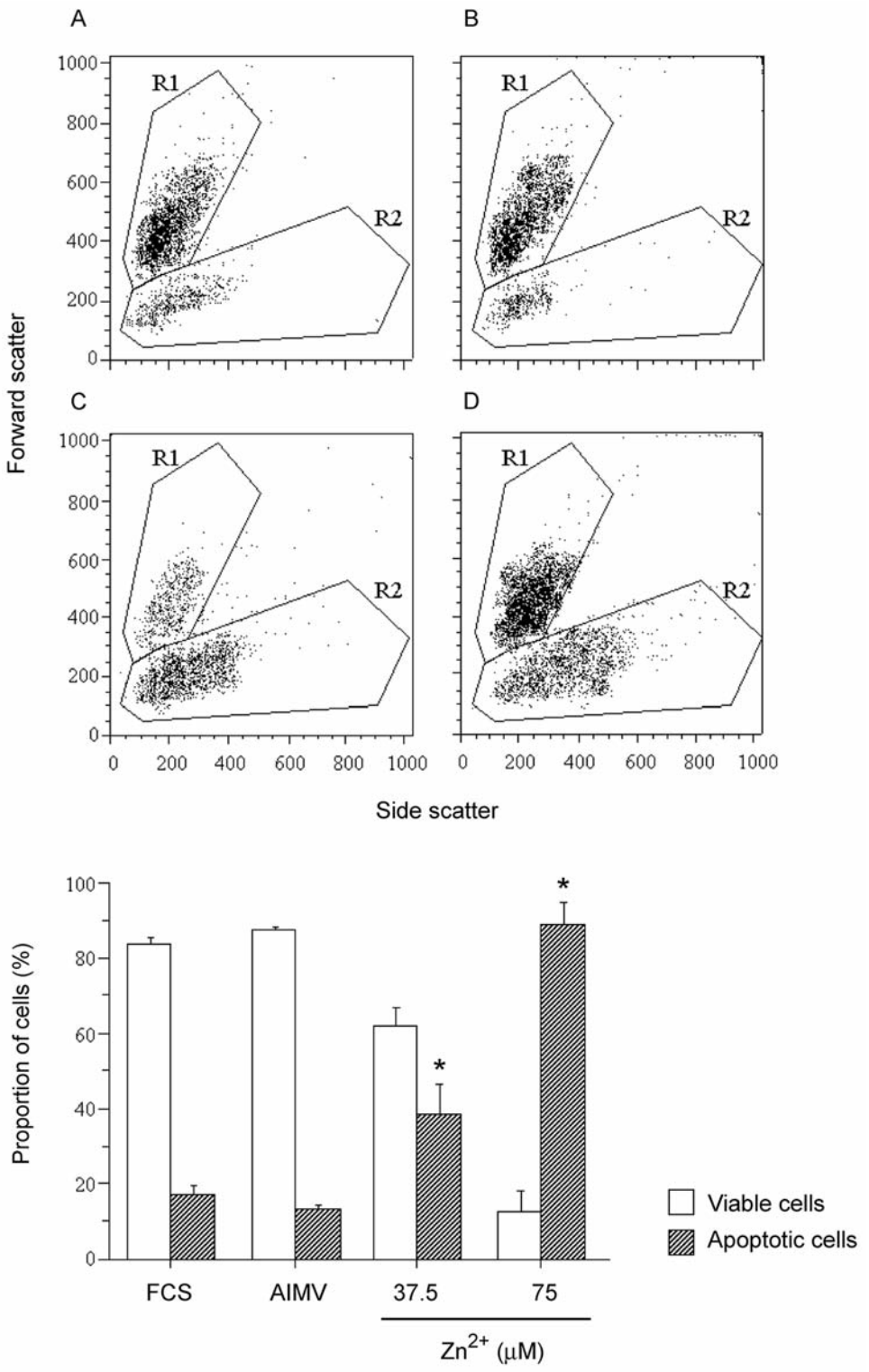

Figure 2. *Typical forward and side scatter dot plots for WM 266-4 cells incubated overnight in complete medium (A), AIMV medium (B), or in AIMV medium supplemented with 37.5 μM Zn^{2+} (C) or 75 μM Zn^{2+} (D) (top). Events in region R1 represent viable cells, whereas those in region R2 represent apoptotic cells. C and D: Zn^{2+}-treated WM 266-4 cells exhibit decreased forward-scatter and increased side-scatter characteristic of apoptotic cells. Percentages of cells in each region were calculated considering the number of cells of the regions R1+R2 as 100% of the cells counted. Data are representative of one of three separate experiments. The histogram (bottom) reports the mean±S.D. of the results obtained in three separate experiments. *p <0.05 versus AIMV.*

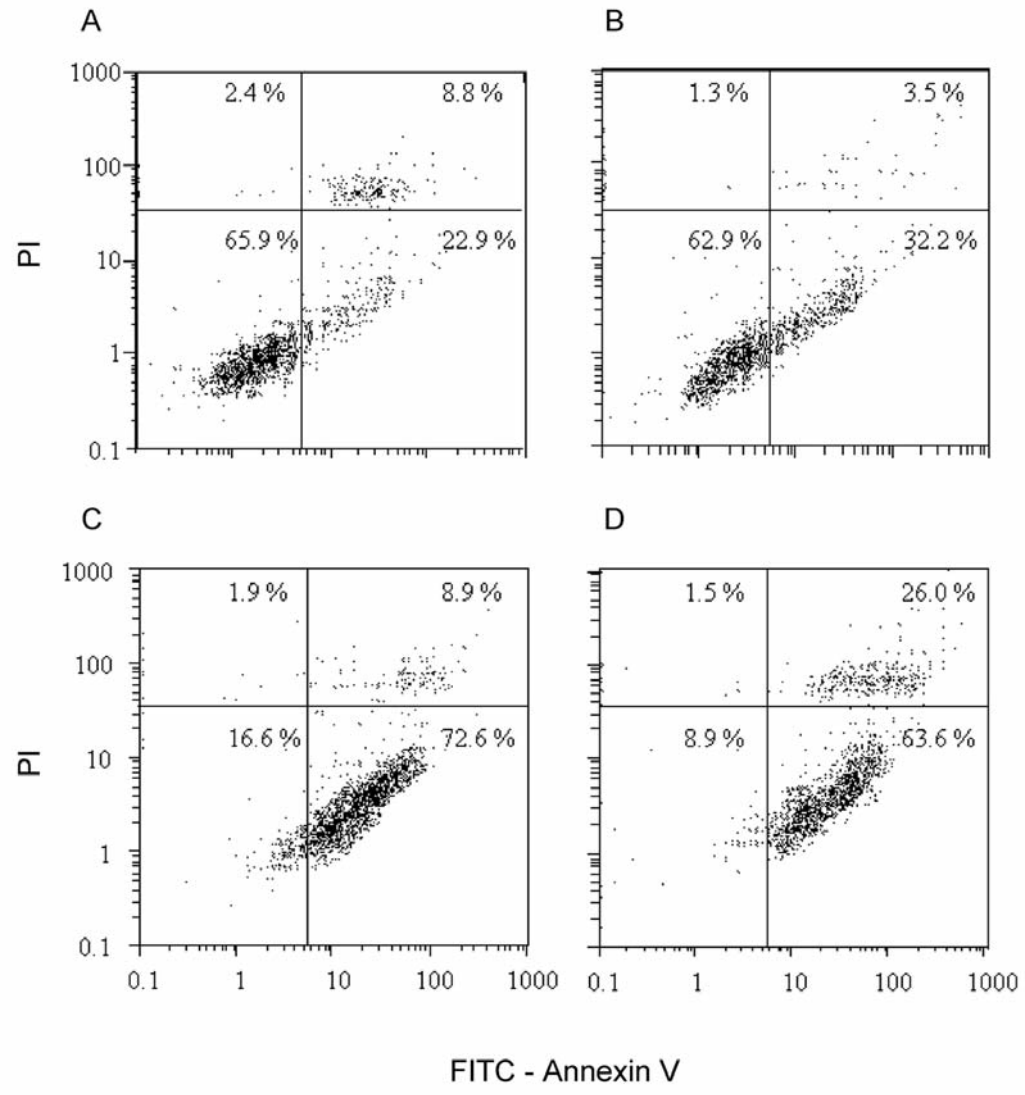

Figure 3. *Effect of Zn^{2+} on the apoptosis of WM 266-4 cells. Untreated (A, complete medium; B, AIMV medium) and 37.5 μM (C) or 75 μM (D) Zn^{2+}-treated WM 266-4 tumor cells, were stained with a fluorescein isothiocyanate (FITC)-labeled recombinant annexin V and propidium iodide (PI) and analyzed for the presence of early apoptotic (FITC+PI−) and late apoptotic or necrotic cells (FITC+PI+) through flow cytometer. An increased number of early apoptotic cells was present after overnight incubation with 37.5 μM (C) or 75 μM (D) Zn^{2+}. Cells treated with 75 μM Zn^{2+} also exhibited an increase of late apoptotic or necrotic cells. Results are representative of one of three experiments.*

appearance of the classical morphological changes of apoptosis (Figure 1B). Apoptotic and viable melanoma cells were then identified on the basis of their light scattering properties. Figure 2 demonstrates the typical forward and side scatter distribution of WM 266-4 cells *in vitro* treated with 37.5 μM or 75 μM Zn^{2+}. A low number of apoptotic cells was present in untreated cells cultured in FCS (R2, Figure 2A) or AIMV (R2, Figure 2B), whereas the percentage of apoptotic cells progressively significantly increased in melanoma cells treated with 37.5 μM (R2, Figure 2C) or 75 μM (R2, Figure 2D) Zn^{2+}.

To evaluate whether the morphological changes described induced by the treatment of WM 266-4 cells with Zn^{2+} were associated with other signs of apoptosis, we analyzed detached and monolayer cells after simultaneous staining with FITC-labeled annexin V and propidium iodide, evaluating the number of early apoptotic and late apoptotic or necrotic cells by cytofluorimetric analysis. As shown in Figure 3, the percentage of early apoptotic cells ($FITC^+PI^-$) was significantly increased after overnight supplementation with 37.5 μM or 75 μM Zn^{2+} (72.6% and 63.6% respectively *vs*. 32.2% or 22.9% found in cells grown in AIMV or CM)

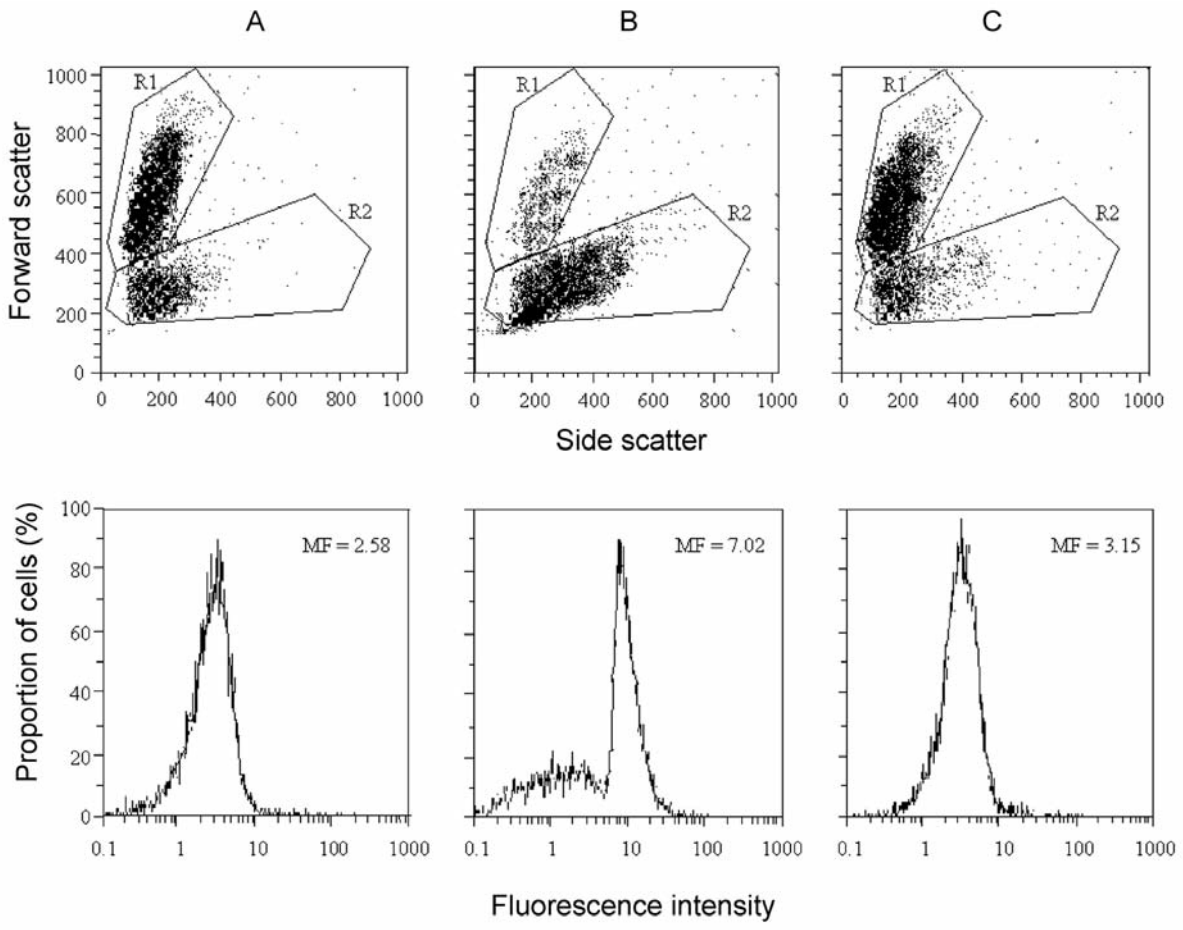

Figure 4. *Evaluation of intracellular reactive oxygen species (ROS) in Zn^{2+}-treated human melanoma cells. WM 266-4 tumor cells were incubated overnight in AIMV medium alone (A) or in AIMV medium supplemented with 75 μM Zn^{2+} (B), or 75 μM Zn^{2+} plus 10 mM N-acetylcysteine (NAC) (C). The percentage of apoptotic cells was evaluated through flow cytometric analysis of forward -and side- scatter dot plots (top); the rate of intracellular ROS production was determined by monitoring the oxidation of hydroethidine to fluorescent ethidium (bottom; MF=median fluorescence). B: WM 266-4 cells treated with 75 μM Zn^{2+} exhibit the morphological characteristics of apoptotic cells and had an increased content of intracellular ROS. C: Pre-treatment for 4 h of WM 266-4 cells with 10 mM NAC completely prevented both apoptosis and the increase of intracellular ROS induced by 75 μM Zn^{2+}. Treatment with 10 mM NAC did not modify apoptosis or the ROS content of WM 266-4 cells (data not shown). Data are representative of one of three separate experiments.*

(Figure 3). Incubation with 75 μM Zn^{2+} also led to an increase in the percentage of late apoptotic or necrotic cells (FITC+PI+) (26% *vs*. 3.5% or 8.8% found in cells grown in AIMV or CM) (Figure 3). The supplementation of human lymphocytes with 37.5 or 75 μM Zn^{2+} significantly inhibited apoptosis found in control lymphocytes.

NAC prevents Zn^{2+}-induced apoptosis of melanoma cells. To determine whether Zn^{2+}-induced apoptosis of tumor cells correlated with an increase in ROS, we quantified the intracellular redox status in Zn^{2+}-treated and untreated WM 266-4 tumor cells by measuring dihydroethidium fluorescence as an indicator of endogenous superoxide levels. As shown in Figure 4, the apoptosis induced by incubation of melanoma cells with 75 M Zn^{2+} was associated with a rise in the intracellular content of ROS (Figure 4B). Similar data were obtained in melanoma cells treated with 37.5 μM Zn^{2+}. The incubation of tumor cells with NAC (10 mM) 4 h before Zn^{2+} administration prevented both Zn^{2+}-induced apoptosis and the increase of ROS level (Figure 4C).

Zn^{2+} induces expression of p53 protein in human melanoma cells. We evaluated the effect of Zn^{2+} treatment on the level of p53 protein in WM 266-4 melanoma cells. As shown in Figure 5, overnight incubation of WM 266-4 cells in medium containing 75 μM Zn^{2+} (Figure 5C) increased the low level of the p53 protein present in untreated cells (Figure 5B).

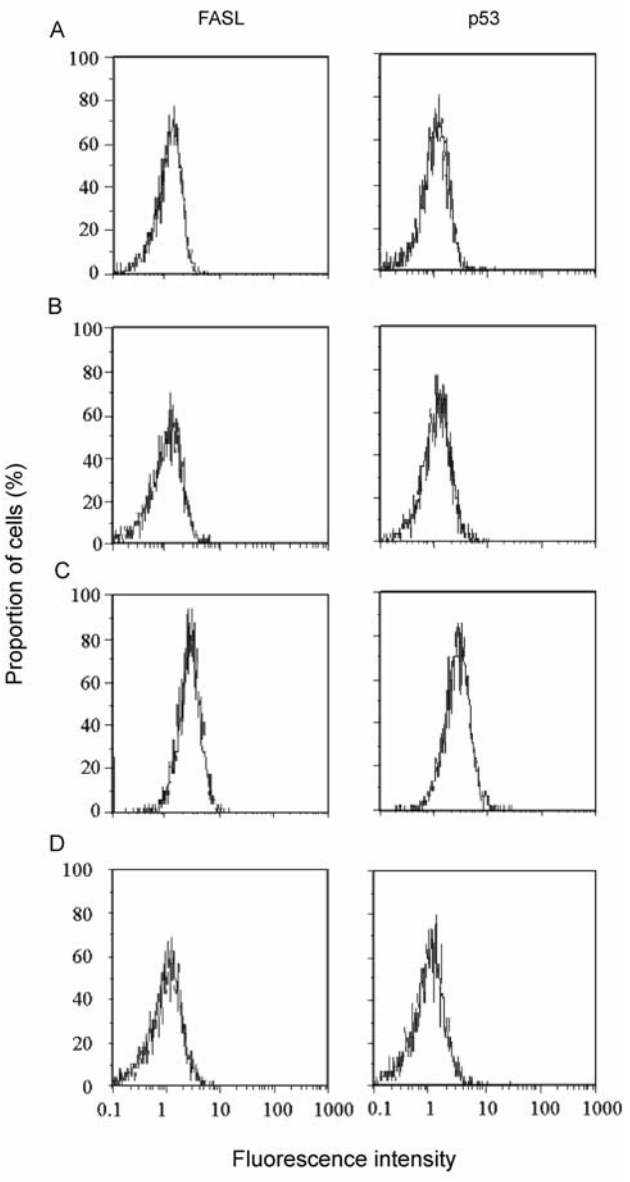

Figure 5. *Effect of Zn^{2+} on p53 and FAS Ligand (FASL) proteins human melanoma cells. WM 266-4 cells were incubated overnight in medium alone (B), in medium supplemented with Zn^{2+} (75 μM) (C), or with Zn^{2+} (75 μM) plus N-acetylcysteine (10 mM) (D). Cells were then stained with monoclonal antibody against p53 or FASL and analyzed by flow cytometry, as reported in Materials and Methods. IgG2a was used as an isotype control (A).*

Zn^{2+} induces FASL protein expression in human melanoma cells. Next, we examined Zn^{2+}-treated WM 266-4 cells for the presence of FASL protein. Flow cytometric analysis showed that the cell surface expression of FASL molecule, when compared to isotype control-stained cells (Figure 5A), was only weakly detected in untreated WM 266-4 cells (Figure 5B), whereas it was significantly induced in cells treated with 75 μM Zn^{2+} (Figure 5C).

NAC prevents Zn^{2+}-induced apoptosis and expression of p53 and FASL proteins. To evaluate the effect of NAC on expression of p53 and FASL induced by Zn^{2+} in WM 266-4 melanoma cells, we performed flow cytometric analysis of Zn^{2+}-treated tumor cells. As shown in Figure 5D, the incubation of melanoma cells with 10 mM NAC completely prevented apoptosis and the increase of both p53 and FASL proteins induced by 75 μM Zn^{2+}.

Discussion

We demonstrated that zinc is able to induce apoptosis of human melanoma cells, while increasing intracellular ROS and modulating p53 and FASL protein expression.

A protective role of zinc in cancer has been indicated by some studies. *In vitro*, zinc has demonstrated efficacy in inducing apoptosis in glioma (3), bladder (4), prostate (5), as well as breast cancer (6) cells. *In vivo*, zinc treatment increased resistance against Ehrlich's ascites tumor challenge in mice (17) and decreased the incidence of spontaneous lung tumors arising in A/J mice (18). Furthermore, pre-treatment with zinc inhibited secondary carcinogenesis by anticancer agents (18).

Data from the literature have clearly defined that pharmacological Zn^{2+} concentrations cause an inhibition of apoptosis of both lymphoid (19) and tumor (20, 21) cells. We were the first to report that Zn^{2+} concentrations equal or lower than the physiological plasma Zn^{2+} concentration induce apoptosis of mouse thymocytes, showing that Zn^{2+} may have opposing effects on apoptosis and that it does not only act as an inhibitor, but may also play a more complex role in the regulation of the apoptotic process (22).

In a previous study, we demonstrated that zinc induces apoptosis of mammary carcinoma cells through modulation of ROS, p53 and FAS/FASL pathways (6). Herein we demonstrated that the Zn^{2+}-induced apoptosis of human melanoma cells is similarly associated with an increase of intracellular ROS, as well as *p53* and FASL proteins. This modulation by Zn^{2+} is abrogated by the anti-oxidant NAC, preventing the increase of intracellular levels of ROS. Our data support recently reported evidence indirectly suggesting a possible positive modulation of apoptosis in cancer cells by zinc.

The effect of Zn^{2+} is obtained at concentrations not toxic to normal cells. In fact, in parallel experiments conducted on human lymphocytes, apoptosis was inhibited rather than induced by the same zinc concentrations able to kill tumor cells through apoptosis.

The increase of apoptosis in Zn^{2+}-treated melanoma cells correlated well with the modulation of intracellular levels of ROS. The potential Zn^{2+}-dependent modulation of ROS has been suggested by findings obtained in both non-tumoral (5-7, 23) and tumoral (6) models. Herein we demonstrated that

the apoptosis induced by Zn^{2+} in melanoma cells may be dependent on induction of intracellular ROS and may involve p53 and FASL modulation. This is supported by the fact that p53 can regulate the intracellular redox state inducing apoptosis by a pathway that is dependent on ROS production (24). Furthermore, data from the literature have shown that a strict relationship also exists between FAS-mediated apoptosis and ROS. ROS may induce *FASL* mRNA expression in cancer cells; the effect is blocked by treatment with antioxidants (25). Furthermore, the activation of FAS induces ROS and apoptosis (26) and treatment with anti-oxidants inhibits FAS-mediated apoptosis (27).

The tumor-suppressor gene *p53* plays a pivotal role in the activation of the apoptotic machinery in damaged cells and in the apoptosis of autologous tumor cells in response to chemotherapy (28). We previously demonstrated in mammary carcinoma cells that Zn^{2+} induces transcriptional activation of *p53* gene, with increased expression of *p53* mRNA and protein, which correlates with the induction of apoptosis. Accumulating evidence has been provided that p53 up-regulates the FAS/FASL-mediated apoptotic pathway. Adenoviral vectors expressing wild-type p53 increased *FASL* and FASR expression in colorectal tumors in *nu/nu* mice (29) and in lung and breast cancer cell lines (30). The transient expression of the wild-type *p53* gene up-regulated *FASL* mRNA, as well as protein expression and induced rapid apoptosis of lung and colon cancer cells (31). In another experimental model, cells possessing functional p53 increased their surface expression of FAS and FASL in response to DNA damage (32).

In agreement with our previous data (6), we demonstrated that Zn^{2+} increases p53 and FASL protein in melanoma cells. One of the most interesting findings of our study is that apoptosis and the modulation of both p53 and FAS/FASL induced by zinc are prevented by treating tumor cells with the anti-oxidant NAC. This clearly demonstrates the central role of free radicals in zinc-induced apoptosis of cancer cells. Further studies are warranted to ascertain the potential usefulness of zinc to correcting the dysregulation of apoptosis in the therapy of melanoma.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

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