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Characterization of the cell death induced by cadmium in HaCaT and C6 cell lines

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Abstract
Cell death resulting from cadmium (Cd) intoxication has been confirmed to induce both necrosis and apoptosis. The ratio between both types of cell death is dose- and cell-type-dependent. This study used the human keratinocytes HaCaT expressing a mutated p53 and the rat glial cells C6 expressing a wild p53 as models to characterize Cd-induced apoptosis, using sub-lethal and lethal doses. At these concentrations, features of apoptosis were observed 24 h after C6 cell treatment: apoptotic DNA fragmentation and caspase-9 activation, whereas Cd did not induce caspase-3. In HaCaT, Cd did not induce apoptotic DNA fragmentation or caspase-9 and -3 activation. The results also showed that the inhibition of p53 led to a resistance of the C6 cells to 20 μM Cd, decreased the apoptosis and increased the metallothioneins in these cells. p53 restoration increased the sensitivity of HaCaT cells to Cd but did not affect the MT expression. The results suggest that Cd induced apoptosis in C6 cells but a non-apoptotic cellular death in HaCaT cells.

Keywords: *Cadmium, metallothioneins, apoptosis, p53, C6 cells, HaCaT cells*

Abbreviations: *CAPS, 3-cyclohexylamino-1-propanesulphonic acid; ROS, reactive oxygen species; Cd, cadmium; PBS, phosphate-buffered saline; ABTS, 2,2′-azino-di-[3-ethylbenzthiazoline-sulphonate(6)]; TBS-T, tris-buffered saline containing tween 20; LEHD-pNA, N-acetyl-Leu-Glu-His-Asp-pNA (p-nitroanilide); DMSO, dimethylsulphoxide; AU, apoptotic units; ELISA, enzyme-linked immunosorbent assay; EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; BCA, bicinchoninic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; BSA, bovin serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; FIENA, fluorometric immunosorbent enzyme assay; AFC, 7-amido-4-trifluoromethyl-coumarin; DEVD-AFC, Acetyl-Asp-Lime-Valley-Asp-7-amido-4-trifluoromethyl-coumarin; DTT, dithiothreitol; DMEM, dulbecco modified eagle's medium; RPMI, roswell park memorial institute medium.*

Introduction

Cadmium (Cd) is a naturally occurring metallic element, widely used in pigments, stabilizers, coatings and above all in nickel-cadmium batteries. This environmental toxicant has a biological half-life exceeding 25 years and can also be found in tobacco smoke, industrial emissions and food. Cd was classified as a type I carcinogenic by the International Agency for Research on cancer [1,2]. Mechanisms of Cd toxicity are multifactorious and dose and cell-type dependent. However, in spite of their diversity, the pathways affected in mammalians by Cd exposure points to a general threat to basic cellular functions and can lead to cell death. The mechanisms by which

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Cd induces cellular death are usually discussed in view of the cell lines studied and the concentrations in this metal. In particular high doses of Cd (acute exposure) induce both necrosis and apoptosis. It seems that the oxygen reactive species formation, the lipid peroxidation, the generation of conditions which decrease the antioxidant levels and the inhibition of DNA repair are necessary for apoptotic induction [3–10].

Apoptosis results from a controlled combination of enzymatic activations and pro- or anti-apoptotic gene expression. This can induce cell self-destruction, without injuring surrounding cells, preserving tissue integrity, even that of the organization [11]. There is no inflammatory reaction [12] and it contradicts necrotic death which assumes an exogenic destruction producing a membrane alteration and the dispersion of the cellular contents. Many disorders in the mechanisms controlling apoptosis can be responsible for the release and progression of several pathologies. These can be characterized either by an apoptosis deficit or an excess of apoptosis. The apoptosis deficit is often associated with autoimmune and lymphoproliferative disease [13,14] development, but also with carcinogenesis [15]. On the other hand, the excess of apoptosis is involved in the development of many pathologies: congenital anomalies and chronic neurodegenerative disease such as Alzheimer's [16] and Parkinson's disease.

Several studies have shown that Cd toxicity leads to apoptosis in many models [17–22] such as HeLa cells [9] or fibroblasts [23]. The pro-apoptotic effect of Cd is mediated by various signalling pathways which trigger caspase-dependent or caspase-independent apoptosis [20]. Recent studies have focused on the role played by signalling cascades in apoptosis after exposure to Cd [17]. In these processes, molecules such as caspases or the p53 protein play an important role. Caspase activation is one of the factors intervening in the disorganization of the cell's cytoskeleton [24]. The p53 protein controls a large variety of genes whose products are involved in cellular cycle arrest and/or apoptosis [25,26] and can direct cellular cycle arrest in the G1 phase through the protein p21 activation. The tumour suppressor p53 is one of the most important factors intervening in the regulation of cellular proliferation and protects the cells against increased tumoural proliferation. It plays a central role in the cellular response to genotoxic stress. Inhibition of p53 is an important stage of carcinogenesis. The p53 mutations have been observed in more than 50% of human cancers [27,28] activated in response to certain stressful situations such as DNA damage, oncogene activation or Cd cell treatment [29,30].

The upstream apoptotic pathways always converge towards a common effector [31] and lead to apoptotic DNA fragmentation. There are two types of DNA fragmentation: high-molecular-weight fragmentation, from 50–300 Kpb [32] and low-molecular-weight fragmentation obtained by internucleosomal cleavage, from 150–200 pb [33,34]. These mechanisms constitute powerful apoptotic programmes present in the majority of cell types and are initiated by a variety of intra- or extracellular signals among which is the cell exposure to toxic heavy metals such as Cd.

The aim of the present study was to compare the apoptotic action of Cd in HaCaT cells expressing mutant p53 and C6 cells expressing wild type p53 and to describe the molecular mechanisms of apoptosis induced by Cd in these cell lines. Finaly, certain authors have suggested a relation between p53 and metallothioneins. It seems that the absence of p53 involves an increase in metallothionein expression [35–37]. The metallothioneins exert antioxidant functions while acting on the homeostasis of essential elements such as zinc and copper and in the detoxification mechanisms of highly toxic metals such as Cd [38]. However, this relation is debated. To better understand the mechanisms of resistance of HaCaT cells against Cd, not only the relation between p53 and metallothioneins, but also the role of p53 in the cell death induced by Cd was studied.

Materials and methods

Cell culture

The spontaneously immortalized human keratinocyte HaCaT cell line came from the DKFZ (Deutsches Krebsforschungszentrum, German Cancer Research Center, Heidelberg, Germany) [39]. The cells were cultured at 37°C in a 5% CO_2 humidified atmosphere in RPMI 1640 medium (Invitrogen, France) containing 10% FCS ([Biotechnologie], France), penicillin (5000 U/mL)/streptomycin (50 mg/L) and 2 mm L-glutamine (Invitrogen, France).

C6 (ATCC, France) are cell lines from a rat glial tumour induced by N-methylnitrosourea [40,41]. The cells were cultured at 37°C in a 5% CO_2 humidified atmosphere in D-MEM medium (Invitrogen, France) containing 10% FCS ([Biotechnologie], France), penicillin (5000 U/mL) and streptomycin (50 mg/mL).

Cells treatment with cadmium

A stock solution of cadmium chloride ($CdCl_2$, 10 mM) was prepared in distilled water and sterilized by filtration. A sufficient quantity of this stock solution was added to the culture medium to obtain the desired final concentrations: 4, 5 and 20 μM Cd for C6 cells and 15, 50 and 100 μM Cd for HaCaT cells. $CdCl_2$ came from Sigma (France).

Cells were seeded in different materials (96-well microplates, petri dishes) for 24 h incubation with each metal concentration. Then cells were harvested

for each experiment. A control with no treatment was systematically included in each experiment.

Apoptotic DNA fragmentation

The DNA fragmentation was quantified by the Cell Death Detection ELISA^{plus} colourimetric assay kit (Roche Diagnostics, France) after cell treatment with Cd for 24 h. After treatment, the floating cells were harvested and mixed with the coldly separated adherent cells. A total of 5×10^4 cells were taken, centrifuged (1500 rpm, 10 min at 4°C) and lysed. Then DNA fragmentation was measured as described according to the manufacturer's recommendations and revealed by ABTS at 405 nm. Results were expressed as the ratio of apoptotic cells in the treated cells vs the untreated cells and defined as the enrichment factor (AU = apoptotic units; [42]).

Caspase-9 activity

Caspases-9 activity was measured by using the Alexis colourimetric assay kit (Alexis, France). Cells were incubated with the different Cd concentrations for 24 h then were collected and lysed by 50 µL of lysis buffer for 10 min in ice. After centrifugation (10 000 *g*, 1 min), the proteins in supernatant were quantitated using the Pierce kit (Pierce, France). We incubated 150 µg of protein in reaction buffer (10 mm of DTT) with caspase-9 substrate (LEHD-pNA) for 1 h at 37°C; then the absorbance levels were measured at 405 nm.

Caspase-3 activity

The caspase-3 activity was specifically and quantitatively measured in microplates using the FIENA (Fluorometric Immunosorbent Enzyme Assay; Roche Diagnostics) fluorescent assay kit. A total of 2×10^6 cells were incubated and apoptosis was induced by Cd for 24 h. Cells were recovered and treated according to the method provided by the FIENA kit. Caspase-3 activity was revealed by the DEVD-AFC substrate. Fluorescence (AFC) was measured between 370–425 nm out of the excitation filter and 490–530 nm out of the emission filter. Caspase-3 activity was given by the standard curve.

Western blot detection of p53

Activation of p53 in HaCaT cells. The HaCaT cells were incubated with cell-permeable p53 activator (Calbiochem, France) in increasing concentrations from 0–30 µg/mL for 24 h. This activator is a synthetic peptide of 46 amino acids corresponding to p53 C-terminal amino acids. This peptide binds to the mutant p53 and restores the removed functions of p53 in the cells. The product and the concentrations used in our study were described by Selivanova et al. [43–45].

Inhibition of p53 in C6 cells. The C6 cells (expressing the wild p53) were incubated with Pifithrin-α cyclic (Calbiochem) in increasing doses: from 0–20 µM for 24 h. This inhibitor and the concentrations used were described by Komarov et al. [46].

Extraction of cellular content. After 24 h of incubation with the activator or the p53 inhibitor, HaCaT and C6 cells were lysed with 100 µL of cytobuster (commercial detergent providing protein insulation without a second treatment; Novagen, France) for 5 min. The cells were harvested and centrifuged at 14 000 rpm, 4°C for 5 min. The supernatants were removed for protein measurement using the Pierce kit and for p53 and metallothionein detection using the western blot assay.

Quantitative protein determination (BCA^{TM} Bicinchoninic acid, Pierce kit). Protein levels were determined using the BCA^{TM} Bicinchoninic acid kit (Pierce) in total and soluble cell lysates, read in 96-well microplates at 580 nm using a microplate reader (Bio-Tek, USA) and its accompanying software (KC Junior), which directly calculated protein concentrations from the calibration curve.

SDS-PAGE for p53. The samples (50 µg) were diluted in Laemmli buffer and were deposited in gel. After migration in SDS/PAGE, the acrylamide gels (15%) were incubated in transfer buffer (192 mM glycine, 25 mM Tris-HCl (pH 8.5) 20% (v/v) methanol [47]) at room temperature for 20 min. The proteins were then transferred on nitrocellulose membrane (PROTAN, France) for 2 h using a 50-V device (Bio-Rad transblotting, France). Then the membranes were saturated in 10% with milk (TBS-1X, Tween-20 at 0.1% and milk at 10% (w/v)) for 1 h at room temperature. After three washings, we cut out the membranes at the 30-KDa molecular weight band. The upper part was incubated overnight at 4°C with primary monoclonal antibody anti-p53 (DO-7; Dako, France) diluted to 1/1000. The lower part was incubated with primary monoclonal antibody WAF1 (Ab1) anti-p21 (Oncogene Research Products, UK) diluted to 1/200. The membranes were washed three times with TBS-Tween (10 min/washing) and the secondary antibody (NA931; Amersham, France) diluted to 1/3000 was added for 1 h. In the end, membranes were washed three times and revealed with the ECL (Enhanced chemiluminescence, Amersham), then exposed on photographic film (Hyperfilm^{TM}ECL^{TM}, Life Science, Amersham).

Western blot detection of metallothioneins [48,49]

Electrophoresis. The SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) was carried out according to the Laemmli [50] protocol using 15% gels in a Bio-Rad mini-gel device. In this protocol modified by McKeon and Lyman [48] and revised again by Mizzen et al. [49], the samples (20 μg total protein) were denatured in 20 μL sample buffer (10 mM Tris-HCl, pH 7.5; 10 mM EDTA; 20% (v/v) glycerol; 1% (w/v) SDS; 0.005% (w/v) bromophenol blue; 100 mm DTT). The samples were heated for 5 min before loading, followed by a migration at 200 V at room temperature [49].

Transfer on membranes and glutaraldehyde treatment. Two transfer buffers were used: (A) After electrophoresis, the gel was incubated in traditional transfer buffer (192 mM Glycine, 25 mM Tris-HCl (pH 8.5) 20% (v/v) methanol [47]) at room temperature for 20 min and (B) in CAPS buffer (10 mM 3-cyclohexylamino-1-propanesulphonic acid (pH 10.8)) in 10% methanol [51] with or without the addition of 2 mm $CaCl_2$ to each buffer. The calcium chloride removes the SDS fixed to proteins, thus maximizing their fixation on membrane [48]. Then the proteins were transferred at 40 V at 4°C on nitrocellulose membrane (PROTAN, France) for 1 h using the Bio-Rad transblotting device. Next, the membranes were incubated for 1 h in glutaraldehyde (2.5% final), which increases the retention of low-weight protein by a factor 1.5–12 compared to the untreated membrane [48,49], then washed three times for 5 min in phosphate buffer (8.1 mM Na_2HPO_4; 1.2 mM KH_2PO_4; 2.7 mM KCl, pH 7.4). With the third washing, we added 50 mM monoethanolamine to stop the residual glutaraldehyde reactivity [49].

Immunodetection. The membranes were blocked in 10% milk in Tris-buffered saline (TBS: 20 mm Tris-HCl, pH 7.4; 154 mM NaCl) and 0.1% (v/v) Tween-20 at room temperature for 2 h. Membranes were incubated overnight in primary antibody (Dako, E9 clone) diluted to 1/1000 in 10% milk-TBS-tween at room temperature before washing in TBS-tween and incubation for 4 h in secondary antibody combined with horseradish peroxidase (goat anti-mouse IgG (Bio-Rad) diluted to 1/1000 in TBS-Tween. After three washings in TBS-Tween, blots were developed by ECL, then membranes were exposed on a photographic film (Hyperfilm^{TM}ECL^{TM}; Amersham).

Cell viability assay

After cell treatment with Cd alone or first with p53 activator (9 μg/ml) or inhibitor (150 μM) for 24 h and with Cd concentrations for another 24 h, cell viability was determined colourimetrically using the 3-(4, 5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma) assay [52]. Cells were washed with phosphate saline buffer (PBS; Invitrogen) and incubated at 37°C for 2 h in fresh culture medium containing MTT (100 μl/mL medium). Then the cells were washed with PBS and lysed by dimethylsulphoxide (DMSO; Sigma). The coloured formazan crystals formed after conversion of the tetrazolium salt MTT by mitochondrial dehydrogenases were solubilized and measured by spectrophotometry at 570 nm.

Statistical analyses

Each experiment was repeated three times. All data are expressed as mean ± standard deviation and were analysed by analysis of variance (ANOVA). Differences were considered significant when $p < 0.05$.

Results

Analysis of apoptotic DNA fragmentation

Apoptosis was evaluated by studying the DNA fragmentation, which is a final step in programmed cell death before cells divide into several apoptotic bodies and are phagocyted, using an ELISA kit (Figure 1A and B). The results showed that Cd did not induce an apoptotic DNA fragmentation in HaCaT cells. The mono- and oligo-nucleosomes enrichment factor remained relatively stable from 0–100 μM Cd (Figure 1A).

In parallel, we observed a very significant ($p < 0.01$ and $p < 0.001$) apoptotic event in C6 cells. The mono- and oligo-nucleosomes increased as a result of a dose dependent DNA fragmentation (Figure 1B).

Determination of caspase-9 activity

Caspase-9 activity characterizes the intrinsic apoptotic or mitochondrial pathway. Its study makes it possible to determine whether this transduction is involved in the apoptotic process induced by Cd. After a 24 h HaCaT cells treatment with Cd (15, 50 and 100 μM), we observed no increase in caspase-9 activity compared to untreated control (Figure 2A). C6 cells treated with Cd (4, 5 and 20 μM) showed a significant ($p < 0.05$ and $p < 0.01$) increase in caspase-9 activity (Figure 2B). These results showed that the mitochondrial pathway (caspase-9) could be the initiating pathway of the apoptotic process in the C6 cells and that caspase-9 was not involved in the high rate of mortality of the HaCaT cells observed with 100 μM Cd.

Determination of caspase-3 activity

The mechanism of Cd action was further studied, looking at the caspases located downstream from

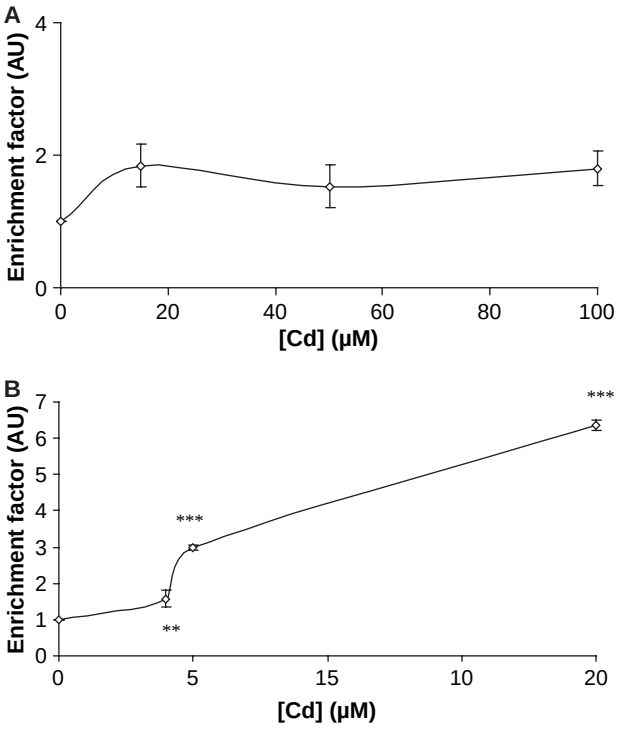

Figure 1. Evaluation of apoptotic DNA fragmentation induced by cadmium (Cd). (A) HaCaT cells were incubated with Cd (0, 15, 50 and 100 µM) for 24 h. (B) C6 cells were incubated with Cd (0, 4, 5, 20 µM) for 24 h. The DNA fragmentation was quantitatively evaluated using a colourimetric cell death detection ELISA kit (Roche Diagnostics), as described in Materials and methods. Apoptosis was reflected by the enrichment of the nucleosomes in the cytoplasm (AU: apoptosis units) and the results were expressed as the mean ± 1 SD of three independent experiments (**: $p < 0.01$, ***: $p < 0.001$ for (B)).

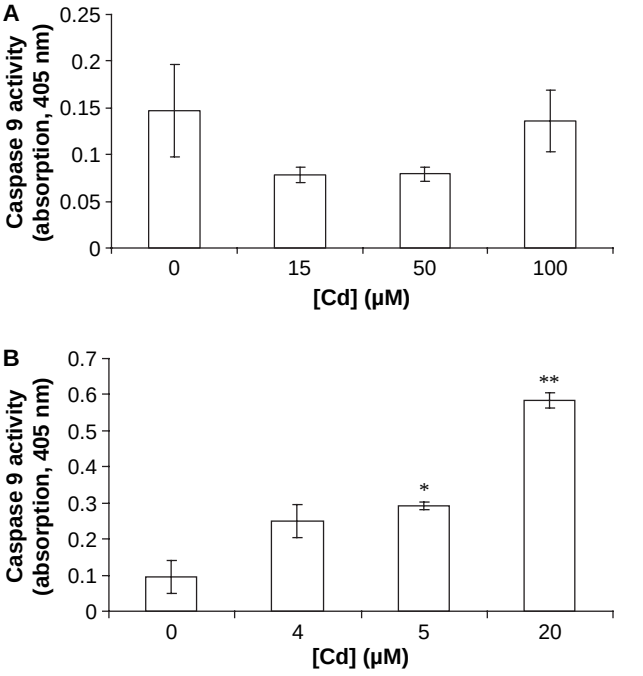

Figure 2. Determination of caspase-9 activity induced by cadmium (Cd). (A) HaCaT cells were incubated with Cd (0, 15, 50 and 100 µM) for 24 h. (B) C6 cells were incubated with Cd (0, 4, 5 and 20 µM) for 24 h. The caspase-9 activity was determined using a colourimetry assay kit (Alexis) as described in Materials and methods. The absorbance was measured at 405 nm and the results were expressed as the mean ± 1 SD of three independent experiments (*: $p < 0.05$, **: $p < 0.01$ for (B)).

caspase-9 such as caspase-3. The activity of caspase-3 was specifically and quantitatively measured using the FIENA assay kit (Roche Diagnostics). After 24 h of treatment with Cd, the caspase-3 activity was not modified between untreated controls and the treated HaCaT cells (Figure 3A) or the treated C6 cells (Figure 3B). Caspase-3 activity did not seem to be induced by Cd.

Role of p53 and p21 in apoptosis induced by cadmium

To explore the possible involvement of p53 and 21 in apoptosis and study the effect of p53 on HaCaT cell resistance or C6 cell sensitivity to Cd and the relation between p53 and metallothioneins, the western blot assay was used. The MTT colourimetric test was used to assess the viability of the cells.

p53 and p21 proteins modulation

Given that the HaCaT cells are p53-mutated, we studied the role of p53 in the resistance of these cells to low Cd concentrations by restoring this protein. The restoration of p53 was made by a chemical activator. The results showed that p53 expression in HaCaT was accompanied by the induction of the protein p21, which is its principal target. We observed a strong restoration of the protein p53 when the activator was used at 9 µg/mL (Figure 4A). This concentration was chosen for the rest of the experiments since at higher concentrations the activator became toxic and the p53 band disappeared.

Contrary to HaCaT, C6 cells express a normal p53. An inhibitor was used to block p53 expression in these cells. The results showed a progressive reduction in the intensity of p53 band with the increase in inhibitor concentrations (Figure 4B). With 150 µM of inhibitor, p53 expression was cancelled, therefore this concentration of inhibitor was used for the rest of the study.

The occurrence of the p21 protein band in the cells treated with activator or inhibitor followed the p53 expression pattern. p21 expression increased with p53 expression (HaCaT cells, Figure 4A) and decreased or disappeared when p53 expression was inhibited (C6 cells; Figure 4B).

Cellular response to cadmium after activation (HaCaT) or inhibition (C6) of p53

In order to specify the role of p53 on the HaCaT cell resistance or the C6 cell sensitivity to Cd, we evaluated not only cellular viability and apoptosis but also metallothioneins (MT) expression.

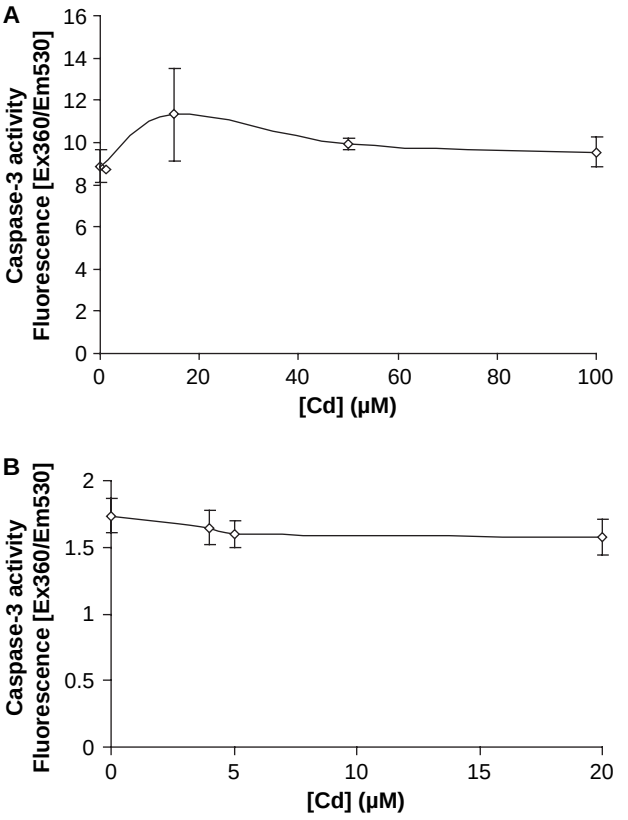

Figure 3. Determination of caspase-3 activity induced by cadmium (Cd). (A) HaCaT cells were treated with Cd (0, 15, 50 and 100 µM) for 24 h. (B) C6 cells were treated with Cd (0, 4, 5 and 20 µM) for 24 h. The caspase-3 activity was measured using the FIENA fluorescent kit (Roche Diagnostics) as described in Materials and methods. The results were expressed as the mean ± 1 SD of three independent experiments.

HaCaT cells. Cells were incubated with Cd alone or first with 9 µg/ml of p53 activator for 24 h and then with Cd (0, 15, 50 and 100 µM) for another 24 h. Cellular viability was evaluated using the colourimetric MTT test. The results showed that the HaCaT cells remained resistant to low Cd concentrations (up to 50 µM) even when the p53 and the p21 proteins were restored (Figure 5). However, we

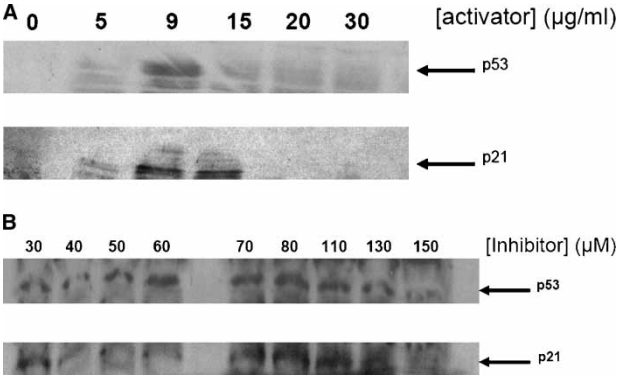

Figure 4. p53 and p21 expression established by western blot. (A) HaCaT cells were incubated with p53 activator 0–30 µg/mL for 24 h. (B): C6 cells were incubated with p53 inhibitor 30–150 µM for 24 h; 50 µg of total protein were deposited in gel.

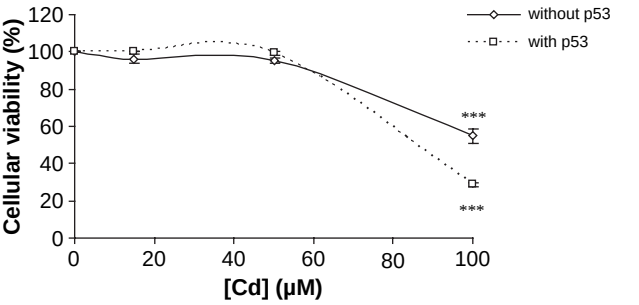

Figure 5. Cell viability after cadmium (Cd) exposure before and after p53 activation. HaCaT cells were incubated with Cd for 24 h or first with p53 activator (9 µg/mL) for 24 h followed by Cd treatment (0, 15, 50 and 100 µM) for another 24 h period. Cell viability was assessed using the colourimetric MTT test: each experiment was repeated three times and data are expressed as mean ± 1 SD (**: $p < 0.01$).

observed a significant reduction in cellular viability after cell treatment with p53 activator and 100 µM Cd (from 54% without p53 to 28% with p53) (Figure 5). This result suggested the involvement of p53 in HaCaT cell sensitivity to 100 µM Cd. The p53 activation in HaCaT cells led to an increase in the enrichment factor of mono- and oligo-nucleosomes released into the cytoplasm (Figure 6). In view of these results and in agreement with Figure 5, we suggest that p53-dependent apoptotic HaCaT cell death is increased when p53 wild type functions are restored.

Concerning MT expression, the results obtained using western blot did not show any significant change in MT expression after Cd treatment in the absence of p53 (Figure 7A). These proteins were constitutively expressed in the untreated HaCaT control. To explore the relation between p53 and MT expression, we also treated HaCaT first with 9 µg/mL of p53 activator for 24 h then with Cd

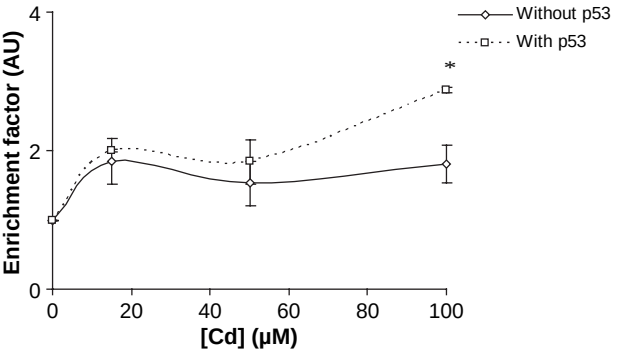

Figure 6. Apoptosis induced by cadmium (Cd) before and after p53 activation. HaCaT cells were treated with Cd or first with p53 activator (9 µg/mL) for 24 h followed by Cd treatment (0, 15, 50, 100 µM) for another 24 h period. The DNA fragmentation was evaluated using a colourimetric cell death detection ELISA kit (Roche Diagnostics), as described in Materials and methods. Apoptosis was reflected by the enrichment of the nucleosomes in the cytoplasm and the results were expressed as the specific enrichment factor and were given as the mean ± 1 SD of three independent experiments (*: $p < 0.05$).

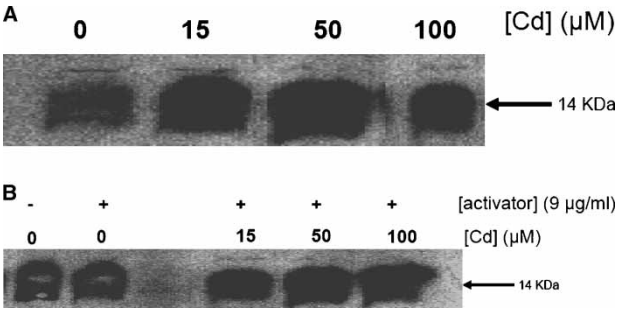

Figure 7. Metallothionein expression. (A) HaCaT cells were incubated with Cd alone (0, 15, 50 and 100 μM) for 24 h. (B) 24 h after HaCaT cell treatment with p53 activator (9 μg/mL), the cells were incubated with Cd (0, 15, 50, 100 μM) for another 24 h period. The effect of p53 restored on metallothionein expression was analysed by western blot, as described in Materials and methods.

(0, 15, 50 and 100 μM) for another 24 h. Any significant changes in MT expression were observed in HaCaT cells in the presence of p53 under our experimental conditions (Figure 7B).

C6 cells. As for the HaCaT cells, we checked the effect of p53 on Cd toxicity in C6 cells. C6 cells were incubated with Cd alone or for 24 h with 150 μM of p53 inhibitor and for 24 h with Cd (0, 4, 5 and 20 μM). Cellular viability was evaluated using the colourimetric MTT assay (Figure 8). The results showed that cellular viability under Cd exposure was significantly increased after p53 inhibition. At 20 μM of Cd, cellular survival amounted to 50% in the presence of p53 while in the absence of p53, the cellular viability reached 83% (Figure 8). In addition, apoptosis decreased significantly with 20 μM Cd ($p < 0.05$) when p53 was inhibited (Figure 9). The enrichment factor of mono- and oligo-nucleosomes released into the cytoplasm of C6 cells reached 6 (with p53) and 2 (without p53). These results suggested that p53 plays a major role in the apoptosis induced by Cd in C6 cells.

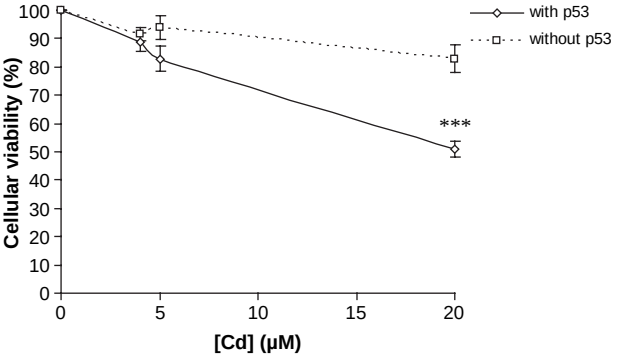

Figure 8. Cell viability after cadmium (Cd) exposure before and after p53 inhibition. C6 cells were incubated with Cd alone for 24 h or with p53 inhibitor (150 μM) for 24 h followed by Cd treatment (0, 4, 5 and 20 μM) for another 24 h. Cell viability was assessed using the colourimetric MTT test and data were expressed as mean ± 1 SD of three independent experiments (***: $p < 0.001$).

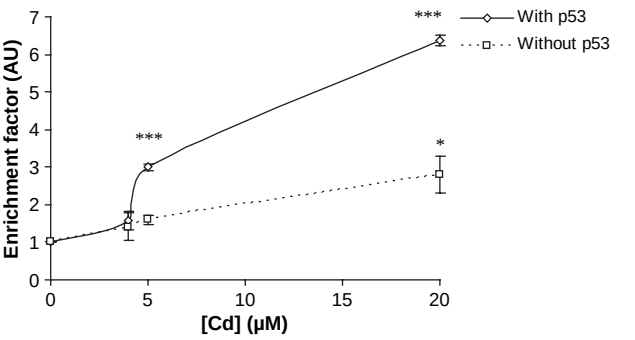

Figure 9. Apoptosis induced by cadmium (Cd) before and after p53 inhibition. C6 cells were incubated with Cd for 24 h or first with p53 inhibitor (150 μM) for 24 h followed by Cd treatment (0, 4, 5 and 20 μM) for another 24 h. The DNA fragmentation was evaluated using a colourimetric cell death detection ELISA kit (Roche Diagnostics). Apoptosis was reflected by the enrichment of the nucleosomes in the cytoplasm and the results were expressed as the specific enrichment factor and were given as the mean ± 1 SD of three independent experiments (*: $p < 0.05$, ***: $p < 0.001$).

MT expression was not observed in the untreated C6 control cells. Cd induced MT expression after 24 h of C6 treatment with 4 and 5 but not with 20 μM Cd (Figure 10A). To study whether p53 inhibition could increase MT expression even in untreated control and consequently if the MT take part in the resistance to Cd toxicity, the C6 cells were first incubated with p53 inhibitor (150 μM) for 24 h then with Cd (0, 4, 5 and 20 μM) for another 24 h period. The results showed no variation in MT expression in C6 treated with p53 inhibitor alone or when C6 cells were incubated with Cd up to 5 μM after p53 inhibition (Figure 10B). The MT expression pattern was relatively the same as that obtained without the p53 the p53 inhibitor (Figure 10A and B). However, when p53 was inhibited, a MT band appeared when C6 cells were incubated with 20 μM Cd (Figure 10B). Thus, the absence of p53 led to a

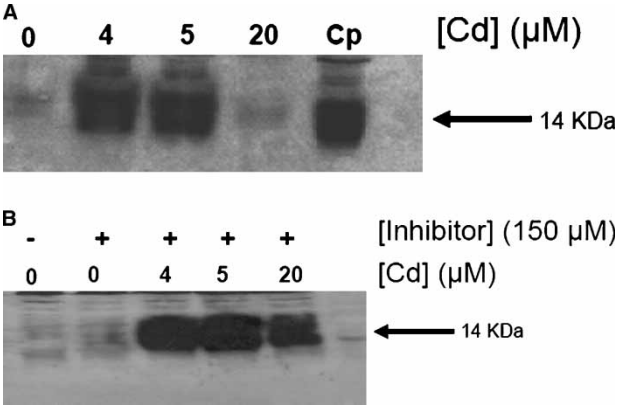

Figure 10. Metallothionein expression. (A) C6 cells were incubated with Cd alone (0, 4, 5 and 20 μM) for 24 h. Cp = positive control (commercial metallothionein; Sigma). (B) 24 h after C6 cell treatment with p53 inhibitor (150 μM), the cells were incubated with Cd (0, 4, 5, 20 μM) for anothor 24 h period. The effect of inhibited p53 on metallothionein expression was evaluated using western blot as described in Materials and methods.

higher MT expression for 20 μM Cd resulting in a lower Cd toxicity.

Discussion

The data from the literature and our preliminary report (data not presented) showed that cadmium (Cd) can induce ROS generation by lipid peroxidation, a decrease in non-enzymatic antioxidant level (glutathione) and a decrease in enzymatic activities (glutathione peroxidase and reductase (GPx and GRase), superoxide dismutases (SODs) and catalase (CAT)), oxidation of proteins, DNA damage and the inhibition of DNA repair. These processes were involved in oxidative stress and in apoptotic cell death [2–4,6–10,53]. However, the mechanisms of Cd-induced apoptosis remain cell-type-dependent. Our results showed that 24 h of exposure to 100 μM Cd does not induce apoptosis in HaCaT cells. However, the C6 glioma cells do show Cd-induced apoptosis.

Caspases activation is one of the factors intervening in the disorganization of the cell's cytoskeleton [24]. A significant increase in the activities of caspase-9 and caspase-3 were observed in many cells [54]. Mitochondria was the cellular compartment responsible for activation of apoptosis by releasing cytochrome C [55], along with other pro-apoptotic proteins, thereby activating caspase-9 and caspase-3, the major apoptosis execution enzymes [56–58]. Our results showed an increase in caspase-9 activity in C6 but not in HaCaT cells after treatment for 24 h. Several studies proposed that the induction of apoptosis in C6 cells by Cd begins with an externalization of the phosphatidylserine interior towards the outside of the plasmic membrane which would result in mitochondrial membrane potential alteration, followed by activation of caspase-9 and internucleosomal DNA fragmentation [59–61]. Our results were in favour of these observations reported in the literature.

Also, it has been shown that Cd disturbs the homeostasis of calcium [21,62,63]. Indeed, these authors described that the exposure to Cd involved an increase in the intracellular calcium levels which could control the cytochrome C release [64] and thus allow caspase-9 activation. Thus, the apoptotic events induced by Cd in C6 cells may derive not only from mitochondrial membrane potential alteration but also from disturbance of intracellular calcium homeostasis as suggested by literature data [65]. In HaCaT cells, the results suggested that Cd did not induce caspase-9 activities.

Our results suggest that HaCaT and C6 cells had different death mechanisms induced by Cd. In the C6 cells, the caspase-9 pathway did not converge downstream towards the activation of the specific major executer caspase of apoptosis, caspase-3, as was described by Green and Kroemer [66] and Thompson [31]. Indeed, we showed that Cd did not induce an increase in caspase-3 activity after C6 line treatment for 24 h. This could be explained by the fact that the apoptosis induced by Cd in C6 did not require the caspase-3 pathway even after caspase-9 activation, suggesting that another apoptotic pathway may be responsible. Studies have shown that Cd could induce apoptosis by the mitochondrial pathway independently of some caspases [21,67]. Thus, the pro-apoptotic effect of Cd is mediated by various signalling pathways that trigger caspase-dependent or caspase-independent apoptosis [20]. We also hypothetized that the activity of caspase-9 generated by Cd could be insufficient to activate caspase-3 downstream or that this caspase-3 could be inhibited by Cd which would confirm Yuan et al.'s [68] observations. Thus, the C6 glioma cells do show Cd-induced apoptosis which is dependent on caspase 9, but not caspase 3. However, 100 μM of Cd did not activate caspase 9 or caspase 3 in HaCaT cells. Cd can induce both necrosis and apoptosis. The ratio between both types of cell killing is dose- and cell-type-dependent.

One of the differences between HaCaT and C6 cells is that the HaCaT cells are human keratinocytes mutated in p53 [39] and C6 cells have a wild type p53. Using the colourimetric MTT assay, we showed that there was a high level of cellular survival (Figure 5) and using the comet assay that the Cd concentrations up to 50 μM did not induce genotoxicity in HaCaT cells (data not shown). At the same time, 50% of C6 cell death was obtained with 20 μM Cd after treatment for 24 h (Figure 8). In fact, in agreement with data from the literature Cd-induced cytotoxicity and the delayed inhibition of DNA replication and apoptosis were p53-dependent. Cell treatment with Cd induced p53 protein accumulation and activity [29]. Thus to understand the role of p53 not only in this HaCaT survival and in the C6 cells sensitivity, but also in apoptotic process and metallothionein expression, we restored this protein by p53 activator cell-permeable or inhibited it by p53 inhibitor (Pifitrin-α cyclic cell-permeable). All three domains—N-terminal, C-terminal and p53 core—are involved in intra- and/or intermolecular interaction, ensuring the proper folding of the specific DNA binding domain. Both the DNA binding and transcriptional transactivation contact function as well as structural mutants were amenable to reactivation by the peptide derived from the p53 C-terminal domain [69]. The C-terminal peptide (derived from the COOH terminus residues 361–382) binds to the mutant p53 core domains [45] and can disrupt the negative regulatory role of the C- and N-terminal domains in mutant p53.

The C-terminal peptide has a direct effect on the DNA binding and/or conformation of the mutant core domain itself because of a high content of basic lysine and arginine residues. The binding of the peptide to the mutant p53 core domain may introduce positive charges in the vicinity of the DNA contacting residues which in turn may create novel contacts with DNA. Secondly, interaction of the C-terminal peptide with mutant p53 core domains might stabilize their folding and thus switch the equilibrium towards the wild type conformation [44,70].

The results showed that the HaCaT cells treated with 9 μg/mL of p53 activator presented an accumulation of p53 and p21 proteins which led to a strong sensitivity of these cells to Cd concentrations up 100 μM (Figure 5). Our results suggested that the absence of p53 intervened in HaCaT resistance to Cd concentrations up to 100 μM , but the high level cellular mortality observed at 100 μM Cd, in the absence of p53, came from a non-apoptotic process. These results are in agreement with Magal et al.'s [71] studies showing that p53 and p21 were not involved in HaCaT cell death. However, the activation of p53 in HaCaT cells by transfection of the C-terminal peptide increases the toxicity of 100 μM Cd by 50% and increases apoptosis. This is evidence that wild type functions of p53 were well restored in HaCaT and that this p53 restoration induced apoptosis via several pathways involving membrane receptors and caspase activation [72–76] until apoptotic DNA fragmentation. Our results also showed that the C-terminal peptide induced toxicity when it was used at high level and in agreement with Senatus et al.'s [77] studies. These authors showed a dose-dependent and cell type-dependent death. Half of this cell death was observed with 30 μM or 50 μM of C-terminal peptide in human and rat glioma cell lines [77]. The process was completely different in C6 cells than in HaCaT cells having mutant p53. The results indicated that mortality (Figure 8) may be related to p53-dependent apoptotic cell death (Figure 9). It was established that the p53 tumour suppressor protein inhibits cell development by inducing cellular cycle arrest and/or apoptosis [27]. In many cell lines, Cd induced p53 expression and later activated transcription and synthesis of the p21 protein, an inhibitor of the cellular cycle [78–80]. The p21 protein belongs to the Cip/Kip family, which controls the checkpoint G1/S transition in the cellular cycle by inhibiting the activity of D/CDK cyclin [27]. The p21 protein is one of the major transcriptional targets of p53 and one of the principal mediators of cellular cycle arrest [81].

However, the increased mortality in C6 cells when p53 is present suggests that the DNA damage to be repaired was substantial in spite of the arrest of the cellular cycle in the G1 phase which would lead to much greater apoptotic cellular death [27,82]. Indeed, p53 may induce the inhibition of the transcription or the translation of repair genes [83,84] when the lesions induced by Cd are severe. The action of p53 in mitochondria could increase its action on nuclei and thus amplify its pro-apoptotic activity. The p53 localization in mitochondria is on the level of the external mitochondrial membrane potential alteration and precedes its translocation in nucleus [85]. The p53 protein can induce the expression of PIG3, a homologous protein of the NADPH-quinone oxidoreductase, which generates free radicals [86]. Marchenko et al. [87] described that p53 was localized in mitochondria after hypoxia or DNA damage. Our results suggest that the mechanisms that directed apoptotic DNA fragmentation in C6 cells came from the intrinsic p53 a nd caspase-9-dependent pathway without caspase-3 activation.

For metallothionein (MT) expression, the p53 inhibition did not increase MT levels in untreated C6 control, as occured with the HaCaT cells. It has been reported in the literature that MTs, a low molecular mass, inducible protein and rich in cysteine residues, direct the detoxification of heavy metals such as Cd [88,89], the storage of essential metals, the scavenging of free radicals and protect cells against oxidative stress [88–95]. Our results showed that the absence of p53 had a significant influence on MT level and these MTs were involved in C6 cells resistance to 20 μM Cd. The results are in agreement with data in the literature [35–37] and also show that normal p53 and the decrease in MT level were involved in the sensitivity of C6 cells (Figures 8–10). These results suggest that the increased MT levels bind and prevent Cd toxicity. In parallel, any changes were observed in MT expression in HaCaT cells in the presence or absence of p53 (Figure 7A and B), which confirms that MT is not involved in HaCaT resistance and that the relation between p53 and MT expression is cell-type-dependent.

In conclusion, the results presented here suggest a model in which Cd induced cell-type-dependent death. In C6 cells, Cd-induced apoptotic DNA fragmentation or oxidative stress induced by Cd causes p53- and caspase-9-dependent apoptotic death without caspase-3 activation. Multiple signalling pathways may be involved in non-apoptotic HaCaT cell death but the apoptosis could be inducible in these cells by p53 restoration. The increased MT levels bind and prevent Cd-induced free radicals which are involving in apoptosis but the data are not sufficient to say that this increased MT is a significant factor in the decrease in apoptotic cell killing.

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