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Cadmium-induced apoptosis in C6 glioma cells: Mediation by caspase 9-activation

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Abstract

The induction of apoptotic cell death by cadmium was investigated in eight mammalian cell lines. Great differences in the cytotoxicity of cadmium were found with different cell lines: Rat C6 glioma cells turned out to be most sensitive with an IC_{50}-value of 0.7 μM, while human A549 adenocarcinoma cells were relatively resistant with an IC_{50}-value of 164 μM $CdCl_2$. The mode of cadmium-induced cellular death was identified to involve apoptotic DNA fragmentation in three cell lines, i.e., in C6 glioma cells, E367 neuroblastoma cells and NIH3T3 fibroblasts. In C6 glioma cells, this process was investigated in detail. Internucleosomal DNA-fragmentation occurred 40 h after application of $CdCl_2$ and was concentration-dependent between 1–100 μM $CdCl_2$, followed by a decrease at higher concentrations due to necrotic processes. Apoptotic chromatin-condensation and nuclear fragmentation was observed 48 h after application of 2.5 μM $CdCl_2$. Furthermore, cadmium (1 μM, 48 h) caused a breakdown of the mitochondrial membrane potential as shown by the decline in mitochondrial uptake of rhodamine 123. Also, we found an activation of caspase 9, a protease known to be activated in apoptotic processes following mitochondrial damage. Besides Cd^{2+}, other toxic heavy metal ions (Hg^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, CrO_4^{2-}, Cu^{2+} or Co^{2+}) did not induce apoptotic DNA fragmentation in C6 cells. The only exception was Zn^{2+} which caused apotosis at high concentrations (>150 μM) whereas it protected against cadmium-induced apoptosis at low concentrations (10–50 μM).

Abbreviations: FBS – fetal bovine serum; HBS – HEPES buffered saline; IC_{50} – 50% inhibition of cell viability; MTT – 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide); pNA – para-nitrophenylacetate; NR – neutral red; PBS – phosphate buffered saline; Rh 123 – rhodamine 123; Tris – tris(hydroxymethyl)methylamine.

Introduction

Chronic cadmium exposure has been involved in a variety of pathological conditions, e.g., nephrotoxicity, neurotoxicity and carcinogenicity. Major effects of cadmium on proteins are binding to thiol groups and substitution of zinc, causing structural changes in these molecules. It has been shown that cadmium affects various cellular processes, including energy metabolism and protein synthesis (Goering *et al.* 1995). The toxicity of cadmium is associated with production of reactive oxygen species and lipid peroxidation (Kumar *et al.* 1996; Szuster-Ciesielska *et al.* 2000). Cadmium inhibits DNA repair processes, e.g., by inactivation of DNA repair enzymes like XPA- and Fpg-protein (Asmuss *et al.* 2000). Cadmium also interferes with the intracellular signalling network and gene regulation on multiple levels (Beyersmann & Hechtenberg 1997). Cadmium ions induce the expression of various genes including the cellular proto-oncogenes c-jun and c-myc (Jin & Ringertz 1990). Furthermore an activation of mitogenic protein kinases is described: Cadmium stimulates nuclear translocation and activity of protein kinase C (Beyersmann *et al.* 1994) and it induces the expression of the immediate early gene c-fos in mesangial cells

through activation of the mitogen activated protein kinase (Ding & Templeton 2000). Cd^{2+}, because of its similarity to Ca^{2+} is able to interfere with calcium-channels, calcium-pumps and the function of calcium-modulated proteins (Verbost *et al.* 1988; Visser *et al.* 1993). Low micromolar concentrations transiently stimulate DNA synthesis and cell growth in various cell lines (Zglinicki *et al.* 1992).

Apoptosis is a central physiological process first described by Kerr *et al.* (1972). In contrast to necrosis, the pathological form of cell death, this programmed cell death is a normal and strategic event with a crucial role in developmental processes which necessitate cell depletion (Hengartner 2000). Apoptotic cell death is characterized morphologically by a shrinkage of the cell, nuclear fragmentation, membrane blebbing and formation of apoptotic bodies. On the molecular level, exposure of phosphatidylserine from the cytoplasmatic to the extracellular site of the plasmamembrane and disruption of mitochondrial membrane potential occurs, followed by efflux of cytochrome C (Cai *et al.* 1998). Furthermore, activation of specific proteases (so-called caspases) occurs and chromatin condensation is observed followed by a nuclear fragmentation. Last, a distinct apoptotic endonuclease is activated resulting in apoptosis-specific DNA-fragmentation. This process is triggered by a variety of agents, including calcium ionophores, radiation, heat shock, cytokines, serum deprivation, oxidants or toxic heavy metals like mercury or cadmium. Several reports have shown that cadmium induces apoptosis in many tissues and cells both *in vivo* and *in vitro*. Cadmium-induced apoptosis was reported e.g., in CEM-C12 human T-lymphocytes (El Azzouzi *et al.* 1994), in LLC-PK1 porcine kidney cells (Ishido *et al.* 1995), in rat testis (Xu *et al.* 1996), mouse liver (Habeebu *et al.* 1998), rat lung epithelial cells (Hart *et al.* 1999), CL-3 human lung carcinoma cells (Chuang *et al.* 2000), human HeLa cells (Szuster-Ciesielska *et al.* 2000) and Rat-1 fibroblast cells (Kim *et al.* 2000). However, the mechanism responsible for the apoptotic action of cadmium is poorly understood. The aim of our study was to compare the apoptotic action of cadmium in different cell lines and to assess molecular mechanisms of cadmium-induced apoptosis in C6 glioma cells. Moreover, effects of the essential metal ion zinc on cadmium-induced apoptosis were investigated and the ability of further heavy metal ions to induce apoptosis in C6 cells was tested.

Material and methods

Chemicals

All tissue culture reagents were purchased from Gibco (Eggenstein, Germany), petri dishes and multiwell plates were obtained from Nunc (Wiesbaden, Germany). Collagen S was obtained from Boehringer (Mannheim, Germany), stock solution of $CdCl_2$ (Titrisol) was from Merck (Darmstadt, Germany). Equilibrated phenol was obtained from Roth (Karlsruhe, Germany), caspase 9-assay from Alexis (Grünberg, Germany), rhodamine 123 from Molecular Probes (Leiden, The Netherlands). All other chemicals were of analytical grade and were purchased from Sigma (Deisenhofen, Germany) or Riedel de Haen (Seelze, Germany).

Cell culture

C6 rat glioma cells, initially derived from a N-nitrosourea-induced astrocytoma (Benda *et al.* 1968) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 4,5 g l glucose and 2 mM L-glutamine, supplemented with 5% fetal bovine serum (FBS). PC12 rat pheochromocytoma cells were cultivated on collagen-coated plastic dishes in RPMI-1640, supplemented with 10% horse serum (heat-inactivated) and 5% FBS; 3T3L1 murine fibroblasts were grown in DMEM (4,5 g l glucose) supplemented with 5% newborne calf serum; E367 rat neuroblastoma cells were cultivated in DMEM (4,5 g l glucose) containing 10% FBS. A549 human fibroblasts and L929 murine fibroblasts were grown in DMEM (1 g l glucose) supplemented with 10% FBS; VH16 human fibroblasts were cultivated in HAM F10 medium with 15% FBS; NIH3T3 murine fibroblasts were grown in DMEM (4,5 g l glucose) supplemented with 10% FBS. The cell culture medium contained 100 units ml penicillin and 100 μg ml streptomycin and was changed twice per week. The cells were maintained in a humidified atmosphere at 37 °C with 10% CO_2 (C6, PC12, 3T3L1 and E367 cells) or 5% CO_2 (A549, L929, VH16 and NIH3T3 cells).

Determination of cytotoxicity

The effect of cadmium on cell viability was determined using the neutral red accumulation assay (modified procedure of Babich *et al.* 1986) and the MTT-assay (Mosmann 1983). Briefly, 50,000

cells/well were plated on a 24-multiwell dish, allowed to attach for 24 h and treated with various concentrations of $CdCl_2$ for different times. After this treatment, the cells were washed with phosphate buffered saline, the medium was changed and cells were incubated for another 24 h. Then the cells were incubated either with 16 μg ml neutral red or 20 μg l MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide) under normal culture conditions for 3 h. The active uptake of neutral red as a marker for cell viability was determined photometrically at 540 nm after solubilisation of the cells in 50% ethanol/49% water/1% acetic acid. As a second marker of cell viability, the conversion of the tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenases was measured. After cell-lysis with isopropanol the absorbance was measured at 570 nm using a Milton Roy 1201 spectrophotometer. Cell viability is shown graphically as percent of the control value (no $CdCl_2$ added). To compare the toxicity in the different cell lines, the IC_{50}-value was taken, describing the cadmium-concentration where 50% of the cells are deficient in the tested viability parameter (neutral red uptake/MTT reduction).

Measurement of apoptotic DNA-fragmentation

To investigate the oligonucleosomal DNA-fragmentation of cadmium-treated cells, the cells were incubated with various concentrations of this heavy metal ion for different times 48 h after seeding of the cells. Attached and floating cells were collected and the DNA was isolated using classical phenol/chloroform-extraction. Briefly, about 10^7 cells were disrupted in 500 μl lysis buffer (10 mM Tris/HCl, 10 mM EDTA, 0.6% SDS) and incubated with 100 μg ml RNAse A (DNAse-free) for 40 min at 37 °C. Proteins were precipitated by addition of 125 μl NaCl (5 M) for 1 h at 4 °C, followed by a centrifugation ($10,000 \times g$, 15 min). The supernatant was extracted with phenol/chloroform (500 μl/250 μl) and subsequently with chloroform (650 μl). The DNA in the supernatant was precipitated overnight (-20 °C) by addition of 1 ml isopropanol. After centrifugation (15 min, $10,000 \times g$) the DNA pellet was resuspended in 30 μl TE-buffer (10 mM Tris/HCl, 1 mM EDTA). DNA-yield was quantified photometrically at 260 nm. The oligonucleosomal fragmentation of DNA as a characteristic feature of the apoptotic cell death was analyzed electrophoretically (1.75% agarose, 3.5 h at 60 V, 4 μg DNA/lane). After staining with ethidium bromide the gel was documented under UV-light using a Herolab UVT-28M gel scanning system.

Microscopic analysis of nuclear fragmentation

To investigate nuclear fragmentation as a second feature of apoptotic cell death, cells were grown in 33 mm culture dishes for 48 h and then incubated with different concentrations of $CdCl_2$ for another 48 h. Then cells were gently washed with HEPES-buffered-saline (25 mM HEPES, 120 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1 mM $MgCl_2$, 1.3 mM $CaCl_2$, 5 mM glucose, pH 7.35) and then nuclei were stained with 100 μM Hoechst 33258 for 15 min. After washing with PBS the cells were photographed at a 400-fold magnification using a Zeiss Axioskop 50 Typ B.

Microscopic analysis of mitochondrial membrane potential

We investigated disruption of mitochondrial membrane potential to determine the mode of apoptotic cell death. Cells were grown in 33 mm culture dishes for 48 h and then incubated with different concentrations of CdCl2 for 48 h. Cells were gently washed with HEPES-buffered-saline and then mitochondria were stained with 3 μM rhodamine 123 for 10 min. After washing with PBS, the cells were photographed at a 400-fold magnification using a Zeiss Axioskop 50 Typ B.

Determination of caspase 9 activity

Caspase 9 activity was measured using a commercial available kit (Alexis). The cells were incubated with different concentrations of $CdCl_2$ for 48 h, then cells were collected, washed with PBS and permeabilized (50 μl lysisbuffer, 10 min on ice). After centrifugation ($10,000 \times g$, 1 min) the protein content of the supernatant was determined. An aliquot containing 150 μg protein was incubated with reaction buffer (including 10 mM dithiothreitol) and caspase 9 substrate (LEHD-pNA). After 60 min (37 °C) the absorption was measured at 405 nm using a Milton Roy 1201 spectrophotometer.

Protein determination

Protein concentration was determined spectrophotometrically by the method of Bradford (1976), bovine serum albumine was used as protein standard.

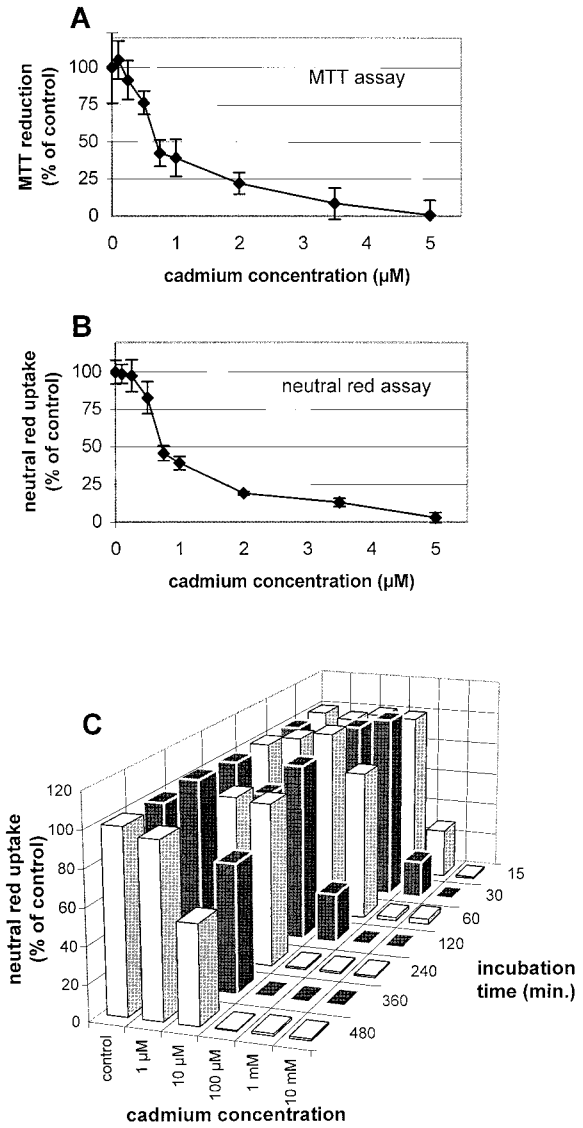

Fig. 1. Cadmium-induced cytotoxicity in C6 glioma cells. Data are means of at least three independent experiments with the standard deviation shown. A: Decrease in cell viability determined using the MTT-assay: incubation with different concentrations of $CdCl_2$ (0.1 μM–5 μM) for 24 h. B: Decrease in cell viability determined using the neutral red accumulation-assay: incubation with different concentrations of $CdCl_2$ (0.1 μM–5 μM) for 24 h. C: Time and concentration dependence of effects of cadmium on cell viability (short time incubation) determined using the neutral red accumulation assay. Incubation was with 1 μM–10 mM $CdCl_2$ for 15–480 min.

Statistics

Experiments were carried out at least in triplicate unless otherwise stated. All values were expressed as the mean ± standard error of the mean (SEM).

Results

Cytotoxicity of cadmium in different cell lines

We used two cell viability assays to analyse the cytotoxic effects of cadmium in different cell lines. The neutral red accumulation assay was chosen as the standard test for the determination of cytotoxicity. To verify these data we also measured the effect of cadmium on the reduction of MTT by mitochiondrial dehydrogenases in several cell lines. Great differences in the sensitivity between these cell lines were found, as depicted in Table 1. Following 24 h exposure to $CdCl_2$, the IC_{50}-values with most tested cell lines were found to be approximately 10–20 μM $CdCl_2$. In contrast, human A549 adenocarcinoma cells were relatively resistant with an IC_{50}-value of 164 ± 13.2 μM $CdCl_2$. Rat C6 glioma cells on the other hand turned out to be the most sensitive to a cadmium-exposure, they showed an IC_{50}-value of 0.7 ± 0.1 μM $CdCl_2$. Dose-response curves of cadmium incubation in C6 cells (neutral red accumulation assay/MTT-assay) and the results of short-time incubation are shown in Figure 1. The experiments indicate that cadmium is an highly cytotoxic agent in C6 glioma cells and the toxicity of $CdCl_2$ is both dose- and time-dependent.

Cadmium-induced DNA-ladder formation

To investigate the mechanism of cadmium-induced cell death, we tested eight different cell lines for the ability of cadmium to induce an internucleosomal DNA-fragmentation. Apoptotic DNA-fragmentation was detected by agarose gel electrophoresis as a so-called *DNA-ladder*. Only in three of the investigated cell lines (C6 glioma cells, E367 neuroblastoma cells and NIH3T3 fibroblasts), the cadmium-mediated cell death was caused by induction of apoptosis, the other cell lines tested (PC12, L929, VH16, 3T3L1 and A549 cells) showed no DNA-fragmentation (Table 2).

For C6 glioma cells, this process was further characterized. Apoptotic cell death was time-dependent, the formation of the DNA-ladder occurred 40 h after application of $CdCl_2$ (Figure 2 B), and this effect was concentration-dependent from 1 μM $CdCl_2$ up to a maximum at 75–100 μM $CdCl_2$, followed by a decrease at higher concentrations (Figure 2 A).

Table 1. Toxicity of cadmium in different cell lines. Exponentially growing cells were treated with different concentrations of $CdCl_2$ for 24 h and then the uptake of neutral red or the reduction of MTT was measured. The IC_{50}-value is shown, describing the $CdCl_2$-concentration where 50% of the cells were deficient in the tested viability parameter (neutral red uptake/MTT reduction).

Cell line	Cell type	Organism	IC_{50} $CdCl_2$ (NR, 24 h)	IC_{50} $CdCl_2$ (MTT, 24 h)
C6	glioma	rat	0.7 ± 0.1 μM	0.68 ± 0.1 μM
A549	adenocarcinoma	human	164 ± 13.2 μM	145 ± 17.8 μM
NIH3T3	fibroblast	mouse	12 ± 2.8 μM	15.6 ± 1.8 μM
VH16	fibroblast	human	65 ± 7.2 μM	n. d.
E367	neuroblastoma	rat	12 ± 0.7 μM	n. d.
3T3L1	fibroblast	mouse	5 ± 0.6 μM	n. d.
L929	fibroblast	mouse	16 ± 3.9 μM	n. d.
PC12	pheochromocytoma	rat	15 ± 3.2 μM	n. d.

n.d., not done.

Cadmium-induced nuclear fragmentation in C6 glioma cells

As a second marker for apoptosis in C6 glioma cells, we used Hoechst 33258 staining to evaluate morphological changes of nuclei following treatment with $CdCl_2$. Whereas nuclei of control cells showed homogeneous staining by the dye (Figure 3 A), nuclei of cadmium-treated cells on the other hand showed a condensation of the chromatin, which was compacted into sharply delinated masses that associate with the nuclear envelope (Figure 3 B). This fragmentation of the nucleus is another characteristic feature of apoptotic cell death.

Disruption of the mitochondrial membrane potential

We used the fluorescent dye rhodamine 123 to analyze changes in mitochondrial membrane potential during the apoptotic cell death. Rh 123 is a cationic fluorescent dye that is transported into mitochondria possessing an active membrane potential. Mitochondria of untreated cells possess the ability to take up this dye and exhibit distinct fluorescent points throughout the cytoplasma of the cell (Figure 3 C). However, in respose to $CdCl_2$, there was no transport of the dye into mitochondria and only a diffuse fluorescence was visible (Figure 3 D). This effect shows that a loss of the mitochondrial membrane potential occurs during cadmium-induced apoptosis in C6 glioma cells.

Activation of caspase 9 by cadmium

To determine wether the mitochondrial pathway is involved in cadmium-induced apoptosis in C6 glioma cells, we tested the activation of caspase 9 in this process. During the mitochondrial-mediated apoptosis a permeability transition pore is opened, followed by an efflux of cytochrome C. This leads to an activation of caspase 9, which in turn activates downstream effector-caspases, like caspase 3 by cleavage to the active enzyme. We incubated cells for 48 h with different concentrations of cadmium and then analysed the activity of caspase 9 using a nitrophenol-labelled caspase 9-substrate. A significant increase in enzyme activity was found after incubation with 5 and 10 μM $CdCl_2$ (Figure 4) suggesting a central role for caspase 9 in cadmium-induced apoptosis.

Effect of zinc on cadmium-induced DNA-ladder formation

Zinc generally is thought to have anti-apototic properties. To investigate, if zinc has a protective effect on cadmium-induced apoptosis in C6 glioma cells, too, we incubated the cells with 5 μM $CdCl_2$ and simultaneous with different concentrations of $ZnCl_2$ (10–250 μM) and analyzed the apoptotic DNA-ladder after 48 h of incubation. We found an bimodal effect of zinc on cadmium-induced apoptosis: Low doses of $ZnCl_2$ (10–50 μM) caused a reduction of the DNA-ladder-formation, whereas higher doses of zinc (100–250 μM $ZnCl_2$) led to an enhancement of this apoptotic feature (Figure 5), suggesting a pro-apoptotic role for zinc besides its anti-apoptotic properties.

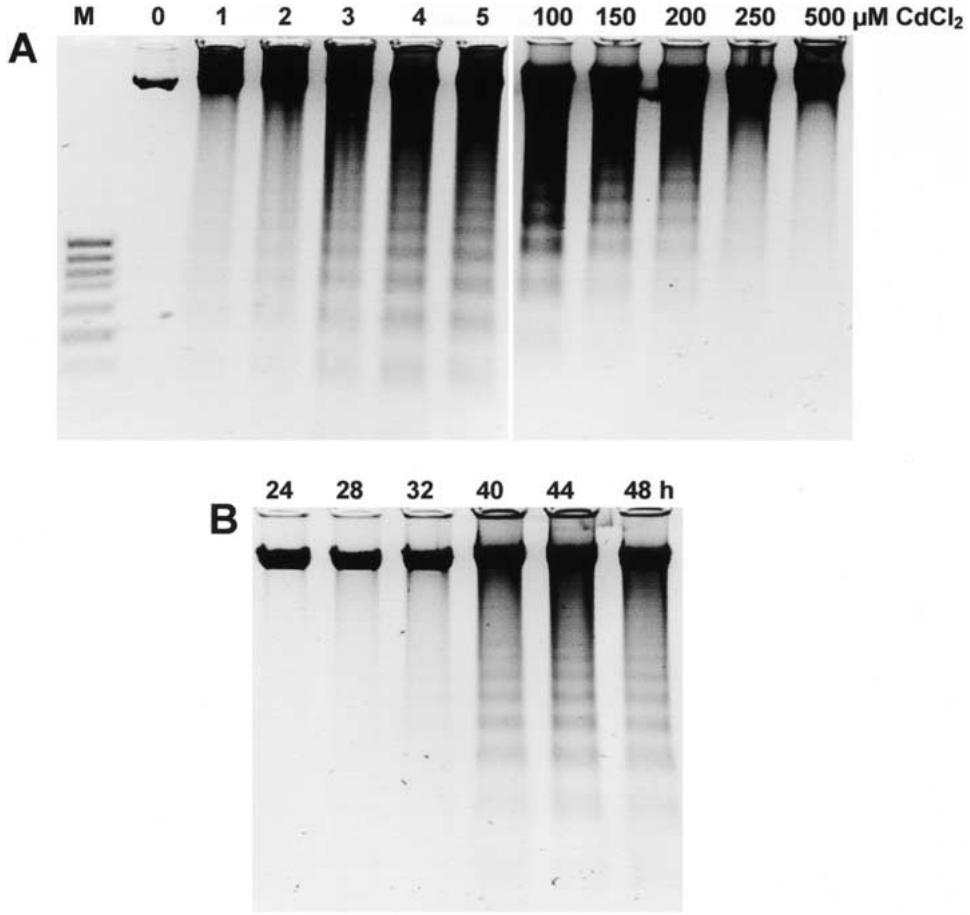

Fig. 2. Generation of internucleosomal DNA-fragments in C6 glioma cells by cadmium. Agarose gel electrophoresis patterns of DNA from cells treated either with different concentrations of CdCl2 (A) or with 10 μM $CdCl_2$ for different times (B). Each frame shows one representative experiment out of three. A: Concentration-dependence of cadmium-induced DNA-ladder formation: C6 glioma cells were incubated with different concentrations of $CdCl_2$ for 48 h and the internucleosomal DNA-fragmentation was analysed electrophoretically. The ladder formation occurred at concentrations starting at 1 μM $CdCl_2$, reaching a maximum at 75–100 μM $CdCl_2$. B: Time-dependence of cadmium-induced DNA-ladder formation: C6 glioma cells were incubated with 10 μM $CdCl_2$ and the generation of internucleosomal DNA-fragmentation was analysed after 24–48 h. The DNA-ladder formation was first observed after an incubation time of 40 h.

Table 2. Effect of cadmium on apoptotic DNA-fragmentation in different cell lines. Exponentially growing cells were treated with different concentrations of $CdCl_2$ for 48 h, then DNA was isolated using phenol/chloroform-extraction and analysed by agarose gel electrophoresis.

Cell line	IC_{50} $CdCl_2$ (NR, 24 h)	Concentrations used	DNA-ladder (48 h)
C6	0.7 ± 0.1 μM	1, 2, 3, 4, 5, 10, 25, 50, 100 μM	starting at 1 μM
A549	164 ± 13.2 μM	50, 100, 150, 200, 250 μM	none
NIH3T3	12 ± 2.8 μM	1, 5, 10, 25, 50 μM	starting at 10 μM
VH16	65 ± 7.2 μM	10, 25, 50, 75, 100 μM	none
E367	12 ± 0.7 μM	1, 5, 10, 15, 20 μM	starting at 10 μM
3T3L1	5 ± 0.6 μM	1, 2,5, 5, 10, 25 μM	none
L929	16 ± 3.9 μM	5, 10, 25, 50, 100 μM	none
PC12	15 ± 3.2 μM	1, 5, 10, 25, 50 μM	none

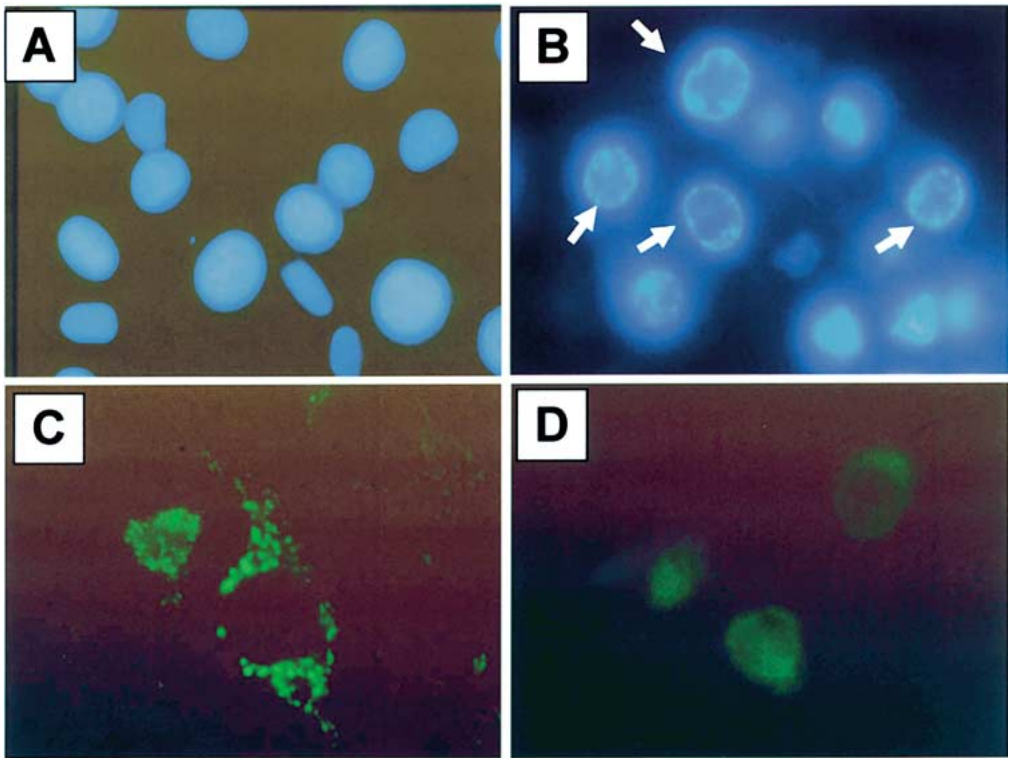

Fig. 3. Nuclear fragmentation and breakdown of mitochondrial membrane potential in C6 glioma cells treated with cadmium. Cells were analysed by fluorescence-microscopy: DNA staining with Hoechst 33258 shows apoptotic fragmentation of nuclei (A, B), and staining of mitochondria with rhodamine 123 shows the decrease of mitochondrial membrane potential (C, D). A: Hoechst 33258-staining of untreated control cells, showing intact nuclei. B: Hoechst 33258-staining of C6 cells incubated with 2.5 μM $CdCl_2$ for 48 h, showing chromatin condensation and nuclear fragmentation. C: Rhodamine 123-staining of untreated control cells, showing functionally active mitochondria. D: Rhodamine 123-staining of C6 cells incubated with 1 μM $CdCl_2$ for 48 h, showing a significant decrease in fluorescence as an indicator of the disruption of the mitochondrial membrane potential.

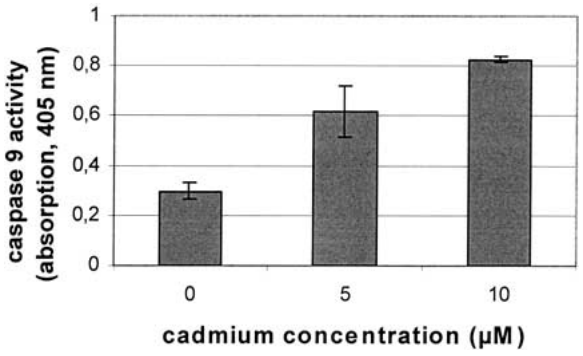

Fig. 4. Activation of caspase 9 by cadmium in C6 glioma cells. Cells were incubated with different concentrations of CdCl2 for 48 h, cells were permeabilized and a nitrophenylacetate-labelled caspase 9-substrate was added. Absorbance was measured at 405 nm after 1 h of incubation.

Effect of various heavy metal ions on DNA-ladder formation in C6 glioma cells

To investigate, if the induction of apoptosis by heavy metal ions in C6 cells was specific for cadmium, we tested the ability of other heavy metal ions to induce an apoptotic DNA-ladder. C6 glioma cells were incubated with different concentrations of various heavy metal ions (Co^{2+}, Ni^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}, Fe^{2+}, Cu^{2+} and CrO_4^{2-}) for 48 h. It was found, that the formation of the DNA-ladder in C6 cells was quite specific for cadmium, other toxic heavy metal ions showed no apoptotic features in our cell system under the conditions applied (Table 3). The only tested metal ion that resulted the formation of an apoptotic DNA-ladder was zinc (starting at 150 μM $ZnCl_2$).

Discussion

We investigated the toxicity of cadmium in eight different cell lines and found great differences in their sensitivity: Rat C6 glioma cells turned out to be very sensitive to a cadmium-exposure, while human A549 adenocarcinoma cells were relatively resistant. A pos-

Table 3. Effect of different heavy metal ions on apoptotic DNA-fragmentation in C6 glioma cells. Exponentially growing cells were treated with different concentrations of various toxic heavy metal ions for 24 or 48 h, then DNA was isolated using phenol/chloroform-extraction and analyzed by agarose gel electrophoresis.

Heavy metal ion	Incubation time	Concentration used	DNA-ladder
$CoCl_2$	24 h	10, 25, 50, 100, 250, 500, 750 μM	none
$CoCl_2$	48 h	10, 25, 50, 100, 250, 500, 750 μM	none
$NiCl_2$	24 h	10, 25, 50, 100, 250, 500, 750 μM	none
$NiCl_2$	48 h	50, 100, 250, 500, 1000 μM	none
$ZnCl_2$	24 h	15, 50, 100, 250, 500, 750, 1000 μM	none
$ZnCl_2$	48 h	50, 100, 150, 200, 250 μM	starting at 150 μM
$HgCl_2$	24 h	1, 2.5, 5, 10, 25, 50, 75 μM	none
$HgCl_2$	48 h	1, 2.5, 5, 10, 20, 25, 50 μM	none
$PbCl_2$	48 h	25, 50, 125, 250, 500 μM	none
$FeSO_4$	48 h	1, 10, 50, 100, 250 μM	none
$CuCl_2$	48 h	100, 250, 500 μM	none
Na_2CrO_4	48 h	100, 250, 500 μM	none

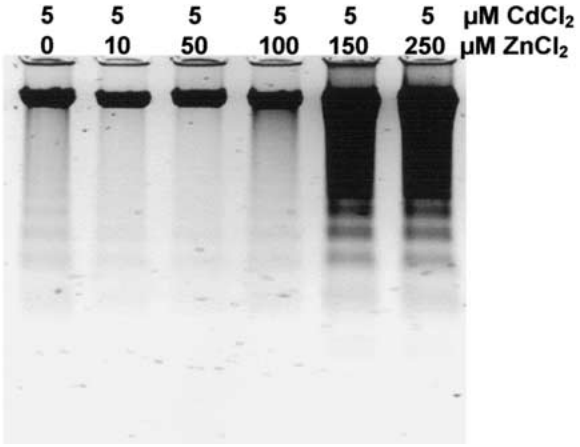

Fig. 5. Bimodal effect of zinc on cadmium-induced DNA-fragmentation in C6 glioma cells. Apoptotic cell death was initiated by incubation with 5 μM $CdCl_2$ for 48 h. To determine the anti-apoptotic effect of zinc, different concentrations of $ZnCl_2$ (10–250 μM) were added at the same time. and the effect of the apoptotic DNA-ladder was investigated. DNA was isolated by phenol/chloroform extraction and analysed by agarose gel electrophoresis. One representative experiment out of three is shown.

sible explanation for these great differences in the cellular sensitivity to cadmium could be the presence of different intracellular GSH-levels, different velocities of cadmium uptake or variations in content of protective enzymes (Hatcher *et al.* 1995). Our results indicate that C6 glioma cells are more sensitive to cadmium compared to all other tested cell lines, suggesting that glial cells, e.g., astrocytes, probably are a sensitive target of cadmium toxicity. This fact may be relevant *in vivo*: Although kidney and liver are major target organs of cadmium toxicity, there are also neurotoxic effects caused by this heavy metal (Kumar *et al.* 1996), possibly mediated by destruction of astrocytes.

Whereas necrotic (pathological) cell death is associated with the disruption of the cellular membrane, apoptosis, being the active form of cell death, is associated with the activation of distinct enzymes and has a widespread biological importance (Cameron & Feuer 2000). Although it was previously assumed that cadmium-induced cell death occurs predominantly necrotically, it was recently shown, that this heavy metal ion can act by induction of programmed cell death, too. The effect of cadmium on apoptotic processes is still discussed controversely. While few authors describe anti-apoptotic properties of this heavy metal ion (Shimada *et al.* 1998; Eneman *et al.* 2000; Yuan *et al.* 2000), several reports have shown that cadmium can induce apoptosis in many tissues and cells both *in vivo* and *in vitro* (Hamada *et al.* 1997). However, there are only few studies that have elucidated mechanistic effects of cadmium-induced apoptosis.

A first aim of our study was to compare the apoptotic action of cadmium in different cell lines. Only in three of the investigated cell lines, the cadmium-mediated cell death was caused by induction of apop-

tosis, indicating that cadmium exposures apoptotic features only in distinct cell lines. This result shows that there is no uniform mode of cell death caused by this heavy metal ion. In C6 glioma cells, the apoptotic process was further investigated. The endonucleolytic cleavage of DNA was analysed by agarose gel electrophoresis. The formation of the DNA-ladder, one of the hallmarks of apoptotic cell death, occurred 40 h after application of $CdCl_2$. A concentration-dependent induction was found from 1 μM up to a maximum at 75–100 μM $CdCl_2$, followed by a decrease of DNA-ladder formation at higher concentrations presumably due to necrotic processes. The apoptotic cell death was further confirmed by fluorescence microscopy.

With respect to understanding the apoptotic properties of cadmium, a most pertinent question is how this heavy metal induces the apoptotic machinery. To obtain further information on the mechanism of cadmium-induced apoptosis in C6 glioma cells, we investigated the effect of a cadmium-incubation on mitochondrial membrane potential and caspase 9-activation. Using fluorescent dye rhodamine 123 we observed a decrease in mitochondrial membrane potential during cadmium-induced apoptosis. Defective mitochondria release cytochrome C through the permeability transition pore, which in turn mediates the activation of caspase 9 (Cai *et al.* 1998). We indeed found that the cadmium-mediated breakdown of the mitochondrial membrane potential was associated with an activation of caspase 9. These results complement findings of other laboratories. Kim *et al.* (2000) described a similar decrease of rhodamine 123 fluorescence during cadmium-induced apoptosis in Rat-1 fibroblasts, suggesting that this heavy metal acts by altering the permeability of the mitochondrial membrane. Other indications for the involvement of mitochondria in cadmium-induced apoptosis were obtained by transfection with bcl-2. Bcl-2 is an anti-apoptotic protein localized in the mitochondrial membrane (Tsujimoto & Shimizu 2000), that protects against various modes of apoptotic cell death, e.g., cadmium-mediated apoptosis in Rat-1 cells (Kim *et al.* 2000). Bcl-2 may suppress cadmium-induced cell death by inhibiting the breakdown of the mitochondrial membrane potential and/or the cytochrome C release.

Zinc at low concentrations is essential for cellular growth and differentiation and exhibits an apoptosis-suppressing function. In various *in vitro* and *in vivo* systems, application of zinc showed anti-apoptotic properties (Barbieri *et al.* 1992, Sunderman, 1995), probably due to inhibition of caspase 3-maturation, antioxidant properties or inhibition of the Ca^{2+}/Mg^{2+}-dependent apoptotic endonuclease (Perry *et al.* 1997; Aiuchi *et al.* 1998; Chai *et al.* 1999). Zinc showed protective effects against cadmium-induced apoptosis, too. Ishido *et al.* (1995) showed that zinc (50 μM) suppresses the apoptotic DNA ladder formation elicited by cadmium in LLC-PK1 cells. We tested the effect of zinc on cadmium-induced apoptosis in C6 glioma cells and found bimodal features of this treatment depending on the applied concentration of zinc: A decrease of cadmium-induced DNA-ladder formation was found after addition of 50 μM $ZnCl_2$, whereas higher concentrations of $ZnCl_2$ led to an increase in apoptotic DNA-ladder formation. We conclude that zinc has a dual effect on cadmium-induced apoptotic cell death in C6 glioma cells. Low concentrations (up to 50 μM) are protective against cadmium-induced cell death, probably mediated by an inhibition of cellular cadmium uptake, whereas zinc at higher concentrations induces apoptotic cell death in C6 glioma cells itself by a yet unknown mechanism (Haase 2000).

A further question was, if the apoptosis-inducing property of cadmium was specific for this heavy metal or if it was a general mode of metal-induced cell death in C6 cells. Therefore, we investigated apoptotic effects of other heavy metal ions on DNA-ladder formation in C6 glioma cells. Various heavy metal ions (Hg^{2+}, Pb^{2+}, Ni^{2+}, Fe^{2+}, CrO_4^{2-}, Cu^{2+} or Co^{2+}) were tested at different concentrations and different times (24 or 48 h). However, besides cadmium only zinc was able to induce apoptosis, starting to cause DNA-ladder formation at concentrations >150 μM. Therefore, we conclude that the induction of apoptosis by heavy metal ions in C6 glioma cells is rather specific for cadmium and zinc. The similar behaviour could be explained by the similar chemical characteristics of this two cations in terms of ionic radii and protein binding abilities, but the apoptosis-inducing ability of zinc remains rather surprising.

Previous results of our laboratory (Lohmann & Beyersmann 1993) showed that cadmium and zinc inhibit DNA-fragmentation in dependence of free Ca^{2+} (100 μM) in isolated bovine liver nuclei. It was found that cadmium alone was able to stimulate DNA fragmentation, but in combination with Ca^{2+}, cadmium was even a stronger inhibitor of the endonuclease than zinc, suggesting an ambivalent role of cadmium on the apoptotic endonuclease: low concentrations of cadmium are able to activate this enzyme mediated e.g.

by binding to a Ca^{2+}-binding site, whereas higher concentrations are inhibitory.

There is increasing evidence that elevation of intracellular free calcium can induce apoptosis. In C6 glioma cells calcium ionophores like A23187 cause an endonucleolytic cleavage of DNA. One explanation for cadmium-induced apoptosis could be, that cadmium promotes cell death via an increase of intracellular free calcium. We tried to test this hypothesis using Fura-2, a fluorescent probe for intracellular free calcium ions, but there was a strong interference by the fluorescent complex of Fura-2 with cadmium, making this measurement impossible (Wätjen 2000).

Cadmium could also cause apoptosis via induction of oxidative stress. Hart *et al.* (1999) found evidence for the participation of oxidative stress in apoptosis caused by cadmium in rat lung epithelial cells. They reported that up-regulation of redox-sensitive transcription factors (NFκB and AP-1) precedes the induction of apoptosis. In C6 glioma cells we tested the ability of various antioxidants to inhibit cadmium-induced apoptosis, but no protective effect was found (Wätjen 2000). Hence, an oxidative mechanism of cadmium-induced apoptosis is not probable.

The results of the present study show that cadmium is a potent inductor of cell death, but the tested cell lines showed different sensitivity. Cell death by apoptosis occurs only in three out of eight tested cell lines as measured by DNA-laddering experiments, suggesting that there is no general mode of cell death caused by this heavy metal. Our data illustrate that cadmium is a potent inductor of apoptosis in C6 glioma cells, representing a model of astroglial cells. This observation may be relevant *in vivo*, for instance to explain the perturbation of processes in the developing brain by cadmium. Because apoptosis also is thought to play an important role in neurodegenerative diseases, low concentrations of cadmium may initiate programmed cell death leading to a selective cell death in distinct brain regions. In conclusion, our results support the consideration that induction of apoptosis by cadmium is an important mechanism for the toxicity of this heavy metal ion.

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