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Cadmium-induced apoptosis in C6 glioma cells: Influence of oxidative stress

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Abstract

Cadmium has recently been shown to induce apoptosis in C6 glioma cells via disruption of the mitochondrial membrane potential and subsequent caspase 9-activation. Here we show that both H_2O_2 and $CdCl_2$ induced apoptotic DNA fragmentation in C6 cells. The employment of glutathione as an antioxidant prevented the induction of apoptotic DNA fragmentation by cadmium completely and catalase strongly reduced cadmium-induced DNA fragmentation suggesting that cadmium exerts its apoptotic effects at least partly via the production of H_2O_2. Apoptosis may be induced by cadmium indirectly through formation of oxidative stress, e.g., by inhibition of antioxidant enzymes. After incubation of C6 cells with cadmium for short times (up to 4 h), we analyzed the formation of intracellular reactive oxygen species and cellular lipid peroxidation. After 1 h of incubation with inreasing concentrations of $CdCl_2$ (1–500 μM), no increase in dichlorofluorescein fluorescence was found. At variance, lipid peroxidation was slightly elevated after 2 h incubation with cadmium (50–100 μM). Furthermore, we analyzed the modulation of markers for oxidative stress after prolonged (24 h) exposure to cadmium. The intracellular glutathione content as measured using the fluorescent probe monobromobimane was decreased after incubation with $CdCl_2$ (0.5–10 μM) for 24 h. Furthermore, we measured the effect of cadmium on the level of oxidized DNA lesions (predominantly 8-hydroxyguanine) using the bacterial Fpg-DNA-repair protein. After 24 h of incubation with 5 μM $CdCl_2$ we found a sixfold increase in Fpg-sensitive DNA-lesions. We conclude that short time incubations with cadmium (up to 4 h) caused only slight or insignificant effects on the generation of reactive oxygen species (formation of thiobarbituric acid reactive substances, fluorescence of dichlorofluorescein), whereas incubation with this heavy metal for 24 h lead to a decrease in intracellular glutathione concentration and an increase in oxidative DNA-lesions. Our data demonstrate that cadmium as similar to H_2O_2 is a potent inducer of apoptosis in C6 cells. Even if cadmium unlike Fenton-type metals can not produce reactive oxygen species directly, the apoptotic effects of cadmium at least in part are mediated via induction of oxidative stress. Because both apoptosis and oxidative stress are thought to play important roles in neurodegenerative diseases, low concentrations of cadmium that initiate programmed cell death may lead to a selective cell death in distinct brain regions via generation of oxidative stress.

Abbreviations: DCF – 2′,7′-dichlorofluorescein; FBS – fetal bovine serum; Fpg – formamido-pyrimidine-glycosidase; GSH – glutathione; H_2DCF – 2′,7′-dichlorodihydrofluorescein; H_2DCF/DA – 2′,7′-dichlorodihydro-fluorescein diacetate; IC_{50} – 50% inhibition of a cell viability parameter; HBS – HEPES buffered saline; MBBr – monobromobimane; MTT – 3,(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide); NR – neutral red; PBS – phosphate buffered saline; ROS – reactive oxygen species; TBARS – thiobarbituric acid reactive substances.

Introduction

Chronic cadmium exposure has been implied in a variety of pathological conditions including neurodegenerative processes (Kumar *et al.* 1996). Therefore we have been interested in the elucidation of mechanisms by which cadmium causes the death of cells in the nervous system. The cytotoxicity of this heavy metal could be explained by a great variety of cellular mechanisms. The necrotic properties of relatively high cadmium concentrations may be understood in terms of inhibition of metabolic enzymes. The apoptotic action of relatively low cadmium concentrations may involve disturbance of cellular signal transduction and/or damage to DNA. One effective mechanism consists of disturbation of intracellular signalling pathways by competitive interference with zinc or calcium-dependent enzymes or perturbation of calcium homeostasis (Beyersmann & Hechtenberg 1997). Another plausible mechanism implies the generation of oxidative stress. Because cadmium is not able to catalyze Fenton-type reactions in biological systems due to its redox potential, it can not generate reactive oxygen species (ROS) directly. However, cadmium can induce oxidative stress indirectly, e.g., by inhibition of antioxidative enzymes, thereby producing an accumulation of endogenous ROS (Shukla *et al.* 1987, Stohs & Bagchi 1995). Another possibility may be, that cadmium disturbs the intracellular prooxidant/antioxidant balance, thereby producing oxidative stress: Cadmium has a high affinity to sulfhydryl groups e.g. in reduced glutathione, which may cause a depletion of reduced glutathione and have implications for the maintenance of the thiol-disulfide balance in the cell (Nigam *et al.* 1999). Several authors have shown, that cadmium is able to induce oxidative stress in various cell lines (Stacey *et al.* 1980; Amoruso *et al.* 1982, Ochi *et al.* 1987; Tatrai *et al.* 2001; El-Maraghy *et al.* 2001; Stohs *et al.* 2001). Cadmium has been reported to induce the production of superoxide anions in J774A.1 cells (Hassoun & Stohs 1996), and the generation of ROS as detected by a fluorescent probe in MRC-5 cells (Yang *et al.* 1997).

In contrast to necrosis, the pathological form of cell death, programmed cell death is a normal and crucial event in developmental processes which necessitate cell depletion. 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 there occurs an externalization of phosphatidylserine from the cytoplasmatic to the extracellular site of the plasmamembrane and a disruption of mitochondrial membrane potential, followed by a efflux of cytochrome C. Furthermore, distinct proteases (so-called caspases) are activated, and there is also chromatin condensation followed by a nuclear fragmentation and activation of a distinct endonuclease resulting in apoptosis-specific DNA-fragmentation. This whole process is triggered by a variety of agents, including calcium ionophores, radiation, heat shock, cytokines, serum deprivation or oxidative stress.

Several reports have shown that cadmium induces apoptosis in various tissues and cells both *in vivo* and *in vitro* (Hamada *et al.* 1997). E.g., cadmium-induced apoptosis was reported in rat testes (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), HeLa human cervix carcinoma cells (Szuster-Ciesielska *et al.* 2000) and Rat-1 fibroblast cells (Kim *et al.* 2000).

In the present study, we have used rat C6 glioma cells because we were interested in the mechanism of cadmium-induced cell death in the nervous system. These cells are very sensitive to cadmium, and apoptosis is the mode of cell death induced by cadmium in these cells. In the neutral red accumulation assay, cadmium exhibited an IC_{50}-value of 0.7 μM (24 h) and observed the following markers of apoptosis: disruption of mitochondrial membrane potential, caspase 9-activation, and internucleosomal DNA fragmentation starting at concentrations as low as 1 μM $CdCl_2$ with a maximal induction at 75–100 μM $CdCl_2$ (Wätjen *et al.* 2002).

However, the induction of apoptosis is a complex process and the mechanisms responsible for the apoptotic action of cadmium are still poorly understood. The aim of our study was to compare the apoptotic action of cadmium in different cell lines with respect of oxidative stress involvement. Reactive oxygen species (ROS), such as hydrogen peroxide, superoxide anions and hydroxyl radicals are involved in initiation of some types of apoptosis. Because in various cell lines it was shown that cadmium exerts its toxic effects via generation of oxidative stress we tested the involvement of oxidative stress in cadmium-induced apoptosis and compared it with H_2O_2-induced apoptotic effects.

Materials 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), H_2O_2 (30%) was obtained from Fluka (Buchs, Germany), equilibrated phenol was from Roth (Karlsruhe, Germany), hydroxylapatite (high resolution) was from Calbiochem (Bad Soden, Germany) and monobromobimane and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF/DA) was from Molecular Probes (Leiden, Netherlands). Fpg protein was a generous gift from Prof. Andrea Hartwig (Karlsruhe, Germany). 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. A549 human fibroblasts were grown in DMEM (1 g/l glucose) supplemented with 10% FBS and NIH 3T3 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 and PC12 cells) or 5% CO_2 (A549 and NIH3T3 cells).

Determination of cytotoxicity

The effect of chemicals on cell viability was determined using the neutral red accumulation assay (modified procedure of Babich *et al.* 1986) and the MTT-assay (Mosman 1983). Briefly, 10 000 cells well were plated on a 96-multiwell dish, allowed to attach for 24 h and treated with different concentrations of $CdCl_2$ or H_2O_2 for 24 h. After this treatment, the cells were washed with phosphate buffered saline and were incubated either with 16 μg/l 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 cell-lysis in 50% ethanol/49% water/1% acetic acid. The conversion of the tetrazolium salt MTT to a colored formazan by mitochondrial dehydrogenases is a second marker of cell viability. The colour development was measured photometrically at 570 nm after cell-lysis in isopropanol. Cell viability is shown graphically as percent of the control value (no $CdCl_2$ or H_2O_2 added). As measure of toxicity in the different cell lines, the IC_{50}-value was taken, describing the concentration where 50% of the cells are deficient in the tested viability parameter (neutral red uptake or MTT reduction).

Detection of thiobarbituric acid reactive substances

Lipid peroxidation was quantified by the thiobarbituric acid assay (Draper & Hadley 1990), which measures the production of malondialdehyde or other related substances, so-called 'thiobarbituric acid reactive substances' (TBARS). This method is a first global measure of lipid peroxidation and was applied because of its sensitivity and simplicity. Cells were washed with HBS and transferred into Eppendorf reaction tubes, then the cell suspension was incubated with different concentrations of $CdCl_2$ or H_2O_2 for 2 h. After incubation, 1 ml of 0.375% 2-thiobarbituric acid and 15% trichloroacetic acid in 0.25 M HCl were added. The tubes were placed in a waterbath and kept at 95 °C for 45 min. After cooling, the suspension was centrifuged at 11 000 $\times$ g for 3 min and the absorbance of the supernatant was measured photometrically at 535 nm using a Milton Roy 1201 spectrophotometer.

Intracellular DCF formation

As a marker for oxidative stress the fluorescent probe 2′,7′-dichlorodihydrofluorescein (H_2DCF) was used. Cells were incubated with H_2DCF/DA (2′,7′-dichlorodihydrofluorescein diacetate) which is cleaved by cytosolic esterases to H_2DCF preventing the back-diffusion of the dye in the extracellular space. The oxidation of intracellular nonfluorescent H_2DCF to highly fluorescent DCF was measured in a Perkin Elmer LS50B luminescence-spectrometer (excitation: 485 nm, emission: 525 nm). Cells were incubated with 100 μM H_2DCF/DA in HBS-buffer containing 0.3% bovine serum albumine for 30 min in the dark. After loading of the cells with the fluorescent probe the cells were washed in HBS-buffer and the cell suspension was transferred in a 96-multiwell plate. In the fluo-

rescence reader first the formation of DCF by endogeneous generation of oxidative stress was measured for 15 min, then $CdCl_2$ was added in different concentrations and the oxidation of intracellular H_2DCF to highly fluorescent DCF was measured. After each measurement, 1 mM H_2O_2 was added to check the experimental system leading to a strong increase in DCF-formation.

Measurement of intracellular SH-groups

An important parameter accompanied by generation of oxidative stress is decrease of the intracellular GSH concentration due to oxidation processes (conversion of GSH to GSSG). The concentration of intracellular SH-groups was measured using monobromobimane (MBBr). This probe, which readily reacts with thiols is essentially non-fluorescent until conjugated. C6-cells were incubated with different concentrations of $CdCl_2$ for 24 h. Then cells were collected, washed in HBS-buffer and incubated with 40 μM MBBr. Fluorescence increase was measured using a Perkin Elmer LS50B luminescence-spectrometer (excitation: 390 nm, emission: 460 nm). The fluorescence-values after 5 min of MBBr-incubation were normalized by protein content. The data were shown as percent of the control value (no cadmium added).

Measurement of apoptotic DNA-fragmentation

In the late process of apoptotic cell death a specific endonuclease is activated which cleaves only in the linkerregion of the DNA. After electrophoresis, this results in an oligonucleosomal-sized DNA fragmentation muster, the so-called 'DNA-ladder' which is a marker of apoptotic cell death. 1.5×10^6 cells were seed in 10 cm dishes, allowed to attach for 48 h and then incubated with either hydrogen peroxide or $CdCl_2$ $\pm$ antioxidative substances for 48 h. Then, attached and floating cells were collected and the DNA was isolated using phenol/chloroform-extraction. Briefly, the 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 (10 000 $\times$ g, 15 min) 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 the Herolab UVT-28M gel scanning system.

Detection of oxidized DNA-bases (Fpg-sensitive DNA-lesions)

Oxidative DNA damage is one of the mechanisms postulated to play an important role in mutagenesis and carcinogenesis. Without DNA repair, oxidative DNA damage prior to cellcell replication, can induce gene mutations and putative 'initiate' the exposed cell, converting it to an irreversibly altered neoplastic lesion which may undergo promotion and progression to potential malignancy. DNA strand breaks and Fpg-sensitive sites were determined with slight modifications as described by Hartwig *et al.* (1996). 10 000 cells were seed in a 33-mm tissue dish, allowed to attach for 24 h and were incubated with different concentrations of $CdCl_2$ for 24 h. Then cells were carefully washed with PBS and 500 μl lysis buffer was added (6 mM Na_2HPO_4, 1 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl and 0.1% Triton X-100). After 5 min on ice, lysis buffer was removed and 500 μl high salt solution was added (4 M NaCl, 10 mM EDTA and 2 mM Tris, pH 8.0) for 10 min on ice. The nucleoids were then incubated with bacterial Fpg-protein (1 μg/ml) in 760 μl enzyme buffer (5 mM Na_3PO_4, 10 mM EDTA, pH 7.5) for 30 min at 37 °C in the dark. For the detection of oxidized DNA bases, the Fpg-protein, a DNA repair protein causing DNA strand breaks at e.g. 8-hydoxyguanine-sites, was omitted. At the end of the incubation, an alkaline solution was added yielding the final concentration of 7 mM NaOH, 13 mM EDTA and 370 mM NaCl, pH 12.3, then the DNA was allowed to unwind for 30 min in the dark. The further steps of unwinding, neutralization and separation of single and double-stranded DNA were performed as described previously (Hartwig *et al.* 1993). Briefly, the solution was neutralized with HCl, sonificated and SDS was added to a final concentration of 0.05%. Separation of single- and double-stranded DNA was performed on 0.5 ml hydroxylapatite columns at 60 °C, where single- and double-stranded DNA were eluated with

1.5 ml of 0.15 M and 0.35 M potassium phosphate buffer, respectively. The DNA content of both fractions was determined by adding Hoechst 33258 (final concentration of 7.5×10^7 M) to 1 ml of each sample and measuring the fluorescence with a luminescence spectrometer (Perkin Elmer LS50B, excitation: 360 nm, emission: 450 nm). The fraction of double-stranded DNA was calculated as described before (Hartwig *et al.* 1993). In order to quantitate the lesion frequencies, the fraction of double-stranded DNA was compared with the amount of double-stranded DNA produced by X-rays in a calibration experiment (Hartwig *et al.* 1996).

Protein determination

Protein concentration was determined spectrophotometrically by the method of Neuhoff *et al.* (1979) using the dye amido black 10B. Bovine serum albumine was used as protein standard.

Statistics

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

Results

Cytotoxicity of H_2O_2 and $CdCl_2$ in different cell lines

Previously we had investigated the cytotoxic effects of cadmium in various cell lines (Wätjen *et al.* 2002). To evaluate, if the toxicity of cadmium was mediated via induction of oxidative stress, we first analyzed the cytotoxic effects of hydrogen peroxide in four selected cell lines: rat C6 glioma cells, rat PC12 pheochromocytoma cells, human A549 adenocarcinoma cells and murine NIH3T3 fibroblasts. We used two different cell viability assays: The neutral red accumulation assay and the MTT-assay. Table 1 shows the cytotoxicity data for cadmium obtained in a previous study (Wätjen *et al.* 2002) and those for H_2O_2 measured in this work. In agreement with previous results on cadmium toxicity, we found great differences in the cellular sensitivity to hydrogen peroxide. C6 cells were most sensitive: after 24 h of incubation, an IC_{50}-value (NR) of 62.8 ± 7.1 μM H_2O_2 was observed. In contrast, A549 cells were relatively resistant to oxidative stress showing an IC_{50}-value (NR) of 750 ± 48 μM H_2O_2.

Induction of apoptosis by H_2O_2 and $CdCl_2$ in different cell lines

In a previous study, we had investigated different mammalian cell lines in respect to the ability of cadmium to induce apoptosis and found an induction of apoptosis only in few cell lines (Wätjen *et al.* 2002). Cadmium induced the formation of oligonucleosomal DNA-ladder patterns in rat C6 glioma cells and murine NIH3T3 fibroblast cells, whereas PC12 and A549 cells showed no apoptotic features after application of cadmium. Also in the case of hydrogen peroxide-induced cell death we found differences between the four investigated cell lines. Apoptotic DNA-ladders were found in C6 glioma and NIH3T3 fibroblast cells after incubation with different concentrations of H_2O_2 for 48 h. In C6 cells, apoptotic DNA-fragmentation by H_2O_2 was starting at 250 μM with a maximum at 750–1000 μM (Figure 1). NIH3T3 cells were less sensitive with oligonucleosomal DNA-fragmentation starting at H_2O_2 concentrations of 400 μM. On the other hand, rat PC12 pheochromocytoma and human A549 adenocarcinoma cells showed no apoptotic features after application of hydrogen peroxide even at concentrations of 1 mM H_2O_2 (results summarized in Table 2).

Lipid peroxidation induced by cadmium

If cadmium exerts its apoptotic action via induction of oxidative stress, an increase in lipid peroxidation products should be detectable. We measured the production of reactive aldehydes, e.g., malondialdehyde, using their reaction with thiobarbituric acid to form coloured thiobarbituric acid-reactive substances (TBARS). We found only a moderate increase in TBARS after 2 h of incubation with cadmium. 50 μM $CdCl_2$ caused an increase to 107.4 ± 2.3% TBARS/mg protein compared to the control value, 100 μM $CdCl_2$ led to an increase to 114.9 ± 8.2. Treatment of C6 glioma cells with 50 μM H_2O_2 under similar conditions led to an increase to 131.4 ± 3.5% (Figure 2).

Generation of intracellular ROS by cadmium

In order to measure the generation of reactive oxygen species by cadmium we used the fluorescent probe DCF. There was no significant increase in DCF-formation after incubation with different concentrations of $CdCl_2$ for up to 4 h. Treatment with 5 μM $CdCl_2$ for 1 h led to a DCF-fluorescence of 105 ± 4.3% compared to the control, 50 μM and

Table 1. Toxicity of cadmium and hydrogen peroxide in different cell lines. After treatment with different concentrations of $CdCl_2$ or H_2O_2 for 24 h, the uptake of neutral red (NR) or the reduction of MTT was measured. As an index for cytotoxicity the IC_{50}-value is shown, describing the concentration of agents causing 50% loss of the tested viability parameter (neutral red uptake or MTT reduction). The data are means ± S.E.M. of three independent experiments.

Cell line	Cell type	IC_{50} (NR) μM $CdCl_2$	IC_{50} (MTT) μM $CdCl_2$	IC_{50} (NR) μM H_2O_2	IC_{50} (MTT) μM H_2O_2
C6	rat glioma	0.7 ± 0.1	0.68 ± 0.1	62.8 ± 7.1	50 ± 11.2
NIH3T3	murine fibroblast	12 ± 2.8	15.6 ± 1.8	186 ± 29.8	190 ± 40.9
PC12	rat pheochromocytoma	15 ± 3.2	n.d.	n. d.	n. d.
A549	human adenocarcinoma	164 ± 13.2	145 ± 17.8	750 ± 48	745 ± 129

n.d., not done

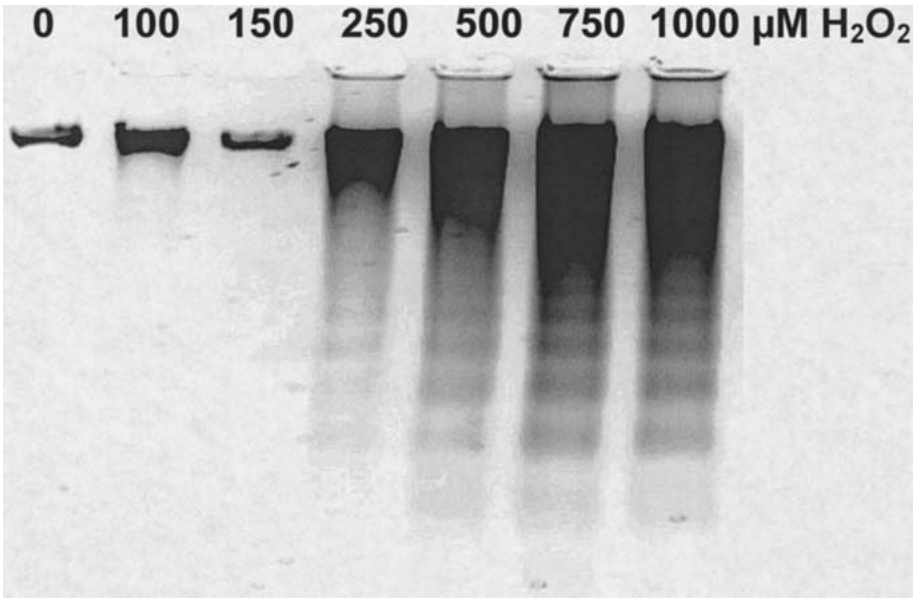

Fig. 1. Induction of apoptosis in C6 glioma cells by H_2O_2. Agarose gel electrophoresis patterns of DNA from cells treated with different concentrations of H_2O_2 for 48 h. Oligonucleosomal-sized DNA fragments as a so-called DNA-ladder occurs at concentrations starting at 250 μM H_2O_2. A representative experiment out of three is shown.

500 μM $CdCl_2$ led to values of 105 ± 7.1% and 99 ± 6.1%, respectively. To check the responsiveness of this experimental system, we added 1 mM hydrogen peroxide, and we found a strong increase in DCF-fluorescence up to 171 ± 10.6% (1 h). Also in two other cell lines, A549 and PC12 cells there was no increase in DCF formation detectable after application of cadmium (up to 250 μM). Furthermore, we investigated if not only H_2O_2 but also redox-active heavy metal ions were able to enhance the level of ROS. Figure 3 shows that $FeSO_4$ evoked a strong increase in DCF-fluorescence in C6 glioma cells, confirming the sensitivity of the experimental system to metal-catalyzed ROS formation. In a similar manner, we found an increase in DCF-fluorescence after incubation with cobalt: 10 μM, 100 μM, and 1000 μM $CoCl_2$ led to a DCF-fluorescence of 118 ± 9.5%, 127.1 ± 12.4% and 141.2 ± 15.6%, respectively, compared to the control. $PbCl_2$ and $ZnCl_2$ which are not catalyzing Fenton-type reactions, were inactive in this assay. These data demonstrate that cadmium does not induce detectable DCF-formation in C6 cells under the conditions employed.

Influence of cadmium on intracellular free SH-groups

Another characteristic feature of oxidative stress is a change in the cellular redox status by the reduction of

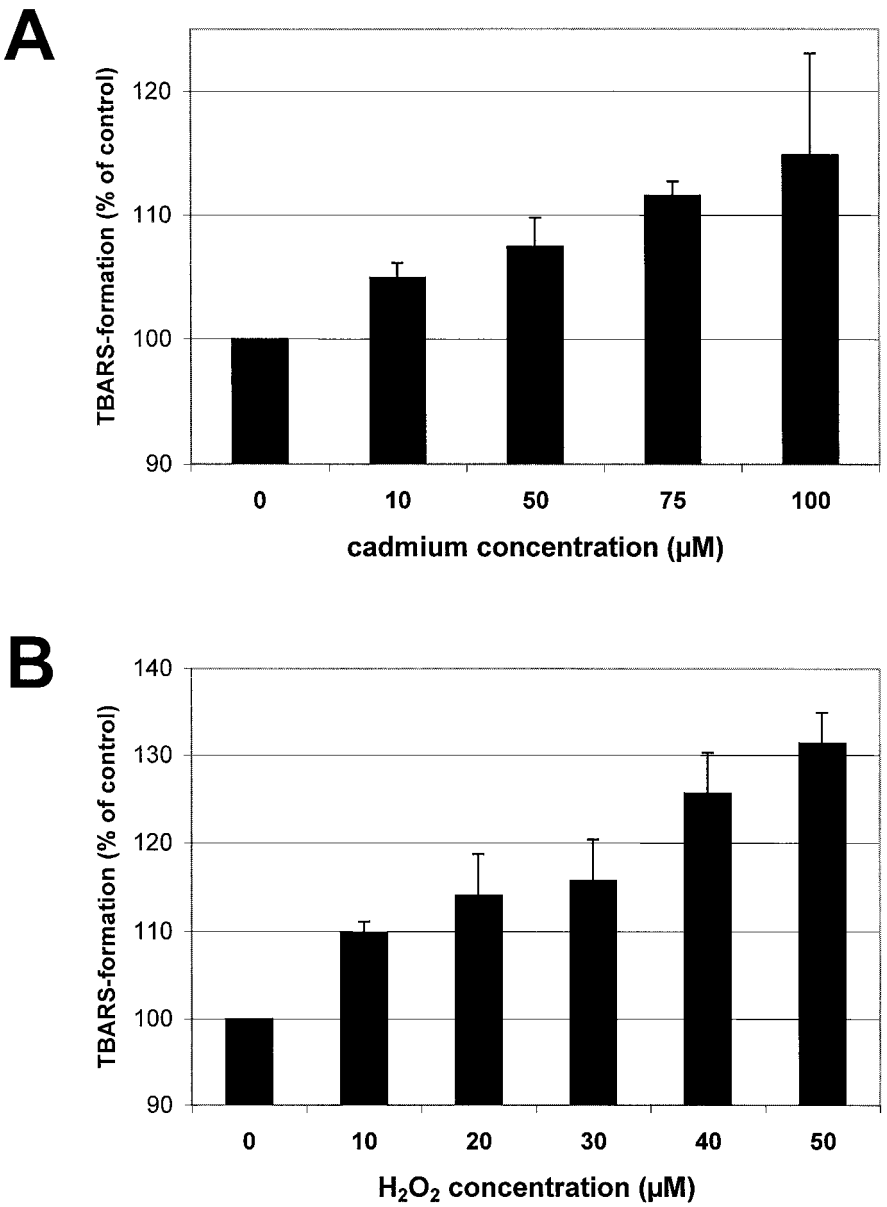

Fig. 2. Formation of TBARS in C6 glioma cells treated with $CdCl_2$ or H_2O_2. Cells were incubated with different concentrations of either $CdCl_2$(A) or H_2O_2 (B) for 2 h and then analyzed for lipid peroxidation products measured as formation of thiobarbituric-acid reactive substances (TBARS). Both $CdCl_2$ and H_2O_2 led to an increased level of TBARS compared to the control. Data are mean values $\pm$ S.E.M. of three independent experiments.

intracellular free SH-groups due to disulfide formation (predominantly by formation of oxidized glutathione). To test this parameter, cells were incubated for an extended time (24 h) with different concentrations of $CdCl_2$, washed and incubated with 50 μM of the fluorescent sulfhydryl probe monobromobimane. Incubation of C6 glioma cells with 0.5 and 10 μM $CdCl_2$ led to a decrease in monobromobimane-fluorescence of 84.9 $\pm$ 3% and 69.8 $\pm$ 4%, respectively, compared to the control levels (Figure 4). We conclude that cadmium causes a decrease in intracellular free sulfhydryl groups as a result of oxidative stress generated by this metal.

Table 2. Effect of cadmium and hydrogen peroxide on apoptotic DNA-fragmentation in different cell lines. Exponentially growing cells were treated with different concentrations of $CdCl_2$ or H_2O_2 for 48 h. DNA was extracted and analyzed by agarose gel electrophoresisas described in Materials and methods.

Cell line	$CdCl_2$ concentrations (μM)	Cadmium-induced DNA-ladder (48 h)
C6	1, 2, 3, 4, 5, 10, 25, 50, 100	starting at 1 μM
NIH3T3	1, 5, 10, 25, 50	starting at 10 μM
PC12	1, 5, 10, 25, 50	none
A549	50, 100, 150, 200, 250	none
Cell line	**H_2O_2 concentrations (μM)**	**H_2O_2-induced DNA-ladder (48 h)**
C6	100, 150, 250, 500, 750, 1000	starting at 250 μM
NIH3T3	100, 200, 300, 400, 500	starting at 400 μM
PC12	10, 50, 100, 150, 250	none
A549	50, 100, 250, 500, 1000	none

Generation of oxidative DNA lesions by cadmium

Oxidation of DNA-bases by ROS is an important mechanism of mutagenesis. We investigated the effect of cadmium on the cellular content of oxidized DNA bases (e.g., 8-hydroxyguanine) in C6 glioma cells. We used the method of incision at oxidative DNA lesions with the bacterial repair enzyme formamidopyrimidine-glycosidase (Fpg) in combination with alkaline unwinding. After 24 h incubation with 5 μM $CdCl_2$, we found a an increase from the basal level of 1015 ± 402 up to 6067 ± 601 Fpg-sensitive DNA-lesions per cell (Figure 5B). This observation suggests that cadmium-induced apoptosis is mediated at least in parts via oxidative mechanisms damaging DNA. In addition, we asked whether cadmium would also cause DNA strand breaks directly, i.e., without employment of the Fpg protein. However, with the same dose and time there was no induction of DNA-strand breaks by cadmium in C6 cells without the incising Fpg protein (Figure 5A). At variance, incubat ion of C6 cells with H_2O_2 led to a massive formation of strand breaks without administration of Fpg protein. A calculation of the extent of DNA lesions resulted in the formation of approximately 30 000 DNA strand breaks per cells after exposure of C6 glioma cells to 100 μM H_2O_2 for 24 h.

Influence of various antioxidants on cadmium-induced DNA-ladder formation

If Cadmium exerted its apoptotic action via generation of oxidative stress, an application of antioxidants should cause a protective effect. To test this hypothesis, we incubated C6 glioma cells with $CdCl_2$ (1 and 5 μM for 48 h) and additionally with the antioxidants glutathione and ascorbic acid. Only glutathione (1 mM) prevented DNA-ladder formation completely. Contrary to our expectations, we did not find a decrease but found a strong increase in DNA-ladder formation after co-incubation with ascorbic acid (750 μM) suggesting that ascorbic acid enhances the apoptotic properties of cadmium. The antioxidants alone were not able to induce apoptotic DNA ladder formation at concentrations up to 1 mM (data not shown). Furthermore, with C6 cells we confirmed a finding of Yang *et al.* (1997) who described a protecting effect of the H_2O_2-scavenging enzyme catalase (1 mg/ml) on cadmium-induced ROS-production in MRI-5 cells. Figure 7 shows that co-incubation of C6 cells wit h cadmium (5 and 10 μM) and catalase (1 mg/ml) strongly reduced DNA ladder formation. We conclude that cadmium at last partially produces oxidative stress via production of H_2O_2.

Discussion

Oxidative stress is an important factor in several neurological disturbances such as Alzheimers and Parkinsons disease. A possible role of reactive oxygen species in these pathologic brain conditions may be the induction of apoptotic cell death (Buttke & Sandstrom 1994). The cellular redox balance is a complex system of enzymatic and non-enzymatic antioxidant defenses and it constitutes an important factor for the

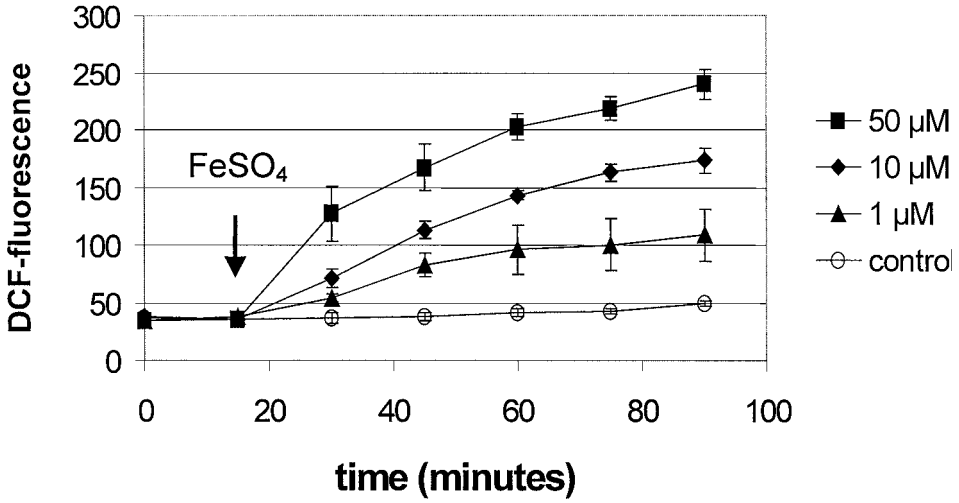

Fig. 3. DCF-formation in C6 glioma cells treated with $FeSO_4$. Cells were incubated with H_2DCF/DA for 30 min, then the increase in DCF fluorescence was measured. After 15 min different concentrations of $FeSO_4$ were added causing a strong increase in fluorescence as a marker of intracellular ROS production. Data are mean values ± S.E.M. of two independent experiments.

modulation of apoptosis. Not only the formation of reactive oxygen species (ROS), but also the generation of conditions which diminish the level of antioxidants is thought to induce apoptosis. Heavy metal ions like Fe^{2+}, Cu^{2+} and Co^{2+} can initiate oxidative damage by enhancing the production of ROS through Fenton-like reactions (Stohs & Bagchi 1995). Because of its redox potential Cd^{2+} itself is not able to catalyze Fenton-type reactions in biological systems. If cadmium induces cellular effects via oxidative stress, the increase in the level of ROS must be caused indirectly, e.g., by inhibition of antioxidant enzymes or by depletion of antioxidant molecules (Stohs *et al.* 2000).

The mechanisms by which cadmium cause cell death can be necrotic or apoptotic depending on investigated cell line and the concentration of this metal. Previous investigations showed, that cadmium-induced cell death in rat C6 glioma cells is mediated by caspase 9-activation suggesting the involvement of a mitochondrial-mediated apoptotic pathway (Wätjen *et al.* 2002; Kondoh *et al.* 2002). A central role for oxidative stress in cadmium-induced apoptosis is suggested by the increased production of superoxide anions in HeLa and bovine aorta endothelial cells (Szuster-Ciesielska *et al.* 2000) and in HL-60 cells (Bagchi *et al.* 2000). Furthermore, cadmium-induced apoptosis in H9 and Jurkat cells is accompained by an inactivation of thioltransferase resulting in oxidative stress (Chrestensen *et al.* 2000). On the other hand, several authors demonstrated protective effects against cadmium toxicity due to an adaptive upregulation of genes coding for protective proteins like metallothionein (Coogan *et al.* 1994). Eneman *et al.* (2000) reported a decrease in the rate of H_2O_2-induced apoptosis in cadmium-adapted alveolar epithelial cells due to an induction of several protective proteins including metallothioneins, glutathione-S-transferase and γ-glutamylcysteinsynthetase.

Although the mechanisms by which cadmium initiates apoptosis are discussed controversely, the generation of ROS and oxidation of critical SH-groups seem to play a pivotal role in this complex process. In the present study, we investigated changes in the cellular redox status during cadmium-induced apoptosis. We found a decrease of the intracellular GSH content and an increase of oxidative DNA-lesions, but only marginal generation of TBARS (indicative of lipid peroxidation) and of ROS. For comparison, we examined the cytotoxicity of hydrogen peroxide and its ability to induce apoptotic cell death in four selected cell lines. The sensitivity of the tested cell lines towards H_2O_2 paralleled their sensitivity towards cadmium: While human A549 adenocarcinoma cells were relatively resistant towards both $CdCl_2$ and H_2O_2, C6 rat glioma cells were comparatively sensitive towards both stressors. A possible explanation for the observed differences in sensitivity towards H_2O_2 and $CdCl_2$ could be a different intracellular GSH-level or a different content of antioxidative enzymes. For example, A549 cells are known to possess a high intracellular concentration of GSH (Gaubin *et al.* 2000) which probably is the cause for the relative resistance of

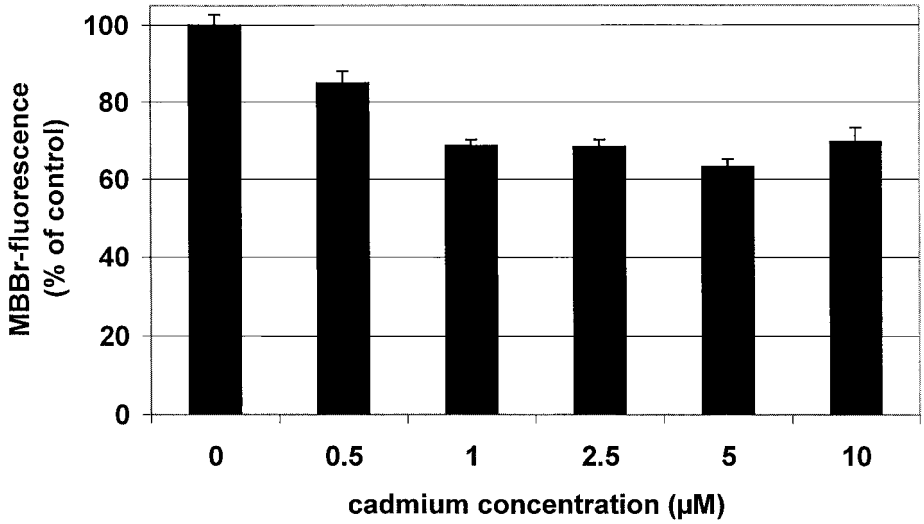

Fig. 4. Decrease of intracellular SH-groups in C6 glioma cells after incubation with $CdCl_2$. Cells were incubated with different concentrations of $CdCl_2$ for 24 h and analysed for free SH-groups using the fluorescent probe monobromobimane. Cadmium led to a decrease in the amount of intracellular SH-groups. Data are mean values ± S.E.M. of two independent experiments.

this cell line against cadmium and H_2O_2-toxicity. To obtain additional information on the apoptotic mechanisms of cadmium-induced apoptosis, we investigated the mode of H_2O_2-induced apoptosis in these cell lines. H_2O_2 was able to cause an oligonucleosomal DNA-fragmentation in rat C6 glioma cells (starting at 250 μM) and murine NIH 3T3 fibroblasts (starting at 400 μM), but not in PC12 and A549 cells. This is consistent with the observation that cadmium induces apoptotic cell death in C6 and NIH3T3-cells but necrosis in PC12 and A549 cells.

Additional evidence that oxidative stress was involved in the apoptotic process caused by cadmium was obtained by determination of lipid peroxidation products. Cadmium was shown to increase the level of malondialdehyde in brain tissue of young albino rats (Kumar *et al.* 1996) and the level of TBARS in isolated rat hepatocytes (Pourahmad & O'Brien 2000). We found a slight increase in TBARS-formation after 2 h incubation with $CdCl_2$, which may either be due to enhanced free radical generation or depletion of antioxidant capacity. This weak response is not unexpected because Cd^{2+} ions unlike Fe^{2+}-ions can not undergo redox-cycling and hence not catalyze Fenton-type reactions.

To establish that changes in the redox-state were associated with cadmium-induced apoptosis, we also investigated alterations in glutathione metabolism. Glutathione participates in a number of significant cellular processes including protection of the cell against certain toxic compounds and oxidative damage. Oxidized glutathione (GSSG) is implicated as mediator of apoptosis. Whereas moderate concentrations of cadmium in mammalan cells induce the synthesis of glutathione (Chin & Templeton 1993; Hatcher *et al.* 1995), more elevated concentrations cause a decrease in the level of reduced glutathione (GSH) (Nigam *et al.* 1999; Pourahmad & O'Brien 2000). With A549 cells, exposure to low concentrations of $CdCl_2$ (1–10 μM) increased the glutathione level whereas high cadmium concentrations (10–100 μM) induced a significant decrease in the GSH-level (Gaubin *et al.* 2000). In C6 glioma cells we found a decrease in GSH-levels after 24 h of incubation with the low concentration of 1 μM $CdCl_2$. Our results are in accord with the work of Nigam *et al.* (1999) who also observed that $CdCl_2$ caused a decrease in GSH, albeit at high doses of the metal ion. Because cadmium is also an inducer of sufhydryl proteins which protect from oxidative stress, the observed glutathione depletion that occurs at the low micromolar cadmium-concentrations used may be due to the production of ROS at a rate that exceeds the ability to regenerate reduced glutathione. The employment of 1 mM glutathione as an antioxidant prevented the induction of apoptotic DNA fragmentation by cadmium completely. However, a co-incubation with ascorbic acid led to a strong increase in cadmium-induced DNA ladder formation even at low concentrations of $CdCl_2$ (1 μM) when only slight effects are caused by the heavy metal ion.

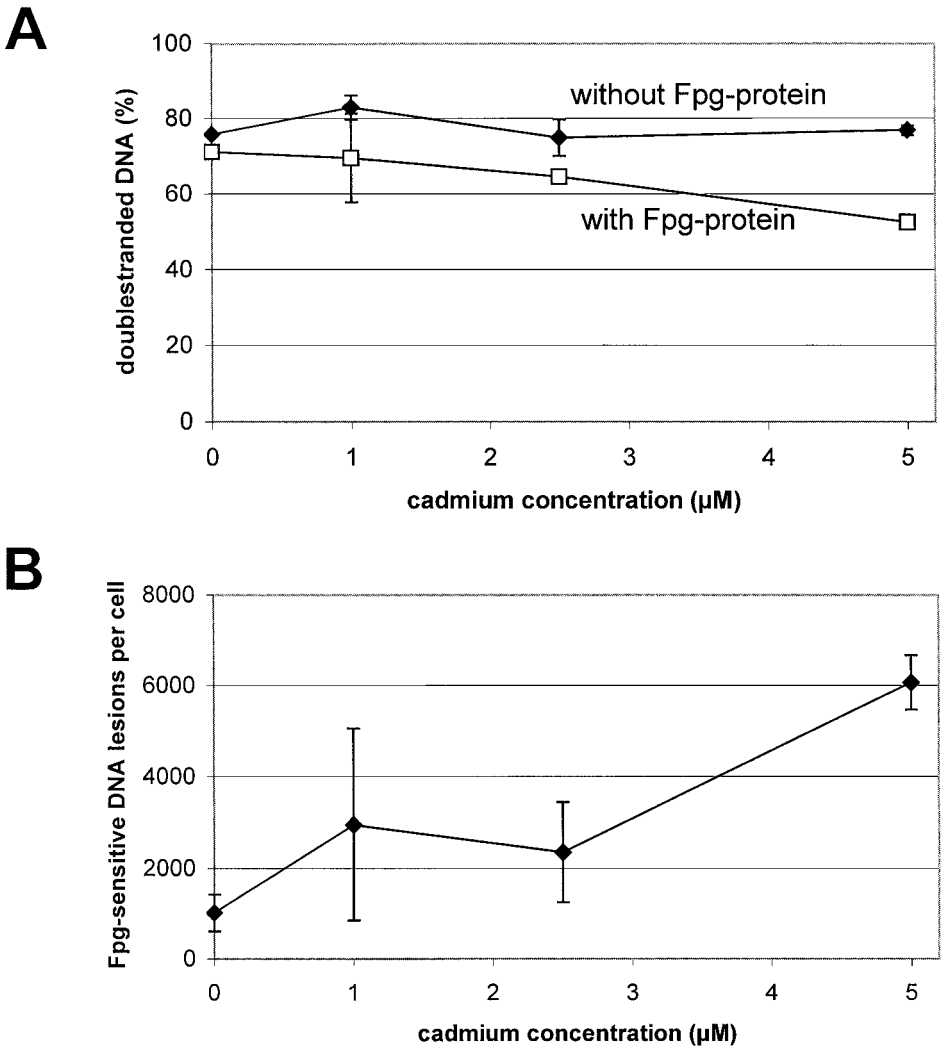

Fig. 5. Fpg-sensitive DNA damage in C6 glioma cells after incubation with cadmium. Cells were incubated with different concentrations of $CdCl_2$ for 24 h, then the percentage of double stranded DNA (± Fpg protein) was measured using the method of alkaline unwinding (A). Out of these data the amount of Fpg-sensitive lesions per cell was calculated (B). Cadmium caused an increase in Fpg-sensitive DNA lesions in C6 glioma cells as a marker of oxidative stress. Data are mean values ± S.D. of two independent experiments.

An interpretation of this phenomenon is that ascorbic acid has not only anti-oxidative properties but also can exert pro-oxidative and pro-apoptotic effects.

A further marker of cadmium-mediated oxidative stress is the increase in oxidative DNA lesions. Incubation of C6 glioma cells with cadmium led to a six-fold increase in oxidative DNA lesions analysed by the alkaline unwinding in combination with Fpg-protein. Oxidative DNA damage is one of the mechanisms postulated to play an important role in mutagenesis and carcinogenesis, but also may trigger programmed cell death. Cadmium may evoke an increase in oxidative DNA base damage by its property to cause an elevation of the cellular level of ROS. Alternatively, cadmium may amplify the accumulation of endogeneous DNA lesions, because it is capable of inhibiting the repair of oxidative base modfication (Dally & Hartwig 1997; Asmuss *et al.* 2000). Within our experimental design we cannot discriminate if the cadmium-mediated increase in DNA-lesions was caused mainly by generation of oxidative stress or by inhibition of DNA repair enzymes. But together with our data on the decrease in GSH evoked by cadmium, these results are additional support for the hypothesis of cadmium-mediated oxidative stress in C6 glioma cells.

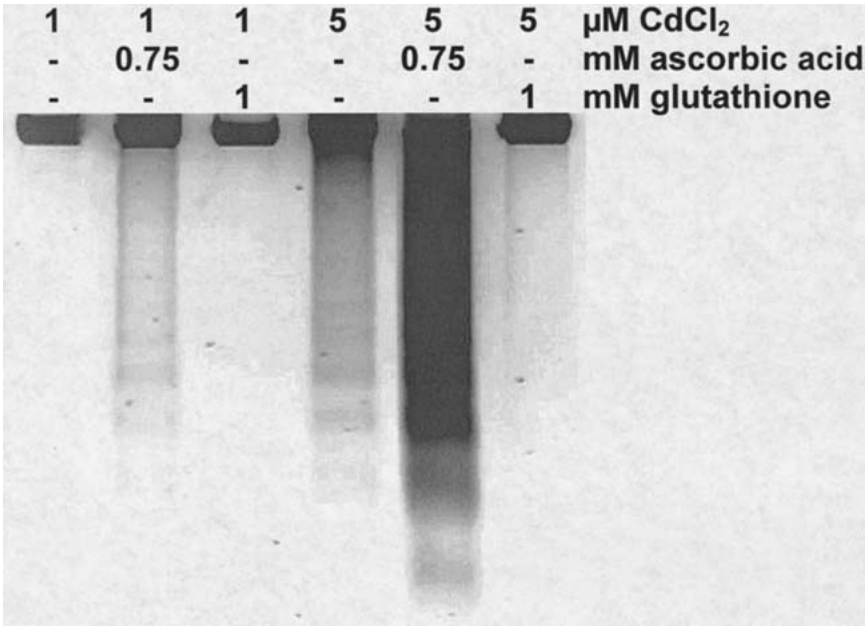

Fig. 6. Effect of antioxidants on cadmium-induced DNA-ladder formation in C6 glioma cells. Agarose gel electrophoresis pattern of DNA from cells co-incubated with $CdCl_2$ and the structural unrelated antioxidants ascorbic acid and glutathione. Ascorbic acid led to a strong increase in cadmium-caused DNA ladder formation. Co-incubation with glutathione diminished DNA ladder formation mediated by $CdCl_2$. A representative experiment out of two is shown.

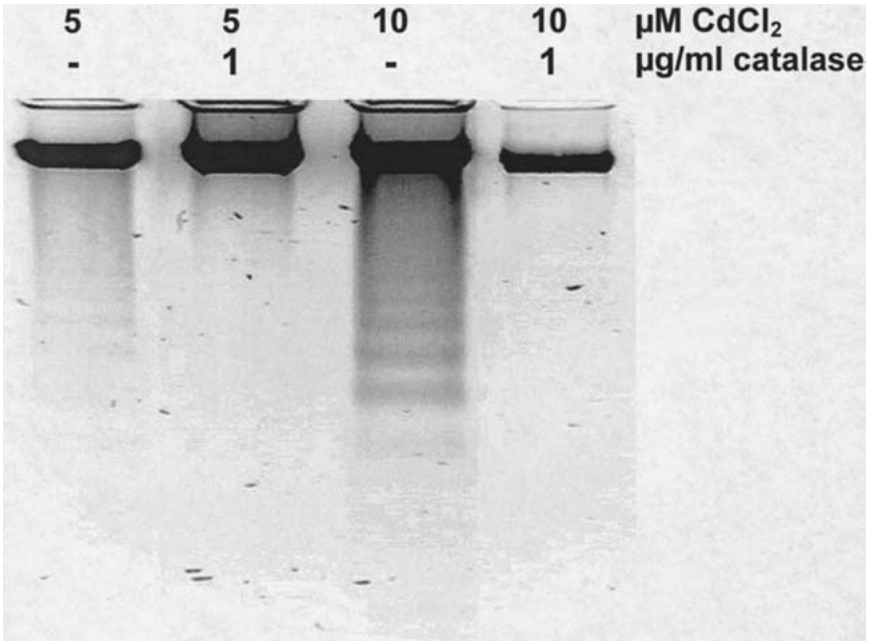

Fig. 7. Effect of catalase on cadmium-induced DNA-ladder formation in C6 glioma cells. Agarose gel electrophoresis pattern of DNA from cells co-incubated with $CdCl_2$ and antioxidative enzyme catalase. Co-incubation with catalase (1 mg/ml) led to a strong decrease in cadmium-mediated DNA ladder formation. A representative experiment out of two is shown.

Formation of dichlorofluorescein (DCF) is a well-known marker of oxidative stress. Cadmium had been shown to increase the fluorescence of DCF in rat hepatocytes (Pourahmad & O'Brien 2000) and MRC-5 human fetal lung fibroblasts (Yang *et al.* 1997). The latter authors tested the influence of the antioxidative enzymes superoxide dismutase (SOD) and catalase on the cadmium-mediated ROS-production. They found a suppression of ROS formation only by incubation with catalase but not with SOD, suggesting that cadmium stimulated the generation of hydrogen peroxide but not superoxide anions. In contrast to these results, we did not detect any significant increase in DCF-fluorescence even after incubation with 500 μM $CdCl_2$ for two hours. One reason for the different results may be that we had to employ a relatively short incubation time because thereafter we observed a spontaneous increase of fluorescence in the control, another reason may be a slower cadmium uptake into C6 cells compared to other cell lines. In contrast to cadmium, the redox-active transition metal ions Co^{2+} and Fe^{2+} caused significant increases in DCF-fluorescence in C6 cells starting already at low concentrations.

However, the induction of ROS by these metals is not related to their capability to induce apoptosis in C6 cells. Besides H_2O_2, only $CdCl_2$ and $ZnCl_2$, were able to cause oligonucleosomal DNA-fragmentation whereas $CoCl_2$ or $FeSO_4$ failed to induce apoptosis in these cells (Haase *et al.* 2001; Wätjen *et al.* 2002). We conclude that $CdCl_2$ does not induce apoptosis via generation of hydroxyl radicals. However, H_2O_2 seems to be at least partially to be involved in the apoptotic activity of cadmium, because co-incubation with catalase completely abolished the generation of DNA-ladders by cadmium in C6 glioma cells.

In summary, the present study has established, that cadmium induced its apoptotic action in C6 glioma cells at least in part via generation of oxidative stress. This was verified by the detection of an increase in oxidative DNA-lesions and a decrease of intracellular glutathione caused by cadmium. The active intermediate seems to be hydrogen peroxide because catalase prevents the generation of apoptotic DNA fragmentation by cadmium. Because both apoptosis and oxidative stress are thought to play important roles in neurodegenerative diseases, low concentrations of cadmium that initiate programmed cell death may lead to a selective cell death in distinct brain regions via generation of oxidative stress.

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