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Cadmium activates a programmed, lysosomal membrane permeabilization-dependent necrosis pathway

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HIGHLIGHTS

- Cadmium causes a necrotic cell death in endothelial cells.
- Cd-induced necrosis is a programmed, BCL-XL-inhibitable process.
- Lysosomal membrane permeabilization and release of DNAse II are central in the execution of cell death.
- DNAse-mediated degradation of nuclear DNA results in "apoptosis mimicry" in several cell death assays.

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ABSTRACT

Cadmium is a highly toxic, carcinogenic, and atherogenic element. A central principle in many Cd-induced pathophysiologies is the induction of cell death. In past studies Cd was shown to cause apoptosis, necrosis, programmed necrosis, or autophagy. This study was conducted to precisely define the end stage processes and outcome of Cd-induced cell death in endothelial cells (ECs). We show that Cd leads to acidification and permeabilization of lysosomes, followed by the release of active DNAse II from lysosomes. The absence of nuclear DNA due to DNAse II activity may have lead to misinterpretations of the type of cell death outcome in previous studies. Further, Cd-induced cell death is characterized by a massive release of lactate dehydrogenase (LDH), a gold standard marker for the occurrence of plasma membrane rupture i.e. necrosis. Importantly, lentivirus-based over-expression of the anti-apoptotic protein BCL-XL abrogates lysosomal rupture, DNA degradation and LDH release, clearly indicating that Cd induces a programmed form of cell death with a necrotic endpoint.

1. Introduction

Cadmium (Cd) is a relatively rare toxic heavy metal and is found in the earths' crust from 0.1 to 0.5 μg/g, and in the atmosphere from 0.1 to 5.0 ng/m^3. Industrial activities, mainly zinc production and the use of Cd in pigments, plastic stabilizers, and batteries have significantly increased the amount of Cd in the biosphere, and as a consequence Cd exposure of humans (The International Cadmium Association; www.cadmium.org). The major source for Cd uptake by (non-smoking) humans is food, and tobacco smoking approximately doubles the daily Cd uptake (ATSDR, 2009; Authority, 2009; Friberg and Nordberg, 1986; Jarup and Akesson, 2009). In the human body Cd has a half-life of 10–30 years and accumulates massively in organs like liver, kidney, and testes. Further, Abu-Hayyeh et al. demonstrated that also the vascular system is another target organ for Cd deposition (Cd concentrations of up to 20 μM were observed in the aortic wall of heavy smokers) (Abu-Hayyeh et al., 2001; ATSDR, 2009; Satarug and Moore, 2004; Staessen et al., 2001). In past decades the health threat of chronic low dose Cd exposure was underestimated. Accordingly, the European Food Safety Authority has reduced the provisional tolerable weekly intake from 7 μg/kg to 2.5 μg/kg in 2009 (Authority, 2011).

Apart from the well known toxic effects of Cd on liver, kidneys and testis, the International Agency for Research on Cancer has classified Cd as a human carcinogen (Achanzar et al., 2001; Benbrahim-Tallaa et al., 2007, 2009; CANCER, 1997; Joseph, 2009; Waalkes, 2003), and recent studies, including ours, clearly indicate that Cd is also a significant risk factor for cardiovascular diseases (Messner et al., 2009; Peters et al., 2010).

At the cellular level Cd was demonstrated to impair cell cycle progression and cell proliferation (Templeton and Liu, 2010), DNA repair (Bertin and Averbeck, 2006), cell–cell adhesion (Prozialeck et al., 2008), and Cd was also shown to cause cell death in a

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large number of different cell types (Templeton and Liu, 2010). Cell death induction by Cd was ascribed to the causation of ER-stress (Wang et al., 2009), mitochondrial depolarization (Messner et al., 2009), increase in ceramides and calpain-activation (Lee and Thevenod, 2008), ROS-production (Yang et al., 2007), and DNA-damage (Liu and Jan, 2000). Intriguingly, the reported final outcome of Cd-induced cell death is highly diverse, ranging from classical apoptosis (Jung et al., 2008) and necrosis (Kaji et al., 1992; Kishimoto et al., 1991) to programmed necrosis (Messner et al., 2009) and autophagy (Dong et al., 2009).

This study was conducted to precisely define the final outcome of Cd-induced cell death in endothelial cells, and to study the cellular processes involved therein with a "bottom up" research strategy. Many previous studies on cadmium-induced cell death focussed primarily on upstream signalling analyses, lacking a hard fact characterization of the ultimate outcome. As the endpoint of cell death defines whether an agent (Cd) causes inflammation (necrosis) or not (apoptosis), the clear definition of the mode of cell death is crucial for the pathophysiological understanding of Cd-caused diseases.

2. Materials and methods

2.1. General

All reagents used were of purissimum or analytical grade quality and were purchased from Sigma–Aldrich (Sigma–Aldrich, Vienna, Austria) unless specified otherwise.

2.2. Cell isolation and culture

The isolation and culture of human umbilical vein endothelial cells (HUVECs) has been described previously (Bernhard et al., 2003). The isolation and analysis of HUVECs were approved by the Ethics Committee of the Medical University of Innsbruck (No.: UN2979) and the Ethics Committee of the Medical University of Vienna (EK Nr. 1183/2012). Cells were routinely passaged in 0.2% gelatine-coated (Sigma, Steinheim, Germany) polysterene culture flasks (TPP, Switzerland) in endothelial growth medium (EGM, Lonza) in a humidified atmosphere containing 5% CO_2. For cell death analyses, 3×10^5 HUVECs per well were seeded onto gelatine-coated 6-well plates (TPP, Switzerland). Prior to each experiment, medium was replaced by fresh medium.

2.3. Generation of lentiviral vectors

BCL-XL viruses: For constitutive over-expression of human BCL-XL in HUVECs, BCL-XL encoding cDNA was PCR amplified and recombined into pDONR-207 (Invitrogen) using Invitrogen's B/P recombination kit. A sequence verified clone was used for L/R recombination with pHR-SFFV-dest-IRES-Puro thereby generating the lentiviral expression plasmid pHR-SFFV-BCLXL-IRES-Puro (Sigl et al., 2009).

2.4. Generation of stable BCL-XL over-expressing HUVECs

For lentiviral transduction, human HEK 293T cells were transiently transfected with lentiviral plasmids containing cDNAs coding for human BCL-XL or eGFP, along with the packaging plasmids pCMV 8.91 and pVSV-G (kindly provided by Didier Trono). Forty eight and 72 h after transfection lentiviral supernatant was sterile filtered (0.2 μM), supplemented with polybrene to a final concentration of 4 μg/ml and added to the target cells overnight.

2.5. Quantification of cell death

For detection and/or quantification of cell death, forward/sideward light scattering analysis and AnnexinV/propidium iodide-staining were used as described (Bernhard et al., 2003). AnnexinV/PI− staining allows the discrimination of intact viable cells (AnnexinV− negative and PI− negative), early apoptotic (AnnexinV− positive and PI− negative) and necrotic cells (AnnexinV− positive and PI− positive).

2.6. Analysis of number of viable cells

The number of viable cells was determined using the XTT assay (Biomol GmbH, Hamburg, Germany). HUVECs were seeded into gelatine coated 96-well plates. After 24 h the medium was replaced by fresh medium and the cells were treated with various Cd concentrations for the indicated times. For further details see manufacturers' instructions.

2.7. Lactate dehydrogenase release assay

The amount of lactate dehydrogenase (LDH) released from cells was quantified using the LDH cytotoxicity kit II (Biovision) according to the manufacturer's instructions.

2.8. Quantification of cellular DNA content

For the detection and quantification of nuclear DNA content, HUVECs were seeded into gelatine coated 6-well plates and allowed to adhere over night. After replacing the medium with fresh medium, the cells were incubated with various Cd concentrations for the indicated times. After enzymatic detachment, the cells were permeabilized with saponin (1 mg/ml), stained with propidium iodide (50 μg/ml) and analysed and quantified by flowcytometry using a Cytomics FC 500 (Beckmann Coulter, Brea, CA, USA).

2.9. Staining of cells and fluorescence microscopy

To analyse the subcellular localization of DNAse II, HUVECs were treated with Cd for the indicated times. After treatment, the cells were washed with PBS and fixed with 4% PFA for 3 min at room temperature. Fixed cells were washed with PBS and permeabilized with 0.3% Triton X-100 for 30 min. Following an additional washing step with PBS, non-specific binding sites were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature followed by staining with primary antibody against DNAse II (mouse polyclonal antibody, Abnova GmbH, Heidelberg, Germany; 10 μg/ml) for 1 h at room temperature. After 3 washing steps with PBS, the cells were incubated with secondary antibody (Alexa Fluor 488, goat anti-mouse, Invitrogen, Carlsbad, CA, USA) for 1 h in the dark and at room temperature. Thereafter, the monolayer was washed 3 times with PBS and nuclear staining was performed using propidium iodide (1 μg/ml) for 8 min at room temperature in the dark. After 3 final washing steps, cells were mounted in ProLong Gold (Invitrogen, Carlsbad, CA, USA) and analysed using a LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software (Zeiss, Oberkochen, Germany).

2.10. In vitro DNA laddering

To analyse cytosolic nuclease activity of Cd treated HUVECs, nuclear DNA was extracted from endothelial cells using a DNA purification kit (Promega GmbH, USA). Nuclear DNA (2 μg) was then incubated with cytosolic extracts of Cd-treated HUVECs and controls (30 μg) for 3 h at 37 °C. DNA fragmentation was analysed by agarose gel electrophoresis (0.5%).

2.11. Lysosomal labelling and pH imaging

For the staining of acidic compartments in HUVECs, LysoTracker green was used (Invitrogen, Molecular Probes, USA). After Cd treatment of cells for the indicated times, HUVECs were stained with LysoTracker dye (75 nM) for 30 min at 37 °C. The cells were then analysed using a LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software (Zeiss, Oberkochen, Germany) or by FACS analysis.

For the imaging of pH-changes, LysoSensor green was used (Invitrogen, Molecular Probes, USA). After Cd treatment of cells for the indicated times, HUVECs were stained with LysoSensor probe (75 nM) for 5 min at 37 °C. Endothelial cells were analysed using a LSM 510 Meta attached to an Axioplan 2 imaging MOT using ZEN software (Zeiss, Oberkochen, Germany) or by FACS analysis.

2.12. Western blotting

Western blotting was performed as previously described (Bernhard et al., 2001). Equal amounts of protein were loaded. Primary antibody used was anti-LC3 (rabbit anti-LC3; Sigma–Aldrich, Cat. No.: L8918). Quantification of bands was performed using Quantity One Software (Quantity one, Biorad).

2.13. In vivo experimentation

The mouse aortic sections analysed in this study were obtained in the course of a previous study (Messner et al., 2009). The animal experiment was approved by the Animal Ethics Committee of the Austrian Federal Ministry for Research and Science. Eight female ApoE knock out mice were divided randomly into 2 groups. The control group received normal water and the Cadmium group was treated with 100 mg/L of $CdCl_2$ in the drinking water. Both groups were fed a Western type diet to induce the development of atherosclerotic plaques. After 12 weeks of treatment the aorta was excised between the aortic arch and the iliac bifurcation. The aorta was cleaned by removing connective tissue and fat, washed in PBS and fixed immediately in 4% paraformaldehyde. After fixation and dehydration aortas were embedded in paraffin and 5 μm sections were prepared.