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Cadmium (Cd^{2+}) exposure differentially elicits both cell proliferation and cell death related responses in SK-RC-45

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ABSTRACT

Cadmium (Cd^{2+}) is a major nephrotoxic environmental pollutant, affecting mostly proximal convoluted tubule (PCT) cells of the mammalian kidney, while conditionally Cd^{2+} could also elicit protective responses with great variety and variability in different systems. The present study was designed to evaluate the molecular mechanism of Cd^{2+} toxicity on human PCT derived Renal Cell Carcinoma (RCC), SK-RC-45 and compare its responses with normal human PCT derived cell line, NKE. Exposure of SK-RC-45 cells with different concentrations of $CdCl_2$ (e.g. 0, 10 and 20 μM) in serum free medium for 24 h generate considerable amount of ROS, accompanied with decreased cell viability and alternations in the cellular and nuclear morphologies, heat shock responses and GCLC mediated protective responses. Also phosphatidylserine externalization, augmentation in the level of caspase-3, PARP, BAD, Apaf1 and cleaved caspase-9 along with decreased expression of Bcl2 and release of cytochrome *c* confirmed that, Cd^{2+} dose dependently induces solely intrinsic pathway of apoptosis in SK-RC-45, independent of JNK. Furthermore, the non-toxic concentration (10 μM) of Cd^{2+} induced nuclear translocation of Nrf2 and increased expression in the level of HO-1 enzyme suggesting that at the milder concentration, Cd^{2+} induces protective signaling pathways. On the other hand, exposure of NKE to different concentrations of $CdCl_2$ (e.g. 0, 10, 20, 30 and 50 μM) under the same conditions elevate stronger heat shock and SOD2 mediated protective responses. In contrary to the RCC PCT, the normal PCT derived cell follows JNK dependent and extrinsic pathways of apoptosis. Cumulatively, these results suggest that Cd^{2+} exposure dose dependently elicit both cell proliferative and cell death related responses in SK-RC-45 cells and is differentially regulated with respect to normal kidney epithelia derived NKE cells.

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1. Introduction

The heavy metal cadmium (Cd^{2+}) is known to be a widespread environmental contaminant due to its extensive use in various anthropogenic and industrial activities like electroplating, pigment, battery, plastic, cigarette smoke, and fertilizer industries (Järup and Åkesson, 2009; Ognjanovic et al., 2008). Furthermore, Cd^{2+} toxicity could be presented in different ways, depending on its chemical form, dose and route of intake, tissue affinity, sex and age, as well as acute or chronic nature of exposure (Johri et al., 2010). It is an established environmental nephrotoxic pollutant that specifically affects human renal health (Ognjanovic et al., 2008; Johri et al., 2010).

However, the mechanisms of Cd^{2+} induced toxicity in a particular species under various conditions are different (Iscan et al., 1994; Waisberg et al., 2003). Chronic exposure to Cd^{2+} leads to accumulation of the metal chiefly in the liver and kidneys, causing membrane damage, altered gene expression etc., and ultimately leads to apoptosis (Lind et al., 1997; Obara et al., 2011; Shaikh et al., 1999). Among these, the renal proximal tubule (PCT) epithelial cells and glomerulus are considered as the main targets for Cd^{2+}-induced cytotoxicity (Hamada et al., 1997; Pari et al., 2007). Oxidative stress and the reactive oxygen species (ROS) mediated apoptosis plays a major role in acute as well as chronic toxicity of Cd^{2+} (Hart et al., 1999; O'Brien and Salacinski, 1998; Prozialeck and Lamar, 1995; Waisberg et al., 2003). Actually Cd^{2+} lowers the endogenous antioxidant glutathione level and inhibits the mitochondrial electron transport chain which in turn leads to generation of superoxide radicals. To minimize oxidative stress related damages, antioxidant defense mechanisms are usually developed in all organisms. Several antioxidants and detoxifying enzymes, such as heme oxygenase-1 (HO-1) (Zhang et al., 2013) etc. protect the cells from the detrimental effects of ROS. HO-1 belongs to the phase II enzymes and is expressed in an inducible manner by transcription factor, nuclear factor erythroid 2 related factor 2 (Nrf2). However, along with its damaging effect, milder forms of Cd^{2+} stress might result in the activation of repair mechanisms in cell involving these cytoprotective enzymes. But the exact role of ROS in the activation of signal transduction pathways involved in

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defense mechanisms during Cd^{2+} stress still need research attention and confirmation within various models due to its great variability (Cuypers et al., 2010). On the other hand, Cd^{2+} might induce different apoptotic pathways among diverse cell types depending on the exposure (*in vitro*) conditions and other factors (Cuypers et al., 2010). So, to promote further understanding about the Cd^{2+} induced signaling pathways (nephrotoxic as well as proliferative mechanisms) in renal cancer cells, we have investigated the effects of $CdCl_2$ on SK-RC-45 cells in the first phase of the study and then compared some selected parameters with a normal human kidney epithelial (NKE) cells to show how differentially these cells (SK-RC-45) handle the Cd^{2+} insult. We selected this particular cell line for this specific study as these cells are generated from a human kidney cancer in the lining of the PCT and these PCT cells of the mammalian kidney are the prime target of cadmium-induced toxicity (Thevenod et al., 2000). In the first phase of this study, we evaluated the effects of $CdCl_2$ on cell proliferation (in time and dose dependent manner), intra cellular ROS generation, effect of N-acetyl-L-cysteine (NAC) on cell proliferation and detail molecular mechanism of Cd^{2+} induced toxicity and cell death (e.g., the changes in Bcl2, BAD, caspase-3 and -9, Apaf1, poly (ADP-ribose) polymerase (PARP) and change in the sub cellular location of cytochrome c). Moreover, we have also investigated Cd^{2+} induced change in the expression level of the phase II detoxifying HO-1 and sub cellular localization of its transcriptional regulator, Nrf2. Finally, we have compared the unique responses to Cd^{2+} stress with respect to glutamate cysteine ligase catalytic subunit (GCLC), phospho-JNK (p-JNK), Hsp70, superoxide dismutase-2 (SOD2) and caspase-8 expression levels which significantly differ from NKE cells' response. The outcome of this study is expected to provide clues on the differential effects of Cd^{2+} in renal carcinoma cell line compared to normal cell line.

2. Materials and methods

2.1. Materials and reagents

RPMI-1640 and fetal bovine serum (FBS) were purchased from HIMEDIA (Mumbai, India) and HyClone (Thermo Scientific HyClone, Logan, Utah), respectively. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and cadmium chloride ($CdCl_2$) was purchased from Sisco Research Laboratory (Mumbai, India). NAC was purchesed from Sigma (Missouri, USA). Fluorescein isothiocyanate (FITC) conjugated Annexin V apoptosis detection kit, 2′, 7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), FITC conjugated anti-rabbit IgG, anti-Bcl2, anti-BAD, anti-HO-1, anti-Nrf2, anti-Hsp70, anti-pJNK, anti-GCLC, anti-SOD2, anti-caspase-8 and anti-cytochrome *c* primary antibodies and HRP-tagged secondary antibodies were obtained from Abcam (Cambridge, UK). Anti-caspase-3 antibody was purchased from Sigma (Missouri, USA). Anti-PARP, anti-caspase-9, anti-Apaf1 and anti-β-actin antibodies were purchased from Cell Signaling (Cell Signaling Technology Inc., Danvers, MA). All additional chemicals used in the study were of the highest experimental grade available.

2.2. Cell culture and treatment

The well-characterized, long-term human Renal Cell Carcinoma (RCC) line (Das et al., 2008; Kudo et al., 2003), SK-RC-45 and NKE cell lines were used (obtained as a gift from Dr. Kausik Biswas, Bose Institute) for the present study. Both the cells were maintained in 75-cm^2 culture flasks at 37 °C using RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicine and 2.5 μg/ml amphotericin B in a CO_2 incubator maintaining CO_2 at 5%. For the experimental purpose, $CdCl_2$ (filter-sterilized solution) exposure was done at the time of approximately 80% confluency (Xie and Shaikh, 2006a). All experiments were carried out under sterile conditions and the results were determined in triplicate for the reproducibility. The Cd^{2+} exposure was done in RPMI-1640 media without serum as it is known that serum proteins form complexes with Cd^{2+} which reduces its uptake and toxicity (Gennari et al., 2003; Xie and Shaikh, 2006a). In dose and time dependent study, the LC_{50} values of $CdCl_2$ for SK-RC-45 and NKE were determined as 20 μM and 50 μM (respectively) and the optimum time of incubation were found to be 24 h for both. Throughout the study for this LC_{50} and a non-toxic concentration (10 μM) and three non-toxic concentrations 10, 20 and 30 μM (20 μM is of primary interest for comparative study and the other two doses for showing gradual response of NKE to Cd^{2+} exposure) have been used as toxicant exposed groups for SK-RC-45 and NKE respectively.

2.3. Dose and time dependent Cell viability assessment

For dose dependent study, SK-RC-45 and NKE cells were incubated with 0–100 μM of Cd^{2+} (at intervals of 10 i.e. 10, 20, 30 μM, etc.) for 24 h. In time dependent study (only for SK-RC-45), viability had been checked at an interval of 6 h starting from 0 h up to 30 h. After incubation period in each case, the media was discarded and the cells were washed gently twice with phosphate buffered saline (PBS). Then cell viability was measured by MTT assay (Madesh and Balasubramanian, 1997).

2.4. NAC pretreatment and cell viability assessment of SK-RC-45

Cells were also pretreated with a very potent widely accepted antioxidant NAC. After an initial dose dependent study, a 2 h pretreatment (Oh and Lim, 2006) with 5 mM concentration of NAC found to be optimum (data not shown). After this pretreatment, cells were incubated with $CdCl_2$ with only LC_{50} concentration for 24 h (selected from dose and time dependent study above). Cell viability was then measured by MTT assay following technique described elsewhere (Madesh and Balasubramanian, 1997).

2.5. Assessment of cellular morphology of SK-RC-45 by phase contrast and nuclear morphology by fluorescence microscopy

SK-RC-45 cells were cultured in 6-well cell culture plates (BD Falcon) for both type of imaging (Mao et al., 2007). The cells were exposed to $CdCl_2$ of 10 and 20 μM concentrations for 24 h. After treatment, the medium was discarded and washed with PBS (pre-warmed to RT). Then for phase contrast microscopy after PBS wash fresh medium was added and cells were then viewed and photographed under phase contrast microscope (Leica Microsystem DN1000; camera: DFC450 C). For studying nuclear morphology, PBS wash was followed by fixation of attached cells with 70% pre-chilled ethyl alcohol (keeping overnight at −20 °C). Next day the cells were again washed twice with chilled PBS and stained with propidium iodide (PI) for 30 min at room temperature, in dark. The cells were again washed with PBS, and analyzed under fluorescent microscope (Olympus BX61).

2.6. Measurement of intracellular ROS production in SK-RC-45

Sets of 2×10^6 cells (approximately) were exposed to $CdCl_2$ at specified doses and for 24 h. At the end of the incubation period, cells were scrapped and pelleted by centrifugation at 300g for 5 min at RT. The pellets were collected and suspended in 1 ml of PBS, pre-warmed to 37 °C. Two (2) μl of H_2DCFDA working solution was then added from stock to make the final concentration of

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