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Mitochondrial fusion: A mechanism of cisplatin-induced resistance in neuroblastoma cells?

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ABSTRACT

Cisplatin induces apoptosis through different pathways. The intrinsic apoptotic pathway is mediated by mitochondria, which, as a result of cisplatin treatment, undergo morphological alterations. The aim of this study was to investigate cisplatin-induced mitochondrial functional and morphological long-term effects in neuroblastoma B50 rat cells. To this purpose, we followed evaluated different several apoptotic markers by means of flow cytometry, confocal and electron microscopy and western blotting techniques. We applied different treatment protocols based on the incubation of the neuroblastoma B50 rat cells with 40 μM cisplatin: (i) for 48 h and harvesting of the cells at the end of the treatment; (ii) further recovery in drug-free medium for 7 days post-treatment; (iii) conditions as in (ii) followed by re-seeding in normal medium and growth for a further 4 days. We observed apoptosis induction after the first treatment and after the recovery from cell death after long-term culture in drug-free medium. Interestingly, the latter phenomenon was characterized by mitochondrial elongation and mitochondrial protein rearrangement. In recovered and re-seeded cells, mitochondrial equilibrium moved toward fusion, possibly protecting cells from apoptosis.

1. Introduction

Cisplatin (cisPt) is one of the most potent anticancer agents used in the treatment of various solid tumors (Galanski, 2006). Its cytotoxicity is mediated by the induction of apoptosis and the arrest of cell cycle resulting from its interaction with DNA and the consequent formation of cisPt-DNA adducts (Wang et al., 2004). CisPt therapy can lead to a number of side effects such as ototoxicity, nephrotoxicity and neurotoxicity (Pasetto et al., 2006), thus limiting the dose that can be administered to patients (Cepeda et al., 2007). Moreover, a significant proportion of initially sensitive tumors eventually develop chemoresistance (Koberle et al., 2010), which severely compromises treatment effectiveness. Mechanisms of resistance are multiple (Siddik, 2003; Galluzzi et al., 2012): some of them are activated before cisPt binds to cytoplasmic targets and DNA (Aida et al., 2005; McWhinney et al., 2009; Ishida et al., 2010; Chen and Kuo, 2010; Yamasaki et al., 2011), or during adduct cisPt/DNA formation (Gifford et al., 2004; Sakai et al., 2008; Tajeddine et al., 2008; Olaussen, 2009; Shachar et al., 2009; Roos et al., 2009; Kamal et al., 2010). Other mechanisms function after the binding to DNA (Brozovic et al., 2004; Feldman et al., 2008; Pinho et al., 2009; Michaud et al., 2009; Wang et al., 2010; Yuan et al., 2010; Janson et al., 2011) or could be sustained by alterations in signaling pathways that are not directly engaged by cisPt (Fijolek et al., 2006; Ren et al., 2008; Shen et al., 2010; Hu and Friedman, 2010).

In cisPt-treated rats, we previously described apoptosis in proliferating neuronal cells of the external granular layer of developing cerebellum (Pisu et al., 2005). This finding added new information about the neurotoxic effects of cisPt in the central (Schiffer et al., 1996) and peripheral nervous systems (Cavaletti et al., 1992; McDonald et al., 2005).

Our research then focused on the study of *in vivo* and *in vitro* molecular apoptotic markers in two areas of the rat developing central nervous system (CNS) (Cerri et al., 2011; Bernocchi et al., 2011) and in cultured neuroblastoma cells (Bottone et al., 2008; Santin et al., 2011). Neuroblastoma represents one of the most common solid pediatric tumors and has been used extensively to

Abbreviation: cisPt, cisplatin.

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screen novel compounds for neurotoxic properties and associated mechanisms.

The purpose of the present study was to determine the long-term effects of cisPt on the undifferentiated neuroblastoma cell line B50, focusing on apoptosis and, in particular, on the mitochondrial pathway.

Mitochondria play an integral role in numerous cellular signaling pathways, including programmed cell death (Braschi and McBride, 2010; Osman et al., 2011; Estaquier et al., 2012), are important targets of cisPt (Bottone et al., 2008) and constantly divide and fuse with each other, thus leading to changes in mitochondrial morphology, length, size and number (Wang et al., 2009). After an apoptotic stimulus, mitochondria go toward fragmentation (fission) (Frank et al., 2001; Reddy et al., 2011; Leboucher et al., 2012), but sometimes the equilibrium between fission and fusion moves to promote fusion and elongated mitochondria appear in the cytoplasm. Mitochondrial fission often plays a proapoptotic role, whereas mitochondrial fusion seems to protect cells from cell death. The processes of fission and fusion are known to be involved in maintaining mitochondria integrity, electrical and biochemical connectivity, turnover, segregation and protection of mitochondrial DNA (Westermann, 2002; Okamoto and Shaw, 2005; Manczak et al., 2010; Anand et al., 2012; Figge et al., 2012; Ferree and Shirihai, 2012).

We analyzed the functionality and the morphological structure of mitochondria in B50 neuroblastoma cells by means of immunocytochemical, cytofluorimetric and western blotting techniques. After a 48 h-cisPt treatment and successive long-term recovery, we monitored the apoptotic effect of cisPt by flow cytometry; then, we observed the morphological structure both at confocal and electron microscopy, and finally we considered different markers, such as cytochrome c and AIF (apoptosis inducing factor), resident in mitochondria in physiological conditions and translocated to the cytoplasm after an apoptotic stimulus; Bax and Bcl 2, with proapoptotic and antiapoptotic activity, respectively; Opa1, a GTPase involved in the inner mitochondrial membrane fusion; Sirt1, a sirtuin with a cellular protective role. The analysis of these parameters could help in defining the nature of mitochondrial alterations discovered in B50 neuroblastoma rat cells after cisPt treatment.

2. Materials and methods

2.1. Cells and treatments

B50 neuroblastoma rat cells (ATCC) were cultured in 75 cm^2 flasks in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, 1% glutamine, 100 U penicillin and streptomycin (Celbio) in a 5% CO_2 humidified atmosphere. Twenty-four hours before the experiments, cells were seeded on glass coverslips for fluorescence microscopy, or grown in 75 cm^2 flasks for flow cytometric analysis.

Cell treatment was performed according to different protocols:

(i) incubation with 40 μM cisPt (Teva Pharma) for 48 h. This concentration was selected considering our *in vivo* experimental design (*i.e.* a single injection of 5 μg/g *b.w.* in 10-day old rats); this concentration corresponds to the dose most commonly used in chemotherapy (Bodenner et al., 1986; Dietrich et al., 2006);
(ii) incubation with 40 μM cisPt for 48 h followed by a recovery of 7 days in drug-free medium;
(iii) incubation with 40 μM cisPt for 48 h, recovery of 7 days in drug-free medium, followed by re-seeding and growth in normal medium for 4 days.

2.2. Flow cytometry

For cell cycle analysis, cells were detached by mild trypsinization to yield single-cell suspensions for flow cytometry, washed in phosphate-buffered saline (PBS), permeabilized with 70% ethanol for 10 min and stained for 10 min at room temperature with 2 μg/ml propidium iodide (PI) (Sigma–Aldrich) for 1 h.

DNA content was measured with a Partec PAS III flow cytometer (Münster) equipped with 100 W mercury lamp. At least 20,000 cells/sample were measured.

For the identification of apoptotic cells through Annexin V/FITC *versus* PI, cells were detached by mild trypsinization as before, incubated with FITC-conjugated Annexin V (3 μL/10^6 cells) (Bender MedSystem, Prodotti Gianni) and counterstained with 2 μg/ml PI. After 10 min incubation, dual parameter flow cytometric analysis was performed with the flow cytometer Partec PAS III, equipped with argon laser excitation (power 200 mW) at 488 nm, 510–540 nm interference filter for the detection of FITC green fluorescence, and a 610 nm long-pass filter for PI red fluorescence detection.

Changes in mitochondrial membrane potential were monitored using JC-1 (5,5V,6,6V-tetrachloro-1,1V,3,3V-tetraethylbenzimidazolcarbocyanine iodide) (Molecular Probes, Invitrogen), a lipophilic, cationic dye that can selectively enter into mitochondria and reversibly change the color from green to red as the membrane potential increases. The green signal indicates depolarized mitochondria and the red signal indicates polarized mitochondria (Reers et al., 1995). Thus, the shift from red to green fluorescence is considered a reliable indication of a drop in mitochondrial membrane potential. Cells grown in flasks were harvested by mild trypsinization with 0.25% trypsin in PBS containing 0.05% ethylenediaminetetraacetic acid and were incubated in culture medium with 2 μM JC-1 for 20 min at 37 °C in the dark. After two washes with PBS at 37 °C, cells in suspension were analyzed by flow cytometry using a Partec PAS III equipped with argon ion laser with 20 mW output power at 488 nm excitation and with 530/30 nm and 585/42 nm band-pass emission filters. Data were analyzed using FlowMax software from the same company.

Three independent experiments were carried out for every technique, and the average of the scores was used. Values are expressed as the mean ± SD (standard deviation) and differences were compared using Student's *t*-test.

2.3. Immunocytochemical reactions at confocal microscope

For immunocytochemical analysis, samples grown on coverslips were fixed with 4% formalin and post-fixed with 70% ethanol for 30 min, at −20 °C. Cells were incubated with of primary antibodies (see Table 1) for 60 min at room temperature in a dark moist chamber; coverslips were incubated with secondary antibodies (see Table 1). Cells were mounted in a drop of Mowiol (Calbiochem) for confocal microscopy analysis. When necessary, cells were counterstained with Hoechst 33258. Three independent experiments were carried out.

The mitochondrial length was measured by means of confocal microscopy software. The bar chart was obtained after counting cells with elongated mitochondria; three observations was made and ten fields were considered. The statistical significance of any differences between control and treated samples was determined by Student's *t*-test.

For confocal laser scanning microscopy, a Leica TCS–SP system (Leica) mounted on a Leica DMIRBE-inverted microscope was used. For fluorescence excitation, an Ar/UV laser at 364 nm was used for Hoechst 33258, an Ar/Vis laser at 488 nm was used for FITC and a He/Ne laser at 543 nm was used for Alexa 594. Spaced (0.5 μm) optical sections were recorded using a 63× oil immersion objective. Images were collected in the 1024 × 1024 pixel format,