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Developmental expression of cellular prion protein and apoptotic molecules in the rat cerebellum: Effects of platinum compounds

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ABSTRACT

Programmed cell death is regulated by prototypes of a large family of Bcl-2-like proteins such as Bax and Bcl-2. A neuroprotective role for cellular prion protein (PrPc) on programmed cell death has been reported, although the cytosolic accumulation of PrPc correlates with toxicity and death of some neurons by apoptosis. In order to understand the signalling function of PrPc in promoting survival or suppressing cell death, we analyzed the expression and co-localization of PrPc, Bax and Bcl-2 proteins in the developing cerebellum of rats treated at PD10 (postnatal day 10) with the chemotherapeutic drug cisplatin (cisPt) or the new platinum (Pt) compound [Pt(*O*,*O*′-acac)(γ-acac)(DMS)] (PtAcacDMS). Differences in the expression of PrPc, Bax and Bcl-2 were found in proliferating cells and immature Purkinje neurons. One day after administration (PD11), cisPt markedly increased the apoptosis of the proliferating cells of the EGL (external granular layer); at the same time, several apoptotic bodies with strong Bax immunoreactivity were noticed. After PtAcacDMS, changes in PrPc and apoptotic proteins, with respect to the controls, were found but Bax immunopositive apoptotic bodies were not detectable, which could mean that apoptotic cell death of proliferating cells is preserved. Co-localization was clearly detected in the Purkinje cell population and may explain better the mechanisms by which PrPc and the apoptotic proteins function, and particularly the role of PrPc. Considering the reactivity of Purkinje neurons to these proteins at PD11 and Pd17, at least PrPc expression increased after cisPt and PtAcacDMS treatments or, if PrPc decreased, balanced itself with Bcl-2. The noteworthiness of this finding is that it emphasizes that most of the post-mitotic Purkinje cells need to be rescued, otherwise they undergo degeneration and are not replaced. Based on the effects of both Pt compounds on Purkinje cell differentiation, it should be emphasized that PrPc, together with the synergistic action of the co-localized anti-apoptotic protein, acts as a neuroprotective protein countering cytotoxicity in the postnatal critical phases of cerebellum development.

1. Introduction

Prion diseases are neurodegenerative disorders that affect animals and humans. The prion pathogen infection consists in an abnormal prion protein which is an altered isoform of a normal cellular prion protein (PrPc), a sialoglycoprotein widely expressed at the cell surface via a glycosyl phosphatidyl inositol anchor (Prusiner, 1996,1998; Stahl and Prusiner, 1991; review: Chiesa and Harris, 2009); cytosolic localized PrPc has also been detected (Mironov et al., 2003). It is now clear, however, that alterations in the normal function of PrPc may play an important role in causing or contributing to the disease phenotype; for this reason, elucidating the physiological activity of PrPc has become a major priority in prion research.

PrPc function remains enigmatic, due to its ubiquitous distribution; not only it is expressed most abundantly in the brain, but has been also detected in non-neuronal tissues (Horiuchi et al., 1995). Some possible biological functions for PrPc have been proposed. A generalized physiological function in the brain as antioxidant (Brown et al., 1997) involvement in copper homeostasis (Pauly and Harris, 1998), signal transduction

Abbreviations: cisPt, cisplatin; EGL, external granular layer; IGL, internal granular layer; ML, molecular layer; PCL, Purkinje cell layer; PD, postnatal day; PrPc, cellular prion protein; Pt, platinum; PtAcacDMS, [Pt(*O*,*O*′-acac)(γ-acac)(DMS)].

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(Mouillet-Richard et al., 2000), neurotransmission and synapse formation (Carleton et al., 2001; Collinge et al., 1994; Zomosa-Signoret et al., 2008), and adhesion to the extracellular matrix (Schmitt-Ulms et al., 2001). The accumulation of PrPc in the cytosol seems to correlate with toxicity in some neuronal cells (Harris and True, 2006; Wollmann and Lindquist, 2002) but, interestingly, a subset of neurons in the hippocampus, neocortex and thalamus of mouse brain appeared healthy even though cytosol-localized PrPc has been detected in these neurons (Barmada and Harris, 2005; Mironov et al., 2003).

Recently, evidences emerged suggesting that PrPc may be cytoprotective, particularly against internal and environmental stimuli that initiate an apoptotic programme (review: Westergard et al., 2007). PrPc over-expression rescues cultured neurons and some mammalian cell lines, from several kinds of death-inducing stimuli (Kuwahara et al., 1999; Li et al., 2007a; Roucou and LeBlanc, 2005; Solforosi et al., 2004).

At present, neuronal roles for PrPc as an anti- and conversely pro-apoptotic protein have been postulated (Kim et al., 2004). It may be speculated is possible that PrPc is a member of the Bcl-2 family, since a similarity between the Bcl-2 homology domain of the Bcl-2 family members and the octapeptide repeats in N-terminal region of PrPc has been noticed (review: Zomosa-Signoret et al., 2008). Investigations on the pro-apoptotic molecule Bax, which plays a major role in regulating neuronal death in the CNS (central nervous system) both during development and after injury (Akhtar et al., 2004; Yuan and Yankner, 2000), indicate that neurodegeneration involves both Bax-dependent and Bax-independent pathways. PrPc could inhibit Bax directly or, indirectly by acting as a signalling molucule (Roucou and LeBlanc, 2005). Bax deletion in mice expressing the neurotoxic form of PrPc slows apoptosis of cerebellar granule cells (Li et al., 2007a,b). In parallel, a protective effect of PrPc has been proposed which depends on signalling function of PrPc to promote neuronal survival or suppress neuronal death (Li et al., 2007a,b; Solforosi et al., 2004).

In the present study, immunocytochemical techniques were used to investigate the trend and the role of PrPc in the developing rat cerebellum in relation to the involvement of the pro- and anti-apoptotic proteins Bax or Bcl-2 before and after treatment with platinum (Pt) compounds cisplatin (cisPt) and a new platinum complex [Pt(*O*,*O*′-acac)(gamma-acac)(DMS)] (PtAcacDMS). Particularly, high levels of PrPc (Lainé et al., 2001; Salès et al., 1998), Bcl-2 and Bax (review: Vogel, 2002) are expressed in the Purkinje neurons.

CisPt, the most commonly used therapeutic agent (review: McWhinney et al., 2009), is a highly effective anticancer drug which has an important role also in the treatment of childish malignancies such as neuroblastoma, osteosarcoma and some brain tumours (Prestayko et al., 1979). Mechanisms of action of cisPt are principally based on binding of DNA of mitotic cells (Ahmad, 2010; Eastman, 1991). In proliferating cells, Pt atoms form covalent bonds to purine bases whose result is to block DNA synthesis. The DNA damage triggers cell-cycle arrest, activation of the tumour-suppressor p53 and apoptosis (Qin et al., 2002). Moreover, cisPt toxicity can also induce death in non mitotic cells by interacting with cytoplasmic proteins (Mandic et al., 2003).

Among the new Pt complexes, PtAcacDMS, which has two acetylacetonate ligands, one *O*,*O*′-chelate and the other sigma-linked by metine in the gamma position, was found to be the most active in rapidly producing a sustained apoptotic response (Muscella et al., 2008). Differently from cisPt, whose activity appears to be at the cellular level and DNA linking, the cytotoxicity of the new complex is associated solely with intracellular accumulation. Moreover, PtAcacDMS has shown low reactivity with nucleobases and a specific reactivity with sulphur ligands. This suggests that its preferred cell target could be amino acid residues of enzymes and other proteins involved in apoptotic induction, thus characterizing it as a compound with non-genomic targets. Recently, the low neurotoxicity of PtAcacDMS was supported by findings on cerebellum postnatal ontogenesis (Bernocchi et al., 2011; Cerri et al., 2011).

The trend of the PrPc, Bax and Bcl-2 markers was followed at two crucial stages of cerebellum postnatal histogenesis, i.e. during the active cell proliferation in the external granular layer (EGL) and during the maturation phase of Purkinje cells (review: Altman, 1972a,b). Therefore, particular attention was paid to changes in the layers of differentiating Purkinje cells and precursors of granule cells.

2. Materials and methods

2.1. Animal and treatments

Ten-day-old Wistar rats were given a single subcutaneous injection of cisPt (0.5 mg/ml; Teva Pharma, Italy) or PtAcacDMS in the nape of the neck at a dose of 5 μg/g b.w. (corresponding to the therapeutic dose suggested by Bodenner et al. (1986) and Dietrich et al. (2006)) or 10 μg/g b.w. Throughout the experiment, the rats were kept in an artificial 12 h light:12 h dark cycle and provided rat chow and tap water ad libitum. One day (PD11), 7 days (PD17) and 20 days (PD30) after drug administration, treated (4 per stage) and untreated control rats (4 per stage) of the same age were deeply anesthetized with an intraperitoneal injection of 35% chloral hydrate (100 μl/100 g b.w.; Sigma, St. Louis, MO, USA); the brains were quickly removed, fixed in Carnoy's solution (6 absolute ethanol/3 chloroform/1 acetic acid) for 48 h, then placed in absolute ethanol and in acetone, and embedded in Paraplast X-tra (Sigma). Sections (8 μm thick) of cerebellar vermis were cut serially in the sagittal plane and collected on silan-coated slides. The slides were then processed for immunohistochemical procedures (as described later). To avoid possible staining differences due to small changes in the procedure, each reaction was carried out simultaneously on slides from treated and untreated animals at different stages.

All experiments were performed according to the guidelines for care and use of laboratory animals as published by the Italian Ministry of Health (DDL 116/92). All efforts were made to minimize the number of animals used and their suffering.

2.2. Single immunoperoxidase stainings

The immunoreaction was performed on cerebellum sections of 11, 17 and 30 day-old-rats. Sections were deparaffinized in xylene, rehydrated through a series of graded alcohol treatments and rinsed in PBS. The endogenous peroxidases were suppressed by incubation of sections with 3% H_2O_2 in 10% methanol in phosphate-buffered saline (PBS; Sigma) for 7 min. Subsequently, the sections were washed in PBS and incubated for 20 min in normal serum (15 μl normal serum/1000 μl PBS) at room temperature in order to block nonspecific antigen binding sites. The serum for blocking is prepared from the same species in which the biotinylated secondary antibody is made. Localization of PrPc was achieved by applying on brain sections a monoclonal mouse antibody anti-PrPc (Sigma) diluted 1:100 in PBS overnight in a dark moist chamber. Thereafter, the sections were sequentially incubated with diluted biotinylated secondary antibody solution (1:200; Vector Laboratories, Burlingame, CA, USA) for 30 min. Sections were washed in PBS and incubated for 30 min at room temperature with Vectastain Elite ABC reagent (Vector Laboratories). Then, 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) with 0.01% H_2O_2 in Tris–HCl buffer (0.05 M, pH 7.6) was used as a chromogen. After each reaction step, sections were washed thoroughly in PBS (two changes of 5 min each). Sections were dehydrated in ethanol, cleared in xylene, and mounted in Eukitt (Kindler, Freiburg, Germany). For control staining, some sections were incubated with PBS instead of the primary antibody. No immunoreactivity was present in this condition.

The slides were observed with an Olympus BX51 microscope, and the images were recorded with an Olympus Camedia C-5050 digital camera and stored on a PC. Corrections to brightness and contrast were made with Paint Shop Pro 7 (Jasc Software Inc.).

2.3. Double immunofluorescence reactions

Localization of PrPc/Bcl-2 and PrPc/Bax was achieved by applying on cerebellum sections of 11 and 17 day-old-rats, respectively:

- a mouse monoclonal anti-PrPc (1:100; Sigma) and a rabbit polyclonal anti-Bcl-2 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and
- a mouse monoclonal anti-PrPc (1:100; Sigma) and a rabbit polyclonal anti-Bax (1:100; Santa Cruz Biotechnology),

in PBS overnight in a dark moist chamber.

Sections were washed in PBS and incubated with the secondary antibodies, respectively: Alexa-Fluor 488 goat anti-mouse (1:100, Molecular Probes, Milan, Italy) and Alexa-Fluor 594 donkey anti-rabbit (1:100, Molecular Probes), in PBS for