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Okadaic acid induces apoptosis in Down syndrome fibroblasts

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ABSTRACT

Down's syndrome (DS) is characterized by several pathological aspects leading to an increased susceptibility to cardiovascular diseases, infections, leukemia, endocrine alterations. DS patients display some of the physiopathological characteristics of aging, observed also in Alzheimer disease (AD), such as abnormalities in lipids metabolism, diabetes, high cholesterol fraction, senile plaques and neurofibrillary tangles. For this reason DS is considered a precocious and accelerated model of senescence, in which increased apoptosis is the main cornerstone. In order to better understand the apoptotic process in pathological cellular aspects of DS, the aim of this study was to investigate the apoptotic response of DS fibroblasts to OA, a toxin that induces malformations and inhibits growth in different cell lines. We focused specifically on the mitochondrial response by investigating changes in mitochondrial membrane potential (evaluate by flow cytometry and fluorescence microscopy using JC-1 probe) and alterations of mitochondrial outer membrane (evaluated by flow cytometry using annexin V/propidium iodide). Results indicates that DS Fibroblasts have a baseline of apoptosis higher than normal fibroblasts and are more susceptible to the pro-apoptotic effect of OA.

Understanding the mechanism of apoptosis in DS fibroblasts could provide new insight in the pathogenic mechanism of this pathology and suggest potential therapeutical targets to the clinical treatment at complex diseases associated to this pathology.

1. Introduction

Down's syndrome (DS) or trisomy 21 is the most common autosomal-aneuploidy that survives after birth and the single most frequent genetic cause of mental retardation. The neuropathology of DS is complex and includes development of Alzheimer's disease (AD) by middle age (Coyle et al., 1986; Mann, 1988; Lott and Head, 2001). DS is characterized by several pathological aspect leading to an increased susceptibility to cardiovascular diseases, infections, leukemia and endocrine alterations (Baird and Sadovnick, 1988). DS displays some of the physiopathological characteristics of aging, observed also in AD, such as abnormalities in lipids metabolism, diabetes, high cholesterol fraction, senile plaques and neurofibrillary tangles (Kuzu et al., 2005). For this reason DS is considered a precocious and accelerated model of senescence (Walford et al., 1981), in which increased apoptosis is the main cornerstone (Martin, 1978). Previous studies reported increased apoptosis in neurons, granulocytes and other peripheral blood cells of DS patients (Busciglio and Yankner, 1995; Antonucci et al., 1997; Yasui et al., 1999), but no data have been described so far about apoptosis in Down's syndrome skin fibroblasts (FDS) (46 XY) compared to normal skin fibroblasts (FCTR) (47 XY).

Apoptosis is a genetically-mediated form of cell death that is characterized by specific morphological and biochemical properties. It can be induced by a great variety of stimuli, including growth factor deprivation, cell detachment (also know as *anoikis*) and exogenous stimuli (toxins) which may be specific for each cell type, leading to differences in the overall process (Kuwana and Newmeyer, 2003).

These series of events can be divided into early and late apoptosis phases and are characterized by specific intracellular modifications. The early stages characteristics of apoptosis include cell shrinkage, nuclear condensation, DNA degradation and breakdown in the nuclear material. Later stages of apoptosis are characterized by deformation of membrane blebs leading to "apoptotic bodies" and cell fragmentation with subsequent phagocytosis by macrophages (Majumdar et al., 2001; Wyllie et al., 1980; Kerr et al., 1972). Two major pathways for inducing apoptosis can be

Abbreviations: DS, Down syndrome; FCTR, fibroblasts of control; FDS, Down syndrome fibroblast; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol carbocyanine-iodide; OA, okadaic acid; PBS, phosphate buffer saline; PI, propidium iodide; PP-1-2A, phosphatase protein 1-2A; PS, phosphatidylserine; TMB, tetramethylbenzidine; $\Delta\Psi_m$, mitochondrial membrane potential variation.

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distinguished: the intrinsic pathway that involves mitochondria, and the extrinsic pathway that runs from death receptors. At the mitochondria level, mitochondrial outer membrane permeabilization (MOMP) is a pivotal event. Following MOMP, pro-apoptotic factors (apoptogens) normally found in the space between the inner and outer mitochondrial membranes are irreversibly released into the cytoplasm. MOMP is often associated to a decrease in the mitochondrial inner transmembrane potential (*D*wm), possibly due to the pore opening in the inner membrane.

Apoptosis can be considered a physiological cell response to stress or senescence (Pothana and Rekha, 2008), but it is also characteristic of some pathological conditions and diseases. In particular, an intense apoptosis is a striking feature of human diseases characterized by early cell senescence, such as Alzheimer or DS (Corsi et al., 2004).

Many alterations have been described in mitochondria of DS subjects (Druzhyna et al., 1998; Lee et al., 2000). Altered free radical metabolism and impaired mitochondrial function are linked to neuronal degeneration of DS cortical neurons in culture (Busciglio et al., 2002) and may be associated with both mental retardation and AD pathology in DS patients. Astrocytes show decreased mitochondrial redox activity and depolarization of mitochondrial membrane potential ($\Delta\Psi_m$) (Lenaz et al., 2006). These dysfunctions could be responsible for a higher vulnerability to exogenous toxic insults (Busciglio and Yankner, 1995), leading to programmed cell death through different pathways. Different stress or toxic agents have been reported to induce apoptosis in DS patient derived cells (Helguera et al., 2005; Zana et al., 2007).

In this study apoptosis of FDS was induced with okadaic acid (OA), a marine toxin produced by dinoflagellates, a black sponge *Halichondria okadaii*, (Mouratidou et al., 2006). OA is a potent apoptosis inducer in several cell systems (Boe et al., 1991; Ishida et al., 1992; Song et al., 1992; Afshari et al., 1994; Benito et al., 1997), but it has never been studied in DS derived cells.

This toxin inhibits Ser–Thr protein phosphatases PP1 and PP2A (Brimfield, 1995; Haystead et al., 1989; Bialojan and Takai, 1988) which are vital to phosphorylation/dephosphorylation within the cell, thus inducing apoptosis in a wide variety of cell types, including human/non-human and primary cultures or established cell lines (Atkinson et al., 2009).

Several biochemical changes have been identified in most apoptotic cells, which could be useful for the early detection of apoptosis. We focused on mitochondria, because they have a central role in the apoptotic mechanism (Green and Reed, 1998; Leira et al., 2001). In this study the mitochondrial response of FDS and FCTR to OA was evaluated by JC-1, a cyanine dye which determines the loss of mitochondrial membrane potential, annexin V/propidium iodide detecting the change in phospholipids asymmetry in the plasma membrane.

2. Materials and methods

2.1. Cell culture for microscopy and cytometry

FCTR and FDS cell lines, obtained from Galliera Genetic Bank (Telethon Research Service, Genoa) were routinely grown in monolayer in 25 cm^2 flasks at a density of 2500 cells/cm^2 at 37 °C in a humidified CO_2 incubator in MEM-EARLE culture medium (Biochrom AG, Berlin) supplemented with 1 mM sodium pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine, 1 mM non essential aminoacids and 10% fetal bovine serum (FBS) (Biochrom). The medium was replaced at 3 day interval. Subconfluent cells were routinely harvested with 0.05% trypsin/0.02% EDTA (Biocrom). For all the assays cells were exposed to 100 nM OA (Calbiochem, Canada) for 20 min, 1 and 4 h. For microscopy assay, cells were grown at the same density on coverslips and fixed in 3% paraformaldehyde at room temperature for 15 min after OA treatment.

2.2. Detection of changes in mitochondrial membrane potential by JC-1

The loss of $\Delta\Psi_m$ is a hallmark for apoptosis. It is an early event preceding phosphatidylserine externalization and caspase activation. In healthy cells, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimidazol carbocyanine-iodide (JC-1) exists as a monomer in the cytosol which fluoresces green and also accumulates into the mitochondria where it can forms aggregates, due to the physiological $\Delta\Psi_m$, which stain red. In apoptotic and necrotic cells, JC-1 exists only in the monomeric form and stains the cytosol green. The absorption/emission maxima of the aggregate red form are 585/590 nm (FL-2). The absorption/emission maxima of the green monomeric form are 510/527 nm (FL-1).

After reaching optimal confluence, cells were treated for different time (see previously section) with OA, than washed with cold PBS, exposed to 0.3 μg/mL JC-1 dye solution (Molecular Probes Inc., Eugene, OR) and finally incubated in the dark for 1hour at 37 °C. After washing and trypsinization, cells were diluted in 500 μL of medium without pH indicator and read by flow cytometry (Cyflow, Partec). The same probe was also used for fluorescence microscopy analysis. Cells were grown until desired confluence and than treated with OA. Cells were than incubated in the dark for 15 min at 37 °C in a 0.3 μg/mL JC-1 dye solution, washed with pre-warmed PBS, fixed with paraformaldehyde and mounted using Mowiol (Sigma, Aldrich, Milan). Cells were than analyzed by fluorescence microscopy (Nikon Eclipse 80i).

2.3. Cell apoptosis analysis by annexin V-FITC and propidium iodide

Apoptosis induced by OA was analyzed by flow cytometry utilizing annexin V-FITC and propidium iodide (PI) staining (SIGMA). In brief, cells were trypsinized, washed with PBS and resuspended with 500 μL of a specific binding buffer containing 10 μL of PI, and 5 μL of annexin V-FITC (Pigault et al., 1994; Kuypers et al., 1996). After exactly 10 min incubation in the dark at room temperature, cells were analyzed for annexin V and PI staining by flow cytometry. Each experiment was run in triplicate. Excitation wavelength was 488 nm and emitted green fluorescence of annexin V (FL-1) and red fluorescence of PI (FL-2) were collected using respectively a 525 and a 575 nm band pass filter. Early apoptosis and late apoptosis/necrosis were expressed as the percentages of annexin V^+/PI^- and annexin V^+/PI^+ positive cells.

2.4. Statistical analysis of biological assays

Statistical analysis was performed using the Prism statistical analysis package (GraphPad Software, San Diego, CA). Data are expressed as mean ± SD. Differences between treatment groups were evaluated by ANOVA and considered significant for $p < 0.05$.

3. Results

3.1. FDS present a lower $\Delta\Psi$m than FCTR before and after OA treatment

OA-induced apoptosis was evaluated monitoring changes in $\Delta\Psi_m$ by the use of JC-1 probe. FDS and FCTR were treated with 100 nM OA for 20 min, 1 or 4 h and at the end of the incubation time JC-1 emission was analyzed by a fluorescence microscopy. In FDS and FCTR not treated with OA, the double red/green staining in the cytosol indicates that JC-1 was present both as monomers