Methods 46 (2008) 319–323

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Assessing mitochondrial outer membrane permeabilization during apoptosis

Zankruti Dave, Maya Byfield, Ella Bossy-Wetzel *

Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 4000 Central Florida Blvd., Orlando, FL 32816, USA

ARTICLE INFO

Article history:
Accepted 17 October 2008
Available online 26 October 2008

Keywords:
Cytochrome *c*
Mitochondrial outer membrane permeability
Cell death
Fluorescence microscopy
Bax and t-Bid

ABSTRACT

Mitochondria play a pivotal role in the regulation of apoptosis. An imbalance in apoptosis can lead to disease. Unscheduled apoptosis has been linked to neurodegeneration while inhibition of apoptosis can cause cancer. An early and key event during apoptosis is the release of factors from mitochondria. In apoptosis the mitochondrial outer membrane becomes permeable, leading to release of apoptogenic factors into the cytosol. One such factor, cytochrome *c*, is an electron carrier of the respiratory chain normally trapped within the mitochondrial intermembrane space. Many apoptotic studies investigate mitochondrial outer membrane permeabilization (MOMP) by monitoring the release of cytochrome *c*. Here, we describe three reliable techniques that detect cytochrome *c* release from mitochondria, through subcellular fractionation or immunocytochemistry and fluorescence microscopy, or isolated mitochondria and recombinant Bax and t-Bid proteins *in vitro*. These techniques will help to identify mechanisms and characterize factors regulating MOMP.

1. Introduction

Apoptosis is a regulated cell death program necessary for the homeostasis of multicellular organisms and normal embryonic development [1]. It also plays a role in diseases such as cancer, where the process is shut down, and neurodegenerative disorders, where excess cell death occurs [2].

There are several pathways that trigger apoptosis in cells. The extrinsic pathway involves extracellular signals that activate receptors from the tumor necrosis factor (TNF) super family cell surface receptors, such as Fas [3]. These "death receptors" contain an intracellular death domain (DD), which upon ligand binding form an intracellular complex called the death inducing signaling complex. Formation of this complex leads to activation of caspase-8, which in turn propagates the activation of downstream effector caspases, and final execution of cell death. Caspase-8 also cleaves the BH3-only protein Bid and truncated Bid (t-Bid) translocates to the mitochondria to activate the intrinsic apoptotic pathway [4].

There are many signals that lead to cell death through the mitochondrial pathway including DNA damage and oxidative stress. Often thought of as "the point of no return", mitochondrial membrane permeabilization (MMP) plays a crucial role in the intrinsic pathway of apoptosis. MMP has several complex mechanisms that involve both the mitochondrial inner membrane (IM) and mitochondrial outer membrane (OM). The IM impermeability is necessary for maintaining the proton gradient that is required for oxidative phosphorylation [5]. However, IM permeabilization to small ions and water causes the mitochondrial transmembrane potential ($\Delta\Psi_m$) to dissipate and promotes osmotic matrix swelling and consequent OM rupture [6]. The mitochondrial permeability transition pore complex (mPTPC) mediates OM rupture. While the composition of this multi-component complex is not entirely defined yet, it includes the adenosine nucleotide translocation (ANT), cyclophilin D (CypD), and voltage-dependent anion channel (VDAC) [7].

There are methods that utilize fluorescent probes to detect permeabilization of the IM. Cell permeant dyes such as Rhodamine 123 and Tetramethylrhodamine ethyl ester (TMRE) measure $\Delta\Psi_m$, but are not considered reliable indicators of IM permeabilization [8,9]. Another method of studying mPTPC opening is the calcein quenching method, which involves monitoring the changes in fluorescence produced by the fluorochrome calcein in the mitochondria and cytosol resulting from redistribution of fluorescence quenching ions [10]. While informative, neither fluorescent probes to measure $\Delta\Psi_m$, nor the calcein quenching method address the initial mechanism of cell death.

OM permeabilization has been proposed to occur independently of IM permeabilization. One possible mechanism is the activation of pro-apoptotic Bcl-2 family proteins (*e.g.,* Bax, Bid), which translocate from the cytosol to the OM favoring the formation of pores or large multimeric channels and allow the release of apoptogenic inner membrane space (IMS) proteins into the cytosol [11–15]. Activation of the mPTPC might also lead to the physical rupture of the OM and release of IMS proteins, preventing inhibition of the permeability transition (PT). [16]. Recent research suggests that mitochondrial fission, another early event in apoptosis, also regulates MOMP. Chemical inhibition of DRP1, a major molecule of the fission machinery, affects Bax-dependent cytochrome *c* release [17].

* Corresponding author. Fax: +1 407 823 0956.
E-mail address: ebossywe@mail.ucf.edu (E. Bossy-Wetzel).

1046-2023/$ - see front matter © 2008 Published by Elsevier Inc.
doi:10.1016/j.ymeth.2008.10.019

In this article, we describe standard methods for assaying MOMP during apoptosis. First, we illustrate subcellular fractionation to detect the release of IMS proteins, cytochrome *c* [18] and Omi/HtrA2 [19]. We also present a cell-free system used to assay cytochrome *c* release from isolated liver mitochondria. Finally, we describe immunocytochemistry and fluorescence microscopy to assess the subcellular localization of cytochrome c *in situ*. Immunoblotting or cell staining methods can be time-consuming especially in regards to drug characterization. For screening methods, a commercial ELISA kit from R&D Systems, Inc. can be used.

2. Description of methods

2.1. MOMP detection by subcellular fractionation and immunoblotting

2.1.1. Equipment and reagents

Glass Dounce homogenizer (2 ml) and tight B-type pestle (Kontes)
Mini-electrophoresis and transfer unit (Invitrogen)
1× Phosphate Buffered Saline (PBS) (EMD)
Mitochondrial isolation buffer (250 mM sucrose, 20 mM HEPES-KOH, pH 7.4, 10 mM KCl, 1.5 mM EGTA, 1.5 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT)
Complete™ Protease inhibitor cocktail (Roche)
Mitochondrial lysis buffer (50 mM HEPES, pH 7.4, 1% NP-40, 10% glycerol, 1 mM EDTA, 2 mM DTT, protease inhibitor cocktail)
Quickstart Bradford assay reagent (Bio-Rad)
Transblot Transfer medium , pure nitrocellulose, 0.2 μM, (Bio-Rad)
Ponceau S solution (Bio-Rad)
12% NuPAGE mini-gels plus running system (Invitrogen)
Mouse anti-cytochrome *c*, clone 7H8.2C12 (BD Biosciences)
Rabbit anti-OMI/HtrA2 (R&D Systems)
West Pico Enhanced Chemiluminescent Substrate (Pierce)
Rabbit anti-COX IV (Cell Signaling)
Mouse anti-β-Actin (Sigma)
Restore™ Western Blot Stripping Buffer (Pierce)
Staurosporine, 2 mM stock in DMSO (Calbiochem)

2.1.2. Procedure

2.1.2.1. Subcellular fractionation. Treat two 100 mm culture dishes of confluent HEK293T cells with the pro-apoptotic drug staurosporine (STS) (1 μM) or carrier DMSO for 4 h. For apoptosis studies it is important to harvest the non-adherent floating cells. Therefore, place the cells directly on ice after STS treatment without removing the medium, and remove the cells from the culture dish using a disposable cell scraper. Transfer the cell suspensions into a 15 ml tube on ice. Collect the cells by centrifugation at 200*g* for 5 min using a tabletop centrifuge at 4 °C. Next, wash the cell pellets twice with 10 ml of ice-cold PBS (pH 7.4). You must remove all PBS from the cell pellet before adding the mitochondrial isolation buffer. Resuspend the pellet with 300 μl of ice-cold mitochondrial isolation buffer and incubate on ice for 20–30 min. Transfer the cell suspension to a glass dounce with a tight pestle (B-type). Apply 50–60 strokes by moving the pestle gently up and down. Transfer the cell homogenates with a Pasteur pipette to an eppendorf tube and spin the sample at 800*g* for 10 min at 4 °C to remove nuclei and unbroken cells. Carefully remove the supernatant, which contains mitochondria, and transfer to a new eppendorf tube and spin at 22,000*g* for 15 min at 4 °C. The pellets contain mitochondria, and are resuspended with 100 μl of mitochondrial lysis buffer. Save the supernatant as the cytosolic fraction. You can save both mitochondrial and cytosolic fractions by freezing at −70 °C. Measure the protein concentration of the cytoplasmic and mitochondrial fraction by using the Quickstart Bradford assay reagent and by comparing to a standard of BSA 0.05–0.5 mg/ml diluted in the mitochondrial isolation buffer.

2.1.2.2. Immunoblotting with apoptogenic factor antibodies. Separate cytosolic and mitochondria-enriched fractions (20 μg) by SDS–PAGE and transfer to nitrocellulose membranes. After transfer, stain the nitrocellulose filters with Ponceau S solution to confirm equal protein transfer. Incubate membranes for 2 h at room temperature with PBS, 5% nonfat milk, 0.1% Tween 20 to block for nonspecific protein binding. Dilute the primary mouse anti-cytochrome *c* (1:1000) or anti-OMI/HtrA2 (1:1000) antibodies in PBS, 0.1% Tween 20, 5% BSA and rock the blots overnight at 4 °C on a shaker. The primary antibody solution can be stored at 4 °C by adding 0.02% NaN_3 and used repeatedly up to five times. The following day, wash membranes three times with PBS, 0.1% Tween 20, for 5 min, and incubate with secondary horseradish peroxidase-linked goat anti-mouse or anti-rabbit secondary antibodies (1:2000) for 2 h at room temperature. To confirm equal protein loading, place membranes in stripping buffer and rock for 5 min on a shaker. After stripping, re-block with PBS, 5% nonfat milk, 0.1% Tween 20 for 2 h. To confirm equal loading of cytosolic and mitochondrial fractions, use mouse anti-β-Actin (1:10,000) and rabbit anti-COX IV (1:1000), respectively. Visualize protein-antibody complexes, using West Pico Enhanced Chemiluminescence reagent.

2.1.3. Data analysis

We used this method in Fig. 1 to demonstrate the release of cytochrome *c* and Omi/HtrA2 from mitochondria in HEK293T cells induced to undergo apoptosis by STS treatment. After a 4 h treatment with STS, we found cytochrome *c* and Omi/HtrA2 in the cytosolic fraction (Fig. 1, upper panel). It is obvious that these proteins moved from the mitochondria to the cytosol because DMSO-control treated cells show retention of these proteins in the mitochondrial fraction (Fig. 1, lower panel). We ran samples in duplicates.

2.1.3.1. Troubleshooting. To study MOMP as evidenced by cytochrome *c* release, you must have adequate amounts of healthy cells to start out. Moreover, proper isolation of mitochondria using the

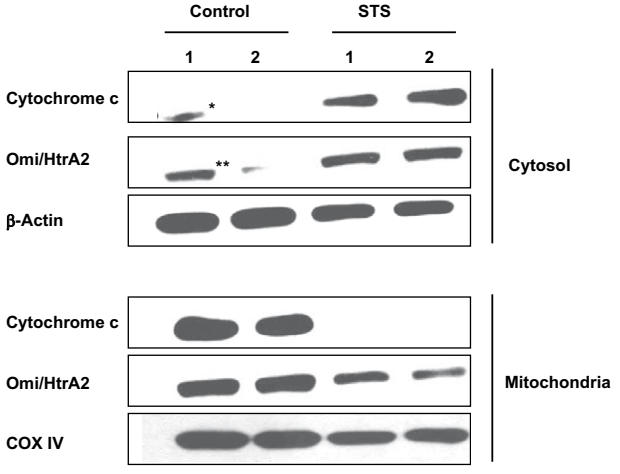

Fig. 1. Analysis of the release of cytochrome *c* and Omi/HtrA2 during apoptosis by subcellular fractionation. HEK293T cells were treated for 4 h with 1 μM staurosporine (STS). The cytosolic and mitochondrial fractions were prepared and analyzed by immunoblotting using anti-cytochrome *c* (1:1000) or anti-Omi/HtrA2 (1:1000) antibodies. To ensure proper loading, blots were reprobed with anti-β-actin antibodies for the cytosolic fraction and the mitochondrial protein Cox IV for the mitochondrial fraction. Results are presented in duplicates (labeled 1 and 2). *Contaminant band seen in cytochrome *c* blot. **Likely contaminant band seen in the Omi/HtrA2 blot.