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A sandwich ELISA for the conformation-specific quantification of the activated form of human Bax

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ABSTRACT

Bcl-2 family proteins are critical regulators of mitochondrial outer membrane permeabilization (MOMP), which represents the point of no return of apoptotic cell death. The exposure of the Bax N-terminus at the mitochondria reflects Bax activation; and this activated configuration of the Bax protein is associated with MOMP. N-terminal exposure can be detected using specific monoclonal and/or polyclonal antibodies, and the onset of activated Bax has extensively been used as an early marker of apoptosis. The protocols of immunoprecipitation and/or immunocytochemistry commonly used to detect activated Bax are long and tedious, and allow semiquantification of the antigen at best. The sandwich ELISA protocol we developed has a 5 ng/mL detection limit and is highly specific for the activated conformation of Bax. This ELISA allows a rapid quantification of activated human Bax in whole cells and isolated mitochondria protein extracts. These properties grant this assay the potential to further clarify the prognostic and diagnostic value of activated Bax in disorders associated with deregulated apoptotic pathways such as degenerative diseases or cancer.

Introduction

Apoptosis is a programmed cell death mechanism that is finely regulated by protein–protein interaction at the mitochondria between pro- and anti-apoptotic members of the Bcl-2 family proteins [1–5]. Defects in this regulation are associated with several diseases, such as neurodegenerative disorders and cancer [6–10].

Cytochrome c release is the point of no return of apoptosis, which promotes and amplifies the cell death cascade [1,2,5,11]. The permeabilization of the mitochondrial outer membrane involves the translocation of Bax to the mitochondria, where it undergoes a change of conformation (Bax activation) that leads to the exposure of the Bax N-terminal region [1,2,4,5,8,12]. Activated Bax forms oligomers that are the principal components of the mitochondrial apoptosis-induced channel (MAC), through which cytochrome c is released to the cytosol [5,8,9,11,13–16].

Activated Bax is extensively used as an early marker of apoptosis [1–5,8,11,12]. Quantification of the accumulation of activated Bax in mitochondria is a valuable tool to clinically diagnose pathologies associated with cell degeneration or tumorigenesis [7,10,22,23]. Antibodies that recognize the Bax N-terminus allow the discrimination of activated/oligomerized from native cytosolic Bax [17,18]. Current methods used to detect activated Bax are based on immunoprecipitation followed by immunodetection by Western blot. These time-consuming techniques provide only qualitative detection or, at best, semiquantification of activated Bax, which in many cases is not reproducible enough to report small but significant differences between control and apoptotic conditions. This article describes a rapid method based on a sandwich ELISA that allows an accurate and reproducible quantification of recombinant

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human activated/oligomerized Bax in vitro. In addition, this ELISA also permits reproducible quantification of activated Bax in mitochondrial or whole cell protein extracts from normal and apoptotic HeLa cells. This sandwich ELISA allows rapid and convenient quantification of recombinant and native activated Bax, a well-recognized early apoptotic marker with potential prognostic value in degenerative disorders and/or cancer therapeutics.

Materials and methods

Cell culture and apoptosis induction

HeLa cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 50 mg/ml streptomycin, and 50 IU/ml penicillin at 37 °C in 5% CO_2. Cells were split 1/2 (v/v) in 175 cm^2 flasks 24 h before the treatment, and then untreated or treated with 0.5 μM staurosporine for 16 h to induce apoptosis [19].

Recombinant proteins

Recombinant monomeric/nonactivated (monomeric) and oligomeric/activated (activated) human Bax of full length, with six histidines at the N-terminus, were expressed in *Escherichia coli* and purified as previously described [19,20]. Activated Bax was obtained by purifying the oligomers after disrupting bacteria in a buffer containing 1% Triton X-100 (v/v), a detergent that artificially induces Bax activation/oligomerization [21]. Once the oligomers were obtained, Triton X-100 was exchanged for 1% octyl glucoside by dialysis. The purified protein was stored in 25 mM Hepes-NaOH, 0.2 mM DTT, 1% octyl glucoside, 30% glycerol, pH 7.5, at −80 °C [19].

Total protein extraction from whole cells and mitochondria

Total proteins were extracted from control (untreated) or staurosporine-treated (apoptotic) HeLa cells (4 × 175 cm^2 flasks each). At the end of the treatment, cells were harvested in phosphate-buffered saline (PBS) containing 1 mM EDTA and recovered by centrifugation at 750*g* for 10 min. Pellets containing cells were washed with 1X PBS and suspended in lysis buffer consisting of a 1x PBS supplemented with complete protease inhibitor mixture (Roche Molecular Biochemicals) and 2% CHAPS (w/v) (Sigma-Aldrich), in order to prevent artificial Bax oligomerization [21]. Cells were incubated on ice for 60 min, sonicated for 4 × 30 s, incubated on ice for 90 min, sonicated again, and centrifuged at 100,000*g* for 30 min. Supernatants contained the extracted proteins.

Mitochondria were isolated from untreated or apoptotic HeLa cells (10 × 175 cm^2 flasks each), as previously described [19]. Pellets containing mitochondria were lyzed in mitochondrial buffer (230 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM Hepes-NaOH, pH 7.5) supplemented with complete protease inhibitor mixture (Roche Molecular Biochemicals) and 2% CHAPS (v/v). Mitochondria were then incubated on ice for 1 h, sonicated for 4 × 30 s, and finally centrifuged for 30 min at 100,000*g*. Supernatants contained the mitochondrial extracted proteins.

The total protein content in both whole cells and mitochondria extracts was measured using a BCA assay (Pierce).

Estimation of total Bax content by Western blot and densitometry

Total protein extracts (10 μg) from untreated and apoptotic cells and mitochondria were run in a SDS-PAGE gel, along with different concentrations of oligomeric recombinant Bax. Proteins were then transferred onto a PVDF membrane (BioRad). Membrane was blocked in 1x PBS, 0.2% (v/v) Tween-20, 5% (w/v) milk for 30 min, followed by incubation with 0.2 ng/μL polyclonal HRP-linked anti-Bax antibody (N20-HRP, Santa Cruz Biotechnology, Inc.) for 1 h at room temperature or overnight at 4 °C. Bax signal was detecting using HRP-dependent luminescence (ECL, Pierce), which was collected on an X-ray film after different exposure times (GE Healthcare).

Nonsaturated Western blot signals were scanned, and the pixel density of the Bax bands was quantified using the software Image J (NIH Open Source). Pixel quantification of the bands corresponding to increasing amounts of recombinant oligomeric Bax were used to build a standard curve, which allowed estimation of the Bax content in the different protein extracts tested [22,23].

Immunoprecipitation of activated/oligomeric BAX

Mitochondrial protein lysates (35–55 μg), HeLa total cell protein lysates (60–140 μg), or recombinant human Bax monomers and oligomers (10 ng total Bax) were resuspended in mitochondrial buffer or in 1x PBS supplemented with complete protease inhibitor mixture and 2% CHAPS (w/v) (Sigma). Samples were incubated overnight at 4 °C with 1 μg of monoclonal (6A7, Sigma-Aldrich) or polyclonal anti-Bax antibody (N20, Santa Cruz Biotechnology, Inc.), or alternatively with control mouse or rabbit total IgGs (Santa Cruz Biotechnology, Inc.). The different samples were then incubated with 1/1 (v/v) protein G-coupled magnetic beads (Invitrogen) at room temperature for 45 min, and the immunocomplexes were precipitated by placing the assay tubes in a holder magnet for 10–30 s. Proteins contained in the pellets were separated from the magnetic beads by washing them twice with 1x PBS, resuspension in sample buffer, incubation at 60 °C for 5 min, and magnetic precipitation of the denatured protein G magnetic beads. Supernatants were directly supplemented with sample buffer (BioRad) and heated at 60 °C for 5 min. Bax content in supernatants and pellets was estimated by Western blot and densitometry using Image J as described in Refs. [22,23].

Sandwich ELISA for the quantification of activated Bax

Activated Bax ELISA was performed in a 96-well plate (Costar). Each well used for the assay was coated overnight with 100 μL of capture antibody solution containing 0.25 μg/mL of the monoclonal anti-Bax 6A7 antibody (Sigma-Aldrich) in DPBS (Fisher) at 4 °C. All the following incubations were performed at room temperature and with continuous plate shaking. Coated wells were washed four times with 100 μl DPBS, 0.05% Tween-20 (Sigma-Aldrich), and incubated for 1 h with 200 μL blocking buffer containing 1% BSA (w/v) (Sigma-Aldrich) in DPBS, followed by four washes with regular DPBS. Both standards and samples were diluted in DPBS 2% (w/v) CHAPS and analyzed in duplicate or triplicate. Activated Bax standards contained 0, 0.3, 0.6, 1.2, 2.5, 5.0, 10, 20, 40, or 80 ng/mL of recombinant activated Bax. Total protein extracts from isolated mitochondria of control and apoptotic HeLa cells were resuspended in DPBS 2% (w/v) CHAPS at a final concentration of 0.2–0.45 mg/mL, whereas proteins extracted from whole HeLa cells were resuspended at a final concentration of 3.5–10 mg/mL in the same buffer. Activated Bax standards and samples were incubated for 2 h at room temperature in the wells precoated with the 6A7 antibody. The plate was then washed four times with DPBS. Each sample was incubated for 1 h with 100 μL of detection antibody solution containing 0.1 μg/mL of the polyclonal anti-Bax N20 antibody (Santa Cruz Biotechnology, Inc.) in blocking buffer. After incubation, the plate was washed four times with DPBS, and then wells were incubated for 1 h with 100 μl of a solution containing 0.12 μg/mL goat anti-rabbit biotinylated third antibody (Abcam) in blocking buffer. Each well was washed four times with DPBS and incubated