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Research Article

Relative timing of redistribution of cytochrome *c* and Smac/DIABLO from mitochondria during apoptosis assessed by double immunocytochemistry on mammalian cells

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ABSTRACT

Redistribution of cytochrome *c* and Smac/DIABLO from mitochondria occurs during apoptosis, although the relative timing of their release is not well characterized. Double immunocytochemistry was utilized here to study quantitatively the patterns of release of cytochrome *c* and Smac/DIABLO from mitochondria in single cells. Human osteosarcoma cells and murine embryonic cortical neurons were analyzed during apoptosis induced by staurosporine. In osteosarcoma cells treated with staurosporine for 24 h, a substantial proportion of cells (36%) released cytochrome *c* from the mitochondria before Smac/DIABLO. In contrast, these proteins were released mostly concordantly in neurons; only a minority of cells (≤15%) released cytochrome *c* without Smac/DIABLO (or vice versa) from mitochondria. Patterns of release in either cell type were unaltered by addition of the caspase inhibitor, zVAD-fmk. The double immunocytochemistry procedure facilitated clear definition of the temporal release of cytochrome *c* and Smac/DIABLO from mitochondria in intact apoptotic cells, enabling us to demonstrate for the first time that their mutual redistribution during apoptosis varies between different cell types.

Introduction

The intermembrane space (IMS) of mitochondria accommodates proteins that can activate the cell death pathway leading to apoptosis. Some of these proteins appear to be bifunctional, such that under physiological conditions, they participate in mitochondrial metabolism and respiration to generate ATP or to protect against oxidative stress, but once released into the cytoplasm of the cell following permeabilization of the outer mitochondrial membrane (OMM), they potentiate the apoptotic death cascade. These events culminate in the activation of downstream caspases (including caspase-3) that are responsible for the characteristic morphological and biochemical features of apoptosis [1]. Two proteins

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released from the mitochondria that activate caspases include cytochrome c (cyt c) and Smac/DIABLO (hereinafter called Smac). Once in the cytoplasm, cyt c (12 kDa) forms the apoptosome complex with Apaf-1 and caspase-9 in the presence of ATP/dATP, while Smac (monomeric size of 23 kDa) antagonizes the endogenous caspase inhibitors, IAPs (inhibitor of apoptosis proteins).

The nature of OMM permeabilization to liberate cyt c and Smac (and other proteins) from the IMS following apoptotic induction, and the factors that control this process remain to be discerned. One hypothesis that has gained much recognition over recent years is that mitochondrial protein redistribution occurs through specific OMM pores/channels modulated by the Bcl-2 protein family, the likely candidates being the pro-apoptotic member Bax or Bak. An alternative theory involves complete rupture of the OMM due to swelling of the mitochondrial matrix following activation of the Permeability Transition (PT) pore [2,3]. Current studies have addressed these issues by establishing the timing and order of release of IMS proteins from the mitochondria during apoptosis. In particular, co-localization studies have shown variable results on the temporal release of both proteins. In some studies, release of Smac was synchronized with that of cyt c [4–8], while in others, it was shown to be delayed [9] or even precede [10,11] cyt c release. These discrepancies may depend on variability in the experimental cellular systems studied as well as the apoptotic stimulus applied.

A number of techniques have been used to study the timing of release of IMS proteins. One of these is western immunoblotting of cellular fractions to determine the overall distribution of proteins (i.e., mitochondrial vs. cytosolic) during apoptosis. This procedure, however, is limited to examining the release process on whole cell populations and does not permit analysis of protein localization at the level of individual cells, which is imperative for studying detailed timing of release. In situ detection of protein distribution by immunocytochemistry has provided the means to carry out such studies so that one can view the location of endogenous proteins in intact cells. Time-lapse confocal microscopy of cyt c-GFP and Smac-YFP has given further insights into the release kinetics of these proteins relative to each other. Results from such studies suggest that the onset of release of these proteins from the IMS occurs at relatively the same time following apoptotic induction [7,12,13]. However, the duration of Smac release has been reported to be the same [8], or significantly longer compared with that for cyt c [12,13].

We have developed a double immunocytochemical staining procedure to monitor the distribution of cyt c and Smac in situ in single cells. We show here that time-course analyses can be performed in order to determine the relative timing of release of both proteins following apoptotic induction in intact cells. These studies were performed on two different cell types including an immortalized culture of human osteosarcoma cells and a primary culture of murine embryonic cortical neurons. The data obtained lead to clear discrimination of the release of cyt c and Smac from mitochondria within single osteosarcoma cells treated with staurosporine (STS). However, in primary neurons likewise treated, cyt c and Smac were found to be released more or less concordantly.

Materials and methods

Reagents

Staurosporine and zVAD-fmk were purchased from Sigma-Aldrich (St. Louis, MO, USA), and Bachem (Basel, Switzerland), respectively. Mouse monoclonal anti-cyt c antibody (clone 6H2.B4) and rabbit anti-active caspase-3 antibody were from BD Pharmingen (San Diego, CA, USA). Rat monoclonal anti-Smac antibody (clone 10G7) was from Alexis Biochemicals (Carlsbad, CA, USA). Secondary antibodies labeled with Alexa 488, 568 or 647, and propidium iodide (PI), were from Molecular Probes (Eugene, OR, USA).

Cell culture

Human osteosarcoma 143B TK^- cells (hereinafter called 143B) were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM HEPES buffer, and 10% (v/v) foetal bovine serum. Cells were maintained in a humidified 37 °C incubator with 5% CO_2. Primary cultures of cerebrocortical neurons (hereinafter called neurons) were obtained from embryonic day 15 Swiss-white mice using previously described procedures [14]. Institutional ethical approval was obtained for these studies. Cells were initially cultured in Neurobasal medium with B-27, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mM L-glutamine, and 10% (v/v) foetal bovine serum. After 24 h in vitro, cells were maintained in serum-free medium, otherwise as above. The cultures were maintained in a humidified 37 °C CO_2/N_2 incubator (5% CO_2, 8.5% O_2). As in previous studies [14], under these conditions >95% of cells were neurons, revealed by immunostaining experiments with antibodies specific for GFAP. For immunostaining experiments, cells were seeded in 24-well plates containing 13 mm round coverslips pre-coated with poly-D-lysine (5 μg/ml) at a density of 0.5×10^6 cells/well.

Apoptotic induction

143B cells were sub-cultured the day prior to STS treatment and seeded into 6-well plates at a density of 1×10^5 cells/well. Cells were then left overnight in the incubator to establish adherence, during which time cells commenced proliferation. Cells were treated with 100 nM STS for various times (0–48 h). Neurons were cultured for 6 days in vitro prior to treatment but did not proliferate. On the day of treatment, the medium was replaced by that lacking B-27 supplements (otherwise as above for neuronal cultures), and cells were exposed to 200 nM STS for various times (0–24 h). For the zVAD-fmk pre-treated cells, 143B cells and neurons were initially incubated with 100 μM zVAD-fmk and 50 μM zVAD-fmk, respectively for 30 min. The cells were then treated with STS while maintaining the zVAD-fmk in the cultures.

Apoptotic detection

Following treatment of 143B cells with STS at appropriate times, adherent cells (i.e., viable) were trypsinized and combined with non-adherent cells (i.e., non-viable) to collect