Detection of pro-caspase-3 in cytosol and mitochondria of various tissues

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Abstract Caspases are a family of cysteine proteases of critical importance in the apoptotic cell death process. They are normally present as zymogens (pro-caspases) in the cytoplasm of vertebrate and other organisms. In this study we have shown that pro-caspase-3 is localized to cytosol and mitochondria of various rat tissues (brain, heart, kidney, liver, spleen and thymus). Although the majority of pro-caspase-3 was localized in the cytosol, the amount of mitochondrial pro-caspase-3 was significant. The ratio of cytosolic and mitochondrial pools of pro-caspase-3 appeared to vary between different tissues. The higher amount of mitochondrial pro-caspase-3 was found in thymus and spleen, i.e. tissues in which spontaneous apoptosis plays an important role. Our findings provide further support for mitochondrial localization of pro-caspase-3 and the critical role of this organelle in apoptosis.

Key words: Apoptosis; Caspase; Mitochondria; Rat; Tissue

1. Introduction

Apoptotic cell death is a general phenomenon occurring in all multicellular organisms. It is recognized that the biochemical machinery of cell death is constitutively expressed [1]. It also appears that distinct pathways leading to apoptosis may converge at the same 'cytoplasmic regulator' of this multistep process. The caspase family of aspartic acid-specific cysteine proteases, which are activated during apoptosis in numerous cell systems, seem to be the most likely candidate for this function. These proteases cleave several proteins localized to cytosol, cytoskeleton, intracellular membranes and nuclei, although the significance of any one particular of these cleavages in the cell death process is still unclear (for review see [2]).

It has been demonstrated that in a cell-free system based on cytosol from normally growing cells, three apoptotic protease activating factors (Apaf-1–3) were required to activate pro-caspase-3 [3–5]. The binding of Apaf-3 (pro-caspase-9) to Apaf-1, which shares similarity with the pro-domain of CED-3 and CED-4, is dependent on the presence of Apaf-2 (cytochrome *c*). Activation of pro-caspase-3 is achieved when caspase-9 cleaves pro-caspase-3. It is likely that activation of pro-caspase-3 is a central event in the caspase cascade during apoptosis, and can be recognized as a 'point of no return' in the multistage apoptotic process.

The fact that release of cytochrome *c*, and possibly other factors, from mitochondria leads to activation of pro-caspase-3 in cytosol, and that Bcl-2 can block this release, has brought mitochondria back into the focus of apoptosis research [6–8]. It was recently demonstrated that pro-caspase-3 is not only present in the cytosol of some cell types, but also in the intermembrane space of the mitochondria of the same cells [9]. However, there have been no reports concerning the tissue specificity of this localization. In the present study we have addressed the question of the intracellular localization of pro-caspase-3 in various tissues. We found the majority of pro-caspase-3 to be localized in the cytosol, but a sizeable fraction had a mitochondrial location in thymus, brain, liver, heart, spleen and kidney of rats. This observation suggests that mitochondrial localization of pro-caspase-3 may be a general phenomenon. The possible physiological roles of this observation are discussed below.

2. Materials and methods

2.1. Antibodies

Anti-p17 antibodies were provided by Dr. Donald W. Nicholson (Merck Frosst Center for Therapeutic Research, Pointe-Claire, Dorval, Quebec, Canada), anti-HSC-70 antibodies were from Stressgen Biotechnologies Corp. (Victoria, Canada) and anti-cytochrome *c* antibodies were kindly provided by Dr. Ronald Jemmerson (University of Minnesota Medical School, Minneapolis, MN, USA). Goat anti-mouse, anti-rat and anti-rabbit IgG-HRP were from Pierce (Rockford, IL, USA). ECL was from Amersham Corp. (Buckinghamshire, UK).

2.2. Isolation of mitochondrial and cytosolic fractions from different tissues

The tissues (brain, heart, kidney, liver, spleen and thymus) were removed from male, 3-week-old Sprague-Dawley rats (50–70 g), which had been killed by cervical dislocation. The cytosolic and mitochondrial fractions were isolated from all tissues according to the protocols previously described [10]. Briefly, all tissues were homogenized in buffer containing 0.25 M sucrose and 1 mM EDTA (pH 7.4). The homogenate was centrifuged at $900\times g$ for 10 min. The supernatant was centrifuged at $10\,000\times g$ for 15 min to pellet the mitochondria. The supernatant was further centrifuged at $100\,000\times g$ to remove any other particulate material (microsomal fraction). The resulting supernatant was designated as the cytosol.

2.3. Percoll-sucrose density gradient centrifugation

Percoll-sucrose density gradient was prepared as previously described [10]. Briefly, 100 μl of thymus mitochondria were layered on the top of the gradient, containing 25% Percoll and 75% sucrose buffer (0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, 2 mM MOPS, pH 7.2, and 10,000 U/ml of heparin), and centrifuged in a SW41 rotor at 24 000 rpm ($62\,000\times g$) for 35 min, with slow acceleration and without break. Different fractions were removed and proteins were prepared for Western blotting. These fractions were further tested for cross-contamination with lysosomes or Golgi vesicles, using specific enzyme markers, i.e. acid phosphatase and UDP-galactosyltransferase, respectively. For acid phosphatase the assay mixture contained in 0.5 ml: 80 mM sodium acetate, pH 5.0, 2% Triton X-100, 0.8% Na/K-tartrate, 40 mM β-glycerophosphate. The reaction was started by the addition of the substrate and incubated for 20 min at 37°C. Reaction was terminated by adding perchloric acid, and after centrifu-

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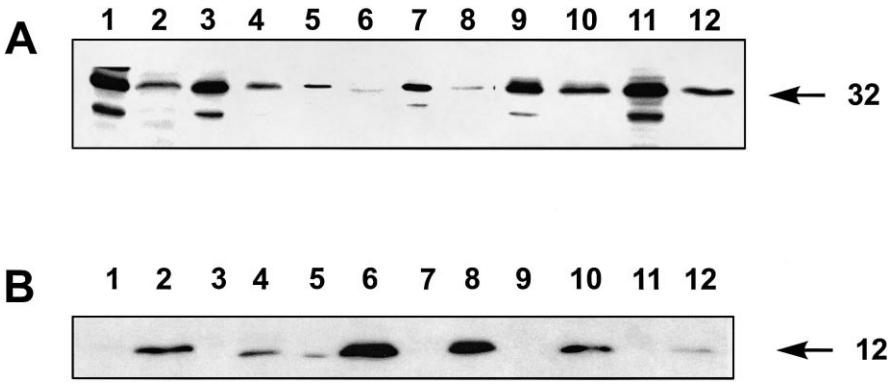

Fig. 1. Subcellular localization of caspase-3 and cytochrome *c* in different tissues. Proteins from cytosolic (1, 3, 5, 7, 9, 11) and mitochondrial (2, 4, 6, 8, 10, 12) fractions isolated from rat liver (1, 2), thymus (3, 4), heart (5, 6), kidney (7, 8), brain (9, 10) and spleen (11, 12) homogenates were prepared for Western blotting. The membranes were probed with (A) anti-p17 and (B) anti-cytochrome *c* antibodies.

gation the supernatant was incubated with Fiske-Subbarow reagent and analyzed at 830 nm. For UDP-galactosyltransferase the incubation mixture contained in 200 μl: 25 μM ATP, 12.5 μM $MnCl_2$, 37 mM 2-mercaptoethanol, 2.5% ovalbumin, 0.5% Triton X-100, 120 mM Na-cacodilate buffer, pH 6.2. Reaction was started by addition of the substrate and UDP[^{14}C]-galactose. Reaction was terminated by adding TCA and the pellet was resuspended in 1% SDS and radioactivity was counted in Bray scintillator.

2.4. Western blot analysis

Laemmli loading buffer was added to the samples and after boiling for 4 min, the polypeptides were resolved on a 12% SDS gel at 130 V. The gels were transblotted onto nitrocellulose membranes (0.2 μm) for 2 h at 100 V. The membranes were blocked overnight in a buffer (50 mM Tris, pH 7.5, 500 mM NaCl) containing 1% bovine serum albumin and 5% non-fat milk powder. They were then probed with primary antibodies in blocking solution without milk, followed by 1 h with secondary antibody in an identical solution, and then visualized by ECL according to the manufacturer's instructions.

3. Results

3.1. Mitochondrial localization of pro-caspase-3 in different tissues

To determine tissue specificity of mitochondrial localization of pro-caspase-3 we tested the cytosol and mitochondria from rat brain, heart, kidney, liver, spleen and thymus. The results show that both the soluble fraction (cytosol) and the pellet (mitochondrial) from all tissues contained pro-caspase-3 (Fig. 1A). Using anti-cytochrome *c* antibody, we were able to demonstrate that the pellet was indeed the mitochondrial fraction (Fig. 1B). These data suggests that mitochondrial localization of pro-caspase-3 is a general phenomena found in all tissues. The ratio of cytosolic to mitochondrial pro-caspase-3 appeared to vary between different tissues. For example, mitochondrial pro-caspase-3 content of thymus and spleen was higher than that of heart and kidney, which contained much lower quantities of this protease. However, the cytochrome *c* content of heart and kidney mitochondria was relatively higher than that of other tissues. In other words, it appears that the amount of cytochrome *c* in mitochondria from different tissues is conversely proportional to the level of pro-caspase-3 (Fig. 1).

3.2. Percoll-sucrose density gradient separation of mitochondria

Peroxisomes, processing vesicles of the Golgi system, and lysosomes can be separated from mitochondria by density gradient centrifugation [11]. To determine whether pro-caspase-3 co-sedimented with the mitochondria, or was present in a lower-density fraction, the 10 000 × *g* pellet, containing mitochondria, was subjected to Percoll-sucrose density gradient centrifugation. Ten different fractions were removed and prepared for Western blotting. Fig. 2A shows that the bottom two fractions (#9 and 10), were stained positive with anti-cytochrome *c* antibodies, indicating the expected presence of mitochondria. Reprobing of membrane with anti-p17 antibodies showed that only these two fractions stained positive (Fig. 2B). The bottom two fractions of Percoll gradient as well as crude 10 000 × *g* pellet were analyzed for the presence of lower-density fractions such as lysosomes or Golgi vesicles. The activity of marker enzymes for lysosomes and Golgi vesicles, acid phosphatase and UDP-galactosyltransferase, respectively, were not detectable in any of these fractions (data not shown). Thus, these results suggest that mitochondria, but not lower-density vesicles derived from other organelles, contained pro-caspase-3.

3.3. Intramitochondrial localization of pro-caspase-3

To determine whether pro-caspase-3 was localized inside mitochondria, or merely associated with the external surface, isolated mitochondria were incubated with proteinase K in presence or absence of NP-40. After incubations, protein sam-

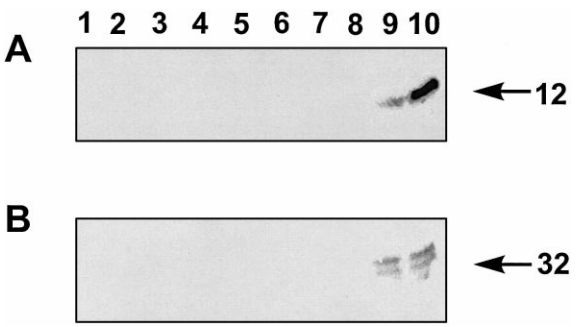

Fig. 2. Analysis of pro-caspase-3 presence in mitochondria-enriched fractions after Percoll-sucrose gradient centrifugation. The 10 000 × *g* pellet, extracted from thymus cells, was loaded on the top of a Percoll-sucrose gradient. After centrifugation, ten fractions (starting from the top of the gradient), were removed and prepared for Western blotting. Membranes were probed with (A) anti-cytochrome *c* and (B) anti-p17 antibodies.

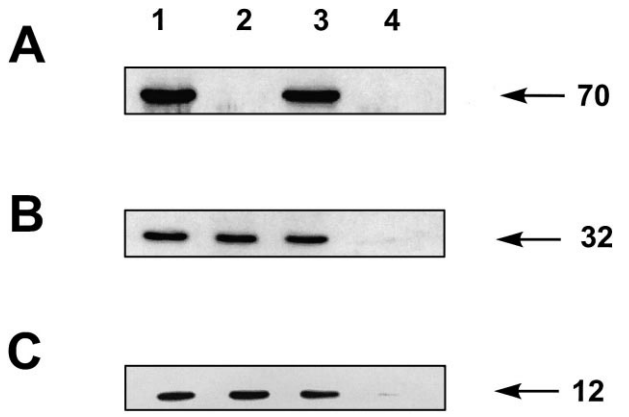

Fig. 3. Analysis of pro-caspase-3 localization inside mitochondria. Mitochondria were incubated alone (lane 1), in presence of proteinase K (lane 2), NP-40 (lane 3) and both (lane 4) for 3 min at room temperature. Samples were prepared for Western blotting and membranes were probed with antibodies against HSC-70 (A), p17 (B) and cytochrome *c* (C).

ples were collected and resolved on SDS gels. Following Western blotting, membranes were stained with antibodies against HSC-70, p17 and cytochrome *c*. It is known that HSC-70 (cytosolic HSP-70) associates with outer membrane of mitochondria [12], whereas cytochrome *c* localizes in the mitochondrial intermembrane space. As shown in Fig. 3A, treatment with proteinase K alone was sufficient to degrade HSC-70, demonstrating its association with outer mitochondrial membrane. Similar results were obtained after reprobing the nitrocellulose membranes with antibodies against Bcl-2 (data not shown). However, pro-caspase-3 and cytochrome *c* were sensitive to proteinase K digestion only after solubilization of outer mitochondrial membrane with NP-40 (Fig. 3B and C). Thus, these results provide further support for the intramitochondrial localization of pro-caspase-3.

4. Discussion

The aim of this study was to determine if localization of pro-caspase-3 to mitochondria is a tissue-specific trait or a general trend that occurs in all tissues. Using biochemical approaches we have demonstrated that pro-caspase-3 is localized not only to cytosol but also to mitochondria from rat brain, heart, kidney, liver, spleen and thymus. A similar pattern of pro-caspase-3 distribution was recently observed using the confocal microscopy techniques in a number of cell lines [9].

It appears that some tissues contain more mitochondrial pro-caspase-3 which is conversely proportional to the levels of cytochrome *c* in these mitochondria. A high level of mitochondrial pro-caspase-3 in lymphoid tissues may be related to the high susceptibility of cells in these tissues to undergo spontaneous as well as inducible apoptosis. Despite the potential importance of these findings, there are a number of questions which need to be addressed regarding the biological significance of this observation. For example, it is still unclear if mitochondrial pro-caspase-3 is activated during apoptosis and, if so, whether it acts directly on mitochondria, or is exported from the mitochondrion to exert its proteolytic activity on extra-mitochondrial target proteins? In this regard, it is of interest to note that there are, as yet, no known mitochondrial proteins reported to be cleaved by caspases. If active caspase-3 is exported from mitochondria, it should be possible to detect the release of an approximately 60 kDa protein from mitochondria of apoptotic cells. It has recently been reported that mitochondria contain a soluble protein, apoptosis inducing factor (AIF) of approximately 50 kDa in size, that is released after induction of permeability transition (PT) and is sufficient to cause nuclear apoptosis [13].

In summary, we have clearly demonstrated that the mitochondrial localization of this pro-caspase is a general phenomenon and may play an important, but yet unknown, role in apoptotic pathway. Finding a functional role for this caspase is the aim of the ongoing research in our laboratory.

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