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Minireview

Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis

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Abstract Apoptosis-inducing factor (AIF) is encoded by one single gene located on the X chromosome. AIF is ubiquitously expressed, both in normal tissues and in a variety of cancer cell lines. The AIF precursor is synthesized in the cytosol and is imported into mitochondria. The mature AIF protein, a flavoprotein (prosthetic group: flavine adenine dinucleotide) with significant homology to plant ascorbate reductases and bacterial NADH oxidases, is normally confined to the mitochondrial intermembrane space. In a variety of different apoptosis-inducing conditions, AIF translocates through the outer mitochondrial membrane to the cytosol and to the nucleus. Ectopic (extra-mitochondrial) AIF induces nuclear chromatin condensation, as well as large scale (∼50 kb) DNA fragmentation. Thus, similar to cytochrome *c*, AIF is a phylogenetically old, bifunctional protein with an electron acceptor/donor (oxidoreductase) function and a second apoptogenic function. In contrast to cytochrome *c*, however, AIF acts in a caspase-independent fashion. The molecular mechanisms via which AIF induces apoptosis are discussed.

Key words: Apoptosis-inducing factor; Bcl-2; Cytochrome *c*; Mitochondrion; Programmed cell death

1. Introduction

Apoptosis is characterized by the permeabilization and/or rupture of the outer mitochondrial membrane [1–3]. As a result, proteins normally confined to the intermembrane space are translocated to non-mitochondrial structures [4–6]. Their translocation from an orthotopic (mitochondrial) to an ectopic (extra-mitochondrial) localization then triggers catabolic reactions which give rise to the apoptotic phenotype. As an example, cytochrome *c* redistributes from the mitochondrion to the cytosol [4] and triggers the formation of a caspase-9/caspase-3 activating complex, the apoptosome, which recruits several cytosolic factors such as Apaf-1 and ATP [7]. Several additional mitochondrial intermembrane proteins (heat shock protein 10, SMAC, and several pro-caspases, in particular pro-caspases-9) are released together with cytochrome *c* and stimulate the 'apoptosome' [7–10]. Once released from the apoptosome, caspase-3 triggers activation of yet another protein, caspase activated DNAse (CAD, also called DNA fragmentation factor, DFF), which mediates oligonucleosomal DNA fragmentation [11–14]. It would be an oversimplification, however, to assume that the pathway leading from mitochondrial membrane permeabilization to nuclear apoptosis relies exclusively on apoptosome formation and caspases. Thus, the mitochondrial intermembrane space contains a protein that provides a direct, caspase-independent molecular link between mitochondria and nuclei: apoptosis-inducing factor (AIF) [15,16]. This review will summarize our current knowledge on the molecular and cellular biology of AIF.

2. The AIF protein

Our group published in 1996 [17,18] that the mitochondrial intermembrane protein fraction contains an activity which suffices to force isolated HeLa nuclei to adopt an apoptotic morphology and to lose at least part of their DNA content. We coined this activity as 'apoptosis-inducing factor' (AIF). Based on a semi-automated cytofluorometric assay designed to measure the frequency of subdiploid nuclei exposed to mitochondrial proteins [19], we purified a protein which maintains its bioactivity in the presence of the pan-caspase inhibitor Z-VAD.fmk [8]. Cloning of the full-length cDNAs corresponding to mouse AIF (612 amino acids) and human AIF (613 aa) [15] revealed that AIF is strongly conserved between the two mammalian species (92% aa identity in the whole protein). The mouse AIF cDNA codes for a protein which is organized in three domains: (1) an amino-terminal mitochondrial localization sequence (MLS) of 100 amino acids; (2) a spacer sequence of 27 amino acids; and (3) a carboxyterminal 485 amino acid oxidoreductase domain with strong homology to oxidoreductases from other vertebrates (*X. laevis*), non-vertebrate animals (*C. elegans*, *D. melanogaster*), plants, fungi, eubacteria, and archaebacteria [20] (Fig. 1).

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Abbreviations: AIF, apoptosis-inducing factor; CAD, caspase-activated DNAse; $\Delta\Psi_m$, mitochondrial transmembrane potential; FAD, flavine adenine dinucleotide; GFP, green fluorescent protein; MLS, mitochondrial localization sequence; NAD, nicotine adenine dinucleotide; NLS, nuclear localization sequence; PT, permeability transition

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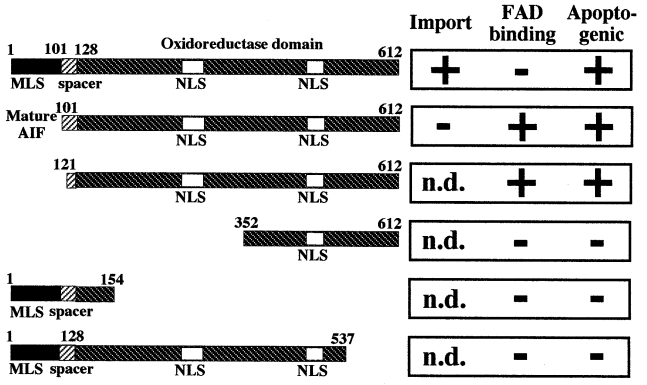

Fig. 1. Structure–function relationship of AIF. The scheme delineates the functional properties (mitochondrial import in vitro, FAD binding, apoptogenic effect on purified HeLa nuclei) of recombinant proteins corresponding to the AIF precursor (which has an N-terminal mitochondrial localization sequence, MLS), mature AIF (which lacks the MLS), as well as several truncation or deletion mutations. NLS denotes nuclear localization sequence. Numbers refer to the amino acids position of the mouse AIF sequence.

Among eukaryotic proteins with known enzymatic function, the strongest homology with AIF was found for plant semidehydroascorbate and ascorbate reductases, which are flavoproteins [20]. Functionally important amino acids involved in the interaction with the prosthetic groups (flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD)) are strongly conserved between AIF and bacterial NADH oxidoreductases [20]. Accordingly, natural AIF purified from mouse liver mitochondria was found to be an FAD-binding protein and recombinant AIF can be refolded in vitro, in the presence of FAD, resulting in stable FAD binding (unpublished results). Recombinant AIF precursor protein does not spontaneously incorporate FAD when purified from inclusion bodies of *Escherichia coli*. In contrast, recombinant AIF proteins lacking the MLS (AIF Δ1–100, mature AIF) or the MLS and part of the spacer region (AIF Δ1–120) spontaneously incorporate FAD when expressed in *E. coli*. Full-length AIF precursor protein (without FAD), AIF Δ1–100, and AIF Δ1–120 (both with FAD) all induced nuclear apoptosis when added to purified HeLa nuclei (Fig. 1). This indicates that the oxidoreductase activity (which depends on the presence of the prosthetic group) is not relevant to the apoptogenic effect of AIF. However, deletion mutations affecting parts of the oxidoreductase domain (Δ1–351, Δ155–612, Δ538–612) abolished the apoptogenic potential of AIF (Fig. 1), indicating that at least some of the structural features of the oxidoreductase domain are important for the apoptogenic effect of AIF [15].

3. Localization and expression pattern of the AIF gene

Fluorescent in situ hybridization (FISH) with an AIF cDNA revealed that only one single mouse chromosome hybridizes with the AIF cDNA (Fig. 2A,B). The AIF gene is localized within mouse X chromosome region A6 (Fig. 2C), which is syntenic to the human X chromosome region Xq25–26, where the human AIF gene is located (EMBL accession no. Z81364). FISH analyses confirmed that human cells only contain one AIF or AIF-related gene, on Xq (not shown). Based on Northern blot analysis using the full-length AIF

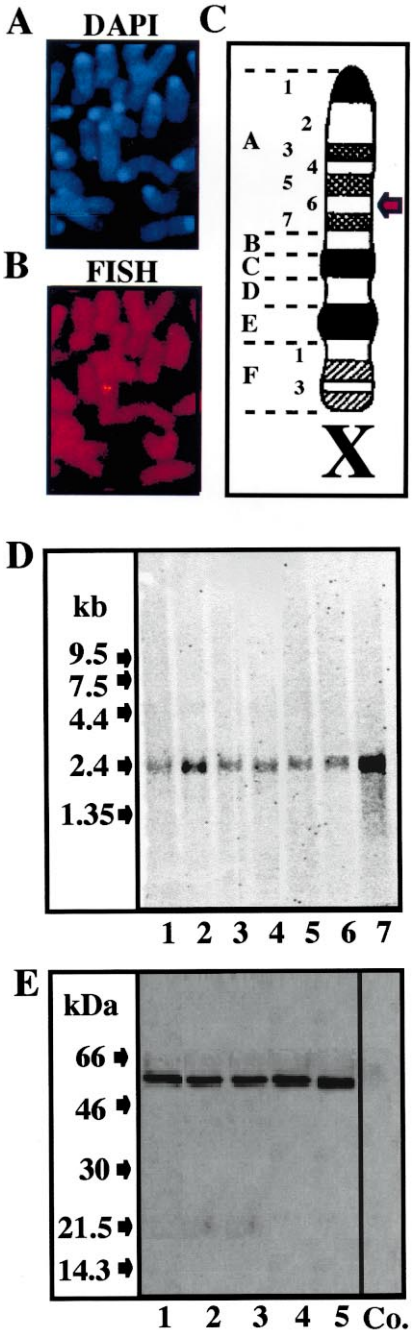

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Fig. 2. Expression pattern of AIF at the DNA, RNA, and protein levels. (A–C) chromosomal localization of the AIF gene. Fluorescent in situ hybridization (FISH) of AIF (red fluorescence, B) on a male mouse karyogram counterstained with DAPI (blue fluorescence, A). The detailed position of the AIF gene was mapped to chromosome X region A6 (10 determinations with identical results, C). (D) mRNA expression pattern of AIF. An AIF cDNA probe was hybridized to a Northern blot of polyadenylated RNA from human pancreas (1), kidney (2), skeletal muscle (3), liver (4), brain (6), placenta (5), and heart (7). (E) Protein expression pattern of AIF. An antiserum raised against aa 151–200 of mAIF was used to detect AIF protein contained in purified mitochondria from mouse liver (1), kidney (2), heart (3), brain (4), or spleen (5). Control blots performed in the presence of the immunogenic peptides covering aa 151–200 yield negative results for liver mitochondria (Co.).

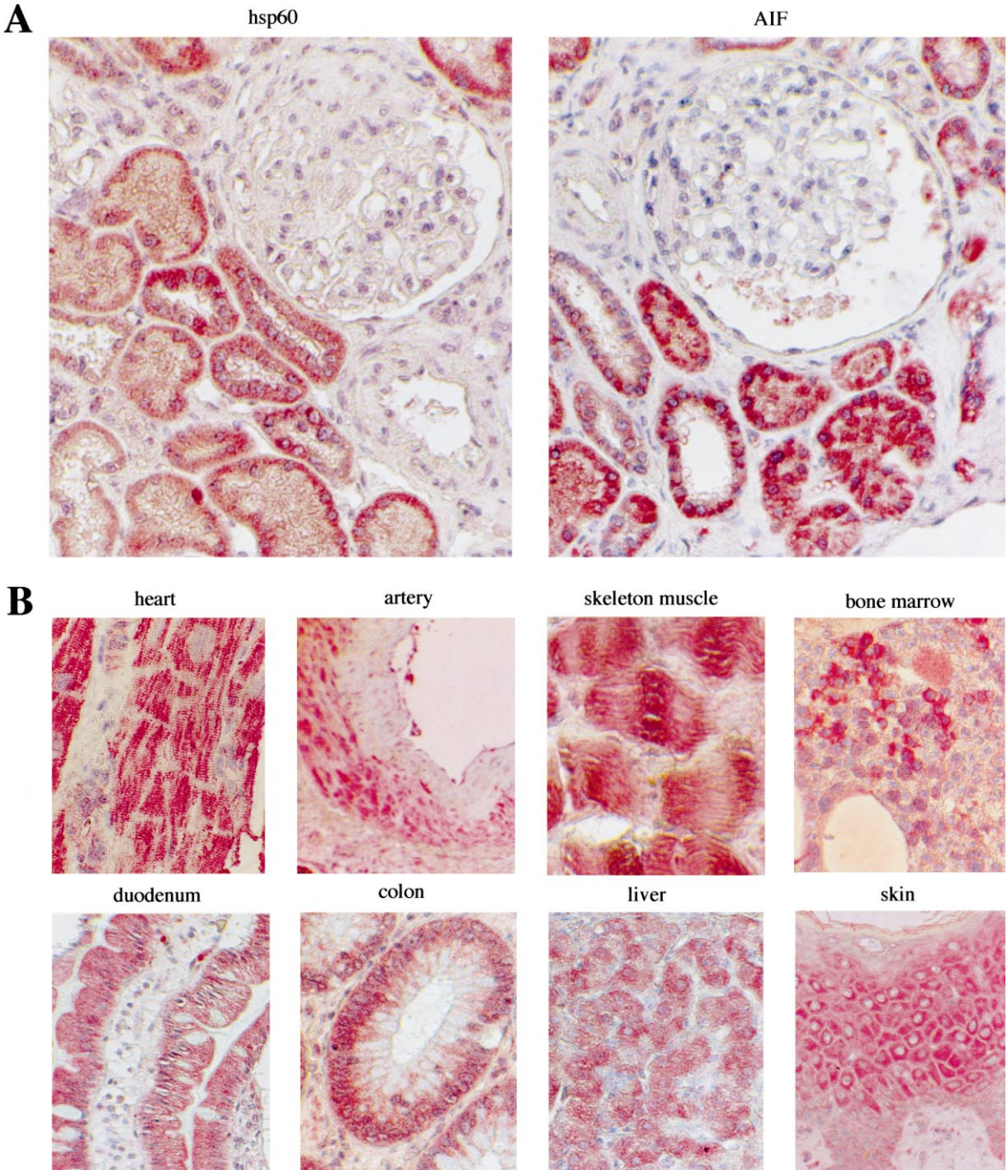
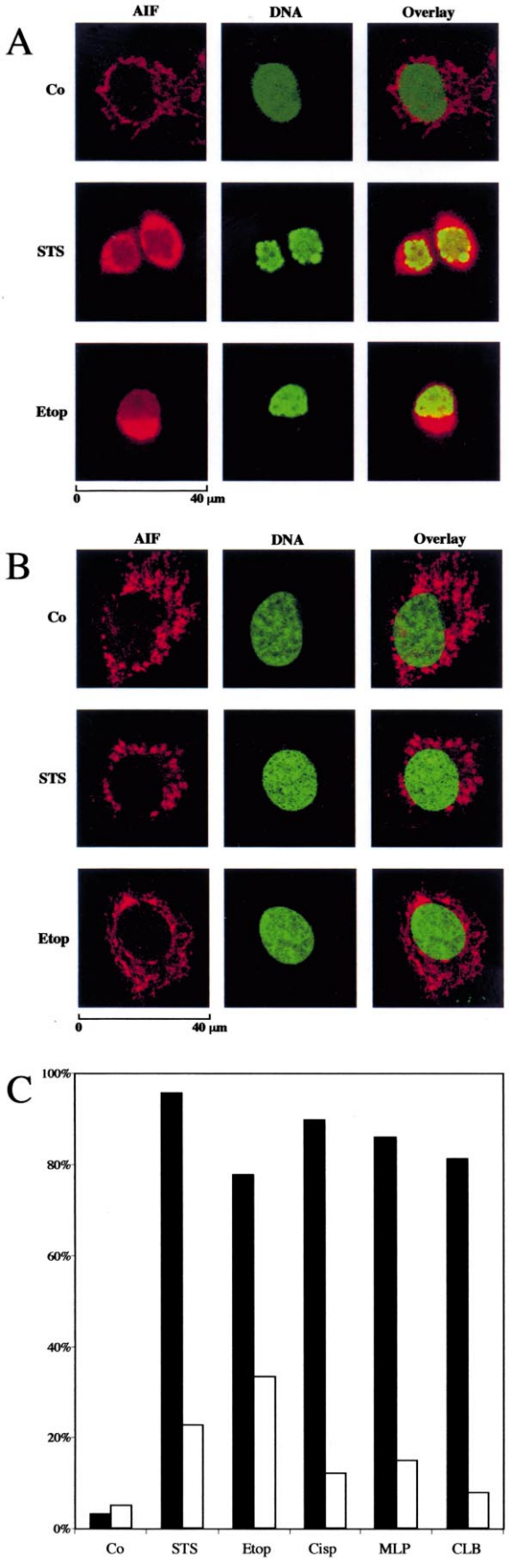


Fig. 3. Tissue distribution of AIF in human tissues. Immunohistochemichal stainings were performed on normal human tissue sections with the AIF (or hsp60) antibody revealed with Fast Red (Dako Envision method) and then counterstained with hematoxylin. AIF appears in red and nuclei in dark blue. (A) Distribution of a mitochondrial marker (hsp60, left panel) and AIF (right panel) localizations on normal kidney sections (×200). The most abundant AIF staining is in tubular epithelial cells predominantly in proximal tubules. At close inspection, all cell types contained an intracytoplasmic AIF staining. Note the strong correlation between AIF staining intensity and the expression level of the mitochondrial proteins hsp60. (B) From left to right and top to bottom. Cardiomyocyte location of AIF (×400), the renal arcuate artery showing a predominant AIF staining in the myocytes of the media (×200), AIF in skeleton muscle myocytes (×200), normal bone marrow showing a strong intracytoplasmic staining in red cell line (predominantly in erythroblasts) and weaker in granular cell line (×400), AIF staining of epithelial cells in duodenum and in Lieberkühn crypts of the colon (×200 for both), AIF location in hepatocytes (×400) and predominant AIF staining in the stratum Malpighii of the skin (×400).

cDNA as a probe, one single 2.4-kb AIF mRNA species was found to be expressed ubiquitously in human tissues (Fig. 2D). This finding was corroborated at the protein level for mouse tissues using an antibody raised against aa 151–200 of AIF, which recognizes a single ~57-kDa protein (Fig. 2E). Thus, despite the fact that several AIF cDNA variants are listed in the GenBank data base (e.g. AF100928, AL049704, and AL049703 for human AIF), suggesting alternative splicing of the primary AIF transcript, only one dominant AIF mRNA species and protein species can be discerned



by Northern and Western blot analyses. Comparative cytohistochemical staining with antibodies specific for AIF and for the mitochondrial matrix protein hsp60 reveal an overlapping expression pattern, as well as an identical subcellular distribution in histological sections of human tissues (exemplified for the renal cortex in Fig. 3A). At close inspection, all cell types were found to express AIF, and a strong correlation between AIF staining intensity and the expression level of mitochondrial proteins such as hsp60 (Fig. 3A) was observed. AIF protein was detected in numerous human tissues including muscle, bone marrow, intestine, liver, and skin (Fig. 3B). As to be expected, AIF was found to be localized in mitochondrion-rich areas (e.g. in the intersegmental areas of striated muscle or in the basal perinuclear area of colon epithelial cells) (Fig. 3B). Hence, AIF is a ubiquitous protein confined to mitochondria, at least in healthy normal tissues. AIF was also found to be expressed in numerous human cancer cell lines (>65 lines tested thus far). Thus far, we have not succeeded in identifying a single cell line or primary tumor in which the AIF protein would be completely absent. This may indicate that, in addition to its apoptogenic function, AIF exerts a yet-to-be-defined vital function, perhaps linked to its oxidoreductase function.

4. Subcellular localization of AIF in healthy versus apoptotic cells

In vitro assays performed with the primary transcription/translation product of full-length AIF cDNA revealed that AIF is imported into mitochondria and that this import depends on the presence of the N-terminal MLS [15]. This result has been recently confirmed in intact cells. Transient transfection of COS cells with green fluorescent protein (GFP) fused to the C-terminus of AIF to generate a chimeric AIF–GFP protein, targets GFP to mitochondria [21]. In contrast, a truncated AIF–GFP fusion protein in which the N-terminal MLS was removed (AIF–GFPΔ1–100), exhibited a diffuse cytoplasmic distribution of GFP, comparable to that obtained with GFP alone [21,22]. Similarly, immunofluorescence staining of untransfected cells with an AIF-specific antiserum (raised against amino acids 151–200) revealed that endogenous AIF is exclusively found in mitochondria [15,16]. Subcellular and submitochondrial fractionation corroborated that, in healthy cells, AIF is confined to the mitochondrial intermembrane space [15].

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Fig. 4. Redistribution of AIF during in vitro chemotherapy. SHEP neuroblastoma cells transfected with the Neomycin (Neo) resistance vectory only (A) or with a Bcl-2-expressing vector (B) were cultured in the absence (Co) or presence of staurosporin (STS, 1 μM, 4 h) or etoposide (Etop, 100 μM, 24 h). Cells were stained with an anti-AIF antibody (revealed by a phycoerythrine conjugate; red fluorescence) and Sytox green (DNA-intercalating dye; green fluorescence). Note the punctate, cytoplasmic AIF staining in untreated control Neo cells that contrasts with the diffuse nuclear and cytoplasmic staining in Neo cells undergong apoptosis (A). No AIF translocation was detectable in Bcl-2 overexpressing cells (B). Frequency of AIF translocation (C) induced by staurosporin, etoposide (same conditions as in A,B), cisplatin (Cisp; 50 μM, 24 h), melphalan (MLP; 100 μM, 20 h), or chlorambucil (CLB; 100 μM, 20 h) in Neo control cells (full columns) and bcl-2 transfected cells (open columns). The percentage of cells exhibiting AIF translocation is shown.

When cells are induced to undergo apoptosis, AIF is released from mitochondria to the cytosol and to the nucleus. This result has been obtained using all available methods for the determination of the subcellular localization of AIF: in situ immunostaining, transfection with AIF–GFP, and subcellular fractionation. As an example (Fig. 4A), AIF translocates from mitochondria (see punctate red immunofluorescence staining sparing the nucleus in control cells, Fig. 4A), when apoptosis is triggered by the protein kinase inhibitor staurosporin, yielding a diffuse cytoplasmic as well as nuclear staining ([15,16] and Fig. 4A). In addition, a wide array of chemotherapeutic agents induce the mitochondrial release of AIF in SHEP neuroblastoma cells. This applies to the DNA-damaging agent cisplatin, the topoisomerase II inhibitor etoposide, and alkylating agents such as melphalan and chlorambucil (Fig. 4A,C), as well as treatment with the stress-induced pro-apoptotic second messengers ceramide and ganglioside GD3 (not shown). The AIF translocation was inhibited by transfection-enforced overexpression of Bcl-2, correlating with the inhibition of chromatin condensation, one of the hallmarks of nuclear apoptosis (revealed by DNA staining with Sytox green; Fig. 4A–C). AIF translocation was also observed in a variety of cell lines induced to undergo apoptosis, including activated peripheral blood T-lymphocytes, CEM and Jurkat lymphoma cells, HeLa cervix carcinoma cells, COS renal cells, and fibroblasts [15,16,21–25]. In conclusion, it appears probable that AIF translocation invariantly accompanies apoptosis.

Kinetic studies performed in staurosporin-treated Rat-1 cells revealed that the release of AIF precedes that of full-blown nuclear apoptosis. In this model, the release of AIF occurs concomitantly with the dissipation of the mitochondrial inner transmembrane potential, and shortly before the release of cytochrome *c* is detectable by in situ immunofluorescence [15,16]. The early signs of nuclear nuclear apoptosis (stage I) consist in rippled nuclear contours and a rather partial chromatin condensation. It appears that AIF is fully translocated during this early stage, when the cytochrome *c* release is still incomplete. Only at a later stage of nuclear apoptosis (stage II), with a marked peripheral chromatin condensation and formation of nuclear bodies, both AIF and cytochrome *c* are released from mitochondria. Pharmacological caspase inhibition arrests nuclear apoptosis at the stage I of apoptosis, yet does not affect the release of AIF and cytochrome *c*. Based on these data, it can be speculated that the (caspase-independent) stage I of chromatin condensation involves the translocation of AIF from mitochondria to the nucleus, whereas the (caspase-dependent) stage II relies on caspase activation by cytochrome *c* [15,16].

5. Apoptogenic effects of ectopic AIF

If microinjected into the cytoplasm of normal cells, recombinant AIF suffices to cause three hallmarks of apoptosis, namely (1) the dissipation of the mitochondrial transmembrane potential and the release of cytochrome *c*, (2) the condensation of nuclear chromatin, (3) and the exposure of phosphatidylserine on the plasma membrane surface [15,21,25]. These alterations are rapid (30–120 min) and are not prevented by addition of the pan-caspase inhibitor Z-VAD.fmk [15,25]. Moreover, they are not affected by overexpression of the anti-apoptotic protein Bcl-2 [15]. Similar in vivo effects have been obtained by transfection-enforced overexpression of a truncated AIF–GFP construct lacking the N-terminal MLS (AIF–GFPΔ1–100) and that is misdirected to the extra-mitochondrial compartment [21]. These data confirm that ectopic (extra-mitochondrial) AIF is a caspase-independent effector of apoptosis that acts beyond the Bcl-2-controlled checkpoint of cell death. Is AIF required for apoptosis to occur? This question has been addressed by microinjecting a specific antiserum into the cytoplasma of Rat-1 cells treated with staurosporin [15]. This manipulation prevents the staurosporin-induced nuclear apoptosis. Control experiments involving a pre-immune antiserum or an anti-AIF antibody blocked by pre-incubation with the AIF-derived immunogenic peptides yielded no inhibition of staurosporin-stimulated apoptosis [15]. Thus, at least in some pathways of apoptosis induction, AIF is both sufficient and required to induce apoptosis (Table 1).

The apoptogenic effects of AIF, as revealed in intact cells, have been recapitulated in cell-free systems. As discussed above, addition of recombinant AIF to purified nuclei causes chromatin condensation [15]. This is accompanied by a large-scale DNA fragmentation, but not by oligonucleosomal DNA fragmentation. In intact cells, Z-VAD.fmk prevents oligonucleosomal DNA degradation, yet frequently has no effect on the large-scale fragmentation pattern [16]. These results are compatible with the hypothesis that AIF is (one of) the mediator(s) responsible for large-scale chromatin degradation. In the cell-free system, the nuclear effects of AIF are observed in the absence of cytosolic extracts, indicating that AIF acts on one or several yet unidentified sessile nuclear target molecules. In strict contrast, AIF has no direct effect if added to purified mitochondria in vitro. To reproduce the membrane-permeabilizing effects of AIF in such a system, a cytosolic heat-labile factor must be added together with AIF [15]. In the presence of cytosol, AIF causes mitochondria to release cytochrome *c* and to lose their transmembrane potential [15]. Thus, AIF has indirect effects on the integrity of mitochondrial membranes.

Table 1
Effects of AIF and AIF neutralization on apoptosis induction

Experimental system	Observations
AIF microinjection	Loss of mitochondrial transmembrane potential Release of cytochrome *c* from mitochondria Release of AIF–GFP fusion protein from mitochondria Nuclear chromatin condensation (stage I) Phosphatidylserine exposure Effects not inhibited by Z-VAD.fmk or Bcl-2
Overexpression of AIF lacking MLS by transfection	Nuclear chromatin condensation (stage I+II) Z-VAD.fmk arrests at stage I No inhibitory effect of Bcl-2
Injection of anti-AIF antibody into cells	Inhibition of nuclear apoptosis induced by staurosporin or actractyloside

6. Open questions and perspectives

The available data suggest that AIF has two opposite functions. On the one hand, AIF is likely to serve as a vital protein, presumably via its oxidoreductase function. The detailed characterization of the redox reactions catalyzed by AIF, as well as the thorough investigation of cells in which AIF has been knocked out by homologous recombination should shed some light on the question why AIF is important for normal cell survival. Alternatively, AIF appears to be a lethal factor as soon as it is released from the mitochondrial intermembrane space, the compartment to which it usually is confined. How AIF mediates this apoptogenic function remains entirely elusive. Thus, the functional relationship between AIF and other factors involved in chromatin condensation and degradation (caspases, CAD, acinus, cyclophilin etc.) remains unknown [26]. Moreover, the AIF-interacting protein(s) in the cytosol (which are required for its membrane permeabilizing effects) and the nuclear AIF target(s) have not yet been identified. Based on our knowledge of other mitochondrial proteins and their pro-apoptotic effects, it may be speculated that endogenous AIF inhibitors exist. Such inhibitors would prevent accidental cell death induction by partial AIF release and set the threshold at which AIF levels suffice to drive the full cell death program. It is tempting to speculate that the identification of AIF targets and AIF inhibitors may lead to the generation of a new class of cytotoxic or cytoprotective agents. The future will reveal whether AIF itself will be useful as a caspase-independent, Bcl-2-independent death inducer, for instance for the gene therapy of cancer.

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