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Letter to the Editor

Caspase inhibition prevents the mitochondrial release of apoptosis-inducing factor

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Dear Editor,

Apoptosis-inducing factor (AIF) was the first identified caspase-independent cell death effector.[1] However, despite a number of interesting studies implicating AIF in apoptosis (*in vitro* experiments with recombinant AIF, microinjection of neutralizing antibody, genetic invalidation, crystal structure, etc.)[2] its role as a caspase-independent effector remains unclear. Moreover, it was recently reported that the release of AIF occurs downstream of the release of cytochrome *c* in response to several proapoptotic stimuli,[3] increasing the controversy about AIF.

When Susin *et al* published the molecular characterization of AIF in 1999, they reported that overexpression of AIF induces programmed cell death (PCD).[1] When we overexpressed full-length AIF in HeLa, Cos-7 or 293T cell lines, we did not observe a significant apoptotic effect (Figure 1a) even 72 h after transfection (data not shown). We observed that AIF accumulates in the mitochondria and that the cells remain viable (Figure 1b), even in the presence of very high amounts of AIF. This suggests that mitochondrial AIF is not toxic for the cell and that only the cytosolic form of AIF is proapoptotic as previously reported.[2]

Intriguingly, Klein *et al*[4] transfected AIF into primary granule cells from mice with an Harlequin mutation (causing about an 80% reduction in AIF expression) and observed that AIF overexpression decreased peroxide-mediated cell death. Moreover, mutant cerebellar granule cells were susceptible to exogenous and endogenous peroxide-mediated apoptosis, but could be rescued by AIF expression suggesting that AIF serves as a free radical scavenger.[4] The conclusion of this study is inconsistent with work by Yu *et al*[5] showing that the mitochondrial release of AIF is involved in caspase-independent peroxide-mediated neuronal cell death.

Bax is one of the main proapoptotic Bcl-2 family proteins and the presence of either Bax or Bak is required for most mitochondria-dependent cell death processes.[6] Bax has been reported to induce cytochrome *c* release by a process that is not inhibited by the pan-caspase inhibitor zVAD-fmk.[3] Very recently, it has been shown that Bax insertion into the mitochondrial outer membrane produces membrane openings that allow the passage of very large (2 MD) dextran molecules.[7] This suggests that Bax, after insertion into the mitochondrial outer membrane, may allow the release of all the soluble mitochondrial inner membrane space proteins. We transfected HeLa cells with a construct for the expression of GFP-Bax in the presence of the broad caspase inhibitor peptide zVAD-fmk and 18 h after transfection cells were immunostained for the detection of cytochrome *c* or AIF. We observed that while GFP-Bax was inserted in the mitochondria (as assessed by colocalization with Hsp60, a protein from the mitochondrial matrix, data not shown), caspase inhibition prevented the release of AIF but not of cytochrome *c* (Figure 1c).

To confirm that the mitochondrial insertion of Bax triggers the release of cytochrome *c* but not of AIF, we incubated freshly purified mitochondria from HeLa cells with recombinant oligomeric Bax (200 nM), and performed Western blot analysis of cytochrome *c* and AIF in both the mitochondria supernatants and pellets. We observed that the mitochondrial release of cytochrome *c* was nearly complete in the presence of Bax, whereas Bax did not induce any significant mitochondrial release of AIF (Figure 1d).

Our data presented here using an anti-AIF monoclonal antibody are consistent with a very recent study where a polyclonal antibody raised against AIF was used,[3] and the description that AIF is not soluble in the mitochondrial inner membrane space[3] may explain why AIF is not coreleased with cytochrome *c* after Bax insertion in the mitochondrial membrane.

Altogether, our results suggest that the release of AIF requires caspase activation, which occurs downstream of Bax-mediated cytochrome *c* release. How activated caspases induce AIF release remains to be determin.

Both proapoptotic drugs staurosporine and actinomycin D require the presence of either Bax or Bak to induce cytochrome *c* release and cell death.[6] HeLa cells were treated with one of these drugs in the presence of zVAD-fmk and the extent of cytochrome *c* and AIF release was analyzed by immunofluorescence. zVAD-fmk prevented the mitochondrial release of AIF, although it had no effect on the release of cytochrome *c* induced by the drugs (Figure 1e) confirming our results in GFP-Bax-transfected cells and in a recent publication where cell fractionations were performed.[3] This is also consistent with the *Caenorhabditis elegans* model where it has been recently described that the release of AIF during apoptosis occurs mainly in a caspase (Ced-3)-dependent manner.[8] Nevertheless, under our conditions, it appears that an average of 10 and 13%, respectively, of the actinomycin D- or staurosporine-treated cells in the presence of caspase inhibitor show a release of AIF with cytochrome *c* (Figure 1e) suggesting that a small part of AIF can be released independently of caspase activation.

Susin *et al*[9] reported that microinjection of a neutralizing anti-AIF antibody into the cytosol of living cells before staurosporine treatment, prevents the type I of nuclear apoptosis (i.e. perinuclear chromatin condensation) and suggested that AIF induces this specific caspase-independent phenotype.[1,9] In the presence of zVAD-fmk, staurospor-

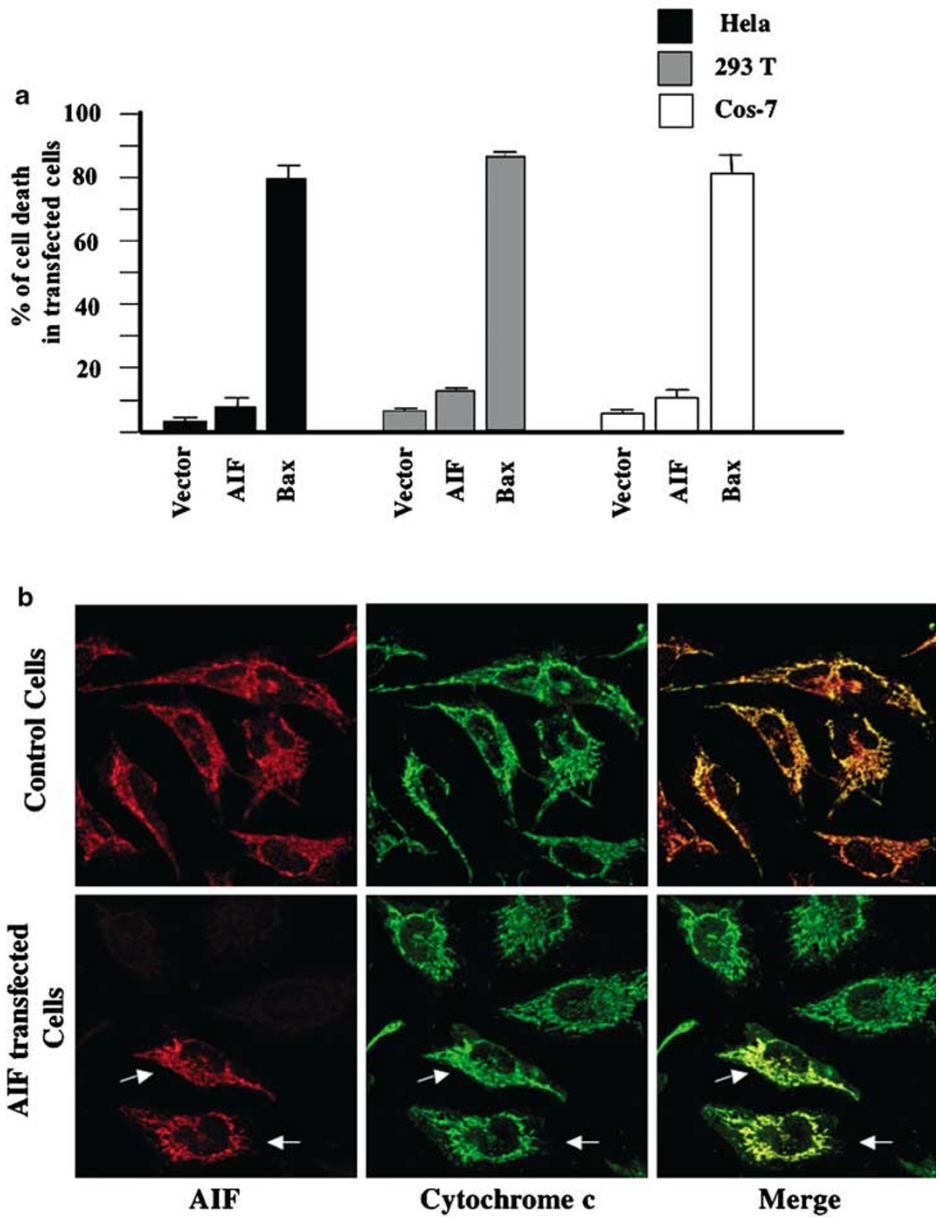

Figure 1 (**a**) Mitochondrial AIF is not toxic in HeLa, 293T and Cos-7 cell lines. HeLa, 293T and Cos-7 cells were transiently cotransfected with a vector for the expression of GFP with either a vector for the expression of AIF, a vector for the expression of Bax or vector alone. At 24 h after transfection, cells were fixed and nuclei were stained with Hoechst. Green cells were counted and cells showing blebbing, shrinkage and chromatin fragmentation were considered as dying. Each histogram indicates mean $\pm$ S.D. of three fields of at least 100 cells within a representative experiment. (**b**) HeLa cells were transiently transfected with a vector for the expression of AIF. At 24 h after transfection, cells were immunostained with a mouse monoclonal anti-AIF (Santa Cruz, clone E1) and a sheep polyclonal anti-cytochrome *c* (Cyt *c*) (Sigma). Cells overexpressing AIF (indicated by arrows) do not show any signs of cell death. Overexpression of AIF also serves as a control to confirm the specificity of the anti-AIF monoclonal antibody. (**c**) Mitochondrial insertion of Bax is sufficient to induce the release of Cyt *c* but not of AIF. GFP-Bax expression, and immunostaining of Cyt *c* and AIF together with nuclear Hoechst staining in HeLa cells 18 h after transient transfection with a vector encoding GFP-Bax in the presence of the caspase inhibitor z-VAD-fmk (100 μM). Arrows indicate GFP-Bax-transfected cells. It is followed by a quantitative analysis of the numbers of GFP-Bax-transfected cells with intracytosolic release of Cyt *c* or AIF in the presence of z-VAD-fmk. Each histogram indicates mean $\pm$ S.D. of three fields of at least 100 cells within a representative experiment. (**d**) Mitochondria isolated from HeLa cells were incubated for 30 min at 30°C with 200 nM of recombinant oligomeric Bax. Mitochondrial pellets and supernatant fractions were separated by SDS-PAGE, and their respective contents in AIF and Cyt *c* analyzed by Western blotting. (**e**) Caspase inhibitor prevents mitochondrial release of AIF in cells treated with proapoptotic drugs. HeLa cells were either left untreated (Control) or treated for 7 h with actinomycin D (20 μM) or with staurosporine (2 μM) in the presence of zVAD-fmk (100 μM), then the cells were immunostained with anti-Cyt *c* and anti-AIF antibodies together with Hoechst nuclear staining. Arrows indicate nuclei with type I of nuclear apoptosis.[9] It is followed by a quantitative analysis of the numbers of actinomycin D- or staurosporine-treated cells with intracytosolic release of Cyt *c* and/or AIF in the presence of z-VAD-fmk. Each histogram indicates mean $\pm$ S.D. of three fields of at least 100 cells within a representative experiment

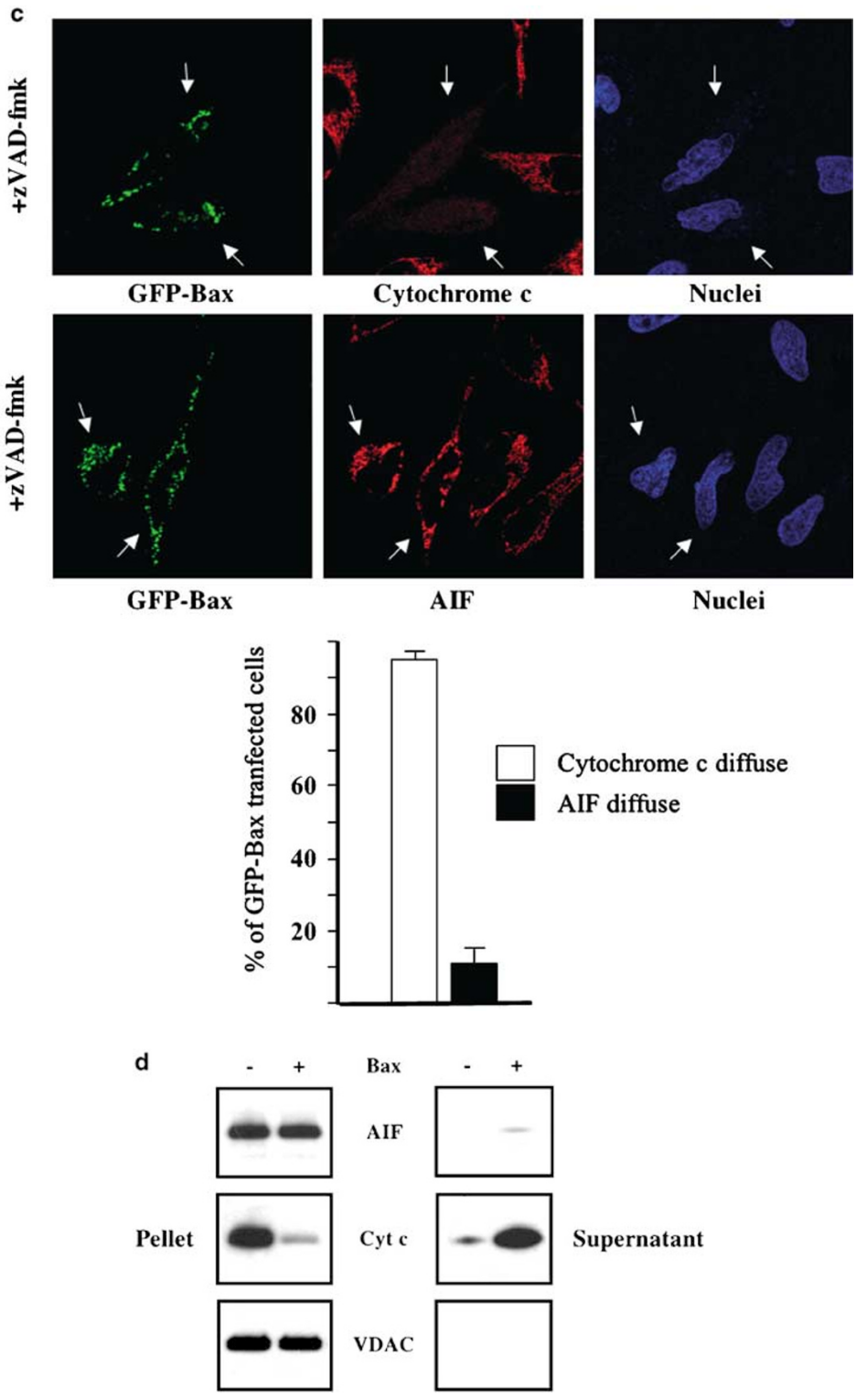

Figure 1 *continued*

ine-treated cells show a type I of nuclear apoptosis while AIF is still localized in the mitochondria (Figure 1e) suggesting that other factors are involved in the induction of the type I of nuclear apoptosis. However, we cannot rule out the possiblity that in our conditions of immunofluorescence, a weak, nondetectable amount of AIF is released.

Recently, it has been shown that the genetic inactivation of AIF renders embryonic stem cells resistant to cell death after

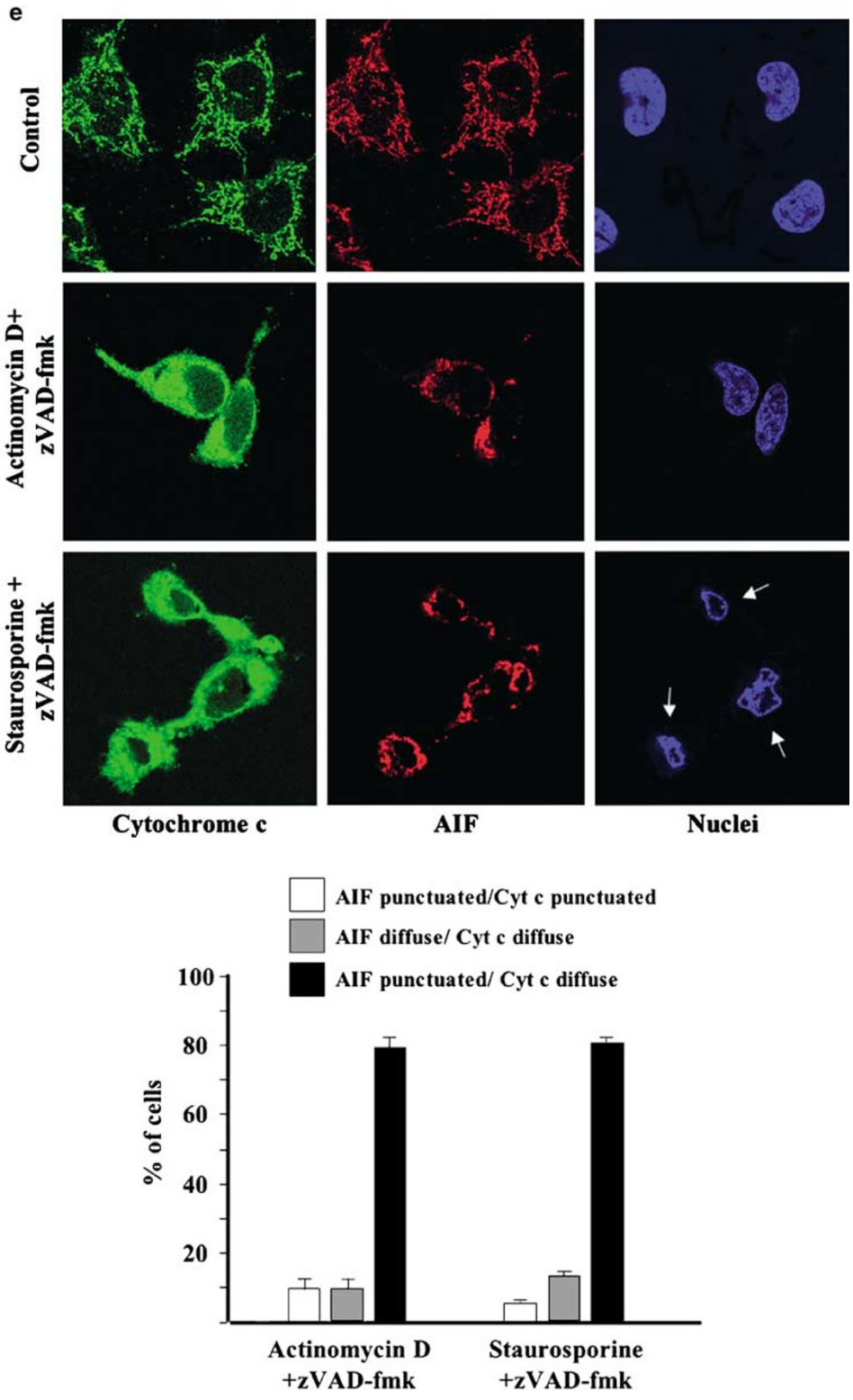

Figure 1 *continued*

serum deprivation and menadione treatment (in this last case, only if caspases are simultaneously blocked).[10] However, these embryonic stem cells are sensitive to cell death induced by staurosporine, etoposide, azide, Ter-butyl hydroperoxide, anisomycin and UV.[10] Moreover, the genetic inactivation of AIF showed that AIF seems to be essential for PCD during cavitation of embryoid bodies, the first wave of cell death indispensable for mouse morphogenesis. Nevertheless, it is

difficult to confirm that the observed phenotype of AIF KO mice is really dependent on a defect in the putative apoptogenic function of AIF or, if the lack of development is because of a defect in the other physiological function of AIF. Di Pietro *et al*[11] performed genetic inactivation of another mitochondrial NAD-dependent oxidoreductase: the mitochondrial NAD-dependent methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase (NMDMC) and the authors observed that this genetic inactivation also impaired mouse development at a very early stage and concluded that this enzyme is essential for embryonic development. Thus, it appears that two mitochondrial NAD-dependent oxidoreductases (AIF and NMDMC) are essential for mouse embryonic development. Interestingly, in *C. elegans*, the inactivation of AIF using RNAi does not impair the embryonic development of this organism, suggesting that AIF is not essential for PCD during the development of this invertebrate organism.[8]

In conclusion, our results suggest that the release of AIF, in opposition to cytochrome *c*, is not a consequence of the Bax/Bak-mediated mitochondrial permeabilization and that the release of AIF requires caspase activation downstream of the release of cytochrome *c*. Nevertheless, AIF has been shown to be released in a caspase-independent manner under some conditions,[2] thus we cannot rule out the possibility that a release of AIF may occur after a Bax/Bak-independent mitochondrial permeabilization. The factors involved in the caspase-independent release of AIF thus remain to be determined.

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