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Letter to the Editor

p53 can promote mitochondria- and caspase-independent apoptosis

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Dear Editor,

The tumour suppressor p53 plays a pivotal role in suppressing tumorigenesis by inducing cell cycle arrest or apoptosis. Cell cycle arrest is mediated by transcriptional induction of genes whose products inhibit cell cycle progression. Conversely, the molecular events that lead to p53-dependent apoptosis are less clear. Transcriptional activation is commonly implicated but growing evidences show that transrepression and transcription-independent functions can also play a central role in p53-dependent apoptosis.[1] At the cellular level, all studies converge to the crucial role of both the mitochondrial pathway (cytochrome *c* release,[2] ROS production or/and $\Delta\Psi$m drop)[3] and caspase activation in p53-induced apoptosis.[4] In this way, the Bcl-2 antiapoptotic protein as well as caspase inhibition were shown to protect cells from p53-induced apoptosis.

We previously showed in rat embryo fibroblasts (e.g. the REtsAF cell line) expressing a temperature-sensitive mutant (tsA58) of the simian virus 40 large tumour antigen (LT) that LT inactivation leads to p53-mediated apoptosis. Moreover, we reported that while *bcl-2* overexpression inhibits apoptosis, caspase inhibition surprisingly accelerates apoptosis and moreover abolishes the protective effect of Bcl-2.[5] These data led us to postulate that caspase inhibition would unmask an alternative route for p53-induced cell death signal, which would lead to a caspase-independent and Bcl-2-insensitive cell death process.

In order to ascertain that this new cell death process observed in the presence of ZVAD is dependent on p53 activity, REtsAF cells were transiently transfected with genes encoding temperature-sensitive dominant-negative mutants of p53 ($p53^{val135}$ and $p53^{ala143}$).[6,7] At restrictive temperature, these mutants are defective in their DNA binding domain and consequently loss their sequence-specific transactivation and transrepression properties. We observed that overexpression of either $p53^{val135}$ or $p53^{ala143}$ led to a decreased rate of apoptosis in the absence or in the presence of ZVAD (Figure 1a), demonstrating that active p53 is required for these two cell death pathways.

Next, we controlled that this unexpected effect of ZVAD on p53-induced cell death could be reproduced in a more physiological model. For this purpose, we induced a p53-dependent apoptosis in primary rat embryo fibroblasts (RE) by addition of 100 μM etoposide, in the absence or in the presence of ZVAD. As observed in REtsAF cells, ZVAD treatment accelerated commitment to death of RE cells. As control, ZVAD reduced staurosporine-induced apoptosis of RE cells, showing its usual protector effect against this p53-independent cell death (Figure 1b). These results argue for a physiological relevance of the proapoptotic effect of ZVAD on p53-mediated apoptosis.

In order to characterise the pattern of the p53-induced death of REtsAF cells in the presence of ZVAD, we examined the associated nuclear and cytological alterations. In the absence of ZVAD, the earliest morphological changes observed are the rounding up, the brightening phase, the shrinkage of the cells as well as the blebbing of the plasma membrane (Figure 1c). These events are associated with typical apoptotic chromatin compaction and fragmentation in globular, crescent-shaped figures (stage II chromatin condensation), and they preceded the breaking up of the cells into fragments as well as the fragmentation of DNA, as judged by the flow cytometric analysis of TUNEL-stained cells. In the presence of ZVAD, light microscopy observation showed that some typical morphological features of apoptosis such as loss of adherence and condensed cytoplasm were evident in dying REtsAF cells. Further fluorescence microscopy examination revealed chromatin condensed in lumps (stage I chromatin condensation), and rounded cells without microvilli or protusions on the surface (Figure 1c). However, specific events of the final degradation phase such as the nucleus and cell fragmentation do not occur whereas cells completely detached from the substratum. These observations indicate that p53 can promote two cell death pathways showing apoptotic features, one that is caspase-dependent and another that is independent of caspases.

Recent data suggest that noncaspase proteases including calpains, cathepsins or serine proteases can also mediate cell death and bring about many of the morphological changes characteristic of apoptosis in a caspase-independent manner.[8] We investigated the possible involvement of these proteases in the novel route by which p53 signal cell death through the use of specific inhibitors: serine protease inhibitors (TPCK, TLCK), calpain protease inhibitors (ALLN, MDL), cysteine protease inhibitors (Lactacystine, Z-FA-fmk). Microscopic studies of cell morphology did not show notable differences in the amount of Z-VAD-dependent cellular death whether the cells were cultured in the presence or in the absence of the drugs. These results suggest that none of the tested proteases are involved in p53-induced apoptosis of REtsAF cells in the presence of ZVAD.

Inasmuch as the mitochondrial pathway plays a central role in many models of p53-dependent apoptosis, we investigated

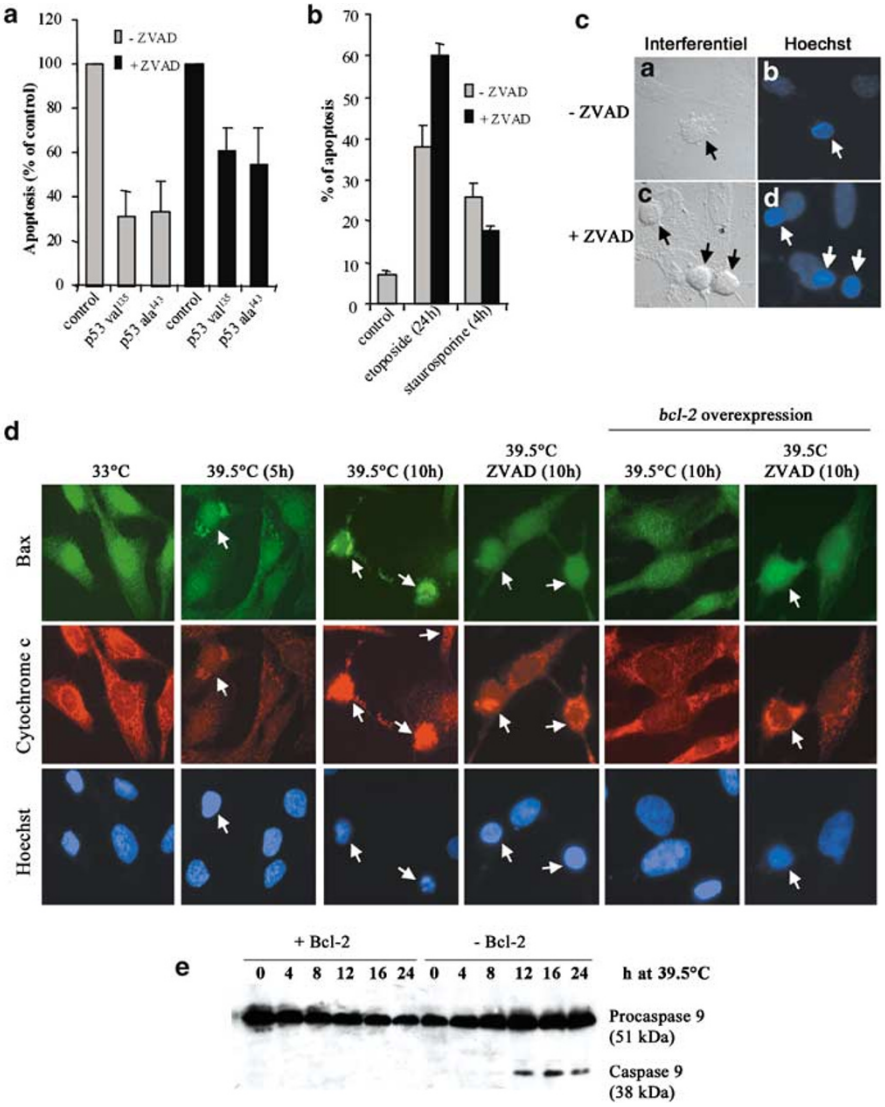

Figure 1 (**a**) Role of p53 in ZVAD-induced REtsAF cell apoptosis. Transient transfections with vectors encoding dominant-negative mutants of p53 ($p53^{va1135}$ and $p53^{ala143}$). An empty vector was used as control. At 24 h after transfections, cells were placed at 39.5°C for 16 additional hours, in the presence or in the absence of ZVAD (100 μM). Results, defined by FDA-BET epifluorescence observation, were presented as the percentage of apoptosis (among transfected cells) referred to control. (**b**) Effect of ZVAD on p53-dependent (etoposide) and p53-independent (staurosporint) apoptosis in RE cells. Graph indicates the percentage of apoptic cells defined by morphological microscopic observation; nontreated cells were used as control. (**c**) Morphological features of REtsAF cell death at 39.5°C. Nuclei status of cells that do not overexpress *bcl-2*, cultured at 39.5°C in the absence (a,b) or in the presence of ZVAD (c,d) by light microscopy observation (a,c) or Hoechst staining of chromatin (b,d). Arrows indicate dying cells. (**d**) Effect of Bcl-2 and ZVAD treatment on Bax and cytochrome *c* localisation in REtsAF cells at 39.5°C. Immunostaining of Bax and cytochrome *c* in REtsAF cells overexpressing or not *bcl-2* placed at 39.5°C in the presence or in the absence of ZVAD. Nuclei are visualised by Hoechst staining. Arrows indicate apoptotic cells. (**e**) Western blot analysis of caspase 9 in REtsAF cells overexpressing or not *bcl-2*

its contribution to the two p53 apoptotic programs described in REtsAF cells. For this purpose, we examined the subcellular distribution of Bax and cytochrome *c* in these cells. We observed that, in the absence of caspase inhibitor, p53 induces Bax relocation/activation to mitochondrial membrane (as soon as 5 h after p53 activation; Figure 1d). This event is followed by cytochrome *c* release from intermembrane space (10 h after p53 activation; Figure 1d) and caspase-9 activation (12 h after p53 activation; Figure 1e). Bcl-2 inhibits this apoptotic program probably in counteracting Bax relocation/activation and thus preventing downstream events such as cytochrome *c* outflow or caspase-9 activation (Figure 1d and e). Surprisingly, although ZVAD treatment accelerates p53-induced cell death, it appeared that the cell death progressed in the absence of Bax relocalisation/activation and cytochrome *c* release, whatever the cells overexpressed or not *bcl-2* (Figure 1d). In support of these observations, we did not detect alteration of the mitochondrial membrane potential nor the release of AIF (data not shown), an other death-promoting factor which can be responsible for a caspase-independent death signalling downstream of mitochondria.[9] These results indicate that p53 can induce in our model, the typical mitochondrial-dependent cell death program and an alternative cell death program, unmasked by the ZVAD treatment, which does not require mitochondrial signalling and which is not inhibited by Bcl-2. Moreover, it can be assumed that one or more ZVAD-sensitive

caspase are involved in the choice between these two apoptotic pathways occurring upstream of mitochondria.

In summary, even though it is clear today that physiological cell death can occur in the complete absence of caspases, only a few cases of apoptotic death without caspase activation have been reported. Most often caspase-independent cell death is related to paraptosis, autophagy or nonlysosomal cell death.[8] Moreover, mitochondrial outer membrane permeabilisation (MOMP) controlled by Bcl-2 family proteins resides at the heart of several alternative death pathways whatever their apoptotic or necrotic feature. Therefore, the p53-induced cell death program in the presence of ZVAD appears to differ from most caspase-independent alternative pathways: on the one hand by its apoptosis-like nature and on the other hand by being MOMP-independent and insensitive to Bcl-2 protection. Then, although nonclassical cell death programs are emerging today, in this study we showed for the first time that p53 can trigger such a caspase- and MOMP-independent apoptotic signalling pathway.

At last, our findings may provide new perspectives in cancer therapy. Direct mutations in the p53 gene occur in about half of these tumours. In many others, the p53 pathway is ablated by the p53 binding to viral proteins or as a result of alterations in genes whose products either regulate p53 activity (MDM2, $p14^{ARF}$) or are downstream mediators of the p53 signalling.[10] Notably, inactivation of Apaf-1, which leads to the blocking-up of apoptosis, may contribute to the low frequency of p53 mutations observed in therapy-resistant melanomas.[4] Therefore, the ability of p53 to promote an alternative mitochondria- and caspase-independent apoptotic program may form the bases for new therapies against tumours in which the p53 gene is wild type and which have acquired defects in the signalling pathways that are downstream of p53.

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