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Letter to the Editor

Endoplasmic reticulum stress-induced cell death requires mitochondrial membrane permeabilization

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Dear Editor,

Accumulating evidence suggests that mitochondrial membrane permeabilization (MMP) is a rate-limiting step of programmed (developmental) cell death as well as stress-induced cell death,[1] including in the context of anti-cancer chemotherapy[2] or viral infection.[3] This notion is re-enforced by the observation that the knock-out of proteins involved in the pathways leading to MMP or closely linked to MMP (Bax, Bak, Bim, AIF, cytochrome *c*, Apaf-1, caspase-9 etc.) results in a major phenotype.[4] Recently, we have launched the working hypothesis[5] that MMP may be a rate-limiting event of apoptosis induction even when cell death is initiated through a primary stimulus affecting other organelles than mitochondria such as nuclei (via p53 activation), lysosomes (via activation of cathepsins) or the endoplasmic reticulum (ER). The pro-apoptotic agents that specifically act on the ER include tunicamycin (TM, which inhibits N-linked glycosylation), brefeldin A (BFA, which inhibits ER-Golgi transport) and thapsigargin (TG, which inhibits the sarcoplasmic/endoplasmic Ca^{2+}-ATPase SERCA). Whereas BFA and TG elicit a local unfolded protein response,[6] TG depletes ER Ca^{2+} and thus impinges on Ca^{2+} signaling.[7] Mouse embryonic fibroblasts lacking both Bax and Bak become resistant to apoptosis induction by TM and BFA, an observation that may be attributed to the obligatory participation of Bax and Bak in MMP induction,[8] or alternatively, suggests an as yet poorly characterized function of Bax and Bak at the ER level. Indeed, Bax redistributes both to mitochondria and to ER upon apoptosis induction, and overexpression of Bax reportedly causes a loss of ER Ca^{2+} content.[9]

To probe the importance of MMP for ER stress-induced cell death, we assessed the effects of two local MMP inhibitors, Bcl-X_L and vMIA on cellular alterations provoked by TM, BFA, and TG (Figure 1). Bcl-X_L is found inserted in intracellular membranes (in particular the outer mitochondrial membrane) and is known to stabilize the mitochondrial membrane barrier function by local interactions with pore forming proteins contained in the permeability transition pore complex, namely the voltage-dependent anion channel (VDAC), the adenine nucleotide translocase (ANT), and pro-apoptotic Bcl-2 family members.[1,10,11] In contrast to Bcl-2,[12–14] no local ER effects have been described for Bcl-X_L.[15] Viral mitochondrial inhibitor of apoptosis (vMIA) is encoded by the Cytomegalovirus UL37 gene, has no obvious structural similarity with Bcl-2-like molecules, and is exclusively found in mitochondrial membranes, where it specifically interacts with ANT but not with VDAC, as shown by mass spectroscopic identification of vMIA-interacting proteins and confirmed by co-immunoprecipitation assays.[16–18]

TM, BFA, and TG induced signs of MMP in several different cell lines including B cell lymphoma BJAB (Figure 1a) and cervical carcinoma HeLa cells (Figure 1b). Such signs consist in the loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$), as determined by means of the $\Delta\Psi_m$-sensitive fluorochrome, $DiOC_6$[3] (Figure 1a), and the release of cytochrome *c* from mitochondria, as determined by immunofluorescence analysis (Figure 1b,c). Thus, both the permeability of the inner membrane (on which the $\Delta\Psi_m$ builds up) and that of the outer membrane (which retains cytochrome *c*) were compromised by the three different ER-targeted toxins. ER stress-induced MMP was an early event since it was detectable in a fraction of cells that still lack signs of chromatin condensation (not shown). Stable transfection with vMIA and Bcl-X_L prevented ER stress-induced MMP (Figure 1a,b,c), both in BJAB and in HeLa cells. It has been reported that, in determined circumstances, for instance in type I cells stimulated by CD95 ligation, Bcl-2-mediated MMP inhibition is not sufficient for the prevention of apoptosis.[19] Therefore, we assessed whether MMP inhibition would suffice to suppress apoptosis induction by TM, BFA, or TG. Clearly, vMIA and Bcl-X_L overexpression did reduce the frequency of cells which manifest caspase activation (not shown), nuclear chromatin condensation (Figure 1b), phosphatidylserine exposure on the outer leaflet of the plasma membrane (as determined with an Annexin V-FITC conjugate, Figure 1d), and loss of viability (as determined by staining with propidium iodide, PI, Figure 1e). In conclusion, it appears that MMP inhibition by Bxl-X_L or vMIA can protect cells against apoptosis induction by ER-specific toxins.

Using a monoclonal antibody specific for the apoptogenic conformation of Bax (6A7), we determined the putative link between vMIA-induced (presumably ANT-mediated[16–18] apoptosis inhibition and the Bax/Bak mediated MMP[8] induced by ER stress. ER stress did induce an apoptosis-associated change in Bax conformation, linked to its aggregation in cytoplasmic spots (Figure 2f), some of which coincide with mitochondria (as determined by confocal microscopy, not shown). vMIA did prevent the apoptosis associated conformational change of Bax, as well as its aggregation, a finding that may link our previous observation that Bax and ANT can interact to induce MMP.[20,21]

In conclusion, it appears that ER stress can induce apoptosis through a reaction that depends on pro-apoptotic members of the Bcl-2 family (Bax, Bak) and which involves MMP as a critical step towards cellular demise. Rather than

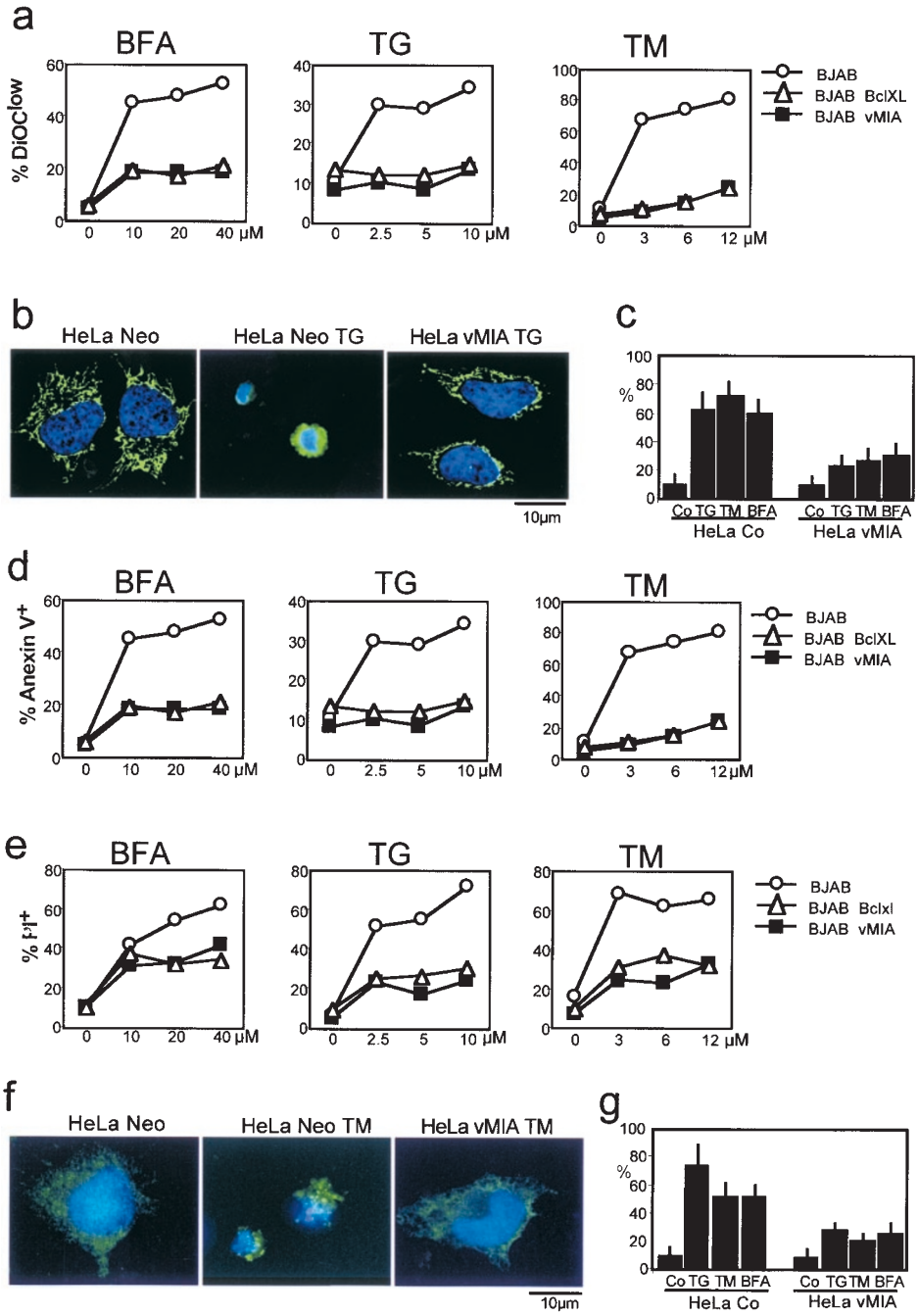

Figure 1 Mechanisms of cell death induced by three ER-specific toxins, (**A**) brefeldin (BFA), thapsigargin (TG) and tunicamycin (TM). BJAB cells (**A,D,E**) or HeLa (**B,C,F,G**) stably transfected with the vector containing the neomycin resistance gene only (Neo), vMIA or Bcl-X_L, were treated with the indicated doses of BFA, TG or TM (36 h in the case of BJAB cells; 18 h in the case of HeLa cells), followed by the determination of different apoptosis-associated parameters. (**A**) The reduction of the mitochondrial transmembrane potential ($\Delta\Psi_m$) induced by ER stress was determined by staining with the $\Delta\Psi_m$ sensitive dye 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6(3)$ (40 nM, 15 min, 37°C), followed by cytofluorometric analysis.[23] Results are shown for Neo-BJAB, Bcl-X_L-BJAB and vMIA-BJAB cells, and similar data were obtained for HeLa cells (not shown). (**B**) Representative immunofluorescence stainings for *in situ* detection of cytochrome *c* in Neo-HeLa cells that were left untreated or cultured 18 h with TG (10 μM), as well as in vMIA-HeLa cells treated with TG. Cells were stained with anti-cytochrome *c* antibody (revealed in green) and counterstained with Hoechst 33342.[24] Note the diffuse cytoplasmic staining of cytochrome *c* in TG-treated Neo-HeLa cells. (**C**) Quantitation of cells with diffuse cytochrome *c* staining. Neo-HeLa and vMIA-HeLa cells were cultured 18 h with TG (10 μM), TM (12 μM) or BFA (40 μM), and the frequency of cells exhibiting mitochondrial release of cytochrome *c* was determined as in **B**. (**D**) Phosphatidylserine exposure induced by BFA, TG or TM. After culture with the indicated agents, Neo-BJAB, Bxl-X_L-BJAB and vMIA-BJAB cells were stained with Annexin V-FITC conjugate and cytofluorometric determination of Annexin V-binding.[25] (**E**) Loss of viability induced by ER-specific toxins. Neo-BJAB, Bcl-X_L-BJAB and vMIA-BJAB cells were stained with propidium iodide (PI, 2 μM) and the percentage of cells incorporating this vital dye was measured by cytofluorometry. Similar data were obtained in HeLa cells, in which vMIA inhibited the loss of viability induced by BFA, TG or TM (not shown). (**F**) Immunofluorescence detection of Bax, using a monoclonal antibody (6A7) specific for the apoptogenic conformation of Bax. Neo-HeLa cells that were left untreated or cultured 18 h with TM (12 μM), as well as vMIA-HeLa cells treated with TM, were stained with mAb, as described.[26] Note the cytoplasmic aggregates induced by TM in Neo but not in vMIA cells. (**G**) Quantitation of the Bax aggregation in different cell lines treated with BFA, TG or TM, as exemplified in **F**. All experiments shown here were repeated at least three times, yielding similar results

causing a direct activation of the caspase activation cascade,[22] which on theoretical grounds might bypass the requirement of MMP for cell death induction,[19] ER stress-induced cell death does rely on MMP. It remains an open question whether a similar MMP requirement will be observed for cell death triggered by lysosomal toxins and atypical forms of programmed cells commonly attributed to autophagy.

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