

Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway

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Summary

Apoptin, a small proline-rich protein derived from the chicken anaemia virus, induces cell death selectively in cancer cells. The signalling pathways of apoptin-induced, cancer cell-selective apoptosis are not well understood. Here, we demonstrate that apoptin triggers apoptosis by activating the mitochondrial/intrinsic pathway, and that it acts independently of the death receptor/extrinsic pathway. Jurkat cells deficient in either FADD or caspase-8 (which are both necessary for the extrinsic pathway) were equally as sensitive to apoptin as their parental clones. This demonstrates that apoptin is likely to act through the mitochondrial death pathway. Apoptin treatment causes a loss of mitochondrial membrane potential, and release of the mitochondrial proteins cytochrome c and apoptosis-inducing factor. Apoptin-induced cell death is counteracted

by the anti-apoptotic Bcl-2 family members, Bcl-2 itself and Bcl-X_L, as shown in Jurkat leukaemia cells. In addition, we describe the processing and activation of caspase-3. By contrast, cleavage of caspase-8, which is predominantly triggered by the death receptor pathway, is not observed. Furthermore, apoptin triggers the cytoplasmic translocation of Nur77, and the inhibition of Nur77 expression by siRNA significantly protects MCF7 cells from apoptin-triggered cell death. Thus, our data indicate that the apoptin death signal(s) ultimately converges at the mitochondria, and that it acts independently of the death receptor pathway.

Key words: AIF, Apoptin, Death receptors, Mitochondrial death pathway, Nur77

Introduction

Apoptosis is a physiological form of cell death characterized by nuclear chromatin condensation, cytoplasmic shrinking and membrane blebbing (Lauber et al., 2004; Schmitz et al., 2000). This form of programmed cell death is predominantly induced by cancer therapy (Kawanishi and Hiraku, 2004; Wesselborg and Lauber, 2005). The two best-studied cell death signalling pathways include the extrinsic/cell death receptor pathway and the intrinsic/mitochondrial-initiated pathway (Greil et al., 2003; Los et al., 1999). The death receptor family [the tumor necrosis factor receptor (TNF-R) superfamily] includes CD95 (Fas/APO-1), TNF-R1, DR3, DR4 (TRAIL-R1) and DR5 (TRAIL-R2) receptors. Upon ligation, these receptors recruit the FADD-adaptor protein and the apical caspase-8 (or caspase-10), resulting in the formation of the death-inducing signalling complex (DISC). Following this, caspase-8 is activated and released from the DISC, and further acts on downstream effector molecules (Los et al., 1999; Sartorius et al., 2001). The mitochondrial death pathway is activated in the presence of diverse apoptotic stimuli, such as anti-cancer drugs, UV and γ -irradiation (Hill et al., 2003). The tumor

suppressor gene p53 and the orphan steroid receptor Nur77 have been proposed as being the main molecules capable of transmitting the apoptotic signal from the nucleus to the mitochondria (Erster and Moll, 2004; Jeong et al., 2003; Lane and Hupp, 2003; Li et al., 2000). The initial common event during activation of the intrinsic/mitochondrial death pathway is the release of cytochrome c. Cytochrome c triggers the formation of a multimeric Apaf-1/cytochrome c/dATP/pro-caspase-9 protein complex called the apoptosome, and leads to the activation of caspase-9. Caspase-9 then cleaves and activates downstream caspases, including caspase-3, caspase-6 and caspase-7. This pathway is regulated at several levels. The mitochondrial apoptotic pathway is negatively modulated by the anti-apoptotic Bcl-2 family members, which block the release of cytochrome c from the mitochondria. Furthermore, caspase activation and the activity of already active caspases can be inhibited by the 'inhibitor of apoptosis proteins' (IAPs). In turn, IAPs can be inactivated by Smac/DIABLO or OMI/HtrA2, two regulatory proteins that are released from mitochondria.

Several strategies have been pursued to develop cancer

selective therapies. The most broadly applied approaches explore defects in the regulation of the cell cycle (cell cycle check-points), which are commonly found in cancer cells (Blagosklonny and Darzynkiewicz, 2005; Westwell, 2004). Other strategies for selective cancer therapy involve molecules that act on and kill cancer cells preferentially, leaving normal cells unaffected. Several viruses require (rapidly) dividing cells for the completion of their lifecycle, thus viral proteins have gained attention as potential cancer selective therapeutics (Cassens et al., 2003; Cornelis et al., 2004; Oro and Jans, 2004).

Apoptin is a viral protein that fulfils these expectations. Derived from the chicken anaemia virus (CAV), this 14 kDa protein selectively induces apoptosis in a wide variety of transformed cells but not in primary cells (Danen-Van Oorschot et al., 1997; Oro and Jans, 2004; Poon et al., 2005). However, the transient acquisition of a transformed-like phenotype (for example, by the regulated expression of the SV40 large T antigen, or by irradiation with ultraviolet-B) renders even primary cells sensitive to apoptin (Oro and Jans, 2004; Zhang et al., 2004). The cancer-selective toxicity of apoptin correlates with its cellular localization. Apoptin enters the nuclei of cancerous cells, whereas in non-transformed cells it remains in the cytoplasm (Guelen et al., 2004). However, the forced targeting of apoptin into the nuclei of primary cells is not sufficient for apoptin's toxicity. The current hypothesis is that nuclear trafficking and tumor-specific phosphorylation of apoptin at Thr-108 are essential for the induction of apoptosis (Guelen et al., 2004; Rohn et al., 2002). Recently, Rohn and colleagues reported that phosphorylation of apoptin is required for apoptosis, but that abolishing the phosphorylation site of apoptin does not significantly disrupt its nuclear input into tumor cells (Rohn et al., 2005).

The signalling pathways involved in apoptin-induced cell death are not well understood. It has been reported that apoptin-induced cell death is independent of the p53 status of the cell. Recently, it has also been shown that apoptin associates with the anaphase-promoting complex and induces G2/M arrest and apoptosis (Teodoro et al., 2004). It has also been shown that apoptin co-localizes with Bcl-10 and FADD (Guelen et al., 2004). Both Bcl-10 and FADD form death effector filaments in the cytoplasm when overexpressed; however, the functional significance of the interaction of apoptin with these proteins still remains to be established.

By using cells with defective FADD or caspase-8 (key players in death receptor signalling) and other molecular tools, we demonstrate that apoptin acts independently of death receptor signalling. Instead, apoptin triggers the activation of the mitochondrial death pathway. In addition, we demonstrate an apoptin-triggered release of cytochrome c and apoptosis-inducing factor (AIF) from mitochondria that can be counteracted by anti-apoptotic Bcl-2 family members. Nur77 translocates to the cytoplasm upon apoptin-triggered cell death, and the inhibition of Nur77 expression by siRNA significantly protects cells against apoptin-triggered cell death.

Materials and Methods

Cell culture and reagents

Jurkat (T-cell leukaemia) cells, Jurkat clones stably transfected with

FADD-DN (a dominant-negative FADD mutant lacking the N-terminal DED), caspase-8 deficient Jurkat cells, BJAB (B-cell lymphoma) cells, a BJAB clone expressing FADD-DN, PC-3 (prostate cancer) cells, and MCF7, MCF7-caspase-3 and MCF7-Bcl-2 (breast adenocarcinoma) cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (Hyclone), 100 µg/ml penicillin and 0.1 µg/ml streptomycin (Gibco BRL). The cells were grown at 37°C with 5% CO₂ in a humidified incubator. The peripheral blood mononuclear cells were isolated by Ficoll-gradient fractionation, as described previously (Los et al., 1995). The following antibodies were used: murine anti-cytochrome c, anti-AIF, rabbit anti-Nur77, anti-haemagglutinin tag (anti-HA, Biomed), anti-tubulin (Santa Cruz Biotechnologies), rabbit anti-caspase-3, mouse anti-caspase-8, anti-CD95 IgM (Upstate Cell Signaling) and anti-CD95 neutralizing (anti-APO-1) IgG₁ (a kind gift from Dr H. Walczak, DKFZ, Heidelberg, Germany).

Purification of TAT-apoptin and TAT-GFP

Plasmids expressing TAT-apoptin and TAT-GFP were a kind gift from Dr Tavassoli (King's College, London, UK). The recombinant plasmids were expressed in a bacterial system, and then purified by nickel-chromatography as described previously (Guelen et al., 2004).

Cell fractionation

Cytoplasmic, nuclear and mitochondrial fractions were separated by differential centrifugation (Wu et al., 2002). Briefly, the cells were treated with apoptin, then harvested and washed once with PBS after the indicated time points. The cells were re-suspended for 5 minutes on ice in a lysis buffer: 10 mM Tris-HCl (pH 7.8), 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 mg/ml aprotinin and 1 mg/ml leupeptin. In some experiments, an equal amount of distilled water was added to the cells in order to increase the cell lysis. Cells were then sheared by passing them through a 22-gauge needle. The nuclear fraction was recovered by centrifugation at 600 g for 5 minutes, and the 'low-speed' supernatant was centrifuged at 10,000 g for 30 minutes to obtain the mitochondrial fraction (pellet) and the cytosolic fraction (supernatant). The mitochondrial fraction was further lysed in the buffer: 10 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA (pH 8.0).

Measurement of mitochondrial membrane potential

Mitochondrial permeability transition was determined by staining the cells with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolyl carbocyanine iodide (JC-1; Molecular Probes), as described previously (Bedner et al., 1999). The mitochondrial membrane potential was quantified by flow cytometric determination of cells with a decreased fluorescence in the FL-2 channel. Data were collected and analysed using a FACSCalibur (Becton Dickinson) equipped with CELLQuest software. Data were given in percent cells with low ΔΨ_m, which reflects the percentage of cells losing the mitochondrial membrane potential.

Immunoblotting

The release of cytochrome c and AIF from mitochondria, and the cleavage of caspase-3, were detected by immunoblotting. The cells were treated with apoptin for the indicated time periods and the extracts were prepared as described above. Protein (30 µg) was separated by denaturing SDS-PAGE and then transferred onto nylon membranes. The membranes were blocked in 5% non-fat dried milk in TBS and then incubated overnight with the primary antibodies at 4°C. Then, the blots were incubated with the corresponding secondary antibodies conjugated with HRP at room temperature for 1 hour. Visualization was carried out by enhanced chemiluminescence (ECL) detection (Amersham-Pharmacia Biotech).

Measurement of apoptosis by flow cytometry

Apoptosis was measured by using the Nicoletti method (Barczyk et al., 2005). Briefly, cells grown in suspension were treated with purified TAT-apoptin (1 μ M) or TAT-GFP (control) for the indicated time periods, and then harvested by centrifugation at 800 *g* for 5 minutes. The cells were washed once with PBS, and then re-suspended in a hypotonic PI lysis buffer (1% sodium citrate, 0.1% Triton X-100, 0.5 mg/ml RNase A, 40 μ g/ml propidium iodide). Cell nuclei were then incubated for 30 minutes at 30°C and the nuclei were subsequently analyzed by flow cytometry. Nuclei to the left of the G1 peak containing hypodiploid DNA were considered to be apoptotic.

MTT-assay

The MTT-assay was performed exactly as described previously (Ghavami et al., 2005). Briefly, the experiment was performed in a 96-well (flat-bottomed) plate. Towards the end of the experiment, 10 μ l of 5 mg/ml tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)] dissolved in PBS was added to the cells in 100 μ l of medium. Cells were then incubated for 4 hours at 37°C. At the end of the incubation time, the cells were spun down to the bottom of the 96-well-plate at 90 *g* for 10 minutes, and the supernatant was discharged by rapid inverting of the plate. 150 μ l of ethanol:DMSO mix (1:1) was added per well and then the plate was placed on a shaker for 20 minutes to solubilize the formazan crystals. Absorbance was measured using a spectrophotometer equipped with $\lambda=570$ nm (measurement) and $\lambda=630$ nm (reference) filters.

Immunocytochemistry and confocal imaging

Cells were grown overnight on coverslips and then transfected with GFP-apoptin. After 36 hours, the cells were washed with PBS and then fixed in 4% paraformaldehyde. Then, the cells were permeabilized with 0.1% Triton X-100. To detect AIF release, the cells were incubated with anti-AIF mouse IgG (Santa Cruz Biotechnology; diluted 1:500). Following three wash steps with PBS, the AIF-antibody complexes were stained with the corresponding Cy3-conjugated secondary antibody (diluted 1:2500), then washed twice with PBS. To visualize nuclei, cells were stained with DAPI (10 μ g/ml). The mitochondria were stained with Mitotracker Red CMXRos (Molecular Probes; 200 nM in RPMI medium for 15 minutes, prior to fixing), a mitochondrial specific dye. The fluorescent images were then observed and analyzed using an Olympus-IX81, a multi-laser confocal microscope.

RNA interference (RNAi)

The target siRNA for Nur77 (CAG UCC AGC CAU GCU CCU) and the scrambled siRNA were purchased from Dharmacon RNA technologies. The cells were grown to 90% confluency and then transfected with the siRNA duplex (100 nM final concentration) using Lipofectamine (Invitrogen) according to the manufacturer's instructions. The expression of the Nur77 was determined by immunoblotting at 0, 12, 24 and 30 hours post-transfection.

Results

Apoptin-induced cell death is independent of the death receptor pathway

To delineate the apoptin-triggered death pathways, we used recombinant TAT-apoptin, which can enter cells as a result of the attached TAT-transport peptide (Guelen et al., 2004). TAT-apoptin accumulated within the nuclei of transformed cells, whereas the TAT-GFP used as a control was evenly distributed within the cell (Fig. 1A). The nuclear accumulation of TAT-apoptin was not restricted to the recombinant protein itself, as

transiently expressed apoptin also accumulated in the nucleus of transformed cells (data not shown). The peripheral blood mononuclear (primary) cells were fully resistant to TAT-apoptin (Fig. 1B). The addition of IL-2 (5 U) to some samples to prevent spontaneous cell death had a much stronger impact on cell viability than did the apoptin itself. By contrast, Jurkat T-cell leukaemia cells were highly sensitive to TAT-apoptin treatment (Fig. 1C,D, Fig. 3B). The resistance to apoptin observed in primary cells was due to the difference between its cellular distribution in the primary cells and in the Jurkat T-cell leukaemia cells. Whereas in primary cells TAT-apoptin was localized mainly in the cytoplasm, in Jurkat cells it was found in the nucleus (Fig. 1F). Thus, the data confirm earlier observations indicating that apoptin is selectively toxic for transformed cells (Danen-Van Oorschot et al., 1997; Oro and Jans, 2004; Teodoro et al., 2004; Poon et al., 2005).

Next, we tested the role of components of the death receptor signal transduction pathway in apoptin-induced cell death. FADD and caspase-8 are the key downstream signalling components of the death-inducing signalling complex (DISC), a multi-protein structure formed upon ligation of the death receptors of the TNFR/NGFR family, which initiate apoptotic signalling through the death receptor pathway. To delineate the role of the DISC-components in apoptin-triggered death, we used Jurkat T cells that lacked the expression of caspase-8 [Jurkat-Caspase-8^(-/-)]. The caspase-8-deficient Jurkat cells were as sensitive to TAT-apoptin-induced apoptosis as the wild-type Jurkat cells were (Fig. 1C). Next, we tested the role of the FADD adaptor molecule in the apoptin-triggered death pathway, by using Jurkat cells overexpressing a truncated, dominant-negative form of FADD (FADD-DN). Overexpression of FADD-DN prevents the formation of a functional DISC, and thus the activation of caspase-8 that is triggered not only by CD95-L/Fas-L, but also by TRAIL or anti-APO-1-antibodies (Sprick et al., 2000; Stroh et al., 2002). The cells were treated with TAT-apoptin and, after the indicated time points, apoptosis was measured by flow cytometry. After 48 hours of apoptin treatment, about 55% of both the parental Jurkat cells and the cell clones overexpressing FADD-DN were killed. Thus, the state of FADD has no effect on the cell death induced by apoptin in Jurkat cells (Fig. 1D). The same pattern is observed with the cells treated for 72 and 96 hours, respectively. In a control experiment, Jurkat cells lacking FADD or caspase-8 activity, and the 'wild type' cells were treated with an agonistic anti-CD95 antibody that crosslinks the CD95/APO-1/Fas receptor molecule and induces cell death through a caspase-8/FADD-dependent mechanism. After 8 hours of treatment with the anti-CD95 antibody, wild-type cells showed an increased amount of cell death, whereas the cells either lacking caspase-8 or overexpressing FADD-DN were completely resistant (Fig. 1E). These results suggest that cell death induced by apoptin does not depend on caspase-8 or FADD. To confirm the data generated using Jurkat cells, we also verified the cell death induced by apoptin in a human B cell line, BJAB, stably expressing FADD-DN (Chinnaiyan et al., 1995). Measurement of the cell death induced by apoptin showed no difference in mutant cell lines when compared with that of the wild-type cells (data not shown).

In order to further confirm the above findings, we inhibited the activation of CD95 by using a neutralizing anti-APO-1 (IgG₁) antibody. Unlike the anti-APO-1 IgG₃ and anti-CD95

IgM antibodies that can exist as polymers and can thus activate CD95/Fas/APO-1, anti-APO-1 IgG₁ exists only in the monomeric form and thus it fails to induce the CD95 trimerization that is required for activation of the CD95-death pathway. The anti-APO-1 IgG₁ antibody strongly reduced the cell death induced by the stimulatory anti-CD95 (IgM) antibody. By contrast, apoptosis induced by apoptin was not affected by the neutralizing anti-CD95 antibody (Fig. 2A,B). Thus, all of the above data indicate that apoptin acts independently of the death receptor signalling pathway.

Mitochondrial membrane potential is lost during apoptin-induced cell death, and anti-apoptotic Bcl-2 family members block apoptin-induced apoptosis

To gain more insight into the mechanism of apoptotic signalling triggered by apoptin, we investigated the

mitochondrial/apoptosome-dependent apoptotic pathway. The mitochondrial pathway is mainly triggered by cell death signals that cause cellular stress. These signals frequently cause the loss of the mitochondrial membrane potential ($\Delta\Psi$). Thus, we studied the effect of apoptin on the mitochondrial membrane potential. The $\Delta\Psi$ was monitored by JC-1, a mitochondrial dye that shows decreased fluorescence when the mitochondrial membrane potential is lost. Cells treated with apoptin showed a significant loss of mitochondrial membrane potential when compared with the untreated cells (Fig. 3A). Furthermore, deficiency of either caspase-8 or FADD had no effect on the loss of mitochondrial membrane potential caused by apoptin. This experiment indicates that apoptin-triggered cell death is associated with the loss of mitochondrial membrane potential, and it further suggests that apoptin kills the cells independently of the death receptor pathway.

The intrinsic/mitochondrial cell death pathway is negatively modulated by the anti-apoptotic Bcl-2 family members. We therefore tested whether overexpression of Bcl-2 or Bcl-X_L affects the apoptin-triggered death pathway. As shown in Fig. 3B, both Bcl-2 and Bcl-X_L strongly inhibited apoptin-induced cell death, whereas about 80% of the parental Jurkat cells were killed by apoptin. To broaden the above conclusion, we tested the effect of apoptin on MCF7 human breast adenocarcinoma cells, which do not express caspase-3, as well as on a clone of MCF7 cells re-transfected with caspase-3 and MCF7 cells overexpressing Bcl-2. MCF7 cells showed significant sensitivity to TAT-apoptin, which was augmented by the expression of caspase-3 but significantly inhibited by Bcl-2 (Fig. 3C). Thus, these data confirm our previous observations, indicating that the mitochondrial death pathway is triggered by apoptin, as well

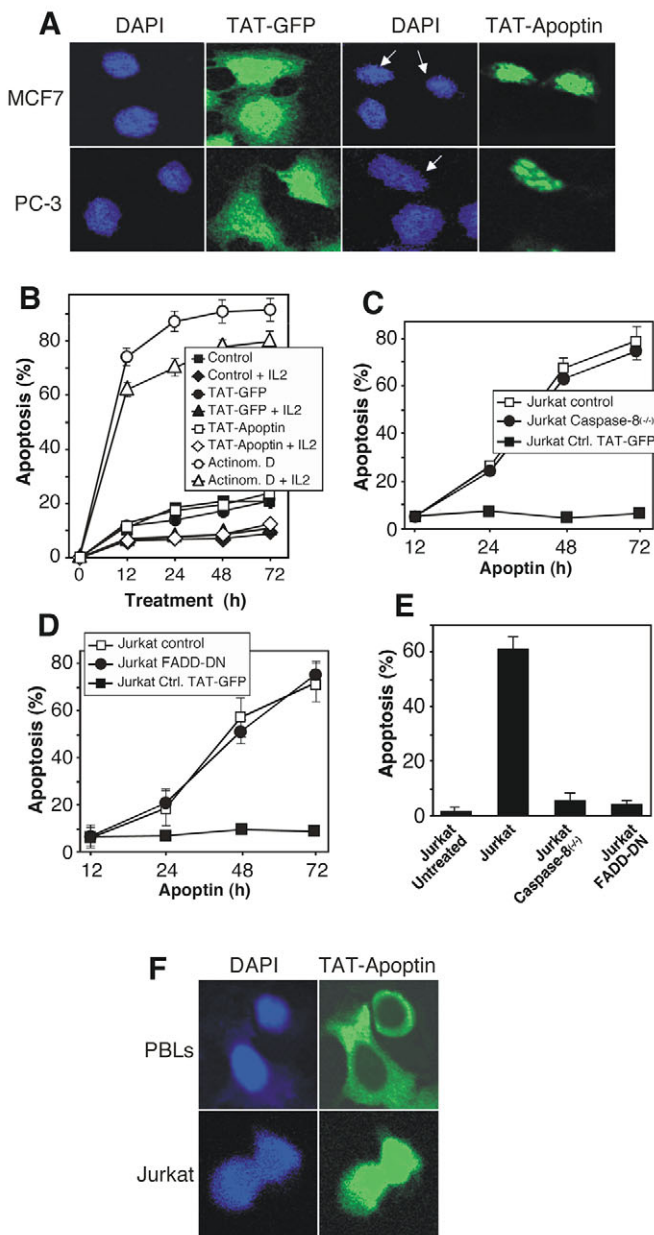


Fig. 1. Apoptin-induced cell death is independent of FADD and caspase-8. (A) MCF7 and PC-3 cells treated with recombinant TAT-GFP (1 μ M) or with recombinant TAT-apoptin (1 μ M), and counterstained with DAPI. TAT-GFP localizes both to the nucleus and to the cytoplasm, whereas TAT-apoptin is found almost exclusively in the nuclei (arrows). TAT-apoptin was detected using an anti-haemagglutinin-tag antibody; the tag is incorporated between TAT and the apoptin itself within the fusion protein. Cells treated with TAT-GFP show normal morphology, whereas some of the cells treated with TAT-apoptin show typical apoptotic morphology (cell shrinkage, nuclear condensation). (B) Apoptosis detection in peripheral blood mononuclear cells treated with recombinant TAT-GFP (1 μ M) or recombinant TAT-apoptin (1 μ M) for the indicated time, or with 5 μ g/ml Actinomycin D as a positive control. Some samples were also treated with IL-2 (5 U) in order to counteract the spontaneous apoptosis. (C,D) Measurement of apoptosis in Jurkat caspase-8^(-/-) cells (C), Jurkat cells expressing FADD-DN (D), and control cells treated with TAT-apoptin for the indicated time periods. To exclude solvent- or TAT-peptide related effects, some control cells were treated with the recombinant TAT-GFP (1 μ M) instead of TAT-apoptin treatment. (E) Apoptosis detection, measured by flow cytometry, in Jurkat cells either left untreated or treated with the agonistic anti-CD95 antibody (0.1 μ g/ml) for 8 hours. Treated Jurkat caspase-8^(-/-) cells and Jurkat cells expressing FADD-DN are also shown. The percentages of apoptotic cells (mean values of three independent experiments) are shown. (F) Jurkat and peripheral blood mononuclear cells (PBLs), isolated by Ficoll-PaqueTM PLUS gradient (Amersham Biosciences) treated with the recombinant TAT-Apoptin (1 μ M) for 12 hours. TAT-apoptin was detected by an anti-HA antibody, as in A. Cells are counterstained with DAPI.

as the previously published data (Danen-van Oorschot et al., 2000). It also shows that although caspase-3 contributes to the apoptin-triggered death pathway, it can be functionally replaced by other family members.

The mitochondrial components cytochrome c and AIF are released upon apoptin-triggered cell death

To further examine the effect of apoptin on mitochondria, we studied the release of various factors from the mitochondria. The apoptin-dependent release of cytochrome c from mitochondria has previously been reported in Saos cells (Danen-van Oorschot et al., 2000). This same group has also reported that the apoptin-dependent cytochrome c release, and the loss of mitochondrial membrane potential, cannot be blocked by expression of the p35 caspase inhibitor. In addition to cytochrome c, other signalling molecules such as AIF and endonuclease G are released from mitochondria in the presence of various apoptotic signals. Cytochrome c, along with dATP, Apaf-1 and caspase-9, forms an apoptosome complex leading to the activation of downstream caspases, including caspase-3 and caspase-7. By contrast, AIF does not require apoptosome formation to induce cell death. Thus, in

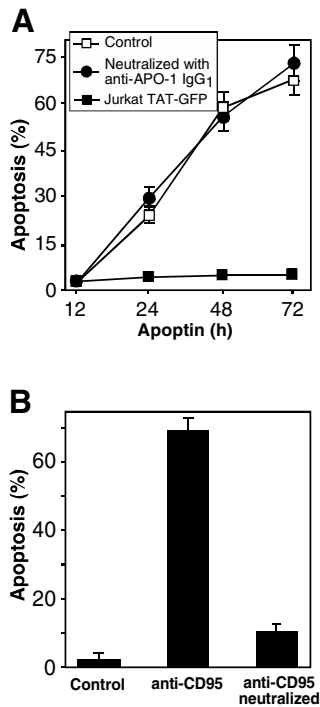


Fig. 2. Blocking of CD95/Fas has no effect on apoptin-induced cell death. (A) Flow cytometry analysis of apoptosis in Jurkat cells, either grown in control medium or incubated with a human anti-APO-1-IgG₁ (a neutralizing antibody that blocks the interaction of CD95/APO-1/Fas and the ligand), treated with TAT-apoptin for the indicated time points. To maintain the blockage, treatment with the blocking antibody was repeated every 8 hours. To exclude solvent- or TAT-peptide-related effects, some control cells were treated with recombinant TAT-GFP (1 μ M) instead of TAT-apoptin. (B) Control experiment to assess cell death mediated by CD95. Both control cells and the Fas-neutralized cells were treated with an anti-Fas (IgM) antibody, and cell death was measured after 8 hours.

order to gain further insight into the pathways triggered by apoptin, we compared the mitochondrial release of both molecules.

To check the release of cytochrome c from mitochondria upon apoptin-induced cell death, we separated various fractions of cells treated with TAT-apoptin and then detected the protein by western blot. As shown in Fig. 4A, after 36 hours

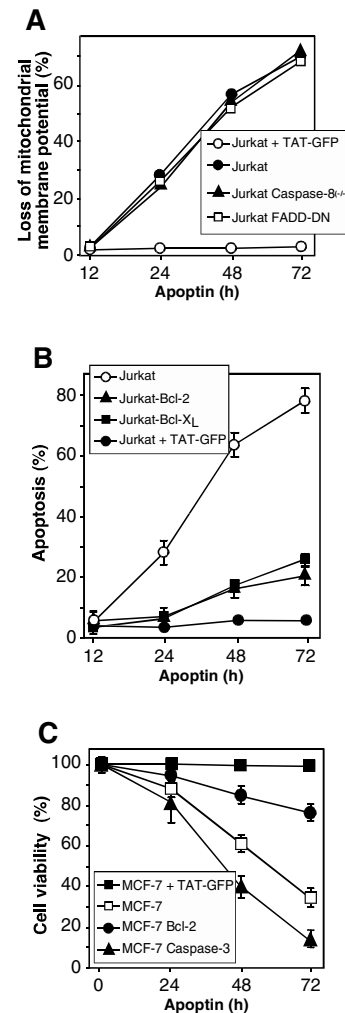


Fig. 3. Mitochondrial membrane potential is lost during apoptin treatment, and anti-apoptotic Bcl-2 family members block apoptin-induced cell death. (A) Mitochondrial membrane potential ($\Delta\Psi$ m) determination in Jurkat control cells, caspase-8^{-/-} Jurkat cells and FADD-DN Jurkat cells either left untreated or treated with TAT-apoptin for the indicated time periods. $\Delta\Psi$ m was determined by using a cationic carbocyanine dye, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), which shows a decrease in the red fluorescence upon loss of membrane potential. (B) Apoptosis (hypodiploid nuclei) detection, measured by flow cytometry, in Jurkat cells and clones stably transfected with either Bcl-2 or Bcl-X_L treated with TAT-apoptin (1 μ M) for the indicated time. (C) MCF7, MCF7-Bcl-2 and MCF7-caspase-3 breast cancer adenocarcinoma cells treated with TAT-Apoptin (1 μ M) for the indicated period of time assessed for cell viability by the MTT assay. To exclude solvent- or TAT-peptide-related effects, some control cells (A-C) were treated with recombinant TAT-GFP (1 μ M) instead of TAT-apoptin.

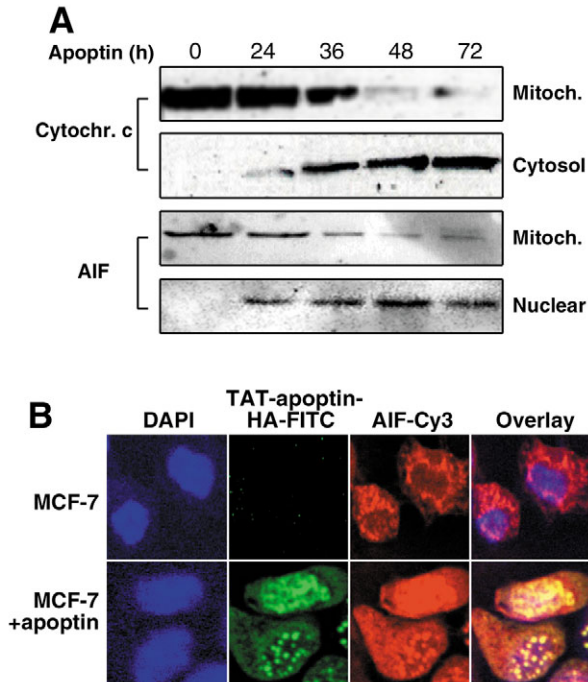


Fig. 4. Apoptin triggers the release of pro-apoptotic mitochondrial proteins. (A) Western blot analysis of cytochrome c and AIF in mitochondrial (Mitoch.), cytosolic (Cytosol) and nuclear fractions of Jurkat cells treated with apoptin. (B) The precise localization of AIF in cells, as determined by confocal microscopy. MCF7 cells were either left untreated or stimulated with TAT-apoptin (1 μ M). After 36 hours, cells were immunostained with an anti-AIF antibody followed by the corresponding Cy-3 conjugated secondary antibody (red) and with an anti-HA-antibody (TAT-apoptin has an incorporated HA-tag) followed by the detection with a FITC-labelled antibody (green). Cells were counterstained with DAPI to visualize nuclei.

of apoptin treatment, significant quantities of cytochrome c are detected in the cytosol.

In a similar way, we studied the release of AIF from mitochondria during apoptin-induced cell death. AIF, similarly to cytochrome c, starts to appear in the cytoplasm around 24–36 hours after apoptin treatment (Fig. 4A). However, unlike cytochrome c, AIF goes to the nucleus after it is released from mitochondria. In the nucleus, AIF causes ‘high-molecular-weight’ DNA fragmentation and chromatin condensation (Loeffler and Kroemer, 2000). Using confocal microscopy, we then investigated whether apoptin caused the nuclear accumulation of AIF. As shown in Fig. 4B, in the absence of apoptin, AIF is localized in mitochondria (Fig. 4B, red staining attributed to Cy3-conjugated mAb). However, when the cells are treated with TAT-apoptin, after 30 hours most of the AIF is released from mitochondria and transferred into the nucleus. Similar results were obtained after transfecting cells with GFP-apoptin (data not shown). Thus, our data indicate that, apart from cytochrome c, AIF is also released from mitochondria and enters the nucleus during apoptin-induced cell death.

Downstream effector caspases are activated during apoptin-induced cell death

To further investigate the role of the mitochondrial pathway

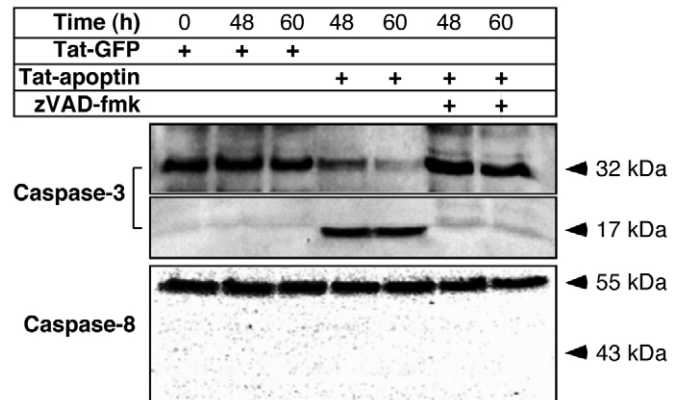


Fig. 5. Apoptin activates caspase-3 but not caspase-8. Western blot analysis of cell lysates from Jurkat cells treated with apoptin in the presence or absence of a caspase inhibitor zVAD-fmk (40 μ M) for 0, 48 and 60 hours to detect the levels of caspase-3 and caspase-8, and cleavage. Jurkat cells treated with TAT-GFP are shown as a control. The molecular sizes of pro-caspase molecules and the active fragments are indicated.

during apoptin-induced cell death, we studied the activation of the downstream effector caspases. Once cell death signals reach the mitochondria, cytochrome c is released to the cytosol where it triggers the apoptosome pathway, which further activates downstream caspases, such as caspase-3 and caspase-7. Thus, we checked the effect of apoptin on the downstream effector molecule caspase-3. Western blot analysis of Jurkat cells treated with apoptin shows that the 32 kDa pro-caspase-3 molecule is cleaved into an active 17 kDa subunit after 48 hours. The cleavage of pro-caspase-3 is inhibited in the presence of a broad range caspase inhibitor, zVAD-fmk. However, western blot analysis of pro-caspase-8 cleavage, which acts proximally at the death receptor pathway, shows no cleavage in the presence of apoptin (Fig. 5). Thus, these data further confirm that the intrinsic/mitochondrial death pathway, and not the death-receptor/extrinsic pathway, is triggered by apoptin.

Nur77 transmits the apoptin-induced death signal from the nucleus to the mitochondria

Phosphorylation-dependent nuclear retention of apoptin occurs only in transformed cells, and it plays an important role in the toxicity of apoptin. Yet, ultimately, the mitochondrial pathway becomes activated by apoptin. Nur77 and p53 are the best-known candidates for nucleo-cytoplasmic apoptotic signalling. Because it has been reported that apoptin-triggered cell death is p53 independent (Zhuang et al., 1995a), we tested whether Nur77, a nuclear orphan receptor and member of the steroid/thyroid receptor family, plays a role in the apoptin-induced pathway. Nur77 is capable of transmitting the apoptotic signal from the nucleus to the mitochondria (Erster and Moll, 2004; Jeong et al., 2003; Lane and Hupp, 2003; Li et al., 2000). Furthermore, Nur77 can modulate apoptosis by activating the transcription of pro- and anti-apoptotic genes (Rajpal et al., 2003). Inhibition of Nur77 expression in MCF7 cells by specific siRNA (Fig. 6A) strongly protected against apoptin-induced cell death (Fig. 6B). Furthermore, transfer of

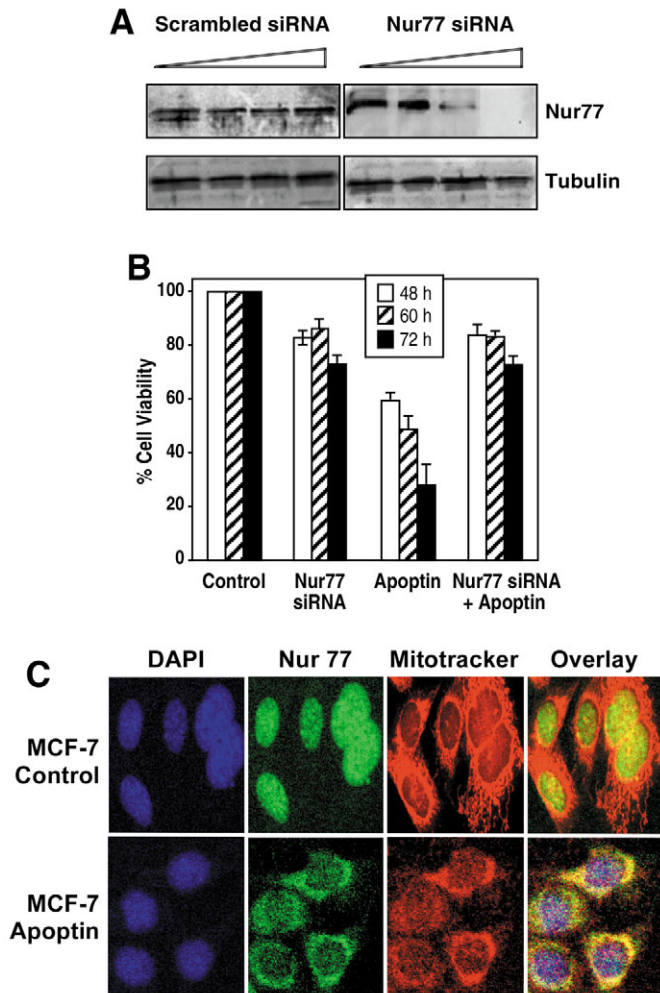


Fig. 6. Apoptin-induced cell death depends on Nur77, and involves its nucleo-cytoplasmic translocation. (A) Detection of Nur77 in MCF7 cells lipofected with scrambled or Nur77-targeting siRNA; protein was extracted at 0, 12, 24 and 30 hours post transfection. Tubulin was included as a loading control. (B) Cell viability of MCF7 cells treated with TAT-GFP (Control), transfected with Nur77 siRNA, or treated with TAT-apoptin (1 μ M) either alone or in the presence of Nur77 siRNA, as determined by MTT assay at the indicated time points. (C) Confocal microscopy visualization of Nur77 (green) in MCF7 cells either left untreated or treated with TAT-apoptin for 30 hours. Mitochondria are stained by Mitotracker® (red), nuclei with DAPI (blue).

Nur77 from the nucleus to the cytoplasm could be observed within 24–30 hours of the stimulation of MCF7 cells with TAT-apoptin (Fig. 6C). Together, these data indicate that Nur77 is an integral component, and a downstream signalling molecule, within the apoptin-triggered cell death pathway.

Discussion

The mechanism of cancer cell-specific apoptosis induction by apoptin is not known in great detail. Several anti-cancer agents use components of the death receptor machinery, such as FADD (Mishima et al., 2003), caspase-8 (Seki et al., 2000), CD95 (Fulda et al., 1998) and TRAIL receptors (Walczak and

Krammer, 2000; Wen et al., 2000), to kill cancer cells (reviewed by Wesselborg and Lauber, 2005). Furthermore, it has been reported previously that apoptin itself partially colocalizes with FADD in mammalian cells (Guelen et al., 2004). Using an array of cell lines that are either defective for or overexpress components of the apoptotic machinery, we have shown that neither caspase-8 nor FADD, both crucial DISC-components of pro-apoptotic TNF-R/NGF-R family members, play a role in apoptin-induced cell death. A number of anti-cancer drugs upregulate CD95L, which in turn may contribute to their anti-cancer activity in an autocrine or paracrine manner (Fulda and Debatin, 2005; Muller et al., 1997). Furthermore, some previous reports indicate that CD95 can still signal atypical apoptotic cell death in the absence of caspase-8 (Holler et al., 2000). Thus, by using an anti-CD95 mAb, (APO-1, IgG₁) that inhibits the interaction between CD95 and CD95L, we have shown that apoptin-induced cell death does not involve the CD95 receptor triggered by autocrine- or paracrine-delivered CD95L. Together, these data exclude a role of the TNF-R/NGF-R family death receptors in apoptin-induced apoptosis.

The mitochondrial/apoptosome death pathway is ultimately activated by a number of anti-cancer drugs and stress stimuli (Creagh and Martin, 2003; Del Bello et al., 2004; Renz et al., 2001). These stimuli frequently cause the loss of mitochondrial membrane potential, and the release of cytochrome c, AIF and other molecules (Antonsson and Martinou, 2000; Hill et al., 2003; Martinou et al., 2000). The mitochondrial/intrinsic death pathway is modulated by pro- and anti-apoptotic Bcl-2 family members (reviewed by Marsden and Strasser, 2003). Apoptin causes the loss of mitochondrial membrane potential both in wild-type cells and in clones devoid of functional caspase-8 or FADD. Apoptin causes the cleavage of caspase-3 and caspase-7, but not of caspase-8. These data show that apoptin kills cancer cells by activation of the mitochondrial death pathway and, furthermore, they show that the caspase-8/Bid-dependent signal-amplification loop is not important for apoptin-induced death.

Apoptin cell death signalling triggers the release of cytochrome c and AIF (which then translocates to the nucleus). Moreover, the anti-apoptotic Bcl-2 family members Bcl-2 and Bcl-X_L efficiently protect against apoptin-triggered apoptosis, thus indirectly confirming their involvement in the mitochondrial death pathway. These data are in contrast to previous reports by Noteborn and colleagues (Danen-Van Oorschot et al., 1999; Schoop et al., 2004; Zhuang et al., 1995b). A possible discrepancy regarding the effect of Bcl-2 family members on apoptin-induced cell death may be attributed to the different cell lines used in our study. Nevertheless, we have also observed a strong inhibition of apoptin-induced cell death in MCF7 breast cancer cell lines stably transfected with either Bcl-2 (Fig. 3C) or Bcl-X_L (data not shown). Thus, anti-apoptotic Bcl-2 family members seem to counteract apoptin-triggered cell death in at least some haematological malignancies and in breast adenocarcinoma cells. These data corroborate well with the experimental results reported here that support the involvement of the mitochondrial death pathway in apoptin-induced cell death.

The cancer-selective toxicity of apoptin, observed by us, and by a number of other groups (Danen-Van Oorschot et al., 1997; Guelen et al., 2004; Poon et al., 2005), is intriguing to

researchers, as this viral protein targets the molecular basis of the transformed phenotype. Targeted cancer therapies interfere with malignant cell growth and division at various points during the development, growth and spread of cancer. It has been proposed recently that apoptin kills cancer cells by interfering with the function of the anaphase-promoting complex (Teodoro et al., 2004). Interference with the final stages of mitosis certainly causes cellular stress that would most likely activate the mitochondrial death pathway. Furthermore, the toxicity towards (rapidly) dividing cells would at least in part explain the cancer-selective toxicity of apoptin. Because apoptin localizes in the cytoplasm of primary cells but accumulates in the nuclei of transformed cells, it is tempting to speculate that the selective nuclear localization in combination with the mitotic interference could largely explain the cancer-selective effects of apoptin. Recent papers indicate that nuclear trafficking and tumor-specific phosphorylation of apoptin at Thr-108 are essential for the induction of apoptosis (Guelen et al., 2004; Rohn et al., 2002). However, the data published by Rohn and colleagues indicate that although phosphorylation of apoptin is required for apoptosis, abolishing the Thr-108 phosphorylation site of apoptin does not significantly disrupt its nuclear input in tumor cells (Rohn et al., 2005). In addition, apoptin can be artificially directed to the nuclei of primary cells by the attachment of a nuclear localization signal. In such cases, apoptin still remains non-toxic to the primary cells (Danen-Van Oorschot et al., 2003; Guelen et al., 2004; Rohn et al., 2002) (S.M. and M.L., unpublished).

We have shown here that, in MCF7 cells, apoptin residing in the nucleus requires Nur77 for its toxicity. Furthermore, we have shown that Nur77 translocates to the cytoplasm between 24-30 hours after treatment with TAT-apoptin. Our data corroborate well with the recent finding (Lee et al., 2005) that Nur77, in concert with RXR α , translocates from the nucleus to mitochondria, and that it can directly activate the mitochondrial death pathway. It is unlikely that the previously proposed scenario of Nur77-mediated conversion of Bcl-2 from an anti-apoptotic to a pro-apoptotic molecule (Lin et al., 2004) is among mechanisms contributing to the toxicity of apoptin, as our experimental data clearly underline the protective role of both Bcl-2 and Bcl-X_L against apoptin-triggered cell death. Nevertheless, it cannot be excluded that Nur77 may exert its pro-apoptotic action by functionally inhibiting the anti-apoptotic properties of Bcl-2 and Bcl-X_L. Conversely, transcriptional activation of pro-apoptotic genes that are controlled by Nur77 cannot be ruled out either.

Thus, considering the above observations, and the rather slow and asynchronous death kinetics triggered by apoptin, we assume that apoptin employs several different mechanisms to kill cancer cells. These mechanisms and pathways ultimately cause cellular stress and converge on the mitochondrial death pathway.

In conclusion, apoptin induces apoptosis independently of the death receptor pathway, but it activates the intrinsic/mitochondrial apoptotic-signalling cascade. At the mitochondrial level, the apoptin-triggered signal(s) causes the release of various mitochondrial pro-apoptotic molecules that either directly propagate the apoptotic process or activate downstream molecules in the apoptotic signalling pathway. The nuclear localization of apoptin in transformed cells and the

ultimate activation of the mitochondrial death pathway suggests that cell death-activating signals must be sent from the nucleus to mitochondria. At least in our experimental system, the cytoplasmic translocation of Nur77 is crucial for the toxicity of apoptin. It remains to be determined whether Nur77 acts here solely as the signalling molecule, or whether it concomitantly activates the expression of pro-apoptotic genes that cooperate in the activation of the apoptotic pathways in cancer cells (Rajpal et al., 2003).

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