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c-IAP1 Blocks TNFα-Mediated Cytotoxicity Upstream of Caspase-Dependent and -Independent Mitochondrial Events in Human Leukemic Cells

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Tumor necrosis factor-α (TNFα) mediates cytochrome *c* release from mitochondria, loss of mitochondrial membrane potential ($\Delta\Psi$m) and apoptosis in sensitive leukemic cells. In the present study, by using the human leukemic U937 cell line, we demonstrate that the cytochrome *c* release is caspase-8-dependent and can be blocked by an inhibitor of caspase-8, Z-Ile-Glu (OMe)-Thr-Asp(OMe)-fluoromethyl ketone (Z-IETD.fmk), or a pan caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD.fmk). However, TNFα-mediated loss of $\Delta\Psi$m was not inhibited by caspase inhibitors. The apoptotic process was blocked by either Z-IETD.fmk or Z-VAD.fmk in cells with lower $\Delta\Psi$m. U937 cells with stable transfection of the cellular inhibitor of apoptosis protein 1 (c-IAP1) are resistant to TNFα-induced activation of caspases, Bid cleavage, cytochrome *c* release and $\Delta\Psi$m collapse. In addition, both c-IAP1 and XIAP were not up-regulated upon prolonged exposure to TNFα. In contrast, there was a caspase-dependent cleavage of XIAP, but not c-IAP1, during treatment with TNFα for 7 days. These results demonstrate that c-IAP1 blocks TNFα signaling at a level controlling both activation of caspase-8 and a signal to cause loss of $\Delta\Psi$m. The sensitive U937 cell line failed to acquire resistance and gain a self-protecting advantage against apoptosis, upon induction of c-IAP1 expression.

Key Words: **apoptosis; caspases; cytochrome *c;* inhibitors of apoptosis proteins (IAPs); mitochondrial membrane potential ($\Delta\Psi$m).**

The binding of tumor necrosis factor-α (TNFα) to the extracellular domains of the TNF receptor-1 (TNFR-p55) initiates either apoptosis or activation of the transcription factor NF-κB (1, 2). Ligation of this receptor leads to oligomerisation and subsequent recruitment of cytosolic adapter and effector proteins to the receptor complex, which consists of the Fas-associated death domain (FADD) and the TNF receptor-associated domain (TRADD), and forms the death-inducing signaling complex (DISC). FADD, then, binds to the upstream initiator molecule procaspase-8 and initiates the apoptotic signaling cascade (3, 4).

It has been reported that TNFα mediates mitochondrial multiple alterations, such as cytochrome *c* release (5–7), loss of mitochondrial membrane potential ($\Delta\Psi$m) (8), inhibition of mitochondrial electron transport chain enzyme activity (9), and generation of reactive oxidative species (ROS). It has been suggested that TNFα-induced mitochondrial multiple dysfunctions are mediated either by the irreversible opening of the mitochondrial permeability transition pore (PT) (8) or the caspase-8-mediated Bid cleavage (6). TNFα-mediated cytochrome *c* release from mitochondria and a loss of $\Delta\Psi$m have been considered to be separate events (5, 6). Neither anti-apoptotic Bcl-2 family protein, such as Bcl-2/Bcl-xL, nor inhibitor of caspases, such as benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD.fmk) alone can block all mitochondrial alterations-mediated by TNFα (6, 10, 11). This indicates that TNFα-mediated signaling is diverged upstream of mitochondria.

Cell lines can respond differently to anti-CD95/Fas antibody or TNF-related apoptosis-inducing ligand (TRAIL) treatment and therefore they are divided into Type I and Type II cells. In Type I cells, the induction of apoptosis is via the extrinsic pathway, e.g., by the activation of caspase-8, followed by the rapid cleavage of caspase-3 prior to loss of $\Delta\Psi$m and release of cytochrome *c.* In contrast, in Type II cells the loss of $\Delta\Psi$m or release of cytochrome *c* precedes the activation of

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both caspase-8 and caspase-3 (12, 13). The pathways activated in response to TNFα-induced apoptosis are still unclear.

It has recently been discovered that the Inhibitors of Apoptosis Proteins, c-IAP1, c-IAP2 and XIAP interfere with the transmission of intracellular death signals, by inhibiting the caspase-dependent apoptotic pathways (14–16), arrest the cascade of proteolysis and therefore provide protection from apoptosis. IAPs bind to and potentially inhibit the activation of caspase-3 (17, 18) and -7 (15, 19) and therefore block the apoptotic process. In contrast, these IAP family proteins could not prevent caspase-8-induced proteolytic activation of procaspase-3. However, they subsequently inhibited active caspase-3 directly (17, 18). The inhibitory effect of c-IAP1 and c-IAP2 on activation of caspase-3 is weaker than XIAP (17). This implies that c-IAP1 and c-IAP2 are less effective for blocking activation of downstream caspases. Although it is known that TRADD, TNF-receptor-2 associated factor (TRAF2) and c-IAP1 are recruited to the TNF receptor-1 complex after TNFα treatment (2), the inhibitory effect of c-IAP1 on TNFα-mediated signaling has not been widely investigated.

In this study, we found that TNFα-induced apoptosis in the U937 cell line can be inhibited by c-IAP1 and inhibitors of either caspase-8 or pan caspases. These caspase inhibitors diminish TNFα-mediated cytochrome *c* release, but not the loss of $\Delta\Psi$m. U937 cells with stable transfection of c-IAP1 are resistant to TNFα-induced processing of caspase-8, Bid and caspase-3. Both cytochrome *c* release and loss of $\Delta\Psi$m, induced by treatment with TNFα, are blocked in cells over-expressing c-IAP1. We demonstrate that c-IAP1 blocks TNFα-initiated signaling upstream of the apical caspase-8 and mitochondria.

MATERIALS AND METHODS

Materials. The human recombinant TNFα, 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), mouse anti-β-actin antibody and all chemicals were purchased from Sigma (Dorset, UK). *Escherichia coli* DH5α strain, DMRIE-C reagent, serum-free OPTI-MEM medium and Geneticin (G418 sulphate) were purchased from GIBCO-BRL (West Sussex, UK) and the Plasmid DNA MiniPrep Kit from QIAGEN (Valencia, CA). Z-Ile-Glu (OMe)-Thr-Asp(OMe)-fluoromethyl ketone (Z-IEDT.fmk) was purchased from Alexis Biochemicals (Nottingham, UK) and Z-VAD.fmk from Promega (Southampton, UK). 3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and Mitotrack red CMXRos were purchased from Molecular Probes (Leiden, The Netherlands). Rabbit anti-caspase-3 antibody was from Stressgen-Bioquote (North Yorkshire, UK). Polyclonal c-IAP1 and Bid antibodies were supplied by R & D Systems (Oxon, UK) and monoclonal anti-XIAP antibody (clone 48) from BD Transduction Laboratories (San Diego, CA). Monoclonal caspase-8 antibody (clone 12F5) was from Alexis Biochemicals. T7-horseradish peroxidase (HRP)-conjugated monoclonal antibody was obtained from Novagen (Madison, WI) and the Native anti-cytochrome *c* antibody (clone 6H2.B4) was purchased from BD Pharmingen (San Diego, CA). SuperSignal enhanced chemiluminescence (ECL) was from Pierce (Rockford, IL).

Cell culture and gene transfection. Human U937 leukemia cells were cultured in the presence of RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator. The full-length T7-tagged c-IAP1 (GenBank Accession No. U37547, MIHB) pcDNA3 plasmid was amplified in *E. coli* DH5α strain and was purified by using a Plasmid DNA MiniPrep Kit. Four micrograms plasmid DNA was transfected into 2×10^6 cells using DMRIE-C reagent in serum-free OPTI-MEM medium. After culturing at 37°C for 5 hours, cell culture condition was recovered by the addition of 15% FCS containing RPMI-1640 medium. After 48 h of transfection, transfectants were selected in the presence of 0.8 mg/ml Geneticin for one month and then further selected by single cell cloning in a 96-well plate. U937/c-IAP1 transfectant, which over-express c-IAP1-pcDNA3-T7, were determined by dot blot using T7-HRP-conjugated antibody.

Apoptosis assays. Cell (10^5) suspension were laid onto a Superfrost slide, air-dried and fixed with acetone/methanol (1:1, v/v) for 10 min at room temperature. Cells were stained with 50 ng/ml DAPI. Nuclear morphology was viewed by the fluorescent microscopy. Intact nuclei showed uniform distribution of chromatin. Upon induction of apoptosis, chromatin condensation was observed accompanied by beading to multiple spherical particles and followed by disintegration of the nucleus. The percentage of apoptotic cells was calculated by counting 100 cells in randomly selected fields. Alternatively, apoptotic cells were also analyzed by flow cytometry. Briefly, cells were permeabilised with 70% ethanol and stained with 100 μg/ml PI. Flow cytometric analysis was carried out on cells gated on an integral channel versus peak channel displayed to exclude cells debris and clumped cells using a FACScan flow cytometer (Becton Dickinson, Oxford, UK) and analyzed with the 'Cell Quest' program.

Assessment of $\Delta\Psi m$ by flow cytometry. Cells were treated with TNFα (10 ng/ml) for 2 and 4 h. A lipophilic and positively charged fluorescent dye was used to evaluate the mitochondrial transmembrane potential (20). Cell Suspension (2×10^5 cells) was incubated with 80 nM $DiOC_6(3)$ at 37°C for 15 min and analyzed by a FACScan flow cytometer.

Immunofluorescence analysis of cytochrome c release. To colocalize cytochrome *c* in mitochondria, intact cells were first labeled with the mitochondrion specific dye, MitoTracker red CMXRos. Cells in culture medium were incubated with MitoTracker red CMXRos (100 nM) at 37°C for 30 min. Cells were washed twice with Ca^{2+}/Mg^{2+}-free PBS and resuspended in 10% FCS containing culture medium. One hundred fifty microliters cell suspension (5×10^5 cells/ml) was laid onto a microscope slide. Slides were air-dried, permeabilised and fixed in acetone/methanol (1:1, v/v) solution for 15 min. Cells were then washed for three times in PBS and incubated with the anti-cytochrome *c* antibody 6H2.B4 for 1 h at room temperature in a humidified chamber. Cells were washed in 0.1% Tween 20 containing PBS (PBST) and then incubated with the FITC-conjugated anti-mouse secondary antibody. Cells were rinsed three times in PBST and stained with 50 ng/ml DAPI. Slides were dried at 4°C in the dark. ImmunoMount solution (Shandon) was added and viewed under a Zeiss Axioskop fluorescence microscope (Zeiss, Germany) attached to a CCD camera (Photometric Ltd. Arizona, U.S.A.) driven by IPLLabs Spectrum and SmartCapture (Cambridge, UK) software. The filter wheel was set at Texas red (excitation 540–580 nm/emission 600–660 nm), fluorescein (excitation 465–495/emission 515–555 nm), and DAPI (excitation 310–380 nm/emission 435–485) (5).

Western blotting. Proteins (50 μg) from whole cells were subjected to standard SDS–PAGE at 20–40 mA/gel and transferred onto PVDF membrane (Sigma). The membranes were blocked with 5% filtered nonfat milk in PBST for 1 h at room temperature, and then probed with specific primary antibody (indicated individually in the figure legends) for 1 h at room temperature. Bound antibodies were further probed using appropriate HRP-conjugated secondary anti-

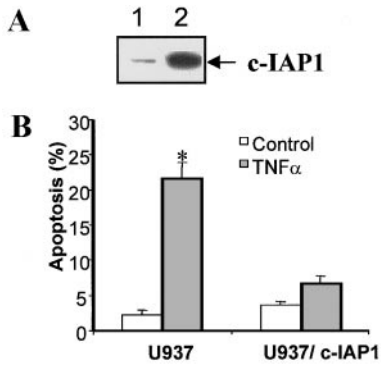

FIG. 1. Inhibitory effect of c-IAP1 on TNFα-induced apoptosis. (A) Expression of c-IAP1. Fifty micrograms of protein lysate was loaded to 10% SDS–PAGE. Polyclonal c-IAP1 antibody was used at 1:1000 dilution for the c-IAP1 expression. Lane 1 represents the U937 cells and lane 2 is the U937/c-IAP1 cells. (B) TNFα-induced apoptosis. Percentages of apoptosis in both resting states and TNFα-treated cells were counted on DAPI stained cells and confirmed by flow cytometry. Data shown are from three independent experiments. The significantly decreased apoptosis was analysed by *t* test. $^*P < 0.001$.

bodies for 1 h followed by detection using SuperSignal ECL for 2 min. Longer exposure was used to detect weaker cleavage bands. The density of each band was analyzed using an AlphaImager 2000 Densitometer (Alpha Innotech Corp., San Jose, CA).

RESULTS

c-IAP1 Blocks TNFα-Induced Apoptosis by Inhibition of Cleavage of Caspase-8, Bid, and Caspase-3

To evaluate the inhibitory effect of c-IAP1 on TNFα-induced apoptosis in the U937 cell line, full-length c-IAP1 gene stable transfectant, named the U937/c-IAP1 cell line was developed by transfecting T7-tagged-c-IAP1-pcDNA3 plasmid DNA into U937 cells and selected by G418 sulphate. Up-regulation of c-IAP1 protein levels (about 17 times) in the U937/c-IAP1 cell line was detected by Western blot analysis (Fig. 1A). To assess the inhibitory effect of c-IAP1 on TNFα-induced apoptosis, cells were incubated with or without 10 ng/ml TNFα for 4 h. TNFα-induced apoptosis was observed by apoptotic morphology on DAPI stained cells using a fluorescence microscope and confirmed by flow cytometry on ethanol fixed/PI stained cells. TNFα-induced apoptosis in the U937/c-IAP1 cell line was significantly inhibited compared to the U937 parental cells ($P < 0.001$). In fact, there was no significant difference in apoptosis between the resting cells and TNFα-treated U937/c-IAP1 cells ($P > 0.05$, Fig. 1B).

We then studied whether the anti-apoptotic protein c-IAP1 could inhibit TNFα-induced cleavage of caspases. Cells were treated with 10 ng/ml TNFα for 2 and 4 h. TNFα-induced processing of procaspases was detected in U937 cells by Western blot analysis. Decreased protein bands of procaspase-8 (55/57 kDa) and procaspase-3 (34/32 kDa) were seen in the U937 cell line after treatment with TNFα for 2 and 4 h (Fig. 2A). The cleavage bands of caspase-8 and -3 were detected at 4 h. Similarly, the decline in the protein band of Bid, a substrate of caspase-8, was detected in the U937 cells in response to TNFα (Fig. 2A). TNFα-induced processing of procaspase-8, -3 and Bid was completely blocked in cells over-expressing c-IAP1 (Fig. 2B). These results indicate that c-IAP1 blocks TNFα-induced apoptosis by inhibiting the activation of procaspase-8 and -3.

Comparison of the Effect of c-IAP1 with Inhibitors of Caspases on TNFα-Induced Mitochondrial Alterations

Accumulating evidence indicates that the signal transduction after engagement of various cell surface death receptors may proceed via a common pathway requiring efficient recruitment and activation of the caspase-8, which is the most apical caspase. The c-IAP1-mediated resistance of U937 cells to TNFα was therefore compared with effects of the specific inhibitor of caspase-8, Z-IETD.fmk, or a pan caspase inhibitor, Z-VAD.fmk. U937 cells were pre-treated with either 100 μM Z-IETD.fmk or 100 μM Z-VAD.fmk for 2 h

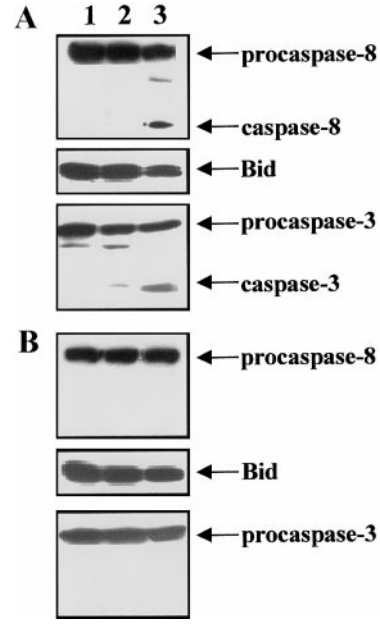

FIG. 2. TNFα-induced cleavage of procaspases and Bid. The U937 (A) and U937/c-IAP1 (B) cells were treated with TNFα. Cell lysates were prepared at 0 (lane 1), 2 (lane 2) and 4 h (lane 3), respectively. Fifty micrograms of protein was loaded to 10% SDS–PAGE. Antibodies for caspase-8 and Bid were used at 1:1000 dilution and antibody for caspase-3 was used at 1:5000 dilution. HRP-conjugated anti-mouse antibody (Santa Cruz) was used at 1:2000 and anti-rabbit antibody (Santa Cruz) was at 1:3000 dilutions. The Bid cleavage band did not appear because the anti-Bid antibody only recognises the pro-protein band.

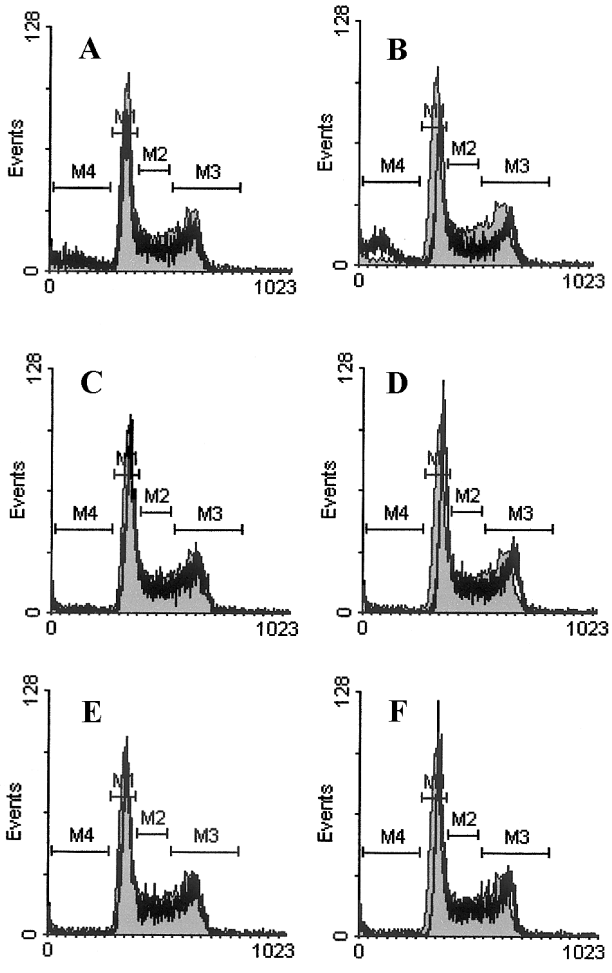

FIG. 3. Inhibitory effect of Z-IETD.fmk or Z-VAD.fmk on TNFα-induced apoptosis. Flow cytometric analysis was performed on ethanol permeabilised and PI stained cells. Cell cycle phases were G0/G1 (M1), S (M2), G2/M (M3) and apoptosis (M4). The solid profile represents cells without TNFα treatment. The line profile shows cells treated with TNFα. (A and B) U937 cells, (C and D) cells treated with 100 μM Z-IETD.fmk; (E and F) cells treated with 100 μM Z-VAD.fmk. (A, C, and E) Cells incubated for 2 h with or without TNFα; (B, D, and F) correspond to 4 h treatment. Data presented show representative results of one of the independently performed experiments.

and then incubated with TNFα up to 4 h. Similarly to c-IAP1, TNFα-induced apoptosis was completely inhibited by either Z-IETD.fmk or Z-VAD.fmk as analyzed by flow cytometry (Fig. 3). This indicates that the inhibition of caspase-8 is sufficient to abrogate TNFα-mediated apoptosis in the U937 cell line.

TNFα-induced cleavage of Bid and the inhibition of c-IAP1 on Bid cleavage were shown in the Fig. 2. We, therefore, tested whether TNFα induces cytochrome *c* release and whether this is correlated with the activation of caspase-8. TNFα-mediated cytochrome *c* release from mitochondria was analyzed by immunostaining. Mitochondria in living cells were first stained with a red mitochondria-selective vital dye, MitoTrack, and then cytochrome *c* was stained green. Cytochrome *c* in control cells localized to mitochondria, as red and green merged pixels appeared orange/yellow (Figs. 4A and 4C). After exposure to TNFα for 3 h, cytochrome *c* release was observed in U937 cells, as shown by the green-labeled cytochrome *c* image separated from the mitochondrial location with a clearly diffuse pattern (Fig. 4B). Cells over-expressing c-IAP1 failed to release cytochrome *c* in response to TNFα (Fig. 4D), as shown cytochrome *c* localization was similar to its control (Fig. 4C). As expected, TNFα-induced cytochrome *c* translocation was also inhibited in the U937 cell line after pre-treatment with either 100 μM Z-IETD.fmk (Fig. 4E) or 100 μM Z-VAD.fmk (Fig. 4F). This suggests that TNFα-mediated cytochrome *c* release is caspase-8 dependent.

The association between cytochrome *c* release and loss of $\Delta\Psi$m in response to TNFα is debated. The reduction of $\Delta\Psi$m can be either a cause or a consequence of cytochrome *c* release (10, 21). As we have found that TNFα-induced cytochrome *c* release was diminished by c-IAP1 or inhibitor of caspase-8, $\Delta\Psi$m collapse in U937 cells was observed after treatment with TNFα for 2 h (Fig. 5A) and a substantial $\Delta\Psi m^{Low}$ cells appeared after 4 hours exposure (Fig. 5B). We reasoned that if the reduction of $\Delta\Psi$m is a consequence of either cytochrome *c* release or activation of caspases, TNFα-mediated loss of $\Delta\Psi$m should also be inhibited by c-IAP1, Z-IETD.fmk or Z-VAD.fmk. Surprisingly, only c-IAP1 showed complete inhibition on the reduction of $\Delta\Psi$m, as it was shown that TNFα failed to induce $\Delta\Psi$m collapse in U937/c-IAP1 cells (Figs. 5C and 5D). Although there was a preventing effect by either 100 μM Z-IETD.fmk or 100 μM Z-VAD.fmk at 2 hours time point, the reduction of $\Delta\Psi$m after 4 hours exposure to TNFα was not inhibited by both caspase inhibitors (Figs. 5F and 5H). We therefore propose that TNFα-induced loss of $\Delta\Psi$m is caspase-independent. The c-IAP1 blocks apoptosis at an apical point upstream of caspase-8 and controls signals responsible for both cytochrome *c* release and loss of $\Delta\Psi$m.

Effect of c-IAP1 on UV Light-Induced Apoptosis

UV light-induced apoptosis is induced via the cytochrome *c*/Apaf-1/caspase-9 pathway after DNA damage (22). IAPs inhibit cytochrome *c*-dependent apoptosis by binding to the activated caspase-9 or caspase-3. To confirm whether c-IAP1 plays a role downstream of cytochrome *c*, we stimulated both U937 parental and U937/c-IAP1 cells with UV light for 5 min and then further culture for 4 h. As shown in Fig. 6, c-IAP1 failed to protect cells against UV light-induced apoptosis. This suggests that c-IAP1 has less inhibitory effect on downstream caspases in the U937 cell line.

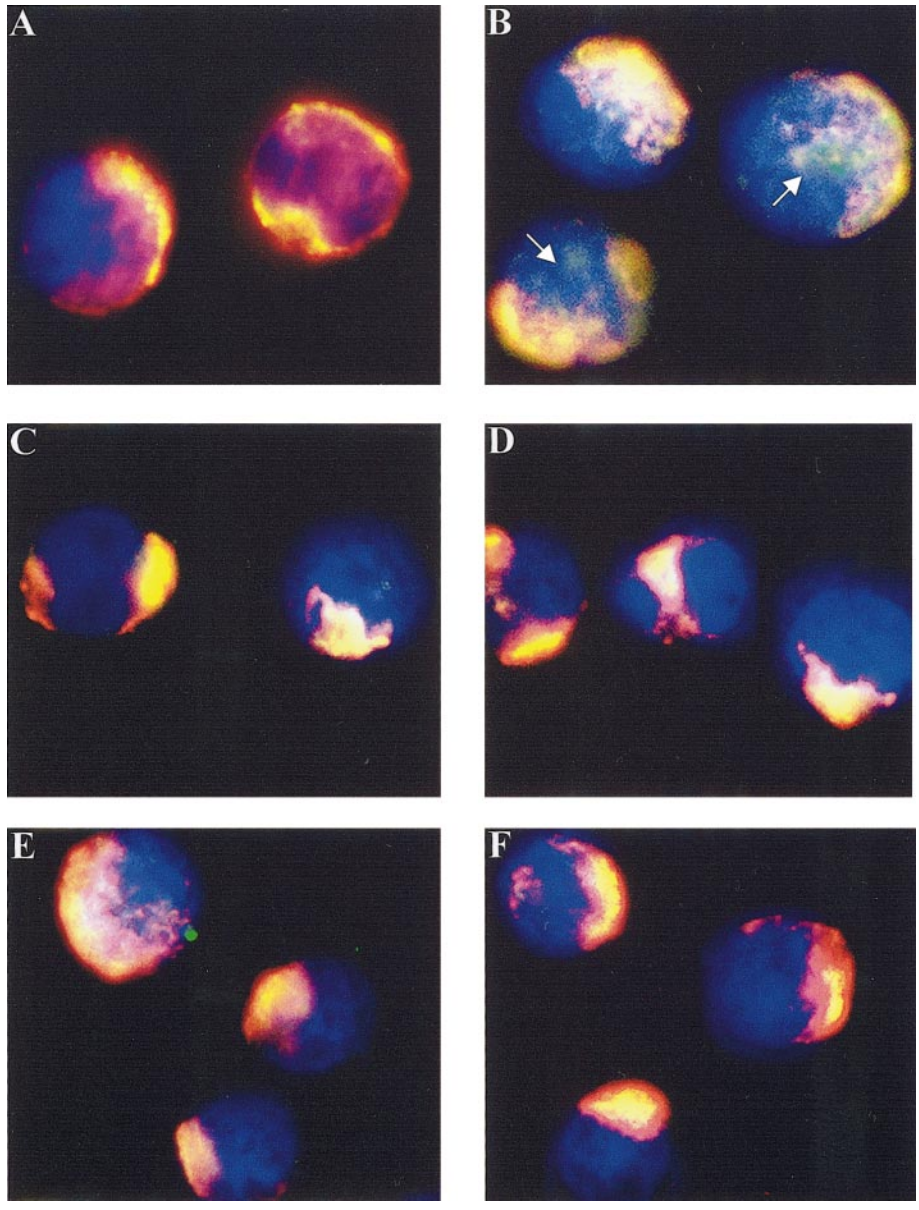

FIG. 4. Inhibition of TNFα-induced cytochrome *c* release by c-IAP1, Z-IETD.fmk or Z-VAD.fmk. Cells were labelled with MitoTrack, fixed, permeabilised and stained with native anti-cytochrome *c* antibody and DAPI (50 ng/ml). The stained cells were examined by fluorescence microscopy. Cytochrome *c* antibody was visualized with a FITC-conjugated anti-mouse IgG and assigned the green color, whereas, mitochondria labelled with MitoTrack were assigned the red color. In the control U937 cells (A), U937/c-IAP1 cells treated with (D) or without (C) TNFα, U937 cells pretreated with Z-IETD.fmk (E) or Z-VAD.fmk. (F) and TNFα, red and green images were merged, overlapping red and green pixels appear orange/yellow. U937 cells treated with TNFα alone (B) displayed diffusion of cytochrome *c* into cytosol.

Regulation of c-IAP1 and XIAP Proteins by TNFα

It has been reported that IAPs can be regulated during apoptosis (23, 24). To study whether c-IAP1 and XIAP could be regulated in U937 cells during long-term exposure to TNFα, U937 cells were continuously exposed to 4 ng/ml TNFα for 7 days. Similar levels of apoptosis were induced in the presence of 4–10 ng/ml TNFα (data not shown). Cells were harvested every 24 h and the protein levels of c-IAP1 or XIAP and their relationship with the activation of caspase-3 was detected by Western blot analysis and analyzed by densitometry. The c-IAP1 protein levels were dramatically decreased at 3 days in the presence of TNFα, but gradually increased thereafter, achieving the original levels after 7 days of exposure (Figs. 7A and 7B). XIAP protein levels gradually decreased in time and reached the lowest levels after 6 days of exposure to TNFα. However, the remarkably increased XIAP levels were seen at the 7 days time point (Figs. 7C and 7D). The decreased full-length XIAP protein band (53-kDa) was

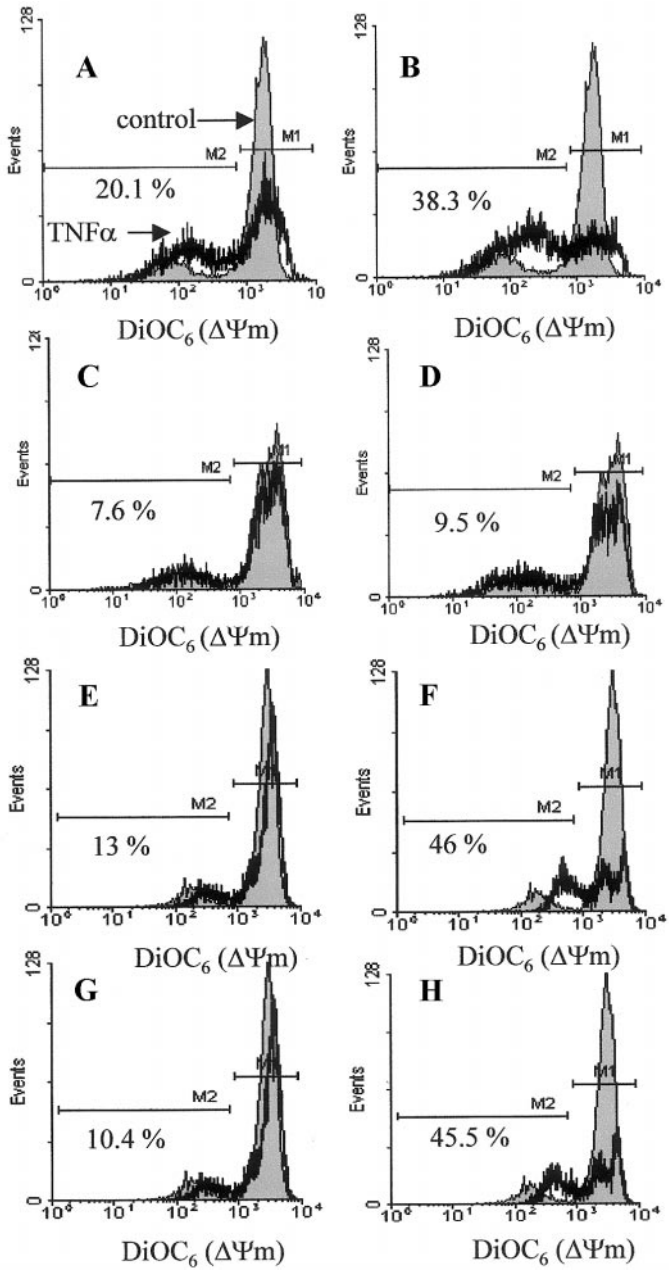

FIG. 5. Inhibition of TNFα-induced loss of $\Delta\Psi$m by c-IAP1, Z-IETD.fmk or Z-VAD.fmk. (A and B) U937 cells treated with TNFα only. (C and D) U937/c-IAP1 cells. (E and F) U937 cells pretreated with 100 μM Z-IETD.fmk. (G and H) U937 cells pretreated with 100 μM Z-VAD.fmk. After incubation with (line profile) or without (solid profile) 10 ng/ml TNFα for 2 h (A, C, E and G) and 4 h (B, D, F and H), cells were incubated with 80 nM $DiOC_6(3)$ for 15 min at 37°C and analysed by flow cytometry (FACScan) at FH1-L channel. Histograms show fluorescence intensity (x-axis) of $DiOC_6(3)$ for 15 min at 37°C and analysed by flow cytometry (FACScan) at FH1-L channel. Histograms show fluorescence intensity (x-axis) of $DiOC_6(3)$ versus cell number (y-axis). Cells were gated as two populations, M1 is cells with high $\Delta\Psi$m ($\Delta\Psi m^{HIGH}$) and M2 is cells with low $\Delta\Psi$m ($\Delta\Psi m^{LOW}$).

associated with the appearance of a 29-kDa fragment (BIR-3-Ring region). We investigated whether the regulation of IAPs is correlated with the activation of caspase-3. TNFα-induced cleavage of procaspase-3 was analyzed simultaneously. The procaspase-3 levels were continuously decreased for 5 days and increased slightly thereafter (Figs. 7E and 7F). The procaspase-3 cleavage was correlated with the decreased full-length XIAP ($P < 0.05$, r = 0.839) but not with c-IAP1 ($P > 0.05$, r = 0.369).

DISCUSSION

In this study, we demonstrated that c-IAP1 blocks TNFα-mediated apoptosis in leukemic U937 cells by inhibiting both the activation of the apical caspase-8 and mitochondrial depolarization. C-IAP1 is less effective than XIAP in the inhibition of downstream of caspases (18, 19). So, the inhibitory effect of c-IAP1 on the apoptotic pathway has attracted less interest than XIAP. In agreement with others, our data showed that U937 cells over-expressing c-IAP1 did not acquire resistance to the DNA damaging agent, UV light.

An early event triggered by death receptor signaling is caspase-8-mediated cleavage of the pro-apoptotic Bcl-2 family member Bid (25, 26). After cleavage, truncated Bid translocates to the mitochondria and induces cytochrome *c* release, ultimately resulting in the activation of effector caspases. We found that the activation of caspase-8 and cleavage of Bid occurred at an early time point in response to TNFα treatment and inhibition of caspase-8 by either Z-IETD.fmk or Z-VAD.fmk blocked TNFα-induced cytochrome *c* release and subsequently apoptosis. This indicates that TNFα-induced cytochrome *c* release is a consequence of procaspase-8 processing and Bid cleavage. However, loss of $\Delta\Psi$m was not blocked by the inhibition of caspases in the U937 cells. It was also found that TNFα-induced dissipation of $\Delta\Psi$m and activation of caspases in U937 and HL60 cell lines were separate events (27).

Unlike TNFα, TRAIL-induced both cytochrome *c* release and loss of $\Delta\Psi$m are caspase-dependent.

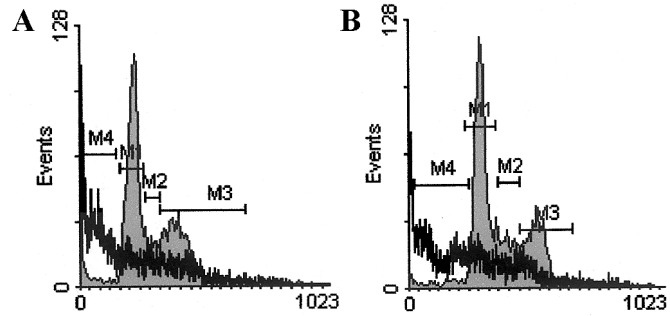

FIG. 6. UV light-induced apoptosis. UV light-induced apoptosis on U937 parental cells (A) and U937/c-IAP1 cells (B) was analyzed by flow cytometry on ethanol fixed and PI stained cells. Solid profile indicates untreated control and line profile shows UV light-treated cells.

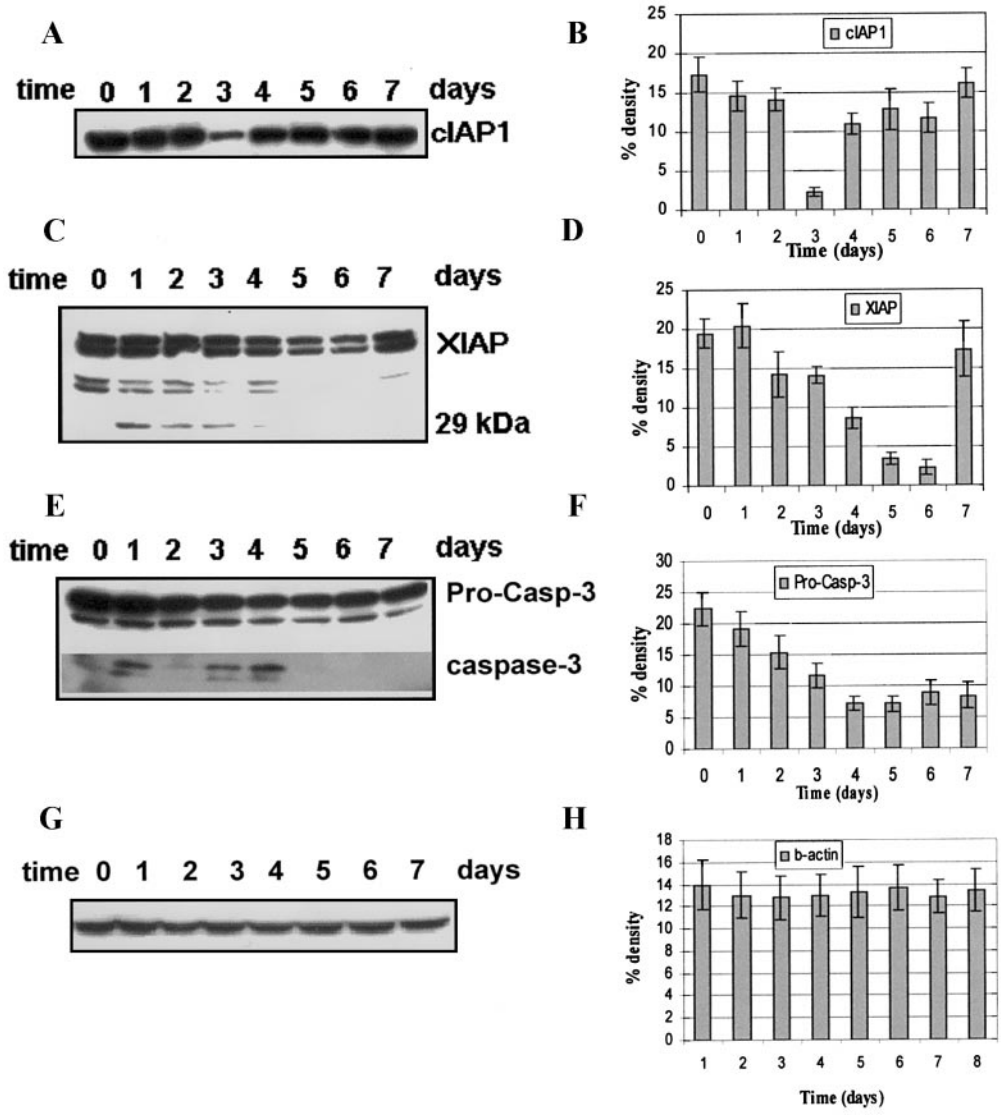

FIG. 7. The association of IAP expression and activation of caspase-3. U937 cells were incubated with TNFα (4 ng/ml) for 7 days. Fifty micrograms protein lysates were subjected to 10% SDS–PAGE. The polyclonal anti-c-IAP1 antibody was used at 1:1000 dilution. The monoclonal anti-XIAP antibody was used at 1:2500 dilution. The polyclonal anti-caspase-3 antibody was used at 1:5000 dilution. The monoclonal anti-β-actin antibody was used at 1:5000 dilution. (A) Western blot for c-IAP1, (C) for XIAP, (E) for caspase-3 and (G) for β-actin. The relative intensity of protein band was analyzed by the densitometry and represented in B (c-IAP1), D (XIAP), F (caspase-3) and H (β-actin). Western blots shown are the typical imaging from three independent experiments. Data shown in bar charts are mean $\pm$ SD analyzed from three blots.

Z-VAD.fmk (100 μM) completely inhibited TRAIL-induced both cytochrome *c* release and mitochondrial depolarization (13). The mechanism of TNFα-mediated mitochondrial damage is unclear. We have previously reported that the inhibition of the mitochondrial electron transport chain enzyme activity occurred after exposure to TNFα for 15 minutes (9). However, TNFα-mediated cytochrome *c* release became apparent after 2 hours treatment and was independent of mitochondrial depolarization (5). It is known that cytochrome *c* release from mitochondria is correlated with caspase-8-dependent cleavage of Bid in Type I cells (11, 13, 28–30). Bid-induced cytochrome *c* release can be mediated by a pathway independent of mitochondrial PT (31). Other reports suggested that opening of PT leads to mitochondrial depolarization and cytochrome *c* release (21, 32). The present data indicate that TNFα-mediated loss of $\Delta\Psi$m and cytochrome *c* release, are indeed separate events in U937 cells. Cells with low $\Delta\Psi$m can escape from TNFα-induced apoptosis, upon blockage of activation of caspases by Z-IETD.fmk or Z-VAD.fmk. This implies that the mitochondrial depolarization may be not crucial for TNFα-mediated apoptosis in U937 cells.

The major finding of this study is that the TNFα-mediated signaling pathway is diverged at a point upstream of the apical caspase-8. From this point, one pathway is via caspase-8/Bid/cytochrome *c* and caspase-8/caspase-3. Another one, which is unclear, leads to loss of $\Delta\Psi$m or PT. Therefore, it could not be determined whether the U937 cell line belongs to type I or type II cells, using TNFα as a stimulus. The reduction of $\Delta\Psi$m can be reversible or irreversible and is involved in both apoptotic and necrotic cell death (33). If loss of $\Delta\Psi$m is irreversible, the cell commits to die, regardless the type of cell death. U937 cells over-expressing c-IAP1 are resistant to TNFα-mediated cytochrome *c* release and $\Delta\Psi$m collapse. This suggests that c-IAP1 blocks TNFα-mediated signal at or above the diversion point, probably by combining with the TRADD/TRAF2 complex (2). It was shown that c-IAP1 has also a direct inhibitory effect on the processing of caspase-3 by caspase-9, but the effect is weaker than XIAP (17). However, we failed to detect protective effect of c-IAP1 on UV light-induced apoptosis, which has been confirmed to be via cytochrome c/Apaf-1/caspase-9/caspase-3 pathway (22). We, therefore, propose that c-IAP1 is a powerful inhibitor of apoptosis upstream of mitochondrial events and the apical caspase-8, which can prevent TNFα-mediated apoptosis and toxicity to mitochondria in U937 cells.

It was reported that IAPs could be regulated or cleaved during exposure to TNFα (24, 34–36). The induction of IAPs by TNFα is associated with the activation of NF-κB (34). Cleavage of XIAP or c-IAP1 was found to be either caspase-dependent (24, 37) or caspase-independent (35). Upon prolonged treatment with TNFα, no induction of IAPs was observed in U937 cells. TNFα induced a time-dependent cleavage of XIAP, which was correlated with activation of caspase-3. Transient loss of c-IAP1 was not associated with caspase-3 activity and the original levels of c-IAP1 were recovered. We, therefore, propose that U937 might become more sensitive to DNA damaging agent-induced apoptosis after prolonged exposure to TNFα due to the loss of full length XIAP. However, the levels of c-IAP1 in U937 cells were virtually not affected by long-term exposure to TNFα.

In conclusion, TNFα-mediated cytochrome *c* release and loss of $\Delta\Psi$m could be regulated by different signaling pathways. The c-IAP1 is a powerful inhibitor of apoptosis upstream of mitochondrial events and the apical caspase-8, which can prevent TNFα-mediated apoptosis and toxicity to mitochondria in U937 cells. Better understanding of the inhibitory mechanism of c-IAP1 on apoptosis could provide crucial information regarding its role in immunotherapy for leukemia.

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