Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways

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Abstract
Staurosporine has long been used in vitro as an initiator of apoptosis in many different cell types, but the mechanism involved remains poorly understood. In the present study, we have examined the apoptosis-inducing potential of staurosporine in cultured melanoma cell lines and dissected the staurosporine-induced apoptotic signaling pathway. We report that although staurosporine activated Bax and the mitochondrial caspase-dependent apoptotic pathway, it also induced apoptosis of melanoma by caspase-independent pathways. The caspase-dependent apoptotic pathway was activated relatively soon after exposure to staurosporine and was associated with release of cytochrome c and Smac/DIABLO from mitochondria and cleavage of poly(ADP-ribose) polymerase and inhibitor of caspase-activated DNase. This pathway was inhibitable by broad caspase inhibitors. A second apoptotic pathway that appeared to be involved in late apoptotic events was caspase independent in that inhibitors of caspases did not prevent the late onset of apoptosis. Overexpression of Bcl-2 inhibited the early onset of apoptosis but not the later, caspase-independent pathway. Apoptosis-inducing factor may be responsible for the late apoptotic execution in that its translocation from mitochondria into the nucleus coincided with the late onset of apoptosis and could not be inhibited by either a pan-caspase inhibitor or overexpression of Bcl-2. Our results indicate that staurosporine is able to bypass resistance of melanoma cells to mitochondrial caspase-dependent apoptotic pathways; hence, derivatives of staurosporine may warrant further evaluation either alone or with other apoptosis-inducing agents. [Mol Cancer Ther. 2004;3(2):187–197]

Introduction
Recent studies have suggested that many therapeutic agents used against cancer, such as immunotherapy, chemotherapy, and irradiation, mediate their effects by induction of apoptosis of the cancer cells (1–3). Three major apoptotic pathways originating from three separate subcellular compartments have been identified as the death receptor-mediated pathway, the mitochondrial apoptotic pathway, and the endoplasmic reticulum pathway (4–6). Although each pathway is initially mediated by different mechanisms, they share a common final phase of apoptosis, consisting of the activation of the executioner caspases and dismantling of substrates critical for cell survival (7, 8).

The mechanisms involved in induction of apoptosis by chemotherapeutic agents such as alkylating agents, topoisomerase inhibitors, and antimitic agents are believed to be largely mediated by the mitochondrial apoptotic pathway (1). This involves release of mitochondrial apoptotic proteins such as cytochrome c (9), apoptosis-inducing factor (AIF; 10), second mitochondrial-derived activator of caspase/direct inhibitor of apoptosis (IAP) protein binding protein with low pI (Smac/DIABLO; 11–12), endonuclease G (13) and Omi1/HtrA2 (14, 15). On release, cytochrome c interacts with apoptotic proteinase-activating factor-1 and pro-caspase-9 to form apoptosomes. The latter activates caspase-9 and downstream effector caspases such as caspase-3 that are responsible for apoptotic destruction of the cells (9, 16). In contrast, AIF and endonuclease G translocate directly to the nucleus where they induce chromatin condensation and/or DNA fragmentation (10, 13). Cytosolic Smac/DIABLO and Omi1/HtrA2 mediate apoptosis by binding to IAP protein family members that inhibit activation of caspase-9 and inhibit the activity of activated caspase-3 (11, 12, 14). Although the mechanism(s) that underlies the release of mitochondrial apoptotic proteins remains uncertain, the Bcl-2 family members play a central role in regulating changes in mitochondrial outer membrane permeability (17–20). Studies have shown that the antiapoptotic Bcl-2 family members such as Bcl-2, Bcl-XL, and Mcl-1 appear to preserve the integrity of the outer mitochondrial membrane by binding to mitochondrial porin channels (17, 18). Apoptosis proceeds when proapoptotic BH3-only proteins such as Bid, Bim, and Noxa bind to the antiapoptotic Bcl-2 family members and promote binding of the multidomain proapoptotic proteins Bax and Bak to the mitochondrial outer membrane, where they initiate changes in mitochondrial outer membrane permeability. Overexpression of Bcl-2 inhibits the mitochondria-dependent pathway to apoptosis (19, 20).

Staurosporine, a protein kinase inhibitor, has been characterized as a strong inducer of apoptosis in many different cell types. The mechanism(s) by which staurosporine induces apoptosis, however, remains controversial. Although it is generally believed that the mitochondrial apoptotic pathway plays a critical role in staurosporine-induced apoptosis (21, 22), some studies found that Bcl-2
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overexpression was ineffective in protecting cells from killing by staurosporine (23). Similarly, while most reports showed a requirement for caspase activation in staurosporine-induced apoptosis, caspase-independent mechanism(s) was also suggested (24, 25). Multiple mechanisms may therefore be involved in staurosporine-induced apoptosis and these may vary between different cell types.

Melanoma continues to increase in incidence in many parts of the world and remains among the top six cancers as a cause of death and morbidity. Treatment of melanoma once it has spread beyond the skin remains unsatisfactory. This is largely due to its unresponsiveness to available chemotherapeutic and biologic reagents, which has been attributed to development of resistance to apoptosis (26, 27). Understanding and overcoming resistance mechanism(s) of melanoma to apoptosis would therefore facilitate identification of new therapeutic targets and development of new treatments.

In the present study, we examined the apoptosis-inducing potential of staurosporine in cultured melanoma cell lines and dissected the staurosporine-induced apoptotic signaling pathway. We report that staurosporine induced relatively high levels of apoptosis in the majority of melanoma cell lines through both caspase-dependent and -independent pathways. While caspas play a determining role in early apoptotic responses, AIF may be involved in apoptotic execution at late stages after staurosporine treatment. Although the mitochondrial apoptotic pathway was activated by staurosporine, overexpression of Bcl-2 could only delay and partially inhibit staurosporine-induced apoptosis. This suggests that staurosporine induces apoptosis of melanoma by nonconventional mitochondrial apoptotic pathways and the latter may be useful to exploit in treatment of melanoma resistant to the conventional apoptotic pathway.

Materials and Methods

Cell Lines

Human melanoma cell lines Me4405, Mel1007, IgR3, Mel-FH, Mel-RMu, Mel-RM, Mel-CV, and MM200 have been described previously (28, 29). The cell lines were cultured in DMEM containing 5% FCS (Commonwealth Serum Laboratories, Melbourne, Victoria, Australia). Melanocytes were kindly provided by Dr. P. Parson (Queensland Institute of Medical Research, Brisbane, Queensland, Australia) and described elsewhere (29).

Antibodies, Recombinant Proteins, and Other Reagents

Staurosporine was purchased from Sigma Chemical Co. (Castle Hill, New South Wales, Australia). It was dissolved in DMSO and made up in a stock solution of 1 mM. The cell-permeable pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (z-IETD-fmk), the caspase-3 specific inhibitor Z-Val-Ala-Asp(OMe)-Val-Ala-Asp(OMe)-CH2F (z-VDVAD-fmk) were purchased from Calbiochem (La Jolla, CA). The antioxidant γ-glutamlycysteinylglycine (GSH) was purchased from Sigma Chemical Co. (St. Louis, MO). The rabbit polyclonal antibodies (Abs) against caspase-3, caspase-8, and Bid, the mouse monoclonal Abs (mAbs) against cytochrome c and poly(ADP-ribose) polymerase (PARP), and the rabbit mAb against the active form of caspase-3 were purchased form PharMingen (Bioclone, Marrickville, New South Wales, Australia). The rabbit polyclonal Abs against inhibitor of caspase-activated DNase (ICAD) and the mouse mAbs against Bcl-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal Ab against Smac/DIABLO was from Calbiochem. The rabbit polyclonal Ab against cleaved caspase-9 was purchased from New England Biolabs (Beverly, MA). The rabbit polyclonal Ab against caspase-2 was from R&D Systems, Inc. (Minneapolis, MN). The rabbit polyclonal anti-Bax against amino acids 1–20 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Isotype control Abs used were the ID4.5 mouse IgG2a mAb against Salmonella typhi supplied by Dr. L. Ashman (Institute for Medical and Veterinary Science, Adelaide, South Australia, Australia), the 107.3 mouse IgG1 mAb purchased from PharMingen (San Diego, CA), and the rabbit IgG from Sigma Chemical (Castle Hill, New South Wales, Australia).

Plasmid Vector and Transfection

Stable Mel-RM transfectants of Bcl-2 were established by electroporation of the PEF-puro vector carrying human Bcl-2 provided by Dr. David Vaux (Walter and Eliza Hall Institute, Melbourne, Victoria, Australia) and described elsewhere (29).

Flow Cytometry

Immunostaining on intact and permeabilized cells was carried out as described previously (28). Analysis was carried out using a Becton Dickinson (Mountain View, CA) FACSscan flow cytometer. The percentage of antigen-positive cells was calculated as the difference in positive area between positive and negative control histograms. The positive area was that to the right of the intersection of the two curves.

Apoptosis

Apoptotic cells were determined by the propidium iodide method as described elsewhere (28).

Mitochondrial Membrane Potential

Tumor cells were cultured in 24-well plates and allowed to reach exponential growth for 24 h before treatment. MitoTracker Red CMXRos (Molecular Probes, Eugene, OR) was added at 100 nM during the last 30 min of treatment. The medium was removed into a 75-mm Falcon polystyrene tube (Becton Dickinson, Sunnyvale, CA), and the adherent cells were trypsinized and collected into the same tube. After washing with PBS, the cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA) for MitoTracker uptake. Untreated cells were used as controls.
Western Blot Analysis
Methods used were as described previously (28), with minor modification. Briefly, the protein content of cell extracts was determined by the Bradford assay (Bio-Rad, Sydney, New South Wales, Australia). A total of 20–30 μg of protein was electrophoresed on 10–15% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary Abs at the appropriate concentration, and subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (1:3000 dilution; Bio-Rad). Labeled bands were detected by Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear Life Science Products, Boston, MA) and exposed on Hyper MP autoradiography film (Amersham, Castle Hill, New South Wales, Australia).

Preparation of Mitochondrial and Cytosolic Fractions
Methods used for subcellular fraction were similar to the methods described previously (29).

Measurement of Reactive Oxygen Species Generation
Generation of reactive oxygen species (ROS) was monitored by measurement of hydrogen peroxide generation. Cells that were seeded in 24-well plates overnight with or without treatment with staurosporine were incubated with the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA; Sigma Chemical, St. Louis, MO) for 30 min. The medium was removed to a 75-mm Falcon polystyrene tube and the adherent cells were trypsinized and collected into the same tube. After washing twice with PBS, the intensity of DCF-DA fluorescence was determined by using a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA), with an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Results
Staurosporine Induces Caspase Activation and Apoptosis in Melanoma Cells
We examined the apoptosis-inducing potential of staurosporine in melanoma by treating IgR3 and Mel-RM cells with a range of concentrations of the compound for 24 h. As shown in Fig. 1A, staurosporine induces apoptosis of the melanoma cells even when used at concentrations as low as 100 nM, with the percentage of apoptotic cells peaking at 1 μM. Figure 1B shows representative flow cytometry histograms of the apoptotic assays. The kinetics of the induction of apoptosis by staurosporine is shown in Fig. 1C. While apoptosis could be detected by 6 h after treatment, the kinetics differed slightly between Mel-RM and IgR3. The
percentage of apoptotic cells in the former peaked at 24 h after treatment, whereas peak apoptosis of the latter was observed at 48 h. A summary of studies on a panel of melanoma cell lines and melanocytes treated with staurosporine at 1 μM for 24 h is shown in Fig. 1D. Staurosporine induced apoptosis in all the melanoma cell lines, with the percentage of apoptotic cells ranging from 33% in Me4405 to 79% in IgR3. Staurosporine induced apoptosis in about 78% of melanocytes.

To study if caspase activation was involved in staurosporine-induced apoptosis, we firstly examined caspase-3 activation in IgR3 and Mel-RM cells by flow cytometry using a mAb that specifically recognizes the active form of caspase-3. Figure 2A shows representative flow cytometry histograms of activated caspase-3 and Fig. 2B shows the kinetics of caspase-3 activation. Caspase-3 activation could be detected by 3 h with peak activation at 16 h after exposure to staurosporine. Activation of caspase-3 by staurosporine was confirmed by Western blot analysis as shown in Fig. 2C.

The functional activity of the activated caspase-3 was examined against two caspase-3 substrates, ICAD and PARP. As shown in Fig. 2C, ICAD expression was reduced by 6 h and was barely detectable by 16 h in both Mel-RM and IgR3 cells after staurosporine treatment. Similarly, PARP cleavage was also detected by 6 h with the appearance of the cleaved 85-kDa fragment after exposure to staurosporine. At 16 h, only the cleaved form of PARP was detectable. Figure 2C also shows the effect of treatment with staurosporine on X-linked IAP (XIAP) expression, which was shown to be cleaved by activated caspase-3 during apoptosis (33). XIAP expression was decreased at 6 h and was no longer detectable by 16 h after exposure to staurosporine.

We examined possible involvement of caspase-8, -9, and -2 in staurosporine-induced apoptosis by Western blot analysis before and after exposure to the compound. As can be seen from Fig. 2C, pro-caspase-8 expression remained unaltered until 6 h after treatment with staurosporine. By 16 h, pro-caspase-8 expression was no longer detectable, but there was no apparent appearance of the active form of caspase-8 (data not shown). The activation status of caspase-9 was examined by using an Ab that specifically recognizes the cleaved p38 fragment of cleaved caspase-9. Treatment with staurosporine for 6 h resulted in expression of the cleaved caspase-9, and there was a marked increase in the expression levels by 16 h after the addition of staurosporine. Figure 2C also shows that pro-caspase-2 was almost completely cleaved as early as 6 h after treatment with staurosporine.

Staurosporine Induces Apoptosis of Melanoma through Caspase-Dependent and -Independent Pathways

To confirm the role of caspase activation in staurosporine-induced apoptosis of melanoma, we treated IgR3 and Mel-RM cells with the pan-caspase inhibitor, z-VAD-fmk, 1 h before adding staurosporine at 1 μM for a further 24 h. Figure 3A shows that while z-VAD-fmk completely inhibited apoptosis of melanoma induced by tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family that is known to induce apoptosis in the melanoma cell lines (28, 29), it only partially blocked staurosporine-induced apoptosis. This suggests that both caspase-dependent and -independent pathways were induced by staurosporine in melanoma cells. To further confirm this, we treated Mel-RM cells with z-VAD-fmk 1 h before adding staurosporine for differing time periods as indicated in Fig. 3B. Staurosporine-induced apoptosis was markedly delayed by z-VAD-fmk, with negligible apoptosis being detected at 6 h. The levels of apoptosis at 12, 16, 24, and 48 h after treatment with staurosporine in the presence of z-VAD-fmk were markedly decreased.

The role of caspase-3, -8, -9, and -2 in staurosporine-induced apoptosis was further studied by using specific inhibitors. Mel-RM and IgR3 cells, were treated with the
caspase-3 specific inhibitor z-DEVD-fmk, the caspase-9 specific inhibitor z-LEHD-fmk, the caspase-8 specific inhibitor z-IETD-fmk, or the caspase-2 specific inhibitor z-VDVAD-fmk 1 h before adding staurosporine for a further 24 h. As shown in Fig. 3C, pretreatment with z-DEVD-fmk, z-LEHD-fmk, or z-VDVAD-fmk resulted in partial reduction in staurosporine-induced apoptosis, whereas pretreatment with z-IETD-fmk, the caspase-8 inhibitor, had only a minor effect on the percentage of apoptotic cells. There was no significant difference in the degrees of reduction of staurosporine-induced apoptosis produced by z-DEVD-fmk, z-LEHD-fmk, or z-VDVAD-fmk (P > 0.05, Fisher’s exact test).

**Staurosporine Activates the Conventional Mitochondrial Apoptotic Pathway**

To study the potential effects of staurosporine on the mitochondrial apoptotic pathway, we measured the

**Figure 3.** Effects of inhibition of caspases on staurosporine-induced apoptosis of melanoma. A, inhibition of staurosporine- and TRAIL-induced apoptosis by a pan-caspase inhibitor, z-VAD-fmk. Mel-RM and IgR3 cells were treated with z-VAD-fmk (20 μM) 1 h before adding TRAIL (200 ng/ml) or staurosporine (1 μM) for another 24 h. Apoptosis was measured by propidium iodide method using flow cytometry. **Columns,** mean of three individual experiments; **bars,** SE. B, effects of z-VAD-fmk on apoptosis induced by staurosporine at different staurosporineges. Mel-RM cells were treated with z-VAD-fmk (20 μM) 1 h before adding staurosporine (1 μM) for indicated time periods. Apoptosis was measured by propidium iodide method using flow cytometry. Data are representative of three individual experiments. C, effects of inhibition of caspase-3, -9, -2, and -8 on staurosporine-induced apoptosis. IgR3 and Mel-RM cells were treated with the caspase-3 specific inhibitor z-DEVD-fmk (30 μM), the caspase-9 specific inhibitor z-LEHD-fmk (30 μM), the caspase-2 specific inhibitor z-VDVAD-fmk (50 μM), and the caspase-8 specific inhibitor z-IETD-fmk (30 μM) 1 h before adding staurosporine (1 μM) for another 24 h. Apoptosis was measured by propidium iodide method using flow cytometry. **Columns,** mean of three individual experiments; **bars,** SE.

**Figure 4.** Staurosporine induces changes in MMP. A, induction of changes in the ΔΨm by staurosporine in Mel-RM and IgR3 lines. Cells were treated with staurosporine (1 μM) for indicated time periods. The ΔΨm was measured by uptake of the MitoTracker Red CMXRos using flow cytometry. Data are representative of three individual experiments. B, staurosporine-induced changes in ΔΨm are independent of caspases. Mel-RM (left) and IgR3 (right) cells were treated with z-VAD-fmk (20 μM) 1 h before adding staurosporine (1 μM) for indicated time periods. The ΔΨm was measured as described in A. **Columns,** mean of three individual experiments; **bars,** SE. C, staurosporine-induced release of cytochrome c and Smac/DIABLO from mitochondria to the cytosol. Mel-RM and IgR3 cells were treated with staurosporine (1 μM) for 6 h before harvest. Mitochondrial and cytosolic fractions were subjected to Western blot analysis. Data are representative of two individual experiments. Western blot analysis of COX IV levels was included to show relative purity of mitochondrial fractions.
mitochondrial membrane potential ($\Delta \Psi_m$) in melanoma cells treated with staurosporine. Figure 4A shows that treatment of melanoma cells with staurosporine induced changes in the $\Delta \Psi_m$ in melanoma cells, which could be detected at 3 h and peaked at 6 h after exposure to staurosporine. The reduction in $\Delta \Psi_m$ was markedly decreased by 12 h and was barely detectable at 16 h after treatment. The changes in $\Delta \Psi_m$ induced by staurosporine appeared caspase independent in that pretreatment of cells with z-VAD-fmk before adding staurosporine had a negligible effect as shown in Fig. 4B.

The induction of changes in mitochondrial membrane permeability (MMP) by staurosporine was further confirmed by studying the release of cytochrome c and Smac/DIABLO from mitochondria into the cytosol in Mel-RM and IgR3 cells as shown in Fig. 4C. Cytochrome c and Smac/DIABLO were localized exclusively in mitochondrial fractions before treatment, but after exposure to staurosporine for 6 h, both cytochrome c and Smac/DIABLO were observed in the cytosolic fractions with a corresponding decrease in the mitochondrial fractions.

We next studied the possible role of AIF in staurosporine-induced apoptosis by immunofluorescence microscopy. As shown in Fig. 5, before treatment, punctuate cytoplasmic staining of AIF was predominantly colocalized with mitochondria that were labeled by CMXRsos. At 6 h after exposure to staurosporine, there was a marked decrease in cellular volume that was associated with perinuclear condensation of mitochondria in most of the cells. AIF staining at this stage was still primarily colocalized with mitochondria. In contrast, AIF staining at 16 h after exposure to staurosporine appeared predominantly associated with the nucleus, as indicated by colocalization with nuclei identified by DAPI labeling. Figure 5 also shows that...

![Figure 5](image-url)

**Figure 5.** Staurosporine induces translocation of AIF from mitochondria to nucleus. **A,** Mel-RM cells growing on coverslips were treated with staurosporine (1 $\mu$M) for indicated time periods. For labeling of mitochondria, the MitoTracker Red CMXRos was added for the last 30 min of treatment. Cells were then incubated with an Ab against AIF followed by labeling with the Alexa 488 secondary Ab. For nuclear labeling, cells were then incubated with DAPI. Coverslips were mounted and examined using a fluorescence microscope. **B,** translocation of AIF induced by staurosporine is independent of caspases. Mel-RM cells were treated with z-VAD-fmk (20 $\mu$M) 1 h before adding staurosporine (1 $\mu$M) for another 16 h. Cells were then stained as described in **A.**
Staurosporine induces a conformational change in Bax and induces its relocation from the cytosol to mitochondria. A, representative flow cytometry histograms of assays of induction of a Bax conformation change by suberic bishydroxamic acid. Mel-RM and IgR3 cells with or without treatment with staurosporine (1 μM) for 6 h were subjected to flow cytometry analyses using a Bax-NH2-terminal epitope-specific Ab in permeabilized cells. B, relocation of Bax from the cytosol to mitochondria. Mel-RM and IgR3 cells were treated with staurosporine (1 μM) for indicated time periods before harvest. The mitochondrial and cytosolic fractions were subjected to Western blot analyses. Data are representative of two individual experiments.

pretreatment of cells with z-VAD-fmk could not inhibit staurosporine-induced relocation of AIF from mitochondria into nuclei. This suggests that translocation of AIF induced by staurosporine is a caspase-independent process.

Staurosporine Induces Conformational Changes of Bax and Its Relocation from the Cytosol to Mitochondria

Bax translocation from the cytosol to mitochondria is believed to play a key role in mitochondrion-mediated apoptosis induced by a variety of apoptotic stimuli (22, 31). Bax translocation involves a conformation change that exposes the NH2 terminus and the hydrophobic COOH terminus that targets mitochondria (31, 32). The NH2-terminal region is occluded in intact cells and hence is not available for binding by Bax-NH2-terminal epitope-specific Abs (31, 32). We studied the conformational status of Bax in melanoma cells with or without exposure to staurosporine by using an Ab directed against the NH2-terminal region of Bax in flow cytometry. As shown in Fig. 6A, a population of the cells was positive for this Ab with weak staining intensity before treatment. After exposure to staurosporine for 6 h, there was a marked increase in the levels of conformationally changed Bax.

We next studied the role of Bax in staurosporine-induced apoptosis of melanoma by examining the expression of Bax in different subcellular fractions of IgR3 and Mel-RM cells with or without exposure to staurosporine. As shown in Fig. 6B, the Bax protein was predominantly in the cytosol with only a negligible amount being detected in the mitochondrial fractions both before and at 3 h after treatment. In contrast, a considerable amount of Bax was observed in the mitochondrial fractions with a corresponding decrease in the levels of expression in the cytosol after treatment with staurosporine for 6 h. This was most evident in the staurosporine-sensitive IgR3 cells.

Overexpression of Bcl-2 Delays Staurosporine-Induced Apoptosis

To further study the role of mitochondria in staurosporine-induced apoptosis, we transfected cDNA encoding Bcl-2 into Mel-RM cells. Bcl-2 expression was measured by Western Blot analyses as shown in Fig. 7A. There was a marked increase in the levels of Bcl-2 in the Bcl-2-transfected cells, but the levels in the cells transfected with the vector alone were similar to those in the parental cells. As shown in Fig. 7B, apoptosis of melanoma induced by TRAIL, which is known to induce apoptosis of melanoma predominantly through the mitochondrial apoptotic pathway (29), was nearly completely inhibited in the Bcl-2 transfectants. In contrast, the percentage of apoptotic cells after treatment with staurosporine for 24 h was only partially decreased in Bcl-2 transfectants compared with cells transfected with the vector alone. Figure 7C shows that staurosporine-induced changes in the AIF were reversed in Bcl-2-transfected cells. Similarly, staurosporine-induced caspase-3 activation was also inhibited by Bcl-2 overexpression (Fig. 7D).

Given that apoptosis induced by staurosporine at different stages may be associated with different apoptotic signaling pathways, we studied the effects of overexpression of Bcl-2 on apoptosis at different time points after exposure to staurosporine. Mel-RM cells transfected with Bcl-2 were treated with staurosporine for indicated time periods as shown in Fig. 7E. Staurosporine-induced apoptosis was markedly delayed in Bcl-2 transfectants with significant apoptosis only being detected at 16 h compared with 6 h in cells transfected with the vector alone. The levels of staurosporine-induced apoptosis were markedly decreased in the Bcl-2 transfectants with the percentages of apoptotic cells reaching about 50–70% of those in the control cells at 16, 24, and 48 h after treatment with staurosporine.

We next studied the effects of overexpression of Bcl-2 on staurosporine-induced translocation of AIF from mitochondria into the nucleus. As shown in Fig. 7F, at 16 h after exposure to staurosporine, AIF staining in Bcl-2 transfectants displayed a diffuse perinuclear staining pattern. Weak diffuse staining was also detected within the nucleus. In contrast, by 24 h after exposure to staurosporine, nuclear staining pattern of AIF was predominant.

To further understand the mechanism(s) by which staurosporine induces changes in mitochondria, we treated Mel-RM and IgR3 cells with staurosporine or TRAIL in the presence of cyclosporine A (Cyc.A), which is believed to be able to inhibit mitochondrial permeability transition induced by various stimuli and thus blocking apoptotic signaling (33, 34). As shown in Fig. 7G, while Cyc.A
Figure 7. Effects of overexpression of Bcl-2 on staurosporine-induced apoptotic events. A, Bcl-2 was overexpressed in Mel-RM cells transfected with the cDNA encoding Bcl-2 but not in the cells transfected with the vector alone. Whole cell lysates were subjected to Western blot analysis. Data are representative of two individual experiments. B, overexpression of Bcl-2 partially inhibited apoptosis induced by staurosporine. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with staurosporine (1 μM) for 24 h before assay of apoptosis by propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. C, overexpression of Bcl-2 inhibited changes in the ΔΨm induced by staurosporine. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with staurosporine (1 μM) for 6 h before the ΔΨm was measured by uptake of the MitoTracker Red CMXRos using flow cytometry. Columns, mean of three individual experiments; bars, SE. D, overexpression of Bcl-2 inhibited caspase-3 activation induced by staurosporine. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with staurosporine (1 μM) for 6 h before activated form of caspase-3 was measured in permeabilized cells using flow cytometry. Columns, mean of three individual experiments; bars, SE. E, overexpression of Bcl-2 delayed staurosporine-induced apoptosis. Mel-RM cells transfected with cDNA for Bcl-2 or vector alone were treated with staurosporine (1 μM) for indicated time periods before assay of apoptosis by propidium iodide method using flow cytometry. Data are representative of three individual experiments. F, overexpression of Bcl-2 delayed staurosporine-induced translocation of AIF. Mel-RM cells transfected with cDNA for Bcl-2 that were grown on coverslips were treated with staurosporine (1 μM) for indicated time periods. Cells were then incubated with an Ab against AIF followed by labeling with the Alexa 488 secondary Ab. For nuclear labeling, cells were then incubated with DAPI. Coverslips were mounted and examined using a fluorescence microscope (×400). G, staurosporine-induced apoptosis of melanoma could not be inhibited by Cyc. A. Mel-RM and IgR3 cells with or without pretreatment with Cyc.A (5 μM) for 1 h were treated with staurosporine (1 μM) or TRAIL (200 ng/ml) for another 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Data are representative of three individual experiments.
Staurosporine-Induced Apoptosis of Melanoma Is Independent of the Generation of ROS. A, representative flow cytometry histograms of assays of ROS production. Mel-RM and IgR3 cells with or without treatment with staurosporine (1 \( \mu \text{M} \)) for indicated time periods. DCF-DA (10 \( \mu \text{M} \)) was added for the last 30 min of incubation. The data shown are the mean ± SE of three individual experiments. B, the antioxidant GSH did not inhibit staurosporine-induced apoptosis. Mel-RM and IgR3 cells were treated with GSH for 2 h before adding staurosporine (1 \( \mu \text{M} \)) for another 24 h. Apoptosis was measured by the propidium iodide method using flow cytometry. Columns, mean of three individual experiments; bars, SE. C, the antioxidant GSH inhibited the generation of ROS induced by staurosporine. Mel-RM and IgR3 cells were treated with GSH (10 \( \mu \text{M} \)) for 2 h before adding staurosporine (1 \( \mu \text{M} \)) for another 16 h. ROS production was measured as described in A. Columns, mean of three individual experiments; bars, SE.

Discussion

Staurosporine has long been used in vitro as an initiator of apoptosis in many different cell types, but the mechanism involved remains poorly understood. A growing body of evidence suggests that staurosporine differs from other apoptotic agents such as chemotherapeutic drugs and death-inducing ligands in that it induces apoptotic cell death in tumor cells normally resistant to these agents (24, 25, 35). We show in the present study that staurosporine induces relatively high levels of apoptosis in all the melanoma cell lines including those resistant to TRAIL, a member of the TNF family that induces apoptosis in about two-thirds of melanoma cell lines (28, 29). At least two apoptotic pathways appeared to be responsible for induction of apoptosis by staurosporine.

Firstly, staurosporine induced changes in MMP, which was evidenced by changes in \( \Delta \Psi_m \) and release of cytochrome c and Smac/DIABLO into the cytosol, and caspase-3 activation, which were maximal at about 6 h after exposure to staurosporine. The mechanism(s) by which staurosporine induced changes in the MMP is not entirely clear. Caspase-8 was degraded at a relatively late stage (after 6 h), and an inhibitor of caspase-8 did not inhibit apoptosis. Cleavage of caspase-2, -3, and -9 was evident by 6 h, consistent with the kinetics of changes in MMP (i.e., they were downstream of or parallel with changes in MMP). Changes in the \( \Delta \Psi_m \) were inhibited by overexpression of Bcl-2, which resulted in a delayed death event (24). In Jurkat cells, a dominant-negative caspase inhibitor blocked early (<3 h) but not delayed (>12 h) apoptotic cell death induced by staurosporine (25). Studies on MCF-7 cells also demonstrated caspase-dependent and -independent events (24). In Jurkat cells, a dominant-negative caspase inhibitor blocked early (<3 h) but not delayed (>12 h) apoptotic cell death induced by staurosporine (25). Studies on MCF-7 cells also demonstrated caspase-dependent and -independent events (24).
caspase-9 mutant inhibited apoptosis induced by FasL or a chemotherapeutic drug, etoposide, but had only marginal effects on apoptosis induced by staurosporine (35). It was reported that release of AIF is a caspase-independent event (36), and in view of this, we examined whether translocation of AIF from mitochondria into the nucleus may be responsible for late apoptotic execution of melanoma cells by staurosporine.

Evidence in support of this possibility was firstly the kinetics of AIF release. Microscopy showed that staurosporine-induced release of AIF from mitochondria did not occur until after 6 h and that its translocation into the nucleus was evident by 16 h. This release was much later than that of cytochrome c and Smac/DIABOL. Secondly, translocation of AIF into the nucleus was not inhibited by the pan-caspase inhibitor (z-VAD-fmk) or by overexpression of Bcl-2. The latter did cause some delay in AIF translocation to the nucleus, consistent with the observed delay in staurosporine-induced apoptosis in melanoma cells with overexpressed Bcl-2. These results suggest that staurosporine induces release of AIF by mechanisms that are independent of changes in the ΔΨm. This is also supported by the kinetics of staurosporine-induced changes in the ΔΨm in that the changes in the ΔΨm had peaked and were reversing prior to AIF entry into the nucleus. Further investigation of the mechanism concerned and its relation to the conformational changes of Bax is needed.

An alternative explanation that we considered for caspase-independent induction of apoptosis by staurosporine was generation of ROS. Some apoptotic agents such as certain histone deacetylase inhibitors can induce apoptosis by the production of ROS independent of caspase activation (37, 38). The present studies, however, showed that although ROS were produced in melanoma cells by treatment with staurosporine, they did not appear to be involved in induction of apoptosis as the antioxidant GSH inhibited generation of ROS but was unable to prevent melanoma from apoptosis induced by staurosporine.

Staurosporine is known to inhibit several protein kinases in cells. Apparently, inhibition of protein kinases may have been responsible for inducing conformational changes in Bax and its translocation from the cytosol to mitochondria (39–44). staurosporine has been shown to inhibit the serine/threonine kinase Akt/protein kinase B leading to decreased phosphorylation of Bad (39, 40). Bad is capable of forming heterodimers with the antiapoptotic proteins Bcl-XL and Bcl-2 and antagonizing their antiapoptotic activity (39). Phosphorylated Bad cannot bind to either Bcl-XL or Bcl-2, so that inhibition of Akt-mediated phosphorylation of Bad would increase sensitivity of cells to apoptosis (44). Overexpression of Akt was shown to inhibit staurosporine-induced movement of Bax and apoptosis in HeLa cells (21). Inhibition of Erk1/2 activation by staurosporine may also play a part in staurosporine-induced changes in Bax (43). We have previously shown that inhibition of Erk1/2 facilitated apoptosis induction in melanoma by promoting conformational changes of Bax and its relocation to mitochondria (44).

Despite the overall relatively high levels of apoptosis induced by staurosporine, there was some variation in sensitivity to staurosporine among the melanoma cell lines, as shown for studies on the IgR3 (sensitive) and Mel-RM (partially resistant) cell lines. The mechanism(s) underlying this is currently not clear but was reflected in increased changes in MMP and caspase-3 activation in the IgR3 compared with the Mel-RM line. Several different kinases are involved in resistance to apoptosis, such as the mitogen-activated protein kinase Erk1/2 and Akt/protein kinase C pathways. It is therefore possible that the different sensitivities of the melanoma cells to staurosporine may reflect the degree to which these pathways were involved in protection of cells against apoptosis.

In summary, these studies show that staurosporine can activate an apoptotic pathway that is dependent on changes in MMP and activation of caspases as well as a pathway that is independent of caspase activation and not inhibitable by overexpression of Bcl-2. The latter may be due to AIF release in that the latter was also independent of caspase activation and overexpression of Bcl-2. Staurosporine is therefore able to bypass resistance of melanoma cells to mitochondrial caspase-dependent pathways, and (non-toxic) derivatives of staurosporine may prove to be valuable agents against melanoma alone or in combination with other agents such as TRAIL.

References


Staurosporine induces apoptosis of melanoma by both caspase-dependent and -independent apoptotic pathways

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