Available online at www.sciencedirect.com
ScienceDirect
Cellular Signalling 19 (2007) 2468–2478
CELLULAR SIGNALLING
www.elsevier.com/locate/cellsig

Bid is not required for Bax translocation during UV-induced apoptosis

Yinyuan Wu [a], Da Xing [a,*], Wei R. Chen [a,b], Xichao Wang [a]

[a] MOE Key Laboratory of Laser Life Science & Institute of Laser Life Science, South China Normal University, Guangzhou 510631, China
[b] Department of Engineering and Physics, College of Mathematics and Science, University of Central Oklahoma, Edmond, Oklahoma 73034, USA

Received 16 July 2007; accepted 26 July 2007
Available online 11 August 2007

Abstract

UV irradiation triggers apoptosis through both the membrane death receptor and the intrinsic apoptotic signaling pathways. Bax, a member of the Bcl-2 family of proteins, translocates from the cytosol to the mitochondrial membrane during UV-induced apoptosis, but the regulation of Bax translocation by UV irradiation remains elusive. In this study, we show that Bax translocation, caspase-3 activation and cell death by UV irradiation are not affected by Z-IETD-fmk (caspase-8 inhibitor), but delayed by Pifithrin-α (p53 inhibitor), although Bid cleavage could be completely abolished by Z-IETD-fmk. Co-transfecting YFP-Bax and Bid-CFP into human lung adenocarcinoma cells, we demonstrate that translocation of YFP-Bax precedes that of Bid-CFP, there is no significant FRET (fluorescence resonance energy transfer) between them. Similar results are obtained in COS-7 cells expressing YFP-Bax and Bid-CFP. Furthermore, using acceptor photobleaching technique, we observe that there is no interaction between YFP-Bax and Bid-CFP in both healthy and apoptotic cells. Additionally, during UV-induced apoptosis there is downregulation of Bcl-x_L, an anti-apoptotic protein. Overexpression of Bcl-x_L in cells susceptible to UV-induced apoptosis prevents Bax translocation and cell death, repression of Bid protein with siRNA (small interfering RNA) do not inhibit cell death by UV irradiation. Taken together, these data strongly suggest that Bax translocation by UV irradiation is a Bid-independent event and inhibited by overexpression of Bcl-x_L.

Keywords: Bax translocation; Bid; Bcl-x_L; UV irradiation; Apoptosis

1. Introduction

UV irradiation is a DNA-damaging agent that activates a p53-dependent apoptotic response [1,2]. DNA damage can change the phosphorylation levels of p53 protein resulting in cell cycle arrest and apoptosis. P53 stimulates a wide network of signals that act through two major apoptotic pathways [3]. The extrinsic pathway is initiated through ligation of the death receptor family receptors by their respective ligands. Amongst others this family includes the tumour necrosis factor receptors, CD95/Fas/APO-1 and the TRAIL receptors [3,4]. Receptor ligation is followed by the formation of the death inducing signalling complex (DISC), which is composed of the adapter molecule FADD and caspase-8 [5,6]. Recruitment to DISC activates caspase-8, which in turn either directly cleaves and activates the effector caspases, or indirectly activates the downstream caspases through cleavage of the BH3 protein Bid, leading to engagement of the intrinsic pathway of apoptosis [7–9]. This intrinsic pathway of caspase activation is regulated by the pro- and anti-apoptotic Bcl-2 family proteins. These proteins induce or prevent the release of apoptogenic factors, such as cytochrome *c* or Smac/DIABLO, from the mitochondrial intermembrane space into the cytosol [10,11]. However, the precise initiating apoptotic mechanisms upstream of mitochondria by UV irradiation remained obscure.

Proapoptotic Bax and Bak are essential regulators of the mitochondrial pathway of apoptosis [12,13]. Bak resides permanently on the outer mitochondrial membrane (OMM), whereas Bax is normally found in the cytosol of healthy cells and

Abbreviations: CFP; YFP; DsRed and GFP; cyan; yellow; red and green fluorescent protein; FRET; fluorescence resonance energy transfer; Bak; Bcl-2 antagonist/killer; Bax; Bcl-2-associated X protein; BH3; Bcl-2-homology domain-3; Bid; BH3-interacting-domain; tBid; truncated Bid; FADD; FAS-associated death domain; MOMP; mitochondrial outer-membrane permeabilization; PUMA; p53-upregulated modulator of apoptosis; TNF; tumor necrosis factor; MEFs; mouse embryonic fibroblasts; DISC; death inducing signalling complex; UV; Ultraviolet; siRNA (small interfering RNA).

* Corresponding author. Tel.: +86 20 85210089; fax: +86 20 85216052.
E-mail address: xingda@scnu.edu.cn (D. Xing).

0898-6568/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.cellsig.2007.07.024

translocates to the OMM during apoptosis [14,15]. After translocation to mitochondria, Bax induces cytochrome *c* release either by forming a pore by oligomerization in the outer mitochondrial membrane, or by opening other channels [16,17]. Studies using recombinant proteins have shown that Bax activation by active Bid [7,18] or BH3 peptides from Bid or Bim is essential and sufficient to permeabilize vesicles composed of mitochondrial lipids in the absence of other proteins [19]. In the process, Bax oligomerizes, and such oligomerization of Bax and Bak coincides with membrane permeabilization and cytochrome *c* release [20,21]. Recent studies have similarly shown that purified or recombinant p53 also has the ability to activate Bax to oligomerize in lipid membranes and cause permeabilization [22]. These studies support a model in which the activation of Bax or Bak by BH3-only "activator" proteins and, perhaps, other proteins (such as p53) with this activator function, is necessary and sufficient for mitochondrial outer-membrane permeabilization and the release of proapoptotic factors from the mitochondrial intermembrane space. This effect is regulated by anti-apoptotic members of the Bcl-2 family that can sequester the activator protein and also bind to activated Bax and Bak to inhibit their ability to oligomerize and permeabilize membranes. It was also reported that the transcription-independent activation of Bax by p53 occurred with similar kinetics and concentrations to those produced by active Bid. Mouse embryonic fibroblast cells (MEFs) deficient in Bax were resistant to UV-induced apoptosis [22]. Thus, the regulation of Bax translocation by UV irradiation is not fully understood.

Bid was first reported in 1996, it is widely expressed in various tissues, with the highest level being in the kidney [18]. In a resting cell, Bid is predominantly cytoplasmic. Following TNF-α or Fas treatment, Bid is cleaved by caspase 8 in an unstructured loop, exposing a new amino terminal glycine residue, which becomes myristoylated, facilitating its translocation to the mitochondria, where it induces the activation of Bax and Bak, resulting in the release of cytochrome *c* [23,24]. Studies with $Bid^{-/-}$ mice have demonstrated that Bid is required for Fas induced apoptosis [25]. On the other hand, $Bid^{-/-}$ MEFs were found to be as susceptible as $Bid^{+/+}$ MEFs to a wide range of intrinsic damage signals [13]. More recently, however, it was demonstrated that $Bid^{-/-}$ MEFs are less susceptible than $Bid^{+/+}$ MEFs to the DNA-damaging reagent adriamycin, as well as to the nucleotide analog 5-fluorouracil [26]. However, the apoptotic pathways in which Bid plays a role are not yet fully characterized.

In order to investigate the relationship between Bid and Bax during UV-induced apoptosis, we monitor these events in real-time. Our results demonstrate that Bax translocation is independent of Bid activation, but delayed by p53 inhibitor, inhibited by Bcl-x_L. Our findings will extend the knowledge about the cellular signaling mechanisms mediating UV-induced apoptosis.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from GIBCO (Grand Island, NY). Z-IETD-fmk (caspase-8 inhibitor) and Pifithrin-α (p53 inhibitor) were purchased from BioVision (Mountain View, CA, USA). Lipofectamine™ Reagent was purchased from Invitrogen (Carlsbad, CA, USA). DNA Extraction kit was purchased from Qiagen (Valencia, CA, USA). pGFP-Bax was kindly supplied by Richard J.Youle [27], pYFP-Bax and pCFP-Bcl-x_L were kindly supplied by Andrew [45]. pDsRed-Mit was kindly supplied by Dr. Y. Gotoh [46]. pBid-CFP was kindly supplied by Dr. K. Taira [47]. Other chemicals were mainly from Sigma (St Louis, MO). The pGPU6/GFP/Neo-shBID-1, pGPU6/GFP/Neo-shBID-2 and pGPU6/GFP/Neo-shNC were purchased from GenePharma (Shanghai, China).

2.2. Cell culture and treatments

The human lung adenocarcinoma cell line (ASTC-a-1) was obtained from Department of Medicine, Jinan University and COS-7 cell line was obtained from Department of Medicine, Zhongshan University. They were cultured in DMEM supplemented with 15% fetal calf serum (FCS), penicillin (100 units/ml), and streptomycin (100 mg/ml) in 5% CO_2 at 37 °C in humidified incubator. Transfections were performed with Lipofectamine™ 2000 reagent according to the manufacturer's protocol. The medium was replaced with fresh culture medium after 5 h. Cells were examined at 24–48 h after transfection. For UV treatment, medium was removed and saved, cells were rinsed with PBS and irradiated, and medium was restored. Unless otherwise specified, cells were exposed to UV irradiation at a fluence of 120 mJ/cm^2 and observed at the time indicated. For experiments with the inhibitors, cells was pretreated with Pifithrin-α (20 μM) or Z-IETD-fmk (10 μM) 1 h before UV irradiation. The inhibitors were kept in the medium throughout the experimental process.

2.3. Time-lapse confocal fluorescence microscopy

GFP, CFP, YFP and DsRed fluorescence were monitored confocally using a commercial laser scanning microscope (LSM 510/ConfoCor 2) combination system (Zeiss, Jena, Germany) equipped with a Plan-Neofluar 40×/1.3 NA Oil DIC objective. Excitation wavelength and detection filter settings for each of the fluorescent indicators were as follows: GFP fluorescence was excited at 488 nm with an argon ion laser and emission was recorded through a 500–550 nm band pass filter. CFP fluorescence was excited at 458 nm with an argon ion laser and emission was recorded through a 470–500 nm band pass filter. YFP fluorescence was excited at 514 nm with an argon ion laser and emission was recorded through a 535–545 nm band pass filter. DsRed fluorescence was excited at 543 nm with a helium-neon laser and emitted light was recorded through a 560 nm long pass filter.

For time-lapse imaging, culture dishes were mounted onto the microscope stage that was equipped with a temperature-controlled chamber (Zeiss, Jena, Germany). During control experiments, bleaching of the probe was negligible.

2.4. GFP-Bax translocation assay

To monitor GFP-Bax translocation in living cells, ASTC-a-1 cells were co-transfected with pGFP-Bax and pDsRed-Mit. Using Zeiss LSM 510 confocal microscope, we imaged both the distribution pattern of GFP-Bax and that of DsRed-Mit simultaneously during UV-induced apoptosis. Bax redistribution was assessed by the matching fluorescence of GFP-Bax and DsRed-Mit emission. The cells exhibiting strong punctate staining of GFP, which overlapped with the distribution of DsRed, were counted as the cells with mitochondrially localized Bax.

2.5. FRET analysis

FRET was performed on a commercial Laser Scanning Microscopes (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, the 458 nm line of an Ar-Ion Laser was attenuated with an acousto-optical tunable filter, reflected by a dichroic mirror (main beam splitter HFT458), and focused through a Zeiss Plan-Neofluar 40×/1.3 NA Oil Dic objective onto the sample. CFP and YFP (FRET-acceptor) emission were collected through 470–500 and 535–545 nm band pass filters, respectively. The quantitative analysis of the fluorescence images was performed using Zeiss Rel3.2 image processing software (Zeiss, Germany). After background subtraction, the average fluorescence intensity per pixel was calculated. During control experiments, bleaching of the probe was negligible.

2.6. Performance of acceptor photobleaching

ASTC-a-1 cells co-transfected with YFP-Bax and Bid-CFP were grown on the coverslip of a chamber. The chamber was placed on the stage of the LSM microscope for performance of acceptor photobleaching. The acceptor photobleaching was performed with the highest intensity of 514 nm laser, the images of YFP and CFP emission in and out of the bleaching area were recorded and processed with Zeiss Rel3.2 image processing software (Zeiss, Germany).

2.7. Confirmation of cell apoptosis

ASTC-a-1 cells were cultured in 96-well microplate at a density of 5×10^3 cells/well for 24 h. The cells were then divided into five groups and exposed to UV irradiation at fluence of 0 (control), 30, 60, 120, and 240 mJ/cm^2, respectively. Cell cytotoxicity was assessed with CCK-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. OD_{450}, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China), and the OD_{450} is inversely proportional to the degree of cell apoptosis.

2.8. SDS-PAGE and Western blotting

At the indicated time after UV irradiation, cells were scraped from the dish, then washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), and lysed with ice-cold lysis buffer (50 mmol/l Tris–HCl pH8.0, 150 mmol/l NaCl, 1% TritonX-100, 100 μg/ml PMSF) for 30 min on ice. The lysates were centrifuged at 12000 rpm for 5 min at 4 °C, and the protein concentration was determined. Equivalent samples (30 μg protein extract was loaded on each lane) were subjected to SDS-PAGE on 12% gel. The proteins were then transferred onto nitrocellulose membranes, and probed with indicated antibody (Cell Signaling Technology, MA), followed by IRDye 800 secondary antibody (Rockland Immunochemicals, Inc.). Detection was performed using the LI-COR Odyssey Infrared Imaging System (LI-COR, Inc., USA).

3. Results

3.1. Cell death induced by UV irradiation is not affected by Z-IETD-fmk, but delayed by Pifithrin-α

To establish a proper UV irradiation dose to induce apoptosis, ASTC-a-1 cells were irradiated with various fluence. Cells apoptosis were analyzed using Cell Counting Kit-8 at 6 h after UV irradiation. The OD450 value, an indicator of cells apoptosis, was measured. The OD450 value decreased as the irradiation fluence increased, which indicated that the effects of UV irradiation on apoptosis of ASTC-a-1 cells were dose-dependent (Fig. 1A).

To observe the effects of Z-IETD-fmk (caspase-8 inhibitor) and Pifithrin-α (p53 inhibitor) on UV-induced apoptosis, we added Z-IETD-fmk (10 μM) or Pifithrin-α (20 μM) to cells 1 h before UV irradiation, cells apoptosis were analyzed using Cell Counting Kit-8 at 0 h (control), 3 h, 6 h, 9 h, 12 h after 120 mJ/cm^2 UV irradiation in the presence or absence of Z-IETD-fmk or Pifithrin-α. The results showed that cells apoptosis were little affected in the presence of Z-IETD-fmk, however, cells apoptosis were delayed by several hours in the presence of Pifithrin-α (Fig. 1B).

3.2. Bax translocation by UV irradiation is not affected by Z-IETD-fmk, but delayed by Pifithrin-α

Bax exists in the cytosol of healthy cells and translocates to the mitochondria during apoptosis. To real-time detection of GFP-Bax translocation from the cytosol to the mitochondria during UV-induced apoptosis, we transiently co-transfected GFP-Bax and DsRed-Mit (a marker for mitochondria) into cells, after transfection, the cells were incubated for 48 h, followed by different treatments as indicated, then performed with the LSM microscope. It has reported that the Bax protein, even when overexpressed well beyond the endogenous level, would translocate completely from the cytosol to the mitochondria [27]. To exclude that overexpression of GFP-Bax in our concentration resulted in apoptosis spontaneously, we examined distribution of GFP-Bax and DsRed-Mit without treatment, the results were shown in Fig. 2A, GFP-Bax had a diffuse distribution in the whole cell for more than 12 h. However, GFP-Bax translocation in typical cells started at 5~6 h after UV irradiation (Fig. 2B).

To investigate the effects of Z-IETD-fmk and Pifithrin-α on GFP-Bax translocation by UV irradiation, we added Z-IETD-fmk (10 μM) or Pifithrin-α (20 μM) to cells 1 h before UV irradiation. As shown in Fig. 2C, there was no significant difference in temporal and spatial redistribution of GFP-Bax as compared with the results of Fig. 2B. The results showed that

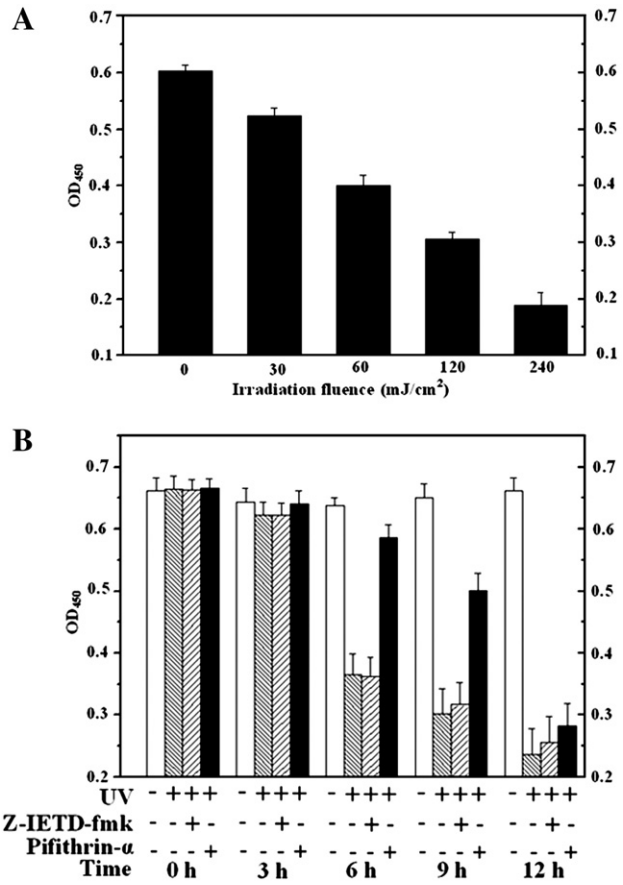

Fig. 1. UV irradiation induces apoptosis in ASTC-a-1 cells. (A) Cells apoptosis was analyzed using Cell Counting Kit-8 at 6 h after various fluence UV irradiation. (B) Cell viability was assessed by the CCK-8 assay at 0, 3, 6, 9 and 12 h after 120 mJ/cm^2 UV irradiation in the presence or absence of Z-IETD-fmk or Pifithrin-α. Error bars are s.e.m. from four independent experiments.

Z-IETD-fmk did not affect GFP-Bax translocation by UV irradiation. However, GFP-Bax translocation by UV irradiation was delayed by about 4 h in the presence of Pifithrin-α (Fig. 2D). These data suggested that Bax translocation by UV irradiation was not affected by Z-IETD-fmk, but delayed by Pifithrin-α. These results were further confirmed by the statistical analysis (Fig. 2E).

3.3. Translocation of YFP-Bax precedes that of Bid-CFP and there is no significant FRET between them

Bid is a BH3-only proapoptotic protein that can be cleaved directly by caspase-8 during apoptosis [7,8,18,28]. The resulting truncated Bid plays a role in the induction of Bax conformational change and subsequent translocation to

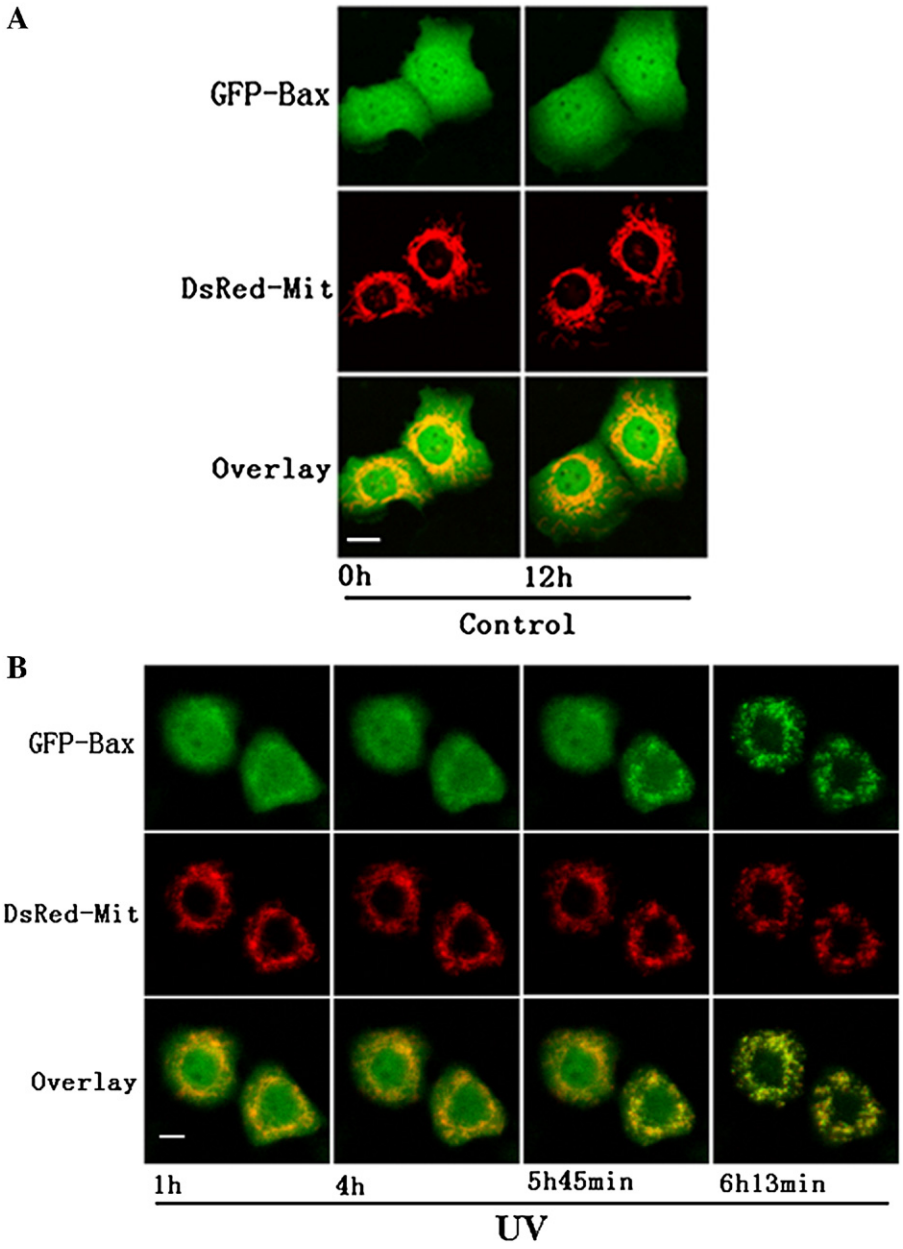

Fig. 2. Dynamics of GFP-Bax translocation during UV-induced apoptosis. (A–D) Showed the typical confocal images of ASTC-a-1 cells co-transfected with GFP-Bax and DsRed-Mit in different conditions. The two panels of GFP-Bax and DsRed-Mit are shown separately and are merged to show the overlay. GFP-Bax localization at mitochondria was determined based on the overlap of GFP-Bax and DsRed-Mit fluorescence images. (A) Confocal images showed the distribution of GFP-Bax and DsRed-Mit in untreated cell. The GFP-Bax typically displayed a diffuse, cytoplamic localization for more than 12 h. (B, C, D) Sample records from time-lapse confocal measurements of GFP-Bax and DsRed-Mit distribution in ASTC-a-1 cells after UV irradiation in the presence or absence of Z-IETD-fmk or Pifithrin-α, respectively. Similar results were obtained from three independent experiments. Scale bar: 10 μm. (E) Quantification of cells showing mitochondrial GFP-Bax. At the indicated time points, the percentage of cells showing Bax translocation to mitochondria was assessed by counting the number of cells exhibiting mitochondrial Bid. Data were collected from $n=150–200$ cells per treatment in 10–15 randomly selected image frames from $n=3$ independent experiments. Data represent the mean±s.e.m.

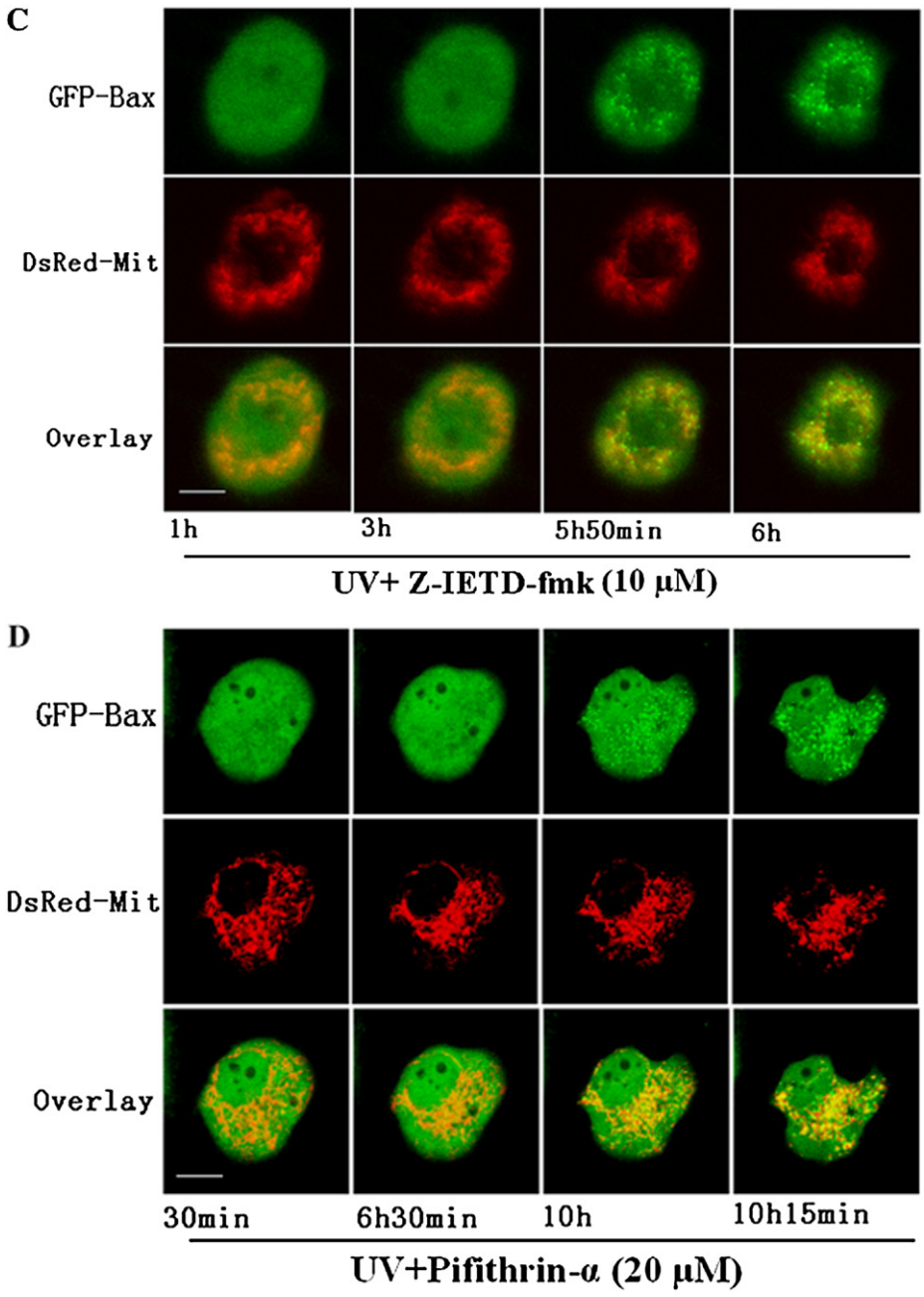

Fig. 2 (*continued*).

mitochondria [7,8,29,30]. Therefore, we examined the role of Bid and Bax during UV-induced apoptosis. To exclude that overexpression of Bid-CFP and YFP-Bax in our concentration resulted in apoptosis spontaneously, we examined distribution of Bid-CFP, YFP-Bax and DsRed-Mit without treatment, the results were shown in Fig. 3A, they remained unchanged for more than 7 h. Interestingly, when we compared the characteristic of Bid and Bax translocation from cytosol to mitochondria during UV-induced apoptosis, we found that Bax translocation differed from that of Bid. In almost all cells, Bax translocation was earlier than that of Bid and the FRET channel remained unchanged in the whole course (Fig. 3B). Similar results were obtained in COS-7 cells expressing YFP-Bax and Bid-CFP (Fig. 3C). Western blotting showed that Bid cleavage started at about 9 h after UV irradiation, which was inhibited by Z-IETD-fmk (Fig. 3D). These results indicated that Bid unlikely served as a direct activator of Bax translocation during UV-induced apoptosis.

3.4. Acceptor photobleaching demonstrated that YFP-Bax doesn't bind to Bid-CFP during UV-induced apoptosis

To further confirm that YFP-Bax did not bind to Bid-CFP during UV-induced apoptosis, the acceptor photobleaching

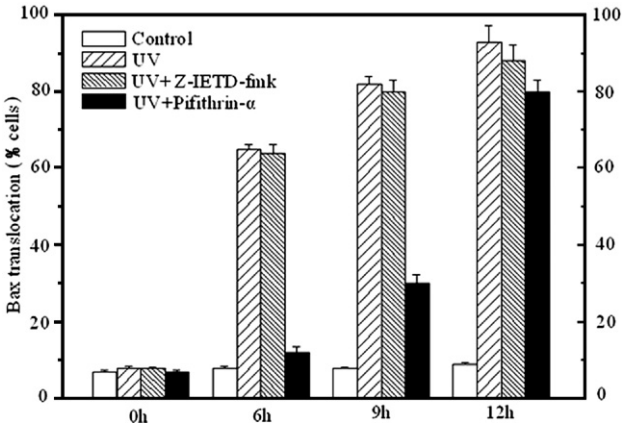

Fig. 2 (*continued*).

technique was recommended. Acceptor photobleaching, one of the techniques for measuring FRET, the acceptor molecule of the FRET pair is bleached, resulting in a unquenching of the donor fluorescence [31]. Choosing a healthy cell co-transfected YFP-Bax and Bid-CFP without UV irradiation, we bleached the acceptor YFP-Bax by strong excitation with 514 nm laser, which does not bleach Bid-CFP, the emission intensity of YFP-Bax decreased while the emission intensity of Bid-CFP remained the same (Fig. 4A). The similar results were obtained in apoptotic cells (Fig. 4B). Out of the bleaching area, fluorescence intensities of both channels had no obvious changes. These results indicated that there was no interaction between YFP-Bax and Bid-CFP in both healthy and apoptotic cells.

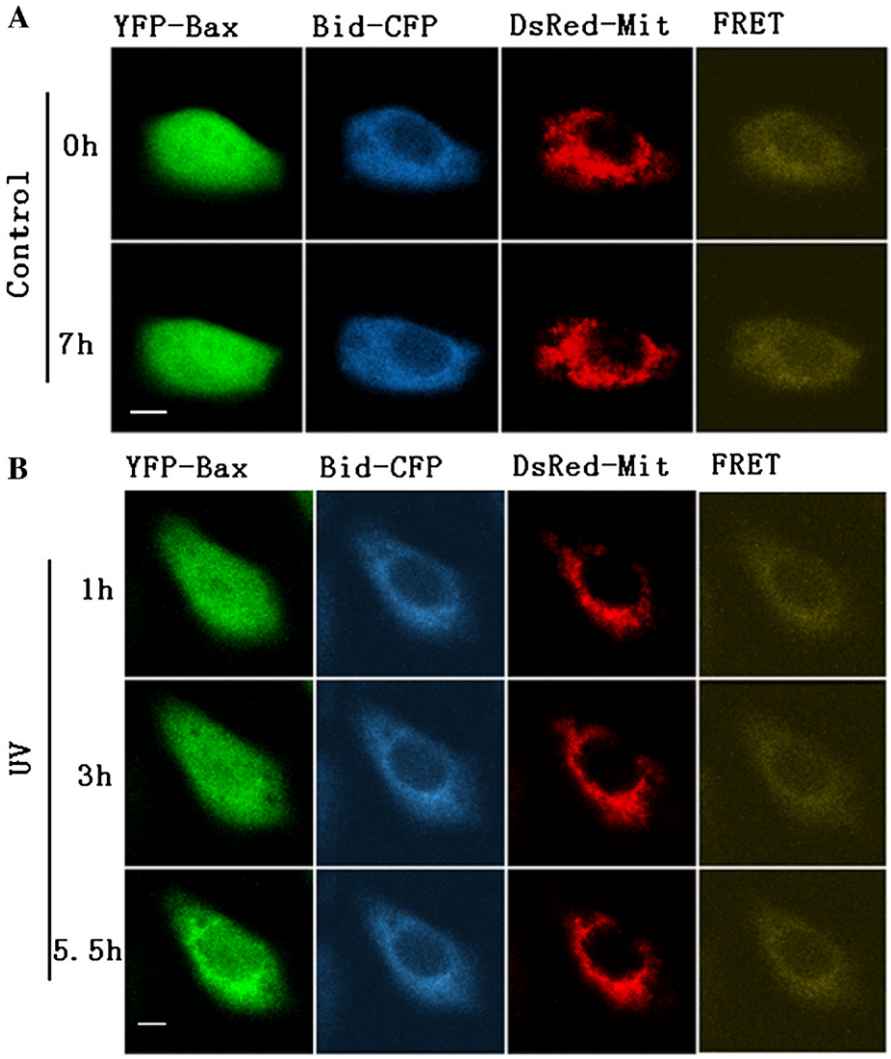

Fig. 3. Single-cell imaging analysis of the interaction between YFP-Bax and Bid-CFP during UV-induced apoptosis. (A–B) Showed the typical confocal images of ASTC-a-1 cells transfected with YFP-Bax, Bid-CFP and DsRed-Mit in different conditions. (A) Confocal images showed the distributions of YFP-Bax, Bid-CFP and DsRed-Mit in untreated cell. Both YFP-Bax and Bid-CFP typically displayed a diffuse, cytoplamic localization, the intensity of FRET remained unchanged for more than 7 h. (B) Sample records from time-lapse confocal measurements of YFP-Bax, Bid-CFP and DsRed-Mit distribution in ASTC-a-1 cells after UV irradiation. YFP-Bax redistributed into mitochondria from cytoplasma while Bid-CFP remained unchanged, and the intensity of FRET remained unchanged. (C) COS-7 cells transfected with YFP-Bax, Bid-CFP and DsRed-Mit were exposed to UV irradiation. Similar results were obtained from three independent experiments. Scale bar: 10 μm. (D) ASTC-a-1 cells were collected at the indicated time after UV irradiation in the presence or absence of Z-IETD-fmk, and were analysed for Bid cleavage by Western blotting. Data were representative of three independent experiments.

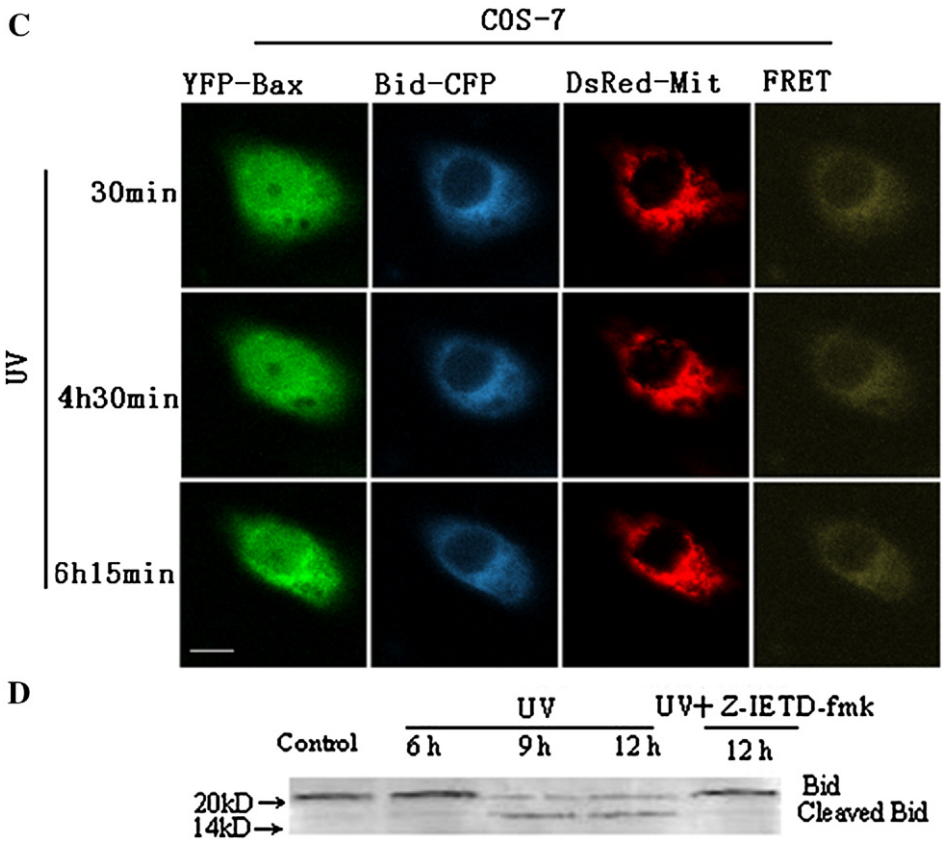

Fig. 3 (*continued*).

3.5. Caspase-3 activation induced by UV irradiation was not affected by Z-IETD-fmk, but delayed by Pifithrin-α

It is known that caspase-3 activation was a major biochemical event for the occurrence of apoptosis. Thus we investigated the effects of Z-IETD-fmk and Pifithrin-α on caspase-3 activation by UV irradiation. Western blotting showed that caspase-3 activation at 6 h after UV irradiation was not affected by Z-IETD-fmk, but inhibited by Pifithrin-α (Fig. 5A). Caspase-3 activation was also occurred in the presence of Pifithrin-α at 12 h after UV irradiation (Fig. 5B). These results revealed that caspase-3 activation induced by UV irradiation was not affected by Z-IETD-fmk, but delayed by Pifithrin-α.

3.6. Bcl-x_L prevents UV-induced apoptosis

It is known that anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-x_L, can block Bax- and Bak-induced apoptosis [32]. Therefore, if Bax plays a significant role in apoptosis induced by UV irradiation, the presence of anti-apoptotic Bcl-x_L proteins should abolish or decrease the rate of apoptosis. To investigate whether Bcl-x_L prevents UV-induced apoptosis, ASTC-a-1 cells co-transfected with YFP-Bax and CFP-Bcl-x_L were treated with UV irradiation, then the real-time monitoring of YFP-Bax and CFP-Bcl-x_L redistribution was performed on LSM microscope. As shown in Fig. 6A, YFP-Bax had a diffuse distribution in the whole cell for more than 20 h, and the cells did not exhibited characteristics of apoptosis. These results were also confirmed by statistical analysis (Fig. 6B and C).

3.7. Knocking down Bid by siRNA cannot inhibit UV-induced apoptosis

The above experiments showed that cell death, Bax translocation and caspase-3 activation induced by UV irradiation is not affected by Z-IETD-fmk. Futhermore, we wanted to examine whether knocking down the endogenous Bid could promote or facilitate the UV-induced apoptosis. To address this question, we used siRNA constructs with specific sequences of Bid (siRNA-Bid). Transfection of these constructs into ASTC-a-1 cells can significantly blocked the expressed Bid protein, whereas the negative control siRNA (siRNA-NC) did not (data not shown). Knowing that ASTC-a-1 cells had a moderate level of endogenous Bid expression, we transfected the siRNA-Bid to ASTC-a-1 cells and observed that transfection of siRNA-Bid reduced the endogenous Bid protein levels. Interestingly, we found siRNA-Bid as well as negative control siRNA had no effect on the UV-induced apoptosis (Fig. 7A). Furthermore, these results were confirmed by the statistical analysis (Fig. 7B). These experiments were repeated

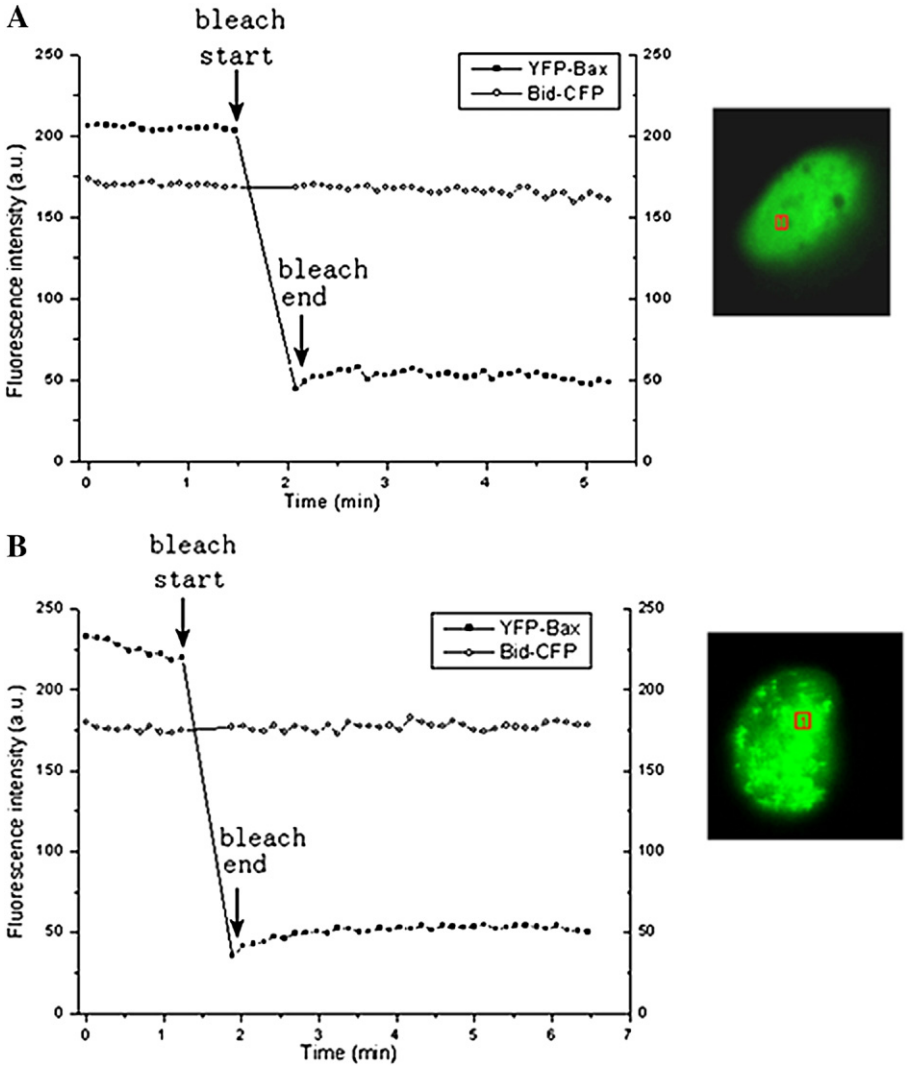

Fig. 4. The binding of YFP-Bax and Bid-CFP was examined by acceptor photobleaching. (A) ASTC-a-1 cells co-transfected with YFP-Bax and Bid-CFP without treatment was bleached with 514 nm high intensity laser, the intensity of YFP decreased while the intensity of CFP remained unchanged. Scale bar: 10 μm. (B). Apoptotic cell co-transfected with YFP-Bax and Bid-CFP was bleached with 514 nm high intensity laser, the intensity of YFP decreased while the intensity of CFP remained unchanged.

three times. Our results indicate that siRNA-Bid cannot reduce UV-induced apoptosis.

4. Discussion

Bax has been shown to be necessary for UV-induced apoptosis, recent studies have demonstrated that purified or recombinant p53 has the ability to activate Bax to oligomerize in lipid membranes and cause permeabilization [22]. It is also reported that Bax activation by active Bid [7,18] or BH3 peptides from Bid or Bim is essential and sufficient to permeabilize vesicles composed of mitochondrial lipids in the absence of other proteins [17,19]. It was demonstrated that $Bid^{-/-}$ MEFs are less susceptible than $Bid^{+/+}$ MEFs to the DNA damage [26]. So, the regulatory mechanism of Bax translocation by UV irradiation has been unclear. We now provide several lines of evidence that demonstrate that Bax translocation

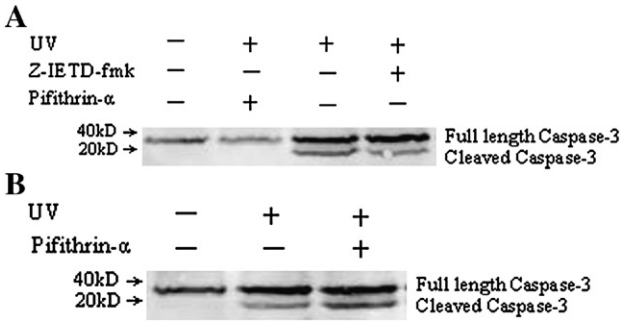

Fig. 5. Caspase-3 activation induced by UV irradiation was not affected by Z-IETD-fmk, delayed by Pifithrin-α. (A) ASTC-a-1 cells were collected at 6 h after UV irradiation in the presence or absence of Z-IETD-fmk or Pifithrin-a, and were analysed by Western blotting for caspase-3 activation. (B) ASTC-a-1 cells were collected at 12 h after UV irradiation in the presence or absence of Pifithrin-α., and were analysed by Western blotting for caspase-3 activation. Data were representative of three independent experiments.

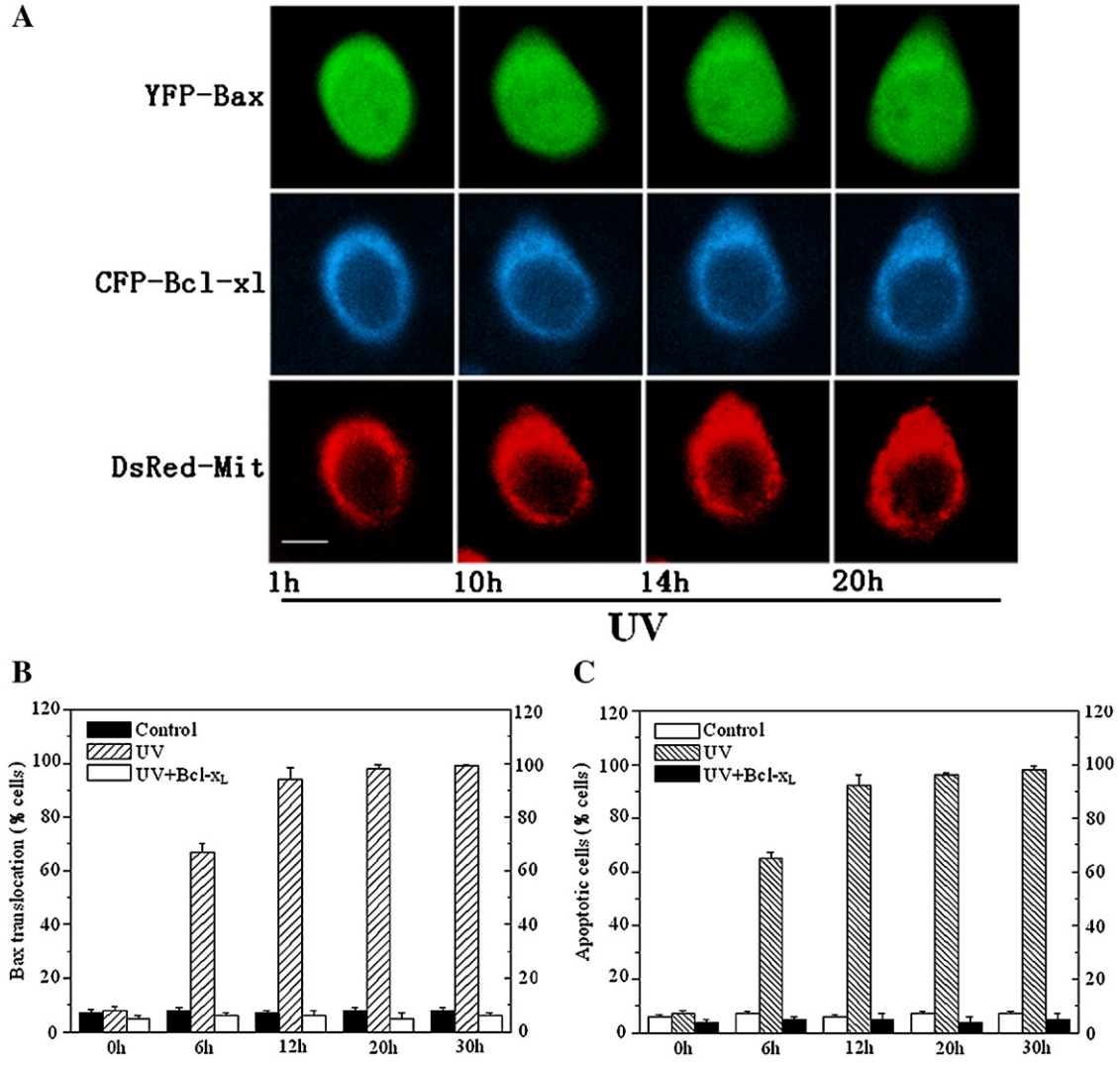

Fig. 6. Bcl-x_L prevents UV-induced apoptosis. (A) Showed the typical confocal images of ASTC-a-1 cells transfected with YFP-Bax, CFP-Bcl-x_L and DsRed-Mit after UV irradiation. Scale bar: 10 μm. (B) ASTC-a-1 cells co-transfected with YFP-Bax, CFP-Bcl-x_L and DsRed-Mit were exposed in different conditions for indicated times and assessed by counting the number of cells exhibiting mitochondrial Bax. (C) ASTC-a-1 cells transfected with CFP-Bcl-x_L were exposed in different conditions for indicated times and assessed by counting the number of cells exhibiting apoptosis. Error bars are s.e.m. from several hundred cells in three independent experiments.

by UV irradiation is a Bid-independent event, delayed by p53 inhibitor, and inhibited by Bcl-x_L: (1) Bax translocation and cell death by UV irradiation were not affected by Z-IETD-fmk, delayed by Pifithrin-α, inhibited by Bcl-x_L (Figs. 1, 2, and 6). (2) Co-transfecting Bid-CFP and YFP-Bax in a single cell, we found that YFP-Bax translocation was earlier than that of Bid-CFP and there was no significant FRET between them (Fig. 3). (3) Using acceptor photobleaching technique, we also demonstrated that there was no interaction between Bid-CFP and YFP-Bax in both healthy and apoptotic cells (Fig. 4). (4) Caspase-3 activation by UV irradiation was not affected by Z-IETD-fmk, but delayed by Pifithrin-a (Fig. 5). (5) Repression of Bid protein with siRNA did not inhibit cell death by UV irradiation (Fig. 7). These results strongly indicate that Bid is not required for Bax translocation during UV-induced apoptosis.

Why Bax translocation, caspase-3 activation and cell death by UV irradiation were not affected by Z-IETD-fmk, delayed by Pifithrin-α? UV irradiation allows stabilization of p53, which accumulates in the nucleus and regulates target gene expression. Numerous genes are regulated by p53, such as those encoding death receptors, for example, FAS (CD95) and proapoptotic Bcl-2 proteins (for example, Bax, Bid, Noxa, and PUMA). In parallel, p53 also accumulates in the cytoplasm, where it directly activates the proapoptotic protein Bax to promote mitochondrial outer-membrane permeabilization (MOMP) [22, 33–35]. Once MOMP occurs, proapoptogenic factors (for example, cytochrome *c*) are released from mitochondria, caspases are activated, and apoptosis rapidly ensues [36]. Thus, p53 possesses a proapoptotic function that is independent of its transcriptional activity [37]. Pifithrin-α is a small molecule inhibitor of p53 transcriptional activity, so it cannot fully inhibited Bax translocation, caspase-3 activation and cell death by UV irradiation. However, Pifithrin-α could block nuclear p53 function, thus inhibit expression of PUMA, which

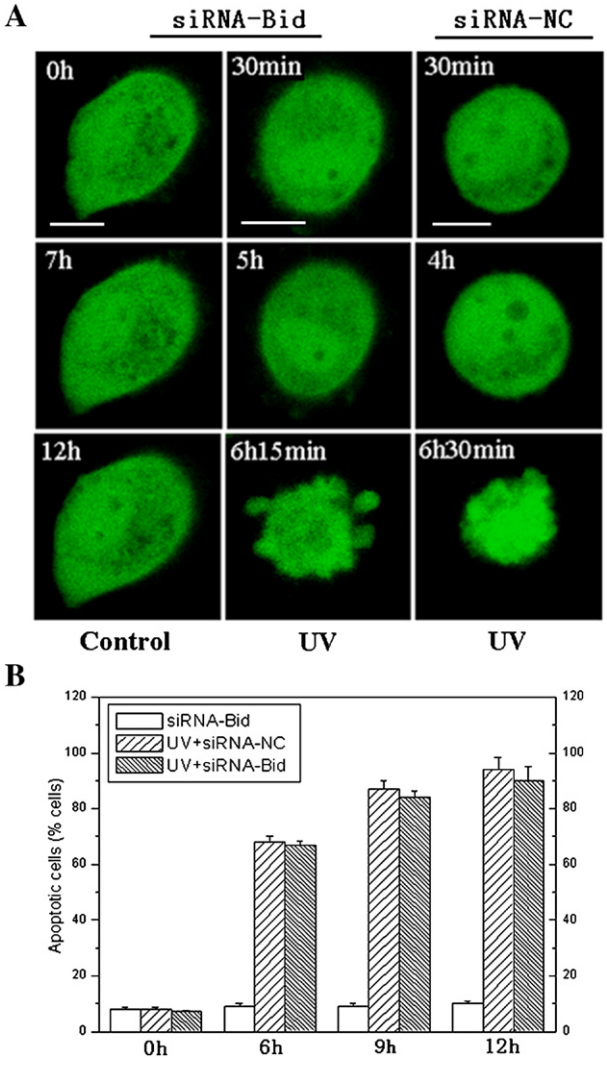

Fig. 7. siRNA knockdown of Bid protein. (A) ASTC-a-1 cells were transfected with siRNA-Bid or siRNA-NC. 48 h later the cells were observed with the LSM microscope. Scale bar: 10 μm. (B) Statistic analysis of apoptotic cells transfected with siRNA-Bid or siRNA-NC with or without UV irradiation. Data were representative of three independent experiments.

could displace p53 from Bcl-x_L, allowing p53 to induce mitochondrial permeabilization, so apoptosis induced by UV irradiation is delayed by Pifithrin-α.

Another related question is how Bcl-x_L prevents Bax transolation? For long, it has been puzzling that Bcl-x_L, which is mainly localized at the intracellular membranes (mitochondria and ER), prevents Bax from translocating from cytosol to mitochondria and ER, undergoing conformational changes and forming oligomers. It is of interest to note that Bcl-x_L could sequester p53 [38]. P53 play a role in Bax conformational change induced by UV irradiation. Our results indicate that Bcl-x_L prevents Bax activation through p53, which leads to Bax conformational change and its translocation. Clearly, additional studies are required to fully delineate the biochemical mechanisms by which Bcl-x_L regulates Bax activation and apoptosis in response to UV irradiation.

The fluorescence resonance energy transfer (FRET) is a process by which transfer of energy occurs from a donor fluorophore molecule to an acceptor fluorophore molecule in close proximity. The emission spectrum of the donor molecule overlaps with the absorption spectrum of the acceptor molecule. When the two fluorophores are spatially close enough there is energy transfer between the donor and acceptor molecules. The excited donor transfers its energy to the acceptor. This results in a reduction in donor fluorescence emission and, at the same time, an increase in acceptor fluorescence emission [39]. FRET is a powerful technique that can provide insight into the spatial and temporal dynamics of protein–protein interactions in vivo [28,40–44]. In our current study, we employ single-cell FRET analyse to monitor the interaction and translocation between YFP-Bax and Bid-CFP by UV irradiation. We clearly demonstrated Bid is not required for Bax translocation during UV-induced apoptosis. It specifically enhances our understanding of the regulatory mechanism of Bax translocation during UV-induced apoptosis that could be potentially missed in population-based studies.

In summary, we demonstrated that Bax translocation by UV irradiation is a Bid-independent event and inhibited by overexpression of Bcl-x_L. Our results imply that Bid is involved, but not the dominant pathway in UV-induced apoptosis.

Acknowledgments

We thank Dr. K. Taira (University of Yokyo, Hongo, Tokyo, Japan) for kindly providing pBid-CFP plasmid, Dr. Y. Gotoh (University of Yokyo, Yayoi, Tokyo, Japan) for kindly providing the pDsRed-Mit plasmid, Andrew kindly providing the pYFP-Bax and pCFP-Bcl-x_L plasmids and we also thank Richard J.Youle for kindly providing the pGFP-Bax plasmid. This research was supported by the National Natural Science Foundation of China (30670507; 30600128; 30470494) and the Natural Science Foundation of Guangdong Province (015012).

References

[1] K.H. Kraemer, Proc. Natl. Acad. Sci. U. S. A. 94 (1) (1997) 11.
[2] A. Ziegler, A.S. Jonason, D.J. Leffell, J.A. Simon, H.W. Sharma, J. Kimmelman, L. Remington, T. Jacks, D.E. Brash, Nature. 372 (6508) (1994) 773.
[3] S. Haupt, M. Berger, Z. Goldberg, Y. Haupt, J. Cell Sci. 116 (20) (2003) 4077.
[4] R. Locksley, N. Killeen, M. Lenardo, Cell 104 (4) (2001) 4487.
[5] M.P. Boldin, T.M. Goncharev, Y.V. Goltsev, D. Wallach, Cell 85 (6) (1996) 803.
[6] M. Muzio, A.M. Chinnayan, F.C. Kischkel, K. O'Rourke, A. Shevchenko, J. Ni, C. Scaffidi, J.D. Bretz, M. Zhang, R. Gentz, M. Mann, P.H. Krammer, M.E. Peter, V.M. Dixit, Cell. 85 (6) (1996) 817.
[7] X. Luo, I. Budihardjo, H. Zou, C. Slaughter, X. Wang, Cell. 94 (4) (1998) 481.
[8] H. Li, H. Zhu, C.J. Xu, J. Yuan, Cell. 94 (4) (1998) 491.
[9] A. Gross, X.M. Yin, K. Wang, M.C. Wei, J. Jockel, C. Milliman, H. Erdjument-Bromage, P. Tempst, S.J. Korsmeyer, J. Biol. Chem. 274 (2) (1999) 1156.
[10] J.M. Jurgensmeier, Z.H. Xie, Q. Deveraux, L. Ellerby, D. Bredesen, J.C. Reed, Proc. Natl. Acad. Sci. U. S. A. 95 (9) (1998) 4997.
[11] D.M. Finucane, E. Bossy-Wetzel, N.J. Waterhouse, T.G. Cotter, D.R. Green, J. Biol. Chem. 274 (4) (1999) 2225.

[12] A.M. Ranger, B.A. Malynn, S.J. Korsmeyer, Nat. Genet. 28 (2) (2001) 113.
[13] M.C. Wei, W.X. Zong, E.H. Cheng, T. Lindsten, V. Panoutsakopoulou, A.J. Ross, K.A. Roth, G.R. MacGregor, C.B. Thompson, S.J. Korsmeyer, Science 292 (5517) (2001) 727.
[14] Y.T. Hsu, K.G. Wolter, R.J. Youle, Proc. Natl. Acad. Sci. U. S. A. 94 (8) (1997) 3668.
[15] A. Gross, J. Jockel, M.C. Wei, S.J. Korsmeyer, EMBO J. 17 (14) (1998) 3878.
[16] M. Saito, S.J. Korsmeyer, P.H. Schlesinger, Nat Cell Biol. 2 (8) (2000) 553.
[17] T. Kuwana, M.R. Mackey, G. Perkins, M.H. Ellisman, M. Latterich, R. Schneiter, D.R. Green, D.D. Newmeyer, Cell. 111 (3) (2002) 331.
[18] K. Wang, X.M. Yin, D.T. Chao, C.L. Milliman, S.J. Korsmeyer, Genes Dev. 10 (22) (1996) 2859.
[19] T. Kuwana, L. Bouchier-Hayes, J.E. Chipuk, C. Bonzon, B.A. Sullivan, D.R. Green, D.D. Newmeyer, Mol. Cell. 17 (4) (2005) 525.
[20] M.C. Wei, T. Lindsten, V.K. Mootha, Genes Dev. 14 (16) (2000) 2060.
[21] S.J. Korsmeyer, M.C. Wei, M. Saito, S. Weiler, K.J. Oh, P.H. Schlesinger, Cell Death Differ. 7 (12) (2000) 1166.
[22] J.E. Chipuk, T. Kuwana, L. Bouchier-Hayes, N.M. Droin, D.D. Newmeyer, M. Schuler, D.R. Green, Science 303 (5660) (2004) 1010.
[23] J. Zha, S. Weiler, K.J. Oh, M.C. Wei, S.J. Korsmeyer, Science 290 (5479) (2000) 1761.
[24] X. Wang, Genes Dev. 15 (22) (2001) 2922.
[25] X.M. Yin, K. Wang, A. Gross, Y. Zhao, S. Zinkel, B. Klocke, K.A. Roth, S.J. Korsmeyer, Nature 400 (6747) (1999) 886.
[26] J.K. Sax, P. Fei, M.E. Murphy, E. Bernhard, S.J. Korsmeyer, W.S. El-Deiry, Nat. Cell Biol. 4 (11) (2002) 842.
[27] A. Nechushtan, C.L. Smith, Y.T. Hsu, R.J. Youle, EMBO J. 18 (9) (1999) 2330.
[28] Y.Y. Wu, D. Xing, L. Liu, T.S. Chen, W.R. Chen, Acta Biochim. Biophys. Sinica. 39 (1) (2007) 37.
[29] S. Desagher, A. Osen-Sand, A. Nichols, R. Eskes, S. Montessuit, S. Lauper, K. Maundrell, B. Antonsson, J.C. Martinou, J. Cell Biol. 144 (5) (1999) 891.
[30] H. Yamaguchi, K. Bhalla, H.G. Wang, Cancer Res. 63 (7) (2003) 1483.
[31] E.V. Munster, G.J. Kremers, M.J. Adjobo-Hermans, T.W. Gadella, J. Microsc. 218 (Pt 3) (2005) 253.
[32] E.H. Cheng, M.C. Wei, S. Weiler, R.A. Flavell, T.W. Mak, T. Lindsten, S.J. Korsmeyer, Mol. Cell. 8 (3) (2001) 705.
[33] M. Mihara, S. Erster, A. Zaika, O. Petrenko, T. Chittenden, P. Pancoska, U.M. Moll, Mol Cell. 11 (3) (2003) 577.
[34] P. Dumont, J.I. Leu, A.C. Della, III. Pietra, D.L. George, M. Murphy, Nat. Genet. 33 (3) (2003) 357.
[35] K.H. Vousden, Science 309 (5741) (2005) 1685.
[36] N.N. Danial, S.J. Korsmeyer, Cell. 116 (2) (2004) 205.
[37] J.E. Chipuk, U. Maurer, D.R. Green, M. Schuler, Cancer Cells 4 (5) (2003) 371.
[38] J.E. Chipuk, L.B. Hayes, T. Kuwana, D.D. Newmeyer, D.R. Green, Science 309 (5741) (2005) 1732.
[39] S. Fields, Nature 340 (6085) (1989) 245.
[40] F. Gaits, K. Hahn, Sci STKE (165) (2003) PE3.
[41] T. Kiwamu, N. Takeharu, M. Atsushi, M. Masayuki, J. Cell Biol. 160 (2) (2003) 235.
[42] F. Wang, T.S. Chen, D. Xing, J.J. Wang, Y.X. Wu, Lasers Surg. Med. 36 (1) (2005) 2.
[43] Y.X. Wu, D. Xing, W.R. Chen, Cell Cycle 5 (7) (2006) 729.
[44] X.J. Gao, T.S. Chen, D. Xing, F. Wang, Y.H. Pei, X.B. Wei, J. Cell. Physiol. 206 (2) (2006) 441.
[45] A.J. Valentijn, A.D. Metcalfe, J. Kott, C.H. Streuli, A.P. Gilmore, J. Cell Biol. 162 (4) (2003) 599.
[46] F. Tsuruta, N. Masuyama, Y. Gotoh, J. Biol. Chem. 271 (46) (2002) 14040.
[47] R. Onuki, A. Nagasaki, H. Kawasaki, T. Baba, T.Q. Uyeda, K. Taira, Proc. Natl. Acad. Sci. U. S. A. 99 (23) (2002) 14716.