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ORIGINAL PAPER

Real-time monitoring full length bid interacting with Bax during TNF-α-induced apoptosis

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Abstract Bid, a member of the pro-apoptotic Bcl-2 protein family, is activated through caspase-8-mediated cleavage into a truncated form (p15 tBid) during TNF-α(tumor necrosis factor α)-induced apoptosis. Activated tBid can induce Bax oligomerization and translocation to mitochondria, triggering the release of cytochrome c, caspase-3 activation and cell apoptosis. However, it is debatable that whether Bid and tBid can interact directly with Bax in living cells. In this study, we used confocal fluorescence microscope, combined with both FRET (fluorescence resonance energy transfer) and acceptor photobleaching techniques, to study the dynamic interaction between Bid and Bax during TNF-α-induced apoptosis in single living cell. In ASTC-a-1 cells, full length Bid induced Bax translocation to mitochondria by directly interacting with Bax transiently in response to TNF-α treatment before cell shrinkage. Next, we demonstrated that, in both ASTC-a-1 and HeLa cells, Bid was not cleaved before cell shrinkage even under the condition that caspase-8 had been activated, but in MCF-7 cells Bid was cleaved. In addition, in ASTC-a-1 cells, caspase-3 activation was a biphasic process and Bid was cleaved after the second activation of caspase-3. In summary, these findings indicate that, FL-Bid (full length-Bid) directly regulated the activation of Bax during TNF-α-induced apoptosis in ASTC-a-1 cells and that the cleavage of Bid occurred in advanced apoptosis.

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Introduction

TNF-α (tumor necrosis factor α) provides a wide variety of biologic signals, which are involved in the regulation of cell death and participate in the governing of immune homeostasis [1]. TNF-α exerts its pleiotropic function through two distinct receptors, TNFR1 (tumor necrosis factor α receptor 1) and TNFR2 (tumor necrosis factor α receptor 2) [2]. Both receptors contain several cysteine repeats in their extra-cellular domains, whereas their intracellular domains contain no significant homology. Apoptosis is mainly induced through TNFR1 by two best characterized pathways. Once engaged by ligand, these receptors initiate the formation of a DISC (death-induced signaling complex) that leads to activation of caspase-8 [3]. Active caspase-8 can initiate two pathways: the activation of a cascade of downstream caspases (caspase-3) leading to irreversible cell death, and the cleavage of the pro-apoptotic Bid protein. Cleavage of cytosolic Bid at Asp-59 yields a p15 C-terminal truncated fragment (tBid) that translocates to the mitochondria to induce the release of Cyt c (cytochrome c) [4–7]. Released Cyt c induces the oligomerization of Apaf-1, which forms a multimeric complex that recruits and leads to the activation of caspase-9. Activated caspase-9 leads to downstream caspase-3 activation resulting in apoptotic cell death [8].

Bid, a BH3-only multi-functional molecule, has been implicated in the TNFR1 and Fas death signal pathways [9]. In death-receptor signal pathway, Bid is post-translationally activated through caspase-8-mediated cleavage of

the p22 FL-Bid (full-length Bid) protein to a truncated form (p15 tBid) [4–6]. Targeting of tBid to mitochondria induces the activation of Bax and Bak in a BH3-dependent manner, resulting in the release of Cyt c [10, 11] by a mechanism that is independent of the induction of the mitochondrial permeability transition [10, 12–14]. BN-PAGE (blue native polyacrylamide gel electrophoresis) technique shows that the interaction between tBid and Bax is a transient ''touch-and-go'' interaction, as the previously proposed mechanism for tBid and Bak [11, 15]. Recent work has demonstrated that a stapled Bid BH3 helix directly binds and activates Bax in vitro and in vivo using a FITC-labeled, cell-permeable Bid [16]. Some other experiments show both FL-Bid and p15 tBid can induce Bax oligomerization to trigger cytochrome c release and the following apoptotic events in vitro [10, 17]. On the other hand, by comparing the time course of Bid and Bax translocation from cytosol to mitochondria [18], Bid is unlikely to serve as a direct activator of Bax translocation during STS (staurosporine)-induced apoptosis in cells co-expressing Bid and Bax. A recent report demonstrates that both tBid and FL-Bid have the capacity to translocate to mitochondria during apoptosis [19]. However, there are still issues yet to be addressed such as the interaction between Bid/tBid and Bax, when and how, or indeed if Bid/tBid interact with Bax.

Genetically encodable fluorescent reporters based on FRET (fluorescence resonance energy transfer) have become powerful tools for monitoring the activities of signaling molecules with high spatiotemporal resolution in living cells [20, 21]. In this study, we used confocal fluorescence imaging, FRET and acceptor photobleaching techniques to investigate the dynamics of Bid cleavage and the interaction between Bid and Bax during TNF-α-induced apoptosis in living cells. Our results indicate that FL-Bid could interact with Bax directly in the cytoplasm after TNF-α treatment. This interaction induces Bax translocation to mitochondria. Dynamic activation of caspase-3 before Bax translocation was visualized and the Bid cleavage was investigated after the cell shrinkage.

Materials and methods

Materials

DMEM (Dulbecco's modified Eagle medium) was purchased from GIBCO (Grand Island, NY, USA). TNF-α was purchased from Sigma (St Louis, Mo, USA). Lipofectamin 2000 was purchased from Invitrogen (Carlsbad, CA, USA). DNA Extraction kit was purchased from Qiagen (Valencia, CA, USA). CHX (Cycloheximide) was purchased from Sigma.

Cell culture and transfection

ASTC-a-1, HeLa, MCF-7 and SCAT3 stable-expressing ASTC-a-1 cells [22] were cultured in DMEM medium supplemented with 15% fetal calf serum, and the cells were maintained at 37°C in a humidified atmosphere (95% air and 5% CO_2). For microscopy purposes, the cells were cultured on a cover slip-bottomed small chamber. Cell cultures at a confluence of 40–60% were used for transfection with 0.55–0.65 μg DNA and 2 μl Lipofectamin 2000 per chamber.

Plasmid DNA of pECFP-C1 was purchased from Clontech (USA). Plasmid DNA of FRET-Bid (a kind gift from Dr. R. Onuki, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan) [23] consisted of YFP (yellow fluorescent protein), FL-Bid and CFP (cyan fluorescent protein). After Bid cleavage, CFP separates from YFP, so the FRET between CFP and YFP decreases (Fig. 1A). Bid-CFP was also a kind gift from Dr. R. Onuki, consisted of CFP and FL-Bid. YFP-Bax was a kind gift from Andrew P. Gilmore (Wellcome Trust Centre for Cell Matrix Research, School of Biological Sciences, University of Manchester, UK), containing YFP and Bax (human Bax α) [24]. Bid-CFP and YFP-Bax were used to monitor the direct interaction between Bid and Bax. Upon Bid interacting with Bax, YFP is in close proximity to CFP, the FRET efficiency between CFP and YFP would increase (Fig. 1B). DsRed-Mit was a kind gift from Yukiko Gotoh (Japan Science and Technology Corporation, Japan) [25], constructed by inserting synthetic double-stranded oligonucleotides spanning a mitochondrial targeting sequence of human cytochrome c oxidase into BamHI and NheI sites of the pDsRed-N1 vector.

FRET imaging and acceptor photobleaching

FRET was performed on a commercial Laser Scanning Microscopes combination system (LSM510/ConfoCor2,

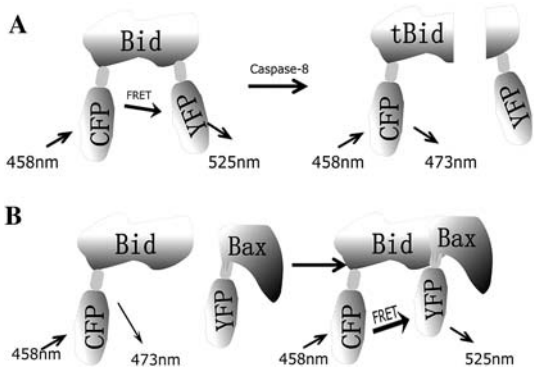

Fig. 1 Schematic diagram of Bid cleavage and the interaction between Bid and Bax

Zeiss, Jena, Germany) equipped with a Zeiss Plan-Neofluar 40 × /1.3 NA Oil Dic objective as described previously [22, 26]. To monitor the fluorescence of FRET-Bid reporter, the 458 nm line of an Ar-Ion Laser (Zeiss, Jena, Germany) was used to excite CFP, reflected by a dichroic mirror (main beam splitter HFT458/514). The emission fluorescence was split by a second dichroic mirror (secondary beam splitter NFT515) into separate CFP (470–500 nm bandpass) and FRET (530 nm longpass) channels, respectively. To monitor the fluorescence of YFP-Bax protein, the 488 nm (or 514 nm) line of the Ar-Ion Laser was used to excite YFP, reflected by a dichroic mirror (main beam splitter HFT488 (or main beam splitter HFT458/514)). A 500–550 nm band pass (or a 530 nm long pass) filter was used to record the emission fluorescence for the YFP channel. To monitor the fluorescence of DsRed-Mit protein, the 543 nm line of a He–Ne Laser (Zeiss, Jena, Germany) was used to excite DsRed, reflected by a dichroic mirror (main beam splitter HFT KP700/543). The 565–615 nm bandpass filter was used to record the emission fluorescence for the DsRed channel.

To monitor the fluorescence of PI (propidium iodide), the 488 nm line of the Ar-Ion laser was used to excite PI, reflected by a dichroic mirror (main beam splitter HFT KP 700/488). A LP650 nm long pass filter was used to record the emission fluorescence for the PI channel. To monitor the fluorescence of Hochest33342 (352/461), a mercury lamp was used to excite Hochest33342, reflected by a filter set (BP 365/12, FT 395, LP397). A BP 390–465 nm bandpass filter was used to record the emission fluorescence for the Hochest33342 channel.

For time-lapse imaging, cover-slip-bottomed small chambers were mounted on the microscope stage equipped with a temperature-controlled chamber (Tempcontrol 37-2 digital, Zeiss). Digital image analysis for quantitative evaluation was performed using Zeiss Rel3.2 image processing software (Zeiss, Germany). In the case of any fluorescent proteins, after choosing the area of interest from the cell images, the average fluorescence intensities of CFP and YFP channels and the background were obtained. The background-subtracted fluorescence intensity of FRET divided by background-subtracted fluorescence intensity of CFP is the ratio of FRET/CFP.

To induce the apoptosis, ASTC-a-1, HeLa and MCF-7 cells transfected with FRET-Bid plasmid or YFP-Bax and Bid-CFP or YFP-Bax and DsRed-Mit were treated with TNF-α (40 ng/ml) plus CHX (15 μg/ml). The cells were then imaged by LSM microscope using the same method as described above.

Acceptor bleaching experiments were carried out to assess the correct expression and the sensitivity of the FRET-Bid probe in single cell. During the experiment, the acceptor (YFP) was selectively bleached by repeated scanning of the cell area with 514 nm laser line. A quantitative analysis of acceptor bleaching showed the absolute fluorescence values for CFP and YFP for a single cell when plotted as a function of time. Upon bleaching there was a marked decrease in the acceptor fluorescence (YFP), which coincided with an increase in the donor fluorescence (CFP) because of an inability of the acceptor to accept energy from the donor after bleaching. Therefore the increase of CFP fluorescence upon YFP bleaching confirmed that FRET exists between the two fluorescent proteins in the FRET-Bid probe in vivo. The same method is also used in YFP-Bax and Bid-CFP co-expressed cells.

SDS-PAGE and Western Blotting

Briefly, cells were washed three times with pre-cooled PBS before being smudged. The treated or untreated cells were lysed in a buffer containing 50 mM Tris·HCl pH8.0, 150 mM NaCl, 1% TritonX-100, 100 μg/ml PMSF and 1 × protease inhibitor cocktail set I (Calbiochem®, cat.No. 539131). The samples were separated by 15% SDS-polyacrylamide gel electrophoresis and transferred onto protran BA85 cellulosenitrat(e) (schleicher&schuell bioscience* D-37586. Dassel, Germany) or BioTrace™ NT Nitrocellulose Membranes (Pall). The resulting membrane was blocked with 5% skim milk, incubated with a designated primary antibody and the secondary antibody. The signals were detected with an ODYSSEY® Infrared Imaging System (Li-Cor, NE, USA). The following antibodies were used for immunoblot: anti-caspase-8 (Cell signaling, Cat. No. 9746), anti-Bid (Cell signaling, cat. No. 2002), IR-Dye®800CW anti-rabbit IgG (Rockland, Gilbertsville, PA, USA), Alexa Fluor 680® goat anti-Mouse IgG (Molecular Probes, OR, USA).

Results

Bid interacting with Bax transiently but not firmly during TNF-α-induced apoptosis

Both Bid and Bax have been involved in the initiation of apoptosis through the mitochondrial pathway [27]. The most popular model of the relationship between these two proteins during apoptosis is that Bid induces conformation changes and oligomerization of Bax and insertion of Bax into the outer mitochondrial membrane, yet none of the efforts have resulted from observing Bid-Bax complex in cells [10–12, 15].

It has been reported that Bid can induce the translocation of Bax to mitochondria by interacting directly with Bax in a cell-free experiment [10]. In order to examine whether there was direct interaction between Bid and Bax

in living cells during TNF-α-induced apoptosis, the ASTC-a-1 cells, co-transfected with Bid-CFP and YFP-Bax plasmids, were performed on confocal fluorescence imaging microscope. Before TNF-α treatment, acceptor photobleaching technique was used to detect that whether Bid bound to Bax in the cytoplasm (Fig. 2A). Fig. 2(A) shows that, the fluorescence intensity of YFP sharply decreased after photobleaching of YFP, but the fluorescence intensity of CFP and FRET kept constant. This result is similar with that in the control, pECFP-C1 and YFP-Bax co-expressing, cell (Fig. 2A inset), which suggests that, Bid didn't bind to Bax before TNF-α treatment. Subsequently, the same cells were treated with TNF-α (40 ng/ml) plus CHX (15 μg/ml) on the microstat. The dynamic interaction of Bid and Bax was investigated with LSM microscope. The images of FRET, CFP and 514 nm-excited YFP channels are shown in Fig. 2(B). YFP-Bax translocated to mitochondria before the cell shrinkage. But Bid-CFP didn't translocate to the mitochondria before the cell shrinkage. Fig. 2(C) is the quantification of fluorescence intensities of CFP and FRET channels (Fig. 2B) and ratio of FRET/CFP emission in the selected region after TNF-α treatment. The curves show that, the emission of FRET and ratio of FRET/CFP increased, and the emission of CFP decreased (Fig. 2C). These results imply that Bid interacted with Bax directly during TNF-α-induced apoptosis and that, they bound to each other not firmly but transiently. To confirm the interaction between Bid and Bax, we repeated the same photobleaching of YFP after the cell shrinkage as the above one (Fig. 3A). An immediate increase of the fluorescence intensity of CFP was observed after photobleaching of YFP. This implies that there existed an energy transfer from CFP to YFP (Fig. 3A) and further verifies the interaction between Bid and Bax during TNF-α-induced apoptosis. These results were further confirmed by the statistical analysis of photobleaching of YFP-Bax in both pECFP-C1 and YFP-Bax co-expressing cells or Bid-CFP and YFP-Bax co-expressing cells (Fig. 3B). The percentage of cells exhibiting Bid interaction with Bax increased largely compared with the control cells after cell shrinkage (Fig. 3B).

To prove whether the cell underwent apoptosis, Hochest33342 and PI staining were performed on the cells after the experiment of second photobleaching of YFP. Figure 3(C) shows the cell nuclear condensation and DNA fragmentation, suggesting that the cell was an apoptotic cell. Simultaneous assessment of nuclear chromatic morphology by Hoechst33342 staining and PI staining verified that the cells eventually manifested typical apoptotic condensation nuclei (Fig. 3C). These results strongly confirmed that there was interaction between Bid and Bax in apoptotic cells and that, the interaction is not firmly but transiently.

Bax oligomerization and translocation to mitochondria during TNF-α-induced apoptosis

To confirm Bax translocation to mitochondria, DsRed-Mit plasmid (to encode a mitochondrial targeting fusion protein [25]) and YFP-Bax plasmid were co-transfected into ASTC-a-1 cells. After TNF-α plus CHX treatment, Bax was observed in punctuate clusters associated with mitochondria (Fig. 4). These perimitochondrial clusters appeared identical to those described for GFP-Bax after staurosporine treatment [18]. In addition, clusters of Bax also appeared near the mitochondria. These results indicate that there existed at least two stages of Bax oligomerization: clusters on mitochondria and clusters near mitochondria. The same results also were captured in MCF-7 (data not shown), a caspase3-deficient cell line [28]. These results also demonstrate that Bax translocated to the mitochondria before cell shrinkage.

FL-Bid, not tBid, interact with Bax during TNF-α-induced apoptosis

We have observed that Bid interacted with Bax during TNF-α-induced apoptosis, but which one of Bid or tBid directly interacted with Bax? To investigate whether Bid was cleaved in TNF-α-induced apoptosis, ASTC-a-1 cells transfected with the FRET-Bid plasmid were treated with TNF-α plus CHX. Cleavage of FL-Bid resulted in a separation of YFP and CFP, thus reduced the efficiency of resonance energy transfer. Acceptor bleaching experiments were carried out to assess the sensitivity of the FRET probe. The acceptor fluorophore YFP was selectively bleached with 514 nm laser (Fig. 5A), and the emission intensity of CFP channel increased, implying that there were FRET between the donor (CFP) and the acceptor (YFP) of the FRET-Bid probe in vivo. After TNF-α plus CHX treatment the CFP and YFP fluorescence distributed evenly in the cytoplasm before cell shrinkage (Fig. 5B). The quantification of the mean fluorescence intensities of CFP and FRET channels showed little change before the cell shrinkage (Fig. 5C). However, both the Bid cleavage and translocation to mitochondria (as the arrow shown in Fig. 5B) were detected about 1 h after cell shrinkage (Fig. 5C). These data show that Bid was neither cleaved nor translocated to mitochondria before cell shrinkage but that Bid was cleaved and Bid translocated to mitochondria after cell shrinkage. The same results were observed in HeLa cells transfected with FRET-Bid plasmid (data not shown). Surprisingly, in MCF-7 cells (caspase-3 deficient) Bid was cleaved and translocated to mitochondria before cell shrinkage (data not shown). This is coincided with the data reported by Manus W. Ward et al. [19].

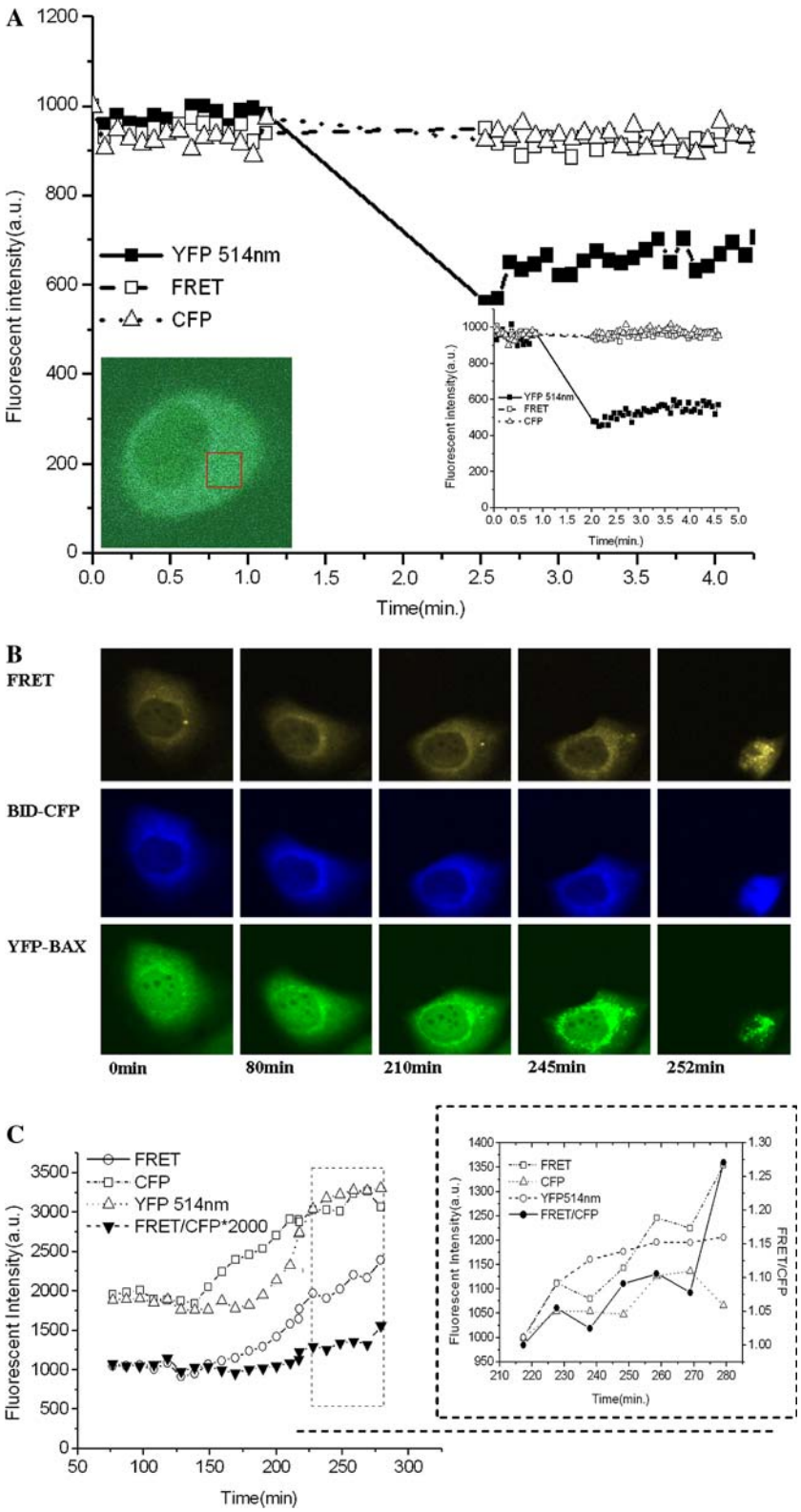

Fig. 2 Dynamic interactions between Bid and Bax. **(A)** Acceptor photobleaching on Bid-CFP and YFP-Bax co-expressing ASTC-a-1 cell before TNF-α treatment. YFP was selectively photobleached of at high laser power at 514 nm. The frame in the cell image indicated the photobleached area. The inset shows the result of selectively photobleaching of YFP in pECFP-C1 and YFP-Bax co-expressed cell, as a negative control. **(B)** Fluorescence image series of FRET, CFP and YFP emission in ASTC-a-1 cell after TNF-α plus CHX treatment. 0 min is when TNF-α plus CHX treatment started. **(C)** Dynamics of FRET, CFP and YFP emission intensities, corresponding to those in **(B)**, respectively. The data in the dashed frame is normalized to the corresponding values at 225 min and re-plotted in figure for easier comparison

In order to confirm the above results, western blotting experiment was performed (Fig. 5D). The shrinking cells were easy to be separated from the adherent cells by blowing with medium. Based on this, the shrinking cells and the adherent cells were separated and harvested after the cells treated with TNF-α plus CHX for 11.5 h. Three cell lines were used in the experiment, including ASTC-a-1, HeLa and MCF-7 cells. The whole cell extract were

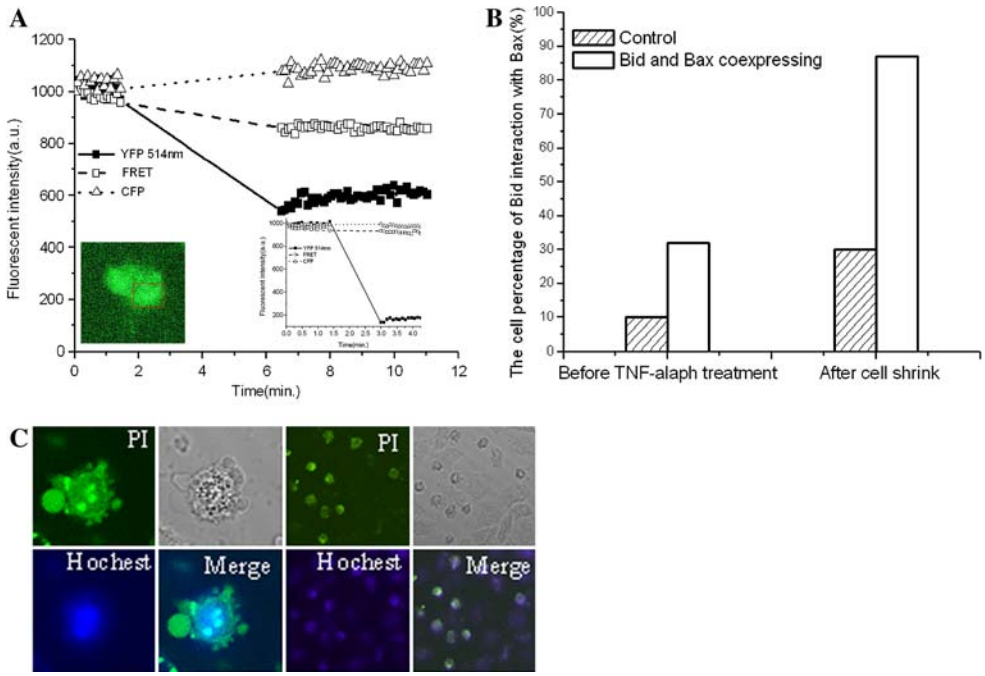

Fig. 3 Bid interact directly with Bax during apoptosis. **(A)** Acceptor photobleaching experiment on shrunk cells as treated with TNF-α, plus CHX. The inset shows the result of selectively photobleaching of YFP in CFP and YFP-Bax co-expressed cell, as a control. **(B)** Statistical analysis of the interaction between Bid and Bax in cells. Bid-CFP and YFP-Bax co-expressing cells were subjected to photobleaching of YFP with the same method as in Figure 2A before TNF-α plus CHX treatment (Before TNF-alpha treatment) and 7–9 hours after the treatment (After cell shrink). The cell percentage of Bid interaction with Bax was calculated by the counts of the FRET decreasing cells versus all the co-expressed cells. The control experiments were performed with the same method in pECFP-C1 and YFP-Bax co-expressed cells. Results were from independent experiments (n = 23–39). **(C)** The shrinking ASTC-a-1cells after TNF-α plus CHX treatment stained with PI and Hochest 33342. The left four images showed the single apoptotic cell, and the right four images showed many apoptotic cells

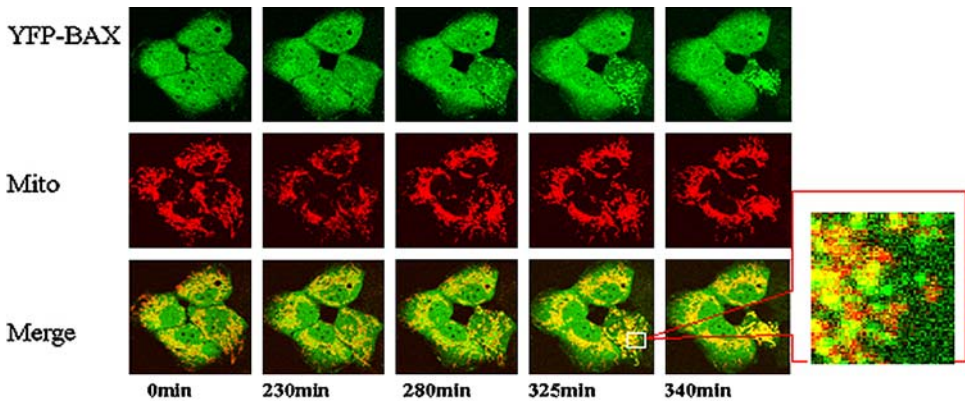

Fig. 4 Bax translocated to mitochondria during apoptosis. The image series of DsRed and YFP fluorescence emission in ASTC-a-1 cells co-transfected with YFP-Bax and DsRed-Mit after TNF-α plus CHX treatment. The time below the pictures indicated the time after the treatment. The image in the mark frame was the zoom-in image of the frame indicated region. Bar for 10 μm

prepared for western blotting. In contrast to the non-cleave Bid in adherent cells (except in MCF-7 cells), Bid was cleaved only in shrinking cells in ASTC-a-1and HeLa cells.

To check whether the observed changes of Bid were accompanied by the activation of initial caspase, activation of caspase-8 in the whole cell extracts with western blotting was detected (Fig. 5D). Caspase-8 is shown to be one of the typical initial caspases in TNF-α-induced apoptosis. Activation of caspase-8 was observed in the samples of both the adherent cell extracts and the shrinking cell extracts in three cell lines. In contrast, the activation of caspase-8 was not observed in control cells.

These results demonstrated that Bid was neither cleaved nor translocated to mitochondria before cell shrinkage during TNF-α-induced apoptosis in ASTC-a-1 cells and imply that FL-Bid, not tBid, interacted with Bax during this process.

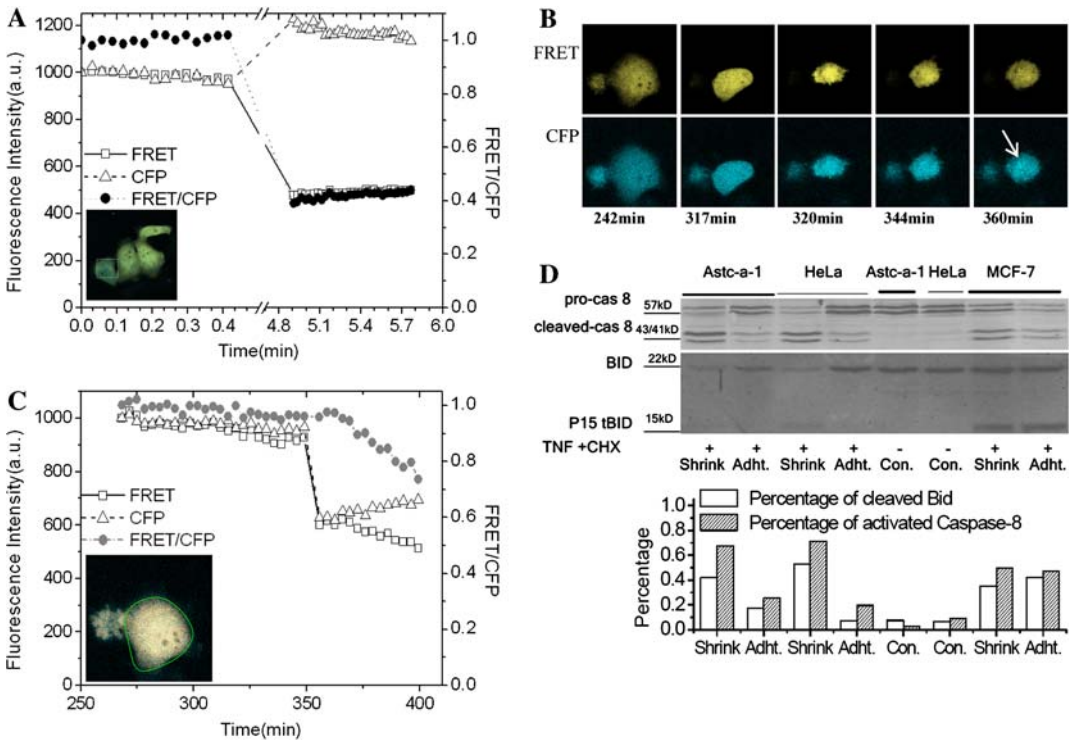

Fig. 5 Bid was not cleaved before cell shrinkage during apoptosis. **(A)** Acceptor photobleaching on ASTC-a-1 cell transfected with YFP-Bid-CFP before TNF-α treatment. YFP fluorescence was photobleached by 514 nm laser. The frame in the cell image indicated the photobleached area. **(B)** Fluorescence image series of FRET and CFP emission intensities in ASTC-a-1 cells after TNF-α plus CHX treatment. The time below the images indicate the time after treatment. The arrow indicated the Bid translocation to the mitochondria. **(C)** Quantitative analysis of the CFP and FRET fluorescent intensities and Ratio of FRET/CFP in B. The frame in the cell image indicated the selected area. **(D)** Whole cell lysate of ASTC-a-1, HeLa and MCF-7 were subjected to 15% SDS-PAGE (30 μg total protein per lane) followed by Western blotting with antibodies against Caspase-8 or Bid. All the cells were treated with TNF-α plus CHX for 11.5 hrs, except the control cell (Con.). The shrinking and the living cells were separated by slightly blowing with medium. After blowing, the shrinking cells (Shrink.) were suspended in the medium, and the living cells kept adherent (Adht.). Then the shrinking cell samples and the adherent cell samples were lysed, respectively. The column graph showed the quantitative analysis of the western blotting

Bid cleavage and Bax oligomerization after caspase-3 activation

In the best characterized pathways of TNFR1 induced apoptosis, tBid translocates to the mitochondria and activates mitochondria pathway leading to downstream caspase-3 activation and resulting in apoptotic cell death [4–6, 8]. However, in our experiment, Bid was cleaved and translocated to mitochondria after cell shrinkage during TNF-α-induced apoptosis in ASTC-a-1 cells. Thus we speculated that Bid cleavage preceded the activation of caspases-3. To test this hypothesis, we used the cell stably expressing SCAT3 [22] to monitor the caspases-3 activation during TNF-α-induced apoptosis. The initiation of apoptosis is usually asynchronous within a population of cells, we thus defined the initial time of cell shrinkage as time zero, after shrinkage as +x min, before shrinkage as –x min. A decrease of FRET/CFP ratio is positively linked to the activation of caspase-3 (Fig. 6A). The quantification of ratio of FRET/CFP shows a biphasic process, began at 150 min ± 57 min and 50 min ± 25 min ($n = 6$) before cell shrinkage (Fig. 6B), respectively. The latter decreasing was more rapidly than the former one. This is consistent with previous reports [29]. These results indicate that the activation of caspase-3 occurred before cell shrinkage and Bid cleavage.

In addition, the dynamics of caspase-3 activation and Bax translocation are combined into Fig. 6B in order to show the temporal relationship between them. Bax oligomerization occurred at 20 min ± 8 min before cell shrinkage and was in the process of the second activation of caspase-3 (Fig. 6B). It seemed that Bax might be involved in the caspase-3 mediated signal-amplification during apoptosis, but the same oligomerization of Bax was also observed in MCF-7, a caspase-3 deficient cell line (data not shown). Thus, the oligomerization of Bax may be independent to the activation of caspase-3 and directly triggered by FL-Bid.

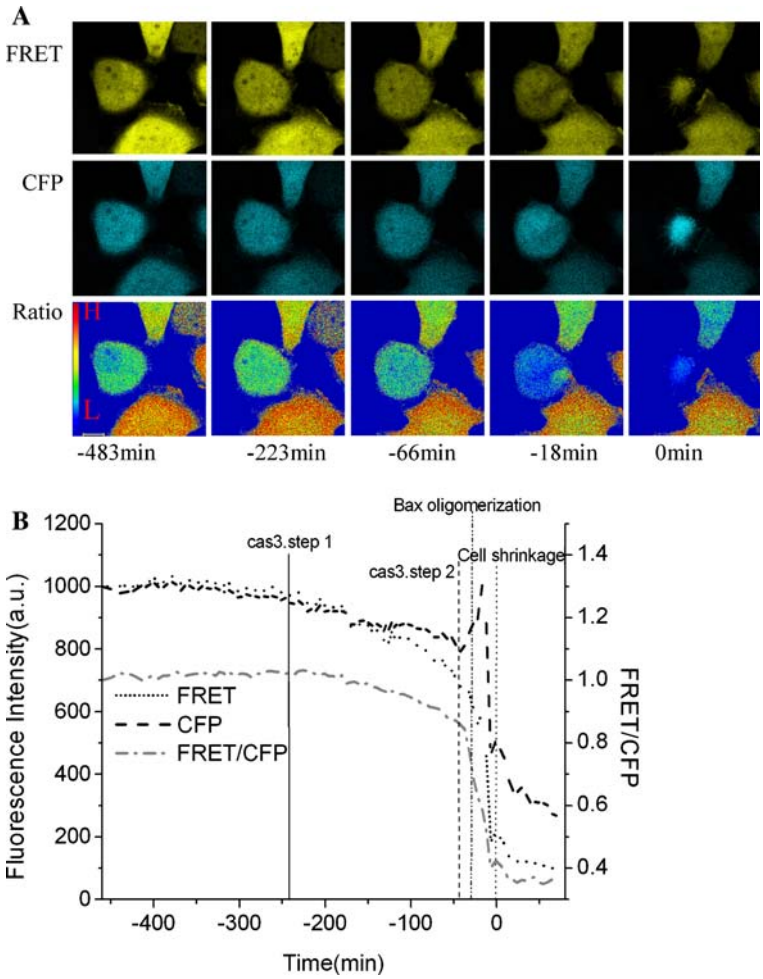

Fig. 6 Activation of caspase-3 during apoptosis. **(A)** Fluorescence image series of FRET, CFP and Ratio emission intensities in SCAT3 stable-expressing ASTC-a-1 cells after TNF-α plus CHX treatment. The initial time of cell shrink was defined as 0 min and before shrinkage as the negative time. The Ratio images are pseudo color images. **(B)** Quantitative analysis of the CFP and FRET fluorescent intensities and Ratio of FRET/CFP in A. The frame in the cell image indicated the selected area. Cas3.step1 stands for the beginning of the first stage of caspase-3 activation; cas3.step2 for the beginning of the second stage of caspase-3 activation; Bax oligomerization for the beginning of the Bax oligomerization; Cell shrinkage for the beginning of the cell shrinkage

Discussion

In the present study, single-cell FRET and acceptor photobleaching techniques were employed to monitor the interaction between Bid and Bax within individual cell during apoptosis. Our results show that FL-Bid/tBid directly interacted with Bax in single living cell during TNF-α-induced apoptosis. On the other hand, Bid-CFP did not co-localize with YFP-Bax clusters, but instead remained circumscribing the mitochondria during apoptosis (Fig. 2). These results strongly support that Bid serves as a direct activator of Bax translocation during TNF-α-induced apoptosis and that this activation must be a ''hit and run'' process. These findings are in agreement with the result of previous in vitro and in vivo studies that Bid and stapled Bid BH3 helix can bind to Bax directly [16, 18]. Surprisingly, our latter experiments demonstrate that FL-Bid, not tBid, directly interacted with Bax in ASTC-a-1 cells, while caspase-8 had been activated (Fig. 5). However, caspase-cleavage is considered to be fundamental in the activation of pro-apoptotic Bid, especially in the pathways of apoptosis triggered by death receptor ligands [4, 5, 11, 30–33]. Thus, these results indicate that there is another mechanism in regulating the cleavage of Bid when caspase-8 has been activated during TNF-α-induced apoptosis in ASTC-a-1 cells.

Why was Bid not cleaved by activated capase-8 in vivo and cleaved after cell shrinkage? More recently, it has been reported that Bid can be phosphorylated both in vitro and in vivo, particularly by enzymes of the casein kinase family, such as CKI and CKII (Casein Kinase I and II). CKI and CKII can phosphorylate Bid, subsequently protects Bid from caspase-mediated cleavage regardless of which stimuli have activated the caspase [34]. Thus, it is possible that the phosphorylated Bid may result in the non-cleavage. On the other hand, we speculate that the cleavage

of Bid may be dependent on the ATP level in cells. It has been reported that activation of caspase-8 can lead to translocation of FL-Bid to the mitochondria with induction of the MPT (membrane permeability transition) and consequently lead to Cyt c loss, caspase-3 activation and apoptosis [34, 35]. Before cell shrinkage, mitochondria had no obvious morphologic changes (Fig. 4), which could induce MPT and the decrease of ATP level [36]. After cell shrinkage, the morphologic changes of mitochondria are likely to lead to the decrease of ATP level. The lower ATP level may result in the dephosphorylation of Bid. This in turn could make Bid cleaved by activated caspase-8 or other caspases (caspase-10 and caspase-3) [35–37]. In our study, Bid cleavage was observed after the activation of caspase-3 (Figs. 5, 6). Based on the kinetics of caspase-3 activation from different cells, we determined the initial time of caspase-3 activation was at –150 min ± 57 min, and a more quickly activation was at –50 min ± 25 min (Fig. 6). But Bid cleavage occurred after cell shrinkage in ASTC-a-1 cells. For this phenomenon, we have an explanation. In cytotoxic drug and UV radiation-induced apoptosis, a small amount of cytochrome c via a caspase-independent mechanism may be sufficient to activate caspase-3. Cleavage of Bid resulted from activation of caspase-3 may provoke a much more significant (caspase-dependent) release of cytochronme c and induce cell apoptosis [38]. Thus, we speculate that the cleavage of Bid in ASTC-a-1 cells during TNF-α-induced apoptosis may be a result of activation of caspase-3 and/or caspase-8 after cell shrinkage (Fig. 7).

To further prove the imaging results that Bid was cleaved after cell shrinkage, we used a special method to harvest the treated cells for blotting experiments. It is known that in traditional western blotting experiment, protein samples are prepared by lysing both the shrinking cells and adherent cells together. The pro- and anaphase of apoptosis could not be discriminated. By flushing the cells lightly with culture medium, we were able to roughly separate the shrinking cells from adherent ones. This method of separation can partially retrieve the defect of the sample preparation. Our results of extraction of shrinking cells and adherent cells strongly support Bid was not cleaved in adherent ASTC-a-1 and HeLa cells. This is in consistent with the microscopy results that Bid was cleaved after cell shrinkage (Fig. 5D). Bid was cleaved both in adherent and shrinking MCF-7 cells (Fig. 5D), as confirmed in the microscope study (data not shown) [19].

According to the above experimental data and the related analysis, we summarize the signaling pathways related to TNF-α-induced apoptosis in ASTC-a-1 cells. Once engaged by TNF-α, two signal pathways are activated. One is the activation of caspase-cascade signal pathway [3]. The other is initiation of interaction between the FL-Bid and Bax that leads to the activation of Bax and the following translocation of Bax to mitochondria (Fig. 7). In addition, Bid is cleaved after cell shrinkage and the biological importance of the cleavage is yet to be determined.

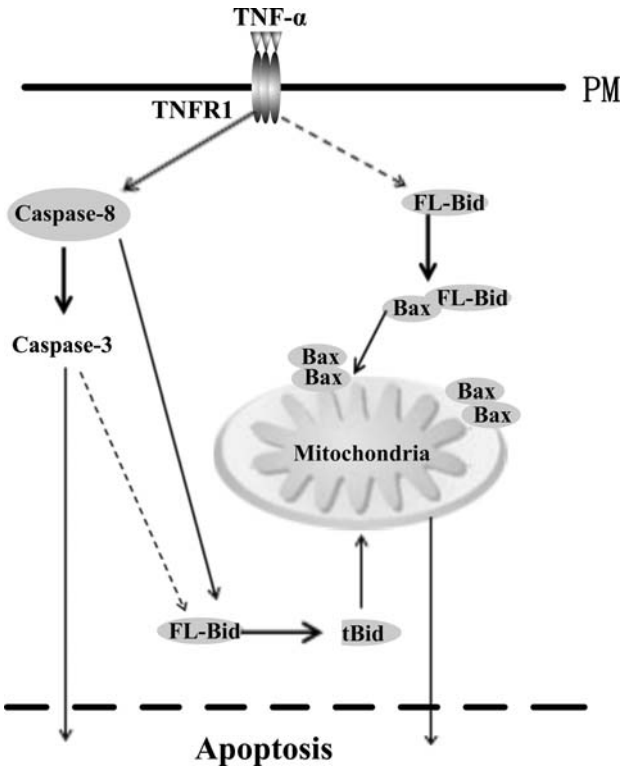

Fig. 7 Schematic representation of TNF-α induced apoptotic pathway in ASTC-a-1 cells

Conclusion

In this study, confocal laser scanning microscopy combined with both FRET and acceptor photobleaching techniques enable us to detect the dynamic interaction between Bid and Bax during TNF-α-induced apoptosis in living cells. The results demonstrate that FL-Bid directly interact with Bax during TNF-α induced apoptosis in ASTC-a-1 cells. Bid is cleaved only after cell shrinkage in both ASTC-a-1 and HeLa cells. However, caspase-8 and caspase-3 are activated before cell shrinkage. Thus, activated caspase-3 and/or caspase-8 may cleave Bid after cell shrinkage. It will be a challenge to find out what initiates interaction between FL-Bid and Bax during apoptosis.

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