Mitochondria from TRAIL-resistant prostate cancer cells are capable of responding to apoptotic stimuli

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Received 17 March 2004; received in revised form 15 July 2004; accepted 16 July 2004
Available online 20 August 2004

Abstract

TNF\textalpha-related apoptosis inducing ligand (TRAIL) has been shown to induce apoptosis in prostate cancer cells. However, some prostate cancer cells, such as LNCaP are resistant to TRAIL. In addition to the involvement of several pathways in the TRAIL-resistance of LNCaP, it has been shown that mitochondrial response to TRAIL is low in these cells. Therefore, in this study, using in vitro cell free and reconstitution models, we have demonstrated that mitochondria from these cells are capable of responding to apoptotic stimuli. Furthermore, experiments to determine the influence of cytochrome \textc{c} on apoptotic response noted that incubation of cytosol with exogenous cytochrome \textc{c} induced truncation of Bid. We have demonstrated that truncation of Bid by exogenous cytochrome \textc{c} is mediated through the activation of caspases-9 and -3. Incubation of cytosol with recombinant caspases-9 and -3 in the absence or presence of inhibitors showed that activation of caspase-9, leading to the activation of caspase-3 was necessary for the truncation of Bid. Published results indicate that in apoptotic cells cytochrome \textc{c} is released from the mitochondria in two installments, an early small amount and a late larger amount. Our results suggest that the initial release of cytochrome generates tBid that is capable of translocation into the mitochondria causing further release of cytochrome \textc{c}. Thus, in addition to providing functional explanation for the biphasic release of cytochrome \textc{c} from mitochondria, we demonstrate the presence of a feedback amplification of mitochondrial apoptotic signal.

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Keywords: Prostate cancer; Apoptosis; Mitochondria; Cytochrome \textc{c}; Bid; Caspases

1. Introduction

Apoptosis is induced either through the activation of an intrinsic pathway or an extrinsic pathway. Well-known examples of the extrinsic pathway are the tumor necrosis factor \textalpha{} (TNF\textalpha) family ligands that activate death domain-containing receptors. Since its discovery in 1995, the newest member of the TNF\textalpha{} family, TNF\textalpha-related apoptosis inducing ligand (TRAIL) has attracted enormous interest as an apoptotic agent in the treatment of cancer [1,2]. Significantly, TRAIL triggers apoptosis preferentially in tumor cells compared to normal cells through the activation of specific death receptors DR4 [3] and DR5 [4–6]. Activation of death receptors by TRAIL recruits the cytoplasmic adapter protein Fas-associated protein with a death domain (FADD), which then activates caspase-8 (FLICE, MACH, Mch5) by induced proximity protease activity [7]. In type I cells, caspase 8 propagates death signal directly through the activation of procaspase-3, while in type II cells, the apoptotic signal is amplified via the mitochondria. In the latter, caspase-8 cleaves Bid, which translocates into the mitochondria and promotes the release of cytochrome \textc{c}. Bid, a member of the BH3 domain only subgroup of Bcl2 family of proteins, is a 22-kDa cytosolic protein that is cleaved by activated caspase-8 in cells treated with variety of apoptotic agents such as TRAIL [8], UV...
that release of cytochrome c into the cytosol. Before its release, in the mitochondria, cytochrome c is attached to the outer surface of the inner mitochondrial membrane, where it is involved in respiratory electron transfer chain. Release of cytochrome c from the mitochondria to the cytosol is fast [12,13] and is required for mitochondria-mediated apoptosis. It has been demonstrated that release of cytochrome c to the cytosol is accomplished in two steps: an early stage that is characterized by the release of only small fractions of cytochrome c, and a late stage that is characterized by the release of larger fractions of cytochrome c [14]. Once released into the cytosol, cytochrome c binds to cytosolic Apaf-1. The Apaf-1–cytochrome c complex undergoes multimerization (estimated to be an octamer) in an ATP-dependent manner [15,16]. After the formation of the multimeric complex, procaspase-9 is recruited into the complex, which is then activated. It is demonstrated that Apaf-1 by itself bound ATP poorly, while in the presence of cytochrome c, ATP binding to Apaf-1 increased at least 10-fold [16]. Interestingly, recruitment of procaspase-9 to the complex further increased the binding of Apaf-1 to ATP. Activation of procaspase-9 is an important and necessary step for the activation of effector caspsases such as caspases-3 and -7. Thus, cytochrome c is critical for the initiation of post-mitochondrial apoptotic events. Not surprisingly, cytochrome-c deficiency reduced caspase-3 activation and rendered the cells resistant to proapoptotic agents [17].

Induction of apoptosis is gaining acceptance as a therapeutic option for cancer. In prostate cancer, androgen depletion is a treatment of choice to induce apoptosis in androgen-responsive cells [18,19]. However, treatment of androgen-independent cells require alternate methods to induce apoptosis. Many apoptogenic drugs have been utilized to induce apoptosis in prostate cancer cells [8,20–26] including TRAIL [27–31]. However, the response of prostate cancer cells to TRAIL depends on the cell-type. For example, we have shown that LNCaP C4-2 were sensitive to TRAIL [8], while LNCaP were resistant [8,27–30]. It was suggested that the lack of response of LNCaP to TRAIL is due to higher levels of constitutively phosphorylated Akt [30] although LNCaP C4-2 cells with similar levels of phosphorylated Akt were responsive to TRAIL [8]. Further experiments indicated that the differential response of the cells to TRAIL reside at the level of mitochondria [8,31]. Therefore, the goal of this study was to utilize in vitro cell-free and reconstitution models to investigate whether the mitochondria of LNCaP cells are capable of responding to apoptotic stimuli. Furthermore, experiments to determine the effects of cytochrome c on the these mitochondria in vitro demonstrated the presence of a cytochrome c-mediated short feedback loop for the activation of Bid enabling increased apoptotic response.

2. Materials and methods

2.1. Cell culture

Prostate carcinoma cell line, LNCaP (passage 24) were obtained from American Type Culture Collection (Rockville, MD) and were grown in RPMI 1640 medium supplemented with 2 mM l-glutamine, 4.5 g/l glucose, 10 mM HEPES, 1.5 g/l sodium bicarbonate, 1.0 mM sodium pyruvate, and 10% fetal bovine serum (Hyclone, Logan, UT) and grown in the presence of 5% CO₂ at 37 °C. Cells were treated with 400 ng/ml of TRAIL (Biomol Research Laboratories, Plymouth Meetings, PA) for 2 h. Upon completion of the experiment, cells were harvested, total proteins, cytosol or mitochondrial fractions were isolated as described below. For experiments using inhibitors, cells were pre-treated with 100 μM each of nonspecific caspase inhibitor Z-VA(D)-FMK or caspase-8–specific (Z-IETD-FMK) inhibitor for 1 h before treating the cells with TRAIL.

2.2. In vitro experiments

In some experiments, 50 μM caspase-9-specific (Z-LEHD-FMK) or caspase-3 specific (Z-DQMD-FMK) inhibitors were added to the cytosol during the in vitro experiments (all inhibitors were purchased from Enzyme Systems Products, Livermore, CA). The inhibitors were dissolved in DMSO, and to limit cellular toxicity, care was taken to see that DMSO concentration never exceeded 0.2% (in these experiments DMSO was typically 0.128%) in the culture medium. Appropriate controls included vehicle-treated cells with or without the inhibitors. In some experiments, 100 μM recombinant caspase-3 and/or caspase-9 (Biomol Research Laboratories) were added to the cytosol during incubation.

2.3. Isolation of live mitochondria

LNCaP cells were harvested after treatment with vehicle or 400 ng/ml TRAIL for 2 h and centrifuged at 200×g for 8 min at 4 °C. Cell pellets were washed twice with cold PBS and re-suspended in Mitochondria Incubating Buffer (MIB buffer) containing 1% protease inhibitor cocktail from Roche Diagnostic, Indianapolis, IN (MIB buffer: 210 mM Mannitol, 60 mM Sucrose, 10 mM KCl, 10 mM Sodium Succinate, 5 mM EGTA, 1 mM ADP, 0.5 mM DTT, 20 mM HEPES-KOH, pH 7.5). Cells were homogenized with 100 strokes, in a Dounce glass homogenizer and centrifuged at 1200×g for 10 min at 4 °C. The supernatant was then transferred to a clean tube and centrifuged at 5500×g for 15 min. The supernatant was discarded and the mitochondrial pellet was washed three times with MIB buffer. Mitochondrial pellet was resuspended in MIB buffer, protein was assayed and 20 μg of live mitochondria was utilized for in vitro experiments.

The purity and integrity of mitochondria were checked using Mitocapture Mitochondrial Apoptosis Detection kit (Alexis Biochemicals). Mitochondrial pellet was diluted in...
Mitocapture solution and incubated at 37 °C, centrifuged at 500 × g and the pellet was resuspended in incubation buffer. The cell suspension was observed in a fluorescence microscope. Intact and mitochondria with membrane damage were recognized due to changes in fluorescence.

2.4. Preparation of cell lysates for Western blotting

LNCaP were harvested by trypsinization, washed twice in 1 × PBS and cell pellets were resuspended in lysis buffer (100 mM Tris–HCl pH 8.0, 0.1% Triton X-100 and protease inhibitor cocktail). Cells were incubated over ice for 30 min and centrifuged at 10,000 × g at 4 °C for 10 min. The supernatant was collected and the protein concentration was assayed using Bio-Rad protein reagent (Bio-Rad Laboratories, Hercules, CA).

2.5. Separation of cytosolic and mitochondrial fractions

LNCaP were harvested after treatment with vehicle or 400 ng/ml TRAIL for 2 h and centrifuged at 200 × g for 8 min at 4 °C. Cell pellets were washed twice with cold PBS and re-suspended in Buffer A containing 1% proteinase inhibitor cocktail (Buffer A: 20 mM HEPES–KOH, pH 7.2, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium–EDTA, 1 mM sodium–EGTA, 250 mM Sucrose). The cells were homogenized with 100 strokes, in a Dounce glass homogenizer and centrifuged at 1200 × g for 10 min at 4 °C. The supernatant was then transferred to a clean tube and centrifuged at 10,000 × g for 15 min at 4 °C to pellet mitochondria. The mitochondrial pellet was re-suspended in lysis buffer (described above). The supernatant was centrifuged at 100,000 × g for 60 min at 4 °C and the S-100 fraction was collected as cytosol.

2.6. Western blotting

Proteins (50 μg, unless stated otherwise) were separated on NuPAGE 10% Bis-Tris gels (Novex pre-cast mini gels, InVitrogen, Carlsbad, CA) at 100 V for 1 h in the presence of 1 × MES-SDS running buffer (InVitrogen). Separated proteins were transferred to (PVDF) membranes (Bio-Rad Laboratories) at 42 V for 2.5 h using a Novex XCell II blotting apparatus in MES transfer buffer in the presence of NuPAGE antioxidant. Transfer of the proteins to the PVDF membrane was confirmed by staining with Ponceau S. The blots were blocked in 5% non-fat dry milk in TBS, washed twice for 10 min each with TBS containing 0.1% Tween-20 and incubated for 2 h at RT with primary antibody diluted in TBS containing 0.5% milk. Cells were harvested, protein was extracted and subject to Western analysis as described recently [8,26]. The following antibodies were used in the immunoblots: cytochrome c (Imgenex, San Diego, CA), BID (BioSource International, Camarillo, CA), caspases-8, -9, and -3 (BD Pharmingen, San Diego, CA), actin (Sigma) and Cox II (Molecular Probes, Eugene, OR). Immunoreactive bands were visualized using ECL detection system (Amersham, Pharmacia Biotech, Arlington Heights, IL) and signals were developed after exposure to X-ray film (X-Omat films, Eastman Kodak, Rochester, NY).

2.7. Assay for caspase-9 activity

Caspase-9 activity was assayed using a colorimetric substrate, Ac-LEHD-pNA using a kit from Chemicon International (Temecula, CA). Cleavage of the C-terminal peptide bond by the enzyme released p-nitroaniline, which was measured at 405 nm. Pure recombinant human caspase-9 was utilized as a positive control.

2.8. Removal of caspase-3 or BID from cytosol by immunoprecipitation

Cytosol (500 μg) extracted as described above was added to 2 × Immuno-Precipitation buffer (2 × IP buffer: 20 mM Tris–HCl pH 7.4, 300 mM NaCl, 2% Triton X-100, 2 mM EDTA, 2 mM EGTA, 0.4 mM Sodium-Orthovanadate with protease inhibitor cocktail) and diluted to 1 × IP. To this mixture, 5 μg/ml of anti-caspase-3 antibody (R&D system) or anti-BID antibody (MBL, Medical Biological Laboratories, Naka-Ku Nagoya, Japan) were added, mixed well, and incubated overnight at 4 °C on a shaker. Protein A (20 μl) was added to the samples mixed and again incubated overnight at 4 °C. Samples were centrifuged at 12,000 × g for 30 s, supernatant was collected, protein was assayed and 35 μg each was utilized in all in vitro reactions.

3. Results

3.1. Release of cytochrome c from mitochondria incubated with TRAIL-treated cytosol

Mitochondria were isolated from untreated LNCaP and were maintained on ice in a mitochondrial incubation buffer. The integrity of isolated mitochondria was confirmed by incubating them with mitocapture reagents. Results indicated that our isolation technique provided greater than 80–85% mitochondria with intact membranes (data not shown). Furthermore, we were able to maintain mitochondria in intact, viable condition for up to 4 h after isolation and therefore, all experiments were completed before this period. Isolated mitochondria were incubated for 10, 30, 60 or 120 min with cytosol isolated from untreated LNCaP (Fig. 1, first four lanes, C10 to C120) or cytosol from cells treated with 400 ng/ml TRAIL for 2 h (Fig. 1, last four lanes, T10 to T120). As expected, incubation of mitochondria from untreated cells with cytosol from untreated cells did not release mitochondrial cytochrome c. However, mitochondrial cytochrome c was released upon incubation with cytosol from TRAIL-treated cells, as these cells are resistant to TRAIL, but not non-responsive. Cytochrome c was released with as little as 10-min incubation, but
significant amount of cytochrome c was noted upon incubation for 60 min. These in vitro results show that in TRAIL-resistant LNCaP cells, TRAIL activated cytosolic factors are capable of releasing cytochrome c from the mitochondria, suggesting that mitochondria in these cells are functionally intact.

3.2. Cytochrome c initiates BID cleavage in cell-free system

Release of cytochrome c is a critical step in mitochondrial apoptotic pathway. It has been recognized that cytochrome c is released in two increments—an initial release of small amounts of cytochrome c followed by release of larger amount of cytochrome c [14] although its functional significance is not clear. We hypothesized that the function of the initial release of cytochrome c from mitochondria is to activate proteins (e.g. Bid), which then is capable of amplifying the apoptotic signal. To test this hypothesis, cytosol from untreated and TRAIL-treated LNCaP were incubated with exogenous cytochrome c. Incubation of cytosol from untreated cells with exogenous cytochrome c resulted in truncation of p22 Bid into p15 tBid (Fig. 2, lane 3). Presence of tBid in TRAIL-treated cytosol was not surprising, although addition of cytochrome c increased the signal for tBid (lane 5). Experiments to determine whether the presence of ATP affected the truncation of Bid showed no tBid when cytosol was treated with ATP alone (lane 2).

3.3. Caspase activation necessary for BID cleavage by cytochrome c

Results of the previous experiments demonstrated that tBid was generated upon incubation with cytochrome c. To confirm that truncation of Bid by cytochrome c is mediated through the activation of caspase(s), the activity of endogenous caspases was blocked by treating LNCaP with pan-specific caspase inhibitor, ZVAD in the presence or absence of TRAIL. Similar to results described in Fig. 2, incubation of cytosol from cells with functional caspases resulted in the truncation of Bid (Fig. 3, lane 3). However, inhibiting the activity of caspases blocked the truncation of Bid upon incubation with cytochrome c (Fig. 3, lane 5), suggesting that cytochrome c-induced truncation of Bid requires the activation of caspases (lanes 6–9).

3.4. Is caspase-8 required for cytochrome c-induced BID cleavage?

It is well established that in TRAIL-induced apoptosis, activation of caspase-8 is a prerequisite for the truncation of Bid. As the previous experiments demonstrated that caspase(s) are required for cytochrome c-mediated truncation of Bid, the role of caspase-8 was examined. LNCaP were
treated with general caspase inhibitor (Fig. 4A) or with specific inhibitor of caspase-8 activity (Fig. 4B), in the absence or presence of TRAIL. Incubation of cytosol from cells with intact caspase activity with cytochrome c, did not activate caspase-8 (Fig. 4A, lane 3), suggesting that the truncation of Bid under these conditions may not be mediated through caspase-8. Similar experiment with cytosol from cells where caspase-8 was specifically blocked generated tBid (Fig. 4B, lane 5), similar to cytosol with no caspase-8 inhibitor (Fig. 4B, lane 3) confirming that caspase-8 activation is not necessary for the truncation of Bid under the present experimental conditions.

3.5. Caspase-9 is activated in response to exogenous cytochrome c

The above experiments demonstrated that truncation of Bid by cytochrome c requires caspase activation, although caspase-8 does not play a role in this process. As cytochrome c forms an apoptosome complex with Apaf-I resulting in the activation of caspase-9, we next determined whether caspase-9 is responsible for this response. Incubation of cytosol from untreated cells with cytochrome c activated caspase-9 (Fig. 5A). When caspase activity was blocked by general caspase inhibitor and the cytosol was incubated with cytochrome c, the activity of caspase-9 was reduced compared to cells where caspase activity was not inhibited. As expected, treatment of cells with TRAIL increased the activity of caspase-9 significantly compared to untreated controls. Incubation of this cytosol with cytochrome c boosted the activity of caspase-9 significantly,

Fig. 4. Is caspase-8 required for cytochrome c-induced Bid cleavage? (A) Cells were incubated for 1 h with 100 μM nonspecific caspase inhibitor (Z-VAD-FMK) in the absence (C) or presence (T) of TRAIL (400 ng/ml) for 2 h. Cytosol was isolated and caspase-8 was examined by immunoblots as described. Cyto C denotes cytochrome c, Casp. Inhib. indicates the presence of inhibitor, PC8 denotes procaspase-8, while Cl 8 represents cleaved caspase-8. (B). Cells were treated as above except that instead of nonspecific caspase inhibitor, cells were treated with 100 μM caspase-8-specific inhibitor (Z-IETD-FMK, Enzyme Systems Products, Livermore, CA). The proteins were analyzed for the presence of Bid.

Fig. 5. (A) Caspase-9 is activated in response to exogenous cytochrome c: cells were treated with TRAIL and general caspase inhibitor as described earlier and the protein was analyzed by in vitro assays. (B) Cells were treated with nonspecific caspase inhibitor and TRAIL as described earlier. Samples were analyzed for the presence of caspase-3. PC3 denotes procaspase-3, while Cl 3 denotes cleaved products.

Fig. 4. Is caspase-8 required for cytochrome c-induced Bid cleavage? (A) Cells were incubated for 1 h with 100 μM nonspecific caspase inhibitor (Z-VAD-FMK) in the absence (C) or presence (T) of TRAIL (400 ng/ml) for 2 h. Cytosol was isolated and caspase-8 was examined by immunoblots as described. Cyto C denotes cytochrome c, Casp. Inhib. indicates the presence of inhibitor, PC8 denotes procaspase-8, while Cl 8 represents cleaved caspase-8. (B). Cells were treated as above except that instead of nonspecific caspase inhibitor, cells were treated with 100 μM caspase-8-specific inhibitor (Z-IETD-FMK, Enzyme Systems Products, Livermore, CA). The proteins were analyzed for the presence of Bid.
while caspase inhibitors reduced caspase-9 activity. These results demonstrate that incubation of cytosol with exogenous cytochrome c activated caspase-9, which may be directly or indirectly responsible for truncation of Bid.

3.6. Exogenous cytochrome c induced activation of caspase-3 in vitro

As caspase-3 is activated by activated caspase-9, the involvement of caspase-3 in cytochrome c-induced truncation of Bid was examined. Addition of cytochrome c to cytosol from untreated cells resulted in activation of caspase-3 as evidenced by the presence of cleaved products of caspases-3 (Fig. 5B, compare lanes 3). Treatment of cells with caspase inhibitor blocked the activation of caspase-3 in the presence or absence of exogenous cytochrome c (compare lanes 3 and 5, Fig. 5B). As expected, caspase-3 was greatly activated in cells treated with TRAIL (Fig. 5B, lane 6) that was further enhanced by the addition of cytochrome c (lane 7). Treatment of cells with caspase inhibitor blocked caspase-3 activity (Fig. 5B, lanes 8 and 9). These results indicate that addition of cytochrome c to cytosol induced activation of caspase-3.

3.7. Caspase-3 is required for the truncation of Bid

Results described above demonstrated that incubation of cytosol with cytochrome c induced activities of caspases-9 and -3. However, these experiments did not demonstrate which of the two caspases was directly responsible for the truncation of Bid. Therefore, cytosol was isolated from untreated cells, incubated with recombinant caspase-9 or caspase-3, and its effect on truncation of Bid was examined. Addition of recombinant caspase-9 to the cytosol resulted in tBid (Fig. 6A, lane 2), while addition of caspase-9-specific inhibitor to the incubation mixture blocked this response (Fig. 6A, compare lanes 2 and 3), suggesting activation of caspase-9 is required for the truncation of Bid. To examine whether the effect of caspase-9 is mediated through the activation of caspase-3, cytosol was incubated with recombinant caspase-3, which showed significant increase in tBid (Fig. 6A, lane 5). This response was lost in the presence of a caspase-3-specific inhibitor (lane 6), suggesting that activation of caspase-3 is a necessary step in the cytochrome c-induced truncation of Bid. Furthermore, tBid was not seen when the cytosol was incubated with recombinant caspase-9 and caspase-3 specific inhibitor (Fig. 6A, lane 4), indicating that addition of cytochrome c to the cytosol activated caspase-9, which in turn activated caspase-3 that is responsible for the truncation of Bid.

To further confirm these interesting results, endogenous caspase-3 was removed from the cytosol by immunoprecipitation (Fig. 6B, inset; compare lane b to lane a). When recombinant caspase-9 was added to caspase-3 depleted cytosol, tBid was not detected (Fig. 6B, lane 2), suggesting that caspase-3 is necessary for truncation of Bid in these experiments. These results were confirmed by the addition of recombinant caspase-3, which restored tBid response (Fig. 6B, lane 4). The signal for tBid disappeared when the effects of recombinant caspase-3 was blocked by caspase-3

Fig. 6. (A) Caspase-3 is required for truncation of Bid: cytosol from control cells was incubated with pure recombinant caspase-9 (Casp 9) or caspase-3 (Casp 3), in the absence or presence of inhibitors for caspases-9 (Casp 9 Inhib.) and -3 (Casp. 3 Inhib.). Samples were processed as described earlier. (B) Endogenous caspase-3 was removed from control cytosol by immunoprecipitation. Caspase-3 depleted cytosol was incubated in the presence of pure recombinant caspase-9 or -3 with or without respective inhibitor. Casp. 9 and Casp. 3 denote addition of pure recombinant caspases-9 and -3, C9I9: both recombinant caspase-9 and inhibitor of caspase-9; C3I3: both recombinant caspase-3 and inhibitor of caspase-3. Inset: To confirm the success of immunoprecipitation, cytosol from control (lane a) and immunoprecipitated (lane b) samples were analyzed for the presence of caspase-3 by Western blots. (C) Cytosol from untreated cells was isolated and incubated with increasing concentrations of recombinant caspase-3 and the samples were incubated as described earlier. Samples were processed for the presence of Bid.
inhibitor (lane 5) confirming the necessity of activated caspase-3 for the cytochrome c-induced truncation of Bid.

The role of caspase-3 in truncation of Bid was further confirmed by the incubation of cytosol with increasing concentrations of recombinant caspase-3 (Fig. 6C). Intensity of bands for truncated Bid increased with increasing concentrations of caspase-3 and reached a maximum with 150 U of recombinant caspases-3.

3.8. Translocation of caspase-induced tBid to the mitochondria

In the preceding paragraphs, we have demonstrated that incubation of cytosol with cytochrome c activated caspase-3 via activation of caspase-9, resulting in the truncation of Bid. To determine the functional importance of tBid generated under these experimental conditions, translocation of truncated Bid to mitochondria was examined. Live mitochondria were isolated from untreated cells and were incubated with cytosol treated with exogenous caspase-3. Cytosolic and mitochondrial fractions were isolated and analyzed for the presence of Bid. For loading controls, the blots were probed for the presence of actin and Cox II (mitochondria-specific marker). As expected, when cytosol was incubated with buffer alone (negative control), only full-length Bid was seen (Fig. 7A, lane 1). Incubation of cytosol with caspase-3 (positive control) showed the presence of tBid, in addition to full-length Bid (lane 2). As expected, analysis of proteins from mitochondria isolated from untreated cells did not show the presence of either full-length or truncated Bid (lane 3). Next, the mitochondria were incubated with cytosol treated with caspase-3 and cytosolic and mitochondrial fractions were separated. Analysis of the mitochondrial fraction showed the presence of tBid, but not full-length Bid (lane 5). However, both full-length and tBid were present in cytosolic fraction (lane 4). These results indicate that tBid generated in response to incubation of cytosol with caspase-3, is capable of translocation into the mitochondria.

In apoptotic cells, translocation of tBid into the mitochondria causes the release of cytochrome c to the cytosol. Fig. 7A demonstrated that caspase-induced tBid translocated into the mitochondria. Therefore, it was of interest to examine whether translocation of tBid into mitochondria resulted in the release of cytochrome c. Very little cytochrome c was observed (Fig. 7B, lanes 1 and 2) in any of the cytosolic fractions in the absence of mitochondria (probably minor contamination due to mitochondrial breakdown). However, incubation of mitochondria with caspase-treated cytosol resulted in significant increase in the levels of cytochrome c in the cytosolic fraction (lane 3), while cytochrome c was not present in the mitochondrial fraction (lane 4) indicating its depletion. These results indicate that in vitro truncated Bid is capable of translocation into the mitochondria, resulting in the release of cytochrome c from the mitochondria.

4. Discussion

TRAIL has been shown to induce apoptosis in several cancer cells. Results from our and other laboratories have noted that some prostate cancer cells, such as LNCaP were resistant to TRAIL-induced apoptosis, although these cells responded to other apoptotic stimuli such as anti-androgens, staurosporine and levostatin [21,22,32]. Experiments to determine the reasons for poor response of LNCaP to TRAIL identified lower mitochondrial response to TRAIL treatment [8,30,31]. The results of the present study, utilizing cell-free and reconstitution models, demonstrate that the mitochondria from LNCaP are capable of responding to apoptotic stimuli.

TRAIL activates death receptors resulting in the activation of caspases-8 and truncation of Bid. Truncated Bid is then translocated into the mitochondria to promote the release of cytochrome c, a key step in the mitochondrial apoptotic pathway. Translocation of cytochrome c from the mitochondria is initiated by an early release of a small amount of cytochrome c, followed by a larger, late stage
release of cytochrome c [14]. As cytochrome c is a crucial member of the mitochondrial apoptotic response, in the present study, we examined the importance of cytochrome c on apoptotic response in vitro. Incubation of LNCaP cytosol with exogenous cytochrome c resulted in the truncation of Bid, suggesting that cytochrome c released from mitochondria in response to caspase-8 mediated translocation of tBid into the mitochondria, ultimately resulting in the activation of caspase-3. The newly formed tBid is added to the pool of tBid generated in response to the activation of TRAIL pathway including activation of caspases-8, translocated to the mitochondria resulting in the release of larger amounts of cytochrome c. Thus, we provide evidence to demonstrate the functional significance of biphasic release of cytochrome c and the presence of a feed back loop involving pre- and post-mitochondrial events.

Acknowledgements

This study was supported by start-up funds of Medical College of Georgia and VA Merit Review to MVK.

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