Introduction

Cytochrome c is an essential component of the mitochondrial respiratory chain. It is a soluble protein, localized in the intermembrane space, and is loosely attached to the surface of the inner mitochondrial membrane. The role of cytochrome c in mitochondria-mediated apoptosis signaling pathway of mammalian cell apoptosis has been thoroughly investigated [1–5]. Cytochrome c release from mitochondria is a key event and plays an important role in initiating apoptosis in the mammalian cells. Once released into the cytosol, cytochrome c binds to apoptotic protease-activating factor-1 (Apaf-1), leading to an unmasking of its caspase recruitment domain and the subsequent binding and autoproteolytic activation of procaspase-9. The complex of procaspase-9, cytochrome c and Apaf-1 is known as the apoptosome. Active caspase-9 then proteolytically activates downstream effector caspases, such as caspase 3, which degrades various cellular proteins propagating the apoptotic signal [5–11]. Because apoptosis is an evolutionarily conserved form and a common way for deleting unwanted and superfluous cells, it is considered that apoptosis regulation of insects has many parallels to vertebrate system. However, the role of cytochrome c in insect cell apoptosis is not yet completely understood. There are conflicting reports on cytochrome c release from the mitochondria to cytosol important event in mitochondria-mediated apoptosis signaling pathway between the Diptera and Lepidoptera. In the Drosophila apoptotic system, cytochrome c is not involved in apoptosis in Drosophila cells such as S2 and BG2 cells [12]. Over-expression of cytochrome c in Drosophila BG2 cells or addition of recombinant cytochrome c to cytosolic BG2 extract did not lead to increased caspase activation or apoptosis, suggesting cytochrome c was not required for apoptosis in Drosophila BG2 cell lines [12]. Silencing of cytochrome c expression did not affect the induction of apoptosis in S2 cells [13]. The role of cytochrome c and other mitochondrial factors in caspase activation in Drosophila S2 cell extracts has been investigated, suggesting caspase activation in cultured Drosophila cell line S2 is regulated solely by cytoplasmic factors and does not involve any mitochondrial factors [14,15]. The studies in Drosophila have demonstrated that cytochrome c is not released from mitochondria. However, the role of cytochrome c in insect cell apoptosis is not yet completely understood. There are conflicting reports on cytochrome c release from the mitochondria to cytosol important event in mitochondria-mediated apoptosis signaling pathway between the Diptera and Lepidoptera. In the Drosophila apoptotic system, cytochrome c is not involved in apoptosis in Drosophila cells such as S2 and BG2 cells [12]. Over-expression of cytochrome c in Drosophila BG2 cells or addition of recombinant cytochrome c to cytosolic BG2 extract did not lead to increased caspase activation or apoptosis, suggesting cytochrome c was not required for apoptosis in Drosophila BG2 cell lines [12]. Silencing of cytochrome c expression did not affect the induction of apoptosis in S2 cells [13]. The role of cytochrome c and other mitochondrial factors in caspase activation in Drosophila S2 cell extracts has been investigated, suggesting caspase activation in cultured Drosophila cell line S2 is regulated solely by cytoplasmic factors and does not involve any mitochondrial factors [14,15]. The studies in Drosophila have demonstrated that cytochrome c is not released from mitochondria.
mitochondria during apoptosis in the insect, and that cytochrome c plays no role in caspase activation in these cells. In contrast with reports in *Drosophila*, the release of cytochrome c from mitochondria and consequently activation of caspase-3 were demonstrated during lepidopteran cell apoptosis. The release of cytochrome c from mitochondria in ultraviolet-induced *Spodoptera frugiperda* Sf-9 cells was observed by western blotting [16]. Antioxidants prevented UV-induced apoptosis by inhibiting mitochondrial cytochrome-c release and caspase activation in Sf-9 cells [17]. Cytochrome c added into the lysate of Sf-9 cells can also activate procaspase-3-like [18]. The study has shown that 2-deoxy-d-ribose is able to induce apoptosis, provoke cytochrome c release from the mitochondria and elicit caspase-3-like activity in *Lymantria dispar* IPLB-LdFB cells [19]. Cytochrome c release from the mitochondria in apoptotic *Spodoptera litura* cells induced with AfMNPV or ultraviolet has been detected by western blotting and cytochrome c may be required for caspase activation during the induction of apoptosis [2,20]. Recent report indicated that cytochrome c release into the cytosol is an important event during lepidopteran Sf9 cells apoptosis induced with actinomycin D and may occur independent of mitochondrial membrane potential loss and MPTP (mitochondrial permeability transition pore) formation [21–23]. Caspase activation in Sf9 cell extracts is exclusively dependent on the release of cytochrome c [21–23].

In studies reporting Lepidopteran cell apoptosis, the release of cytochrome c during cell apoptosis was mainly based on western blot analysis. Here, we used morphology observation, co-localization analysis, cell-free extract system and the technique of RNA interference to investigate the role of cytochrome c in apoptotic Sl-1 cells induced by AfMNPV.

**Results**

**Mitochondrial morphological changes in cells undergoing apoptosis**

Mito-Tracker Green staining showed that mitochondria disturbed in cytoplasm in normal Sl-1 cells (Fig. 1A). However, the infection of AfMNPV caused mitochondria to aggregate at 4 h post-infection (Fig. 1B). At 8 h of post-infection, Mito-Tracker green staining showed a diffusion of fluorescence, suggesting the disruption and depolarization of mitochondria (Fig. 1C).

The ultrastructure of mitochondria was observed to determine whether morphological changes of mitochondria occurred in cells undergoing apoptosis induced with AfMNPV. Virus infection resulted in a very obvious alteration in mitochondrial morphology (Fig. 2). In normal cells, mitochondria are in an elongated form with intact cristae (Fig. 2A). In cells infected with AfMNPV, these cells displayed abnormal mitochondrial morphology. The electron micrographs showed that some mitochondria appeared swollen and rounded at 4 h post-infection (Fig. 2B). Mitochondria were further swollen and cristae of mitochondria disappeared in cells at 8 h post-infection (Fig. 2C). Mitochondria showed swollen and vesicular-swollen morphology at 12 h post-infection (Fig. 2D).

These observations demonstrated that mitochondria were disrupted in apoptotic SL-1 cells induced by AfMNPV. Similar to mammalian cell apoptosis, the morphological changes in mitochondria was also an important feature in insect apoptotic cells.
Cytochrome c co-localization in Sl-1 cells undergoing apoptosis

To determine cytochrome c localization during apoptosis, Sl-1 cells were stained by double fluorescence staining. In control cells, the co-localization of cytochrome c and mitochondria was observed by both Mito Tracker red and coupled –FITC cytochrome c antibody. In cells undergoing apoptosis, cytochrome c did not colocalized with the mitochondria (Fig. 3), suggesting cytochrome c localization was altered in Sl-1 cells undergoing apoptosis induced by AMNPV.

Cytochrome c induces caspase -3 activation in Sl-1 cell-free extracts

Cytochrome c is critical for caspase activation and the resulting consequence of the cytochrome c release generally gives rise to caspase activation in mammalian cells. To test whether cytochrome c can induce caspase activation in a cell-free extracts, cytochrome c was added to Sl-1 cell extracts and caspase-3 activation was tested by a fluorescent assay using the substrate Ac-DEVD-AFC. It was found that the addition of cytochrome c increased caspase-3 activity. Caspase-3 activity evidently increased with incubation time extension in the presence of cytochrome c (Fig. 4). Under similar conditions, no increasing of caspase-3 activity was measured in the absence of cytochrome c. These results revealed that cytochrome c could induce and promote caspase-3 activation in Sl-1 cell-free extracts. It is suggested that the signal for caspase-3 activation is directly related to cytochrome c.

DsRNA silences cytochrome c expression in Sl-1 cells

Based on RT-PCR amplification, sequence alignment and sequencing, the recombinant plasmid pLitmus-cyt c which contained partial sequence of Spodoptera litura cell cytochrome c gene and the T7 promoter sequence on both ends of the insert had been constructed and was identified by electrophoresis (Fig. 5A). DsRNAs corresponding to the coding regions of cytochrome c were transcribed in vitro and the transcription productions were identified by electrophoresis (Fig. 5B). To investigate the effect of dsRNA on cytochrome c expression, semi-quantitative RT-PCR was used to verify cytochrome c expression levels. Sl-1 cells were treated with dsRNA at different time points and total RNA was isolated. Cytochrome c mRNA levels were determined with semi-quantitative RT-PCR. Levels were relative to mRNA expression in cells treated with GFP dsRNA and normalized with an internal control actin gene. Results showed that cytochrome c mRNA levels decreased and was down-regulated after transfection with cytochrome c dsRNA (Fig. 5C).

Western-blot was further used to confirm the down-regulation of cytochrome c after transfection with cytochrome c dsRNA. The total protein of cells were extracted at different time points after transfection and determined according the methods of BCA. β-tubulin was used as an internal control to normalize the relative level of cytochrome c. The results demonstrated that the abundance of cytochrome c decreased gradually during 72 h of post-transfection with cytochrome c dsRNA (Fig. 6).

Silencing of cytochrome c prevents AfMNPV –induced cell apoptosis

To determine whether silencing of cytochrome c expression affected apoptosis in Sl-1 cells induced with AMNPV, we treated cells with double–stranded RNA transcribed from cytochrome c cDNA at different time points. Cells treated with dsRNA had been infected by AMNPV for 10 h and then visualized. Morphological observation showed that a large number of apoptotic bodies appeared in cells infected with the virus (Fig. 7A1). After treatment with cytochrome c dsRNA, the number of apoptotic cells induced with AMNPV declined significantly, and apoptotic bodies decreased gradually with time extension after cytochrome c dsRNA treatment (Fig. 7 A2, 3 and 4). Morphological feature showed that cytochrome c dsRNA interfered with the induction of apoptosis with the virus in Sl-1 cells. To detect quantitatively apoptotic cells, cells transfected with dsRNA at 48 h were analyzed by flow cytometry. The flow cytometry analysis showed that cytochrome c dsRNA caused the decline of the apoptotic rate in AMNPV-induced Sl-1 cells (Fig. 7A and B). These results indicated that cytochrome c dsRNA treatments resulted in a significant reduction of apoptotic rate. Apparently, silencing of cytochrome c inhibited apoptosis induced by AMNPV in Sl-1 cells.

Silencing of cytochrome c inhibited the activation of procaspase 3 and pro-caspase 9 in Sl-1 cells induced by AMNPV

To examine the effect of the expression knockdown of cytochrome c on caspase-3 activation in Sl-1 cells, we conducted cytochrome c dsRNA inference as well as caspase-3 activity assays. Cells were treated with dsRNAs corresponding to the coding region of cytochrome c for 24, 48 and 72 h, followed by infection with AMNPV at 5 moi for 10 h, cytotoxic fractions from Sl-1 cells undergoing apoptosis were used as caspase-3 activation assay. The caspase-3 activity assay performed for the cells incubated in the presence of cytochrome c dsRNA and AMNPV revealed a significant decrease in caspase-3 activity with respect to the control cells treated with GFP dsRNA and without cytochrome c dsRNA and other treatments. At 24 h post treatment of cytochrome c dsRNA, cells infected with the virus showed that caspase-3 activity decreased significantly. At 72 h after incubation, the caspase-3 activity still was markedly lower than the control cells after various treatments (Fig. 8A).

To determine whether the decreased levels of cytochrome c expression would also affect caspase-9 activity in Sl-1 cells, we measured caspase-9 activity in cells infected with AMNPV in the presence of dsRNA. The result showed that interfering cytochrome c expression caused the reduction of the level of caspase-9 activity (Fig. 8B).

Figure 3. Cytochrome c localization in Sl-1 cells was altered during apoptosis. Sl-1 cells infected with AFMNPV for 6 h were co-stained with anti-cytochrome c monoclonal antibody and Mito-Tracker red. After incubation with FITC-coupled secondary antibodies, cells were visualized by confocal laser scanning microscopy. Con: Control cells treated without AFMNPV; Inf: Cells infected with AFMNPV for 6 h. doi:10.1371/journal.pone.0040877.g003
Silencing of cytochrome c expression remarkably influenced the caspase-3 and caspase-9 activity in apoptotic Sl-1 cells infected with AfMNPV, suggesting that the caspase-3 and caspase-9 activity in Sl-1 cells is closely associated with cytochrome c.

Caspase-9 was involved in the activation of pro-caspase-3 in Sl-1 cells infected with AfMNPV

In order to determine the relationship between caspase-9 and caspase-3 cascade, Sl-1 cells were infected by AfMNPV in the presence or absence of caspase-9 inhibitor (Z-LEHD-FMK) and caspase-3 activities were measured by a spectrofluorometer at 10 h post-infection. The results demonstrated that the inhibition of caspase-9 activity significantly blocked the activation of pro-caspase-3 and the effect was in a dosage-dependent manner (Fig. 9), suggesting the activation of procaspase-9 was the upstream event during the process of apoptosis in Sl-1 cells induced with AfMNPV.

Inhibition of the function of Apaf-1 blocked apoptosis

5′-p-fluorosulfonylbenzoyl adenosine (FSBA), an ATPase inhibitor, is known to inhibit the function of Apaf-1/CED-4-like molecules and could result in the blockage of the caspase activity in the embryo of Drosophila and Sf9 cells of Spodoptera frugiperda [24,25]. To determine the function of S. litura Apaf-1 in the process of insect cell apoptosis, the relative caspase-3 activity and the percentage of cell apoptosis were measured as described above after Sl-1 cells were treated with FSBA and the inducers of apoptosis (AfMNPV). The results demonstrated that FSBA could inhibit the AfMNPV-induced apoptosis in Sl-1 cells (Fig. 10). In addition, FSBA could also inhibit the actinomycin D-induced apoptosis in Sl-1 cells (data not shown). These results strongly suggest that Apaf-1 plays an important role during apoptosis of Sl-1 cells, which was known to involve in the cytochrome c-induced apoptosome formation.

Discussion

Cytochrome c has a key role in the mitochondrial–mediated apoptosis pathway and triggers caspase activation as well as caspase cascade in mammalian cells [8,26,27]. Our previous work has demonstrated the release of cytochrome c from mitochondria into cytosol and subsequent activation of caspase-3 in S. litura cells (Sl-1 cell line) undergoing apoptosis induced by various stimuli, such as baculovirus or UV, but these results were primarily based on western blot analysis [2,20]. In this paper, we further demonstrated the release of cytochrome c from mitochondria into the cytosol in apoptotic Sl-1 cells by using immunofluorescence staining. Meanwhile, electron microscopy revealed that mitochondria were disrupted in SL-1 cells undergoing apoptosis induced with AfMNPV. In Drosophila, expression of Hid or Kpr resulted in mitochondrial disruption [28], which indicates the morphological change of mitochondria is a common feature of apoptotic insect cells. Our data suggests that the release of cytochrome c could be associated with the alteration of mitochondria morphology in Lepidopteran cell apoptosis. Because the role of cytochrome c on apoptosis signaling is an important and controversial issue regarding insect cell apoptosis, we used the technique of RNA interference and a cell-free extract system to further confirm the role of cytochrome c on apoptosis induced by AfMNPV in Lepidopteran Sl-1 cells. The present study showed the addition of exogenous cytochrome c to Sl-1 cell-free extracts was sufficient to induce caspase-3 activation which indicates that cytochrome c is required for caspase-3 activation in Lepidopteran Sl-1 cells. More recent studies showed the addition of purified cytochrome c or mitochondria lysates to Sf9 cell-free extracts induced DEVDase activity [21]. When cytochrome c was deleted with anti-cytochrome c antibody, DEVDase had been not observed [21]. The role of cytochrome c in both Sl-1 cell-free and Sf9 cell-free extracts is consistent and has parallel to mammalian cell-free extracts. In Drosophila, one study had shown that cytochrome c
induced a small amount of caspase activation in embryo extracts but there was a 2.5-fold induction of caspase activity, which contrasts with the 50–100-fold induction or more in vertebrate cell extracts in that study [29]. However, the two cytochrome c species, DC3 and DC4, failed to induce caspase activation and promote caspase activation in extracts from human cells [30,12].

There is distinctive difference on the role of cytochrome c in both Lepidopteran cell-free and Drosophila cell-free systems.

The dsRNA-mediated gene silencing has been used to silence the expression of targeted genes associated in apoptosis, such as cytochrome c, Apaf-1-related killer (ARK, apoptosis-related killer) in Drosophila cells [12,13]. In order to facilitate the functional study of cytochrome c on apoptosis in insect cells, we silenced the expression of cytochrome c by RNA interference in Spodoptera litura cells with dsRNAs to examine the effects of silencing of cytochrome c on apoptosis induced with AlMNPV. Silencing of cytochrome c expression in Sl-1 cells resulted in the inhibition of

**Figure 5. Cytochrome c dsRNA silenced cytochrome c expression in SI-1 cells.** (A) Identification of recombinant plasmid pLitmus-cytc containing cytochrome c DNA fragment. pLitmus-cytc was digested with BamH I and EcoR I and then analyzed by agarose gel electrophoresis. M1: 1 kb maker, M2: 100 bp maker, S: plasmid DNA products. (B) Electrophoretic analysis of dsRNA transcribed in vitro. pLitmus-cyt c was digested with BamH I and EcoR I respectively and transcribed in vitro, and RNA products were electrophorised on agarose gel. M: 100 bp marker, S: RNA transcription products. (C) Cytochrome c mRNA level after treatment with dsRNA. SI-1 cells treated with dsRNA for 0, 24, 48, and 72 h, and after inoculated with AlMNPV for 10 h, total RNA in each treatment was isolated. Cytochrome c mRNA was determined by semi-quantitative RT-PCR.

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apoptosis induced with AMNPV. Once dsRNA was introduced into the cells, the apoptotic rate significantly decreased, indicating silencing of cytochrome c protected the cells from the death infected by AMNPV. The mechanism underlying the protective effect of silencing cytochrome c appears to involve an activation of caspase or change of caspase activity. Therefore, we also set out whether silencing of cytochrome c affected caspase-3 and caspase-9 activity in Sl-1 cells. Knockdown of cytochrome c led to a significant decrease of caspase-3 and caspase-9 activity suggesting the activity of caspase-3 and caspase-9 activity is dependent upon cytochrome c in Lepidopteran cells.

For the studies reported in Drosophila, silencing both dc3 and dc4 in BG2 cells had no effect on the processing of Dronc (a homologue of mammalian caspase-9) and Drice (a homologue of mammalian caspase-3) or caspase activity after induction of apoptosis by cyloheximide, indicating cytochrome c is not required for caspase activation in Drosophila cells [12]. Although Drosophila cytochrome c protein may be not required for apoptosis and caspase activation, accumulating evidence has shown that cytochrome c involved in apoptosis and caspase activation in Lepidopteran insect apoptosis [2,16,17,19,20,21]. It is considered that the molecular mechanisms of apoptosis are highly conserved throughout evolution. Why does a distinctive difference exist on the role of cytochrome c in apoptosis in Lepidopteran and Diptera insects? The report pointed that the findings in Drosophila are based largely, if not exclusively, on studies in cultured S2 cells which are phagocytic cells derived from embryonic macrophages/hemocytes, suggesting that macrophages are uniquely resistant to apoptosis among embryonic cell types, and that S2 cell line is not a suitable general model for Drosophila cell death [31]. We do not know whether the differences of the cytochrome c role between two kinds of insects during apoptosis are due to different cell types. Perhaps this difference reflects the differences of the evolution between Lepidopteran and Diptera insects. Therefore, the role of cytochrome c during insect cell apoptosis should be investigated in more insect cell lines or tissue types.

Cytochrome c plays an important role for apoptotic signaling and may be required for Lepidopteran insect apoptosis. We speculate that the role of cytochrome c in Lepidopteran cells and mammalian cells apoptosis is homologous. The function of cytochrome c in mammalian cell apoptosis involves Apaf-1 activation and apoptosome formation. We have obtained the EST of Slapaf-1 (Unpublished) in Sl-1 cells, which shares high homology with Bombyx mori Bmapaf-1 [32] and Manduca sexta Msapaf-1 (Unpublished), and the elucidation of the apoptosome formation is undergoing in Lepidopteran cells.

In summary, based on the present studies and the previous reports [2,17,20,21,22,33,34], the pathway of apoptosis mediated by cytochrome c has been given a clue in AMNPV-infected Sl-1 cells. In the present studies, we have provided further evidence that cytochrome c is essential for AMNPV- or actinomycin D-induced apoptosis through cytochrome c-induced apoptosome formation (Fig. 11).

Materials and Methods

Cell and virus

Spodoptera litura cell line (SL-ZSU-1 or Sl-1) was obtained from the Institute of Entomology, Zhongshan University [35]. Anagapha falcifera multiple nuclear polyhedrosis virus (AMNPV) was a gift of Dr. McIntoch in Department of Agriculture, USA [36].

Cell culture and induction of apoptosis

SL-1 cells were propagated in GIBCO™ Grace’s medium (Invitrogen, Grand Island, N.Y., USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, N.Y., USA), 0.3% yeast extract and 0.3% lactalbumin hydrolysate at 28°C. For induction of apoptosis, 5 x 10⁵ cells/ml were seeded into flasks or 6-well plates, after cells attached the bottom of the plate, the medium was removed and cells were inoculated with AMNPV at multiplicity of infection (MOI) of 5 for 1.5 hr. The virus was removed and cells were washed twice with serum-free medium and then cultured in Grace’s medium supplemented with 10% FBS. The morphological changes of apoptotic cells were visualized with microscopy.
Observation of mitochondrial morphology

Sl-1 cells were grown on coverslips (20 mm x 20 mm) in 6-well plates overnight. After infection with AfMNPV for different time periods, cells were incubated with 300 nM Mito-Tracker Green (Molecular Probes Company) for 15 min at room temperature. After the stained cells were washed twice with PBS, they were visualized under confocal microscopy according to the instruction provided by the manufacturer.

Sl-1 cells cultured in flasks were washed three times in PBS buffer after cells were infected with AfMNPV at 0 h, 4, 8 and 12 h post infection respectively and then cells were pre-fixed in 2.5% glutaraldehyde for 30 min and fixed in 2.5% glutaraldehyde for 2 h at 4°C. The specimens were conventionally dehydrated, infiltrated, embed and sectioned. Thin sections were stained with uranyl acetate and observed under a transmission electronic microscopy (Philips 2 Tecnai 10).

Immunofluorescence staining

Cells were grown on coverslips (10 mm x 10 mm) in 24-well plates. After apoptosis induced with AfMNPV, cells were washed twice with PBS and incubated with 300 nM Mito-Tracker Red CMXRos in PBS with 0.8% BSA for 15 min at room
temperature. Cells were washed twice with PBS and fixed with 4% cold paraformaldehyde for 10 min at room temperature. The washed cells were permeabilized with 0.1% Triton X-100 for 5 min at room temperature and washed three time with PBS and then were blocked with 1% BSA for 30 min at room temperature. Cells were incubated with anti-cytochrome c mouse monoclonal antibody (BD Biosciences) overnight at 4°C. After cells were washed three times in PBS, cells were stained with FITC-conjugated secondary antibody for 90 min at room temperature and then washed three times with PBS. Coverslips were mounted onto slides and observed with confocal laser scanning microscopy at an excitation wavelength of 488 nm and an emission wavelength of 515 nm for FITC, and at 595 nm excitation wavelength and 570 nm emission wavelength for Mito-Tracker Red CMXRos.

Figure 8. Down-regulation of cytochrome c resulted in the reduction of caspase 3 and caspase-9 activity in AfMNPV-induced Sl-1 cell. (A) Sl-1 cells treated with dsRNA for different time points, apoptosis was induced with AfMNPV, and then caspase-3 activity was measured at 10 h post-infection. 1. Control cells treated alone with AfMNPV; 2. Cells treated with GFP dsRNA and virus; 3. Cells without any treatment; 4. Cells treated with dsRNA, 24 h later, infected by AfMNPV for 10 h; 5. Cells treated alone with dsRNA for 24 h; 6. Cells treated with dsRNA, 48 h later, infected by AfMNPV for 10 h; 7. Cells treated alone with dsRNA for 48 h; 8. Cells treated with dsRNA, 72 h later, infected by AfMNPV for 10 h; 9. Cells treated alone with dsRNA for 72 h. (B) Caspase-9 activity in cytochrome c dsRNA-treated Sl-1 cells after infection with AfMNPV for 10 h, compared with control cells. 1. Normal cells; 2. Cells infected with AfMNPV; 3. Cells infected with AfMNPV for 10 h after GFP dsRNA treatment for 48 h; 4. Cells infected with AfMNPV for 10 h after cyt c dsRNA treatment for 48 hr. *, p<0.05.

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Preparation of cell-free extracts
SL-1 cells were harvested and washed twice with ice-cold PBS. The cells were suspended in 5 ml lysis buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA-Na2, 1 mM EGTA-Na2, 1 mM DTT, 250 mM sucrose), supplemented with protease inhibitors (0.1 mM PMS, 6 mg/ml aprotinin, 8 mg/ml leupeptin and 10 mg/ml pepstatin). After sitting on ice for 10 min, the cells were homogenized with a glass homogenizer. The homogenates were centrifuged at 100,000 g for 1 h at 4°C. The supernatant was further centrifuged at 100,000 g for 30 min. The resulting supernatants were saved as cell-free system and stored at −80°C in multiple aliquots for the in vitro apoptosis assay.

Apoptosis assay in cell-free system
Cytochrome c was added into the cell-free system at a final concentration of 5 μmol/l and incubated at 28°C. The cell-free system treated with cytochrome c was designated as the apoptotic reaction system. The reactive system treated without cytochrome c was used as the control. Apoptosis in the cell-free system was determined and confirmed by the caspase-3 activity assay.

Construction of recombinant plasmid containing cytochrome c DNA fragment
RNA was isolated from SL-1 cells (1.5×10^6 cells) with Trizol (GIBCO-BRL) extraction according to manufacturer’s instruction. cDNA was generated using 1 μg total RNA and 1 μl M-MLV reverse transcriptase (3 U/μl) (Promega). Based on the sequence homology from Lepidopterans such as *Bombyx mori*, *Trichoplusia ni*, *Manduca sexta* cytochrome c genes, A pairs of degenerate primers for cytochrome c (forward primer, 5′-GGGTGTWCCCTGCWGGMAATGCT-3′; reverse primer, 5′-GRTCTGCACGYTCRTTKGCCT-3′) was designed for PCR. Total volume of 50 μl PCR reaction system consisted of 5 μl 10×PCR buffer,1 μl cDNA template, 1 μl forward primer, 1 μl reward primer, 0.5 μl Taq DNA polynmarase(4 U/μl), 2 μl 10 mM dNTP and ddH2O. The PCR product was electrophorised on 1.5% agarose gel and recovered from gel by Axygen kit according to protocol outlined by manufacturer. The purified DNA was cloned into T-vector and sequenced. The DNA sequences with 293 bp were analyzed with the cytochrome c genes from *Bombyx mori* and *Trichoplusia ni* with NCBI-Blast software and the identity of Spodoptera litura cytochrome c DNA fragments compared with silkworm and cabbage lopper cytochrome c genes were 83% and 84% respectively. According the sequence of Spodoptera litura cytochrome c DNA fragment, the sequence specific primer containing BamH I and EcoR I sites for cytochrome c, forward primer, 5′-CGGGATCCGATCTGCACGCTCGTTTGCCT-3′ (underline, BamH I site) and reverse primer, 5′-CGGAATTCTGCCACACTGTTGAAGCCGGT-3′ (underline, EcoR I site) were redesigned. Spodoptera litura cytochrome c DNA fragments (248 bp) were amplified by PCR and analyzed by 1.5% agarose gel electrophoresis and recovered. The plasmid pLitmus28i (New England BioLabs., Inc.) DNA was extracted and then plasmid DNA and cytochrome c DNA fragments were digested with BamH I and EcoR I. The plasmid DNA and cytochrome c DNA fragments were ligated with DNA ligase (Toyobo) and the resulting recombinant plasmid, pLitmus-cytc, was transformed into E. coli DH5α.
was transformed into *E. coli* DH5α strain. The pLitmus-cyt c plasmid was identified with BamH I and EcoR I digestion and DNA sequence analysis. The plasmid containing GFP gene fragment, pLitmus-GFP, was constructed as described similarly for negative control.

**DsRNA synthesis and RNAi**

The pLitmus-cyt c plasmids were extracted using QIAGEN spin miniprep kit (QIAGEN GmbH, Hilden, Germany) and linearized with BamH I and EcoR I digestion. Then the target DNA fragment was purified and used as a template to produce dsRNA. RNA was synthesized using a T7 transcription kit. The dsRNA products were ethanol precipitated and suspended in water and quantified. 5 μg of dsRNA was visualized by 1.5% agarose gel. The dsRNA was stored at −80°C. For *Spodoptera litura* cytochrome c dsRNA mediated RNA interference assay, 5 × 10⁵ cells/ml were seeded into 24-well plates and cultured in serum-free medium for 2 hr. Cells in each well were transfected with a mixture of 1 μg dsRNA, 5 μl Cellfectin reagent (Invitrogen, USA) and 25 μl serum-free medium according to manufacture’s protocol. After cells were incubated for 6 h at 28°C, serum-free medium were replaced with medium supplemented with 10% FBS. Cells were grown at various time intervals and then infected with αMNPV at MOI of 5. After virus inoculum was removed at 2 h post infection, cells in new medium were cultured for 10 hr. Cells were transfected with pFBDC plasmid and GFP dsRNA as a negative control. Cytochrome c mRNA expression in Sl-1 cells was measured with semi-quantitative RT-PCR with β-actin gene used as an internal control. Briefly, the cells were infected with αMNPV after the cells had been treated with dsRNA for 24, 48 and 72 h. Then the total RNA in each treatment was isolated at 10 h post infection. cDNAs were synthesized by reverse transcription and a forward primer (5-TGCCACACTGTT-...
GAAGCCGGT-3) and a reverse primer (5-GATCTGG-CACGCCTGTTTGCCGCT-3) for cytochrome c gene were used together to produce PCR products. The PCR products were electrophoresed on 1.5% gel and analyzed with Bio-Rad software Quantity One.

To further confirm the effect of RNAi of cytochrome c in SI-1 cells, the abundance of cytochrome c was demonstrated by western-blot at the different time points after the transfection of cytochrome c dsRNA. The protocol was briefly described as follow. The cells were harvested at 0, 24, 48, and 72 h after the transfection as described above. The concentration of total protein was determined according to the method of BCA after addition of lysis buffer containing protease inhibitors and centrifugation. Total cellular proteins were separated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membrane was blocked with TBS containing 5% skimmed milk for 1 h at room temperature and then incubated with a monoclonal anti-b-tubulin (Sigma-Aldrich, St. Louis, MO, USA) and anti-cytochrome c mouse antibody (BD Biosciences) as primary antibody at a 1:1000 dilution. Finally, the membrane was washed with blocking buffer and then incubated with peroxidase-conjugated goat anti-mouse immunoglobulin G (Promoter biotechnology, China) as secondary antibody for 1 h at room temperature. The specific bands were visualized by revelation with 3-amino-9-ethylcarbazole (AEC).

Assay of caspase activity

To assay caspase activity in SI-1 cells interfered with dsRNA, after cells were transfected with dsRNA at different times, the cells had been infected with AfMNPV at MOI of 5 for 10 h. The cells were collected by centrifugation at 13,500g for 5 min at 4°C. The cells were suspended in cell lysis buffer and incubated on ice for 10 min and the cell lysates were centrifuged at 13,500g for 20 min at 4°C. The resultant supernatant was collected and quantified by Biophotometer (Eppendorf, Germany). Caspase 3 and caspase 9 activities were determined by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC and Ac-LEHD-AMC (BD Sciences), respectively. The reaction mixtures consisted of 25 μg of extracts and 100 mM substrate in 250 μl assay buffer (50 mM Hepes-KOH, pH 7.5, 75 mM NaCl, 1 mM EDTA-Na2, 2 mM DTT, 0.5% Chaps, 10% sucrose) were mixed, incubated for 50 μM substrate in 250 μl assay buffer (50 mM Hepes-KOH, pH 7.5, 75 mM NaCl, 1 mM EDTA-Na2, 2 mM DTT, 0.5% Chaps, 10% sucrose) were mixed, incubated for 20 min at 4°C. The resultant supernatant was collected and quantified by Biophotometer (Eppendorf, Germany). Caspase 3 and caspase 9 activities were determined by measuring the proteolytic cleavage of the fluorogenic substrate Ac-DEVD-AMC and Ac-LEHD-AMC (BD Sciences), respectively. The reaction mixtures consisted of 25 μg of extracts and 100 mM substrate in 250 μl assay buffer (50 mM Hepes-KOH, pH 7.5, 75 mM NaCl, 1 mM EDTA-Na2, 2 mM DTT, 0.5% Chaps, 10% sucrose) were incubated at 37°C for 1 h. The result was terminated with a dilution of 1 ml ice-cold assay buffer (pH7.5), and fluorescence was measured using a spectrofluorimeter at an excitation wavelength of 395 nm and an emission wavelength of 460 nm.

Flow cytometric analysis of apoptotic cells

The Annexin V-FITC kit was used for flow cytometric analysis of apoptosis in SI-1 cells. Briefly, after cells were transfected with dsRNA at different time points, the cells were inoculated with AfMNPV for 10 h. The cells were pelleted by centrifugation at 800×g for 10 min and then washed twice with PBS, and then resuspended with preluted binding buffer. The concentration of cells was adjusted to 1×10⁶ cells/ml. 5 μl Annexin V-FITC was added to 195 μl cell supernatant and gently mixed, incubated at room temperature for 10 min then stained with 10 μl of 20 μg/ml propidium iodide for 20 min. The cells were examined using fluorescein isothiocyanate (FITC) on a flow cytometer and analyzed by flow cytometry on 488 nm fluorescence/area histogram.

Treatment of AfMNPV-induced SI-1 cells with FSBA

5×10⁵ cells/ml were seeded into flasks or 6-well plates and grown over night. Then the medium was replaced with the fresh medium containing 10% FBS and FSBA at different concentrations (0, 50, and 100 μM). After 3 h of the pretreatment of the cells with FSBA, the cells were washed twice with fresh medium and inoculated at AfMNPV at multiplicity of infection (MOI) of 5 for 3 h. The virus inoculum was removed and cells washed twice with serum-free medium and then cultured in Grace’s medium supplemented with 10% FBS and FSBA at different concentrations. The relative caspase-3 activity and the percentage of apoptotic cells were measured at 12 h post-infection according to the methods described as above.

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Author Contributions

Conceived and designed the experiments: KL JP. Performed the experiments: KL YY DS ZG JL ML NS. Analyzed the data: JP HH. Contributed reagents/materials/analysis tools: KL YY DS ZG SG. Wrote the paper: KL JP.

References

1. Liu X, Kim CN, Yang J, Temmerson R, Wang X (1996) Induction of apoptotic program in cell-free extract: requirement for dATP and cytochrome c. Cell 86: 147–157.
2. Liu L, Peng J, Liu K, Yang H, Hong H (2007) Influence of cytochrome c on apoptosis induced by Anaglyph (Syngrapha) falcifera multiple nuclear polyhedrosis virus (AMNPV) in insect Spodoptera litura cells. Cell Biol Int 31: 996–1001.
3. Bossy-Wetzel E, Newmeyer DD, Green DR (1998) Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J 17: 37–49.
4. He J, Xiao Y, Carlos A, Zhang L (2000) Role of mitochondrial cytochrome c in copper-induced apoptosis in coronary artery endothelial cells. J Pharmacol Exp Ther 295: 896–903.
5. Arnould D, Parone P, Martinou J, Antonsson B, Estauquier J, et al. (2002) Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. J Cell Biol 159: 923–922.
6. Yang J, Liu X, Bhalra K, Kim CN, Ibrazo AM, et al. (1997) Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. Science 275: 1129–1132.
7. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science 275: 1132–1136.
8. Zou H, Henzel WJ, Liu X, Lutsch G, Wang X (1997) Apaf-1, a human protein homologous to C. elegans Ced-4, participates in cytochrome c dependent activation of caspase-3. Cell 90: 405–413.
9. Nunez G, Benedikt MA, Hu Y, Inohara N (1998) Caspas: the proteases of the apoptotic pathway. Oncogene 17: 3237–3245.
10. Jiang X, Wang X (2000) Cytochrome c promotes caspase 9 activation by inducing binding to Apaf-1. Biol Chem 273: 3199–3203.
11. Joza N, Susin SA, Daugas E, Stanford WL, Cho SK, et al. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. Nature 410: 549–554.

12. Dorstyn L, Milh K, Lazebnik Y, Kumar S (2004) The two cytochrome c species, DC3 and DC4, are not required for caspase activation and apoptosis in Drosophila cells. J Cell Biol 167: 405–410.

13. Zimmermann KC, Ricci JE, Drein NM, Green DR (2002) The role of ARK in stress-induced apoptosis in Drosophila cells. J Cell Biol 156:1077–1087.

14. Means JC, Muro I, Clem RJ (2006) Lack of involvement of mitochondrial factors in caspase activation in a Drosophila cell-free system. Cell Death Differ 13: 1222–1234.

15. Dorstyn L, Kumar S (2006) A cytochrome c-free fly apoptosome. Cell Death Differ 13: 1049–1051.

16. Sahdev S, Taneya TK, Mohan M, Sah N, Khar AK, et al. (2003) Baculoviral p35 inhibits oxidant-induced activation of mitochondrial apoptotic pathway. Biochem Biophy Res Commun 307: 483–90.

17. Mohan M, Taneya TK, Sahdev S, Moharre R, et al. (2003) Antioxidants prevent UV-induced apoptosis by inhibiting mitochondrial cytochrome-c release and caspase activation in Spodoptera frugiperda (Sf9) cells. Cell Biol Int 27: 483–490.

18. Beem E, Holliday LS, Segal MS (2004) The 1.4-MDa apoptosome is a critical intermediate in apoptosome maturation. Am J Physiol Cell Physiol 287: 664–672.

19. Malagoli D, Iaconi I, Marchesini E (2005) Cell-death mechanisms in the IPLB-LdFb insect cell line: a nuclear located Bel2-like molecule as a possible controller of 2-deoxy-D-ribose—mediated DNA fragmentation. Cell Tissue Res 320: 337–343.

20. Shan S, Liu K, Peng J, Yao H, Li Y, et al. (2009) Mitochondria are involved in apoptosis induced by ultraviolet radiation in lepidopteran Spodoptera litura cell line. Insect Science 16: 485–491.

21. Kumarswamy R, Seth RK, Dwarkannah BS, Chandna S (2009) Mitochondrial regulation of insect cell apoptosis: evidence for permeability transition pore-independent cytochrome-c release in the Lepidopteran Sf9 cells. Int J Biochem Cell Biol 41:1430–1440.

22. Tian L, Liu S, Liu H, Li S (2012) 20-hydroxyecdysone upregulates apoptotic genes and induces apoptosis in the Bombyx fat body. Arch Insect Biochem Physiol 79:207–219.

23. Wei W, Gai Z, Ai H, Wu W, Yang Y, et al. (2012) Baculovirus infection triggers a shift from amino acid starvation-induced autophagy to apoptosis. PLoS ONE 7: e37457.

24. Wei W, Wei Y, Ahlimit M, Ma Y, Fu T, et al. (2011) Responses of two insect cell lines to starvation: autophagy: prevents them from undergoing apoptosis and necrosis, respectively. J Insect Physiol 57: 723–734.

25. Liu K, Tang Q, Fu C, Peng J, Yang H, et al. (2007) Influence of glucose starvation on the pathway of death in insect cell line Spodoptera frugiperda cellul: apoptosis follows autophagy. Cytotechnology 54: 97–105.