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Synchronized Expression of Two *Bombyx mori* Caspase Family Genes, *ice-2* and *ice-5* in Cells Induced by Ultraviolet Irradiation

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The caspase family proteins play an important role in programmed cell death (apoptosis). To date, the expression profiles of the caspase family genes in *Bombyx mori* (Bm) are poorly known. In this study, we examined the expression profiles of two novel Bm caspase family genes (*ice*-2 and *ice*-5), the potential change of the mitochondrial membrane and the morphology in Bm cells after stimulation of ultraviolet (UV) irradiation. The results showed the potential change of the mitochondrial membrane occurred at 5 hours after UV irradiation treatment. Analysis of fluorescent quantitative RT-PCR demonstrated that both the *ice*-2 and *ice*-5 might be involved in UV induced apoptosis in Bm cells. Notably, after UV irradiating, expression pattern of *ice*-2 and *ice*-5 were remarkably different. The *ice*-2 gene was highly expressed at two time points, 0.5 and 5 hours after UV stimulating, while the expression level of *ice*-5 only peaked at 5 hours after UV stimulating. It indicated that apoptosis induced by UV irradiation was involved in the mitochondrial pathway and the two isoforms of Bm *ice* may act but play different role during the apoptosis of Bm cells.

Key words: *Bombyx mori*, Caspase, *ice*-2, *ice*-5, apoptosis, ultraviolet irradiation.

Introduction

Apoptosis, an essential physiological process required for normal development of multi-cellular organisms and tissue homeostasis, is also apparent in the process which dying cells and their nuclei shrink and fragment, and the chromosomal DNA is cleaved into internucleosomal repeats (Parrish *et al.*, 2001). The Mammalian cells apoptotic pathways were wildly researched in the past, and could be divided into two major ways: the extrinsic and intrinsic apoptotic pathway. The majority of apoptotic stimuli such as irradiation, cytotoxic drugs, granzyme B and DNA damage initiate the intrinsic apoptotic pathway (mitochondrial pathway) by causing the loss of mitochondrial membrane potential followed by the release of cytochrome c from the mitochondrial (D'Costa and Denning, 2005; Twomey and McCarthy, 2005). Therefore, loss of mitochondrial membrane potential could be used for monitoring the initiation of apoptosis.

Ultraviolet (UV) irradiation induced apoptosis through a large number of unrelated pathway, including p53, Bax, caspase-3, and PARP cleavage (Srivastava and de Larrea, 2008). Interlukin-1-converting enzyme (ICE) was first discovered in mammal as a member of caspase (cys-teinylaspartate specific proteinase) family and named caspase-1, which is the key mediator of caspase-dependent apoptosis. Several years later ICE was identified as a CED-3 like protein in *C. elegans* (Yuan *et al.*, 1993). In lepidopteran insects, *ice* was identified as a label gene of pro-death in the *Heliothis virescens* midguts developmental apoptotic process (Parthasarathy and Palli, 2007). In silkworm, three isoforms of *ice* homological gene, named *ice*, *ice*-2 and *ice*-5, were described in GenBank (Accession number: *ice*, AY88522; *ice*-2, DQ360829; and *ice*-5, DQ360830). In our previous study, *ice*-2 and *ice*-5 were cloned, with an open reading frame of 852 and 936 base pairs (bps), respectively. The difference between the two genes was that *ice*-2 contained an extra exon with 86 bp and lost two nucleotides at the beginning of the exon 6 (Song *et al.*, 2007). However, the function of two isoforms of Bm *ice* gene is still missing.

In this study, we attempt to characterize the genes of

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ice-2 and *ice*-5 in the early phage of UV induced apoptosis, morphological and mitochondrial membrane potential changes of Bm cells were observed, meanwhile, time course transcriptional profiles of these two genes were investigated by real time RT-PCR. Since there are few studies on insect ICEs, the present study on UV induced apoptotic expression will provide new insight into the function of ICEs in insects.

Materials and Methods

Bm cells culture and treatment

The Bm cells were cultured in TC-100 insect cell culture medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) at 27°C. UV irradiation was applied to treat Bm cells. The cells were plated at a density of 2×10^5 cells in 6-well plates. The cells, with a very thin layer of PBS were irradiated for 20 s under UVA and UVB lamps. The total dosage irradiated to these cells, measured by an International Light Inc. (Newburyport, MA) radiometer fitted with a UV detector, was 20 mJ/cm^2. After irradiation, the cells were again cultured in TC-100 insect cell culture medium and were by turns collected and observed under microscope after incubation in different time.

RNA extraction

Total RNA was extracted from the collected cells by use of Trizol (Invitrogen) according to the manufacturer's protocol. Contaminating genomic DNA was removed by Rnase-free Dnase I (Promega). The concentration of the RNA was assessed using Genspec III (Hitachi Genetic systems), a spectrophotometer, and the integrity of the RNA was assessed by running 2 μl of RNA on a 1% EB/agarose gel. And the RNA was stored at −70°C until ready to use.

Reverse transcription

Two microgram DNase-treated RNA was reverse-transcribed to single-stranded cDNA in a 20 μL reaction containing 0.2 μmol/L oligo-dT, 0.5 mmol/L of each dNTP, 5 μL M-MLV 5×reaction buffer and 200 U M-MLV reverse transcriptase (Promega). The thermal cycling profiles are as follows: 65°C for 5 min, 37°C for 60 min, 75°C for 5 min. The resultant cDNA was stored at −20°C until ready to use.

Real-time PCR

Primers used for the real time PCR amplification of *ice*-2, *ice*-5 and Bm actin were selected based on the sequences available in GenBank. Primers were designed for the specific detection (for *ice*-2 Forward: 5'- tct gtt gac ggt tat ctt tc -3'; Reverse: 5'- tat tgt tgg tct cct gac at -3'; for *ic*e-5, Forward: 5'- tgt tga cga gct tgt gac tc -3'; Reverse: 5'- cac cat cgt gat cat atg ca -3'). Primers for Bm *actin*A3 (Forward: 5'- atc cag cag ctc cct cga gaa gtc t -3'; Reverse: 5'- aca atg gag gga cca gac tcg tcgt -3') were used as endogenous reference gene in real time PCR. Real-time PCR amplifications were performed to examine the relative expression of *ice*-2 and *ice*-5 in treated Bm cells in the sequence detection system (Stratagene, USA). Duplicates of 0.5 μL cDNA from each reverse transcription reaction were used as templates. The reactions were performed in a total volume of 50 μl using SYBR premix EXTaqTM perfect Real time kit (TaKaRa, Dalian, China) as recommended by the manufacturer. The following MX3000P thermocycling program was used: denaturation program (3 min at 95°C), amplification and quantification program repeated 40 times (10 s at 95°C, 30 s at 58°C and 20 s at 72°C with a single fluorescence measurement), melting curve program (55°C to 95°C with a heating rate of 0.1°C/s). Relative expression levels of *ice*-2 and *ice*-5 were calculated with the comparative Ct (2-ΔΔCt) method. Means and standard errors for each time point were obtained from the averages of three independent sample sets.

JC-1 Assay for Mitochondrial Membrane Potential

Mitochondrial potential change was assessed in live Bm cells by using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol-carbocyanine iodine (JC-1) (*Smiley et al*., 1991). For quantitative fluorescence measurement, cells were rinsed once after JC-1 staining and scanned with a fluorescence spectromety (Molecular Devices SPECTRA MAX, GEMINI EM) at 485-nm excitation and 530 and 590 nm emission, to measure green and red JC-1 fluorescence, respectively. Each well was scanned at 25 areas rectangularly arranged in 5' 5 pattern with 1-mm intervals and an approximate beam area of 1 mm^2 (bottom scanning).

Results

Morphological change of Bm cells after UV stimulation

Using microscope, UV stimulated Bm cells were observed at 0, 0.5 to 6 h, respectively. As time passed by, the morphology of cells was changed. However, in the first 4 hours after stimulating, there were few cells that had different morphology with normal cells (Fig. 1). Then several cell membranes wrinkled at 5 hours after stimulating. At 6 hours after stimulating the wrinkle became more remarkable. Meanwhile, apoptotic bodies could be observed at this stage.

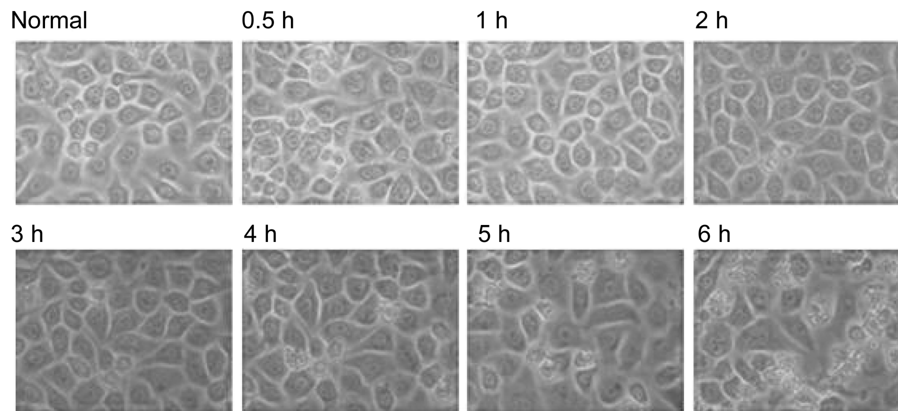

Fig. 1. Progression of Bm cells after UV stimulating. Morphological change of Bm cells were observed at 0.5 to 6 h, respectively. Normal Bm cells were used as a control. The photos were taken at 200 × magnification.

Mitochondrial membrane potential change of Bm cells
Bm cells were acutely exposed to 20 mJ/cm^2 UV irradiation and were collected at different time. The results showed that during the first five hours, the 590 : 530 fluorescence ratio of JC-1 dye declined slightly, and the change could be omitted compared to the later change (Fig. 2). The red-green JC-1 fluorescence ratio started to decrease at 5 hours after UV stimulating. Correspondingly, the rate of dying cells began to increase at 5 hours after UV stimulating. Thus, the potential change of Bm cells was consistent with the morphological change of the cells.

Expression profiles of the ice-2 and ice-5 genes
The relative expression of mRNA of *ice*-2 and *ice*-5 of UV stimulated Bm cells were analyzed by quantitative real-time RT-PCR. The *ice*-2 gene was highly expressed at two time points, 0.5 and 5 hours after UV stimulating, while the expression level of *ice*-5 only peaked at 5 hours after UV stimulating (Fig. 3). In other time stages, however, very low levels of both *ice*-2 and *ice*-5 mRNAs were detected. Interestingly, the mRNA level of *ice*-2 was higher than *ice*-5 at the majority time stages from 0 to 6 hours except for the 5 hours.

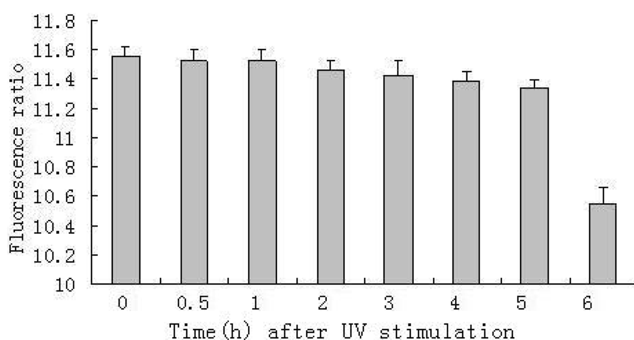

Fig. 2. Change of mitochondrial membrane potential of UV stimulated Bm cells. Mitochondrial membrane potentials of Bm cells in different time (0 to 6 h) after UV stimulating were measured by JC-1 assay. Data are the mean ± S.E. of results of three separate experiments; each experiment was performed in triplicate.

Discussion

UV induced apoptosis includes multiple signaling pathways. The results in this study implied that the majority of cells which began dying at 6 hours with a notable decrease of mitochondrial membrane potential might be via intrinsic apoptotic pathway (mitochondrial pathway). Bm *ice*, *ice*-2 and *ice*-5 resemble human caspase-3, which plays a

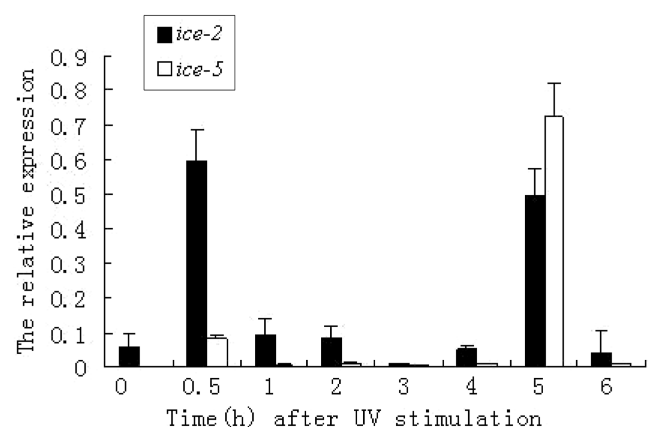

Fig. 3. Expression profiles of *ice-2* and *ice-5* in Bm cells. Real-time RT-PCR analyses were performed using total RNA from the cells which were collected at 0, 0.5 to 6 h after UV stimulating, respectively. The relative *ice-2* and *ice-5* expression levels as calculated by $2^{-\Delta\Delta Ct}$ were shown for each group and the bar charts (mean ± S.E.) represented three independent experiments with three replications.

role as an effector and depends on the release of cytochrome c from the mitochondrial. However, expression of the *ice* isoform was not detected in this study, since no copies of the *ice* were found in dozens of the sequenced clones (data not shown). The peaks level of *ice*-2 and *ice*-5 reached when cellular morphology and mitochondrial membrane potential remained unchanged (Fig. 1 and 2), suggesting that the activation of Bm ICE-2 and ICE-5 might be related to the release of cytochrome c from the mitochondrial. Therefore, they maybe initiators rather than effectors in UV induced apoptosis in Bm cell line. As mentioned in the introduction, *ice*-2 and *ice*-5 were transcribed from the same gene but spliced differently under UV irradiation, and they both have a QACRG active site which belongs to caspase family (Song *et al*., 2007). The fact that they are different from each other exclusively in one exon implied the important role of the exon. From 0 to 0.5 h post UV irradiation, while the level of *ice*-2 increased from low level to the highest level, the level of ice-5 increased from an undetectable level to low level (Fig. 3), suggesting that *ice*-2 may also play an more important role in the early phage of UV induced apoptosis than *ice*-5, that the *ice*-2 and *ice*-5 may be the upstream members of apoptotic pathway induced by UV irradiation in Bm cells, and that *ice*-2 and *ice*-5 might induce expression of themselves in the later phage. The level of both *ice*-2 and *ice*-5 decreased significantly at 1 h post UV irradiation and remained from 1 to 4 h post UV irradiation. Moreover, the significant increase in the level of both the *ice*s at 5 h post UV irradiation followed by a remarkable decline of mitochondrial membrane potential made us to speculate that the two *ice*s might be the initiator of intrinsic apoptotic pathway induced by UV irradiation. Thus, the one exon difference may be the reason for different roles of *ice*-2 and *ice*-5 in apoptotic pathway.

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