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# Characterization of Programmed Cell Death in the Silkworm Thoracic Ganglia during Postembryonic Periods

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**Abstract:** Programmed cell death was characterized in the silkworm thoracic ganglia TG1, TG2 and TG3 during postembryonic periods by TUNEL assay. Apoptotic cells were detected in the three TGs of all larval stages except for day-1, 2 1st instar larvae, in which no apoptotic cells were found. From day-7 5th larva, the numbers of apoptotic cells were dramatically increased and peaked on day-1 pupa and day-2 pupa and then abruptly decreased. Apoptotic cells finally disappeared in day-1 adult. *In-vivo* injection of 20-hydroxyecdysone (20E) into day-8 5th larva resulted in a striking decrease of apoptotic cells. Actinomycin D (Act D) or cycloheximide (CHX), injected into hemolymph of day-8 5th larva, resulted in a decrease of apoptotic cells in the three TGs. Injection of caspase-8 and -3 inhibitors also blocked cellular apoptosis. These results will provide valuable information for understanding of cellular changes in the three TGs during metamorphosis of the insect species.

**Key words:** Cell apoptosis, thoracic ganglia, silkworm, 20E, TUNEL assay

Programmed cell death, or apoptosis, plays an important role in eliminating excess, outmoded or damaged cells during embryonic and postembryonic development (Jiang et al., 1997; Aravind et al., 1999; Liu and Hengartner, 1999). The term "programmed cell death" (PCD) was established to distinguish cell death from necrotic cell destruction (Lockshin and Zakeri, 1990). PCD usually proceeds through a stereotypical series of distinct morphological stages that include cell shrinkage, chromatin condensation, DNA cleavage, ultimately cellular fragmentation and formation of apoptotic bodies (Kerr et al., 1972; Wyllie, 1980). Because PCD plays such a critical role in both normal and abnormal development, extensive studies have focused on its regulation.

PCD is an integral component of maintenance of homeostasis and host defense against pathogens in animals (Jacobson et al., 1997; Vaux and Korsmeyer, 1999). In insect, imaginal tissues and organs are newly formed from primordial cells, or imaginal discs, within larva, while most of larval tissues are degenerated during pupal stage. PCD occurs simultaneously in various differentiating tissues (Holman et al., 1996; Kaellen, 1965). In case of development of both vertebrate and invertebrate nervous systems, PCD of mature and differentiating cells has been clearly demonstrated and extensively studied (Caldero et al., 1998; D'Mello, 1998; Truman, 1984; Truman et al., 1992), whereas only few studies reported PCD in cellular precursors (Carr and Simpson, 1981; Champlin and Truman, 1998; Monsma and Booker, 1996; Nordlander and Edwards, 1969). On the other hand, studies on mammals and birds suggested that death of cellular precursors might be common in the development of the central nervous system (Blaschke et al., 1998; Diaz et al., 1999).

Factors that influence whether particular cells die or survive include the levels of, and developmental timing of exposure to, hormones and neurotrophic factors (Burek and Oppenheim, 1996). Among many extrinsic factors that regulate PCD are steroid hormones, represented by 20-hydroxyecdysone (20E) (Breedlove, 1992; Mills and Sengelaub, 1993; Nordeen et al., 1985, Truman et al., 1992). Steroid hormones influence many aspects of cellular phenotype. During insect metamorphosis, 20E regulates cellular survival, dendritic and axonal growth or regression of nerve cells and other changes in neuronal development (Hoffman and Weeks, 2001). In amphibians, metamorphic

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changes are regulated by thyroid hormones (Hoskins and Grobstein, 1984; Tata, 1994). In avian and mammalian species, gonadal steroid hormones generate sexually dimorphic regions of the nervous system through sex- and region-specific regulation of cellular birth, growth, and survival (Garcia-Segura et al., 1994; Kawata, 1995). In most cases it is unknown whether an effect is mediated by a direct action on the neurons of interest or by indirect signals from other hormonally sensitive cells or a combination of the two effects. This information is crucial in understanding how PCD is activated or repressed within individual cells.

This study showed pattern of cellular apoptosis in the three silkworm TGs during postembryonic life and how apoptotic TG cells respond to 20E, including implication of caspases in PCD (Thornberry and Lazebnik, 1998; Susin et al., 1998).

## MATERIALS AND METHODS

### Animals

Cold-treated eggs from silkworm, *Bombyx mori*, which were kindly provided by Department of Agricultural Biology in National Institute of Agricultural Science and Technology (Suwon, Korea), were hatched to 1st larvae about 8-10 days after incubation under 12 : 12 h light/dark photoperiod at 29°C and relative humidity of 70%. Larvae were then reared on artificial diet of "Silk-mate" (Nihon Nosan Kogyo, Yokohama, Japan). After hatching, 1st larvae were incubated at 28°C, 2nd and 3rd larvae at 27°C and 26°C, respectively, and 4th larva to adult at 25°C. Day-8 5th instar larvae were mainly used.

### Analysis of apoptotic TG cells by TUNEL assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method was used to visualize cells undergoing apoptosis. Tissue preparations were performed according to the method described by Lee et al. (1998) and Kim et al. (1998). Thoracic ganglia were dissected from each developmental stage in 0.1 M sodium phosphate buffer (PB, pH 7.4). The isolated tissues were then fixed in 4% paraformaldehyde (PFA) in 0.1 M PB for 6-10 h at 4°C, depending on size of each tissue. They were then immersed in phosphate-buffered saline (PBS) with 1% Triton X-100 (PBST) at 4°C overnight, followed by dehydration in a graded ethanol series, clearing in xylene, and rehydration in the reverse order to dehydration.

TUNEL assay was performed using a DeadEed™ Fluorometric TUNEL System Kit (Promega). Three isolated TGs of designated stages were digested with 50 μl Proteinase K solution (20 μl/ml Proteinase K in buffer solution) for 10 min to increase their permeability. After washing with PBS for 5 min, the TGs were treated with kit-provided equilibration buffer for 5-10 min at room temperature and incubated in the TdT solution buffer consisting of equilibration buffer (45 μl), nucleotide mix (5 μl), and rTdT (1 μl) in a humidified chamber for 1 h at 37°C. The tissues were then incubated for 15 min in stop solution (2 × SSC), washed three times in PBS for 15 min, and treated with propidium iodide (PI, 0.5-1.0 μl/ml in PBS). Tissues were then rinsed three times in PBS for 30 min, transferred onto slide glasses, exposed to one drop of anti-fade solution to prevent from exposure to light, and mounted on slides with coverslip (Boatright et al., 2003; Bossy-Wetzel and Green, 2000). Apoptotic cells were observed under confocal laser scanning microscope (Zeiss LSM 310) with fluorescence optics, using identical pinhole, brightness and contrast setting.

### Injections of 20E and chemicals

Larvae were injected with 20E and other chemicals as described by Mitchell (1978). 20E (Sigma) was dissolved in ethanol and stored at –20°C until use. Day-8 5th instar larvae (average weight 1.3g) were injected with 20E, while the same volume of PB was injected into controls. Cycloheximide (CHX; Sigma) and actinomycin D (ActD; Sigma) were dissolved in ethanol at 10 mg/ml and 0.1 mg/ml, respectively, and stored at –20°C until use. Caspase-8 and -3 inhibitors (Calbiochem) were kept as stock solutions of 100 mM in dimethyl sulfoxide (DMSO) solution. Through abdominal cavity, 1 μl of suitably diluted compounds were injected into day-8 5th larva with Hamilton syringe.

### Western blot

Protein samples were prepared from day-8 5th instar larvae that were injected with PB (control), 20E, ActD, CHX, and caspase-8 and -3 inhibitors. Dissected TGs were homogenized in 2× sample buffer (125 mM Tris, 4% SDS, 20% glycerol, 0.005% bromophenol blue, 10% β-2-mercaptoethanol), boiled for 3 min, and centrifuged at 10,000 *g* at 4°C. The supernatant was boiled again for 3 min, and equilibrated to room temperature before loading. Equal amounts of total protein were loaded per lane and separated on 12% gels. Western blot transfer of the separated proteins was performed at 4°C, using nitrocellulose membranes at 100 V for 1.5 h. The blots were blocked for 1 h in TBST containing 5% nonfat dried milk. The membranes were probed with monoclonal anti-cleaved caspase3 antibody (Cell signaling) diluted 1 : 1000 in TBST containing 5% nonfat dried milk at 4 overnight. They were then washed for 1 h with frequent changes of TBST, followed by incubation in a peroxidase-coupled secondary antibody (1 : 2500) for 1 h in TBST containing 5% nonfat dried milk. The blots were then rinsed as before and developed using an enhanced chemi-luminescence detection system (ECL, Amersham Corp, UK). Membranes were stripped and reprobed with β-actin (1 : 1000) to confirm equal loading of the sample.

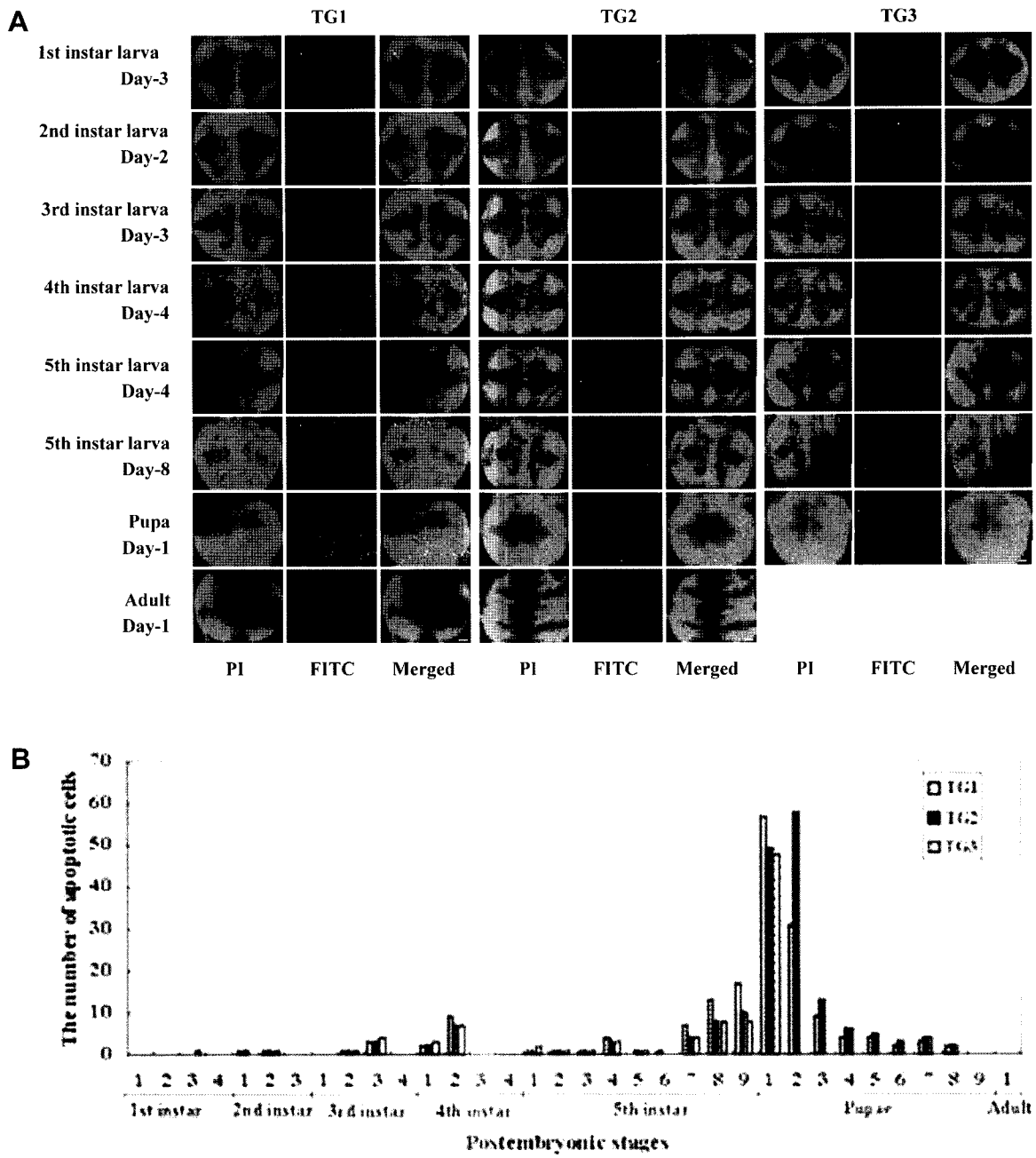


**Fig. 1.** Patterns of cellular apoptosis in TG1, TG2 and TG3 of silkworm during postembryonic stages. (A) Confocal micrographs showing apoptotic cells in TG1, TG2 and TG3 of day-1 1st instar larvae to day-1 adults. No cellular apoptosis was found in the three TGs of day-1 1st instar larva and day-1 adult. Apoptotic cells began to gradually increase after the day-7 5th instar larva. Apoptotic cell number in the three TGs in reached maximum pupa. Subsequently, apoptotic cells sharply decreased at day-2 pupa. Nuclei with fragmented DNA were detected. Intact nuclei were counter-stained with PI. Scale bar represents 100 μm. (B) Histogram showing apoptotic cell number in the three TGs during postembryonic periods. In most larval stages, apoptotic frequency moves within narrow limits. In day-7 to -9 5th instar larval stages, however, apoptotic cells were rapidly increased until day-1 or -2 pupal stages. Thereafter, apoptotic cells were decreased drastically. Developmental days of larva, pupa and adult are indicated as Arabic numbers along the horizontal axis.

## RESULTS

### Apoptotic cells in three TGs during postembryonic periods

In postembryonic stages, all apoptotic cells harbored nuclei that appeared green under the confocal microscope when treated with FITC in TUNEL assay, but appeared red when stained by PI. When PI and FITC staining images were superimposed, the apoptotic nuclei appeared yellow (Fig. 1A). TG1, TG2 and TG3 showed a similar pattern of cellular apoptosis except for the day of the largest apoptotic cell number.

In the three TGs of day-1 1st instar larva, there were no apoptotic cells, which began to appear in the TGs of the day-3 1st instar larva (Fig. 1B). In the mid-day of each instar period, TGs showed a small rise in apoptotic cells. However, apoptotic cells of TGs began to gradually increase in day-7 5th instar larva and dramatically rised and culminated in day-1 and -2 pupae; the highest number apoptotic cells appeared in dya-1 pupa. Day-2 pupa was ruled out because the 2nd thoracic ganglion (TG2) cells and 3rd thoracic ganglion (TG3) were fused into a larger ganglion, forming a thoracic neuron center. Cellular apoptosis of the three TGs decreased sharply 2 days after pupation, stayed at a low level for a few days and disappeared in day-1 adult.

In order to monitor the pattern of apoptotic cells in all postembryonic stages, at least 7 and at most 23 TGs were isolated from larvae, pupae or adult of each postembryonic day (Table 1). The average number of apoptotic cells in each postembryonic stage was determined by dividing the total number of apoptotic cells counted in all TGs by the number of TGs investigated at each stage.

**Table 1.** Apoptotic cells of three TGs in various postembryonic developmental stages investigated for pattern of TG cell apoptosis in the silkworm, *Bombyx mori*

| Developmental stages | Total investigated TGs | Average apoptotic cells in a TG1 | Average apoptotic cells in a TG2 | Average apoptotic cells in a TG3 |
|---|---|---|---|---|
| 1st larva Day-1 | 16 | 0 | 0 | 0 |
| Day-1 | 19 | 0 | 0 | 0 |
| Day-3 | 19 | 0 | 0 | 1 |
| Day-4 | 19 | 0 | 0 | 0 |
| 2nd larva Day-1 | 16 | 1 | 1 | 0 |
| Day-2 | 19 | 1 | 1 | 1 |
| Day-3 | 16 | 0 | 0 | 0 |
| 3rd larva Day-1 | 16 | 0 | 0 | 0 |
| Day-2 | 19 | 1 | 1 | 1 |
| Day-3 | 22 | 3 | 3 | 4 |
| Day-4 | 16 | 1 | 0 | 0 |
| 4th larva Day-1 | 16 | 2 | 2 | 3 |
| Day-2 | 23 | 9 | 7 | 7 |
| Day-3 | 16 | 0 | 0 | 0 |
| Day-4 | 16 | 0 | 0 | 0 |
| 5th larva Day-1 | 16 | 1 | 1 | 2 |
| Day-2 | 16 | 1 | 1 | 1 |
| Day-3 | 19 | 1 | 1 | 1 |
| Day-4 | 22 | 4 | 3 | 3 |
| Day-5 | 22 | 1 | 1 | 1 |
| Day 6 | 19 | 1 | 0 | 0 |
| Day-7 | 18 | 7 | 4 | 4 |
| Day-8 | 15 | 13 | 8 | 8 |
| Day 9 | 18 | 17 | 10 | 8 |
| Pupa Day-1 | 15 | 56 | 49 | 48 |
| Day-2 | 15 | 31 | 58 | |
| Day-3 | 12 | 9 | 13 | |
| Day-4 | 12 | 4 | 6 | |
| Day-5 | 10 | 4 | 5 | |
| Day-6 | 11 | 2 | 3 | |
| Day-7 | 10 | 3 | 4 | |
| Day-8 | 7 | 2 | 2 | |
| Day-9 | 7 | 0 | 0 | |
| Adult Day-1 | 7 | 0 | 0 | |

*Average apoptotic cell number in a TG, obtained by dividing total number of apoptotic cells in all TGs by total number of TGs investigated.

## Cellular apoptosis in TGs exposed to 20E

20E inhibited cellular apoptosis in the three TGs of day-8 5th instar larva (Fig. 2A). 20E at various concentrations led to similar results. Apoptotic cells induced by lower concentrations of 20E (0.1-1.0 μg/ml) showed no remarkable contrast in its number compared to those by higher concentrations (5.010 μg/ml). As shown in Fig. 2B, all concentrations of 20E inhibited cellular apoptosis in the three TGs (Fig. 2B). The inhibition was stronger in the TG2 and TG3 than in TG1.

## Inhibition of apoptosis by ActD and CHX

In many cases, apoptosis required synthesis of new proteins that were believed to activate preexisting cell death machinery.

In order to investigate whether synthesis of new proteins was a prerequisite for cellular apoptosis, 100 ng/ml ActD or 5 μg CHX was injected into day-8 5th larva larva. The presence of apoptotic cells was then monitored in the three TGs by confocal microscopy 48 h after the injection, and cleaved caspase-3 was detected by western blotting 24 h later (Fig. 3A).

As shown in Fig. 3B, the number of apoptotic cells in three TGs injected with ActD were significantly lower (about 8 (TG1), 5 (TG2) and 4 (TG3) and about 86% (TG1), 90% (TG2) and 92% (TG3) decreases) than those of the control (about 56 (TG1), 49 (TG2) and 48 (TG3)). When the three TGs were injected with CHX, the number of apoptotic cells was also decreased significantly (about 6 (TG1), 4 (TG2) and 3 (TG3) and about 89% (TG1), 92% (TG2) and 94% (TG3) decreases), as compared to those from the control (about 56 (TG1), 49 (TG2) and 48 (TG3)). Results from western blotting, indicated no cleaved caspase-3 within the TGs of the specimens (Fig. 3C). These results implied that specific RNA or protein synthesis might constitute the cause of death in these TG cells.

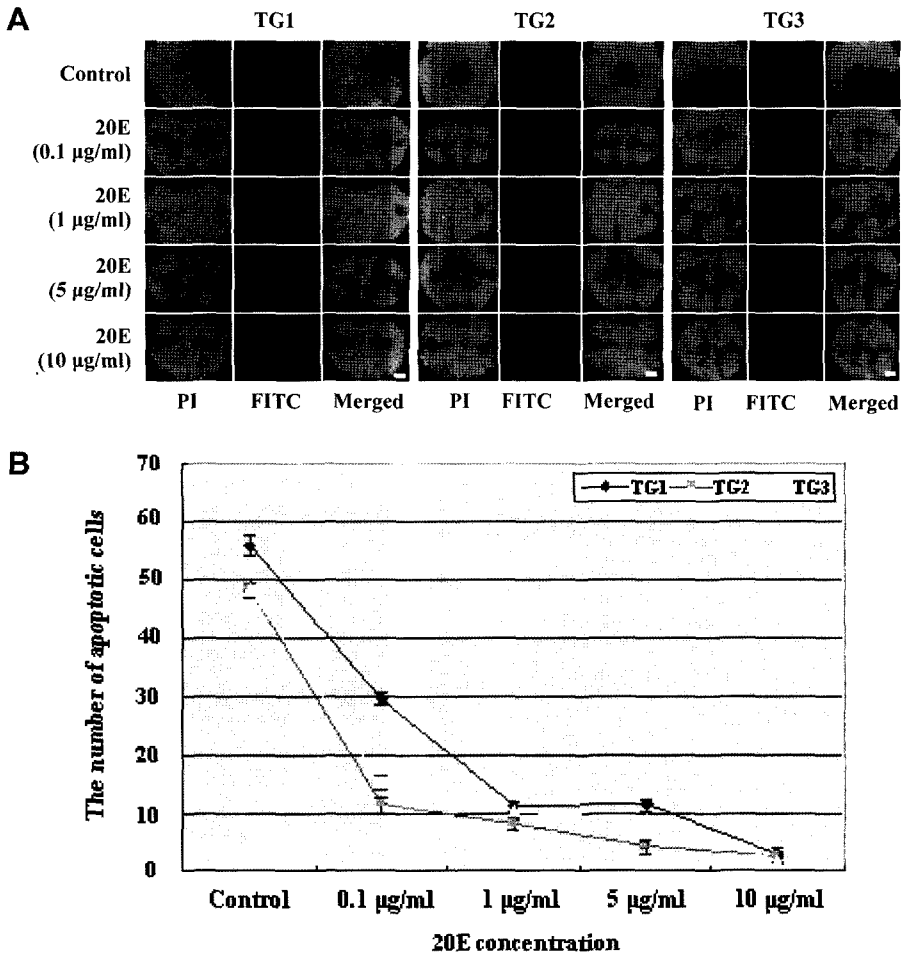


**Fig. 2.** Dose effects of 20E on cellular apoptosis in TG1, TG2 and TG3. (A) Confocal micrographs of the three TGs of day-8 5th instar larva after injection with various concentrations of 20E (0.1, 1, 5, 10 mg/ml). In all 20E concentrations, cellular apoptosis in the three TGs was shown to be effectively inhibited. Apoptotic cells were rarely found in larva after injection of 10 mg/ml 20E. Scale bar represents 100 μm. (B) Histogram presenting the number of apoptotic cells in the three TGs. Diamonds, squares and triangles represent numbers of apoptotic cells in TG1, TG2 and TG3, respectively.

### Effect of caspase inhibitors on PCD of TG cells

In order to clarify whether caspase-8 and caspase-3 were involved in signal pathway of cellular apoptosis in the *B. mori* TGs, caspase inhibitors were injected into hemolymph of day-8 5th instar larva. In both cases cell death was efficiently blocked (Fig. 4A). As shown in Fig. 4B, the number of apoptotic cells was significantly decreased to about 89% (TG1), 92% (TG2) and 85% (TG3) and about 93% (TG1), 88% (TG2) and 94% (TG3), respectively.

Results of western blotting, performed 24 h after the injection of caspase-8 or -3 inhibitors, indicated no cleaved caspase-3 within the three TGs of the specimens (Fig. 4C). These showed that caspase -8 and -3 inhibitors effectively blocked cellular apoptosis in the three TGs.

## DISCUSSION

### Inhibition of apoptosis by 20E in TGs during metamorphosis

During metamorphic reorganization of insect CNS, fluctuations in the 20E titer in hemolymph evoke PCD, proliferation, maturation, and remodeling of larval neurons into adult forms (Schubiger et al., 1998). In *B. mori*, 20E was found to be responsible for three titer peaks in the hemolymph, from 4th instar larval stage until day-1 of the adult stage: the first, smallest 20E peak appeared between day-2 and day-3 4th instar larval periods, and the second, medium-sized peak between day-6 and day-8 5th instar larval stage, and the largest peak between day-2 and day-3

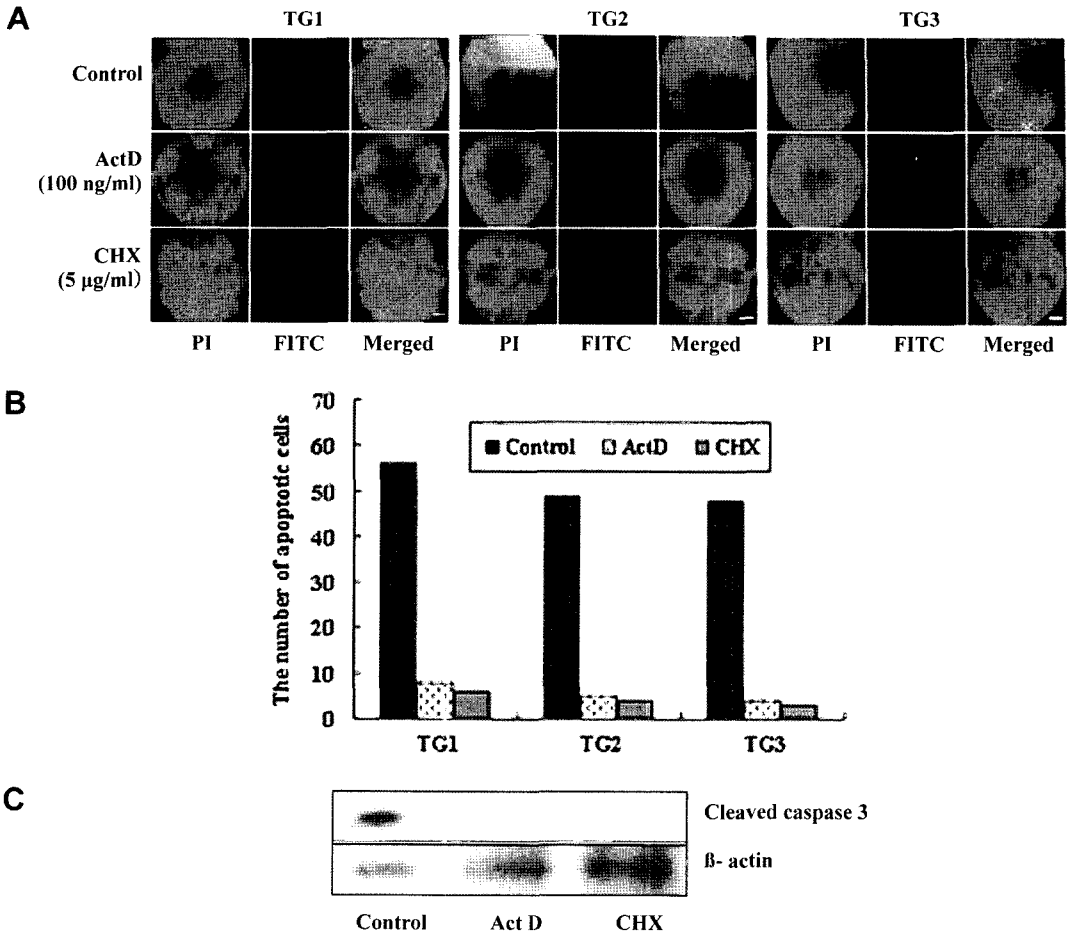


**Fig. 3.** Effects of Act D and CHX on cellular apoptosis in TG1, TG2 and TG3 of day-8 5th instar larva. (A) Confocal images of three TGs obtained 48 hours after injection of 100 ng/ml ActD or 5 mg/ml CHX). TGs injected with either Act D or CHX exhibited very few apoptotic cells, whereas those of control larva exhibited a sizeable number of apoptotic cells. Scale bar indicates 100 ìm. (B) The numbers of apoptotic cells in the three TGs injected with Act D or CHX were about 8 (TG1), 5 (TG2) and 4 (TG3), and about 6 (TG1), 4 (TG2) and 3 (TG3). (C) Results of western blot in day-8 5th instar larval TGs, 24 hours after injection of Act D or CHX suggested that activation of cleaved caspase-3 was completely inhibited by both treatments.

pupal stages (Mizoguchi et al., 2001). In this study, the *Bombyx* TGs exhibited only one large peak in apoptotic cell number in the day-1 or day-2 pupae (see Fig. 1). Other peaks were detected at very low levels.

Data obtained from this study had no corresponding relation with changes in the titer of 20E in the hemolymph. Exposure to 20E resulted in inhibition of cellular apoptosis in the three TGs. Many previous studies suggested that 20E induces cellular apoptosis by increasing its titer within the hemolymph in various tissues of insect species (Dorstyn et al., 1999; Draizen et al., 1999; Fujiwara and Ogai, 2001; Kinch et al., 2003; Lee et al., 2002; Robinow et al., 1997; Terashima et al., 2000). Therefore, the results from this study were in contrast to the previous studies. This remains to be solved, because the relationship between the increase in 20E titer and inhibition of cellular apoptosis could not be explained by the results of this study.

### RNA and protein synthesis during PCD in TGs

Previous studies showed that PCD is related with 20E level (Streichert et al., 1997; Weeks et al., 1992). 20E, which is located in hemolymph, transfers to cytosol where if combines with a specific receptor, forming a complex of 20E and receptor (EcRs). This complex translocates to nuclei to bind to a specific site on DNA, triggering transcription of specific genes (Hoffman and Weeks, 2001). These series of events occur during apoptosis in various insect species. In this study, however, cellular apoptosis in the three TGs was shown to be inhibited by increased 20E. Therefore, the way how increase of 20E titer inhibited cellular apoptosis in the three TGs remains to be further studied in future.

### Effects of caspases in apoptosis of TG cells

Caspases (a family of cysteine proteases) are important components of the death machinery in many vertebrates

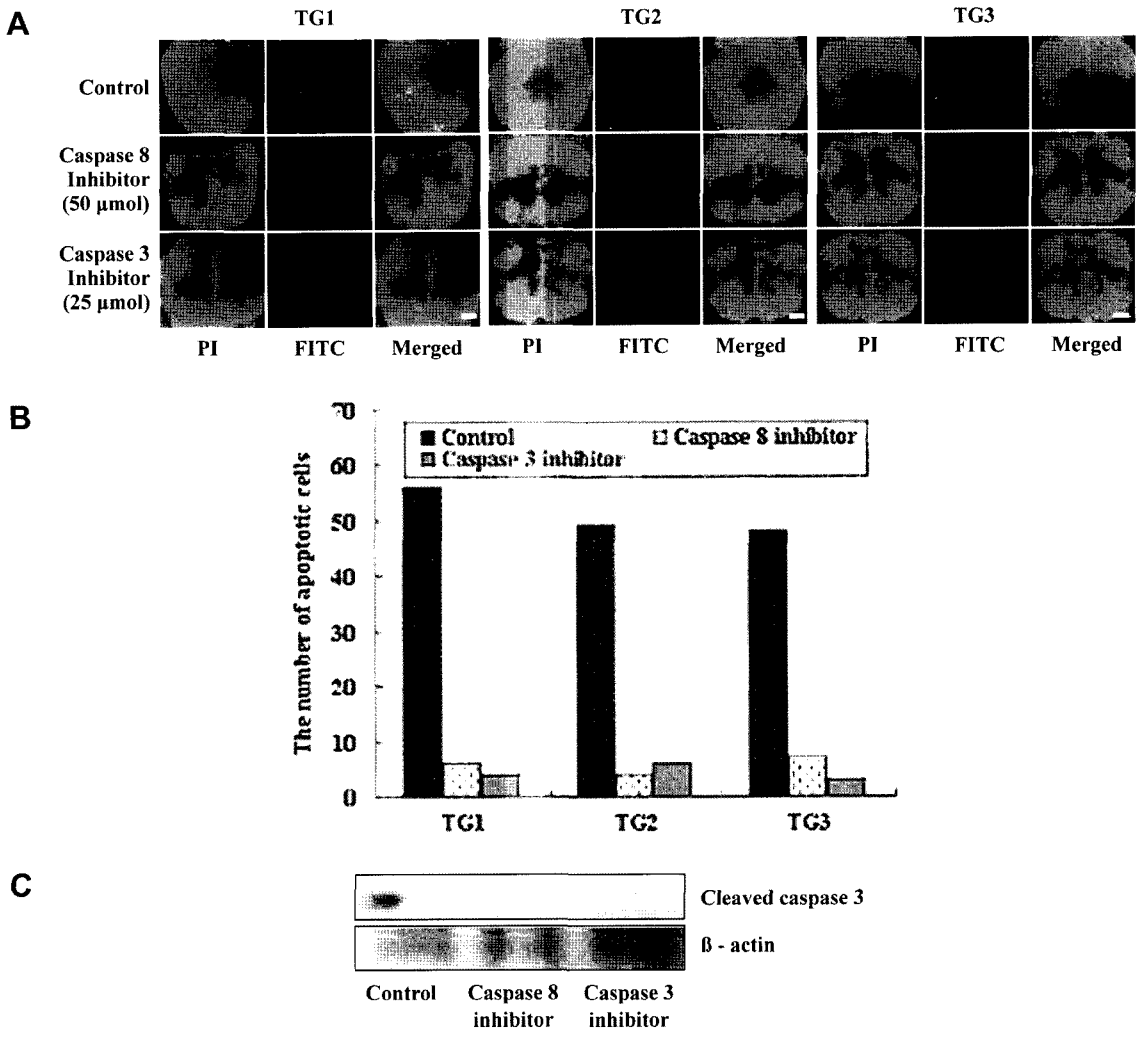


**Fig. 4.** Effects of caspase-8 and -3 inhibitors on cellular apoptosis in TG1, TG2 and TG3 of day-8 5th instar larva. (A) Confocal images obtained 48 hours after injection of 50 iM caspase-8 inhibitor or 25 iM caspase-3 inhibitor. After injection of the inhibitors, apoptotic cells were significantly decreased compared to those of control larva. Scale bar represents 100 mm. (B) The numbers of apoptotic cells in the three TGs injected with caspase-8 and -3 inhibitors were about 6 (TG1), 4 (TG2) and 7 (TG3), and about 4 (TG1), 6 (TG2) and 3 (TG3). (C) Western blot of the three TGs after injection of the inhibitors suggested that activation of cleaved caspase-3 was completely inhibited not only by the casapase-3 inhibitor but also by the caspase-8 inhibitor.

and invertebrates (Thornberry and Lazebnik, 1998). Caspases have a unique requirement for an aspartate residue at the site of proteolytic cleavage; specific pharmacological inhibitors of caspases contain either a single aspartate or an aspartate within a short peptide sequence (Livingston, 1997). Caspase inhibitors could block apoptosis in both insect and mammalian cells (Clem and Miller, 1994; Kondo et al., 1997; Milligan et al., 1995; White et al., 1996).

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