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Teratocyte-secreting proteins of an endoparasitoid wasp, *Cotesia plutellae*, prevent host metamorphosis by altering endocrine signals

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ABSTRACT

An endoparasitoid wasp, *Cotesia plutellae*, parasitizes young larvae of the diamondback moth, *Plutella xylostella*, with its parasitic factors of polydnavirus, venom, ovarian proteins, and teratocytes (TCs). TCs are originated from embryonic serosal membrane at hatch of *C. plutellae* egg. Injection of *in vitro* cultured TCs significantly prolonged a larval period of nonparasitized *P. xylostella* and impaired a larva-to-pupa metamorphosis. This developmental alteration was also induced by injection of TC-cultured medium (TCM). However, heat-treated TCM significantly lost the inhibitory activity against larval development of *P. xylostella*. Larvae treated with TC or TCM appeared to undergo abnormal endocrine conditions. Juvenile hormone esterase activity was significantly suppressed at early last instar by injection of TC or TCM. In addition, expression of ecdysone receptor at final instar was lost, but that of insulin receptor was maintained until the end of the larval period in TC or TCM treatment. A proteomic analysis of TCM predicted several teratocyte-secreting proteins (TSPs). The inhibitory effect of host development by TCs was significantly enhanced by an addition of another parasitic factor, *C. plutellae* bracovirus. These results suggest that *C. plutellae* TC plays a crucial role in alteration of host development by secreting TSPs.

1. Introduction

Endoparasitoid wasps alter host physiological processes for their survival and growth. In particular, koinobiotic parasitoids suppress host immunity and delay host development (Beckage and Gelman, 2004). An endoparasitoid wasp, *Cotesia plutellae*, parasitizes young larvae of the diamondback moth, *Plutella xylostella* (Bae and Kim, 2004). Parasitized host larvae undergo significant alterations in immune and developmental processes (Lee and Kim, 2004; Ibrahim and Kim, 2008). For a successful parasitization, *C. plutellae* transfers several parasitic factors derived from maternal and embryonic sources (Lee et al., 2005). Maternal parasitic factors include ovarian protein (OP) localized in oviduct lumen, venom protein in venom gland, and polydnavirus (PDV) synthesized in ovarian calyx cells (Kim, 2006). Embryonic factors include teratocytes (TCs) and wasp larva (Basio and Kim, 2005a). During parasitization, *C. plutellae* females lay eggs in host hemocoel and also transfer maternal factors. First two days before egg hatch, maternal factors play crucial roles in inducing host immunosuppression to protect wasp eggs from encapsulation of host hemocytes (Nalini et al., 2008). OP and venom protein suppress host immunity independently or cooperatively with PDV products (Basio and Kim, 2006). *C. plutellae* PDV is classified as a bracovirus (BV) called CpBV, which encodes 157 putative genes in its episomal genome (Chen et al., 2011). Among various CpBV genes, CpBV-lectin is expressed at the early parasitization period and significantly inhibits hemocyte encapsulation of *P. xylostella* by protecting wasp eggs from nonself recognition by the parasitized host (Nalini et al., 2008). Other CpBV genes are also expressed in the parasitized host and play crucial roles in parasitism (Kim et al., 2007).

TCs are derived from extra-embryonic membrane (=serosa) during parasitoid egg hatch (de Buron and Beckage, 1997). TCs have been reported in some endoparasitoid wasps, such as Braconidae, Scelionidae, Mymaridae, Trichogrammatidae, Aphelinidae, and Platygastridae (Dahlman and Vinson, 1993; Hotta et al., 2001; Basio and Kim, 2005a). In addition, TC-like cells also have been recorded in Ichneumonidae (Roulex-Bonnin et al., 1999). Due to their relatively large cell size, TCs can be easily discriminated from hemocytes in hemolymph of parasitized insects. TCs are assumed to have several functions, such as inhibitory effects on synthesis of juvenile hormone esterase (JHE) and storage proteins as well as altering host endocrine titers (Zhang et al., 1992, 1997). They also play a role in supplying nutrition for their host parasitoids by releasing specific proteins that would benefit the development of the parasitoid (Okuda and Kadono-Okuda, 1995; Zhang et al., 1997). These physiological alterations may be induced by the secretory activity of TC (Dahlman, 1990). For example, TSP14

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(14 kDa teratocyte-secreting protein) is secreted from TCs of *Microplitis croceipes* and inhibits host gene expression at a post-transcriptional level (Rana et al., 2002).

TCs of *C. plutellae* are released from wasp embryos at hatch and inhibit lepidopteran host immune response (Basio and Kim, 2006). Immunosuppressive activity of *C. plutellae* TCs was further enhanced by the addition of OP and venom (Basio and Kim, 2005b). However, little is known about an inhibitory effect of TCs on host immature development in *P. xylostella*. Parasitized larvae exhibit a prolonged larval period especially at the last instar by two days at 25 °C, in which TCs are released from wasp embryos when *P. xylostella* is at penultimate instar and appear to survive until wasp egression from host for cocooning (Kwon et al., 2010). These suggest that TCs may contribute to delaying host larval development and preventing a larva-to-pupa metamorphosis. To test this inhibitory effect of *C. plutellae* TCs, we further analyzed TC development in parasitized larvae with reference to their development under *in vitro* culture conditions. Also, we injected the cultured TCs to nonparasitized larvae to see if they could interfere with larval development of *P. xylostella*. Finally, we investigated TSPs using a proteomic approach.

2. Materials and methods

2.1. Insect rearing and parasitization

Adults of *P. xylostella* were fed 10% sucrose solution and allowed to mate for 24 h to lay eggs on aluminum foil. After hatching, late first instar larvae of *P. xylostella* (4 days after oviposition) were parasitized by *C. plutellae* adults at 2:1 (host: wasp) ratio for 24 h, and then the parasitized larvae were allowed to feed on cabbage leaves at 25 ± 1 °C under a photoperiod of 16:8 (L:D) h until the end of parasitoid larval development. Adults emerged from the cocoons (11 days after parasitization at 25 ± 1 °C) were collected for next cycles. For TC collections under *in vitro* conditions, *C. plutellae* eggs were collected from the parasitized larvae of *P. xylostella* at 36 h after parasitization because parasitoid eggs hatched in host body at around 48 h after parasitization.

2.2. Observation of parasitoid egg development and TC release

Different ages (0–48 h) of parasitized larvae were collected and surface-sterilized by soaking in 70% ethanol for 30 s. After rinsing the larvae in sterile distilled water, the larvae were dried on absorptive tissue paper. Before dissection all instruments (e.g. glass wares and forceps) were sterilized with 70% ethanol. The treated larvae were placed on glass slide containing 50 μL TC culture medium (an insect cell culture medium) TC-100 (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (WelGENE) and ampicillin (100 μg/mL, BioShop, Burlington, ON, Canada). Under a stereomicroscope (SZ4045, Olympus, Tokyo, Japan), the larvae were dissected by opening hemocoel to collect parasitoid eggs. The eggs were transparent and their embryo development was observed under a phase contrast microscope (BX51, Olympus, Tokyo, Japan).

For TC collection, *C. plutellae* eggs were collected from host larvae at 36 h after parasitization. The collected eggs were washed five times with TC culture medium. Each egg was cultured in 20 μL TC culture medium in a sterile petri dish (35 × 10 mm, SPL Life Sciences, Pocheon, Korea) at 25 °C. The petri dish was kept in a wet chamber to prevent desiccation. Usually, parasitoid eggs hatched at 12–18 h after collection. Thus, the egg-collecting day was referred to as day 0. As the serosa was dissociated, most TCs were released into the medium early on day 1 either as individual cells or as sheets of cells. However, some TCs remained closely associated with the first instar parasitoid larvae, often attached just behind the head capsule. By day 2, all TCs were dissociated from the larvae. The parasitoid larvae were then removed with capillary tubes. Free form of TCs was collected from the culture medium at day 1 or at day 5 by pipetting and subsequent centrifugation at 150 *g* for 2 min. The TC cells were then rinsed three times with 100 mM phosphate-buffered saline (PBS, pH 7.4). TCs were counted using a hemocytometer (Superior, Marienfeld, Germany) with a tally counter.

2.3. TC developmental analysis in both in vivo and in vitro conditions

For *in vivo* TC developmental analysis, parasitized larvae were sequentially dissected from the first day of parasitization ('P1') to the eighth day of parasitization ('P8') at 25 °C. By opening the hemocoel, the exuding hemolymph was collected. The remaining cadaver hemocoel was washed with PBS. Both hemolymph extracts were combined and observed under a phase contrast microscope to count total TCs and measure TC diameters. TC density was expressed by TC number per parasitoid egg. In each host age, five different larvae were used to represent mean TC density.

For *in vitro* TC developmental analysis, TCs were collected using 36 h parasitoid eggs as described above. TC density and size assessment were analyzed from day 1 to day 21 at 25 °C culture conditions using five different cultures. TC density represents a total TC cell number per egg by counting total cells released from a parasitoid egg. Randomly chosen 30 TC cells were used to measure cell diameter. TC survival was assessed by 1% trypan blue dye exclusion test (Park and Kim, 2000).

2.4. TC-cultured medium (TCM) collection and protein fractionation

TCs were isolated as described above and cultured in the insect cell culture medium at 25 °C. 'TCMD1' represents TC medium cultured TCs for 1 day after collecting mature (36 h old) parasitoid egg. 'TCMD5' represents TC medium cultured TCs for 5 days after collecting the mature egg. Newly hatched larvae were removed from all TC cultures. Proteins of TCM were fractionated with different concentrations of ammonium sulfate (Duksan, Ansan, Korea). Briefly, 10 mL of TCM was slowly mixed with 1.44 g of ammonium sulfate for 10 min to give 0–25% concentration gradient. After 1 h incubation on ice, the slurry was centrifuged at 12,500 ×*g* for 25 min at 4 °C. The pellet was saved for 0–25% fraction and the supernatant was used for subsequent sequential fractionations of 25–50% using 1.57 g, 50–75% using 1.72 g, and 75–100% using 1.90 g of ammonium sulfate. All collected pellets were resuspended with PBS and dialyzed against PBS for 24 h at 4 °C to remove ammonium sulfate. Protein amount of each fraction was measured by the Bradford method (Bradford, 1976).

2.5. Collection of C. plutellae bracovirus (CpBV)

CpBV was isolated from ovary of adult female *C. plutellae*. Briefly, 10 female wasps were dissected under microscope and the isolated ovaries were washed with PBS and the ovarian calyx area was cut using sharp tip of needle. CpBV suspension in PBS was collected using pipette and centrifuged 800 *g* for 3 min to exclude large cellular debris. The supernatant was then passed through 0.45 μm filter (Pall Corporation, Ann Arbor, MI, USA) using a 3 mL syringe. Viral particles were collected by centrifugation at 12,500 *g* for 30 min and resuspended in PBS.

2.6. Microinjection of TC, TCM, and CpBV to P. xylostella larvae

To analyze the parasitic factors, TC, TCM, and CpBV were prepared as described above. To inactivate proteins, TCM was heated at 70 °C for 30 min. These samples were micro-injected with a volume of 500 nL to each nonparasitized third instar *P. xylostella*. For micro-injection, glass capillary needles were prepared by a micropipette puller (PN-30, Narishige, Tokyo, Japan). Injection was performed through the intersegmental abdominal membrane of the larvae with the capillary, which was micro-manipulated by an Ultra Micropump (Four) with SYS-microcontroller (World Precision Instruments, Sarasota, FL, USA)