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Effects of high temperature on the hemocyte cell cycle in silkworm larvae

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Abstract

To understand the inhibitory effects of high temperature on insect growth at the cellular level, we investigated the influence of high temperature on the proliferation and division of larval hemocytes in the silkworm, *Bombyx mori*. Although the total number of hemocytes in the larval body increased enormously over time at 26 °C, no increase was observed at 38 °C. The number of mitotic hemocytes in circulation increased between days 1 and 2 of the fourth larval stage at 26 °C, whereas fewer hemocytes were observed at 38 °C. Laser scanning cytometry revealed that the DNA content of hemocytes collected from the fourth-stadium larvae was predominantly 2C, 4C, and 8C, and the proportion of each type of hemocyte changed dynamically with development during the fourth instar. Specifically, the proportion of hemocytes with a higher DNA content increased gradually during the feeding phase then decreased during the molting phase at 26 °C; in contrast, no decrease was observed at 38 °C. The heat-induced accumulation of 8C hemocytes was mainly detected in granulocytes and plasmatocytes. These data suggest that high temperatures induce a G_2 arrest in larval hemocytes.

Keywords: *Bombyx mori*; Cell cycle; Cell division; Hemocyte; High temperature

1. Introduction

A close relationship exists between insect development and temperature. In a number of insect species, the growth rate is increased and the developmental period is shortened as the temperature increases (Wigglesworth, 1972). However, each species has its own range of optimal temperatures for growth, and extremely high temperatures inhibit rather than promote growth by inducing growth delays and developmental failures, such as larval ecdysis and adult emergence (Chapman, 1998). The mechanisms underlying these effects are unknown. In this study, we sought to understand the effects of high temperature on insect growth at the cellular level.

We previously investigated the effects of high temperatures on cultured cells derived from the silkworm, *Bombyx mori* (Kiuchi et al., 2007). At 38 °C, which is an unusually high temperature for silkworms, the cells did not die but were arrested in G_2. From this result, we speculated that the induction of a cell cycle arrest by high temperatures is closely related to the suppression of insect growth and development. To confirm this speculation, we investigated whether the cell cycle arrest observed in cultured cells in response to high temperatures also occurs in silkworms.

Cell division in insects depends on the developmental stage of the individual and is strongly regulated by the hemolymph concentration of such hormones as 20-hydroxyecdysone (20E) and juvenile hormone (JH). Gardiner and Strand (2000) showed that lepidopteran hemocyte growth tended to be higher at the feeding stage (i.e., the middle of each instar) than at the molting stage. Koyama et al. (2004) demonstrated the control exerted by 20E on the cell cycle in *Bombyx* wing discs in detail, while Truman et al. (2006) showed that JH suppresses the growth and development of imaginal tissues in starved *Manduca* larvae after ecdysis. These results suggest that cell cycle varies over the course of development in response to endocrine signals. For these reasons, the status of the cell cycle during each growth phase must be understood to analyze the effects of high temperatures on insect cells.

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It is difficult to monitor the progression of insect cells through the cell cycle based on the DNA content, because endoreplication (i.e., the replication of DNA during S phase without the subsequent completion of mitosis) is a widespread phenomenon in arthropods, meaning that many insect cells are polyploid (Smith and Orr-Weaver, 1991; Edgar and Orr-Weaver, 2001). This poses a very serious problem for cell cycle analysis, since the DNA content of a diploid cell at G_2 is equal to that a of tetraploid cell at G_1.

In this study, cell proliferation and division were studied using *Bombyx* hemocytes, which are easily isolated. Silkworm hemocytes are classified into five morphotypes: granulocytes, plasmatocytes, prohemocytes, spherulocytes, and oenocytoids (Nittono, 1960; Akai and Sato, 1973; Wago, 1991). Larval hemocytes are produced in the hematopoietic organ (Akai and Sato, 1971), and proliferate by mitosis in the hemolymph (Arnold and Hinks, 1976, 1983; Beaulaton, 1979; Feir, 1979; Gardiner and Strand, 2000). Mitosis has been observed in prohemocytes, granulocytes, and, rarely, in spherulocytes (Nittono, 1960). The number of hemocytes and their components fluctuates during growth (Gardiner and Strand, 2000).

Here, we investigated hemocyte proliferation and division in *Bombyx mori* through the larval molting cycle, and we assessed the effects of high temperatures on these events. To understand the effects of temperature on the hemocyte cell cycle, we began by characterizing hemocyte proliferation and division at each stage of larval development under normal conditions. We then analyzed the DNA content of each type of hemocyte and its progression through the cell cycle.

2. Materials and methods

2.1. Silkworms

Shoon *Bombyx mori* silkworms were used in this study. The larvae were reared on an artificial diet (Nihonnosankogyo Co., Japan) under a continuous cycle of 12 h light and 12 h darkness at 25 ± 2 °C until the third instar. Twenty newly ecdysed larvae in the fourth instar were then transferred to a plastic case ($20.5 \times 15.0 \times 5.2\,cm^3$) and reared at 26 or 38 °C without light. Fluctuations in temperature in the incubators were controlled within 0.5 °C and the humidity was maintained at 60–70%. Fresh food was administered each day to avoid deterioration, and the weight of each larva (three males and three females) was measured until ecdysis to the fifth instar.

2.2. Hemocyte quantitation

Hemolymph was collected onto Parafilm™ from incisions made in the caudal horn of each larva, and 5 μl of the fluid were placed immediately into a single well of a 96-well microplate with 100 μl of phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 138 mM NaCl, and 2.7 mM KCl, pH 7.4) containing 10% formalin and 0.001% 1-phenyl-2-thiourea to prevent melanization of the hemolymph. The hemocytes, which were fixed at the bottom of the well, were then counted (five random areas, $6.35 \times 10^{-4}\,cm^2$ each) under an inverted microscope, and the density of the hemocytes was calculated. Our preliminary results showed that almost all of the hemocytes were fixed at the bottom. The amount of hemolymph contained in a fourth-instar larva was previously estimated at 30% of the total body weight (Nagata et al., 1980). Thus, the total number of hemocytes per larva was estimated by multiplying the hemocyte density by the estimated hemolymph volume.

2.3. Detection of mitotic hemocytes

The number of hemocytes undergoing mitosis was assessed immunohistochemically as described by Champlin and Truman (1998) and Koyama et al. (2004) with minor modifications. Hemolymph was collected from the larvae (three males and three females) by the above-mentioned method and mixed by gently pipetting on Parafilm™. A 50–100 μl aliquot of the mixture was then placed on the $1 \times 1\,cm^2$ area of a slide coated with poly-Lysine (POLY-PREP™ SLIDES; Sigma-Aldrich Co., Ltd., Dorset, UK) and 0.001% 1-phenyl-2-thiourea was added. The hemocytes were allowed to attach to the slides at room temperature for 10 min; subsequently, they were fixed for 10 min in PBS containing 3.7% paraformaldehyde. After three washes in Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, and 2.7 mM KCl, pH 7.4) for 5 min each, the hemocytes were permeabilized in TBS containing 0.1% Triton X-100 for 5 min, rinsed in TBS, and blocked for 15 min in 1% bovine serum albumin-solubilized TBS. The hemocytes were then rinsed in TBS for 5 min and immunostained with a 1:200 dilution of anti-phosphohistone-H3 (Ser10) antibody (Upstate Biotechnology, Lake Placid, NY) for 24 h at 4 °C. After being washed three times in TBS for 5 min each, the hemocytes were incubated with a 1:400 dilution of FITC-conjugated anti-rabbit IgG secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 1 h at room temperature. After immunostaining, 100 μl of a solution containing 50 μg/ml RNase (Ribonuclease; Roche Diagnostics Co., Indianapolis, IN) and 5 μg/ml propidium iodide (PI; Sigma) were added to the hemocytes and incubated for 1 h at 37 °C. Finally, the hemocytes were washed three times in TBS for 5 min each and mounted in Vectashield (Vector Laboratories, Burlingame, CA). The immunostained hemocytes were observed by fluorescence microscopy using a UV excitation filter (BX51, Olympus, Tokyo, Japan) and photographed with a CCD camera (DP70, Olympus).

2.4. Cell cycle analysis and classification of the hemocytes

Fixed hemocytes were washed three times with 100 μl of PBS; 100 μl of RNase-PI (50 μg/ml RNase and 5 μg/ml PI) were then added, and the cells were incubated for 24 h at 37 °C. Finally, the hemocytes were mounted in Vectashield,