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Apoptosis and Adhesion of Hemocytes During Molting Stage of Silkworm, *Bombyx mori*

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To clarify the regulatory mechanism of the rapid changes in the hemocyte density in the silkworm, *Bombyx mori*, during ecdysis, we evaluated the relationship between the hemocyte density and the incidence of apoptosis during this stage. We also evaluated the role of the sugar chains on the adhesion of hemocytes by analyzing the effects on the hemocyte density of the injection of enzymes that cut sugar chains and monosaccharides into the body cavity.

The hemocyte density was increased in the molting stage and spinning, and then decreased after the ecdysis. During spinning, the diameter of the granulocytes markedly increased, in which fatty granules in the cytoplasm increased, becoming foamy. They were identified to be apoptotic hemocytes using the Hoechst staining and the Comet assay. The decrease in the hemocyte density during spinning was mainly caused by the apoptosis of granulocytes. Next, we focused on the fluctuation of hemocyte density during the molting stage. Examination of the changes in the hemocyte density induced by injecting glycoside hydrolases, neuraminidase, sialic acid, or monosaccharides into the body cavity during the fourth molt stage and the third day in fifth instar larva demonstrated that the alteration of hemocyte density was regulated by the attachment and detachment of hemocytes via a selectin ligand, sugar chains. As with the injection of glycoside hydrolase, neuraminidase, sialic acid and fucose raised the hemocyte detachment, and it was assumed that the selectin ligands include the sialyl Lewis x like sugar chains, the same as mammalian lymphocytes.

Keywords: *Bombyx mori*, hemocyte, apoptosis, adhesion, sialyl Lewis x

INTRODUCTION

In holometabolous insects, the body structures and physiological systems change drastically during metamorphosis (Mirth, 2005; Nijhout and Williams, 1974). Most insect hemocytes have a phagocytic function against tissue debris resulting from developmental processes as well as foreign substances in their immune systems (Wigglesworth, 1973). Hemocytes play a crucial role of scavenging and their density alters during metamorphosis (Wigglesworth, 1973; Yamashita and Iwabuchi, 2001). The hemocyte density in the silkworm, *Bombyx mori* (*B. mori*), peaks in the molting stage, and rapidly decreases after the molting stage, then increases during the next molting stage. The hemocyte density is highest during the pupal stage, and rapidly decreases upon pupation (Nittono, 1960). There are marked changes in the hemocyte density with molting and spinning, but the mechanism remained unclarified. In the pupa, pupal specific granulocyte-derived foamy cells appear simultaneously (Jones, 1956; Akai, 1964; Nardi *et al*., 2001), and the hemocyte density becomes lower during pupation, suggesting that the death of foamy granulocytes causes the rapid decrease in the hemocyte density during pupation.

Gowans and Knight (1964) discovered the lymphocyte homing phenomenon, whereby lymphocytes that are collected from specific lymphoducts and injected into animals return to the initial lymphoducts. This homing phenomenon is induced by homing receptors on the surface of lymphocytes and hemocyte adhesion molecules on the surface of endothelial cells, and the density of circulated lymphocytes may be markedly affected. These homing receptors are called selectin, and its ligands are specific sugar chains, such as sialyl Lewis x containing N-acetylneuraminic acid (sialic acid) and fucose (Gallatin *et al*., 1983; Kobzdej *et al*., 2002; Haselhorst *et al*., 2001). Selectin has not been identified in insects, but the hemocyte density in insects might be regulated by the homing phenomenon via selectin ligands as in mammalian lymphocytes.

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In this study, to analyze the factors regulating the changes in the hemocyte density of *B. mori* during molting and spinning, when the hemocyte density vigorously fluctuates, we evaluated the relationship between hemocyte apoptosis and changes in the hemocyte density, and also evaluated the effects of the injection of glycoside hydrolases, neuraminidase or monosaccharides on the hemocyte density, referring to mammalian *in vivo* examinations for lymphocyte homing, *e.g.* the intravenous injection of neuraminidase (True *et al*., 1990; Marin *et al*., 1995) and the neuraminidase treatment of the high endothelial venule (Rosen *et al*., 1985).

MATERIALS AND METHODS

Animals

Silkworms, *B. mori* (Fuyo × Tsukubane), which were purchased from Ehime Sanshu Inc., were fed with artificial food (Silkmate, Nihon Nosan Kogyo Inc.) in an incubator at a temperature of 25.0°C and a humidity of 75–80%.

Collection of hemolymph

Larva were chilled in crushed ice for 15 min, and immersed in 70% ethanol for 1 min. Then, 1.0 ml of cold anticoagulant (0.098 M NaOH, 0.146 M NaCl, 0.017 M EDTA and 0.041 M citric acid, pH 4.5 and osmolality 370 Osm kg^{-1}) was injected via a 27G needle inserted into the body. Hemolymph, obtained by cutting a proleg, was collected into a micro tube.

Giemsa staining

A drop of hemolymph was spread on a glass slide using a cover slip, dried for several hours, and subjected to Giemsa staining. The Giemsa staining solution was prepared by diluting 1.875 ml of Giemsa stock solution in 50 ml of 1/225 M phosphate buffer (pH 6.4).

Calculation of the hemocyte density

The hemocyte density was obtained in males at each stage using the hemolymph diluted 5-fold with 0.8% physiological saline. Hemocytes were counted according to the white blood cell counting method for human blood, using a Burker- Turk hemocytometer, and the hemocyte density was calculated from the hemocyte counts per μl.

Hoechst staining for the apoptotic hemocyte identification

Hemocytes were stained on a slide glass using a Vybrant™ Apoptosis Assay Kit (Molecular Probes, Eugene, OR) containing Hoechst 33342 (Hoechst) and propidium iodide (PI), and observed at 400-fold magnification under a fluorescence microscope. Apoptotic hemocyte nuclei with strong white blue fluorescence and necrotic hemocyte nuclei with red fluorescence were counted and the ratio of apoptotic hemocytes to the total hemocyte count was calculated.

Comet assay for the apoptotic hemocyte identification

Apoptotic hemocytes were detected using a Comet Assay kit (Trevigen Instructions, MD). To prepare the hemocyte suspension, the hemolymph collected was diluted to a hemocyte density of 100 cells/μl with Ringer solution for insects. The hemocyte suspension was mixed with 1% Comet LMAgarose in Reagent Kit of Comet Assay™ at 37°C at a ratio of 1:10, and spread evenly on a glass slide, then left still horizontally in a cool and dark place at 4°C for 30 min. The glass slide was immersed in a lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% Triton x-100) at 4°C, and left to stand in a cool and dark place at 4°C for 1 hour. Subsequently, the glass slide was immersed in a freshly prepared alkaline solution (0.6 g NaOH, 250 μl of 200 mM EDTA, 49.75 ml dH_2O), and left to stand in a cool and dark place at 4°C for 1 hour. The slide was removed from the alkaline solution, the excess buffer was gently tapped from the slide and washed by immersing in TBE buffer (10.8 g Tris base, 5.5 g Boric acid, 0.93 g EDTA-2Na, 900 ml dH_2O). The slide was transferred to a horizontal electrophoresis apparatus and set to 1 volt per cm. The comet slide was stained using a Silver staining kit (MoBi Tec GmbH, Göllingen).

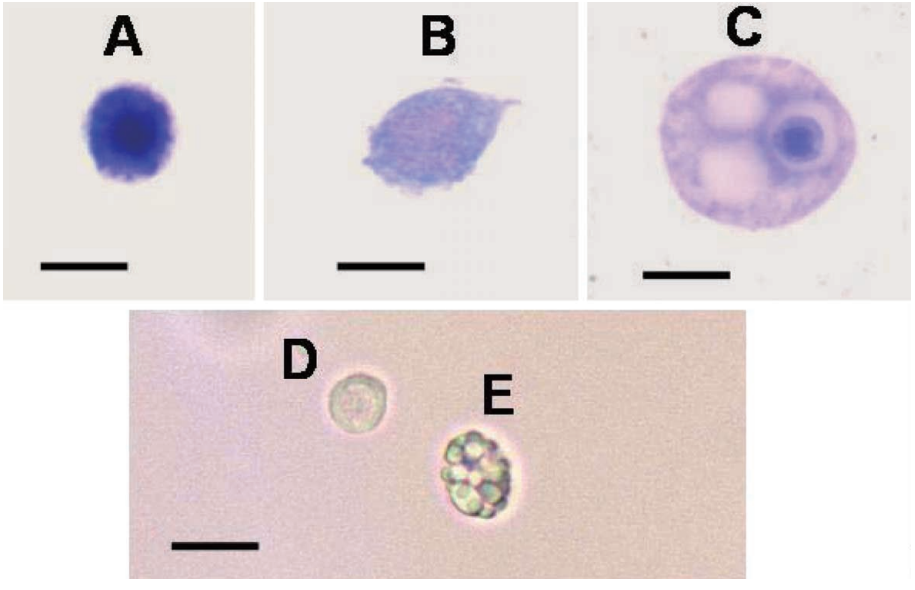

Fig. 1. Hemocytes observed in the fifth instar larva of silkworm, *Bombyx mori*. A: granulocyte (Giemsa staining); B: plasmocyte (Giemsa staining); C: oenocyte (Giemsa staining) ; D: prohemocyte (unstaining); E: spherule cell (unstaining). Scale bar=10 μm

Injection of glycoside hydrolase, neuraminidase or monosaccharides to the body cavity

Ten μl of glycoside hydrolases, neuraminidase or monosaccharides was injected via the intersegmental membrane of the larva using a 23 G needle, and hemostasis was performed using superglue. The glycoside hydrolase used was 0.5 U/ml glycopeptidase F (Takara, Otsu), and 1 U/ml neuraminidase (Roche Diagnostics GmbH, Penzberg) was used. The monosaccharides used were 0.013 M N-acetylneuraminic acid (sialic acid) (Nakarai Tesque, Kyoto), 0.026 M N-acetylneuraminic acid, 0.026 M galactose (Nakarai Tesque, Kyoto), 0.026 M N-acetylglucosamine and 0.026 M fucose (Wako, Osaka) dissolved in 9.6 mM PBS (pH 7.2). After injection of these solutions, the hemocyte density was measured at certain intervals. These data were compared to the control data after injection of the same volume of 9.6 mM PBS (pH 7.2) without enzymes and monosaccharides.

RESULTS

Hemocyte classification and hemocyte density

Nittono (1964) reported five types of *B. mori* hemocytes. We also identified these hemocytes: granulocyte (GR), plasmocyte (PL), oenocyte (OE), spherule cell (SP) and prohemocyte (PRO) (Fig. 1), and changes in the hemocyte den-

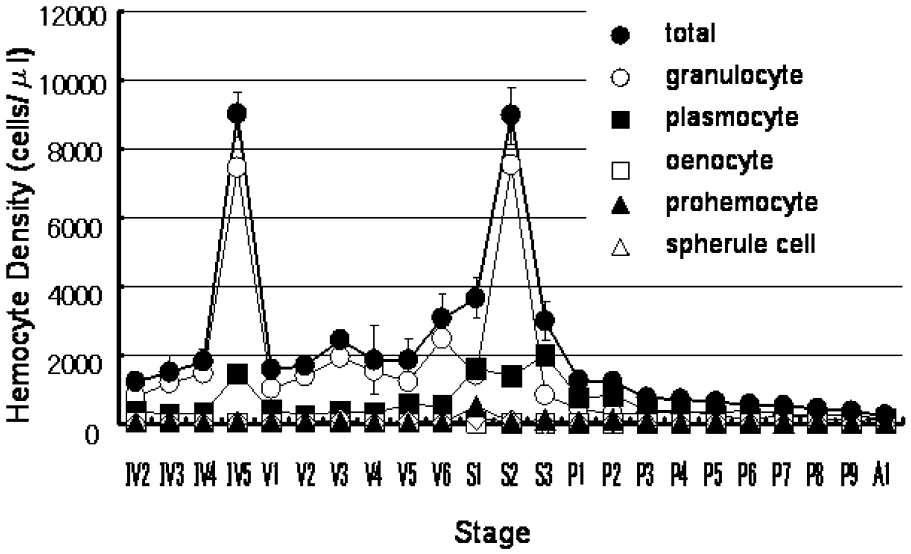

Fig. 2. The fluctuation of hemocyte density from the fourth molt stage to adult stage. IV: the fourth instar larva; V: the fifth instar larva; S: spinning; P: pupa; A: adult. Arabic numerals indicate the number of days for each stage. Each datum comes from males only, and each point represents an averaged value for 5 samples±SD.

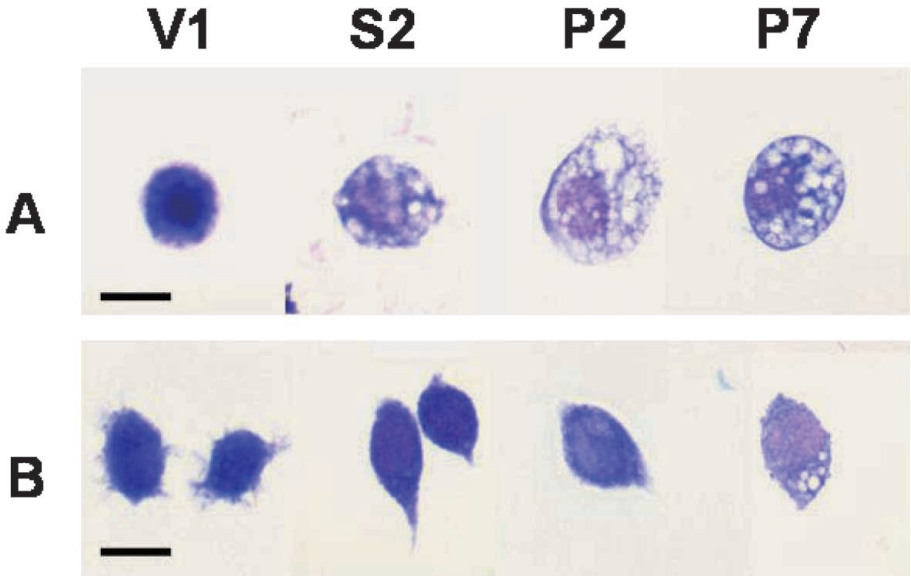

Fig. 3. Morphological change of granulocyte and plasmocyte from larval stage to pupal stage. A: granulocyte; B: plasmocyte. V1: the first day of the fifth instar; S2: the second day of spinning; P2: the second day of pupa; P7: the seventh day of pupa. Scale bar=10 μm

sity in the fourth molt to adults of the silkworm, *B. mori* were observed using a Burker-Turk hemocytometer (Fig. 2). The hemocyte density was increased in the molting stage, the fifth day of the fourth instar, and indicated a peak of 9,000 cells/μl. The hemocyte density was then rapidly decreased after molting and reached 1,800 cells/μl on the first day of the fifth instar larva. In late fifth instar larvae, the hemocyte density was gradually increased again to reach 9,000 cells/μl on the second day of spinning. The hemocyte density was decreased rapidly after spinning, then decreased slowly until reaching the adult stage. Regarding the hemocyte density of each type, the GR and PL comprised 90% or more at all stages, and changes in the total hemocyte density were almost entirely due to changes in the two hemocyte types.

Morphological change of hemocytes during ecdysis

We observed the morphological change of hemocytes at each stage in the fourth molt to adults using Giemsa staining. Almost all hemocytes observed by Giemsa staining were GR or PL, and only a few OE were detected (Fig. 2). SP was observed shortly at the beginning of spinning, and few PRO were detected. In pupa, the cytoplasm of PL contained vacuoles, which were absent in the larvae. More marked morphological changes were observed in the GR. The cell diameter of the GR began to increase in spinning and reached about two fold of that in larva at the beginning of pupa. Though no fatty granules were observed in GRs in larva, a few granules began to be observed in spinning, and increased in size and number in pupa (Fig. 3).

Identification of apoptotic hemocytes

Using an apoptotic detection kit containing Hoechst and PI, apoptotic hemocytes were observed (Fig. 4A), and changes in the apoptotic hemocyte percentage were calculated for each stage (Fig. 5). The nucleus of apoptotic hemocytes showed a strong white blue fluorescence with Hoechst. The apoptotic hemocyte ratio was increased to about 20% in the fourth molt, but it was decreased to 5% or lower in the fifth instar larvae. During spinning, the apoptotic hemocyte ratio was rapidly increased again and reached the highest ratio, about 30%, on the second day in pupa (Fig. 5). As in the observation of apoptotic hemocytes by Hoechst staining, Comet assay-positive hemocytes were detected in each stage (Fig. 4B). The apoptotic hemocyte ratio determined by Comet assay was the highest (about 28%) on the second day in the pupa, agreeing with the result obtained by

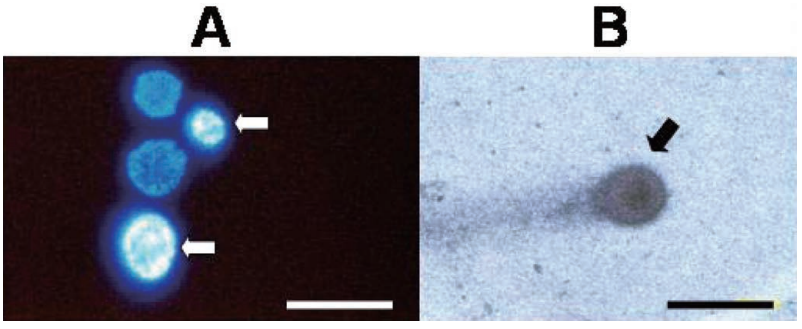

Fig. 4. Identification of apoptotic hemocytes. A: Hoechst 33342 and propidium iodic staining; B: Comet assay. Arrows indicate the nucleus of apoptotic hemocytes. Scale bar=10 μm

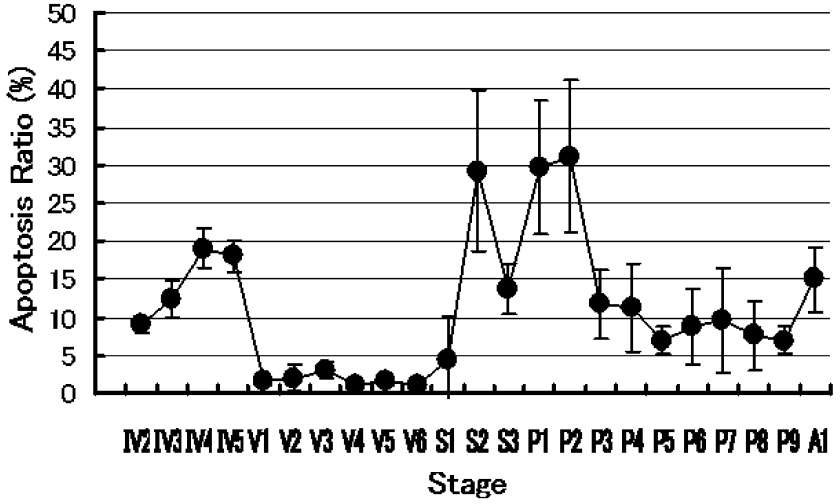

Fig. 5. Apoptotic hemocyte ratio from the fourth molt stage to adult stage. IV: fourth instar larva; V: fifth instar larva; S: spinning; P: pupa; A: adult. Arabic numerals indicate the number of days for each stage. Each datum comes from males only, and each point represents an averaged value for 5 samples±SD.

Hoechst staining.

The injection of glycoside hydrolases, neuraminidase or monosaccharides

The changes in the hemocyte density were examined by injecting glycoside hydrolase, neuraminidase or monosaccharides into the body cavity of silkworms in the fourth molting stage or on the third day of the fifth instar larva (Fig. 6). Generally, the hemocyte density was rapidly decreased after the molting stage, but the decrease in the hemocyte density was suppressed by injecting a glycoside hydrolase, such as glycopeptidase F, and neuraminidase into the body cavity. 0.026 M sialic acid or 0.026 M fucose injection also suppressed the decrease in the hemocyte density, but the suppression caused by the injection of sialic acid diluted to half the concentration was weaker. The injection of 0.026 M galactose or N-acetylglucosamine did not suppress the decrease in the hemocyte density.

The hemocyte density was suddenly decreased after the fourth molting stage, and it was low during the fifth instar larva. The hemocyte density was rapidly increased by the injection of glycoside hydrolase or neuraminidase into the body cavity on the third day of the fifth instar larva, and reached 8,000 cells/μl 8-12 hours after injection. The injection of 0.026 M sialic acid or 0.026 M fucose increased the hemocyte density, but the increase in the hemocyte density was little with 0.013 M sialic acid injection. The injection of 0.026 M galactose or 0.026 M N-acetylglucosamine did not increase the hemocyte density.

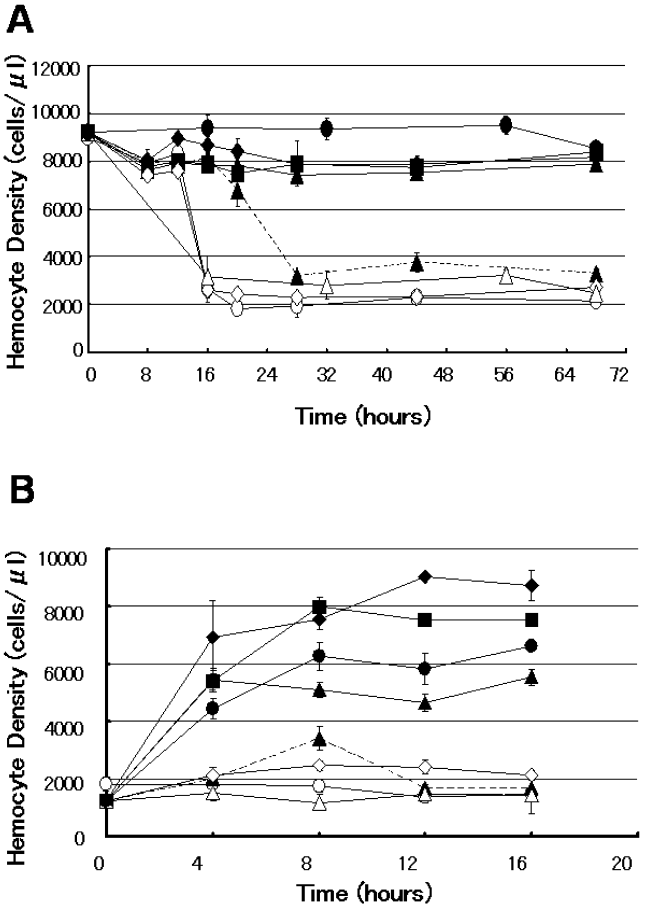

Fig. 6. Changes in hemocyte density by injection of glycoside hydrolase (glycopeptidase F), neuraminidase or monosaccharides on the fourth molting stage (A) and the third day of the fifth instar larva (B). Each point represents an averaged value for 3 samples±SD. —○—: PBS; —■—: glycopeptidase F; —◆—: neuraminidase; —●—: 0.026 M fucose; – –▲– –: 0.013 M N-acetylneuraminic acid; —▲—: 0.026 M N-acetylneuraminic acid; —◇—: 0.026 M galactose; —△—: 0.026 M N-acetylglucosamine

DISCUSSION

The hemocyte density in insects markedly varies with metamorphic stages. As metamorphosis is regulated by the endocrine system (Otaki *et al*., 1968; Sakurai *et al*., 1977), the changes in the hemocyte density may be regulated by juvenile hormone and ecdysone (Ling *et al*., 2003), but the regulatory mechanism has not been clarified. It is assumed that hemocytes are adhering to the surface of organs in the body cavity (Tsukushi, 1972), but the adhesion mechanism remains unclear.

In the hemolymph of *B. mori,* the hemocyte density was rapidly increased during molting and spinning, and the apoptotic hemocyte ratio was also increased at the same stage (Fig. 2). After molting and spinning, the hemocyte density and apoptotic hemocyte ratio were decreased. However, the apoptotic hemocyte ratio was instantly increased after spinning, the beginning of pupa, again. In this stage, fatty granules were enlarged in the cytoplasm and the cell diameter was increased (Fig. 3). Examination of hemocyte apoptosis and fragmentation of DNA during this stage by fluorescence staining of nucleus and Comet assay demonstrated a high percentage of apoptotic hemocytes (Figs. 4, 5). In human atherosclerosis, macrophage-derived foamy cell formation occurs and forms lipid-enriched atherosclerotic plaques (Sakihama *et al*., 1995), and the apoptosis is caused by incorporation of oxidized low-density lipoprotein (oxidized LDL) (Goyert *et al*., 1988). It was assumed that granulocytes incorporate lipoproteins derived from apoptotic tissues debris during larval-pupal ecdysis, and undergo death themselves during pupation, like the macrophage-derived foamy cells. The decrease of the hemocyte density after spinning will be caused by the apoptosis of foamy hemocytes.

It has been clarified that sugar chain structures and cell adhesion molecules, i.e. selectin, on the cell surface play important roles in the extravascular migration of human lymphocytes (Suzuki *et al*., 1993), and many selectin families have been identified (Chou, 1996). The properties of sugar chain structures of selectin ligands have also been clarified. These sugar chains have structures containing sialic acid, such as sialyl Lewis x (sLe x) and sialyl Lewis a (sLe a) (Kobzdej *et al*., 2002; Ito *et al*., 2001).

Recently, strong evidence emerged for the existence of sialic acid in the larval hemolymph of *Bombyx mori* (Kato *et al*., 1999). In order to clarify the relationship between the hemocyte density and hemocyte adhesion to the organ surface in the body cavity via selectin-ligands, we examined the changes in the hemocyte density at intervals after injection of glycopeptidase F, which cuts N-glycan as glycoside hydrolase, or neuraminidase, which cuts sialic acid on the sugar chains, at the terminal in the fourth molting stage when the hemocyte density peaks due to a temporary increase, and on the third day of the fifth instar when the

hemocyte density is lower than the molting stage. As a result, the decrease in the hemocyte density after the fourth molting stage was inhibited by injection of the glycoside hydrolase or neuraminidase, and the hemocyte density was rapidly increased within several hours after injection on the third day of the fifth instar larva (Fig. 6). These findings suggested that the silkworm hemocytes were adhering with N-glycan containing sialic acid, like human leukocytes, which may play an important role in the alteration of the hemocyte density. As observed by injection of glycoside hydrolase or neuraminidase, the hemocyte adhesion was inhibited by injecting sialic acid or fucose into the body cavity, suggesting that there were sialic residue or fucose in the terminal of the sugar chains relating to the hemocyte adhesion. In other words, it was the homing phenomenon in insects.

These findings suggested that the hemocyte density fluctuation in *B. mori* was regulated by the attachment and detachment of hemocytes to organs in the body cavity via selectin-ligands, such as sialyl Lewis during larval-larval ecdysis, GR homing, and the decrease of hemocyte density in during larval-pupal ecdysis was mainly caused by the apoptosis of GRs.

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