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Flow Cytometric Analysis of the Effect of Silkworm Hemolymph on the Baculovirus-Induced Insect Cell Apoptosis

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Abstract The effect of silkworm hemolymph on the inhibition of baculovirus-induced insect cell apoptosis was quantitatively investigated using a flow cytometric analysis. *Spodoptera frugiperda* (Sf9) cell and *Autographa californica* nuclear polyhedrous virus (AcNPV) were used as a model for insect cell and baculovirus in this study, respectively. Compared with a mammalian cell cycle, the fraction of G1 cells was relatively small in the Sf9 cell cycle. Silkworm hemolymph did not affect the Sf9 cell-cycle distribution before the baculovirus infection. However, the fraction of cells which are not in the sub-G1 phase remained at a high level for 3 days after the infection in the medium without silkworm hemolymph, while it remained at a high level for 7 days after the infection in the medium supplemented with silkworm hemolymph. The fractions of apoptotic cells in the sub-G1 phase were 4.7%, and 4 days after infection, 22.7%, in the media with and without silkworm hemolymph, respectively.

Key words: Flow cytometric analysis, silkworm hemolymph, apoptosis, insect cell, baculovirus

The insect cell-baculovirus system has been used for the production of recombinant proteins [1, 16]. This system offers several advantages, including a high expression owing to a strong polyhedrin promoter, the production of functionally and immunogenetically active recombinant proteins due to proper post-translational modifications, and the nonpathogenicity of the baculovirus to vertebrates and plants [4]. To maximize the production of recombinant proteins, a high density culture of insect cells is important prior to the viral infection [12, 13, 14], and a high expression of the cloned gene is essential after the infection [15]. Recombinant gene expression begins at approximately 1 day post-infection and continues until the host cells die. Therefore, host cell viability and longevity after the baculovirus infection are important for replication of the baculovirus DNA containing a recombinant gene and for expression of the cloned gene.

The baculovirus-induced cell death was found to be apoptosis, which is a morphologically and biochemically defined type of programmed cell death [3]. *Spodoptera frugiperda* and the *Autographa californica* nuclear polyhedrosis virus (AcNPV) have been widely used as the model insect cell and baculovirus, respectively, for studying the baculovirus regulation of host apoptosis. A specific gene product, P35, which prevents apoptotic response, was identified in AcNPV and in the silkworm baculovirus *Bombyx mori* nuclear polyhedrosis virus (BmNPV) [3, 8].

Silkworm hemolymph has been effectively used for the culture of an insect cell-baculovirus system. It was used as a substitute for fetal bovine serum (FBS) [6, 9, 10]. FBS concentration in the medium could be reduced to 1% without any decrease in cell growth rate, and the cell concentration maximized by adding 5% silkworm hemolymph. Silkworm hemolymph, when added at 5% to the medium, increased the production of recombinant protein by 4.5-fold in an insect cell-baculovirus system [5]. Park and co-workers found that host insect cell longevity increased by supplementing the medium with silkworm hemolymph [5, 20]. There are two forms of cell death mechanisms, apoptosis and necrosis, which have different biochemical and morphological characteristics. Necrosis involves cell swelling, rupture of the plasma membrane, and release of cytoplasmic materials when cells are exposed to certain acute and accidental stimuli. Apoptosis, on the other hand, is a genetically programmed form of cell death mechanism that involves cell shrinkage, nuclear condensation, membrane blebbing, and internucleosomal cleavage of DNA. The apoptosis is a negatively influencing factor in mammalian and insect cell cultures [2, 7, 18, 19]. In a previous study [11, 17, 21], it was reported that silkworm hemolymph inhibited baculovirus-induced insect cell apoptosis, and the

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inhibition of apoptosis was observed qualitatively by agarose gel electrophoresis and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. In this article, the effect of silkworm hemolymph on the inhibition of baculovirus-induced insect cell apoptosis was quantitatively investigated using a flow cytometric analysis.

MATERIALS AND METHODS

Cell Culture and Infection with Baculovirus

Spodoptera frugiperda (Sf9) cells were cultivated in a Grace medium (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 0.35 g/l $NaHCO_3$, and antibiotic-antimycotic (Gibco). The cells were maintained at 28°C. Silkworm hemolymph was added to the medium for the purpose of investigating its effect on the host cell apoptosis. Cells in the late exponential growth phase (4 days in culture) were infected with a recombinant baculovirus. The recombinant baculovirus used was the *Autographa californica* nuclear polyhedrous virus (AcNPV) that produces β-galactosidase under the control of a polyhedrin promoter. For the infection, the medium was aspirated and a virus stock solution was added. A multiplicity of infection (MOI) of 13 was used for all experiments. After incubating for 1 h, the virus solution was replaced with the medium used before the infection.

Collection of Silkworm Hemolymph

The silkworm hemolymph was collected from the fifth instar larvae by clipping the side of an abdominal leg. Silkworms were kindly provided by Dr. Sam-Eun Kim, Department of Sericulture and Entomology, National Institute of Agricultural Science and Technology, Suwon, Korea. The collected hemolymph was heat-treated at 60°C for 30 min, then chilled and centrifuged. The supernatant was filtered with a 0.2-μm membrane filter and used for supplementing the medium.

Flow Cytometric Analysis

Cells were washed twice with phosphate buffered saline (PBS, pH 7.4). Cell pellets were resuspended in PBS, mixed with cold 70% ethanol for fixation, and then stored at −20°C until the FACS analysis. Fixed cells were washed twice with PBS, incubated with 100 μg/ml RNase A at 37°C for 1 h, and stained with 50 μg/ml propidium iodide for 15 min. Measurements of DNA fluorescence in individual cells were carried out using a FACSCalibur flow cytometer (Becton-Dickinson) according to the manufacturer's instructions.

Measurement of Cell Viability

The cell concentration was measured using a hemocytometer and viable cells were detected by the trypan blue exclusion test. Since dead cells absorb trypan blue (Sigma), they can be identified under a light microscope. The cell viability was defined as the ratio of viable cells to the total cells.

RESULTS AND DISCUSSION

The baculovirus-induced insect cell death process is divided into two characteristic phases: a delay phase and a first-order death phase. In the delay phase, the host cell viability is constantly maintained. In the first-order death phase which follows the delay phase, cell viability decreases exponentially. Therefore, the cell longevity is characterized by the delay period. The delay period of Sf9 cell/AcNPV system is usually 3 days in the medium without the silkworm hemolymph. It increases with the concentration of silkworm hemolymph in the medium [20]. This increase is caused by the inhibition of host cell apoptosis, and the extent of apoptosis can be quantitatively analyzed by the flow cytometric method.

Flow cytometric analysis of the cell death and the cell-cycle progression was carried out for the Sf9 cell/AcNPV system. Figure 1 shows the characteristic peaks in the flow cytometric analysis of Sf9 cells. Compared with a mammalian cell cycle, the fraction of G1 cells was relatively small in the Sf9 cell cycle. The sub-G1 fraction represents apoptotic bodies. Therefore, the extent of apoptosis can be quantified by the percentage of sub-G1 area. Figure 2 shows the flow cytometry results of Sf9 cells cultured in the medium with or without the silkworm hemolymph. Fractions of cells in G1, S, and G2/M phases were 22%, 45%, and 33%, respectively, when cells were cultured in the medium without silkworm hemolymph. Nearly the same cell-cycle distribution was observed when cells were cultured in the medium supplemented with 5% silkworm

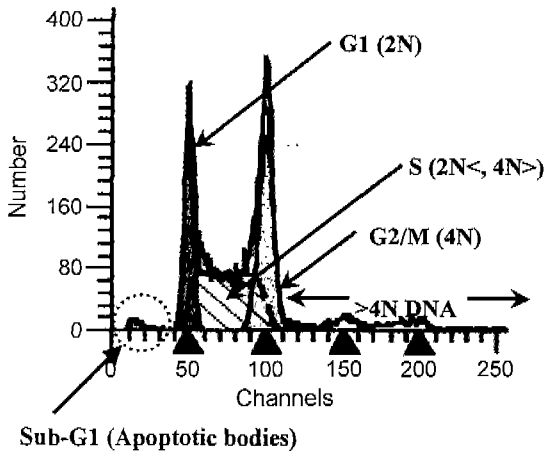

Fig. 1. Characteristic peaks in flow cytometric analysis of Sf9 cells.

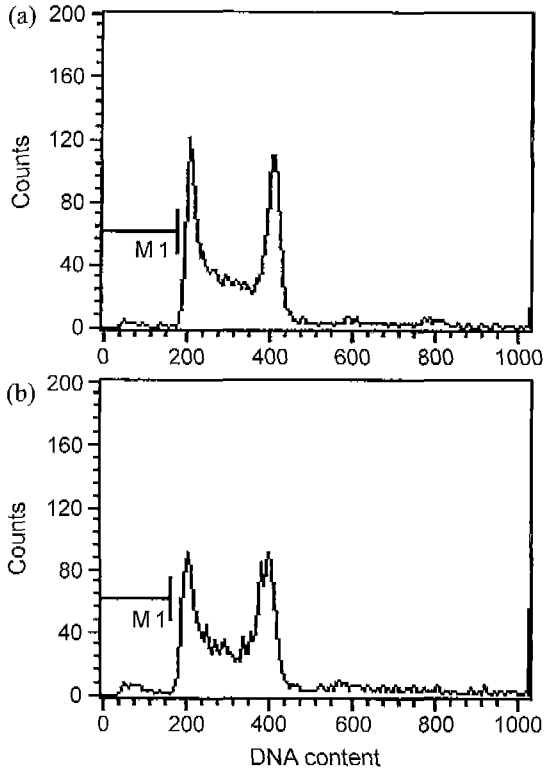

Fig. 2. Flow cytometric analysis of Sf9 cells cultured in the medium with or without silkworm hemolymph.
(a) Supplemented with 10% FBS, (b) supplemented with 5% FBS and 5% silkworm hemolymph.

hemolymph: Those were 20%, 44%, and 36%, respectively. These data indicate that silkworm hemolymph does not affect the Sf9 cell-cycle distribution before the baculovirus infection.

Cells were infected with baculovirus in the late exponential growth phase, and the flow cytometric results after infection are shown in Fig. 3. Within 1 day after the infection, the fraction of cells at G1 and S phases decreased and the cell cycle was arrested at the G2 phase. Consequently, most of the cells were shifted to the G2 phase. This phenomenon is usually observed in the insect cell/AcNPV system, and these G2 phase-arrested cells have more DNA than 4 N. This is due to the viral DNA replication. Silkworm hemolymph was not likely to change the cell-cycle arrest and viral DNA replication. In fact, silkworm hemolymph did not affect the baculovirus production (data not shown). This flow cytometric pattern was maintained until 3 days after the infection. The fraction of apoptotic cells was 22.7% after 4 days of the infection in the medium without the silkworm hemolymph, while it was only 4.7% in the medium supplemented with 5% silkworm hemolymph. The fraction of apoptotic cells was much lower in the medium supplemented with silkworm hemolymph, also after 5 or 6 days of the infection.

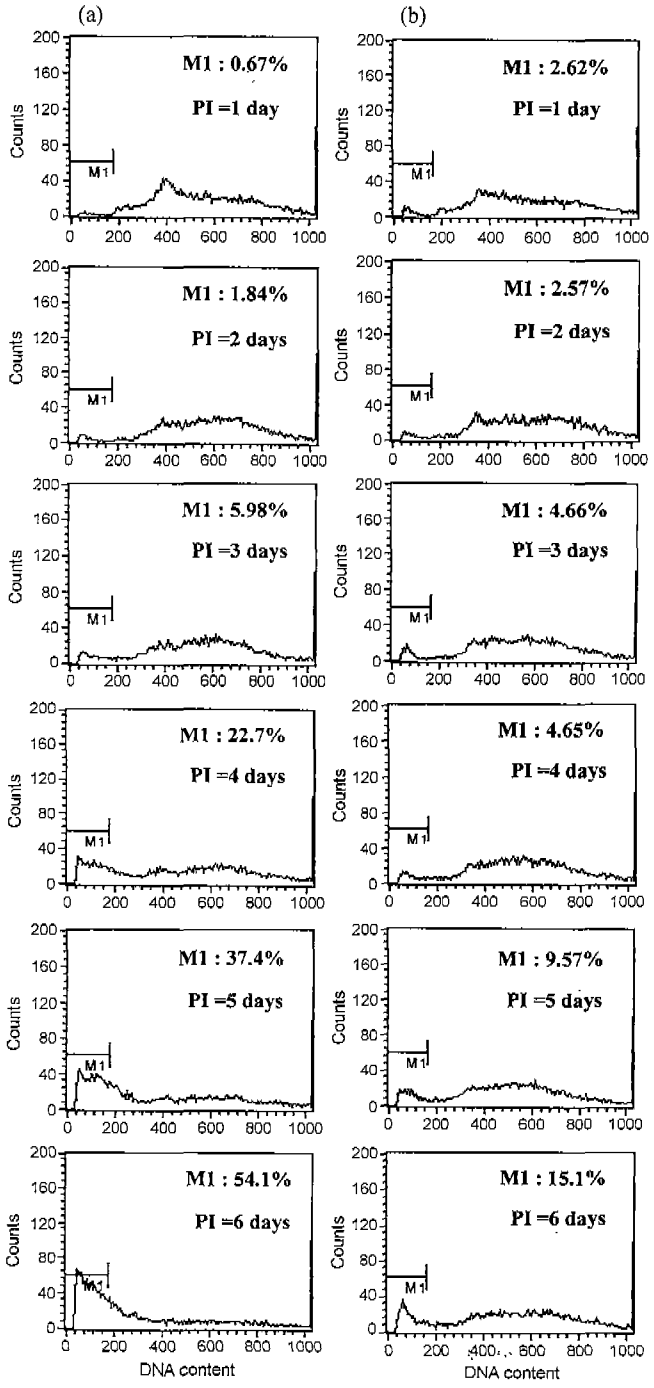

Fig. 3. Effect of silkworm hemolymph on AcNPV-induced Sf9 cell apoptosis.
(a) Medium supplemented with 10% FBS, (b) medium supplemented with 5% FBS and 5% silkworm hemolymph.

Cell viability, which is defined as the ratio of viable cells to total cells, can be measured by the trypan blue exclusion method. Dead cells can be identified under a light microscope in the trypan blue exclusion method, since they absorb trypan blue. However, when cells begin to undergo apoptosis,

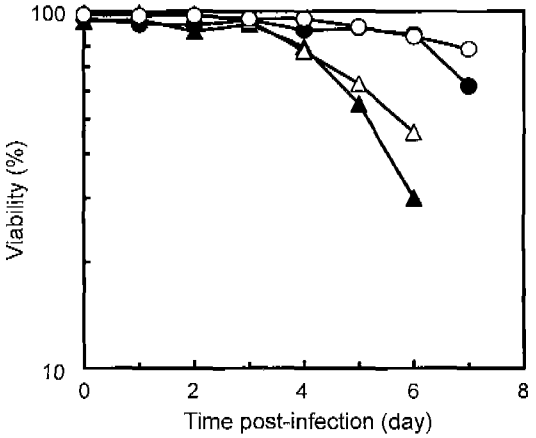

Fig. 4. Effect of silkworm hemolymph on the cell viability measured by the trypan blue exclusion method and the flow cytometric fraction of cells which are not in the sub-G1 phase.
Open symbol: flow cytometric method. Closed symbol: trypan blue exclusion method. Circle: medium supplemented with 5% FBS and 5% silkworm hemolymph. Triangle: medium supplemented with 10% FBS.

it is difficult to count the total cell number because the apoptotic bodies are not stained with trypan blue. Therefore, we averaged the total cell number in the delay phase and used this value as the total cell number in the first-order death phase. The stained cells having a normal size of infected-Sf9 cell were counted as viable cells. The cell viability measured by the trypan blue exclusion method was compared with the flow cytometric fraction of cells which are not in the sub-G1 phase. The results obtained by these two methods were similar, as shown in Fig. 4. The viability remained at a high level for 3 days after the infection without silkworm hemolymph in the medium. With the addition of silkworm hemolymph, the host cell viability remained at a high level for 6 or 7 days after infection. However, in the first-order death phase, the flow cytometric results were slightly higher than the viability measured by the trypan blue exclusion method.

This deviation can be explained by the increased amount of DNA due to G2 phase arrest and viral DNA replication after the infection. The flow cytometric viability might be overestimated. In the flow cytometric method, particles having less amount of DNA than normal cells are counted as apoptotic bodies. However, some cells undergoing apoptosis may have a normal amount of DNA, since the DNA increased already as a result of the G2 phase arrest and viral DNA replication after the infection.

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