Kinetic Effect of Silkworm Hemolymph on the Delayed Host Cell Death in an Insect Cell-Baculovirus System

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The kinetic effect of silkworm hemolymph on host cell viability during a baculovirus-induced insect cell death process was investigated. Host cell viability after viral infection is important for replication of the baculovirus DNA containing a recombinant gene and expression of the cloned gene. The baculovirus-induced insect cell death process can be divided into a delay phase and a first-order death phase, which are characterized by a delay time (t_d) and a specific death rate (k_d), respectively. For 0–10% silkworm hemolymph in the media, higher concentrations resulted in longer delay times and lower specific death rates. By adding 10% silkworm hemolymph, the delay time increased from 72 to 164 h, and the specific death rate was reduced from $13.8 \times 10^{-3}$ to $6.0 \times 10^{-3}$ h$^{-1}$. In addition, host cell viability correlated with DNA fragmentation, which is the biochemical hallmark of apoptosis. This indicates that the silkworm hemolymph inhibits the baculovirus-induced insect cell apoptosis. However, the silkworm hemolymph did not affect the number of hypothetical targets, which represents host cell susceptibility to the baculovirus. The concentration of fetal bovine serum (FBS) in the medium did not affect the delay time, while lower concentrations of silkworm hemolymph resulted in shorter delay times. This means that the substance which increases the longevity of the host cell is not in the FBS but in the silkworm hemolymph.

Introduction

The insect cell-baculovirus system has been increasingly used for the production of recombinant proteins (1, 2). This system has several advantages, including high expression owing to a strong polyhedrin promoter, production of functionally and immunogenetically active recombinant proteins due to proper post-translational modifications, and nonpathogenicity of the baculoviruses to vertebrates and plants (3). To maximize the production of recombinant proteins, a high-density culture of insect cells is important prior to viral infection (4), and high expression of the cloned gene is essential after the infection (5, 6). Recombinant gene expression begins at approximately 1 day post-infection and continues until the host cells die. Host cell viability decreases with time post-infection during production of the recombinant protein. Host cell viability is important for replication of the baculovirus DNA containing a recombinant gene and 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 (7). A specific gene product, p35, of the baculovirus Autographa californica multiply embedded nuclear polyhedrosis virus (AcMNPV) was identified as being responsible for preventing premature cell death (7), and Kamito et al. (8) identified the p35 gene also in the silkworm baculovirus Bombyx mori nuclear polyhedrosis virus (BmNPV). The p35 gene also accelerates the expression of other virus genes either directly or indirectly (9, 10).

Silkworm hemolymph is the most studied insect hemolymph. Insect hemolymph was used as a culture medium in the early stage of insect tissue culture. On the basis of the chemical analysis of insect hemolymph, a synthetic medium was formulated for insect cell culture (11, 12), but it still had to be supplemented with insect hemolymph (13). However, insect cell medium has been currently supplemented with FBS instead of insect hemolymph ever since FBS was proven to be beneficial for the growth of insect cells. In a previous study (14), we reported that, by supplementing the medium with silkworm hemolymph, we improved the production of recombinant β-galactosidase up to 4.5-fold. We also found that host cell longevity increased by supplementing the medium with silkworm hemolymph. For the higher expression of recombinant proteins, the host cell needs to remain viable longer after the viral infection. In this article, the kinetic effect of silkworm hemolymph on the host cell viability during a baculovirus-induced insect cell death process was investigated.

Materials and Methods

Cell Line and Culture Conditions.

Spodoptera frugiperda (Sf9) cells were cultured at 28 °C in 25 cm$^2$ tissue culture flasks (Falcon) containing 6 mL of Grace’s medium (Gibco) supplemented with 0.35 g/L NaHCO$_3$ and antibiotic-antimicotic (Gibco). Various amounts of silkworm hemolymph and FBS (Hydene) were added to the medium to investigate their effect on the host cell viability. 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 (Division of Genetics, Sericulture & Insect Research).
Institute, Suwon, Korea). The collected hemolymph was heat-treated at 60 °C for 30 min, then chilled and centrifuged (15). The supernatant was used for supplementing the medium.

**Baculovirus and Infection Conditions.** Cells in the late exponential phase (5 days in culture) were infected with recombinant baculovirus Autographa californica nuclear polyhedrosis virus producing β-galactosidase (β-gal-AcNPV) at a multiplicity of infection (MOI) of 13. For the infection, the medium was aspirated and 1 mL of virus stock solution was added. After incubating for 1 h, the virus solution was replaced with the medium used before the infection.

**Analytical Procedures.** Ten or more culture flasks were prepared initially for one set of experiments. After taking a sample every day for the assay of cell concentration, cell viability, and β-galactosidase activity from one culture flask, the flask was discarded and the next one was used for the next sample. Cell concentration was measured using a hemacytometer, and cell viability was determined by the trypan blue exclusion test. DNA fragmentation was measured by electrophoresis in an agarose gel (16). The activity of β-galactosidase was assayed by measuring the hydrolysis of o-nitrophenyl-β-D-galactoside (ONPG) (17). The extracellular activity was determined from the supernatant of the sample after centrifugation. To measure the intracellular activity, the precipitated cells were washed twice with PBS (phosphate buffered saline), and the resuspended cells in PBS were disrupted at 0 °C using a ultrasonic homogenizer (US-150T, Nissei). Cell debris was removed by centrifugation at 4200g for 5 min. The intracellular activity of β-galactosidase was determined from the supernatant of the cell extract.

**Results and Discussion**

Figure 1 shows Sf9 cell growth and baculovirus-induced cell death in Grace’s medium supplemented with 5% FBS. After baculovirus infection, total cell density is maintained at a constant level, whereas viable cell density begins to decrease exponentially 3 days after the infection. The baculovirus-induced insect cell death process can be divided into two characteristic phases: a delay (or constant viability) phase and a first-order death phase (18, 19). After the baculovirus infection, the host cell viability defined by the ratio of viable cells to total cells is constantly maintained in the delay phase, which is characterized by a delay time \( t_d \). In the first-order death phase which follows the delay phase, cell viability decreases exponentially. This phase is characterized by a specific death rate \( k_d' \), which is a rate constant in a first-order kinetic equation, and can be determined from the slope \( k_d' = k_d/2.303 \) as shown in Figure 1.

Viability is constantly maintained at high level for 3 days after infection in the medium supplemented with 5% FBS. Similar results were observed when the medium was replaced with fresh one containing 5% FBS after infection as shown in Figure 2. Higher FBS concentration (10%) also did not increase the delay time. However, the addition of silkworm hemolymph increased the longevity of host cell. This indicates that FBS concentration or other medium components do not affect host cell longevity, but silkworm hemolymph increases it.

**Kinetic Effect of Silkworm Hemolymph on Host Cell Viability.** To investigate the effect of silkworm hemolymph on host cell viability, we added various amounts of silkworm hemolymph to Grace’s media supplemented with 5% FBS and adapted cells to growth in each medium for more than three months through an adaptation process (15). The adapted cells were grown in 25 cm² culture flasks containing each medium and were infected with the recombinant baculovirus when the cell density reached about 1.5 × 10⁶ cells cm⁻² in the middle of the exponential growth phase. Figure 3 shows the effect of silkworm hemolymph on the host cell viability after the viral infection. Higher concentrations of silkworm hemolymph result in increased longevity of the host cell. The delay times and specific death rates at various concentrations of silkworm hemolymph are listed in Table 1. The delay time increases and the specific death rate decreases as the silkworm hemolymph concentration is higher.

Viability is constantly maintained at a high level for about 7 days after infection in the medium supplemented with 10% silkworm hemolymph, while host cells begin to die 3 days after infection in the medium without hemolymph. The delay time increases from 3 to 4.9 days by adding even 1% silkworm hemolymph. The specific death rate \( k_d' \) decreases gradually from 13.8 × 10⁻³ to 6.0 × 10⁻³ h⁻¹ with the concentration of hemolymph in the medium. By adding 10% silkworm hemolymph, the
specific death rate was reduced to 1/2.3 of that without silkworm hemolymph, while the delay time increased 2.3-fold. Table 1 shows that \( t_d \) is nearly constant for every concentration of silkworm hemolymph. This result can be interpreted by the \( n \)-target inactivation model which was originally derived to explain the survival rate of cells upon irradiation (20, 21, 22) and applied to the baculovirus-induced insect cell death (18).

**Extrapolation Number.** Viability (\( V \)) is expressed in the following equation by the \( n \)-target inactivation model. It assumes that each cell has \( n \) inactivation sites and that every site must be hit for the cell to be inactivated.

\[
\ln V = \ln(nV_0) - k_d t_d
\]  

(1)

where \( V_0 \) is an initial viability. The number of inactivation targets \( n \) can be determined by extrapolating the straight line in the first-order death phase to the viability axis as shown in Figure 4. The original meaning of the \( n \) was the number of inactivation targets on the cell; however, “extrapolation number” has been proposed to be used as a term for the \( n \) since the model is a highly simplified one (20). The extrapolation number is considered as a measure of the virus-host interaction (18). Figure 4 shows that the extrapolation numbers are the same \( (n = 2.7) \) at the various concentrations of silkworm hemolymph. This indicates that the silkworm hemolymph does not affect the number of hypothetical targets representing a host cell susceptibility to the baculovirus.

This equation represents that \( k_d t_d \) remains constant if \( n \) is constant. Therefore, the result that \( k_d t_d \) remains the same in every concentration of silkworm hemolymph in Table 1 means that \( n \) remains constant for these cases. A more detailed mathematical model for the mechanistic steps in infection such as attachment, internalization, endosomal sorting, endosomal fusion, and nuclear accumulation was developed (23).

**Inhibition of Apoptosis by Silkworm Hemolymph.**

The baculovirus-induced insect cell death was found to be apoptosis (7), and cells undergoing apoptosis activate an endonuclease that cleaves DNA between nucleosomes to give fragments. This DNA digestion is the biochemical hallmark of apoptosis. We investigated whether silkworm hemolymph inhibits the DNA fragmentation in Sf9 cells infected with baculovirus. Figure 5 shows the results when cells were cultured in the medium supplemented with 5% FBS. The DNA fragmentation was not observed during the first 3 days after infection, while it occurred from the fourth day. When 5% silkworm was added to the 5% FBS medium, the DNA fragmentation began to be detected on the seventh day after infection as shown in Figure 6. The time when the DNA digestion begins corresponds to the time when viability begins to decrease in each case. These results represent that the silkworm hemolymph inhibits the baculovirus-induced insect cell apoptosis.

A specific gene product, p35, which prevents the apoptotic response, was identified in Autographa californica multiply embedded nuclear polyhedrosis virus (AcMNPV) and silkworm baculovirus Bombyx mori nuclear polyhedrosis virus (BmNPV) (7, 8). Some other genes are also known to regulate apoptosis in cells of the mammalian immune system. The proto-oncogene bcl-2

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**Table 1. Effect of Silkworm Hemolymph on Kinetic Parameters**

<table>
<thead>
<tr>
<th>silkworm hemolymph (%)</th>
<th>( t_d ) (h)</th>
<th>( k_d (10^{-3} \text{ h}^{-1}) )</th>
<th>( t_d k_d )</th>
<th>( n )</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>72</td>
<td>13.8</td>
<td>0.99</td>
<td>2.7</td>
</tr>
<tr>
<td>1</td>
<td>117</td>
<td>8.5</td>
<td>0.99</td>
<td>2.7</td>
</tr>
<tr>
<td>3</td>
<td>125</td>
<td>8.0</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>149</td>
<td>6.7</td>
<td>1.0</td>
<td>2.7</td>
</tr>
<tr>
<td>10</td>
<td>164</td>
<td>6.0</td>
<td>0.98</td>
<td>2.7</td>
</tr>
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</table>

Figure 3. Effect of silkworm hemolymph on host cell viability in the medium supplemented with 5% FBS; (●) 0% hemolymph, (■) 1% hemolymph, (◆) 3% hemolymph, (▲) 5% hemolymph, (▼) 10% hemolymph.

Table 1. Effect of Silkworm Hemolymph on Kinetic Parameters

\[
k_d = \frac{\ln(nV_0) - \ln V_o}{t_d}
\]  

(2)

\[
k_d t_d = \ln n
\]  

(3)

Figure 4. Extrapolation of straight lines in the first-order death phase: (●) 0% hemolymph, (■) 1% hemolymph, (◆) 3% hemolymph, (▲) 5% hemolymph, (▼) 10% hemolymph.
and the Epstein–Barr virus latent gene LMP1 prevent the apoptotic response of B lymphocytes (24, 25) while the tumor-suppressor gene encoding p53 induces apoptosis in a myeloid leukemic cell line (26). The baculovirus-induced cell apoptosis was significantly delayed by adding silkworm hemolymph to the medium. This means that silkworm hemolymph contains components preventing apoptosis. Further study is required to identify those components.

Location of Recombinant β-Galactosidase. We have reported previously that the production of recombinant β-galactosidase was improved up to 4.5-fold by supplementing the medium with silkworm hemolymph (14). The β-galactosidase produced in the cell is released into a medium as the cell viability decreases. Figure 6 shows the percentage of β-galactosidase released into the medium at various concentrations of silkworm hemolymph. For the higher concentration of silkworm hemolymph which gives longer $t_d$ and lower $k_d$, more β-galactosidase remains inside the cell. The released β-galactosidase is less than 30% in the delay phase ($t < t_d$) in every case. This means that the recombinant protein remains inside the cell longer in the silkworm hemolymph medium since high viability is maintained longer.

Kinetic Effect of FBS on Host Cell Viability. Various amounts of FBS were added to the Grace’s media supplemented with 5% silkworm hemolymph to investigate the effect of FBS on the host cell viability. The adapted cells in each medium were infected with the recombinant baculovirus by the same method as described earlier. Figure 7 shows that the delay times are almost same regardless of FBS concentration; it does not decrease at lower concentrations of FBS, whereas lower concentrations of silkworm hemolymph resulted in shorter delay times. As shown in Figure 2, the delay time was the same in 5% FBS and 10% FBS media. These results show that the substance that increases the longevity of the host cell is not in the FBS but in the silkworm hemolymph. The extrapolation number can be obtained by the same method as in Figure 4. Unlike the case of silkworm hemolymph, the extrapolation numbers are not the same at different FBS concentrations.

The delay times, specific death rates, and extrapolation numbers at various concentrations of FBS are listed in Table 2. The specific death rate and extrapolation number increase as the FBS concentration decreases in the range of 5–2%. In contrast to these results, the specific death rate and extrapolation number are rela-
Table 2. Effect of FBS on Kinetic Parameters

<table>
<thead>
<tr>
<th>FBS (%)</th>
<th>t₀ (h)</th>
<th>k₀ (10⁻³ h⁻¹)</th>
<th>n</th>
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<tbody>
<tr>
<td>1</td>
<td>154</td>
<td>14.8</td>
<td>9.9</td>
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<tr>
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<td>160</td>
<td>28.8</td>
<td>101.3</td>
</tr>
<tr>
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<td>155</td>
<td>20.2</td>
<td>22.0</td>
</tr>
<tr>
<td>5</td>
<td>149</td>
<td>6.7</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Conclusion

We showed the kinetic effect of silkworm hemolymph on the host cell viability during a baculovirus-induced insect cell death process. The host cell longevity increased more than twice from t₀ = 3 days to t₀ = 7 days by adding 10% silkworm hemolymph to the medium, while a host cell susceptibility to the baculovirus remains the same. The increased host cell longevity favors the longer production of recombinant proteins, which results in the higher productivity. Silkworm hemolymph enables host cells to produce recombinant β-galactosidase for a longer period by increasing the longevity of the cells, whereas the production stops earlier without silkworm hemolymph (14). The kinetic study of the baculovirus-induced cell death delayed by silkworm hemolymph is important not only for the optimization of recombinant protein production but also for better understanding of a regulation mechanism of cell death. The baculovirus-induced insect cell death was found to be apoptosis (7); our results showed that the silkworm hemolymph inhibits the baculovirus-induced insect cell death. Cell viability correlated with DNA fragmentation, which is the biochemical hallmark of apoptosis. A more detailed assay for the apoptosis in this system is in progress. The kinetic analysis of baculovirus-induced cell death inhibited by silkworm hemolymph shown in this article may be useful for better understanding of insect cell apoptosis.

Acknowledgment

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References and Notes


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