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Mitochondria-Targeted Apoptosis in Human Cytomegalovirus-Infected Cells

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Mitochondria often play central roles in apoptotic pathways, and disruption of the mitochondrial transmembrane potential (ΔΨm) has been observed in various cells undergoing apoptosis. Human cytomegalovirus (HCMV) infection induces apoptosis in permissive cells; however, investigations of mitochondria-targeted apoptosis in HCMV-infected human foreskin fibroblast (HFF) cells have been limited. Here, we investigated the mitochondrial apoptosis pathway in HCMV-infected HFF cells. Flow cytometry analysis using JC-1 revealed that HCMV infection induces disruption of ΔΨm in HFF cells when administered 24 h post-infection (hpi), and this disruption was maximized at 48 hpi. Moreover, cytochrome *c*, normally a mitochondrial inner membrane protein, was detected in cytoplasmic extracts of HCMV-infected cells, but not mock-infected cells, by western blot analysis at 24 hpi. A caspase activity assay based on fluorescence spectrophotometry using a fluorogenic substrate revealed an increase in caspase-3 activity at 48 hpi in HCMV-infected cells. Caspase-8 activity was increased at 72 hpi in HCMV-infected cells. These results imply that HCMV infection induces mitochondria-mediated apoptosis in HFF cells.

Keywords: Apoptosis, caspase, cytomegalovirus, mitochondria

Introduction

Apoptosis, or programmed cell death, is generally characterized by distinct morphological characteristics such as cell shrinkage, convolution, pyknosis, and energy-dependent biochemical mechanisms [1, 12, 36]. Apoptosis occurs normally as a homeostatic mechanism during development and aging [11]. Apoptosis also occurs as a defense mechanism during immune reactions or when cells are damaged by diseases or infectious agents [23], including virus infections. Virus-infected cells choose to kill themselves in order to curtail the release of progeny virus and thereby protect neighboring cells from second-round virus infections. Naturally, many viruses develop diverse mechanisms to modulate host-cell apoptosis. Viruses may interfere with either the highly conserved effector mechanisms of apoptosis or specific regulatory mechanisms [22, 29, 35].

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen with a mostly asymptomatic clinical outcome. HCMV, like other members of the Herpesviridae, is capable of establishing life-long persistent infections, and viral inhibition of apoptosis has been proposed as one of the main mechanisms allowing this persistence [2, 8, 16, 24, 37]. Inhibition of apoptosis by HCMV is thought to be mediated by HCMV-encoded immediate early (IE) genes such as IE1, IE2, viral inhibitor of caspase-8-induced apoptosis (vICA) [34, 37], and mitochondria-targeting viral mitochondria-localized inhibitor of apoptosis (vMIA) [15, 17, 33].

On the other hand, virus-induced apoptosis is involved in both the disease process and transmission of the virus [25, 29]. Viral transmission is enhanced by inhibiting apoptosis in the early stage of virus proliferation, since this allows complete progeny production. In the case of HCMV, some reports have indicated that apoptosis is induced during corneitis in AIDS patients as well as in the rejection

of transplanted organs [9, 19]. Pleskoff *et al*. [26] proposed that HCMV US28, a CC chemokine receptor, is related to apoptosis. Although the mechanism of apoptosis induction by HCMV US28 has not been clearly determined, it does involve the activation of caspases. Moreover, HCMV induces mitochondria fragmentation in nonsusceptible cells like HeLa and NIH3T3 cells [30], and this presents the possibility that mitochondria contribute to the process of HCMV-induced apoptosis during the late stage of infection in susceptible cells. The present study presents experimental evidence supporting the involvement of mitochondria in the process of HCMV-induced apoptosis in susceptible cells.

Materials and Methods

Cell Culture

In this study, HCMV-susceptible human foreskin fibroblast (HFF) cells (kindly provided by the Waterborne Virus Bank in the Republic of Korea (http://knrrb.knrrc.or.kr/english/index.jsp?rrb=wava)) were used. The HFF cells were cultured in Dulbecco's Minimum Essential Medium (DMEM, Gibco BRL, USA) containing 10% fetal bovine serum (FBS; Gibco BRL), 100 μg/ml streptomycin, and 100 U/ml penicillin, and maintained in DMEM containing 2% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin, in an incubator with an atmosphere of 5% CO_2 at 37°C.

Virus Proliferation

The HCMV TOWNE strain (obtained from Dr. G. Hayward, Johns Hopkins University, Baltimore, MD, USA) was used in this study. To obtain infectious HCMV stocks, 0.01-0.05 plaque forming units (pfu)/cell were inoculated into an HFF cell monolayer cultivated in a 100 mm cell culture dish, as described above. The dish was gently shaken with an interval of 15 min, to facilitate adsorption, and then incubated at 37°C for 1 h. Then, the medium was washed using phosphate-buffered saline (PBS) and replaced with DMEM with 2% FBS. As cytopathic effects were observed 4 to 5 days after infection with the virus, the medium was changed and the virus was harvested 8 to 10 days post-infection (dpi). The HCMV-infected HFF cells were detached with a cell scraper and the supernatant containing the detached HFF cells was moved into a 50 ml centrifuge tube. After centrifugation at 180 ×*g* for 5 min, the supernatant including the HCMV was moved into a new tube and the pellet was resuspended in 1/10 of the entire volume. Then, the pellet was frozen in liquid nitrogen and thawed twice in order to lyse the cells. After centrifuging, the supernatant was added to the pre-supernatant including HCMV and centrifuged at 24,650 ×*g* for 1 h at 4°C. Then, it was resuspended using serum-free DMEM, aliquoted, and stored at −70°C.

Plaque Assay

For virus quantification, the virus was serially diluted in 10-fold increments, and 0.2 ml of 10^{-3} to 10^{-5} diluted virus were inoculated into the HFF cell monolayer in a 6-well cell culture plate. The virus was allowed to adsorb for 1 h, and then the virus inoculum was removed and replaced with the primary overlay medium (DMEM with 2% FBS, 0.37% $NaHCO_3$, 0.25% agarose, 100 μg/ml streptomycin, 100 U/ml penicillin, and 1 μg/ml amphotericin B deoxycholate (Fungizone)). At 7 dpi, a secondary overlay medium, with the same composition as the primary medium, was added to the primary overlay medium. After 7 more days of culture, the cells were fixed for 6 h using 10% formalin diluted by PBS. The formalin and overlay media were then removed, the cells were stained using 0.03% methylene blue, and the number of plaques was counted.

HCMV Infection and Caspase-3 and -8 Inhibitor Treatments

HCMV TOWNE strains were used in this study and the multiplicity of infection (moi) was 1 pfu/cell. The cells were infected by allowing the virus to adsorb to the monolayer of HFF cells for 1 h. After being infected with the virus, the cells were washed using PBS, and DMEM with 2% FBS was then added. To characterize the contributions of caspase-3 and caspase-8 to mitochondria-targeted apoptosis induced by HCMV, the inhibitors Ac-DEVD-CHO (BD Biosciences, USA) and Ac-IETD-CHO (BD Biosciences) were used for caspase-3 and caspase-8, respectively. Each inhibitor was diluted to a final concentration of 25 μM in DMEM with 2% FBS and preprocessed for 1 h before infecting HFF cells, cultivated as a monolayer, with virus. After 1 h of preprocessing, the cells were washed with PBS. Then, the cells were infected with virus using the same method as described above, and were maintained in DMEM with 2% FBS before harvesting.

Propidium Iodide Staining

For DNA fragmentation analysis, propidium iodide (PI) (Molecular Probes, USA) was used. HCMV-infected and mock-infected HFF cells were gently detached, moved to a tube, and centrifuged at 180 ×*g* for 5 min. Then, PBS was added to the pelleted cells and the tube was centrifuged again under the same conditions. The cells were fixed at −20°C for 1 h by adding 200 μl of cold 70% ethanol to the pellet. The fixed cells were then centrifuged at 180 ×*g* for 3 min, the ethanol was removed, and the pellet was washed with PBS. The cells were then resuspended in 300 μl of PI staining solution (PBS, pH 7.4, 0.1% Triton X-100, 0.1 mM EDTA, pH 7.4, 0.05 mg/ml RNase A (50 U/mg), and 50 μg/ml PI) and stained for 1 h at room temperature. The fluorescence of 10^4 cells was then analyzed using a flow cytometer (FACSCalibur, BD Biosciences).

JC-1 Staining

HCMV-infected and mock-infected cells were centrifuged at 1,000 ×*g* for 5 min under the same conditions as detailed above. PBS was then added to wash the pelleted cells. The cells were then resuspended in 100 μl of 0.1% bovine serum albumin-PBS with 2.5 μg/ml of JC-1 (Molecular Probes), a lipophilic cationic probe,

the monomer of which emits at 527 nm, and allowed to hybridize at room temperature for 20 min. After completing the reaction, 10^4 cells resuspended in 100 μl of PBS were analyzed using a flow cytometer.

Western Blotting

To detect cytochrome *c* released into the cytoplasm, the Mitochondria Isolation Kit for Cultured Cells (Pierce, USA) was used to remove the mitochondria and obtain protein extracts from the cytoplasm. Proteins were extracted using NP-40 buffer (150 mM NaCl, 1% NP-40, pH 8.0, and 500 mM Tris) and the protein was quantified using the Bradford method [7]. Then, 20 μg samples of the protein were electrophoresed on 15% SDS-PAGE gels and electrically transferred to a polyvinylidene difluoride membrane (Millipore, USA). The membrane was then gently washed using PBS with 0.1% Tween-20 (PBS-T), and blocked with PBS-T, which included 5% skim milk, at room temperature for 1 h. After completing the blocking process, the membrane was incubated with monoclonal anti-cytochrome *c* antibodies (Oncogene Research Products, USA), diluted 1:2,000, for 1 h. Then, it was washed three times with PBS-T for 10 min each wash, and then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (Promega, USA) diluted 1:5,000 in 5% skim milk/PBS-T. After again washing the membrane three times with PBS-T for 10 min each, a luminescence reaction was generated using an enhanced chemiluminescence system (iNtRON Biotechnology, Korea), and the membrane was exposed to X-ray film to obtain images.

Caspase Activity Analysis

After detaching the cells and washing them with PBS, 1×10^6 cells were counted and centrifuged. The pelleted cells were lysed by adding 50 ml of cell lysis buffer (BD Biosciences; 10 mM Tris-HCl, 27 mM KCl, pH 7.5, 100 mM $NaH_2PO_4/NaHPO_4$, 130 mM NaCl, 1% Triton X-100, and 10 mM sodium pyrophosphate (NaPPi)) to the pellet, followed by centrifugation at 180 ×*g* for 5 min. Then, the supernatant was removed. To measure caspase-3 activity, 1 ml of HEPES buffer (BD Biosciences; pH 7.5, 40 mM HEPES, 20% glycerol, and 4 mM DTT) was added to 15 μl of Ac-

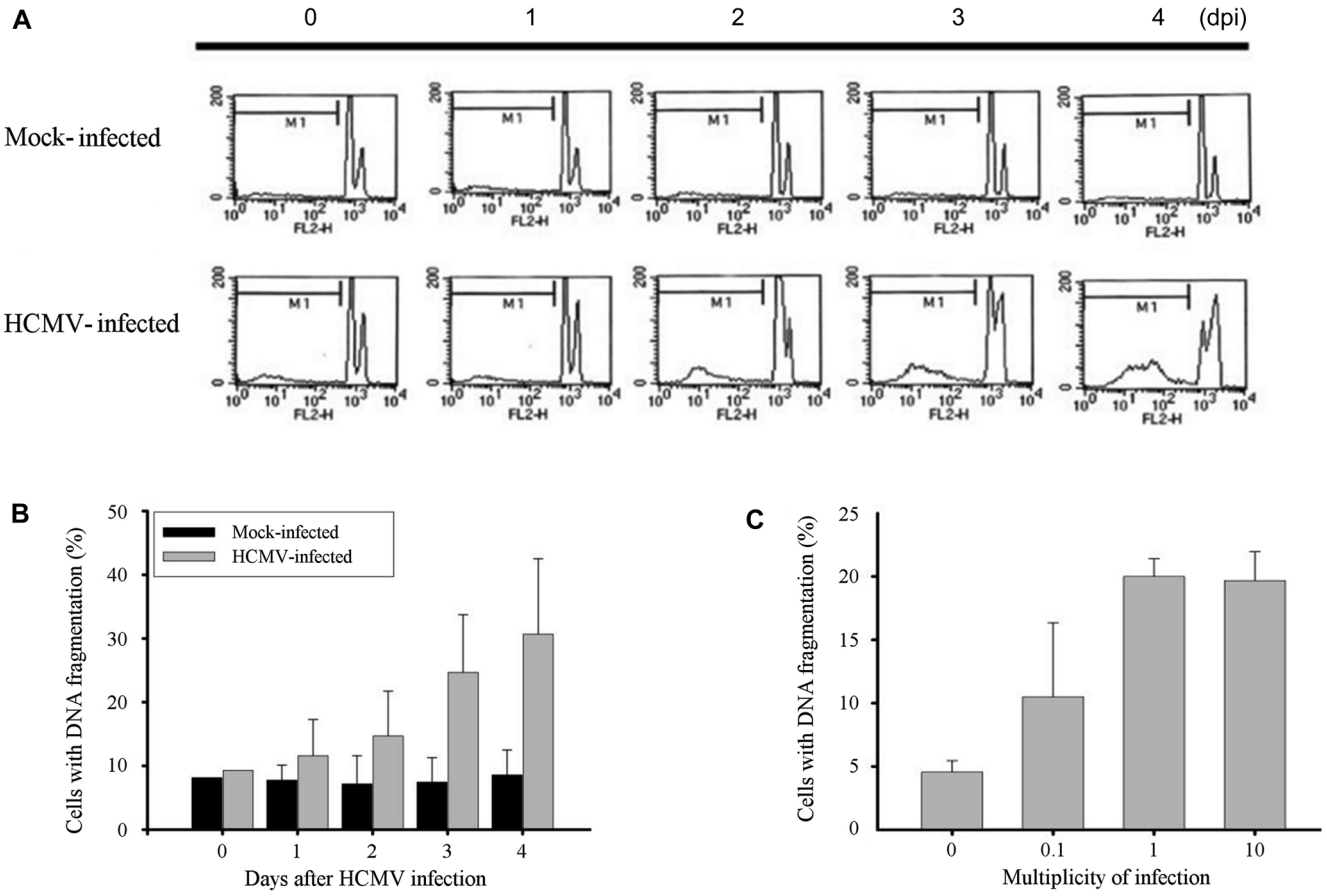

Fig. 1. Increase in fragmented DNA, indicating HCMV-induced apoptosis, in HFF cells.
(**A**, **B**) Time-dependent increase: HFF cells were infected with HCMV (moi = 1 pfu/cell), harvested every 24 h, stained with propidium iodide, and analyzed by flow cytometry. (**C**) Moi-dependent increase: HFF cells were infected with HCMV at various moi, and at 3 days post-infection, cells were stained with propidium iodide and analyzed using a flow cytometer.

DEVD-AMC (BD Biosciences), mixed with 10 µl of cell extracts, and allowed to react at 37°C for 1 h. Then, excitation at 380 nm and emission at 420 nm were measured using a fluorescence spectrophotometer. To measure the activity of caspase-8, 1 ml of assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (w/v) CHAPS, and 10% sucrose, pH 7.2) was added to 10 µl of Ac-IETD-AFC (BD Biosciences), mixed with 10 µl of cell extracts, and allowed to react at 37°C for 1 h. Then, excitation at 400 nm and emission at 480 nm were measured using a fluorescence spectrophotometer.

Statistical Analysis

The data used for measurement of the activity of caspases were mean values calculated from three independent experiments. The error bars represent standard deviations. Numerical analysis was performed *via* independent sample *t*-tests with SPSS (version 12.0) for Windows. *P*-values below 0.01 were considered statistically significant.

Results

Apoptosis Induction in HFF Cells Upon Infection with HCMV

To test whether HCMV infection induces apoptosis in HFF cells, which are HCMV-permissive, the degree of DNA fragmentation was analyzed using PI stain and flow cytometry. After infecting HFF cells with moi = 1 using HCMV TOWNE, the HCMV-infected cells were harvested every day for 4 days and compared with mock-infected HFF. Section M1 in Fig. 1A shows that infected cells developed more fragmented DNA than mock-infected cells. This increase can be considered an index of apoptosis because DNA fragmentation results from apoptosis. In the mock-infected cells, DNA fragmentation was observed at an almost uniform level of 8%, whereas the HCMV-infected cells showed continuous increases in the number

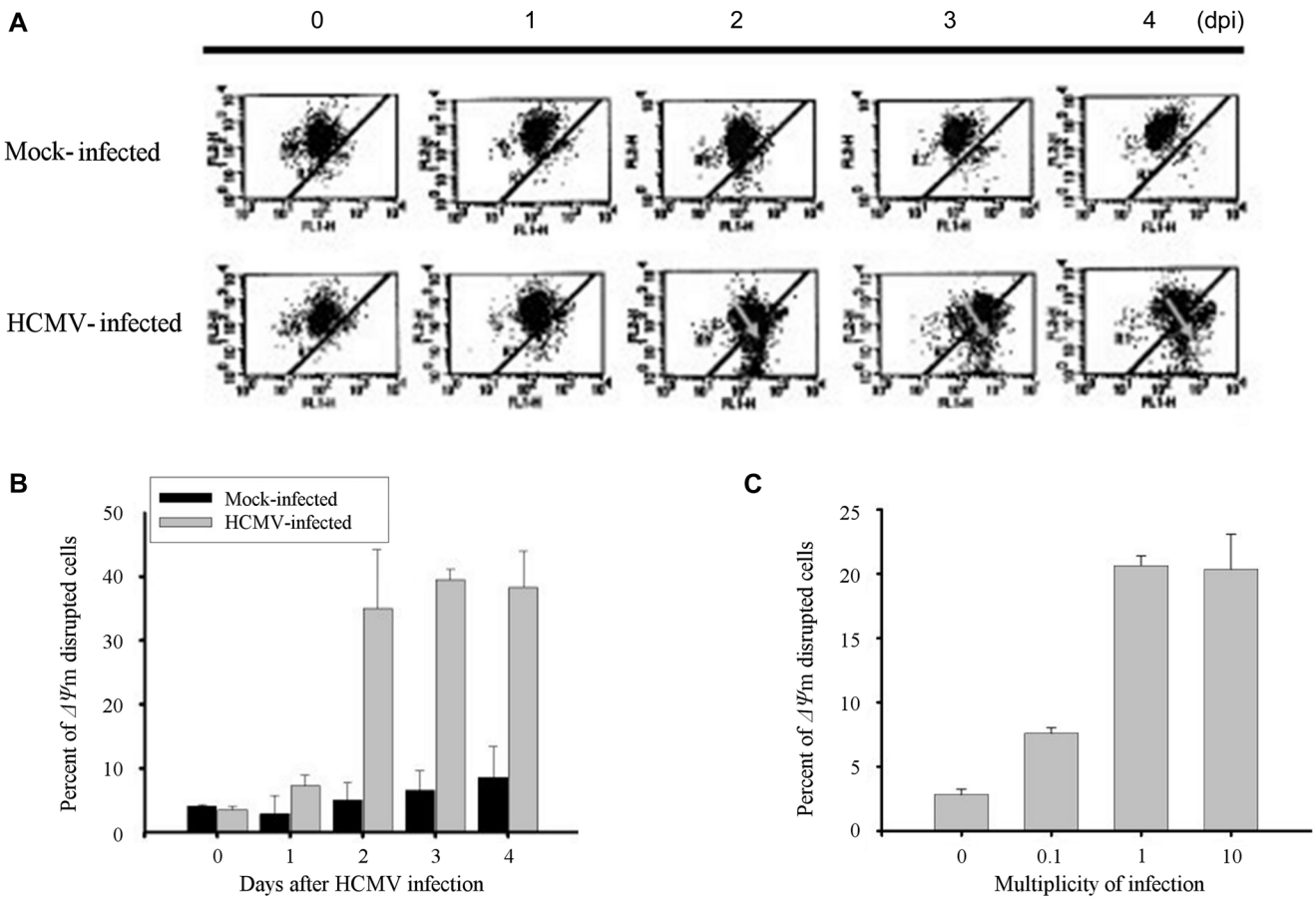

Fig. 2. Flow cytometry analysis of HCMV-induced mitochondrial transmembrane potential disruption (ΔΨm).
(**A**, **B**) HFF cells were infected with HCMV at a moi of 1 pfu/cell and harvested 0, 1, 2, 3, and 4 days post-infection. Cells were treated with JC-1 (2.5 µg/ml) for 20 min at room temperature and analyzed by flow cytometry. (**C**) Moi-dependent increase: HFF cells were infected with HCMV at various moi, and at 3 days post-infection, cells were analyzed for ΔΨm based on JC-1 using a flow cytometer.

of cells showing DNA fragmentation, in a time-dependent manner: 9.3% (right after the infection), 11.6% (1 dpi), 14.7% (2 dpi), 24.7% (3 dpi), and 30.7% (4 dpi) (Fig. 1B). Moreover, in an experiment in which the moi of HCMV infections was varied, the number of cells exhibiting DNA fragmentation was proportionally increased along with increases in the quantity of virus (Fig. 1C). This indicated that infection with HCMV directly affected DNA fragmentation.

Changes in Mitochondrial Transmembrane Potential Caused by HCMV Infection

To verify whether mitochondria play a role in the apoptosis caused by HCMV infection, changes in the mitochondrial transmembrane potential were investigated. In a normal state, JC-1 combines with the mitochondrial lipid membrane, resulting in coagulation of its matrix. In our experiment, this can be detected as red fluorescence (the *y* axis in Fig. 2A). However, if, as part of the apoptosis process, the potential differences are removed owing to the disruption of the transmembrane potential due to increased permeability of the external membrane, JC-1 monomers will be increased and this will give rise to green fluorescence (the *x* axis in Fig. 2A). It is possible to detect changes in the transmembrane potential by examining the ratio between these two fluorescence values and to verify whether apoptosis in these cells takes place *via* the mitochondria [5]. That is, if the mitochondrial transmembrane potential is disrupted, the movement of fluorescence values from the upper left to the lower right of the scatter plot can be detected (the arrow mark in Fig. 2A). In the case of mock-infected HFF cells, no significant increases or decreases in the ratio between these two fluorescence values were detected. However, although the HFF cells infected with HCMV showed no increases in JC-1 monomers with elapsed time, JC-1 aggregates were observed to decrease over time, suggesting depolarization in the mitochondrial membrane of HFF cells due to HCMV (Fig. 2B). In addition, increases in the moi of HCMV caused increases in the polarization of the mitochondrial membrane. This suggested that HCMV plays a direct role by disrupting the mitochondrial transmembrane potential (Fig. 2C).

Discharge of Cytochrome *c* Caused by HCMV Infection

Cytochrome *c* is located on the inner membrane of mitochondria, and cannot be found in the cytoplasm of normal cells. It is discharged from the mitochondrial membrane to the cytoplasm when apoptosis is induced. To determine the amount of cytochrome *c* in the whole cells, the cells were harvested for 4 days after infection, and a western blot assay was conducted. The results indicated no

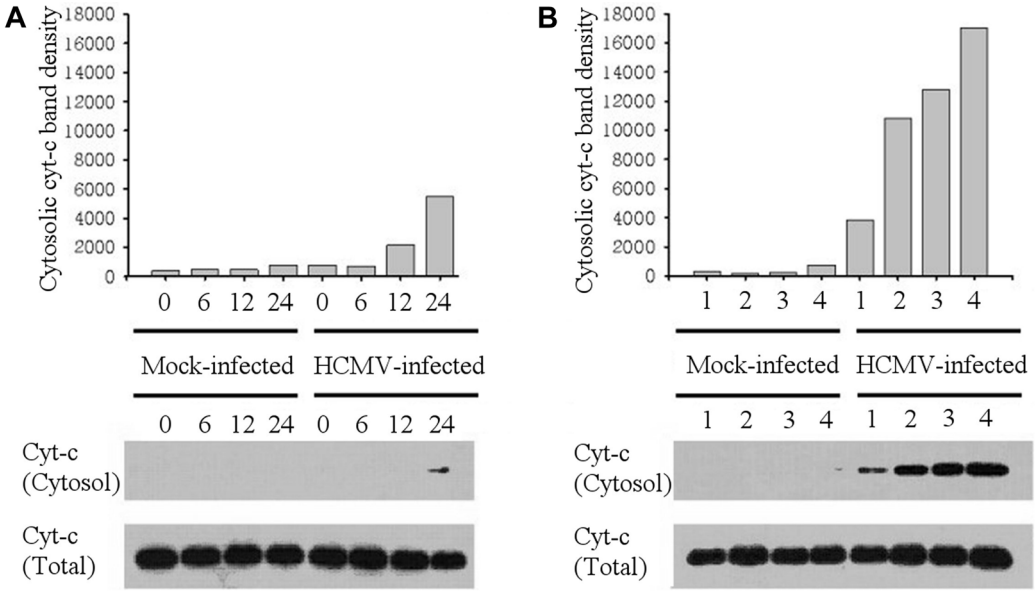

Fig. 3. Western blot analysis of cytosolic cytochrome *c*.

Cells were infected with HCMV at moi = 1, harvested at the indicated times, and the amounts of cytosolic and total cytochrome *c* were measured by western blotting. (**A**) Cells were harvested at 0, 6, 12, and 24 h post-infection and analyzed for cytochrome *c*. (**B**) Cells were harvested at 1, 2, 3, and 4 days post-infection and analyzed for cytochrome *c*. The amount of cytosolic cytochrome *c* was quantified by densitometry of the western blot bands, measured using the Scion Image program.

change in total cytochrome *c* quantity regardless of elapsed time and moi (Fig. 3). Cytochrome *c* was only detected in the cytoplasm in the HCMV-infected cells. Only low levels were detected at 1 dpi, but increased substantially by 2 dpi (Fig. 3B). Then, densitometry was used to quantify the bands in the western blot, and the results were transformed into numbers that are presented as a graph.

Activity of Caspase-3 Caused by HCMV Infections

To test whether HCMV infection leads to caspase-3 activity, Ac-DEVD-AMC was used to measure this activity. Although caspase-3 exists in an inactive stage under normal conditions, it can be activated by a signal and dissolves AMC. As the isolated AMC emits fluorescence, the amount of caspase-3 can be determined by measuring activated fluorescence values. Activated caspase-3 was measured in samples of cells collected for 4 days after infection with HCMV. In the mock-infected cells, there was no caspase-3 activity. However, the activity of caspase-3 was increased in the infected cells and was inhibited by the caspase-3 inhibitor. This showed that caspase-3 activity was increased 2 days after infection and then continued to increase over time (Fig. 4A).

Activity of Caspase-8 Caused by HCMV Infection

Changes in the activity of caspase-8 caused by HCMV infection were measured using Ac-IETD-AFC. Caspase-8 activity was measured using the same methods used to measure caspase-3 activity. More fluorescence was emitted by isolated AFC than by isolated AMC, indicating greater activity by caspase-8 than by caspase-3. There was no increase of caspase-8 activity in the mock-infected cells, and the activity of caspase-8 was increased in the infected cells. In the mock-infected cells, the activity of caspase-8 was inhibited by the caspase-8 inhibitor. Caspase-8 activity began 3 days after infection and then was maintained over 4 days (Fig. 4B).

Measurement of the Activity of Caspases Using Caspase Inhibitors

Ac-DEVD-CHO, an inhibitor of caspase-3, was preprocessed for 1 h and then the cells were infected with HCMV. The cells were then withdrawn 3 days after the infection and the activities of caspase-3 and -8 were measured. It was verified that the activities of both caspase-3 and -8 were inhibited by the inhibitor of caspase-3 (Figs. 5A and 5B). Whereas caspase-8 was significantly inhibited by the caspase-3 inhibitor, the inhibition of caspase-3 by the caspase-3 inhibitor was not statistically significant. The activity of caspase-3 was not affected by Ac-IETD-CHO, which is an inhibitor of caspase-8 (Fig. 5C), but the activity of caspase-8 was inhibited by Ac-IETD-CHO (Fig. 5D). This suggests that caspase-3 is upstream of caspase-8 in this pathway.

Discussion

Because HCMV exhibits high infection rates and can become fatal in immune-deficiency patients, it is important to develop a better understanding of this virus. Apoptosis

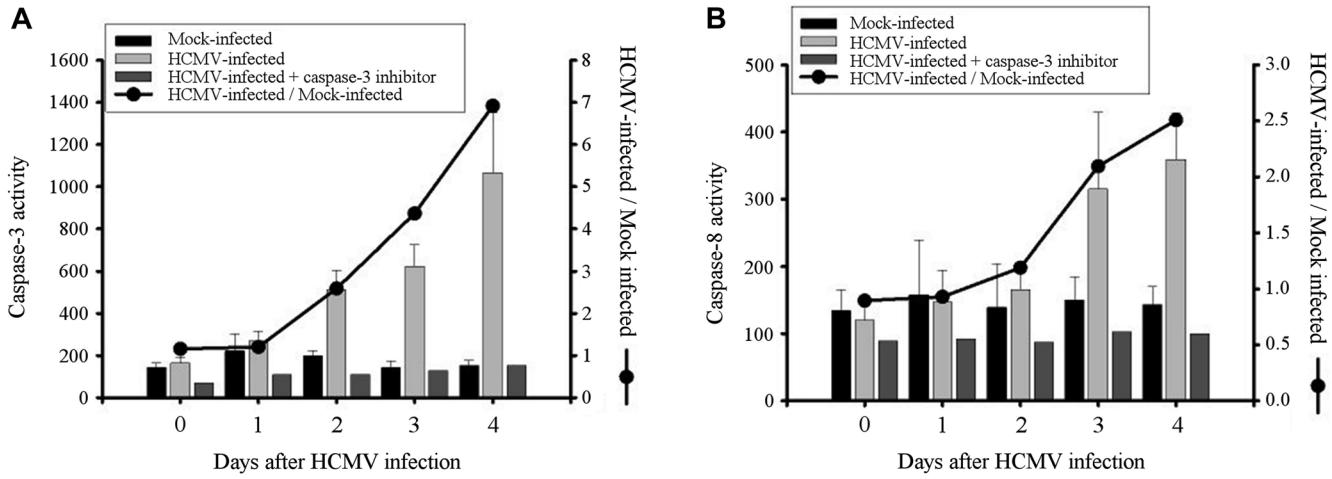

Fig. 4. Increase in caspase-3 and caspase-8 activities in HCMV-infected cells.
Mock- or HCMV-infected cells were harvested and lysed. The cell lysate was incubated with the appropriate fluorogenic substrate, Ac-DEVD-AMC for caspase-3 (**A**) and Ac-IETD-AFC for caspase-8 (**B**), and analyzed using a fluorescence spectrophotometer.

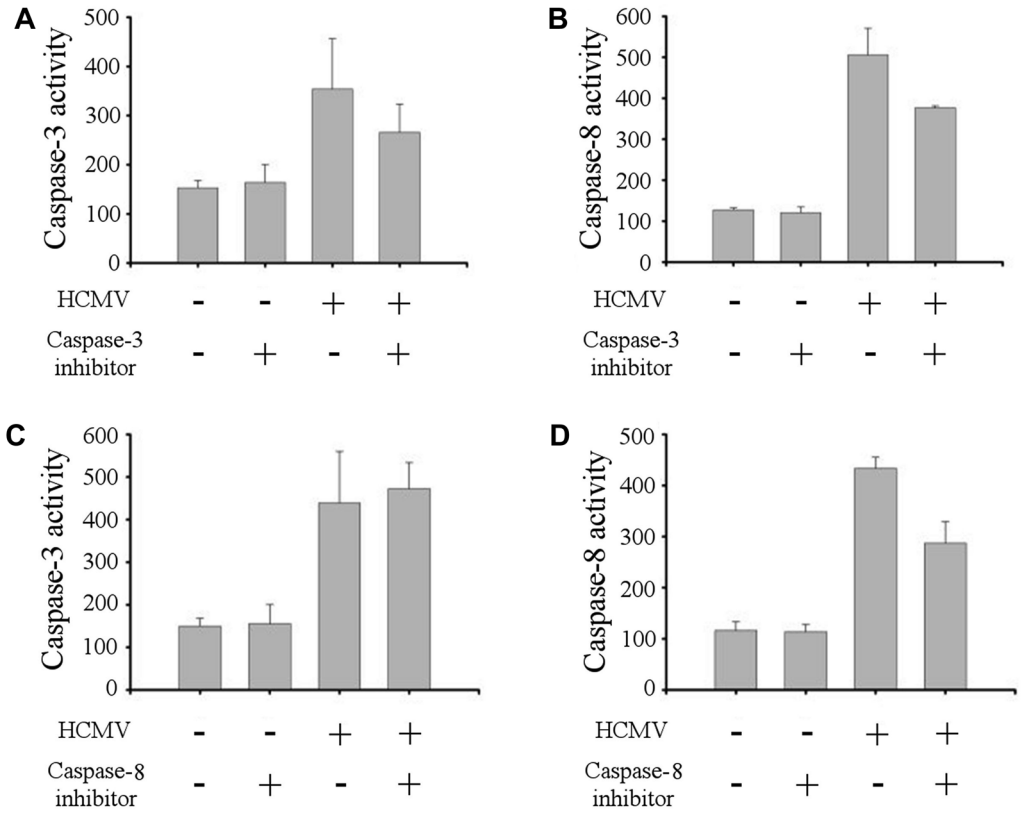

Fig. 5. Inhibition of caspase-3 or caspase-8 activity by the caspase-3 inhibitor, Ac-DEVD-CHO, and the caspase-8 inhibitor, Ac-IETD-CHO.

(**A**) Caspase-3 activity was decreased by the caspase-3 inhibitor. (**B**) Caspase-8 activity was decreased by the caspase-3 inhibitor. (**C**) Caspase-3 activity was not decreased by the caspase-8 inhibitor. (**D**) Caspase-8 activity was decreased by the caspase-8 inhibitor.

is a form of programmed cell death caused by genetic expression in cells and can be induced when cells are infected with viruses or are otherwise damaged. In many studies, it has been reported that HCMV inhibits apoptosis by expressing anti-apoptosis proteins like vICA and vMIA [2, 4, 6, 15, 16, 25, 27]. vICA is expressed from pUL36 and prevents the generation of caspase-8 by combining with pro-caspase-8, leading to the inhibition of apoptosis [21, 33]. vMIA is expressed from pUL37 exon 1 and inhibits the permeabilization of mitochondria through combining with Bax, which facilitates apoptosis [10, 15, 16]. The present study was guided by the hypothesis that cells infected by HCMV inhibit apoptosis at an early stage of infection and then induce apoptosis at later stages, in order to easily discharge virus particles. Experiments were implemented based on this hypothesis.

First, to determine whether apoptosis occurs in HFF cells, a DNA fragmentation assay was performed using the PI stain. DNA fragmentation is a biochemical phenomenon that can identify apoptosis and has been largely used to verify apoptosis [5, 13]. HFF cells infected by HCMV exhibited increased DNA fragmentation, indicating that HCMV-infected cells undergo apoptosis. In a similar result to this, a DNA fragmentation phenomenon caused by an infection of vesicular stomatitis virus (*i.e.*, induction of apoptosis) was reported [20].

The process of apoptosis can be divided into an extrinsic (death receptor) pathway and an intrinsic (mitochondrial) pathway [28]. The extrinsic pathway is initiated *via* a death receptor, when an initiator caspase such as caspase-8 binds to it and activates a death-inducing signaling complex. Activated caspase-8 may activate an effector caspase directly (type I), or indirectly, *via* mitochondria (type II) [27, 31]. The intrinsic pathway induces mitochondrial membrane permeabilization (MMP), and is initiated by various death signals. Caspases are activated through the discharge of a pro-apoptotic factor, such as cytochrome *c*, by MMP to the cytoplasm. The disruption of the mitochondrial transmembrane potential

and the amount of cytochrome *c* were measured over the course of 4 days after infection of HFF cells with HCMV. They were found to reach their maximum values 2 days after infection. This suggests that the pathway for HCMV-induced apoptosis involves mitochondria. The discharged cytochrome *c* forms an apoptosome by combining apf-1 with caspase-9, and this activates caspase-3 [3, 14, 18]. Next, the effect of HCMV infection on the activity of caspase-3 was investigated, and caspase-3 activity was detected 2 days after infection. Because caspase-3 was a representative effector caspase, this clearly showed that the apoptosis was induced by HCMV.

Because the type I extrinsic pathway does not involve mitochondria, it was excluded. Then, to verify which type II pathway, extrinsic or intrinsic, was responsible for HCMV-induced apoptosis, the activity of caspase-8 was measured. The activity of caspase-8 was found to be initiated 3 days after infection with HCMV. Both the disruption of mitochondrial transmembrane potential and the discharge of cytochrome *c*, however, exhibited maximum values just 2 days after infection, and caspase-3 activation was also initiated 2 days after infection. Caspase-8 was activated in the early stage as an initiator caspase. However, activation of caspase-8 is thought to take place after activation of caspase-3. Because a previous report found that the activity of caspase-8 is facilitated by caspase-3 [32], activation was measured after treatment with caspase inhibitors in order to verify whether caspase-8 activity is initiated by a receptor or by caspase-3. As a result, the activity of caspase-8 was inhibited by the inhibitor of caspase-3, but the activity of caspase-3 was not affected by the inhibitor of caspase-8. Thus, caspase-8 appears to be activated through activation of caspase-3 and not *via* the receptor.

The results described above verified that the mitochondrial transmembrane potential can be disrupted by transmitting a death signal to mitochondria upon infection of cells by HCMV. In addition, cytochrome *c* is discharged to the cytoplasm. Then, caspase-3 is activated by combining it with caspase-9. In addition, it was shown that caspase-8 is activated by caspase-3. Thus, the present results indicate that HCMV-induced apoptosis takes place *via* an intrinsic pathway.

Acknowledgments

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