The Journal of Microbiology, September 2002, p.224-229
Vol. 40, No. 3

Induction of Apoptosis in Human Monocytes by Human Cytomegalovirus is Related with Calcium Increase

Myung Sook Moon[1], Gyu Cheol Lee[1], and Chan H. Lee[1,2]*

[1]Division of Life Sciences, and [2]Biotechnology Research Institute, Chungbuk National University, Cheongju, Chungbuk 361-763, Korea

(Received July 16, 2002 / Accepted September 4, 2002)

The effect of human cytomegalovirus (HCMV) on three human monocyte cell lines at different stages of differentiation was investigated. While the viability of HL-60 cells or U-937 cells was not significantly affected by HCMV infection, the viability of THP-1 cells was reduced. Acridine orange/ethidium bromide staining revealed that the reduction of THP-1 cell viability was due to increased apoptotic death following HCMV infection. Apoptosis in HL-60 cells was not affected by HCMV infection, and induction of apoptosis of U-937 cells by HCMV was intermediate between HL-60 and THP-1 cells. Since HL-60 cells are the least differentiated and THP-1 cells are the most differentiated, the induction of apoptosis of human monocytes appears to be related to the degree of cell differentiation. Flow cytometric and confocal microscopic studies using fluorescent calcium indicator Fluo-3 suggested a significant increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) in THP-1 cells undergoing apoptosis by HCMV infection. Again $[Ca^{2+}]_i$ in HCMV-infected HL-60 cells was not critically altered, and that in HCMV-infected U-937 cells was intermediate between THP-1 cells and HL-60 cells. Calcium influx blockers such as verapamil and nifedipine partially reversed HCMV-induced apoptosis in THP-1 cells.

Key words: human cytomegalovirus, apoptosis, monocytes, calcium

Human cytomegalovirus (HCMV) is a ubiquitous human pathogen and is acquired early in life, mostly without any significant clinical symptoms. After primary infection, HCMV remains in the human body and establishes a latent infection. Later in life, with natural or acquired dysfunctions of immunity, reactivation of HCMV from latency may occur and this often causes severe clinical outcomes with high morbidity and mortality (Britt and Alford, 1996). Because of the clinical importance of the latency and reactivation of HCMV, many investigators have tried to identify the cell types involved in HCMV latency *in vivo*. The blood cells, especially peripheral blood mononuclear cell (PBMC) have been suggested to involve in the transmission of latent HCMV (Meyers, 1986; Tegtmeier, 1989; Nelson *et al.*, 1990). Further analyses of PBMC have suggested monocytes as possible reservoirs for HCMV from which reactivation and dissemination occur (Nelson *et al.*, 1990; Taylor-Wiedeman *et al.*, 1991). Reactivation from latency often requires differentiation or maturation of latently-infected cells (Weinshenker *et al.*, 1988; Ibanez *et al.*, 1991; Taylor-Wiedeman *et al.*, 1994).

* To whom correspondence should be addressed.
(Tel) 82-43-261-2304; (Fax) 82-43-273-2451
(E-mail) chlee@cbucc.chungbuk.ac.kr

In response to HCMV infection, host cells develop innate and adaptive defense mechanisms. One of these well-known innate defense mechanisms is the induction of apoptosis in virus-infected cells (Everett and McFadden, 1999; Roulston *et al.*, 1999). In contrast to the adaptive immune defense system, which occurs only after a viral infection and requires the expansion of effector cell clones that specifically recognize foreign antigens, the innate immune defense system is pre-existing and antigen-nonspecific (Flint *et al.*, 2000). Since viruses require live cells for successful replication, the apoptotic death of virus-infected cells would be an efficient means to limit multiplication of viruses in a host.

Recently, HCMV has been reported to induce apoptosis in hematopoietic progenitor MO7e cells (Sindre *et al.*, 2000), which may explain the HCMV-induced suppression of the growth of the CD34+ bone marrow hematopoietic progenitor cells (Holberg-Petersen *et al.*, 1996; Sindre *et al.*, 1996). Since these cells are regarded as the possible site for HCMV latency, an investigation of whether HCMV induces apoptosis in the cells of myeloid/monocyte lineage such as HL-60, U-937 and THP-1 cells was merited. In this study we report that HCMV induced apoptosis in the cells of myeloid/monocyte lineage in a differentiation-dependent manner, and that the increase of intracellular free calcium concentration was involved in

the process of apoptosis.

Materials and Methods

Cells and Virus

Three human cell lines in myeloid/monocyte lineage were used in this study. HL-60 cells are promyeloid progenitor cells and least differentiated among the three cell lines. U-937 and THP-1 cells are promonocyte and monocyte cells, respectively. THP-1 cells are the most differentiated among the three. These cells were grown in RPMI supplemented with 10% fetal bovine serum (FBS), 100 μg/ml penicillin and 100 U/ml streptomycin. For production of infectious virus stocks and quantitative assay of viral infectivity, human foreskin fibroblast (HFF) cells were used. HFF cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Human cytomegalovirus (HCMV) strain Towne stock was prepared by infecting HFF cells at low multiplicity of infection (MOI) and harvesting by three cycles of freezing and thawing. The amount of infectious virus was determined by plaque assay. Titers of HCMV stock prepared in this way ranged between 1 and 7×10^6 plaque forming units (PFU) per ml.

MTT assay

The effect of HCMV on the viability of the monocyte cells was estimated using the microculture tetrazolium (MTT) assay. The assay is based on the reduction of the soluble tetrazolium salt by mitochondria of viable cells. The reduced product, an insoluble colored formazan, is dissolved in dimethyl sulfoxide (DMSO) and measured spectrophotometrically. Under the experimental conditions of this study, the amount of formazan formed was proportional to the number of viable cells. Mock- or HCMV-infected cells were seeded in each well of 96-well microplates. At 24 h, 50 μl of MTT solution (2 mg/ml) was added to each well. After incubation for an additional 4 h at 37°C, plates were centrifuged at 450×g for 5 min. The formazan crystal precipitates were dissolved with 150 μl of DMSO and the absorbance at 540 nm was measured with a spectrophotometer.

Acridine orange and ethidium bromide (AO/EB) staining

The ability of HCMV to induce apoptosis in monocyte cells was investigated by staining cells with acridine orange and ethidium bromide (AO/EB). Cells were mock- infected or infected with HCMV at an MOI of 1 PFU/cell for 1 h at 37°C. The virus inoculum was removed and cells were incubated for 24 h in RPMI1640 medium supplemented with 2% FBS. Twenty-five μl of cell suspension was mixed with 1 μl of AO/EB staining solution (acridine orange 100 μg/ml, ethidium bromide 100 μg/ml) and examined under a fluorescence microscope (BX-50F-3; Olympus Optical Co., Tokyo, Japan).

Fluo-3 staining

The concentration of intracellular free calcium ($[Ca^{2+}]_i$) was measured indirectly by using a cell-permeable fluorescent calcium indicator Fluo-3/AM (Molecular Probes, Eugene, OR, U.S.A.). Cells were mock-infected or infected with HCMV at MOI of 1 PFU/cell. Cells were collected by centrifugation at 24 h after infection, washed with PBS and 5×10^5 cells were stained with 2 μM Fluo-3/AM and 0.02% Pluronic F-127 (Molecular Probes) for 40 min at room temperature. Cells were then washed with PBS containing 0.1% bovine serum albumin (BSA), resuspended in 200 μl BSA/PBS and analyzed with a Flow cytometer (FACS Calibur-S, Beckon-Dickinson, San Jose, CA, U.S.A.) or laser scanning confocal microscope (MRC 1024, Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Results and Discussion

Decrease in THP-1 cell viability by HCMV infection is due to apoptosis

Human cell lines at different stages in the myeloid/monocyte lineage were infected with HCMV. HL-60 cells are the least differentiated, and THP-1 cells are the most differentiated. The differentiation stage of U-937 cells could be located between HL-60 and THP-1 cells. Twenty-four h after HCMV infection, the viability of the cells was determined by MTT assay. As shown in Fig. 1, the viability of THP-1 cells was significantly reduced ($p<0.01$), while the viability of HL-60 or U-937 cells was not significantly altered.

The viability of the cells can be influenced by the balance among cell proliferation, cell death and cell differentiation. In this study we investigated the possibility of

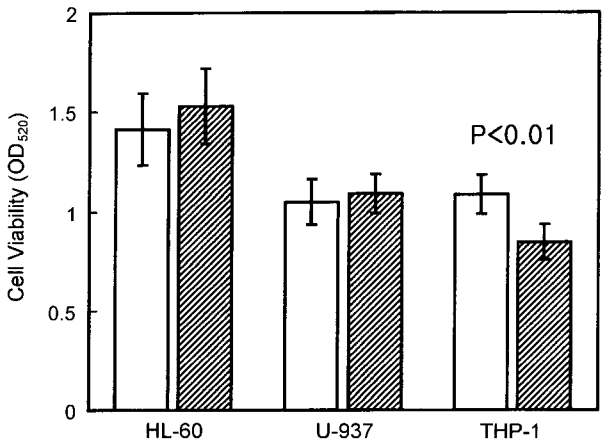

Fig. 1. The effect of HCMV infection on the viability of the three cell lines in human myeloid/monocyte lineage. Cells were infected or mock-infected with HCMV, and the numbers of viable cells were determined by MTT assay. (□), mock-infected cells; (▨)-HCMV-infected cells.

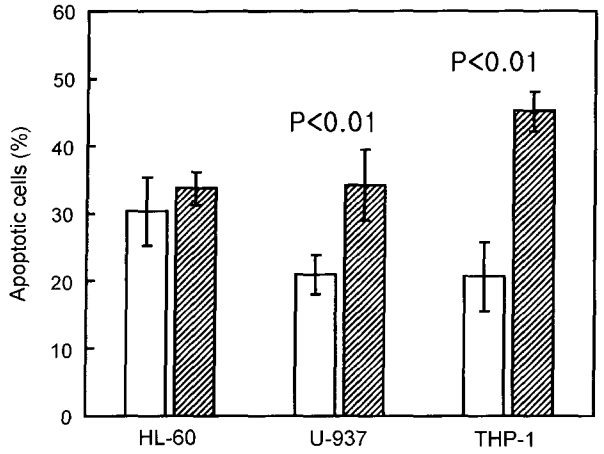

Fig. 2. The effect of HCMV infection on the apoptosis of the three cell lines in human myeloid/monocyte lineages. Cells were infected or mock-infected with HCMV, stained with acridine orange/ethidium bromide, and the numbers of apoptotic cells were counted under fluorescence microscope. (□), mock-infected cells; (▨), HCMV-infected cells.

Table 1. The effect of HCMV infection on the number of early and late apoptotic cells in the three human monocyte cell lines

Cells	Mock-infected	HCMV-infected	p-value[1]
HL-60			
Early apoptotic	22.25±5.99	28.78±7.69	>0.1
Late apoptotic	7.75±4.30	5.06±3.01	>0.1
Total	30.00±5.42	34.02±2.23	>0.1
U-937			
Early apoptotic	15.43±5.77	27.23±4.21	<0.01 (*)
Late apoptotic	4.30±2.84	7.22±3.60	>0.1
Total	19.76±5.64	34.47±4.55	<0.01 (*)
THP-1			
Early apoptotic	13.01±2.55	37.43±5.56	<0.001 (**)
Late apoptotic	6.25±3.01	8.44±2.27	>0.1
Total	19.27±4.72	45.91±4.33	<0.001 (**)

[1]The p values were calculated by paired student t test. Asterisks indicate statistically significant (*, greater than 99% confidence level; **, greater than 99.9% confidence level) differences between mock- and HCMV-infected cells.

apoptotic death of the cells infected with HCMV as the potential cause for the decrease in cell viability. Cells were infected or mock-infected with HCMV and the relative number of each cell line undergoing apoptosis was determined by acridine orange/ethidium bromide (AO/EB) staining. Normally a certain portion (20 to 30%, depending on cell types) of the uninfected cells was undergoing apoptosis when they were maintained in the culture media containing 2% FBS. In HL-60 cells, the percentage of apoptotic cells remained essentially unaltered (30% in mock-infected cells to 34% in HCMV infected cells, $p>0.05$) (Fig. 2). The percentage of apoptotic cells of U-937 cell line increased greatly with statistical significance (20% in mock-infected cells to 34% in HCMV-infected cells; 70% increase, $p<0.01$). The most significant effect by HCMV infection in the number of cells undergoing apoptosis was observed with THP-1 cells. More than 2-fold increase in apoptotic cells (19% in mock-infected cells to 46% in HCMV-infected cells; 119% increase, $p<0.01$) resulted from HCMV infection. Next we wanted to know whether the apoptotic U-937 or THP-1 cells following HCMV infection were at early or late phase of apoptosis. In U-937 and THP-1 cells, the increase in apoptosis following HCMV infection was mostly attributed to the increase in the number of early apoptotic cells (Table 1). These observations could be supported by our findings that some of the early events in apoptosis such as the translocation of phosphatidylserine to the outer leaflet of plasma membrane and the dissipation of mitochondrial transmembrane potential were observed in THP-1 cells infected with HCMV (data not shown).

Apoptosis is the term describing a special type of cell death with characteristic morphologic changes (Kerr *et al.*, 1972). In contrast to necrosis, apoptosis is an active process that requires gene expression. Cells are continuously exposed to internal and external damage and various repair systems are turned on to fix the damage. If the repair systems do not work properly, or the damage is too serious to be repaired, suicide programs are turned on. Cells with irreversible damage, for example by productive infection of virus, kill themselves when they recognize that they have become useless or dangerous to a living system of a higher hierarchical position. These relationships are regarded as examples of the Samurai Law of Biology: "It is better to die than to be wrong" (Skulachev, 2001). In viral infections, apoptosis plays a role in the innate immune defense system (Everett and McFadden, 1999). In contrast to the adaptive immune defense system, which occurs only after a viral infection and requires the expansion of effector cell clones that specifically recognize foreign antigens, the innate immune defense system is pre-existing and antigen-nonspecific. Examples of the innate immune defense system include phagocytosis, production of cytokines, the complement system and apoptosis (Flint *et al.*, 2000). Since viruses require live cells for successful replication, the apoptotic death of virus-infected cells would be an efficient means to limit multiplication of viruses in a host. Although virus-infected cells die due to apoptosis, many more neighboring cells would be protected from secondary viral infections. Thus, the apoptotic death of THP-1 cells infected with HCMV, could be interpreted as a natural means of defense against viral infection.

Then why did HL-60 cells not undergo apoptosis while THP-1 and U-937 cells became apoptotic following HCMV infection? The answer might be because HCMV infection could be a potential threat to THP-1 or U-937 cells but not to HL-60 cells. Previous reports suggested

that HCMV gene expression and replication are differentiation-dependent (Weinshenker *et al.*, 1988; Ibanez *et al.*, 1991; Taylor-Wiedeman *et al.*, 1994; Lee *et al.*, 1999). The lack of permissiveness for HCMV is due to the presence of differentiation-specific negative regulators which disappear upon differentiation to a permissive phenotype (Kothari *et al.*, 1991; Sinclair *et al.*, 1992). Furthermore, it was shown that only the HCMV infected THP-1 cells, but not the HCMV infected HL-60 cells, would support viral gene expression and replication after the treatment of the cells with TPA (Lee *et al.*, 1999). When HL-60 cells were induced to differentiate by treating cells with 12-O-tetradecanoyl-phorbol 13-acetate (TPA), HCMV infection induced an increase in apoptosis (data not shown). Therefore, HCMV-induced apoptosis in the cells of the myeloid/monocyte lineage appears to depend on the degree of cell differentiation. Therefore, HCMV infection could result in more serious consequences in the more differentiated THP-1 cells than in the less differentiated HL-60 cells. In this situation, apoptotic death of virus-infected cells might be the method of choice to protect uninfected naïve cell populations (Everett and McFadden, 1999: Roulston *et al.*, 1999).

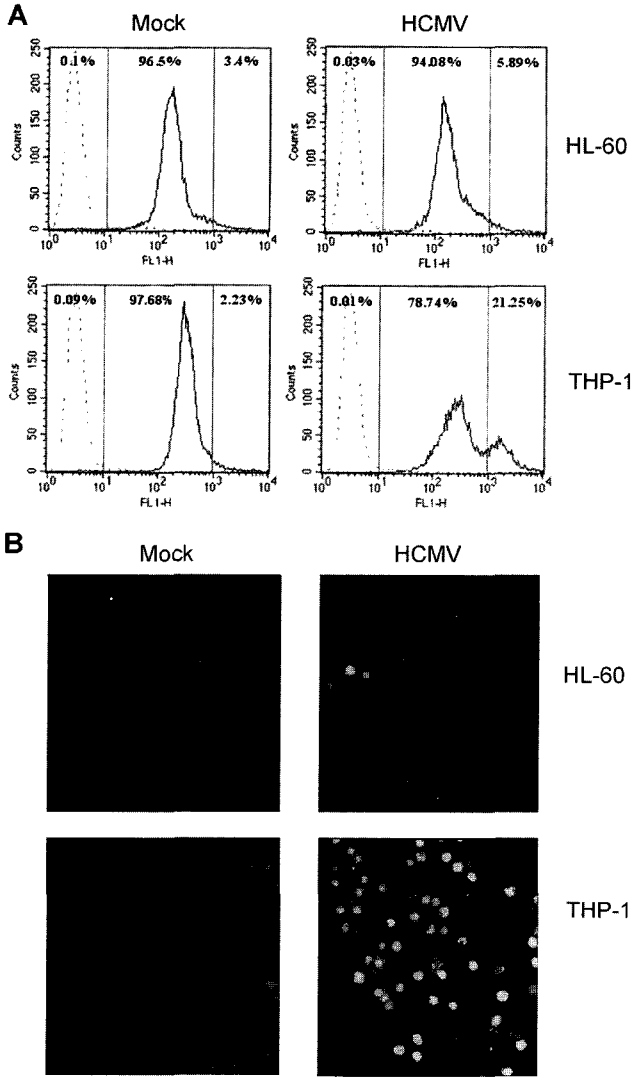

Fig. 3. $[Ca^{2+}]_i$ increase in THP-1 cells infected with HCMV. (A), HL-60 and THP-1 cells were infected with HCMV. Cells were stained with fluorescent calcium probe Fluo-3/AM (2 μM), and analyzed by flow cytometer. (B), HL-60 and THP-1 cells were infected with HCMV. Cells were then loaded with Fluo-3/AM and the fluorescence was observed by confocal laser scanning microscope.

HCMV-induced apoptosis is associated with the increase in intracellular free calcium concentration

One of the early events in apoptosis is the increase in intracellular free calcium concentration ($[Ca^{2+}]_i$) (Yu *et al.*, 2001). Accordingly, it was assumed that HCMV-induced apoptosis might be associated with the increase of $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ was measured indirectly using fluorescent Ca^{2+} indicator fluo-3. The mean fluo-3 fluorescence of HCMV-infected cells relative to the mock-infected cells did not change significantly in HL-60 cells (Fig. 3A). On the other hand, the mean fluorescence increased significantly in THP-1 cells by HCMV infection (from 356 in mock-infected cell to 647 in HCMV-infected cells, Fig. 3A). The cell populations were arbitrarily divided into three fractions with low (M1), medium (M2) and high (M3) fluorescence. The cells in the M1 fraction represent the cells with low $[Ca^{2+}]_i$ at background concentration. The cells in the M3 fraction represent the cells with higher $[Ca^{2+}]_i$ than most of the mock-infected cells (M2 fraction). While the relative proportion of the cells in M1, M2 and M3 fractions in HL-60 cells was essentially unaltered by HCMV infection, the proportion of M3 fraction in THP-1 cells increased significantly by HCMV infection (2.23% to 21.25%). Further studies with a laser scanning confocal microscope revealed that the number of THP-1 cells with higher fluo-3 fluorescence remarkably increased by HCMV infection (Fig. 3B). Relation between the increase of $[Ca^{2+}]_i$ in THP-1 cells following HCMV infection and HCMV-induced apoptosis was investigated by treating THP-1 cells with specific Ca^{2+} influx blockers after HCMV infection. The proportion of apoptotic cells decreased dramatically when the HCMV-infected THP-1 cells were treated with calcium influx blockers such as verapamil or nifedipine (Fig. 4).

Elevation in $[Ca^{2+}]_i$ may mediate apoptosis in many cell types, both at early stages and late stages (Yu *et al.*, 2001). In certain cases, an early increase in $[Ca^{2+}]_i$ may induce the cellular injury that then triggers apoptosis. In HCMV infection, an early increase in $[Ca^{2+}]_i$ was observed and this early increase did not require HCMV gene expression (Nokta *et al.*, 1987; Fortunato *et al.*, 2000). Additionally this increase in $[Ca^{2+}]_i$ may account for the subsequent HCMV gene expression and replication (Kang *et al.*, 1995). Although it is not clear from our study whether HCMV gene expression is required or not for the apop-

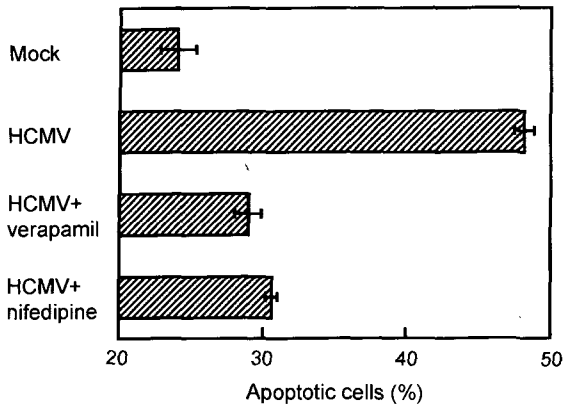

Fig. 4. Reversal of apoptosis in HCMV-infected THP-1 cells by calcium influx blockers. THP-1 cells were infected with HCMV and treated with verapamil (10 μM) or nifedipine (10 μM). The number of apoptotic cells was determined by acridine orange/ethidium bromide staining.

tosis in THP-1 cells, the increase in $[Ca^{2+}]_i$ may correlate with HCMV gene expression since RT-PCR analysis revealed that HCMV UL122 gene transcription was observed in THP-1 cells but not in HL-60 cells (data not shown). The increase in $[Ca^{2+}]_i$ appears to be responsible, at least in part, for the apoptosis in THP-1 cells following HCMV infection since calcium influx blockers such as verapamil and nifedipine blocked HCMV-induced apoptosis in THP-1 cells (Fig. 4). Influx of calcium from the extracellular medium, however, does not seem to be the sole reason for the increase in $[Ca^{2+}]_i$ in apoptotic THP-1 cells. There are several possibilities for the source of the increased $[Ca^{2+}]_i$ in HCMV-infected cells: 1) stimulation of calcium entry from the extracellular medium, 2) stimulation of calcium release from the intracellular calcium sequestering compartment such as endoplasmic reticulum and mitochondria, 3) release of calcium from intracellular calcium-binding proteins, and 4) block of calcium efflux to extracellular medium or intracellular calcium sequestering compartment by a specific calcium pump (Carafoli, 1987; Tsien and Tsien, 1990). In this study we only explored the first possibility and other possibilities are likely since calcium influx blockers did not inhibit HCMV-induced apoptosis completely. Further studies are merited to elucidate the relationship between HCMV-induced apoptosis and calcium.

Acknowledgment

This work was supported by a grant from the Korea Science and Engineering Foundation (Grant No. R01-2000-00072).

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