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HCMV infection attenuates hydrogen peroxide induced endothelial apoptosis – involvement of ERK pathway

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Abstract Human cytomegalovirus (HCMV) exerts anti-apoptotic effect during early stage of infection, which provides HCMV time for propagation. We investigated pathways mediating the resistance to H_2O_2-induced cell death – a self-defense mechanism to remove infected cells. We found that human aortic endothelial cells (HAECs) infected with VHL/E strain of HCMV during first 3 days were resistant to H_2O_2 (0–2 mM) induced apoptosis. This anti-apoptotic effect may be mediated by the upregulation of Bcl-2, an anti-apoptotic protein through the activation pro-survival pathway extracellular signal regulated kinase (ERK). Through this mechanism, HCMV is able to propagate and causes endothelial dysfunction, hence vascular disease.

Keywords: Endothelium; Apoptosis; Hydrogen peroxide; Cytomegalovirus

1. Introduction

Human cytomegalovirus (HCMV) – a ubiquitous β-herpesvirus – has been associated with atherogenesis and coronary restenosis by its infectivity in endothelial and smooth muscle cells [1–3]. It is the most common pathogen affecting more than 50% human population in most parts of the world [4]. While majority of infected individuals are asymptomatic, HCMV infection can cause severe illness in neonates and in immunocompromised individuals, e.g. organ transplant recipients. Understanding the mechanisms mediating HCMV-induced pathological changes has major biological significance to human health.

Apoptosis is a highly regulated self-defense mechanism involving the removal of senescent, abnormal, potentially harmful cells such as virally infected cells from body system. The deregulation of apoptotic mechanism contributes to a variety of diseases. HCMV employs multiple strategies to suppress apoptosis during the early stage of infection in order to create an optimal environment for the viral propagation [5,6]; and to promote apoptosis at the late stage of infection to facilitate viral dissemination [7]. Although the HCMV-mediated anti-apoptotic effect is a well-described phenomenon, the mechanisms for the effect are not clear. Recent studies have shown that HCMV encodes at least two proteins UL36 and UL37 to directly interfere with the apoptotic signaling pathways [8,9]. However, these two molecules cannot satisfactorily explain the anti-apoptotic process involved in HCMV infection to human cells [2,10]; and their involvements in endothelial cells are also not clear.

Endothelial cells undergo apoptosis in response to stress, such as growth factor withdrawal, UV irradiation, chemotherapy and oxidative stress. When the apoptosis is moderate, it can be protective; when it is excessive, it can be harmful and pro-atherogenic. Hydrogen peroxide (H_2O_2) is a secondary messenger in many cells to execute extracellular signals, such as inflammation process. In response to infection, monocytes or macrophages can produce a large amount of H_2O_2, as a part of self-defense mechanisms, to eliminate cells infected with pathogens, possibly by apoptosis induction. Of course, H_2O_2 as a major component of reactive oxygen species (ROS) is also elevated in hypercholesterolemia or inflammation and promotes atherogenesis. With these double-edged sword effects, it is biologically and clinically relevant to investigate whether and how HCMV-infected endothelial cells would respond to H_2O_2-induced apoptotic process. Understanding the mechanisms responsible for these effects will be of great value in appreciating how HCMV infection triggers or accelerates endothelial dysfunction and vascular diseases. In the current study, we investigated the interactions between HCMV infection and H_2O_2 exposure in endothelial apoptosis. We have demonstrated that HCMV-infected HAECs are resistant to H_2O_2-induced apoptosis during the early stage of infection by upregulating Bcl-2 through ERK pathway.

2. Materials and methods

2.1. Reagents and antibodies

Bcl-2 (Cat#2872), phospho-Bcl-2 (Cat#2871), Bcl-xl (Cat #2762), Bax (Cat #2772), Bad (Cat #9292), phospho-p44/42 MAPK (Thr202/Tyr204) (Cat #4377), p44/42 MAPK (Cat #9012), Phospho-Akt (Ser473) (Cat #9271), Akt(Cat #9272) antibodies and MEK1/2 inhibitor U0126 (Cat #9903) were purchased from Cell Signaling (Beverly, MA). Mouse anti-HCMV immediate early antigen and late antigen monoclonal antibody were from Chemicon International, Inc. (Temecula, CA). Hydrogen peroxide 30% solution was purchased from Sigma (St. Louis, MO).

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2.2. Cell culture

Primary human aortic endothelial cells (HAECs) were purchased from Cell Applications, Inc. (San Diego, CA) and cultured in EGM-2 medium (Cambrex, Walkersville, MD) supplemented with 2% fetal bovine serum, hydrocortisone, hFGF, VEGF, R-3IGF, hEGF, GA-1000 and heparin. All experiments were carried out using HAECs in passages 3–8.

2.3. HCMV infection and hydrogen peroxide treatment

The VHL/E (a generous gift from Dr. James Waldman) strain of HCMV was used in the study [11]. For infection, HAECs were grown to confluence and infected with HCMV at 1.0 multiplicity of infection (MOI). Cells were incubated at 37 °C for 3 h for virus absorption. Cells were then washed by PBS and continued the culture in EGM-2 growth medium for 3 days. H_2O_2 was added to the cells at indicated doses and time points. Following the treatment, cells were then washed with PBS for three times and further cultured in EGM-2 growth medium for 16 h before subjected to further analyses.

2.4. Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling (TUNEL) assay and immunocytochemistry staining

The TUNEL assay was performed to identify the apoptotic cell using the in situ cell detection kit (BD Biosciences). In brief, HAECs grown on gelatin-coated coverslips were washed twice by PBS, and fixed by 4% paraformaldehyde solution in PBS for 15 min at room temperature. Coverslips were then washed with PBS and permeabilized in 0.2% Triton X-100/PBS for 10 min. Each coverslip was added 50 μl of the TUNEL reaction mixture and incubated in a dark humidified chamber for 1 h at 37 °C. The reaction was terminated by adding 2× SSC and incubated at room temperature for 15 min. The cells were then blocked with 1% BSA/PBS for 1 h, followed by incubating with anti-HCMV immediate early (IE) antibody (Chemicon) and Texas Red conjugated secondary antibody. The DNA dye DAPI (4′6′ diamidino-2-phenylindole dihydrochloride) was used to label the nuclei at the concentration 0.1 μg/ml for 30 min. The slides were examined with a Leica DMLS Epifluoresence microscope (20 ×–40× magnification). The data were analyzed with the Image-Pro Plus V4.5 software (Media Cybernetics, Inc).

2.5. Caspase-3 activity

The caspase-3 activity was determined using the Caspase-3 Assay Kit (BD Biosciences). Briefly, HAECs were grown in 6-well plates to confluence, infected with HCMV, and treated with H_2O_2 as indicated. For each reaction, HAECs were washed with PBS and re-suspended in cold Cell Lysis Buffer (10 mM Tris–HCl, 10 mM NaH_2PO_4/K_2HPO_4, pH 7.5, 130 mM NaCl, 1% Triton-X 100, and 10 mM sodium pyrophosphate) for 30 min on ice. Five microliter reconstituted caspase-3 fluogenic substrate Ac-DEVD-AMC [*N*-acetyl-Asp-Glu-Val-Asp-AMC (7-anmino-4-methylcoumarin)] was added to each well containing 0.2 ml of the 1× HEPES buffer before 30 μl of the cell lysate was added. The reaction mixtures were incubated at 37 °C for 1 h. The amount of AMC liberated from Ac-DEVC-AMC was measured using ultraviolet spectrofluorometry plate reader with the excitation wavelength of 380 nm and an emission wavelength range of 420–460 nm. Apoptotic cell lysates containing active caspase-3 yielded a considerable emission as compared to non-apoptotic cell lysates. Relative caspase-3 activities were calculated according to the protein concentrations of the cell lysates.

2.6. Mitochondrial membrane potential

The loss of mitochondrial membrane potential is a hallmark for apoptosis. We used JC-1 Assay kit (Invitrogen, Carlsbad, CA) to measure the mitochondrial membrane potential in HCMV- and mock-infected HAECs treated with or without 1 mM H_2O_2 for 3 h. In brief, HAECs were grown in 6-well plates to confluence. Cells were then mock- or HCMV-infected, and treated with or without H_2O_2 as described above. Cells were detached from the plate by short treatment with trypsin, which was inactivated by adding trypsin neutralizing solution (TNS, Cambrex). We then centrifuged the cell suspension at $400 \times g$ for 5 min to remove the supernatant. Cells were resuspended in 0.5 ml 1× JC-1 Reagent solution and incubated at 37 °C in a 5% CO_2 incubator for 15 min. This was followed by a brief centrifugation and the removal of the supernatant. Cells were then washed twice with 2 ml of 1× assay buffer. We resuspended the cell pellet in 0.5 ml of 1× assay buffer; and the JC-1 aggregation in mitochondria was quantified using Flow Cytometry. JC-1 (5,5′6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolycarbocyanine iodide) stains mitochondria bright red in normal cells. A loss in mitochondrial membrane potential prevents JC-1 aggregation; and JC-1 remains as a monomer in the cytoplasm and exhibits green fluorescence.

2.7. Western blot analysis

Cells were lysed in Cell Lysis buffer (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1% Nonidet P-40, 2.4 mM sodium pyrophosphate, 1 mM DTT, 1 mM PMSF 2 mM vanadate and 1× protease inhibitors cocktail from Sigma) by incubation on ice for 1 h. The total protein concentration was determined with the protein assay kit (Bio-Rad Laboratories, Hercules, CA). Fifty micrograms of protein samples was separated on 12% SDS–PAGE gels for 1 h at 80 v, which was then transferred to the nitrocellulose membrane. The membrane was blocked in 5% non-fat powdered milk in TBST (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). The membrane was incubated with the primary antibody at 4 °C overnight, followed by incubation with HRP-conjugated secondary antibody and detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Equal loading was confirmed by the house-keeping control β-actin band.

2.8. RNA isolation and quantitative real-time RT-PCR

Mock- and HCMV-infected HAECs were treated with H_2O_2 as described above. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). One microgram of total RNA was used for the synthesis of cDNA by iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed using iQSYBR Green Supermix Kit (Bio-Rad) following the manufacturer's instruction. The quantitative gene expression by real-time PCR was evaluated using the comparative threshold cycle (C_T) method and was normalized against the endogenous control β-actin. The PCR primer sequences are Bcl-2 forward: 5′-GGG GAG GAT TGT GGC CTT C-3′, reverse: 5′-CAG GGC GAT GTT GTC CAC C-3′; Bcl-xl forward: 5′-GGT CGC ATT GTG GCC TTT TTC -3′, reverse: 5′- GCT GCA TTG TTC CCA TAG AG-3′; Bax forward: 5′- TGA GCA GAT CAT GAA GAC AGG -3′, reverse: 5′- GAC ACT CGC TCA GCT TCT TG -3′; β-actin forward: 5′-CTG GAA CGG TGA AGG TGA CA-3′, reverse: 5′-AAG GGA CTT CCT GTA ACA ATG CA-3′. All samples were run in duplicates and were repeated at least three times.

2.9. Statistical analyses

Quantitative variables are presented as means ± S.D. of three separate experiments, and compared by independent Student's t test between groups and by ANOVA for comparisons among three or more groups. Two-tailed $P < 0.05$ is regarded as statistically significant. The statistical analyses were conducted using SPSS 12.0 for Windows (SPSS Inc., Chicago, IL).

3. Results

3.1. HCMV attenuated H_2O_2-induced apoptosis in HAECs

It has been shown that HCMV suppresses cell death in many cell types during early stage of infection [12–15]. Among several laboratory strains (AD169, Towne), our previous data demonstrated that HCMV VHL/E strain, an originally clinical isolate, had a high rate of infection to the primary HAECs [7]. HAECs were mock- or HCMV-infected at MOI 1.0. Three days after infection, cells exposed to 0 or 1 mM of H_2O_2 for 3 h. We identified cells undergoing apoptosis by in situ TUNEL staining with fluorescein; and HCMV-infected cells were identified with anti-IE antibody (Fig. 1). TUNEL is a sensitive method to examine apoptosis via DNA fragmentation. By microscopic examination, we detected very few TUNEL positive apoptotic cells (shown as green color, condensed nuclei co-localized with DAPI nuclei stain) in HCMV-infected cells without H_2O_2 treatment. After exposure to 1 mM H_2O_2 for

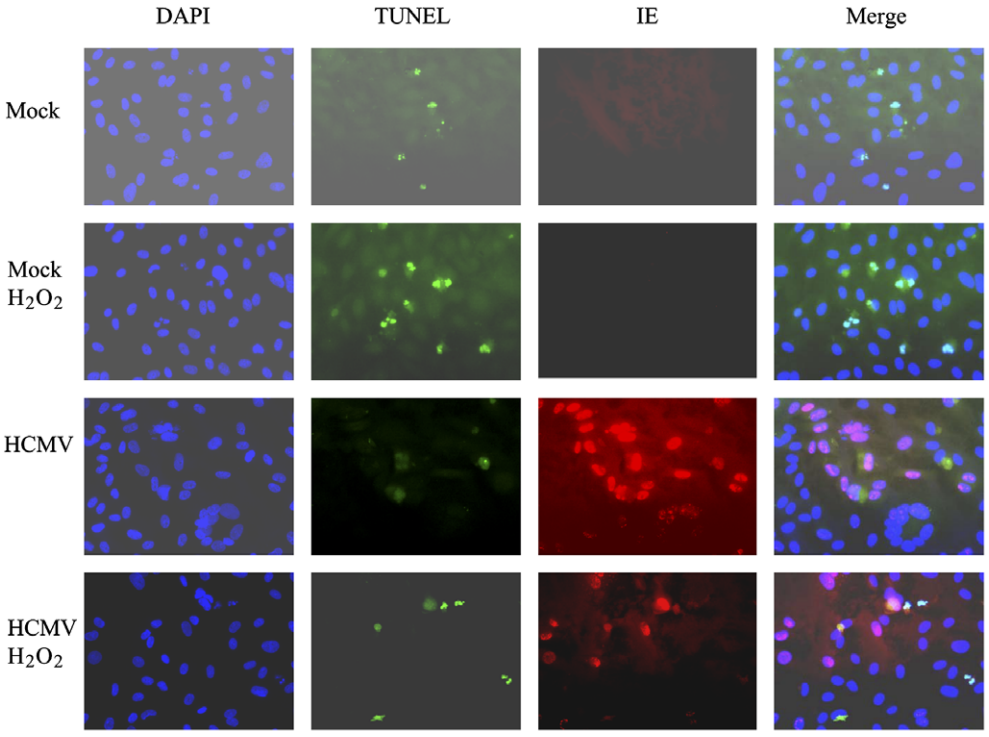

Fig. 1. HCMV infection protects HAECs from H_2O_2-induced apoptosis. HAECs were mock- and HCMV-infected at 1.0 MOI, and were treated with 0 or 1 mM H_2O_2 for 3 h on 3 day post-infection. Cells were then stained with DAPI (blue) for nuclei, TUNEL (green) for apoptosis, and anti-IE antibody for HCMV immediate early antigen (red). There were more TUNEL positive cells in mock-infected than in those in HCMV-infected cells. Merged images show minimal co-localization between TUNEL and IE. The pictures shown are the representative of three separate experiments repeated.

3 h, both mock-infected and HCMV-infected cells had more TUNEL positive stained cells than non-H_2O_2-treated cells. However, more apoptotic cells were observed in the mock-infected cells than those in the HCMV-infected cells (Fig. 1).

We further quantified the number of the positively stained cells by randomly choosing at least four different visual fields on each phase of the coverslips under microscope with low magnification (20×), and counted the TUNEL positive cells (green), HCMV IE positive cells (red), and DAPI nuclear staining (blue) by Image-Pro Plus V4.5 software (Media Cybernetics, Inc). The apoptosis rate and HCMV infection rate were calculated by the total numbers of TUNEL positive cells and IE positive cells divided by the total number of cells as measured by the DAPI staining. At 3 days post-infection, approximately 40% HAECs were infected by HCMV as indicated by the positive IE staining.

HAECs were exposed to different doses of H_2O_2 (0, 1, 2 mM). The percent of apoptotic endothelial cells was approximately 2-fold higher in the mock-infected HAECs than those in the HCMV-infected cells for each dose of H_2O_2 treatment. The percentage of apoptotic cells was about 2.5-fold higher in HAECs exposed to 2 mM H_2O_2 comparing to the cells without the treatment (Fig. 2A). We also evaluated the time-dependent changes in HAECs treated with 1 mM H_2O_2. At 0.5 h post treatment, there was a slight induction of apoptosis in the mock-infected culture. By the 2, 4 and 6 h post-H_2O_2 treatment, there was a significant increase in the positive staining for apoptosis in the mock-infected cells. The HCMV-infected cells, on the other hand, had significantly less apoptotic cells (Fig. 2B). These results indicate that HCMV infection can effectively attenuate H_2O_2-induced endothelial apoptosis.

3.2. Caspase-3 activation by the H_2O_2 exposure

Caspase activation is a crucial step in executing the apoptotic process. Among known caspases, caspase-3 activation often indicates an irreversible stage of apoptotic induction and plays an important role in various drug-induced apoptosis. We investigated whether caspase-3 was involved in the H_2O_2-induced endothelial apoptosis. We measured the intracellular levels and activities of caspase-3 in the experimental HAECs. As shown in Fig. 2C, caspase-3 activity increased significantly by the H_2O_2 treatment in a dose-dependent manner in the mock-infected cells, which was about 20–40% higher than that in the HCMV-infected cells with the treatment of 0–2 mM H_2O_2.

3.3. Loss of mitochondrial membrane potential

The mitochondrial permeability transition is an important step in the induction of apoptosis. We used JC-1 assay kit to quantify the mitochondrial membrane potential in HCMV- and mock-infected HAECs treated with or without H_2O_2. The relative mitochondrial membrane potential was calculated by comparing to the mock-infected control without H_2O_2 exposure (Fig. 2D). In the mock-infected HAECs, H_2O_2 treatment reduced the mitochondrial membrane potential by 75.6% comparing to the non-treatment control cells. On the other hand, the mitochondrial potential was reduced only 31.6% after the exposure to 1 mM of H_2O_2 for 3 h in the HCMV-

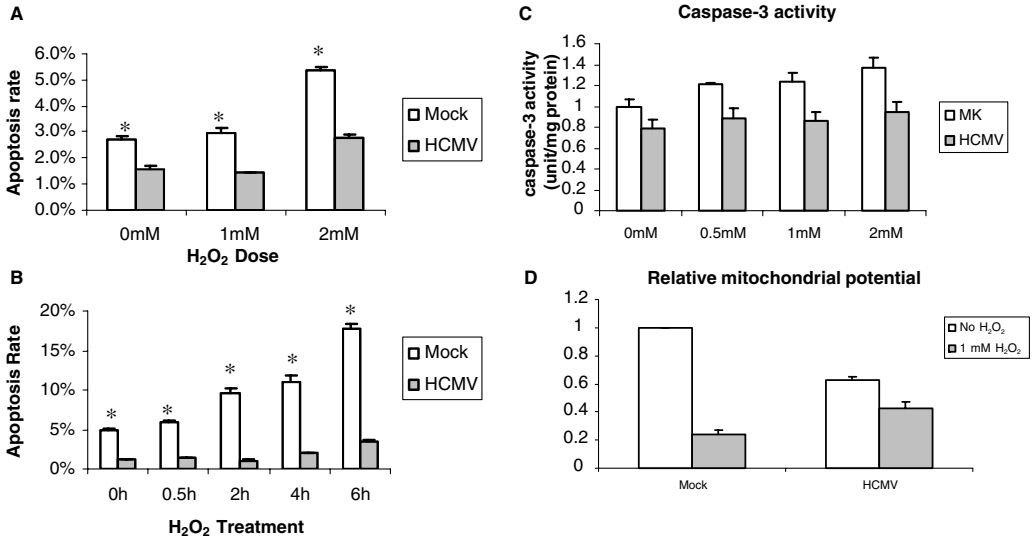

Fig. 2. H_2O_2induces apoptosis in HAECs. (A) *Dose dependent effects of H_2O_2-induced apoptosis*. HAECs were treated with H_2O_2 at indicated doses for 3 h. TUNEL positive cells and total cells were counted in at least four randomly selected areas per coverslip under fluorescence microscope (20×). HCMV-infected cells had significantly lower apoptosis rate compared to the mock-infected cells. (B) *Time course of H_2O_2-induced apoptosis.* HAECs were treated with 1 mM H_2O_2 for 0–6 h. The percentage of apoptotic cells increased rapidly in the mock-infected cells, but remained low in HCMV-infected cells. (C) *Caspase-3 activity increased in a dose-dependent manner in the mock-infected cells.* But there was virtually no change in HCMV-infected cells. (D) *Mitochondrial potential decreased when treated with 1 mM of H_2O_2.* While HAECs infected with HCMV showed a lower mitochondrial potential comparing to the mock-infected HAECs, further H_2O_2 treatment did not change the potential as significant as that in the mock-infected cells. All results presented in the figure were expressed as means + S.D. ($n = 3$) at each dose or time point from three individual experiments. $^*P < 0.01$ for differences between mock- and HCMV-infected groups by independent Student's t test. For within group differences in relation to dose- and time-dependent changes, oneway ANOVA was used, and $P < 0.001$ for the mock-infected cells, $P > 0.05$ for HCMV-infected cells.

infected HAECs, which was 43% higher than that in the mock-infected HAECs with the same H_2O_2 treatment.

3.4. HCMV infection upregulated Bcl-2 expression in endothelial cells

Our results showed that H_2O_2 increased endothelial cell apoptosis by activating caspase-3 and loss of mitochondrial potential, which appeared to be attenuated by HCMV infection. To investigate the mechanism of anti-apoptotic effect by the HCMV, we measured the protein levels of Bcl-2 family members: Bcl-2, Bcl-xl, Bax and Bad (Fig. 3A). HCMV immediate early antigen (HCMV IE) and HCMV late antigen were also examined to verify the HCMV infection status (Fig. 3A). Bcl-2, a well-known anti-apoptotic protein, was elevated in the HCMV-infected cells. Using Student's t tests, we found that Bcl-2 protein levels (expressed as density ratios relative to the density of β-actin band) in HCMV-infected cells treated with 0, 1 and 2 mM H_2O_2 were significantly higher than those mock-infected cells receiving the same doses of H_2O_2 treatments (1.12 ± 0.13 vs 1.59 ± 0.21, $t = 5.88$, $P = 0.028$; 1.16 ± 0.13 vs 1.53 ± 0.21, $t = 4.38$, $P = 0.045$; 0.95 ± 0.13 vs 1.54 ± 0.19, $t = 9.89$, $P = 0.01$, $N = 3$, respectively). Bcl-2 protein level decreased, although non-significantly, when mock-infected cells were exposed to 1 or 2 mM H_2O_2 for 3 h. But HCMV-infected cells did not show significant changes in Bcl-2 level under the same treatment of H_2O_2. The Bcl-xl - the other pro-survival protein, was not affected by the HCMV infection or the H_2O_2 treatment. The same findings were also observed for Bax and Bad – the pro-apoptotic proteins in the Bcl-2 family.

We further measured the Bcl-2, Bcl-xl and Bax mRNA levels in these experimental cells. Similar to the protein levels, there was a significant increase in Bcl-2 mRNA in HCMV-infected cells compared to the mock-infected cells (Fig. 4A). However, H_2O_2 treatment did not change Bcl-2 mRNA levels in either experimental group. There was virtually no difference in Bcl-xl and Bax mRNA levels between HCMV- and mock-infected cells with or without H_2O_2 treatment (Fig. 4B and C). These results indicate that increased Bcl-2 mRNA transcription and protein expression may contribute to the anti-apoptotic effect by the HCMV infection in endothelial cells against H_2O_2-induced apoptosis.

3.5. Activation of ERK mediates the anti-apoptotic effects in HCMV-infected cells

We next sought to determine which cellular signal transduction pathways were involved in preventing HCMV-infected HAECs from apoptosis. Various members of the MAPK family have been implicated for their influences in cell survival responding to stress. We attempted to find out whether HCMV infection might inhibit apoptosis through blocking MAPK cascades in H_2O_2-treated cells. We examined the effects of H_2O_2 treatment and HCMV infection on the phosphorylation of ERK1/2 using antibodies specific for the phosphor-ERK1/2(Thr202/Tyr204). HAECs treated with 1 or 2 mM H_2O_2 for 3 h had significantly reduced phospho-ERK 1/2 in the mock-infected cells. However, the phospho-ERK 1/2 level was unchanged with the same H_2O_2 treatment in the HCMV-infected cells (Fig. 5). Thr202/Tyr204 are the conserved phosphorylation sites for ERK1/2; and are critical for ERK1/2 activation. Our results suggest that H_2O_2 treatment inhibits ERK1/2 activation, hence induces HAEC apoptosis. HCMV infection, however, blocks the H_2O_2-initiated ERK1/2 inhibition, hence attenuates apoptotic process in HCMV-infected endothelial cells.

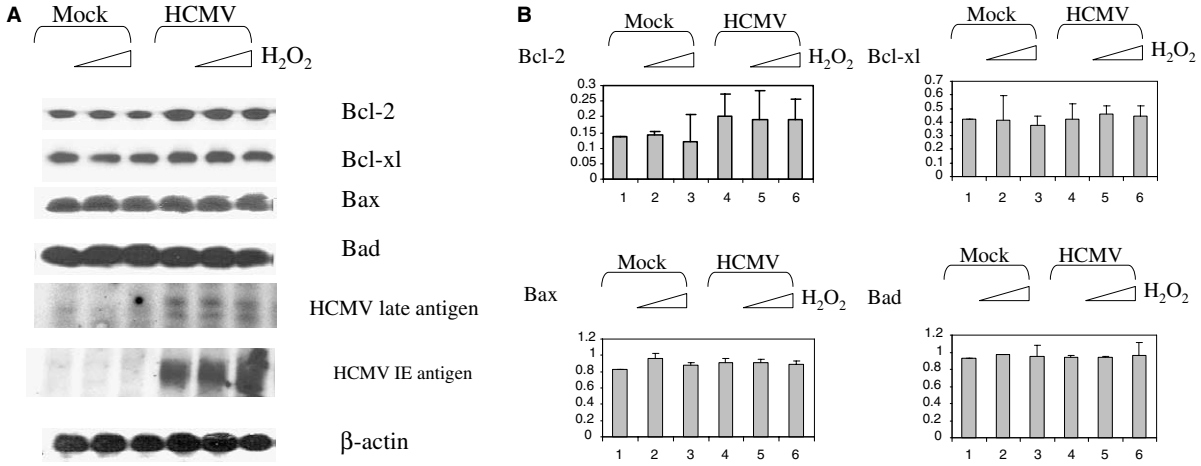

Fig. 3. Changes in levels of Bcl-2 family proteins in mock-and HCMV-infected cells. (A) *Western blotting analyses in mock-and HCMV-infected HAECs.* HAECs were grown to confluence before infected with HCMV for 72 h. Cells were then treated with 0, 1 and 2 mM H_2O_2 for 3 h (corresponding three lanes in mock- and HCMV-infected cells in the Western blot). Cell lysates containing 50 μg proteins were separated in a 12% SDS–PAGE electrophoresis before transferred to a nitrocellulose membrane for immunoblotting against specific proteins as indicated. The representative results from three separate experiments are shown. (B) *Quantitative analyses of the Bcl-2, Bcl-xl, Bax and Bad levels in Western blot.* The relative band intensities are means + S.D. ($n = 3$) from at least three individual experiments and normalized against β-actin protein level. Columns 1–6 represent cells treated with 0, 1 and 2 mM H_2O_2 for 3 h in mock-infected (lanes 1–3) and HCMV-infected (lanes 4–6) cells. While Bcl-2 levels were higher in HCMV-infected cells compared to the mock-infected cells ($P < 0.05$ by the Student's t test between mock-infected and HCMV-infected of the same H_2O_2 treatment), the changes in the levels of Bcl-xl, Bax and Bad were small with the indicated doses of H_2O_2. There was also a reduction in Bcl-2 levels with the increased doses of H_2O_2.

3.6. ERK1/2 activation involved in Bcl-2 upregulation in HCMV-infected endothelial cells

MAPK-ERK is an important cell signal pathway involved in cell survival. HCMV infection induced phosphorylation and activation of ERK1/2, and thus increased the ability to prevent H_2O_2-induced apoptosis. Since Bcl-2 expression was also upregulated in HCMV-infected HAECs, we next examined whether activation of ERK1/2 could contribute to the Bcl-2 expression in the course of HCMV infection. We analyzed the changes in phospho-ERK and phospho-Bcl-2 (thr56) after pre-incubation with U0126 – a chemical compound act as MEK inhibitor [16]. HAECs were mock- and HCMV-infected as described above. Cells were pre-incubated with 10 μM U0126 for 2 h. The same volume of solvent DMSO was added to the control wells. After 2 h of incubation, H_2O_2 at the indicated doses was added to the cells and incubated for an additional 3 h. The cells were harvested for the preparation of cell lysates for the Western blotting analysis and for total RNA isolation. We showed a complete block of the ERK1/2 phosphorylation by the U0126 treatment in both mock- and HCMV-infected cells, while the total ERK protein levels were unchanged (Fig. 6A). Bcl-2 phosphorylation was also significantly inhibited in the U0126-treated cells; but total Bcl-2 as well as Bcl-xl and Bax were not affected by the U0126 treatment (Fig. 6A). On the other hand, Bcl-2 mRNA levels were significantly reduced in mock- and HCMV-infected cells with the treatment of U0126. However, Bcl-xl and Bax mRNA levels did not change with the U0126 treatment (Fig. 6B). TUNEL staining proved that HAECs pre-incubated with U0126 had significant higher apoptosis rate than those pre-incubated only with DMSO (Fig. 6C). Taken together, these data suggest that there is a correlation between the level of ERK activity and Bcl-2 gene expression; and that ERK1/2 is involved in regulating the Bcl-2 gene expression. This ERK-Bcl-2 pathway may play an important role in HCMV-mediated anti-apoptotic function in HAECs.

4. Discussion

One of the unique features for viruses like HCMV is to evade the host immune detection and elimination, which enables the virus to infect a large number of people and co-exit with humans for life time. Because of this lifelong existence, HCMV plays an important role in the pathogenesis of vascular diseases including atherosclerosis and restenosis [3,17,18]. In addition, HCMV also appears to be able to hijack cell cycle and cell survival machineries for the benefit of viral propagation and dissemination. During early infection, HCMV makes the host cells resistant to cell death so that the virus carrying cells will not be removed from the body system. This gives the HCMV sufficient time and material to propagate. Once this process is completed, at the late stage of infection, HCMV makes the infected cells more likely to die so that it helps the viral dissemination. When the condition is not favorable, HCMV can also stay dormant in latent form with minimal viral activity so that it can stay under the radar screen of immune surveillance and wait for the right condition, e.g. during immunocompromised stage, to propagate again. Therefore, it is vital to understand the molecular mechanisms mediating these processes and to break the cycles that favor HCMV existence and growth.

One of the significant findings in the present study is that HCMV-infected HAECs are resistant to H_2O_2-induced cell death. H_2O_2 is an active ROS produced during the aerobic metabolism under physiological conditions. In moderate "physiological" amount, H_2O_2 appears to promote cell growth via ERK 1/2 pathway [19,20]. However, when produced in

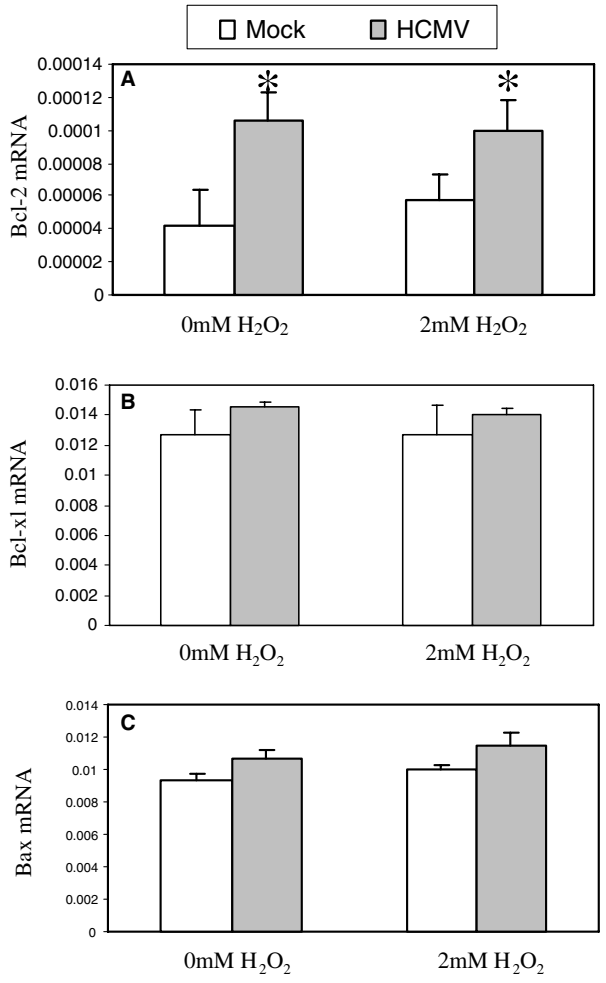

Fig. 4. Levels of mRNA of the Bcl-2 family members in HCMV-infected cells. HAECs were grown to confluence, and infected with HCMV at MOI 1.0. On 3 days post-infection, cells were treated with indicated doses of H_2O_2 for 3 h. Total RNA were isolated and used for cDNA synthesis. Bcl-2 (A), Bcl-xl (B) and Bax (C) mRNA levels were quantified by using quantitative real-time RT-PCR. The housekeeping gene β-actin was used as an internal control. Levels of Bcl-2 mRNA were significantly higher in the HCMV-infected cells than the mock-infected cells with or without H_2O_2 treatment. Levels of Bcl-xl and Bax mRNA were not affected by HCMV infection or H_2O_2 treatment. All data are presented as means + S.D. of three individual experiments ($n = 3$).

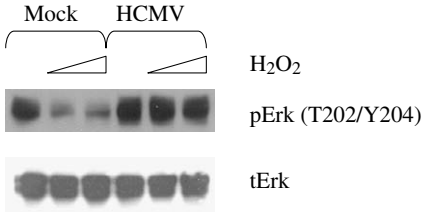

Fig. 5. Activation of ERK after HCMV infection. HAECs were grown to confluence before infected with HCMV at MOI 1.0 for 3 days. Cells were then treated with 0, 1, 2 mM H_2O_2 for 3 h (corresponding three lanes in the Western blot) before harvested for the analyses of phosphorylated and total ERK. While phospho-ERK was significantly elevated, the total ERK showed no change in HCMV-infected cells. Representative Western blot gels from three separate experiments ($n = 3$) are shown.

excess (>200 μM), e.g. under inflammatory condition [20], H_2O_2 can cause tissue injuries, e.g. endothelial cell apoptosis, and is associated with atherogenesis. It appears that H_2O_2 can increase mitochondria permeability or activate JNK/c-Jun pathway, hence triggers endothelial apoptosis [21–23]. However, when the excess H_2O_2, e.g. released by the activated monocytes or macrophages, is targeted at cells carrying pathogens, the cell death initiated by the H_2O_2 is beneficial. Even this relatively non-selective cell death pathway initiated by H_2O_2 is blocked in cells with active HCMV propagation. HCMV-infected HAECs had significantly less cells undergoing apoptosis when exposed to H_2O_2 (Fig. 2A and B). Cells that did go apoptosis were mostly not yet infected by the HCMV as illustrated by co-localized staining of TUNEL for apoptosis and IE for HCMV infection (Fig. 1).

We have further shown that the expression of Bcl-2 – a pro-survival protein, which was inhibited by H_2O_2 treatment, was increased by the HCMV infection (Fig. 3A). The increase in Bcl-2 mRNA levels in HCMV-infected endothelial cells suggests that the HCMV will either promote Bcl-2 transcription or stabilize the Bcl-2 mRNA. While no previous studies have shown the relationship between HCMV infection and Bcl-2 expression in endothelial cells, Bcl-2 expression was increased by the infection of other virus, e.g. human T-cell lymphotropic virus type I, in endothelial cells [24]. Bcl-2 as a pro-oncogene plays an important role in endothelial cell death [25–27]. In addition to sustain cell survival for viral proliferation, elevated Bcl-2 in endothelial cells could have an additional effect on angiogenesis, hence carcinogenesis [28]. This role could be especially relevant when HCMV already produces its own anti-apoptotic proteins, e.g. vMIA [29–31], which is unrelated to Bcl-2 but capable of prolonging cell survival for pro-viral proliferation environment. On the other hand, the Bcl-xl – the other pro-survival Bcl-2 family protein, Bax and Bad – the pro-apoptosis Bcl-2 family proteins, did not show changes. However, these results cannot rule out the possibilities that these Bcl-2 family proteins are involved in the anti-apoptotic process during the HCMV infection. HCMV could regulate these apoptosis-related proteins by altered subcellular translocation, integration and oligomerization with the mitochondrial membrane without causing level changes in the total cell lysates. More experiments are needed to explore these changes.

Endothelial cells possess a multitude of redox-sensitive signaling systems, including the extracellular signal-regulated kinase (ERK1/2) and p38 MAPK, Akt, JNK and caspases [32,33]. The best characterized among MAPKs is extracellular signal regulated kinase (ERK). This kinase is activated by a cascade of phosphorylation that involves initially in Ras interaction with Raf-1. Activation of Raf-1 in turn phosphorylates MEK1 followed by phosphorylation of p44 (ERK1 isoform) and p42 (ERK2 isoform) on tyrosine and threonine residues. Mitogen-activated ERK is known to act as an anti-apoptotic factor by mediating cell proliferation and survival [34]. Under low level of oxidative stress, e.g. H_2O_2 concentration lower than 100 μM, it induces activations of ERK, Akt, NF-κB and acts as a pro-survival factor. When the concentration of ROS (e.g. H_2O_2) is high, however, it promotes pro-apoptotic changes. In our experiment, high concentrations of H_2O_2 (1 or 2 mM) resulted in a significant reduction in phospho-ERK in the mock-infected endothelial cells. However, this repression was not evident in the HCMV-infected cells. These data suggest that H_2O_2 may induce apoptosis by inhibiting

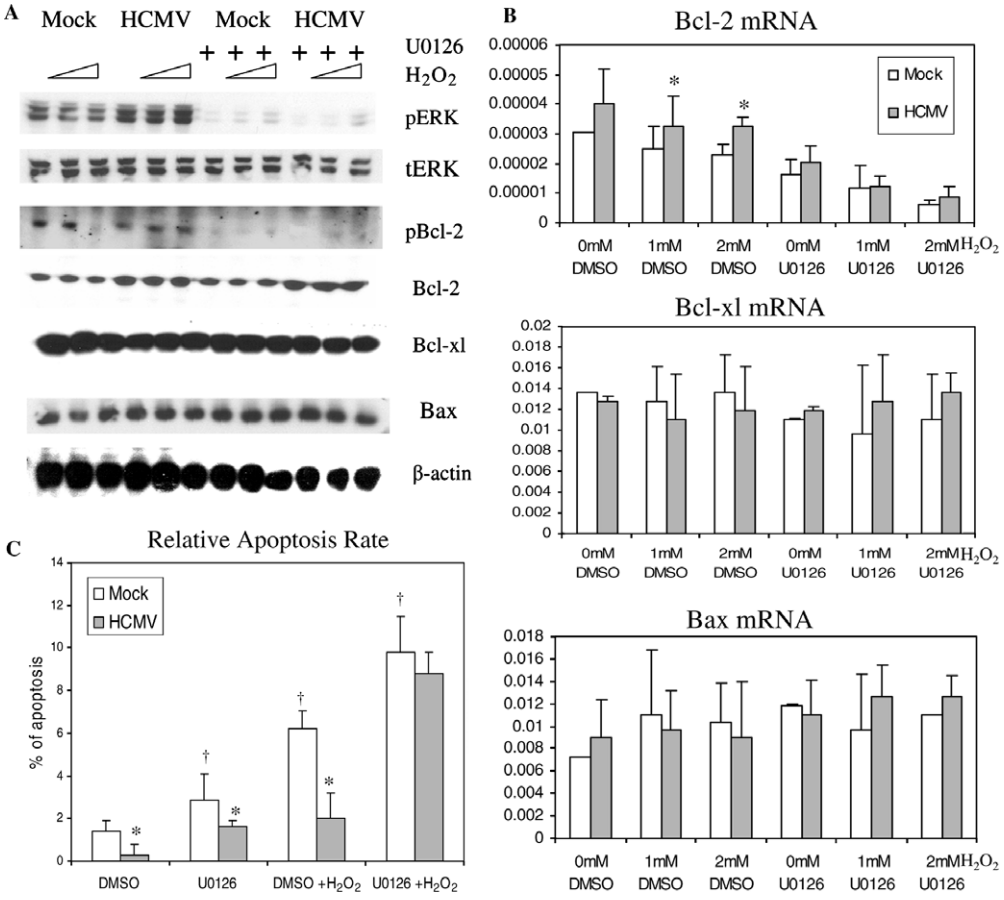

Fig. 6. HCMV infection increased total Bcl-2 levels and maintained the Bcl-2's phosphorylation status; MEK inhibitor U0126 attenuated HCMV-mediated effects on Bcl-2. HAECs were infected with HCMV for 3 days. Cells were pre-incubated with DMSO or 10 μM of U0126 for 2 h, and were treated with indicated amounts of H_2O_2 for 3 h. (A) *Western blot for phospho-ERK, tERK, phospho-Bcl-2, Bcl-2, Bcl-xl and Bax.* Fifty micrograms of the cell lysate proteins was loaded and separated by 12% SDS–PAGE. Cells were treated with 0, 1 and 2 mM H_2O_2 in the corresponding lanes. (B) *Changes in mRNA levels of Bcl-2 in cells treated with U0126.* Total RNA was isolated and used for the cDNA synthesis. Bcl-2, Bcl-xl and Bax mRNA levels were quantified by using real-time PCR and were normalized against β-actin. Data represent the means + S.D., $n = 3$. While the Bcl-2 mRNA levels were similar between mock-infected and HCMV-infected cells treated with U0126, they were elevated by the HCMV infection in cells treated with vehicle DMSO only ($P < 0.05$ for 1 or 2 mM H_2O_2, respectively). (C) *MEK inhibitor U0126 increased apoptosis in both mock-infected and HCMV-infected HAECs.* HAECs were infected and pre-incubated with DMSO or 10 μM of U0126 as described in Section 2; and then treated with 1 mM of H_2O_2 for 3 h. Cells were fixed and stained by the TUNEL kit to evaluate the apoptosis rate. All percentages of apoptosis are means + S.D. of three separate experiments ($n = 3$). In all treatment conditions, except those treated with U0126 + H_2O_2, the rates of apoptosis were significantly lower in the HCMV-infected HAECs than those in the mock-infected cells ($^{*}P < 0.01$). Yet, the U0126 treatment abolished the HCMV infection-mediated difference in H_2O_2-induced HAEC apoptosis. On the other hand, U0126 pre-incubation with or without simultaneous H_2O_2 treatments significantly increased apoptosis rate in both mock- and HCMV-infected HAECs ($^{\dagger}P < 0.05$). While HCMV infection significantly inhibited H_2O_2-induced endothelial apoptosis, the difference was no longer present by the simultaneous U0126 treatment.

ERK pathway in endothelial cells. HCMV infection activates the ERK1/2, thus attenuates the H_2O_2-induced apoptosis. The MAP kinase pathway is known to affect the expression of genes involved in cell survival [35,36]. The fact that the Bcl-2 and the ERK signaling pathways are both implicated in controlling cell viability suggests that MAP kinase could be the upstream regulator of Bcl-2, as suggested in several recent studies [37–39]. Indeed, our experiment in cells (with or without HCMV infection) treated with U0126 – a chemical inhibitor of MKK1/2 [16] has demonstrated the reduced Bcl-2 mRNA and protein levels with the decreased phosphor-ERK.

Recent studies have established that Bcl-2 function is primarily modulated by heterdimerization with other pro-apoptotic members in the Bcl-2 family. Phosphorylation of Bcl-2 could play an important role in this regulatory mechanism. Several types of Bcl-2 phosphorylation have been reported [40], and are associated with Bcl-2 activation [41–45]. Our results showed that H_2O_2 treatment was able to reduce the Bcl-2 phosphorylation at the Thr 56 residue in mock-infected cells. However, H_2O_2 was unable to change the phospho-Bcl-2 levels in the HCMV-infected cells (Fig. 6A). Pre-incubation of HAECs with U0126 – an ERK inhibitor, completely blocked the Bcl-2 phosphorylation. Bcl-2 mRNA level was also decreased after pre-treatment with U0126 (Fig. 6B). These changes were translated into an increased endothelial apoptosis with the H_2O_2 exposure (Fig. 6C). Indeed, the percentages of endothelial apoptosis were similar in mock- and HCMV-infected cells when treated with U0126 and H_2O_2 (Fig. 6C). Our results indicate that ERK1/2 may act as an upstream regulator of Bcl-2 and is involved in the maintenance of the Bcl-2 phosphorylation status at the Thr 56 residue. Functional relevance of this phosphorylation

requires further investigation, especially in endothelial cells infected with HCMV.

In conclusion, we have shown that HCMV interferes with endothelial apoptosis initiated by H_2O_2. Because HCMV replicates to high titers during first few days post-infection in endothelial cells, this anti-apoptotic effect by HCMV confers the virus a significant survival advantage. Our study has further suggested that pro-survival pathways initiated by the ERK activation and Bcl-2 may be responsible for the anti-apoptotic effect when challenged by oxidative stress. Indeed, this pro-survival environment initiated by the HCMV infection may be a universal mechanism for the virus-mediated apoptosis resistance. Rather than directly interfering with the pro-apoptotic pathways, HCMV may counter-balance the effect by boosting up the pro-survival machineries. It may be effective not only to H_2O_2-initiated apoptotic process, but also to other apoptotic triggers including UV irradiation, growth factor withdraw or TNF-α stimulation. Although more investigations are needed, interrupting the pro-survival pathways rather than activating pro-apoptotic pathways could be more effective in breaking the propagation cycle initiated by HCMV infection, hence minimizing the pathological changes.

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