Upregulation of Nrf2 expression by human cytomegalovirus infection protects host cells from oxidative stress

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INTRODUCTION

Human cytomegalovirus (HCMV/HHV-5) is a widespread pathogen that infects the majority of the world’s population and establishes life-long latency with periodic reactivation. Primary infection as well as spontaneous reactivation usually remains asymptomatic in healthy people, but can cause life-threatening diseases in immunologically immature or compromised individuals (Britt, 2008). It is the major cause of retinitis and blindness in AIDS patients and also of graft loss in recipients of bone marrow and solid-organ transplants (Steininger, 2007; Streblow et al., 2007). HCMV is also associated with a variety of other human diseases, including birth defects resulting from congenital infection, atherosclerotic vascular diseases and some cancers (Söderberg-Nauclér, 2006; Tsutsui, 2009). The pathogenesis of diseases associated with acute HCMV infection is primarily attributable to lytic virus replication or host immune response to virus-infected cells (Cope et al., 1997; Deayton et al., 2004; Emery et al., 1999, 2000; Varani & Landini, 2011). However, only a subset of patients develops invasive HCMV-related diseases, and the course of infection is known to be highly variable even in the most severely immunocompromised individuals. Therefore, as yet undefined aspects of HCMV-host interaction might contribute to the pathogenesis during acute or chronic viral infection.

Nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) belongs to the cap’n’collar (CNC) subfamily of basic region leucine zipper (bZIP) transcription factors that include NF-E2, Nrf1–3 and Bach1–2 in vertebrates (Sykiotis & Bohmann, 2010). Nrf2 plays a crucial role in regulating cellular responses to a variety of oxidative and xenobiotic stresses. Under non-stressed conditions, Nrf2 is expressed at low levels because of its interaction with Keap1, resulting in the ubiquitination and degradation of Nrf2. When activated, Nrf2 is stabilized by its dissociation from Keap1. Free Nrf2 binds to DNA sequences termed antioxidant-responsive elements (ARE) as a heterodimer with Maf, which subsequently activates the transcription of downstream target genes including haem oxygenase-1 (HO-1), which protects cells against the detrimental effects of different stresses (Niture et al., 2010). The oxidative modification of Keap1 and protein kinase C (PKC)-mediated phosphorylation of Nrf2 are known to disrupt the Keap1–Nrf2 interaction, thereby activating the Nrf2 pathway (Niture et al., 2009). The involvement of other kinases including the phosphatidylinositol 3-kinase (PI3K) and casein kinase 2 (CK2) in the activation of Nrf2 has also been reported (Afonyushkin et al., 2011; Kim et al., 2012; Nakaso et al., 2003; Wang et al., 2008).

The Nrf2 pathway has been implicated in various viral infections. The downregulation of Nrf2 increased the entry...
and replication of influenza virus in human nasal epithelial cells. However, treatment with Nrf2 activators had the opposite effect(s), thus suggesting an antiviral role for Nrf2 (Kesic et al., 2011). Hepatitis B virus (HBV) infection also activated the Nrf2 pathway, leading to increased host cell protection against oxidative stress-induced protein modification (Schaedler et al., 2010). The effect of hepatitis C virus (HCV) infection on the Nrf2 pathway is still controversial. HCV infection has been reported to prevent host cell apoptosis through the activation of Nrf2, but it has also been claimed that HCV inhibits the Nrf2 pathway by the delocalization of small Maf proteins (Burdette et al., 2010; Carvajal-Yepes et al., 2011).

It has recently been reported that HCMV infection does not activate the Nrf2 pathway in life-extended fibroblasts, which have been engineered to constitutively express the catalytic subunit of telomerase (Tilton et al., 2011). HCMV infection prevented the nuclear translocation of Nrf2 and failed to activate HO-1 expression. However, other investigations have shown that HO-1 expression is increased upon HCMV infection at the RNA or protein level when primary fibroblasts are used (Browne et al., 2001; Stanton et al., 2007). Therefore, the effect of HCMV infection on the Nrf2 pathway needs to be further clarified.

The goal of this study was to investigate the regulation and biological significance of Nrf2 during HCMV infection of primary fibroblasts. These studies indicate that HCMV infection influences Nrf2 expression and activates HO-1 expression through Nrf2. The induction of Nrf2 expression appears to be dependent on viral gene expression and the activity of the cellular kinase CK2. Our data involving buthionine sulfoximine (BSO) suggest that HCMV infection might protect host cells against oxidative stress-induced cell death through the activation of Nrf2.

**RESULTS**

**HCMV activates the transcription factor Nrf2**

To test the effect of HCMV infection on Nrf2, human foreskin fibroblasts (HFFs) were infected with HCMV (Towne strain), and cell monolayers were immunostained using immediate early (IE) 1/IE2- and Nrf2-specific antibodies. As a positive control, HFFs were treated with 100 μM tert-butylnhydroquinone (tBHQ), a well-known Nrf2 inducer, for 24 h. Nrf2 protein levels were undetectable in normal HFFs (Fig. 1a, panel 1), but in cells treated with tBHQ, Nrf2 protein levels increased significantly in the nucleus (Fig. 1a, compare panels 1 and 2). In the HCMV-infected cells, Nrf2 protein levels (red) started to increase at 24 h post-infection (p.i.), and reached their highest levels at 48 h p.i. (Fig. 1b, panels 5–8). Nrf2 induction was observed in both the nuclear and cytoplasmic compartments (Fig. 1b, panels 13–16). These results indicate that HCMV infection can activate Nrf2. To corroborate these results, a clinical HCMV strain (JHC) was also used. HFFs were infected with either the Towne or JHC strain under the same experimental conditions and analysed using immunofluorescence using IE1/IE2- and Nrf2-specific antibodies. This clinical strain also increased Nrf2 expression in both compartments (Fig. 1c, compare panels 4 and 6).

Previously, in studies using HFFs immortalized by the constitutive expression of the telomerase catalytic subunit (TERT), it was reported that HCMV infection increased Nrf2 expression but prevented its nuclear translocation, which is inconsistent with our results generated with the use of normal primary HFFs (Tilton et al., 2011). To directly compare these two types of HFFs, we also constructed HFFs that express TERT using retroviral vectors expressing TERT and GFP as a bicistronic message or GFP alone as a control. Transduced cells were cultured for five passages, and FACS analysis was used to measure the transduction efficiency, which was over 90%. The TERT expression was confirmed by immunostaining using a TERT-specific antibody. Control cells expressed an undetectable level of TERT, whereas it was readily visible in the nucleus of TERT-expressing cells (Fig. 1d, compare panels 5 and 6). Both cell types were then infected with HCMV and analysed by immunofluorescence to observe IE1/IE2 and Nrf2 protein levels. In both the control and TERT-expressing cells, HCMV infection could upregulate the level of nuclear Nrf2 (Fig. 1e, compare panels 6 and 8). Our data indicate that the expression of TERT does not affect HCMV-mediated Nrf2 activation. Additional studies are needed to determine what caused the differences between these two studies.

**HCMV infection upregulates HO-1 expression**

HO-1 is one of the major antioxidative stress genes, and its gene expression is regulated primarily by Nrf2. To test the effect of HCMV infection on HO-1 expression, HFFs were infected with HCMV, and total proteins were prepared at appropriate time points and subjected to immunoblotting analysis. HCMV infection induced the expression of the HO-1 protein in a transient manner (Fig. 2a). The level of the HO-1 protein peaked at 48 h p.i., then decreased to a basal level. To investigate whether HCMV infection regulated the induction of HO-1 expression at the transcriptional level, the RNA level of HO-1 was determined by Northern blot hybridization. The steady state mRNA level of HO-1 was lower at 6 h p.i. than at basal level, but increased thereafter, peaking at 24 h p.i. followed by the decrease (Fig. 2b). These data suggested that HCMV infection could alter HO-1 expression at the RNA level.

To exclude the possibility that HO-1 induction might have occurred in uninfected cells rather than in the infected cells (for example, by the action of cytokines or other materials secreted from neighbouring HCMV-infected cells), HFFs were infected with HCMV, and HO-1 expression was analysed by immunofluorescence using IE1/IE2- and HO-1-specific antibodies. Uninfected
cells expressed undetectable levels of HO-1 (Fig. 2c, panel 4). At 12 h p.i., some cells expressed IE1/IE2. Increased HO-1 protein expression was only observed in IE1/IE2-positive cells and was undetectable in neighbouring IE1/IE2-negative cells (Fig. 2c, panel 11). At 24 h p.i., nearly all the cells expressed both IE1/IE2 and HO-1 (Fig. 2c, panel 12). These data indicate that HO-1 expression is induced only in infected (IE1/IE2-positive) cells.

To confirm these results, the effect of HCMV infection was also tested on another cell line, SK-N-SH, known to be susceptible to this herpesvirus. Cells were infected with HCMV and examined by immunofluorescence at 24 h p.i. HO-1 was not detected in uninfected cells (Fig. 2d, panel 3). In infected cultures, nearly all IE1/IE2-positive cells showed increased HO-1 expression (Fig. 2d, panel 8). These data indicate that our observations are not restricted to one particular cell type.

Fig. 1. Nrf2 activation during HCMV infection. (a) HFFs were treated with 100 μM tBHQ for 24 h, and immunofluorescence (IF) was performed using an Nrf2-specific antibody (red). Nuclei were stained with Hoechst stain (blue). (b) HFFs were infected with HCMV and examined by IF at the time points indicated using IE1/IE2-specific (green) and Nrf2-specific (red) antibodies. (c) HFFs were infected with a laboratory-adapted strain (Towne) or a clinical isolate (JHC) of HCMV, and the localization of Nrf2 was analysed by IF at 48 h p.i. (d) HFFs stably expressing the catalytic subunit of telomerase (TERT) were immunostained using TERT-specific antibodies (red). (e) HFFs expressing TERT and normal HFFs were infected with HCMV and examined by IF using IE1/IE2-specific (green) and Nrf2-specific (red) antibodies at 48 h p.i. Bar, 20 μm.
The possible involvement of viral late gene products in the HCMV-mediated regulation of HO-1 expression was also tested. HFFs were infected with HCMV in the absence or presence of 30 μM ganciclovir (GCV). Cells were harvested at the indicated time points, and Western blot analysis was performed on whole-cell extracts to monitor HO-1

**Fig. 2.** HO-1 induction in HCMV-infected cells. (a) HFFs were infected with HCMV and harvested at the time points indicated. HO-1 protein levels were measured by immunoblotting using IE1/IE2-, HO-1- and β-actin-specific antibodies. (b) HO-1 mRNA levels were determined by Northern blot hybridization. 28S rRNA levels served as a loading control. (c) HFFs were infected with HCMV, and cell monolayers were analysed by IF using IE1/IE2-specific (green) and HO-1-protein-specific (red) antibodies. Bar, 20 μm. (d) SK-N-SH, a neuroblastoma cell line, was infected with HCMV and examined by IF at 24 h p.i. (e) HFFs were infected with HCMV, and at 72 h p.i., they were treated with cycloheximide (CHX; 100 μM) for 1 h, then incubated with MG132 (2 μM) for the times indicated. Protein levels were measured by immunoblotting using HO-1- and β-actin-specific antibodies. (f) HFFs were infected with HCMV in the absence or presence of 30 μM ganciclovir (GCV). Cells were harvested at the indicated time points, and Western blot analysis was performed on whole-cell extracts to monitor HO-1.
expression levels. As expected, GCV treatment inhibited the expression of a late gene, pp28 (UL99), as well as the expression of IE2, whose expression during the late stage of infection has been shown to be dependent on viral DNA synthesis (Fig. 2f, compare lanes 4 and 5, and lanes 6 and 7) (Fehr & Yu, 2011). The kinetics of HO-1 protein expression were transient as observed above, and this expression pattern was not altered by the addition of GCV (Fig. 2f, compare lanes 2, 4, 6 with lanes 3, 5, 7). Our data suggest that the expression of viral late genes might not be involved in regulating HO-1 expression.

HO-1 protein levels were almost undetectable at 72 h p.i., whereas HO-1 mRNA levels were still significantly upregulated (compare Fig. 2a, lane 5 with lane 6 of Fig. 2b), suggesting the possibility that HO-1 may be controlled post-translationally, including enhanced proteasome degradation, during the late stages of infection. The stability of HO-1 was investigated in the absence or presence of the proteasome inhibitor MG132 following cycloheximide treatment to inhibit further HO-1 protein synthesis. HFFs were infected with HCMV, treated with 100 μM cycloheximide at 72 h p.i. for 1 h, and then treated with 2 μM MG132 for the times indicated. After cycloheximide treatment, HO-1 protein levels gradually decreased over time (Fig. 2e, lanes 1–4), and the addition of MG132 did not affect the stability of HO-1 protein levels (Fig. 2e, compare lanes 2, 3, 4 with lanes 5, 6, 7). These data suggest that proteasome activity may not be involved in controlling HO-1 protein levels during this stage of infection. Additional studies will be needed to determine how HCMV infection decreases HO-1 protein levels at this time.

**Nrf2 is required for HCMV-mediated induction of HO-1 expression**

To test the involvement of Nrf2 in HCMV-mediated regulation of HO-1, Nrf2-specific small hairpin RNAs (shRNA) were used. HFFs were transduced with retroviral vectors expressing an shRNA specific for Nrf2 (Nrf2sh) or luciferase (LUCsh) in a bicistronic message with GFP. Overall, the transduction efficiency was approximately 90 % as determined by FACS. HFFs expressing either type of shRNA were infected with HCMV and immunostained using IE1/IE2- and Nrf2-specific antibodies. Nrf2 protein levels were reduced in Nrf2sh cells (Fig. 3a, compare panels 3 and 4). In LUCsh cells, HO-1 protein levels were increased in response to HCMV infection (Fig. 3b, compare lanes 1 and 2). The basal levels of the HO-1 protein expression were lower in Nrf2sh cells than in LUCsh cells (Fig. 3b, compare lanes 1 and 3), and the HCMV-mediated increase in HO-1 protein levels was suppressed in Nrf2sh cells (Fig. 3b, compare lanes 3 and 4). These data indicate that the HCMV-mediated induction of HO-1 expression is controlled, at least in part, by Nrf2.

UV-irradiated virus is known to be taken up by cells, but does not support the expression of viral proteins due to the damage of viral DNA. To test whether viral gene expression was necessary for HCMV-mediated Nrf2 activation, the effect of UV-irradiated virus was used. HFFs were treated with normal or UV-irradiated virus, and cells were analysed for the IE1/IE2 and Nrf2 proteins by immunofluorescence at 48 h p.i. UV irradiation completely abolished the ability of the virus to induce the expression of Nrf2 and viral proteins, indicating that newly synthesized viral protein(s) might be needed for the upregulation of Nrf2 expression (Fig. 4a, compare panels 2 and 3, and panels 5 and 6).

Reactive oxygen species (ROS) have been shown to activate antioxidative genes through the activation of Nrf2. Therefore, Nrf2 is required for HCMV-mediated induction of HO-1. (a) HFFs expressing either type of shRNA were infected with HCMV and examined by IF using IE1/IE2-specific (green) and Nrf2-specific (red) antibodies. Bar, 20 μm. (b) HFFs expressing shRNAs were infected with HCMV, and HO-1, GCLC and β-actin protein levels were measured at 48 h p.i. by immunoblotting.

**The expression of viral gene products is required for HCMV-mediated activation of Nrf2**

That HO-1 expression increases the expression of various antioxidative genes through the activation of Nrf2.
HCMV induces expression of Nrf2

Fig. 4. Effect of UV-irradiated HCMV on Nrf2 and intracellular ROS. (a) HFFs were treated with normal or UV-irradiated virus, and examined by IF using IE1/IE2-specific (green) or Nrf2-specific (red) antibodies at 48 h p.i. Bar, 20 μm. (b) The level of intracellular ROS was determined using CM-H2DCFDA. The geometric mean fluorescence intensity of cells was expressed as a fold induction relative to mock-infected cells. Data are presented as mean ± so of triplicate samples; ns, non-significant; **, P<0.01 (one-way ANOVA and Tukey’s multiple comparison test).

the indirect consequence of the increased ROS levels (Speir et al., 1996). HFFs were treated with normal or UV-irradiated virus, and the amount of intracellular ROS was measured, using 2′,7′-dichlorodihydrofluorescein diacetate (DCFDA), a fluorescence-based ROS indicator. The level of ROS was increased in both cells treated with normal or UV-irradiated virus (Fig. 4b). Because Nrf2 activation was not observed in cells treated with UV-irradiated virus, these data suggest that Nrf2 was activated by HCMV infection, not by the increased level of intracellular ROS that might have occurred in response to viral infection.

CK2 kinase is involved in HCMV-mediated activation of Nrf2

The PI3K pathway has been shown to mediate Nrf2 activation in various experimental settings (Nakaso et al., 2003; Wang et al., 2008). Because HCMV infection has been reported to activate the PI3K pathway, we tested whether this pathway is involved in HCMV-mediated activation of Nrf2 (Johnson et al., 2001). HFFs were infected with HCMV, and at 24 h p.i., the cells were incubated with increasing doses of two different PI3K inhibitors, Wortmannin and LY294002, for an additional 24 h. Total protein extracts were prepared, and immunoblot analysis of Akt phosphorylation (Ser473), a downstream substrate of PI3K, was performed. Uninfected cells showed high levels of Akt phosphorylation, and HCMV infection did not significantly affect these levels (Fig. 5a, compare lanes 1 and 2). Treatment with Wortmannin decreased Akt phosphorylation, indicating that the PI3K pathway was indeed inhibited by this chemical, which did not affect the HCMV-mediated induction of HO-1 (Fig. 5a, lanes 2–5). These results suggest that the PI3K pathway is likely not to be involved in HCMV-mediated Nrf2 activation.

To be certain, the effect of LY294002 on HO-1 expression was also tested. Unlike Wortmannin, LY294002 treatment (20 μM) suppressed the HCMV-mediated induction of HO-1 expression (Fig. 5b). Because LY294002 is known to inhibit not only PI3K but also CK2, we tested whether inhibiting CK2 would affect HCMV-mediated HO-1 induction (Gharbi et al., 2007). HCMV-infected HFFs were treated with a CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB) and examined using immunoblot analysis. TBB treatment inhibited the HCMV-mediated increase of HO-1 expression in a dose-dependent manner, suggesting that CK2 may be involved in Nrf2 activation by HCMV (Fig. 5c).

To confirm these results, Nrf2 expression in LY294002- and TBB-treated cells was also analysed by immunofluorescence detection of IE1/IE2 and Nrf2. In untreated control cells, HCMV infection increased Nrf2 protein levels (Fig. 5d, compare panels 5 and 6), but LY294002 (20 μM) and TBB (60 μM) treatments abolished this increase without affecting IE1/IE2 expression (Fig. 5d, compare panel 6 with panels 7 and 8, and panel 2 with panels 3 and 4). Together, these data suggest that CK2 activity is required for HCMV-mediated Nrf2 activation.

Nrf2 is involved in HCMV-mediated protection of host cells against oxidative stress

Nrf2 and its downstream genes are known to play a central role in cellular defence against oxidative stress. Based on these data, the possible role of the HCMV-mediated Nrf2 activation in the context of oxidative stress was investigated. HFFs were infected with HCMV for 2 h and then cultured in new medium containing 2 mM BSO, a potent inducer of oxidative stress that functions by inhibiting the synthesis of the antioxidant glutathione (GSH). One day later, cellular GSH levels were measured. HCMV infection increased the level of GSH by approximately threefold compared with the uninfected control (Fig. 6a, compare bars 1 and 2). BSO treatment reduced the GSH levels in both control and HCMV-infected cells to similar low levels,
indicating the synthesis of GSH was effectively inhibited (Fig. 6a, compare bars 1 and 3, and bars 2 and 4).

The effect of BSO-induced oxidative stress on cell viability was measured using an MTT assay. BSO treatment resulted in massive cell death and reduced cell viability to 20% compared with the untreated control cells (Fig. 6b, compare bars 1 and 3). In the absence of BSO, HCMV infection did not affect cell survival under the experimental conditions used (Fig. 6b, compare bars 1 and 2). In the presence of BSO, however, HCMV infection significantly increased the percentage of viable cells (Fig. 6b, compare bars 3 and 4). The difference was almost threefold, indicating that HCMV may protect host cells from BSO-induced cell death.

The possible involvement of Nrf2 in this process was also examined using shRNAs. HFFs expressing a control luciferase shRNA (LUCsh) or Nrf2 shRNA (Nrf2sh) were infected with HCMV and treated with BSO as described above. In HFFs expressing the control shRNA, the HCMV-infected cells were more resistant to BSO-induced cell death (Fig. 6c, compare bars 1 and 2). When Nrf2 expression was inhibited, however, HCMV no longer had a protective effect on the cells (Fig. 6c, compare bars 3 and 4). In the absence of BSO, HCMV infection did not affect the viability of HFFs expressing either type of shRNA (data not shown).

The role of Nrf2 in viral replication under normal experimental conditions was also investigated. Nrf2sh cells were infected with HCMV, together with three control cell types including normal, mock-transduced and LUCsh cells. The culture supernatants were collected at various time points, and viral titres were determined using an infectious centre assay. The amount of progeny virus was similar between all four samples (Fig. 6d). Similar data were obtained using a primary JHC strain (data not shown). These data suggest that HCMV-mediated Nrf2 activation may protect cells from oxidative stress but does not seem to play a noticeable role under normal culture conditions.

**DISCUSSION**

Nrf2 is one of the key transcription factors involved in regulating the expression of antioxidative stress genes. In this study, we demonstrated that HCMV infection of HFFs could activate Nrf2, thus resulting in increased HO-1 expression by the CK2 pathway. When host cells were...
stressed by BSO, HCMV-infected cells survived more efficiently than uninfected control cells through the activation of Nrf2. Our data suggest that HCMV may control cellular Nrf2 for its own benefit, e.g. to overcome the oxidative stress imposed by itself or the host’s disease status.

It has recently been reported that hsp90 expression is increased following antioxidant or heat shock treatment and subsequently interacts with Keap1, which is phosphorylated by CK2, leading to the dissociation of the Keap1–Nrf2 complex and the stabilization of Nrf2 (Niture & Jaiswal, 2010). HCMV has been shown to increase hsp90 expression, and our data indicate that HCMV-mediated Nrf2 activation was abolished following treatment with CK2 inhibitors (Stanton et al., 2007). Altogether, these data would suggest that HCMV activates Nrf2 by manipulating the hsp90 and Keap1 interaction. These data may also explain how HCMV infection activates Nrf2 independently of ROS production.

It has recently been reported that Nrf2 is not activated by HCMV infection in HFFs expressing the catalytic subunit of human telomerase. It is not yet clear what contributed to this discrepancy (Tilton et al., 2011). One possibility is the difference in the manner in which the telomerase-expressing cells were constructed. In our case, HFFs were used after only five or six passages following the initial retroviral transduction, while Tilton et al. established their cell line through drug selection and subcloning.

Viral infections in general have been known to generate oxidative stress in host cells, which would impose negative effects on the virus because the host cell would be damaged, as has been shown in the case of human immunodeficiency virus, herpes simplex virus, HBV and HCV infections (Joyce et al., 2009; Liu et al., 2008; Valyi-Nagy & Dermody, 2005). Oxidative stress can be caused directly by the actual viral infection and/or an indirect

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Fig. 6. The possible role of Nrf2 in HCMV-mediated cell protection against oxidative stress. (a) HFFs infected or mock-infected with HCMV were treated or left untreated with BSO (2 mM) at 2 h p.i. The intracellular concentration of GSH was measured at 24 h following BSO treatment. (b) The viability of mock-infected and HCMV-infected cells was analysed at 4 days following BSO treatment. (c) HFFs expressing either type of shRNA were treated as mentioned above, and the cell viability was measured. (d) HFFs expressing shRNAs, normal HFFs and mock-transduced HFFs were infected with HCMV. The titres of progeny virus in culture supernatant were measured at the times indicated using infectious centre assays. ICU, Infectious centre unit. Data are presented as mean ± SD of triplicate samples; ns, non-significant; *, P<0.05; ***, P<0.001 (one-way ANOVA and Tukey’s multiple comparison test).
consequence of the host inflammatory response. HCMV infection has been reported to induce oxidative stress in cell culture as well as a systemic inflammatory response in patients who present with a primary HCMV infection, suggesting that HCMV infection generates oxidative stress (Speir et al., 1996; van de Berg et al., 2010). Pre-existing inflammation could be another source of oxidative stress because HCMV has been shown to be reactivated from latency in several inflammatory diseases (Varani & Landini, 2011). To cope with these stresses, HCMV may have developed the means to survive or reduce the detrimental effects of oxidative stress, as in the case of HBV, which has also been shown to activate Nrf2 (Schaedler et al., 2010).

Neither the viral growth rate nor the cell viability were significantly affected in host cells engineered by knock-down to express Nrf2 at low levels. Therefore, it appears that Nrf2 does not play a significant role under unstressed conditions, even in the context of HCMV infection. The magnitude of the oxidative stress imposed by in vitro HCMV infection might not be severe enough for Nrf2 to show any visible effects. Indeed, it has been shown that Nrf2 does not have any clear phenotypic effects under unstressed conditions, as evidenced in experiments involving mice that were genetically modified to lack or overexpress Nrf2 (Calkins et al., 2010; Chan et al., 1996). When the cells were treated with BSO, however, the expression level of Nrf2 was important for the survival of host cells under stressful conditions. Taken together, it is possible that HCMV infection may render cells more resistant to oxidative stress, such as that induced by inflammatory responses, for its own benefit.

Our findings have implications for understanding the chronic inflammation characteristic of HCMV-related diseases. The cell-protective effect of Nrf2 might lead to increased survival of HCMV during oxidative stress and thus HCMV persistence under these conditions. Persistence would result in further oxidative stress, contributing to the establishment of chronic inflammation.

METHODS

Cell culture and reagents. For HFFs, cells of passage 12–20 were used. Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % (v/v) FBS and 1 % (v/v) penicillin/streptomycin solution (Invitrogen-Gibco) at 37 °C in a humidified atmosphere of 5 % CO2. Human neuroblastoma cell line SK-N-SH was obtained from the Korean Cell Line Bank, and cultured in Eagle’s minimum essential medium (Sigma) supplemented with penicillin-streptomycin-glutamine (Invitrogen-Gibco). LY294002 and Wortmannin were obtained from Cayman Chemicals, and all other chemicals were from Sigma.

Viruses and infections. For preparation of HCMV strain Towne, HFFs were infected at an m.o.i. of 0.01 and incubated for 10–14 days until visible cytopathic effects were observed. Culture supernatants were collected, filtered through a 0.45 μm membrane, and concentrated by ultracentrifugation at 92 600 g for 1.5 h at 4 °C. Viral stocks were aliquoted and stored at −70 °C. The titres of viral stocks were determined as an infectious centre unit (ICU) after the measurement of the IE1/IE2-positive cells by infectious centre assay (Huh et al., 2008). Clinical strain JHC (passage 3), originally isolated from patients undergoing bone marrow transplantation, was provided by Dr Chan Hee Lee (Chungbuk National University, Cheongju, Korea), and passed one more time in HFFs (Jung et al., 2011). To prepare UV-treated HCMV, viral stocks were placed in 1.5 ml tubes and exposed to 254 nm UV light (40 W) for 1 h. For HCMV infection, cells seeded on the previous day were incubated with viral inocula for 2 h at 37 °C in the CO2 incubator. Unless otherwise mentioned, all infections were performed at an m.o.i. of 3.

Plasmids. The plasmid expressing the catalytic subunit of human telomerase (pCS2-htERT) was provided by Han-Woong Lee (Yonsei University, Seoul, Korea). The hTERT gene was PCR amplified from this plasmid using the following primers: forward, 5’-AGATCTCTAT-GCCGGCGGCTCCCGCGTCGCGGA-3’; reverse, 5’-AGATCTTCTCAGGATTGGTCTTTAGTAAGTC-3’.

The PCR product was cloned into the BanHI site of the retroviral vector MSIG (Jang et al., 2012). This vector contains the LTR originally from a murine stem cell virus, and eGFP is expressed as a bicistronic message using the internal ribosome entry site (IRES) from encephalomyocarditis virus.

The shRNA expression vector was constructed using the plasmid MSIG as a backbone. Optimal shRNA sequences targeting human Nrf2 were designed using web-based software (siDESIGN Center). A 19 nt target sequence and its corresponding position were designed as follows: Nrf2 (human), 5’-TGCAGAAGTTGACATTTA-3’, nucleotides 659–677. The shRNA sequence targeting luciferase (5’-GGCCGGTTGCTAGTACCAAC-3’) was used as the control. After annealing complementary single-stranded oligonucleotides, double-stranded fragments were inserted into the BglII and HindIII sites of the vector pSUPER.retro.puro (OligoEngine). The H1 promoter-driven shRNA expression cassette was excised using the EcoRI and Xhol sites from this construct and subcloned into MSIG.

Generation of transduced cells. Retroviral vectors were prepared by the three-plasmid transfection method using Lipofectamine and Plus reagent (Invitrogen) according to the manufacturer’s protocols. The packaging constructs include pVM-gp for the gag-pol genes from murine leukemia virus (MLV) and pCA-VSVG for the env gene (Yu et al., 2003). Two days later, supernatants were collected and concentrated as described in the Viruses and infections section. The retroviral vector titres were determined by measuring the percentage of GFP-positive HFFs transduced with different dilutions of the viral stock. To generate gene transferred HFFs, 105 cells were seeded in six-well plates on the previous day and transduced with the retroviral vectors at an m.o.i. of 5. Cells were expanded, and the transduction efficiency was measured as described above. Polybrene (8 μg ml−1) was included to increase the transduction efficiency.

Northern blot hybridization. Total RNAs were isolated from HFFs using Trizol reagent (Invitrogen). Twenty micrograms of RNA were subjected to 1 % (w/v) formaldehyde–agarose gel electrophoresis and transferred to nylon membrane (GE Healthcare) by overnight capillary blotting. Specific cDNA probes for human HO-1 gene were prepared by random priming using the Klenow fragment of DNA polymerase I (Stratagene) and [α-32P]dCTP (GE Healthcare). The membranes were hybridized with labelled probes (106 c.p.m. ml−1) for 1 h at 68 °C, washed with 0.2 × SSC and 0.1 % (w/v) SDS at 55 °C for 30 min, and then exposed to Image plate (Fuji Film).

Immunoblotting. Cells were lysed with Cytobuster (Novagen) supplemented with protease and phosphatase inhibitor cocktail (Roche Diagnostics) for 15 min at 4 °C. Protein concentration was determined by a Bradford assay (Bio-Rad). Equal amounts of protein were resolved by 9–12 % (w/v) SDS-PAGE and transferred to PVDF

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membranes (GE Healthcare). The membranes were blocked for 1 h at room temperature with TBST (150 mM NaCl, 10 mM Tris/HCl, 0.1% (v/v) Tween 20, pH 8.0) containing 1% (w/v) BSA (Invitrogen-Gibco). The membrane was incubated with primary antibodies diluted in block overnight at 4°C. After being washed with TBST for 20 min three times, the membranes were incubated with HRP-conjugated goat anti-mouse or rabbit IgG secondary antibody (A0168 or A0545, Sigma) for 1 h at room temperature. The blots were then washed three times with TBST, and the protein bands were visualized with the enhanced chemiluminescence system (Millipore).

Immunoblotting was performed using the following primary antibodies: anti-HO-1 (SPA-894; Virusys), anti-IE (MAb810; Millipore), anti-pp28 (CA004; Virusys), anti-β-actin (A5441; Sigma), anti-phospho Akt (Ser473) (4060; Cell Signalling), and anti-Akt (610876; BD Pharmingen).

Immunofluorescence. The procedure for immunofluorescence with HCMV-infected fibroblasts was as described by Buchkovich et al. (2009). Immunofluorescence was performed using the following primary antibodies: anti-IE, anti-HO-1, anti-Nrf2 (ab62352; Abcam), anti-hTERT (600-401-252; Rockland).

Detection of intracellular ROS. The level of intracellular ROS was determined using fluorescence-based ROS indicator, 5-(and-6)-(chloromethyl)-2’,7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA; Invitrogen). Cells were washed in pre-warmed PBS, and incubated with 1 μM of CM-H2DCFDA diluted in Hank’s balanced salt solution (HBSS) (Sigma) for 5 min. After incubation, cells were washed in PBS and further incubated in HBSS for 30 min at 37°C. Then, cells were rapidly trypsinized and resuspended in HBSS. The fluorescence intensity of cells was measured using FACS with the aid of CellQuest software (BD).

GSH and cell viability assay. Intracellular GSH concentration and cell viability were measured using the Glutathione Assay kit (Cayman) and the Cell Proliferation kit I (Roche), respectively, according to the manufacturers’ protocols.

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