Human cytomegalovirus immediate early proteins upregulate endothelial p53 function

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Abstract Infected endothelial cells are found to be resistant to apoptosis possibly mediated by p53 cytoplasmic sequestration. We explored whether the immediate early 84 kDa protein (IE84) of cytomegalovirus (CMV) is responsible for p53 cytoplasmic sequestration. The endothelial cells were transfected with plasmids containing IE1 and 2 coding regions which are known to synthesize IE84 and 72 proteins. Our study found that p53 expression was significantly elevated in endothelial cells transfected with IE1 and 2 plasmids. However, p53 was only found in the nucleus rather than sequestered in the cytoplasm. We have demonstrated that IE84 and 72 are not responsible for p53 dysfunction caused by CMV infection, rather they upregulate p53 function and promote endothelial apoptosis.

Key words: p53; Cytomegalovirus; Immediate early gene; Endothelial apoptosis

1. Introduction

Response to injury is one of the major hypotheses on the origin of atherosclerosis, which is marked as endothelial dysfunction and followed by development of fatty streaks composed of lipid laden macrophages, T cells and smooth muscle cells [1,2]. Early lesions may progress into fibrous plaques and complicated plaques [3]. A common viral infection may be the first step in the complex process of the development of atherosclerosis, a concept that is consistent with the response-to-injury hypothesis [4]. Human cytomegalovirus (CMV), a member of the herpesviruses, can infect human vascular endothelial cells and induces changes relevant to atherogenesis [5]. CMV infection increases expression of endothelial surface adhesion molecules, which upregulate the recruitment of granulocytes [6–8]. CMV infection also changes endothelium from anticoagulant to procoagulant status [8]. Recently, it has been found that actively proliferating CMV increases resistance of endothelial cells to apoptosis, which provides a period long enough for CMV propagation and for the initiation of atherosclerosis [9]. The mechanism of resistance to apoptosis was suggested to be cytoplasmic sequestration of p53 [9]. However, the molecular mechanism responsible for such sequestration is still not clear.

p53, a short-lived protein, is maintained at low levels in an inactivate state in unstressed cells [10,11]. In response to stimulation by cellular stress, p53 transcription and expression are enhanced. The p53 protein is transiently stabilized as a tetramer and functions as a transcriptional factor in the nucleus, where it induces the transcription of genes including $p21^{WAF1/CIP1}$, *bax*, and *hdm2* (*mdm2* in mouse) [12–14]. $p21^{WAF1/CIP1}$ and *bax* mediate the anti-proliferative function of p53 by blocking cell cycle progression and provoking apoptosis [13,15]. However, *hdm2* encodes a cellular negative regulator of p53, which constantly transfers p53 dimers and monomers that split from the tetramer in the nucleus to the cytoplasm. It also targets p53 to the cytoplasmic proteasome for degradation [16]. Studies have shown that the blocking of hdm2 nuclear export can stabilize p53 in the nucleus, and the increasing expression of hdm2 can sequester most of the p53 in the cytoplasm [17].

A number of immediate early (IE) proteins of CMV are expressed following entry of the virus into cells. IE2 encoded IE84 is one of the major CMV immediate early proteins and appears to be a promiscuous transactivator of viral and cellular gene expressions [18]. IE84 has been found to bind to the C-terminus of p53 that has the tetramerization function [19]. It also has been found that actively proliferating CMV increases hdm2 expression but not p21 in vivo, which in turn sequesters p53 in the cytoplasm for degradation [20]. We hypothesize that CMV immediate early protein IE84 could contribute to p53 cytoplasm sequestration possibly by preventing p53 from forming tetramers.

2. Materials and methods

2.1. Endothelial cells culture

Primary human umbilical vein endothelial cells (HUVEC) were isolated as previously described [9] and were grown at 37°C in a humidified 5% CO_2/95% air atmosphere in M199 medium, containing 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), sodium pyruvate (1 mmol/l), L-glutamine (4 mmol/l), heparin (30 μg/ml) and supplemented with endothelial cell growth factor (100 μg/ml). Cells cultured up to three passages were used in the experiment.

2.2. Plasmids

Plasmids containing the CMV major IE promoter upstream of IE84 (IE2 plasmid, a kind gift from Y.S. Huang, University of North California), and IE84 and 72 coding regions (IE1 and 2 plasmid, a kind gift from Linda Burns, University of Minnesota) have been previously described and verified in synthesis of the appropriate IE proteins. Control plasmid does not encode CMV immediate early proteins.

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2.3. Transfection

Confluent HUVEC in a 100 mm petri dish were harvested by 0.1% trypsin. 10 ml full medium was added to inactivate trypsin. The suspension was centrifuged at $300 \times g$ for 2 min to collect the cells. The supernatant was discarded and cells were resuspended in full culture medium to obtain a cell density of 5×10^6 cells/ml. 400 μl of the cell suspension was transferred to a 4 mm Gap cuvette. The IE2 or IE2 and IE1 72 containing plasmid (50 μl, 36 μg/ml) was added in the cuvette and mixed by aspiration. The cuvette was placed in an electroporation apparatus (Genetronics) with the setting of 400 V, 975 μF capacitance, 24 Ω resistance, 12.5 ms pulse length. After electroporation, cells were transferred to a 6 well plate containing 1% gelatin coated coverslips and incubated at 37°C 5% CO_2/95% air for 48 h before fixing them.

2.4. Infection of HUVEC with CMV

The VHL strain of CMV was used in the study (a kind gift from Dr. W.J. Waldman, Ohio State University). The virus was propagated as previously described [21]. Titration of the stock CMV was determined by a rapid quantitation assay using an immunofluorescent detection method for CMV major IE viral protein. CMV was thawed at 37°C and sonicated in a cold water bath for 10 min. After removing the growth medium, the confluent HUVEC were washed with Hanks' balanced salt solution (HBSS). CMV suspended in HUVEC culture medium inoculated HUVEC at a multiplicity of infection of 0.1 plaque forming units/cell.

2.5. Immunofluorescent staining and confocal microscopy

HUVEC grown on coverslips were washed with phosphate buffered solution (PBS), fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with cold methanol for 20 min. The non-specific binding was blocked by 1% bovine serum albumin (BSA) and incubated with mouse anti-CMV immediate early protein antibody (1:10 000, Chemicon, Temecula, CA), and rabbit anti-p53 antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) at 37°C for 1 h. After washing with PBS, FITC conjugated goat anti-mouse IgG (1:100, Sigma-Aldrich) and TRITC conjugated goat anti-rabbit IgG (1:100, Sigma-Aldrich) were added and incubated in the dark at room temperature for 40 min. After washing three times with PBS, the coverslips were mounted with fluorescent-G (Southern Biotech Assoc., Birmingham, AL) and put face down onto slides. The edges of the coverslips were sealed with nail polish. The fluorescent image was visualized with an MRC 2000 confocal imaging system.

2.6. Viability assay

Viability was measured by a neutral red inclusion assay [22]. After serum starvation for 48 h, transfected and non-transfected HUVEC grown in 48 well cell culture plates were washed with HBSS buffer to remove serum, and serum free M199 was added to one-half of the wells, whereas serum containing medium was added to the other half. After 48 h incubation at 37°C in 5% CO_2/95% air, the wells were washed with HBSS. The culture medium (0.5 ml) containing the lysosomally accumulated vital dye neutral red (50 μg/ml) was added to each well for 2 h. The cells were washed with HBSS, and the dye absorbed by viable cells was extracted with acidified ethanol (50:50 mixture of absolute ethanol and 1% acetic acid). The optical density (OD) of the supernatant was measured at 540 nm. Viability was calculated as the percentage of OD ratio of serum starved cells to non-serum starved cells. In each experiment, four test wells per group were used.

2.7. Apoptosis and co-localization with CMV infection

After 48 h serum starvation, HUVEC grown on coverslips were washed with HBSS, fixed with 4% paraformaldehyde, and permeabilized with cold methanol. The apoptotic cells were detected by TdT-mediated dUTP nick end labeling (TUNEL), using the In Situ Death Kit (Cat. #1684795, Roche Molecular Biochemicals, Germany). After TUNEL staining, the cells were washed with PBS, and incubated in 1% BSA for 20 min to block non-specific binding of antibody. Then the cells were incubated with mouse anti-CMV immediate early protein antibody (1:10 000, Chemicon, Temecula, CA) for 1 h at 37°C before TRITC conjugated goat anti-mouse IgG (1:500, Sigma-Aldrich, St. Louis, MO) was added onto cells and incubated for 0.5 h. The cells were then washed with HBSS three times and the images were visualized under confocal microscopy.

2.8. Statistics

Results are expressed as the mean ± S.E.M. and Student's *t*-test was applied using the Sigmastat program.

3. Results

3.1. Effects of IE on p53 expression and cytoplasm sequestration

For the primary cultured HUVEC, transfection efficiency is often a problem and most methods can only obtain 1 or 2% cells transfected. With the optimized electroporation method in our lab, we were able to achieve 10% HUVEC transfection (data not shown). HUVEC were fixed and double labeled for CMV IE84 and p53 protein after 48 h transfection with IE2. As shown in Fig. 1.1A–C, p53 expression could not be detected in non-transfected HUVEC and HUVEC transfected with control plasmids. However, p53 expression in the IE2 transfected HUVEC was significantly elevated (Fig. 1.1D–F). Most p53 in transfected cells distributed in the nucleus rather than in the cytoplasm.

To explore whether IE84 needs the cooperation of IE72 to sequester p53 in the cytoplasm, HUVEC was transfected with IE1 and 2 plasmids (Fig. 1.1G–I). p53 was also upregulated in IE1 and 2 transfected HUVEC. Most of the p53 was still localized in the nucleus in the transfected cells. These findings indicate that immediate early gene encoded proteins elevated p53 expression, but did not sequester p53 in cytoplasm.

We further confirmed the findings in CMV directly infected endothelial cells. CMV genes were sequentially expressed following the infection to mammalian cells. Expression of CMV genes is divided into immediate early, early, and late stages according to the type of gene expressed. Only the immediate early genes are expressed in the first 6 h after the initial infection. The immediate early proteins function as transcriptional factors, which are essential for the early and late genes. Since we found that IE gene encoded proteins did not sequester p53 in cytoplasm, we explored whether the same phenomena could be seen in the CMV infected HUVEC. After 4 h infection, we localized p53 in the CMV infected cells by double immunofluorescent staining as described in Section 2. As shown in Fig. 1.1J–L, p53 expression was elevated in CMV infected HUVEC, and most p53 distributed in the nucleus rather than the cytoplasm.

3.2. Effects of IE on endothelial apoptosis

It is known CMV abrogates p53 dependent apoptosis induced by serum starvation. To explore whether IE84 alone or IE84 and IE72 could inactivate p53 function, serum starvation for 48 h was applied to IE2 or IE1 and 2 transfected HUVEC to induce p53 dependent apoptosis. Apoptotic cells were demonstrated using the TUNEL assay. As shown as Fig. 1.2, most serum starvation induced apoptosis co-localized with IE2 alone (Fig. 1.2a–c) or IE1 and 2 (Fig. 1.2d–f) transfected HUVEC. IE2 alone and IE1 and 2 transfected HUVEC were more likely to undergo p53 dependent apoptosis. This implies that IE84 alone or IE84 with IE72 did not inactivate the p53 function of inducing apoptosis. In contrast, p53 upregulated by IE84 made more endothelial cells undergo apoptosis.

However, for endothelial cells infected with intact CMV virus, p53 started to be sequestered to the cytoplasm when the infected cells were observed at 48 h post-infection (data

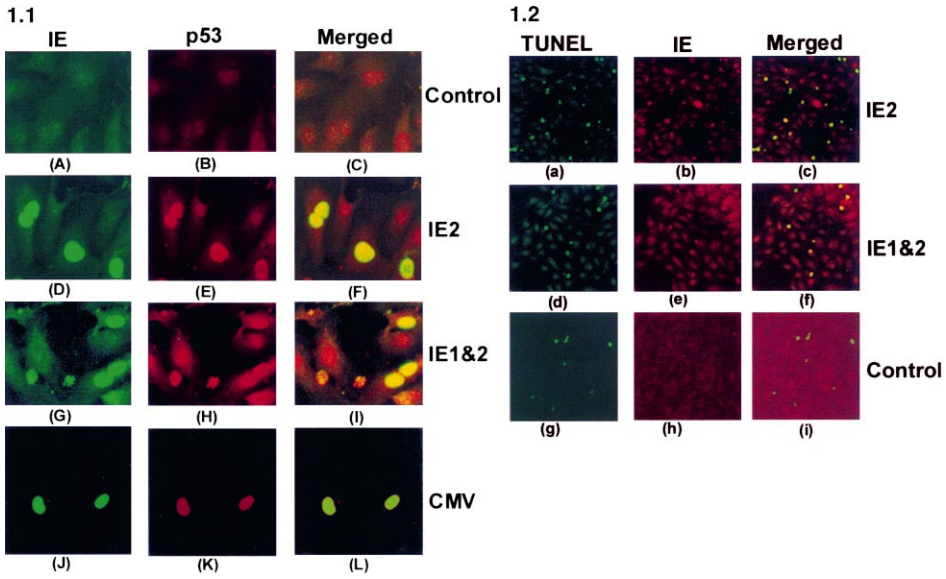

Fig. 1. CMV IE2 alone or IE1 and 2 increased p53 expression in HUVEC but did not sequester p53 in the cytoplasm. HUVEC were transfected with IE2 or IE1 and 2 plasmids which encode IE84 and IE72 and 84 proteins respectively. We co-localized p53 and IE84 or IE72 and 84 in transfected HUVEC with immunofluorescence staining as described in Section 2. p53 expression was increased in IE2 or IE1 and 2 transfected HUVEC compared to non-transfected HUVEC, and all p53 localized in nucleus (1.1A–I). At the immediate early stage of CMV infection, p53 expression was upregulated and localized in the nucleus of HUVEC. HUVEC were infected with VHL CMV for 4 h, in which only CMV immediate early proteins were expressed. p53 was upregulated in CMV infected cells compared to non-infected cells, and all the p53 localized in the nucleus of HUVEC (J–L). Serum starvation induced apoptosis co-localized with IE2 and IE1 and 2 transfected cells. After 48 h transfection with IE2, IE1 and 2, non-specific plasmid, HUVEC were serum starved for 48 h. Most of the apoptotic cells co-localized with IE2 or IE1 and 2 transfection (1.2a–f, i).

not shown). During this late stage, CMV infected endothelial cells were clearly more resistant to apoptosis than controls. These endothelial cells were also serum starved for 48 h after 48 h infection with CMV. The apoptosis which was identified with TUNEL co-localized with CMV infection that was identified with immunofluorescent staining of IE. As shown in the merged photo of Fig. 2, no CMV infected HUVEC co-localized with apoptosis. In other words, CMV infection protected HUVEC from serum starvation induced apoptosis, which is consistent with a previous report [9].

We also measured the cell viability after 48 h serum starvation. The viability assay was conducted three times in triplicate. As shown in Fig. 3, IE84 alone and IE84 with IE72 did not protect HUVEC from serum starvation induced apoptosis ($P < 0.05$). Fewer IE2 or IE1 and 2 transfected cells were viable compared to control plasmid transfected and non-transfected cells. In contrast, more viable cells were found in CMV infected endothelial cells than in controls.

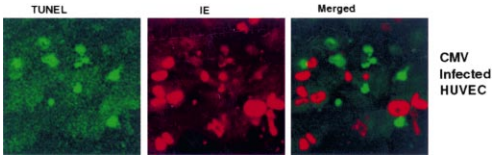

Fig. 2. CMV infection protected HUVEC from serum starvation induced apoptosis. After 48 h infection, HUVEC were serum starved for 48 h. The CMV infection was identified with immunofluorescent staining of IE, and apoptosis was identified with TUNEL staining. The same images were merged together to co-localize the apoptosis and CMV infection. As shown in the merged photo, no CMV infected HUVEC co-localized with apoptosis.

4. Discussion

In exploring the mechanism of CMV induced resistance to p53 dependent apoptosis, we have shown that IE84 alone or IE84 and 72 upregulated p53 expression but were unable to sequester it in the cytoplasm. The upregulation of p53 expression is consistent with the report by Muganda et al. [23]. This result was also confirmed in CMV infected HUVEC. At the immediate early stage of infection, p53 was upregulated but still localized in the nucleus. At this stage, IE proteins have made cells more likely to undergo apoptosis possibly mediated by upregulated p53. Humans may benefit from this increased p53 expression and apoptosis at the immediate early stage of CMV infection by eliminating CMV infected cells. This increased apoptosis was also demonstrated in both IE2 and IE1 and 2 transfected endothelial cells, which had increased p53 expression and were more likely to undergo apoptosis. The IE1 and 2 transfection also tended to co-localize with positive apoptosis as demonstrated by the TUNEL assay.

Although Fortunato et al. [24] suggested that CMV infection increased the p53 level by reducing p53 degradation, reports by Muganda et al. [23] indicated that IE84 could increase p53 transcription and expression. Our results are consistent with an increased expression. p53 has to be transported to the proteasome in the cytoplasm for degradation. However, in IE2 or IE1 and 2 transfection and the immediate early stage of CMV infection, all the elevated p53 was localized in the nucleus rather than the cytoplasm.

Although IE84 has the capacity to bind to the C-terminus of p53 [25], which could potentially inhibit p53 tetramerization, and thus p53 function, our experiments were unable to demonstrate whether there was any effect on p53 tetramerization. Nevertheless, we have clearly shown that p53 function

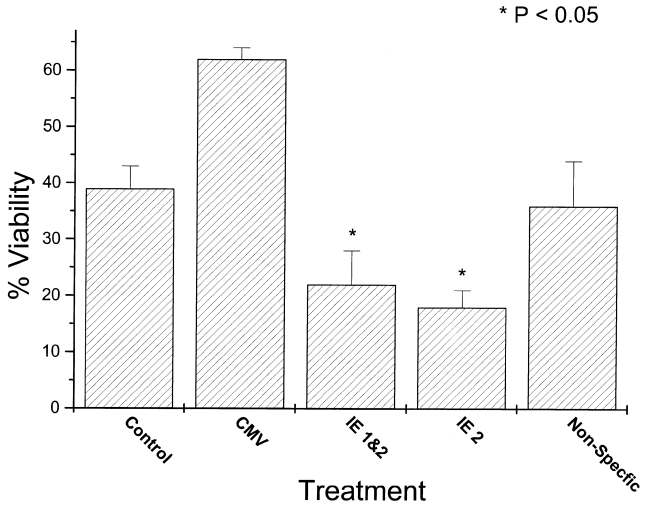

Fig. 3. IE2 or IE1 and 2 plasmids transfected HUVEC were not resistant to serum starvation induced apoptosis compared to control cells. After 48 h transfection with IE2 or IE1 and 2, half of the HUVEC were serum starved for 48 h. Half of the cells were maintained in normal medium as control. Half of the HUVEC cultured in normal medium and HUVEC infected with CMV for 48 h were also serum starved for 48 h. The viability of serum starved cells was measured with the neutral red inclusion assay and calculated as percentage of OD ratio of serum starved test cells to non-serum starved cells. The viability of IE2 alone or IE2 with IE1 transfected cells was significantly lower than the control cells. The CMV infected cells (48 h), however, had increased viability.

was upregulated rather than inactivated in IE transfected cells. This could imply either that IE84 did not bind to the C-terminal of p53 and inhibit p53 tetramerization, or that tetramerization was not essential for p53 function.

In conclusion, IE84 upregulated p53 expression but did not sequester p53 in the cytoplasm to inactivate p53 function. Endothelial cells transfected with IE plasmids or at the early stage of CMV infection were more likely to undergo apoptosis, which may be a protective mechanism for humans to eliminate CMV infected cells. It should be noted, however, that more studies will be needed to directly link the nuclear p53 accumulation induced by CMV immediate early proteins to the accelerated endothelial apoptosis.

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