Effects of Human Cytomegalovirus Immediate-Early Proteins on p53-mediated Apoptosis in Coronary Artery Smooth Muscle Cells

Koichi Tanaka, MD, PhD; Jian-Ping Zou, MD, PhD; Kazuyo Takeda, MD, PhD; Victor J. Ferrans, MD, PhD; Gordon R. Sandford, PhD; Thomas M. Johnson, PhD; Toren Finkel, MD, PhD; Stephen E. Epstein, MD

Background—Restenotic and atherosclerotic lesions often contain smooth muscle cells (SMCs), which display high rates of proliferation and apoptosis. Human cytomegalovirus (HCMV) may increase the incidence of restenosis and predispose to atherosclerosis. Although the mechanisms contributing to these processes are unclear, studies demonstrate that one of the immediate-early (IE) gene products of HCMV, IE2-84, binds to and inhibits p53 transcriptional activity. Given the role of p53 in mediating apoptosis, we studied the ability of IE2-84 to inhibit p53-dependent apoptosis in human coronary artery SMCs.

Methods and Results—Apoptosis of SMCs was induced either by use of an adenovirus vector encoding human wild-type p53 protein or by treatment with doxorubicin. HCMV IE1-72 and IE2-84, the major IE proteins of HCMV, were overexpressed separately with adenovirus vectors encoding each protein, and the effects on p53-induced apoptosis were examined by both nick end-labeling (TUNEL) assay and flow cytometry. Expression of IE2-84, but not IE1-72, protected SMCs from p53-mediated apoptosis.

Conclusions—These data indicate that an HCMV IE protein antagonizes p53-mediated apoptosis, suggesting a pathway by which HCMV infection predisposes to SMC accumulation and thereby contributes to restenosis and atherosclerosis. (Circulation. 1999;99:1656-1659.)

Key Words: viruses ■ proteins ■ apoptosis ■ restenosis

Evidence implicates human cytomegalovirus (HCMV) as a contributing agent in the pathogenesis of restenosis and atherosclerosis.1–3 One mechanism to account for these effects is through the known ability of HCMV to enhance cellular proliferation.4 This proliferative signal may relate to the ability of the immediate-early (IE) gene product of HCMV, IE2-84, to bind p53 and inhibit its transactivational activity.1,4 Because p53 transcriptionally regulates gene products such as the cyclin-dependent kinase inhibitor p21, which in turn regulates cell cycle progression,5 the inhibitory effect of IE2-84 on p53 could increase smooth muscle cell (SMC) proliferation and thereby increase SMC accumulation. However, the extent of SMC accumulation is not simply a function of proliferation rate. Apoptosis occurs in restenotic and atherosclerotic lesions,6 and alterations in this process could contribute to SMC accumulation.6 Because wild-type p53 activates an apoptotic program in SMCs,7,8 HCMV-induced p53 inhibition might prevent p53-modulated apoptosis, providing a mechanism whereby HCMV may augment SMC accumulation in developing lesions. Previous studies demonstrated that HCMV infection inhibits apoptosis in human endothelial cells, an effect apparently mediated by exclusion of p53 from the nucleus.9

In the present investigation, we further elucidated the role of HCMV in modulating apoptosis. Specifically, we determined whether IE1-72 and IE2-84, the major HCMV IE proteins, inhibit p53-induced apoptosis in human coronary artery SMCs.

Methods

Cells
Human coronary artery SMCs were purchased from Clonetics Corp and cultured in smooth muscle basal medium (Clonetics Corp) with insulin 5 μg/mL, human epidermal growth factor 0.5 ng/mL, human fibroblast growth factor-B 2 ng/mL, penicillin 10 000 U/mL, streptomycin 10 000 μg/mL, and 5% FBS.

Adenovirus Vectors
The adenoviruses Ad.IE1 and Ad.IE2 contain the HCMV IE1-72 and IE2-84 genes, respectively, under control of the HCMV major IE
promoter (MIEP) and upstream of a polyadenylation signal, inserted into an Ad5 vector. During transgene insertion, homologous recombination results in deletion of the entire E1A region and ~90% of the E1B coding sequence of wild-type adenovirus. The adenovirus Ad.p53 (a gift from Silvia Bacchetti, McMaster University, Hamilton, Ontario, Canada) contains an expression cassette consisting of the HCMV MIEP promoter and SV40 T-antigen polyadenylation signal flanking coding sequences for human wild-type p53 protein, inserted into an E1-deleted Ad5 vector. Ad.βGal, used as a control adenovirus, is an E1-deleted Ad5 vector containing the Escherichia coli LacZ gene controlled by MIEP.

Apoptosis Induced by p53 Through Adenoviral Gene Transfer in Coronary Artery SMCs

Twenty-four hours after plating, cells were infected with Ad.IE1, Ad.IE2, or Ad.βGal at 200 multiplicity of infection (MOI). Viruses were removed from the medium 24 hours after infection, and then cells were superinfected with 100 MOI of Ad.p53 to induce apoptosis. As a control, cells were superinfected with 100 MOI of Ad.βGal instead of Ad.p53. The total amount of adenovirus infection in each group was 300 MOI. Virus was removed from the medium 24 hours after superinfection. Apoptosis was assessed by nick end-labeling (TUNEL) assay at 48 hours or by flow cytometry at 72 hours after superinfection.

Doxorubicin-Induced Apoptosis in Coronary Artery SMCs

Coronary artery SMCs were infected with Ad.IE1, Ad.IE2, or Ad.βGal at 200 MOI 24 hours after plating. Virus was removed from the medium 24 hours after infection, and cells were cultured in regular medium for an additional 24 hours before addition of 1.0 μmol/L doxorubicin (Sigma Chemical Co). Adherent cells were harvested 24 hours after addition of doxorubicin for the expression of endogenous p53 and 48 hours after for quantification of the proportion of apoptotic cells by flow cytometry.

Assays for Apoptosis

For demonstration and quantification of apoptosis, TUNEL assay was performed with an ApopTag Direct In Situ Apoptosis Detection Kit—Fluorescein (Oncor) according to the manufacturer’s recommendations. Apoptotic nuclei were labeled with fluorescein, and all nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, Sigma). Samples were examined with a laser scanning confocal fluorescence microscope.

Flow cytometric quantification of apoptotic cells was performed as described by Nicolletti et al. Adherent cells were harvested and stained in hypotonic fluorochrome solution (propidium iodide 50 μg/mL in sodium citrate plus 0.1% Triton X-100, Sigma). Cells were analyzed by a fluorescence-activated cell sorter (FACSscan) flow cytometer (Becton Dickinson). Apoptotic nuclei were identified as a subgenomic DNA peak and were distinguished from cell debris on the basis of both forward light scatter and fluorescence of propidium iodide.

Western Blot Analysis

Western blot was performed with 10 μg of cell lysate with mouse monoclonal antibodies against IE1-72 and IE2-84 (Vancouver Biotech), p53 (DO-1; Santa Cruz Biotechnology), or p21 (Santa Cruz Biotechnology). A monoclonal antibody against α-tubulin (Calbiochem) was used to confirm equal protein loading. Immune complexes were detected by enhanced chemiluminescence (Amersham).

Statistical Analysis

Differences between groups were analyzed by 1-way ANOVA. A value of P<0.05 was considered statistically significant.

Results

After adenovirus-mediated gene transfer, IE1 or IE2 expression was detected by Western blot of SMC lysate (Figure 1).

To understand the biological effects of these IE gene products, we used a model of p53-dependent apoptosis. Certain cells, including primary human SMCs and transformed colonc epithelial cells, undergo apoptosis after adenoviral gene transfer of wild-type p53. As demonstrated in Figure 2A, expression of p53 significantly increased the percentage of TUNEL-positive nuclei in SMCs. Coexpressed IE2-84 markedly decreased, while coexpressed IE1-72 potentiated the level of p53-induced apoptosis. Similar results were obtained by FACS analysis (Figure 2B and 2C). The protective effects of IE2-84 were not mediated by a change in the level of p53 expression (Figure 2D).

The ability of p53 to induce apoptosis may depend on its function as a transactivator of gene expression. Although p53 regulates several gene products, the best-characterized product is the cyclin-dependent kinase inhibitor p21. Using p21 levels as an indicator of p53 transcriptional activity in SMCs, we assessed the effects of IE gene expression on p53 transcriptional activity. P53 expression significantly increased p21 levels (Figure 3). Coexpression of IE1-72 potentiated this increase, whereas expression of IE2-84 inhibited it. The degree of inhibition by IE2-84 was dependent on the level of IE2-84 expression. These results are consistent with the notion that IE gene products modulate p53-dependent apoptosis by regulating p53 transcriptional activity.

To assess whether other agents that induce p53-mediated apoptosis are also altered by IE gene products, we treated SMCs with doxorubicin. When added to SMCs at a concentration of 1.0 μmol/L for 24 hours, doxorubicin increased p53 levels (Figure 4) and induced apoptosis in Ad.βGal-infected cells (the number of apoptotic cells increased 4-fold above control values). This is consistent with results in other cell types demonstrating that doxorubicin can induce apoptosis through a p53-dependent pathway. Consistent with our results with direct p53-mediated apoptosis, IE1-72 increased and IE2-84 suppressed doxorubicin-induced apoptosis (Figure 5).
Discussion

This investigation demonstrates that the IE gene products of HCMV, IE1-72 and IE2-84, have the capacity to influence apoptosis in coronary artery SMCs. It is of interest that the functional roles of IE1-72 and IE2-84 are remarkably similar to those of oncoproteins encoded by DNA tumor viruses such as E1A and E1B of adenovirus or E7 and E6 of human papillomavirus. In each case, one of the major IE proteins appears, like IE2-84 of HCMV, to have antiapoptotic properties. The capacity of IE2-84 to inhibit a p53-dependent signaling pathway that leads to p21 induction and apoptosis is consistent with previous studies indicating that IE2-84 binds to p53 and inhibits its capacity to transactivate a promoter controlling a downstream reporter gene. In contrast, the other viral IE proteins appear, like IE1-72, to have proapoptotic effects.

That these observations are not limited to experimental conditions in which p53 is overexpressed through adenoviral gene transfer is indicated by our experiments using doxorubicin-induced apoptosis. This model uses, at least in part, the intrinsic p53-modulated apoptotic pathway. Exposure of SMCs to doxorubicin increased p53 levels and led to apoptosis. As in the model of apoptosis caused by exogenous

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Effects of HCMV IE proteins on Ad.p53-induced apoptosis. A, Analysis by TUNEL assay. Approximately 150 nuclei were counted in each of 5 fields, and percentage of TUNEL-positive nuclei was calculated. Results are expressed as mean±SD. *P<0.05. B and C, Analysis by flow cytometry. Cells were harvested, stained with hypotonic propidium iodide solution, and analyzed. Percentage of apoptotic nuclei was determined by calculating ratio of subgenomic DNA content (M2) to total DNA content (M1+M2). Results are expressed as mean±SD. n=3, *P<0.05. D, p53 levels in cells infected with Ad.βGal, Ad.IE1, or Ad.IE2 with or without coinfection of Ad.p53. Equal protein loading was confirmed by examination of α-tubulin (αT) expression.

![Figure 3](http://circ.ahajournals.org/)

**Figure 3.** Effects of IE proteins on p53-mediated p21 induction. Levels of p21 were determined by Western blot. Cyclin-dependent kinase inhibitor p21 was induced after p53 expression and was positively or negatively modulated by respective coexpression of IE1-71 or IE2-84.

![Figure 4](http://circ.ahajournals.org/)

**Figure 4.** Doxorubicin induces p53 expression in SMCs. Levels of p53 under basal conditions or 24 hours after doxorubicin (1.0 μmol/L) treatment. The latter were ~33% of that observed after Ad.p53 infection.
p53 gene transfer, IE1-72 increased and IE2-84 decreased apoptosis.

Although the proapoptotic effect of IE1-72 and the functionally homologous proteins of other DNA tumor viruses is difficult to understand from the point of view of viral evolution, a reasonable explanation derives from an evolutionary context of competing strategies for host versus pathogen survival. To survive, a virus infects a cell and forces it to replicate; the virus uses the cell’s replicative machinery to drive its own replication. HCMV accomplishes this, in part, through expression of IE1-72, which interacts with the Rb gene product, eliminating its inhibition of E2F.15 E2F in turn activates genes required for cell cycling. The resulting proliferation signal drives the cell to replicate in an inappropriate context (inadequate growth factors present or other influences in play that signal the cell to remain quiescent), leading the cell to activate p53 and initiate its apoptotic cascade. This is analogous to what occurs with c-myc overexpression, which induces cellular proliferation in the presence of growth factors but induces p53-dependent apoptosis when the cell is deprived of growth factors.13

In summary, p53-induced apoptosis in coronary artery SMCs is inhibited by HCMV IE2-84 and stimulated by IE1-72. These data, together with those of other studies, provide mechanistic support for a role of HCMV in restenosis and atherosclerosis.

References

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