Human Cytomegalovirus Immediate-Early Protein IE2–86, but not IE1–72, Causes Graft Coronary Arteriopathy in the Transplanted Rat Heart

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Background—Graft coronary arteriopathy (GCA) after heart transplantation is a major factor limiting the long-term survival of the recipients. Human cytomegalovirus (HCMV) infection is a possible cause of this disease which is characterized by diffuse intimal thickening resulting from smooth muscle cell migration and proliferation. It has been reported that HCMV immediate-early (IE) proteins, IE1 and IE2, could play an important role in the development of this disease; however, the precise in vivo role of these proteins in causing GCA has not been clarified.

Methods and Results—Excised Lewis rat hearts were transfected with HCMV IE1–72, IE2–86 or control plasmid by intra-coronary infusion of Hemagglutinating Virus of Japan-liposome, and transplanted into syngeneic recipients’ abdomens. All cardiac grafts continued to beat well throughout the incubation period in the absence of immunosuppression. Exclusive expression of IE1–72 or IE2–86 protein in coronary artery walls was demonstrated after IE1–72 or IE2–86 gene transfection, respectively. Luminal occlusion as a consequence of intimal thickening of graft coronary arteries developed in the IE2–86 transfected hearts at day 21 after transplantation (30.1±3.4% occlusion, P<0.0001), compared with the IE1–72 and control transfected ones (8.2±1.6 and 6.8±1.1%, respectively). In contrast, there was no significant difference in luminal occlusion between the IE1–72 and control transfected hearts.

Conclusions—We have demonstrated that expression of IE2–86 alone, but not IE1–72, causes intimal hyperplasia after cardiac transplantation. IE2–86 protein may therefore prove to be a useful target in therapies aimed at preventing HCMV-related GCA and improving the long-term result of cardiac transplantation. (Circulation. 2002;106[suppl I]: I-158-I-162.)

Key Words: transplantation ■ viruses ■ arteriosclerosis ■ coronary disease ■ rejection

Graft coronary arteriopathy (GCA) in cardiac transplantation, recognized as a type of chronic rejection, is a major limiting factor in the long-term survival of recipients.¹ This rapidly progressive disease, characterized by diffuse intimal hyperplasia resulting from migration and proliferation of vascular smooth muscle cells (SMCs), is estimated to affect more than 40% of recipients who survive beyond 4 years after transplantation.² The pathogenesis of GCA is believed to involve complex pathways within or between various cells of arterial walls, which would be activated by both immunologic and non-immunologic stimuli.² Some stimuli may directly influence migration/proliferation of SMCs, resulting in intimal thickening. Some stimuli may act on endothelial cells and inflammatory cells, contributing cytokines and growth factors to the vascular wall. This, in turn, may accelerate SMC migration/proliferation and extracellular matrix synthesis, and lead, ultimately, to GCA.

There is growing clinical evidence indicating the role of human cytomegalovirus (HCMV) infection in the pathogenesis of GCA.¹–³ The replication cycle of HCMV is characterized by the expression of immediate-early (IE), early and late genes. IE gene products appear in the nucleus of HCMV-infected cells 1 to 6 hour after infection to regulate the expression of early and late genes, and remain present even in latent infection.⁴ It has been shown that HCMV-infected cells have potential pathogenicity without its virion production, and that major IE proteins, IE1 and IE2 (predominantly IE1–72 and IE2–86, respectively), play an important role in modifying expression of both viral and host cellular genes.⁴ These IE proteins have been shown to modulate the cellular expression of onco-genes⁵ and cytokines⁶,⁷ and accelerate migration/proliferation of vascular SMCs.⁸ This is likely to be important in the mechanism of HCMV-mediated GCA; however, the details remain unknown. In the present study, we investigated the direct in vivo effect of IE1–72 or IE2–86 protein in causing GCA using a syngeneic rat heart transplantation model.
Methods

Animal Care
All studies were performed with the approval of the institutional ethics committee of Harefield Heart Science Centre at Imperial College, UK. The investigation conforms to the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication No. 85 to 23, 1996).

Ex Vivo Gene Transfection and Heterotopic Transplantation of Donor Hearts
Full-length cDNA for HCMV IE1–72 or IE2–86 (kind gifts from Professor Thomas Stammering, Institut fur Klinische und Molekulare Virologie, Universitat Erlangen-Nurnberg, Germany) was subcloned into the pcDNA3 expression vector (Invitrogen). As a control plasmid, pcDNA3 without any gene insert was used. Gene transfection to the coronary arterial walls was mediated by intra-coronary infusion of Hemagglutinating Virus of Japan (HVJ)-liposome as described before. Briefly, 200 μg DNA mixed with 64 μg high mobility group 1 nuclear protein (Wako) was enclosed in liposome composed of phosphatidylserine, phosphatidylcholine and cholesterol (Sigma, St. Louis MO). The liposome was then incubated with 30,000 U of inactivated HVJ (Z strain), finally producing 4 mL of purified HVJ-liposome. Male Lewis rats (200 to 220 g, Charles River) were systemically heparinized (200 USP units, IV), the hearts arrested with cold cardioplegia (St. Thomas’ Formula II, Martindale Pharmaceuticals) and removed under anesthesia with sodium pentobarbitol (50 mg/kg, IP). The hearts were infused with 1 mL of ice-cold HVJ-liposome containing pcDNA3 with either IE1–72 or IE2–86 cDNA, or the pcDNA3 control plasmid, via the coronary arteries with the venae cava and pulmonary arteries ligated. After 10 minute incubation under increased intra-coronary pressure on ice, the hearts were transplanted into the abdomens of male Lewis rats (250 to 275 g). The aorta and the pulmonary artery of donor hearts were anastomosed with the abdominal aorta and the inferior vena cava of recipient rats, respectively, in an end to side manner with the aid of an operating stereo microscope. After reperfusion, the surgical wounds were repaired and the rats returned to the cages to recover. No immunosuppressive reagent was given. This syngeneic transplant model does not undergo rejection in theory, thus emphasizing the importance of the independent effect of IE proteins in GCA. Rats were killed at 7 or 21 days after transplantation under anesthesia described above and the transplanted hearts collected for evaluation.

LacZ-Gene Transfection
To clarify the efficiency and localization of protein expression by the present gene transfection method, LacZ-gene transfection was performed and the protein expression examined with in situ staining for β-galactosidase (β-gal). Full-length cDNA for the Escherichia coli β-gal, a generous gift from Dr Y Sawa (First Department of Surgery, Osaka University, Japan), was subcloned into pcDNA3 expression vector. Transfection of lacZ-gene was performed by intra-coronary infusion of HVJ-liposome exactly as described above. At day 7 after LacZ-gene transfection, heart samples (n=5) were cut into 4 segments transversely to be frozen in embedding medium. As a control, samples of control-plasmid transinfected hearts, as described above, were used. Cryo-sections (10 μm) were cut, fixed in 2% formaldehyde and 0.2% glutaraldehyde, and incubated with 1 mg/mL 5-bromo-4-chloro-3-indoly-β-D-galactopyranoside, 5 mMol/L potassium ferricyanide, 5 mMol/L potassium ferrocyanide and 2 mMol/L MgCl2, for 12 to 15 hours at 37°C. The sections were counter-stained with Elastica van Gieson to highlight coronary arterial walls. The aorta and the pulmonary artery of donor hearts were anastomosed with the abdominal aorta and the inferior vena cava of recipient rats, respectively, in an end to side manner with the aid of an operating stereo microscope. After reperfusion, the surgical wounds were repaired and the rats returned to the cages to recover. No immunosuppressive reagent was given. This syngeneic transplant model does not undergo rejection in theory, thus emphasizing the importance of the independent effect of IE proteins in GCA. Rats were killed at 7 or 21 days after transplantation under anesthesia described above and the transplanted hearts collected for evaluation.

Statistical Analysis
All values are expressed as mean±SEM. Comparison between groups was assessed by one-way ANOVA followed by Bonferroni’s post hoc test. A value of P<0.05 was considered statistically significant.

Results

Operative Mortality
Rat hearts were transinfected with HCMV IE1–72, IE2–86, LacZ, or control plasmid, mediated by intra-coronary infusion of HVJ-liposome, and transplanted into syngeneic recipient abdomens (total 32 procedures). No animals were lost as a consequence of the operative procedures or post-operative complications, all cardiac grafts beating well throughout the 7- or 21-day incubation period without immunosuppression.

Efficiency of Gene Transfection
To clarify the localization of protein expression after the described gene transfection method mediated by intra-coronary infusion of HVJ-liposome, in situ staining for β-gal was performed after LacZ-gene transfection. At day 7 after LacZ-gene transfection, β-gal expression was found in all layers of the coronary arterial walls; endothelium, medial layer and adventitia (Figure 1A). Quantification demonstrated that 30.6±2.5% of the total medial area (n=59 arteries) was positively stained. Widespread expression of β-gal positive cardiomyocytes was also found in the LacZ-gene transinfected hearts, confirming the previous reports. In contrast, there were no β-gal stained cells found in any control-transfected hearts (Figure 1B).

Expression of IE-Gene Products After Gene Transfection
Western blotting demonstrated expression of IE1–72 or IE2–86 protein in the hearts at day 7 after IE1–72 or IE2–86 gene transfection, respectively. Further, at day 21 after transfection, IE1–72 or IE2–86 protein expression was also observed in the respective groups, although the expression...
level was considerably reduced, compared with that at day 7 (Figure 1C). No expression of IE1–72 or IE2–86 was observed in the control-transfected hearts at any time points.

Intimal Thickening in Graft Coronary Arteries
At 7 or 21 days after transplantation, the grafted hearts were collected and stained with Elastica van Gieson to highlight neointimal formation in graft coronary arteries. The IE2–86 gene transfected hearts developed luminal occlusion as a consequence of concentric intimal thickening of coronary arteries at day 7 (Figure 2C) with further development by day 21 (Figure 2F). Neointimal formation was not clear in the IE1–72 (Figure 2B, 2E) or control transfected hearts (Figure 2A, 2D) at either time point. Quantification demonstrated that luminal occlusion at day 7 was significantly enhanced in the IE2–86 transfected hearts (15.9 ± 1.9% occlusion), compared with both the IE1–72 (6.4 ± 1.2%, P = 0.004) and control transfected ones (4.3 ± 0.8%, P < 0.001; Figure 3). Furthermore, by day 21 after transplantation, luminal occlusion in the IE2–86 gene transfected hearts was further developed (30.1 ± 3.4%, P < 0.001) as compared with day 7 samples, with significant differences from day 21 samples of the IE1–72 (8.2 ± 1.6%, P < 0.001) and control transfected ones (6.8 ± 1.1%, P < 0.001). Incidence of arterioles showing more than 50% occlusion at day 21 after gene transfection was 10/51, 0/56 and 0/54 for IE2–86, IE1–72 and control transfected hearts, respectively. There was, in contrast, no significant difference in luminal occlusion between the IE1–72 and control transfected hearts at either day 7 or 21. The IE1–72 or control transfected hearts also showed no significant difference in luminal occlusion between day 7 and 21.

Discussion
We have demonstrated that HCMV IE2–86 protein alone, introduced by gene transfection, causes rapidly progressive, concentric intimal thickening in graft coronary arteries in a syngeneic rat cardiac transplantation model. In contrast, IE1–72 expression did not result in luminal occlusion of the graft coronary arteries after transplantation, compared with the controls. These findings suggest that IE2–86 protein, but not IE1–72, plays a causative role in HCMV-mediated GCA in cardiac transplant recipients.

HCMV is capable of infecting vascular SMCs and become latent after acute infection. During the latency period, the virus can modulate cellular metabolism of SMCs, for example, production of growth factors, which can lead to migration/proliferation of SMC via interaction with p53, initiating accelerated intimal hyperplasia. There is growing evidence that this pathogenic feature of HCMV is largely because of the abilities of IE gene products to transactivate or repress viral and host cellular genes. HCMV expresses 2 major IE gene products, IE1 and IE2, that have some similar
functions and are sometimes synergistic. Both proteins are able to influence expression of their own promoter, but with profoundly opposite effects. While IE1 acts as a transactivator for the major IE promoter, IE2 represses it via direct binding with a target DNA sequence that spans the CAP site. Both IE1 and IE2 can also transactivate expression of host cellular genes, such as c-myc and c-fos, but in different ways. In contrast to IE1, which appears to act indirectly via NF-κB, IE2 modulates host cellular gene expression by direct interaction with cellular genes or proteins. Recent studies have revealed physical association of IE2 with a number of transcription factors, including TATA-binding protein, TFIID, and Rb. These data indicate that IE2 may be more accountable for the HCMV-modulated host cellular profile, compared with IE1. Castillo et al demonstrated that expression of IE2, but not IE1, induces quiescent cells into S phase and delays cell cycle exit, suggesting that IE2 may be responsible for HCMV-induced proliferation by altering cell cycle control. It has also been demonstrated that IE2, but not IE1, interacts with p53, and this interaction results in the downregulation of the p53 transactivating effect on certain genes such as the cyclin-dependent kinase inhibitor p21, which in turn regulates cell cycle progression. Further, Tanaka and co-workers have reported that p53-mediated apoptosis in coronary artery SMCs is inhibited by IE2, but stimulated by IE1 in vitro. Such ability of IE2 to inhibit a p53-dependent signaling pathway that leads to p21 induction and apoptosis could accelerate SMC proliferation and accumulation, resulting in intimal thickening in the coronary arteries. These differences in the direct effects on SMCs between IE1 and IE2 may help to explain our finding that IE2–86, but not IE1–72, induces intimal thickening in graft coronary arteries after cardiac transplantation.

In addition to the above-mentioned direct effect on SMC proliferation, major IE proteins may increase graft coronary arteriosclerosis indirectly by enhancing the production of cytokines and growth factors in endothelial and/or inflammatory cells, which lead to paracrine effects on coronary artery SMC division. In the present study, the degree of infiltration of inflammatory cells into myocardial interstitium was higher in the IE2–86 and IE1–72 transfected hearts compared with the control transfected ones. This might support the potential proinflammatory effects of IE proteins. It has also been shown that IE proteins can introduce changes in the endothelium relevant to atherogenesis, such as expression of endothelial surface adhesion molecules. As endothelial cells as well as medial cells of coronary arteries are probably transfected to express IE proteins in our study as suggested by the pattern of protein expression after β-gal gene transfection, one could speculate that such an effect of IE proteins (presumably including IE2–86) on endothelial cells might also be involved in the mechanism of IE2–86-induced intimal thickening of graft coronary arteries observed in our study. Further work is needed to clarify the independent role of IE1 and IE2 in these endothelial-related mechanisms for developing intimal thickening of graft coronary arteries.

GCA is a major factor limiting the long-term survival of the recipients of cardiac transplantation. Thus, urgent development of a treatment or preventative strategy against this HCMV-related disease would be of great value. One noticeable point may be that the current drugs would have no impact on IE gene products, although the regulation of IE proteins has been revealed to be the key to vascular SMC migration/proliferation that leads to intimal thickening. In the present study, we have demonstrated that IE2–86 alone leads to intimal hyperplasia in coronary arteries after cardiac transplantation, suggesting that inhibiting this protein could be useful in the prevention of GCA. Antisense oligonucleotide against IE2 mRNA has been developed for attenuating expression or function of IE proteins and evaluated for its in vitro effects on HCMV infection. Furthermore, this phosphoroyhioate oligonucleotide has already been applied to HCMV retinitis patients with a favorable result. Suzuki and co-workers have reported that intracoronary infusion of HVJ-liposome results in a highly effective delivery of antisense cdk2 kinase oligonucleotides into coronary vessel walls, leading to GCA prevention in mice. Thus, we would propose that a novel therapeutic strategy directed against IE2 protein: gene therapy using antisense IE2 oligonucleotide mediated by intra-coronary infusion of HVJ-liposome, might be useful for preventing HCMV-mediated GCA.
Clinical studies have indicated an association between the presence of HCMV infection and the development of not only GCA but also atherosclerosis in native arteries. Hendrix et al demonstrated that major arteries obtained from HCMV seropositive patients contained HCMV nucleic acid sequences, which are equally distributed among arteries with and without atherosclerotic changes, indicating that the vascular wall is the site of latent HCMV infection and that there is no tropism of HCMV for the sites of predilection for atherosclerotic lesions. The influence of prior HCMV infection and the development of not only atherosclerotic lesions but also atherosclerosis in native arteries has been reported. Hendrix et al demonstrated that major arteries obtained from HCMV seropositive patients contained HCMV nucleic acid sequences, which are equally distributed among arteries with and without atherosclerotic changes, indicating that the vascular wall is the site of latent HCMV infection and that there is no tropism of HCMV for the sites of predilection for atherosclerotic lesions. The influence of prior HCMV infection and the development of not only atherosclerotic lesions but also atherosclerosis in native arteries has been reported. Hendrix et al demonstrated that major arteries obtained from HCMV seropositive patients contained HCMV nucleic acid sequences, which are equally distributed among arteries with and without atherosclerotic changes, indicating that the vascular wall is the site of latent HCMV infection and that there is no tropism of HCMV for the sites of predilection for atherosclerotic lesions.

In conclusion, we have demonstrated that HCMV IE2–86 protein, but not IE1–72, leads to intimal thickening in graft coronary arteries after syngeneic cardiac transplantation, suggesting a causative role of IE2–86 in HCMV-mediated GCA. IE2–86 protein may therefore, prove to be a useful target in therapies aimed at preventing HCMV-related GCA and improving the long-term result of cardiac transplantation.

Acknowledgments

This work was supported by the harefield Research Foundation and the British Heart Foundation. Ken Suzuki is supported by the research fellowship from the uehara Memorial Foundation.

References


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_Circulation_. 2002;106:I-158-I-162
doi: 10.1161/01.cir.0000032881.55215.db

*Circulation* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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