Inhibition of Rat Vascular Smooth Muscle Cell Proliferation In Vitro and In Vivo by Recombinant Replication-Competent Herpes Simplex Virus

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Background and Purpose—The proliferation of vascular smooth muscle cells (VSMCs) is a common feature associated with vascular proliferative disorders such as atherosclerosis and restenosis after balloon angioplasty. We examined the antiproliferative effects of recombinant replication-competent herpes simplex virus (HSV), hrR3, to proliferative VSMCs both in vitro and in vivo.

Methods—Early passages of Sprague-Dawley rat VSMCs were infected with hrR3 at a low multiplicity of infection (0.01 to 1.0) to examine the in vitro cytotoxic activity of this recombinant HSV to VSMCs in a proliferative state. Sprague-Dawley rats underwent balloon dilatation injury of the left carotid artery to induce neointimal formation. The injured carotid arteries were infected with hrR3 five days after balloon injury. Two weeks after injury, the left carotid arteries were fixed, and the areas of the neointimal and medial layers were analyzed microscopically. Because the reporter Escherichia coli lacZ gene in hrR3 is expressed only in infected cells in which the virus is actively replicating, virus replication was confirmed by X-gal staining.

Results—A morphometric analysis revealed that there were significant differences in the intima/media ratio between the HSV-treated group and mock-infected group (0.354 ± 0.068 and 1.08 ± 0.055, respectively). In the histological study (X-gal staining), positive X-gal staining was observed chiefly in the VSMCs in the medial layer just beneath the internal elastic lamina, indicating active viral replication.

Conclusions—Virus-mediated cytocidal therapy using recombinant HSV vector is a promising modality for the treatment of the restenosis after balloon angioplasty. (Stroke. 1999;30:2431-2439.)

Key Words: balloon embolization ■ herpesvirus ■ muscle, smooth

Vascular smooth muscle cell (VSMC) proliferation contributes to the intimal thickening of the arteries, playing an important role in atherosclerosis and especially in restenosis after percutaneous transluminal angioplasty and endarterectomy.1–6 The rates of recurrent narrowing after percutaneous transluminal coronary angioplasty have been reported to be as high as 30% to 50%.3,4 Strategies to prevent the proliferation of VSMCs have focused on growth factors and their receptors, suicide genes, and suppressor oncogenes with and without various vector systems such as viruses, liposomes, and fusions of the two.7–17 However, almost all strategies using gene technology reported so far involve the transfer of therapeutic genes into target VSMCs.

We and others have reported the use of conditionally replication-competent herpes simplex virus (HSV) mutants for the experimental treatment of malignant tumors.18–24 Certain HSV mutants, including those that are deficient for the virus-encoded enzyme ribonucleotide reductase (RR), can replicate in and ultimately destroy dividing cells but are severely impaired for replication in nondoning cells because RR is a key enzyme in the de novo synthesis of DNA precursors, catalyzing the reduction of ribonucleotides to deoxyribonucleotides.25 We hypothesized that such an HSV mutant would replicate in the proliferative phase of VSMCs, which is responsible for the pathogenesis of restenosis after balloon injury, and have a therapeutic effect on this pathology. Therefore, we studied the efficacy of the HSV-1 mutant hrR3,26,27 which contains an Escherichia coli lacZ gene insertion in the ICP6 gene, on restenosis. The presence of the lacZ gene in hrR3 allows identification of the cells in which the virus actively replicates using β-galactosidase (β-gal) histochemistry (X-gal staining). In the present study, we examined the antiproliferative effect of this recombinant HSV, hrR3, on...
proliferative VSMCs both in vitro and in vivo in balloon injured carotid arteries.

Materials and Methods

Cell Culture

Primary cultures of rat VSMCs were prepared by enzymatic digestion, modified as follows. Briefly, the thoracic aortas of Sprague-Dawley rats (Shinshu Laboratories Co Ltd, Kusatsu, Japan) were isolated aseptically, and the adventitia and outer media of the isolated aortas were cleanly stripped off and discarded. Then, the vessels were minced, placed in collagenase (Sigma Chemical Co) and submerged in cold PBS. Samples were then placed in a substrate solution (containing 1 mg/mL X-gal, 5 mmol/L potassium ferricyanide, 2 mmol/L magnesium chloride, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40) for 3 hours. Cross-sectional samples of the injured carotid arteries were taken from the center of the injured and treated area. Then they were refixed in 4% paraformaldehyde at 4°C for 6 to 12 hours, paraffin-embedded, stained with hematoxylin and eosin, and photographed. The cross-sectional areas of the intimal and medial regions of the sections were measured by use of an image-analyzing software package (NIH Image). The intimal to medial (I/M) area ratio of each injured artery was determined. In some experiments, the injured arteries were harvested 3 days after HSV infection and were processed in the same way as described above.

Statistical Analysis

All data are expressed as mean±SEM. Statistical differences of thickness in neointimal hyperplasia, medial layer, and I/M ratio of hrR3 and mock-infected samples were assessed by ANOVA followed by the Scheffé test, using Statview-J4.02 (BrainPower). Statistical significance was accepted at P<0.05.

Immunohistochemical Staining for Detection of Factor VIII–Related Antigen and Ki-67

To identify the endothelial cells, immunohistochemical staining for factor VIII–related antigen was applied for the untreated or balloon-injured and virus-treated arteries. Briefly, the paraffin-embedded samples were sectioned and treated with protease K for 4 minutes. Endogenous peroxidase was quenched with methanol/peroxidase solution. The specimens were treated with 50 mmol/L Tris HCl (pH 7.6) containing 0.15 mol/L NaCl and 0.1% Tween 20 for 5 minutes, followed by incubation with 1:200 diluted peroxidase-conjugated rabbit anti-factor VIII–related antigen antibody (Dako Japan), which is cross-reactive to rodent factor VIII–related antigen. The specimens were then processed by the incubation with 3,3′-diaminobenzidine tetrahydrochloride (DAB) substrate solution (Dako Japan).

The balloon-injured artery was mock-infected 5 days after injury, sampled on day 8, and processed for the immunohistochemical staining for the detection of Ki-67 in the injured vessel wall. Ki-67 is well known as a marker of proliferating cells. The tissue sections were subjected to microwave-treated epitope retrieval. The section was incubated with 1:100 diluted mouse monoclonal antibody for Ki-67 (Dako Japan), incubated with horse anti-mouse IgG antibody,
and then processed with the incubation with the avidin-biotin peroxidase complex reagent (Vectastain ABC kit, Vector Laboratories). DAB was used as the final chromagen. In both immunohistochemical stainings, hematoxylin was used as the nuclear counterstain.

**Results**

**In Vitro Cytopathic Efficacy of hrR3 and X-gal Staining**

The ICP6 gene belongs to the early (β) genes, and its promoter is active and effective in the replication phase of the virus. Consequently, β-gal protein expression driven by the ICP6 promoter in hrR3 serves as an indicator of both virus spread and replication. We could thus confirm in vitro virus infection, spread, and replication by means of X-gal staining of hrR3-infected VSMCs. VSMCs were infected with hrR3 at an MOI of 0.01, 0.1, or 1.0 on day 0. X-gal staining of VSMCs infected with hrR3 at an MOI of 0.1, 2 days after infection, is illustrated in Figure 2E. Many cells were stained blue by X-gal, and they were rather shrunken or swollen in shape, likely a result of cell death, compared with the nonstained cells. The photographs presented in Figure 2A to 2D were taken 3 days after mock or virus infection at

*Figure 2.* In vitro cytopathic efficacy of hrR3 and X-gal staining. VSMCs (1 × 10⁵) were infected with hrR3 at MOI values of 0.01, 0.1, and 1.0, whereas controls were mock-infected. Cells were photographed on day 3. X-gal histochemistry was performed on hrR3-infected cells (MOI of 0.1) on day 2 after infection. A, Mock infection; B, hrR3 MOI 0.01; C, hrR3 MOI 0.1; D, hrR3 MOI 1.0; and E, hrR3 MOI 0.1 (X-gal staining). Original magnifications of the objective lens are ×5 for panels A, B, C, and D and ×10 for panel E. In panels A to D, VSMCs were destroyed in a dose-dependent fashion. In panel E, active viral replication was detected in X-gal-positive cells.
different MOIs. VSMCs were destroyed by this recombinant HSV in a dose-dependent fashion, and X-gal staining showed active virus replication. It is noteworthy that even the cells infected with hrR3 at an MOI of 0.01 were completely destroyed within 5 days (data not shown).

Inhibition of Balloon Injury–Induced Neointimal Hyperplasia by Recombinant HSV

We next determined whether hrR3 could inhibit VSMC proliferation in vivo as well. We introduced 50 to 75 μL of either hrR3 or virus buffer alone into injured rat left carotid arteries 5 days after balloon injury. Two weeks after the balloon injury, the injured arteries were harvested to measure the cross-sectional areas of the intima and media. The representative photographs are shown in Figure 3. In the early stage of HSV infection (3 days after hrR3 infection, on day 8 after injury), β-gal activity was found in the most internal part of the media, ie, just beneath the internal elastic lamina (Figure 3B). Even at a later stage of hrR3 infection (on day 14 after injury), β-gal activity remained in the same part of some infected arteries, although the activity was rather weak compared with that on day 3 after infection (data not shown). The administration of hrR3 reduced the area of intimal mass of an injured artery (Figure 4B and 4B') compared with that of the administration of virus buffer alone (Figure 4A and 4A').

The area of medial mass was similar in the 2 groups (hrR3-infected and mock-infected); however, the area of no treatment is somewhat small (Figure 5A). Statistical analysis also revealed that the area of neointima in hrR3-infected arteries is markedly reduced compared with that in mock-infected arteries (Figure 5B). As a result, the I/M area ratio was substantially reduced by the administration of hrR3 (P<0.001) (Figure 5C). In addition, if the virus was applied just after the balloon injury, almost no X-gal–positive cells were observed on day 8 or 14 after infection. Also, no therapeutic effects were observed if the virus was administered just after the balloon injury (Figure 4C).

Immunohistochemical Staining of Ki-67 for Proliferating Cells and Factor VIII–Related Antigen for Remodeled Endothelium

hrR3 can replicate only in cells in the proliferative phase. We tried to identify the cells in the proliferative phase directly by immunohistochemistry using monoclonal antibody for Ki-67. Several days after injury, some cells in the neointima and internal part of the medial layer of balloon-injured artery were positive for Ki-67 (Figure 3C). No Ki-67 immunoreactivity was observed in the nontreated vessel wall (data not shown). As described above, hrR3 attacks cells in the proliferative phase. Therefore, it might be possible that the virus exhibited cytocidal activity not only on the proliferative VSMCs but also on remodeled endothelium. Using the antibody for factor VIII–related antigen, we performed immunohistochemical staining for the detection of remodeled endothelium in the virus-treated arteries. Figure 4B" demonstrates the intact remodeled endothelium of the virus-treated vessels. The specimen shown in Figure 4B" is identical to that in Figure 4B'.

Discussion

The proliferation of VSMCs is thought to contribute to restenosis after arterial injury, and several growth factors are thought to be involved in this event.1–6 Many therapeutic approaches using modern genetic engineering technology have been applied to this pathogenesis. Recently, we reported that the adenovirus-mediated gene transfer of truncated form of fibroblast growth factor receptor could inhibit the in vitro cell growth of VSMCs.32 Other than truncated forms of fibroblast growth factor receptor, antisense basic fibroblast growth factor, Rb (retinoblastoma suppressor oncogene), bone morphogenetic protein 2, and HSV-thymidine kinase gene have been introduced to inhibit neointimal hyperplasia.33–36 Many investigators have adopted nonviral vectors, such as liposomes, or viral vectors, such as retroviruses; however, most recent attempts have used replication-defective recombinant adenovirus vectors for high transduction efficiency. No attempts using replication-competent virus vectors have been reported so far for the treatment of restenosis after balloon injury model. In the present study, we introduce a new concept to inhibit this neointimal hyperpla-
Figure 4. Representative photographs of balloon injury–induced neointimal hyperplasia treated with hrR3. hrR3 (5 × 10^8 pfu/mL) or virus buffer alone (50 to 75 μL) was introduced into injured rat left carotid arteries 5 days after balloon injury in panels A, A’, B, B’, and B”’. Two weeks after balloon injury, the injured arteries were harvested and stained with X-gal, except for those shown in panel B”’. A and A’, Mock infection. B, B’, and B”’, hrR3 treatment. B”’ shows immunohistochemical staining for the identification of endothelial cells using antibody against factor VIII–related antigen. C, Artery infected with hrR3 just after the balloon injury and sampled 2 weeks later. D, No treatment. Original magnifications of the objective lens are ×10 (A, B, C, and D) and ×40 (A’, B’, and B”’). In panels A, A’, B, B’, and C, the neointima is demarcated between 2 arrows. Contralateral right carotid arteries of mock-infected rats were sampled as “no treatment.”
sia, not by transfer of therapeutic genes but by direct killing of the cells in hyperplasia by conditionally replication-competent HSV.

HSV deletion mutants have been constructed to attenuate toxicity to nondividing cells, including the thymidine kinase gene, DNA polymerase α, dUTPase, and RR. In dividing cells, these HSV deletion mutants can use host enzymes to complement the defects in nucleotide metabolism. They can replicate in dividing cells but are severely impaired for replication in nondividing cells. Some of these HSV deletion mutants have been used for the treatment of malignant tumors in experiments as described above, and some protocols are ongoing for the treatment of malignant brain tumors in humans.

In the present study, we demonstrated the effectiveness of this virus-mediated cytocidal approach by using conditionally replication-competent virus vectors for restenosis of balloon-injured rat carotid artery. We selected hrR3 as a candidate vector for this treatment experiment because of deletion of the RR gene and the presence of the reporter E. coli lacZ gene under control of the viral ICP6 promoter. This reporter gene is expressed preferentially in cells in which the virus replicates. From the data presented here, hrR3 could infect the injured arterial wall and replicate, with detectable β-gal activity distinctly in the early stages of virus infection. It is believed that balloon angioplasty activates VSMCs in the medial layer and that the activated VSMCs release some cytokines. Then, as a result, the proliferation and migration of themselves occur in the initial stage of restenosis. This is also supported from our data in Figure 5A, which shows that the area of medial mass of the no-treatment group is small compared with that of the 2 balloon-injured groups (hrR3-infected and mock-infected). The infected virus seemed to replicate and spread in the same layer surrounding the infected portion. It is also speculated that the infected cells would be destroyed and that hyperplasia of the neointima would finally be inhibited. It is noteworthy that no β-gal activity was observed when the virus was administered to a noninjured artery or even to an injured artery just after the injury (Figure 4C). This suggests that hrR3 might infect the intima or internal part of the medial muscle layer; however, it cannot replicate in the tissue if the infected cells are not in a proliferative state. Using immunohistochemical staining for Ki-67, we directly demonstrated that several days after injury, some cells in the neointima and in the internal part of the medial layer of balloon-injured artery were proliferating (Figure 3C). Using anti—proliferating cell nuclear antigen antibody, we also observed the same results, and no Ki-67 activity was observed in nontreated vessel walls (data not shown). In some parts of the treated arteries, no neointimal formation was found at all, whereas in other parts of them, hyperplasia of the neointima could still be observed. To obtain complete inhibition of this pathology, repetitive infection or higher titers of the virus challenge may be necessary.

It should be stressed that replication-competent vectors have advantages over replication-defective vectors for cancer.
gene therapy. Even with the use of higher titer recombinant adenovirus vector (replication defective), 100% of gene transduction in target tissue in vivo is almost impossible. Therefore, the same advantage can be anticipated even for the treatment of neointimal hyperplasia after balloon injury. However, the most important consideration for the use of replication-competent vectors is that the replication should be safely controlled and confined to the target cells alone. It might be possible that the virus attacked not only the proliferative VSMCs but also the remodeled endothelium. Therefore, we performed immunohistochemical staining for the detection of remodeled endothelium in the virus-treated arteries by using the antibody for factor VIII–related antigen (Figure 4B’). By this investigation, we could confirm the existence of remodeling of endothelium in the virus-treated vessel. Some inflammatory cell infiltration was observed adjacent to the adventitia in hrR3- or mock-treated arteries. However, there was no positive X-gal staining outside of the treated vessels. Also, we observed several rats for >6 months after treatment with hrR3, and no clinical complications such as hemiparesis or weight loss were observed. In addition, hrR3 is hypersensitive to the antiviral agent of gancyclovir or acyclovir, compared with wild-type HSV. Before the clinical application of this vector, a more thorough examination of safety should be performed. It is possible that multiattenuated virus vectors, with reduced possibility of reversion to wild type, might be safer. As we reported recently, it is possible to construct dividing cell-specific and also tissue-specific replication-competent HSV. Proliferative VSMC-specific HSV might be the ideal weapon for this strategy.

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The last 2 decades have seen a tremendous increase in the use of percutaneous transluminal angioplasty and intravascular stenting to treat atherosclerotic coronary and peripheral vascular disease and, more recently, carotid disease. Together with carotid endarterectomy, it is estimated that close to 1 million of these procedures were performed in the United States in 1996, with considerable impact on subsequent morbidity from these diseases. Additionally, among patients with coronary artery disease, percutaneous approaches have spared numerous individuals from surgery. In spite of progressive improvements in interventional techniques, a persistent problem has been subsequent restenosis. Until the advent of intracoronary stenting, the rates of restenosis following angioplasty had remained relatively constant at approximately 30% to 50%. One recent clinical trial demonstrated that stenting reduced the rate of restenosis to as low as 18%.1 As with angioplasty, however, the early clinical trials may underestimate the restenosis rates ultimately seen in clinical practice. Thus, restenosis following percutaneous interventions and endarterectomy remains a profound clinical problem affecting a substantial number of patients at considerable cost.

Restenosis results from dysregulated and unchecked proliferation of vascular smooth muscle cells in the neointima.2,3 As a result, it is likely that successful prevention and/or treatment of this disorder will eventually be achieved by agents that target the molecular machinery of proliferating smooth muscle cells. Many recent experimental approaches to inhibit restenosis have attempted to inhibit neointimal smooth muscle cell proliferation by targeting vascular growth factors (PDGF and FGF), transcription factors (c-myc and c-myc), cell cycle regulators (cdc2 kinase), and other proteins (proliferating cell nuclear antigen).2 Other approaches have utilized the vascular delivery of toxins or ionizing radiation to kill proliferating cells.4 Many newer approaches are forms of gene therapy, in which genetic material is delivered to target cells and expression of the foreign gene results in a biologic effect.5 Currently, a leading candidate vector for vascular gene therapy is the adenovirus, in part because of its high transduction efficiency in vascular cells.6,7 However, gene therapy is the adenovirus, in part because of its high potential biologic determinants and role of intimal hyperplasia.8,9 In part because of this effect, gene therapy with adenoviral vectors requires the use of replication-deficient vectors that are incapable of establishing a productive infection in a host cell, which is followed by host cell lysis and release of daughter viral particles into the local environment.10

Editorial Comment

The so-called cytotoxic effect is the result of viral replication in a host cell, which is followed by host cell lysis and release of daughter viral particles into the local environment. In part because of this effect, gene therapy with adenoviral vectors requires the use of replication-deficient viruses to achieve desirable levels of target gene expression without cytotoxicity and virus-induced inflammation. In contrast to the use of replication-defective adenoviruses for gene therapy, Miyatake et al have capitalized on the cytotoxic effect commonly seen after infection by a number of different viruses and effectively redirected it toward cells that are actively proliferating.
adult vasculature. Indeed, similar replication-competent
HSVs have been used experimentally to inhibit the growth of
malignant gliomas, a setting in which there is likely to be
little proliferation in the surrounding brain parenchyma.8,9

In the current study, rat carotid arteries were balloon-
injured and 5 days later infected with HSV or sham infected.
HSV-infected arteries demonstrated expression of the re-
porter gene β-galactosidase 2 days after viral infection,
indicative of viral replication in proliferating vascular smooth
muscle cells. Even at this stage many of the infected cells
were dying due to the cytopathic effect, and by 5 days after
infection no β-galactosidase staining was seen, presumably
because all of the infected, proliferating cells were dead. HSV
significantly reduced neointimal thickness by approximately
70% compared with that of uninfected vessels, which suggest
that this may be a viable approach to inhibit restenosis.

However, an important consideration is whether this ge-
etically altered HSV might also infect and kill proliferating
endothelial cells during endothelial remodeling. Re-
endothelialization after balloon injury is vital to prevent
subsequent thrombosis as well as to restore endothelium-
dependent vasoreactivity; therefore, HSV-mediated destruc-
tion of the endothelium might result in significant thrombotic
complications. Importantly, immunohistochemical staining
demonstrated intact, and likely remodeled, endothelium.
Furthermore, no β-galactosidase staining was seen in the endo-
thelium, suggesting that re-endothelialization of the injured
vessels took place during the interval between balloon injury
and viral infection. These findings suggest that the prolifera-
ting smooth muscle cells in the neointima were the primary,
and perhaps only, target of HSV. Furthermore, the authors
observed no evidence of viral infection outside of the treated
vessels, suggesting that the cytopathic effect was entirely
local.

The results presented here provide evidence for an encour-
aging new approach to treat restenosis. As pointed out, the
safety of these conditionally replication-competent viruses
needs to be more thoroughly evaluated prior to their use in
humans. However, the focal nature of the restenotic lesion
provides a unique and readily accessible target for such
therapeutic agents, and they have the potential to significantly
reduce the impact of this important disease process.

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