Improved Vascular Gene Transfer With a Helper-Dependent Adenoviral Vector

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Background—Adenoviral vectors are the most widely used agents for vascular gene transfer. However, the utility of adenoviral vectors for vascular gene transfer is limited by brevity of expression and by the induction of a significant host inflammatory response. Third-generation or “helper-dependent” adenoviral vectors have achieved prolonged recombinant gene expression in liver and muscle with minimal associated inflammation; however, they have never been tested for vascular gene transfer.

Methods and Results—We constructed a helper-dependent adenoviral vector expressing rabbit urokinase plasminogen activator (HD-AduPA). HD-AduPA was compared, in a rabbit model of carotid gene transfer, with a first-generation adenovirus, also expressing rabbit uPA (FG-AduPA). uPA expression and vector DNA were measured in arteries harvested from 3 to 56 days after gene transfer. Vector-specific mRNA, vascular inflammation, and neointimal formation were assessed 14 days after gene transfer. uPA expression was lost, and vector DNA declined rapidly in arteries infused with FG-AduPA. In contrast, uPA expression and vector DNA persisted in HD-AduPA arteries for ≥56 days, with stable expression from 14 to 56 days. Increased uPA expression in HD-AduPA arteries was accompanied by high levels of vector-specific uPA mRNA. Moreover, HD-AduPA arteries had significantly less inflammation and neointimal formation than FG-AduPA arteries.

Conclusions—Helper-dependent adenoviral vectors can stably express a therapeutic gene in the vascular wall for ≥8 weeks, with minimal associated inflammation. Helper-dependent adenoviral vectors will be useful agents for vascular gene transfer and gene therapy. (Circulation. 2004;110:GGG-GGG.)

Key Words: carotid arteries ■ endothelium ■ gene therapy ■ plasminogen activators ■ urokinase

Gene transfer is a powerful tool for investigating the roles of gene products in the vessel wall and for improving blood vessel function.1,2 First-generation adenoviral (FG-Ad) vectors are the most commonly used vectors for vascular gene transfer. Advantages of FG-Ad vectors include an ability to confer high-level transgene expression after only brief contact with vascular tissue and to transfer genes to the (generally) nondividing endothelial and smooth muscle cells of the blood vessel wall.3,4 Shortcomings of FG-Ad vectors for vascular gene transfer include brevity of transgene expression (1 to 2 weeks) and induction of an inflammatory response in the vessel wall.5-7 These shortcomings, believed to result from destructive host immune responses to viral proteins expressed from the nearly complete adenoviral genomic sequences in FG-Ad vectors,8 must be overcome if Ad vectors are to realize their full potential as experimental and therapeutic agents.

To attempt to overcome the limitations of FG-Ad vectors, several groups have generated modified Ad vectors in which open reading frames encoding adenoviral proteins are deleted or anti-inflammatory genes are inserted. These modified vectors are called “second-generation” Ad vectors.9-13 Other groups have generated Ad vectors in which all viral open reading frames are replaced with noncoding “stuffer” DNA. Vectors that lack all viral open reading frames are known as “third-generation or “helper-dependent” (HD) vectors.14,15 Theoretically, cells infected by second-generation and HD-Ad vectors are less easily detected by the immune system than cells infected with FG-Ad. Second-generation and HD-Ad vectors yield long-term, stable transgene expression with minimal associated inflammation in animal models of liver, lung, and muscle gene transfer.16-18 In contrast, in animal models of vascular gene transfer, second-generation vectors prolonged transgene expression only marginally or not at all.12,13,19,20 The ability of second-generation vectors to lessen the host inflammatory response has also been inconsistent.12,13,19,20 Use of HD-Ad vectors for vascular gene transfer has not yet been reported, and it has been uncertain whether HD-Ad vectors would achieve prolonged, stable transgene expression in the vessel wall. The rapid loss of vector DNA and transgene expression after infusion of FG-Ad vectors into arteries of both immune-deficient mice21 and profoundly immunosuppressed rabbits12 cast doubt on whether immune system evasion—the basis on which HD-Ad...
vectors are thought to be advantageous—can ensure persistence of transgene expression in the artery wall.

To determine the potential for HD-Ad vectors to confer prolonged transgene expression in the artery wall while avoiding induction of a local host inflammatory response, we infused FG- and HD-Ad vectors into rabbit carotid arteries. The arteries were harvested up to 8 weeks later, and persistence of vector and transgene expression and the local host inflammatory response were measured.

**Methods**

**Construction of an HD-Ad Vector Expressing Urokinase Plasminogen Activator**

We constructed an HD-Ad vector that expresses rabbit urokinase plasminogen activator (HD-AduPA; Figure 1) by ligation of the uPA expression cassette from the FG-Ad AdrBuPA22 (hereafter called FG-AduPA) into the SwaI site of the HD-Ad backbone plasmid pC4HSU, digestion of the product with PmeI, transfection into 293Cre4 cells, and infection of the cells with "H14," a helper virus that is a null, first-generation, E1-negative adenovirus. HD-AduPA was expanded by serial passage and purified by CsCl ultracentrifugation. We used a rabbit uPA transgene rather than a human transgene or a marker gene such as /H9252-galactosidase to avoid confounding host immune responses against a foreign protein.24 Expression of an endogenous protein such as uPA also enables assessment of whether overexpression has been achieved and, if so, to what extent. uPA is also potentially a therapeutic gene,25 although not under all circumstances.22

Preparations of HD-AduPA, FG-AduPA, and H14 were characterized by spectrophotometry to measure vector particle (part) concentration.26 E1A-positive virus was quantified by real-time polymerase chain reaction: primers, 5'-CATCTACGTTGTTAGCATCGCTATTACCA, 5'-TGGAATCCCGGTAGCTCA; probe, TCAGCCAGTACCTCTTCGATCAGCTGGT.

**In Vitro uPA Expression**

Cultured rabbit aortic endothelial cells (ECs) were infected by incubation for 48 hours with HD-AduPA or FG-AduPA at 2000 parts per cell. Plasminogen activator (PA) activity was measured in media conditioned for 4.5 hours by the ECs using human plasminogen (American Diagnostica) and the plasmin substrate S-2390.27 PA activity was detected as a change in optical density at 405 nm and converted to international units of uPA with reference to a standard curve constructed using human single-chain uPA (American Diagnostica). Northern analysis was performed on RNA harvested 48 hours after infection. RNA was quantified with radiolabeled probes for uPA and GAPDH and a phosphorimager.28

**In Vivo Gene Transfer**

We performed in vivo gene transfer to carotid artery endothelium of specific pathogen-free male New Zealand White rabbits (3 to 4 kg, Western Oregon Rabbit Co).29 Vector stocks were infused at 2×10^10 to 5×10^11 parts/mL. All animal protocols were approved by the Animal Care and Use Committee of the University of Washington.

**In Vivo uPA Expression**

Arteries were harvested 3, 14, 28, and 56 days after gene transfer. Each artery was divided into 4 equal segments. Typically, the most proximal and distal parts of these segments were used for protein, RNA, and DNA analysis. These segments were opened longitudinally, weighed, and placed in M199 medium at 37°C for 1 hour; then they were rinsed 3 times and placed in medium for 4.5 hours. Conditioned media and arterial tissue were frozen for later determinations of PA activity, uPA mRNA, and vector DNA.

**Quantification of Viral Genomes**

HD-AduPA and FG-AduPA genomes were quantified by use of real-time polymerase chain reaction amplification of CMV promoter sequence22: primers, 5'-CATCTACGTTGTTAGCATCGCTATTACCA, 5'-TGGAATCCCGGTAGCTCA; probe, TCAGCCAGTACCTCTTCGATCAGCTGGT.
Histological and Morphometric Analyses
The middle 2 segments of each artery were divided in half, yielding 4 smaller segments. These were embedded side by side in OCT and frozen. Serial cryostat sections were stained to detect expression of CD43 (a T-cell antigen) and vascular cell adhesion molecule-1 (VCAM-1). Sections were photographed with a digital camera and the stained area quantified using image analysis software (Image-Pro Plus, Media Cybernetics). The stained area of each section was normalized to artery size by dividing the measured area (in mm²) by the length (in mm) of a circumferential line drawn through the middle of the arterial media. The values from the 4 sections were used to calculate a mean value for the artery. Neointimal and medial expression from FG-AduPA arteries was due primarily to adenovirus-infused arteries with FG-AduPA at a lower dose: 2×10⁹ parts/mL. uPA expression from these low-dose FG-AduPA arteries at 3 days was equivalent to expression from 3-day arteries infused with 5×10¹¹ parts/mL HD-AduPA (7.1 IU·g⁻¹·h⁻¹ [3.9 to 10 IU·g⁻¹·h⁻¹] versus 7.4 IU·g⁻¹·h⁻¹ [5.5 to 14 IU·g⁻¹·h⁻¹]; P=0.8; Figure 2A). However, by 14 days, uPA expression from these low-dose FG-AduPA arteries also declined to background levels (1.5 IU·g⁻¹·h⁻¹ [0.80 to 1.7 IU·g⁻¹·h⁻¹]). Thus, 14-day HD-AduPA arteries expressed uPA at a level 3-fold higher than 14-day FG-AduPA arteries (both doses) and H14 arteries (P=0.001 by ANOVA). HD-AduPA arteries also expressed significantly more uPA than low-dose FG-AduPA and H14 arteries at 28 and 56 days (P=0.032 and 0.012 by ANOVA; high-dose FG-AduPA was not tested beyond 14 days). Moreover, uPA expression from HD-AduPA arteries did not change significantly between 14 and 56 days (P=0.46 by ANOVA).

Humoral Immune Response to Adenovirus
Serum antibodies to adenoviral capsid proteins were measured on the day of gene transfer and 14 days later by ELISA.²\¹²

Statistical Analysis
Normally distributed data are presented as mean±SD and compared with the t test or 1-way ANOVA. Data either not normally distributed or for which group variances were unequal are expressed as median (25% to 75% range) and compared by rank-sum test or Kruskal-Wallis ANOVA.

Results
Preparation of HD-Ad
Purified stocks of HD-AduPA were 3 to 4×10¹² parts/mL. The FG-AduPA stock was 4.7×10¹⁰ parts/mL and 1.4×10¹¹ plaque-forming units/mL. E1A-positive genomes were below 1 in 10⁶ viral genomes in all vector stocks. Because HD-AduPA does not form plaques, the particle concentrations of the vectors were used for all studies.

In Vitro uPA Expression
ECs infected with HD-AduPA or FG-AduPA expressed vector-specific uPA mRNA and secreted increased amounts of active uPA (Figure 1). PA activity was typically 2- to 3-fold higher in cells infected with FG-AduPA compared with HD-AduPA.

Persistent In Vivo uPA Expression by HD-AduPA
Three days after vector infusion at 5×10¹¹ parts/mL, FG-AduPA arteries expressed 10-fold more uPA than HD-AduPA arteries (75 IU·g⁻¹·h⁻¹ [25% to 75% range, 27 to 150 IU·g⁻¹·h⁻¹] versus 7.4 IU·g⁻¹·h⁻¹ [5.5 to 14 IU·g⁻¹·h⁻¹]; P<0.001; Figure 2A). On day 14, PA activity of HD-AduPA arteries declined by 44% to 4.1 IU·g⁻¹·h⁻¹ (2.6 to 4.5 IU·g⁻¹·h⁻¹). In contrast, PA activity of FG-AduPA arteries declined by 98% to “background” levels (1.4 IU·g⁻¹·h⁻¹ [1.1 to 1.8 IU·g⁻¹·h⁻¹]; P=0.006 versus 14-day HD-AduPA arteries). Background PA activity of adenovirus-infused arteries was determined by measuring PA secretion from arteries infused with H14 (5×10¹¹ parts/mL) and harvested 3 to 56 days later. For example, 14-day H14 arteries secreted 1.2 IU·g⁻¹·h⁻¹ (0.35 to 1.7 IU·g⁻¹·h⁻¹; Figure 2A).

To exclude the possibility that the rapid loss of transgene expression from FG-AduPA arteries was due primarily to higher initial uPA expression by FG-AduPA, we infused arteries with FG-AduPA at a lower dose: 2×10⁹ parts/mL. uPA expression from these low-dose FG-AduPA arteries at 3 days was equivalent to expression from 3-day arteries infused with 5×10¹¹ parts/mL HD-AduPA (7.1 IU·g⁻¹·h⁻¹ [3.9 to 10 IU·g⁻¹·h⁻¹] versus 7.4 IU·g⁻¹·h⁻¹ [5.5 to 14 IU·g⁻¹·h⁻¹]; P=0.8; Figure 2A). However, by 14 days, uPA expression from these low-dose FG-AduPA arteries also declined to background levels (1.3 IU·g⁻¹·h⁻¹ [0.80 to 1.7 IU·g⁻¹·h⁻¹]). Thus, 14-day HD-AduPA arteries expressed uPA at a level 3-fold higher than 14-day FG-AduPA arteries (both doses) and H14 arteries (P<0.001 by ANOVA). HD-AduPA arteries also expressed significantly more uPA than low-dose FG-AduPA and H14 arteries at 28 and 56 days (P=0.032 and 0.012 by ANOVA; high-dose FG-AduPA was not tested beyond 14 days). Moreover, uPA expression from HD-AduPA arteries did not change significantly between 14 and 56 days (P=0.46 by ANOVA).
We hypothesized that the persistence of uPA expression from HD-AduPA arteries was due to persistence of HD-AduPA vector DNA. To test this hypothesis, we measured vector DNA in the same HD-AduPA and FG-AduPA arteries used for the PA activity time course experiment illustrated in Figure 2A. Indeed, 96% (92% to 97%) of low-dose and 98% (95% to 98%) of high-dose FG-AduPA DNA was lost between days 3 and 14, whereas only 64% (54% to 74%) of HD-AduPA DNA was lost during this time (P<0.001 by ANOVA; Figure 2B). The percentage of remaining vector DNA was also significantly higher in HD-AduPA than in low-dose FG-AduPA arteries at days 28 and 56 (P=0.004 and 0.002).

Rabbit arteries also express endogenous uPA. To exclude the unlikely possibility that elevated uPA expression in HD-AduPA arteries represented induction of endogenous uPA expression, 14-day HD-AduPA and FG-AduPA arteries (both high and low vector doses) were subjected to Northern analysis (Figure 3). The endogenous uPA mRNA and transgene uPA mRNA are different sizes and can therefore be discriminated by Northern analysis.22 In 11 of 12 FG-AduPA arteries, uPA transgene mRNA was less abundant than endogenous uPA mRNA. In contrast, in all HD-AduPA arteries, uPA transgene mRNA was more abundant than endogenous uPA mRNA. HD-AduPA transgene mRNA was 12-fold more abundant than FG-AduPA transgene mRNA (2.2 arbitrary units [1.8 to 3.1 arbitrary units] versus 0.18 arbitrary units [0.12 to 0.27 arbitrary units]; P<0.001).

Vascular Inflammation and Neointimal Formation
Fourteen days after vector infusion, HD-AduPA arteries had significantly less staining for T cells and VCAM-1 (Figures 4 and 5) and smaller neointimas than arteries infused with the same dose of FG-AduPA (P=0.01 to 0.001; Figure 5). HD-AduPA arteries had more T-cell staining and more neointimal formation than arteries infused with buffer alone (P≤0.05 for both). To exclude the unlikely possibilities that HD-AduPA arteries were damaged to such an extent that a robust inflammatory response was not possible or that HD-AduPA had intrinsic anti-inflammatory activity, we infused H14 (an FG-Ad virus) along with HD-AduPA. Coinfusion of H14 with HD-AduPA increased T-cell infiltration, VCAM-1 expression, and neointimal formation significantly (P<0.05 for all; Figures 4 and 5).

Humoral Immune Response to Adenovirus
Between days 0 and 14, rabbits infused with either FG-AduPA (n=7) or HD-AduPA (n=5) all developed significant titers (>1:100) of antibodies to adenoviral capsid proteins.

Expression from HD-AduPA Is Upregulated by FG-Ad
ECs transduced in vitro with HD-AduPA expressed 50% to 60% less uPA than ECs transduced with the same number of FG-AduPA particles (Figure 1). In vivo, however, 3-day HD-AduPA arteries expressed 90% less uPA than 3-day arteries infused with the same number of FG-AduPA particles (Figure 2). Because the 2 vectors contain the same uPA expression cassette, it seemed likely that HD-AduPA could express at a higher level in vivo. We hypothesized that a contribution of FG-AduPA that was independent of the CMV-uPA expression cassette increased transgene expression significantly in vivo. To test this hypothesis, we added H14 (an FG-Ad that does not encode uPA) to HD-AduPA (both at 4×10^{11} parts/mL) and infused this mixture into 6 arteries. Three days later, arteries infused with H14 and
HD-AduPA expressed 60-fold more uPA than arteries infused with 5×10^11 parts/mL of HD-AduPA alone (420 IU · g^{-1} · h^{-1} [138 to 522 IU · g^{-1} · h^{-1}] versus 7.4 IU · g^{-1} · h^{-1} [5.5 to 14 IU · g^{-1} · h^{-1}]; n=6, 12 respectively; P<0.001; Figure 6A). Indeed, uPA expression from HD-AduPA under these conditions exceeded uPA expression from FG-AduPA (Figure 2A). Northern analysis confirmed that the addition of H14 to HD-AduPA increased the level of CMV promoter-driven uPA transcripts (7.4-fold [5- to 12-fold]; n=4; Figure 6B) but had no effect on endogenous uPA transcripts (data not shown). However, the high level of uPA expression in H14+ HD-AduPA arteries did not persist. At day 14, PA activity in medium conditioned by H14+ HD-AduPA arteries declined to 5.6 IU · g^{-1} · h^{-1} (1.2 to 9.5 IU · g^{-1} · h^{-1}; n=6), a 99% (98% to 99.7%) decline from day 3. This decline in uPA expression was accompanied by a similar loss of HD-AduPA genomes. When infused with H14, 90% (86% to 95%) of HD-AduPA genomes were lost between days 3 and 14 compared with 64% (54% to 74%) when HD-AduPA is infused alone. Thus, addition of H14 increases expression from HD-AduPA at day 3 but significantly accelerates the rate of loss of both expression and vector genomes (P=0.001 for both). To test whether H14 also upregulated expression from HD-AduPA in vitro, we infected rabbit ECs with HD-AduPA alone or with H14. Addition of H14 upregulated expression of uPA protein and mRNA only minimally (Figure 6C and 6D and data not shown).

**Discussion**

We tested the ability of HD-AduPA to achieve prolonged transgene expression in rabbit carotid arteries. Our major findings were the following. First, in vitro, HD-AduPA expressed less uPA protein than FG-AduPA. Second, in vivo, HD-AduPA initially expressed uPA protein at lower levels than FG-AduPA. However, uPA expression from FG-AduPA was essentially lost within 14 days, whereas expression from HD-AduPA persisted for at least 56 days, with stable expression from 14 to 56 days. Third, HD-AduPA DNA was significantly more persistent in vivo than FG-AduPA DNA. Fourth, infusion of HD-AduPA was associated with minimal vascular inflammation compared with FG-AduPA. Fifth, uPA expression from HD-AduPA was upregulated significantly by coinfusion of FG-Ad. Finally, coinfusion of FG-Ad with HD-AduPA increased inflammation and accelerated loss of both HD-AduPA genomes and transgene expression. We conclude that HD-Ad can achieve persistent, high-level transgene expression in large-vessel endothelium while minimizing the host inflammatory response.

Experiments performed with FG-Ad provide insights into the biological roles of gene products and suggest gene therapy strategies for preventing or reversing vascular disease.1,2 However, inflammation induced by infusion of FG-Ad can confound interpretation of experiments. In experiments with FG-Ad, the role of the transgene product is revealed on a background of inflammation; this role might differ in an uninflamed vessel. Vector-induced inflammation also limits the utility of FG-Ad for gene therapy. Inflammation plays a central role in the pathogenesis of atherosclerosis,30 and vectors that induce inflammation would be expected to worsen vascular disease. Brevity of expression of FG-Ad is also problematic for both experimental and clinical applications. Use of FG-Ad to express a transgene in an animal model, while informative, may reveal only effects of a short, intense pulse of transgene expression. Use of FG-Ad clinically would not be expected to alter the course of most chronic human vascular diseases, which are lifelong and will

![Figure 5](http://circ.ahajournals.org/)

*Figure 5. Quantification of inflammation and neointimal formation. Arteries were infused with indicated vectors and concentrations (parts/mL) or buffer alone and harvested 14 days later. Areas within intima staining for CD43 (A; a T-cell marker), VCAM-1 (B), and total neointimal area (C) were measured by computer-assisted planimetry and normalized to artery size. Data points represent individual arteries; bar heights are group medians. Statistical significance of intergroup differences is indicated.*
likely require years of therapy. For these reasons, many groups have attempted to develop vectors for vascular gene transfer that do not cause inflammation and that stably express transgenes.

We were initially uncertain whether HD-Ad would confer stable transgene expression in the artery wall. This uncertainty derived in part from our inability to achieve stable transgene expression after infusion of FG-Ad in arteries of immune-deficient mice and immunosuppressed rabbits.12,21 Mice in these earlier studies were RAG-2 null and therefore completely deficient in antigen-specific immunity. The rabbits were sufficiently immunosuppressed by cyclophosphamide that they did not form antibodies to the infused adenovirus and had virtually no T cells in the transduced artery segments. These data suggested that evasion of the antigen-specific immune system, eg, with HD-Ad, might be inadequate to ensure stable transgene expression. Here, we report relatively stable, prolonged transgene expression with HD-AduPA despite activation of the antigen-specific immune system, evidenced by the presence of high titers of anti-adenoviral antibodies. Persistence of HD-AduPA expression does not de-\refeqref{circulation:6}cline after day 14 and because promoter shutdown typically occurs after transgene expression.

The kinetics of loss of uPA expression from HD-AduPA arteries (Figure 2A) and the dramatic increase in HD-AduPA expression in arteries coinfused with H14 (Figure 6A) suggest 3 mechanisms to account for the decline in HD-AduPA expression between days 3 and 14. First, the decline in HD-AduPA expression could be due entirely to loss of transcriptionally active HD-AduPA genomes. Indeed, the loss of HD-AduPA genomes between days 3 and 14 (64% [54% to 74%]) is similar to the decline in uPA expression from HD-AduPA arteries during the same period (44% [38% to 64%]). However, because some of the lost HD-AduPA genomes may not have ever been transcriptionally active, the contribution of genome loss to the decline in expression could be underestimated. A second plausible mechanism for the decline in HD-AduPA expression is that on day 14, the remaining HD-AduPA genomes are, on average, expressing fewer uPA transcripts than they expressed on day 3. Loss of expression from these genomes could be due to an active process of “promoter shutdown,” caused, for example, by promoter methylation.31 However, promoter shutdown seems unlikely here because HD-AduPA expression does not decline after day 14 and because promoter shutdown typically
leads to complete loss of transgene expression.\textsuperscript{32} A third possible mechanism is that the decline in HD-AduPA expression between days 3 and 14 is more a function of upregulated transcription at day 3 than of either loss of transcriptionally active HD-AduPA genomes or promoter shutdown at day 14. This mechanism is supported by data from the experiment in which addition of H14 to HD-AduPA increased expression from HD-AduPA 60-fold at day 3. Thus, the 2-fold difference in uPA expression from HD-AduPA arteries harvested on day 3 versus 14 may reflect a modest and transient upregulation of HD-AduPA expression on day 3, possibly because of stimulation of expression from the CMV promoter, as described below.

The dramatic upregulation of HD-AduPA expression 3 days after coinfusion of H14 suggests that expression from HD-AduPA could also be upregulated by application of an appropriate transcriptional stimulus at a later time point. Upregulation would simply require an intervention that duplicated the effects of H14 on CMV promoter–driven transcription. These effects may be mediated through an H14-induced local inflammatory response (Figures 4 and 5) with cytokine-mediated upregulation of CMV promoter activity\textsuperscript{33} or by activation of NF-κB-mediated transcription.\textsuperscript{32} As a first step toward testing the hypothesis that HD-AduPA expression could also be upregulated at a later time point, we removed 6 arteries 14 days after HD-AduPA infusion and exposed half of each artery ex vivo to phorbol myristate acetate (PMA; 200 ng/mL) and half to buffer alone. Others have shown that PMA stimulates CMV promoter activity in adenovirus-infected vascular cells.\textsuperscript{34} Indeed, PMA upregulated artery wall PA activity by 6-fold compared with the control segments \(P<0.05\) to a level 2-fold above the PA activity of untransduced arteries treated with PMA. These in vitro data are preliminary and require in vivo confirmation; however, they suggest that if a less toxic and more specific intervention than PMA infusion could be identified, uPA expression from HD-AduPA could be upregulated in vivo at later time points.

Previous studies have reported both upregulation of HD-Ad by addition of FG-Ad and upregulation of CMV promoter activity by adenoviral gene products,\textsuperscript{35,36} In both cases, upregulated expression was due to transactivation of the CMV promoter by adenoviral E4 proteins expressed by a FG-Ad. Consistent with this model, both studies reported significant upregulation in vitro and in vivo. In contrast, although our data do not exclude a modest role for transactivation of the CMV promoter by FG-Ad proteins, the large difference between in vivo upregulation (60-fold) and in vitro upregulation by FG-Ad (1.2– to 2-fold) suggests that an in vivo, non–cell-autonomous process, such as inflammation resulting from the FG-Ad, is primarily responsible for upregulating HD-AduPA expression. A final, important conclusion from the H14 addition experiments is that, when stimulated, HD-AduPA can express more uPA in vivo than FG-AduPA. On the basis of our previous study in which a 10-fold increase in uPA expression was sufficient to decrease thrombus accumulation in an ex vivo AV shunt,\textsuperscript{25} stimulated HD-AduPA expression (which achieves PA activity >100-fold above endogenous levels) is more than adequate to produce a local antithrombotic effect.

In summary, HD-Ad, infused into rabbit carotid arteries, can express uPA, a potentially therapeutic gene, at a level 3-fold above endogenous expression levels for at least 8 weeks with only low levels of associated inflammation. HD-Ad will be useful for carrying out longer-term, mechanistic investigations in vascular biology. HD-Ad might also be useful in a clinical setting to express therapeutic genes that prevent thrombosis,\textsuperscript{37,38} reverse lipid accumulation,\textsuperscript{39} or retard inflammation in the artery wall.\textsuperscript{40}

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