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Construction of a novel expression cassette for increasing transgene expression *in vivo* in endothelial cells of large blood vessels

Nagadhara Dronadula, PhD, Liang Du, MD PhD, Rowan Flynn, PhD, Joshua Buckler, MD, Jordan Kho, BS, Zhilong Jiang, MD PhD, Shinji Tanaka, MD PhD, and David A. Dichek, MD
Department of Medicine, University of Washington, Seattle, Washington, USA

Abstract

The success of gene therapy hinges on achievement of adequate transgene expression. To ensure high transgene expression, many gene-therapy vectors include highly active virus-derived transcriptional elements. Other vectors include tissue-specific eukaryotic transcriptional elements, intended to limit transgene expression to specific cell types, avoid toxicity, and prevent immune responses. Unfortunately, tissue specificity is often accompanied by lower transgene expression. Here we use eukaryotic (murine) transcriptional elements and a virus-derived posttranscriptional element to build cassettes designed to express a potentially therapeutic gene (interleukin-10) in large vessel endothelial cells (EC) at levels as high as obtained with the CMV immediate-early promoter, while retaining EC-specificity. The cassettes were tested by incorporation into helper-dependent adenoviral vectors, and transduction into bovine aortic EC *in vitro* and rabbit carotid EC *in vivo*. The murine endothelin-1 promoter showed EC-specificity, but expressed only 3% as much IL-10 mRNA as CMV. Inclusion of precisely 4 copies of an EC-specific enhancer and a posttranscriptional regulatory element increased IL-10 expression to a level at or above the CMV promoter *in vivo*, while retaining—and possibly enhancing—EC specificity, as measured *in vitro*. The cassette reported here will likely be useful for maximizing transgene expression in large vessel EC, while minimizing systemic effects.

Keywords

adenovirus; endothelium; carotid artery; interleukin-10; gene expression regulation

Introduction

Endothelial cells (EC) of large arteries play major roles in several important physiologic and pathologic processes including regulation of hemostasis and thrombosis, control of

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Correspondence: David A. Dichek, Department of Medicine, University of Washington, 1959 NE Pacific St, Box 357710, Seattle, WA 98195-7710. ddichek@uw.edu.

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leukocyte trafficking, regulation of vascular tone, and modulation of lipoprotein entry to the vascular wall.¹ Durable, high-level expression of recombinant genes in large-vessel EC, using *in vivo* gene transfer, is potentially a powerful tool for understanding these biological processes and for preventing and treating human diseases such as atherosclerosis and thrombosis.²⁻⁵ However, achievement of durable, high-level transgene expression in EC *in vivo* has been challenging.⁶⁻⁹

Several groups have focused on the problem of increasing transgene expression levels in EC while limiting expression in other cell types, in which transgene expression could be deleterious.^{10,20} Expression cassettes constructed by these groups contain EC-specific eukaryotic promoter and enhancer elements, expected to be active in EC and less active in other cell types. These cassettes express transgenes at high levels in EC in tumor vessels and in small vessels of ischemic muscle, estrogen-stimulated uterine tissue, liver, and lung; often with evidence of relative EC-specific expression.^{11, 13, 15, 17, 20} However, far less attention has been devoted to maximizing transgene expression in EC of large blood vessels, in which transcriptional regulation is likely different than in smaller vessels.²¹ Many investigators use the cytomegalovirus (CMV) immediate early promoter to drive transgene expression in large vessel EC despite its lack of tissue-specificity and its susceptibility to attenuation and shutdown.^{16, 22} The need for an EC-specific expression cassette that outperforms CMV in large vessel EC is well enunciated; however, attempts to construct such a cassette have not been successful.¹⁶

Several years ago, we reported that helper-dependent adenovirus (HDAd) can express a transgene in EC of large arteries for at least 2 months—far longer than first or second-generation Ad vectors—and with negligible local inflammation.²³ These results suggest that HDAd will be extremely useful for expressing transgenes in EC of large vessels and that HDAd is therefore an attractive platform for developing and testing highly active, EC-specific expression cassettes. Here we report use of a 1.36 kb enhancer-promoter fragment from the murine endothelin-1 (mET-1) gene,²⁴ a truncated version of the ET-1 enhancer-promoter that contains 3 additional copies of a 45 bp EC-specific ET-1 enhancer element (“ETE”), a genomic (intron-containing) DNA sequence, and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE)²⁵ to attempt to build a cassette that expresses at least as well as CMV in large-vessel EC *in vivo* while retaining significant EC-specificity.

Results

Use of the mET-1 enhancer-promoter and a genomic IL-10 clone as a strategy to obtain high-level, EC-specific transgene expression

We used the potentially therapeutic²⁶ rabbit interleukin-10 (IL-10) cDNA and gene as reporters in experiments aimed at developing improved expression cassettes. Our initial hypotheses were that the native mET-1 enhancer-promoter would express at a lower level than the CMV promoter (as is typically the case for cell-type-specific promoters),^{12, 16} and that the native mET-1 enhancer-promoter would show relative EC-specificity compared to the 750 bp CMV promoter. Because inclusion of introns can boost transgene expression,^{16, 27} we also hypothesized that vectors containing the complete IL-10 exon-intron sequence

would express at higher levels than vectors containing the IL-10 cDNA. We began to test these hypotheses by transducing bovine aortic EC (BAEC) with HDAd containing four different IL-10 constructs (CMV-cIL-10, CMV-gIL-10, mET1-cIL-10 and mET1-gIL-10) as well as HDAdNull, as a negative control (Figure 1). We measured IL-10 mRNA in transduced cells and normalized expression both to GAPDH mRNA and to vector DNA measured in cells from the same well. Expression from CMV-cIL-10 was 30-fold higher than mET1-cIL-10 and was approximately 3-fold higher than CMV-gIL-10 (Figure 2a). Expression from the two mET-1 vectors (mET1-cIL-10 and mET1-gIL-10) was not significantly different. Two-way ANOVA confirmed overall effects of CMV versus mET-1 promoters (CMV > mET-1; $P < 0.001$); this effect was present for both cDNA and gDNA. There was also an overall effect of cDNA versus gDNA (cDNA > gDNA; $P < 0.001$); however, this was driven by the CMV results and was not evident at the low levels of expression obtained with the mET1 promoter.

We next looked for evidence of EC specificity of the mET-1 enhancer-promoter. The same vectors were used to transduce bovine aortic smooth muscle cells (BASMC), and IL-10 expression was again measured. For both BAEC and BASMC, IL-10 expression from the CMV promoter-containing vectors (both cDNA and genomic) was assigned a value of 100%. Expression from each of the mET-1-containing vectors was then calculated as a fraction of the expression of the analogous CMV-containing vector in the same cell type. Using this calculation, EC specificity would manifest as a higher percentage of CMV-mediated expression in EC than in SMC. The mET1-gIL-10 cassette expressed IL-10 mRNA at a higher relative level in BAEC than BASMC (4-fold higher; $P = 0.009$) whereas the mET1-cIL-10 cassette expressed at a similar level relative to CMV in both cell types (Figure 2b; $P = 0.6$). Two-way ANOVA confirmed an overall effect of cell type (EC > SMC) on level of transgene expression from the mET-1 promoter ($P = 0.03$).

We next performed this same 4-vector comparison in our *in vivo* EC gene transfer model.^{9,28} The four IL-10-expressing vectors and the control vector HDAdNull were infused into surgically isolated rabbit carotid arteries and IL-10 mRNA was measured in arterial extracts 3 days later. In contrast to the *in vitro* results, expression from HDAd-CMV-gIL-10 was highest *in vivo* (2-fold above HDAd-CMV-cIL-10; $P = 0.06$; Figure 2c). Similar to the *in vitro* results, IL-10 mRNA levels in arteries infused with either of the mET-1 vectors were far below IL-10 mRNA levels in arteries infused with the CMV vectors. The background signal in this experiment was the q-RT-PCR signal from arteries transduced with HDAdNull; the signal from arteries transduced with both of the mET-1 vectors was at this level (Figure 2c). Therefore, if either of the mET-1 vectors expressed IL-10 mRNA *in vivo*, the level of mRNA was too low for us to detect.

Increased IL-10 expression and preserved EC-specificity in vitro after addition of EC-specific enhancer elements

To increase expression from the mET-1 promoter, we constructed plasmids in which the ET-1 promoter sequence 5' of the native 45 bp EC-specific ET-1 enhancer (the ETE; located at -364 to -319 in the mouse ET-1 gene; Figure 1)²⁴ was replaced by 2, 3, or 4 additional copies of the ETE. In these plasmids the ETE were located upstream of a minimal (318 bp)

mET-1 promoter and the rabbit IL-10 cDNA (Supplementary Figure 1). These plasmids were transfected into BAEC and expression of IL-10 mRNA quantified. The plasmid containing 3 copies of the ETE (the native ETE with 2 additional copies: “3XETE”) expressed 4–5-fold more IL-10 mRNA than the plasmid containing 1 ETE copy. The plasmid with 4 copies of the ETE (“4XETE”) expressed twice as much IL-10 mRNA as plasmids containing 3 or 5 copies ($P < 0.001$ for all comparisons; Figure 3a).

Based on this result we constructed a new HDAd in which 3 copies of the ETE were added upstream of the endogenous ETE and minimal mET-1 promoter (4XETE-cIL-10; Figure 1). EC were transduced with one of 3 HDAd: 4XETE-cIL-10, CMV-cIL-10, or mET1-cIL-10 and IL-10 mRNA measured. In these experiments, in relation to CMV-cIL-10 expression set at 100%, inclusion of 4 ETE copies increased IL-10 expression by about 7-fold compared to HDAd-mET1-cIL-10 (Figure 3b; $P < 0.001$). However, this increased level of expression was still only about 30% of levels obtained with HDAd-CMV-cIL-10 ($P = 0.02$).

To determine whether addition of 3 copies of the ETE and removal of the ET-1 promoter sequences 5' of the native ETE (in HDAd4XETE-cIL-10) affected EC-specificity, we compared IL-10 expression from HDAd4XETE-cIL-10 in BAEC and BASMC. When normalized to CMV-directed expression in the same cell type, HDAd4XETE-cIL-10 expressed approximately 4-fold more IL-10 mRNA in BAEC than in BASMC ($P < 0.001$; Figure 3c). By comparing these data to data generated with the mET1-driven vectors (Figure 2b) we noted that addition of the 4X-ETE increased IL-10 expression not only in EC, but also in SMC. Therefore, the 4X-ETE is not completely EC-specific. To assess this observation more quantitatively, we repeated the measurements of IL-10 expression using a randomly selected subset of samples from the *in vitro* experiments with the mET1-cIL10 and 4XETE-cIL-10 vectors (samples from the experiments shown in Figure 2b and 3c). We measured IL-10 expression simultaneously in all samples, without normalization to CMV expression in the same cell type (Figure 4). These measurements revealed—as in Figure 2b—that expression from mET1 was low in both EC and SMC. However, here we detected significantly higher expression from mET1 in EC versus SMC (2-fold; $P < 0.001$). Addition of the 4X-ETE increased IL-10 expression in both cell types but far more in EC than SMC (280-fold versus 28-fold). Therefore, although the 4X-ETE is not completely EC-specific, it is far more active in EC than SMC and therefore contributes both higher expression and increased EC-specificity above that achieved with the mET1 promoter.

Increased IL-10 expression and preserved EC-specificity *in vitro* after addition of a posttranscriptional regulatory element

To further increase vector-mediated transgene expression while hopefully maintaining relative EC-specificity, we inserted both the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE)²⁵ and an optimized version of this element (oPRE; in which the potentially oncogenic X protein sequences are deleted)²⁹ into the IL-10 3' UTR in plasmids containing the CMV-cIL-10 and 4XETE-cIL-10 expression cassettes. We tested both PRE in both orientations in plasmids containing both the CMV and 4XETE-mET-1 promoters. Results of these EC transfections were generally unimpressive, with increased IL-10 mRNA

(< 2-fold) only from the WPRE in the forward orientation, and only with the CMV promoter (Supplementary Figure 2a,b).

Because our *in vitro* plasmid transfection data did not always predict relative expression levels when the same cassettes were transduced by HDAd *in vivo* (compare CMV-cIL-10 and CMVgIL-10 in Figure 2a versus 2c), before abandoning the PREs we tested one of them in an HDAd. We selected the oPRE in the forward orientation, and used it to construct HDAd4XETE-cIL-10-oPRE (Figure 1). In this experiment, in relation to CMV-cIL-10 expression set at 100%, BAEC transduced with HDAd4XETE-cIL-10-oPRE expressed about 50% more IL-10 mRNA than BAEC transduced with HDAd4XETE-cIL-10 (Figure 5a; $P = 0.01$). This level of expression was about 60% of that obtained in parallel transductions with HDAd-CMV-cIL-10. At this stage of our experiments, we developed a western blot method for detection of rabbit IL-10 protein. Western analysis of media conditioned by BAEC transduced with HDAd4XETE-cIL-10-oPRE and HDAd-CMV-cIL-10 revealed that HDAd4XETE-cIL-10-oPRE produced at least as much IL-10 protein as HDAd-CMV-cIL-10 (Supplementary Figure 3), possibly reflecting a post-transcriptional effect of the oPRE.

To determine whether addition of the oPRE affected EC-specificity, we compared IL-10 expression from HDAd4XETE-cIL-10-oPRE in BAEC and BASMC. When normalized to CMV-directed expression in the same cell type, HDAd4XETE-cIL-10-oPRE expressed approximately 3-fold more IL-10 mRNA in BAEC than in BASMC ($P < 0.001$; Figure 5b). Therefore, significant EC-specificity was retained after addition of the oPRE. To further examine the potency and specificity of the oPRE, we measured IL-10 mRNA in a randomly selected subset of samples from those portrayed in Figure 5b, and compared IL-10 mRNA in these samples with IL-10 mRNA in samples from EC and SMC transduced with the mET1-cIL-10 and 4XETE-cIL-10 vectors (Figure 4). This analysis revealed that addition of the oPRE increased IL-10 expression equally (3-fold) in both cell types. Therefore, unlike the 4XETE, the oPRE does not act preferentially in EC.

Increased *in vivo* IL-10 expression from vectors containing 4XETE and the oPRE

We next tested whether addition of the 4XETE and oPRE to the mET-1 promoter also increased transgene expression in rabbit EC *in vivo*. IL-10 mRNA was measured in rabbit carotids infused with one of 4 HDAd: CMV-cIL-10, mET1-cIL-10, 4XETE-cIL-10 and 4XETE-cIL-10-oPRE (Figure 6). As before, expression from mET1-cIL-10 was far below expression from CMV-cIL-10. Addition of the 3 ETE copies to the native mET-1 enhancer-promoter greatly increased IL-10 expression (9-fold; $P < 0.001$), yielding expression that was not significantly different from that obtained with the CMV promoter-containing vector ($P = 1$). Addition of the oPRE further increased IL-10 expression by 2-fold ($P = 0.01$ versus 4XETE-cIL-10) to a level 35% higher than CMV-cIL-10-transduced arteries, although this difference was of only borderline statistical significance ($P = 0.1$).

Lack of systemic vector dissemination and transgene expression

If vectors are released from the site of infusion, disseminate throughout the animal, and transduce cells in distant tissues, and if the transduced cells in these distant tissues are not

EC, then transgene expression in distant tissues could provide an *in vivo* read-out of promoter specificity. We therefore determined whether vector DNA was present in distant tissues after carotid gene transfer, and if so whether the IL-10 transgene was expressed.

We measured vector DNA and IL-10 expression in tissues from rabbits after carotid artery infusion of either HDAd-CMV-cIL-10 or HDAd-Null. Vector DNA was below background levels (as determined by measurement of DNA in tissues of unoperated animals) in all tissues other than the carotids (Figure 7a). Because vector-directed RNA (present as numerous copies per cell) might be more easily detected than vector DNA (fewer copies per cell), we also measured IL-10 mRNA in these organs. IL-10 mRNA was not significantly above background levels (as determined by measurement of IL-10 mRNA in tissues from HDAdNull-infused rabbits) at any site other than the common carotid artery (Figure 7b).

Discussion

The objective of this study was to construct an expression cassette that would drive high-level transgene expression in large vessel EC transduced by an HDAd vector and also provide significant cell-type specificity. The need for an EC-specific cassette that achieves expression levels in large vessel EC at least as high as those obtained with the CMV promoter is well stated in a recent article by White *et al.*¹⁶ We pursued this goal using rabbit IL-10 cDNA and genomic clones, murine ET-1 promoter and enhancer sequences, and a post-transcriptional regulatory element (PRE). Our major findings were: 1) The native murine 1.36 kb ET-1 enhancer-promoter expresses at a far lower level than the CMV promoter in EC both *in vitro* and *in vivo*; 2) inclusion of rabbit IL-10 introns in the expression cassette leads to a modest increase in IL-10 expression in rabbit EC *in vivo*, but lowers expression in BAEC *in vitro*; 3) The 1.36 kb native murine ET-1 enhancer-promoter provides modest EC-specificity of transgene expression; 4) Addition of ET-1 enhancer elements substantially increases transgene expression in EC both *in vitro* and *in vivo* and increases EC-specificity; 5) Addition of a PRE further increases transgene expression in EC; 6) Transgene expression from a cassette including a 364 bp ET-1 enhancer-promoter, 3 additional ET-1 enhancers, and a PRE is at least as high as is obtained with the CMV promoter *in vivo*, and—based on parallel *in vitro* studies—is more EC-specific.

The CMV promoter is often incorporated into gene-transfer vectors because of its high activity, small size, and ability to express transgenes in a wide range of cell types. CMV is especially useful for proof-of-concept studies that require only a burst of transgene expression, especially those carried out only *in vitro*. However, the utility of CMV *in vivo* is limited by its lack of tissue specificity, which allows transgene expression in nontargeted cell types including antigen-presenting cells.^{22,30} The CMV promoter is also susceptible to promoter attenuation and shutdown, far more so than eukaryotic promoters.^{22,31} Other disadvantages of CMV for cardiovascular applications include downregulation by β -blockers³² (drugs that are taken by many patients with cardiovascular diseases) and upregulation by inflammation,²³ which many cardiovascular therapies aim to reduce.³³

Because of the limitations of CMV and other viral promoters for EC-targeted gene transfer, several groups have constructed and tested vectors with putative EC-specific promoters

including Flt-1, ICAM-1, ICAM-2, von Willebrand factor, Tie-1, Tie-2, flk-1, vascular endothelial cadherin (VE-cadherin), lysyl oxidase-1 (LOX-1), and endoglin.¹¹⁻¹⁶ In several cases relative EC-specificity was achieved compared to viral or broadly active eukaryotic promoters such as phosphoglycerate kinase. However, EC-specificity was acquired at the cost of reliably lower transgene expression.^{12, 14, 16}

In work published largely since we began this project, the ET-1 promoter and enhancer were used to target EC—with apparent success—by at least two groups. One group used the ET-1 promoter and a multimerized 3X ETE to construct Ad for use in EC-targeted gene therapy aimed at blocking tumor angiogenesis or promoting ischemic angiogenesis.^{17, 18, 20, 34} The vectors appeared relatively EC-specific and dramatically outperformed the CMV promoter both *in vitro* (in BAEC and HUVEC) and *in vivo*. However, the exact sequences of these ET-1-based promoter/enhancer constructs are not published,^{17, 18, 20, 34} and the *in vivo* expression data are largely limited to small vessels, with no quantitation of absolute expression levels achieved in large vessel endothelium.^{17, 18, 34} For these reasons it is difficult to compare our results with these precedents. A second group fused multimers of the ETE (4X and 7X) to the human Cdc-6 and cyclin A promoters, to generate expression cassettes specific for proliferating EC.^{19, 35} The ETE multimers added EC specificity to these proliferation-sensitive promoters and conferred upon the Cdc-6 promoter an ability to express a reporter gene in tumors and estrogen-stimulated uterus at a level similar to that obtained with the CMV promoter. However, these expression levels appeared low; only about 1% of CMV expression in normal lung. Our study is the first to introduce a eukaryotic expression cassette *in vivo* in large vessel EC and unequivocally achieve transgene expression at least as high as obtained from the CMV promoter.

Several groups have shown that inclusion of introns in plasmid and viral vectors increase transgene expression.^{16, 27, 30, 36, 37} We were therefore surprised that the IL-10 cDNA vector expressed significantly more IL-10 mRNA *in vitro* than the genomic IL-10 vector (Figure 2a). In contrast, the genomic vector expressed more IL-10 mRNA than the cDNA vector *in vivo* (Figure 2c). The reason for this discrepancy is unclear, but could include use of EC from different species (bovine *in vitro* versus rabbit *in vivo*) or alteration in EC transcriptional regulation after removal from the vessel wall.²¹ It is possible that bovine—but not rabbit—EC express a transcriptional regulator that suppresses expression by binding to the genomic rabbit IL-10 sequences. We could not test the role of species specificity because rabbit endothelial cells are no longer available to us. Another example of *in vitro/in vivo* discrepancy is evident in Figures 5a and 6, in which the 4XETE-oPRE vector compares far more favorably to CMV *in vivo* than *in vitro*. This might be due to presence of ETE-binding transcription activators in rabbit EC *in vivo* versus their absence in cultured BAEC. Because we constructed the final 2 vectors (4XETE-cIL-10 and 4XETE-cIL-10-oPRE; Figure 1) based on *in vitro* results showing superiority of the IL-10 cDNA (Figure 2a) we may have missed an opportunity to construct vectors that—because they contained the genomic IL-10 sequence—expressed higher than both 4XETE-cIL-10-oPRE and CMV-cIL-10 *in vivo* (Figure 6). However, the increased expression level provided by the genomic DNA versus cDNA was relatively small (2-fold; Fig. 2c), of only borderline significance ($P = 0.06$) and was not found in transduced arteries of fat-fed rabbits (data not shown). Based

on the comparatively dramatic effect of adding 3 copies of the ETE (> 200-fold increase; Figure 4) introduction of additional 5' regulatory elements to the expression cassette is likely to be a more fruitful approach for increasing transgene expression.

Some of the features of the rabbit carotid model used here are worthy of comment. First, compared to the rat carotid gene transfer model with which we investigated transcriptional regulation in EC,³⁸ gene transfer in the rabbit model is more reproducible and transgene expression levels are farther above background. Second, there is no evidence in the rabbit model of vector dissemination beyond the site of gene transfer (Figure 7). Although it is likely that a small amount of vector is released systemically and is present in distant tissues below the limit of assay detection, our data nevertheless suggest that surgically isolated blood vessels can be efficiently transduced *in situ* by Ad without causing a potentially toxic systemic exposure to adenovirus.³⁹ Confinement of transgene expression to the site of gene transfer is particularly important when expressing immunosuppressive genes such as IL-10 which—if released systemically—could cause immunosuppression and susceptibility to opportunistic infection. The absence of detectable vector DNA and RNA at distant sites also suggests that local vascular IL-10 gene therapy can be accomplished without systemic side effects.

In summary, we report an animal model and a general approach (incorporation of EC-specific transcriptional elements and other cis-acting sequences) that dramatically increases HD-Ad-driven transgene expression in EC (Figure 6). *In vivo* expression levels with the final construct are at least as high as those obtained with CMV. To our knowledge expression from a eukaryotic-based promoter at a level equivalent to CMV has never been unequivocally demonstrated in EC of a large vessel. Moreover, *in vitro* studies (Figures 4 and 5b) provide strong support for preservation of substantial EC-specificity, even at the highest expression levels. This model and approach will be useful for testing additional vector modifications aimed at further increasing transgene expression,^{30,40} adding physiologic regulation,⁴¹ and enhancing duration of expression. Improved vectors, designed and tested with this approach, will increase the likelihood that vascular gene therapy will be clinically useful.

Materials and methods

Construction of IL-10 expression cassettes and HDAd vectors

A plasmid containing a rabbit IL-10 cDNA was a kind gift from Dr. Harvey Perkins (Australian National University, Canberra, AU).⁴² We cloned the rabbit IL-10 gene by PCR amplification of genomic DNA extracted from the skin of a New Zealand White rabbit. Plasmid pqET-1, containing the murine endothelin-1 (mET-1) promoter, was obtained from Dr. T. Quertermous (Stanford University, Palo Alto, CA). To increase expression from cassettes containing the mET-1 promoter, we inserted additional copies of a 45-bp endothelium-specific enhancer element (“ETE”)²⁴ present in the mET-1 promoter upstream of the native ETE in the mET-1 promoter. Multimers of the ETE were constructed using standard recombinant DNA methods. The WPRE was obtained from Dr. David Russell (University of Washington, Seattle).⁴³ We also used a truncated version of the WPRE, termed “oPRE” or “optimal Post-transcriptional Regulatory Element”, obtained from Dr.

Axel Schambach (Hannover Medical School, Germany).²⁹ The oPRE lacks the X Protein coding sequence and promoter, which are associated with oncogenic activity.⁴⁴ To enable efficient transfer of the IL-10 expression cassettes into the HDAd backbone plasmid pC4HSU (Microbix Biosystems, Toronto, ON, Canada) we constructed a shuttle plasmid that we used to insert cloned sequences into pC4HSU by homologous recombination (Supplementary Figure 4). A detailed description of cloning of the rabbit IL-10 gene, construction of all of the expression cassettes (Figure 1), as well as generation and production of the HDAd vectors is provided in the Supplementary Information.

Characterization of HDAd vectors

Viral particle (part) concentration was measured by spectrophotometer and was typically $1 - 3 \times 10^{12}$ part/ml.⁴⁵ E1A and helper virus (H14) contamination were measured with a quantitative (q) PCR-based approach in which viral genomes were measured by qPCR for SV40 polyA sequences and both E1A-containing and H14 sequences were measured by qPCR for sequences present only in E1A or H14. Absolute quantification of copies was achieved by reference to standard curves constructed by performing qPCR on known amounts of plasmid DNA containing the target sequences. Probes for qPCR were from Applied Biosystems (Foster City, CA) or IDT (Coralville, IA), primers were from Invitrogen (Carlsbad, CA) or Bioneer (Alameda, CA), and qPCR reagents were from Abgene/Thermo Fisher (Waltham, MA). E1A contamination in the viral preparations was below 1 in 10^6 HDAd genomes, and helper virus contamination was less than 1% of HDAd genomes.

Quantification of IL-10 expression in vitro

Bovine aortic endothelial cells (BAEC) and bovine aortic smooth muscle cells (BASMC) were purchased (Cell Applications Inc, San Diego, CA) and expanded in culture. Cells were transduced at passages 6 – 8 by addition of HDAd at 1×10^{10} part/ml (MOI approximately 2×10^4) at $t = 0$. Six hrs later, the vector-containing medium was aspirated, and the cells washed and incubated with fresh medium. The cells were harvested at $t = 24$ hr. RNA was extracted and purified using Trizol (Invitrogen) or the RNeasy mini kit (Qiagen, Valencia, CA); DNA was extracted and purified with the DNeasy kit (Qiagen). RNA was first digested with DNase I to remove contaminating genomic DNA, then 100 ng of RNA was reverse-transcribed and amplified with a one-step qRT-PCR protocol and reagents from Abgene/Thermo Fisher. qRT-PCR probes were from Applied Biosystems or IDT and the primers from Bioneer. The IL-10 specific sequences are: forward primer, 5'-AGA ACA GCT GCA TTC ACT TTC CA-3'; reverse primer, 5'-CCT TCG ATT GAA AGA AAG TCT TCA C-3'; probe, 5'-6-FAM-CTC CGC GAG CTC CGT GCT GC-TAMRA-3'. Cycle threshold (Ct) values for IL-10 were normalized for the amount of input RNA using Ct values for GAPDH obtained with the same RNA sample. IL-10/GAPDH values were then normalized for transfection/transduction efficiency by dividing by the number of vector copies in the transfected/transduced cells, obtained by qPCR for the SV40 polyA sequence. In each set of experiments, IL-10 expression from a CMV promoter-containing vector (containing the IL-10 cDNA for all experiments except for the cell-type-specificity experiments, in which both the cDNA and genomic DNA-containing vectors were used) was assigned a value of 100%. Expression from mET-1 promoter-containing vectors was calculated as percentage of the expression of the analogous CMV promoter-containing vector, measured in the same cell

type. All *in vitro* experiments were performed three times with an $n = 3$ per treatment each time. All data points ($n = 9$) were pooled for final analyses.

In vivo gene transfer

Specific pathogen-free male New Zealand White rabbits (3 – 4 kg, Western Oregon Rabbit Co.) were fed a normal chow diet. After at least 1 wk of adaption to new housing, rabbits underwent bilateral gene transfer to their common carotid arteries. This procedure, carried out under general anesthesia, includes surgical isolation and vector infusion into the lumens of isolated carotid segments for 20 min, and achieves gene transfer almost exclusively to luminal endothelial cells.^{9,28} All animal protocols were approved by the Office of Animal Welfare of the University of Washington.

Quantification of vector copies and IL-10 expression in vivo

Carotid arteries were harvested 3 d after gene transfer and cut into 4 equal segments designated A, B, C, and D from the proximal to distal end. To measure HDAd vector copy number, DNA was isolated from both segment B and segment D, using the DNeasy Blood and Tissue kit (Qiagen). Segments A and C were snap-frozen in liquid nitrogen for later extraction of RNA with the RNeasy mini kit (Qiagen).

To evaluate whether this carotid gene transfer protocol resulted in vector dissemination and ectopic expression, in some rabbits we harvested RNA and DNA from several organs (brain, heart, lung, liver, spleen, kidney, muscle, and intestine) at the same time the carotids were harvested. RNA and DNA from these tissues were extracted as described above.

Western analysis of IL-10

BAEC were transduced with 1×10^{10} part/ml of HDAdNull, HDAd-CMV-cIL-10, or HDAd-4XETE-cIL-10-oPRE for 6 hr. Medium was changed to DMEM and collected after 24 hr. Vector DNA isolated from the cells and quantified by qPCR revealed equivalent transduction in all 3 groups (data not shown). Equal volumes of conditioned medium were resolved under reducing conditions by electrophoresis on 0.1% SDS 15% polyacrylamide gels. Proteins were transferred electrophoretically to an Immobilon-P membrane (Millipore, Billerica, MA). After blocking in 10 mM Tris-HCl buffer, pH 8.0, containing 150 mM sodium chloride, 0.1% Tween 20, and 5% (w/v) nonfat dry milk, IL-10 protein was detected with goat anti-human IL-10 primary antibodies (R&D Systems, Minneapolis, MN) followed by incubation with HRP-conjugated Donkey anti-goat secondary antibodies (Rockland, Gilbertsville, PA). Bound antibody was detected with a chemiluminescence reagent kit (Immun-Star WesternC kit; Bio-Rad Laboratories, Hercules, CA).

Statistical methods

Data are expressed as Mean \pm SD. Group means were compared by t-test. Multiple groups were compared by one or two-way ANOVA. Sigma plot 11 software (Systat Software Inc., San Jose, CA) was used for statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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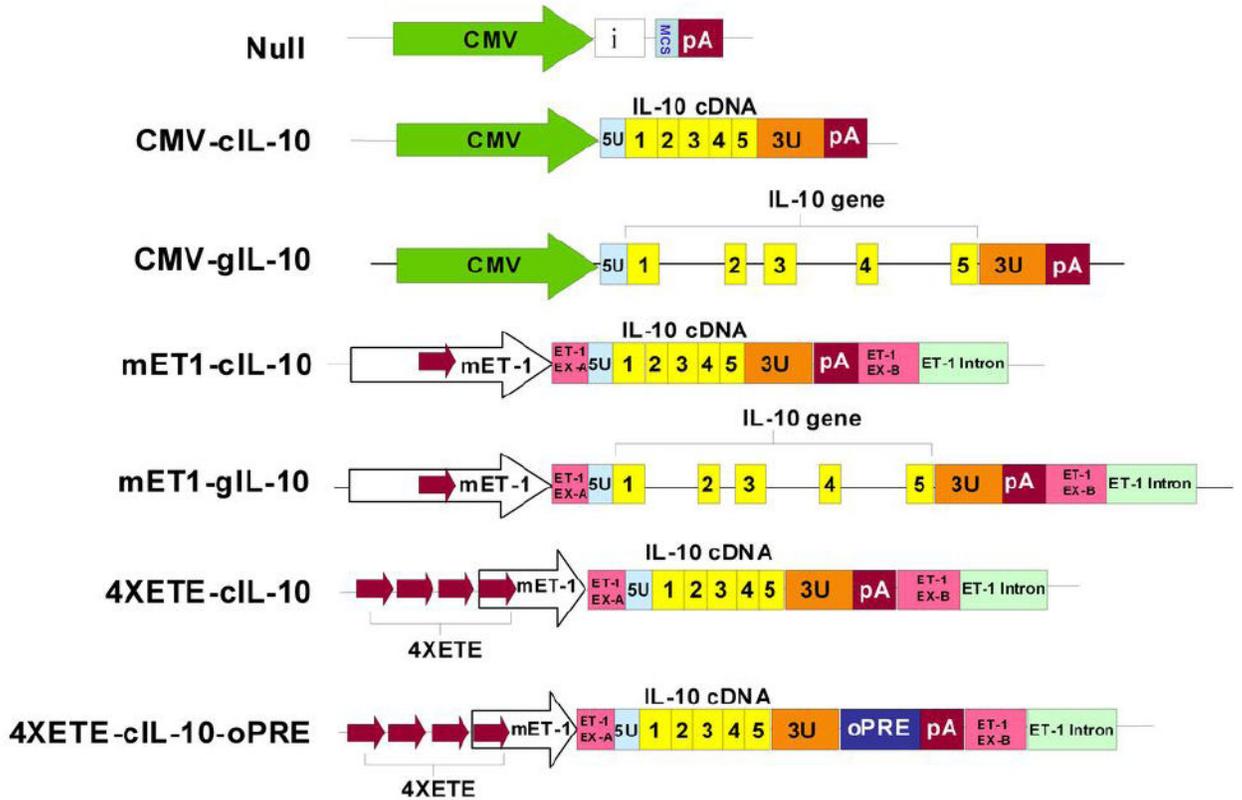
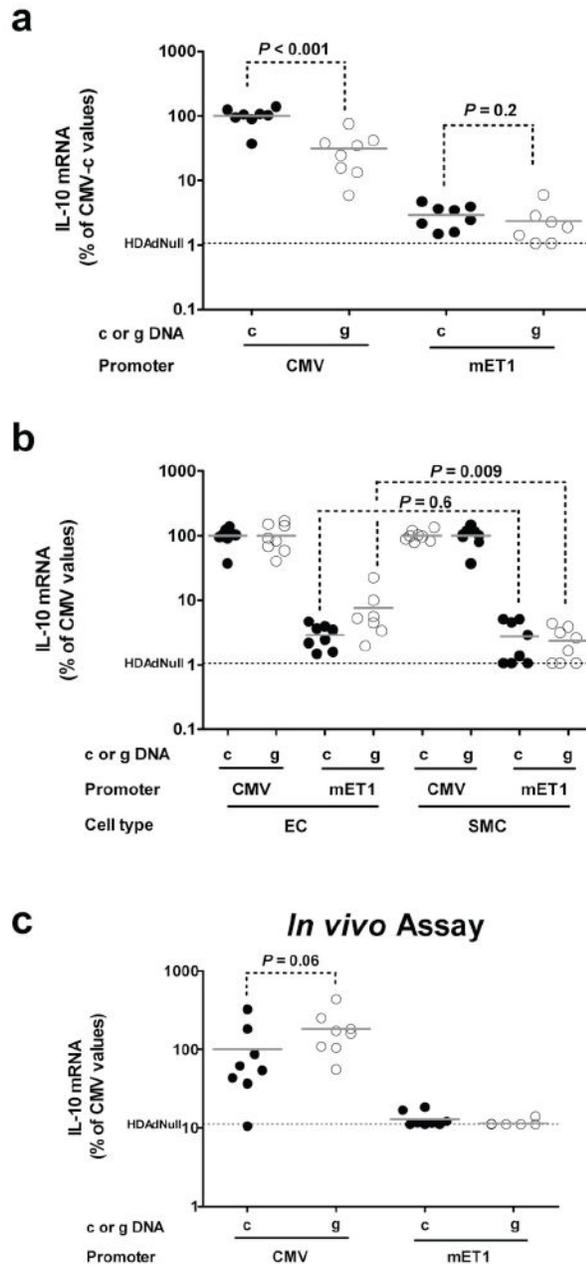


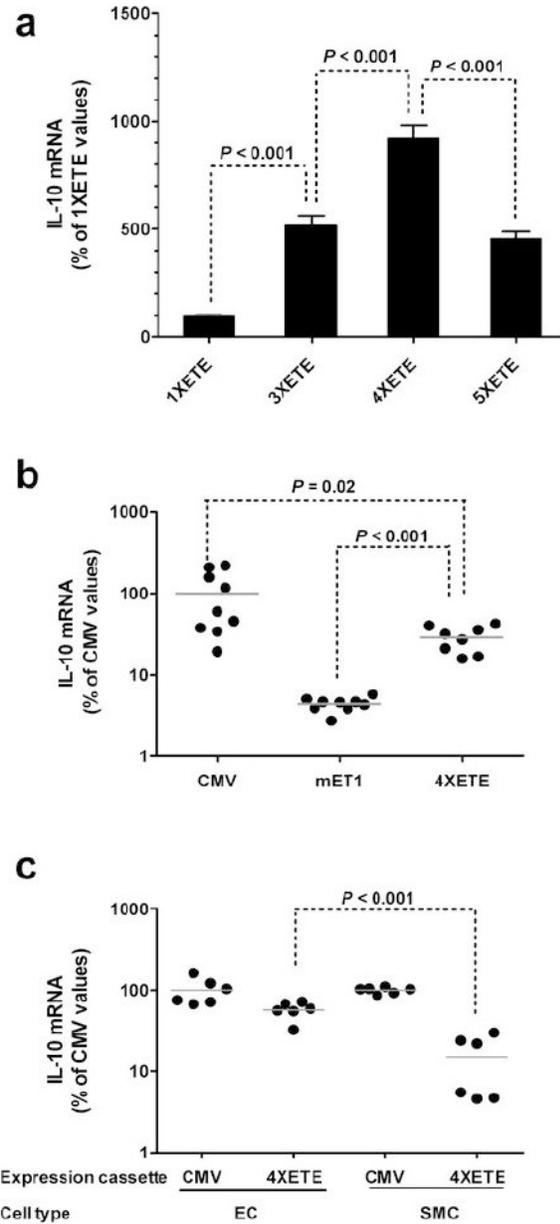
Figure 1.

Expression cassettes. Cassettes were tested both in plasmid vectors *in vitro* and in HDAd *in vitro* and *in vivo*. Symbols: CMV = CMV promoter from pCI plasmid; i = β -globin/IgG chimeric intron from pCI; MCS = multiple cloning site; pA=SV40 poly A signal; 5U = 5'-untranslated region of rabbit IL-10 gene; 1, 2, 3, 4, 5 (yellow blocks) = rabbit IL-10 exons; 3U = 3'-untranslated region of rabbit IL-10 gene; mET-1 = native murine endothelin-1 enhancer-promoter; red arrows = ET-1 enhancer (ETE) in native position and with 3 additional ETE added as direct repeats (4XETE); ET-1 EX-A and ET-1 EX-B = 5' and 3' segments of mouse ET-1 exon 1 (untranslated); ET-1 intron = 5' segment of mouse ET-1 intron 1; oPRE = optimized woodchuck hepatitis virus post-transcriptional regulatory element. Drawing is not to scale.

**Figure 2.**

Expression of IL-10 cDNA (“c”) or genomic DNA (“g”) from the CMV promoter or the mET-1 enhancer-promoter *in vitro* and *in vivo*. **(a)** Bovine aortic endothelial cells (EC) were transduced with each of the 4 vectors, mRNA harvested after 24 hr, and IL-10 mRNA measured by qRT-PCR with mean expression from CMV-cIL-10 assigned a value of 100%. **(b)** EC or bovine aortic smooth muscle cells (SMC) were transduced, mRNA harvested after 24 hr, and IL-10 mRNA measured by qRT-PCR. For each cell type, mean IL-10 expression in each of the 2 CMV-containing-vector-transduced groups was assigned a value of 100%. Expression in individual wells transduced with this CMV-containing vector or with the analogous mET-1-containing vector is calculated as a percentage of this value. The EC data

in **(b)** are the same data as in **(a)**, replotted after making these calculations. Data points in **(a)** and **(b)** are individual wells. **(c)** *In vivo* IL-10 expression. RNA was harvested from carotid arteries 3 d after transduction and IL-10 mRNA was measured by qRT-PCR. Mean IL-10 expression in the CMV-cIL-10 group was assigned a value of 100%. Expression in each artery is plotted as a percentage of this value. The background IL-10 qRT-PCR signal [measured in cells **(a-b)** or in arteries **(c)** transduced with HDAdNull] is indicated. Bars in all panels are group means.

**Figure 3.**

Enhancement of IL-10 expression and preservation of EC-specificity after addition of ET-1 enhancer (ETE) sequences to the mET-1 promoter. **(a)** EC were transfected with plasmids containing the 318 bp mET-1 promoter, the IL-10 cDNA, and 1, 3, 4, or 5 copies of the ETE (see Supplementary Figure 1). IL-10 mRNA was measured by qRT-PCR. Mean IL-10 expression for the 1XETE construct was assigned a value of 100%. Expression measured in individual wells was calculated as a percentage of this value and represented here as mean \pm SD. **(b)** EC were transduced with HDAd containing the indicated expression cassettes (see Figure 1) and IL-10 mRNA measured by qRT-PCR. Mean IL-10 expression for the CMV-cIL-10 group was assigned a value of 100%. Values from individual wells are expressed as a percentage of this value. Bars are group means. **(c)** EC or SMC were transduced with HDAd

containing the CMV-cIL-10 or the 4XETE-cIL-10 cassettes (Figure 1) and IL-10 mRNA measured by qRT-PCR. For each cell type, mean IL-10 expression for the CMV-cIL-10 group was assigned a value of 100%. IL-10 expression from individual wells is expressed as a percentage of this value. Bars are group means; SD are shown in **(a)**.

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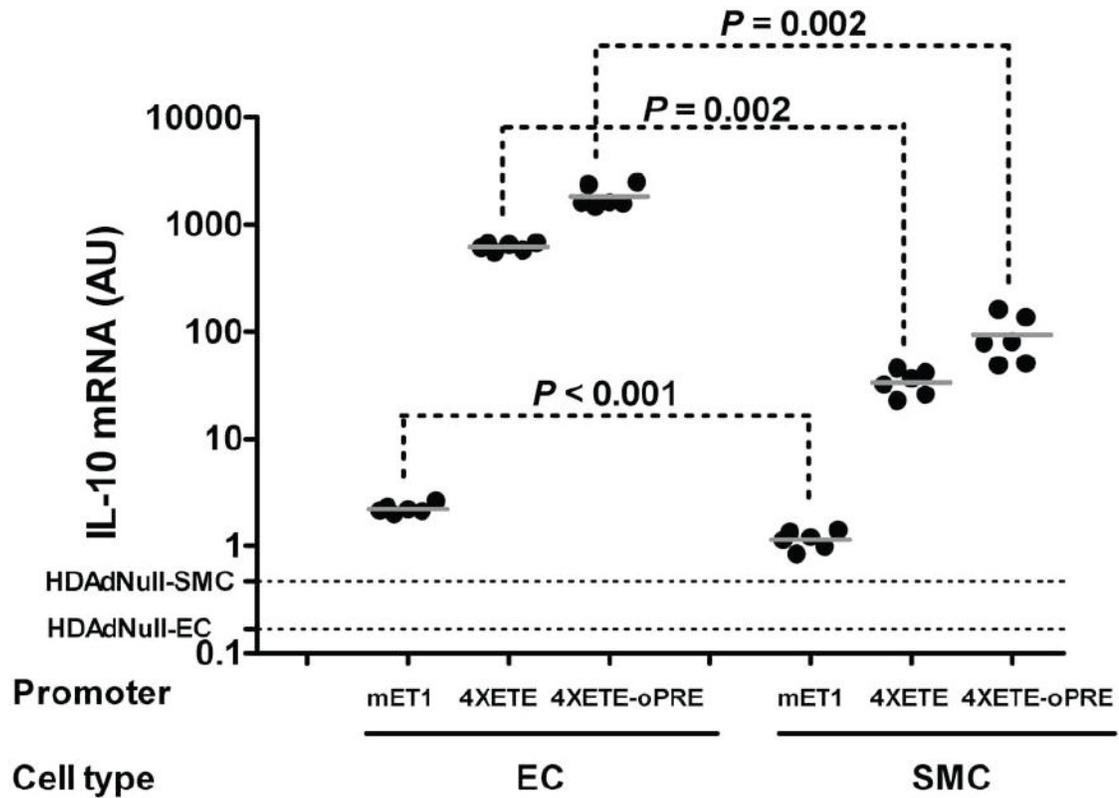
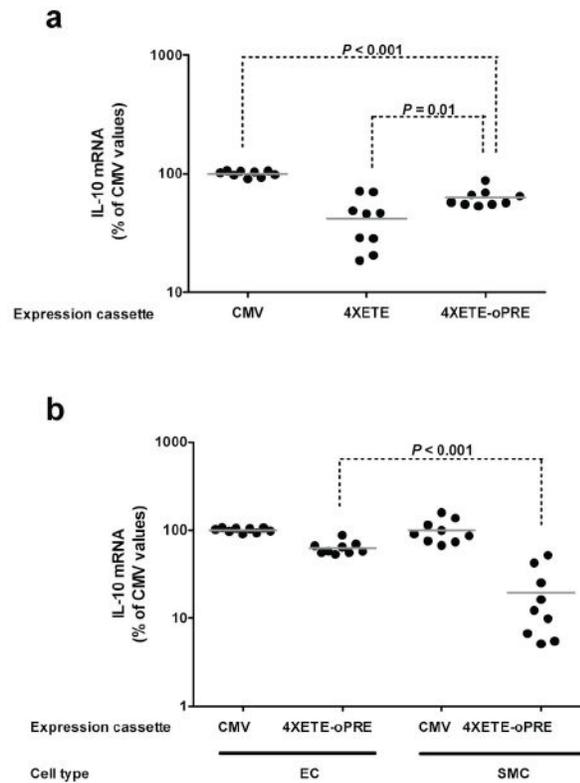


Figure 4.

Relative activities of the 4XETE and oPRE in EC and SMC. Bovine EC or SMC were transduced with HDAd in which expression of the IL-10 cDNA is driven by the mET1 enhancer-promoter, the truncated mET1 enhancer-promoter with 3 additional copies of the ETE (4XETE), or the 4XETE enhancer-promoter with the oPRE added (see Figure 1). IL-10 mRNA was measured by qRT-PCR, normalized to GAPDH mRNA in the same extracts, and expressed as arbitrary units (AU). Background signal in this assay was determined by measurement of IL-10 mRNA in extracts of EC and SMC transduced with HDAdNull. Data points are from individual wells, bars are group means. The cell extracts assayed here are a subset of those represented in Figures 2b, 3c, and 5b. We repeated our measurements of IL-10 and GAPDH mRNA in these 3 sets of extracts (qRT-PCR was performed on all 3 sets simultaneously here) to generate data for this figure.

**Figure 5.**

Enhancement of IL-10 expression and preservation of EC-specificity after addition of the optimized woodchuck hepatitis virus posttranscriptional regulatory element (oPRE). **(a)** EC were transduced with HDAd containing the CMV-c-IL-10 expression cassette or the 4XETE-cIL-10 cassette both without and with the oPRE. IL-10 mRNA was measured by qRT-PCR. Mean IL-10 expression for the CMV-cIL-10 cassette was assigned a value of 100%; IL-10 expression in individual wells was calculated as a percentage of this value. **(b)** EC or SMC were transduced with HDAd containing the CMV-cIL-10 or the 4XETE-cIL-10-oPRE cassettes (Figure 1) and IL-10 mRNA measured by qRT-PCR. For each cell type, mean IL-10 expression for the CMV-c-IL-10 group was assigned a value of 100%; IL-10 expression in individual wells was calculated as a percentage of this value. Bars in all panels are group means.

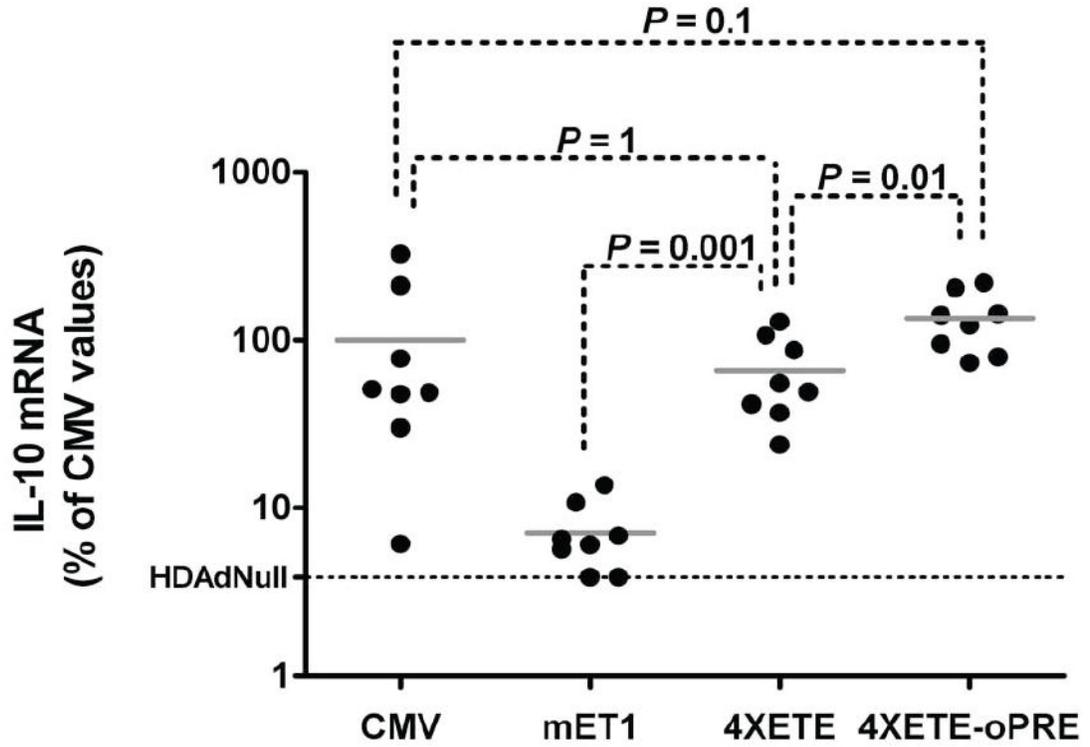
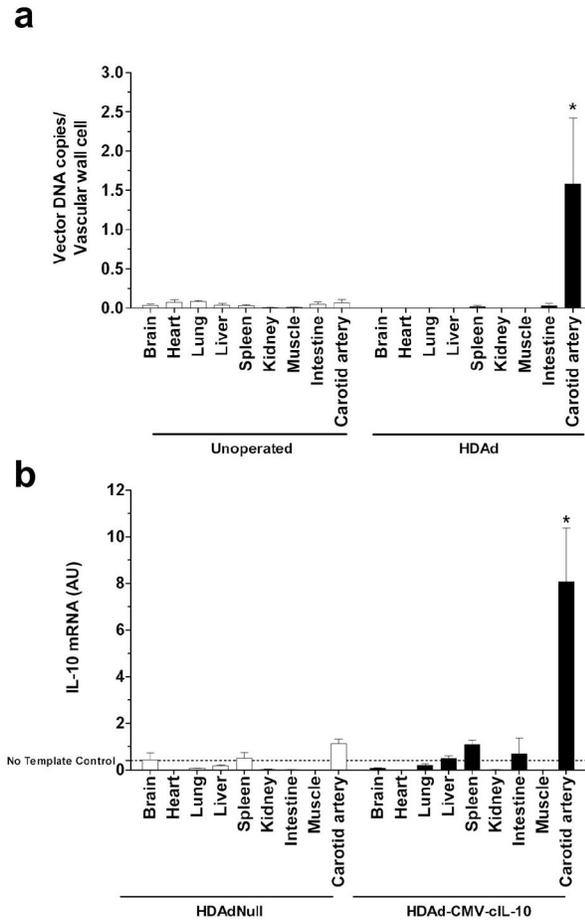


Figure 6. Enhancement of IL-10 expression *in vivo* after addition of enhancers and the oPRE. Rabbit EC were transduced *in vivo* by infusion of HDAd containing one of the four indicated expression cassettes, each containing the IL-10 cDNA (Figure 1). Three days later, transduced arteries were removed and IL-10 mRNA measured by qRT-PCR. Mean IL-10 expression for the CMV-cIL-10 group was assigned a value of 100%; IL-10 expression from individual arteries was calculated as a percentage of this value. The background IL-10 qRT-PCR signal (measured in arteries transduced with HDAdNull) is indicated. Data points are individual arteries; bars are group means.

**Figure 7.**

Lack of detectable systemic vector distribution and transgene expression in tissues distant from site of gene transfer. Carotid arteries were infused locally with an HDAd (either HDAdNull or HDAd-CMV-cIL-10). Three days later, DNA and RNA were isolated from carotid arteries and various distant tissues. Unoperated arteries were also included as controls. **(a)** Vector DNA ($n = 16$ per group) was measured by qPCR with probe/primers specific for the CMV promoter. * $P < 0.01$ compared to unoperated carotid artery. **(b)** IL-10 mRNA was measured by qRT-PCR of RNA from carotid arteries ($n = 5$ per group) infused with either HDAdNull or HDAd-CMV-cIL-10, and from other tissues ($n = 4 - 5$ per group) harvested from the same rabbits. The background q-RTPCR signal in this assay is that obtained with the “no template control” sample. Values near the no-template control signal are likely noise rather than signal. Among the HDAd-CMV-cIL-10 samples, only the signal from carotid artery was significantly different from the analogous HDAdNull sample (* $P < 0.03$). Values were normalized to GAPDH expression, expressed as arbitrary units (AU), and are shown as mean \pm SD.