Adeno-associated virus serotype 9 efficiently targets ischemic skeletal muscle following systemic delivery

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Targeting therapeutic gene expression to the skeletal muscle following intravenous (IV) administration is an attractive strategy for treating peripheral arterial disease (PAD), except that vector access to the ischemic limb could be a limiting factor. As adeno-associated virus serotype 9 (AAV-9) transduces skeletal muscle at high efficiency following systemic delivery, we employed AAV-9 vectors bearing luciferase or enhanced green fluorescent protein (eGFP) reporter genes to test the hypothesis that increased desialylation of cell-surface glycans secondary to hindlimb ischemia (HLI) might help offset the reduction in tissue perfusion that occurs in mouse models of PAD. The utility of the creatine kinase-based (CK6) promoter for restricting gene expression to the skeletal muscle was also examined by comparing it with the cytomegalovirus (CMV) promoter after systemic administration following surgically induced HLI. Despite reduced blood flow to the ischemic limbs, CK6 promoter-driven luciferase activities in the ischemic gastrocnemius (GA) muscles were ~34-, ~28- and ~150-fold higher than in the fully perfused contralateral GA, heart and liver, respectively, 10 days after IV administration. Furthermore, luciferase activity from the CK6 promoter in the ischemic GA muscles was ~twofold higher than with CMV, while in the liver CK6-driven activity was ~42-fold lower than with CMV, demonstrating that the specificity of ischemic skeletal muscle transduction can be further improved with the muscle-specific promoters. Studies with Evans blue dye and fluorescently labeled lectins revealed that vascular permeability and desialylation of the cell-surface glycans were increased in the ischemic hindlimbs. Furthermore, AAV9/CK6/Luc vector genome copy numbers were ~sixfold higher in the ischemic muscle compared with the non-ischemic muscle in the HLI model, whereas this trend was reversed when the same genome was packaged in the AAV-1 capsid (which binds sialylated, as opposed to desialylated glycans), further underscoring the importance of desialylation in the ischemic enhancement of transduction displayed by AAV-9. Taken together, these findings suggest two complementary mechanisms contributing to the preferential transduction of ischemic muscle by AAV-9: increased vascular permeability and desialylation. In conclusion, ischemic muscle is preferentially targeted following systemic administration of AAV-9 in a mouse model of HLI. Unmasking of the primary AAV-9 receptor as a result of ischemia may contribute importantly to this effect.

KEYWORDS: Adeno-associated virus; gene therapy; hindlimb ischemia; muscle-specific promoter

INTRODUCTION

Peripheral arterial disease (PAD) is mainly caused by atherosclerosis, which results in obstructions in arterial beds other than the coronary arteries, and the most common site is the lower extremity where occlusive disease leads to impaired perfusion. PAD affects about 3–10% of adults in the world and 15–20% in extremity where occlusive disease leads to impaired perfusion. The coronary arteries, and the most common site is the lower extremity where occlusive disease leads to impaired perfusion. PAD occurs in mouse models of PAD. The utility of the creatine kinase-based (CK6) promoter for restricting gene expression to the skeletal muscle was also examined by comparing it with the cytomegalovirus (CMV) promoter after systemic administration following surgically induced HLI. Despite reduced blood flow to the ischemic limbs, CK6 promoter-driven luciferase activities in the ischemic gastrocnemius (GA) muscles were ~34-, ~28- and ~150-fold higher than in the fully perfused contralateral GA, heart and liver, respectively, 10 days after IV administration. Furthermore, luciferase activity from the CK6 promoter in the ischemic GA muscles was ~twofold higher than with CMV, while in the liver CK6-driven activity was ~42-fold lower than with CMV, demonstrating that the specificity of ischemic skeletal muscle transduction can be further improved with the muscle-specific promoters. Studies with Evans blue dye and fluorescently labeled lectins revealed that vascular permeability and desialylation of the cell-surface glycans were increased in the ischemic hindlimbs. Furthermore, AAV9/CK6/Luc vector genome copy numbers were ~sixfold higher in the ischemic muscle compared with the non-ischemic muscle in the HLI model, whereas this trend was reversed when the same genome was packaged in the AAV-1 capsid (which binds sialylated, as opposed to desialylated glycans), further underscoring the importance of desialylation in the ischemic enhancement of transduction displayed by AAV-9. Taken together, these findings suggest two complementary mechanisms contributing to the preferential transduction of ischemic muscle by AAV-9: increased vascular permeability and desialylation. In conclusion, ischemic muscle is preferentially targeted following systemic administration of AAV-9 in a mouse model of HLI. Unmasking of the primary AAV-9 receptor as a result of ischemia may contribute importantly to this effect.

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The natural tissue tropism of the various AAV serotypes can be exploited to favor gene delivery to one organ over another. This tropism is based on the viral capsids recognizing specific viral receptors expressed on specific cell types, thus allowing a degree of cell-specific targeting within a given organ. Cell-specific expression may be further aided by the use of tissue-specific promoters conferring gene expression restricted to a specific cell type. This is desirable for gene therapy applications targeting organ-specific diseases, as this will help avoid any possible harmful side effects due to gene expression in off-target organs. Recently, several muscle-specific promoter constructs based on the muscle creatine kinase (MCK) regulatory region were shown to provide striated muscle-restricted gene expression. Of the several regulatory cassettes based on the MCK regulatory element, the CK6 promoter has been shown to provide skeletal muscle-restricted gene expression with reduced expression in cardiac muscle. This is particularly desirable in the context of using AAV-9 for PAD gene therapy via systemic administration as AAV-9 has a known preference for cardiac over skeletal muscle. However, the use of skeletal muscle-specific promoters in combination with the most recent AAV serotypes in the context of PAD is largely unexplored and indeed the entire approach could, in theory, be limited by the fact that blood flow to the ischemic limb is reduced thus creating a barrier to intravascular gene delivery. Recently, cell-surface N-linked glycans with terminal galactosyl residues were shown to serve as the primary receptor for AAV-9. Desialylation of these galactosylated glycans was shown to markedly increase cell-surface binding and transduction by AAV-9 and significantly decrease that by other AAV serotypes, like AAV-1. We hypothesized that ischemia induces desialylation of the cell-surface glycans, resulting in increased availability of AAV-9 receptors, and this together with the increase in vascular permeability characteristic of the ischemic tissue might suffice to overcome the barrier of reduced blood flow in the ischemic tissues. In the present study, we first sought to compare the magnitude and specificity of reporter gene expression driven by the human cytomegalovirus (MCK) regulatory region early and the minimal CK6 promoters packaged into AAV-9 and administered by IV injection in a mouse model of hindlimb ischemia (HLI). Using a novel approach that combines a muscle-specific promoter with an AAV serotype that preferentially transduces skeletal muscle, we show here that targeted expression of reporter genes in the ischemic muscles following systemic delivery is not only possible, but that it is also markedly enhanced relative to the non-ischemic muscles and other tissues.

RESULTS

Magnitude and specificity of gene expression from IV injection of AAV-9 harboring the CMV promoter

The perfusion ratio of ischemic to non-ischemic hindlimbs in C57Bl/6 mice (n = 5) immediately post HLI was 0.34 ± 0.12 (mean ± s.e.m). Consistent with previous reports from our group, the perfusion ratio recovered to 0.48 ± 1.4 by post-operative (post-op) day 7, at which time the mice received IV injections of AAV9/CMV/Luc (4.15 × 10^11 vector genomes (vgs)/animal) via the right internal jugular vein. Luciferase expression was then monitored by non-invasive in vivo bioluminescence imaging. Age-matched C57Bl/6 male mice that did not undergo HLI and that did not receive any vector served as negative controls (Figure 1a). As indicated by the purple color-coding, luciferase expression from the CMV promoter was observed throughout the body on post-AAV days 7 (Figure 1b) and 14 (Figure 1c), However, luciferase expression appeared strongest in the upper abdominal region corresponding to liver. Interestingly with regard to hindlimb expression, bioluminescence signals appeared stronger in the ischemic hindlimbs (mouse’s left side, rightmost hindlimb in Figure 1) than in the non-ischemic, contralateral hindlimbs on both days 7 and 14 post-AAV injection. Region of interest analysis was then used to estimate relative luciferase signal intensity in each hindlimb and the upper abdomen (corresponding to liver). On post-AAV day 7, the mean bioluminescence signal in the ischemic hindlimbs was 2.7-fold higher than in the non-ischemic limbs but 17.7-fold lower than in the liver (Figure 1d). On post-AAV day 14, bioluminescence in the ischemic limbs was 4.3-fold higher than in the non-ischemic limbs but 4.5-fold lower than in the liver.

Although bioluminescence imaging provides a non-invasive estimate of relative luciferase activities in serial studies, it is difficult to compare values between tissues due to differences in tissue depth and the differential absorption of photons by different tissues. For this reason, rigorous quantitative measurement of luciferase activity was performed in tissue extracts from the various organs as shown in Figure 1e. Luciferase activity in the ischemic gastrocnemius (GA) muscles of mice treated with AAV9/CMV/Luc was sevenfold higher than in the contralateral GA, 2.0-fold higher than in the heart and 1.8-fold higher than in the liver. These results demonstrate that AAV-9 is highly effective for delivering gene(s) to the ischemic skeletal muscle following systemic delivery, even when using a non-tissue-specific promoter.

Magnitude and specificity of gene expression from IV injection of AAV-9 harboring the CK6 promoter

HLI was surgically induced in the left hindlimbs of adult C57Bl/6 mice (n = 4). Immediately after surgery on post-op day 0, the ratio of perfusion as measured by laser Doppler between the ischemic and non-ischemic hindlimbs was 0.34 ± 0.12 (mean ± s.e.m.). On post-op day 7, the perfusion ratio had partially recovered to 0.48 ± 3.5. On post-op day 8, all mice received IV injections of AAV9/CK6/Luc (4.15 × 10^11 vgs/animal) via the right internal jugular vein. Luciferase expression was again monitored by bioluminescence imaging. Bioluminescence signals appeared strongest in the ischemic hindlimbs on post-AAV days 6 (Figure 1f) and 10 (Figure 1g). Using region of interest analysis, the mean bioluminescence signal in the ischemic limbs was 50.5-fold higher than in the non-ischemic limbs but 17.7-fold lower than in the liver (Figure 1h). On day 10 post-AAV, bioluminescence was 37.8-fold higher in the ischemic vs non-ischemic limbs and 9.8-fold higher than in the liver (Figure 1h). Similar results were obtained in parallel experiments performed in BALB/c mice (data not shown).

The more rigorous, quantitative measurement of luciferase activity in tissue extracts from selected organs harvested on day 10 post AAV is presented in Figure 1i. Again, luciferase activity was significantly higher in the ischemic hindlimbs muscles compared with the contralateral non-ischemic muscles or liver. Luciferase activity in the ischemic GA muscle of mice treated with AAV9/CK6/Luc was 34.1-fold higher than in the contralateral GA, 28.1-fold higher than in the heart and 150-fold higher than in the liver (all comparisons P < 0.05). Luciferase activity in the non-ischemic GA was 1.2-fold higher than in the heart and 6.6-fold higher than in the liver. Furthermore, luciferase activity in the non-ischemic GA was 1.9-fold higher, whereas that in the liver was 41.7-fold lower in the CK6 group when compared with the CMV group. These results clearly demonstrate that the combination of AAV-9 and the CK6 promoter is highly efficient and selective for delivering genes to the ischemic skeletal muscle following systemic delivery.

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Distribution of enhanced green fluorescent protein (eGFP) expression in the ischemic hindlimb muscle confirms the efficiency of AAV-9

Vectors carrying the eGFP gene driven by the CMV or CK6 promoters (AAV9/CMV/eGFP and AAV9/CK6/eGFP) were systemically administered to adult C57Bl/6 mice (n = 5 per group) via jugular vein at a dose of 4.15 × 10^{11} vgs/mouse on the seventh day following HLI surgery. In vivo bioluminescence (IVIS) images were obtained on the seventh (b) and fourteenth day (c) following vector administration. In the CK6 group, (f, g), adult C57Bl/6 mice (n = 4 per group) were injected with 4.15 × 10^{11} vgs/mouse via jugular vein. In vivo bioluminescence (IVIS) images obtained on the sixth (f) and tenth day (g) following vector administration. (d, h) For each group of mice, the mean values of bioluminescence as average radiance (photons s^{-1} cm^{-2} sr^{-1}) were obtained from the regions of interest in panels (b, c, f, g) and plotted as the ratios of ischemic limb to non-ischemic limb and ischemic limb to upper abdomen (corresponding to liver). (e, i) Bar graph showing luciferase activities in tissue extracts from the CMV and CK6 groups, respectively. Protein extracts from various tissues were collected 10–14 days after vector administration for homogenization and in vitro luciferase assays. Luciferase activities are expressed as relative light units per mg protein (RLU per mg protein; mean ± s.e.m, *P < 0.05).

Figure 1. Time course and tissue distribution of CMV- and CK6-mediated luciferase expression from AAV-9 following IV injection 7–8 days after HLI surgery. (a) Negative control consisting of an age-matched C57Bl/6 male mouse that did not undergo HLI and did not receive any vector. In the CMV group, (b, c), adult C57Bl/6 mice (n = 5 per group) were injected with 4.15 × 10^{11} vgs/mouse via jugular vein. In vivo bioluminescence (IVIS) images were obtained on the seventh (b) and fourteenth day (c) following vector administration. In the CK6 group, (f, g), adult C57Bl/6 mice (n = 4 per group) were injected with 4.15 × 10^{11} vgs/mouse via jugular vein. In vivo bioluminescence (IVIS) images obtained on the sixth (f) and tenth day (g) following vector administration. (d, h) For each group of mice, the mean values of bioluminescence as average radiance (photons s^{-1} cm^{-2} sr^{-1}) were obtained from the regions of interest in panels (b, c, f, g) and plotted as the ratios of ischemic limb to non-ischemic limb and ischemic limb to upper abdomen (corresponding to liver). (e, i) Bar graph showing luciferase activities in tissue extracts from the CMV and CK6 groups, respectively. Protein extracts from various tissues were collected 10–14 days after vector administration for homogenization and in vitro luciferase assays. Luciferase activities are expressed as relative light units per mg protein (RLU per mg protein; mean ± s.e.m, *P < 0.05).
results using an independent reporter system confirm that the combination of AAV-9 capsid and CK6 promoter is highly efficient for the selective delivery of gene(s) to the ischemic skeletal muscles following systemic delivery.

Vascular permeability is markedly increased by HLI
HLI was surgically induced in the left hindlimbs of C57Bl/6 mice (n = 6). On post-op day 7, the mice received intraperitoneal injections of Evans blue dye and the animals were euthanized 24 h later. Ex vivo images of the hindlimb muscles (Figure 3a) show that the ischemic TA muscles (I) were markedly blue compared with the non-ischemic (NI) ones. The quantitative analysis of the muscle extracts is summarized in Figure 3b. The Evans blue dye content in the ischemic TA muscles (2.6 ± 0.3%) was nearly fivefold higher than in the non-ischemic TA muscles (0.3%, P < 0.05).

HLI induces marked desialylation of cell-surface N-linked glycans, thereby unmasking the primary receptor for AAV-9 binding
HLI was surgically induced in the left hindlimbs of adult male C57Bl/6 mice (n = 3). Seven days following HLI, the distribution of sialylated desialylated cell-surface glycans in the mouse hindlimb skeletal muscles was assessed by fluorescence microscopy using lectin staining. Of the two lectins used, Maackia amurensis lectin (MAL-I) binds to α2,3-sialylated glycans whereas Erythrina cristagalli lectin (ECL) binds to the desialylated galactose residues of cell-surface glycans. Myofibers from the ischemic TA showed abundant ECL staining along the cell surface compared with a weaker staining seen in the non-ischemic TA muscles (Figure 4, top). Conversely, MAL-I staining was readily detected in the non-ischemic TA muscles but was much weaker in the ischemic TA muscles (Figure 4, bottom). For each of the two lectins (MAL-I and ECL), four × 40 fields of view were quantified for mean fluorescence intensity from each ischemic and non-ischemic muscle from each of the three mice entered into the study. The mean ratio of ECL fluorescence intensity in the ischemic vs non-ischemic muscle was 1.6, while the same fluorescence intensity ratio in MAL-I-stained tissue was 0.8. To incorporate an internal standard, the ratio of ECL to MAL-I fluorescence intensity in the
ischemic muscle (4.3 ± 0.6) was then compared with the same ratio in the non-ischemic muscle (1.9 ± 0.1), and the difference was found to be significant (P < 0.05). These results demonstrate that the induction of HLI causes marked desialylation of cell-surface N-linked glycans, thus unmasking the primary cell-surface attachment factor for AAV-9.

Magnitude of gene expression and tropism of tissue distribution following IV injection of AAV-1 and -9 harboring the CK6 promoter further implicates HLI in the unmasking of cell-surface receptors, thereby facilitating selective transduction by AAV-9.

HLI was surgically induced in the left hindlimbs of adult C57Bl/6 mice (n = 5 per group) 7 days before the injection of AAV/CK6/Luc genomes packaged in either AAV-1 or AAV-9 capsids. On post-op day 7, the ratio of perfusion in the ischemic vs non-ischemic hindlimbs as measured by laser Doppler was 0.44 ± 0.03 (mean ± s.e.m) for the AAV-9 group and 0.29 ± 0.01 for the AAV-1 group. After laser Doppler measurement on post-op day 7, five mice received IV injections of AAV9/CK6/Luc (4.15 x 10^11 vgs/mouse) via jugular vein, while the remaining five mice were similarly treated with AAV1/CK6/Luc. Luciferase expression was again monitored by bioluminescence imaging. In the AAV-9 group, bioluminescence signals again appeared strongest in the ischemic hindlimbs on post-AAV days 7 (Figure 5a) and 14 (Figure 5b), with markedly reduced expression in the liver and little luciferase expression, if any, detected elsewhere. In the AAV-1 group, bioluminescence signal intensities in the ischemic hindlimbs were visibly less intense than that seen in mice injected with the same vg packaged in AAV-9 capsids (Figures 5c and d). Furthermore, the marginal bioluminescence signals from the ischemic hindlimbs in the AAV-1 group were not stronger than those generated by the liver.

The more rigorous, quantitative measurement of luciferase activity in tissue extracts from the selected organs is presented in Figure 5e. Again, luciferase activity in the ischemic muscle was significantly higher in the ischemic hindlimb muscle compared with the contralateral non-ischemic muscle, liver or other organs. Luciferase activity in the ischemic muscle from mice injected with AAV9/CK6/Luc reached a mean of 2.0 x 10^6 ± 1.2 x 10^6 RLU per mg protein (mean ± s.e.m), whereas Luc activity in the same mice declined to only 4.4 x 10^4 ± 1.2 x 10^4 RLU per mg protein in the non-ischemic muscle, 4.0 x 10^4 ± 1.8 x 10^4 RLU per mg protein in the liver, 1.4 x 10^4 ± 3.7 x 10^3 RLU per mg protein in the heart, 1.5 x 10^3 ± 1.0 x 10^3 RLU per mg protein in the kidney and 7.9 x 10^2 ± 2.7 x 10^2 RLU per mg protein in the brain (see Figure 5e). Although luciferase activity in the ischemic muscle was nearly 24-fold higher in the AAV-9 group as compared with the AAV-1 group (all comparisons vs AAV-9 in the ischemic muscle P < 0.05 by analysis of variance), luciferase
activity from AAV-1 nevertheless trended higher in the ischemic vs non-ischemic muscle.

We next compared the vg copy numbers persisting in tissue samples at 14 days post-AAV injection using real-time PCR (Figure 5f). Interestingly, AAV-9 vg copies were significantly higher in the liver \((3.3 \times 10^5 \pm 1.8 \times 10^4 \text{ vg copies per } \mu\text{g host genomic DNA})\) than in any other tissue examined (mean ± s.e.m., all comparisons \(p<0.05\) by analysis of variance). Nevertheless, the next highest concentration of vgs was found in the ischemic muscle \((1.7 \times 10^5 \pm 2.4 \times 10^4 \text{ vg copies per } \mu\text{g host genomic DNA})\) followed by kidney, brain, non-ischemic muscle and heart. By contrast, AAV-1 did not exceed \(3 \times 10^4\) vg copy numbers per \(\mu\text{g host genomic DNA}\) in any tissue examined; with the highest copy number found in the liver \((2.6 \times 10^5 \pm 6.2 \times 10^4 \text{ vg copies per } \mu\text{g host genomic DNA})\), followed by non-ischemic muscle, brain, ischemic muscle, kidney and heart. Finally, the vg per \(\mu\text{g genomic DNA}\) copy numbers for AAV-9 were 5.6-fold higher in the ischemic vs non-ischemic muscle; whereas this trend was reversed for AAV-1 which had sixfold higher copy numbers in the non-ischemic vs ischemic muscle.

These results clearly demonstrate that AAV-9 selectively targets the ischemic hindlimb muscle, and that the AAV serotype 9 capsid, in combination with the CK6 promoter, is highly efficient and selective for delivering genes to the ischemic skeletal muscle following systemic delivery.

**DISCUSSION**

PAD is a major health-care problem, and more than a decade of clinical trials of gene therapy for PAD has failed to bring this approach forward in any meaningful way. Some of the plausible explanations for previous failures in human studies include: gene delivery vectors with inherently low magnitudes and durations of gene expression and intra-muscular injection methods, which are far more effective in pre-clinical studies of small animals with limited muscle mass where most of the muscle is accessible to the needle. In humans, studies have found no evidence of transgene expression or, when present, was limited and heterogeneous in distribution. Therefore, systemic delivery offers numerous theoretical advantages for treating patients with PAD, although two major concerns exist. First, blood flow to the ischemic limb is reduced in PAD, and this may limit access of the vector to the ischemic tissue. Second, it is desirable to restrict gene expression to the cell type of interest as the expression of therapeutic genes in off-target tissues could potentially lead to deleterious side effects. The results of the current study show, for the first time, that gene expression in the ischemic hindlimb muscle can be achieved by systemic injection of an AAV-based vector system with a skeletal muscle-tropic capsid (AAV-9) and a tissue-specific promoter (a compact version of the muscle-specific MCK promoter/enhancer). In the present study, using an AAV serotype 9-based vector in an adult mouse model of HLI, we demonstrate that (1) the CMV promoter is adequate to achieve ischemia-tropic gene expression in the skeletal muscle following IV administration. Using the CK6 promoter provides for more robust and highly specific gene expression in the ischemic skeletal muscle, (2) vascular permeability and desialylation of cell-surface glycans are both increased in the post-ischemic hindlimbs, (3) luciferase activity in the ischemic skeletal muscle, (4) luciferase protein expression from AAV-9 is significantly higher in the ischemic tissues as compared with the same vg packaged in an AAV-1 capsid, and (5) vg copy numbers for AAV-9 (which binds desialylated glycans) are elevated in the ischemic vs non-ischemic muscle, whereas copy numbers for AAV-1 (which binds sialylated glycans) are reduced in the ischemic vs non-ischemic muscle. These last three findings are complementary, and together indicate that ischemic desialylation synergizes with increased vascular permeability to yield preferential transduction of the ischemic muscle tissue following IV delivery of AAV-9.

Strong, non-selective, viral promoters such as CMV are typically used in animal studies as well as clinical trials of gene therapy for PAD. Although tissue-specific promoters may be efficient at restricting gene expression to a particular cell or tissue type, their widespread use has not been realized because of a generally lower level of gene expression that is considered suboptimal for gene therapy applications. Furthermore, the ‘payload capacity’ of the AAV capsid effectively limits the size of the recombinant AAV genome to approximately 4.9–5.3 kb. The choice of promoter for AAV-mediated, organ-specific gene expression should therefore be based on the size, specificity and strength of the promoter. Previous work in the field of gene therapy for muscular dystrophy led to the creation of hybrid promoter/enhancers in which various enhancers (including the MCK enhancer) have been introduced adjacent to the minimal MCK promoter. In a recent comparison of five such hybrid constructs, Hauser et al. identified a compact combination of the MCK enhancer and promoter (CK6) that was sixfold stronger than the full-length 3.3-kb MCK promoter/enhancer and almost 12% as strong as the CMV promoter in the muscle cells. Accordingly, we used the minimal CK6 promoter/enhancer in this study to achieve high-level, muscle-specific gene expression. Finally, in gene therapy protocols, the viral vector burden should be kept to a minimum to avoid vector-related side effects. Although the specificity of gene expression needed for clinical efficacy will depend largely upon the nature of the therapeutic transgene, this study achieved efficient transduction of the ischemic skeletal muscle without detectable adverse effects using a dose of \(1.4 \times 10^{13} \text{ vg kg}^{-1}\), which is comparable with IV doses of AAV vectors used in other small and large animal studies.

One might anticipate lower expression levels in the ischemic limbs compared with the non-ischemic limbs based on the fact that the ischemic limbs in this study had approximately one-half of the relative perfusion compared with the non-ischemic limbs. Contrary to this expectation, the luciferase reporter gene and in vivo bioluminescence imaging (IVIS) clearly indicated that the ischemic hindlimbs had higher luciferase activity than the non-ischemic hindlimbs following IV delivery (Figures 1b, c, f and g). The ratios of in vitro luciferase activity in the ischemic skeletal muscle vs the other key organs such as liver and heart are summarized in Figures 1e and i. Using the CK6 promoter, luciferase activity in the ischemic GA muscle was found to be ~34-fold higher than in the contralateral GA and ~150-fold higher than in the liver. Luciferase activity in the ischemic GA was also ~twofold higher while that in the liver was ~42-fold lower in the CK6 group when compared with the CMV group. Thus by combining the CK6 promoter with the AAV-9 capsid, we were able to harness the superior transduction efficiency of the AAV-9 capsid while retaining high selectivity for the ischemic skeletal muscle over liver. Using in vivo bioluminescence imaging, similar data were obtained in a second inbred mouse strain (BALB/c), which has been previously documented to have extremely poor perfusion recovery after HLI (data not shown). These results confirm that the preferential transduction of the ischemic skeletal muscle was not restricted to mouse strain.

To the best of our knowledge, our results are the first to show robust and homogeneous gene expression in the ischemic limbs compared with the non-ischemic (contralateral) limbs following systemic delivery of an AAV vector. In further comparing the CMV and CK6 promoters, we found that the apparent tropism for the ischemic skeletal muscle was much more pronounced with the CK6 promoter. One plausible explanation for this observation is that the increased vascular permeability and desialylation of cell-surface glycans associated with ischemia may act in synergy with the natural muscle tropism of the AAV-9 capsid and the specificity of the CK6 promoter for the skeletal muscle. The eGFP reporter gene was then used to characterize the distribution of gene expression and the rate of transduction in the ischemic skeletal
muscle after IV administration of AAV-9 vectors driven by the CMV and CK6 promoters. Using the CK6 promoter, the transduction rate in the ischemic skeletal muscle was > 50% at the dose used in this study. These results also demonstrated that AAV-9 achieves a relatively homogeneous distribution of gene expression in the ischemic skeletal muscle after IV administration, particularly when deployed in combination with a muscle-specific promoter.

Studies with Evans blue dye were then conducted to test the hypothesis that increased vascular permeability may be one of the factors contributing to the preferential transduction of the ischemic skeletal muscle. Consistent with previous studies, the results indicated that vascular permeability was indeed increased in the ischemic hindlimbs in our HLI model, suggesting a potential role in the increase in transduction efficiency observed under ischemic conditions. Recently, Shen et al. showed that N-linked glycans with terminal galactosyl residues serve as the primary receptor for AAV-9 in Chinese hamster ovary cells. Although sialylated glycans serve as the cellular receptors for other AAV serotypes such as AAV-1, it was the desialylation of the N-terminal galactosylated glycans that increased cell-surface binding and infectivity of AAV-9. Using two lectins, MAL-I (which binds to N-linked glycans) and ECL (which binds to the desialylated galactose residues of cell-surface glycans), we report here, for the first time, that ischemia markedly increases the desialylation of cell-surface glycans in the mouse HLI model of PAD, suggesting a second contributing mechanism for the increase in transduction efficiency under ischemic conditions. Studies comparing the vg copy numbers and luciferase gene expression levels driven by the CK6 promoter between the AAV-9 and AAV-1 serotypes were then conducted to evaluate further the role of desialylated cell-surface glycans in this system. The AAV-1 capsid was selected for this comparison because AAV-1 utilizes sialylated glycans for cell-surface binding and transduction. We report here, for the first time, that the AAV-9 capsid is far more efficient at targeting gene expression to the ischemic skeletal muscle, demonstrating a 24-fold advantage over AAV-1 in tissue luciferase activity after IV administration. These findings are consistent with results obtained from an analysis of vg copy number in various tissues, where the AAV-9 vg per µg genomic DNA copy numbers were 5.6-fold higher in the ischemic vs non-ischemic muscle; whereas this trend was reversed for AAV-1, which had sixfold higher copy numbers in the non-ischemic vs ischemic muscle. These results provide convincing evidence in support of the hypothesis that ischemia-induced desialylation of galactosylated N-glycans unmasks the primary cellular receptor for AAV-9, thus promoting cell-surface binding and transduction after IV injection, ultimately resulting in markedly increased and selective transgene expression in the ischemic as compared with the non-ischemic myofibers and other tissues, well above and beyond that afforded by increased vascular permeability alone. Although these two mechanisms may complement one another in vivo, the effect of ischemia on AAV9-mediated gene expression is so pronounced that other mechanisms may also contribute. For example, the stress induced by fasting was recently shown to increase in vivo transduction efficiency by AAV vectors, including AAV9, and the local stress induced by ischemia may function similarly to enhance the intracellular post-entry processing of AAV genomes.

CONCLUSIONS

This study shows, for the first time, that transgene expression is targeted to the ischemic muscle following systemic administration of muscle-tropic AAV vectors. The specificity of ischemic skeletal muscle transduction can be further improved with the use of a muscle-specific promoter. Increased desialylation of the cell-surface N-glycans is a mechanism that likely contributes to the ischemic enhancement of AAV9-mediated gene transfer after systemic delivery. These findings will be of immediate utility in pre-clinical studies examining the role of various genes in the recovery from HLI and may ultimately prove valuable in clinical gene therapy protocols targeting PAD.

MATERIALS AND METHODS

Plasmids

The AAV vectors bearing the CMV promoter driving the expression of firefly luciferase (AAV/CMV/Luc) or eGFP (AAV/CMV/eGFP) have been described previously. Construction of AAV vectors bearing the CK6 promoter driving the expression of firefly luciferase (AAV/CK6/Luc) or eGFP (AAV/CK6/eGFP) was accomplished in two steps. First, the CMV promoter was removed from AAV/CMV/Luc and AAV/CMV/eGFP by double digestion with XbaI and HindIII. Second, a PCR-amplified 571-bp CK6 MCK enhancer/promoter was directionally inserted as an Xbal-HindIII fragment. The 571-bp muscle-specific CK6 MCK enhancer/promoter construct was a kind gift of Dr S D Hauksha. The AAV-1-pseudotyped AAV/CK6/Luc vector used for the comparison of AAV-9, and AAV-1 was obtained from the Penn Vector Core in the School of Medicine Gene Therapy Program at the University of Pennsylvania.

AAV vector production

AAV vectors were packaged in AAV-293 cells by the triple transfection method, then purified by ammonium sulfate fractionation and iodixanol gradient centrifugation as described previously. Titers of the AAV vectors (vgs per ml) were determined by real-time PCR as described previously.

Animal procedures

Animal protocols used in this study were approved by the Institutional Animal Care and Use Committee and conformed to the ‘Guide for the Care and Use of Laboratory Animals’ (NIH Publication 85-23, revised 1985). All mice (C57BL/6 and BALB/c) (15–21-weeks old) were purchased from The Jackson Laboratories (Bar Harbor, ME, USA). Age-matched (15–21-week old) male mice were used for all the experiments to exclude estrogen as a potential confound in the HLI model described below.

Induction of HLI

Mice underwent unilateral femoral artery ligation and excision on the left hindlimb as described previously. Necrosis was visually assessed each day. Blood flow in the ischemic and contralateral non-ischemic limbs was measured as described previously with a laser Doppler perfusion imaging system (Perimed, Stockholm, Sweden).

AAV vector delivery

For IV injection, mice were anesthetized with isoflurane as described above and the AAV-1 or -9 solutions (50–100 µl containing 4.15 × 1011 vgs) were slowly injected via the right jugular vein on days 7–8 following HLI surgery.

Bioluminescence imaging in vivo

Bioluminescence imaging was performed using an IVIS 100 system (Caliper Life Sciences, Hopkinton, MA, USA). Luciferase expression in live mice was non-invasively detected after the intraperitoneal injection of luciferin, and images were processed as described previously. Equal-sized regions of interest were selected over each hindlimb and upper abdomen to obtain estimates of bioluminescence intensity.

Quantitative in vitro luciferase activity assays

Luciferase activity was measured using luciferase assay reagents from Promega Corp. (Madison, WI, USA). After bioluminescence imaging and euthanasia at 10–14 days post-vector injection, the heart, liver, skeletal muscle and other tissues were collected from experimental mice. Protein extracts were prepared and luciferase activities (RLU) were determined using a FLUOstar Optima micro-plate reader (BMG Labtech, Durham, NC, USA).

Fluorescence imaging

eGFP expression and desialylation of cell-surface glycans in mouse tissues were documented by fluorescence microscopy using an LSM 700 confocal microscope (Carl Zeiss AG, Gottingen, Germany). For eGFP expression 14 days following vector administration, animals were euthanized for muscle collection and fixation in 3.7% paraformaldehyde at 4°C for 1 h. After (3 × )
S-min phosphate-buffered saline washes, tissues were equilibrated with 30% sucrose in phosphate-buffered saline overnight. In all, 15-μm thick cryosections were then cut and used for quantifying eGFP expression by fluorescence signal intensity.

For assessing sialylated and desialylated cell-surface glycans, animals were euthanized 7 days post HLI. Ischemic and contralateral muscles were harvested and placed in optimal cutting temperature (OCT) compound for snap freezing in liquid nitrogen. A total of 7-μm cryostat sections were prepared to assess the differential distribution of sialylated or desialylated glycans in the ischemic vs non-ischemic muscles. Staining was performed using the biotinylated lectins, MAL-I and ECL (Vector Laboratories, Burlingame, CA, USA). Lectins were visualized using Streptavidin-Alexa Fluor-555 (Invitrogen, Carlsbad, CA, USA). Quantification of the fluorescence signal intensity of the lectin staining was done using Image J software (US National Institutes of Health, Bethesda, MD, USA).

Evans blue dye assay
Animals were anesthetized and maintained on 1–1.2% isoflurane in oxygen. Evans blue dye, 50 μl of a 20 mg ml⁻¹ solution in saline, was injected intraperitoneally, and tissues were harvested 24 h later. Evans blue dye was extracted from tissue following a protocol adapted from Bohmer et al. Briefly, the 28–70 mg samples were freeze-dried overnight. Formamide (300 μl) were added, and the samples were placed in a water bath at 60 °C for 2 h to extract Evans blue dye. After an additional 10 h at room temperature, the absorption of Evans blue dye was measured using a microplate reader (Model 3550; Bio-Rad, Hercules, CA, USA) set at 595 nm. Sample concentrations were determined using a calibration curve generated in parallel from dilutions of Evans blue dye in formamide.

Determination of AAV vg copy number per μg genomic DNA
The AAV2 genomic backbone AAV/Ck6/Luc was cross-packaged into capsids from AAV serotypes 9 and 1 for injection as described above. Two weeks after vector administration, total genomic DNA from a panel of tissues was prepared using a QiAamp DNA Mini Kit (Qiagen, Inc., Valencia, CA, USA). Real-time PCR using the Bio-Rad iTaq Universal SYBR Green Supermix was performed on a Bio-Rad CFX Connect system (Bio-Rad, Hercules, CA, USA). The following primers were used for amplifying the firefly luciferase gene: 5’-TTGCCTGATACCTGGCAGATGGAA-3’ (forward) and 5’-TTCCTGATACCTGGCAGATGGAA-3’ (reverse). Known copy numbers (10⁻¹⁰⁻¹⁰⁸) of the plasmid AAV/Ck6/Luc were used to construct the standard curve. The results were expressed as mean AAV vg copy numbers per μg of host genomic DNA.

Statistical analysis
Data were expressed as mean ± s.e.m. For statistical comparisons of luciferase activity and vg copy number, values from various tissues were compared using one-way analysis of variance and a Tukey’s post test was used to assess significance. P < 0.05 was considered statistically significant in all of the comparisons.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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