DELIVERY OF VEGF165 WITH HYPOXIA-REGULATED ADENO-ASSOCIATED VIRUS (AAV) SEROTYPE 9 PRODUCES STABLE CONDUCTION VESSELS IN MOUSE ISCHEMIC LIMBS

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INTRODUCTION Therapeutic angiogenesis is being tested as a possible treatment for peripheral and coronary artery diseases (1, 2). Encouraging results from preclinical studies have not been well reproduced in the more than 1000 patient trials that have been completed. Clinical trials to date focused on different isoforms of VEGF or FGF delivered to ischemic heart or limb by direct injection of proteins or genes. The latter include plasmid and adenoviral (Ad) vectors in which expression of the cloned gene is directed by the ubiquitous cytomegalovirus (CMV) promoter. The protein and gene therapy approaches have been questioned because of the transient nature of growth factor expression and the absence of directional cues for new vessel growth. In support of this, we recently demonstrated in the rabbit hind-limb model that the delivery of VEGF165 by adenovirus generated transient expression of VEGF protein that peaked within 2-days. Endothelial cell (EC) activation was rapid and strong with areas of up to 25% of ECs registering Ki67-positive staining. This effect was very transient and was followed closely by EC apoptosis suggesting that VEGF delivery by adenovirus is short-lived and supports negligible production of stable conducting vessels (3). To overcome the transient expression typical of adenoviral vectors and to limit VEGF expression to ischemic muscle, in the current study we evaluated the angiogenetic response of VEGF165 driven by an hypoxia-regulated AAV vector in the mouse hind-limb ischemia model.

METHODS We generated recombinant AAV9 where human VEGF165 expression is directed by a phosphoglycerate kinase (PGK) promoter containing a tandem series of Hypoxia response elements and Silencers (AAV9-H/S-VEGF). The latter constitutes a regulatory unit wherein gene expression is silenced under aerobic conditions and activated by hypoxia and ischemia; this novel regulation constitutes “conditional silencing”, which efficiently turns off gene expression when perfusion is reestablished in the muscle. AAV9 is a newly isolated serotype that is reported to have strong tropism and highly efficient penetration in muscle (J. Wilson, personal communication). Use of AAV9 has not been described in any ischemia model. We used laser Doppler imaging to compare the return of blood flow in the mouse hind-limb after induction of ischemia by ligation and excision of the femoral artery and its branches. Following surgery, limb muscles were injected with equal amounts (10⁹) of Ad-VEGF, AAV9-H/S-VEGF, AAV9-GFP or PBS as controls. Doppler was recorded at 7, 14, 21, 28, and 60 days. In a parallel study fibroblast growth factor (FGF) was delivered by time-release gel matrices and perfusion imaged at the same time points as for the viral delivery.

RESULTS Muscles injected with either Ad-VEGF or AAV9-H/S-VEGF retained significantly higher perfusion indices than control muscles at all time points (n=6). At 1-week, the Doppler ratio for Ad-VEGF and AAV9-H/S-VEGF were not significantly different. However after 1-week the perfusion indices of the two groups diverged. Whereas the Ad-VEGF treatment group showed no further increase in perfusion index, the scores for AAV9-H/S-VEGF treatment group continued to increase up to 2 months returning to control perfusion within this time.
At the 28d time point, the Doppler ratio of the AAV9-H/S-VEGF group was significantly higher than that of the Ad-VEGF group (0.719 versus 0.552, respectively, n=3; p<0.02). Auto amputation was not seen in any of the treated limbs but occurred in 20% of untreated (PBS) limbs. The Doppler imaging results were supported by capillary density analyses. VEGF expression directed by the different viral vectors was measured in vivo. Significant levels of VEGF were present in both ischemic and non ischemic muscles injected with Ad-VEGF 1-2 weeks (n=6); this result has been consistently reproduced in rat, mouse and rabbit muscles. In contrast expression of viral vectors in which VEGF is regulated by conditional silencing never expressed in normally perfused tissue and expressed at low level in all ischemic muscles. These results confirm our in vitro studies showing that hypoxia is essential to activate gene expression from the hypoxia-conditionally silenced promoters.

Results with AAV9-H/S-VEGF were also compared with FGF delivery from time-release gel matrices. In the latter experiments Doppler perfusion indices increased more rapidly than either Ad-VEGF or AAV9-H/S-VEGF up to 28 days (n=4). After this time point, the scores from limbs with time-release FGF ceased to increase and in fact Doppler ratios decreased significantly at the 60 day time point. Therefore after 60 days only the AAV9-H/S-VEGF treatment groups had sustained increases of Doppler score that returned perfusion almost back to that of control limbs.

**DISCUSSION AND CONCLUSIONS** Our results confirm an overriding importance of sustained and regulated growth factor expression for productive and successful therapeutic angiogenesis for the treatment of critical limb ischemia. To date, proteins, plasmids, and adenoviruses have been used in clinical trials to deliver VEGF and FGF, but none of these treatments produce the sustained levels of growth factors that are required to generate stable functional new vessels in the ischemic muscles. Results using time-release matrices for FGF indicate that vessels begin to deteriorate when growth factors are withdrawn even after strong angiogenesis for up to 1-month. This may also explain the “leaky vessel” phenomenon that has been attributed to single factor gene therapy. Our results support the implementation of new gene therapy trials using a conditionally silenced AAV expressing one or more pro-angiogenic genes.

**REFERENCES**

(2) Webster, KA. Therapeutic Angiogenesis for Heart Disease. Future Cardiology. 1: 99-110, 2005.