Inducible adeno-associated virus vectors promote functional angiogenesis in adult organisms via regulated vascular endothelial growth factor expression

Sabrina Tafuro¹, Eduard Ayuso², Serena Zacchigna¹, Lorena Zentilin¹, Silvia Moimas¹, Franca Dore³, and Mauro Giacca¹*

¹Molecular Medicine Laboratory, International Centre for Genetic Engineering and Biotechnology (ICGEB), Padriciano, 99, 34012 Trieste, Italy; ²Centre of Animal Biotechnology and Gene Therapy (CBATEG), Universitat Autonoma de Barcelona, Barcelona, Spain; and ³Struttura Complessa di Medicina Nucleare, Azienda Ospedaliero-Universitaria Ospedali Riuniti, Trieste, Italy

Received 25 January 2009; revised 18 April 2009; accepted 5 May 2009; online publish-ahead-of-print 14 May 2009

Time for primary review: 23 days

Aims Members of the vascular endothelial growth factor (VEGF) family are among the most promising cytokines to induce neovascularization of ischaemic tissues; however, their unregulated expression often results in major undesired effects. Here, we describe the properties of inducible vectors based on the adeno-associated virus (AAV), allowing precise control of VEGF expression, and exploit these vectors to define the kinetics of the angiogenic response elicited by the factor.

Methods and results Based on a tetracycline-inducible transactivator, we designed an AAV vector system allowing the pharmacological regulation of VEGF production in vivo and tested its efficacy in inducing functional neoangiogenesis in both normoperfused and ischaemic skeletal muscle in mice by a combination of histological, immunofluorescent, and molecular imaging techniques. We observed that a prolonged expression of VEGF was required to determine the formation of stable vessels, able to persist upon withdrawal of the angiogenic stimulus. However, the vessels formed in the presence of continuous VEGF expression consisted mainly of dilated and leaky capillaries. As determined after pinhole scintigraphy, this abnormal vasculature accounted for a significant drop in functional tissue perfusion. In contrast, transient VEGF expression, followed by a period of VEGF withdrawal, allowed maintenance of functional perfusion under resting conditions and during exercise. This VEGF-inducible system was highly effective in improving vascularization and function in a hind-limb ischaemia model.

Conclusion Together, these results clearly indicate that the fine tuning of VEGF expression is required to achieve the formation of a stable vasculature able to sustain functional neovascularization.

KEYWORDS
AAV vectors; Angiogenesis; Gene therapy; VEGF; Vessel maturation

1. Introduction
The formation of new blood vessels in adult organisms (angiogenesis) occurs through a multistep process requiring the orchestrated action of a variety of soluble factors.¹ ²

One of the essential players in this process is the 165-amino acid isoform of vascular endothelial growth factor-A (VEGF), which acts as a powerful inducer of endothelial cell migration and proliferation through interaction with its receptors on the surface of endothelial cells (for recent reviews, cf. references 3–5).

Over the last several years, VEGF has been extensively exploited to induce therapeutic angiogenesis for the treatment of both peripheral artery occlusive disease and myocardial ischaemia. Despite promising results obtained in animals models,³ the short half-life of the VEGF protein hampers its successful use in a clinical setting.³ Thus, most clinical experimentations have relied on gene transfer using either plasmid DNA or adenoviral vectors to drive VEGF expression, but their overall outcome has been largely disappointing.⁷ The limited therapeutic benefit induced by VEGF gene transfer is probably related to the transient nature of gene expression induced by the used vectors. Even injecting high doses of naked plasmid DNA, the levels of the expressed protein remain relatively low, and its production fades after 1–2 weeks.⁸ With first-generation adenoviral vectors, expression levels are very high the first few days after transduction, but because of the powerful immune response elicited by the adenovirus infection itself, the transduced cells are eliminated within a few weeks from injection.⁹,¹⁰ Thus, the short burst of VEGF
synthesis obtained by either of these transient expression systems is presumably insufficient to generate a stable and mature vascular network.

On the other hand, the prolonged, unregulated expression of VEGF using genetically engineered myoblasts,11,12 or vectors based on the adeno-associated virus (AAV),13–15 leads to the formation of a persistent vasculature, but also induces massive vessel leakiness, due to the well-recognized permeabili zing activity of the factor,16 to the formation of vascular lacunae17 or blunt hemangiomas,18 and, finally, to the inhibition of pericyte coverage and blood vessel maturation.19

Together, these observations leave an outstanding question, namely what is the proper timing for VEGF expression to induce a stable and functional vasculature? To answer this question, here we exploit an AAV-based inducible system allowing VEGF expression in vivo in a tight pharmacologically tunable manner.

2. Methods

2.1 AAV vectors

The reverse tetracycline-controlled transactivator (rtTA)20 was obtained from the pTet-On vector (Clontech) and cloned into pAAV-MCS (Stratagene) to obtain pAAV-rtTA. The pAAV-TRE2/VEGF vector was generated by cloning the coding sequence of VEGF in the TRE2 plasmid (Clontech). Infectious AAV vector particles were generated by the AAV Vector Unit at ICGEB Trieste (www.icgeb.org/avu-core-facility.html) according to established procedures.21 The viral preparations had titres ~2 × 10^{12} genome copies (gc)/mL.

2.2 Animals


When indicated, doxycycline was supplied at 2 mg/mL together with 5% sucrose in the drinking water.

2.3 Histology and immunofluorescence

Histological, immunohistochemical, and immunofluorescence analysis were performed as already described,14,22,23 at least 10 sections from three different regions of each sample were analysed. For whole vasculature staining, mice were injected with 100 μL FITC-conjugated lectin (Molecular Probes) and, after 20 min, perfused with 1% paraformaldehyde. Thick sections of 80 μm were treated with RNase for 30 min at 37°C and stained with propidium iodide (Sigma); z-stacks acquisitions at 0.25 μm intervals on a Zeiss LSM 510 confocal laser-scanning microscope were used to generate 3D images. Analysis of vessel permeability was performed by a modified Miles assay as described.14

2.4 VEGF mRNA and protein quantification

Human VEGF protein and mRNA in AAV-transduced muscles was quantified by VEGF immunoassay (R&D Systems) and real-time PCR using TaqMan probes.22

2.5 Hind-limb ischaemia

Animals were injected 15 days before surgery with 5 × 10^{10} gc of the viral mixture AAV-rtTA and AAV-TRE2/VEGF in both the tibialis anterior and the gastrocnemius muscles. Hind-limb ischaemia was induced by ligation of the left femoral artery at its proximal origin. Doxycycline treatment was initiated few hours after surgery. Evaluation of limb function was performed from days 1 to 12 post-ischae mia by assigning a numeric score to progressive impairment of leg function.24

2.6 Pinhole scintigraphy

Static scintigraphy was performed by a gamma camera equipped with a pinhole collimator (Siemens Ecam). Functional images of the mouse legs were acquired after the injection of 3.7 mBq of 99mTc-Tetrofosmin (MioViewTM GE Healthcare) in basal condition and 37 mBq after 10 min hind-limb muscle contraction as described.17

For quantitative analysis, a polygonal region of interest (ROI) was manually drawn over the hind limbs paying attention to avoid abdominal radioactivity. ROI was drawn first on the ischaemic limb thigh and then symmetrically also applied to the contralateral leg. To exclude inter-individual differences as well as investigator-dependent bias, activity concentration per area unit was calculated as a measurement of perfusion in a standardized manner. ROI selection and count ratio determination was repeated twice and showed good reproducibility (variation <5%); three investigators confirmed placement of the ROIs prior to calculation; statistics were evaluated using ANOVA. Data are presented as the ratio between the left and right hind limbs including the 95% confidence interval. The method was validated by perfusion assessment in a group (n = 6) of non-operated and non-treated animals, which had equal perfusion in both hind limbs (ratio 1.06 ± 0.14).

2.7 Statistical analysis

One-way ANOVA and Bonferroni/Dunnett’s post hoc test was used to compare multiple groups. Pairwise comparison between groups was performed using Student’s t-test. Statistical analysis was applied after assessment that the variables had a normal distribution using the Kolmogorov-Smirnov test.

3. Results

3.1 An inducible AAV vector system for the in vivo conditional expression of VEGF

We designed an AAV vector system allowing the inducible expression of VEGF. The system consists of the combination of two AAV vectors, one constitutively expressing the inducible rtTA (AAV-rtTA),20 and another one expressing the VEGF cDNA under the control of the regulatory region TRE2, representing the binding site for activated rtTA, upstream a minimal CMV promoter (AAV-TRE2/VEGF) (Figure 1A). In cultured cells, the production of VEGF from this dual-vector system was found to be strictly dependent on cell treatment with doxycycline (see Supplementary material online, Figure S1A).

The two AAV vectors were injected (1 × 10^{11} gc total) into the tibialis anterior muscle of mice at a 1:3 ratio—a ratio that minimizes the risk of leakage (data not shown)—and doxycycline was added to the animals’ drinking water for various intervals of time (n = 6 per group). The injected muscles were then isolated and the levels of transgene mRNA measured by real-time RT–PCR. As shown in Figure 1B, transgene expression was undetectable in the absence of doxycycline, while it progressively increased at days 3 and 7 after transduction in the presence of the antibiotic, remaining constantly elevated from day 14 onwards (>4000-fold increase expression). VEGF transcription
returned to control levels in animals that received doxycycline for 14 days and in which the treatment was then interrupted for the subsequent 3 or 7 days. Most strikingly, in animals in which transcription was first activated and then turned off, expression could be activated again by the re-administration of doxycycline. The amount of VEGF mRNA and protein expressed upon inducible AAV gene transfer was comparable to that obtained using the constitutive immediate-early CMV promoter (Figure 1B and C, respectively). In the same animals, the persistence of vector DNA after transduction was quantitatively assessed by real-time PCR and found to be independent from VEGF induction (see Supplementary material online, Figure S1B). Of notice, the levels of expression of VEGFR-1 and VEGFR-2, both of which mediate the VEGF-A response,3,5 were upregulated in muscles in which expression of the ligand was turned on, possibly reflecting the increase in capillary density induced by the factor—see below (Figure 1D and E, respectively).

3.2 Conditional VEGF-induced angiogenesis

To assess the angiogenic response to doxycycline-induced VEGF expression, additional mice (n = 6 per group) were injected with the two vectors, treated with doxycycline for 30 days, and either analysed immediately or after a period of doxycycline withdrawal of 15 or 30 days. At each time point, mice were perfused in vivo with FITC-lectin to stain the vascular endothelium. Three-dimensional images of 80 μm-thick muscle sections were generated by confocal microscopy after nuclear staining using propidium iodide. The microvasculature of a mouse injected with the inducible AAV vectors but not treated with doxycycline is shown at two magnifications in Figure 2A, in which the regular spatial organization of the capillaries surrounding each muscle fibre is clearly evident. This appearance was indistinguishable from that obtained by the analysis of muscles from uninjected animals (data not shown). After 30 days of continuous VEGF expression (Figure 2B, upper panels), muscle fibres were surrounded by abnormally enlarged capillaries, with a sack-like morphology. In contrast with normal capillaries, lectin staining of these VEGF-induced vessels appeared fuzzy, consistent with its extravasation into the surrounding tissue. Typical of VEGF-induced angiogenesis,22 a massive cellular infiltration was also present. Fifteen days after the cessation of VEGF expression (Figure 2B, middle panels), the newly formed capillaries had lost their cloudy appearance but were clearly increased in number,
with a diminished cellular infiltration. This increased capillary density persisted at day 30 after VEGF withdrawal (Figure 2B, lower panels), when capillaries showed a normalized structure.

Vessel density ~200 muscle fibres was quantitatively evaluated in 80 μm-thick sections. Each fibre was surrounded by 2.3 ± 0.2 capillaries/section in muscles injected with AAV vectors but not treated with doxycycline, analogous to uninjected muscles (Figure 2C). After 30 days of antibiotic treatment, quantification was not possible due to the very diffuse pattern of the lectin staining. However, at day 15 after cessation of doxycycline administration, the number of capillaries bearing normal appearance was significantly increased (7.0 ± 0.9 capillaries/section; P < 0.01; approximately three-fold increase). This increase persisted unaltered over 30 days from the cessation of antibiotic treatment. Supplementary material online, Movie 1 shows 3D reconstructions of serial confocal microscopy images captured along the z-axis at 0.25 μm intervals. In addition, Supplementary material online, Figure S2 shows that the angiogenic effect of VEGF strictly depended on the dose of doxycycline administered to the animals.

A modified Miles assay was used to determine the permeability of the newly formed vasculature. After 30 days of continuous VEGF induction, vessels underwent a approximately three time increase in permeability, while returned to physiological levels after 15 days from doxycycline withdrawal (Figure 2D). No modification of vascular permeability compared with controls was detected in animals injected with the two vectors but not treated with doxycycline (data not shown).

3.3 Time-dependent arterial formation induced by VEGF

One of the striking consequences of prolonged VEGF expression is the induction of a marked angiogenic effect, resulting in the formation of large set of new blood vessels, mainly consisting of 20–150 μm-diameter arterioles, surrounded by α-SMA- and NG-2-positive cells. We have already shown that this effect of VEGF is paralleled by the recruitment of NP-1-positive myeloid cells from the bone marrow, which essentially contribute to pericyte recruitment through a paracrine action. Both artery formation and cellular infiltration were clearly visible in the animals transduced by the inducible vector and continuously treated with doxycycline for either 15 or 30 days (Figure 3Aa and Ba, respectively). At both time points, VEGF expression determined a ~10-fold increase in arterial density compared with the untreated control leg (Figure 3C).

Strikingly, in the animals treated with doxycycline for 15 days and subsequently exposed to VEGF withdrawal for additional 15 days, the newly formed vasculature completely regressed (Figure 3A). In contrast, when the initial VEGF stimulus was protracted for 30 days, the newly formed arterioles persisted for the subsequent 15 or 30 days, independent of the continuation of antibiotic treatment (approximately seven-fold increase; see Figure 3B.
for representative images and Figure 3C for quantification). Consistent with the stability of the vessels formed after prolonged (30 days) VEGF expression, no endothelial cell apoptosis was detected at different times after doxycycline withdrawal (Supplementary material online, Figure S3).

During development, vessel stabilization depends on the exit of endothelial cells from the cell cycle. Consistently, VEGF-expressing muscles contained a higher number of BrdU-positive endothelial cells at 15 days compared with 30 days (see Supplementary material online, Figure S4A and B). By real-time PCR, a marked upregulation of several factors involved in vessel maturation was detected at both 15 and 30 days. Of interest, out of these factors, ephrin-B2 was selectively overexpressed at day 30 compared with day 15, whereas thrombospondin-1 (TSP-1) and TSP-2 showed an opposite trend (see Supplementary material online, Figure S4C).

3.4 Analysis of VEGF-induced vessel function by pinhole scintigraphy

To assess the functional competence of the vasculature formed in response to VEGF, muscle perfusion was determined by measuring muscle metabolism by pinhole scintigraphy using 99mTc-tetrofosmin (Figure 4A). Animals injected with the AAV-rtTA-AAV–TRE2-VEGF vector combination (n = 10) were analysed after 1 month of continuous doxycycline treatment, as well as after an additional 15 days of antibiotic withdrawal. At both time points, data were acquired in both resting conditions and after exercise, induced by 10 min of pacing-induced hind-limb muscle contraction. After 30 days of VEGF stimulus, 99mTc-tetrofosmin uptake was comparable in both legs in basal conditions, whereas a decrease in the perfusion of the treated leg was observed during exercise, indicative of a poor functionality of the neovasculature upon continuous VEGF stimulation. In contrast, when VEGF expression was switched off for an additional 15 days, perfusion after exercise was remarkably increased in the VEGF-treated limb (Figure 4B).

Figure 4C shows the increase in muscle perfusion in the treated leg after exercise relative to the basal condition in each individual mouse. Most VEGF-expressing mice showed impaired perfusion of the treated leg after exercise at day 30 of doxycycline treatment. However, after cessation of the VEGF stimulus, perfusion of the treated limb invariably
improved (mean ratio 0.86 ± 0.27 at the end of doxycycline treatment vs. 1.8 ± 0.73 15 days later; \(P < 0.01\)), indicative of proper maturation of the newly formed vasculature in the absence of VEGF.

3.5 Inducible activation of VEGF restores function after hind-limb ischaemia

Next, we wished to assess the therapeutic potential of the inducible system in the context of tissue ischaemia. Animals (\(n = 30\)) were injected with the AAV vectors into the left tibialis anterior and gastrocnemius muscles and, after 15 days in the absence of doxycycline treatment, the left femoral artery was ligated. Half of the animals received doxycycline immediately after surgery, whereas the other half was left untreated. An additional group (\(n = 10\)) underwent surgery for ligation of the femoral artery but was not injected with the vectors.26

Limb function was analysed daily up to day 14 by assigning a progressive score ranging from 3 (leg dragging) to 0 (normal motility), according to established parameters.24 The doxycycline-treated animals showed a remarkably faster recovery of the limb function (\(P < 0.001\); Figure 5A). By pinhole scintigraphy, a large perfusion deficit was clearly evident at 3 h after artery ligation; this defect was largely reversed at day 15 in the animals treated with doxycycline (3.4- vs. 1.9-fold at day 15 compared with day 0 in treated and untreated animals, respectively; \(P < 0.001\); Figure 5B and C).

Histological analysis was performed after sacrifice at day 15. In the animals that did not receive the antibiotic, muscles presented extensive necrosis with adipose substitution and inflammation (Figure 5D). In sharp contrast, the injured muscles in the doxycycline-treated animals showed large areas of fibres with central nuclei, a hallmark of muscle regeneration (inset in Figure 5D). The area occupied by regenerating fibres was \(\approx 10\%\) in the untreated animals, whereas it rose to \(30\%\) in the animals that received doxycycline (\(P < 0.001\); Figure 5E). In the same sections, the number of \(\alpha\)-SMA-positive arterial vessels was significantly increased in the animals treated with doxycycline (\(\approx 1.6\) times; \(P < 0.001\)) compared with untreated animals (Figure 5F).

Taken together, these results indicate that the activation of the otherwise silent VEGF expression by antibiotic treatment immediately after ischaemia remarkably improves the functional recovery of the ischaemic limbs.

4. Discussion

We have developed a dual AAV vector system, based on the Tet-On approach, for the in vivo conditional expression of VEGF. Expression of the factor could be protracted for several months by simply adding doxycycline to the animals’ drinking water and rapidly turned off upon
withdrawal of the antibiotic. Even when shut down for several months, the system could be promptly and fully reactivated by re-administration of the antibiotic. These features represent a main progress made over the previously used Tet-regulated reporters, delivered by plasmids, retroviral, or lentiviral vectors, which are highly susceptible to positioning effects and prone to chromatin-mediated silencing. Consistent with other reports, the high responsiveness of our AAV vector system is probably due to their episomal persistence in the nucleus of non-dividing cells in the absence of integration, and thus free from the constrains imposed by the host DNA chromatin. Preclinical experimentation using AAV vectors similar to ours but carrying other transgenes has already proven the tight transcriptional control of the therapeutic gene also in non-human primates.

By using this inducible system, we found that transient (\(<15\) days) VEGF expression induced the formation of a large number of unstable vessels that regressed spontaneously as soon as VEGF production was halted. These results, which are fully consistent with analogous conclusions obtained using transgenic animals, challenge the usefulness of all gene delivery systems driving short-term transgene expression, such as plasmids and adenoviral vectors, and might explain, at least in part, the overall failure of the gene therapy clinical trials for the induction of therapeutic neovascularization attempted so far.

In contrast, sustained VEGF expression for at least 1 month induced the formation of stable vessels, which, however, were leaky and poorly functional. These observations support the notion that vessel formation in adult organisms relies on two subsequent phases: an early phase...
strictly dependent on the presence of VEGF, followed by a second one, in which VEGF is not only no longer required but even detrimental for vessel functionality, possibly to the inhibition of pericyte function exerted by the factor. During the VEGF-dependent period, endothelial cells, which mainly originate from the local, pre-existing vasculature, become activated to form a set of leaky, immature, and irregularly shaped vessels. This newly formed vasculature mainly consists of dilated capillaries and large vascular spaces surrounded by a thin endothelial layer, often invading the muscle fibre parenchyma. Following this period, a second phase of vessel maturation is required to ensure the proper acquisition of functional competence of the newly formed vasculature. In trying to understand which are the molecular events involved in vessel stabilization, it is of interest to note that the notable differences between the tissues analysed at 15 or 30 days of continuous VEGF expression were the upregulation, at 15 days, of TSP-1 and TSP-2—two matricellular glycoproteins that modulate cell matrix interaction and are endowed with antiangiogenic activity—and, at 30 days, of ephrin-B2, a protein known to favour the interaction between endothelial and mural cells. These changes correlated with a significant reduction of the extent of endothelial cell proliferation at the later time point. Together, these modifications appear indicative of an overall quenching of the angiogenic phenotype at day 30 compared with day 15, concomitant with a progressive stabilization of the interaction between endothelial and mural cells.

If, on one side, our AAV-based dual system, stands as a powerful tool to dissect the kinetics of the molecular pathways involved in angiogenesis, on the other side, it might hold promise in a therapeutic perspective for patients at risk of developing critical ischaemia. In the mouse hind-limb ischaemia model, pre-treatment with silenced vectors and their activation immediately after the induction of ischaemia determined a significant functional improvement. The animals treated with the antibiotic showed a considerable increase in vessel density and, most notably, extended muscle regeneration. In keeping with these findings, tetrofosmin uptake was increased in the treated animals, indicative of both higher perfusion and improved metabolism. These results are consistent with a direct effect of VEGF on both vessel formation and muscle regeneration and can thus explain why a striking protective effect was observed at 15 days in ischaemic conditions, despite no increased perfusion was detected by inducing VEGF expression in normoxic muscles at the same time point.

In conclusion, our system provides a powerful molecular tool to switch VEGF expression on and off in vivo, allowing its delivery only for a desired period of time, thus improving the efficacy of VEGF gene transfer. The developed approach might thus stand as an appealing alternative to the simultaneous delivery of VEGF together with other factors promoting vessel maturation, such as angiopoietin-1, possibly further increasing the safety of angiogenic gene transfer. In addition, our finding that ischaemic hind limbs could be successfully treated by activating pre-injected AAV vectors by simple doxycycline administration also suggests that these vectors might be considered for preventive gene therapy applications in high-risk patients, in which therapeutic gene expression might be rapidly turned on at need and only for an optimal treatment time. Obviously, the clinical translatability of our system will need further validation in a large animal model, in which important aspects of human pathology can be reproduced, including the occurrence of co-morbidities, which might impact on the efficiency of VEGF-driven therapeutic angiogenesis.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
The authors are grateful to Marina Dapas, Sara Tomasi, and Michela Zotti for technical support, to Mauro Sturnega for help in animal experimentation, and to Suzanne Kerbavics for editorial assistance.

Conflict of interest: none declared.

Funding
This work was supported by a grant from the "Fondazione CR Trieste", Trieste, Italy, by grants from the Regione Friuli Venezia Giulia, Italy, and by a grant from the World Anti-Doping Agency (WADA), Montreal, Canada.

References
15. Ferrari M, Arsic N, Recchia FA, Zentilin L, Zacchigna S, Xu X et al. Adeno-Associated Virus-mediated transduction of VEGF165 improves...


