Arteriolar Genesis and Angiogenesis Induced by Endothelial Nitric Oxide Synthase Overexpression Results in a Mature Vasculature

Andrew V. Benest, Oliver A. Stone, William H. Miller, Colin P. Glover, James B. Uney, Andrew H. Baker, Steven J. Harper, David O. Bates

Background—Generation of physiologically active vascular beds by delivery of combinations of growth factors offers promise for vascular gene therapy.

Methods and Results—In a mesenteric model of physiological angiogenesis, combining endothelial nitric oxide synthase (eNOS) (and hence NO production) with VEGF and angiopoietin-1 overexpression resulted in a more functional vascular phenotype than growth factor administration alone. eNOS gene delivery upregulated eNOS, VEGF, and Ang-1 to similar levels as gene transfer with VEGF or Ang-1. eNOS overexpression resulted in neovascularization to a similar extent as VEGF and Ang-1 combined, but not by sprouting angiogenesis. Whereas combining Ang-1 and VEGF increased both exchange vessels and conduit vessels, neither growth factor nor eNOS alone resulted in vessels with smooth muscle cell (SMC) coverage. In contrast, combining all three generated microvessels with SMCs (arteriolar genesis) and further increased functional vessels. Use of a vasodilator, prazosin, in combination with Ang1 and VEGF, but not alone, also generated SMC-positive vessels.

Conclusion—Coexpression of eNOS, VEGF, and Ang-1 results in a more mature vascularization of connective tissue, and generates new arterioles as well as new capillaries, and provides a more physiological therapeutic approach than single growth factor administration, by combining hemodynamic forces with growth factors. (Arterioscler Thromb Vasc Biol. 2008;28:1462-1468)

Key Words: angiogenesis ■ arteriogenesis ■ VEGF ■ Ang-1 ■ eNOS ■ pericyte ■ vascular smooth muscle

Therapeutic angiogenesis remains a promising tool in the treatment of ischemic diseases, but as yet clinical trials remain largely unsuccessful.1 For ultimate efficacy of proangiogenic gene therapy, the neovessel bed generated must be functional, stable, and homeostatic. This can be achieved not only by the generation of capillaries, but also by the recruitment of pericytes and vascular smooth muscle cells (VSMCs) to the vessels, so that the vascular bed can regulate perfusion. Current treatment relying on the overexpression of individual growth factors has not generated the growth of a complete vascular tree.2 A contributing factor to this could be the focus on single growth factors, which are unable to stimulate all components of vessel growth.

Combining different growth factors that stimulate different parameters of angiogenic growth results in an additive3 or even synergistic effect on angiogenesis.4 Although a combination of the destabilizing vascular endothelial growth factor-A (VEGF) and stabilizing angiopoietin-1 (Ang-1) in the adult rat mesentery induced a greater degree of functionality than either factor alone, the vessels formed were still of capillary phenotype (ie, microvessels with no VSMC coverage). Generating arterioles that contain smooth muscle cells (arteriolar genesis) is a requirement for revascularization of poorly vascularized tissues if the revascularized tissues are to be able to regulate their perfusion through normal physiological feedback mechanisms.

Increased blood flow, for instance by inducing vasodilatation with prazosin, is known to induce angiogenesis in skeletal muscle5-6 and increased shear stress is a critical mediator of vessel wall thickness and composition.7-11 A reduction in sprouting angiogenesis in favor of capillary splitting coincides with the highest blood flow in experimental models of hindlimb ischemia.12 Taken together, increased blood flow initiates both vessel growth and remodeling of angiogenic vessels.

Nitric oxide is a potent vasodilator. In the endothelium, it is produced by catalysis of l-arginine by endothelial nitric oxide synthase (eNOS), which is a calcium dependent en-
zyme involved in growth factor mediated angiogenesis. We aimed to test the hypothesis that angiogenic blood vessels could be remodeled by increased nitric oxide, presumably acting as a vasodilator to result in arteriolar genesis (formation of muscular arterioles) as well as angiogenesis (formation of new blood vessels, mainly capillaries). Using the mesenteric angiogenesis assay, a detailed and specific analysis of the microvessel phenotype was produced and a measurement of vessel function was made. To locally increase blood flow through the vessels around the mesenteric panel, we locally overexpressed eNOS to generate a continuous gradient of NO.

Therefore, we tested the hypothesis that by inducing angiogenesis in a nonischemic and well-validated model, overexpression of eNOS will induce both angiogenic growth and the recruitment of VSMC to the neovessels.

**Materials and Methods**

In brief, we used the adult rat mesenteric model of angiogenesis, as previously described. In brief (see online supplement for full methods at http://atvb.ahajournals.org), adult male rats (350g) were anesthetized and a laparotomy performed under sterile conditions. The mesenteric panels were imaged intravitally. Adenoviruses expressed eGFP, VEGF, Ang-1, or eNOS were injected into the mesenteric fat pad. In experiments where prazosin was used, prazosin was administered at 50 mg/L in the rat’s drinking water. The animal was sutured and allowed to recover. Seven days later the same mesenteric panel was located and imaged. It was then fixed in vivo and staining for angiogenic markers was carried out. Imaging of angiogenic blood vessels was carried out by immunofluorescence and confocal microscopy.

Confirmation of viral transfection was carried out by ELISA from mesenteric fat panels. To support in vivo data, primary human adipocytes were cultured and infected with the same adenoviruses as described previously. ELISA was used to confirm transfection and to determine whether this was attributable to overexpression in adipocytes or for other reasons, cultured adipocytes were transfected with GFP or eNOS virus and expression compared. Whereas eNOS virus transfection upregulated expression of eNOS protein (see supplemental Table 1), doubled nitrite levels, and increased VEGF levels 46±5% (P<0.05 for each compared with control, t tests), it did not significantly affect Ang-1 expression.

**ENOS Overexpression Increases Functional Vessel Area**

To determine whether the increased protein levels in the mesenteric fat pad resulted in a functional change in vessel perfusion, the total area of patent vessels was measured (functional vessel area, FVA, Figure 1A). Overexpression of eGFP did not increase the FVA on day 1 compared with day 7 (Figure 1B, P=0.58) but Ang-1+VEGF (Figure 1B, P=0.002), eNOS (Figure 1B, P=0.03) and eNOS+Ang-1+VEGF (Figure 1B, P=0.0003) did significantly increase FVA. Furthermore, eNOS+Ang-1+VEGF induced the greatest response compared with eGFP (Figure 1C, P<0.001), Ang-1+VEGF (Figure 1C, P<0.001), and eNOS (Figure, 1C, P<0.001). None of the other proangiogenic groups were significantly different from each other (Figure 1C, P>0.05). In summary, overexpression of eNOS, Ang-1, or VEGF (or together) stimulates an increase in the functional vessels available for flowing blood.

**Results**

**Increased Protein Levels After Adenovirus Injections**

Consistent with other reports, injection of multiple adenoviruses into the mesenteric fat pad results in overexpression of the virus’s respective proteins as a percentage of total protein extracted 3 days post-infection. Treatment groups containing Ad-Ang-1 and Ad-eNOS all increased expression of Ang-1 and eNOS compared with control (see supplemental Table 1; P<0.001). VEGF protein was also increased by Ad-eNOS, Ad-Ang-1+Ad-VEGF, and Ad-Ang-1+VEGF+eNOS. No increase in eNOS levels were observed after administration of any combination of growth factors without Ad-eNOS being present. However, eNOS overexpression did induce the expression of the other two critical determinants of blood vessel growth—VEGF and Ang-1. To determine whether this was attributable to overexpression in adipocytes or for other reasons, cultured adipocytes were transfected with GFP or eNOS virus and expression compared.

**ENOS Overexpression Remodelled Sprouting Vessels**

To determine whether the increase in FVA induced by eNOS was a result of higher blood flow (attributable to subsequent
vasodilatation induced by nitric oxide), we investigated the phenotype of the vessels produced. Confocal microscopy of the mesenteric panel stained for isolectin IB4, antibodies to NG2 and αSMA enabled detailed analysis of the endothelial and periendothelial composition of the vessel (Figure 2). All treatments that increased the FVA also increased the microvessel density compared with control (Ang-1+VEGF \( P < 0.05 \), eNOS, eNOS+Ang-1+VEGF \( P < 0.01 \); Figure 3A). None of the angiogenic groups differed from each other significantly. In addition, all the angiogenic groups increased the proliferating endothelial cell (PEC) density (Figure 3B) compared with control (\( P < 0.01 \)), but not when compared with each other (\( P > 0.05 \)). Thus all three treatments resulted in an angiogenic response (increased number of capillaries and proliferation of endothelial cells).

Different angiogenic agents have previously been demonstrated to induce different vessel branching patterns, which are likely to reflect the presence or absence of sprouting angiogenesis.\(^3,14–16\) Overexpression of eNOS alone did not significantly increase sprouting in the microvasculature (Figure 3C). Although overexpression of eNOS+Ang-1+VEGF induced a significantly higher sprout point density compared with control (Figure 3C, \( P < 0.01 \)), this sprouting was still significantly lower than the sprouting seen after VEGF overexpression (demonstrated to induce vessel sprouting) alone in the adult rat mesentery,\(^3,13\) and was no different to VEGF and Ang-1 in the absence of eNOS. Although sprouting did not appear to be stimulated by eNOS overexpression, there was also an increase in branch point formation after eNOS administration and eNOS+Ang-1-VEGF compared with control (\( P < 0.01 \)) and this was no different from Ang-1+VEGF. Taken together, despite VEGF and Ang-1 levels being approximately equal in all angiogenic groups measured, there appeared to be differences in how blood vessels...
were forming. Ang-1+VEGF appeared to remodel sprouts into vessels and eNOS overexpression appeared to induce vessel growth through a sprouting independent mechanism, as sprouting was not increased, but branch formation was.

**eNOS Overexpression Induced Muscle Coated Vessel Formation**

Increased blood flow is considered as a leading determinant in the arteriogenic mechanisms that underlie collateral formation and also arterialization of microvessels. The process of arteriogenesis results in a change in the composition of the periendothelial wall. The mesenteric connective tissue panels are perfused with small order capillaries, generally <20 μm diameter, and their periendothelium tends to be pericyte, but not smooth muscle. After treatment with Ad-eGFP, Ad-eNOS, or combined Ang-1 and VEGF treatment, the vessels of the mesentery remained free of smooth muscle cells. In stark contrast however, administration of combined eNOS, Ang-1, and VEGF induced a highly significant coverage of vessels with smooth muscle cells (Figures 2 and 4A; \( P < 0.001 \) compared with the other groups). To determine whether the VSMC coverage was restricted to larger diameter microvessels, we investigated the density of VSMCs on conduit microvessels (16 to 35 μm diameter) and on exchange capillaries (<16 μm diameter). Although there was no significant difference between the 2 groups (\( P > 0.05 \); Figure 4B) there was a trend for VSMCs to be present on the larger vessels. Approximately 30% of each vessel is covered by pericytes (fractional pericyte area [FPA]) in control vessels and this was not significantly altered after eNOS or Ang-1+VEGF treatment (Figure 4C). The pericyte coverage was significantly reduced, however, after eNOS+Ang-1+VEGF treatment compared with all other groups (\( P < 0.05 \); Figure 4C). This reduced pericyte coverage matched the increase in coverage by aSMA resulting in an approximately equal mural cell coverage of blood vessels after triple adenovirus treatment (Figure 4C). There was no correlation, however, between fractional pericyte area and fractional smooth muscle cell area (Figure 4D). Although VSMCs were only present after Ad-eNOS+Ang-1+VEGF, eNOS treatment alone induced an equal frequency distribution of vessel diameters compared with control (Figure 4E; \( P > 0.05 \)). All other treatment groups resulted in a shift to the right in distribution, indicating a greater number of larger diameter vessels (Figure 4D; \( P < 0.001 \) versus eGFP). This suggests that eNOS overexpression was sufficient to generate a “normal” distribution of vessels.

**Muscle Coated Vessel Formation Was Also Induced by Combined Growth Factor and Vasodilatation**

To determine whether the induction of a mature vasculature that resulted in higher blood flow and more new vessel formation was attributable to the vasodilatory effects of eNOS transduction, we repeated the experiment using a pharmacological vasodilator, prazosin. Rats were treated with eGFP virus or both Ang1 and VEGF as above, and prazosin (50 mg/L) was administered in drinking water for the duration of the experiment. Rats treated with prazosin and eGFP virus increased functional vessel area to 349 ± 43% compared with day1. This was significantly greater than eGFP (\( P < 0.0043 \)), but significantly less than eNOS alone (\( P > 0.001 \)), and indicates that the prazosin resulted in an increase in blood flow, presumably attributable to vasodilation. However, it did not increase FVA in conjunction with Ang1 and VEGF compared with the 2 growth factors alone (\( P > 0.05 \)), in contrast with eNOS (Figure 5A). Estimation of angiogenic parameters, such as sprout point density, indicated that there was no significant angiogenic response with prazosin alone (Figure 5B; \( P > 0.05 \)). Moreover, it did not
enhance FVA when combined with VEGF and Ang1 compared with the two growth factors alone \( (P > 0.05) \). This was also true for branch point density, vessel density, and pericyte coverage \( (P > 0.05) \). In contrast with this lack of angiogenic response, however, prazosin did induce smooth muscle cell coverage of the microvessels when combined with Ang1 and VEGF (although not alone, Figure 5C). This response was predominantly limited to the larger \( (>16 \mu m) \) diameter vessels (Figure 5D). Thus arteriolar genesis, but not angiogenesis, in this model was enhanced by a vasodilator in the presence of VEGF and Ang1.

**Discussion**

This study determined that overexpression of multiple growth factors, in addition to the overexpression of a vasodilatating agent, resulted in both an angiogenic response (as determined by an increased number of capillaries) and an arteriogenic response as demonstrated by an increase in VSMC covered vessels.

**Increased Blood Flow Induces a Different Angiogenic Phenotype Despite Approximately Equal Protein Levels Compared With Ang-1+VEGF**

Injection of Ad-VEGF and Ad-Ang-1 into the mesenteric fat pad increased levels of VEGF and Ang-1, respectively, as has previously been determined.\(^3\) Furthermore, Ad-eNOS injection resulted in increased levels of eNOS protein, which is qualitatively similar to others in multiple tissue types.\(^19-21\) Of interest, however, Ad-eNOS also increased VEGF expression both in the fat pad and in cultured adipocytes. This could be a result of increased HIF-1α stabilization\(^22,23\) or via increased heme-oxygenase levels,\(^24\) both shown to upregulate VEGF in response to increased NO. In addition, Ang-1 levels were also increased in the fat pad, but not in adipocytes. Ang-1 upregulation has so far only been reported in isolated cerebral endothelial cells after NO donor treatment,\(^25,26\) so it is still unclear which cells in the fat pad provide the source of Ang-1. Furthermore, the phenotype of the vessels produced by eNOS overexpression alone largely represent those produced by a overexpression of Ang-1+VEGF coincidentally.\(^3\)

The stabilizing action of NO during angiogenesis (in our setting produced by eNOS) has been previously demonstrated in NO producing tumor cell line, and also in eNOS-deficient engineered blood vessels,\(^27\) which reported enhanced perfusion and association between the EC and the periendothelium. Consequently, we suggest that in the setting of angiogenesis into a largely avascular area, increased eNOS levels (and therefore increased NO levels) are likely to mediate vessel remodeling and stabilization via VEGF and Ang-1 production. Other studies have reported that EC stimulated with VEGF result in increased eNOS levels.\(^28-30\) We did not observe such findings, possibly as we are primarily transfecting adipocytes (which do not appear to express eNOS\(^31\)), or the mechanisms regulating eNOS expression are not common to both EC and adipocytes. It will be interesting to elucidate the effect of VEGF antagonists on the eNOS-mediated neovascularization, as it has been demonstrated that NO-induced angiogenesis can be inhibited by the use of VEGF-TRAP in models of skeletal muscle angiogenesis.\(^32\)

**Increased Blood Flow Remodels Angiogenic Vessels**

eNOS overexpression increased branch point density but did not increase sprout point density. Given that increased branching in an absence of increased sprouting is evidence of nonsprouting angiogenesis, mediated by increased blood flow,\(^5,33,34\) we therefore provide indirect evidence of intussusceptive vessel growth or vessel splitting. Increasing blood flow is known to be a critical driving force in the recruitment of contractile mural cells to the vessel wall of preexisting microvessels.\(^17,33,35,36\) eNOS is a critical mediator of vascular hemostasis\(^12\) and produces NO which is a potent vasodilator,\(^19\) which leads to increased blood flow and a consequent hyperaemia induced angiogenesis.\(^6,18,33,37\) Furthermore, eNOS has recently been demonstrated to be a critical component in the induction of arteriogenesis as eNOS-
deficient mice were able to mount an angiogenic response, but failed to recruit VSMCs into the vessels, suggesting that remodeling of vessels into larger conduit vessels was not possible.38 Using the same angiogenesis model it was recently demonstrated that the mesenteric microcirculation of rats of this age and strain comprised pericyte coated vessels, with no VSMCs present.3 However, after eNOS + Ang-1 + VEGF overexpression we show here that there was a significant increase in the coverage of VSMCs. Thus eNOS, in the presence of VEGF and Ang-1 expression, was able to generate new arterioles, (arteriolar genesis). Thus we demonstrate a mechanism by which arteriolar formation from preexisting capillaries can occur.

It is not clear from where the VSMCs have originated. Increased blood flow is known to stimulate proliferation of fibroblasts in the mesenchyme,33 many of which had assumed a position within the vessel wall. Furthermore, enhanced shear stress increased arteriolar development, with increased VSMCs present on smaller order vessels.33 It is also possible that VSMC progenitors are present in the circulating blood—bone marrow–derived circulating progenitor have been found to be increased in the periendothelium of tumor vessels.39,40 Furthermore, in models of low hypoxia, tissue resident progenitor cells appear to migrate to sites of active angiogenesis, again within the periendothelium but not expressing periendothelial markers. A long held view is that pericytes might develop into VSMCs.41 Current immunohistochemical data supports pericytes and VSMCs as not being continuous. In the tracheal and retinal microcirculations, larger macrovessels express αSMA and NG2, but NG2 is only present in the microvessels.42,43 Taken together, in studies whereby both NG2 and αSMA have been used, different cell types are identified and therefore, it is likely there are different cell populations. We saw vessels with both pericytes and VSMC, therefore we do not have evidence supporting a trans-differentiation mechanism.

Increased blood flow via prazosin (and consequently α1-adrenergic receptor blockade) leads not only to increased shear stress but also increased wall stress. In skeletal muscle beds, the expression of mature VSMCs (myosin heavy chain positive) and immature and mature VSMCs (αSMA positive) is different between terminal arterioles and higher order arterioles, and this changes through development.36 The authors interpreted the different expression patterns to demonstrate that VSMCs might originate from the higher order vessels and migrate along the vascular tree. As proof of principle, increasing blood flow with prazosin44 or via skeletal muscle extirpation45 led to quantitatively similar findings. Increasing blood flow through ligation of arterioles in the mesentery, leading to increased hemodynamic forces in the downstream and connecting vessels also leads to an increase in proliferation and presence of VSMCs.10 Interestingly, prazosin in this model led to increased muscle cell investment without contribution to angiogenesis, further suggesting that additional pathways are regulated by eNOS, which have an effect on vessel remodeling and are not limited specifically to the vasodilatatory actions of NO.

In summary we show here that coexpression of an angiogenic molecule (VEGF), an endothelial stabilizing factor (Ang-1), and the artificial increase in blood flow, mediated through NO (eNOS) results in a more robust and mature vascularization of connective tissue, and generates new arterioles as well as new capillaries. This therefore provides a mechanism for generating new vascular beds that can regulate their flow and provide homeostatic feedback for perfusion regulation. Current therapy has largely failed to generate the growth of a whole vascular tree, and we demonstrate that the combination of different aspects of vascular growth can induce such vessel remodeling. Methodologies to do so surrounding an ischemic environment may therefore lend themselves to therapeutic relevance.

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Disclosures
None.

References


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Arteriolargenesis and angiogenesis induced by endothelial nitric oxide synthase overexpression results in a mature vasculature.

Andrew V. Benest\textsuperscript{1}, Oliver A Stone\textsuperscript{1}, William H. Miller\textsuperscript{3}, Colin J. Glover\textsuperscript{2}, James B. Uney\textsuperscript{2}. Andrew H. Baker\textsuperscript{3}, Steven J. Harper\textsuperscript{1}, David O. Bates\textsuperscript{1}.

\textbf{MATERIAL AND METHODS}

Adenovirus expressing VEGF\textsubscript{165} (VEGF) was generated as described in\textsuperscript{1,2} and eGFP as described in\textsuperscript{16}. Ang-1* virus was a kind gift of Regeneron\textsuperscript{3} (Tarrytown, NY) and amplified as per VEGF, Ad-eNOS was amplified and verified as previously reported\textsuperscript{4}.

\textbf{Verification of protein production and activity.}

Ang-1, VEGF and eNOS protein levels were quantified using commercially available ELISA kits on protein extracted from mesenteric fat pads, or adipocytes. ELISA kits for VEGF, Ang-1 and eNOS were provided by R&D systems (DY293, DY923 and DEN00 respectively). To ensure that the virally expressed eNOS was active nitrite levels were measured using a commercial nitrite assay (RnD Systems KGE001). Nitrite increased from 164±9.3\textmu M in GFP transfected CHO cells in culture to 472±11.8 in Ad-eNOS infected cells, indicating that over-expression of eNOS could increase NO production.
Antibodies
NCL-L-Ki-67-MM1, (1.5µg/ml), NG2 (Chemicon, Temecula, USA, MAB5384, 5µg/ml), or αSmooth Muscle Actin (DAKO, Glostrup, Denmark M 0851, 1.4µg/ml), and rabbit anti mouse NG2 (AB5320, Chemicon, 5µg/ml) and *Griffonia simplicifolia* lectin IB4 ((GS-IB4) 10µg/ml, Molecular Probes, Cambridge, UK) were used. TRITC labelled streptavidin (1µg/ml, S-870, Molecular Probes, Cambridge, UK) and Alexa Fluor 488, 350 goat-anti-mouse IgG (2µg/ml, Molecular Probes, Cambridge, UK) were used for detection to Lectin, and smooth muscle actin respectively. Hoechst 33324 (1µM, Mol. Probes) to stain mesenteric nuclei.

**Adipocyte Culture**
Human visceral white preadipocytes (C-12730, Promocell, Germany) were grown at 37°C in an atmosphere of 5% CO₂ in preadipocyte growth medium (C-27410, Promocell, Germany) containing 5% Fetal calf serum, 0.4% endothelial cell growth supplement/heparin, 10ng/ml epidermal growth factor and 1µg/ml hydrocortisone. When cells reached full confluence, differentiation was induced by the addition of preadipocyte differentiation medium (C-27436, Promocell, Germany) containing 8µg/ml d-biotin, 0.5µg/ml insulin, 400ng/ml dexamethasone, 44µg/ml IBMX, 9ng/ml L-thyroxine and 3µg/ml ciglitazone. After 72 hours, the differentiation medium was changed to adipocyte nutrition medium (C-27438, Promocell, Germany) containing 3% fetal calf serum, 8µg/ml d-biotin, 0.5µg/ml insulin and 400ng/ml dexamethasone, and cells were maintained for a total of 15 days, fed every 3 days. After 15 days, fully differentiated cells were incubated with either Ad-eNOS or Ad-eGFP for 24 hours, the cells were then washed and media replaced. 72 hours post infection, ice cold lysis buffer (1 mmol/l...
phenylmethylsulfonyl fluoride, 1 mmol/l Na$_3$VO$_4$, 1 µg/ml aprotinin and 1 µg/ml pepstatin, in radioimmunoprecipitation assay buffer) was added to cells and homogenised on ice for 10 minutes. Cells were then centrifuged at 13,000 rpm and the supernatant collected and stored at -20°C. Protein concentrations were determined by standard BCA assay.

**Surgical Procedure**
Surgery was performed as previously described(15, 19) on male wistar rats (300-350g). All surgical procedures were performed under sterile conditions. A mesenteric panel with few vessels was exposed under an intravital microscope (Leica DMIL). The panel was imaged and virus (10$^7$ plaque forming units (PFU)) injected into the nearby fat pad. We have previously shown that this results in transfection of adipocytes(15). Individual adenovirus-expressing-growth factors were injected as 25µl doses and combined as a 50µl or 75µl. Ten microlitres of Monastral blue (0.6%, diluted in saline), was injected into the fat pads on either side of the virus-injected panel. The intestine was replaced and the animal sutured and recovered. Six days later (day 7), the animal was re-anaesthetised with halothane, the mesentery exposed and the virus-injected panel located. and imaged as above$^1$.

**Immunofluorescent Staining**
The mesenteric panel was fixed in vivo with 4% paraformaldehyde for 5 mins, excised, fixed with the same fixative, washed 6 times with 0.5% Triton-X100 in phosphate buffered Saline (0.5% PBX), blocked in 1.5% normal goat serum (Sigma) in 0.5% PBX and incubated with biotinylated *Griffonia simplicifolia* lectin IB4 and/or antibodies as described. The mesentery was washed with 0.5% PBX and incubated with TRITC-
Streptavidin and Alexa Fluor 350 and 488-labelled goat-anti-mouse IgG, washed, incubated with Hoechst 33324, mounted as flat as possible with VectaShield (Vector Lab, Peterborough, UK), and imaged with a Leica Confocal Microscope (Leica Confocal SP2 system, Leica, Bucks, UK).

**MICROVESSEL ANALYSIS**

% Vessel area increase

The vessel area was measured using Openlab software (Improvision, Coventry, UK). The area of these vessels was recorded as previously described\(^1\). Fractional vessel area (FVA) was calculated from the vessel area as a percentage of the mesenteric area. Percent vessel area increase (%AI) was expressed as the difference between the FVA on day 7 and the FVA on day 1 as a percentage of the FVA on day 1.

**MICROVESSEL MEASUREMENT**

For each mesentery, 8 to 12 views were selected randomly using a 40\(\times\) objective and Openlab software (Improvision, Coventry, UK) used to measure vessel parameters between two adjacent branch points. The total vessels were counted and labelled, and branch points, proliferating endothelial cells and sprouts in each image counted. The diameter and length of each vessel were measured. Branch point density, sprout density, and proliferating endothelial cell density were calculated as the number per unit area within five randomly selected fields of view (x40 objective) containing vessels as previously described\(^2\). Vessels were classified into two groups: less than 16\(\mu\)m (exchange vessels), and >16\(\mu\)m (conduit or resistance vessels – generally arterioles and venules).
To determine if any effect observed was due to the overexpression of multiple growth factors and not as a result of the presence of adenovirus, a combination of Ad-eGFP+Ad-Ang-1+VEGF was used, which did not induce any significant difference in any of the parameters measured when compared with coadministration of Ad-Ang-1+Ad-VEGF (data not shown).

**Statistical Analysis**

All data presented as mean±SEM, and all groups n=5 unless explicitly stated. P<0.05 was considered statistically significant. Data was analysed by ANOVA unless stated with post-hoc Neuman Keuls tests when ANOVA showed an overall p<0.05.
REFERENCES


Figure 1. Intravital imaging of the rat mesentery demonstrates increased vessel area following growth factor or haemodynamic factor overexpression but not control (A). Quantification of the FVA on day 1 vs day 7 demonstrated eGFP did not induce any increase in tissue perfusion, but over-expression of all other angiogenic groups did (B, t-test). Comparison of the increase in FVA reveals that a combination of eNOS+Ang-1+VEGF induced the greatest response. *** p<0.001 vs eGFP, ∆∆ p<0.01, ∆∆∆ p<0.001 vs eNOS+Ang-1+VEGF. Data presented as mean±SEM, One-Way ANOVA.

Figure 2. Confocal images of mesenteric vessels following control or angiogenic treatment. eGFP vessels do not sprout, with pericytes wrapped around the length of the vessel. eNOS treatment induces radial enlargement of the vessel and increases the vessel number and branch point density. Ang-1+VEGF induced vessels with a similar phenotype. eNOS+Ang-1+VEGF was the only group to increase the presence of vSMC, as found by the presence of αSMA positive cells in the presence of distinct NG2 positive cells.

Figure 3. Analysis of confocal images. Growth factor and eNOS overexpression induced an angiogenic response, as indicated by an increased density of blood vessels (A) and an increase in endothelial proliferation (B). Increased blood flow induced by eNOS increased sprout point density (C) and branch point density (D). * p<0.05, ** p<0.01, *** p<0.001 vs eGFP, ∆ p<0.05 vs eNOS. Data presented as mean±SEM, One-Way ANOVA with post-hoc Neuman-Keuls. N=5 all groups except PEC density where n=3 for eNOS+Ang-1+VEGF.
Figure 4. Analysis of periendothelium components from confocal image data. The degree of pericyte coverage as a percentage of the vessel area in addition to the fractional smooth muscle coverage demonstrates that the total periendothelium complement to the vessel does not change but the relative composition of pericyte to vSCM does (A). There is a greater degree of coverage in vessels of a larger mean diameter (B). The overall wall coverage was not altered (C). The presence of vSCM does not correspond with an absence of pericyte support (D). Frequency histogram of the vessel diameters produced demonstrates that a combination of growth factors shifts the distribution of vessels produced to a larger diameter, however eNOS overexpression produced a distribution equal to that of control treated vessels (E). ∆ P<0.05 VS eNOS+Ang-1+VEGF.

Figure 5. Effect of Prazocin on VEGF and Ang1 induced neovascularisation. A. Rats administered prazocin during the six days of the experiments in drinking water had a significantly greater functional vessel area than control (A), but did not enhance the effect of Ang1 and VEGF. There was no evidence of angiogenesis in prazocin treated rats (no increase in sprouting (B), branching or vessel density (data not shown). However, prazocin treatment did result in smooth muscle cell coverage in the presence of Ang1 and VEGF (C). This was due to predominantly to an increase in smooth muscle cell coverage of large, conduit vessels (D). *=p<0.05, **=p<0.01, ***=p<0.001 compared with control, +=p<0.05, ++=p<0.01 compared with eNOS, VEGF+Ang1. A-C = ANOVA and SNK, D=unpaired t test.
Table 1.

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<td>Mean ±SEM per mg</td>
<td>Ang1 (pg/mg)</td>
<td>VEGF (pg/mg)</td>
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<td>protein</td>
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<tr>
<td>eGFP</td>
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<td>0±0</td>
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<tr>
<td>Ang-1+VEGF</td>
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<tr>
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<td>274±63.2</td>
<td>16.3±2.55</td>
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<tr>
<td>eNOS+Ang-1+VEGF</td>
<td>17.9±1.87</td>
<td>260±41</td>
<td>12.1±2.44</td>
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|                  | Cultured adipocytes| Ang1 (pg/mg)           | VEGF (pg/mg)           | eNOS (pg/mg)           | nitrite |
|                  |                    |                        |                        |                        |         |
| protein          | Mean ±SEM per mg   | Ang1 (pg/mg)           | VEGF (pg/mg)           | eNOS (pg/mg)           | nitrite |
| eGFP             | 2.37±0.1           | 1.58±1.4               | 0±0                    | 85.6±6.8               |
| eNOS             | 3.2±0.34           | 2.6±0.44               | 2.34±0.96              | 176±8.4                |
Table Legend 1

Protein levels extracted from mesenteric fat pad 3 days post-infection or from cultured human adipocytes as indicated. All data presented as mean±SEM.

Table 1.

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