

ONLINE SUPPLEMENT FOR

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

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MATERIAL AND METHODS

Adenovirus expressing VEGF₁₆₅ (VEGF) was generated as described in^{1,2} and eGFP as described in(16). Ang-1* virus was a kind gift of Regeneron³ (Tarrytown, NY) and amplified as per VEGF, Ad-eNOS was amplified and verified as previously reported⁴.

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 $164 \pm 9.3 \mu\text{M}$ 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 Na₃VO₄, 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 (10⁷ 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 above¹.

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 described¹. 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× 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 described². Vessels were classified into two groups: less than 16µm (exchange vessels), and >16µm (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 \pm 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

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FULL FIGURE LEGENDS

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, $\Delta\Delta$ $p < 0.01$, $\Delta\Delta\Delta$ $p < 0.001$ vs eNOS+Ang-1+VEGF. Data presented as mean \pm 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 α SMA 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. Δ $p < 0.05$ vs eNOS. Data presented as mean \pm 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). $\Delta P < 0.05$ 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.

Mesenteric fat pad				
Mean \pm SEM per mg protein	Ang1 (pg/mg)	VEGF (pg/mg)	eNOS (pg/mg)	n
eGFP	0.44 \pm 1.51	56.7 \pm 20.7	0 \pm 0	3
Ang-1+VEGF	13.7 \pm 4.7	203 \pm 12.7	0 \pm 0	3
eNOS	13.2 \pm 3.78	274 \pm 63.2	16.3 \pm 2.55	3
eNOS+Ang-1+VEGF	17.9 \pm 1.87	260 \pm 41	12.1 \pm 2.44	3
Cultured adipocytes				
Mean \pm SEM per mg protein	Ang1 (pg/mg)	VEGF (pg/mg)	eNOS (pg/mg)	nitrite
eGFP	2.37 \pm 0.1	1.58 \pm 1.4	0 \pm 0	85.6 \pm 6.8
eNOS	3.2 \pm 0.34	2.6 \pm 0.44	2.34 \pm 0.96	176 \pm 8.4