Introduction: A third of patients with critical limb ischaemia (CLI) eventually require amputation. In spite of clinically successful revascularisation patients rarely return to their pre-morbid status, and often report no improvement in their functional outcomes, which may be due to an underlying musculopathy. Non-haematopoietic EPO-derivatives have been designed to retain only tissue-protective functions of EPO. We hypothesised that ARA-290 (EPO-derivative) may have tissue-protective potential that would represent a novel therapeutic adjunct in patients with CLI.
Methods: The effect of EPO and ARA-290 in mediating cytoprotection was assessed firstly in vitro using skeletal myoblasts isolated from CLI and control donors, and a model of simulated ischaemia. Characterisation of CLI myoblasts was also performed, to assess their contractile, migratory and proliferative ability. Subsequently, an in vivo murine model of hindlimb ischaemia, which recapitulates the muscular pathology observed in CLI patients, was used to assess the potential of ARA-290 to improve functional, histological and perfusion outcomes.
Results: Skeletal myoblasts were successfully isolated from CLI patients for the first time. CLI myoblasts and myotubes exhibited increased proliferative capacity but reduced migratory and contractile function and importantly a reduced susceptibility to a second ischaemic-insult compared with control myoblasts and myotubes. EPO and ARA-290 treatment led to significant improvements in myoblasts and myotube function and survival via the JAK2/STAT3, PI3k/Akt and NFκB signalling pathways. In vivo, animals treated with EPO and ARA-290 demonstrated improved functional, histological and perfusion outcomes compared to vehicle-control treated animals.
Conclusion: These studies demonstrate the potential of EPO and its derivatives to protect tissues and cells from ischaemic-injury and encourages the development of novel pharmacological therapies for use in patients with "no option" CLI or severe functional deficit.

VEGFR2 Blockade in Murine Vein Graft Results in Reduced Intraplaque Hemorrhage and Stable Atherosclerotic Lesions

M.R. de Vries [1,2], R.C.M. de Jong [1,2], H.A.B. Peters [1,2], J.F. Hamming [1], M.J. Goumans [2,3], P.H.A. Quax [1,2]

[1] *Department of Surgery, Leiden University Medical Center, Leiden, The Netherlands*
[2] *Einthoven Laboratory for Experimental Vascular Medicine, Leiden University Medical Center, Leiden, The Netherlands*
[3] *Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, The Netherlands*

Introduction: Immature plaque neovessels contribute to atherosclerotic plaque instability and intraplaque hemorrhage by leaking erythrocytes and leukocytes in the plaque. Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), together with the angiopoietin (Ang)-Tie2 system, regulates the maturation of growing neovessels. We have previously shown that murine vein graft lesions exhibit massive plaque neovascularization and that leaky vessels and intraplaque hemorrhage contribute to lesion growth. We hypothesized that blockade of VEGFR2 results in more mature plaque microvessels and less intraplaque hemorrhage.
Methods: Donor caval veins were engrafted in carotid arteries of recipient hypercholesterolemic ApoE3*Leiden mice ($n = 14$/group). Mice were treated at day 14, 17, 21 and 25 with VEGFR2 blocking antibodies (DC101) or control IgG antibodies (10 mg/kg). At day 28 mice were sacrificed for histological analysis of the vein grafts.
Results: Morphometric analysis revealed a striking 50% decrease in vein graft segments that expressed intraplaque haemorrhage in the form of leaky vessels in the DC101 treated group. This was accompanied by a significant 25-fold decrease in extravasated erythrocytes. Furthermore, lesions that exhibit intraplaque hemorrhage showed a strong increase in Ang-2, indicative for immature neovessels. VEGFR2 blockade however, did not affect the neovessel density in the lesions (control 52 $\pm$ 19 neovessels/section; DC101 63 $\pm$ 25 neovessels/section). Interestingly, the vein graft lesion area in the DC101 group was significantly reduced with 32% compared to the control group. Moreover, plaque stability was clearly increased in DC101 treated mice, determined by a 25% reduction in macrophage content, a 50% increase in collagen content and a 120% increase in SMC content.
Conclusion: Blockade of VEGFR2 leads to reduced intraplaque hemorrhage, decreased vein graft lesion area and increased plaque stability. This identifies plaque neovascularization as an attractive target for the treatment of unstable atherosclerotic diseases.

TLR4 Accessory Molecule RP105 (CD180) Regulates Arteriogenesis and Angiogenesis

T. Bastiaansen, J. Karper, A. Wezel, H. de Boer, S. Welten, Z. Aref, R. de Jong, E. Peters, M. de Vries, A. van Oeveren-Rietdijk, A.J. van Zonneveld, J. Hamming, Y. Nossent, P. Quax

Leiden University Medical Center, Leiden, The Netherlands

Introduction: TLR4-mediated mobilisation and activation of pro-inflammatory Ly6Chi monocytes is crucial for effective post-ischemic neovascularisation, i.e. arteriogenesis and angiogenesis. Therefore, we aimed to investigate the role of the TLR4-accessory molecule RP105 (CD180) in neovascularisation. RP105 has been identified as a negative regulator of TLR4 signalling in monocytes. Using immunohistochemical analyses, we found that RP105+ monocytes are present in the perivascular space of remodelling collateral arterioles. As RP105 inhibits TLR4 signalling, we hypothesized that RP105 deficiency would lead to an unrestrained TLR4-mediated inflammatory response and hence to enhanced neovascularisation and blood flow recovery after ischemia.
Methods: RP105−/− and wildtype mice were subjected to hind limb ischemia and blood flow recovery was followed by Laser Doppler Perfusion Imaging during four weeks.

Results: In contrast to our original hypothesis, we found that blood flow recovery was severely impaired in RP105−/− mice. Immunohistochemistry showed that both arteriogenesis and angiogenesis were reduced in these mice compared to wildtype animals. However, both in vivo and ex vivo analyses showed that circulatory pro-arteriogenic Ly6Chi monocytes were more readily activated in RP105−/− mice. FACS analyses in blood and tissue samples from wildtype mice showed that Ly6Chi monocytes migrate to the affected muscle tissues following induction of hind limb ischemia. Although Ly6Chi monocytes were more readily activated in RP105−/− mice, migration into the ischemic tissues was severely hampered and instead, Ly6Chi monocytes accumulated in their storage compartments, bone marrow and spleen, of RP105−/− mice. In vitro studies showed that activation of monocytes with LPS reduces their migratory ability, indicating that a timely, well-regulated activation of monocytes is crucial for effective neovascularisation.
Conclusion: The lack of TLR4 regulation in RP105−/− mice results in an unrestrained inflammatory response, leading to systemic monocyte over-activation. Monocyte activation reduces their migratory ability and thus, this premature, systemic activation of pro-inflammatory Ly6Chi monocytes results in reduced infiltration of Ly6Chi monocytes in affected tissues after ischemia and consequently in reduced arteriogenesis and angiogenesis. Therefore, a tightly regulated inflammatory response is crucial for effective neovascularisation and blood flow recovery after ischemia.

Preclinical Small Animal Model for Studying Ischemia-reperfusion Injury of the Spinal Cord After Crossclamping of the Aorta and the Beneficial Effect of EPO on the Neuronal Function

F. Simon, A. Oberhuber, P. Erhardt, H. Schelzig

Department of Vascular and Endovascular Surgery, University of Düsseldorf, Düsseldorf, Germany

Introduction: Ischemia and reperfusion (I/R) of the spinal cord are central problems of aortic surgery procedures e.g. aneurysm repair. In a large animal model we showed in the past that erythropoietin (EPO) attenuates the morphological signs of spinal cord I/R-injury and improves neurological function, but observation time was only 10 hours short. This small animal model with optimized clamping time evaluates the benefit of perioperative i.v. EPO administration and gives answer to the question of the clinical outcome up to 4 days.
Methods: To evaluate the optimal clamping time 36 New Zealand White Rabbits were used. Unlike other animal species rabbits have a pure segmental blood supply for the spinal cord, therefore reproducible paraplegia can be performed by infrarenal clamping alone. The ischemic times ranged from 15 over 17 to 20, 22 and 25 min to define best fitting clamping time. Afterwards rabbits received either vehicle (control, $n = 10$) or EPO ($n = 10$; 5000 IU/kg) over the last 30 min before clamping and during the first 30 minutes of reperfusion. Intraoperatively blood pressure (invasive), heart rate, oxygen saturation and temperature were recorded. In addition, blood samples were taken before and after aortic clamping for studies on senescence and apoptosis parameters. Clinical neurological examinations were performed using a modified Tarlov score every twelve hours. After 96 h the entire spinal cord was harvested for histological examination.
Results: After 96 h postoperative observation period animals of the 15 min clamping group showed a Tarlov score of 3.65 $\pm$ 1.55; 17 min 4 $\pm$ 1.75; 20 min 1.5 $\pm$ 2.22; 22 min 0 $\pm$ 1.59; 25 min 0 $\pm$ 0. Histological and clinical findings were significantly correlated, $p = 0.007$. Therefore a clamping time of 22 minutes was defined for further experiments. The following study groups consisted of control animals (NaCl; $n = 10$) and EPO treated animals ($n = 10$) challenged with 22 minutes aortic clamping. While the control animals showed no neuronal function at all (median = 0), the EPO group standed out with improved spinal cord function (median = 4,25) after 36 hours of reperfusion. This benefit was lost again after 96 hours of reperfusion (median = 0).
Conclusion: This model is an ideal setup for therapeutic studies on ischemia and reperfusion injury (I/R) after aortic crossclamping. EPO showed impressive benefits in ischemic neuronal tissue by improving spinal cord function.

Shear Stress Induces Vasoprotective Gene Upregulation in Pericytes

C. Schrimpf, K. Tim, U. Böer, M. Klingenberg, O. Teebken, M. Wilhelmi

Division of Vascular and Endovascular Surgery, Department of Cardiothoracic Transplantation and Vascular Surgery, Hannover Medical School, Hannover, Germany

Introduction: Fibrosis is an initial step in the development of atherosclerotic lesions, hallmarked by myofibroblasts entering the tunica media and forming a fibrous cap. Vasa vasorum in the adventitia consist of endothelial cells, surrounded by pericytes. Pericytes can be regarded as cells with stem cell like properties and the capacity to differentiate into myofibroblasts. Although it is known that shear stress induces atherosclerotic lesions, nothing is known about its impact on pericytes. Herein, we investigate the effect of shear stress on pericytes and focus on differentiation into myofibroblasts.
Methods: Primary human pericytes (PCs) were isolated from donor fatty tissue and cultured in DMEM + 10% FCS. Primary cells were characterised by immunofluorescent (IF) staining (NG2, CD90, CD105, CD44, CD45, CD73, CD146, CD31, and PDGFR-beta). Endothelial cells (HUVEC) or PC were seeded into flow chambers and subjected to laminar flow at low 10 dyn, high 30 dyn and no shear stress rates for 48 h ($n = 3$/group). RNA was extracted and analysed by qPCR for tissue inhibitor of metalloproteinase 3, versican