preclinical and clinical studies of cilostazol on the other ischemic situations such as myocardial infarction will be justified.

P2196 Impact of erythroblasts in bone marrow cells on limb salvage after cell implantation in patients with critical limb ischemia

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Objective: Therapeutic angiogenesis with bone marrow mononuclear cells (BMCs) has recently been developed as a less invasive intervention for patients with chronic critical limb ischemia (CLI). There have been no earlier findings, however, on which factors affect the long-term outcome after BMC implantation (BMI). The aim of this study is to identify which factors influence limb salvage after BMI.
Methods: Fifteen no-option CLI patients treated with BMI were enrolled in the present study. Limb ischemia was assessed with the use of the ankle-brachial index (ABI), transcutaneous oxygen tension (TcO2), and rest pain score. The cell populations among the implanted cells were determined by May-Giemsa staining and flow cytometry.
Results: The limb salvage rate after BMI was approximately 53% (n= 7 in the amputation group and n= 8 in the salvage group). There were no significant differences between the groups in clinical characteristics, or in the ABI, TcO2 level, or rest pain score before implantation. The number of implanted BMCs was the same in the two groups. In the cytological studies, the percentages of erythroblasts and neutrophils in the salvage group were significantly higher and significantly lower, respectively, than those in the amputation group (p= 0.02, p= 0.03, respectively). There were no significant differences, however, in the percentages of myeloblasts, myelocytes, monocyte, or lymphocytes. The calculated erythroblast count was significantly higher in the salvage group than in the amputation group (p= 0.03), and the number of CD34-positive cells was somewhat greater in the salvage group than in the amputation group (p= 0.06). Logistic regression analysis revealed that the percentage of erythroblasts was significantly associated with limb salvage (95%CI 0.00-0.79, p= 0.03). In vitro angiogenesis assay demonstrated that CD235a (erythroid marker)-positive cells from BMCs significantly promote endothelial proliferation compared with the CD235a-negative cells ($p< 0.05$).
Conclusions: The cellular composition of the BMCs injected into the ischemic limbs may contribute more to long-term limb outcome after the implantation than the severity of limb ischemia or background factors. The favorable effects of BMI appear to reflect the impact of the erythroblast doses.

P2197 The initial down-regulation of collateral shear force allows perivascular macrophage accumulation and enhances collateral proliferation

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Purpose: Elevated shear force (SF) and increased perivascular macrophage accumulation are believed to be hallmarks of collateral growth (arteriogenesis). NOS expressions and activations are SF dependent. Endothelial monocyte/macrophage adhesion, however, is counteracted by increased NO availability and has been observed under low SF conditions.
Methods: In order to resolve this paradox we first investigated the time course of SF and NOS expressions in growing rat collateral vessels after femoral artery occlusion. Secondly we examined the interdependency of SF, macrophage recruitment and collateral proliferation 1) after increasing collateral blood flow using peripheral nitroglycerin (GTN) infusions and 2) after enhancing macrophage recruitment under NO depletion (oral L-NAME).
Results: (values are given as mean ± SEM, * $p<0.05$): SF was significantly down-regulated post occlusion (SF in dyn/cm^2: pre- 20±2.5 vs. post-occlusion 14±3.7*; n=10) correlating to reduced iNOS and eNOS expression (12 h after occlusion). Acute peripheral application of GTN led to a rise of collateral SF to pre-occlusion levels (SF in dyn/cm^2: pre- 20±2.5 vs. post-occlusion + GTN 22±2.8; n=10). Ongoing low SF conditions (continuous peripheral GTN infusion) reduced collateral macrophage recruitment (macrophages per collateral section: post- 42.5±4.4 vs. post-occlusion + GTN 26.3±1.9*; n=10) and diminished collateral proliferation (proliferative index: post- 0.54±0.04 vs. post-occlusion + GTN 0.19±0.08*; n=10) 3 days after occlusion. Chronic NO depletion led to a significant increase in pericollateral macrophage amounts (macrophages per collateral section: post- 118.9±7.3 vs. post-occlusion + L-NAME 164.6±14.7*; n=19) but not in proliferation (proliferative index: post- 0.6±0.06 vs. post-occlusion + L-NAME 0.59±0.08; n=19) 7 days after occlusion.
Conclusions: Based on these results we propose following resolution of the "Monocyte/NO Paradox": An initial phase characterized by low SF conditions allows the recruitment of circulating cells that are locally activated during a second phase of elevated hemodynamic forces.

P2198 Fibrin improves human peripheral blood endothelial progenitor cells stemness and paracrine function

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Purpose: Fibrin is a natural biopolymer appealing for cell-based regenerative therapies, supporting growth, migration and differentiation of several cell types. Endothelial progenitor cells (EPC) can be easily isolated from peripheral blood, eliminating donor morbidity and used to promote in vivo angiogenesis. Aim of this study was to investigate if fibrin is a good alternative to traditional matrices for EPC growth and function.
Methods: Fibrin was obtained from fibrinogen (9 mg/ml) and thrombin (25 U/ml). Ultrastructure was investigated by scanning electron microscopy (SEM), cryogenic SEM (CRYO-SEM) and atomic force microscopy (AFM). EPC were obtained from peripheral blood and cultured on fibrin (1×10^6 cell/cm^2) for 7-14 days. Fibronectin was used as a control. Metabolic activity was assessed by WST1 assay and viability by confocal microscopy (calcein incorporation). The expression of endothelial (CD31, KDR, vWF, Ve-Cadherin) and embryonic stem cell markers (nanog, oct-4) was assessed by flow cytometry, confocal microscopy and Real Time RT-PCR. For NANOG gene oligos unable to recognize the sequences encoded by pseudogenes were used. Angiogenesis was assessed on matrigel by incorporation of EPC into HUVEC tubules. Finally, the release of 50 cytokines was evaluated by a multiplexable bead system.
Results: SEM and AFM revealed a nanometric fibrous structure, with mean fiber diameter of 165±4 nm and mean density of 95.9±0.2%, while CRYO-SEM showed micropores of different size (10-100 μm). WST1 assay showed an increased metabolic activity of EPC cultured on fibrin as compared to fibronectin (fibrin: 0.519±0.06 a.u. vs. fibronectin: 0.243±0.06, n=5, $p\leq0.01$), up to 14 days. Flow cytometry showed no difference on the expression of endothelial markers (CD31=24±9%; vWF=28±11%; KDR=57±20%; VE-Cadherin=24±7%) as compared to fibronectin. Interestingly the culture on fibrin elicited a marked induction of Oct 4 and Nanog mRNA levels, being 5.5±1.3 and 20.5±3.1 fold enriched on fibrin than fibronectin, $p<0.005$. Angiogenesis assay revealed no significant difference between EPC grown on fibrin or fibronectin. Finally, a significative release of the following cytokines: IP-10, PDGF-bb, IL-8, IL-16, MIG, MIF, SDF-1 α, GRO-α, MCP-1, M-CSF and HGF was detected only from EPC grown on fibrin.
Conclusions: Fibrin is a suitable scaffold for EPC growth, viability and differentiation. The paracrine release of cytokines involved in cell recruitment suggests that EPC grown on fibrin might accelerate blood vessel formation. The stemness more expressed by EPC grown on fibrin adds a surplus value to EPC-based therapies.

P2199 Composite scaffolds for a controlled delivery of bioactive pro-angiogenetic growth factors

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Purpose: The aim of this study was to develop a novel composite scaffold that, combining good mechanical properties with a controlled and sustained release of bioactive pro-angiogenetic growth factors, should be useful for regenerative medicine applications in which a significant tissue distensibility is necessary, such as myocardial infarction.
Methods: The scaffold, constituted by a synthetic biocompatible material, the polyetherurethane-polydimethylsiloxane (PEtU-PDMS), and a biological polymer, the fibrin, was fabricated by spray phase inversion in an original way. In brief, the thrombin solution was sprayed simultaneously to the PEtU-PDMS solution and then incubated overnight at 37°C with the fibrinogen solution at 10 or 20mg/ml, to reach a deep permeation of fibrin into wall thickness. During the fibrin polymerization vascular endothelial growth factors-165 (VEGF165), basic fibroblast growth factors (bFGF), and 5 or 10μg of heparin were incorporated in the fibrin layer. Structural-mechanical properties of scaffolds and the effect of fibrinogen and heparin concentration on growth factors release were evaluated. The in vitro VEGF and bFGF bioactivity was assessed using HUVEC culture. Finally, mRNA expression of IL-8, L-SEL, LFA-1 and iNOS in human monocytes was measured to determine the immune response induced by scaffolds.
Results: Morphological analysis of scaffolds surface showed an homogeneus fibrin layer, constituted by a network of randomly oriented nanofibers, firmly adherent onto the synthetic material. Tensile tests highlighted isotropy, handling and elasticity of the scaffolds. The rate of growth factors release from scaffolds was controlled by the fibrinogen concentration (20mg/ml of fibrinogen determined the slowest release rate), whereas it was not affected by heparin concentration; in addition, bFGF was retained for a longer time than VEGF and thus delivered more slowly. The biological activity of the released growth factors was maintained. Finally, scaffolds induced a slight immune response in vitro as showed by low mRNA expression levels of inflammatory markers.
Conclusion: The results of the present work suggest that the new developed composite scaffold once implanted, providing a co-localization and temporal dis-

tribution of bioactive VEGF and bFGF in addition to handling and elasticity, may be able to stimulate new vessels formation in the target tissue. Implants of composite scaffolds in ischemic hindlimb and in the dorsal subcutaneous tissue of Wistar rats are under investigation to assess their potential to induce angiogenesis.

P2200 The impact of erythropoietin on local balance of angiopoietins and VEGF in a murine model of hind-limb ischemia

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Purpose: Angiopoietin (Ang) -1 and -2, their receptor Tie-2, and vascular endothelial growth factor (VEGF) regulate angiogenesis and may be important in myocardial collateral development. Ang-2 and VEGF act synergistically to produce a stable and functional microvasculature while Ang-1 can also be antiangiogenic, offsetting VEGF-induced angiogenesis. We investigated whether erythropoietin (EPO) alters the local balance of the angiopoietins and VEGF in a murine model of hind limb ischemia.
Methods: Wild type C57BL/6 male mice were anesthetized and underwent surgically induced unilateral hind-limb ischemia with ligation and excision of the left femoral artery. Mice were divided in a randomised blinded manner in two groups and received either EPO (400IU/kg for 5 days in 0.2ml solution, IM) or normal saline (0.2ml for 5 days, IM). At day 28 they were sacrificed and muscle tissues from the both limbs were snap frozen in liquid N2 for RNA extraction. Mice underwent laser Doppler perfusion imaging after surgery on days 1, 7 and 28 for the estimation of the bilateral hind-limb perfusion. Quantitative real time RT-PCR was performed to analyze the differential gene expression between these two models of several angiogenic factors such as VEGF, Ang-1 and Ang-2.
Results: There was no significant difference in the expression of Ang-1 and VEGF between the ischemic (13.2±1.0 and 8.4±0.6 RLU) and non-ischemic (13.4±2.39 and 8.0±0.8 RLU, p=NS for both) limb of control animals. However, the ischemic limb expressed significantly lower Ang-2 (9.1±1.5 RLU) compared to the non-ischemic limb (11.6±1.5RLU, p=0.004) in the control animals. On the contrary, EPO induced a significant elevation of VEGF expression in the non-ischemic limb (10.4±1.0 RLU) compared to the ischemic limb (7.6±1.1 RLU, p=0.008). Importantly, erythropoietin prevented the elevation of Ang-2 in the non-ischemic limb (6.0±1.9RLU) compared to the ischemic (7.6±3.1RLU, p=NS) limb. The expression of Ang-1 was still not significantly different between the two limbs in the erythropoietin-treated animals (12.5±2.3 RLU in ischemic vs 11.3±1.0 RLU in the non-ischemic limb).
Conclusion: Erythropoietin treatment increases VEGF and decreases Ang-2 expression at the non-ischaemic limb in animals with unilateral limb ischaemia. This finding suggests that erythropoietin may play a critical role in neoangiogenesis by interfering in the mechanisms regulating remote post-conditioning.

P2201 Development of a 3D nanostructured scaffold with angiogenic potential in cardiovascular applications

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Purpose: Medical devices realized with a biocompatible polymer, the poly(ether)urethane-polydimetilsiloxane (PEtU-PDMS), may have important cardiovascular applications such as vascular prostheses or cardiac patches. Fibrin is an optimal matrix to promote the in situ release and maintaining of cells widely used in tissue engineering. Endothelial progenitor cells (EPC) are bone marrow cells able to contribute to the vascular repair. Aim of this work was to realize a nanocomposite 3D scaffold composed by PEtU-PDMS, coated with fibrin, able to support EPC growth and differentiation and to promote in vivo angiogenesis.
Methods: Scaffolds were fabricated by spray-phase inversion technique (Advanced Spray Machine Technology). Surface morphology was analysed by stereo-microscopy (Ponceau Red staining) and scanning electron microscopy (SEM). EPC obtained from peripheral blood of healthy donors were cultured on scaffold (1x106 cell/cm^2) in endothelial culture medium containing 5% FBS and specific growth factors. Fibronectin coating was used as control. Cell viability (Calcein-AM incorporation) and endothelial markers expression was assessed by confocal microscopy. VEGF and bFGF release was evaluated by Elisa assay. Four types of scaffolds (A: PEtU-PDMS, B: PEtU-PDMS and fibrin, C: PEtU-PDMS, fibrin and growth factors, D: PEtU-PDMS, fibrin and EPC) were implanted subcutaneously in the dorsal right and left side (angiogenesis model) or in the unilateral hindlimb (ischemia model) of female nude rats for up 14 days. In vivo neo-angiogenesis was evaluated by histology and immunohistochemistry (CD31 staining) and by Laser Doppler imaging.
Results: Pounceau staining showed that fibrin coating was homogeneous and tightly bound to the synthetic polymer surface. SEM showed a well organized layer of fibrin fibres in a nanometric scale (mean diameter ~ 140 nm). EPC viability and the endothelial markers expression was as high as on fibronectin. VEGF and bFGF release was maintained until 14 days. The histological analysis of implanted scaffolds revealed a well organized network of neovessels around the scaffolds C and D as compared with controls (A and B). In the hindlimb ischemic model Laser Doppler blood perfusion was significantly higher with scaffold C implantation.
Conclusions: The spray technology can realize a nanostructured 3D scaffold made of a biocompatible polymer and fibrin as matrix to allows EPC adhesion and differentiation. This new biodegradable support has the potential of an angiogenic "sticker" able to promote neo vessels formation in vivo.

P2202 In vivo characterization of the angiogenic properties of T-cadherin

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Purpose: T-cadherin (T-cad) is an atypical GPI-anchored member of the cadherin superfamily which is upregulated in endothelial cells of vasa vasorum in atherosclerotic lesions and in endothelial cells of tumor-derived blood vessels. Pro-angiogenic properties for T-cad have been demonstrated in vitro using the endothelial cell spheroid model and the Nicosia heart model. Myoblast-mediated delivery of soluble T-cad to mouse skeletal muscle in vivo was shown to facilitate VEGF-induced angiogenesis, and T-cad gene ablation in a mouse mammary tumor model was shown to limit tumor angiogenesis. The effects of T-cad on angiogenesis in vivo remain poorly characterized. In this study we aim to exploit two in vivo models to further investigate and characterize the angiogenic potency of T-cad expressed on endothelial cells.
Methods: We have generated an array of lentiviral vectors to overexpress native T-cad protein, to express different domain-deletion mutants of T-cad protein, and to downregulate T-cad protein. The first in vivo angiogenesis model constitutes a human vasculature in mice engineered by implanting primary human endothelial cells as spheroids embedded within a matrigel-fibrin matrix. In this model endothelial cells are transduced with lentivirii prior to preparation of spheroids. The second in vivo model is the shell-less chick embryo chorioallantoic membrane and here the vasculature is directly infected with lentivirii.
Results: All engineered lentiviral vectors have been tested for efficient and reproducible modulation of T-cad protein in primary endothelial cells (EC's) and a variety of cell lines (endothelial cells, keratinocytes, squamous cell carcinoma cells, melanoma cells). Spheroids composed of T-cad overexpressing EC's or T-cad-silenced EC's (and corresponding control EC's) have been implanted into mice and the relevance of T-cad to building a new vasculature in mice is under analysis. Spheroids composed of EC's transduced with domain-deletion mutants of T-cad have been analysed for sprout outgrowth in vitro. Domains relevant to the ability of T-cad to either stimulate angiogenesis or inhibit angiogenesis in a dominant negative manner) have been identified and investigations on their relevance to vessel development in vivo are under way.
Conclusion: Different in vivo models are being successfully used to characterize the angiogenic impact of T-cad expressed on endothelial cells. This underestimated molecule might be of use as a potential future target for several therapeutic approaches, e.g. during tumor angiogenesis.

P2203 Gene therapy with AdPDGF-C and -D induces proliferation of fibroblasts and impairs cardiac function

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Platelet derived growth factors (PDGFs) are a family of proteins that regulate pericyte proliferation and stabilisation of vessels. Recently two new members of the family PDGF-C and PDGF-D were identified and reported to have angiogenic potential. Thus, they might be useful in revascularisation of ischemic tissues with gene therapy.
We have created adenoviruses encoding PDGF-C and PDGF-D, and tested the angiogenic potential of these growth factors in rabbit skeletal muscle and mouse myocardium. An AdLacZ marker-gene was used as a control. An ischemia model consisting of the ligation of the superficial femoral artery was used in rabbit hindlimb. Closed-chest, trans-thoracic myocardial injections were used in mice. High resolution CPS-ultrasound was used to evaluate changes in blood flow non-invasively in both models. High frequency ultrasound was used to quantify ejection fraction and other cardiac measures in mice. Histology was used in both models for the assessment of microvascular changes.
AdPDGF-C and PDGF-D were found to induce proliferation of fibroblasts and inflammatory cells in the rabbit hindlimb six days after gene transfer. In mice myocardium a similar expression of the growth factors was found to impair cardiac function, such as ejection faction and fractional shortening. Additionally, an increase in the left-ventricular inner-volume was detected implicating possible cardiac insufficiency. The angiogenic changes induced by the growth factors in either model were quite modest compared to the fibrotic and inflammatory changes and functional defects.
In conclusion, PDGF-C and PDGF-D have angiogenic potential in some models but hinder the function of target tissues after gene therapy in mice and rabbits. In