26 Identification of CMTM3 as a new pro-angiogenic factor essential for vessel stabilization

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Introduction: Interactions between vascular endothelial cells (ECs) and perivascular cells (like vascular smooth muscle cells and pericytes) are essential in the regulation of vascular remodelling and stabilization. Defects in the molecular signalling during ECs-pericytes interaction can lead to deregulated growth of the vasculature which subsequently contribute to cardiovascular disease (CVD).

Purpose: Therefore we focus on unearthing molecular pathways which are involved in ECs-pericytes interactions.

Methods and Results: Based on a genome-wide microarray screen of human umbilical vein endothelial cells (HUVECs) co-cultured with pericytes, we identified CKLF-like MARVEL transmembrane domain containing 3 (CMTM3) as a new target gene involved in ECs-pericytes interactions. In coculture CMTM3 was significantly upregulated in HUVECs compared to single cultured HUVECs. To evaluate the function of CMTM3 in in vitro neo-vascular formation and stabilization, a 3D collagen-based co-culture of HUVECs and pericytes was conducted. In this co-culture model, knockdown of CMTM3 in HUVECs with short interference RNA (siCMTM3) significantly reduced endothelial tubule and junction formation. In vivo, morpholino-based silencing of CMTM3 in zebrafish larvae led to defects in sprouting of intersegmental vessels from the dorsal aorta. To assess whether the reduced vascular growth in vitro and in vivo is due to defects in migration of the sprouting ECs, a transwell migration assay was performed on HUVECs. A dramatic decrease in migration of HUVECs was observed in the siCMTM3 treated group compared to control conditions. Furthermore, using immunohistochemistry and western blot we assessed the cell-cell adherence junctions in HUVECs, in which CMTM3 was suppressed with siRNA or overexpressed with adenovirus (adCMTM3). VE-cadherin expression was decreased in the siCMTM3 HUVECs and increased in the adCMTM3 treated HUVECs. Interestingly, VE-cadherin was accumulating in the cytoplasm of adCMTM3 treated HUVECs. Moreover intracellular staining showed a co-localization of CMTM3 with VE-cadherin and the early endosomes markers. Clathrin and Early Endosome Antigen 1.

Conclusion: These findings indicate that CMTM3, which is upregulated in ECs after direct contact with pericytes, plays a pro-angiogenic role both in vitro and in vivo, potentially due to its involvement in endothelial migration. Furthermore, CMTM3 is involved in VE-cadherin synthesis and distribution, a key molecule in vascular stabilization, involved in the regulation of ECs barrier function.

27 Regulation of pulmonary vascular PW1+ progenitor cells recruitment during early chronic hypoxia-induced vessel neomuralization

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Background: Resident pulmonary PW1+/CD34+/+ progenitor cells were identified in human and mouse lung tissue and were shown to differentiate into VSMC in vitro and in vivo. They are recruited to participate in the vascular remodelling during chronic hypoxia (CH)-induced pulmonary hypertension.

Aim: We studied the factors involved in the regulation of pulmonary progenitor cells proliferation and differentiation during CH.

Methods: PW1+/CD34+/+ cells were co-cultured with b-galactosidase as a reporter gene for PW1 expression allowing to follow the lineage of PW1+ cells. These mice were exposed to CH to induce PH and lung vessels neomuralization. Pulmonary PW1+ progenitor cells were measured by flow cytometry.

Results: CH for 4 days induced formation of new VSMC and arteriole neomuscularization. The number of PW1+ /CD34+/+ progenitor cells was increased (p<0.03 vs normoxia, p<0.001). In small pulmonary arteries, the proportion of b-Gal+/VSMC derived from PW1+ cells was also increased after 4 days of CH (4% vs 35% in normoxia, p=0.05). Using a recruitment and differentiation of PW1+ cells into lung VSMC. CXC4R4 inhibition using AMD3100 during CH did not modify their proliferation but completely prevented their differentiation into b-Gal+ and VSMC (p<0.05). Depletion of lung alveolar macrophages using clonodeper liposomes during CH completely prevented their proliferation (p<0.05) and their differentiation into b-Gal+ and VSMC (p<0.05).

Conclusion: These results show that the proliferation of lung resident PW1+ progenitor cells during CH is dependent on lung inflammation and that their differentiation into new VSMC is dependent on the CXCR4/SDF-1 pathway. Our results highlight new regulatory mechanisms of pulmonary vessels remodeling.

28 Impaired interleukin-10 production in response to CpG and depletion of the regulatory CD19+CD24hiCD38hi B cell compartment in patients with coronary atherosclerosis

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Background/Introduction: B-lymphocytes have critical roles in the inflammatory process that drives atherosclerosis. In animal models, conventional B2 B cells promote atherosclerosis, whilst innate B1a B cells are protective. B cells with regulatory function (Bregs) have been identified in animals and humans and have been implicated in the pathogenesis of autoimmune. Whether Bregs have a role in human atherosclerosis is currently unknown.

Purpose: Our aim was to characterise the frequency, phenotype and function of Bregs in atherosclerosis patients.

Methods: CD19+CD24hiCD38hi Bregs were quantified in patients with atherosclerosis (myocardial infarction (MI), n=60; stable angina, SA, n=40), and in healthy subjects (n=30) using flow cytometry. Interleukin-10 (IL-10) production was quantified by intracellular staining.

Results: The percentage and absolute number of circulating Bregs were markedly reduced in MI and SA patients compared to healthy subjects. No differences were noted in total, mature or memory B cells, suggesting a specific depletion of the B cell subset. Bregs from MI and SA patients produced significantly less IL-10 in response to CpG but not CD40L compared to Bregs from healthy subjects. IL-10 production by mature and memory B cells was not impaired. Molecular mechanisms that underlie defects in Bregs in patients with atherosclerosis are being characterised.

Conclusions: Our data show for the first time that patients with atherosclerosis harbour marked numerical and functional defects in Breg cells that may tip the balance in favour of pro-inflammatory B and T lymphocytes. A better understanding of these defects may reveal novel targets for therapies to tackle inflammation in atherosclerosis.

29 Inflammatory effects of serum amyloid A via TLR2 and TLR4 in vascular cells

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Background and purpose: During inflammatory conditions, the plasma levels of serum amyloid A (SAA) increases, e.g., in patients with chronic renal failure (CRF). As apolipoprotein, SAA is mainly
osteoblast-like cells as occurs during physiological osteogenesis. Mineralization in vascular calcification may exert regulatory effects on VSMC differentiation into osteoblast commitment.

Results: SAA plasma concentration increases in patients during conditions of CRF in a stage-dependent manner. SAA dose-dependently increase MCP-1 expression and secretion in VSMC as well as THP-1 and RAW264.7 cells. In addition, IL-6 secretion increases in a dose-dependent manner in macrophage-like cells (THP-1, RAW264.7). Both, MCP-1 and IL-6 secretion induced by SAA were regulated via TLR activation. The TLR2/4 antagonist oxPAPC significantly diminished the SAA-induced MCP-1 and IL-6 production. Stimulation with TLR2/4 agonists (HKS, LPS) confirmed these findings. The agonists/antagonists had no significant influence on cell viability in the concentration used for the experiments.

Conclusion: The finding reveal that the pro-inflammatory reaction of SAA in macrophages in vitro depends on TLR2/4 activation. The accumulation of SAA plasma levels during CRF may substantially contribute to the increased cardiovascular risk of these patients.

30 Collagen cross-linking enzymes are involved in vascular smooth muscle cells calcification
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Background: Vascular calcification shares several commonalities with osteogenic processes including osteoblast-like cell differentiation of vascular smooth muscle cells (VSMC) and extracellular matrix (ECM) synthesis and mineralization. Intracellular and extracellular hydroxylase enzymes, such as PLOD1 and LOX1, render collagen enriched-ECM maturation and stabilization. We aimed to assess the involvement of those enzymes in hyperphosphatemia-dependent calcification in vitro.

Methods: Hyperphosphatemia (HPM) was used to induce active differentiation of VSMC onto function- al osteoblast-like cells as demonstrated by extracellular matrix mineralization and osteoblast markers expression. The experiments were carried out in primary cultured mouse and human VSMC. Two lines of mouse VSMC (mVSMC) were used: wild-type mVSMC and transgenic mVSMC over-expressing human receptor LOK1. Well-known inhibitors for LOX and PLOD1 activity, including glyoxalaminopiridinol (BAPN) and 2,2'-dipyridil, respectively, were used in combination with HPM media.

Results: The over-expression of LOX was associated with a significant increase of ECM mineralization and osteoblast markers expression in mVSMC. Thereby, using BAPN on hVSMC leded to a significant reduction of ECM mineralization, osteoblast-related gene expression, and lowered type-altered phenotype transformation as demonstrated by SM22α and osteocalcin levels. BAPN was also associated with lower levels of soluble collagen compared to HPM. Using 2,2'-dipyridil on mVSMC or hVSMC was associated with lowered ECM mineralization and regulates the synthesis of collagen and osteoblast commitment.

Conclusions: Our findings arise the importance of ECM regulation during vascular calcification. ECM mineralization in vascular calcification may exert regulatory effects on VSMC differentiation into osteoblast-like cells as occurs during physiological osteogenesis.

31 miR-504 inhibits venous smooth muscle cell proliferation and migration by targeting LAMTOR1
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Background and Purpose: Venous graft failure resulting from luminal narrowing and occlusion after coronary artery bypass grafting is a major clinical problem. Aberrant vascular smooth muscle cell proliferation and migration play an essential role in neointimal formation. MicroRNAs have been identified as key regulators of vascular biology. The miR-504 is involved in proliferation of several non-vascular cell types. However, the role of miR-504 in vascular calcification is currently unknown. In this study, we intended to investigate the role of miR-504 on venous smooth muscle cell proliferation and migration.

Methods and Results: The left jugular vein of male SD rats was harvested and then interposed in the carotid artery. In vivo studies demonstrated that miR-504 was down-regulated in graft vein, while LAMTOR1 was up-regulated. Using primary venous SMC, we found that miR-504 expression decrease could result in a significant increase of venous SMC proliferation. We further demonstrated that the expression of LAMTOR1 was attenuated through SM-specific gene such as smooth muscle a-actin (ACTA2) and smooth muscle myosin heavy chain (MYH11) but promoted SMC proliferation and migration. Conversely, over-expression of miR-504 promoted SM contractile gene expression while attenuating SMC migration and proliferation. We further demonstrated that miR-504 specifically suppresses LAMTOR1 at protein level. By luciferase reporter assay, we validated the LAMTOR1 as a direct target of miR-504 in venous SMC. Additionally, Gain-of-function studies showed that over- expression of LAMTOR1 promoted VSMC proliferation and migration, whereas the opposite effect was obtained with the in vitro inhibition of LAMTOR1.

Conclusions: This study demonstrates that miR-504 is crucial for venous SMC proliferation and migra- tion via modulating the LAMTOR1 or miR-504 can be potent anti-proliferative agent for vascular dis- eases such as neointima formation and restenosis after vein graft.

32 Diaphanous related form 2 (DRF2) is essential for KLF2-induced resistance of endothelial cells to flow forces.
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Introduction: Athero-protective effects of laminar blood flow on endothelial cells are predominantly mediated by the transcription factor Krippe-Like Factor 2 (KLF2). Previously we have shown that shear stress exposure as well as KLF2 expression induces actin shear fiber structures that contribute to anti-inflammatory gene expression. Purpose: We searched for KLF2-downstream effectors essential in formation of shear fibers and assessed the functional (mechanical) consequences of shear-induced cytokinetic reorganization. Methods and Results: Using a gene silencing approach we identify DRF2 as specific diaphanous related form 2 essential for KLF2-dependent shear fiber formation in endothelial cells. Traction force mapping and motility assays of shear fiber expressing endothelial cells reveal a KLF2-dependent, but DRF2-independent reduction in both traction force and motility. However, DRF2 is essential for a significant (p < 0.01) 4-fold increase in surface area of individual focal contacts observed in shear-fiber expressing cells. Prolonged (4 days) in vitro exposure of DRF2-silenced endothelial cells to laminar shear stress (15 dyn/cm²) shows a dispersion of these strong matrix contacts in the absence of shear fibers, leading to a marked decline in cellular mecha- nical resistance to the forces imposed by laminar flow, resulting in cell erosion. Thus, in endothelial cells, DRF2 activity is essential for shear fiber formation and promotes focal adhesion maturation to a larger and mechanically more resistant type. Conclusions: We identify DRF2 as novel protein crucially involved in shear fiber formation to sta- bilize the strong cell-matrix adhesion by pronounced large, KLF2-induced focal adhesion plaques, ne- cessary for endothelial cells to withstand the traction force of arterial levels of shear stress.

33 Inhibition of TGFβ axis and renin-angiotensin system in human ascending aorta aneurysms
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Aneurysms of the ascending aorta are usually noninflammatory and characterised by dilation of the artery, with loss of extracellular components and smooth muscle cell (SMC) apoptosis as a result of the action of metalloproteases. We recently demonstrated that the TGF-β/SMAD-2 pathway, that modulates the processes of tissue synthesis and repair, is inhibited. There are evidences indicating that the linkage of caveolin-1 with TGF-β receptor is involved such inhibition. Another point to take into account is that most patients have systemic arterial hypertension – thus, it is expected that the renin-angiotensin system (RAS) may contribute to the lesion by actions on the SMC. The RAS shares intracellular signaling ligands with elements of the TGF-β axis, and may trigger intracellular events via the angiotensin II receptor 1 (AT1). The action of RAS associated to the inhibition of the TGF-β pathway may be pivotal events in the pathogenesis of the aneurysms. The objective of the present study is to evaluate the presence of caveolin-1, angiotensin converting enzyme (ACE), and AT1 in the medial layer of human ascending aorta aneurysms. Samples of ascending aortas from hypertensive patients with aneurysms submitted to surgical correlation (n=10) and, as controls, from normotensive patients submitted to coronary artery bypass surgery (n=10) underwent standard histological...
preparation and immunohistochemical reactions to caveolin-1, ACE, and AT1. Medial positive immuno-
staining was quantified by using a image analysis system coupled to a light microscope. Groups were
compared by t or Mann-Whitney tests; differences with $p \leq 0.05$ were taken as significant. All three
components were increased in patients with aneurysms: means of 14.17%, 3.76%, and 7.77% (medians
13.54%, 3.60%, and 7.29%) respectively for caveolin-1, ACE, and AT1 in aneurysms; and 5.47%, 1.77%,
and 2.61% (medians 5.24%, 1.37%, and 2.55%) in controls, with $p < 0.01$ for all analyses. The expres-
sive increase of caveolin-1 at the medial layer in cases of aneurysms reinforces the hypothesis of the
inhibition of the TGF-β axis in this disease, considering that TGF-β receptor can bind this molecule,
thus impairing the processes of synthesis and tissue repair. The increases in ACE and AT1 indicate the
presence of angiotensin and its probable participation in the SMAD-2 signaling cascade throughout a
TGF-β-independent, which would result in an intense activation of the genetic transcription of mole-
cules such as metalloproteases. The inhibition of TGF-β axis plus the action of angiotensin II would
result in weakening and dilatation of the arterial wall, thus explaining the role of hypertension in the
pathogenesis of the aneurysms of ascending aorta.