Atherosclerosis newsletter

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Our Newsletters frequently summarize articles on clinical or population research. In this letter, we provide summaries of basic research articles to remind readers and authors that the Editors of *Atherosclerosis* are very interested in publishing high quality papers on the foundations of subsequent clinical research projects.

Stabilin 1 and 2 are important regulators for cellular uptake of apolipoprotein B-containing lipoproteins in zebrafish

Scavenger receptors form a superfamily of membrane-bound receptors that bind to and internalize different types of ligands, including pro-atherogenic oxidized low-density lipoproteins (oxLDLs). In atherosclerosis, scavenger receptor-mediated uptake of oxidatively modified lipoproteins by endothelial cells and macrophages in the sub-endothelial space of the arterial wall stimulates the progression of disease. *In vitro* studies have indicated a role for the liver sinusoidal endothelial cell receptors stabilin 1 (stab1) and 2 (stab2) in oxLDL clearance. Verwilligen et al. evaluated the potential role of stab1 and stab2 in lipoprotein uptake in zebrafish, a novel animal model for the study of different aspects of atherosclerosis.

Lipoproteins were injected in the duct of Cuvier of wild-type (ABTL) or stab1 and stab2 mutant (*stab1*$^{-/-}$*stab2*$^{-/-}$) zebrafish larvae at 3 days post-fertilization. To examine the effect of stabilin deficiency on lipoprotein and cholesterol metabolism, zebrafish larvae were challenged with a high cholesterol diet (HCD) for 10 days.

Lipoprotein injections showed impaired uptake of LDL and oxLDL into the vessel wall of caudal veins of *stab1*$^{-/-}$*stab2*$^{-/-}$ zebrafish, which was paralleled by redistribution to tissue macrophages. Total body cholesterol levels did not differ between HCD-fed *stab1*$^{-/-}$*stab2*$^{-/-}$ and ABTL zebrafish. However, *stab1*$^{-/-}$*stab2*$^{-/-}$ larvae exhibited 1.4-fold higher mRNA expression levels of low density lipoprotein receptor a (*ldlra*) involved in (modified) LDL uptake, whereas the expression levels of scavenger receptors *scarb1* and *cd36* were significantly decreased.

The results show that stabilins 1 and 2 have an important scavenging function for apolipoprotein B-containing lipoproteins in zebrafish and that combined deficiency of these two

proteins strongly upregulates the clearance of lipoproteins by macrophages within the caudal vein. Zebrafish is a potential good model to study liver sinusoidal endothelial cell function.

Isolation and culture of murine aortic cells and RNA isolation of aortic intima and media: Rapid and optimized approaches for atherosclerosis research

Atherosclerosis is a chronic inflammatory disease characterized by the formation and growth of a lipid-laden plaque within the vasculature. This multifactorial process is characterized by endothelial cell (EC) dysfunction, monocyte recruitment and adhesion, foam cell formation, smooth muscle cell (SMC) migration and remodeling, and extracellular matrix remodeling and fibrosis. Isolation of the cellular constituents of the mouse aorta is commonly used for expression or functional analyses to investigate different aspects of plaque formation in experimental atherosclerosis models. However, research in this area is hampered by the inefficiency of current isolation protocols, often requiring the use of separate mice for each cell type desired. RNA extraction from aortic intima and media for transcriptomic analysis is also considered difficult with mixed RNA yields. To address these gaps, in this study Chen et al. provide a rapid protocol to isolate and culture diverse cell types concomitantly from the mouse aorta using immunomagnetic cell isolation, and an optimized aortic intimal peeling technique for efficient RNA isolation from the intima and media.

Aortic cells were obtained using an enzymatic solution and different cell types were isolated by magnetic beads conjugated to antibodies targeting endothelial cells ($CD31^+$), leukocytes ($CD45^+$), and fibroblast cells (CD90.2+), and smooth muscle cells were isolated by negative selection. This allows the isolation of relatively large numbers of cells (10,000 cells per aorta) in a predictable manner with high purity (>90%) verified by cell-marker gene expression, immunofluorescence, and flow cytometry. These cells are all functionally active when grown in cell culture. The authors also provide a rapid method to collect aortic intima-enriched RNA from *Ldlr*$^{-/-}$ mice using an intima peeling approach and assessing the transcriptomic profiling associated with accelerated lesion formation.

This protocol provides an effective means for magnetic bead-based isolation of different cell types from the mouse aortic wall, and the isolated cells can be used for functional and mechanistic studies for a range of vascular diseases including atherosclerosis.

Nrf2 deficiency attenuates atherosclerosis by reducing LOX-1-mediated proliferation and migration of vascular smooth muscle cells

Oxidative stress and abnormal proliferation and migration of vascular smooth muscle cells (VSMCs) influence atherosclerosis formation and development. Oxidative stress significantly influences the abnormal proliferation and migration of VSMCs, and nuclear factor erythroid 2-related factor 2 (Nrf2) is a major antioxidant factor. However, the precise function of Nrf2 in the regulation of

abnormal proliferation and migration of VSMCs and atherosclerosis is unclear. Li et al. investigated the proliferation and migration of VSMCs in atherosclerosis in male *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Nrf2*$^{-/-}$ mice fed a high-fat diet for 12 weeks. In cultured mouse VSMCs, the effect of Nrf2 on ox-LDL-stimulated proliferation and migration was studies using siRNA treatment to silence *Nrf2*. Dual luciferase reporter and immunoprecipitation assays were performed to study the interaction between Nrf2 and the promoter sequence of lectin-like oxidized low-density lipoprotein receptor-1 (*LOX-1*).

The results demonstrate that Nrf2 expression levels were increased in the aorta and VSMCs of mice in the atherosclerosis model group compared with the control group. Nrf2 deficiency attenuated atherosclerotic plaque burden, diminished proliferation, and migration of VSMCs but enhanced VSMC-specific marker gene expression *in vitro* and *in vivo*. Furthermore, Nrf2 downregulation contributed to restrain both transcriptional and translational activities of *LOX-1*.

This data indicates that Nrf2 insufficiency is linked to attenuation of atherosclerosis, and could diminish the pathological process by blunting LOX-1-mediated proliferation and migration of VSMCs.

Transcriptome analysis revealed a two-step transformation of vascular smooth muscle cells to macrophage-like cells

Vascular smooth muscle cells (VSMC) have been recognized as the biggest contributors to the growth of the atherosclerotic plaque, rupture of which is the etiological reason underneath most cardiovascular diseases such as myocardial infraction, angina and stroke. In addition to forming fibrous cap cells that stabilize the atherosclerotic plaque, VSMCs *trans*-differentiate into macrophage-like cells that exacerbate the necrotic core. Zhang et al. aimed to address the question of how VSMCs are selected to perform distinct functions under a similar environmental stress, and how much cellular reprogramming happens during VSMC-to-macrophage-like transformation.

Cellular reprogramming during VSMC-to-macrophage-like cell transformation was analysed by by single-cell RNA-Sequencing (scRNA-Seq), transcriptional and metabolic studies of *in vitro* models, and examinations of pathological specimen.

VSMC-derived macrophage-like cells were found to promote the expression of lysosome-related and inflammation-related genes. Transcriptional studies further confirmed that suppression of NOTCH signaling was the prerequisite for VSMCs to undergo sufficient genetic and metabolic reprogramming to a macrophage-like state and perform macrophage-like functions, while high-lipid treatment alone only promoted VSMCs into a pro-inflammatory state without gain of lysosome-related functions. Mechanistic studies showed that NOTCH inhibition shifted VSMCs into a de-differentiated state by suppressing the developmental program, including key factor Myocd, leading to complete transformation into macrophage-like cells.

The study shows that NOTCH signaling serves as a brake to prevent VSMCs transformation from a pro-inflammatory state to a macrophage-like state by affecting key VSMC phenotypic regulators. These results add some insights into the mechanism of VSMC-to-macrophage-like cell transformation and pleiotropic functions and related transcriptome profiles of VSMCs during atherosclerosis development.

Blocking the NLRP3 inflammasome reduces osteogenic calcification and M1 macrophage polarization in a mouse model of calcified aortic valve stenosis

Senile calcific aortic valve stenosis (CAVS) is a disease in which degenerative fibrosis, thickening and severe calcification of aortic valve leaflets lead to abnormal valve structure and function. In this study, Lu et al. investigated how NLR family pyrin domain-containing 3 (NLRP3) inhibition with CY-09 (a specific NLRP3 inflammasome inhibitor) reduces aortic valve stenosis and calcification.

ApoE $^{-/-}$ mice were fed a high-fat diet for 24 weeks with or without intraperitoneal injection of 2.5 mg/kg/day NLRP3 inhibitor CY-09 for 42 consecutive days, while the control group mice were fed a normal diet. The valve function was monitored by echocardiography; calcified nodules were assessed by Von Kossa staining; and calcification-related molecules, inflammatory factors, and white leucocyte influx into the valve were assessed by immunohistochemistry, TUNEL assay, and PCR.

Mice treated with CY-09 exhibited improved aortic valve function and reduced valve calcification deposition. CY-09 intervention significantly downregulated the elevated expression of the NLRP3 inflammasome pathway molecules NLRP3, caspase-1, and interleukin 1β (IL-1β) and the osteogenic calcification markers RUNX family transcription factor 2 (RUNX2), secreted protein acidic and cysteine rich (SPARC), and bone morphogenetic protein 2 (BMP2) in stenotic valves while the number of apoptotic cells and dystrophic calcification markers cadherin 11 (CDH11) and smooth muscle alpha-actin (αSMA) did not change significantly. Inhibition of NLRP3 activity also reduced the ratio of M1/M2 macrophages, prevented the shift of macrophages towards the M1 phenotype, and downregulated the levels of the proinflammatory factors interleukin 6 (IL-6) and tumor necrosis factor (TNF)-α.

Blocking NLRP3 might be a therapeutic target for cardiovascular calcification.