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Atheroprotective role of Caveolin-1 and eNOS in an innovative transplantation model is mainly mediated by local effects

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ABSTRACT

Endothelial dysfunction is crucial in the initiation of atherosclerosis, which is associated with a lack of nitric oxide. The endothelial NO synthase (eNOS) is responsible for constitutive synthesis of NO and inhibited by caveolin-1 (Cav1). In the current study, we examined the influence on intima formation through single and combined deletion of eNOS and Cav1 with a focus on differentiation of local and systemic effects.

A sex-mismatch transplantation of denudated aortae from female C57BL/6n (WT), Cav1$^{-/-}$, eNOS$^{-/-}$ and Cav1$^{-/-}$/eNOS$^{-/-}$ (C/e$^{--/--}$) mice in common carotid artery of male WT mice was performed. After six weeks on Western-type diet, the aortae were explanted and intimal lesions were quantified by determining the intima-media-ratio (IMR). Significantly larger plaques were observed in all knockout mice compared to WT. The highest IMR was detected in Cav1$^{-/-}$ arteries associated with an increased expression of α-smooth muscle actin (αSMA) and the proliferating cell nuclear antigen (PCNA). Both were reduced in aortae from C/e$^{--/--}$. Galectin-3 (Gal3) immunostaining revealed only small infiltrations of macrophages. Systemic cell invasion was detected by Y chromosome fluorescence in situ hybridization (Y-FISH), which showed only small numbers of systemic cells and no differences between the genotypes.

Loss of Cav1 increased vascular lesion by enhancing neointimal proliferation. The combined loss of Cav1 and eNOS, compared to Cav1$^{-/-}$, lowered intima formation, suggesting an increasing effect of eNOS in the absence of Cav1 on vascular lesion. Furthermore, these effects seem to be mediated by local cells rather than by systemically invaded ones.

1. Introduction

Atherosclerosis is a chronic inflammatory disease, which is substantially influenced by migrating cells [1,2] like monocytes, macrophages, neutrophil granulocytes, dendritic cells as well as T and B lymphocytes [3]. Additionally, the systemic migration of endothelial and smooth muscle progenitor cells has been described [4], but its impact on atherogenesis is controversially discussed [5–7].

Nevertheless, cells derived from the local vessel wall are required for atherosclerosis. Ross and Glomset described neointimal proliferation through vascular smooth muscle cells (VSMC) migrating from the media for the first time [8]. Furthermore, new studies prove the impact of local proliferating macrophages in atherosclerotic plaque [9,10], challenging the theory that these cells are derived from migrated monocytes [11]. Another discovery that strengthens the importance of local influences is the phenotypic plasticity of the migrating VSMC [12], which enables them to transdifferentiate into macrophage-like cells [13,14].

Crucial for the development of vascular lesion sites with enhanced inflammation is the endothelial dysfunction, which is induced by the lack of NO [15], resulting in an increased influx and oxidation of LDL [16,17], an increased adhesion and migration of leukocytes [18] and an increased aggregation of platelets [19]. Furthermore, NO is known for a direct inhibition of neointimal proliferation [20]. Despite that, an evidence for a proatherogenic influence of the single knockout is still missing.

The eNOS is primarily responsible for the constitutive synthesis of NO in endothelium [21] and strongly regulated by a multiplicity of mechanism. Acetylcholine, serotonin or ATP increases activity by an enhanced intracellular calcium concentration or shear stress through protein phosphorylation [22].

Cav1 is a 22 kDa integral membrane protein, which is prevalent in many cells including endothelial cells, macrophages and smooth muscle cells [23]. It is known for structuring caveolae, 50–100 μm large membranous invaginations, which hold a high content of signal molecules and receptors [24]. Thereby, the protein interacts in signal transduction

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of G protein-coupled receptors [25], tyrosine kinase receptors [26], JAK/STAT and Ras/Raf/ERK1/2 pathway [27,28]. Moreover, Cav1 has a caveolin scaffolding domain with high affinity to a conserved motif of eNOS. Interaction of both inhibits constitutive NO synthesis [29], whereas a lack of Cav1 increases it resulting in a direct influence on endothelial dysfunction [30,31]. In contrast, a decreased expression of eNOS is described in epithelial cells of the airway of Cav1$^{-/-}$ mice [32] suggesting a cell-specific regulation of this pathway.

The absence of Cav1 is also known to induce an uncoupling of eNOS, leading to increased formation of reactive oxygen species (ROS) in the heart [33,34]. These oxidative metabolites are able to decrease NO bioavailability, induce expression of endothelial adhesion molecules and increase neointimal proliferation [35]. Furthermore, Cav1 has eNOS independent effects on intima formation, particularly through the p42/p44 mitogen-activated protein kinase, which is important in the growth control of vascular smooth muscle cells [28,36].

However, it is still unknown whether Cav1 or eNOS has an enhancing or a decreasing effect on vascular lesion. In the current study, we examined the *in vivo* influence on intima formation through single and combined deletions of Cav1 and eNOS, using an innovative transplantation model that enabled us to differentiate between systemic and local influences.

2. Material and methods

2.1. Animals and cervical aortic graft transplantation

WT (Charles River Laboratories, Sulzfeld, Germany, hereafter called WT), eNOS$^{-/-}$ [37], Cav1$^{-/-}$ [38] and newly generated Cav1$^{-/-}$/eNOS$^{-/-}$ (C/e$^{--/--}$) were used in the current study. Standard genotyping methods were performed to characterize knockout mice. All experiments were approved by the local animal research ethics committee at the TU Dresden and the Regional Council (Landesdirektion) Dresden and performed in accordance to institutional guidelines and the German animal welfare regulations (AZ 24-9168.11-1/2013-30).

The animals were kept on a 12 h light/dark cycle with access to food and water ad libitum until an average age of 10 weeks at the start of the experiments.

The cervical aortic graft transplantation was combined with a denudation treatment to induce vascular lesion in mice [39,40]. Murine thoracic aortae were denudated using 0.64 mm standardized wire after an anesthesia with Ketamin (100 mg/kg) and Xylazin (2%, 10 mg/kg). Afterwards, donor aortae were rinsed with 100 IE/ml Heparin and transplanted immediately into the common carotid artery (CCA) of narcotized WT mice (Ketamin, 100 mg/kg, Xylazin 2%, 10 mg/kg). A detailed procedure is illustrated in Supplemental Fig. 1. The cuff technique was used for anastomosis, which is shown schematically in Supplemental Fig. 2. The whole surgical procedure was performed under aseptic conditions and with help of a stereo microscope (Leica S6D Stereo Zoom). After operation, all mice were fed with the Western-type diet (ssniff®EF R/M acc. TD88137 mod., crude fat 21.2%, sugar 33.2%), analgized with buprenorphine (subcutaneous, 0.1 mg/kg) and treated with ofloxacin hydrochloride in order to reduce perioperative infection risk.

A time series was conducted through an allogenic transplantation in WT mice. Influences on intima formation of Cav1 and eNOS were investigated by using aortae from eNOS$^{-/-}$, Cav1$^{-/-}$ and C/e$^{--/--}$ for transplantation in WT mice. Intima formation was monitored after six weeks (Fig. 1). All procedures were performed in a sex-mismatch manner that involved transplanting female aortae in male WT mice. Thus, we were able to differentiate between local and systemic influences.

Moreover, denudated thoracic aortae from ten-week-old WT, eNOS$^{-/-}$, Cav1$^{-/-}$ and C/e$^{--/--}$ mice as well as contralateral CCAs of animals, which obtained an aortic graft transplantation, were processed histologically for excluding a preexisting intima formation.

2.2. Tissue processing and histology

After euthanization (pentobarbital, intraperitoneal, 100 mg/kg) and heparinization (intracardiac, 50 IE), transplanted grafts were fixed in 4% formaldehyde (pH 7.0), embedded in paraffin and sliced into 4 μm thick sections according to a defined protocol, shown in Supplemental Fig. 3. To quantify intima formation, sections were stained with EvG and digitalized with a microscope camera (SPOT RT KE, model 7.4 slider; Carl Zeiss AG Axiovert S100 microscope, Supplemental Fig. 4). Using the open source software NIH Image J, the area of the lumen (A1) and the area surrounded by the internal elastic lamina (A2) and external elastic lamina (A3) were determined in the images for calculating the IMR (Supplemental Fig. 5).

2.3. Fluorescence in situ hybridization

To detect the migration of systemic cells to lesion sites, Y chromosomes were stained in mice that received a sex-mismatched aortic graft transplantation by using a IDYE™ conjugated paint probe (Mouse IDetectTM Chromosome Paint Probe Red, ID Labs™ Inc., London, Canada). The procedure was performed according to the manufacturer's instructions and modified after Sin et al. [41]. The specificity was proved by Y-FISH in male as well as female tissue (data not shown).

Afterwards, the sections were digitalized with fluorescence compact microscope (Keyence® BZ-9000 BIOREVO, Keyence Germany GmbH, Neu-Isenburg, Germany) and quantified by determining the ratio of Y chromosome positive cells (Y^+) to all intimal cells (Q_{y+}).

2.4. Immunohistochemistry

VSMCs were identified by staining of αSMA, while monocytes and macrophages were detected through Gal3 staining as described previously [42]. In accordance to the same protocol, a rabbit polyclonal antibody (anti-PCNA, HPA030522, Sigma-Aldrich, Munich, Germany, 1:50) was used for staining of PCNA for detection of intimal proliferative activity. Later, the sections were counterstained with Mayer's hemalum.

Endothelial cells were identified through the von-Willebrand-factor (vWF) using a rabbit polyclonal primary antibody (anti-vWF, A0082, Dako, Hamburg, Germany, 1:200, 1 h). Detection was performed using an anti-rabbit antibody conjugated with indocarbocyanine (Cy3) from goat (Goat IgG anti-Rabbit IgG (H + L)-Cy3, 111-165-144, DIANOVA, Hamburg, Germany, 1:200, 1 h). Cell nuclei were stained with 4′,6-Diamidin-2-phenylindol (DAPI, D9542, Sigma-Aldrich, Munich, Germany, 1:2000).

Purified serum proteins from non-immunized rabbits (ChromPure Proteins, rabbit, Jackson ImmunoResearch Laboratories Inc., West Grove, USA, 1:2000) instead of the primary antibody were used as negative controls. Afterwards, the percentage of positively stained area in the intima was determined by using a RGB color space deconvolution (Supplemental Fig. 6). In case of PCNA staining, the number of positive stained cell nuclei per square millimeter was quantified.

2.5. Statistics

Values are reported as the mean ± standard error of the mean. A one-way analysis of variance (ANOVA) followed by Fisher's least significance post hoc test was performed for comparisons between WT, eNOS$^{-/-}$, Cav1$^{-/-}$ and C/e$^{--/--}$ mice. The level of significance is indicated in the following: * and § $p < 0.05$; ** and §§ $p < 0.01$ and *** and §§§ $p < 0.001$.