The tight junction protein ZO-2 mediates proliferation of vascular smooth muscle cells via regulation of Stat1

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Aims Recent evidence suggests that the zona occludens protein 2 (ZO-2) might have additional cellular functions, beyond regulation of paracellular permeability of epithelial and endothelial cells. Deregulation of ZO-2 in response to ischaemia, hypertensive stress, and vascular injury implies its involvement in cardiovascular disorders, most likely via regulating the functional behaviour of vascular smooth muscle cells (VSMC). However, a role of ZO-2 in VSMC biology has yet to be established. Our study was designed to understand the specific functions of ZO-2 in human VSMC.

Methods and results The expression of ZO-2 and Stat1 upon vascular injury was studied using ex vivo organ culture of coronary arteries combined with immunohistochemistry. ZO-2 silencing in human primary VSMC was achieved by means of lentiviral gene transfer. Cell proliferation was assessed by analysing DNA synthesis and by cell counting. Stat1 expression was examined using immunoblotting, immunocytochemistry, TaqMan, and fluorescence activated cell sorting (FACS) analysis. Functional relevance of Stat1 up-regulation was studied using a Stat1 promoter-luciferase reporter assay and intracellular microinjections of a Stat1 specific antibody. ZO-2 was highly expressed in the media and neointima of dilated but not of control arteries, whereas expression of the transcription factor Stat1 was inversely regulated upon injury. Analysis of VSMC with down-regulated ZO-2 revealed increased expression of Stat1 in these cells, whereas Stat1 phosphorylation was not affected. Stat1 up-regulation in VSMC with ZO-2 silencing resulted in a coordinate activation of Stat1-specific genes and consequently led to inhibition of cell proliferation. This effect was restored by microinjection of a Stat1 neutralising antibody.

Conclusion Our data suggest that the tight junction protein ZO-2 is involved in regulation of VSMC growth control upon vascular injury that is mediated by the transcription factor Stat1. Our findings point to a novel function of ZO-2 in VSMC and implicate ZO-2 as a novel important molecular target in pathological states of vascular remodelling in cardiovascular diseases.

1. Introduction

Vascular remodelling processes in cardiovascular diseases are determined by the activation of vascular smooth muscle cells (VSMC) leading to increased migration and proliferation (reviewed in1). Among multiple factors regulating VSMC behavioural changes, a strong and prolonged up-regulation of the zona occludens protein 2 (ZO-2) in VSMC after injury has been documented.2 However, a functional role for this up-regulation of ZO-2 has not been reported yet.

Differential regulation of ZO-proteins in human breast and pancreatic adenocarcinoma suggests that they play a role in cell transformation and proliferation.3 Patients with heart failure display decreased expression of ZO-1 in their cardiomyocytes.4 In spontaneously hypertensive rats, diminished expression of ZO-2 is observed in endothelia after brain ischaemia.5 In endothelial cells, leucocyte adhesion triggers disorganization of cell-to-cell-adherens junctions, which promotes trans-endothelial migration and vascular inflammation.6 These observations suggest a functional involvement of ZO-proteins in vascular remodelling processes.

The tight junction proteins ZO-1, ZO-2, and ZO-3 belong to the membrane-associated guanylate kinase (MAGUK) family of proteins and share three PDZ, a SH3 and a guanylate kinase-like domain.7 These multiple domains allow them to act as scaffolding molecules that organize multi-molecular signalling complexes at the sites of cell–cell contact, to modulate para-cellular permeability and leucocyte transmigration.8–10 By interaction with transcription factors like Jun, Fos, and C/EBP, they are also able to modify gene

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and participate in cell differentiation and transformation.\textsuperscript{12-14} Whereas ZO-3 is exclusively expressed in epithelia,\textsuperscript{15} ZO-1 and ZO-2 are also found in other cell types, such as fibroblasts, astrocytes, glioma, cardiomyocytes, and VSMC.\textsuperscript{16-18} Although members of ZO-protein family show high sequence conservation, they cannot replace each other functionally.\textsuperscript{19}

Silencing of ZO-2 in epithelial cells resulted in perturbation of tight junction functions by altering expression and/or distribution of occludin and E-cadherin.\textsuperscript{20} Interestingly, in these experimental settings, ZO-2 silencing did not alter the number of cells in the monolayer, proliferation, or the rate of apoptosis among epithelial cells. During mouse blastocyst formation, ZO-2-down-regulation resulted in delayed blastocoel cavity formation with normal cell proliferation and outgrowth morphogenesis.\textsuperscript{21} In contrast, nuclear accumulation of ZO-2 lead to increased epithelial and endothelial proliferation,\textsuperscript{22} whereas overexpression of ZO-2 in MDCK cells diminished cell proliferation via negative regulation of cyclin D1 transcription.\textsuperscript{23} Deletion of ZO-2 in mice is embryo lethality. The ZO-2 deficient embryos died shortly after implantation due to an arrest in early gastrulation and display decreased proliferation and increased apoptosis.\textsuperscript{24}

Although the published results about the influence of ZO-2 on epithelial cell proliferation remain controversial, nothing is known about its impact on VSMC behaviour, beyond the documented up-regulation of ZO-2 gene expression after injury. Therefore, our study was designed to clarify about the role of ZO-2 protein regulation after vascular injury and its functional role in primary human VSMC.

2. Methods

2.1 Organ culture of coronary artery, thin layer frozen section preparation, and immunofluorescence staining

Organ culture of porcine coronary arteries, deep-frozen artery sections, their staining, and fluorescence microscopic studies were performed as described previously.\textsuperscript{25} Briefly, right ventricular coronary arteries were isolated from fresh swine hearts. Vascular injury was performed by dilation with a 2-2.5 mm standard coronary artery balloon catheter under the pressure of 8 a.t.m. for 30 s. The vessels were perfused with Dulbecco modified Eagle medium (Biochrom Berlin) supplemented with 10% foetal calf serum, 5000 IE/mL streptomycin, and 5000 U/mL penicillin, cut into 4-5 segments and cultured for 12 days. Then, coronary artery segments were washed with PBS, deep frozen in liquid nitrogen and stored at −80°C prior to preparation of tissue sections.

ZO-2 and Stat1 polyclonal antibodies (Santa Cruz Biotechnology, Inc.), rabbit IgG (Upstate) for control staining, and DRAQ5 (Biostatus Ltd and oligonucleotides:

ZO2_969S 5'-ACGGTCTTCTCAGGCAGATGCGG-3' \( \text{ZO-2} \text{antisense} \)

ZO2_969A 5'-TATCATTCCTCTCGCACTTGGC-3' \( \text{ZO-2} \text{sense} \)

ZO-2-si or control vector-infected cells were seeded in 96-well plates at a density of 5 × 10^3 cells per well, serum starved overnight, and then incubated in SmGM2 for 16 h. Cell number was determined by luminescence using heosaminidase reagent containing 100 mM sodium citrate buffer, pH 5.0, 0.5% Triton X-100, 15 mM p-nitrophenol-N-acetyl-beta-D-glucosaminide (Sigma).

2.2 Cell culture, plasmid construction, transfection, and lentiviral infection

Human primary VSMC from coronary artery (Lonza, Bio Science Walkersville, Inc.) were grown in SmGM2 medium (Lonza). They were used between passages 4 and 6 and serum starved for 8-12 h prior to the experiments.

pLVTHMΔH-2K+ vector was generated by replacing the sequence GFP by the ΔH-2K sequence from pMACS Kk.II (Miltenyi Biotec GmbH) into pLVTHM (Tronolab, Switzerland). Then, pLVTHMΔH-2K+ was constructed by cloning the shRNA duplex downstream of H1 promoter. pLVTHMΔH-2K+ was generated by ligation of

pLVTHMΔH-2K+ digested MluI and Clal and oligonucleotides: ZO2_969S 5'-CGGTCCCTCCCGGGGATCTCTCAGGGACACttt acgaatctctcctctcccccTTTTTTGGAAAT, ZO2_969A 5'-CGATTCCAAAAat ggggtagccttctctcttgaaagTGCTTCTGAActcctgacagctccccagGGGA. siRNA Selection Program developed at Whitehead Institute was used for the design.\textsuperscript{26} All recombinant lentviruses were produced as described previously.\textsuperscript{27}

2.3 Proliferation analysis and cell count

VSMC were cultured either on glass coverslips for immunostainings or directly in culture dishes for analysis of BrdU-incorporation by fluorescence activated cell sorting (FACS) and were infected with control or ZO-2si lentiviruses. On the third day after infection, cells were serum starved for 8 h and then subjected to proliferation assay as described.\textsuperscript{27} ZO-2-staining was performed with rabbit polyclonal antibody (4 μg/mL, Santa Cruz Biotechnology, Inc.) and anti-rabbit Alexa 568 secondary antibody. For BrdU-detection by FACS analysis, cells were detached using 5 mM EDTA in PBS prior to fix-
3. Results

3.1 ZO-2 is up-regulated in VSMC of porcine coronary arteries upon vascular injury

To assess the expression of ZO-2 upon vascular injury, we used an organ culture model of porcine coronary arteries. Immunohistochemical analysis of frozen vascular sections revealed a marked increase in the expression of ZO-2 in the enlarged medial layer of the vessel wall in dilated arteries. This effect was observed in α-smooth muscle actin-positive cells, whereas no change in ZO-2 expression in dilated arteries was observed in endothelial cells (Figure 1).

3.2 Generation of ZO-2 silencing in VSMC

The deregulation of ZO-2 in VSMC upon vascular injury confirmed the functional importance of ZO-2 in these cells. This evidence prompted us to explore the role of ZO-2 in VSMC. We employed the induction of ZO-2 silencing using a lentiviral construct, which was designed and generated by us for this purpose. Co-expression of the mouse cell surface receptor ΔH-2Kملع (control vector or ZO-2-si-vector-infected VSMC allowed us to achieve equal infection levels in control and ZO-2-si-infected VSMC. The infection rate was > 90% (Figure 2A) and therefore high enough to induce effective down-regulation of ZO-2 (Figure 2B).

3.3 ZO-2 silencing results in inhibition of cell proliferation

Increased proliferation of medial VSMC is one of the hallmarks of negative vascular remodelling. To test whether ZO-2 might be involved in the regulation of VSMC proliferation, we assessed the effect of ZO-2 silencing on the growth rate of VSMC using single cell proliferation assays (Figure 3A) and FACS analysis (Figure 3B). We found a dramatic decrease in the proliferation rate of ZO-2-si VSMC corresponding to 25% of that found in control-infected cells (P < 0.001). Furthermore, ZO-2-si VSMC showed a 70% inhibition of cell number compared with control-infected VSMC after 16 h growth (P < 0.001) (Figure 3C). No signs of increased apoptosis or cell death were found in ZO-2-silenced cells during the observed growth period (data not shown).

3.4 ZO-2 silencing induces expression of the transcription factor Stat1

VSMC growth control is mediated by multiple mechanisms. An essential role has been attributed to the signal transducer and activator of transcription Stat1. To investigate a possible interference between ZO-2 and Stat1 in VSMC, we first examined Stat1 expression in ZO-2-si VSMC. We found an increased expression of Stat1 protein in ZO-2-si VSMC analysed by immunoblotting and FACS (Figure 4A and B) (P < 0.007). Additionally, immunocytochemical studies were performed using double-immunostaining of cells with anti-ZO-2 and anti-Stat1 antibodies. These experiments revealed an increased Stat1 cytosolic and nuclear staining in VSMC with down-regulated ZO-2 (Figure 4C). The levels of serine- and tyrosine-phosphorylated Stat1 as quantified by FACS analysis increased absolutely in ZO-2-silenced cells (P < 0.05). However, the differences were equalized when normalising phosphorylated levels to the increased Stat1

2.7 Microinjection assay

Serum-starved VSMC with ZO-2 silencing (ZO-2-si VSMC) were microinjected with anti-pSer727Stat1 rabbit polyclonal antibody (Bio-source) at a concentration of 25 μg/mL in PBS containing 1 mg/mL Ca2+ and Mg2+ or 25 μg/mL non-specific rabbit polyclonal IgG (Sigma) using micromanipulator type MO-8 (Nikon, Melville, NY, USA) with microinjector PL-188 (Nikon). In each experiment, 300-400 cells were microinjected. Microinjected cells were used for proliferation assay, as indicated.

2.8 Statistical analysis

Results are expressed as mean ± SD of at least three independently performed experiments or as a representative result of at least three independently performed experiments. Significant differences were established by two-tailed paired t-test using the computer program Graph Pad Prism Software version 4.03 (Graph Pad Software Inc., USA). Differences were considered significant at * P < 0.05, ** P < 0.01, *** P < 0.001.
3.5 ZO-2 modulates Stat1 transcriptional activity

Transcription factors of the Stat family exert their biological activity by specifically affecting gene transcription. Therefore, we next examined whether ZO-2-directed changes in Stat1 expression affect Stat1-dependent, ISRE-driven, gene transcription. For this purpose, control and ZO-2-si VSMC were transfected with a luciferase reporter plasmid, under the control of ISRE. As shown in Figure 5A, ZO-2 silencing led to a robust induction of a Stat-dependent transcription (P < 0.005). Cells nucleofected with pGL3-vectors lacking or containing eukaryotic promoter and enhancer sequences showed suppressed or up-regulated transcriptional activity correspondingly.

3.6 Stat1 mediates the growth inhibitory effect of ZO-2 silencing

To prove the hypothesis that the increased Stat1 expression and transcriptional activity are responsible for the growth inhibitory effect of ZO-2 silencing in VSMC, we neutralized Stat1 in ZO-2-si VSMC by microinjecting specific antibodies blocking Stat1 Ser727 phosphorylation. VSMC with microinjected unspecific rabbit IgG served as a control. Microinjection of the active antibody restored VSMC proliferation to more than two-fold compared with control IgG microinjected cells (P < 0.003), whereas microinjection of control IgG had minor effects resulted from unspecific cellular response to mechanical manipulation (Figure 5B).

3.7 Stat1 is down-regulated in VSMC of porcine coronary arteries upon vascular injury

As shown in Figure 1, dilation of porcine coronary arteries led to an increased ZO-2 expression. To assess the expression
level of Stat1 in this model, immunohistochemical analysis of frozen vascular sections of respective coronary arteries was performed. These experiments revealed a marked decrease in the expression of Stat1 in the enlarged medial layer of the vessel wall in dilated arteries (Figure 5C).

4 Discussion

The Stat protein family members are among the best studied of the latent cytoplasmic signal-dependent transcription factors modulating gene expression in development, cell growth, and homeostasis (reviewed in33). Stat1 is a downstream target of certain cytokines, whose essential function in regulation of gene expression in tumourigenesis and host defences has been well documented. Increasing evidence points to a novel function of Stat1 and suggests that this member of the Stat family plays a role in the pathogenesis of cardiovascular diseases (reviewed in34,35). The underlying cellular events are, however, sparsely explored and several mechanisms implying a multiple mode of Stat1 activation and of its specific functions in VSMC are widely discussed. Of interest, fludarabine, a nucleoside analogue with both anti-inflammatory and antiproliferative cellular effects abolished VSMC proliferation in vitro and reduces neointimal formation after balloon injury in vivo through specific inhibition of Stat-1 activation.36

The work reported here adds a novel component to these schemes by demonstrating an essential role for the tight junction protein ZO-2 in Stat1 regulation and cell growth in human VSMC. Recent evidence suggests that tight junctions constitute multifunctional complexes involved in various cellular events and are not limited to controlling cellular permeability alone. Involvement of tight junction proteins in regulation of gene expression, signal transduction, cytosolic-nuclear transport, differentiation, and morphogenesis has been studied mainly in epithelial and endothelial cells, which are a traditional experimental model in this field.7,20,22,37 However, nothing is known about the role of MAGUK proteins in VSMC.

Using ex vivo organ culture of porcine coronary artery, we found that ZO-2 protein was strongly up-regulated in VSMC of the vessel wall in response to injury. To date, there has been difficulty in establishing reproducible models of vascular remodelling after arterial injury in small animals. Endo-luminal manipulation of small arteries is technically challenging. This has led to the use of ex vivo organ culture models. These models are attractive because of their relative simplicity and reproducibility and the fact that they simulate major aspects of human vascular wall remodelling.25 The data from our ex vivo organ culture model confirm the findings of others who demonstrated already a decade ago an increase in ZO-2 in injured vasculature.4 Our study has now revealed that cell growth is strongly inhibited in VSMC with
down-regulated ZO-2. Potential involvement of MAGUK proteins in cell growth and proliferation has been suggested, and these studies initiated research on the previously unrecognized roles of junctional contacts in regulating the growth of epithelial tissues. It has been demonstrated that in epithelial cells ZO-1 and ZO-2 interact with other proteins, such as connexin 43 in a cell cycle stage-specific fashion. One other report documented ZO-2-mediated transcriptional regulation of cyclin D1.

The mechanisms utilized by MAGUK proteins to regulate cell proliferation have not been clarified so far. ZO-1 and ZO-2 are unlikely to participate directly in the regulation of genes related to cell growth. It is believed that they influence gene expression indirectly, by regulating transcription factors, such as AP-1, Jun, Fos, c-Myc, C/EBP, ZONAB/DbpA, and other proteins controlling gene expression. Our study provides further insight into the various mechanisms by which ZO proteins might affect cell proliferation process. We demonstrate, using multiple approaches, the existence of a functional link between ZO-2 and the transcription factor Stat1, which is one of the key regulators of VSMC functions contributing to vascular remodelling. We show that ZO-2 down-regulation resulted in increased Stat1 expression and transcriptional activity. Consequently, in functional studies, we observed impaired proliferation of VSMC with down-regulated ZO-2. Our microinjection experiments provide evidence that the inhibition of cell growth was indeed mediated by Stat1, since injection of a Stat1 blocking antibody restored cell proliferation. A minor effect observed after microinjection of control IgG might reflect VSMC response to mechanical manipulation, which has been shown to affect the Jak/Stat pathway. We provide functional evidence that Stat1 could play a role in the vascular remodelling process, because a marked decrease in Stat1 expression was observed upon vascular injury, paralleled by up-regulation of ZO-2 in these arteries. Interestingly, we found no changes in Stat1 tyrosine or serine phosphorylation but rather an absolute increase in phosphorylated Stat1 that was proportional to changes in its expression. Although Stat1 phosphorylation is believed to be the main prerequisite for its functional activity, recent studies reported on phosphorylation-independent functional effects of Stat1. However, our experiments using antibody microinjection point to a requirement for at least serine
phosphorylation in the propagation of ZO-2-directed effects of Stat1 on cell growth. These results support a model whereby vascular injury initiates up-regulated expression of ZO-2 in VSMC leading to inhibition of Stat1 expression in these cells and finally to accelerated cell growth and negative vascular remodelling. The proposed scheme reflects the situation in vivo and explains the prolonged up-regulation of ZO-2 in injured vessels. Thus, the very first response to injury is characterized by increased VSMC migration from the media to the intima, whereas proliferation of these migrating cells is inhibited. In contrast, the later steps of remodelling are related to up-regulated proliferation of VSMC and their negligible migratory activity.44–46 Our studies suggest that the ZO-2 protein is one important regulator of these processes.

The molecular mechanisms underlying the ZO-2–Stat1 interference remain to be determined. MAGUK proteins are characterized by several conserved modules indicating that these proteins may serve multiple purposes.37 ZO proteins contain some unique motifs not shared by other MAGUK family members, including nuclear localization and nuclear export signals and a leucine zipper-like sequence (reviewed in37). Which of these domains and interacting signalling molecules are involved in the propagation of the ZO-2 effect on Stat1 expression in VSMC is a question for exciting future research.

5 Supplementary material

Supplementary material is available at Cardiovascular Research online.

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