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Apoptosis Signal—Regulating Kinase 1 Deficiency Attenuates Vascular Injury—Induced Neointimal Hyperplasia by Suppressing Apoptosis in Smooth Muscle Cells

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Apoptosis signal—regulating kinase 1 (ASK1) is a mitogen-activated protein kinase kinase kinase that plays a crucial role in stress-induced apoptosis. Recently, we have reported that suppressed macrophage apoptosis in ASK1 and apolipoprotein E double-knockout mice accelerates atheromatous plaques in the hyperlipidemia-induced atherosclerotic model. However, the pathogenic role of smooth muscle cell (SMC) apoptosis in atherosclerosis still remains unclear. We investigated neointimal remodeling in ligated carotid arteries of *ASK1*-deficient mice (*ASK1*$^{-/-}$) for 3 weeks. *ASK1*$^{-/-}$ mice had significantly more suppressed intimal formation, inversely manifesting as potential anti-atherogenic aspects of *ASK1* deficiency, characterized by fewer SMCs and less collagen synthesis; and fewer apoptotic SMCs, infiltrating T lymphocytes, and microvessels, associated with decreased apoptosis of luminal endothelial cells, compared with those of wild-type mice. Injured arteries of *ASK1*$^{-/-}$ mice also showed significantly down-regulated expression of pro-apoptotic markers, adhesion molecules, and pro-inflammatory signaling factors. Moreover, tumor necrosis factor-α—induced apoptosis was markedly suppressed in cultured aortic SMCs from *ASK1*$^{-/-}$ mice. These findings suggest that ASK1 accelerates mechanical injury—induced vascular remodeling with activated SMC migration via increased neovascularization and/or enhanced SMC and endothelial cell apoptosis. ASK1 expression, especially in the SMCs, might be crucial, and reciprocally responsible for various pro-atherogenic functions, and SMC apoptosis seems to be detrimental in this model. *(Am J Pathol 2013, 182: 597—609; http://dx.doi.org/10.1016/j.ajpath.2012.10.008)*

Investigations of arterial reaction against altered blood flow or mechanical injury offer potential clues for human atherosclerosis etiology and restenosis, a major cause of morbidity in humans.[1,2] *In vivo* atherosclerotic vascular lesions and remodeling, induced by unilateral carotid artery ligation and predominantly composed of migrating and/or replicating vascular smooth muscle cells (SMCs), with subsequent production of extracellular matrix, have been used as an injury model.[3] In this model, partial blood stasis, enhanced wall tension, and reduced shear stress induce vascular remodeling proximal to the ligature site, similar to atherosclerosis or restenosis after angioplasty.[2–5]

Apoptotic cells, including SMCs, endothelial cells (ECs), and macrophages, are reportedly evident in atherosclerosis.[6,7] Although there are few reports correlating SMC apoptosis with neointimal vascular remodeling in ligation-mediated atherosclerotic models, apoptosis of medial SMCs might accelerate injury-induced neointimal formation, probably by triggering migration and proliferation of SMCs.[8] On the other

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hand, apoptotic intimal SMCs are often not scavenged because of weakened phagocytic clearance (ie, efferocytosis) by macrophages; therefore, they could be the source of extracellular matrix, such as collagen and calcifying vesicles, or thrombin, resulting in the intimal thickening, calcification, or thrombogenicity, respectively.[9,10] Apoptosis of ECs could also be important in the ligation model, because the initial step for injury-induced atherosclerosis includes EC damage and subsequent inflammatory cell migration.[6,11] Despite this, the role of apoptosis by vascular SMCs and/or ECs in the progression of neointimal hyperplasia is still debatable; apoptosis might also be a part of normal vascular wound healing.[12]

Apoptosis signal–regulating kinase 1 (ASK1), which is a mitogen-activated protein kinase kinase kinase family member, is stimulated via distinct mechanisms in response to various cytotoxic stressors, such as oxidative stress mediated by hydrogen peroxide, endoplasmic reticulum stress, and immune system mediators, such as tumor necrosis factor (TNF), IL-1, or Fas ligands.[13,14] ASK1 activates the c-Jun N-terminal kinase and p38 mitogen-activated protein kinase signaling pathways, which subsequently induce intrinsic apoptosis through mitochondria-dependent caspase activation.[13–15] More recently, our research has shown that suppressed macrophage apoptosis in ASK1 and apolipoprotein E (apoE) double-knockout ($ASK1^{-/-}/apoE^{-/-}$) mice accelerates macrophage-rich atheromatous plaques, coincident with fewer necrotic cores in the hyperlipidemia-induced atherosclerotic model,[15] unlike in the current injury-induced neointimal remodeling. Reported data indicate that ASK1 signaling attenuates macrophage-rich atherosclerosis and enhances necrotic core formation by stimulating macrophage apoptosis, which might cause plaque vulnerability via necrotic core development. ASK1 plays a critical role in the regulation of macrophage apoptosis from the anti-atherogenic aspects.[15]

However, little is known about the relationship between apoptosis in ASK1-induced SMCs and/or ECs and vascular remodeling of atherosclerotic models. Actually, another group has reported ASK1 activation to play a key role in vascular neointimal hyperplasia[16]; however, their balloon injury model on rat carotid arteries is not suitable to examine EC apoptosis and, inconsistently, their data have not demonstrated that dominant-negative ASK1 mutant significantly decreases vascular SMC apoptosis. By contrast, we suggested that the mechanisms responsible for atherosclerosis in vascular injury–induced arteries would be fundamentally different from those at play in the high-cholesterol diet–induced aortas in rabbit models.[17] Thus, in the present study, we examined the roles of ASK1 and apoptosis of SMCs and/or ECs in ligation injury–induced neointimal hyperplasia.

Materials and Methods

Animals

The experiments were performed on young male C57BL/6 wild-type (WT) and $ASK1^{-/-}$ mice,[15] weighing 20 to 25 g. To produce the ligation-induced vascular injury model, we ligated the left common carotid artery with a 7-0 silk suture at a site proximal to the carotid bifurcation in two groups of mice at the age of 6 to 8 weeks under anesthesia: an i.p. injection of 100 mg/kg ketamine (Daiichi Sankyo Co, Tokyo, Japan) and 2 mg/kg medetomidine (Meiji Yakuhin Co, Tokyo). The animals were euthanized at 1, 2, or 3 weeks after injection, by an i.p. injection of an overdose of ketamine-medetomidine, and the carotid arteries were excised (each was bisected at both sides of the ligation site to obtain an approximately 3-mm segment). They were then stained with H&E, elastica van Gieson (EVG), or Masson's trichrome stain, or immunohistochemical (IHC) preparations in sequential sections as well, after fixation in 10% neutral-buffered formalin for 24 hours.[15,17,18] Analyses were performed in the ligated left common carotid arteries in all experiments, whereas the contralateral nonligated right carotid arteries served as a control.

The carotid arteries embedded in paraffin for histological examination were cut systematically in sequential sections (4 μm thick) by using a sliding microtome (Leica SM2010R; Leica Microsystems, Wetzler, Germany). The specimens of carotid artery were thoroughly examined by preparing sequential 500- to 750-μm cross sections for H&E or IHC stainings. A robust and reproducible vascular remodeling in WT mice reliably started at a distance of 500 μm from the ligature; small amounts of thrombus were observed at the ligation site,[3] and persisted up to 1500 μm from the ligature. Of each 20 sequential sections (one set), one was stained with H&E staining at least (ie, 80 μm thick intervals).[15,17,18] Quantifying analyses were performed in these 15 serial H&E sections collected from segments of approximately 500 to 1500 μm proximal to the site of ligation, as shown in Supplemental Figure S1. Images of the 15 sections were captured, and the thickened neointimal or medial areas and the intima/media (I/M) ratio were evaluated by a NanoZoomer Digital Pathology Virtual Slide Viewer (Hamamatsu Photonics Corp, Hamamatsu, Japan). The EVG staining clearly revealed both internal and external elastic lamina (EEL). In addition, the extent of constrictive vascular remodeling (reduction in vascular cross-sectional area) was calculated by the reduction in the EEL length in the ligated compared with the control artery (Hamamatsu Photonics Corp).[17,18] These indices were quantified by averaging all measurements counted in an individual mouse; we then selected one representative (mean) section per mouse.[15,17,18] To calculate proportions of collagen content to neointima, we measured intimal lesion areas and Masson's trichrome–positive blue areas in at least three sequential sections of each previously mentioned set, including the representative one, as previously described.[15,18]

The Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health (Kitakyushu, Japan) approved the protocols. They were performed according to the Institutional Guidelines for