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Review

"Jnking" atherosclerosis

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Abstract. Numerous studies in animal models established a key role of the C-jun N-terminal kinase (JNK) family (JNK1, JNK2 and JNK3) in numerous pathological conditions, including cancer, cardiac hypertrophy and failure, neurodegenerative disorders, diabetes, arthritis and asthma. A possible function of JNK in atherosclerosis remained uncertain since conclusions have mainly been based on in vitro studies investigating endothelial cell activation, T-effector cell differentiation and proliferation of vascular smooth muscle cells, all of which represent crucial cellular processes involved in atherosclerosis. However, recent experiments demonstrated that macrophage-restricted deletion of JNK2 was sufficient to efficiently reduce atherosclerosis in mice. Furthermore, it has been shown that JNK2 specifically promotes scavenger receptor A-mediated foam cell formation, an essential step during early atherogenesis, which occurs when vascular macrophages internalize modified lipoproteins. Thus, specific inhibition of JNK2 activity may emerge as a novel and promising therapeutic approach to attenuate atheroma formation in the future. In this review, we discuss JNK-dependent cellular and molecular mechanisms underlying atherosclerosis.

Key words. JNK1; JNK2; SR-A; atherogenesis; macrophage; foam cell formation; phosphorylation; LDL.

Introduction

So far, three distinct MAPK pathways have been described in mammalian cells: the extracellular signal-regulated kinase (ERK) pathway, the c-Jun-N-terminal kinase (JNK) pathway and the p38 MAPK pathway. In general, the ERKs are activated by mitogenic stimuli, whereas the JNKs and p38 MAPKs respond to environmental stress, including ultraviolet light, heat, osmotic shock and inflammatory cytokines [1, 2].
The JNK protein kinases are encoded by three genes: JNK1, JNK2 and JNK3. JNK1 and JNK2 are expressed ubiquitously. In contrast, expression of JNK3 is largely restricted to brain, heart and testis. Transcripts derived from these genes are alternatively spliced to create four JNK1 isoforms, four JNK2 isoforms and two JNK3 isoforms [3]. Extracellular stimuli, including stress and cytokines, lead to the activation of mitogen-activated protein kinase kinase kinases (MAPKKKs) that in turn activate MAP kinase kinase 4 or 7 (MKK4 and MKK7), both of which phosphorylate the tyrosine and threonine residues within the dual phosphorylation motif Thr-Pro-Tyr located in the activation loop of JNK [2, 4]. JNK activation results in phosphorylation of a number of transcription factors, including the c-Jun component of the activator protein-1 (AP-1) transcription family, but also other cellular proteins. JNK inactivation is regulated by serine and tyrosine phosphatases, and by a family of dual specificity MAPK phosphatases [4]. Loss-of-function

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studies in mice provided major insights into the function of JNK genes in development and disease. JNK1, JNK2 as well as JNK3 null mice appeared morphologically normal, but simultaneous disruption of JNK1 and JNK2 resulted in embryonic lethality [5, 6]. More detailed analysis revealed that JNK1 and JNK2 knockout mice are immunocompromised due to defects in effector T cell differentiation and cytokine production [7]. Further studies using animal disease models defined specific roles of JNK genes in a remarkable variety of pathologic conditions, such as cancer development [8], cardiac hypertrophy and heart failure [9], neurodegenerative disorders such as Parkinson's disease [10, 11] and Alzheimer's disease [12], obesity-associated type II diabetes [13], type I diabetes [14], arthritis [15, 16] and asthma [17–19]. In this review, we summarize recent findings about JNK and its function in atherogenesis [20].

JNK2 but not JNK1 represents a key player in atherogenesis

In broad terms, atherosclerosis represents a systemic inflammatory and metabolic disease triggered by a complex interplay of numerous cell types, including T cells, macrophages, hepatocytes, enterocytes and vascular cells [21]. Recent studies indicated that JNK becomes highly activated in atherosclerotic lesions in humans [22] and rabbits [23]. Activated JNK was shown to localize mainly in vascular smooth muscle cells and macrophages. In the study conducted by Ricci et al., a very prominent phosphorylation and thus activation of JNK in total protein extracts from murine atherosclerotic lesions compared with normal vessels was observed [20]. The 10 known splicing variants of the three JNK genes give rise to proteins that have either a molecular weight of 46 kDa (smaller isoforms) or 55 kDa (bigger isoforms). Interestingly, only the bigger isoforms were phosphorylated, while similar levels of total JNK was detected at both molecular weights in diseased and control vessels.

Ricci et al. then investigated the in vivo function of JNK1 and JNK2 in atherogenesis using the $ApoE^{-/-}$ mice as a model for atherosclerosis and could demonstrate genetically that JNK2 but not JNK1 represents a key player in atherosclerosis in mice. Furthermore, pharmacological inhibition of JNK activity for a relatively short time period (4 weeks) using the small molecule inhibitor SP600125 efficiently inhibited atherosclerosis.

A more detailed pharmacological study will be necessary to consider JNK inhibition as a possible treatment modality in patients. An important question is whether treatment with a JNK inhibitor leads to inhibition or even regression of atherosclerotic lesions. For that purpose, different time points and treatment periods need to be included in the study. Given the broad activity of JNK in many cellular processes, treatment with global JNK inhibitors might lead to severe side effects in patients. Therefore, studies with JNK2-selective inhibitors should be considered. At present, most scientists focus on development and testing of JNK1-specific inhibitors. However, the study by Ricci et al. [20] and other recent studies [14, 16] clearly provide evidence for the requirement of inhibitors against JNK2 in specific disease settings. However, one should keep in mind that existing mouse models for atherosclerosis do not fully reflect the situation of human atherosclerosis and that inhibition of JNK2 alone, although probably associated with fewer side effects when compared with broader spectrum inhibitors, will not be sufficient to efficiently attenuate atherosclerosis in humans.

In the remaining part of this review we analyze the possible cellular and molecular mechanisms by which JNK exerts its pro-atherogenic functions.

The role of JNK in endothelial cell activation

Vascular endothelial cell activation upon pro-inflammatory stimuli such as tumor necrosis factor α (TNFα) and interlekin (IL)-1 is manifested through expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and selectins (P-, E- and L-selectins) [24]. Genetic experiments in vivo revealed importance for these molecules in the early pathogenesis of atherosclerosis [25–28].

Previous work mainly established an important role for JNK in TNFα-triggered apoptosis [29–31]. However, several laboratories also reported a possible role for JNK in TNFα-mediated endothelial cell activation. In two studies, it has been demonstrated that the JNK signaling pathway is specifically required for the induction of E-selectin expression induced by TNFα [32, 33]. Moreover, AP-1 transcription factors, in particular the JNK target c-jun, mediate the effect of TNFα-induced VCAM-1 expression in endothelial cells through interaction with NF-kappaB [34]. Another research group reported that activation of the JNK pathway leads to ICAM-1 expression [35]. To our knowledge, none of the experiments addressing endothelial cell activation have been conducted in primary JNK1 or JNK2 knockout endothelial cells. Nevertheless, several experiments suggest that TNFα-mediated cellular processes seem to be controlled mainly by JNK1 and not JNK2 [29, 31, 33]. Consistently, deletion of JNK2 did not result in altered E-selectin, VCAM-1 and ICAM-1 expression in atherosclerotic vessels. As mentioned above, inactivation of JNK1 did not significantly inhibit atherosclerosis in ApoE-deficient mice. Hence, although a possible role for JNK1 in endothelial cell activation has not been excluded

in the study by Ricci et al. [20], it is reasonable to assume that this process will not be of major relevance in the context of atherosclerosis.

Impact of JNK on vascular smooth muscle cell proliferation and migration

Transition of early plaques to more complex lesions is characterized by migration and proliferation of vascular smooth muscle cells (VSMCs) into the intima [21].
Importantly, the AP-1 member c-jun seems to be required for both platelet-derived growth factor (PDGF)-induced VSMC migration and proliferation [36, 37]. Furthermore, inhibition of JNK activity using recombinant adenovirus containing a dominant-negative mutant of JNK attenuates PDGF-induced VSMC migration and proliferation in vitro [38]. Although studies in mouse embryonic fibroblasts showed specific roles for JNK1 and JNK2 in proliferation [39], the study by Ricci et al. demonstrated that inactivation of JNK1 and JNK2 did not lead to a significant change in proliferation of VSMCs in vitro in response to PDGF. It has also been demonstrated that migration was not affected in the absence of JNK2. However, it should be noted here that other potential mitogenic factors, such as thrombin, have been shown to activate JNK in VSMCs [40]. But immunohistochemistry using a VSMC-specific marker did not reveal increased amounts of VSMCs in plaques of $ApoE^{-/-}$ compared with $ApoE^{-/-}$ $JNK2^{-/-}$ mice [20]. Thus, inactivation of JNK2 seems not to be sufficient to attenuate VSMC proliferation and migration, at least not in the context of atherosclerosis.

JNK is a key player in T effector cell differentiation

The role of adaptive immunity in development of atherosclerosis has been broadly investigated [41]. Within the atheroma, CD4-positive T-helper cells can polarize into those secreting generally pro-inflammatory cytokines (known as Th1 cells) and those secreting predominantly anti-inflammatory cytokines (denoted as Th2 cells). In general, Th1 cells predominate in the atheroma. They secrete interferon-γ (IFNγ), IL-2, and TNF-α and -β, which cause macrophage activation, vascular activation and inflammation. Th2 cytokines such as IL-4, IL-5 and IL-10 are less abundant than cytokines of the Th1 type in end-stage human atherosclerotic lesion [42].
The JNK signaling pathway has been implicated in the immune response that is mediated through differentiation of $CD4^+$ helper T (Th) cells into Th1 and Th2 effector cells. JNK1-deficient T cells hyperproliferated, exhibited decreased activation-induced cell death, and preferentially differentiated to Th2 cells, which in turn led to enhanced production of Th2 cytokines such as IL-4, IL5 and IL-10. Likewise, the differentiation of precursor $CD4^+$ T cells into Th1 but not Th2 effector cells is impaired in JNK2-deficient mice. The inability of IL-12 to differentiate JNK2-deficient $CD4^+$ T cells fully into Th1 effector cells is caused by a defect in IFNγ production during the early stages of differentiation [7, 43–46].
Although clear evidence for a role of T cells has been obtained in mouse models of atherogenesis [47-49], complete lymphocyte deficiency did not alter development of atherosclerosis in ApoE-deficient mice in which acceleration of atherogenesis was induced by feeding mice with a high-cholesterol diet [50, 51]. Therefore, the atherosclerosis model which was used by Ricci et al. very likely was T cell-independent. This is also supported by the fact that there was no effect on atherosclerosis in the absence of JNK1, although very similar T cell defects have been reported for both JNK1 and JNK2, and identical genetic backgrounds have been used. However, these findings do not exclude a possible JNK-dependent effect on lymphocyte differentiation in more chronic and immunogenic murine and human atherosclerotic lesions.

Impaired lipid processing in $JNK2^{-/-}$ macrophages

In normal circumstances, the endothelial monolayer resists firm adhesion of monocytes upon contact with flowing blood. However, upon exposure with pro-inflammatory factors, endothelial cells increase the expression of various leukocyte adhesion molecules, which enable monocytes to adhere to the endothelial cell membrane [52]. Adhesion is followed by transmigration of monocytes through the endothelial layer into the intima. This is governed by chemotactic factors produced in the subendothelial layer. Migrated monocytes become tissue-resident macrophages which in turn develop into lipid-loaded foam cells upon exposure to modified lipoproteins [53]. In foam cells, the membranes become enriched in unesterified cholesterol and cytoplasmic cholesteryl ester droplets as well as lysosomal cholesterol crystals form. On the other hand, the macrophages are able to remove at least partially excessive cellular cholesterol. The cholesterol efflux process is mediated by high-density lipoproteins (HDLs) and especially particles containing only apolipoprotein A-I (apoA-I). Reverse cholesterol transport from macrophages to the liver is regarded as a central anti-atherogenic process. Recently it has become clear that cholesterol efflux is a highly regulated process that is mediated by specific molecules, including ATP-binding cassette (ABC) transporters [54]. At the same time, macrophages are important regulators of the innate immune system, and inflammatory pathways induced in activated macrophages are pivotal for the pathogenesis of atherosclerois [55].

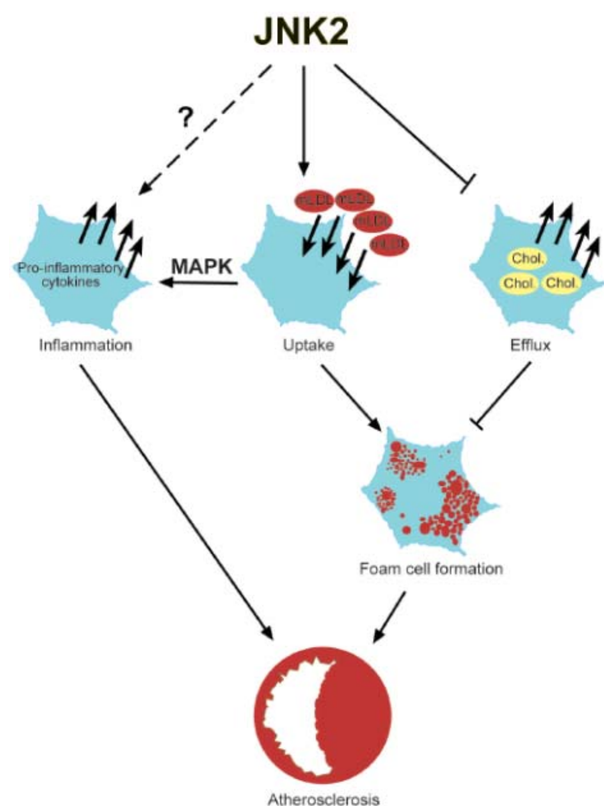

Figure 1. The role of JNK2 in lipid homeostasis and inflammation in macrophages. JNK2 promotes uptake of modified lipoproteins (mLDLs) and suppresses cholesterol (Chol.) efflux in macrophages. Extensive uptake of modified lipoproteins triggers foam cell formation, while reverse cholesterol transport from macrophages attenuates this process. Foam cell formation is a crucial step in promoting early atherogenesis. JNK2 might also promote inflammation and plaque formation by regulating secreation of pro-inflamatory cytokines in macrophages loaded with cholesterol.

As described above, the study by Ricci et al. could largely exclude a function of JNK2 in endothelial cells and vascular smooth muscle cells in the context of atherosclerosis. Importantly, inactivation of JNK2 also did not alter the plasma lipid profile in ApoE-deficient mice. However, this study presented clear evidence that JNK2 but not JNK1 is required for efficient foam cell formation in vitro. Defective foam cell formation in JNK2-deficient macrophages was caused by defective uptake and degradation of modified lipoproteins (acetylated LDL). Interestingly, JNK2-deficient macrophages also showed a two-fold increase in binding of acetylated LDL (acLDL) (see below). Intriguingly, cellular apoprotein AI (ApoAI)-mediated cholesterol efflux was markedly increased in the absence of JNK2, which might also contribute to defective foam cell formation in JNK2-deficient macrophages. Yet the molecular mechanism by which JNK2 attenuates cholesterol efflux in macrophages is currently unknown. Most important, it has also been shown that macrophage-restricted deletion of JNK2 was sufficient to reduce atherosclerosis in mice using bone marrow transplantation experiments [20]. In fact, the bone marrow transplantation experiments further exclude any effect of the absence of JNK2 on endothelial cells and VSMCs. However, it is not clear whether JNK2 is also involved in activation of macrophages and controls secretion of inflammatory cytokines in this cell type, which might also contribute to the development of atherosclerosis. A recent intriguing study suggests that MAPKs, including JNKs, convert cholesterol loading of cells into expression of pro-inflammatory cytokines at the RNA and protein level [56]. Therefore, more work addressing the role of JNK2 in macrophage-dependent inflammation is required.

Overall, these data provide evidence that JNK2-dependent foam cell formation could represent a crucial event in atherosclerosis.

JNK2 controls the abundance and phosphorylation of scavenger receptor A

Transition of macrophages into foam cells is triggered by extensive uptake of modified lipoproteins [57]. Uptake of modified lipoproteins has been shown to be mediated mainly by scavenger receptor A (SR-A) and CD36 since uptake of modified low-density lipoproteins (LDLs) is reduced by almost 90% in macrophages lacking SR-A and CD36 simultaneously [58]. Importantly, SR-A alone mediates approximately 30–50% of uptake of modified lipoproteins [58]. However, the role of SR-A in atherogenesis remains controversial. The most striking evidence that demonstrates a pro-atherogenic role for this receptor resulted from the initial study by Suzuki et al. [59] in which atherosclerosis-prone ApoE$^{-/-}$ mice were crossed with SR-A$^{-/-}$ mice. These double mutant mice showed reduced atherosclerosis compared to ApoE$^{-/-}$ SR-A$^{+/+}$ mice. Since this initial report, a number of conflicting data on the role of SR-A in atherogenesis have been published. In a study by de Winther et al. [60], mice expressing Apolipoprotein E3Leiden, a dysfunctional ApoE variant associated with familial dysbetalipoproteinemia in humans, were crossed with SR-A$^{-/-}$ mice. Surprisingly, these mice showed slightly increased lesion formation in the absence of SR-A. Consistent with the study of Suzuki et al. [59], another study showed that mice deficient for total or macrophage-specific expression of SR-A exhibited decreased atherosclerosis [61]. Gain-of-function experiments in mice also led to inconsistent results. One study demonstrated no effect on atherosclerosis when human SR-A was ectopically expressed in macrophages in LDL receptor-deficient mice [62]. Another study even demonstrated decreased atherosclerosis upon macrophage-specific overexpression of bovine SR-A in LDL receptor-deficient mice [63]. Overall, the experiments

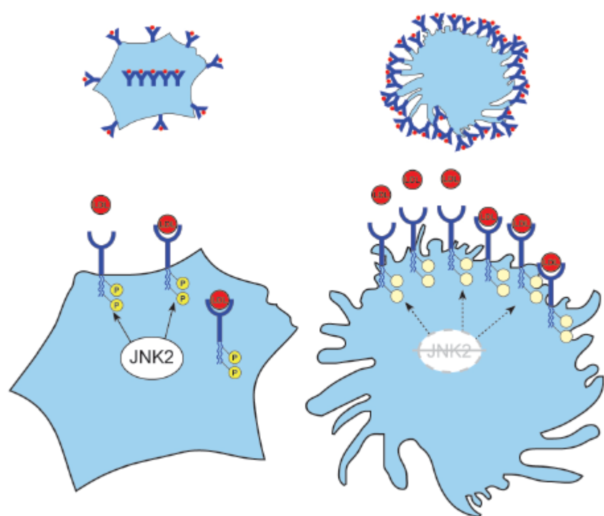

Figure 2. JNK2 promotes SR-A-mediated lipid internalization in macrophages. Left panel: JNK2 indirectly or directly phosphorylates SR-A (receptor in blue), which allows internalization of receptor-bound lipoproteins (in red). Right panel: In the absence of JNK2, the receptor is less phosphorylated and is more abundant on the surface, allowing more binding of lipoproteins and increased adherence of macrophages. However, internalization is no longer efficient.

in mice have been conducted in different genetic backgrounds and different mouse models of atherosclerosis. Moreover, the expression of human or bovine SR-A in mice might lead to completely misleading results since even small amino acid sequence differences might give rise to completely aberrant functions in foreign species. Therefore, these experiments should be interpreted very carefully.

The study by Ricci et al. has demonstrated that protein abundance of SR-A is increased in macrophages lacking JNK2 compared with wild-type cells, which has been confirmed by immunofluorescence, Western blotting and immunohistochemistry, while RNA levels were unchanged. In addition, JNK2-deficient macrophages displayed filopodia-like projections, which has been associated with increased SR-A expression [64]. Since SR-A is responsible for binding and uptake mainly of the acetylated form of LDL (acLDL), these results would imply that binding and uptake of acLDL would be increased in JNK2-deficient cells. Indeed, increased binding of acLDL could be observed. However, it was clearly demonstrated by two independent series of experiments that uptake and degradation of acLDL was severely decreased in the absence of JNK2. Thus, although the receptor seems to be more abundantly expressed and can bind more acLDL, it cannot be internalized efficiently. Three studies indicated that phosphorylation of specific serines located in the cytoplasmic tail of SR-A were required for efficient SR-A-mediated uptake of modified LDLs [65–67]. Indeed, experiments revealed that SR-A phosphorylation was markedly reduced in macrophages lacking JNK2. Thus, JNK2 promotes phosphorylation of SR-A and is required for efficient SR-A-mediated lipid internalization in macrophages. Moreover, decreased phosphorylation of SR-A could lead to increased stability of this receptor and thus could explain the increased abundance of SR-A. However, the exact mechanisms affecting stability of SR-A in the absence of JNK2 need to be further investigated. It also needs to be determined whether JNK2 is phosphorylating SR-A directly, or whether it induces downstream pathways which in turn lead to phosphorylation of SR-A.

Involvement of JNK in the advanced stage of atherosclerosis

In the advanced stage of plaque formation, atherosclerotic lesions can rupture at least partially due to degradation of the extracellular matrix by various proteases such as collagenases and matrix metaloproteinases (MMPs), exposing tissue factors to blood components, which leads to coagulation and thrombosis [21, 68].

Various studies reported that MMP9, which is highly expressed in atherosclerotic plaques, is regulated by JNK [69–72]. The expression of MMP1 was found to be at least partly mediated by JNK [73], and likewise MMP13 was shown to be controlled by JNK in a murine arthritis model [15]. These studies indicate that JNK might be involved in the process of plaque rupture.

Platelets play a key role in hemostasis and thrombosis. The formation of a platelet plug is accompanied by the generation of thrombin, which results in the generation of fibrin required for stabilization of the platelet plug. Thrombin is a potent platelet activator, which proceeds through proteolysis of the protease activated receptors (PARs) [74]. Indeed, thrombin as well as von Willebrand factor, another potential activator of platelets, have been shown to activate JNK in human platelets [75, 76]. However, the lack of a well-established mouse model for plaque rupture and thrombosis complicates experimental design, and therefore a possible role for JNK in late-stage atherosclerosis remains highly speculative [77].

Conclusions

JNK has been shown to be implicated in most of the cellular processes involved in the development of atherosclerosis. The recent in vivo study by Ricci et al. revealed that JNK2 controls lipid homeostasis in macrophages and thus represents a key player in atherosclerosis. These findings rather imply that JNK-dependent effects observed in endothelial cells, VSMCs and T cells probably

play a minor role in the context of atherosclerosis even though compensatory functions of JNK1 and JNK2 could not be excluded in these cells. Overall, specific inhibition of JNK2 could be considered to prevent lesion development in humans.

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