Familial atherosclerosis and neuroimmune guidance cues
From in vitro assessments to clinical events
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Netrin-1 in Coronary Artery Disease: Mechanism of Action and Potential as Therapeutic Target

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Published in Expert Opinion on Therapeutic Targets.
Sept. 2019
1 INTRODUCTION
Cardiovascular disease remains a major public health problem worldwide, and novel therapeutic targets are needed to reduce the occurrence of fatal cardiac events. The discovery of Netrin-1 and its receptors expressed by cells outside of the central nervous system indicates that Netrin-1 has additional functions beyond the central nervous system. Pre-clinical data supports the role for Netrin-1 in the atherosclerosis progression, and more effects of Netrin-1 in mouse studies have been shown to be equally potent in vitro on cells of human origin, or are being validated in human cohorts [1-3].

2 NETRIN-1 IN VESSEL LUMEN
Both Netrin-1 and UNC5B are moderately expressed and regulated by human coronary artery endothelial cells [4, 5]. Netrin-1 expression is increased by atheroprotective laminar flow, while decreased by inflammatory cytokines [4, 5]. Regarding the regulation of endothelial Netrin-1 expression in vivo, mouse studies observed a reduction in the levels of Netrin-1 within the vasculature of atherosclerotic mice [5, 6]. In line with this, Netrin-1 plasma levels were significantly lower in subjects with subclinical atherosclerosis compared to a group with no atherosclerosis [2]. This indicates a role for Netrin-1 in maintaining endothelial barrier function. Indeed, Netrin-1 has been shown to have an anti-inflammatory effect on endothelial cells [4, 7] as well as by inhibiting monocyte adhesion and migration [4, 5]. Supporting evidence for anti-inflammatory endothelial Netrin-1 is found in mouse models with acute lung inflammation due to Staphylococcus aureus infection in which Netrin-1 was found to be expressed on the luminal surface of lung endothelial cells and acted to block the migration of monocytes [1]. Consistent with this, increased levels of Netrin-1 in the circulation has been shown to be protective in atherosclerotic mouse models [7, 8]. Intriguingly aspirin therapy elevates Netrin-1 expression, which may be beneficial in murine and human disease. For example, aspirin treatment protected against disease burden via a reduction of monocyte plaque infiltration and lesion growth in mice [6]. Additionally, healthy human subjects receiving influenza immunization showed vascular activation-induced Netrin-1 depletion, which was counteracted following 300 mg aspirin for 28 days [3].

3 NETRIN-1 IN VESSEL WALL
While Netrin-1 in the circulation and produced by endothelial cells exert an atheroprotective function, Netrin-1 produced by accumulated macrophages within the plaque has a contradicting function (Figure 1). Both Netrin-1 and UNC5B expression by macrophages is induced by oxidized low-density lipoprotein, mediated by CD36 and nuclear factor NFκB, or induced by HIF1A following hypoxia [9, 10]. Macrophages overexpressing active HIF1A reduces migratory capacity compared...
with control cells [10]. Consistent with these studies, Netrin-1 potently inhibits chemotaxis of mouse macrophages *in vitro* [10]. This inhibitory effect is dose-dependent with maximal inhibition on migration (>90%) at a dose of 250 ng/ml. In contrast to its effect on macrophages, Netrin-1 promotes chemotaxis of coronary artery smooth muscle cells [9]. This suggests expression of Netrin-1 in plaques simultaneously prevents the egress of inflammatory cells and induces the recruitment of smooth muscle cells into the intima, thereby promoting lesion progression. Indeed, deletion of Netrin-1 in myeloid cells resulted in 55% smaller and less complex atherosclerotic lesions in LDLR−/− mice following a high fat Western diet. In these studies, the retentive function of Netrin-1 on macrophages in the plaque was confirmed by utilizing a bead method of tracking monocyte accumulation and macrophage exit [9].

**4 EXPERT OPINION**

Netrin-1 plays an important role in atherosclerosis and ischemia-reperfusion injury by acting as cardioprotective agent [6, 8, 9, 11]. The key finding, particularly in atherosclerosis, is the anti-inflammatory effect of Netrin-1 on the endothelium, causing impaired adhesion and influx of monocytes. In contrast, Netrin-1 produced by macrophages leads to their retention in atherosclerotic plaques, suggesting a pro-atherogenic function for Netrin-1. Besides the *in vitro* and *in vivo* mouse studies, an important addition to Netrin-1 studies are serum and plasma measurements in different human cohorts. These suggest Netrin-1 may serve an additional role as a potential biomarker. When interpreting these results, however, we would like to suggest some caution. In our experience not all commercially available ELISA assays measure Netrin-1 and in some reported cases plasma levels are measured below the detection limit.

Another challenge in this research field is the lack of complete knowledge of receptor-ligand interaction, which is of crucial relevance for potentially therapeutic targets. Besides binding to several receptors (UNC5, DCC, Neogenin, MCAM, DSCAM, Adenosine A2b, and several integrins), Netrin-1 can also bind to heparin and heparan sulphate proteoglycans. Binding to proteoglycans tether Netrin-1 to the cellular surface, preventing release into the circulation. Since different receptors are expressed by various cells involved in coronary artery disease, Netrin-1 potentially exhibits diverse and dynamic cellular consequences. However, in our understanding the specific effects of differential ligand-receptor interactions remains limited.

To fully understand Netrin-1, research should also focus on isoforms of Netrin-1. As a result of an alternative internal promoter, a shorter Netrin-1 is produced and localizes to the nucleolus [12]. Upon inflammatory stimulation, endothelial cells show enhanced Netrin-1 nuclear localization and reduced secretion of full-length Netrin-1 [6]. The function of the nuclear Netrin-1 in endothelial cells, immune cells, or vascular smooth muscle cells is yet to be elucidated. In addition to nuclear Netrin-1, truncated Netrin-1 has been reported. Cleavage by matrix metalloprotease results in truncated Netrin-1, which is hypothesized to be more soluble and to contribute to vascular permeability [13]. Enhanced vascular permeability in lesions increases inflammatory cells recruitment, making these plaques more prone to rupture.

In terms of drug development targeting Netrin-1, we suggest a distinction should be made for disease stage. In early stage atherosclerosis, Netrin-1 could have a protective function by repelling monocyte adhesion onto the arterial wall. Therefore, a peptide mimicking Netrin-1 could be a preventive measurement during atherosclerosis treatment. However, during later stages of atherosclerosis plaque-produced Netrin-1 enhances disease progression by inhibiting macrophage egression. Therefore, during...
advanced disease local inhibition of Netrin-1 with an antibody could be a desirable target for therapy. For example, Netrin-1 antibody within a drug eluting stent could be placed during percutaneous coronary intervention. Whether activation or blockade of Netrin-1 signaling will provide clinical benefit in different atherosclerotic stages remains to be determined. Until that time, the first trial with a humanized monoclonal antibody targeting Netrin-1 is being used for patients with locally advanced or metastatic solid tumors. While safety, tolerability, pharmacokinetic, pharmacodynamics are currently assessed, it is promising that no clinical, hemotological or biochemical signs of toxicity were noted in mice and monkeys who received a humanized Netrin-1 antibody, which disrupted the interaction between Netrin-1 and UNC5B (www.clinicaltrials.gov, NCT02977195). Long-term follow-up of these patients could provide valuable information for the possible use of this antibody for other diseases such as cardiovascular disease. Alternatively, targeting specific Netrin-1 receptors or isoforms, potentially in combination with targeted delivery, should be taken into consideration.

In summary, the precise cellular source and targets of Netrin-1 are crucial in determining its effect on disease progression (Figure 1). There are good pre-clinical, mouse, and in vitro studies to elucidate a role for Netrin-1 as a therapeutic target. Understanding the limitations of currently available therapies for coronary artery disease, we await the first pharmacological studies targeting Netrin-1.

REFERENCES