Neuroimmune guidance cues in vascular (patho)physiology

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Chapter 7

Summary and discussion

Summary

Tissue homeostasis is critical for health and preventing disease. As the first interface between blood and underlying tissues, endothelial cells are important regulators of vascular homeostasis. Neuroimmune guidance cues (NGCs) are expressed in a variety of cells, including endothelial cells, extramural cells and immune cells. They are known to regulate the vascular system pre- and postnatally and could influence the function and cellular interactions of cells. This thesis aimed to study the precise contributions of NGCs to vascular physiology and disease and unravel potential novel targets to alleviate and/or prevent (cardio)vascular disease.

An important determinant of (cardio)vascular disease is atherosclerosis. Atherosclerosis is driven by inflammation, which is also known to modulate the expression of some NGCs. In ***Chapter 2*** we provided an extensive overview of regulation of NGC gene expression under pro-inflammatory conditions. Endothelial cells and monocytes, both important cell types in atherosclerosis, showed differential expression of NGC genes. Most changes were observed in the Semaphorin and Ephrin families of NGCs. A profound downregulation of PlexinA4 (PLXNA4), one of the Semaphorin receptors, was observed in endothelial cells after both short and long-term exposure to pro-inflammatory cytokines. Reduction of PLXNA4 expression in endothelial cells resulted in stretched cells with increased actin stress fibers, decreased cell-cell junctions, and a more inflammatory phenotype. In addition, reduction of PLXNA4 resulted in decreased endothelial barrier function and capillary network formation of the endothelial cells, while vascular leakage to both solutes and cells was increased. These data support the hypothesis that NGCs are regulated under pro-inflammatory conditions and show that PLXNA4 is an important regulator of vascular integrity.

Eph receptors and their ephrin ligands are important guidance molecules in vascular development and are also expressed and highly regulated under pro-inflammatory conditions postnatally (chapter 2). Therefore, in ***Chapter 3*** the currently available data of Ephrin ligands and receptors in adult endothelial cell biology was reviewed. We summarized the current knowledge of the expression and regulation ephrin family members and their effect on cellular proliferation, migration, barrier formation and cell-cell interactions. This showed that Ephrin ligands and EPH receptors are involved in both the physiology and pathophysiology of adult vascular biology.

Further exploring the role of Ephrin family members in vascular biology, the function of the EPH receptor B2 (EPHB2) and its EphrinB ligands in atherosclerosis is described in ***Chapter 4***. Immunohistochemical staining of human aortic sections at different stages of atherosclerotic plaque formation, revealed that EPHB2 expression increases with plaque progression, while EphrinB ligand increased primarily in the most severe stage of plaque formation. Double staining of severe atherosclerotic plaques revealed EPHB2 to be mainly present in cells of the monocyte lineage. *In vitro,* activation of EPHB2 signaling in monocytes had no effect on monocyte-endothelial interactions. Knockdown of EPHB2 in monocytes, however, significantly impaired monocyte adhesion and transmigration, implicating a ligand-independent function of EPHB2 in these processes. These cells showed alterations in their actin cytoskeleton and a decreased ability to phosphorylate focal adhesion kinases, which we predict to be causative to the impaired adhesion and migration of these cells. Altogether, this data revealed the accumulation of EPHB2 during atherosclerotic plaque formation and its importance in monocyte adhesion and migration.

In **Chapters 5 and 6**, we further examined the role of NGC in human atherosclerosis by studying the pathological effects of genetic variants in NGC genes in patients with premature atherosclerosis. In ***Chapter 5*** two genetic variants in the ligand-receptor pair EphrinB2-EPHB4 were studied. The identified EphrinB2 variant has a substitution of one of its threonine residues within the intracellular domain of the protein. This threonine residue is a predicted phosphorylation site and substitution could potentially alter EphrinB2 ligand signaling. The variant identified in the EPHB4 receptor results in an amino acid change within the ligand binding domain of the receptor and could potentially disrupt ligand-receptor interactions. While no effect of the genetic variant in EphrinB2 on endothelial function was found, the genetic variant in EPHB4 indeed resulted in impaired ligand-receptor binding. In addition, endothelial-pericyte interactions were impaired in the presence of the mutated variant of EPHB4, implicating a decrease in vascular stability. This decreased vascular stability could, in turn, increase vascular leakage enabling the formation and progression of atherosclerotic plaques.

In ***Chapter 6*** a genetic variant in the NTR domain of the NTN1 gene was explored. Binding assays revealed differential binding capacity of the mutant form of NTN1 to several of its binding partners compared to the wildtype form. At the cellular level, the mutant form of NTN1 showed decreased anti-inflammatory effect on endothelial cells compared to wildtype NTN1. Expression of IL-6, CCL2, and ICAM-1 was increased and

monocyte-to-endothelial cell adhesion was enhanced promoting atherosclerotic plaque formation and growth. Migration of macrophages and vascular smooth muscle cells, on the other hand, was decreased in the presence of mutant NTN1, which reduces plaque stability and increases the risk of plaque rupture and clinical manifestations.

Discussion

NGCs contribute to adult vascular (patho)physiology

Over the years, indications for a role of NGCs in cardiovascular disease (CVD) have been accumulating. Focusing on atherosclerosis, a major contributor to CVD, a study of van Gils et al. already showed that in mice NTN1, SEMA3A and EphrinB2, among others, are differentially expressed in 'atheroprone' regions with disturbed blood flow compared to 'atheroprotective' regions with laminar flow. Besides hemodynamics, pro-inflammatory cytokines also regulated the expression of NTN1, SEMA3A and EphrinB2 and these NGCs were shown to modulate leukocyte chemotaxis, providing the initial clues for a role of these NGCs in atherosclerosis (1). Furthermore, a number of NGCs have been detected in atherosclerotic plaque tissues including several Ephrin (2-4) and Semaphorin (5) family members. NGCs have also been described to have functional implications in atherosclerosis-related processes. SEMA3E and NTN1, for example, promote macrophage retention within atherosclerotic plaques (5, 6) while EphrinA1/EPHA2/EPHA4 and SLIT3 modulate leukocyte adhesion (2, 3, 7). Interestingly, also genetic variants in genes related to the axonal guidance pathway are enriched in CVD and several NGC genes are located on novel genetic risk loci for CVD (8-10). Taken together these studies demonstrated a potential role for NGCs in (endothelial) cell function and immune cell trafficking suggesting involvement in vascular (patho)physiology. However, only a small subset of NGCs was studied so far and little human validation was available. Therefore, this thesis continued the efforts to expand the knowledge about NGCs in human vascular (patho)physiology.

As inflammation is an important inducer of endothelial dysfunction and activator of immune cell trafficking, it was of interest to gain insight in the regulation of NGCs under pro-inflammatory conditions in human endothelial cells and monocytes. For a small subset of NGCs, expression and regulation of expression by (pro-)inflammatory cytokines has been described (1, 2, 4, 11). Here, we were able to provide a complete overview of the expression of a large number of NGCs in primary monocytes as well as endothelial cells, strengthening the association between NGCs and inflammation and their potential involvement in vascular (patho)physiology. Several NGCs, especially within the Semaphorin and Ephrin families of NGCs were regulated. The most striking

observation was PLXNA4 which was consistently decreased upon inflammatory conditions. Plexins have been described to regulate not only the developing and mature nervous and vascular system but also to modulate immune responses (12, 13). We were able to provide evidence for an important role of PLXNA4 in maintaining the endothelial barrier. The endothelial barrier prevents against excessive vascular leakage to solutes but also uncontrolled extravasation of immune cells, which are associated with atherosclerosis (14). Knockdown of PLXNA4 in endothelial cells indeed revealed increased vascular leakage to both solutes and cells, suggesting PLXNA4 to be protective against atherogenesis.

Interestingly, in the course of our studies other studies were published describing a role for some of the inflammation-regulated NGCs in vascular biology and disease. For example EphrinA1, of which we showed its expression to be increased in endothelial cells upon inflammation, was shown to be an important regulator of endothelial proliferation and migration (15). A study of Finney and colleagues described EPHA2, one of the EphrinA1 receptors, to promote atherosclerosis by regulating both inflammation and plaque progression. In endothelial cells EPHA2 was shown to induce an inflammatory phenotype that enhanced monocyte adhesion, while in smooth muscle cells EPHA2 enhanced vascular smooth muscle cell proliferation and extracellular matrix deposition leading to unstable atherosclerotic plaques (16). Another study showed SEMA7A to promote atherogenesis. By binding to integrinβ1, SEMA7A induces expression of adhesion molecules, promoting monocyte adhesion and potentially plaque formation. Indeed, knockdown of SEMA7A in mice showed reduced expression of adhesion molecules, decreased leukocyte adhesion and smaller atherosclerotic plaque sizes, confirming its importance in atherogenesis (17). In addition, SEMA3E and SEMA3D were shown to modulate neutrophil and endothelial migration respectively (18, 19), and SEMA6D, via PLXNA4, to regulate lipid metabolism and anti-inflammatory polarization of macrophages (20). Also, within our own research group, additional functional implications of inflammation-regulated NGCs were discovered. Endothelial SEMA3F for example, which is regulated both by inflammatory cytokines but also hemodynamic factors, regulates the endothelial barrier, while activation of SEMA3F signaling impairs migration of TNFα-stimulated monocytes (21). NTN4, which is decreased upon exposure to inflammatory cytokines, was shown to be important for endothelial homeostasis as it acts as an anti-senescence and anti-inflammatory factor, therewith contributing to vascular health (22).

While not being increased directly by (pro-)inflammatory cytokines, the observation of increased expression of EPHB2 in monocytes differentiating to macrophages in combination with EPHB2 being located on a myocardial infarction locus, led to our investigation of EPHB2 in atherosclerosis. Although EPHB2 has been shown to be expressed in atherosclerotic plaques before (4), our study adds a plaque burden-dependent increase of EPHB2 in atherosclerosis, which was mainly found in monocytes and macrophages. Since reduced levels of EPHB2 led to decreased monocyte adhesion and migration, independent from its EphrinB ligands and via phosphorylation of focal adhesion kinases, also EPHB2 can contribute to atherogenesis by promoting monocyte accumulation.

Besides expanding the knowledge of the functional consequences of NGCs vascular (patho)physiology, also more translational steps have been made in linking NGCs to human pathology. For example, NTN1 levels in human plasma were found to be associated to atherosclerosis (23-25). Not only did NTN1 plasma levels (negatively) correlate to arterial wall inflammation, but subjects with (subclinical) atherosclerosis had significantly lower plasma levels of NTN1 compared to subjects without atherosclerosis. NTN1 plasma levels were also negatively correlated to plaque volume, further strengthening the association between low levels of NTN1 with the initiation and progression of human atherosclerosis (25). In this thesis we were able to link a genetic variant found in the NTN1 gene, to (premature) atherosclerosis. The mutated NTN1 protein not only promoted monocyte adhesion but also blocked smooth muscle cell migration and inhibited macrophage egression. This study confirmed previous findings that NTN1 is important in preventing the initiation and progression of atherosclerosis. The observed genetic variant of NTN1 described here is much less sufficient in providing this anti-atherogenic effect and could be causative of a (premature) atherosclerosis phenotype, further strengthening the association between NTN1 and atherosclerosis.
A genetic variant found in EPHB4 and described in this thesis, also showed potential of being responsible for a premature atherosclerotic phenotype as it distorted ligand-receptor interactions. Using a novel co-culture model, we showed distorted endothelial cell-pericyte interactions, resulting in a decrease in endothelial barrier function. These results could ultimately result in impaired vascular integrity and increased vascular leakage, promoting atherogenesis. Despite the fact that we found implications of this genetic variant in atherogenesis, the data is still too preliminary to make any firm conclusions about the precise contributions of (the genetic variant in) EPHB4 on

atherosclerosis and additional studies are needed to confirm its causation of the premature atherosclerosis phenotype.
Furthermore, using various machine-learning methods, expression of the NGCs SEMA6B, SEMA6C and EPHA2 in circulating monocytes have been shown to be of predictive value to cardiovascular disease, proposing NGC profiling as a potential strategy to predict the development of cardiovascular disease (26). Overall, the past couple of years, we and others have proven functional involvement of NGCs in vascular biology and have strengthened the association between NGCs and their implications in vascular (patho)physiology.

New challenges and future perspectives
Despite the growing understanding of NGC function in the vascular system, many questions remain and several hurdles need to be taken before NGCs could be considered as therapeutic targets to prevent and/or alleviate cardiovascular diseases such as atherosclerosis. One of the challenges lies within the large size the NGC family and its expression in multiple cell types. With our screen for inflammation-regulated NGCs we aimed to specify which NGCs are of importance to atherogenesis. Although this led to further investigation of PLXNA4 and EPHB2, still many of the other genes that were regulated remain to be investigated. A better insight in the contribution of all the different NGCs but also their potential interplay with other ligands and receptors is of great interest to further specify the function of NGCs in vascular biology. This immediately proposes another challenge, namely the interchangeability of ligands and receptors. Almost all NGC ligands can bind multiple receptors with different affinities e.g. EphrinB2 can bind all EPHB receptors but also EPHA4 (27) and SEMA3A can bind PLXND1 as well as several PLXNA receptors (28). This interchangeability clearly complicates distinguishing between the separate effects of different receptors and ligands. The differences in ligand-receptor binding affinities stresses the importance of (local) concentrations of both ligands and receptors for determining the particular effect it will have on a cell. NTN1 for example only prevents leukocyte adhesion at a certain concentration. Higher dosages lead to a shift in receptor-ligand interactions negating the anti-migratory effect of NTN1 at lower dosages (chapter 6 of this thesis).

To complicate things even further, NGCs can induce signaling in more ways than just the traditional ligand-receptor interaction that conveys a signal into the receptor bearing cell known as forward signaling. Several NGC ligands can also convey outside-to-inside signals and induce so-called ‘reverse signaling’. More recently some NGCs have been described to function ligand-independently and crosstalk between NGC

signaling and other signaling systems like e.g. Akt can occur. Induction of signaling can occur both in *trans* (signaling between cells) and in *cis* (signaling within cells). In some cases, signal induction results in cluster formation. The composition of a certain cluster with different ligand/receptor combinations influences the ultimate effect on downstream pathway activation and cellular effects (28, 29). In addition to the many ways of signal induction, much is still unknown or incompletely understood about the downstream pathways of NGC signaling. NGCs have a broad range of downstream binding partners and signaling pathways. For example, EPH receptors have several phosphorylation sites as well as a protein tyrosine kinase domain and a PDZ domain domains and can associate with several effectors proteins, including Src family kinases and Ras/Rho family of GTPases (30). The same holds for PLXN receptors that contain an intracellular GTPase activating protein (GAP) domain as well as a Rho GTPase binding domain (RBD) that can regulate a large variety of small GTPases (28). Although it is quite a challenge, a better understanding of (individual) NGC signaling and its associated downstream targets would greatly advance the progress being made in better understanding NGCs and their implications in vascular pathophysiology.

Another challenge lies within good and representative models to conduct translational research. Studying vascular disease is complicated due a general lack of these models. Human vascular material is generally difficult to obtain and when available often only in the end-stages of disease. Functional vascular research therefore primarily relies on *in vitro* and *in vivo* models. *In vitro,* the use of different cell types, different models, different concentrations and ways of ligand/receptor presentation (e.g. coating, soluble, clustered) hampers the interpretation of the precise contributions of, in this case, NGCs in vascular (patho)physiology. Besides these technical differences, the use of endothelial cells in a 2D setting without being exposed to natural components such as hemodynamics and mural cells complicates its translatability to the human situation. The same holds for animal models. The most often used animal model in atherosclerosis is the mouse model (31). As mice naturally do not develop atherosclerosis, these mice need to be genetically modified to enable the development of 'artificial' atherosclerotic plaques. How well these unphysiological circumstances still relate to the human situation can be debated. Therefore, in this thesis we used families with premature atherosclerosis and verify the effect of genetic variants found in NGC genes. Although this research is still rather based on *in vitro* work and direct human observations are lacking, it at least links patient-derived observations to functional consequences. With the upcoming field of 3D cell culture and increasing knowledge on culturing of patient-derived (stem)cells, hopefully soon, more physiologically relevant

studies can be performed to further strengthen the association between NGCs and human (patho)physiology.

Despite these remaining challenges we did show functional implications and a potential as new therapeutic target for EPHB2, PLXNA4, NTN1 and EPHB4 in vascular biology and atherosclerosis. Inhibition of EPHB2 in monocytes could prevent excessive trans-endothelial migration of monocytes and subendothelial accumulation of macrophages preventing plaque formation and/or destabilization. Ensuring a certain level of PLXNA4 in endothelial cells, protects the endothelial barrier and prevents against vascular leakage of e.g. lipids and immune cells and the formation of atherosclerotic plaques. Availability of NTN1 at atheroprone regions in the vasculature, also could protect against excessive trans-endothelial migration of monocytes and plaque formation. EPHB4 could be important to stabilize vascular structures and maintaining a stable endothelial barrier, preventing against vascular leakage and plaque formation. If in the future cell specific targeting or local distribution of the protein or an inhibitor of the protein could be warranted with e.g. an protein releasing stent or targeted antisense oligonucleotides, these NGCs are still potential promising therapeutic targets for vascular disease and should definitely not be dismissed as regulators of vascular (patho)physiology.

Conclusion

In this thesis I have shown that expression of NGCs, in human vascular cells is influenced by inflammatory conditions. We have confirmed a role for PLXNA4 and EPHB2 in endothelial cells and monocytes, respectively, in vascular (patho)physiology and linked genetic variants in the EPHB4 and the NTN1 gene to dysfunctional proteins that could contribute to (premature) atherosclerosis. Overall, this thesis demonstrates that NGCs are widely involved in postnatal vascular biology and strengthens the association between NGCs and atherosclerosis.

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