TR3 nuclear orphan receptor in cardiovascular disease
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Chapter 8

General discussion
Introduction

Atherosclerosis, (in-stent) restenosis and vein-graft disease are common vascular disorders that involve excess proliferation of smooth muscle cells (SMCs) in the arterial wall. Accordingly, many therapeutic approaches aim at the inhibition of SMC proliferation. In our search for genes involved in atherogenesis we identified a family of transcription factors, being the Nerve Growth Factor Induced-B (NGFI-B) family comprising TR3, MINOR and NOT. In this thesis we demonstrate an important role for this family in SMC proliferation in a range of vasculo-proliferative diseases. We established that the nuclear receptor TR3 is involved in inhibition of SMC and endothelial-cell proliferation and affects the expression of proteins involved in regulation of the cell cycle. As the cell cycle is a final common pathway in cellular proliferation, proteins of the cell cycle have emerged as logical targets for the treatment and prevention of vascular hyperplasia. Pharmacologic activation of TR3 in vascular cells may provide a novel therapeutic approach to retard vascular disease by influencing downstream genes in regulation of the cell cycle and potentially other cellular processes.

TR3-like factors in apoptosis

Evidence accumulated over the past decade indicates that TR3-like factors are involved in important physiological processes. For example, the facilitating role of TR3 in T-cell receptor mediated T-cell apoptosis is well documented. A dominant-negative variant of TR3, which lacks the transactivation domain, inhibits T-cell hybridoma apoptosis and protects thymocytes against antigen-induced apoptosis in vivo. Furthermore, it was shown in human prostate cancer cells that TR3, in response to apoptotic agents, specifically translocates to the mitochondrial membranes to induce mitochondrial membrane permeabilization. Recombinant TR3 has been shown to induce release of cytochrome C when added to purified mitochondria. It should be emphasized that this specific feature of TR3 is independent of its DNA-binding domain as is illustrated by a mutant of TR3, which lacks the DNA-binding domain and consequently acts as a dominant-active variant but still induces cytochrome C release. In the cell types that we studied, no induction of apoptosis upon overexpression of TR3 was observed. Furthermore, the effects of TR3-like factors on SMC and EC proliferation are mediated through DNA binding, which is demonstrated by the counteracting activity of the dominant-negative variant (ΔTA).

Orphan nuclear receptors and ligands

The superfamily of nuclear receptors comprises 83 transcription factors grouped in 7 subfamilies that regulate gene expression in a ligand-dependent manner. Nuclear receptors activate or repress target genes by binding directly to DNA-response elements as homo- or
heterodimers or by binding to other classes of DNA-bound transcription factors. These activities have been linked to the formation of complexes with molecules that appear to serve as co-activators or co-repressors, causing local modification of chromatin structure in order to regulate expression of their target genes.

Many of the nuclear receptors are ligand-activated transcription factors that act as the cognate receptors for steroid, retinoid, and thyroid hormones. In addition to these well-characterized endocrine hormone receptors, there are a large number of orphan receptors of which less is known about the nature and function of their ligands. The task of deciphering the physiological function of these orphan receptors has been aided by a new generation of genomic technologies. Through application of chemical, structural, and functional genomics, several orphan nuclear receptors (such as PPARs, LXR and RXR) have emerged as pharmaceutical drug targets for the treatment of important human diseases. However, until now, no ligands have been identified for TR3, MINOR and NOT and the mechanism of co-activation and ligand-independent trans-activation remains unclear. In several studies it has been described that these nuclear receptors are ligand-independent transcription factors. Wang et al. recently crystallized and resolved the 3D-structure of the ligand-binding domain of NOT and concluded that it contains no cavity in the region usually occupied by ligands. Furthermore, these structural data revealed that NOT lacks a “classical” binding site for the LXXLL motif of co-activators. By contrast, in a recent study it was found that the amino-terminal transactivation domain of MINOR harbors an AF-1 domain which is essential for co-activator recruitment.

6-Mercaptopurine (6-MP), the active metabolite of azathioprine (Imuran) is a nucleotide analogue that selectively blocks de novo DNA synthesis in proliferating cells. 6-MP is widely used at relatively low dose as an immunosuppressive drug in inflammatory bowel disease, organ transplantation and rheumatoid arthritis. Recently, it has been discovered that this drug enhances the transcriptional activity of MINOR and NOT, and to a lesser extent that of TR3, in an AF1-dependent manner. The ability of 6-MP metabolites to regulate the activity of a class of transcription factors broadens the possible mechanisms of action of this drug. In our laboratory, we investigated the effect of 6-MP in relation to TR3-like factors in vein-graft disease and demonstrated that 6-MP inhibits stretch-induced DNA synthesis in cultured SMCs. We propose that the underlying mechanism of the 6-MP effect involves the activation of endogenous TR3 activity (Chapter 5). These data encourage us to embark on studies in which the effect of relatively low doses of 6-MP on atherosclerosis in mice will be assessed.
General discussion

Regulation of TR3 expression

TR3-like factors are strongly induced by a variety of stress stimuli in multiple cell types. In summary: the function of TR3 is well-described in T-cell apoptosis and in the HPA axis, NOT is especially well-studied in brain and MINOR was recently shown to be essential in early mouse embryogenesis. Our studies implicate, for the first time, involvement of TR3, MINOR and NOT in atherosclerosis. In our current working model, the successive events in the development of atherosclerosis are formulated by the "response to injury" theory. In this theory, oxidative stress and subsequent cellular damage are potential noxious events that induce expression of TR3, MINOR and NOT. However, the exact upstream triggers leading to TR3 expression in vascular cells are unknown. So far, we have identified supernatant of oxidized LDL-stimulated macrophages, serum, cyclic mechanical strain and tumor necrosis factor (TNF) a as important stimuli for expression of TR3-like factors in vascular cells. Interestingly, a recent paper confirmed our results showing that TNFα treatment induces TR3 expression in endothelial cells and MINOR expression in SMCs. Recently, TR3 was shown to be induced in a pluripotent mesenchymal embryonic mouse cell line, C3H/10T1/2a by using a discovery screen specifically designed to identify targets genes of the Hedgehog transcription factor. Members of the Hedgehog family are potent secreted morphogens, involved in developmental processes and in cancer. Genes downstream of Hedgehog are thought to regulate cellular growth and differentiation. Whether Hedgehog signalling also plays a role in atherosclerosis, similar to cancer, remains to be established. Future research will also incorporate studies to decipher upstream events preceding TR3 expression in atherosclerosis to identify the signalling pathways involving endogenous protective TR3-mediated responses.

Genes induced downstream of TR3

In this thesis, a first perception of the function of TR3 in vascular cells is given, but more experiments are required to elucidate its exact mechanism of action. We believe that the identification of downstream targets of TR3 in genome-wide transcription profiling experiments are crucial to reach this goal. We initiated such experiments and our data provide further insight into molecular mechanisms in TR3 regulation of the cell-cycle (Chapter 6). Specifically, we showed that adrenomedulin and protein kinase C-delta (PKCδ) may be important effector genes of TR3. Both genes have been shown to inhibit SMC proliferation in vivo and to decrease SMC-rich lesion formation. Whether TR3, MINOR or NOT each specifically target a defined subset of downstream genes or that these genes display substantial functional redundancy with regard to which protein actually activates a defined
target promoter remains to be elucidated. Amino-acid sequence comparisons of the DNA binding domains of these factors, and to a lesser extent of the ligand-binding domains, demonstrate evolutionary conservation of these domains among the subfamily members. The amino-terminal activation domain, however, shows far less homology between the family members, allowing the binding of distinct co-activators/repressors to each factor domain. Moreover, downstream target gene analysis can aid the implementation of TR3 agonists in preclinical models. Potential, unexpected complexities in the (pre)clinical development of target-based therapies may (in part) be revealed beforehand by target gene identification. Possible strategies for targeting TR3-like factors and progression of TR3 modulators to the clinic will be subject of future studies.

Involvement of $p27^{kip1}$ in cell-cycle arrest and inhibition of migration

One of the important discoveries described in this thesis that TR3 is involved in regulation of cell-cycle arrest both in endothelial cells and SMCs. Previous studies have demonstrated the protective effect of the cyclin-dependent kinase (CDK) inhibitor $p27^{kip1}$ against atherosclerosis and restenosis. Deletion of the $p27^{kip1}$ genes, in an apolipoprotein E-null genetic background, enhances arterial cell proliferation and accelerates atherogenesis compared with apolipoprotein E-deficient mice with intact $p27^{kip1}$ genes. The therapeutic effects of $p27^{kip1}$ may result from suppression of both proliferation and migration. In the search for potent inhibitors of SMC proliferation and migration several compounds were shown to inhibit SMC proliferation. One of these compounds is rapamycin (Sirolimus), which is reversibly in coated on stents, which results in reduction of in-stent restenosis from 30% to less than 5% in large clinical trials. Rapamycin-mediated inhibition of SMC proliferation is, like reduction of SMC hyperplasia by TR3 overexpression, also associated with upregulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$ and vice-versa lack of $p27^{kip1}$ reduces rapamycin-mediated inhibition of SMC migration and proliferation. We have shown that rapamycin does not affect TR3 expression in SMCs and, consequently, we concluded that both rapamycin and TR3 upregulate $p27^{kip1}$, although through distinct signaling pathways (data not shown in this thesis).

Interestingly, as mentioned above, $p27^{kip1}$ is also involved in the inhibition of migration of SMCs. Although we did not show that TR3 inhibits migration, the murine restenosis model used in this thesis (i.e. the carotid artery ligation model) in part relies on migration of SMCs from the media to the (neo)intima. It is tempting to propose that TR3 is also involved in migration and it would be of interest to explore this possibility in future experiments.
Rupture-prone atherosclerotic plaques are characterized by a thin fibrous cap, containing numerous macrophage-derived foam cells and few SMCs. Since TR3 decreases SMC proliferation, it may be anticipated that a lower number of SMCs in the fibromuscular cap is related to plaque destabilization. Overexpression of TR3 and consequent inhibition of SMC proliferation may be undesirable under these circumstances. However, TR3 activation may contribute to inhibition of post-intervention SMC-proliferation (restenosis, vein-graft disease). Therefore, the effects of TR3 overexpression in mice in an apolipoprotein E-null genetic background will be investigated.

Not only in SMCs, but also in endothelial cells, p27Kip1 inhibits proliferation and migration.\(^{19}\) We show expression of TR3-like factors in endothelial cells overlying atherosclerotic plaques (Chapter 4). Furthermore, we established that TR3 overexpression in endothelial cells results in p27Kip1 upregulation and cyclin A downregulation. We concluded that in endothelial cells TR3 induces also anti-atherogenic properties, similar to SMCs. However, to appoint the anti-atherogenic properties of TR3 in endothelial cells \textit{in vivo}, dedicated experiments in mice should include overexpression of TR3 in endothelial cells using an endothelial cell-specific promoter like Flt-1 or Tie-2. Finally, mice expressing TR3 in an endothelial cell-specific way should then be crossed into a ApoE-/- or ApoE3 Leiden genetic background to determine the role of TR3 in diet-induced atherogenesis.

**Vascular reactivity of vessel segments overexpressing TR3**

Data in this thesis show no influence of TR3 overexpression on aorta- and small resistance mesenteric arteries reactivity to vasoactive substances (Chapter 7). Moreover, no difference in hind-limb perfusion was seen when TR3- or dominant-negative variant (ATA) overexpressing mice were subjected to femoral artery occlusion. In conjunction with recently published data\(^{21}\), we investigated the effect of TR3 overexpression or full inhibition on small resistance arteries. In knock-out mice of yet another nuclear receptor ROR\(\alpha\) markedly altered vascular function was observed in mesenteric arteries, while there were no changes on vascular function of large arteries.\(^{20}\) ROR\(\alpha\) has been shown to play a major role in vascular biology. Mice lacking ROR\(\alpha\), and fed a high-fat diet, develop more atherosclerosis than wild-type mice. In conclusion, no such role for TR3 on vascular reactivity could be established, which observation will aid possible future clinical implementation of TR3 agonists.
Conclusion

Our studies implicate that TR3-like factors may have a significant protective function in cardiovascular disease. TR3 is expressed in atherosclerosis, in-stent restenosis and vein-graft disease. Moreover, we demonstrated that TR3 inhibits vascular SMC proliferation \textit{in vitro} and \textit{in vivo}. TR3 is a transcription factor and may regulate multiple genes and consequently distinct cellular processes, which assigns TR3 as an attractive target for intervention. TR3 agonists, such as the recently discovered 6-MP, may mediate protective changes in the expression of several proteins that are implicated in the endogenous defense mechanisms during the pathogenesis of atherogenesis.
General discussion

References


