Thiopurines and inhibition of Rac1 in vascular disease
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CHAPTER 7

General Discussion
GENERAL DISCUSSION

The aim of this thesis is to explore the function of immunosuppressive drug azathioprine by inhibition of GTPase Rac1 in different inflammatory diseases. Metabolites of azathioprine have a beneficial impact on aneurysm development and progression, vascular inflammation and on gut epithelial cells, which are diseased in IBD. Many other immunosuppressive drugs are targeting mainly the immune cells, which is indeed also shown for azathioprine and its metabolites, which inhibit T-cell activation and inhibit macrophage activation in a Rac1 dependent and independent manner. In addition, other cell types are targeted in a Rac1 dependent fashion by 6-MP and 6-T-GTP, the main metabolites of azathioprine. Moreover, we show that 6-T-GTP inhibits Rac1 by docking to a groove on the Rac1 surface and thereby inhibiting interaction with a Rac1 activating GEF. These findings shed light on (part of) the actual function of azathioprine, which has been used in the clinic for over 60 years without this knowledge.

Positive effect of Rac1 inhibition in the vasculature

The role of Rac1 and other Rho GTPases, RhoA and Cdc42 guiding various cellular processes in the vasculature is intricate and diverse. As discussed in Chapter 2, Rac1 is in control of NOX enzyme activation and subsequent ROS generation, pro-inflammatory gene expression, fibrosis, proliferation and migration, maintenance of functional endothelial barrier and guidance of leukocyte transmigration. Unbalanced Rac1 activity has been implicated in many vascular pathologies such as hypertension, vascular leakage, angiogenesis, atherosclerosis and aneurysm formation. This thesis in part, provides evidences for Rac1-mediated endothelial cell involvement in aneurysm onset and propagation, by promoting the influx of inflammatory cells into the vessel wall. Macrophage influx is often depicted as a primary trigger for aneurysm formation and progression. In animal models of AAA development, whether we talk about CaCl2-, elastase- or AngII-induced aneurysm formation, accumulation of macrophages within the medial layer of the aorta represents a common denominator for initial as well as for advanced stages of the disease. This is one of the reasons why many of the approaches in treatment of aneurysm formation were based on anti-inflammatory drugs, which almost always resulted in effective prevention of AAA in these animal models. We showed decreased aneurysm formation, and to a lesser extent decreased aneurysm progression by using the immunosuppressive drug azathioprine. In response to angiotensin-II treatment, endothelial cell activation and macrophage influx was observed in the aorta (Chapter 3). In addition, we demonstrated that azathioprine and its metabolites show Rac1-inhibition and Rac1-independent anti-inflammatory effects in endothelial cells (Chapter 4) and macrophages (Chapter 5). In human AAA patients a limited number of (anti-inflammatory) clinical trials have been performed; in the most recent meta-analysis, statins have been described to modestly diminish aneurysm growth, while ACE-inhibitors and doxycycline increased aneurysm growth unexpectedly. An ACE-inhibitor does not only decrease AngII-mediated signaling via the AT1R, but also via the AT2R. Signaling via the latter receptor has been suggested to be beneficial to prevent aneurysm formation in various murine AAA studies, thus should not be inhibited although later experiments in AT2R deficient mice downsize the relevance of this receptor. Statins are very effective against arterial inflammatory disease atherosclerosis, and have been shown to reduce Rac1 activation amongst other pleiotropic anti-inflammatory effects (Chapter 2). Still, the statin effect on aneurysm progression is not overwhelming in human AAA patients. In addition, the pleiotropic anti-inflammatory effects of doxycycline did not prevent aneurysm
growth, but promoted it. In line with these data, Lindeman et al.\textsuperscript{14} presented a case study in which a patient with AAA had a sudden increase in aortic dilatation upon immunosuppressive therapy after kidney transplantation. In addition, in 18 patients with abdominal or thoracic aneurysms, the aneurysm dilatation rate was increased 2-fold after transplant operation and the start of immunosuppressive drugs\textsuperscript{15}. Similarly, in the Blotchy mouse aneurysm model, aortic rupture occurred upon anti-inflammatory glucocorticoid treatment\textsuperscript{16}. These data suggest that not all anti-inflammatory strategies may be helpful against aneurysm growth.

The lack of success in translating anti-inflammatory drug regimens to effective pharmaceutical strategies in human AAA patients may also be attributed to the limited number of progression studies in animal models. As observed in Chapter 3 of this thesis, we conducted a prevention and progression study, and indeed it was more difficult to influence already existing aneurysms with the immunosuppressive drug azathioprine. The aneurysm severity score was mainly decreased in the progression experiment due to less secondary aneurysm development, while the primary AAA did not decrease in size after (only) 3 weeks treatment. In another progression model, mice develop AAA for one month and are then treated for an additional 2 months. This strategy showed that doxycycline, which prevented AAA in all animal models, did not reduce aneurysm progression in already existing AAA\textsuperscript{17}. It illustrates that preventing AAA may be a different process than inhibiting AAA progression, and probably requires promotion of vascular repair responses, rather than inhibition of inflammation per se. In that light, the finding that SMC proliferation was important to reduce AAA formation\textsuperscript{16} may be considered a repair response to compensate for SMC loss in the medial layer of the aorta. Interestingly, it is also known that 6-MP, a metabolite of azathioprine, inhibits SMC proliferation\textsuperscript{19,20}, which may be considered detrimental in reducing AAA progression. Thus, the combined effect of a drug on all the different cell types of the inflamed vascular wall will probably determine if the drug is able to promote vascular repair.

Recently, more AAA progression studies are performed in mice, revealing that renin inhibitor Aliskiren has potential to reduce AAA growth\textsuperscript{21}. Fenofibrate, a drug commonly used to reduce low-density lipoprotein and triglycerides levels, and increase high-density lipoprotein concentrations, was reported to inhibit AAA progression\textsuperscript{22}. Inhibition of pro-inflammatory Cyclooxygenase-2 by Celecoxib, leads to reduced AAA incidence and severity\textsuperscript{23}. In addition, it was recently demonstrated that Celecoxib application also effectively reduces progression of AngII-induced AAA, by preserving a “healthy” SMC phenotype\textsuperscript{24}. Also reduction in testosterone levels was linked with arrested progression of AngII-induced AAA, since castration of male mice, which are more prone to AAA development, led to reduced aortic lumen expansion\textsuperscript{25}. Despite the modest effect of azathioprine on AAA progression, azathioprine and its downstream metabolites very effectively prevented AAA formation by inhibiting transendothelial migration of macrophages. Interestingly, both in endothelial cells and macrophages, 6-MP and 6-T-GTP inhibit Rac1-mediated JNK activation, which seems an important Rac1-mediated signaling route (Chapter 3-5). In other cell types, such as epithelial cells and fibroblasts\textsuperscript{26,27} Rac1-mediated JNK activation has been reported. However, in the gut epithelial cells, the JNK pathway was not the prominent Rac1-mediated signaling route upon activation (Chapter 5). In those cells, STAT3 signaling was influenced strongly, and Rac1 inhibition by 6-MP and 6-T-GTP resulted in decreased epithelial proliferation, as we also observed in SMC\textsuperscript{19,20}. Rac1-mediated STAT3 activation has also been reported in endothelial cells and cardiomyocytes\textsuperscript{28,29}. Rac1-mediated proliferation is an important issue in
cancer, which is a field with an interest in Rac1 inhibitors. The long history of azathioprine use in the clinic has shown that it is relatively safe to use this drug chronically, and thus shows that moderate Rac1 inhibition over a long period of time is feasible.

Interestingly, the fact that 6-MP and 6-T-GTP have anti-inflammatory properties beyond Rac1 inhibition in macrophages and epithelial cells is illustrated by the observation that not all anti-inflammatory effects, such as reduced cytokine expression, are inhibited with Rac1 inhibitor NSC23766 (Chapter 5). NSC23766 inhibits the interaction of Rac1 with GEF Trio or GEF Tiam-1. For endothelial cells we have not demonstrated yet whether all anti-inflammatory effects of 6-MP and 6-T-GTP are exclusively regulated through Rac1 inhibition, or other GTP-dependent pathways. Based on the macrophage and epithelial cell results, it seems that there are other GTP-dependent pathways influenced by 6-T-GTP that decrease the activation status of these cells. This implies that the search for additional GTP-dependent mechanisms that modulate inflammation and are blocked by 6-T-GTP is still relevant. Poppe and colleagues\(^1\) already excluded an effect of 6-T-GTP on the Rho GTPase family members RhoA and CDC42. However, they did demonstrate that 6-T-GTP inhibits Rac2\(^1\). Since 6-T-GTP, NSC23766 and Rac1 inhibitor EHop-016 bind in the same groove on the Rac1 surface (Chapter 6), and it has been shown that EHop-016 inhibits Rac3\(^30\), we hypothesize that 6-T-GTP may also inhibit this third Rac member.

Apart from GTP dependent pathways, the use of azathioprine also results in the formation of 6-T-AMP, 6-T-ADP and 6-T-ATP, which have not been studied yet. Azathioprine decreases normal purine synthesis in endothelial cells\(^31\). We have also observed this phenomenon in endothelial cells (data not shown) as well as in monocytes\(^32\). Such metabolic changes may influence the cells and turn them from physiological into pathological cells\(^33\). The potential anti-inflammatory effects of azathioprine via the AMP pathway deserves future research.

Finally after 60 years of azathioprine use as immunosuppressive drug in the clinic, it has become clear that part of its working mechanism is via inhibition of Rac1 in multiple cell types, via direct binding to Rac1 and preventing the Rac1-GEF binding that is required for Rac1 activation. Understanding this working mechanism will drive development of more specific drugs to inhibit Rac1 or other GEF-dependent GTPases.
Reference List


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