Inflammatory actions of chemokines and extracellular versicles in pathological tissue remodeling

Appendix

Valorisation

According to the Dutch Research Council 'Valorisation is the process of creating value from knowledge, by making knowledge suitable and available for societal or economic application and by transforming it into products, services, processes and new business.' To put it simply, valorisation is a process in which interaction between theory and practice plays an important role. There is another very valid reason to valorise. The research is financed with public funding. It is therefore logical that researchers share their results with their financers, such as taxpayers (commit-nl.nl). The work presented in this thesis aimed to investigate fundamental basic processes such as cellular communication in the clinically relevant context of cardiovascular diseases (CVDs). It is estimated that 17.9 million people die each year of CVDs what accounts for 32% of all deaths worldwide (WHO; June, 2021). The underlying cause of CVDs is atherosclerosis, a disease that is characterized by a slowly progressing lesion formation of the arterial wall. These slowly growing plaques expand gradually, tend to calcify and are prone to rupture. This may lead to a complete occlusion of the vessel, resulting in myocardial infarction or stroke[1]. The basic research, focused on understanding the mechanisms of CVD is important and can help to develop new treatment strategies and may lead to an earlier detection. Most clinical tools that are currently available diagnose at a late stage i.e. vascular calcification is diagnosed overdue, when only treatment is focused on resolving the symptoms, rather than curing the disease.

Platelets are usually known as the key players in thrombosis and hemostasis and their pro-inflammatory role in the vasculature is understudied. In this thesis the role of three platelet-derived chemokines in the vessel wall remodeling (CXCL4, CXCL4L1 and CCL5) was investigated. During atherosclerosis CXCL4 interacts with VSMCs, endothelial cells and leukocytes. In this thesis we show the functional differences between two structurally similar variants of platelet factor 4, which is often neglected in the literature, therefore expanding our knowledge on how platelet-derived molecules modulate the cells in the vasculature. Also, we hypothesize that CXCL4 neutralization could potentially hamper or even decrease calcification of the blood vessels, as indicated by our results, but this aspect of our research needs to be more thoroughly investigated. However, it is important to remember that such interventions may lead to thrombosis or bleeding, since most probably they would affect platelet function as well. Besides VSMCs, CXCL4 interacts also with studied in this thesis endothelial cells (ECs), which are important players in the development of atherosclerosis. It has been shown that CXCL4 may play a pro-atherogenic role by interacting with endothelial cells and monocytes[2] and its neutralization inhibits neutrophil extracellular traps (NETs) formation in ANCA-associated vasculitis[3].

The other investigated chemokine – CCL5 (RANTES) is a model chemokine of relevance to a myriad of diseases. CCL5 can have detrimental effects via the recruitment of immune cells that enhance inflammatory processes[4]. Studies in humans and murine models have demonstrated a central role of CCL5 in the formation of atherosclerotic plaque[5]. In this thesis we showed that endothelial cells rapidly and actively internalize CXCL4 and CCL5 and observed the interdependency between the uptake of CXCL4 and CCL5, which may imply the existence of overlapping pathways for both chemokines in ECs. The investigation of the mechanism of endocytosis, involvement of receptors and downstream signalling pathways is the first step to understand the onset of the disease.

It is known that CXCL4 and CCL5 tend to form heteromers that facilitate leukocyte recruitment[6,7,8]. Blocking those interactions attenuates vascular remodeling processes e.g. atherosclerosis, aortic aneurysm, lung injury and myocardial infarction[7,8,9,10,11]. Interestingly, our observations imply that after the uptake into ECs, CCL5 and CXCL4 appear to be directed to the nucleus, and CXCL4 seemed to accumulate in the nucleolus. The significance of these findings is currently unknown, but it is tempting to speculate on an active involvement of these

chemokines in transcriptional processes. Even though the relevance of these results needs to be further characterized, this adds a further dimension to the cell regulating activities of the chemokine system.

The knowledge of platelet-derived chemokines can provide valuable directions for the development of novel strategies, such as anti-chemokine therapy for various medical indications (i.e. cancer). CXCL4 displays potent anti-angiogenic activity that results in diminished tumour growth and metastasis in vivo by inhibition of ECs[2]. In contrast to CXCL4, CCL5 is rather associated with the promotion of cancer (e.g., prostate and breast)[4]. However, experiments in mice showed that neutralization of CXCL4[12] and CCL5[13] may be of therapeutic potential in colorectal cancer treatment. The understanding how CXCL4 and CCL5 modulate ECs seems to be of high clinical relevance, because it might have a direct effect on cancer patients as well.

The chemokine signalling network consists of more than 50 chemokines and 20 chemokine receptors. This network is redundant in nature, implying that some chemokines can bind to several receptors and vice versa[14]. Chemokine receptor antagonists represent an extremely fruitful intervention therapy in the treatment of inflammatory and autoimmune diseases[15]. However, despite a great therapeutic potential, the success rate of the clinical trials is low. Currently only three drugs directed against chemokine system reached the market: (i) plerixafor (cancer treatment); (ii) maraviroc (HIV treatment); and (iii) mogamulizumab (lymphoma treatment). Of note, phase 3 trials are ongoing to evaluate many other promising potent candidates[14]. Therefore, a more detailed understanding of the biology of chemokines and their receptors in homeostasis and disease is clearly required to enable their targeting with greater efficacy.

After successful blocking/neutralization experiments in mice, researchers hypothesize that blocking CCL5 may be of therapeutic benefit for Parkinson's disease[16] and colorectal carcinoma[13]. Blocking CXCL4 protects against chronic liver allograft dysfunction (CLAD) by reducing liver fibrosis[17] and has beneficial effect on alopecia[18] as well as colorectal cancer re-growth[12]. On the other hand, CXCL4 and CCL5 can either suppress or enhance HIV replication, which is dependent on the ability to self-aggregate and bind to glycosaminoglycans[19,20].

In the context of a recent COVID-19 pandemic, CXCL4 and CCL5 gained even more attention. It was recently reported that autoantibodies to CXCL4 contribute to thrombotic thrombocytopenia, which occasionally occurs during COVID-19 or after vaccination. Although the reported incidence remains very low, if left untreated, it can be debilitating or even fatal[21,22,23]. The mechanism of autoantibodies production in COVID-19 resembles the process that occurs in well-characterized heparin-induced thrombocytopenia (HIT). In regards to CCL5, it was recently reported that blocking CCL5-CCR5 interactions may reduce inflammation and viremia levels in critical COVID-19 patients[24]. However, in some circumstances, anti-CCL5 treatment might exert unwanted effect i.e. by exacerbating other viral infections[25].

Thus, it becomes more evident that platelets play an important role in various physiological processes, beyond thrombosis and hemostasis and can be considered as the inflammatory/immune cells as well. Taken together, above mentioned reports highlight the importance of platelet-derived chemokines in various CVD pathologies, immunology, cancer as well as virology.

Additionally, the role of extracellular vesicles (EVs) in the development of liver disease was investigated in this thesis. Non-alcoholic fatty liver hepatitis (NASH) is the main cause of the liver disease in the western world[26] that may lead to fibrosis and cirrhosis[27,28]. During the disease development, stress responses in hepatocytes and Kupffer cells promote the differentiation of hepatic stellate cells[29] that are central in the development of liver fibrosis. Here, we described the role of EVs from steatotic hepatocytes in the modulation of stellate cells. EVs are small cell fragments (up to 1000 nm), carrying a specific cargo that may be transported

by the bloodstream to distal areas of the organism and influence the behaviour of the recipient cells. Our results showed that hepatic cells released more EVs after treatment with fatty acids and that the chemotactic migration of stellate cells was increased specifically towards FA-EVs. Also, prolonged incubation of stellate cells with FA-EVs induced the expression of proliferation markers and a myofibroblast-like phenotype. We propose that EVs are operational intercellular communicators that provoke pro-fibrotic responses in stellate cells. Moreover, while most of the studies investigating the effect of EVs derived from FA-treated hepatocytes focused on macrophages[30,31,32], our study characterized for the first time the consequences of FA-treatment on hepatic stellate cells. These results will help to understand the mechanisms of intercellular communication in the development of NASH and liver fibrosis. Thus, future investigation of this process may lead to novel therapeutic intervention as well as improved diagnostics through analysis of plasma EV number and content.

Recently, the EV field gained considerable interest. The possibility of isolating EVs from different biofluids makes EVs valuable biomarkers. The concept of a "liquid biopsy" emerged in the field. In this context, EVs may serve as circulating biomarkers with a potential role in the detection of the early stages of various diseases[33]. The application of EVs as immunotherapeutics for cancer is promising, especially the use of EVs derived from immune cells, however clinical trials have shown that using immune cell-derived EVs alone is often insufficient to induce an effective immune response in vivo[34]. EVs are also novel candidates for drug delivery systems because of their high bioavailability, exceptional biocompatibility, and low immunogenicity[35]. Mesenchymal stem cell-derived EVs (MSC-EVs) as therapeutics were shown to have beneficial outcomes in a variety of chronic liver disease models[36]. For example, MSC-EVs exerted anti-fibrotic and anti-inflammatory effects in a rat model of chronic liver fibrosis[37] and ameliorated cirrhosis in chronic rat liver injury[38].

Even though EVs have a growing future as biomarkers, drug delivery systems, or therapeutic targets, there remain milestones to be achieved on the path to their clinical application. There are also some technical issues concerning standardization to be resolved. The application of the study of EVs in a daily clinical setting requires suitable technologies and quality controls that could be managed by the hospital itself or delegated to specific facilities. For that, close collaboration between clinicians from hospitals, the biotech industry, and basic researchers is necessary to turn what is currently an idea into a product[39].

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