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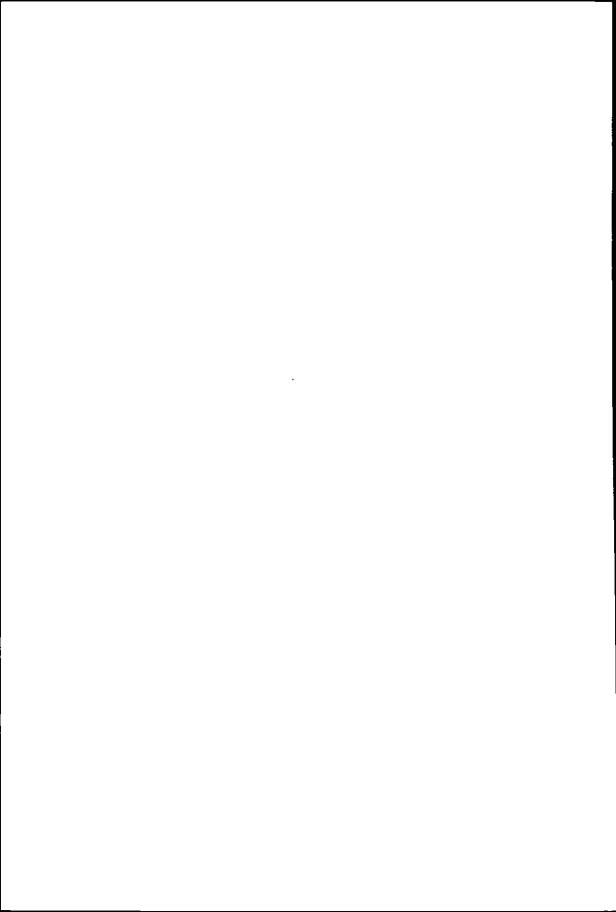|	
Type	PhD thesis
Title	Chemokine receptor signals: role in migration, invasion and cancer metastasis
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Summary

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Metastasis, the spread of tumor cells through the body, is the main cause of cancer deaths. The metastatic process consists of a number of steps. Cancer cells detach from the primary tumor, enter blood or lymphatic vessels, and then extravasate and invade into a distant organ, where they grow out to form a secondary tumor. Part of the metastatic process, the migration of cells from the blood into tissues, resembles the movement of lymphocytes into inflamed areas. This movement is regulated by small proteins, called chemokines (chemoattractant cytokines). Chemokines are produced in inflamed areas, but some of them are also constitutively expressed in hematopoietic and non-hematopoietic organs. Our hypothesis at the start of this research project was that the dissemination of tumor cells to these organs is directed by these constitutively expressed chemokines. The first goal of the research described in this thesis was to demonstrate this involvement of a chemokine receptor in metastasis of T cell lymphomas. Secondly, we studied the signaling pathways activated by chemokines, and required for lymphoma metastasis. The last goal was to determine whether chemokine receptors are also involved in metastasis of carcinomas and, if so, which signaling pathways are involved.

In **chapter 1**, a general introduction is given about chemokines. Chemokines bind to seven-transmembrane G-proteins coupled receptors. All chemokine receptors are briefly discussed, with emphasis on CXCR4 and CXCR5. G-protein and other signal pathways, induced by chemokines, are described. The normal physiological function of the chemokines is discussed, as well as their role in diseases such as AIDS and cancer. In cancer, chemokines are involved not only in metastasis, but also in the influx of leukocytes into the tumor and in angiogenesis, the formation of blood vessels in the tumor.

The research described in **chapter 2** aimed to demonstrate the involvement of a particular chemokine receptor, CXCR4, in the dissemination of T cell lymphomas. As a model, we use a T cell hybridoma, which was generated by fusion of non-invasive lymphomas and invasive activated T cells. The T cell hybridomas metastasize to many tissues. This metastasis is blocked by an inhibitor of G_i proteins, indicating that a G-protein coupled receptor is involved, and we assumed that it was a chemokine receptor. The main candidate receptor was CXCR4, since it is expressed on the T cell hybridoma and its ligand, CXCL12, is present at the sites of metastasis. To generate CXCR4-deficient cells, an "intrakine" method was used: CXCL12, fused to an endoplasmic reticulum (ER) retention signal, KDEL, was expressed in the hybridoma. This CXCL12-KDEL is retained in the ER by the KDEL receptor. The CXCL12-KDEL also binds to CXCR4, which is consequently also sequestered in the ER, so that the cells have no CXCR4 on their surface. These cells indeed do not migrate towards CXCL12, but they do migrate towards another chemokine, CCL17, which binds to another chemokine receptor, CCR4. They are also no longer able to invade into fibroblast monolayers, an in vitro model for the invasion of these cells into tissues in vivo. Moreover, in mice injected with these cells, no metastases are formed. These results demonstrate an essential role for CXCR4 in the dissemination of T-lymphomas.

The dissemination and invasion induced by the chemokine CXCL12, is a complex process that can be divided into two separate steps. Firstly, a signal induced by the chemokine results in the activation of the integrin, LFA-1. Secondly, the binding of the activated integrin to its ligand ICAM-1 results in the amplification of the signal by activating additional LFA-1 molecules. The first signal depends on G_i proteins and the second signal on the tyrosine kinase ZAP-70. In **chapter 3** we show that another heterotrimeric G-protein, $G_{q/11}$, is involved in the first signal. Furthermore, the small GTPases Cdc42 and RhoA are needed for the activation of LFA-1 induced by the chemokine CXCL12. Cdc42 is also required for migration towards high concentrations of chemokine, which is not dependent on the activation of LFA-1. Remarkably, actinomyosin contraction is only required for the activation of LFA-1 induced by the chemokine, and not for the second amplification signal.

The α-subunits of the heterotrimeric G-proteins play a role in migration and invasion, as discussed above. The Gβγ dimer, however, may also promote migration because it can activate phospholipase C (PLC) and phosphatidylinositol-3-kinase (PI3K). These signaling molecules are required for the migration and invasion of the T-cell hybridoma. The Gβγ dimer can, however, also cause downregulation of the signal, by inducing desensitization and internalization of the receptor. In **chapter 4** we block the function of the Gβγ dimer in the T cell hybridoma. We show that this leads to enhanced migration and persistent invasion, due to reduced desensitization and

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internalization. This implies that the Gβγ dimer is not required for the activation of PLC and PI3K. Indeed, PLC can also be activated by the α-subunit of $G_{\alpha/11}$ which is involved in CXCL12-induced chemotaxis. Strikingly, migration of the Gβγ-blocked cells was independent of PI3K, whereas migration of control cells was strongly reduced by PI3K inhibitors. This shows that PI3K is not essential for chemotaxis, as has been proposed, based on results obtained with other cell types. In the hybridoma cells, it is only required when the CXCR4 signal is rapidly desensitized.

Migrating cells display intense exocytosis of vesicles at the front of the cells, which is considered to be necessary for the supply of membrane and signaling molecules. Vesicle fusion is mediated by SNAREs. A v-SNARE on the vesicle membrane interacts with two t-SNAREs on the target membrane. They form a stable complex that promotes the association of the vesicle with the membrane. In neurons, the fusion of vesicles with neurotransmitter is blocked and the vesicles remain docked at the membrane awaiting a calcium signal. The influx of calcium immediately results in vesicle fusion, which is regulated by the calcium-sensor synaptotagmin. Since the response to chemokines is rapid, we hypothesized that the vesicles necessary for migration should also be docked at the membrane. We proposed that their release is regulated by synaptotagmin and occurs immediately after a stimulus arrives. In **chapter 5** we show that the T cell hybridomas indeed express the calcium-sensor synaptotagmin. When its function is blocked, migration is completely inhibited. Moreover, overexpression of synaptotagmin enhances migration, suggesting that the amount of endogenous synaptotagmin is a limiting factor. Finally, we found complexes of v- and t-SNAREs in the T cell hybridomas. Since such complexes are formed when vesicles dock at the membrane, this result indicates that docked vesicles are present. The complexes are dissociated when fusion is induced by calcium influx, but also when cells are treated with the chemokine CXCL12. We propose that docked vesicle fusion, regulated by the calcium sensor synaptotagmin, is essential for chemokine-induced migration.

In the last two chapters we focus on a different tumor cell type, colon carcinoma. In **chapter 6** we show that the chemokine receptor CXCR4 is also essential for metastasis of this tumor type. However, the role of CXCR4 is clearly different from that in the dissemination of the T cell hybridoma. In contrast to the T-cell hybridoma, metastasis of the colon carcinoma does not depend on G_i proteins, which are essential for CXCR4-induced migration and invasion. Furthermore, whereas the T-cell hybridoma expresses CXCR4 constitutively, the levels of CXCR4 are very low on the colon carcinoma cells in vitro. However, the levels are strongly upregulated in vivo, but only after the cells have invaded. Therefore, we conclude that CXCR4 is not necessary for invasion, but required at a later stage, most likely to promote proliferation of the tumor cells. Indeed, CXCR4-deficient cells did colonize the lungs to the same extent as control cells and survived, but did not expand, whereas control cells did grow out. This suggests that CXCR4 inhibitors have potential as anti-cancer agents to suppress outgrowth of micrometastases.

In **chapter 7** we describe our surprising finding that the chemokine receptor CXCR5 is expressed on colon carcinoma cells. This is remarkable since CXCR5 was so far only found on lymphocytes. Similarly as for CXCR4, described in chapter 6, the surface levels of CXCR5 are low in vitro, but upregulated in vivo. The role of this chemokine receptor in metastasis remains to be established, but we found that the chemokine that binds to this receptor, CXCL13, promotes proliferation of CXCR5-expressing colon carcinoma cells. Furthermore, CXCL13 was found to be present in the metastases, so that it can potentially promote proliferation of the metastatic tumor cells.

The results described in this thesis show that the chemokine receptor CXCR4 and its ligand, CXCL12, play an essential role in the dissemination of T cell lymphomas as well as colon carcinomas. CXCR5 and CXCL13 may also promote proliferation in carcinoma metastases, but this remains to be established. Finally, the elucidation of the signaling pathways underlying this complex process will contribute to a better understanding of the dissemination of tumor cells. In particular, differences in metastatic capacity between individual tumors may be explained by the expression of these chemokine receptors and/or the many components of these signal transduction pathways. Finally, the receptors and signal pathway components are potential targets for therapeutic invention.