Mechanisms of Ewing sarcoma metastasis : biochemistry and biophysics

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Summary

The first description of cancer dates back to the ancient Papyrus Ebers in 1500 B. C. The term 'metastasis', the uncontrolled cell growth at distant sites, was first introduced by Lobstein in the beginning of 19th century. In 1889 Stephen Paget suggested a predisposition for sites of metastasis based on 735 fatal cases of breast cancer in multiple reports. He introduced the 'seed and soil' concept, where 'seeds' are the cancer cells with metastatic capacities, and 'soil' is the tissue or organ providing the proper microenvironment for growth. To date this concept is widely accepted with a stunning amount of molecular insights, which have led to a better understanding of both the 'seed' and the 'soil'.

Ewing sarcoma, discovered in 1921, is a neoplasm in the bone, which forms metastasis even at an early stage of tumor development. The development of metastasis is correlated with poor patient prognosis and, hence, low long-term survival rate. Interestingly, Ewing sarcoma metastasis exhibits a well-defined distribution along the body. Ewing sarcoma metastasis is predominantly restricted to the lungs, followed by bone/bone marrow localization. In some cases simultaneous combined lung/bone/bone marrow metastasis localization have been reported. Metastatic sites in other locations rarely occur. As such, the microenvironment of Ewing sarcoma metastasis can be either that of the primary tumor (bone) or it can be drastically different (lung). Hence, the consistency of Ewing sarcoma metastasis localization implies a cellular predisposition.

Many studies attempted to obtain an understanding of the mechanism underpinning the metastatic behavior of Ewing sarcoma. The metastatic capacity was found to be correlated with the expression of the chemokine receptor CXCR6, the G-protein coupled receptor GPR64, increased level of interleukin 6, Caveolin 1, IGF-1R and an enhanced activity of the metalloproteinases 2 and 9. A special role was attributed

to the chemokine receptor CXCR4, which is overexpressed in metastatic Ewing sarcoma, and is regulated by the Ewing sarcoma-specific fusion protein EWS/FLI1. EWS/FLI1 was previously shown to modulate the architecture and integrity of the cytoskeleton, which is viewed as further prerequisite for ES metastasis. Although a significant amount of data is available, a comprehensive model of Ewing sarcoma metastasis is yet to be developed. In this thesis the biochemical and biophysical factors involved in Ewing sarcoma metastasis are further explored.

In **Chapter 2** a single-molecule imaging technique was applied to examine the dynamics of the chemokine receptor CXCR4 on the plasma membrane of an Ewing sarcoma-derived cell line (A673). The CXCR4 mobility on the plasma membrane was detected at 30 nm positional accuracy and 50 ms temporal resolution. This temporal and positional resolution enabled distinguishing different mobility fractions/states of CXCR4 and following the transition between the mobile and immobile state of the receptor upon different conditions. Immobilization of CXCR4 occurred upon stimulation with its ligand CXCL12 in a concentration-dependent manner. The receptors' mobility change during activation appeared to be dependent on its signaling both through G-protein dependent and independent pathways. Signaling through G-proteins resulted in CXCR4 immobilization potentially into a supramolecular scaffold (signalosome), which could then enhance signaling. In contrast, activation of the G-protein independent pathway caused receptor immobilization into clathrin-coated vesicles leading to its internalization and, thus, receptor desensitization. As signaling through G-proteins appeared to interfere with receptor endocytosis, the findings of this study indicate a functional cross-talk between different biochemical cascades.

The involvement of G-protein activation in regulation of CXCR4 was further addressed in the study described in **Chapter 3**. The dynamics of two G_α subunits was examined in A673 cells. Both $G_{\alpha q}$ and $G_{\alpha i}$ exhibited a fast response on activation of CXCR4 by the formation of cluster-like structures. Single-molecule imaging revealed that the two G_α subunits follow a different coupling mode with the receptor. $G_{\alpha q}$ appeared pre-coupled to the receptor and exhibited uncoupling upon receptor activation. In contrast, $G_{\alpha i}$ was not coupled to CXCR4 prior to receptor activation and exhibited elevated coupling with the receptor upon stimulation with CXCL12. Such difference in coupling modes of $G_{\alpha q}$ and $G_{\alpha i}$ suggested a sequential coupling of the G-proteins to

CXCR4. This finding is in agreement with experiments that analyze the time needed for $G_{\alpha q}$ and $G_{\alpha i}$ to initiate downstream cascades. Taken that signaling through $G_{\alpha q}$ is a prerequisite for enhanced $G_{\alpha i}$ signaling, the ability of CXCR4 to subsequently activate both G_{α} subunits might act as a receptor signaling regulatory mechanism.

In **Chapter 4** the development of a light-activated chimeric receptor, usable for high temporally and spatially resolved activation and detection was described. The base construct was developed by exchange of the intracellular loops and C-terminus of the light-activated GPCR, rhodopsin, with the intracellular loops and the C-terminus of the chemokine receptor CXCR4. The cloning steps resulted in a chimeric receptor, optoCXCR4, which presumably was able to initiate CXCR4-specific signaling cascades by light-activation. The optoCXCR4 was further modified on both N- and C-terminals. All the constructs were tested for plasma membrane localization and functionality properties. A successful construct representing a promising tool for further biophysical investigation of chemokine receptor CXCR4 was developed. A key to proper functionality of the construct was the correct light-protection settings during all experimental procedures. The applicability of this promising approach in optogenetics was further discussed.

The involvement of the stiffness of the micro-environment in Ewing sarcoma metastasis was addressed in the study described in **Chapter 5**. The development of Ewing sarcoma in bones with metastasis sites to either bones or lungs, suggested the existence of a mechanical cue for Ewing sarcoma metastasis. This hypothesis was tested using Ewing sarcoma-derived cell lines, which preferred a largely disparate metastatic niche of bone (CHP100) and lungs (6647). In 2D and 3D assays 6647 cells exhibited a strong preference towards the mechanically soft micro-environment. Only on soft substrates 6647 cells were able to develop adhesions and developed an organized actin cytoskeleton. In contrast, CHP100 cells exhibited a higher adhesion potential on stiff substrates. Thus, the mechanical properties of the micro-environment caused a differential behavior of Ewing sarcoma cells of different metastatic profile. It was further evident that this mechanical phenotype was independent of CXCR4 receptor activation.

Taken together, the results presented in this thesis provide deeper insights into the mechanisms controlling signaling of the chemokine receptor CXCR4 and into the role of the micro-environment in Ewing sar-

coma cells behavior. Through various experimental approaches it was shown that both biochemical and biophysical guidance control how Ewing sarcoma develops into its distinct metastatic phenotype.