Chapter 7

Summary and discussion
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Multiple myeloma (MM) is an incurable plasma cell malignancy characterized by a monoclonal proliferation of plasma cells in the bone marrow (BM), associated with osteolytic bone lesions, aberrant angiogenesis and pancytopenia. In the BM, MM cells receive signals to survive and proliferate due to the existence of functional, bi-directional interactions between the MM cells and the other cells in the BM microenvironment. In this thesis, the interaction between the BM environment and MM cells was extensively studied. In particular, we identified novel proteins, N-cadherin and adrenomedullin, contributing to osteolytic bone disease and aberrant MM-induced angiogenesis, respectively. Furthermore, the research presented in this thesis sheds a new light on the role of DKK1 in the progression of MM. Up to now, it was well established that elevated level of DKK1 inhibit osteoblast differentiation and is an important trigger of osteolytic bone disease, contributing to the creation of a niche for MM progression. Interestingly, however, our data revealed that DKK1 has a dual pathogenic role in MM since it can also inhibit tumor growth, by suppressing Wnt signaling in malignant plasma cells. During the progression of MM this inhibitory role of DKK1 is lost as a result of aberrant promoter methylation. In search for additional genetic and epigenetic events involved in aberrant Wnt pathway activation in MM, we uncovered downregulation of CYLD expression as a novel prognostic factor in MM. In particular, we showed that the loss of CYLD enhances the Wnt and NF-κB signaling, sensitizing MM cells to the Wnt and NF-κB ligands. Of note, our data identified downregulation of CYLD as a trigger of malignant plasma cells proliferation and strongly indicates that loss of CYLD enhances MM aggressiveness, through a mechanism involving Wnt pathway hyperactivation.
THE Wnt PATHWAY AND ITS NEGATIVE-FEEDBACK REGULATOR DKK1 IN MALIGNANT PLASMA CELLS

Aberrant activation of Wnt signaling plays a crucial role in the pathogenesis of several types of cancer and is most often caused by mutations in Wnt pathway components such as adenomatous polyposis coli (APC) or β-catenin (CTNNB1). However, although the Wnt pathway is frequently hyperactivated in MMs, pathway intrinsic mutations have not (yet) been found, and the aberrant Wnt activity is the consequence of auto- and/or paracrine stimulation by Wnt ligands. This activation of canonical and non-canonical Wnt signaling promotes dissemination, proliferation, and drug resistance of malignant plasma cells. Interestingly, the malignant plasma cells also express high level of the DKK1, a feedback inhibitor of Wnt signaling. DKK1 can promote bone disease by inhibiting Wnt signaling in osteoblasts thereby preventing their differentiation. This may indirectly promote MM growth, since immature osteoblasts express high levels of IL-6, a central growth and survival factor for myeloma plasma cells. Furthermore, DKK1 enhances the expression of receptor activator of NF-kappa B ligand (RANKL) and downregulates the expression of osteoprotegerin (OPG) in immature osteoblast. The increased RANKL/OPG ratio leads to osteoclast activation promoting osteolytic bone disease. Osteoclasts may also support the growth of myeloma cells through secretion of IL-6 and osteopontin, and by adhesive interactions, stimulating the proliferation of malignant plasma cells. Thus, DKK1 can both exercise paracrine effects on the BM microenvironment, and affect the MM growth by creating an optimal niche for tumor progression.

In chapter 2 we review the literature on the role of the Wnt signaling and DKK1 secretion in MM. In particular, we carefully assess the literature on the potential value of Dickkopf-1 (DKK1) as a new therapeutic target for MM. As shown by several studies, anti-DKK1 antibody treatment can inhibit MM-bone disease in various animal models, strongly suggesting that it might present a valuable therapeutic asset for patients suffering from MM bone disease. Importantly, the data presented in chapter 3 suggest an alternative scenario for the role of DKK1 in the MM. We demonstrate that DKK1 is expressed from the initial phase of the disease onwards and that MM cells are fully responsive to DKK1, confirming that Dickkopf-1 can downmodulates the activity of Wnt pathway in malignant plasma cells. Interestingly, however, in advanced stage MMs and MM cell lines, when the bone marrow independence is acquired, DKK1 expression is lost as a result of aberrant promoter methylation. This observation clearly points to DKK1 as a tumor suppressor for malignant plasma cells. Thus, the data pre-
sented in chapter 3, suggest that therapy based on DKK1 inhibition needs to be carefully monitored since it may not only suppresses the osteolytic bone disease but also promotes the MM tumor growth, especially at extramedullary locations. Indeed, using the 5TGM1 mouse model, it was shown that stimulation of Wnt pathway by lithium chloride significantly increases subcutaneous MM growth.27

LOSS OF CYLD EXPRESSION UNLEASHES Wnt SIGNALING IN MM

The BM environment supports the growth and survival of malignant plasma cells, via adhesion molecules, cytokines and growth factors. Among the different signaling pathways activated in MM cells, by external stimulation, the NF-κB and Wnt pathway presumably are of major importance.15–17,19,20,28–31 Interestingly, the prevalence of aberrant genetic and epigenetic events in NF-κB and Wnt pathway seems to be substantially higher in MM cell lines (MMCLs) compared to primary MMs,28,30–32 which suggests that constitutively active NF-κB and Wnt signaling renders the tumor cells independent of the BM microenvironment. In chapter 4, we focused on cylindromatosis (CYLD) gene; loss and inactivating mutations of this gene were recently described in MM.28,30,33,34 The CYLD gene was initially identified as a tumor suppressor, mutated in familial cylindromatosis and multiple familial trichoepithelioma patients.35 CYLD is a deubiquitinating enzyme acting as a negative regulator of NF-κB and Wnt signaling, by removing lysine-63-linked polyubiquitin chains from NF-κB and Wnt activating proteins.36–38 We established that CYLD acts as an important negative regulator of Wnt and NF-κB signaling in malignant plasma cells. Furthermore, by studying a gene expression data set containing mRNA samples of MMs, we found a significantly lower expression of CYLD in the proliferation subgroup (PR) of MM patients, characterized by poor prognosis and high expression of genes involved in cell growth.39 In support of this observation, introduction of CYLD into UM-3 cells, which lack CYLD expression due to a homozygous deletion, resulted in growth inhibition and increased cell death. Moreover, the proliferation subgroup is characterized by the high expression of Wnt target genes, suggesting that genetic alteration of CYLD expression, together with epigenetic silencing of Wnt inhibitors, contribute to aberrant activation of the Wnt pathway in malignant plasma cells. Importantly, in line with this observation, low expression of CYLD was correlated with inferior progression-free and overall survival in a large cohort of primary MMs confirming that the downregulation or complete loss of this gene plays an instrumental role in the aggressive behavior of malignant plasma cells.
A ROLE OF N-CADHERIN IN MM INTERACTION WITH THE BM MICROENVIRONMENT

In chapter 5 we studied the role of N-cadherin in MM. We observed high N-cadherin expression in 50% of MM patients and MM cell lines. Analysis of N-cadherin in the MM subgroups revealed that it is highly, but not exclusively, expressed in MMs bearing a t(4;14) translocation. This subgroup is characterized by a poor prognosis, but high N-cadherin expression is not an independent prognostic factor. The data point to N-cadherin as an important motility protein in malignant plasma cells. Indeed, in solid tumors overexpression of N-cadherin is part of the switch that occurs during epithelial-mesenchymal transition (EMT). The process of EMT is directly related to cancer invasiveness, reflected by enhanced cell migration and invasion, resulting in metastatic dissemination. Non-motile polarized epithelial cells, embedded via cell-cell junctions, convert into individual, non-polarized motile and invasive mesenchymal cells. However, in MM overexpression of N-cadherin did not affect the (trans-endothelial) migration, and our data suggest that N-cadherin may be involved in the BM retention rather than in dissemination of malignant plasma cells. N-cadherin mediated interaction between MM cells and osteoblasts partially inhibited osteoblast differentiation, suggesting a contribution to osteolytic bone disease. Of note, N-cadherin is expressed by osteoblasts during all stages of the bone formation and N-cadherin-mediated interactions between osteoblasts have shown to be crucial for their differentiation and function. In addition, the N-cadherin mediated adhesion between osteoblast and malignant plasma cells might contribute to another feature of MM, i.e. induction of pancytopenia. Since osteoblasts have a central role in the organization of the endosteal hematopoietic stem cell niche, it is conceivable that overexpression of N-cadherin by malignant plasma cells, facilitates their access to this niche, leading to dysregulation of hematopoiesis and pancytopenia.

THE HYPOXIA TARGET ADRENOMEDULLIN IS ABERRANTLY EXPRESSED IN MULTIPLE MYELOMA AND PROMOTES ANGIOGENESIS

The pathogenesis of MM-induced BM angiogenesis is not yet fully understood. At the verge of progression of MGUS to active MM, elevated levels of pro-angiogenic factors in BM plasma and blood result in an “angiogenic switch”. Since several of the crucial angiogenic factors secreted by MM cells, including
VEGFA and bFGF, are equally expressed by tumor cells from MGUS, smoldering MM and active MM, it has been suggested that the angiogenic switch could be the consequence of increasing tumor burden, rather than aberrant expression of pro-angiogenic factors per se. On the other hand, chronic hypoxia may also play an important role in BM angiogenesis in MM. This is suggested by studies demonstrating that stabilization and nuclear localization of HIF1α affects the transcriptional and angiogenic profiles of myeloma cells, leading to increased expression of VEGFA and IL-8 among other pro-angiogenic factors. In chapter 6, we further explored the role of hypoxia in MM, by studying the transcriptional response of MM cells to low oxygen. Interestingly, by the use of gene expression microarray, we identified the pro-angiogenic factor adrenomedullin as the most highly hypoxia-induced gene in MM cells. Adrenomedullin is involved in blood vessel morphogenesis, vasculogenesis, and tumor angiogenesis. AM stimulates angiogenesis by binding the calcitonin-receptor-like receptor (CRLR), which is widely expressed on normal and hypoxic endothelial cells. Importantly, binding of AM to the CRLR/RAMP2 can also transactivate the VEGFR-2, which is responsible for most pro-angiogenic effects of VEGFA, including the stimulation of endothelial cell differentiation, proliferation, migration and morphogenesis. This AM-induced VEGFR-2 transactivation does not require VEGFA, suggesting that AM can functionally mimic VEGFA, and thereby contribute to MM-induced angiogenesis. Interestingly, however, although AM is a well-established HIF1α target gene, containing HRE sites in its promoter as major regulatory sequences, we observed that several HMCLs and primary MMs also expressed high levels of AM under normoxic conditions. Importantly, these MM cells with normoxic AM expression did not show aberrant basal HIF1α stabilization and displayed no overexpression of other HIF1α/hypoxia target genes, suggesting normoxic regulation of AM expression by HIF1α-independent mechanisms. In line with this notion, our analysis of a large MM gene-expression data set revealed no consistent correlation between AM expression and expression of other hypoxia/HIF1α target genes. These findings imply that mechanisms other than hypoxia can contribute to AM expression in malignant plasma cells, and are consistent with a scenario in which both HIF1α-dependent and independent mechanisms contribute to the “angiogenic switch” in MM. The functional studies presented in chapter 6 strongly support the angiogenic role of AM in MM progression since they demonstrate that forced overexpression and hypoxia-induced AM in MM cells strongly promotes the pro-angiogenic activity of MM cells, as revealed by enhanced endothelial cell proliferation and mesh formation, whereas blockage of endogenously produced as well as hypoxia-triggered AM strongly
reduces the pro-angiogenic activity of MM cells. Thus, the data, demonstrate that MM cells, both in a hypoxia-dependent and independent fashion, aberrantly express and secrete AM, which can mediates MM-induced angiogenesis. This aberrant AM expression could be a major driving force for the angiogenic switch observed during MM progression, which renders AM a novel target for anti-angiogenic therapy in MM.

REFERENCES


