Heparan sulfate proteoglycans in B cell maturation and myeloma plasma cell survival
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General discussion
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Heparan sulfate proteoglycans (HSPGs) are ubiquitously expressed by most cells, and are abundantly found in the extracellular matrix (Bishop et al., 2007; Lindahl et al., 1998). In addition, genetic defects in man and studies with genetically modified animals have indisputably demonstrated the vital role of HSPGs in development, organogenesis, physiology and disease (Bishop et al., 2007; Campos-Xavier et al., 2009; Li et al., 2003). It is therefore remarkable that very little is known about the contribution of HSPGs and their modification by heparan sulfate (HS) modifying enzymes in the development of lymphoid organs or the maturation and differentiation of lymphocytes. Even more so, because nearly all growth factors and chemokines involved in immunology, contain HS-binding domains (Lindahl and Li, 2009).

Modification of heparan sulfate proteoglycans by Glce in lymphoid organogenesis

Already in 1995, Nakato and colleagues have demonstrated, using D. Melanogaster as a model organism, that HSPGs are required for cell division patterning during development (Nakato et al., 1995). In the last two decades, accumulating data on Drosophila provided ample evidence for the reciprocal relation of morphogens (e.g. BMP and WNT) and HSPGs in the patterning of the body axis, regulating organogenesis and wing formation (Penton et al., 1997; Tsuda et al., 1999). In view of that, lymphoid organ development is a complex process that also requires tightly controlled spatio-temporal expression of morphogens, chemokines and angiogenic factors (Manley and Blackburn, 2003; Mebius, 2003; Mebius and Kraal, 2005). Nevertheless, this thesis describes for the first time a function for HSPGs, and in particular HS modification by C5-epimerase (Glce), in the development of lymphoid organs including thymus, spleen, and lymph nodes. Glce causes a release of the conformational constraints of the HS polysaccharide, creating chain flexibility and allowing the access of protein ligands to specific regions of the HS chains (Mulloy et al., 2000). In chapter 2 and 3, we show that this is crucial for binding to HS for an array of morphogens and chemokines, including BMP4, FGF2, FGF10, and SDF-1. Reduced interaction with HSPGs lacking IdoA might therefore cause variation in ligand availability, leading to misregulation of cell fate decisions, which could contribute to the defects we observed in lymphoid organ development. It is therefore surprising that despite the profound abnormalities, once the lymphoid organs are formed, all the expected cell types could be identified and were located at the expected locations in normal numbers. Moreover, we show that transplantation of the Glce-deficient lymph node primordium is capable of attracting lymphocytes and provides a stromal environment with distinct B and T cell areas, sufficient for the generation of class-switched B cells. Apparently, production and presentation of chemokines by the Glce-deficient vessels is not severely disturbed, enabling (wild type) lymphocytes to enter the lymph node via the high endothelial venules. Notably, in chapter 3 we demonstrate that binding of the homeostatic chemokines CXCL13 and CCL21 that guide B and T lymphocytes
to their destined compartment, respectively (Cyster, 2005), is only marginally affected in the absence of IdoA, which could explain their normal distribution within the Glce-deficient stromal environment.

**Glce function in B cell development and plasma cell survival**

Although there is still debate on the contribution and expression of HSPGs in T cells, several laboratories, including our own, have started to reveal a role for HSPGs in B cells (Borgehsi et al., 1999; Garner et al., 2008; Sanderson et al., 1989; van der Voort et al., 2000). Specifically, it was shown that B cells express syndecan-1 during different stages of B cell development (Sanderson et al., 1989), and that early B cells can bind IL-7 via HS, which is an important proliferation inducing cytokine (Borgehsi et al., 1999). In addition, we found that upon simultaneous ligation of CD40 and the B cell receptor (BCR) a very strong increase of cell surface HS expression was induced (van der Voort et al., 2000). Therefore, it is puzzling that conditional loss of EXT1 expression in B cells, by crossing the EXT1\(^{flox/flox}\) mice with CD19-Cre mice, resulted in only minor changes in B cell development (Garner et al., 2008). As discussed by the authors, because only 50-85% of the cell surface HS on the B cells was lost, it is possible that either the remaining amount of HS is sufficient for the selection of B cells, or this selection process may have favored the more normal B cells. The latter is supported by the observation that, in comparison to the immature B cells in the bone marrow, a larger proportion of the B cells in the peripheral blood expressed HS. Nevertheless, there was a tendency towards lower B cell counts in peripheral blood of the mice with EXT1 gene inactivation (Garner et al., 2008). Indeed, in this thesis we show that during B cell development, correctly modified HS is required for the maturation of B cells. In chapter 3 we find that the follicular mature B cell pool in Glce-deficient mice is smaller, which is accompanied by lower basal immunoglobulin levels. Importantly, throughout the lifespan of a B cell, signaling by the BCR is essential, guiding growth, selection, and differentiation. However, over the last years it has become apparent that another major pathway is essential for driving transition into, and survival of follicular mature B cells, that is signals emanating from the interaction of BAFF-R (BR3) with its ligand BAFF (BLyS) (Hoek et al., 2009; Kraus et al., 2004; Mackay and Schneider, 2009; Monroe and Dorschkind, 2007; Stadanlick et al., 2008; Treml et al., 2009).

We demonstrate in chapter 2 and 3 that Glce deficiency can lead to diminished binding of growth factors and chemokines to HS, up to 90%. This could potentially interfere with B cell maturation and/or survival by affecting growth factor signaling. However, since BAFF, which is one of the most potent and crucial mediator in follicular mature B cell survival, has the capacity to auto-multimerize and therefore does not require HSPGs for signaling (Hendriks et al., 2005; Ingold et al., 2005), it seems unlikely that impaired growth factor signaling contributes to the defect we observe in mature B cell survival or maturation. We therefore suggest a novel role for HSPGs in B cell survival. Once matured, naive B cells require signals of the BCR. This BCR signaling is antigen independent, and is known as tonic signaling (Monroe, 2004). HSPGs could affect B cell maturation by interfering with the organization of the BCR signalosome, responsible for generating these tonic survival signals.
potential key player in this scenario is CD19, which possesses a heparan sulfate binding domain (de Fougerolles et al., 2001), and during B cell activation, acts as an adaptor which transiently recruits signaling molecules like Vav, PI3K and Lyn into BCR microclusters (Harwood and Batista, 2010). Altered interaction between CD19 and the abnormally modified HS on Glce−/− B cells could affect CD19 dynamics, leading to defective recruitment of CD19 to the BCR complex. This could result in an imbalance of cell surface molecules determining the outcome of the BCR signal, favoring cell death over cell survival (Monroe, 2006).

Figure 1. Heparan sulfate proteoglycans in B cell maturation and (myeloma) plasma cell survival. Proposed model for the contribution of C5-epimerase (Glce) and EXT1 in the maturation and/or survival of B cells and (myeloma) plasma cells. HSPGs can form microclusters with, amongst others, the B cell receptor (BCR) and CD19. If the HS chains are not properly modified (without Glce modification; Glce−/−) the microclusters cannot be formed to provide the necessary (tonic) survival signal. Normal (bone marrow) plasma cells require Glce for HSPG-dependent binding of APRIL to induce survival signals. Lack of Glce expression renders the plasma cell incapable of APRIL binding. Loss of EXT1 in myeloma plasma cells prevents the cells to accumulate survival factors, including APRIL and SDF-1. See text for more details.

At the end of the germinal center (GC) reaction, a point of divergence is reached at which a GC B cell can differentiate into a memory B cell or plasma cell. After antigen-dependent differentiation, plasma cells migrate to the bone marrow, where they reside in specific niches to maintain protective serum antibody levels (Manz et al., 2005). A key difference between plasma cells and all other B cells is the status of the BCR complex. While the B cells have a cell surface-bound receptor type BCR, plasma cells delete the membrane-associated portion of the BCR,
thereby producing secreted BCRs, better known as immunoglobulins or antibodies. Consequently, plasma cells lack cell surface-associated BCRs, and may therefore be more dependent upon growth factors that provide survival supporting signals (Melchers et al., 1994). Indeed, plasma cells fully depend on the microenvironment for their survival. Importantly, plasma cells are characterized by expression of high levels of the HSPG syndecan-1 (Sanderson et al., 1989). Thus, by engaging soluble factors produced in the plasma cell niche, syndecan-1 can control the survival of plasma cells in the BM. In line with this, it was recently found that a proliferation inducing ligand (APRIL), which is key to the survival of bone marrow plasma cells (O’Connor et al., 2004; Belnoue et al., 2008; Bossen et al., 2008), requires HSPG interaction for effective signaling (Hendriks et al., 2005). Interestingly, in chapter 3 we show that Glce-deficient plasma cells were unable to respond to APRIL-mediated survival signals in vitro, which could be attributed to the strongly reduced capacity of APRIL to interact with HS-moieties lacking IdoA. This suggests that Glce deficiency could impede the longevity of plasma cells, a notion that is supported by the reduced numbers of BM plasma cells, explaining the reduced basal- and antigen-specific antibody levels in Glce−/− mice we observe.

Taken together, we propose a novel role for HSPGs in B cell development, i.e. facilitating the generation of BCR microclusters to induce tonic survival signals, and we demonstrate for the first time that plasma cells require properly modified HS to accumulate soluble factors, e.g. APRIL, for survival (Figure 1).

**EXT1-controlled heparan sulfate expression in myeloma**

A hallmark for the malignant plasma cells in multiple myeloma (MM) is the expression of the HSPG syndecan-1 (Wijdenes et al., 1996). Syndecan-1 is a pleiotropic proteoglycan, which can affect cell fate by strengthening integrin-mediated adhesion via its core protein or by accumulating and presenting soluble factors via its HS side chains (Derksen et al., 2002; Kramer et al., 2003; Morgan et al., 2007; van der Voort et al., 2000). In this thesis we provide unique direct evidence that the cell surface HS chains attached to syndecan-1 are crucial for MM cell growth. By applying RNAi-mediated knockdown of EXT1, the MM cells lose their cell surface HS expression, which results in increased apoptosis and reduced growth. In addition, we show that suppressing syndecan-1 results in the loss of HS on the cell membrane of MM cells, which also impairs cell growth. In support of our observations, others have demonstrated that subcutaneous myeloma tumors treated with bacterial heparitinase, an HS degrading enzyme, caused a reduction in tumor size and significantly interfered with the MM growth. This demonstrates that HS in the microenvironment can support the tumor, regardless of the cell expressing it, since heparitinase also targets non-MM-derived HS, which could be equally important (Yang et al., 2007). Nonetheless, in vitro studies revealed that treating MM cells with heparitinase is sufficient to induce cell death and inhibit cell signaling required for survival (Derksen et al., 2002; Mahtouk et al., 2006).

Furthermore, overexpression of Hsulf-1 and -2, which clip of 6-O sulfate groups on HS chains, resulted in a retarded MM tumor growth in mice as well (Dai et al., 2005). Importantly, as the authors stated, Hsulf activity was only found on the
MM cell surface, and not within the microenvironment, since it is a cell membrane bound protein. As MM cells can shed syndecan-1 into the microenvironment, this could explain why eventually (within seven weeks) in all cases tumors were found, albeit smaller: the shedded syndecan-1 is unaffected by the sulfatase activity and could accumulate in the extracellular matrix to eventually create a sufficiently rich tumor environment. Nevertheless, this study supports the fact that (modified) HS, generated by MM cells, is essential for the growth of MM cells, and that Hsulf-1 and -2 could be potent inhibitors (Dai et al., 2005). Hence, although ample evidence points towards a pivotal role for HS expression by MM, we are the first to demonstrate that cell autonomous expression of HS (EXT1) and not the syndecan-1 core protein per se, is absolutely required for the growth of MM. In our study, MM cells express syndecan-1 without any HS chains. Consequently, syndecan-1 is unable to accumulate growth enhancing and proliferation inducing (soluble) factors, rendering it incapable to support MM growth in the BM (Figure 1).

Besides cytokines and growth factors, another important type of interaction that can control the communication between MM cells and the BM environment is direct cell-cell contact via adhesion- and other cell membrane molecules. Specifically, recruitment of integrins can lead to outside-in signaling, resulting in a series of biological responses, including assembly of focal contact, modulation of cell migration, and proliferation (Katz, 2010). Moreover, adhesion to bone marrow stromal cells (BMSCs) triggers their production of molecules that can modulate growth, survival, and adhesion of MM cells. For example, adhesion of MM cells to BMSCs induces the release of IL6 from the BMSCs. In addition, CD40 triggering on MM cells results in production of IL6 by MM cells. Furthermore, bFGF (FGF2), produced by the MM cells, can also induce IL6 secretion by BMSCs (Bisping et al., 2003; Cauhan et al., 1996). This underscores that even though MM cells are allowed to interact with the BM microenvironment, e.g. via integrins, cadherins, but also the syndecan-1 core protein, it is not sufficient to overcome the loss of HS expression. In chapter 4 we provide evidence that this indeed could be the case, because human MM cells localized in the bone marrow could no longer sustain growth, after the induction of EXT1 knockdown. Furthermore, the loss of heparan sulfate could also sensitize MM cells to drug-induced cell death. Together, the findings in this thesis indicate the HS biosynthesis machinery is a potential novel treatment target for MM (Lindahl, 2007).
Conclusions

Since HS modification consists of a complex series of enzymatic reactions, ultimately leading to a polysaccharide with highly sulfated regions, it is tempting to speculate that each individual modifying enzyme contributes to the generation of specific protein binding domains (Lindahl and Li, 2009). As demonstrated by studies with full knockout mice, specific loss of different HS modifying enzymes leads to distinct, but specific defects in development and organogenesis. Loss of heparan sulfate 2-O sulfotransferase, N-deacetyl/N-sulfotransferase-1, or glucuronyl C5-epimerase results in polysaccharides with aberrant sulfation patterns, which likely determines the strength and outcome of HS-ligand interactions (Bullock et al., 1998; Li et al., 2003; Merry et al., 2001; Ringvall et al., 2000). This is especially interesting, because Bret and colleagues have shown that different B cell subsets have a distinct expression profile of HS modifying enzymes. For example, plasmablasts and plasma cells expressed high levels of heparan sulfate 2-O-sulfotransferase (HS2ST) and EXT1 as compared to memory B cells, whereas HS3ST2 and HS6ST1 expression was higher in memory B cells than in plasmablasts. In addition, compared to normal bone marrow plasma cells, MM cells also display an altered expression pattern (Bret et al., 2009). It is interesting to note that EXT2 was overexpressed in MM compared to normal plasma cells, and patient studies revealed that the overexpression of HS2ST and EXT1 was linked to a bad prognosis (Bret et al., 2009). Together with the studies described in this thesis, it has become clear that the regulation of spatio-temporal expression of HS modifying enzymes is essential in determining the outcome of cell fate, and plays a critical role in a variety of biological processes, including cell adhesion and migration, cell survival, angiogenesis, and tissue remodeling.

References


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