Three childhood malignancies with striking morphologic and phenotypic similarities
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Chapter 7

Concluding remarks & general discussion
Anaplastic Large Cell Lymphoma (ALCL), Hodgkin Lymphoma (HL) and Langerhans Cell Histiocytosis (LCH) are three hematologic diseases that occur relatively frequent in children. To identify genes that are involved in the pathogenesis, Serial Analysis of Gene Expression (SAGE) was applied to generate comprehensive gene expression profiles of the ALCL derived cell line Karpas299, Hodgkin derived cell line L428 and Langerhans cells (LC). Our original goal, i.e. to generate SAGE libraries of laser microdissected tissues of different kinds of LCH entities, was not successful due to difficulties in harvesting sufficient good quality mRNA. Application of the SAR-SAGE procedure, which allows construction of SAGE libraries from as little as 50 ng of total RNA, was successful on good quality RNA isolated from cell lines, but unfortunately not from the laser micro-dissected LCH tissues. As an alternative we generated a SAGE library of LC generated from umbilical cord blood CD34+ progenitor cells (MatTek corporation, Ashland, MA, USA). The recently generated DOR-1 cell line derived from a bone LCH lesion presenting in a 3-year-old girl, might provide a good alternative in future studies. However, the DOR-1 cells exhibited a phenotype, i.e. pleomorphism and consistent immuno-reactivity for CD10 (50%), CD13 (55%), CD68 (65%), and CD117 (70%) while CD1a, Langerin and HLA-DR were not detected, which is not characteristic of LCH lesions.

The first and largest part of this thesis focused on gene expression in ALCL. Chapter 2 provides an overview of the genes upregulated in the neoplastic cells of ALCL as compared to their normal counterpart CD4+ T-cells. Among the upregulated genes are 3 calcium binding proteins S100A10, S100A11 and CALM2. Ca\textsuperscript{2+} released from the ER plays a role in a host of critical signaling reactions, including exocytosis, contraction, metabolism, regulation of transcription, fertilization, and apoptosis. The high expression of 3 calcium binding proteins suggests that altered intracellular signaling might be associated with the oncogenesis of ALCL.

In chapters 3 and 4 we describe a further analysis of two genes, Mcl-1 and TIMP-1, highly expressed in the Karpas299 cell line as demonstrated with SAGE profiling. Mcl-1 was expressed in all ALK\textsuperscript{+} and ALK\textsuperscript{−} ALCL cases whereas the other anti-apoptotic Bcl-2 members, Bcl-2 and Bcl-X\textsubscript{L}, were not or only infrequently expressed in ALK\textsuperscript{+} ALCL, but present in a higher proportion of the ALK\textsuperscript{−} ALCL cases. This strongly suggests that Mcl-1 rather than Bcl-2 or Bcl-X\textsubscript{L} is the main anti-apoptotic protein of the Bcl-2 family expressed in ALCL and in particular in ALK\textsuperscript{+} ALCL. TIMP-1 expression was present in all ALK\textsuperscript{+} ALCLs and generally demonstrated a lower level
of TIMP-1 expression in ALK− ALCLs. Remarkably, TIMP-1 expression was restricted to macrophages in most cases. In the past year one additional study has been performed to identify differentially expressed genes in ALK+ and ALK− ALCL cases using microarray analysis. They demonstrated that several signal transduction intermediates (SYK, LYN, CD37) and cell cycle regulators (cyclin D3, p19INK4D), were overexpressed in ALK+ ALCL, whereas a number of transcription factors were overexpressed in ALK− ALCL (HOXC6, HOX A3). This study was a comparison of ALK+ and ALK− ALCL, whereas we compared ALK+ ALCL derived cell line Karpas 299 with CD4+ T-cells. Different approaches might explain why the genes observed by Thompson et al. were not present in our SAGE library.

SAGE on LC and the subsequence analysis of the highly expressed genes in LCH revealed high expression of actin involved genes FSCN1 and GSN in all LCH cases and of metalloproteinase MMP12 in the majority of the LCH cases. Fascin and Gelsolin may play a role in the movement of LCH cells out of the epidermis while MMP12 was most abundant in multi-system disease LCH which has the worst prognosis, suggesting a role for MMP12 in the progression of LCH (Chapter 5).

Chapter 6 describes the similarities in gene expression between LC and cHL cell lines. We show that HRS cells share many similarities with LC that are not shared with the postulated counterparts CB for many genes. Especially several genes involved in actin modulation (CFL1 and GSN) showed a high expression in LC and cHL. The potential functional importance of these genes in the pathogenesis of cHL warrants further studies elucidating the relevance of these genes. QRT-PCR analysis on a tissue panel of cHL, LCH and ALCL revealed a high expression only in LCH and cHL for 11/13 genes which were not expressed/or lower expressed in ALCL. These genes might represent new markers to discriminate between ALCL and cHL.

ALCL and cHL have immunophenotypical similarities such as the expression of CD30. Stimulation of CD30, however, induces opposite effects since this leads to apoptosis in ALCL but not in cHL. Staber et al. performed gene expression analysis in CD30-stimulated ALCL (Karpas299) and HL (KM-H2) cell lines using cDNA microarrays to identify changes in the transcriptional program responsible for these opposing effects. In HL, there were only minor CD30-specific alterations, whereas ALCL unequivocally showed a pronounced CD30-specific transcriptional response. Ninety-three genes (6.6% of total) were deregulated by more than a factor of two after CD30 stimulation in ALCL cells. The majority of genes identified are involved in cell cycle regulation and apoptosis. The mRNA expression patterns further indicate that
in contrast to HL, CD30 stimulation in ALCL induces cell death via the CD95-CD95 ligand (CD95L) pathway and the TNF-R1/TNF-R2 crosstalk. These data provide a detailed view on the transcriptional changes upon CD30 stimulation and may explain the observed functional differences of HL and ALCL.

In summary, the gene expression study presented in this thesis revealed new insights in the pathobiology of ALCL, LCH and cHL and the results might serve as new starting points for further investigation in the pathogenesis of these hematologic disorders. The most striking finding of this study is the discrepancy between the relation of ALK expression and its downstream targets in ALCL derived cell lines, which appears to be absent in ALCL tissue samples.

**Bcl-2 family members and ALK**

A relation has been described between ALK and the regulation of several anti-apoptotic Bcl-2 family members, e.g. Mcl-1, Bcl-2 and Bcl-X<sub>L</sub> through the activation of signal transducer and activator of transcription (STAT)3 in ALCL cell lines. In chapter 3 we demonstrated that Bcl-2 and Bcl-X<sub>L</sub> are not or only infrequently expressed in ALK<sup>+</sup> ALCL, whereas these genes are present in a higher proportion of the ALK<sup>−</sup> ALCL cases. In contrast, Mcl-1 was expressed in all ALK<sup>+</sup> and ALK<sup>−</sup> ALCL cases. The overexpression of Mcl-1 in ALCL may result from activation of signal transduction pathways including the Jak/STAT pathway in the ALK<sup>+</sup> cases, but it is unclear which mechanism triggers Mcl-1 expression in the ALK<sup>−</sup> cases which generally lack activated STAT3. The complete lack of Bcl-2 and Bcl-X<sub>L</sub> in tumor cells of ALK<sup>+</sup> cases demonstrates that there is no correlation between expression of ALK and that of Bcl-2 and Bcl-X<sub>L</sub>. These data suggest that ALK induced signaling in ALCL derived cell lines is different from the signaling cascade in the tumor cells of ALCL cases.

**TIMP-1 and ALK**

We demonstrated high expression of TIMP-1 in more than 50% of ALCL cases, which is generally produced by macrophages. Despite the higher TIMP-1 expression levels observed in ALK<sup>+</sup> ALCL cases, lack of TIMP-1 expression in the tumor cells of ALK<sup>+</sup> ALCL cases argues against a direct role for ALK induced activation of STAT3 in the regulation of TIMP-1 expression in ALCL cases (chapter 4). Lai et al. demonstrated that ALK-induced activation of STAT3 directly contributes to a high level of TIMP-1 in ALCL derived cell lines. Induction of TIMP-1 through ALK induced STAT3 activation
is, however, not a very likely mechanism in ALCL cases because TIMP-1 expression is usually restricted to the macrophages. It can be speculated that other indirect mechanisms are involved in the induction of TIMP-1 expression in the macrophages of ALCL cases. In response to our publication the group of Lai published a comment to further elaborate on the discrepancy between their and our results. The following part contains our response to their comments.

**Response to comments of R. Lai, V. Leventaki and GZ Rassidakis.**


Based on the general features of anaplastic large cell lymphomas (ALCLs), e.g. tumor cell areas admixed with a variable percentage of macrophages, a careful interpretation of immunohistochemical staining results is warranted. In the case of TIMP-1 expression, which is known to be present in macrophages, application of double staining procedures is a prerequisite to determine the origin of the positively staining cell type reliably. In our recent study, we performed double staining for TIMP-1 with CD30, which identifies the tumor cell population, as well as with CD68, which identifies the macrophages, and noticed that, in most cases, TIMP-1 expression was restricted to the macrophages. These data appear to be in disagreement with a previous study of Lai et al. Whether or not these differences are related to the different antibodies or to the use of paraffin tissue sections in the study of Lai et al., and frozen tissue sections in our study, is not clear. To our knowledge, no studies have been performed to compare the various antibodies on both frozen and paraffin tissue material.

The discrepancy is most pronounced in the ALK+ ALCL cases, which were scored TIMP-1 negative in the vast majority of tumor cells in four of five cases in our study and in only four of 19 cases in the study by Lai et al. In the single positive case in our study, the vast majority of tumor cells demonstrated strong cytoplasmic staining (Rust et al., Fig 2A), whereas in the negative cases, the vast majority of tumor cells lacked TIMP-1 expression (Rust et al., Fig 2B). In contrast, Lai et al. stated that they considered cases to be positive when more than 20% of tumor cells, as identified by morphology, stained positive. The illustrations in their article (Lai et al., Fig 5C and 5D), however, demonstrate only a minority of tumor cells (approximately 20% in 5C and less than 5% in 5D) staining positive for TIMP-1.
This suggests that part of the difference is caused by differences in cut-off levels. In the same ALK+ cases, a vast majority of tumor cells stained positive for pSTAT3 (Fig 5A and B), demonstrating a considerable discrepancy between the percentage of pSTAT3 and TIMP-1 positive cells. Despite the statistically significant correlations reported by Lai et al.\textsuperscript{7} between TIMP-1 expression and presence of ALK and pSTAT3, a substantial number of ALCLs lacked co-expression of ALK and TIMP-1: 4/19 were ALK+TIMP-1− and 5/24 were ALK−TIMP-1+. Co-expression of pSTAT3 and TIMP-1 was also absent in a considerable proportion of the cases: 9/23 were pSTAT3+TIMP-1− and 3/16 pSTAT3−TIMP-1+. Also, pSTAT3 was found in 23% of the tumor cells of TIMP-1− cases. This indicates that despite a statistically significant correlation, there are still several issues to be resolved with respect to TIMP-1 expression in pSTAT3-positive tumor cells in ALCL tissues. Lack of pSTAT3 staining in CD68+ macrophages with TIMP-1 expression restricted to the macrophages (case 2 in Rust et al.\textsuperscript{10}), as illustrated in figure 1 suggests the presence of other pathways leading to induction of TIMP-1.

**Figure 1.** Lack of pSTAT3 and CD68 co-expression in an ALK+ ALCL. Double immunostaining shows that the CD68 (blue)-positive macrophages consistently lack expression of pSTAT3 (red) in case 2 from Ref 1. This case demonstrated TIMP-1 expression only in the macrophages and not in the tumor cells.

In summary, based on the data presented by Lai et al.\textsuperscript{7} and our own data\textsuperscript{10}, we conclude that (1) TIMP-1 positive tumor cells are generally absent or only present in small numbers in ALCL cases, in contrast to most ALCL cell lines; and (2) in addition to ALK-induced phosphorylation of STAT3, other mechanisms may also contribute to the induction of TIMP-1 expression.
Chapter 7

References


