Antigen receptor triggering and apotopic pathways in neoplastic B cells
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CHAPTER 7

General discussion
INTRODUCTION

The primary novel findings of the studies described in this thesis involve the functional consequences of BCR triggering and the identification of aberrations in the apoptosis pathways of B-CLL cells. The studies described in chapter 4 show that the distinct response induced after BCR triggering of B-CLL cells correlates with IgV_H mutation status and provide a link between BCR triggering and the sensitivity to spontaneous or drug-induced apoptosis. In addition, expression of ZAP-70 strongly correlates with the unmutated IgV_H status of patients (chapter 4). A comprehensive analysis of both anti-and pro-apoptotic regulators via the novel RT-MLPA technique identified both protective and sensitising aberrations as the cause for disturbed apoptosis in B-CLL. The anti-apoptotic balance may be tipped via activation of the p53-responsive Puma-mediated apoptosis pathway. The differential activation of this pathway associated to the IgV_H mutation status (chapter 5). Together, these studies might provide clues to the differential clinical outcome among B-CLL subgroups. The implications of our findings are discussed in light of recent studies on BCR triggering, apoptosis regulation and novel developments on B-CLL. Finally, our data will be discussed with respect to emerging strategies on novel anti-cancer therapeutics.

APOPTOSIS REGULATION AND BCR RESPONSIVENESS OF B-CLL CELLS ARE ASSOCIATED TO IgV_H MUTATION STATUS

In B-CLL p53-dysfunction has been correlated with IgV_H mutation status,\textsuperscript{72,84} in addition to adverse clinical outcome and poor response to therapy.\textsuperscript{64,65} Our data associated both the differential functional outcome of BCR triggering (chapter 4) and the differential capacity to induce the p53-responsive apoptosis pathway (chapter 5) to the IgV_H mutation status. Together, these data imply that the differential clinical outcome of B-CLL patients might be related to a different capacity to signal via the BCR and/or to activate the p53-mediated pathway.

It is not precisely known how signals mediated by the BCR interfere with activation of the p53-mediated pathway in B-CLL cells. Differences in BCR-responsiveness of B-CLL patients have been associated with differential signaling via both Syk and ZAP-70.\textsuperscript{215,216} Whereas a previous study found differential Syk protein expression associated to BCR signaling capacity,\textsuperscript{96} we did not observe a difference in Syk protein expression in relation to IgV_H mutation status (chapter 4). Although a strong correlation of ZAP-70 expression and IgV_H mutation status was detected, direct involvement of ZAP-70 with regard to functional consequences of BCR triggering remains to be shown. It appears possible to transfec-t CLL B cells ex-vivo by nucleofection to introduce RNA-interference (RNAi) constructs inducing a targeted deletion of Syk and ZAP-70. In this way, the contribution of these kinases can be investigated.
ZAP-70 AS A B CELL LINEAGE MARKER

ZAP-70 as a surrogate marker for IgVH mutation status
Although previously ZAP-70 has been reported as a kinase solely expressed by T cells and NK cells, it has now been shown that ZAP-70 is also expressed in unmutated IgVH CLL B cells (chapter 4) and correlates with their unfavorable clinical outcome. Determination of IgVH mutation status is quite laborious. It thus has been proposed that ZAP-70 might serve as a surrogate marker for IgVH mutation status in predicting the prognosis for the individual patient. Our studies showed that in CLL ZAP-70 could be reliably detected via multi-color FACS-analysis in combination with CD5 and CD19 indicating that this technique can easily be incorporated in clinical analysis (chapter 4). Exceptions in the association of ZAP-70 and IgVH mutation status have been detected in 5-10% of patients both detecting false-positive and false-negative cases. Among 18 patients tested, we detected one unmutated patient which was ZAP-70-negative and one mutated patient which was ZAP-70-positive (11%). Thus, use of ZAP-70 as a sole surrogate marker for IgVH mutation status is not completely reliable. The BCR activation gene signature in IgVH unmutated patients also has been correlated with high gene expression of the activation induced c-type lectin (AICL), a protein with unknown function. Our investigations of AICL expression in both IgVH subgroups via semi-quantitative PCR confirmed these observations (W. Mackus, J. Hamann and E. Eldering, unpublished observations). In addition to ZAP-70, quantification of AICL expression might be included in clinical analyses of CLL patients.

ZAP-70+ B cells as the normal counterpart of unmutated IgVH B cells
Syk and ZAP-70 are essential for lymphocyte development and signal transduction via their immune receptors. Previous studies using mice deficient for ZAP-70 and Syk showed that both kinases are essential in the early stages of T cell development. Only recently, the same redundant function of ZAP-70 in B cells has been reported. Syk+ mice show a partial block at the pro- to pre-B cell stage of B cell development and fail to induce further maturation of B cells. Unexpectedly, deletion of both Syk and ZAP-70 resulted in a complete block in early B cell development, suggesting the requirement not only of Syk but also of ZAP-70 in the B cell lineage. Indeed, ZAP-70 expression has been detected by RT-PCR analysis in some B cell lines representing early stage in B cell development. We are currently investigating via multi-color FACS analysis whether ZAP-70-positive B cells are present in normal B cell populations. Next, analysis of IgVH mutation status on sorted B cells expressing this phenotype will be required to designate such cells as potential representatives of the normal counterpart of unmutated IgVH B-CLL cells.
Chapter 7

APOPTOSIS REGULATION OF B-CLL CELLS

Introducing a model for apoptosis regulation in B-CLL
Using the RT-MLPA technique a comprehensive overview of the expression of a specific set of genes with a particular focus on a cellular response such as apoptosis or inflammation can be obtained\(^\text{237}\). Because a predefined set of genes is targeted by RT-MLPA instead of the whole genome, a trade-off is made between scope of the analysis and ease of interpretation. Although micro array techniques potentially sample the whole transcriptome, in practice functional interpretation is limited when hundreds of genes are differentially expressed. RT-MLPA provides a quick and reliable method to quantify expression of a limited number of genes. A drawback is that RT-MLPA analysis will not identify involvement of unexpected novel genes in the particular response investigated as only known genes are incorporated in the probeset. Thus, RT-MLPA is excellently used complementary to arrays.

We used the RT-MLPA analysis to investigate expression of apoptosis regulators in B-CLL. The observed aberrations provide an explanation for the block in both apoptosis pathways in B-CLL (chapter 5). Nevertheless, it remains possible to eliminate B-CLL cells via apoptosis. Our studies strongly indicated a major role for BH3-only genes Bmf, Noxa and Puma in apoptosis regulation of B-CLL cells. Recently, various reports have suggested different models for the function of BH3-only proteins\(^\text{30,187,205,300-302}\). A plausible model was proposed by the group of Korsmeyer suggesting that BH3-only proteins either act as sensitizers/activators of apoptosis\(^\text{204}\). Sensitizing BH3-only proteins bind Bcl-2-like members blocking their ability to sequester Bax-like proteins and as such sensitize for apoptosis. Activating BH3-only proteins induce Bax oligomerization via direct binding to Bax leading to cytC release. The recent findings combined with our new data are incorporated in the following model regarding apoptosis regulation in B-CLL (Fig. 1).

The in vivo characteristics of circulating B-CLL cells are represented in Fig. 1A. B-CLL cells express high levels of the anti-apoptotic protein Bcl-2\(^\text{106,107}\) as well as of apoptotic BH3-only members Bmf and Noxa. In circulating cells the apoptotic capacity of these latter proteins is rather limited. Being bound to the cytoskeleton Bmf is trapped in an inactive complex\(^\text{244}\) and the high amounts of Noxa located at the mitochondria\(^\text{231}\) are insufficient to occupy the excess of Bcl-2. Mitochondrial membrane integrity is preserved by the surplus of Bcl2 complexed to Bax-like proteins and the non-apoptotic mode of the B-CLL cell is maintained.

When B-CLL cells undergo spontaneous and cytostatic drug-induced apoptosis modifications in the expression and location of apoptotic regulators are induced (chapter 5) as implicated in the second part of our model (Fig. 1B). Upon in vitro culture Bmf might dissociate from the cytoskeleton and translocate to the mitochondria. Simultaneously, the p53-responsive BH3-only member Puma is upregulated which is profoundly enhanced upon incubation of cells with cytostatic drugs. After translocation to the mitochondria Puma binds to Bcl-2 and induces Bax-mediated cytC release. Due to the high availability of these apoptotic proteins, only a minuscule trigger is required to set off massive apoptosis induction. This trigger allegedly is mediated by the cytostatic drug-induced switch from growth
General discussion

Figure 1. Apoptosis regulation in B-CLL
Based on our observations regarding the aberrant expression of anti-and pro-apoptotic regulators and the functional consequences of BCR triggering in B-CLL cells a model for the regulation of apoptosis in B-CLL is proposed.

(A) In circulating B-CLL cells Bmf is sequestered to the cytoskeleton (1) and Noxa is located at the mitochondria (2). High levels of Bcl-2 preserve mitochondrial integrity by binding of Noxa and Bax blocking their apoptogenic capacity (3).

(B) Upon in vitro culture Bmf is activated, dissociates from the cytoskeleton and translocates to the mitochondria (4). Bmf and Noxa start with occupying the reservoir of Bcl-2 (5). In vitro culture and especially cytostatic drug treatment induce a switch from p53-dependent growth arrest to Puma upregulation (6). Puma translocates to the mitochondria and either discharges, together with Bmf and Noxa, the surplus of Bcl2 from Bax, or, directly complexes with Bax, mediating cytC release resulting in execution of apoptosis (7). BCR triggering might provide a direct link in the inhibition of p53-responsive upregulation as suggested by the protective effect on spontaneous and drug-induced apoptosis in cells of IgVH unmutated patients (8).

arrest to p53-responsive Puma upregulation (Fig. 1B). As the surplus of Bcl-2 is absorbed by Bmf, Noxa and Puma, Bax-like proteins are discharged from their anti-apoptotic family members mediating the release of cytC. So far, it remains elusive whether Puma can directly activate Bax-mediated cytC release or is dependent on coordinated sensitizing actions of Bmf and Noxa.

Although initial studies on Noxa as well as several reviews describe that Noxa is under transcriptional control of p53 our data do not support these findings. In contrast to the strong induction of Puma, expression of Noxa was not enhanced after cytostatic drug treatment of B-CLL cells. RT-MLPA analysis of T cells
activated in a p53-independent manner show induction of high Noxa mRNA levels (unpublished observations within our laboratory). Furthermore, thymocytes from Noxa<sup>−/−</sup> mice do not display altered responses to radiation (A. Strasser, oral communication). Together these results indicate that Noxa is not involved in p53-dependent apoptosis.

**Future directions on apoptosis regulation in B-CLL**

Based on the above considerations several suggestions for further investigations can be made. Bmf is known to be unleashed by loss of cell attachment (anoikis), allowing it to translocate to the mitochondria and bind Bcl-2-like proteins<sup>244</sup>. It can be hypothesised that in case of B-CLL, due to lack of contact with specific factors in the microenvironment, in vitro culture mimicks an anoikis-like signal resulting in dissociation and subsequent translocation of Bmf. Thus, differences in subcellular localisation need to be monitored in B-CLL cells. The high levels of Bcl-2 may be required to bind Bmf and Noxa to prevent their apoptogenic actions, indicating the importance to investigate if and when these proteins interact with each other.

It is as yet not known according to which model Bmf, Noxa and Puma function, but some predictions can be made. Our data suggest that Puma is most important in initiating the final execution of apoptosis. Puma may act as a sensitizing BH3-only protein displacing Noxa or Bmf from Bcl-2, or directly activates the Bax-like proteins<sup>204</sup>. Targeted disruption by introducing RNAi-constructs via the nucleofection method will allow monitoring the requirement of these proteins for the induction of apoptosis. These effects should be investigated comparing viable and apoptotic CLL B cells.

Additionally, survival signals mediated by cells in the CLL microenvironment such as CD40 triggering might influence the apoptosis regulators. Using RT-MLPA, differences in the apoptosis gene expression profile of B-CLL cells stimulated by different survival signals can be monitored. The obtained information can be used to extend the experiments on targeted deletion of apoptosis regulators.

**FUTURE STRATEGIES ON NOVEL ANTI-CANCER THERAPEUTICS**

Recently, the applicability of particular compounds that specifically target the abnormal cell death pathway of cancer cells has been investigated. By restoring the apoptosis pathway at a point downstream of the molecular defect chemoresistance can be overcome which might have important implications in cancer therapy strategies. Several classes of compounds which possibly could fullfill this function have been presented: (1) small peptides mimicking the BH3-only proteins or even the BH3 domains<sup>204</sup> (2) compounds that directly target Bcl-2- or Bax-like proteins<sup>205,303,304</sup>, and (3) compounds promoting apoptosome formation via cytC and Apaf oligomerization<sup>302</sup>. All these compounds aim at attacking cancer cells without harming normal cells.

Hypothetically, BH3-domain peptides could be used either for binding of anti-apoptotic Bcl-2 family members preventing the inhibition of apoptosis but also for activating the Bax-like proteins, thus promoting the induction of apoptosis. Binding
of the BH3-peptide alone appears insufficient for direct activation of Bax-like proteins\textsuperscript{205}, suggesting that both options need to be combined for a BH3-peptide to become effective in modulating apoptosis. By interfering with the ability of Bax to interact with and be suppressed by Bcl-2 family members, BH3-peptides have been shown to promote apoptosis\textsuperscript{205}.

It is intriguing that compounds that act on the basic apoptosis machinery do not induce apoptosis in all cells\textsuperscript{302}; this may be due to the differential expression of various pro- and anti-apoptosis factors in normal versus malignant cells and may provide a strategy for selectively targeting malignant cells. This is in concordance with Korsmeyers model of sensitizing/activating BH3-only proteins predicting that ‘sensitised’ cancer cells might be triggered to undergo apoptosis by activating BH3-only proteins, while normal cells (when “non-sensitised”) remain unharmed\textsuperscript{204}. Structure-based computer screening enabled the identification of specific BH3-like compounds which specifically target Bcl-2 and thus have little effect on cells expressing low levels of Bcl\textsubscript{2}\textsuperscript{303}. These compounds might be used specifically in targeting B-CLL cells expressing high levels of Bcl-2 and simultaneously minimalizes possible cytotoxicity of surrounding non-B-CLL cells. So far, these experiments have been performed using in-vitro cell systems and clinical use of these molecules will take many years of additional research. The expanded knowledge about apoptosis mechanisms in normal and malignant cells will provide opportunities for future directions on treatment modalities against cancer.