Discussion and future perspectives
8. Discussion and future perspectives

Diffuse large B-cell lymphoma (DLBCL) is the most common type of Non-Hodgkin lymphoma with an annual incidence of 950 occurrences in the Netherlands.\(^1\) Although DLBCL is recognized as a distinct entity by the WHO classification, it is a heterogeneous group of tumors with diverse clinical and biologic characteristics.\(^2\) Many patients may be cured with current chemotherapy treatment (cyclophosphamide, doxorubicin, vincristine and prednisone, CHOP) in combination with the CD20 chimeric monoclonal antibody rituximab (R), however 30-40% will eventually die as a result of the disease.\(^3,4,5,6\) Recent studies have shown that inhibition of the apoptosis signaling pathways is strongly related to response to chemotherapy and eventual clinical outcome.\(^7,8,9\) In this thesis, we investigated possible mechanisms responsible for apoptosis resistance in DLBCL and alternative targeted therapies that could restore apoptosis sensitivity. In the next sections we will discuss our findings and their clinical implications.

8.1. Predictive biomarkers

Investigation of mechanisms involved in DLBCL lymphomagenesis has led to the development of numerous biochemical markers. Many studies have tried to associate the survival of DLBCL patients with specific biomarkers that underlie the features of the lymphoma cell. These individual biomarkers have provided valuable additional prognostic information to the IPI and have improved the understanding of the pathogenesis of DLBCL. However the role in clinical practice of these markers as reliable predictors of prognosis and the role in development of new specific targeted therapies is limited due the complexity of biologic processes and involvement of multiple genes. Therefore, recent studies have explored the relation between DLBCL prognosis and molecular features of the tumor cells using gene expression profiling by microarray analysis or RT-PCR. Two major subtypes of DLBCL, one resembling normal germinal center B-cells (GCB-like) and the other resembling \textit{in vitro} activated B-cells (ABC-like) were identified.\(^10,11,12,13\) Patients with a GCB-like DLBCL had a significantly better prognosis than patients with an ABC-like DLBCL. A third subgroup comprised cases that did not express genes characteristic of the GCB-like nor the ABC-like group. This not-otherwise-specified group had a poor outcome similar to that of the ABC-like subtype. However, even some DLBCL with GCB profile respond poorly to chemotherapy and the disease runs a fatal course in these patients.

We have previously confirmed these data in our own group of DLBCL patients by genome wide expression analysis. In addition, we could demonstrate that expression profiling using only genes involved in regulation and execution of apoptosis also resulted in three separate DLBCL groups with different clinical outcome, partly overlapping with GCB- and ABC-like phenotype. These DLBCL subtypes included a hyperplastic lymphoid tissue (HLT) group (containing most GCB-like DLBCL), a cellular cytotoxic response (CCR) group (containing most not-otherwise-specified DLBCL) and an activated apoptosis cascade (AAC) group (containing most ABC-like DLBCL). Both the CCR and the AAC subtype showed a poor clinical outcome in contrast with the HLT subtype that demonstrated a favorable outcome.\(^14\) Thus, expression profiling based on expression of only apoptosis regulating genes appeared to be at least as informative as genome wide expression profiling, which suggests that clinical outcome is directly related to differences in expression levels of these apoptosis regulating genes. This hypothesis was the basis for most of the studies included in this thesis.
8.2. Mechanisms of apoptosis resistance in DLBCL

8.2.1. The intrinsic pathway is constitutively activated in a subset of chemotherapy DLBCL with downstream disruption of this pathway.

Two major apoptosis pathways have been identified: the intrinsic, caspase 9-mediated apoptosis pathway, and the extrinsic, caspase 8-mediated apoptosis pathway. Most chemotherapeutic drugs used in the treatment of DLBCL primarily induce cell death via activation of the intrinsic apoptosis pathway.\(^\text{15,16,17,18}\) In this thesis, we have found that inhibition of the extrinsic apoptosis pathway correlated with an excellent clinical outcome, whereas disruption of the intrinsic apoptosis pathway was correlated with a poor clinical outcome (Chapter 2). In addition, we and others have demonstrated that expression of apoptosis inhibiting proteins of the intrinsic pathway are predictive of poor clinical outcome.\(^\text{19,20,21,22}\)

In a previous study, using microarray analysis, we have shown that a subset of DLBCL with poor clinical outcome was characterized by high expression levels of pro-apoptotic genes, in particular genes that are under transcriptional control of p53 and are involved in the intrinsic apoptosis pathway. These DLBCL samples also demonstrated high expression levels of apoptosis-inhibiting genes.\(^\text{14}\) We confirmed these observations in isolated lymphoma cells of a subset of DLBCL samples (Chapter 4). Using RT-MLPA analysis we identified two groups of DLBCL: one group with low expression levels of both pro- and anti-apoptotic genes and one group with high expression levels of these genes. Most of the DLBCL with high expression levels of pro- and anti apoptotic genes appeared to be refractory to clinical chemotherapy. Functional analysis of DLBCL cells with a low apoptosis profile demonstrated an intact intrinsic apoptosis pathway and caspase activation in combination with apoptosis induction after treatment with chemotherapy (Figure 1). In contrast, DLBCL cells with a high apoptosis profile showed constitutive induction of the intrinsic apoptosis pathway with inhibition downstream of caspase 9 activation due to high expression levels of members of the IAP family, including XIAP. This notion was supported by our previous observation that XIAP expression in neoplastic cells of primary nodal DLBCL is associated with a poor clinical outcome (Chapter 2). Chemotherapy failed to induce apoptosis in these tumor cells, due to the disruption downstream in the pathway, thereby explaining the clinical resistance to chemotherapy (Figure 1). Thus, in a subset of chemotherapy-refractory DLBCL, resistance is not caused by failure to activate the intrinsic pathway, but by downstream inhibition of this pathway.
Figure 1. The intrinsic pathway is constitutively activated in a subset of chemotherapy DLBCL with downstream disruption of this pathway. Previous RT-MLPA analysis revealed two groups of DLBCL: one with relatively low expression levels of both pro- and anti-apoptotic genes (low apoptosis profile) and one group of DLBCL with relatively high expression levels of pro-apoptotic genes in combination with high expression levels of anti-apoptotic genes (high apoptosis profile). DLBCL with a high apoptosis profile frequently appeared to be chemotherapy-refractory and were characterized by spontaneous caspase 9 activation without induction of caspase 3 activation or apoptosis. (A) Chemotherapy failed to induce apoptosis in DLBCL with a high apoptosis profile, due to inhibition of caspase 3 by XIAP protein expression. (B) Low apoptosis profile cells showed no spontaneous caspase activation or apoptosis, only after induction with chemotherapy, induction of apoptosis was observed. (dark gray = activated; light gray = inactivated)

The association between high expression levels of anti-apoptotic genes and a poor response to chemotherapy has been demonstrated in many other studies, however the high expression levels of pro-apoptotic genes in chemotherapy-refractory cases and the upstream induction of the intrinsic apoptosis pathway was unexpected. For several years, the hypothesis was that in tumor cells the balance between levels of pro- and anti-apoptotic regulators was disrupted in favor of the anti-apoptotic proteins resulting in a survival advantage and allowing malignant transformation. In this thesis, we clearly show in a subset of DLBCL samples that the existence of tumor cells and their resistance to chemotherapy is not due to imbalances in expression levels of pro- and anti-apoptotic proteins, but that it is caused by a downstream inhibition of the intrinsic apoptosis pathway which can not be restored by the high expression levels of pro-apoptotic proteins and activation of the pathway upstream. This new insight in the cause of chemotherapy resistance provides new possibilities for improvement of therapy for chemotherapy refractory DLBCL patients.
8.2.2. Possible causes for constitutive activation of the intrinsic apoptosis pathway

In non-neoplastic lymphoid cells, the intrinsic pathway can be triggered by many stimuli, including growth factor deprivation, oxidants, Ca\(^{2+}\) overload, oncogene activation, DNA-damaging agents, and microtubule-attacking drugs.\(^{21,23,24}\) These death stimuli activate the BH3-only proteins and subsequently the Bax/Bak-like members, mostly in a p53-dependent manner.\(^{25,26,27}\)

One possible mechanism for upstream constitutive activation of the intrinsic pathway is stabilization of p53 expression. In normal cells, p53 is present at extremely low levels because the protein is rapidly degraded following synthesis.\(^{28}\) Stabilization of p53 can occur by multiple proteins in response to different stress stimuli. MDM2 has been established as one of the most important regulators of p53 stability that targets the degradation of p53. Most of the stress stimuli inhibit MDM2-mediated degradation of p53, but this is achieved by different independent pathways. P53 can be protected from MDM2 by preventing the interaction between the two proteins via post-translational modifications, including phosphorylation, methylation, or acetylation of p53.\(^{29}\) Furthermore, p53 can be stabilized by activation of expression of the small tumor suppressor protein p14\(^{arf}\). P14\(^{arf}\) binds directly to MDM2, thereby inhibiting the ubiquitin ligase activity of MDM2 and by sequestering MDM2 into the nucleolus, thus preventing nuclear export which is necessary for degradation.\(^{30,31}\) Several oncogenes, including Ras, c-Myc and E1A have been shown to stabilize p53 through p14\(^{arf}\).\(^{32,33,34}\) In our previous microarray experiments, high expression levels of pro-apoptotic genes correlated with high expression levels of E2F1 and BRCA1.\(^{14}\) E2F1 is an oncogene that can induce apoptosis via p53 as a “failsafe” program, which can prevent tumor development when the intrinsic pathway is intact.\(^{27,35,36}\) P53 is stabilized by E2F1 via direct transcriptional activation of p14\(^{arf}\), which inhibits MDM2.\(^{37}\) BRCA1 is a tumor suppressor gene, that can also induce apoptosis in a p53 dependent manner through regulation of its phosphorylation and MDM2 expression.\(^{18}\) Upregulation of one of these proteins could result in constitutive activation of the intrinsic pathway.

Another mechanism that could be involved in constitutive activation of the intrinsic pathway is the endoplasmic reticulum (ER) stress-induced pathway. Accumulation of unfolded and misfolded proteins or an aberrant ER Ca\(^{2+}\) equilibrium results in ER stress and triggers unfolded protein response signaling.\(^{39}\) Prolonged unfolded protein response signaling and ER stress can lead to activation of the intrinsic pathway and caspase 9 activation.\(^{40,41}\) Constitutive activation of the intrinsic pathway might occur, when cells remain exposed to excessive levels of stimuli causing ER stress. Which of the above mechanisms causes the constitutive activation of the intrinsic apoptosis pathway remains uncertain and is currently further investigated.

8.3. Targeting therapy

The improved understanding of mechanisms of resistance to apoptosis and the identification of molecular targets in DLBCL have provided new insights for targeted therapies. ABC-like DLBCL require constitutive activation of the NF-κB pathway for survival. A recent study has shown that the small molecule IκB kinase (IKK) inhibitor PS1145 is toxic for ABC-like DLBCL but not for GCB-like DLBCL cell lines.\(^{42,43}\)

In this thesis we tested alternative targeted therapies that could circumvent downstream disruption of the intrinsic apoptosis pathway or restore sensitivity to apoptotic cell death in DLBCL.
**Ofatumumab and rituximab:** An alternative way to circumvent downstream disruption of the convergence apoptosis pathway is to induce cell death independent of apoptosis by CD20 mAbs. We showed that both ofatumumab and rituximab induced CDC in chemotherapy-refractory DLBCL, with ofatumumab being more effective (Chapter 7). Sensitivity of DLBCL cases to ofatumumab- and rituximab-induced CDC was dependent on expression of complement defense molecules CD55 and CD59, but not on expression of CD46 or apoptosis inhibitors Bcl-2 and XIAP.

**hsTRAIL/Apo2L:** We showed that DLBCL samples, including chemotherapy-refractory lymphomas, can be sensitive to hsTRAIL/Apo2L. Sensitivity to hsTRAIL/Apo2L was not dependent on expression levels of the Bcl-2 and/or XIAP (Chapter 6).

**Small-molecule XIAP antagonist:** We found that the small-molecule XIAP antagonist can induce apoptosis in both chemotherapy and responsive DLBCL cells, but did not affect peripheral blood mononuclear B-cells and tonsil germinal center B-cells from healthy donors. Sensitivity to the XIAP antagonist was characterized by high expression levels of XIAP, relatively low expression levels of Bcl-2, and by constitutive caspase-9 activation (Chapter 5). The small-molecule XIAP antagonist should be used as a single agent therapy. Chemotherapy did not give an additional increase in cell death in combination with the XIAP antagonist in DLBCL sensitive cells. Moreover, apoptosis might be induced in normal non-malignant cells, when treated with a combination of the XIAP antagonist and chemotherapy.

If we compare the efficacy of hsTRAIL/Apo2L and the XIAP antagonist in DLBCL cells, we found that inhibition of XIAP expression by small-molecule XIAP antagonists led to much higher levels of apoptosis induction than treatment with hsTRAIL/Apo2L. The very efficient killing of the XIAP antagonist can be explained by our observation that the intrinsic pathway appears to be maximally activated, and is only disrupted downstream by XIAP. Neutralizing the function of XIAP results in an intact intrinsic apoptosis cascade and in instantaneous cell death. The relatively inefficient killing of hsTRAIL/Apo2L is probably due to the fact that hsTRAIL/Apo2L targets the TRAIL receptors upstream in the extrinsic pathway and is more dependent on downstream apoptosis regulators. Although chemotherapy DLBCL cells were very sensitive to ofatumumab or rituximab induced apoptosis in the small group of samples tested, XIAP is a more specific target than CD20. We found that normal PBMC B-cells from healthy donors showed higher levels of toxicity to the CD20 mAbs than to the small-molecule XIAP antagonist.

**8.4. Tailoring of therapy for the individual patient**

The new insights in the pathogenesis and mechanisms of resistance in DLBCL are not only important for the improvement of therapeutic strategies but it can also be used for tailoring of therapy for the individual patient (Figure 2). Being able to predict which patients are likely to benefit from a specific therapy would: 1) save patients from unnecessary toxicity, and enhance their chance of receiving a drug that helps them and 2) help control medical costs.

In this thesis, we could predict sensitivity for the small-molecule XIAP antagonist, however prediction of sensitivity to hsTRAIL/Apo2L was not possible, although it seems that cases with high expression of TRAIL receptors were more sensitive to hsTRAIL/Apo2L induced apoptosis (chapter 5,6). Prediction of sensitivity for ofatumumab and rituximab in DLBCL patients was not possible (chapter 7). Sensitivity to the IKK inhibitor PS1145 can be predicted by determination of the GCB/ABC-like DLBCL profile.\(^4\)
Figure 2. Example of tailoring therapy for the individual patient based on the experience of DLBCL apoptosis profiles. After diagnosing of DLBCL, caspase 9 activity, Bcl-2 and XIAP expression are determined using FACS analysis or RT-MLPA analysis, respectively. Based upon the obtained apoptosis profile the patient is treated with a specific therapy.

8.5. Concluding remarks and future plans

We demonstrated in this thesis that upstream activation of the intrinsic apoptosis pathway with concomitant downstream inhibition of this pathway is a key-event in chemotherapy refractory DLBCL. The exact mechanisms, that are responsible for constitutive caspase 9 activation are probably related to oncogene activation and/or DNA damage. This item is currently investigated in isolated DLBCL patient cells and DLBCL cell lines. Downstream inhibition of caspase 3 activity can be restored by targeting XIAP with small-molecule XIAP antagonists, either or not in combination with a Bcl-2 antagonist. Small-molecule XIAP antagonists and Bcl-2 antagonists are currently designed for clinical trials and results of the clinical trials remains to be seen in the future. In vitro sensitivity to the XIAP antagonist can be predicted based on biological markers suggesting the possibility of pre-defining patients most likely to benefit from XIAP antagonist therapy.

The studies presented in this thesis included mainly patients treated with CHOP-based chemotherapy regimens. We are currently making an effort to further validate the prognostic value of apoptosis profiling in DLBCL patients uniformly treated with CHOP in combination with rituximab. Based on the results presented in this thesis we expect that R-CHOP refractory patients will benefit from monotherapy with the small-molecule XIAP antagonist and will obtain no adverse side effects, because constitutive activation of the intrinsic apoptosis pathway is probably restricted to malignant cells.
References


