Chapter

1

General Introduction

1. Diffuse large B-cell lymphomas

1.1. General introduction

Lymphomas represent a group of heterogeneous malignancies that originate in lymphocytes. Lymphoma tumor cells are clonal malignant equivalents of normal lymphocytes and their precursors, arrested at a specific stage of differentiation. Traditionally, lymphomas are categorized as Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL). HL is characterized by the presence of low numbers of Reed-Sternberg tumor cells and mononucleated Hodgkin tumor cells.[1,2] All other types of lymphomas are defined as NHL and are distinct from HL in their pathologic features, epidemiology, common sites of involvement, clinical behaviour and treatment. NHL can be divided into two large groups, based upon lineage markers and gene rearrangements expressed by the neoplastic cells: B-cell lymphomas and T-cell/natural killer cell lymphomas. Most NHL arise in the lymph nodes (nodal), however 30-40% of the tumors arise in lymphoid tissue outside the lymph nodes (extranodal), often in mucosa associated lymphoid tissue. Extranodal lymphomas can occur in the gut, skin, brain, lungs and testis. In the Netherlands, NHL accounts for 3% of all cancers diagnosed with an annual incidence of approximately 2400 cases.[www.ikc.nl]

Since 2001, the World Health Organization (WHO) classification is generally used to classify NHL.[3] This classification divides NHL in different types defined by morphological, immunological, genetic and clinical features. The clinical course of these different lymphoma types is highly variable from very indolent lymphomas to highly aggressive lymphomas. The treatment of indolent lymphoma may initially involve a period of observation, while aggressive or highly aggressive NHL is typically treated with chemotherapy and/or radiotherapy, frequently in combination with Rituximab.

1.2. Diffuse Large B-cell Lymphoma (DLBCL)

Diffuse large B-cell lymphoma (DLBCL) accounts for approximately 40% of adult NHL.[4] Although this lymphoma is classified as one disease according to the WHO criteria, DLBCL is clinically, morphologically and genetically a heterogeneous group of tumors that is composed of large B-cells. Within this group of lymphomas, primary mediastinal large B-cell lymphoma (PMBCL), intravascular large B-cell lymphoma and primary effusion lymphoma (PEL) are listed as separate entities. All the other large B-cell lymphomas are joined under the term DLBCL because clear criteria to differentiate them from each other are lacking.[3]

DLBCL can affect any age group, but occur mainly in older people (average of most patients is mid-60). The usual symptoms are a quickly growing mass in an internal lymph node, (e.g. chest, abdomen) or in a perceptible lymph node (e.g. neck, armpit). In general, the tumor starts in lymph nodes (nodal), however in 40% of the cases it begins in other areas such as the intestines, skin, bone, and even the brain or spinal cord (extranodal).[5] DLBCL mainly arise *de novo*, but it can also represent progression of transformation of an indolent lymphoma (most commonly, a follicular lymphoma).
Based on gene expression profiling studies different subtypes of DLBCL can be identified, even though the tumors look the same microscopically. DLBCL cells are considered to arise from normal antigen-exposed B-cells of either germinal center (GC) or post-GC origin.[6] Several microarray studies performed on untreated *de novo* DLBCL samples have identified two major subtypes, one resembling non-neoplastic germinal center B-lymphocytes (GCB-like), the other resembling *in vitro* activated B-cells (ABC-like).[7,8,9,10] The GCB-like subgroup was correlated with a significantly better prognosis in

comparison to the ABC-like subgroup. 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.

1.3. Normal B-cell development

B-lymphocytes originate from precursor B-cells in the bone marrow and undergo a number of maturation steps before they can play an essential role in the humoral immune response. In the bone marrow, B-cell development is initiated by rearrangement of the immunoglobulin (Ig) genes, mediated by V(D)J recombination. During V(D)J recombination, DNA located between the rearranging gene elements is deleted from the chromosome (or sometimes inserted).[11,12] Each B-cell generates a particular pair of genes for its H chain and L chain variable region that differ from those of other B-cells.[13,14] These distinct gene rearrangements encode a specific antibody and also equip each B-cell with individual, molecular clonal markers, resulting in an enormous antigen receptor diversity. Before the immature B-cells leave the bone marrow, cells that are stimulated by self antigens are eliminated by programmed cell death or apoptosis. After leaving the bone marrow the cells enter the blood circulation and home into the secondary lymphoid organs: lymph nodes, spleen, and mucosa-associated lymphoid tissue (MALT). In the secondary lymphoid organs, B-cells localize together with follicular dendritic cells (FDC) in primary follicles. When the B-cells encounter the appropriate antigen, presented on the surface of the FDCs , they migrate to the center of the primary follicles to establish germinal centers (GC).[15] In the dark zone of the GC, the proliferating B-cells (centroblasts) may be subjected to somatic hypermutation (SHM), receptor editing and class switching. The centroblasts give rise to small non-dividing centrocytes that are localized in the light zone of the GC. Here, the centrocytes with the highest affinity for the antigen presented by the FDC, are positively selected for survival.[16] Centrocytes that fail to bind antigen, are eliminated via inhibition of cellular FLICE inhibitory protein (c-Flip) which results in apoptosis.[17] Eventually, B-cells that have successfully bound antigen and survived selection leave the GC to become either memory B-cells or (high affinity) antibody-producing plasma B-cells.[18]

1.4. B-cell lymphomagenesis

Similar to other types of cancer, lymphomagenesis is considered to be a multistep process, during which an accumulation of genetic changes leads to activation of proto-oncogenes or inactivation of tumor suppressor genes.[19] In lymphomas, the activation of oncogenes due to cytogenetic abnormalities is frequently detected and strongly associated with specific lymphoma types.

1.4.1. Oncogene activation

In DLBCL, cytogenetic abnormalities: including translocations, gene amplifications, deletions and point mutations often occur. The origin of the DLBCL tumor cells (GC-B cells, or post GC-B-cells) suggests that the malignant transformation takes place at the GC or post-GC stage. Below are given frequently occurring genetic abnormalities resulting in oncogene activation in DLBCL:

Bcl-2: Bcl-2 is a very potent inhibitor of the intrinsic apoptosis pathway (paragraph 4.1.2) In 20-30% of DLBCL, t(14;18)(q32;q21) translocation occurs that juxtaposes the Bcl-2 locus with the immunoglobulin heavy chain (IgH) locus, resulting in enhanced Bcl-2 expression.[20,21]

Bcl-6: Bcl-6 is a sequence-specific transcriptional repressor involved in regulation of lymphoid development. In 30-40% of DLBCL cases the Bcl-6 proto-oncogene at 3q27 is translocated, resulting in repression of genes involved in B-cell activation, differentiation, cell cycle arrest and apoptosis.[22,23]

c-Myc: c-Myc induces cell proliferation via inhibition of the cyclin kinase inhibitor p27 that is involved in G1 cell arrest. In 15% of DLBCL the c-Myc proto-oncogene, localized at 8q24 is translocated. Overexpression of c-Myc results in uncontrolled and continuous cell proliferation.[24,25]
NF-κB family: The nuclear factor (NF)-κB family includes several dimeric transcription factors that belong to the c-Rel family.[26] NF-κB is involved in regulation of innate and adaptive immunity, inflammatory response, lymphoid organ development as well as apoptosis inhibition. 23% of DLBCL show amplification of the c-REL proto-oncogene (2p12-16), however amplification of this locus did not necessarily correlate with activation of the NF-κB pathway.[27,28]

1.4.2. Antigenic stimulation

Chronic antigenic stimulation might play an important role in lymphomagenesis. Under normal conditions the immune system is in a state of dynamic equilibrium and responds to an antigenic stimuli in either a poly-, oligo or monoclonal way depending on the antigen. The normal immune system can become deregulated when chronic antigenic stimulation occur, resulting in a higher risk of receiving other oncogenic hits, leading to uncontrolled proliferation and malignant transformation of immune cells. Several pathogens causing a chronic inflammation, have been linked to the risk of lymphoma, including Epstein-Barr virus, human immunodeficiency virus, human T-cell lymphotropic virus-1, Helicobacter pylori, hepatitis C, Borrelia Burgdorferi, simian virus 40 and food allergens (gluten in celiac disease).[29,30,31,32]

1.4.3. Escape from immune response

In immune competent patients, expression of tumor antigens causes an immune response. Mature T-cells, either expressing CD8 or CD4 recognize the tumor antigen presented by major histocompatibility complex (MHC) molecules class I or II, respectively. CD4+ T-cells can be divided into two types based on their cytokine expression profile: T helper 1 (Th1) cells, that induce cell-mediated immunity via activation of cytotoxic T-lymphocytes (CTLs), necessary to eradicate virus-infected cells or tumor cells and Th2 cells that activate B-cells to produce antibodies against extracellular antigens. After activation, CD8+ T-cells can induce apoptosis in the tumor cells via induction of the granzyme B induced pathway or the extrinsic apoptosis pathway (paragraph 3.2 and 3.3).[33,34] Tumor cells can escape from CTL induced apoptosis by expression of the granzyme B antagonist protease inhibitor 9 (Pi9) that can effectively inhibit granzyme B.[35] Furthermore, CTLs and natural killer (NK) cells can express killer inhibitory receptors (KIR). Ligation of KIR by MHC-class I molecules on the tumor cells also results in inhibition of CTL or NK cell mediated cell death.36 Other potential mechanisms to escape immune surveillance resulting in survival of the tumor cell are downregulation of MHC class I molecules and Fas mutations.[37,38,39] Patients with a deficient immune status (e.g. AIDS, after transplantation due to immunosuppressive therapy) have an increased risk to develop lymphomas.[40]

1.5. Treatment of DLBCL

DLBCL is the most common aggressive lymphoma. Without any treatment patients die within weeks or months. For several decades, the standard treatment for primary nodal DLBCL has been a combination of chemotherapeutical agents, including cyclophosphamide, doxorubicin, vincristine and prednisone (CHOP).[41] In the last decade the standard treatment for these lymphomas has become CHOP in combination with a chimeric CD20 mAb rituximab (R).[42,43,44] Rituximab targets the CD20 cell surface protein, present on mature B-cells and most B-cell lymphoma cells. Cross linkage

and subsequent homodimerization results in severe B-cell depletion by antibody-dependent cellular cytotoxicity (ADCC)[45], complement-dependent cytotoxicity (CDC)[46] and induction of apoptosis. Monotherapy with rituximab is of limited benefit since virtually al patients experienced a relapse after an initial favorable response. The combination therapy R-CHOP has been shown to be more effective for all DLBCL patients, especially for patients with Bcl-2 expressing DLBCL and for elderly patients presenting with advanced stage.[47,48,49]
The prognosis of primary nodal DLBCL is highly variable. Although many patients benefit from R-CHOP treatment, the disease remains fatal in 30-40% of the patients.[50] Fatal outcome usually results from failure to achieve complete remission or the occurrence of an early relapse. The success of the treatment partially depends on localization of the tumor, stage of the disease, serum lactate dehydrogenase (LDH) levels, age and the general condition of the patient. These prognostic factors are combined in the international prognostic index score (IPI). Although the IPI divides patients in low and high risk groups, the predicted response to therapy has limited clinical relevance. If initial treatment fails or when a relapse occurs, the patient usually has a very poor prognosis. As continuing treatment, patients obtain additional intensified treatment, such as myeloablative chemotherapy or combined chemo-radiotherapy followed by autologous bone marrow transplantation or peripheral stem cell transplantation.[51,52]

2. Causes of poor clinical outcome

Despite significant advances in treatment of DLBCL, resistance to R-CHOP remains a major cause of poor clinical outcome.
Resistance to rituximab: Multiple mechanisms of action have been reported for rituximab and it remains unclear which mechanism is most important in patients. Therefore, it is difficult to understand the potential mechanism of resistance.[53] Possible mechanisms for rituximab resistance might be: poor CD20 surface antigen expression, rapid metabolism of rituximab, low serum levels, reduction of direct apoptosis effect in cases of elevated Bcl-2 protein expression, inhibition of CDC by complement defense molecules (CD46, CD55, CD59) and alteration of cell-mediated immunity. [54,55]
Resistance to CHOP: Chemotherapy resistance is a complex and dynamic process that is related to cell cycle, apoptotic pathways, cellular differentiation and the microenvironment. Potential mechanisms that might be involved in resistance to CHOP include: expression of members of the glutathione and ABC transporter family[56], point mutations, gene deletions, low expression and alterations in distribution of topoisomerase II· (enzyme, involved in sensitivity to topoisomerase II inhibitors)[57], and inhibition of apoptosis pathways.[58]

3. Apoptosis signaling pathways

Programmed cell death or apoptosis is an ATP dependent, physiological form of cell death which can be triggered by a variety of stimuli. Apoptosis is involved in morphogenesis of embryonic tissues and in homeostasis of adult organs and tissues.[59,60] Furthermore, virally infected cells and cells with damaged DNA are removed via apoptosis.[61,62] Dysregulation of the apoptotic pathways contributes to

many diseases, including cancer. The apoptotic process is characterized by typical morphological changes, including: cellular shrinkage, membrane blebbing, nuclear chromatin condensation and fragmentation.[63,64] Eventually, the cell disintegrates into membrane surrounded fragments (apoptotic bodies) which are taken up by phagocytes. Most of the morphological changes during apoptosis are caused by cystein aspartic acid-containing proteases (caspases). Caspases are expressed as inactive zymogens, that are proteolytically cleaved upon induction of apoptosis.[65] According to their structure and function, caspases can be divided into effector caspases (caspase 3, 6 and 7) which carry out cell death, and initiator caspases (caspase 2, 8, 9, and 10) that mainly activate effector caspases.[66] Once activated, effector caspases execute cell death through cleavage of key cellular proteins, including substrates involved in cell structure, signaling, cell cycle control and DNA repair.[67] Four distinct apoptosis pathways for triggering caspase activation have been elucidated; an intrinsic stress-induced pathway, an extrinsic death receptor-induced pathway, a granzyme B induced pathway and an endoplasmic reticulum stress-induced pathway.

3.1. Intrinsic apoptosis pathway

The intrinsic or stress-induced pathway can be triggered by many stimuli, including growth factor deprivation, oxidants, Ca^{2+} overload, oncogene activation, DNA-damaging agents, and microtubule-attacking drugs.[68,69,70] These death stimuli activate BH3-only (Bcl-2-homology domain 3) proteins, a pro-apoptotic subgroup of the Bcl-2 family, that initiate apoptosis via direct or indirect activation of Bax (Bcl-2 associated X protein) and Bak (Bcl-2 antagonist/killer).[71,72] Once activated, Bax and Bak insert in the mitochondrial outer membrane and form oligomers, resulting in mitochondrial outer membrane permeabilization (MOMP) and release of cytochrome c in the cytosol.[73,74] In addition to cytochrome c, mitochondria also release several other proteins, including AIF (Apoptosis Inducing Factor), and IAP antagonists Smac/DIABLO and Omi/HtrA2.[75,76,77] In the cytosol, cytochrome c binds Apaf-1 (apoptotic protease activating factor-1) and in the presence of dATP/ATP it oligomerizes into a heptomeric complex that binds procaspase-9 with its caspase recruitment domains (CARD), resulting in a large protein complex, also designated as the apoptosome.[78,79,80] Pro-caspase 9 becomes activated and active caspase 9 in turn cleaves and activates downstream effector caspases such as pro-caspase 3 (Figure 1).[81]
Almost all cytotoxic anticancer drugs currently in clinical use, induce cell death via induction of the intrinsic apoptosis pathway, including cyclophosphamide, adriamycin, vincristine and etoposide.[82,83,84,85]

3.2. Extrinsic apoptosis pathway

The extrinsic or death receptor-induced pathway is triggered by binding of a ligand to death receptors (DR) of the TNF (tumor necrosis factor) -receptor super family. These death receptors contain a cytosolic death domain (DD) and include TNFR1 (binding with TNF-α), Fas/CD95 (binding with FasL), DR3 (binding with APO3L), TRAIL receptor R1/DR4 and R2/DR5 (both binding with TRAIL/Apo2L).[86] Upon ligand binding, death receptors cluster in the membrane and recruit FADD (Fas Associated Death Domain) protein that contains both a death domain and a death effector domain (DED).[87,88] FADD binds directly or indirectly via TRADD (which binds to TNFR1) the DED-containing pro-caspases 8 and/or 10, forming a death inducing signaling complex (DISC) that results in the activation of caspase 8 and 10.[89,90] Once caspase 8 and/or 10 are activated, cells can undergo apoptosis by signaling through two different pathways. 1) Caspase 8 and/or 10 can cleave and activate pro-caspase 3 directly.[91] 2) When the amount of caspase 3 initially activated by caspase

8 and/or 10 is insufficient to trigger the apoptotic process[92], caspase 3 can be activated indirectly through caspase 8 mediated cleavage of Bid, a pro-apoptotic member of the Bcl-2 family.[93] Cleaved Bid (truncated Bid) interacts with Bax or Bak resulting in disruption of the mitochondrial membrane and induction of the intrinsic apoptosis pathway (Figure 1).[94,95]

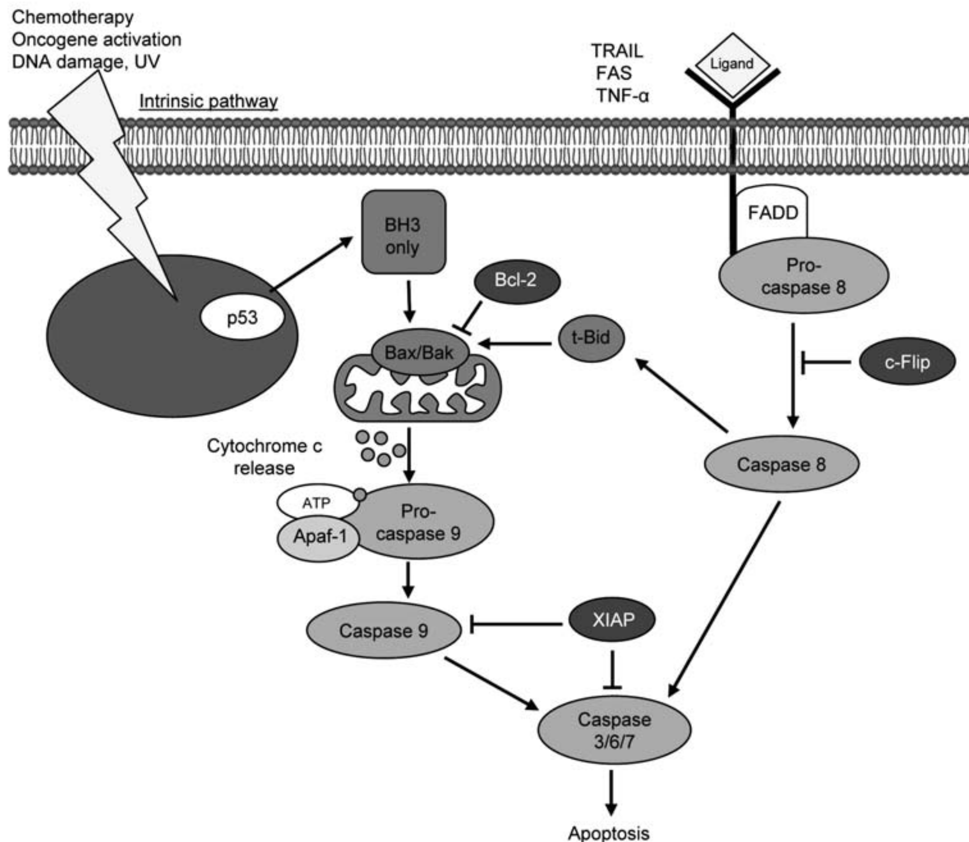

Figure 1: Schematic representation of the two major apoptosis pathways: the intrinsic and extrinsic pathway.

3.3. Granzyme B induced pathway

The granzyme B induced pathway is specific to cytotoxic T-lymphocytes (CTL)s and natural killer (NK) cells.[96,97] CTLs and NK cells eliminate target cells by secretion of cytotoxic granules that contain the protein perforin and a variety of granule-associated proteases, including granzyme B.[98,99] Granzyme B enters the target cell via the mannose-6-phosphate receptor (IGFR2) and the protein perforin that forms pores in the membrane of the cell.[100,101] In the target cell, granzyme B can cleave and activate pro-caspase 3[102] and the pro-apoptotic protein Bid, which activates Bax and Bak to change the membrane permeability of the mitochondria, resulting in induction of the intrinsic apoptosis pathway.[103]

3.4. Endoplasmic reticulum (ER) stress-induced pathway

A fourth pathway for induction of apoptosis is linked to the endoplasmic reticulum (ER), though many details are still unknown. The ER is responsible for proper synthesis, folding and modification of secretory and transmembrane proteins and lipids, and for intracellular Ca^{2+} storage.[104] Accumulation of unfolded and misfolded proteins or an aberrant ER Ca^{2+} equilibrium leads to ER stress and triggers unfolded protein response (UPR) signaling.[105] Prolonged UPR signaling and ER stress induce apoptosis via the intrinsic pathway and via activation of pro-caspase 12 in the ER. Current models suggest that pro-caspase 12 can be cleaved by calpain, caspase 3 or 7.[106,107,108] Activated caspase 12 can directly process caspase 9, leading to caspase 3 and 7 activation.[109] However, caspase-12 has only been cloned in the mouse and rat so far, and therefore it is controversial whether similar mechanisms operate in humans.[110] Recent studies have shown that caspase 4 activation is involved in ER stress-induced apoptosis in humans.[111]

4. Inhibition of the two major apoptosis pathways

The two major pathways to induce apoptosis are the intrinsic and extrinsic apoptosis pathway. These two pathways have been elucidated in great detail in recent years. Regulation of the intrinsic and extrinsic apoptosis pathway occurs by various proteins at many levels by different mechanisms. The levels of pro- and anti-apoptotic regulators have to be in balance, ensuring the survival of long-lived cells and the proper turnover of short-lived cells in a variety of tissues. In cancer, imbalances in levels of pro-apoptotic and anti-apoptotic proteins often occur, in favor of anti-apoptotic proteins, giving cells a survival advantage that promotes malignancy. A summary of apoptosis disrupting mechanisms observed in DLBCL is given below.

4.1. Inhibition of the intrinsic apoptosis pathway

4.1.1. Defective p53 activation

The intrinsic apoptosis pathway can be upstream inhibited by defective p53 activation. P53 can be activated by oncogenes via induction of $p14^{ARF}$ that binds and inhibits p53 inhibitor MDM2.[112] In DLBCL, deletions of the $p14^{ARF}$ encoding INK4a/ARF locus have been found, resulting in defective p53 activation[113,114,115] BMI-1, a negative regulator of the INK4a/ARF locus is detected in DLBCL and related to an unfavorable prognosis.[116] Deletions and mutations of p53 itself have been detected in DLBCL with unfavorable effect on clinical outcome.[117,118,119]

4.1.2. Bcl-2 family members

The members of the Bcl-2 family are important regulators of the intrinsic apoptosis pathway. These proteins determine the cellular decision to live or die by their ability to modulate mitochondrial function.[120] All Bcl-2 family members contain one or more Bcl-2 homology (BH) domains and can be divided into three groups, according to their structure and function.[121] The anti-apoptotic members include Bcl-2, Bcl-X_L, Bcl-W, Bcl-2A1, Bcl-2L10 (DIVA) and MCL-1, and all contain four distinct BH domains, except for MCL-1 (has only two BH domains). Overexpression of each of these anti-apoptotic proteins results in protection of cultured cells against a variety of apoptotic stimuli.[122] The pro-apoptotic members are divided into two groups, the Bax/Bak-like proteins and the BH3-only

proteins. The Bax/Bak-like proteins include Bax, Bak, Bok and Bcl-X_S, and most of them contain three BH domains. Overexpression of each of these proteins results in mitochondrial outer membrane permeabilization and release of cytochome c.[122,123] The BH3-only proteins include Bad, Bik, Bid, Hrk, Bim, Noxa, Puma and BMF, and share only a short BH3 domain with each other. All BH3-only proteins can bind with high affinity to (at least some) anti-apoptotic Bcl-2 proteins and in the presence of Bax/Bak-like members they trigger apoptosis when overexpressed.[124] The relative amounts of active anti- and pro-apoptotic Bcl-2 family proteins determine the sensitivity or resistance of cells to many apoptotic stimuli.

Protein expression of Bcl-2, Bax and Bcl-X alone or in combination is associated with chemotherapy resistance and short-term survival.[125] Enhanced expression of Bcl-2 can be caused by t(14,18)(q32;q21) translocation that juxtaposes the Bcl-2 locus with the immunoglobulin heavy chain (IgH) enhancer.[126] In 11-31% of the DLBCL cases, amplification of the Bcl-2 gene at 18q can be found.[127] In ABC-like DLBCL, high expression of Bcl-2 might be due to NF-κB activation.

4.2. Inhibition of the extrinsic apoptosis pathway

4.2.1. Mutation and inhibition of death receptors

The extrinsic apoptosis pathway is activated via ligation of one of the death receptors, including the receptors for Fas/CD95 ligand, TNF-α and TRAIL/Apo2L. Upstream inhibition of this pathway may occur through mutations, down-regulation of the specific death receptors or through expression of decoy receptors. Fas/CD95 mutations have been found in 6-20% of DLBCL.[128,129,130,131] Loss of TRAIL receptor 1 and 2 expression due to allelic deletions on 8p21-22 is observed in up to 29% of DLBCL.[132,133] Expression of decoyreceptors 1 and 2 is detected in 36% and 25% of DLBCL, respectively.[134]

4.2.2. Inhibition of caspase 8 and 10 by cellular Fas-associated death domain-like interleukin 1β converting enzyme inhibitory protein (c-Flip)

Caspase 8 and 10 activation can be inhibited by expression of c-Flip (Fas-associated death domain-like interleukin 1β converting enzyme inhibitory protein). Eleven distinct splice variants of c-Flip have been reported, including c-$Flip_S$ and c-$Flip_L$, which are expressed as proteins.[135] c-$Flip_S$ competes via its DEDs with caspase 8 and/or 10 for recruitment to the adaptor protein FADD.[136] Blocking of the recruitment of caspase 8 and/or 10 to FADD results in inhibition of processing and activation of these caspases.[137] c-$Flip_L$ also inhibits caspase 8 and 10 activation, but in addition it can form a heterodimer with caspase 8 or 10 through interactions between the DEDs and the caspase-like domains of the two proteins, thereby activating caspase 8 or 10.[136,138,139]

4.3. Inhibition of the downstream convergence apoptosis pathway by the inhibitors of apoptosis proteins (IAPs)

The intrinsic and extrinsic apoptosis pathways converge on downstream effector caspases. Direct suppressors of effector caspases are the inhibitors of apoptosis proteins (IAPs). At present, eight members of the IAP family have been identified in humans: XIAP, cIAP1, cIAP2, NIAP, survivin, ML-IAP, apollon and ILP-2.[140] Although IAPs also posses other functions, most of them bind and potently inhibit effector caspases 3 and 7 and/or initiator caspase 9 (intrinsic pathway). All members of the IAP family contain one or more copies of a domain called the baculoviral IAP repeat (BIR), that is important for their anti-apoptotic activity.[141,142] Different BIR domains are responsible for suppression of the different caspases. IAPs themselves can be negatively regulated by IAP-binding protein

Smac/DIABLO, a mitochondrial protein that can be released in the cytosol during apoptosis induction and IAP antagonist Omi/HtrA2.[143,144]
The best characterized human IAP is XIAP, which binds and inhibits processed caspase 3, 7 and 9.[145,146] XIAP contains three BIR domains of which the second BIR domain of XIAP (BIR2) binds and inhibits caspases 3 and 7, while the third BIR domain (BIR3) inhibits caspase-9.[147,148] XIAP suppresses apoptosis induced by many agents, including TNF, TRAIL, Fas-L, staurosporine, etoposide and paclitaxel.[149,150]
XIAP protein expression is found in 26% of DLBCL.[151] 90% and 37% of DLBCL show cIAP1 and cIAP2 protein expression, respectively. In patients with survivin expression the overall 5-year survival rate is significantly lower than those without survivin expression.[152]

5. Targeting of apoptosis pathways for therapy

Recently, chemotherapy in combination with rituximab has become the standard treatment for DLBCL. Chemotherapy induces apoptosis, primarily via damaging proliferating cells at the level of DNA replication or cell division.[153,154] Treatment with chemotherapy results in the cure of approximately 60% of the patients, however it causes considerable toxicity to normal tissues, such as the bone marrow, gut or kidney.[155,156] Therefore, in recent years anti-cancer agents that specifically target biological properties of tumor cells have been developed, that might overcome tumor resistance as well as minimize toxic side effects.
The improved understanding of mechanisms of apoptosis and resistance to apoptosis have provided new insights for the development of apoptosis-targeted therapies. Several components of the apoptosis cascade are attractive targets for restoration and induction of apoptosis, including TNF, TRAIL/Apo2L, Bcl-2 family members, caspases and IAPs.[157]

5.1. Targeting of the intrinsic pathway for therapy

5.1.1. Therapeutic targeting of members of the Bcl-2 family

Anti-apoptotic members of the Bcl-2 family are frequently over expressed in malignancies, including DLBCL.[126] Expression of these anti-apoptotic proteins is associated with resistance to chemotherapy. Therefore, the anti-apoptotic Bcl-2 members are attractive targets for anti-cancer therapy.
Four different approaches have been applied to target the anti-apoptotic Bcl-2 family members: 1) blocking of gene transcription; 2) degradation of mRNA with anti-sense oligonucleotides; 3) inhibition with small-molecule drugs and 4) inhibition with endogenous antagonists.[158,159]

Inhibition of gene transcription. Gene transcription of anti-apoptotic Bcl-2 members can be inhibited by several drugs. Some synthetic retinoids reduce levels of Bcl-2 and Bcl-X_L mRNA expression and these agents are approved for clinical use.[160] Histone deactylases (HDAC) inhibitors decrease also the expression of Bcl-2, Bcl-X_L and MCL1 at transcriptional level and clinical trials with HDAC inhibitors are now in progress.[161,162] Peroxisome-proliferatory activated receptor γ (PPARγ)-modulating drugs also reduce expression of Bcl-2 and other anti-apoptotic Bcl-2 family genes and these drugs are evaluated in clinical trials.[163]

Bcl-2 mRNA degradation with anti-sense oligonucleotides. Protein expression of anti-apoptotic Bcl-2 members can be reduced using anti-sense oligonucleotides. Anti-sense oligodeoxynucleotides targeting Bcl-2 (oblimersen: G3139, Genasense, oblimers sodium) have been shown to induce or enhance apoptosis when applied alone or in combination with cytotoxic therapies in preclinical studies.[164] Oblimersen have been tested in clinical trials, however they were in some but not in all studies successful.[165,166,167] Anti-sense oligonucleotides targeting Bcl-X_L and Bcl-2/Bcl-X_L are tested in preclinical trials.[168,169] Bcl-X_L can also be inhibited via an other approach. Two protein forms of Bcl-X result from alternative splicing: pro-apoptotic Bcl-X_S and anti-apoptotic Bcl-X_L. Making use of this property, the splicing machinery can be targeted to redirect Bcl-X mRNA processing into pro-apoptotic Bcl-X_S.[170]

Inhibition with small-molecule drugs. Peptides and small-molecules mimicking the BH3-domain bind Bcl-2 or related anti-apoptotic proteins thereby promoting the disruption of Bax/Bcl-2 complexes, followed by the release of cytochrome c and apoptosis. Cell-permeable based BH3 peptide mimetics including SAHBs (stabilized α-helix of Bcl-2 domains), ABT-737,[171] TW-37,[172] and other chemical inhibitors of Bcl-2 members: HA14-1 analogues,[173] antimycin A3,[174] certain theaflavins and epigallechatechins (EGCGs)[175] are currently tested in preclinical trials. Gossypol is a natural compound that also interacts with the BH3-binding pockets of the anti-apoptotic Bcl-2 family proteins resulting in apoptosis of cancer cells in mice models, and is currently being evaluated in clinical trials.[176]

Inhibition with endogenous antagonists. Activation of endogenous proteins that bind and neutralize the anti-apoptotic function of Bcl-2 induce apoptosis in cultured cancer cells. 3Cl-AHPC/MM11453 induces expression of TR3 (Nur77) that can bind and change the conformation of Bcl-2 resulting in conversion of Bcl-2 from a protector into a killer.[177]

5.1.2. Therapeutic targeting of mitochondria

Permeabilization of mitochondria is a crucial step in the intrinsic apoptosis pathway leading to release of cytochrome c in the cytosol. A variety of experimental therapeutic agents can directly target mitochondria causing apoptosis induction and are tested in preclinical studies. Lonidamine, arsenite and CD437 induce channel formation and specifically permeabilize mitochondrial membranes *in vitro*.[178] Avicins, betulinic acid and resveratrol disturb the lipid composition of mitochondrial membranes and induce lipid rafts, which in turn may favor mitochondrial outer membrane permeabilization.[179] Redox-active compounds including thiol-crosslinking agents, promote oxidative reactions and permeabilize the mitochondrial membrane.[180] Dichloroacetate (DCA) shifts metabolism from glycolysis to glucose oxidation and activates channels in cancer cell lines thereby inducing apoptosis.[181]

5.2. Targeting of the extrinsic pathway for therapy

5.2.1. Therapeutic targeting of death receptors

Ligands and receptors of the TNF-family have been studied in detail for their use as anti-cancer therapy, particularly because chemotherapy-resistant tumor cells often have defects in the intrinsic pathway, leaving the extrinsic pathway intact. Two strategies have been applied to target the death receptors and trigger the extrinsic pathway: 1) with recombinant ligands or 2) with agonistic monoclonal antibodies.

TNF family ligands. Despite exciting perspectives from preclinical studies, TNF and FasL compounds could not proceed to the clinic. TNF compounds could not be used in clinical trials due to inflammatory reactions and toxicity, whereas FasL compounds were highly toxic for the liver.[182,183] Triggering of the TRAIL receptors seems to be the most promising approach. TRAIL/Apo2L is well tolerated and specifically damages tumor cells and not healthy cells.[184] Moreover, TRAIL/Apo2L often synergize with chemotherapy. Human soluble TRAIL/Apo2L is currently evaluated in phase I and II clinical trials.

Receptor targeting antibodies. Agonistic antibodies directed against TRAIL receptors exhibit anti-tumor activity and often show synergistic effects with chemotherapy.[185,186] Agonistic TRAIL R1 and TRAIL R2 monoclonal antibodies are currently being evaluated in clinical trials. A phase II clinical trial using an agonistic monoclonal antibody directed against TRAIL receptor-1 (ETR-1/mapatumumab) has recently been completed in Non-Hodgkin lymphomas. Agonistic TRAIL receptor R2 monoclonal antibody (lexatumumab) is currently evaluated in a phase I clinical trial in lymphomas and phase II trials are underway.[187]

5.2.2. Therapeutic targeting of c-Flip

Many lymphomas exhibit intrinsic resistance to TNF-, TRAIL/Apo2L- or FasL-induced apoptosis, due to over expression of the anti-apoptotic protein c-Flip. Targeting of c-Flip might restore the extrinsic apoptosis pathway thereby providing an option to bypass blocks in the intrinsic apoptosis pathway. So far, no compounds have been described that can inhibit c-Flip$_L$ or c-Flip$_S$ expression, however experimental agents have been reported that reduce c-Flip protein expression. Synthetic triterpenoids 2-cyano-3,12-dioxoolean-1,9-bien-28-oic acid (CDDO) and CDDO-methyl ester (CDDO-me) can induce ubiquitination and proteasome-dependent destruction of c-Flip$_S$ in cultured cancer cells.[188] CDDO and CDDO-me are currently being evaluated in phase I trials. Additional possible approaches to suppress c-Flip expression include NF-κB pathway inhibitors and HDAC inhibitors.[189]

5.3. Targeting of the convergence pathway for therapy

5.3.1. Therapeutic targeting of effector caspases

The convergence apoptosis pathway can be directly induced by targeting effector caspases, resulting in their activation and induction of cell death of tumor cells. Adenoviral vectors expressing chimeric inducible caspase 3 were specifically targeted to cancer cells and demonstrated rapid killing of these cells *in vitro*.[190] A chimeric anti-HER2 antibody linked to caspase 3 or 6 demonstrated induction of apoptosis in tumor cell lines that over express the erbB2/HER2 receptor.[191] Small cell permeable drugs that can bind a specific site of pro-caspase 3 have been shown to activate caspase 3 and induce apoptosis in cultured cells.[192]

5.3.2. Therapeutic targeting of Inhibitors of Apoptosis (IAPs)

Over expression of IAPs have been reported in many malignancies. Therefore, targeting of members of the IAP family could provide substantial benefits for anti-cancer therapy.
Three different strategies have been applied to target IAPs 1) degradation of mRNA with anti-sense oligonucleotides; 2) inhibition with small-molecule drugs and 3) inhibition with endogenous antagonists.

Degradation of mRNA using anti-sense oligonucleotides. IAP mRNA can be targeted by anti-sense oligodeoxynucleotides, resulting in knockdown of IAP protein expression and induction of apoptosis. Anti-sense oligodeoxynucleotides targeting survivin[193] (ISIS 23722; LY21 81308) and XIAP[194] (AEG35156/GEM640) have been evaluated and designated for clinical trials.

Inhibition with small-molecule drugs. Several compounds that can inhibit the function of XIAP by targeting the XIAP BIR domains are being tested currently in preclinical studies. Two classes of small-molecule XIAP antagonists that target the BIR2 domain and relieve caspase 3 have been identified, including phenylureas and benzenesulfonamide derivatives.[195,196] Phenylureas suppress tumor growth in xenograft models without toxicity to normal tissues. Moreover these compounds induce apoptosis independent of Bcl-2 and Bcl-X_L expression. An antagonist targeting the BIR3 domain also showed cytotoxic activity and anti-tumor activity in xenograft models.[197]

Inhibition with endogenous antagonists. Another approach to functionally inhibit the IAP family is mimicking of endogenous antagonists of IAPs. Several groups have initiated preclinical studies of Smac mimics, however little details are currently available. Reported antagonists targeting the Smac-binding site include embelin, various peptidomimetics and non-peptide small-molecules.[198,199,200,201] Other endogenous antagonists that have been described are: peanut-like protein 2 (PNUTL2, ARTS), X-linked inhibitor of apoptosis protein associated factor 1 (XAF1) and neutrophin receptor interacting melanoma antigen homolog (NRAGE).[202,203,204]

5.3.3. NF-κB inhibitors

Elevated levels of NF-κB expression occur in many lymphoid malignancies. Strategies interfering with signal transduction events that generate active NF-κB have emerged.
IKKs, that are responsible for ubiquitination and subsequent proteasome-dependent degradation of NF-κB inhibitors IκB, can be targeted, resulting in induction of apoptosis in cultured cancer cells. A broad range of chemical IKK inhibitors have been described including: PS1145,[205] BMS-345541,[206] Bay 11-7082,[207] SC-514,[208] sulfasalazine,[209] β-carbolines[210]. An other promising therapeutic approach also leading to suppression of IκB activity is proteasome inhibition. Proteasome inhibitors MG-132,[211] CEP-1612,[212] PR-171,[213] NPI-0052,[214] and bortezomib (PS-341/velcade)[215] demonstrated blockage of the NF-κB pathway followed by induction of apoptosis in preclinical studies. PR-171, NPI-0052 and bortezomib are currently tested in clinical trials of several types of cancer. Recently, bortezomib has been approved for treatment of refractory myeloma.

6. Aim and outline of the thesis

The main goal of this thesis was to investigate possible mechanisms responsible for apoptosis resistance in DLBCL, allowing identification and tailoring of targeting therapies. The first aim we addressed:

Chapter 2: Is the expression of effector caspase 3 and the apoptosis inhibitory proteins Bcl-2, XIAP and c-Flip related to differences in clinical outcome in DLBCL?

Using immuno-histochemisty, we demonstrated expression profiles that were suggestive for caspase 8 and/or caspase 9 inhibition in all cases. A caspase 8 inhibition only profile (low percentages of active caspase 3 positive DLBCL cells, and expression of c-Flip) was related to a favorable outcome whereas a caspase 9 inhibition only profile (expression of Bcl-2 and XIAP) was strongly related to an unfavorable outcome.

Most of the chemotherapeutic drugs used in the treatment of DLBCL induce apoptosis primarily via the intrinsic apoptosis pathway, further indicating that only inhibition of the intrinsic caspase 9 mediated pathway is seriously involved in resistance to chemotherapy induced apoptosis.

To investigate possible mechanisms that underlie disruption of chemotherapy-induced caspase 9 mediated apoptosis, functional analysis of apoptosis pathways in DLBCL cells was necessary. However, detection of apoptosis in primary DLBCL cells remained unreliable and inefficiently and was frequently hampered by high levels of spontaneous apoptosis. This led to the following question:

Chapter 3: Can we design a rapid and reliable assay to detect apoptosis in isolated lymphoma cells of DLBCL?

In this study we developed an easy and highly sensitive method for detection of apoptotic cells using 7AAD staining in combination with fluorescent beads and the pancaspase inhibitor zVAD-FMK. We showed that this method required few cells and that it was possible to detect apoptosis reproducibly in isolated lymphoma cells of DLBCL biopsies and in hematopoietic cell lines.

In a recent study, using micro array analysis, we have demonstrated that a subset of DLBCL with poor clinical outcome was characterized by a gene expression profile reflecting constitutive activation with concomitant inhibition of the intrinsic apoptosis pathway. It seemed that intrinsic cellular resistance to chemotherapy and predicted clinical outcome depended on the balance between pro- and anti-apoptotic genes. These results prompted us to raise the following question:

Chapter 4: Is chemotherapy resistance caused by disruption of the intrinsic apoptosis pathway?

Here, we showed that expression profiles of apoptosis related genes as determined in isolated lymphoma cells divide DLBCL cases into one group with low expression levels of both pro- and anti-apoptotic genes and one group with high expression levels of these genes. DLBCL with high expression levels of pro- and anti-apoptotic genes frequently appeared to be chemotherapy-refractory and were characterized by high levels of spontaneous caspase 9 activity and mitochondrial membrane depolarization without induction of apoptosis, indicating a disruption of the apoptosis pathway downstream of caspase 9 activation.

In the next three chapters (5, 6 and 7) we tested alternative therapies that could circumvent downstream disruption of the intrinsic apoptosis pathway and restore sensitivity to cell death. First, we hypothesized that inhibition of the anti-apoptotic protein XIAP with targeting therapeutics would restore apoptosis sensitivity and this led to the next question:

Chapter 5: Can the small-molecule XIAP antagonist induce apoptosis in isolated lymphoma cells of DLBCL patients?

We demonstrated that the small-molecule XIAP antagonist induces apoptosis in isolated DLBCL cells, including chemotherapy-refractory cases. In addition, we found that XIAP antagonist-sensitive

cases were characterized by high expression levels of XIAP, relatively low expression levels of Bcl-2, and by constitutive caspase-9 activation. The XIAP antagonist did not affect peripheral blood mononuclear cells and tonsil germinal center B-cells from healthy donors.

An other pathway to induce apoptosis is the extrinsic apoptosis signaling pathway that can be triggered by members of the TNF family, including TRAIL/Apo2L. We hypothesized that it might be possible that the extrinsic apoptosis pathway is still intact in chemotherapy-refractory DLBCL and that these DLBCL cells would still be sensitive to apoptosis. This led to the following aim of our study:

Chapter 6: Can hsTRAIL/Apo2L induce apoptosis in isolated lymphoma cells of DLBCL patients with chemotherapy refractory DLBCL?
In this study we showed that a subset of DLBCL samples including chemotherapy-refractory lymphomas was sensitive to hsTRAIL/Apo2L. Furthermore, hsTRAIL/Apo2L induced apoptosis in DLBCL cells and in B-cell lines demonstrated high expression levels of the apoptosis inhibitors Bcl-2 and/or XIAP, suggesting that these anti-apoptotic proteins did not confer resistance to hsTRAIL/Apo2L-induced apoptosis in DLBCL.

An alternative way to circumvent disruption of the apoptosis pathways is to induce cell death independent of apoptosis in DLBCL. In the last decade, this notion is supported by improvements in clinical outcome in DLBCL following the introduction of rituximab (R) in combination with CHOP. However, a subset of R-CHOP treated patients have shown progressive disease or relapsed rapidly and this resulted in the development and characterization of a panel of new human CD20 mAb, including ofatumumab that might improve outcome. Thus, the final aim of our study was:

Chapter 7: Can ofatumumab induce complement dependent cytotoxicity (CDC) of isolated lymphoma cells of chemotherapy-refractory DLBCL?
Here we showed that the novel human type I CD20 mAb ofatumumab induced CDC in DLBCL cell lines and all chemotherapy-refractory DLBCL cases tested. Ofatumumab was more effective in inducing CDC of DLBCL tumor cells compared to rituximab. Sensitivity of DLBCL to ofatumumab- and rituximab-induced CDC was dependent of CD55 and CD59 expression, although ofatumumab-induced CDC was less inhibited by these molecules.

In the final chapter 8 we discuss possible mechanisms responsible for intrinsic resistance to apoptosis in DLBCL and how these mechanisms can be used in development and application of targeting therapies that can improve clinical outcome in DLBCL patients. Furthermore, we propose a model explaining how apoptosis expression profiles can be used in tailoring of therapy for the individual DLBCL patient.

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