General Introduction
LEUKEMIA

The highly coordinated process of blood cell development and homeostasis, termed hematopoiesis, is essential for the development and survival of a normal individual. Normal hematopoiesis takes place in the bone marrow (BM), where primitive hematopoietic stem cells (HSC) are located. HSC usually are in a quiescent stage but have the potential to undergo polarized division to form new blood cells belonging to different cell lineages. One of the daughter cells retains the lifelong capacity for self-renewal and the omnipotence of the mother cell. The other daughter cell differentiates into a primitive progenitor cell that is able to further divide and differentiate into progeny belonging to one of the hematopoietic lineages, including red blood cells, platelets, and a variety of lymphoid and myeloid cells (Figure 1.1). 1-3

![Figure 1.1 Normal human hematopoiesis.](Image)

Ultimately, terminally differentiated cells are produced that cannot divide and undergo apoptosis after a period of time ranging from hrs (neutrophils) to years (memory B and T lymphocytes). However, if one or more transformations occur at any stage of the differentiation process, this may lead to uncontrolled proliferation of a malignant cell population that is arrested in its maturation. When these malignant cells accumulate in the blood it is known as leukemia. Due to accumulation of leukemic cells in the BM, the production of red blood cells, platelets, and normal leukocytes is
hampered. If untreated, the surplus leukemic cells overwhelm the bone marrow, enter the bloodstream, and eventually invade other parts of the body, such as the lymph nodes, spleen, liver, and central nervous system (brain, spinal cord). Several types of leukemia exist, depending on the maturation state and the lineage commitment of the malignant clone. The most common types of leukemia are acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphocytic leukemia (CLL).

**Treatment of leukemia**

Because of the different nature of the disease, patients with acute leukemia are treated differently from patients with chronic leukemia. Therapy frequently consists of a combination of different cytostatic agents. If patients receive high-dose chemotherapy and total body irradiation, stem cell transplantation (SCT) is required to achieve immunologic reconstitution in these patients. In addition, donor T cells from the transplant may cause a beneficial graft-versus-leukemia (GVL) effect.

**Acute leukemia**

Acute leukemia is characterized by aggressive outgrowth of immature blasts committed to the myeloid (AML) or lymphoid lineage (ALL). Accumulation of these cells in the BM, but later on also in the blood compartment, leads to replacement of normal hematopoiesis.

Treatment regimens of acute leukemia generally involve separate phases. Induction chemotherapy consists of the administration of a combination of chemotherapeutic agents, such as anthracyclines (doxorubicin, daunorubicin), methotrexate, and cytarabine (Ara-C), leading to reduction of proliferating hematopoietic cells (both malignant and benign) from BM and blood, and aiming at obtaining a remission. Due to their quiescent state, HSC are saved from chemotherapy-induced cell death, which allows repopulation of the marrow with normal cells after therapy. A complete remission (CR) requires normalization of the BM (<5% marrow blasts) and recovery of cell counts in the peripheral blood (neutrophil count of $\geq 1 \times 10^9/L$, platelet count of $\geq 100 \times 10^9/L$). A partial response (PR) is achieved when blood count recovery is similar to a CR, but with the persistence of 6% to 25% bone marrow blasts. Once remission is achieved, intensive consolidation therapy is required to destroy the low levels of remaining leukemic cells, known as minimal residual disease (MRD), hence making long-term disease-free survival (DFS) possible.

Despite aggressive induction and consolidation therapy, the overall 5-year survival rates of patients with AML and ALL are low mainly due to the high incidence of relapses of the leukemia (50-60%). In AML, long-term survival occurs in approximately 30% of younger adults, compared to only 5-15% long-term DFS in adults over the age of
60 years. In ALL, the cure rate in children is 70-75%, whereas only 30-40% long term survivors in adults have been reported.

Chronic leukemia
Chronic leukemia can be subdivided in CLL and CML, which are two completely different disorders. CLL is an indolent malignancy, characterized by slow but progressive expansion of a leukemic lymphoid clone with a prolonged life span (>1 year) due to decreased apoptosis, resulting in the accumulation of immature leukemia cells in bone marrow, blood, spleen, liver and lymph nodes. Because of the indolent progression of the disease, the incurability of the disease, and the relatively high age of the majority of patients, many CLL patients initially do not receive therapy. Currently the decision to treat patients is based on multiple factors including advanced clinical staging (Rai-Binet), symptomatic disease, disease activity (lymphocyte doubling time), patient age, and probably in the near future the presence of unfavorable prognostic factors such as ZAP-70 expression, unmutated variable heavy chain immunoglobulin (IgV\_H) status and some genetic aberrations. Therapy regimens frequently consist of different combinations of chlorambucil, fludarabine, cyclophosphamide and rituximab. Rituxumab is a genetically engineered mouse-human chimeric monoclonal antibody directed against the human B-cell-restricted cell surface antigen CD20, which induces apoptosis by complement-dependent and antibody-dependent cell-mediated cytotoxicity.

CML is caused by a reciprocal t(9;22)(q34;q11) translocation in HSC. In the t(9;22) translocation, a major part of the ABL proto-oncogene, including the catalytic domain, is translocated onto the BCR region of chromosome 22, giving rise to the oncogenic bcr/abl fusion protein. Treatment of CML in chronic phase (CML-CP) is aimed at inhibiting the effects of this translocation. Imatinib mesylate (Gleevec) is a compound designed to specifically inhibit the kinase activity of the bcr/abl fusion product. In newly-diagnosed CML-CP patients imatinib showed significantly higher major and complete cytogenetic responses (90 and 82% respectively) when compared with the formerly used treatment regimen consisting of IFN-\alpha and Ara-C (35 and 15% respectively).

Although chronic phase CML can be relatively indolent, progression to accelerated phase CML (CML-AP) or blastic phase (CML-BP) is imminent. CML-BP can be of myeloid, lymphoid or other hematopoietic lineage. Clinically, CML-BP progresses with similarly aggressive kinetics as AML or ALL. CML-AP and CML-BP are usually treated as an acute leukemia, but the results of this treatment are extremely poor.

Allogeneic stem cell transplantation and donor lymphocyte infusions
SCT can be considered as a rescue procedure to achieve immunologic reconstitution in patients of whom both malignant and normal hematopoiesis were destroyed by high-
dose chemotherapy and total body irradiation. SCT can be classified as autologous or allogeneic, based on the origin of the HSC. Autologous SCT is a procedure in which a patient’s own HSC, which were removed from the BM before initiation of intensive treatment regimens, are infused. A disadvantage of this type of transplant is that it is very difficult to guarantee that normal stem cells have been separated from leukemic cells, even after *in vitro* treatment with drugs, immunologic agents, or other methods to kill or remove leukemic cells.

Allogeneic SCT is a form of transplant in which the stem cells are gathered from a HLA-identical healthy donor, which may be a relative (brother, sister, child) or a matched unrelated donor. Besides causing immunologic reconstitution, allogeneic SCT is also an effective anti-leukemic therapy, since donor T cells in the graft may recognize and attack the residual leukemic cells resulting in the possible generation of a GVL effect. However, besides the beneficial effect of allogeneic SCT, several disadvantages of this therapy exist including the potential failure of engraftment, organ toxicities caused by graft-versus-host disease (GVHD) and prolonged immunosuppression with its concomitant risks of post-SCT infectious complications.

Nonmyeloablative or reduced-intensity SCT relies upon the GVL effect of the allograft rather than the direct tumoricidal activity of the conditioning regimen. This regimen results in a shortened duration of cytopenia and a minimal mucosal toxicity, providing a reasonably safe transplant option for patients 20 years older than the population usually treated with traditional fully ablative high-dose chemotherapy regimens.

Many studies showed that the beneficial GVL reactivity observed after allogeneic SCT was induced by donor T cells in the graft. In patients with a relapse of their leukemia after allogeneic SCT, the GVL effect of donor T cells can be utilized by administration of single or multiple doses of lymphocytes from the original stem cell donor. The curative effect of donor lymphocyte infusions (DLI) was first reported in patients with relapsed CML. DLI demonstrated to have curative potential in many hematopoietic malignancies, but the efficacy of DLI varied depending on the nature of the disease. In CML-CP, DLI was highly successful showing complete molecular remissions in 70-80% of the treated patients. In contrast, DLI was less effective in patients with more aggressively proliferating diseases like CML-AP, CML-BP, AML and ALL showing remission rates of 33%, 17%, 15-29% and 0-13%, respectively. Following HLA-identical SCT or DLI, allogeneic T cells recognizing minor histocompatibility antigens (mHags) are thought to be mainly responsible for the observed clinical immune response. MHags are immunogenic polymorphic peptides derived from genetically polymorphic intracellular proteins that may differ between donor and recipient. A number of mHags have been identified till now including the hematopoiesis-specific mHags HA-1 and HA-2. HA-1 and HA-2 specific T cells have been shown to contribute to the clinical response observed after DLI.
Mechanisms of action of frequently used therapies

Chemotherapy and cellular immunotherapy induce different mechanisms in the target cell eventually leading to the death of the target cell.

Chemotherapy
For many cytostatic agents used for the treatment of leukemia specific mechanisms of action have been presumed. The broadly used chemotherapeutic agent Ara-C, a pyrimidine analogue of deoxycytidine (dC), enters the cells in its nucleoside form by passive diffusion, and is then converted by a series of cellular enzymes to the active metabolite Ara-C triphosphate (Ara-CTP). In proliferating cells, Ara-CTP competes with the natural nucleoside triphosphate dCTP for incorporation into replicating DNA. This incorporation causes termination of the daughter strand DNA synthesis at the sites of drug incorporation. Furthermore, Ara-C inhibits DNA repair by blocking topoisomerase I-mediated DNA religation.

Purine analogues like fludarabine (F-Ara-A) and cladribine (2-CdA) have been reported to inhibit DNA polymerase and ribonucleotide reductase.

One of the expected mechanisms of action of anthracyclines like daunorubicin is intercalation of the anthracycline between adjacent base pairs of the double helix of DNA causing inhibition of DNA transcription and replication due to the formation of DNA breaks. Anthracyclines can also inhibit DNA helicases or interact with DNA topoisomerase II, blocking optimal DNA unwinding and strand segregation during DNA transcription and translation.

All these chemotherapeutic agents are supposed to exert their action preferably in cycling cells. In line with this, a substantial number of leukemic blasts and leukemic precursor cells which are in dormant G0-phase of the cell cycle escape from treatment with these drugs. However, also examples can be given that contradict this assumption. Firstly, whereas HSC are indeed saved from chemotherapy-induced cell death, quiescent (G0) lymphocytes in peripheral blood are easily killed by chemotherapeutic treatment. Secondly, although the brains mainly consist of cells in resting phase of the cell cycle, which are expected to be insensitive to chemotherapeutic treatment, cerebral cytotoxicity is a rare complication of chemotherapy treatment. Finally, CLL can be effectively treated with fludarabine, an agent mainly acting by inhibiting DNA synthesis, which seems to be an irrelevant mechanism of killing resting CLL cells. How all these discrepancies must be explained is not clear.

Most chemotherapeutic agents have been reported to kill leukemic target cells via activation of apoptosis pathways. The apoptotic pathways that are thought to be induced after treatment with chemotherapy are described in more detail in the next paragraph (Figure 1.4).
Chapter 1

DNA fragmentation became evident in leukemic cells 6 hrs after exposure to Ara-C and other anti-cancer agents. 56-59 It seems to be unlikely that inhibition of DNA synthesis occurs within 6 hrs of incubation suggesting that other mechanisms of action may be involved in Ara-C-induced apoptosis. Conversely, although first apoptotic signals are already visible after 6 hrs of exposure to Ara-C, in many cells eventual cell death can only be observed after 24 to 48 hrs of incubation, which corresponds to the time required for incorporation of Ara-C into DNA.

Cellular Immunotherapy

The allogeneic antileukemic activity of DLI and HSCT is mainly caused by donor-derived natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). These killer lymphocytes can mediate target cell death using two different effector mechanisms: granule-mediated cell death and death receptor-mediated apoptosis. 60 Cytotoxic granules consist of the pore-forming protein perforin (PFN) and various granzymes, of which Granzyme B (GrB) is the most prominent member. High concentrations of PFN may cause pores in the target cell membrane, which may directly result in target cell death independent on an active apoptosis machinery (necrosis). Low concentrations of PFN enable the introduction of GrB, which in turn can activate different apoptotic cascades in the target cell. The role of the death-receptor pathway in CTL-induced tumor cell death remains to be further elucidated, as described in more detail in the next paragraph.

APOPTOSIS

Apoptosis is a general mechanism for removal of unwanted cells from the body, without causing inflammation, and is therefore essential during development and in the maintenance of tissue homeostasis. 61 Examples of processes of the immune system in which apoptosis plays an important role include the death of cells with short half-lives (neutrophils), 62 and the elimination of self-reactive T cells 63 and low responsive B cells in the germinal center. 64 Deregulation of apoptotic cell death can disrupt the delicate balance between cell proliferation and cell death and contributes to the pathogenesis of a number of human diseases including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (acquired immunodeficiency syndrome). 65-67

Characteristics of apoptosis

Two forms of cell death have been described in vertebrate tissues. 68 When cells die from severe and sudden injury, such as ischaemia, sustained hyperthermia or physical or chemical trauma, this is called necrosis or accidental cell death.
During necrosis there are early changes in mitochondrial shape and function, and the cell rapidly becomes unable to maintain homeostasis. The plasma membrane probably is the major site of damage: by losing its ability to regulate osmotic pressure, the cell swells and ruptures. The cell contents are lost in the surrounding tissue space and provoke an inflammatory response.

Apoptosis is a more subtle form of cell death, often called programmed cell death. Apoptosis refers to a series of morphological changes during cell death that are different from those seen in necrosis and which occur when cell death is physiologically determined or acceptable. Caspases play an important role in the apoptotic process. Initiator caspases are important regulators of apoptosis by inducing a signaling cascade, whereas effector caspases cleave various structural proteins resulting in an ordered disassembly of the cell.

**Morphology**

Although the exact pattern changes from cell type to cell type, in general cells undergoing apoptosis are characterized by the morphological changes indicated in Figure 1.2 for a lymphocyte (adapted from Cohen 62). The various stages of apoptosis are best seen in isolated culture, since in vivo phagocytosis will intervene.

![Figure 1.2. Various stages of apoptosis in a lymphocyte (adapted from Cohen) on page 17.](image-url)

(a) A normal cell has a sparse cytoplasm and heterogeneous nuclear chromatin, and cell volume is about 90 fl.

(b) The cell loses some volume, and its cytoplasmic organelles are now tightly packed. There is clumping of chromatin. At this stage, membrane changes that can lead to phagocytosis such as exposure of phosphatidylserine at the cell surface are present. 69 Loss of membrane phospholipid asymmetry may be caused by changes in energy metabolism, bivalent cation concentrations or cytoskeleton organization. 70

(c) The cell exhibits zeliosis: the plasma membrane becomes ruffled and blebbed.

(d) The cell and nucleus shrink and the chromatin becomes very dense. The chromatin collapses into patches, and subsequently into crescents along the nuclear envelope. Cell volume is now about 70 fl.

(e) The nucleus has collapsed into a black hole.

(f) The collapsed nucleus frequently breaks up into several spheres. This change is often accompanied by fragmentation of the DNA into a ladder of regular subunits, which is the result of apparently random double-stranded breaks in the linker regions between nucleosomal cores. 71 Since there may be over a million of such breaks, this never can be repaired and cessation of transcription is imminent.

(g) Eventually, the cell fragments into membrane-enclosed apoptotic bodies.
Regulation of apoptosis

Apoptosis is tightly regulated and orchestrated at the molecular level by pro-apoptotic members of the caspase family including caspase-3, -6, -7, -8, -9 and -10. Caspases are synthesized in the cell as inactive precursors (zymogens) composed of three distinct domains: an N-terminal polypeptide or prodomain, a large subunit containing the active site cysteine with a conserved QACXG motif, and a C-terminal small subunit. Caspases show an unusual and absolute requirement for cleavage after aspartic acid (Asp) residues. An aspartate cleavage site separates the prodomain from the large subunit, and an interdomain linker containing one or two aspartate cleavage sites separates the large and small subunits. The presence of Asp at the maturation cleavage sites is consistent with the ability of caspases to auto-activate or to be activated by other caspases as a part of an amplification cascade.

Caspases are activated to fully functional proteases by two cleavage events (see Figure 1.3). The first proteolytic cleavage divides the chain into large and small caspase subunits via removal of the linker region, and a second cleavage removes the N-terminal prodomain. The active caspase is a tetramer of two large and two small subunits, with two active sites.

Each active site is formed by sequences supplied by both the large and small subunits, providing amino acids necessary for substrate recognition and catalysis. Despite their shared requirement for cleavage after aspartic acid residues, caspases are highly specific in their substrate preferences. Most caspase substrates can be divided into two general categories: (1) regulators of apoptosis which are either activated or inactivated by cleavage; and (2) housekeeping or structural proteins whose cleavage is required for the ordered disassembly of the cell.

The mammalian caspases have been divided into upstream (initiator) and downstream (effector) caspases based on their sites of action in the proteolytic caspase cascade. Initiator caspases (caspase-8, -9 and -10) function upstream of effector caspases.
as signaling molecules, whereas effector caspases (caspase-3, -6 and -7), which are mammalian homologues of the C. elegans CED-3 caspase, account for the morphological features of apoptosis.

In line with their function, initiator and effector caspases have different prodomains. Initiator caspases have long prodomains containing structurally related protein modules that physically link these proteases to their specific activators, hence enabling oligomerization of procaspases. Two types of interaction modules have been detected: death effector domains (DEDs) and caspase recruitment domains (CARDs). Initiator caspases have substrate specificities that are similar to caspase recognition sites present in their own sequence, implying that these caspases can utilize autocatalysis for activation. In contrast, effector caspases have small prodomains, and are activated via cleavage by initiator caspases or other, already activated, effector caspases.

**Therapy and apoptosis**

Apoptosis can be initiated by many different stimuli including chemotherapy and (cellular) immunotherapy. The different signaling cascades eventually leading to apoptosis that may be induced by the different forms of therapy against leukemia are summarized in Figure 1.4. Chemotherapy treatment may induce apoptosis via activation of the mitochondrial (intrinsic) pathway of apoptosis. In addition to the mitochondrial pathway of apoptosis, involvement of the death receptor pathway, also called extrinsic apoptotic pathway, in chemotherapy-induced apoptosis has been postulated by several investigators, after showing that several anti-cancer drugs induce upregulation of Fas receptor (FasR) and Fas ligand (FasL), followed by subsequent autocrine or paracrine induction of Fas-mediated apoptosis. It is unclear whether both pathways are induced simultaneously or whether preferentially one of the two is activated. Moreover, the pathway of apoptosis likely depends on the nature of the target cells and the cytostatic agent used.

Cytotoxic lymphocytes may induce apoptosis of target cells both via release of granules containing perforin and granzyme B, and via activation of the death receptor pathway. Granule-mediated cell death has been reported to play an important role in the elimination of virus-infected cells and tumor cells. In contrast, death receptor-mediated cell death has primarily been described to be involved in eliminating autoreactive T cells and downsize immune responses after infections. The involvement of the death receptor pathway in the elimination of tumor cells remains to be elucidated.

**Mitochondrial pathway**

The mitochondria play an important role in the initiation of the intrinsic pathway of apoptosis, also called the mitochondrial pathway, by functioning as stress sensors
and determining the ‘point of no return’. Upon stress signals, as caused for instance by growth factor withdrawal, UV- or γ-irradiation, or chemotherapeutic agents, the permeability of the mitochondria is increased. This process is regulated by the B-cell lymphoma 2 (Bcl-2) family of proteins, containing both pro-apoptotic members such as Bcl-2 associated X protein (Bax) and Bcl-2 antagonist/killer (Bak), and anti-apoptotic members such as Bcl-2 and Bcl-XL. Bcl-2 and Bcl-XL reside on the mitochondrial outer membrane, while the pro-apoptotic family members may be either cytosolic or present on the mitochondrial membrane. Mitochondrial outer membrane permeabilization leads to the release of factors such as cytochrome C from the mitochondrial intermembrane space into the cytosol. In the cytosol, cytochrome c binds to and induces oligomerization of apoptotic protease activating factor-1 (Apaf-1). In the presence of ATP or dATP, Apaf-1 and procaspase-9 associate via their CARDs which results in the formation of an “apoptosome” consisting of cytochrome c, Apaf-1, and pro-caspase 9. In this heptameric complex caspase 9 is activated. The apoptosome can then recruit procaspase-3, which is cleaved and activated by the active caspase-9. Active caspase-3 in turn cleaves various death substrates, eventually leading to disassembly of the cell. Along with cytochrome c, two other proteins are released from the mitochondria during apoptosis: SMAC/DIABLO and OMI. These proteins increase apoptosome function by inhibiting the inhibitor of apoptosis protein (IAP) family of caspase inhibitors.
and thus promoting caspase activation, as depicted in Figure 1.4. Besides these relatively well-known pro- and anti-apoptotic proteins, mentioned in Figure 1.4, probably many other proteins are involved. Moreover, different apoptotic pathways may share similar components and in this way influence each other making the actual apoptotic mechanisms very complex.

**Death receptor pathway**

One of the alternative or additional pathways that may play a role in chemotherapy-induced apoptosis is the death receptor pathway. The death receptor pathway is initiated when death ligands of the tumor necrosis factor (TNF) superfamily (FasL, TNF-α, TNF-related apoptosis inducing ligand (TRAIL)), bind to transmembrane proteins called death receptors present at the cell surface of the target cell. Upon extracellular triggering, trimerization of the death receptor occurs, and Fas Associated Death Domain (FADD) is recruited to the intracellular death domain (DD) of the death receptor, followed by binding of pro-caspase 8, also called FADD-like IL-1 converting enzyme (FLICE), to the DD of FADD. In this complex of receptor-bound FADD with pro-caspase-8, called the Death Inducing Signaling Complex (DISC), pro-caspase-8 is proteolytically cleaved. The active caspase-8 that is formed either directly cleaves effector caspases resulting in apoptosis without mitochondrial depolarization, or cleaves BH3-interacting-domain death agonist (Bid), a member of the pro-apoptotic Bcl2 family. Truncated Bid (tBid) relocalizes to the mitochondrial outer-membrane and activates the intrinsic pathway of apoptosis (see Figure 1.4). Cellular caspase-8 (FLICE)-like inhibitory protein (cFLIP), which contains two DEDs and a non-catalytic pseudo-caspase domain, is also recruited to the DISC, where at high concentrations it can block caspase-8 activation (Figure 1.4). At lower, and probably more physiological, concentrations cFLIP is thought to function as a scaffolding molecule that aids caspase-8 oligomerization and activation.

**T-cell induced cell death**

T cell receptors (TCR) of cytotoxic CD8+ T cells recognize a complex formed between class I Major Histocompatibility Complex (MHC) molecules and antigenic peptides, mainly derived from intracellular processing of endogenously synthesized proteins. Binding of different peptides in different class I alleles is based on the length of the specific peptide and the presence of crucial anchor residues. Upon recognition of target antigen by membrane TCRs, CTLs can mediate target cell death potentially using two effector pathways. Target cell death either occurs via TCR-triggered exocytosis of preformed cytotoxic granules or, alternatively, via activation of the death receptor pathway in the target cell. PFN and granzymes are stored in the lytic granules of most CTLs. After specific interaction and recognition...
of a target cell these granules are released, resulting in formation of pores in the cell membrane of the target cell. These pores themselves may either directly cause leakiness of the target cell resulting in necrosis, or enable the introduction of granzymes including GrB into the target cell. GrB in turn can activate different apoptotic cascades in the target cell (Figure 1.4). It directly cleaves effector caspases such as caspase-3,\textsuperscript{122} or it activates the mitochondrial pathway via cleavage of Bid.\textsuperscript{123} Although target cells can internalize GrB in the absence of PFN, sublytic concentrations of PFN are required to induce apoptosis.\textsuperscript{124} The death receptor pathway is induced when apoptosis inducing ligands (FasL, TNF-\textalpha, TRAIL), expressed on the membrane of TCR-activated T cells, cross-link death receptors expressed on the target cells.\textsuperscript{120,125}

Many factors likely affect the susceptibility of leukemic target cells to T cell-mediated cell death. The cell cycle status of the target cell is important, since resting cells are less sensitive to T-cell-mediated apoptosis. Furthermore, the strength of interaction between target and effector cell probably affects the kinetics of target cell death but may also influence the efficacy of T-cell treatment, since not all effector mechanisms may be induced in case of weak effector-target interactions. The strength of interaction between effector and target is largely determined by the affinity of the T cell receptor (TCR), the avidity of the MHC/TCR complex, and the expression of adhesion and costimulatory molecules on both leukemic and T cells. These and probably also other factors should be studied in more detail to improve the effectiveness of T cell-based immunotherapeutic interventions in patients with leukemia.

Resistance to therapy-induced apoptosis

After occurrence of a relapse, many patients are unresponsive to further therapy. Moreover, some patients never reach a state of complete remission. Different mechanisms may underlie this resistance, including expression of drug efflux pumps by leukemic cells,\textsuperscript{126-129} cell cycle status of the leukemic cell, and inability of leukemic cells to undergo apoptosis in response to therapy due to aberrant expression of (anti-) apoptosis proteins. Expression levels of proteins involved in the induction or inhibition of apoptosis have been described to contribute to the response against a variety of drugs used for the treatment of leukemia.\textsuperscript{130-132}

The anti-apoptotic protein Bcl-2\textsuperscript{133} is overexpressed in many cancers including CLL.\textsuperscript{134} One of the mechanisms of Bcl-2 to inhibit apoptosis is prevention of cytochrome c release from the mitochondria.\textsuperscript{135,136} For most cancers, the overexpression of Bcl-2 or Bcl-X\textsubscript{L} correlates with poor survival and progression of the disease.\textsuperscript{137-141} One approach for targeting anti-apoptotic Bcl-2 proteins is by reducing their expression levels using anti-sense oligonucleotides. An antisense
oligonucleotide against BCL2, oblimersen, \(^{142}\) has been in phase III clinical trials for melanoma, CLL and multiple myeloma, as well as in phase II clinical trials for other cancers. \(^{143-145}\) So far, the clinical results are not very promising. \(^{144,146}\) Another important protein family that regulates apoptosis is the IAP family of proteins (see Figure 1.4). \(^{147,148}\) XIAP, c-IAP1, and c-IAP2 bind to and inhibit active caspase-3 and -7, and also procaspase-9, but not caspase-1, -6, -8 or -10. The XIAP protein is overexpressed in many cancers, and the expression of XIAP correlates with apoptotic resistance. Survivin \(^{149}\) is not present in normal adult tissues but is overexpressed in many different tumors, and is important for tumor cell viability. \(^{150}\) Many different strategies to inhibit these IAPs are currently developed. Also overexpression of anti-apoptotic proteins that specifically cause resistance to immunotherapy like FLICE inhibitory protein (FLIP) and proteinase inhibitor-9 (PI-9) have been reported. FLIP is an enzymatically inactive homologue to caspase 8 and interacts with FADD, preventing pro-caspase 8 to bind to the death domain of the death receptors (see Figure 1.4). \(^{151,152}\) Overexpression of FLIP by tumor cells was demonstrated to be an escape mechanism for malignant cells to survive CTL attack. \(^{153}\) PI-9 specifically binds to GrB and was shown to efficiently inhibit GrB-mediated apoptosis in both \textit{in vitro} and \textit{in vivo} studies. \(^{154}\) Besides elevated levels of anti-apoptotic proteins, also defects in pro-apoptotic molecules may cause resistance to chemotherapy, since functional blocks in caspase activation pathways in patients with leukemia have been correlated with poor clinical response to induction chemotherapy. \(^{155}\) Moreover, downregulation of procaspase-8 expression caused resistance to doxorubicin-induced apoptosis in several malignancies. \(^{156-158}\) Finally, recent patient studies showed that absence or low expression of FADD in cells from patients with AML is associated with resistance to chemotherapy treatment and poor clinical outcome. \(^{159-161}\) Because both caspase-8 and FADD are involved in death-receptor-induced apoptosis, this suggests that at least part of the death-receptor signaling pathway is involved in resistance to chemotherapy.

**AIM OF THE STUDY**

Failure of therapy due to acquired resistance is still a main cause of death of patients with leukemia. Aberrations in apoptotic pathways in leukemic cells have been shown to contribute to unresponsiveness of leukemic cells to chemotherapy and may also be involved in decreased sensitivity of these cells to cellular immunotherapy. Before defects can be detected, the apoptotic pathways itself should be unraveled in more detail. Although general therapy-induced apoptotic pathways have been described (Figure 1.4), still many controversies exist which will be highlighted in this thesis.
Chapter 1

The general scope of this thesis is to acquire more insight into the complexity of the apoptotic pathways that are induced in leukemic cells after treatment with conventional cytostatic drugs or with cytotoxic T cells. A better understanding of the apoptotic mechanisms itself and the factors that influence these pathways may help to improve treatment outcomes in patients with different types of leukemia.

Cells in quiescent stage of the cell cycle have been reported to be unsusceptible to most chemotherapeutic drugs including Ara-C. However, resting lymphocytes in peripheral blood are destroyed by chemotherapeutic treatment. Chapter 2 describes a study in which G0 cells from patients with B-CLL were tested for their responsiveness to Ara-C, an agent considered to be S-phase-specific. CFSE-based cytotoxicity assays combined with cell cycle analysis were used to perform long-term analysis of Ara-C-mediated killing of B-CLL cells. We analyzed the mechanisms that were involved in Ara-C-mediated apoptosis of these B-CLL cells, focusing at the effect of Ara-C on DNA and RNA synthesis.

Chemotherapy-induced apoptosis leads to activation of the mitochondrial pathway of apoptosis, although involvement of the death receptor pathway has also been postulated by several investigators (Figure 1.1). In Chapter 3 we investigated in more detail the role of the death receptor pathway and caspase-8 activation in chemotherapy-induced apoptosis in patient-derived myeloid and lymphoblastic leukemia cell lines. For this purpose, we introduced retroviral constructs encoding the anti-apoptotic proteins Bcl-2 and FLIP into these leukemia cell lines, and examined the effect on chemotherapy-induced apoptosis and caspase-8 activation.

Although T cells often express all prerequisites for death receptor-mediated apoptosis, the contribution of this effector mechanism to T-cell-mediated cell death is still largely unclear. Chapter 4 addresses the role of the death receptor pathway in CTL-mediated cell death of human target cells. Since PFN/GrB-mediated apoptosis and death receptor-induced apoptosis differ in time required to eliminate target cells, lysis of target cells was analyzed using CTL clones with slow and rapid kinetics of killing derived from a patient with chronic myeloid leukemia (CML). To determine the involvement of the death receptor pathway, a retroviral construct encoding the anti-apoptotic gene FLIP was introduced into these target cells, and the inhibitory effect of FLIP expression on CTL-induced target cell death was assessed.

PI-9 is considered to be a specific inhibitor of granzyme B-induced cell death, and should therefore not affect Fas-induced apoptosis. In Chapter 5, a more basic analysis of the specificity of PI-9 is performed in which the inhibitory effect of enhanced PI-9 expression on Fas-induced apoptosis is evaluated.
Chapter 6 describes two distinct mechanisms of T-cell-mediated target cell death. We studied whether both mechanisms resulted in the production of IFN-γ by the T cell. Furthermore, it was assessed whether these two forms of CTL-induced target cell death were induced via activation of different apoptotic pathways in the target cell.

Finally, in Chapter 7 the most important findings of this thesis are summarized. These data are compared with the general existing insights on apoptosis induction by chemotherapy and cellular immunotherapy. Finally, some suggestions are done on how these results may be applied for clinical applications.

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General Introduction


