Complexity of apoptotic pathways in leukemia treated with chemotherapy or cellular immunotherapy

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CHAPTER 1

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]

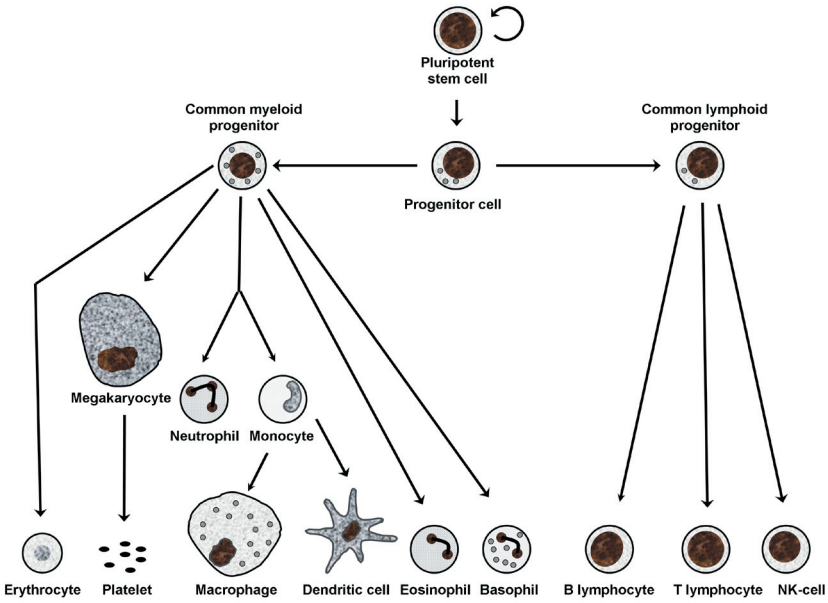

Figure 1.1 Normal human hematopoiesis.

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), [4-6] 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 ≥ 1 x 10^9/L, platelet count of ≥ 100 x 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%). [7] 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. [8-11] In ALL, the cure rate in children is 70-75%,[12] whereas only 30-40% long term survivors in adults have been reported. [13]

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. [14-18] Therapy regimens frequently consist of different combinations of chlorambucil, fludarabine, cyclophosphamide and rituximab. [19-21] 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. [22-24]

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. [25] 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. [26] 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-α and Ara-C (35 and 15% respectively). [27]

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. [28-30] 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. [31] DLI demonstrated to have curative potential in many hematopoietic malignancies, but the efficacy of DLI varied depending on the nature of the disease. [32] In CML-CP, DLI was highly successful showing complete molecular remissions in 70-80% of the treated patients. [32-35] 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. [32;33;36-38] 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 [39] including the hematopoiesis-specific mHags HA-1 and HA-2. [40] HA-1 and HA-2 specific T cells have been shown to contribute to the clinical response observed after DLI. [41;42]

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. [43] 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. [44]
Purine analogues like fludarabine (F-Ara-A) and cladribine (2-CdA) have been reported to inhibit DNA polymerase and ribonucleotide reductase. [45]
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. [46] Anthracyclines can also inhibit DNA helicases [47] or interact with DNA topisomerase II, [48;49] 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. [50-52] In line with this, a substantial number of leukemic blasts and leukemic precursor cells which are in dormant G_0-phase of the cell cycle escape from treatment with these drugs. [53] However, also examples can be given that contradict this assumption. Firstly, whereas HSC are indeed saved from chemotherapy-induced cell death, quiescent (G_0) 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. [45;54;55] 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).

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.

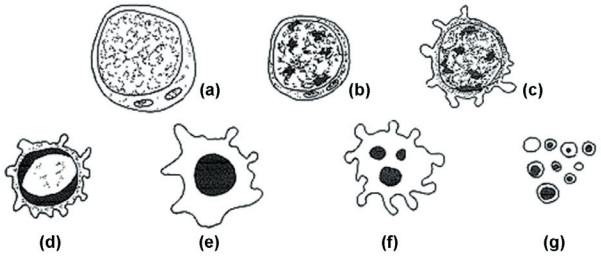

Figure 1.2. Various stages of apoptosis in a lymphocyte (adapted from Cohen[62]).

(a) A normal cell has a sparse cytoplasm and heterogeneous nuclear chromatin, and cell volume is about 90 fL.
(b) The cell looses 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 zeiosis: 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. [72] 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. [73] 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. [74-76]

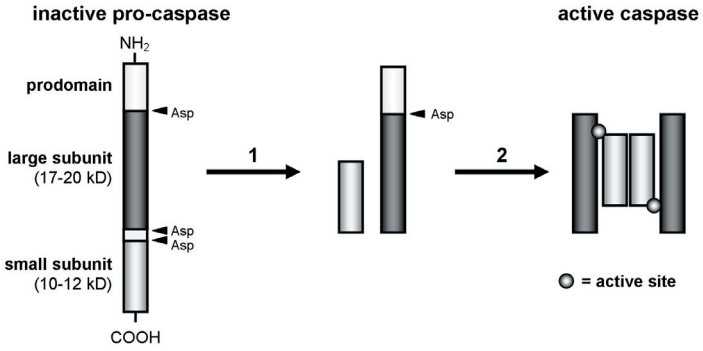

Figure 1.3. Formation of an active caspase.

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. [77;78] 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, [79] account for the morphological features of apoptosis. [73;80]
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). [80-82] Initiator caspases have substrate specificities that are similar to caspase recognition sites present in their own sequence, [78] 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. [83-87] 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. [88;89] 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. [90-93] 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

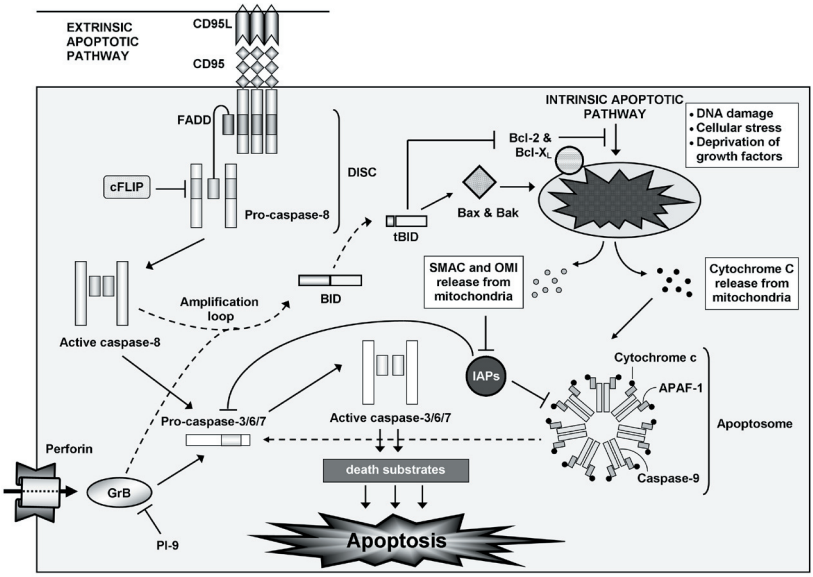

Figure 1.4. Apoptotic pathways induced by chemotherapy or (cellular) immunotherapy (partly adapted from Siegel [94]).

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-X_L. [95;96] Bcl-2 and Bcl-X_L 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 [97;98] 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). [99] 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. [100;101] 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. [102,103]

Along with cytochrome c, two other proteins are released from the mitochondria during apoptosis: SMAC/DIABLO [104;105] and OMI [106]. 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, [80] followed by binding of pro-caspase 8, also called FADD-like IL-1 converting enzyme (FLICE), to the DED of FADD. [107] In this complex of receptor-bound FADD with pro-caspase- 8, called the Death Inducing Signaling Complex (DISC), [108] procaspase-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). [109;110 111;112]
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). [113] At lower, and probably more physiological, concentrations cFLIP is thought to function as a scaffolding molecule that aids caspase-8 oligomerization and activation. [114;115]

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. [116;117]
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. [118;119]
Upon recognition of target antigen by membrane TCRs, CTLs can mediate target cell death potentially using two effector pathways. [60;120] 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. [60;89;121] 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, [122] or it activates the mitochondrial pathway via cleavage of Bid. [123] Although target cells can internalize GrB in the absence of PFN, sublytic concentrations of PFN are required to induce apoptosis. [124] The death receptor pathway is induced when apoptosis inducing ligands (FasL, TNF-α, TRAIL), expressed on the membrane of TCR-activated T cells, cross-link death receptors expressed on the target cells. [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, [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. [130-132]

The anti-apoptotic protein Bcl-2 [133] is overexpressed in many cancers including CLL. [134] One of the mechanisms of Bcl-2 to inhibit apoptosis is prevention of cytochrome c release from the mitochondria. [135;136] For most cancers, the overexpression of Bcl-2 or Bcl-X_L correlates with poor survival and progression of the disease. [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 *in vitro* and *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.

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 G_0 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 1.4). 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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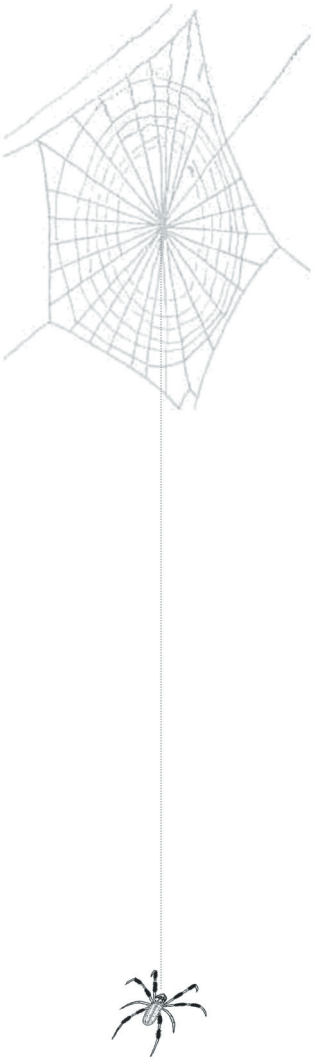
CHAPTER 2



The mechanisms of Ara-C-induced apoptosis of resting B-chronic lymphocytic leukemia cells

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ABSTRACT

Background and objective. Cytarabine (Ara-C) is commonly used for the treatment of acute leukemia. Incorporation of Ara-C into DNA is a key event in the mechanism of killing of proliferating leukemic cells. Previously, we demonstrated cytotoxicity of Ara-C to proliferating, but unresponsiveness to resting (G_0) malignant cells from patients with acute leukemia. In contrast, here we show unexpected apoptosis of G_0-B-CLL cells by Ara-C in a dose dependent manner. In this study we analyzed which cellular processes were involved in Ara-C-mediated killing of G_0-B-CLL cells.

Experimental design. Using primary B-CLL cells (>98% in G_0), we examined the mechanisms of Ara-C-mediated apoptosis in resting G_0 cells. 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. The effect of Ara-C on DNA and RNA synthesis effects were studied using various ^{3}H-incorporation experiments.

Results. Ara-C-mediated cell death of B-CLL cells showed the characteristics of normal apoptosis, such as phosphatidyl serine exposure and caspase activation. The mechanism of killing of quiescent B-CLL cells by Ara-C was shown not to be dependent on DNA replication. In contrast, CD40L-activated B-CLL cells showed S-phase specific depletion of proliferating CLL cells. We demonstrated Ara-C to be converted into its active triphosphate by G_0-B-CLL cells, coinciding with 30% inhibition of RNA synthesis.

Conclusions. In conclusion, our data indicate that Ara-C can induce apoptosis in resting G_0-B-CLL cells using a mechanism independent of cell proliferation and DNA replication but associated with inhibition of RNA synthesis and downregulation of Mcl-1.

INTRODUCTION

Ara-C, an analogue of deoxycytidine (dC), has been the most important compound in the chemotherapy regimens of patients with acute myeloid leukemia for 40 years. The close structural similarity of Ara-C with dC allows it to be metabolized by the same metabolic pathways as used for the normal processing of dC. [1] Cytarabine is transported into cells in its nucleoside form (Ara-C), and is then converted by a series of cellular enzymes to the triphosphate Ara-CTP, the active metabolite of cytarabine. 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. [2] Since the cytotoxicity of Ara-C is dependent on DNA synthesis, maximal effect of Ara-C on cell death is observed during the S-phase of the cell cycle. In line with this,

we previously demonstrated selective lysis of leukemic cells from patients with acute leukemia in active stage of the cell cycle, whereas G_0 cells were unresponsive to several cytostatic agents including Ara-C. [3;4]

In contrast, here we show Ara-C-mediated cell death of resting cells derived from patients with chronic lymphocytic leukemia (CLL). CLL is an indolent malignancy, characterized by progressive expansion of a leukemic lymphoid clone, resulting in the accumulation of leukemia cells at the G_0 quiescent stage of the cell cycle in bone marrow, blood, and other extramedullary sites such as spleen, liver and lymph nodes. There is only a small pool of proliferating cells, [5] as determined by the extremely low incorporation rate of ^{3}H-thymidine by leukemic lymphocytes, [6] or by flow cytometric analysis of newly synthesized DNA. [7]

Recently, nucleotide analogues like fludarabine (F-Ara-A) have been introduced as front-line therapy in the treatment of CLL. [8] Since the incorporation of nucleotide analogues like fludarabine and cytarabine (Ara-C) into DNA has been considered to be a key event in causing cytotoxicity in proliferating leukemic cells, we hypothesized that nucleotide analogues induce cell death of B-CLL cells by a different mechanism. Some studies already revealed that mechanisms of killing resting cells include inhibition of cellular DNA repair, inhibition of RNA synthesis, activation of pro-apoptotic proteins (Apaf-1), and downregulation of survival proteins (Bcl-2). [9;10]

In this study, we analyzed which cellular processes were involved in Ara-C-mediated killing of B-CLL cells. We first examined the relation between Ara-C-mediated apoptosis and the cell cycle status of the B-CLL cells using both quiescent and proliferating (CD40L-activated) B-CLL cells. Since we observed that different mechanisms were involved in Ara-C-mediated killing of G_0-B-CLL cells compared to proliferating B-CLL cells, we studied whether Ara-C could be converted into Ara-CTP in quiescent B-CLL cells, a reaction essential for the cytotoxicity of Ara-C in proliferating cells. [2;11] Furthermore, the effect of Ara-C on RNA synthesis in G_0-B-CLL cells was investigated. Finally, we analyzed whether Ara-C-mediated killing of G_0-B-CLL cells was initiated via downregulation of survival proteins and dependent on caspase activation. In contrast to the mechanism of killing by Ara-C of proliferating leukemic cells, our results demonstrate that Ara-C-mediated killing of B-CLL cells is not dependent on cell proliferation and DNA incorporation, but probably requires Ara-CTP formation and may involve inhibition of RNA synthesis.

MATERIALS AND METHODS

Cells and culture conditions

Peripheral blood was obtained from 4 CLL patients (3 female, 1 male) after informed consent. The materials we used were collected at times that patients had not received any form of therapy concerning their CLL, except one patient who was mildly treated with chlorambucil 2 years earlier. The percentage of CLL cells in peripheral blood samples was in all cases over 90% and these cells showed typical CLL characteristics including expression of CD19, CD5 and CD23. Mononuclear cells were separated by density centrifugation using Ficoll Isopaque, and either cryopreserved in liquid nitrogen or used directly for experiments. Isolated B-CLL cells were cultured at 37°C and 5% CO_2 for a maximum of 10 days in IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium), and 10% pooled human serum. To induce proliferation, B-CLL cells were cultured for 7 days on irradiated (70 Gy) CD40L-transduced mouse fibroblasts [12] (LTK-CD40L; kindly provided by Dr. C. van Kooten, Department of Nephrology, Leiden University Medical Center). Irradiated LTK-CD40L cells were cultured overnight at 37°C and 5% CO2 in a 24 wells plate at a concentration of 2 x 10^5 cells/well in IMDM supplemented with 10% fetal bovine serum (FBS, Cambrex), 3 mM L-glutamine, 50 μg/mL streptomycin and 50 U/mL penicillin. After one day, the medium was removed, and B-CLL cells were added at a concentration of 1x10^6/mL/well, and cultured in the medium described for resting B-CLL cells, supplemented with 500 IU/mL IL-4 (Schering-Plough, Amsterdam, The Netherlands).

Reagents

Apoptosis was induced with the following agents: cytosine arabinoside (Ara-C), 9-β-D-arabinosyl-2-fluoradenine (fludarabine) (both from Sigma-Aldrich, St. Louis, MO, USA), camptothecin (Alexis Corp., Lausanne, Switzerland), and as a negative control deoxycytidine (Sigma) was used.

Cytotoxicity assays

Cytotoxicity was measured using 24 or 40-hr ^{51}Cr release assays as described previously [3] or using carboxyfluorescein diacetate succinimidyl ester (CFSE) -based cytotoxicity assays as described by Jedema *et al*., [13] with some alterations. Target cells were labeled with 5 μM CFSE (Molecular Probes Europe, Leiden, The Netherlands). For the cytotoxicity assay, 25,000 cells/well (100 μL) were plated in 96-well microtiter plates (all in triplicate). Ara-C (10^{-5} M) was added in a volume of 50 μL/well. After 24, 48, 72 and 120 hrs, FACS analysis was performed. To exclude dead cells from the analysis, 7-amino-actinomycin D (7-AAD) (2 μg/mL final concentration) or Propidium Iodide (PI) (1 μg/mL) (both Sigma-Aldrich) was added, and samples were mixed properly and directly analyzed on a flowcytometer (Becton Dickinson (BD), San Jose, CA, USA). To allow quantitative analysis of the viable cells, the wells were harvested and transferred to FACS tubes containing 10,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA). This was done immediately prior to the analysis to avoid the formation of complexes between the cells and the beads. For each sample 5,000 microbeads were acquired, facilitating the calculation of absolute numbers of viable (7-AAD^-/PI^-) $CFSE^+$ target cells. The percentage of specific lysis was calculated as follows:

[(mean absolute number of viable $CFSE^+$ target cells in control medium - absolute number of viable $CFSE^+$ target cells ^{experimental}) / (mean absolute number of viable $CFSE^+$ target cells in control medium)] * 100.

To determine the role of caspases in the induction of Ara-C-mediated cell death, CFSE-labeled B-CLL cells were pre-incubated for 2 hrs with 100 μM of irreversible cell-permeable broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK), caspase-3- (z-DEVD-FMK), caspase-8 (z-LETD-FMK) or caspase-9 inhibitor (z-LEHD-FMK) (all Alexis) prior to apoptosis induction. After 48 hrs of exposure to Ara-C, inhibition of apoptosis was determined using the CFSE-based cytotoxicity assay. To specifically calculate the effect of the caspase-inhibitors on Ara-C-induced apoptosis, caspase-inhibitors were also added to the medium control, and a correction was performed for inhibition of spontaneous cell death by the caspase-inhibitors.

Annexin V / PI staining
Apoptosis was determined by Annexin V and PI staining. Annexin V specifically binds to phosphatidyl serine (PS), a phospholipid that becomes exposed on the surface of cells undergoing apoptosis. Dual staining with PI enables the identification of early apoptotic cells that have not yet lost their membrane integrity. [14] Cells were collected and resuspended in binding buffer 10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) at a concentration of 10^6 cells/mL. Cells were incubated at room temperature for 20 min with 0.1 μg/mL FITC-labeled Annexin V (Bender MedSystems, Vienna, Austria), washed, and resuspended in binding buffer. PI was added at a final concentration of 1 μg/mL, and the cells were directly analyzed on a flowcytometer.

Cell cycle analysis
For cell cycle analysis, 10^6 cells were washed with PBS, fixed and permeabilized for 10 min at 4°C using a solution of 80 μg/mL L-alpha-lysolecithin (Sigma-Aldrich) in 1% paraformaldehyde, washed with PBS containing 0,8 g/L human albumin, and stained for 30 min at 4°C with FITC-conjugated anti-Ki-67 MoAbs (clone MIB-1; Immunotech, Marseille, France) or isotype controls. After washing, cells were incubated with 20 μg/mL RNase (Sentra Systems, Minneapolis, USA) for 5 min at 37°C, and finally stained with 50 μg/mL PI. Cells were subjected to flow cytometric analysis of DNA content using a Becton Dickinson flow cytometer. The percentages of cell cycle distribution were calculated by CellQuest software. [3;4]

Determination of dCK activity
dCK activity, expressed in amounts of substrate converted in time per mg of total protein (pmol min^{-1} mg^{-1}), was measured in duplicate experiments using a dCK protocol as originally described by Cheng *et al*. [15] with minor modifications described by Veuger *et al*. [11] [^{3}H]-labeled Ara-C (specific activity 24 Ci $mmol^{-1}$; Campro Scientific BV, Veenendaal, The Netherlands) was used as substrate for dCK. Specific Ara-CTP formation was measured using more stringent washing of the DEAE-coated paper discs with a buffer consisting of 0.6 M HCl and 1.5 M NaCl.

^{3}H-thymidine and ^{3}H-uridine incorporation assay
B-CLL cells were incubated with either medium, or 10 μM of Ara-C or fludarabine in a round-bottom 96-wells plate ($0.5x10^6$ cells/150 μL) in a humidified atmosphere at 37°C and 5% CO2. To measure DNA or RNA synthesis, after 4 hrs of exposure ^{3}H-thymidine or ^{3}H-uridine (1 μCi/well; Amersham BioSciences, Freiburg, Germany) was added, respectively, and the cells were cultured for an additional 16 hrs. Prior to harvesting, from each ^{3}H-thymidine-incubation 4 wells were pooled (total amount of cells is $2x10^6$/incubation). Using an automatic microharvester cells were harvested on 96-wells Unifilter type GF/C plates (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA), which specifically bind DNA and RNA. After drying, scintillation fluid (MicroScint-O; Perkin Elmer) was added and incorporation of radioactively labeled precursors was determined in a liquid scintillation counter and expressed in counts per minute (CPM).

SDS PAGE and Western Blot analysis
Apoptosis was induced in B-CLL cells using 10^{-5} M Ara-C using a time range from 0 to 48 hrs of exposure. At each time point, whole cell lysates of $6x10^6$ cells were obtained by freeze-thawing the cells in 100 μL NP40-lysisbuffer (50 mM Tris-HCl, pH 7.6, 5 mM dithiotreitol, 20% v/v glycerol, 0.5% v/v Nonidet P40, and 25% v/v Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany). SDS PAGE and Western Blot analysis using PVDF membranes (Millipore Corp., Bedford, MA, USA) were performed as previously described. [11] Primary antibody incubations were performed for 2 hrs in 1% Ecl-blocking reagent. Cyclin D3 specific antibody (clone Ab-2) used for cell cycle analysis was purchased from Oncogene Research Products (San Diego, USA). Bcl-2 antibody (1:50,000) was from Pharmingen (San Diego, CA, USA), Bax (1:50,000) and Mcl-1 (1:1,000) specific antibodies were from SantaCruz Biotechnology (Santa Cruz, CA, USA). Caspase cleavage was detected using antibodies specific for caspase-8 (1:2,000; Pharmingen), caspase-9 (1:8,000; Calbiochem, San Diego, CA, USA), or cleaved caspase-3 (1:1,000; Cell Signaling Technology, Inc., Beverly, MA, USA). After 3 washing steps, membranes were incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:3,000; Promega, Madison, USA). β-actin expression was determined on the same blots after stripping for 30 min at 65°C with buffer containing 0,5% SDS using anti-β-actin clone AC-15 moAbs (1:100,000; Sigma-Aldrich).

RESULTS

Sensitivity of B-CLL cells to various apoptotic agents

We analyzed the sensitivity of B-CLL cells to Ara-C-, deoxycytidine-, camptothecin- and fludarabine-induced apoptosis using both ^{51}Cr release and CFSE-based cytotoxicity assays. A representative result of a CFSE-based cytotoxicity assay showing lysis after 40 hrs of exposure is illustrated in Figure 2.1. B-CLL cells were sensitive not only to fludarabine, but also to other chemotherapeutic agents tested including the S-phase-specific agent Ara-C. Deoxycytidine (dC) used as a non-specific control, did not induce cell death.

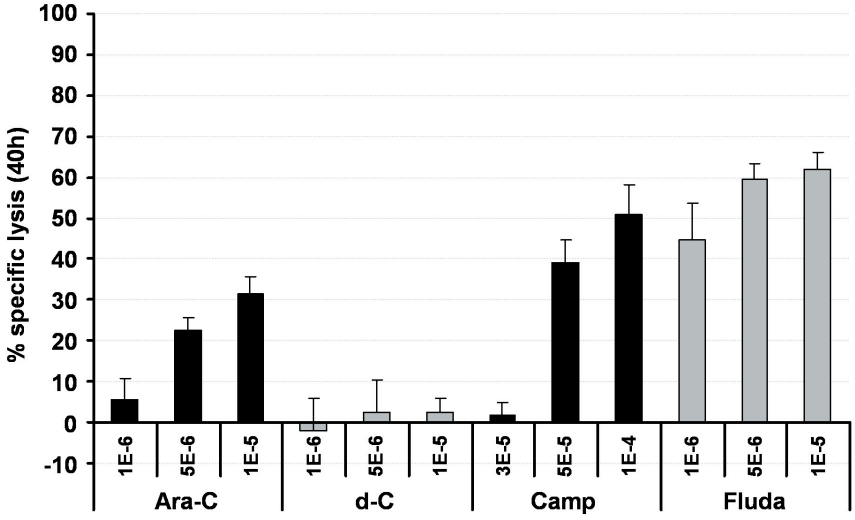

Figure 2.1. Susceptibility of B-CLL cells to various apoptotic agents.
Lysis of B-CLL cells after exposure for 40 hrs to different concentrations of Ara-C, deoxycytidine (dC), camptothecin (Camp) and fludarabine (Fluda) was determined using CFSE-based cytotoxicity assays. A representative result (in triplicate + SD) of 3 independent experiments is shown.

Since Ara-C has been considered to be S-phase specific and B-CLL is characterized by the presence of mainly quiescent cells, we investigated the characteristics of Ara-C-mediated cell death of B-CLL cells in more detail.

Long-term analysis of Ara-C-treated B-CLL cells using a quantitative CFSE-based cytotoxicity assay was performed to study the kinetics of Ara-C-mediated killing of B-CLL cells (Figure 2.2A). When B-CLL cells were cultured for up to 5 days in the absence of Ara-C, the percentage of viable B-CLL cells did not diminish the first 48 hrs, and only after 5 days declined from 100 to 83%, due to spontaneous apoptosis. CFSE intensity of the B-CLL cells was constant, showing that culturing in medium

did not induce cell proliferation. Culturing in presence of Ara-C caused a reduction of 50% of the cells after 48 hrs, and almost a complete deletion of B-CLL cells after 5 days, demonstrating the susceptibility of B-CLL cells to the S-phase-specific agent Ara-C.

To exclude a direct non-specific cytolytic effect of Ara-C to B-CLL cells, we investigated whether Ara-C-mediated cell death of B-CLL cells was characterized by PS exposure, an early characteristic of apoptosis. Annexin V binding and counterstaining with Propidium Iodide (PI) was used to distinguish between dead and early apoptotic cells. Figure 2.2B shows that B-CLL cells first became annexin V positive before staining with PI, illustrating that these cells died via an apoptotic mechanism.

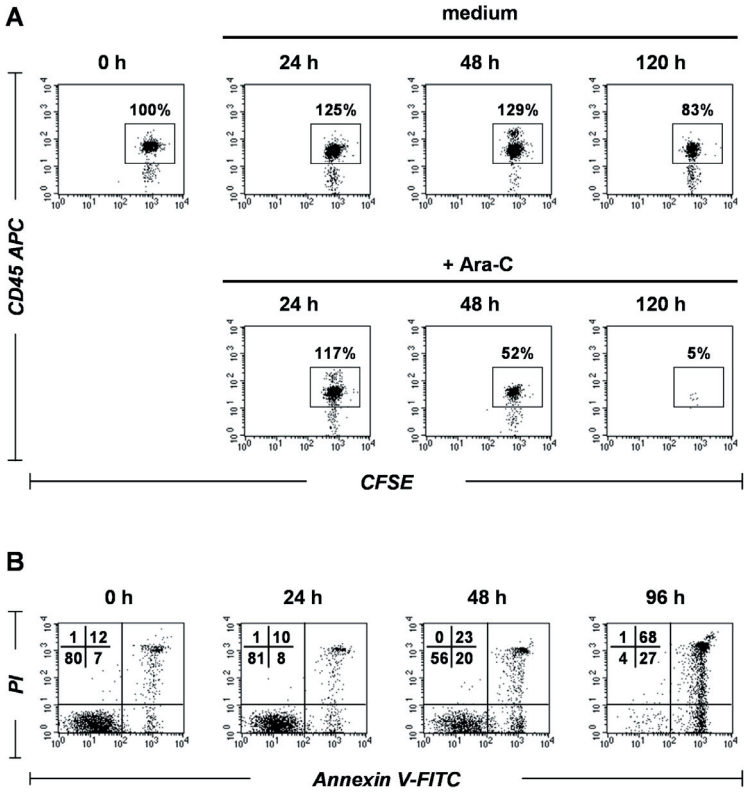

Figure 2.2. Long-term analysis of Ara-C-mediated apoptosis of B-CLL cells.
(A) Kinetics of Ara-C-mediated and spontaneous lysis of B-CLL cells using the CFSE-based cytotoxicity assay. For each time point, the percentage of surviving ($CFSE^+$, $CD45^+$, 7-AAD^-) cells, compared to the medium control at day 0, is indicated. A respresentative result of 3 independent experiments is shown. (B) Phosphatidyl serine exposure by B-CLL cells treated for 0-96 hrs with 10^{-5} M Ara-C, as determined by FACS analysis using annexin V-FITC staining combined with PI. Dotplots are representative of 2 independent experiments.

Relation between Ara-C-induced apoptosis and cell cycle status of B-CLL cells

To investigate the relation between Ara-C-induced apoptosis and the cell cycle status of B-CLL cells, we exposed both resting (unmanipulated) and proliferating B-CLL cells (cultured for 7 days on CD40L) [12] to Ara-C for 0 to 120 hrs, and performed cell cycle analysis combining FITC-labeled anti-Ki-67 antibodies and Propidium Iodide (PI) DNA staining (Figure 2.3A). The percentages of cells in G_0/G_1, S or G_2/M phase were determined based on DNA content. Double staining with Ki-67-FITC was used to discriminate between cells in resting G_0 phase defined as cells lacking expression of the nuclear protein Ki-67, [16;17] and cells in activated G_1 phase. At day 0 of the experiment, resting B-CLL cells were almost all (> 98%) in G_0-phase of the cell cycle, whereas proliferating B-CLL cells showed a cell cycle distribution of 15% of cells in S/G_2M, 69% in G_1, and only 16% in G_0 phase of cell cycle. Both populations of B-CLL cells responded in a different way to Ara-C. Although after 48 hrs of incubation already 50% of primary B-CLL cells were killed, no evidence of alteration in cell cycle status was observed (Figure 2.3Ai), indicating that these cells were killed by Ara-C in G_0-phase of cell cycle. In contrast, in proliferating B-CLL cells slow lysis but specific depletion of cells in S- and G_2/M phase of the cell cycle was observed during exposure to Ara-C.

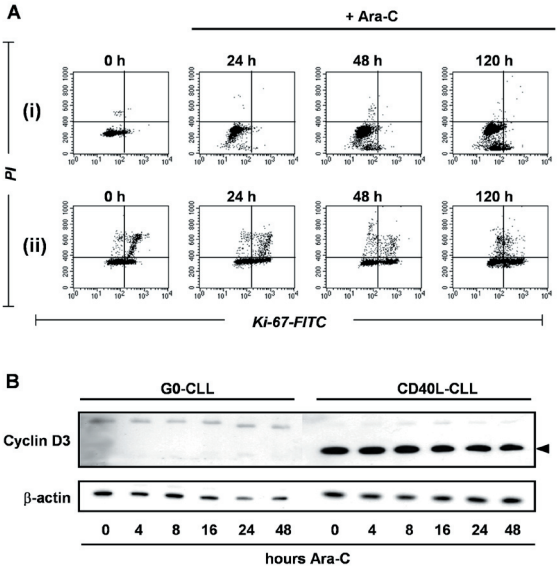

Figure 2.3. Cell cycle analysis of primary and proliferating B-CLL cells after treatment with Ara-C. (A) Cell cycle status of (i) primary B-CLL cells and (ii) CD40L-CLL cells after treatment with Ara-C for 0, 24, 48 or 120 hrs determined by flow cytometric analysis using Ki-67-FITC and PI staining. Representative results of 3 independent experiments are shown. (B) Cyclin D3 expression determined by Western Blot analysis of primary B-CLL cells and CD40L-activated B-CLL cells (cultured for 7 days on CD40L) treated for 0 to 48 hrs with Ara-C. Expression patterns were similar in 2 independent experiments.

To completely ensure that primary B-CLL cells did not leave G_0-phase of the cell cycle before being eliminated by Ara-C, cyclin D3 protein expression, which is mainly found in G_1 phase of the cell cycle, was analyzed (Figure 2.3B). Cyclin D3 could not be detected in primary and Ara-C-treated B-CLL cells, but was present in CD40L-activated B-CLL cells exposed to Ara-C for the times indicated. These data confirm that Ara-C used a different mechanism to kill quiescent (G_0) B-CLL cells than to kill proliferating B-CLL cells.

Ara-C-induced apoptosis of G_0-B-CLL cells is associated with inhibition of RNA synthesis

To study the mechanism of Ara-C-mediated apoptosis of G_0-B-CLL cells, we examined whether Ara-C induced lysis could be blocked by dC, which is a structural analogue of Ara-C and the normal metabolite of DNA synthesis. As shown in Figure 2.4A, lysis of G_0-B-CLL cells by 10^{-5} M Ara-C was inhibited by dC in a dose dependent manner, showing complete inhibition of lysis between 3 x 10^{-5} M and 6 x 10^{-5} M dC.

To investigate whether this inhibition of Ara-C-mediated lysis of G_0-B-CLL cells by dC could be caused by competition for dC kinase (dCK), an enzyme involved in the phosphorylation of both Ara-C and dC, we investigated the capacity of G_0-B-CLL cells to phosphorylate Ara-C, by measuring dCK activity using ^{3}H-labeled Ara-C as a substrate. dCK activity of G_0-B-CLL cells was 81 (pmol $min^{-1}mg^{-1}$), illustrating that quiescent B-CLL cells show higher enzyme activity than expected based on their inactive state. The total amount of phosphorylated Ara-C (mono-, di- and triphosphate) produced in time is shown in Figure 2.4B (■). Specific conversion by G_0-B-CLL cells of Ara-C into Ara-CTP, the active metabolite of Ara-C required for incorporation into DNA or RNA, was demonstrated as well (data not shown). Figure 2.4B further shows that increasing concentrations of dC caused complete inhibition of Ara-C conversion due to competition for dCK. Since both the phosphorylation of Ara-C in G_0-B-CLL cells (Figure 2.4B) and Ara-C-induced cell death of G_0-B-CLL cells (Figure 2.4A) could be completely blocked by increasing concentrations of dC, formation of Ara-CTP is likely to be important for the mechanism of Ara-C-mediated apoptosis of G_0-B-CLL cells.

To determine whether Ara-C affected the amounts of RNA or DNA repair synthesis in G_0-B-CLL cells, ^{3}H-uridine and ^{3}H-thymidine incorporation experiments were performed. G_0-B-CLL cells were cultured for 20 hrs in either medium alone, or in the presence of Ara-C or fludarabine. After 4 hrs of pre-incubation, ^{3}H-labeled uridine or thymidine was added to all incubations, and incorporation into respectively RNA or DNA was measured after a ^{3}H-pulse of 16 hrs. Specific ^{3}H-thymidine incorporation (corrected for background) in 2 x 10^6 quiescent G_0-B-CLL cells cultured in medium was only around 300 CPM (Figure 2.5A), compared to over 4000 CPM in 100,000

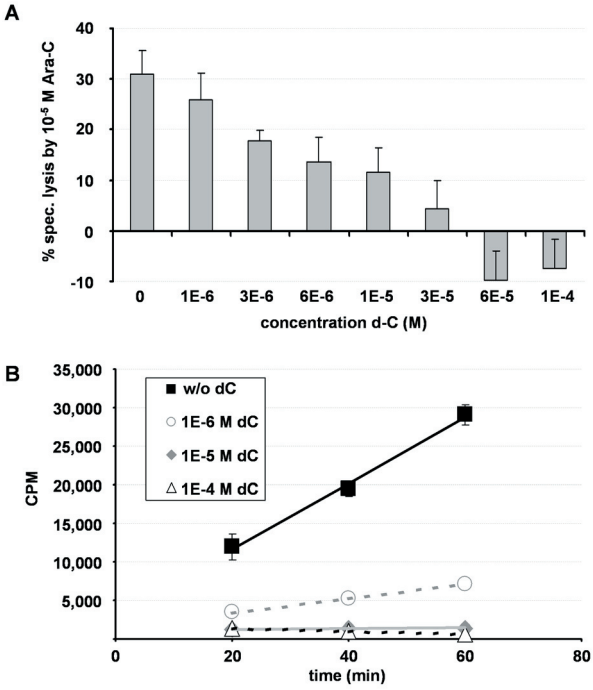

Figure 2.4. Mechanism of Ara-C-mediated killing of G_0-B-CLL cells.
(A) Competition assay using increasing amounts of dC to block Ara-C-mediated cell death of G_0-B-CLL cells after exposure for 72 hrs to 10^{-5} M Ara-C as determined by a CFSE-based cytotoxicity assay. A representative result (in triplicate) of 3 independent experiments is shown. (B) *In vitro* capability of G_0-B-CLL cell extracts to convert Ara-C into its active metabolite Ara-CTP, determined by dCK activity measurement using ^{3}H-labeled Ara-C as a substrate. Total amounts of ^{3}H-labeled Ara-CMP, -CDP and -CTP generated in time are depicted in the figure and indicated in CPM. Increasing amounts of dC were added to the cell extracts to compete for dCK activity and inhibit phosphorylation of ^{3}H-Ara-C. The graph shows mean values from duplicate measurements ± SD. Results are representative of 2 independent experiments.

proliferating CD40L-B-CLL cells (data not shown). These low levels of specific ^{3}H-thymidine incorporation were blocked by both Ara-C and F-Ara-A, showing 40% inhibition at 10^{-6} M, and 60% inhibition at 10^{-5} M.

In contrast, RNA synthesis was much more pronounced than DNA (repair) synthesis, showing basal ^{3}H-uridine incorporation of approximately 30,000 CPM in 0.5 x 10^6 G_0-B-CLL cells (Figure 2.5B), which was only 5-10 times lower than in proliferating B-CLL cells (data not shown). ^{3}H-uridine incorporation was hardly blocked by 10^{-6} M Ara-C or fludarabine (8%), but 10^{-5} M resulted in 30% inhibition of RNA synthesis after 20 hrs of exposure.

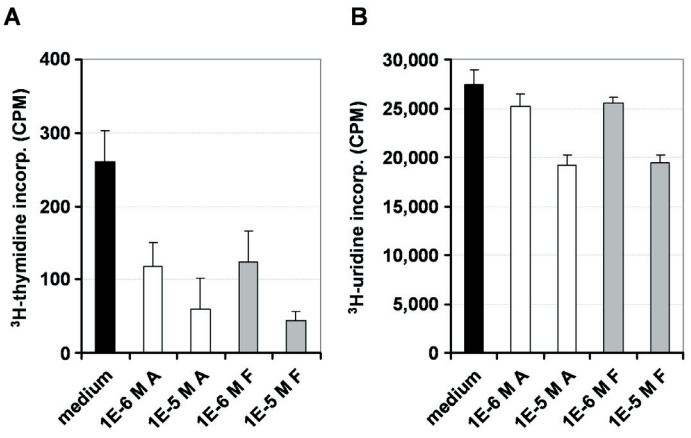

Figure 2.5. Inhibition of RNA synthesis by Ara-C or fludarabine.
G_0-B-CLL cells (0.5 x 10^6/150 µL) were cultured for 20 hrs in absence or presence of 10^{-6} or 10^{-5} M Ara-C (A) or fludarabine (F). The last 16 hrs of incubation, ^{3}H-uridine or ^{3}H-thymidine incorporation was measured. To determine ^{3}H-thymidine incorporation, prior to harvesting on Unifilter plates 4 wells were pooled (total amount of cells is 2.0 x 10^6 per incubation). The data presented are mean values (+ SD) of triplicate ^{3}H-uridine (A) and ^{3}H-thymidine (B) incorporations (in CPM). Inhibition of ^{3}H-uridine or ^{3}H-thymidine incorporation by Ara-C or fludarabine was similar in three independent experiments.

Effect of Ara-C on Bcl-2, Bax and Mcl-1 expression and caspase-activation in B-CLL

One of the initiating mechanisms for drug-induced apoptosis in B-CLL cells has been reported to be alteration of the Bcl-2/Bax ratio, [9] either by downregulation of Bcl-2 or by upregulation of Bax. Moreover, downregulation of other survival proteins such as myeloid cell leukemia sequence 1 (Mcl-1) might contribute to the mechanism of Ara-C-mediated cell death of G_0-CLL cells. Since exposure of G_0-CLL cells to Ara-C resulted in decreased RNA synthesis in these cells, we analyzed whether this inhibition of RNA synthesis resulted in alterations in Bcl-2, Bax or Mcl-1 protein levels. Therefore, G_0-B-CLL cells were exposed to Ara-C in a time range from 0 to 24 hrs, a time interval in which no cell lysis was observed (Figure 2.2). Bcl-2 and Bax expression levels were not significantly altered upon Ara-C treatment, whereas Mcl-1 expression was decreased after 16 hrs of exposure to Ara-C (Figure 2.6A), suggesting that downregulation of Mcl-1 may play an important role in Ara-C-induced apoptosis of G_0-B-CLL cells.

To reveal whether caspases were involved in the induction of Ara-C-mediated cell death of G_0-B-CLL cells, either indirectly occurring as a consequence of cellular stress caused by inhibition of RNA synthesis or by downregulation of the survival protein Mcl-1, or directly induced by Ara-CTP via activation of the apoptosome as described for F-Ara-ATP, [18] caspase activation patterns were assessed in the same cell lysates

by Western Blot analysis (Figure 2.6B). Modest caspase-3 and -8 cleavage was observed after 16 hrs exposure to Ara-C, whereas caspase-9 cleavage could not be detected after apoptosis induction with Ara-C in a time interval from 0 to 24 hrs of exposure (data not shown).

To investigate whether this modest caspase activation was required for the execution of Ara-C-mediated apoptosis of G_0-B-CLL cells, we examined the effect of various caspase-inhibitors on Ara-C-induced cell death. G_0-B-CLL cells were exposed to Ara-C in presence or absence of caspase-3, -8, -9 or the pan-caspase-inhibitor z-VAD-FMK, and Ara-C-induced apoptosis was determined after 48 hrs exposure using quantitative CFSE assays (Figure 2.6C). In the presence of 10^{-4} M z-VAD-FMK, 50% inhibition of Ara-C-induced lysis was obtained (p<0.01), whereas no significant effect (0-25% inhibition) was observed with caspase-3, -8 or -9 inhibitor. These data suggest that caspases were at least partially involved in Ara-C-induced disassembly of the B-CLL cells, although we could not pinpoint which specific caspase plays a key role in Ara-C-mediated apoptosis of G_0-B-CLL cells.

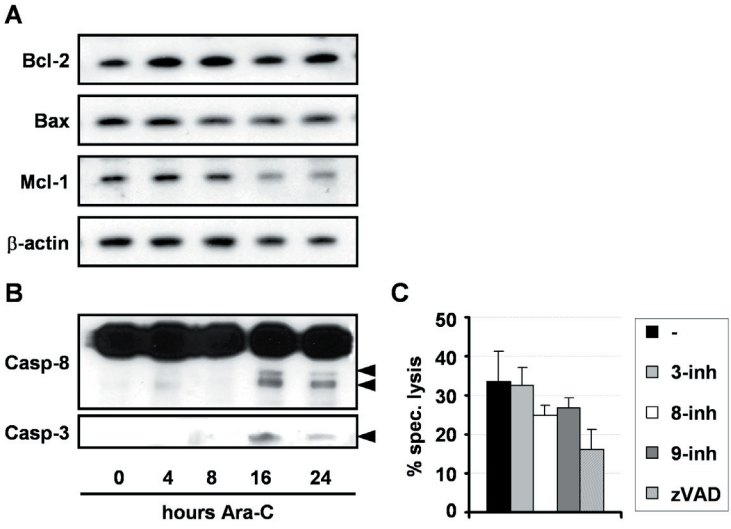

Figure 2.6. Expression of apoptotic proteins and involvement of caspases in Ara-C-mediated apoptosis of G_0-B-CLL cells.
Cell lysates of G_0-B-CLL cells were prepared after apoptosis induction with 10^{-5} M Ara-C in a time course from 0 to 24 hrs of exposure. Expression of various pro- and anti-apoptotic proteins (A) and caspase activation patterns (B) were determined using Western Blot analysis, and the data shown are representative for 3 independent experiments. Active subunits of caspase-8 (p40/42) and caspase-3 (p17/19) are indicated with arrows; caspase-9 cleavage could not be detected (data not shown). (C) Effect of various caspase-inhibitors (10^{-4} M) on Ara-C-induced apoptosis. Mean percentages of Ara-C-induced lysis (n=4) after 48 hrs exposure in presence or absence of specific caspase-inhibitors (caspase-3, -8, -9 inhibitor and zVAD-FMK) were determined using quantitative CFSE assays. Only inhibition by zVAD was significant (p<0.01).

DISCUSSION

Ara-C is the most important agent in the treatment of patients with acute leukemia. Incorporation of Ara-C into DNA is considered a key event in the mechanism of killing of proliferating leukemic cells. [2] We previously demonstrated specific depletion of leukemic cells in S- and G_2/M phase of the cell cycle by Ara-C, whereas resting (G_0) acute leukemic cells did not respond. [3;4] We now observed unexpected lysis of B-CLL cells, which are mainly (>98%) in G_0 phase of the cell cycle, by Ara-C, being independent of cell proliferation and DNA replication. In this study we analyzed which cellular processes were involved in Ara-C-mediated killing of G_0-B-CLL cells. We showed that, in response to Ara-C treatment, G_0-B-CLL cells died via an apoptotic mechanism characterized by PS exposure and caspase activation, which was not initiated via downregulation of Bcl-2. Furthermore, G_0-B-CLL cells were able to convert Ara-C into Ara-CTP, and increasing concentrations of dC completely inhibited Ara-C-mediated apoptosis of G_0-B-CLL cells, indicating that phosphorylation of Ara-C is important for its cytotoxicity. Since unexpectedly high RNA synthesis was present in G_0-B-CLL cells, which was blocked by Ara-C for 30%, we hypothesize that inhibition of RNA synthesis is an important mechanism of killing G_0-B-CLL cells.

For Ara-C, only mechanisms of killing of proliferating cells have been described, such as inhibition of cellular DNA replication by inhibition of DNA polymerase or incorporation of Ara-CTP into DNA, and inhibition of DNA repair. [2] The absence of both ki-67 and cyclin D3 expression in Ara-C-treated B-CLL cells (Figure 2.3) revealed that Ara-C used a different mechanism to eliminate G_0-B-CLL cells. One of the mechanisms, demonstrated to be important in Ara-C- in contrast to fludarabine-mediated cell death of proliferating cells, [19] that also may play a role in quiescent cells, is inhibition of DNA repair synthesis. In this study, we showed that despite the quiescent stage of the cell cycle, B-CLL cells possessed unexpectedly high dCK activity (81 pmol $min^{-1}mg^{-1}$), comparable to primary healthy donor cells and AML cells (84 and 114 pmol $min^{-1}mg^{-1}$, respectively), [11] and that Ara-CTP could be formed. In 1986, Carson´s group already showed that quiescent lymphocytes break and rejoin DNA at a slow and balanced rate. [20] Exposure to DNA repair blocking agents such as Ara-C will cause an accumulation in DNA strand breaks eventually leading to apoptosis. In our experiments, however, basal DNA repair synthesis was hardly detectable (260-400 CPM in 16 hrs) in 2 x 10^6 G_0-B-CLL-cells, whereas Seto *et al*. showed ^{3}H-thymidine incorporations of 2,000 CPM in 3 x 10^6 normal resting lymphocytes within 2 hrs of incubation. Huang *et al*. [10] found slightly higher levels of DNA repair synthesis in CLL cells measuring incorporation of ^{3}H-deoxycytidine during 4 hrs (2,500 CPM in 5 x 10^6 cells). Moreover, they showed that fludarabine inhibited DNA repair synthesis in B-CLL cells (75%), but that this inhibition did not contribute to F-Ara-ATP-induced cytotoxicity observed in these cells. Similarly, we showed that

10^{-6} M Ara-C did result in 40% inhibition of DNA synthesis after 16 hrs (Figure 2.5A), but not in lysis after 48 hrs of exposure (Figure 2.1). Overall, we suppose that inhibition of DNA repair is less important in the mechanism of Ara-C-mediated killing of G_0-B-CLL cells than of proliferating cells.

A potential mechanism of killing of G_0-B-CLL cells by Ara-C is inhibition of RNA synthesis, which is also described for fludarabine. [21] We demonstrated that a remarkable amount of RNA was synthesized within 20 hrs in G_0-B-CLL cells (10-20% of RNA synthesis of proliferating B-CLL cells) and addition of 10^{-5} M Ara-C or fludarabine for 20 hrs resulted in 30% less incorporation of ^{3}H-uridine compared to control G_0-B-CLL cells. Addition of 10^{-6} M Ara-C hardly resulted in inhibition of RNA synthesis (8%, Figure 2.5B), which correlated well with lack of cytotoxicity at this concentration after 48 hrs (Figure 2.1). Our RNA inhibition data were in line with the results of the group of Huang *et al*. [10] showing a decrease in RNA synthesis of 25-50% in B-CLL cells treated for 24 hrs with 3 x 10^{-6}-10^{-5} M fludarabine. Although we showed that Ara-C treatment resulted in 30% inhibition of RNA synthesis, we did not clarify whether this was due to incorporation of Ara-CTP into RNA or via inhibition of RNA polymerase. Some studies in proliferating cells revealed that Ara-CTP was not incorporated into RNA, [22;23] whereas F-Ara-ATP incorporated into both RNA and DNA. [23] Therefore, Ara-C probably inhibits RNA synthesis via inhibition of RNA polymerase, which was also shown by some other groups in a non-human setting. [24;25]

Inhibition of gene transcription by Ara-C may reduce expression of proteins that are important for survival of B-CLL cells such as Bcl-2 and Mcl-1, and in this way lead to apoptosis of G_0-B-CLL cells. Therefore, we investigated levels of Bcl-2 and Mcl-1 protein expression, and observed significant downregulation of Mcl-1 but not of Bcl-2 upon exposure to Ara-C, as was found by several groups after chemotherapy treatment. [9;26]

We demonstrated that caspases play a role in Ara-C-mediated apoptosis of G_0-B-CLL cells, showing that the general caspase-inhibitor z-VAD-FMK reduced Ara-C-mediated cell death of G_0-B-CLL cells by 50%. In contrast to our findings, other groups argued that caspase activation was only a secondary event of fludarabine-induced apoptosis in G_0-B-CLL cells, showing that caspase inhibition only prevented specific manifestations of apoptosis, such as PARP cleavage and DNA fragmentation, but did not prevent cytotoxicity. [27-29] We speculated that caspase-activation might be the consequence of the 30% inhibition of RNA synthesis by Ara-C, which will result in major cellular stress in G_0-B-CLL cells. Moreover, caspases might be activated in response to downregulation of the survival protein Mcl-1. An alternative pathway for fludarabine to induce caspase activation and apoptosis, proposed by Genini *et al*., [18] is activation of the apoptosome pathway. They demonstrated that,

similar to dATP, F-Ara-ATP can cooperate with cytochrome C and apoptosis protein-activating factor-1 (Apaf-1) to form an apoptosome, and directly trigger apoptosis via caspase-9 activation. However, if a similar mechanism plays an important role in Ara-CTP-induced apoptosis as well, a larger inhibition of apoptosis would be expected using caspase-9 inhibitor (Figure 2.6C). Moreover, this pathway is only described for purine analogues such as F-Ara-A and 2CdA, and not for pyrimidine analogues as Ara-CTP.

In conclusion, here we provide evidence for Ara-C-mediated apoptosis of G_0-B-CLL cells, using a different mechanism of killing than proliferating B-CLL cells being independent of cell proliferation and DNA replication. A potential mechanism of killing of resting G_0-B-CLL cells by Ara-C is inhibition of RNA synthesis, either by direct RNA incorporation or by inhibition of RNA polymerase. This decreased RNA synthesis was coincided with downregulation of the survival protein Mcl-1, which may partly explain Ara-C-mediated apoptosis of G_0-B-CLL cells.

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CHAPTER 3

Involvement of caspase-8 in chemotherapy-induced apoptosis of patient derived leukemia cell lines independent of the death receptor pathway and downstream from mitochondria

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ABSTRACT

Resistance of leukemic cells to chemotherapy frequently occurs in patients with acute leukemia, which may be caused by alterations in common apoptotic pathways. Controversy exists whether cytostatic agents induce the mitochondrial or death receptor pathway of apoptosis. In the mitochondrial pathway cytochrome C release and caspase-9 activation play a central role in the induction of apoptosis, while formation of a Death Inducing Signaling Complex (DISC) and caspase-8 activation have been reported to be essential in death receptor-induced apoptosis. Here, we show in human derived myeloid and lymphoblastic leukemia cell lines that caspase-8 plays a more important role than previously expected in apoptosis mediated via the mitochondrial pathway. We demonstrated in these malignant cells chemotherapy-induced apoptosis independent of the death receptor pathway, since blocking this pathway using a retroviral construct encoding Flice inhibitory protein (FLIP) did not inhibit drug-induced apoptosis or caspase-8 activation, while overexpression of Bcl-2 completely inhibited both events. Furthermore, we showed that activation of caspase-8 by cytostatic agents occurred downstream from mitochondria. Since caspase-8 plays a central role in both death receptor- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia, therapeutic strategies focusing at modulation and activation of caspase-8 may be successful in the treatment of drug-resistant malignancies.

INTRODUCTION

The treatment of choice for patients with acute leukemia is high-dose chemotherapy followed by allogeneic or autologous stem cell transplantation. [1] From these patients, approximately 70-80% will achieve complete remission. However, 50-60% of all patients experience a relapse of the disease. [2;3] The majority of these patients is resistant to further cytotoxic treatment. Resistance of leukemic cells to chemotherapy may be caused by overexpression of drug efflux pumps like P-glycoprotein or the multidrug-resistance protein MRP-1, [4] or of anti-apoptotic proteins, such as B cell lymphoma-2 (Bcl-2), [5] since overexpression of these proteins has been associated with poor clinical prognosis. [6-8] Functional blocks in caspase activation pathways are found in patients with leukemia and predict poor clinical response to induction chemotherapy, [9] suggesting that alterations of the intrinsic programmed cell death (apoptosis) pathways of the leukemic cells are also a cause of resistance to chemotherapy.

Cytostatic agents initiate the mitochondrial pathway of apoptosis, also called the intrinsic pathway. Due to cellular stress, mitochondria membranes become permeable

and cytochrome c is released. In the cytosol, cytochrome c binds to pro-caspase 9 and apoptotic protease activating factor (Apaf-1). In this complex, called the apoptosome, caspase 9 is activated and in turn cleaves various effector caspases, eventually leading to disassembly of the cell. [10,11]

In addition to the mitochondrial pathway of apoptosis, involvement of the death receptor 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. [12-16] The death receptor pathway is initiated when death ligands, including FasL and tumor necrosis factor alpha (TNF-α), bind to 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, followed by binding of pro-caspase 8, also called FADD-like IL-1 converting enzyme (FLICE). [17] In this complex of receptor-bound FADD with pro-caspase 8, called the Death Inducing Signaling Complex (DISC), proteolytic cleavage of pro-caspase 8 takes place, initiating a caspase cascade resulting in apoptosis. [18;19]
Recent patient studies showed that absence or low expression of FADD in cells from patients with acute myeloid leukemia is associated with resistance to chemotherapy treatment and poor clinical outcome. [20,21;22] Moreover, downregulation of procaspase-8 expression, by DNA methylation as well as through gene deletion, resulted in several malignancies in resistance to doxorubicin-induced apoptosis. [23-25] Based on these results it may be hypothesized that at least part of the death-receptor signaling pathway is involved in resistance to chemotherapy.

In the present study, 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. We observed remarkable caspase-8 cleavage and similar amounts of caspase-8 and caspase-9 activity upon apoptosis induction using cytostatic agents indicating that the death-receptor pathway may be involved in chemotherapy-induced apoptosis of these cells. To examine whether this caspase-8 activation was due to activation of the death receptor pathway or could alternatively be involved in the mitochondrial pathway, as previously suggested by Wesselborg *et al*., [26] we introduced the anti-apoptotic proteins FLIP and B-cell lymphoma-2 (Bcl-2) into leukemic cell lines, to specifically inhibit the death receptor or mitochondrial pathway, respectively. 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. [27;28] Bcl-2 was used to specifically inhibit the mitochondrial pathway, since one of the mechanisms of Bcl-2 to inhibit apoptosis, is prevention of cytochrome c release from the mitochondria. [29;30]

Our results show that in these human acute leukemic cells chemotherapy-induced apoptosis is mediated through the mitochondrial pathway and not via the death receptor pathway. The considerable caspase-8 activation upon chemotherapeutic treatment is part of the intrinsic apoptotic pathway and occurs downstream of the mitochondria.

MATERIALS AND METHODS

Cell lines and culture conditions

The human GM-CSF-dependent acute monocytic leukemia cell line AML-193 [31] was obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The human acute lymphoblastic leukemia cell line Leiden ALL-HP (ALL-HP) and the human CML-derived lymphoblastic cell line Leiden ALL-CM (ALL-CM) were generated in our laboratory from primary human ALL cells. Both cell lines displayed the karyotype of the primary malignant clone, as ALL-HP cells contained i(21)(q10) and ALL-CM cells contained t(9;22)(q34;q11). The cell lines displayed a precursor B immune phenotype and expressed CD10, CD19 and CD79a (both) and CD20 (ALL-CM).

All cell lines were cultured in serum-free medium consisting of IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium), 0.4% human serum albumin (HSA) (wt/vol) (CLB, Amsterdam, The Netherlands), 20 μg/mL cholesterol (Sigma-Aldrich, St Louis, MO, USA), 20 μg/mL transferrin (Serva, Heidelberg, Germany), 5 x 10^{-5} M β-mercaptoethanol, and 10 μg/mL insulin (both Sigma-Aldrich). Addition of 20 ng/mL recombinant human GM-CSF (Novartis, Basle, Switzerland) induced persistent proliferation of the AML-193 cells.

Apoptosis inducing agents

Apoptosis was induced with the cytostatic agents camptothecin (Alexis Corp., Lausanne, Switzerland) or daunorubicin (Sigma-Aldrich) using concentrations varying from 0.1 to 100 μM. Death-receptor-mediated apoptosis was induced using Fas agonistic antibodies (10 to 1000 ng/mL) that cause crosslinking of the Fas receptor (Fas Ab, 7C11; Beckman Coulter Inc., Fullerton, CA, USA), or by TRAIL (rhs*Killer*TRAIL™) (Alexis) using concentrations varying from 1 to 1000 ng/mL.

Cytotoxicity assays

Cytotoxicity was measured using 16 or 24-hr ^{51}Cr release assays as described previously [32] or using CFSE-based cytotoxicity assays as described by Jedema *et al*., [33] with some modifications. Target cells were labeled with 5 μM CFSE (Molecular Probes Europe, Leiden, The Netherlands), and incubated overnight in a humidified atmosphere of 5% CO_2 and 37°C. For the cytotoxicity assay, 25000 cells/well (100 μL) were plated in 96-well microtiter plates (all in triplicate), and apoptosis-inducing agents were added in a volume of 50 μL/well. After 16 or 24 hrs of exposure, FACS analysis was performed to determine numbers of viable cells. To exclude dead cells from the analysis, Propidium Iodide (PI) (1 μg/mL; Sigma) was added. To allow quantitative analysis of the viable cells, the wells were harvested, and transferred to FACS tubes containing 10000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA). For each sample 3000 microbeads were acquired, facilitating the calculation of absolute numbers of viable (PI^-) $CFSE^+$ target cells. The percentage of specific cell death was defined as:

[(mean absolute number of viable $CFSE^+$ target cells in control medium - absolute number of viable $CFSE^+$ target cells ^{experimental}) / (mean absolute number of viable $CFSE^+$ target cells in control medium)] x 100.

To determine the role of caspases in chemotherapy- or death receptor-induced apoptosis, ^{51}Cr-labeled target cells were pre-incubated for 2 hrs with 100 μM of irreversible cell-permeable broad-spectrum caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (z-VAD-FMK), caspase-3-inhibitor (z-DEVD-FMK), caspase-8 inhibitor (z-LETD-FMK) or caspase-9 inhibitor (z-LEHD-FMK) (all Alexis) prior to apoptosis induction.

Annexin V / PI staining

Apoptosis was determined by Annexin V and PI staining. Annexin V specifically binds to phosphatidyl serine (PS), a phospholipid that becomes exposed on the surface of cells undergoing apoptosis. Dual staining with PI enables the identification of early apoptotic cells that have not yet lost their membrane integrity. [34] Cells were collected and resuspended in binding buffer (10 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$) at a concentration of 10^6 cells/mL. Cells were incubated at room temperature for 20 min with 0.1 μg/mL FITC-labeled Annexin V (Bender MedSystems, Vienna, Austria), washed, and resuspended in binding buffer. PI was added at a final concentration of 1 μg/mL, and the cells were directly analyzed on a flowcytometer.

SDS PAGE and Western Blot analysis

Apoptosis was induced using camptothecin (50 μM) or Fas Ab (500 ng/mL for AML-193 or 100 ng/mL for ALL-HP cells) in a time range from 0 to 24 hrs of exposure. At each time point, whole cell lysates of 2 x 10^6 cells were obtained by freeze-thawing the cells in 100 μL NP40-lysisbuffer (50 mM Tris-HCl, pH 7.6, 5 mM dithiotreitol, 20% v/v glycerol, 0.5% v/v Nonidet P40, and 25% v/v Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany). SDS PAGE and Western Blot analysis using PVDF membranes (Millipore Corp., Bedford, MA, USA) were performed as previously described. [35] Primary antibody incubations were performed for 2 hrs in 1% Ecl-blocking reagent. Caspase cleavage was detected using antibodies specific for caspase-8 (1:2000; BD BioSciences Pharmingen, San Diego, CA, USA), caspase-9 (1:8000; Calbiochem, San Diego, CA, USA), or cleaved caspase-3 (1:1000; Cell Signaling Technology, Inc., Beverly, MA, USA). Antibodies specific for the FLAG epitope tag, used to detect introduced FLIP (1:1000), were purchased from Sigma.

After 3 washing steps, membranes were incubated for 1 hr with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies (1:3000; Promega, Madison, USA). β-actin expression was determined on the same blots after stripping for 30 minutes at 65°C with buffer containing 0,5% SDS using anti-β-actin clone AC-15 moAbs (1:100 000; Sigma-Aldrich).

Caspase activity assay using colorimetric substrates

After apoptosis induction, for each time point 4 x 10^6 cells were washed and resuspended in 100 μL of ice-cold lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT (freshly added), 0.1 mM EDTA). After 5 min of incubation on ice, cells were centrifuged at 10,000 x *g* for 10 min at 4°C, and supernatants (= cell extracts) were either directly used for the assay or quickly frozen in liquid nitrogen, and stored at -80°C, for later use. Caspase activity assays included 50 μL of assay buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT (freshly added), 0.1 mM EDTA and 10% glycerol), 40 μL of cell lysate (~ 60-70 μg of protein) and 10 μL of substrate (200 μM final concentration of DEVD-pNA, IETD-pNA or LEHD-pNA, all purchased from Alexis). Samples were incubated at 37°C, and enzyme-catalyzed release of p-nitroanilide was monitored for several hrs at 405 nm using a microtiter plate reader. Absorbance at 405 nm was plotted versus time for each sample. The slope (OD/time) was calculated for the initial time period over which the plot was linear, and this slope was used as a measure for caspase activity.

Flow cytometric analysis of intracellular Bcl-2

To determine Bcl-2 expression, cells were permeabilized at room temperature for 5 minutes using 0.25% saponine/PBS. After centrifugation at 1400 rpm, cells were stained for 30 min at 4°C with FITC-conjugated anti-Bcl-2 (Dako, Glostrup, Denmark) or IgG1-FITC isotype control (BD) MoAbs. After washing with PBS containing 0,8 g/L human albumin, cells were subjected to flow cytometric analysis using a BD FACScan.

Construction of retroviral vectors and generation of retroviral supernatants

The complete coding region of human FLIP-long (U97074) with a FLAG tag in front of the start codon, was amplified from plasmid pCR3.V64 (kindly provided by Dr. J.P. Medema (Leiden University Medical Center, Leiden, Netherlands) already containing the long form of FLIP with a FLAG sequence at the N-terminus, by PCR using the forward primer 5'-tatagaagatctaccatggattacaaagacgatgac-3' and reverse primer 5'-ta taccgctcgagttatgtgtaggagag-3'. PCR products were sequenced to exclude mutations and were cloned

into the Moloney murine leukemia virus-based retrovirus vector LZRS (G. Nolan, Stanford University, Palo Alto, CA) containing truncated nerve growth factor receptor (ΔNGF-R) as the marker gene. [36] The Bcl-2 construct was kindly provided by Dr. J.P. Medema, and cloned into the pLZRS vector with green fluorescence protein (eGFP) as the marker gene. Retroviral pLZRS vectors encoding eGFP or ΔNGF-R alone were used as control vectors (mock) in the experiments. The pLZRS constructs were transfected into packaging cells ϕ-NX-A (G. Nolan, Stanford University, Palo Alto, CA) [36] using calcium phosphate (Life Technologies, Gaithersburg, MD) and retroviral supernatants were obtained as previously described. [37]

Retroviral transduction of leukemic cells with anti-apoptotic genes
Exponentially growing AML-193, ALL-HP and ALL-CM cells were transduced with retroviral supernatants based on the method described by Hanenberg *et al*. [38] with minor modifications, [37] using recombinant human fibronectin fragments CH-296 (RetroNectin; Takara, Otzu, Japan). ΔNGF-R expression was detected using a phycoerythrin (PE)-conjugated anti-human NGF-R mAb (Pharmingen). Transduced cells were purified by FACS® sort based on marker gene expression using a FACSVantage (Becton Dickinson, Mountain View, CA), and cultured in serum free medium.

RESULTS

Sensitivity of various leukemic cell lines to various apoptosis inducing agents

To determine sensitivity of various leukemic cell lines to various apoptotic stimuli, both ^{51}Cr release assays (AML-193 and ALL-HP) and CFSE assays (ALL-CM) were performed. In Figure 3.1 representative percentages of cell lysis after 24 hrs of exposure to various concentrations of camptothecin (A), daunorubicin (B), Fas agonistic antibody (Fas Ab) (C) or TRAIL (D), are depicted for each cell line. In AML-193 (white bars) and ALL-HP cells (grey bars), the cytostatic agents camptothecin and daunorubicin were equally effective in inducing apoptosis, while ALL-HP cells were slightly more sensitive to the death receptor triggering agents Fas Ab and TRAIL. ALL-CM cells (black bars) were effectively killed by chemotherapeutic agents and TRAIL, but not by Fas Ab due to absent Fas-receptor expression (data not shown). Concentrations of apoptotic agents resulting in at least 40% lysis after 24 hrs of exposure, indicated with an asterisk (*), were used in further experiments.

To investigate whether these leukemic cells died via an apoptotic mechanism in response to these different agents, we performed annexin V/PI stainings after 4, 6, 16 and 24 hrs of exposure (partially shown in Figure 3.2). We counterstained the cells with Propidium Iodide (PI) to distinguish between early apoptotic and dead cells. These data demonstrate that chemotherapy-induced cell death was mediated via apoptosis in all cell lines studied, since cells became annexin V positive after 4 hrs of treatment, while at these time points no cell death was observed (PI negative and no ^{51}Cr release). Triggering of death receptors by either Fas Ab (AML-193 and ALL-HP) or TRAIL (ALL-CM) also resulted in the induction of apoptosis. The kinetics of Fas/TRAIL-induced apoptosis were slower than of chemotherapy-induced apoptosis, especially for ALL-HP cells, which became annexin V positive after 16 to

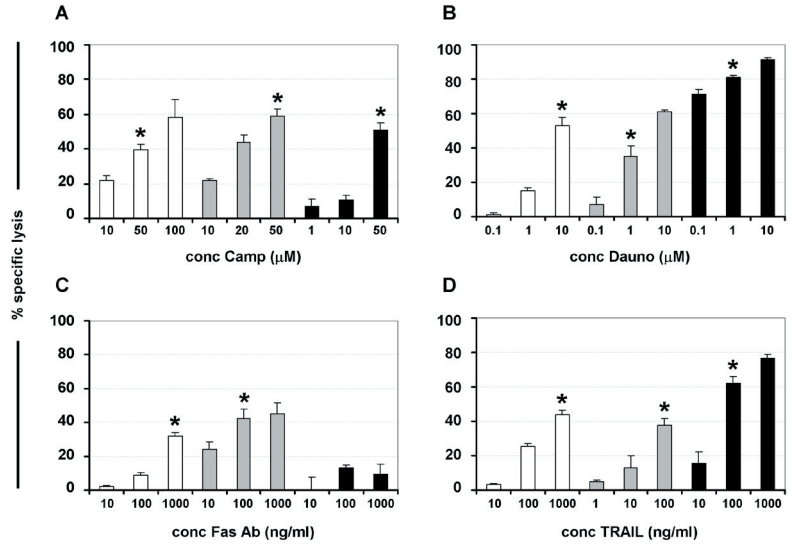

Figure 3.1. Sensitivity of various leukemic cell lines to various apoptotic agents.
AML-193 (white bars), ALL-HP (grey bars) and ALL-CM cells (black bars) were exposed for 24 hrs to various concentrations of (A) camptothecin (Camp), (B) Daunorubicin (Dauno), (C) Fas agonistic antibody (Fas Ab) or (D) TRAIL. Percentages of specific lysis were determined using both ^{51}Cr release assays (AML-193 and ALL-HP) and CFSE assays (ALL-CM). The data shown are representative of three independent experiments. Concentrations of apoptotic agents resulting in at least 40% lysis, marked with an asterisk (*), were used in further experiments.

24 hrs of exposure to Fas Ab. Percentages of cell death after 24 hrs of incubation were comparable between the quantitative annexinV/PI analyses (using Flow-Count Fluorospheres; data not shown) and the ^{51}Cr release assays (Figure 3.1).

Caspase-8 and caspase-9 are involved in chemotherapy-induced apoptosis of various leukemic cell lines

To investigate the contribution of the various apoptotic pathways in chemotherapy-induced apoptosis, we first assessed caspases -8, -9 and -3 activation patterns upon apoptosis induction using camptothecin. Since other cytostatic agents such as daunorubicin caused similar caspase-activation patterns as campthothecin, these results were not shown. Figure 3.3A shows that in all cell lines rapid and substantial caspase-8, -9 and -3 cleavage was detected after treatment with camptothecin (left panel). Caspase-activation was maximal after 4 or 6 hrs of exposure to camptothecin in all cell lines. In AML-193 cells, the first cleavage products of caspase-8 and caspase-9 were present after 2 hrs of exposure, while caspase-3 activation was observed after 4 hrs of treatment. In ALL-HP cells, first cleavage products of caspase-8, -9 and -3 were observed simultaneously after 2-4 hrs of exposure. Kinetics of caspase-activation was the fastest in ALL-CM cells, showing

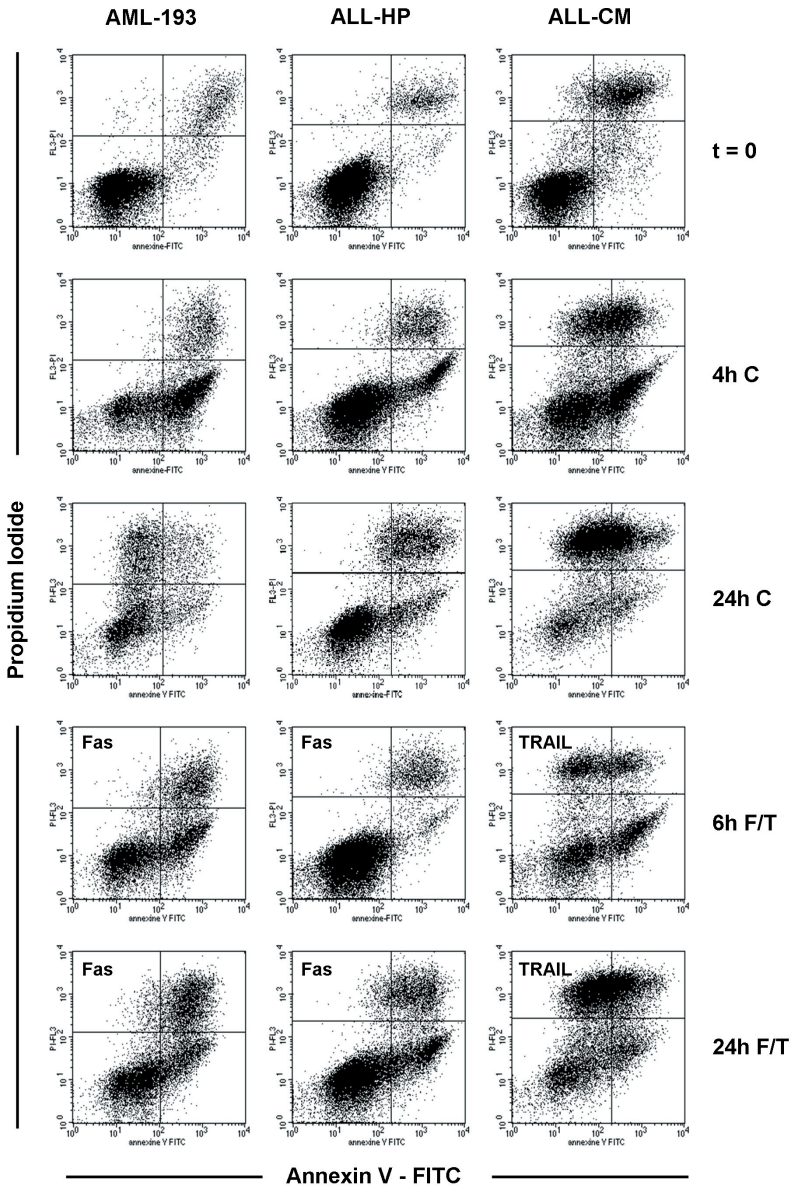

Figure 3.2. Leukemic cells die via an apoptotic mechanism in response to cytostatic agents and Fas Ab or TRAIL.

AML-193, ALL-HP and ALL-CM cells were exposed for 4 or 24 hrs to camptothecin (C), and for 6 or 24 hrs to Fas Ab (F) or TRAIL (T) in case of ALL-CM. Phosphatidyl serine exposure was determined by FACS analysis using annexin V-FITC staining combined with PI. Dotplots are representative of at least 2 independent experiments.

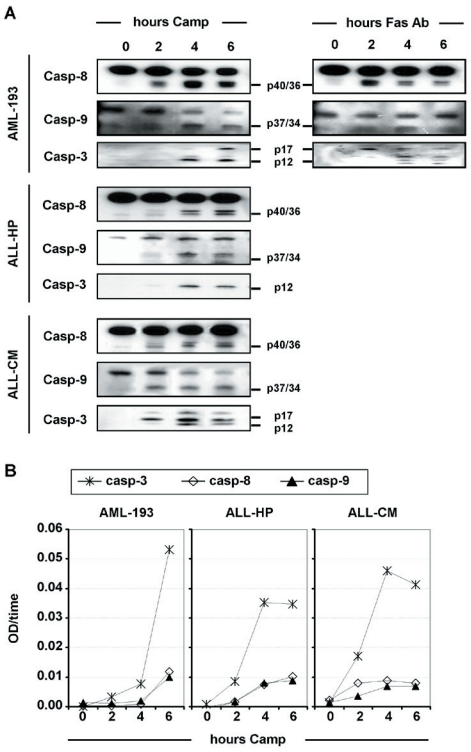

Figure 3.3. Caspase-activation patterns upon chemotherapy or death receptor-induced apoptosis in various leukemic cell lines.
(A) Caspase cleavage patterns for AML-193, ALL-HP and ALL-CM cells exposed to 50 μM of camptothecin (Camp, left panel) for indicated periods of time, and for AML-193 cells treated with 500 ng/mL of Fas Ab (right panel). Cell lysates (15 μg total protein per lane) were separated by 12% SDS-PAGE. Western Blot analysis for caspase-8, -9 and -3 was performed as described in Materials and Methods. The migration position of full-length caspase-8 (Casp-8), its cleavage intermediate p40/36, full-length caspase-9 (Casp-9), its cleavage intermediate p37/34, and the active subunits p17 and p12 of caspase-3 (Casp-3) are indicated. (B) Caspase activity assays in AML-193, ALL-HP and ALL-CM cells treated with 50 μM of camptothecin (Camp) for indicated periods of time. Cell extracts were prepared as described under Materials and Methods and assayed for caspase activity (in duplicate) using the colorimetric substrates DEVD-pNA (caspase-3-like protease), IETD-pNA (caspase-8-like protease), and LEHD-pNA (caspase-9-like protease). Data shown are representative of 2 independent experiments.

cleavage of all three caspases already after 2 hrs of treatment with camptothecin. As illustrated in AML-193 cells, the kinetics of caspase-8 activation upon triggering with camptothecin (left panel) was comparable to Fas Ab-induced caspase-8 activation (right panel), although maximal caspase-8 activation was already observed after 2 hrs of exposure to Fas Ab.
To study the kinetics of caspase-8, caspase-9 and caspase-3 activation in more detail upon exposure to cytostatic agents, we performed caspase-activity assays using

colorimetric caspase substrates (Figure 3.3B). These assays showed similar kinetics of cleavage of caspase-8 and caspase-9 substrates in all cell lines suggesting that both caspases are activated in the same time interval after apoptosis induction with camptothecin. In none of the cell lines cleavage of caspase-8 or caspase-9 substrates occurred earlier than cleavage of caspase-3 substrates (Figure 3.3B), suggesting that all three caspases were simultaneously activated. Conversion of caspase-8 and caspase-9 substrates was much lower than caspase-3 activity in all three cell lines.

To determine whether both caspase-8 and caspase-9 play an important role in the execution pathways induced by chemotherapeutic agents the effect of specific caspase-inhibitors on the percentages of camptothecin and Fas Ab- or TRAIL-induced apoptosis was studied. Percentages of specific inhibition, defined as the percentage of decrease in apoptosis induction in presence of specific caspase-inhibitors, were corrected for inhibition of spontaneous apoptosis mainly observed using the broad-spectrum caspase-inhibitor zVAD-FMK (zVAD). Percentages of specific inhibition of apoptosis by zVAD, and by caspase-3, -8 and -9 inhibitor after 24 hrs of exposure to various apoptotic agents are shown in Figure 3.4. In general, apoptosis was most effectively inhibited by zVAD (black bars). As expected, caspase-8 inhibitor caused 70-80% inhibition of death receptor-induced apoptosis by Fas Ab and TRAIL in all cell lines. Inhibition of death-receptor-induced apoptosis by caspase-9 inhibitor ranged from 20-50%.

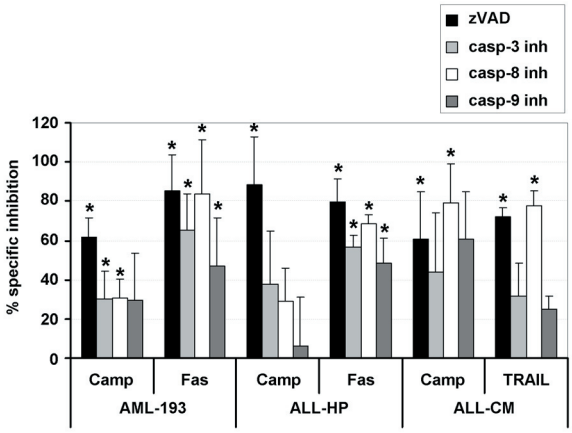

Figure 3.4. Effect of various caspase-inhibitors on chemotherapy or death receptor-induced apoptosis in various leukemic cell lines.
AML-193, ALL-HP and ALL-CM cells were exposed for 24 hrs to 50 μM camptothecin (Camp) or Fas agonistic antibody (Fas) (1000 ng/mL for AML-193 and 100 ng/mL for ALL-HP) in presence or absence of specific caspase-inhibitors (100 μM). Since ALL-CM cells were insensitive to Fas (Figure 3.1), TRAIL (100 ng/mL) was used to activate the death receptor pathway. Percentages of inhibition of lysis by the broad-spectrum caspase-inhibitor zVAD-FMK (zVAD), and by caspase-3, -8, or -9 inhibitors were calculated. Results are the mean percentage of inhibition by each caspase-inhibitor + SD of 3 independent experiments; *, $p<0.05$ compared to control cells without inhibitor.

Inhibition of camptothecin-induced apoptosis by caspase-8, caspase-9 and caspase-3 inhibitors varied between the cell lines studied. In AML-193 and ALL-HP cells minimal inhibition ranging from 10 to 40% was observed, whereas in ALL-CM cells inhibition varied from 40 to 80%. Within each cell line only marginal differences in inhibition with caspase-8, caspase-9 and caspase-3 inhibitors were found. However, in none of the cell lines inhibition with caspase-9 inhibitor was significant, whereas in AML-193 and in ALL-CM caspase-8 inhibitor significantly inhibited camptothecin-induced apoptosis. These data and the simultaneous activation of both caspase-8 and caspase-9 upon exposure to camptothecin suggested that both the death receptor and the mitochondrial pathway play a role in chemotherapy-induced apoptosis of these cell lines.

Chemotherapy-induced apoptosis is not linked to the death receptor pathway but completely dependent on the mitochondrial pathway in leukemic cell lines

To investigate whether the concurrent activation of caspase-8 and caspase-9 indicated that both pathways play a role in chemotherapy-induced apoptosis, we introduced anti-apoptotic constructs into our panel of leukemic cell lines. FLIP expression was used to block the death receptor pathway, whereas the Bcl-2 encoding construct was introduced to specifically inhibit the mitochondrial pathway. Transduced cells were FACS sorted on the basis of ΔNGFR or GFP expression, which resulted in >90% pure populations. Protein expression of the transduced cell lines was determined to verify proper translation of the introduced anti-apoptotic constructs, which is depicted in Figure 3.5A for the various transduced ALL-CM cells.

To investigate whether these anti-apoptotic constructs could prevent early-apoptotic processes, we analyzed annexin V expression in the different cell lines upon apoptosis induction with various apoptotic agents. An example is given in Figure 3.5B, in which ALL-HP cells transduced with mock, Bcl-2 or FLIP were exposed to camptothecin or Fas agonistic antibody. Increased Bcl-2 expression largely prevented phosphatidyl serine exposure on the cell surface after apoptosis induction with camptothecin, but not after treatment with Fas Ab. In contrast, enhanced FLIP expression resulted in specific inhibition of Fas Ab-induced apoptosis, while chemotherapy-induced apoptosis could not be prevented. An overview of the inhibitory effect of both elevated Bcl-2 and FLIP expression on the amount of chemotherapy- and death receptor-induced apoptosis in the three different cell lines is given in Figure 3.5C. All FLIP-transduced cell lines studied showed around 70% inhibition of lysis induced with Fas Ab or TRAIL, illustrating effective inhibition of the death receptor pathway by FLIP. In contrast, FLIP-transduced cells and mock-transduced cells were equally sensitive to camptothecin and daunorubicin also using other concentrations than shown in the figure, demonstrating that the death receptor pathway is not involved in chemotherapy-induced apoptosis in AML-193, ALL-HP and ALL-CM cells. To investigate the involvement of the mitochondrial pathway in chemotherapy-induced

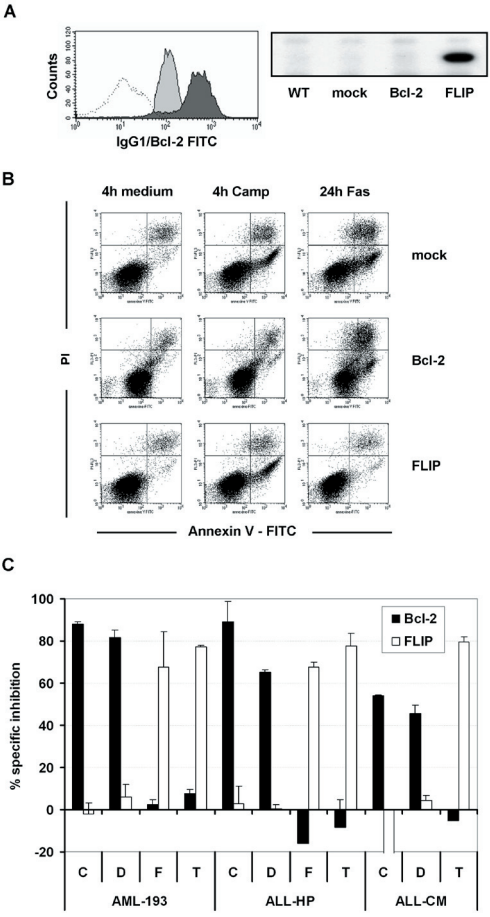

Figure 3.5. Selective inhibition of chemotherapy-induced apoptosis by Bcl-2 and of death receptor-induced apoptosis by FLIP in various cell lines.
(A). Bcl-2 expression (left) of mock (light grey) and Bcl-2 (dark grey) transduced ALL-CM cells was determined using flow cytometry. As a control, IgG1-FITC staining is shown (dotted line). FLAG-tagged FLIP expression (right) of WT, mock-, Bcl-2-, or FLIP-transduced ALL-CM cells is shown, as determined by Western Blot analysis using FLAG-tag specific antibodies. (B) The effect of elevated Bcl-2 and FLIP expression on apoptosis induction after treatment for 4 hrs with camptothecin (Camp) or for 24 hrs with Fas Ab is shown for ALL-HP cells, as determined by FACS analysis using annexin V-FITC staining combined with PI. Note the elevated FL-1 intensity of untreated (4 hrs medium) ALL-HP-Bcl-2 cells caused by GFP marker gene expression of the Bcl-2 construct. (C) The effect of increased Bcl-2 and FLIP expression on total cell death of various leukemic cells after exposure to various apoptotic agents. AML-193, ALL-HP and ALL-CM cells transduced with vector control (mock), Bcl-2, or FLIP were exposed for 24 hrs to camptothecin (Camp), Daunorubicin (Dauno), Fas agonistic antibody (Fas) or TRAIL using concentrations as indicated in Figure 3.1 (*). Because of insensitivity, ALL-CM cells were not treated with Fas. Cytotoxicity was determined using both ^{51}Cr release assays (AML-193 and ALL-HP) and CFSE assays (ALL-CM). Percentages of inhibition by increased expression of Bcl-2 (black bars) or FLIP (white bars) on Camp-, Dauno-, Fas- or TRAIL- induced apoptosis were calculated, and a representative result of three independent experiments is shown in the figure.

apoptosis in these cell lines, the inhibitory effect of Bcl-2 expression was determined. As illustrated in Figure 3.5C, Bcl-2 almost completely inhibited apoptosis induced by 16 to 24 hrs exposure to various cytostatic agents in AML-193 and ALL-HP cells, and caused 50% inhibition in ALL-CM cells, emphasizing an important role of the mitochondria in the execution of chemotherapy-induced apoptosis. The same results were found using other concentrations of cytostatic agents (data not shown).
In conclusion, despite substantial activation of caspase-8 in all 3 leukemic cell lines studied, chemotherapy-induced apoptosis was not executed via the death receptor pathway but was completely dependent on the mitochondrial pathway.

Chemotherapy-induced caspase-8 activation occurs downstream from the mitochondria in all leukemic cell lines studied

To verify that chemotherapy-induced caspase-8 activation was not death-receptor-mediated, we tested the FLIP-transduced cell lines for their capacity to inhibit camptothecin-induced caspase-8 cleavage. Figure 3.6A shows that caspase-8 activation upon camptothecin induction was not affected by increased expression of FLIP in AML-193 and ALL-HP cells, despite decreased basal expression of procaspase-8 in FLIP-transduced AML-193 cells compared to mock- or Bcl-2-transduced cells. Expression of β-actin was used to confirm equal protein loading. As a control, FLIP-transduced AML-193 and ALL-HP cells showed a complete block in Fas-induced caspase-8 activation compared to the mock-transduced cells (Figure 3.6B). These data indicate that caspase-8 activation upon camptothecin-triggering likely occurs in the cytosol, independent of death receptors and DISC formation.
To reveal whether caspase-8 cleavage occurred upstream or downstream from the mitochondria, the effect of Bcl-2 expression on camptothecin-induced caspase-8 activation was assessed (Figure 3.6A). Caspase-8 cleavage was almost completely blocked in all Bcl-2 expressing cell lines, indicating that caspase-8 was mainly activated downstream from the mitochondria upon chemotherapy-induced apoptosis. In contrast, caspase-8 cleavage induced by Fas Ab was mainly occurring independent of mitochondrial activation (Figure 3.6B), since Fas-induced caspase-8 activation in Bcl-2-transduced cells was almost similar as in mock-transduced cells.
To confirm that Bcl-2 completely inhibited caspase-8 activation after treatment with cytostatic agents, we performed in addition caspase-8 activity assays (Figure 3.6C). Although conversion of IETD-pNA substrate was low in both cell extracts of AML-193 and ALL-HP cells treated with camptothecin, clear inhibition of caspase-8 activation was observed in the Bcl-2- compared to the mock- and FLIP-tranduced cell lines.

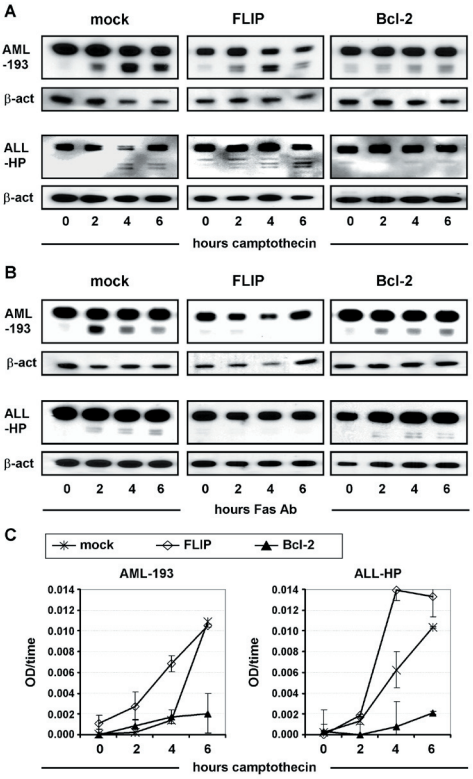

Figure 3.6. Selective inhibition of chemotherapy-induced caspase-8 activation by Bcl-2 and of death receptor-induced caspase-8 activation by FLIP expression.
AML-193 and ALL-HP cells transduced with vector control (mock), Bcl-2, or FLIP were exposed for indicated periods of time to (A) camptothecin (50 μM) or (B) Fas Ab (500 ng/mL). Cell lysates (15 μg total protein per lane) were separated by 12% SDS-PAGE. Western Blot analysis for caspase-8 was performed as described in Materials and Methods. Full-length caspase-8 and its cleavage intermediate p40/36 are shown in the figure (see Figure 3.3A). Equal protein loading was confirmed by expression of β-actin. Note that increased expression of FLIP appeared to decrease procaspase-8 expression in AML-193 but not in ALL-HP cells, compared to mock or Bcl-2 expressing cells. (C) Caspase-8 activity assay in AML-193 and ALL-HP cells transduced with vector control (mock), Bcl-2, or FLIP, and treated with 50 μM of camptothecin (Camp) for indicated periods of time. Cell extracts were prepared as described under Materials and Methods and assayed for caspase-8 activity (in duplicate) using the colorimetric substrate IETD-pNA. Data shown are mean values (+/- SD) of 2 independent experiments.

DISCUSSION

Chemotherapy-induced apoptosis has been considered to be induced via the mitochondrial pathway of apoptosis, in which cytochrome C release and caspase-9 activation play a central role. Some studies, however, described that chemotherapy-induced apoptosis is mediated via the death receptor pathway, in which DISC

formation and caspase-8 activation are key events. In this study, using 3 different cell lines derived from patients with acute leukemia, we investigated the role of the death receptor pathway, and in particular of caspase-8, in apoptosis induced by cytostatic agents. We observed that caspase-8 was rapidly cleaved upon exposure to camptothecin, comparable to caspase-9 and caspase-3 activation. Moreover, inhibition experiments using specific caspase-inhibitors revealed that camptothecin-induced apoptosis was at least equally effectively blocked by caspase-8 as by caspase-9 inhibitor. To examine whether this caspase-8 activation was due to activation of the death receptor pathway, we compared sensitivity of mock-transduced leukemic cell lines with FLIP-transduced cell lines. No inhibitory effect of elevated expression of FLIP on camptothecin-induced apoptosis or caspase-8 activation was observed, whereas the effective inhibition of Fas- or TRAIL-induced apoptosis in the same cell lines proved functionality of the introduced retroviral construct. In contrast, introduction of Bcl-2 resulted in almost complete inhibition of camptothecin-mediated cell death in all 3 cell lines studied, showing that chemotherapy-induced apoptosis is completely dependent on the mitochondrial pathway. Moreover, caspase-8 activation was largely inhibited indicating that caspase-8 activation is occurring downstream from the mitochondria, independent of receptor binding or DISC formation.

Whether the death receptor pathway plays a role in anticancer drug-mediated cytotoxicity had been considered controversial. Several investigators showed that anti-cancer drugs induced upregulation of Fas receptor (FasR) and Fas ligand (FasL), followed by subsequent autocrine or paracrine induction of Fas-mediated apoptosis. [12-16] Furthermore, the Fas signaling pathway could also be activated by Fas trimerization in the absence of Fas-L in cells exposed to UV irradiation [39] or various cytostatic agents. [40] Studies by Friesen *et al*. indicated that extracellular blocking of the Fas-R resulted in inhibition of doxorubicin-induced apoptosis, [12] while overexpression of FADD-DN did not provide protection against drug-induced apoptosis in Jurkat and CEM cells, [18] emphasizing the complexity of the involvement of the CD95 system on chemotherapy-induced apoptosis. The same group argued that triggering of death receptor and/or mitochondrial pathways upon drug treatment is cell type specific, proposing that in so-called type I cells both the receptor and the mitochondrial pathway are activated upon drug treatment, whereas in type II cells apoptosis is predominantly controlled by mitochondria. [18]

Here, we demonstrate that despite considerable caspase-8 activation chemotherapy-induced apoptosis of leukemic cells is not dependent on the death receptor pathway, as previously also reported by Wesselborg *et al*. for the human leukemic T-cell lines CEM and Jurkat. [26] We observed this both in an AML cell line (AML-193) and in two ALL cell lines (ALL-HP and ALL-CM) that closely resembled the primary ALL cells from which they were derived, suggesting that chemotherapy-induced apoptosis

of primary leukemic cells is also not mediated via the death receptor pathway of apoptosis.
Although recent clinical studies identified low or absent expression of FADD protein in AML cells at diagnosis as a new independent prognostic factor for poor response to chemotherapy, [20] decreased FADD expression was only associated with resistance to chemotherapy. Therefore, it is likely that another component of the death receptor pathway is responsible for the poor clinical response observed in these patients. As illustrated in our study, retroviral introduction of FLIP into AML cells seemed to be coincided with downregulation of procaspase-8 (Figure 3.6A and B). Based on the results of this study and on other studies showing that downregulation of procaspase-8 expression resulted in several malignancies in resistance to doxorubicin-induced apoptosis, [9;23-25] we suppose that defects in caspase-8 activation may underlie unresponsiveness of leukemic cells to chemotherapy treatment.

In our panel of cell lines, cleavage of caspase-8 occurred predominantly downstream of mitochondria (Figure 3.6). The mechanism of caspase-8 activation independently of death receptors and downstream of mitochondria is unknown. An early event in the induction of chemotherapy-induced apoptosis is the release of cytochrome C into the cytosol, followed by formation of the apoptosome, consisting of Apaf-1, ATP and procaspase-9, and subsequent cleavage of caspase-9 as the most apical caspase. [11;29] Caspase-8 might also bind to Apaf-1, since it has been found that caspase-8 can interact with the *Caenorhabditis elegans* cell death regulator Ced-4, a homologue of human Apaf-1, in cell free systems. [41] The long prodomain of procaspase-8 would then serve as caspase recruitment domain (CARD), which can bind to the CARD motif of Apaf-1. Procaspase-8 would compete with procaspase-9 for binding to Apaf-1, which may explain the partial inhibition of camptothecin-induced apoptosis obtained with both caspase-8 and caspase-9 inhibitor (Figure 3.4). Procaspase-8 might also be cleaved by active caspase-3 in an amplification step. [42] The results shown in Figure 3.3 do not reveal the order of caspase activation after chemotherapy-induced apoptosis, since in all 3 cell lines studied caspase-8, caspase-9 and caspase-3 activation occurred almost simultaneously. Caspase-3 activity was probably higher than caspase-8 and caspase-9 activity (Figure 3.3B) because more effector caspases (casp-3) than initiator caspases (casp-8 and -9) are required to execute apoptosis.
As expected, Bcl-2 expression did not inhibit death-receptor-mediated apoptosis (Figure 3.5B and C). Unexpectedly, we observed inhibition of death receptor-induced apoptosis by caspase-9 inhibitor (Figure 3.4), which may be explained by non-specific inhibition by irreversible caspase-inhibitors. Therefore, we suppose that in our panel of cell lines apoptosis induced by extracellularly triggering of death receptors is not dependent on the mitochondrial pathway.

In summary, understanding the mechanisms of anticancer drug-induced apoptosis is of major importance for developing effective strategies in tumor therapy.

Since caspase-8 plays a central role in both immuno- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia, and is downregulated in certain tumors, [23-25] therapeutic strategies focusing at modulation and activation of caspase-8 may sensitize drug-resistant malignancies to radiation or combination chemotherapy. Moreover, we assume that cross resistance of CD95 resistant cells to anticancer agents which is commonly observed in leukemia patients, can be explained by defects in a common element of both pathways, such as caspase-8, instead of by activation of the death receptor pathway by cytostatic agents. To investigate this, small interfering RNA (siRNA)-mediated knockdown of caspase-8 expression or genetic ablation in mice are preferable ways of interfering with the function of caspase-8 instead of inhibition assays using caspase-inhibitors.

CONCLUSION

In conclusion, here we show that caspase-8 plays a more important role in apoptosis of leukemic cells induced by cytostatic agents than previously expected. Chemotherapy-induced apoptosis is independent of the death receptor pathway, and activation of caspase-8 occurs downstream from mitochondria, independently of death receptor triggering or DISC formation.

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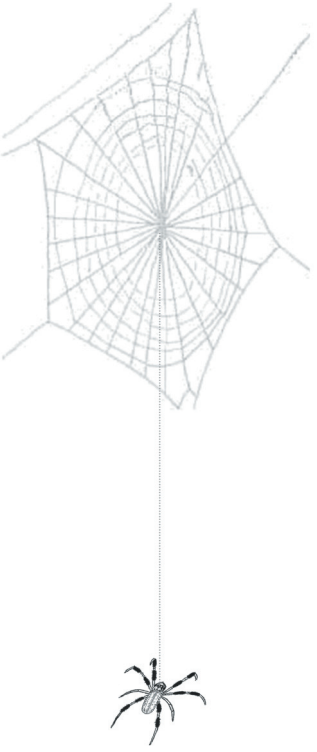
CHAPTER

4



Differential activation of the death receptor pathway in human target cells induced by cytotoxic T lymphocytes showing different kinetics of killing

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ABSTRACT

Background and objectives. Cytotoxic T lymphocytes (CTLs) may use two effector mechanisms to kill their target cells: perforin (PFN) and granzyme B (GrB) dependent granule-mediated cell death and death receptor-mediated cell death. Controversy exists whether in addition to PFN/GrB-mediated apoptosis death receptor-induced apoptosis contributes to the elimination of human tumor cells by CTLs.
Design and Methods. Since the two CTL-mediated effector mechanisms 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 FLICE inhibitory protein (FLIP) was introduced into these target cells.
Results. A CTL clone capable of killing 50% of the target cells within two hrs of incubation primarily acted by release of PFN and GrB. In contrast, two CTL clones showing slower target cell killing kinetics partially used the death receptor pathway (~30% inhibition by FLIP).
Interpretation and Conclusions. In conclusion, we demonstrate that the death receptor pathway contributes to T cell mediated cell death if not all target cells are destroyed by release of PFN and GrB.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) mediate target cell death using two effector pathways: granule-mediated and death receptor-mediated killing. [1;2] Both pathways are activated by membrane T cell receptors (TCRs) recognizing target antigen. In granule-mediated killing, TCR-triggering induces exocytosis of preformed cytotoxic granules, containing perforin (PFN) and a family of serine proteases termed granzymes, [3;4] of which granzyme B (GrB) is the most potent member. Intracellular delivery of GrB results in the initiation of a caspase cascade via proteolytic activation of caspase-3, directly [5] or through a mitochondrium-dependent pathway. [6] In death receptor-mediated killing, TCR-triggering induces surface membrane expression of apoptosis inducing ligands of the tumor necrosis factor (TNF) superfamily (Fas ligand (FasL), TNF-α, TRAIL), which cross-link death receptors expressed on the target cells. [1;7] Upon trimerization of the death receptor, Fas Associated Death Domain (FADD) is recruited to the intracellular death domain, followed by engagement of pro-caspase 8, also called FADD-like IL-1 converting enzyme (FLICE). [8] In this complex, procaspase-8 is proteolytically cleaved, and the active caspase-8 that is formed either directly cleaves effector caspases resulting in apoptosis, or activates the mitochondrial pathway via cleavage of Bid, a member of the pro-apoptotic Bcl2 family. [9;10]

Granule-mediated cell death has been reported to play an important role in the elimination of virus-infected and tumorigenic cells. [11;12] In line with this, mice with defects in PFN expression develop spontaneous lymphoma and have a diminished ability to clear many viruses or tumor cells. [13] The kinetics of granule-mediated cell death is very rapid, lytic concentrations of PFN induce virtually immediate cell death (10 min). GrB combined with sublytic concentrations of PFN also results in rapid target cell death (in approximately 90 min), as recently demonstrated in a mouse fibroblast cell line. [14]

Death receptor-mediated cell death has primarily been described to be involved in eliminating autoreactive T cells and downsize immune responses after infection, [15-18] as clearly demonstrated in various murine studies showing that mice genetically deficient in Fas (*lpr*) or Fas ligand (*gld*) develop lymphoproliferative disorders. [19;20] In other murine studies, Fas/FasL-mediated killing has also been reported to play a role in elimination of virus-infected cells. [21-23] Kinetics of death receptor-induced apoptosis are slow compared to PFN-GrB-mediated cell death, [24;25] which was underlined by a study of Shresta *et al*, who showed in a murine model that apoptosis induced by $GrB^{-/-}$ CTL was delayed for 4 hrs compared to lysis by $GrB^{+/+}$ CTL. [26] In the human setting it is largely unknown whether besides secretion of PFN and GrB, cytotoxic T cells also use the death receptor pathway to kill virus-infected or tumor cells.

In the current study, we investigated the role of the death receptor pathway in CTL-mediated cell death of human target cells. Because of the differences in kinetics of cell death between granule-induced and death receptor-induced cell death, we used not only rapidly lysing CTL but also T cell clones displaying slow kinetics of killing that have been isolated from a patient with chronic myeloid leukemia (CML) at the time of clinical response to donor lymphocyte infusion. We investigated the involvement of the death receptor pathway in CTL-mediated lysis by introduction into EBV transformed B cells (EBV-LCL target cells) of a retroviral construct encoding the anti-apoptotic gene FLICE inhibitory protein (FLIP), which 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. [27;28] In this study we demonstrate that leukemia-reactive T-cell clones at least partially use the death receptor pathway to kill their target cells.

MATERIALS AND METHODS

Cells and culture conditions

HY-A1 is a $CD8^+$ HLA-A1-restricted anti-HY T cell clone isolated from a patient with a bone marrow rejection, recognizing a peptide derived from the DFFRY protein. [29] B57-2 and C6-2 are two mHag-specific $CD8^+$ leukemia-reactive T cell clones isolated from a CML patient (JTO) at the time of clinical response to donor lymphocyte infusion (DLI). [30] These clones are HLA-B57- and Cw-6-restricted, respectively, and

recognize EBV-LCL derived from the patient. The T cell clones were cultured in IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin, 5% pooled human serum, 5% FBS, and 100 IU/mL IL-2 (Chiron, Amsterdam, the Netherlands), and stimulated every 2 weeks with a mixture of irradiated allogeneic peripheral blood mononuclear cells, 800 ng/mL phytohemagglutinin (PHA, Murex Biotech Limited, Dartford, UK), and 120 IU/mL IL-2.
EBV-LCL were cultured in IMDM supplemented with 10% fetal bovine serum (FBS), 3 mM L-glutamine, 50 μg/mL streptomycin and 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium).

CFSE cytotoxicity assay
Cytotoxicity was measured using CFSE (carboxyfluorescein diacetate succinimidyl ester) -based cytotoxicity assays as described by Jedema *et al*. [31] Target cells were labeled with 5 μM CFSE (Molecular Probes Europe, Leiden, The Netherlands), and incubated overnight in a humidified atmosphere of 5% CO_2 and 37°C. For the cytotoxicity assay, 5,000 target cells/well (50 μL) were plated in 96-well microtiter plates (all in triplicate), and 5,000 effector T cells were added in a volume of 100 μL/well. After 2, 5 and 24 hrs of coculture, FACS analysis was performed to determine numbers of viable target cells. To exclude dead cells from the analysis, propidium iodide (PI) (1 μg/mL; Sigma-Aldrich, St Louis, MO, USA) was added. To allow quantitative analysis of the viable cells, the wells were harvested, and transferred to FACS tubes containing 10,000 Flow-Count Fluorospheres (Coulter Corporation, Miami, FL, USA). For each sample 3,000 microbeads were acquired, facilitating the calculation of absolute numbers of viable (PI^-) $CFSE^+$ target cells. The percentage of specific cell death was defined as:
[(mean absolute number of viable $CFSE^+$ target cells in control medium - absolute number of viable $CFSE^+$ target cells ^{experimental}) / (mean absolute number of viable $CFSE^+$ target cells in control medium)] x 100.

Activation of the death-receptor pathway
Death-receptor-mediated apoptosis was induced with Fas agonistic antibodies (10 to 1000 ng/mL) that cause crosslinking of the Fas receptor (Fas Ab, 7C11; Beckman Coulter Inc., Fullerton, CA, USA), or with recombinant human TRAIL (rhs*Killer*TRAIL™) (Alexis Corp., Lausanne, Switzerland).

Generation of retroviral constructs and transduction of EBV-LCL cells
The complete coding region of human FLIP-long (U97074) with a FLAG tag in front of the start codon was amplified from plasmid pCR3.V64 (kindly provided by Dr. J.P. Miedema (Leiden University Medical Center, Leiden, The Netherlands)) by PCR using the forward primer 5'-tatagaagatctaccatggattacaaagacg atgac-3' and the reverse primer 5'-tataccgctcgagttatgtgtaggagag-3'. FLAG-FLIP encoding PCR products were cloned into the Moloney murine leukemia virus-based retrovirus vector LZRS (G. Nolan, Stanford University, Palo Alto, CA) containing truncated nerve growth factor receptor (ΔNGF-R) as the marker gene. [32] Retroviral pLZRS vector encoding ΔNGF-R alone was used as a control vector (mock) in the experiments. Generation of retroviral supernatant and retroviral transduction of EBV-LCL were performed as previously described. [33] Transduced cells were purified by FACS® sort based on marker gene expression using a FACSVantage (Becton Dickinson, Mountain View, CA, USA).

SDS PAGE and Western Blot analysis
Cell lysates of 2 x 10^6 cells were obtained by freeze-thawing the cells in 100 μL NP40-lysisbuffer (50 mM Tris-HCl, pH 7.6, 5 mM DTT, 20% v/v glycerol, 0.5% v/v Nonidet P40, and 25% v/v Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany). SDS PAGE and Western Blot analysis using PVDF membranes (Millipore Corp., Bedford, MA, USA) were performed as previously described. [34] Primary antibody incubations were performed for 2 hrs in 1% Ecl-blocking reagent. Horseradish peroxidase (HRP) -conjugated antibodies specific for the FLAG epitope tag (Sigma) were used to detect introduced FLIP (1:1,000) .

Statistical analysis
Statistical analysis was performed using a two-paired Student's *t*-test to calculate whether lysis in WT-EBV-JTO cells significantly differed from lysis observed in EBV-JTO cells stably expressing FLIP. Differences were considered statistically significant when p values were ≤ 0.05.

Intracellular Perforin and Granzyme B analysis
20,000 T cells were stimulated for 2 hrs with 20,000 specific (EBV-JTO) or a-specific (EBV-JY) target cells. To discriminate between effector and target cells, T cells were stained with CD8 specific antibodies (BD, San Jose, CA, USA). Cells were fixed and permeabilized using Fix Buffer I and Perm/Wash Buffer I (both BD), respectively, according to the manufacturer's protocol. Intracellular amounts of PFN and GrB present in the T cells were determined by flow cytometry using PFN (1:20) and GrB (1:20) specific antibodies (BD). IgG antibodies were used to correct for background staining. The differences in PFN and GrB staining (ΔMFI) after specific and a-specific stimulation of the T cells reflect the amounts of PFN and GrB released by the different T cell clones within 2 hrs of specific stimulation with EBV-JTO.

RESULTS

mHag-restricted CTL clones show different killing kinetics towards the same EBV-LCL

We examined 3 different CTL clones for their capacity to kill EBV-LCL from patient JTO (EBV-JTO). Percentages of specific lysis of EBV-JTO cells were determined after 2, 5 and 24 hrs of coculturing with the T cell clones using the CFSE-based cytotoxicity assay. CTL clone HY-A1 showed very fast kinetics of killing, resulting in 50% of target cell lysis after 2 hrs of incubation and maximal lysis (90%) after 24 hrs (Figure 4.1). CTL clone B57-2 showed 15% of lysis after 2 hrs, almost 40% after 5 hrs, and 70% after 24 hrs of incubation, which we classified as intermediate killing kinetics. CTL clone C6-2 caused no EBV-JTO-specific cell death after 2 and 5 hrs, but 50% lysis after overnight incubation, which we regarded slow killing kinetics.

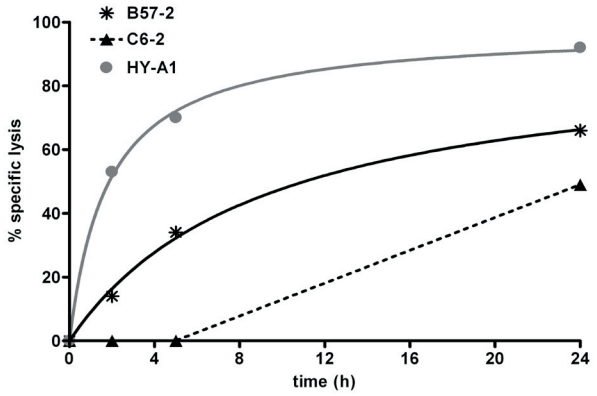

Figure 4.1. Kinetics of minor Ag-restricted CTL-mediated killing of EBV-JTO cells.
EBV-LCL cells from patient JTO (EBV-JTO) were exposed for 2, 5, and 24 hrs to the HLA-A1-restricted CTL clone HY-A1, the B57-restricted CTL clone B57-2 and the Cw6-restricted CTL clone C6-2, at an E/T ratio of 1/1. Percentages of specific lysis were determined using CFSE-based cytotoxicity assays, and the mean percentages of 3 (HY-A1), 5 (B57-2) and 7 (C6-2) independent experiments are indicated in the figure.

Kinetics of Fas-mediated cell death of EBV-JTO cells

To study the kinetics of death-receptor-mediated apoptosis in EBV-JTO cells, we performed cytotoxicity experiments in time using various concentrations of Fas Ab or TRAIL to activate the death receptor pathway. The EBV-JTO cells did not respond to various concentrations of TRAIL (data not shown) suggesting that the target cells do not express the receptor for TRAIL. As shown in Figure 4.2, in the first 2 hrs of exposure to Fas Ab, no lysis was observed. After 5 hrs of exposure, high concentrations of Fas Ab (≥500 ng/mL) caused 20-30% apoptosis of EBV-JTO cells, whereas hardly any (0-10%) target cells died in response to low concentrations of Fas Ab (≤100 ng/mL). Exposure to Fas Ab for 24 hrs resulted in 60% apoptosis at high concentrations, and also significant cell death (~40%) at low concentrations (100 ng/mL) of Fas Ab (Figure 4.2). These data show a correlation between the concentration of Fas Ab and the rate of Fas-induced apoptosis.

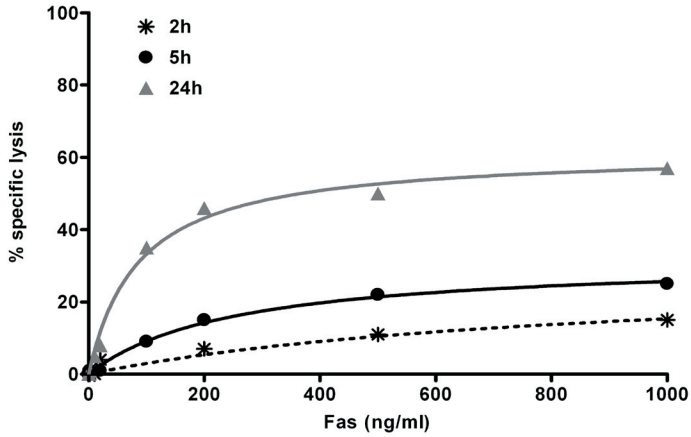

Figure 4.2. Kinetics of Fas Ab-induced cell death of EBV-JTO cells.
Mean percentages of lysis, as determined by CFSE-based or ^{51}Cr-release cytotoxicity assays, obtained in 4 independent experiments are shown. Percentages of lysis after 2, 5 or 24 hrs exposure to various concentrations of Fas Ab (Fas) are represented by dashed black lines, solid black lines, or solid grey lines, respectively.

Elevated FLIP expression efficiently inhibits the death receptor pathway

To study the role of the death-receptor pathway in CTL-mediated apoptosis, we introduced a retroviral construct into EBV-JTO target cells encoding the anti-apoptotic gene FLICE inhibitory protein (FLIP), which specifically blocks death-receptor-induced apoptosis. As a control, target cells were also transduced with empty vector (mock). Transduced EBV-JTO cells were FACS sorted on the basis of NGFR expression, which resulted in >90% pure populations. Protein expression of the transduced cell lines was determined to verify proper

translation of the introduced FLIP-encoding construct, as depicted in Figure 4.3A. To test the functionality of the FLIP construct, wildtype (WT), mock- and FLIP-transduced EBV-JTO cell lines were exposed for 5 or 24 hrs to Fas Ab, and cell death was determined using the CFSE-based cytotoxicity assay (Figure 4.3B). Enhanced FLIP expression resulted in approximately 80% inhibition of lysis induced with Fas Ab, both after 5 and 24 hrs of exposure, compared to WT and mock-transduced EBV-JTO cells, illustrating effective inhibition of the death receptor pathway by FLIP.

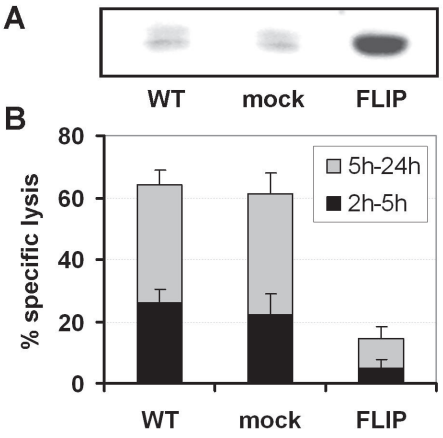

Figure 4.3. Functionality of EBV-JTO cells transduced with the anti-apoptotic gene FLIP.
A. FLIP protein expression of WT, mock-, and FLIP transduced EBV-JTO cells as determined by Western Blot analysis. B. Effective inhibition of Fas-induced apoptosis in EBV-JTO cells stably expressing FLIP. WT, mock-, and FLIP-transduced EBV-JTO cells were exposed to Fas Ab (500 ng/mL), and cytotoxicity was determined after 5 and 24 hrs of exposure using a CFSE-based assay. Mean percentages of lysis (+SD) of 3 independent experiments are shown.

Involvement of the death-receptor pathway in CTL-mediated killing of EBV-JTO cells

To determine the importance of the death receptor pathway in the execution mechanisms of the different T cell clones, we tested WT, mock and FLIP expressing EBV-JTO cells for their sensitivity to CTL clones HY-A1, B57-2 and C6-2 (described in Figure 4.1). Specific lysis was determined after 2, 5 and 24 hrs of incubation with the CTL clones. Lysis of the WT and mock-transduced EBV-JTO cells was identical (data not shown). In our experiments we therefore compared the lysis of FLIP-transduced EBV-JTO cells with the lysis of WT-EBV-JTO cells (Figure 4.4). Expression of FLIP did not significantly affect the lysis of the EBV-LCL by HY-A1, as shown in Figure 4.4A. Moreover, the majority of the target cells were killed within 2 hrs of exposure, in which Fas-mediated apoptosis hardly takes place (Figure 4.2), suggesting that HY-A1-induced cell death of EBV-JTO is mainly directly PFN or PFN/GrB-mediated.

As illustrated in Figure 4.4B, FLIP did not inhibit B57-2-induced lysis of the EBV-LCL in the first two hrs of incubation, which correlates with the absence of Fas-induced cell death in this time interval (Figure 4.2). In the time intervals where Fas-induced apoptosis theoretically plays a role (based on the results of Figure 4.2), an inhibitory effect of FLIP was observed. Significantly lower percentages of lysis of the FLIP expressing EBV-JTO compared to the WT EBV-JTO cell line (mean inhibition of 27±19%, p=0.05) were observed between 2 and 5 hrs of exposure to CTL clone B57-2. Similar results were found in the time interval from 5 to 24 hrs. These data show that the death receptor pathway plays a role in the execution mechanisms used by CTL clone B57-2.

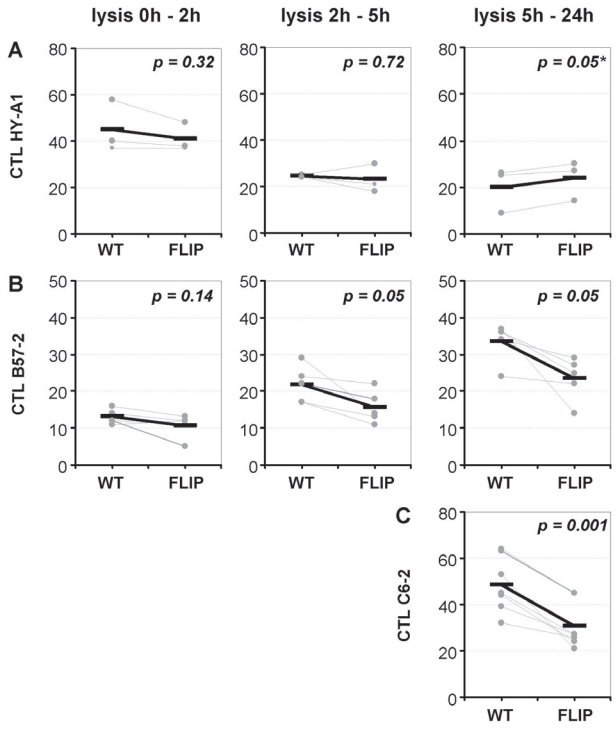

Figure 4.4. The effect of enhanced FLIP expression on CTL-mediated target cell death.
Both wildtype (WT) and EBV-JTO cells stably expressing FLIP were exposed to CTL clones HY-A1 (A), B57-2 (B) and C6-2 (C), At an E/T ratio of 1:1. Percentages of specific lysis were determined after 2, 5 and 24 hrs of exposure using CFSE-based cytotoxicity assays. In the figures, lysis is compared between WT and FLIP-expressing cell lines, and is shown in 3 time-intervals: lysis from 0 to 2 hrs, lysis from 2 to 5 hrs, and lysis from 5 to 24 hrs of exposure. Concerning clone C6-2, lysis in the intervals 0-2 hrs and 2-5 hrs was negligible and is not shown in the figure. Single experiments are represented in the figure by grey lines (n=3 for HY-A1, n=5 for B57-2 and n=7 for C6-2). Mean percentages of lysis of these independent experiments are indicated with a bold black line. Statistical analysis was performed, and p-values are shown in the figures. Lysis in EBV-JTO-FLIP was significantly lower than lysis in EBV-JTO WT if p-values were ≤0.05. * means that lysis was significantly higher in the FLIP-expressing cells than in the WT cells.

Clone C6-2 was not capable of lysing EBV-JTO cells in the first 5 hrs of incubation (Figure 4.1). In the time interval from 5 to 24 hrs, 50% target cell death was observed, which was even higher than with clone B57-2 in the same time interval (35%). To investigate whether this lysis was caused by activation of the death receptor pathway in the target cell, we tested the effect of FLIP on C6-2-induced cell death after 24 hrs of coculture. As depicted in Figure 4.4C, inhibition of the death receptor pathway by FLIP resulted in significantly decreased cell death (p=0.001) compared to WT EBV-JTO cells (mean % inhibition = 36 ± 14).

Involvement of PFN/GrB release in CTL-mediated killing of EBV-JTO target cells

To investigate whether CTL HY-A1 which showed very fast kinetics of killing indeed rapidly secreted high levels of PFN and/or GrB, intracellular PFN and GrB stainings were performed. The amounts of PFN and GrB present in the three CTL clones after 2 hrs stimulation with an irrelevant target cell (JY) compared to the specific target JTO were determined. We suppose that upon recognition of a specific target cell, the CTL will release PFN and GrB, resulting in lower amounts of intracellular PFN and GrB. The differences in PFN and GrB staining (ΔMFI) after specific and a-specific stimulation of the T cells were calculated, and are shown in Figure 4.5A and B, respectively. Thesę ΔMFI values reflect the amounts of PFN and GrB released by the different T cell clones within 2 hrs of specific stimulation with EBV-JTO.

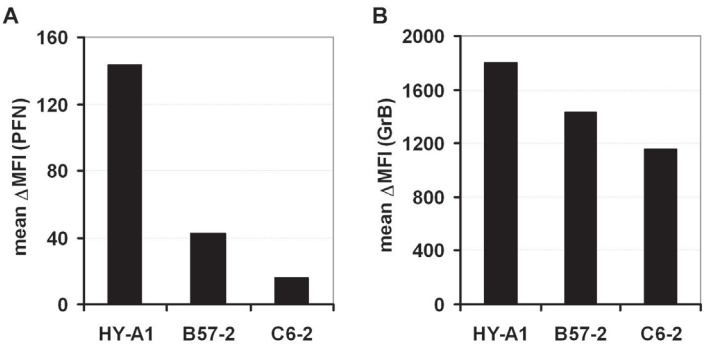

Figure 4.5. CTL-induced PFN and GrB release.
CTL clones HY-A1, B57-2, and C6-2 were stimulated for 2 hrs with the specific target EBV-JTO or with an irrelevant target EBV-JY. Intracellular levels of PFN and GrB were determined in the various $CD8^+$ T cells by flow cytometry (n=2). MFI values were corrected for background staining using control IgG antibodies. The differences in PFN (A) and GrB (B) staining (ΔMFI) after specific and a-specific stimulation of the T cells were calculated, and are shown in the figures. Thesę ΔMFI values reflect the amounts of PFN and GrB released by the different T cell clones within 2 hrs of specific stimulation with EBV-JTO.

In line with our assumption, we found that intracellular levels of both PFN and GrB in HY-A1 cytotoxic T cells were lower after 2 hrs of stimulation with EBV-JTO cells compared to coculture with EBV-JY cells, suggesting that HY-A1 released both PFN and GrB in this time-interval. Compared to the other two CTL clones HY-A1 secreted the highest amount of PFN, which may explain why JTO cells are killed within 2 hrs by HY-A1 but not or barely by B57-2 or C6-2. All three clones secreted remarkable amounts of GrB within 2 hrs of coculture with EBV-JTO, but without PFN this apparently does not lead to apoptosis of the target cell.

DISCUSSION

In this study, we investigated the role of the death receptor pathway in CTL-mediated cell death of human target cells. To determine the contribution of this slow execution mechanism, we introduced the anti-apoptotic protein FLIP, a specific inhibitor of the death receptor pathway. We used CTL clones isolated from a patient with CML that showed slow (C6-2, Figure 4.1), intermediate (B57-2) and fast (HY-A1) kinetics of killing EBV-LCL target cells. Concerning the rapidly lysing CTL clone HY-A1 no involvement of the death receptor pathway could be demonstrated (Figure 4.4A). Since 50% of target cells was already killed within 2 hrs of exposure, a time-interval in which HY-A1 was shown to release PFN and GrB, whereas control experiments indicated that Fas-induced apoptosis did not occur during this time-interval (Figure 4.2), we argued that this CTL clone mainly acted by secretion of PFN and GrB. In contrast, the EBV-LCL target cells were at least partially killed via the death receptor pathway upon exposure to the other two T cell clones (B57-2 and C6-2), suggesting that this pathway is important in the execution mechanism of a T cell if not all target cells have been destroyed by release of PFN/GrB.

Although cytotoxic T cells can mediate target cell death via different effector mechanisms, it is still questionable whether CTL clones preferentially use secretion of PFN and GrB or also use death ligands to kill human tumor cells. [15-18] [11;12] Since kinetics of death receptor-induced apoptosis are slow compared to PFN-GrB-mediated cell death, we analyzed CTL-mediated target cell death in different time intervals (0-2 hrs, 2-5 hrs, and 5-24 hrs). We could not use the standard ^{51}Chromium release assay, [35] because this assay is hampered by spontaneous release of ^{51}Cr, making it impossible to analyze cell death in EBV-LCL for longer than 4-10 hrs of incubation. Therefore we used a CFSE-based cytotoxicity assay which can be used for longer time periods. Results obtained with the CFSE assay have been demonstrated to correlate well with the results obtained in the conventional ^{51}Cr release assay in 4-hrs incubations. [31]

To study the contribution of the death receptor pathway in T-cell-mediated target cell death, we introduced the anti-apoptotic gene FLIP into the target cell via retroviral transduction, and demonstrated that enhanced expression of FLIP caused almost complete inhibition of Fas-induced apoptosis (Figure 4.3B). The inhibitory effect of FLIP was variable when target cell death was induced with the different CTL clones. No inhibition was achieved when EBV-JTO cells were exposed to the very rapidly killing CTL HY-A1. Already 40-50% of target cells were killed within 2 hrs of incubation which was coincided with PFN and GrB release by HY-A1 (Figure 4.5). Although besides PFN also high amounts of GrB were released by CTL HY-A1 after 2 hrs incubation with EBV-JTO, we hypothesize that these target cells may be killed via a PFN-dependent mechanism of cell death, since PFN has been shown to induce direct lysis of the target cell. [36;37] Although most of these studies were performed *in vitro* using non-physiological concentrations, Simon *et al*. illustrated in a murine study, in which CTLs were used that lacked both GrA and GrB, the *in vivo* importance of direct PFN-induced lysis. [38] We observed that the death receptor pathway plays at least a partial role in the effector mechanism of the CTL that killed its EBV-LCL target cells after 5 hrs of exposure (B57-2) and of the CTL being cytotoxic only after 24 hrs of incubation (C6-2). This suggests that this pathway is important at a later time point after T cell-target cell interaction if not all cells have been destroyed by immediate release of PFN/GrB.

Two of the three T cell clones used in this study (B57-2 and C6-2) were isolated from a patient (JTO) during the clinical response after the second DLI. [39] Analysis of the reactivity of the different CTL clones against different subsets of bone marrow (BM) cells from the patient revealed that B57-2 lysed only monocytic cells of the patient, whereas C6-2 was capable of lysing both monocytic and mature myeloid cells ([40], data not shown). The authors showed that resting B and T cells and immature CD34-positive cells representing BM progenitor cells were not or only marginally recognized by these T-cell clones. The T-cell clone HY-A1 recognized all target cell populations. Since both B57-2 and C6-2 CTL clones were capable of rapidly killing monocytes of patient JTO ([41], data not shown), we postulate that the differential kinetics of killing EBV-LCL of the same patient by these two CTL clones may be caused by differences in the strength of interaction between effector and target cell. We hypothesize that Cw6-restricted CTL clones recognize minor antigens highly expressed on monocytes, but poorly expressed on EBV-LCL from the patient resulting in a low avidity interaction between TCR and MHC/peptide complex. In case of a low avidity interaction between TCR and MHC/peptide complex, the CTL probably releases only low amounts of PFN and GrB, as illustrated for C6-2 in Figure 4.5, but may still activate the death receptor pathway, causing slow elimination of the target cell. The strength of triggering of the Fas-receptor on the target cell may determine the kinetics of the apoptosis induction, as was also illustrated by the correlation between the concentration of Fas Ab used to induce target cell death, and the rate of Fas-mediated apoptosis (see Figure 4.2).

In conclusion, in this study we demonstrate that not only PFN/GrB release but also the death receptor pathway plays a role in the execution mechanism of cytotoxic T cells derived from a patient with CML.

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CHAPTER 5

The Granzyme B-binding serpin PI-9 interferes with Fas-induced apoptosis: implications for interpretation of prognostic factors in relation to therapy of cancer

JF de Vries, MHM Heemskerk, R Willemze, JHF Falkenburg and RMY Barge

SHORT REPORT

Patients with leukemia and other hematological malignancies can be successfully treated with chemotherapy followed by allogeneic hematopoietic stem cell transplantation. Chemotherapy mainly kills malignant cells via activation of the mitochondrial pathway of apoptosis, but has also been described to activate the death receptor pathway of apoptosis. [1] After allogeneic stem cell transplantation natural killer (NK) cells and cytotoxic T lymphocytes (CTLs) can mediate target cell death using two different effector mechanisms: perforin (PFN) and granzyme B (GrB) dependent granule-mediated cell death and death receptor-mediated apoptosis. [2] Malignant cells protect themselves from chemotherapy- and CTL-induced apoptosis using several escape mechanisms including upregulation of anti-apoptotic proteins such as B cell lymphoma-2 (Bcl-2), FLICE (caspase-8) Inhibitory Protein (FLIP) and serine protease inhibitor-9 (PI-9). Bcl-2 is a specific inhibitor of the mitochondrial pathway of apoptosis, and overexpression has been shown to be an adverse prognostic factor for outcome of chemotherapeutic interventions. [3] FLIP, an enzymatically inactive homologue to caspase-8, has been shown to efficiently block the death receptor pathway of apoptosis, and enhanced FLIP expression has been associated with poor clinical outcome after treatment with chemotherapy and immunotherapy. [4] PI-9, a protein that specifically binds to GrB, was shown to efficiently inhibit PFN/GrB-mediated apoptosis in both *in vitro* and *in vivo* studies, [5] and is considered to be a specific prognostic marker for outcome of cellular immunotherapy. [6] However, due to the similarity between PI-9 and the viral serpin cytokine response modifier A (CrmA), an efficient inhibitor of Fas-mediated and to a lesser extent of GrB-induced cell death, [7] we hypothesized that PI-9 may also protect cells against Fas-mediated apoptosis, and hence may be less specific for certain immunological processes than expected. In this study, we therefore examined the effect of enhanced PI-9 expression on Fas-induced apoptosis.

Both EBV-transformed B cells (EBV-JTO) and Jurkat J16 target cells were transduced with retroviral constructs encoding FLIP or PI-9. Transduced cells were purified (>95%) on basis of NGFR or GFP marker gene expression using cell sorting. Wildtype (WT) and enhanced FLIP- or PI-9 expressing EBV-JTO and Jurkat cells were exposed to various concentrations of Fas agonistic antibody (Fas Ab, 7C11; Beckman Coulter Inc., Fullerton, CA, USA), and cell death was determined after 5 and 24 hrs of exposure using a CFSE-based cytotoxicity assay. Fas Ab titration curves for both EBV-JTO and Jurkat cells are shown in Figure 5.1A. EBV-JTO cells were less sensitive to Fas Ab treatment than Jurkat cells, since maximal lysis of EBV-JTO cells after 5 hrs of exposure was 30% using 500 ng/mL of Fas Ab, whereas 10 ng/mL of Fas Ab was sufficient to kill 30% of Jurkat cells. The IC_{50} value for EBV-JTO cells after 5 and 24 hrs of exposure was approximately 100 ng/mL of Fas Ab, while IC_{50} values for Jurkat

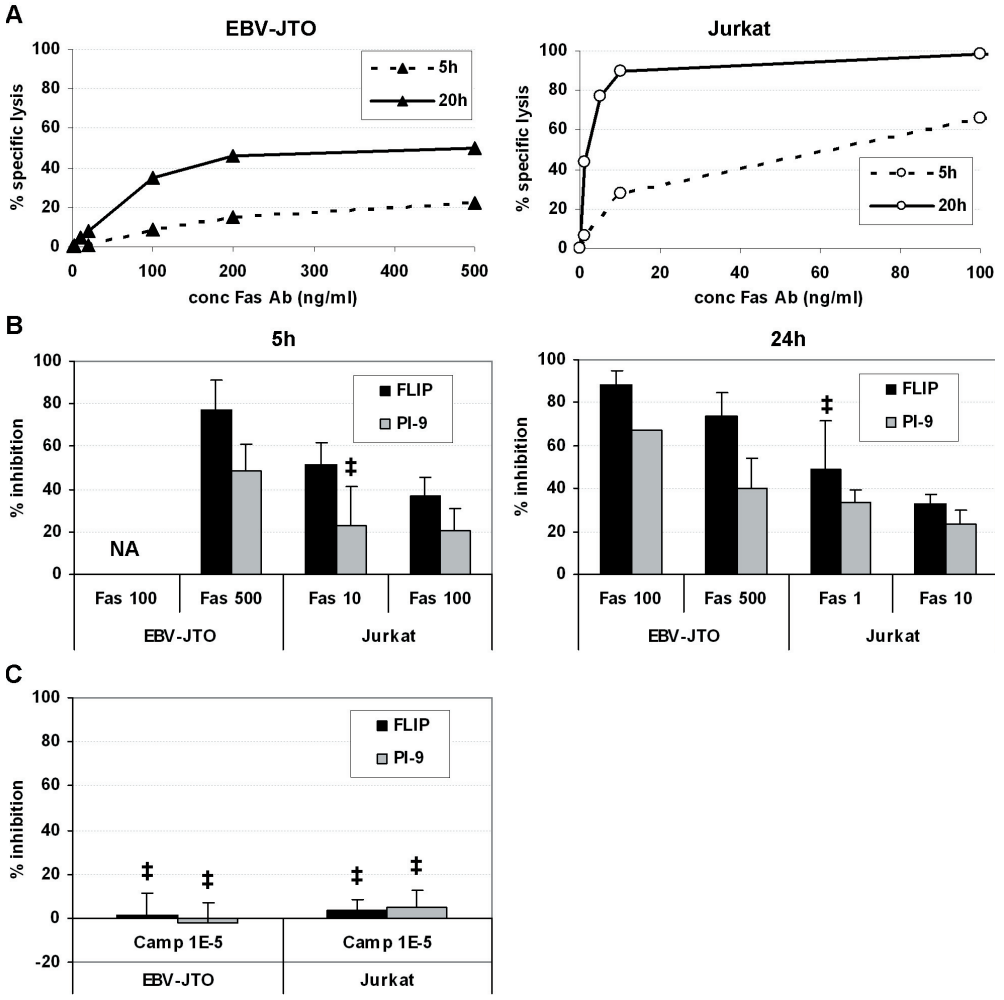

Figure 5.1. Effect of FLIP and PI-9 expression on Fas- and camptothecin (Camp)-induced apoptosis.

(A) Fas Ab titration curves for EBV-JTO (left panel) and Jurkat (right panel) cells after 5 and 24 hrs of exposure. The IC_{50} value for EBV-JTO was 100 ng/mL of Fas Ab; IC_{50} values for Jurkat were 10 and 1 ng/mL of Fas Ab after 5 and 24 hrs of exposure, respectively. (B) Percentages of inhibition of Fas-induced apoptosis by FLIP and PI-9 after 5 hrs (left panel) and 24 hrs (right panel) of exposure of EBV-JTO and Jurkat cells. Percentages of lysis were determined with CFSE-based cytotoxicity assays using IC_{50} concentrations and concentrations of Fas Ab causing maximal lysis of EBV-JTO and Jurkat cells. Data shown are mean values (+SD) of 9 (EBV-JTO) or 4 (Jurkat) independent experiments. NA; not applicable, lysis in WT < 10%. (C) Percentages of inhibition of Camp-induced apoptosis by FLIP and PI-9 after 24 hrs of exposure of EBV-JTO and Jurkat cells. Lysis was 83% and 88% in wild type EBV-JTO and Jurkat cells, respectively, using 10^{-5} M Camp. Data shown are mean values (+SD) of 2 (EBV-JTO) or 4 (Jurkat) independent experiments.

‡ $p > 0.05$; inhibition was considered significant if $p \leq 0.05$.

cells after 5 and 24 hrs of exposure were 10 and 1 ng/mL of Fas Ab, respectively. Percentages of inhibition of Fas-induced apoptosis by FLIP and PI-9 after 5 hrs (left panel) and 24 hrs (right panel) of exposure are shown in Figure 5.1B. FLIP expression resulted in efficient and significant (p<0.05) inhibition of Fas-mediated apoptosis in EBV-JTO cells (75-90%), while the inhibitory effect of the same construct was less pronounced in Jurkat cells (30-50%). Unexpectedly, PI-9 also caused 40-70% and 20-30% inhibition of Fas-induced apoptosis in EBV-JTO and Jurkat cells, respectively. The lower the concentration of Fas Ab used, the higher the inhibitory effect of both FLIP and PI-9 we observed.
To ensure that introduction of PI-9 did not render EBV-JTO and Jurkat cells into less responsive cells in general, we exposed the WT, FLIP- or PI-9-transduced cell lines to various concentrations of the cytostatic agent camptothecin (Alexis Corp., Lausanne, Switzerland). Camptothecin-induced cell death was not inhibited by FLIP or PI-9 in both cell lines (Figure 5.1C). Moreover, upregulation of PI-9 in EBV-JTO cells did not influence lysis caused by other chemotherapeutic agents such as Ara-C or daunorubicin (data not shown).

We demonstrated that in both EBV-JTO and Jurkat cells, PI-9 significantly inhibited Fas-induced apoptosis. This is in contrast with results from other groups showing that PI-9 selectively inhibits GrB-mediated apoptosis without affecting Fas-induced cell death in Jurkat cells. [8;9] We showed that the inhibition of Fas-induced cell death by PI-9 was much more pronounced in EBV-JTO cells than in Jurkat cells, suggesting that the inhibitory effect of PI-9 on Fas-induced apoptosis is dependent on the cell line studied. Jurkat cells are so-called type II cells, in which Fas-induced apoptosis is partially executed via the mitochondria through caspase-8-mediated cleavage of BH3-interacting-domain death agonist (Bid), a member of the pro-apoptotic B-cell lymphoma 2 (Bcl-2) family. [1] This explains the partial inhibition of Fas-induced apoptosis we observed in Jurkat cells by FLIP and also by Bcl-2 (data not shown). In EBV-JTO cells, Fas-induced apoptosis was completely mediated via the death receptor pathway, since FLIP expression almost completely inhibited Fas-induced apoptosis (Figure 5.1B).

It is not known at what level PI-9 interferes with the death receptor pathway. We speculate that PI-9 perturbs the death receptor pathway downstream from caspase-8 activation. Fas-induced apoptosis in Jurkat cells will be less affected by this intervention, since apoptosis can still be executed via the mitochondrial bypass. Bird *et al*. demonstrated that PI-9 poorly inhibited caspase-8 activation *in vitro* (10%), but efficiently blocked activation of caspase-4 (70%), and to a less extent (35%) of caspase-10. [8] Caspase-4 has a thus far unknown function, but intervention of Fas-induced apoptosis by PI-9 may occur at the level of caspase-10, since this protein has been reported to play a role in the death receptor pathway of apoptosis. [10]

In conclusion, here we demonstrate that PI-9 interferes with the Fas cell death pathway, and hence is less specific for the PFN/GrB pathway than previously reported. Consequently, poor clinical outcomes associated with elevated PI-9 levels may not automatically implicate disruption of T-cell-induced PFN/GrB-mediated apoptosis, but can also be the result of blockade of the death receptor pathway which may affect chemotherapy treatment.

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CHAPTER

6

Cytotoxic T cells induce target cell death via an MHC-restricted granule-mediated and a non MHC-restricted death-receptor-mediated mechanism of killing

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ABSTRACT

Background and objectives. Cytotoxic T lymphocytes (CTL) are considered to selectively kill target cells via granule-mediated or death-receptor-induced apoptosis after T cell receptor (TCR) dependent recognition of a specific peptide presented in the context of the correct HLA molecules. In this study, we examined a CTL-mediated mechanism of killing target cells which is non Major Histocompatibility Complex (MHC)-restricted and independent of TCR-triggering.

Design and Methods. We investigated lysis of various HLA-A2 positive and negative target cells derived from patients with acute lymphoblastic leukemia (ALL) by HLA-A2-restricted CTL clones via MHC-restricted and non MHC-restricted interactions. We studied the involvement of granule-mediated and death-receptor-induced apoptosis using blocking studies with ethylene glycol tetraacetic acid (EGTA) and introduction of caspase-8 (FLICE) like inhibitory protein (FLIP) overexpression in the different target cells, respectively.

Results. We showed that HLA-A2-restricted T cell clones killed HLA-A2-expressing target cells via classical TCR/MHC-dependent interactions resulting in 40-70% lysis after 4 hours of incubation (E/T ratio = 10/1). As a consequence of this specific TCR triggering, the T cells downregulated their expression of TCR and CD8 and produced interferon (IFN)-γ. This MHC-restricted target cell death was completely inhibited by pre-incubating the T cells with EGTA. In addition to MHC-restricted target cell death at the same E/T ratio the CTL clones exerted non MHC-restricted target cell death (10-30%) after 10 hours of coculture, which was not accompanied by IFN-γ release and TCR downregulation, and could be specifically inhibited by blocking the death-receptor pathway by overexpression of FLIP in the target cells.

Interpretation and Conclusions. In conclusion, we demonstrate that CTLs can mediate target cell death via a MHC-restricted and non-restricted interaction. MHC-restricted lysis is mediated via the perforin (PFN)/Granzyme B (GrB) pathway of killing, whereas non MHC-restricted cell death is mediated via the death receptor pathway of killing.

INTRODUCTION

T cell receptors (TCR) of cytotoxic $CD8^+$ T cells recognize a complex consisting of class I Major Histocompatibility Complex (MHC) molecules and antigenic peptides, mainly derived from intracellular processing of endogenously synthesized proteins. [1] In response to TCR-dependent recognition of target antigen, cytotoxic T lymphocytes (CTLs) potentially use two effector pathways to mediate target cell death: granule-mediated and death receptor-mediated killing. [2;3] In granule-mediated killing, TCR-triggering of the T cell induces exocytosis of preformed cytotoxic granules,

containing perforin (PFN) and various granzymes including granzyme B (GrB). [4;5] Intracellular delivery of GrB into the target cell results in apoptosis via direct proteolytic activation of caspase-3 or via induction of the mitochondrial pathway of apoptosis. [5] In death receptor-mediated killing, TCR-triggering induces expression of death ligands (Fas ligand (FasL), tumor necrosis factor (TNF)-α, TNF-related apoptosis inducing ligand (TRAIL)) on the T-cell membrane, which cross-link death receptors expressed on the target cells. [2;6] Upon trimerization of the death receptor, a signaling cascade is initiated in which caspase-8, also called Fas Associated Death Domain (FADD)-like IL-1 converting enzyme (FLICE), [7] plays a vital role, eventually leading to apoptosis. [8;9]

Granule-mediated cell death has been reported to play an important role in the elimination of virus-infected cells and malignant cells. [10;11] In contrast, death receptor-mediated cell death has primarily been described to be involved in eliminating autoreactive T cells and in downsizing of immune responses after infections. [12-15] After clearance of an infection, the surplus of T cells has to be deleted to prevent collateral damage. Since activated T cells express both death receptors and death ligands, neighboring T cells are able to kill each other via induction of death-receptor-induced apoptosis, a process leading to termination of the immune response. To prevent that the T cells kill each other while the infection is still present, this process has been reported to only take place in the absence of a specific TCR/MHC-peptide interaction. [16] As long as the cytotoxic T cells are actively engaged via a specific TCR/MHC-peptide interaction, several survival mechanisms have been described that protect them from Fas-mediated deletion. [16-18]

Exocytosis of preformed cytotoxic granules by $CD8^+$ T cells is only induced after specific activation of the TCR by the appropriate MHC/peptide complex. This specific triggering of the TCR will lead to internalization of the TCR complex, [19] and in addition to cytokine production by the activated T cell. [20;21] In contrast, death-receptor-induced apoptosis is not directly dependent on TCR/MHC interaction. [22] Previously, we observed that in addition to killing via specific TCR-mediated recognition of target antigen in the context of the appropriate HLA, *in vitro* $CD8^+$ CTL frequently exert non MHC-restricted crossreactivity against B cell malignancies including acute lymphoblastic leukemia (ALL). [23] Non MHC-restricted target cell lysis has also been reported by other groups. Von Geldern *et al*. showed that besides a classical MHC-restricted mode of killing, the same CTLs can display an NK-like non MHC-restricted activity towards targets, dependent on the functional status of the T cell. [24] The group of Reisner reported on TCR-independent cell death of B-cell lineage chronic lymphocytic leukemia (B-CLL) cells by allogeneic anti-third-party CTLs, although the mechanism of killing of the B-CLL cells was not completely elucidated. [25] We hypothesized that this non MHC-restricted lysis may be mediated via a similar mechanism of killing as used for the removal of T cells after clearance of an infection.

We postulate that activated (death ligand expressing) CTLs may kill death receptor-expressing (neighboring) target cells irrespective of their TCR/MHC specificity. In the absence of specific TCR triggering this effector-target interaction will not result in downregulation of the TCR or its co-receptor CD8, neither in IFN-γ release by the T cell.

In this study, we investigated the recognition of various HLA-A2 positive and negative ALL target cells by HLA-A2-restricted CTL clones via MHC-restricted and non MHC-restricted interactions. To investigate the involvement of the TCR in the recognition of these different target cells, we measured TCR and CD8 downregulation and IFN-γ production by the T cells. We studied whether MHC-restricted and non MHC-restricted effector-target interactions resulted in induction of different apoptotic pathways in the target cells. Our results demonstrate that T cells can mediate target cell death of ALL cells via two distinct mechanisms. MHC-restricted lysis is mediated via the PFN/GrB pathway of killing, whereas non MHC-restricted cell death is mediated via the death receptor pathway of killing.

MATERIALS AND METHODS

Cells and culture conditions

The human acute lymphoblastic leukemia cell lines Leiden ALL-HP (ALL-HP) and Leiden ALL-KW (ALL-KW), and the human CML-derived lymphoblastic cell line Leiden ALL-CM (ALL-CM) were generated in our laboratory from primary human ALL cells, and kindly provided by Dr. B.A. Nijmeijer. These cell lines were phenotypically and karyotypically similar to the primary malignant clone. ALL cell lines were cultured in serum-free medium consisting of IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium), 0.4% human serum albumin (HSA) (wt/vol) (CLB, Amsterdam, The Netherlands), 20 μg/mL cholesterol (Sigma-Aldrich, St Louis, MO, USA), 20 μg/mL transferrin (Serva, Heidelberg, Germany), 5 x 10^{-5} M β-mercaptoethanol, and 10 μg/mL insulin (both Sigma-Aldrich).

EBV-LCL were cultured in IMDM supplemented with 10% fetal bovine serum (FBS), 3 mM L-glutamine, 50 μg/mL streptomycin and 50 U/mL penicillin (all Cambrex Bio Science, Verviers, Belgium).

HA1.83 [26] and HA2.27 [27] are $CD8^+$ HLA-A*0201-restricted T cell clones recognizing the minor histocompatibility antigens HA-1 and HA-2, respectively. MBM-15 is a $CD8^+$ HLA-A2-restricted alloreactive T cell clone. [28] The T cell clones were cultured in IMDM supplemented with 3 mM L-glutamine, 50 μg/mL streptomycin, 50 U/mL penicillin, 5% pooled human serum, 5% FBS, and 100 IU/mL IL-2 (Chiron, Amsterdam, the Netherlands), and stimulated every 1-2 weeks with a mixture of irradiated allogeneic peripheral blood mononuclear cells and 800 ng/mL phytohemagglutinin (PHA, Murex Biotech Limited, Dartford, UK).

Cytotoxicity assay and various blocking strategies

Cytotoxicity was measured using ^{51}Cr release assays as described previously. [29] Effector cells and target cells were incubated at an E/T ratio of 10/1. For exogenous peptide loading, ^{51}Cr-labeled target cells were pulsed with 1 μM peptide for 1 hr at 37°C in 100 μL. The following peptides were used in this study: HA-1 (VLHDDLLEA) and HA-2 (YIGEVLVSV). After labeling, excess of peptide was washed away, and the target cells were added to the effector cells.

Blocking of Ca^{2+}-dependent PFN/GrB-mediated cytotoxicity was performed by preincubation of the effector T cells with 5 mM EGTA (Sigma-Aldrich) for 30 min at 37 °C, prior to addition of the ^{51}Cr-labeled target cells.

Death-receptor-mediated apoptosis was induced with recombinant human TRAIL (rhsKillerTRAIL™) (Alexis Corp., Lausanne, Switzerland) using concentrations from 10 to 100 ng/mL. To determine the role of various caspase-8 in CTL-or TRAIL-induced cell death, ^{51}Cr-labeled target cells were pre-incubated for 1 hr with 100 μM of irreversible cell-permeable caspase-8 inhibitor (z-LETD-FMK) (both Alexis) prior to addition of effector T cells.

TCR downregulation
For analysis of TCR and CD8 downregulation, 20,000 T cells were stimulated with 20,000 target cells. These incubations were performed in medium containing low concentrations of IL-2 (30 instead of 100 IU/mL) to prevent spontaneous activation of T cells by high amounts of IL-2. After 5 hrs, reactions were stopped by placing the samples on ice, and FACS analysis was performed in duplicate using PECy5-labeled TCRαβ (clone BMA031, Immunotech, Marseille, France) and APC-labeled CD8α (clone 3B5, Caltag, Burlingame, CA) specific antibodies. TCR and CD8 surface expression on the T cells after incubation with non-stimulator (HLA-disparate) cells was set as 100%. Percentages of downregulation were calculated as follows:
% of downregulation (stimulator) = [1-(mean MFI stimulator / mean MFI non-stimulator)] * 100.

IFN-γ production
For analysis of IFN-γ production, 5,000 T cells were stimulated with 30,000 stimulator cells. After 24 hrs, supernatant was harvested, and the concentration of IFN-γ was measured in duplicate by standard ELISA (CLB, Amsterdam, The Netherlands).

Generation of retroviral constructs and transduction of target cells
FLAG-tagged FLICE inhibitory protein (FLIP) encoding PCR products were generated as described previously, [30] and cloned into the Moloney murine leukemia virus-based retrovirus vector LZRS (G. Nolan, Stanford University, Palo Alto, CA) containing the truncated nerve growth factor receptor (ΔNGF-R) as the marker gene. [31] Retroviral pLZRS vector encoding ΔNGF-R alone was used as a control vector (mocκ) in the experiments. Generation of retroviral supernatant and retroviral transduction of target cells were performed as previously described. [28] FLIP-transduced cells were purified by cell sorting using NGFR-specific antibodies (BD BioSciences, San Diego, CA, USA).

RESULTS

MHC-restricted and non MHC-restricted CTL-mediated target cell death

We examined the HLA-A*0201-restricted CTL clones HA1.83 and HA2.27 recognizing the minor histocompatibility antigens HA-1 and HA-2, respectively, as well as the alloreactive HLA-A2-restricted CTL MBM-15 for their capacity to kill various HLA-A*0201 positive and negative target cells. Percentages of lysis were determined after 4 and 10 hrs of coculture using standard ^{51}Cr release assays, and are shown in Figures 6.1A and B, respectively. After 4 hrs of incubation the HLA-A*0201 and HA-1 and HA-2 expressing cell lines ALL-CM and EBV-BDV were recognized by all three CTL clones resulting in 40-70% of lysis (Figure 6.1A). EBV-Z cells expressing HLA-A*0201 but not the minor antigens HA-1 and HA-2 were killed by MBM-15. Specific lysis by the CTL clones HA1.83 and HA2.27 was observed after exogenously pulsing with specific peptides (EBV-Z + pep). HLA-A*02 negative target cells (ALL-KW) were not killed by any of the three CTL clones. HA-1 and HA-2 positive target cells expressing the HLA-A2 subtype variant HLA-A*0205 (ALL-HP) were not

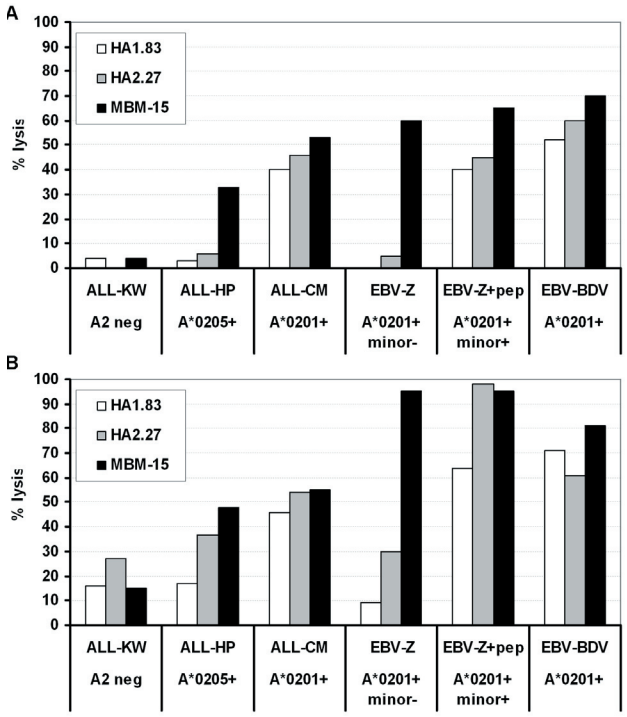

Figure 6.1. HLA-A*0201 specific CTLs mediate target cell death via a MHC-restricted and non MHC-restricted interaction.
Percentages of lysis were determined after 4 hrs (A) and 10 hrs (B) of incubation of different HLA-A*0201 positive and negative target cells with the HA-1 and HA-2 specific CTL clones HA1.83 and HA2.27 and the alloreactive CTL MBM-15, at an E/T ratio of 10/1. Results are representative of 3 or more independent experiments. EBV-Z is a HLA-A*0201^{+} HA-1^{-} HA-2^{-} target cell. EBV-Z +pep indicates EBV-Z exogenously loaded with HA-1 and HA-2 peptide.

recognized by HA1.83 and HA2.27, whereas 30% of specific lysis of these cells was observed after 4 hrs of coculture with the alloreactive CTL MBM-15, illustrating that this CTL clone can also recognize other HLA-A2 subtype variants. These data show that after 4 hrs of incubation CTL clones only display specific MHC-restricted target cell death, and are not reactive with HLA-disparate or mHag negative target cells. In contrast, after 10 hrs of incubation, in addition to MHC-restricted target cell death all three HLA-A*02-restricted CTL clones showed lysis of HLA-A*02 negative target cells, varying from 10 to 30%. These data suggest that T cell clones are not only capable of lysing target cells via a specific TCR-MHC/peptide interaction, but also via a non MHC-restricted mechanism.

Differential TCR involvement and T cell activation in MHC-restricted and non MHC-restricted target cell recognition

One of the hallmarks of TCR-dependent effector-target interaction is internalization of the TCR-MHC/peptide complex by the T cell resulting in decreased expression of the TCR and its co-receptor CD8 on the membrane of the T cell. To investigate whether TCR triggering is involved in the recognition of non MHC-restricted target cells, the T cell clones HA2.27 and MBM-15 were cocultured for 5 hrs with the same HLA-A*0201 positive and negative target cells as mentioned in Figure 6.1. FACS analysis showed that expression levels of TCR$\alpha\beta$ and CD8 on HLA-A2-restricted CTLs were similar after culturing for 5 hrs in medium alone or in presence of HLA-A2 negative ALL-KW cells (data not shown). Therefore, the HLA-A2 negative ALL-KW cells were used in these experiments as non-stimulator cells. The percentages of TCR and CD8 downregulation were calculated as described in the Materials and Methods section, and representative results are shown in Figure 6.2A and B, respectively. Downregulation of both TCR$\alpha\beta$ and CD8 was only observed in HA2.27 when stimulated with the HLA-A*0201$^+$ HA-2$^+$ target cells ALL-CM and EBV-BDV, or with the HLA-A*0201$^+$HA-2$^-$ EBV-Z target cells exogenously loaded with HA-2 peptide. In contrast, MBM-15 also internalized its TCR complex when stimulated with the HLA-A*0205 expressing cell line ALL-HP, which correlated with the lysis found with the same T cell clone, as shown in Figure 6.1.

Because TCR triggering will result in T cell activation and concomitant cytokine release, we also determined IFN-γ production by HA2.27 and MBM-15 after stimulation with the same target cells for 24 hrs. As shown in Figure 6.2C, no IFN-γ production was found by the T cells without antigen-specific stimulation. HA2.27 produced high amounts of IFN-γ (1500-2500 pg/mL) in case of TCR/MHC specific interactions (ALL-CM, EBV-BDV and EBV-Z loaded with HA-2 peptide), whereas no IFN-γ release was measured after stimulation with ALL-KW (HLA-A2$^-$), ALL-HP (HLA-A*0205) or EBV-Z (HLA-A*0201$^+$ HA-2$^-$) cells. MBM-15 produced IFN-γ in response to both HLA-A*0201 and HLA-A*0205 expressing target cells but not to the HLA-A2 negative cell line ALL-KW.

In conclusion, these data show that T cell clones may kill target cells via a non MHC-restricted mechanism, which is not accompanied by downregulation of the TCR complex or by IFN-γ production. Apparently, in contrast to TCR/MHC-specific recognition TCR triggering is not involved in CTL-induced cell death of non MHC-restricted target cells.

CTL-induced MHC-restricted and non MHC-restricted target cell death are executed via induction of two distinct effector pathways

To investigate whether MHC-restricted and non MHC-restricted CTL-mediated target cell death were induced via activation of differential effector pathways, we selectively blocked the two main potential effector pathways, and examined the inhibitory effect

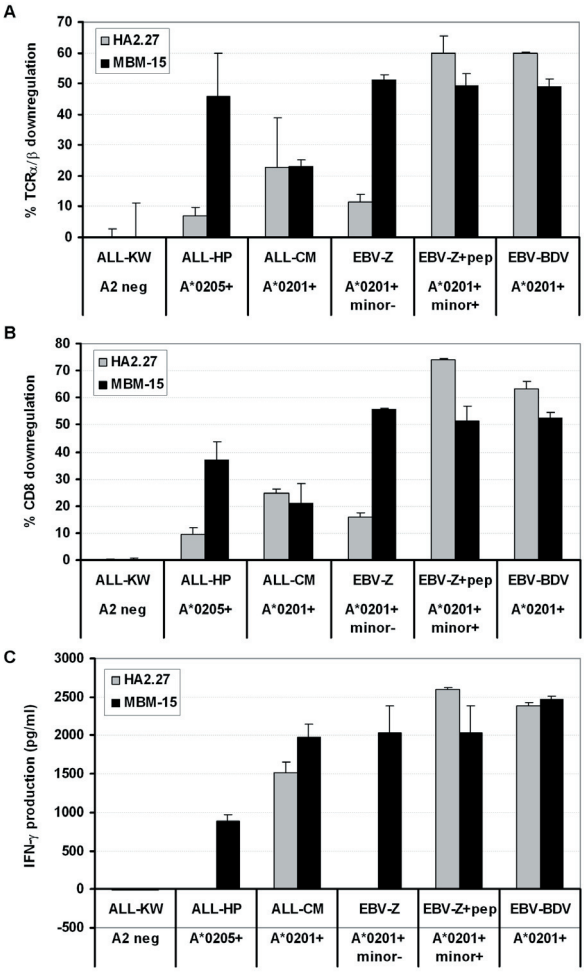

Figure 6.2. Non MHC-restricted effector-target interactions do not result in internalization of the TCR complex or in CTL-mediated IFN-γ production.
The HLA-A*0201-restricted HA-2 specific CTL HA2.27 and the HLA-A2 specific alloreactive CTL MBM-15 were stimulated with various HLA-A*0201 positive and negative target cells. After 5 hrs, expression levels of TCRαβ and CD8 were determined by FACS analysis, and percentages of TCR (A) and CD8 (B) downregulation were calculated as described in the Materials and Methods. ALL-KW cells were used as non-stimulator cells. After 24 hrs, IFN-γ production (pg/mL; in triplicate + SD) was measured using standard ELISA reactions (C).

on CTL-mediated lysis of ALL-CM (A*0201$^+$) versus ALL-HP (A*0205$^+$) target cells. ALL-HP target cells were used instead of ALL-KW cells since both MHC-restricted and non MHC-restricted cell death could be analyzed in this cell line using the CTL clones MBM-15, and HA1.83 or HA2.27, respectively.

PFN/GrB-mediated lysis was blocked by incubating CTLs with the Ca^{2+}-chelator EGTA, which has been reported to efficiently inhibit all secretion pathways in the T cell. [32] Death-receptor-induced apoptosis was inhibited by introduction of a retroviral construct encoding FLIP, an efficient inhibitor of death-receptor-induced apoptosis, [33] into ALL-HP and ALL-CM target cells (more than 90% pure populations after enrichment using cell sorting). Death-receptor-induced apoptosis was previously shown to be efficiently inhibited in these FLIP-expressing ALL cell lines. [30]
Previous studies showed that granule-induced and death-receptor-mediated apoptosis differ in time required to eliminate target cells. PFN/GrB-mediated target cell death can already take place within 2 hrs of incubation, whereas death-receptor-

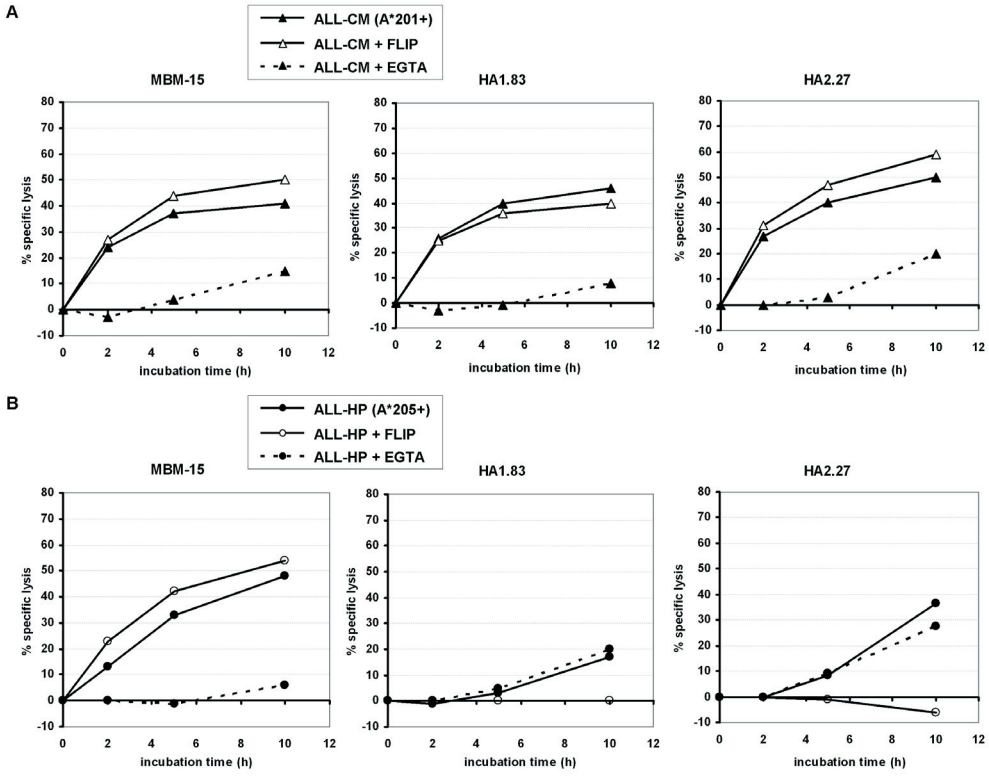

Figure 6.3. CTL-induced MHC-restricted and non MHC-restricted target cell death are mediated via activation of two distinct effector pathways.
The effect of enhanced expression of the anti-apoptotic protein FLIP or addition of EGTA on the kinetics of lysis of (A) ALL-CM (HLA-A*0201$^+$) and (B) ALL-HP (HLA-A*0205$^+$) target cells by various CTLs was studied. Percentages of lysis of WT or FLIP-expressing ALL-CM (▲) and ALL-HP (●) cells by CTL clones HA1.83, HA2.27 and MBM-15 (E/T ratio = 10/1) in presence or absence of 5 mM EGTA were determined after 2, 5 and 10 hrs of incubation using standard ^{51}Cr release assays. Results are representative of at least 3 independent experiments.

mediated apoptosis was observed after 5-24 hrs of coculture. [34] Therefore, in these experiments specific lysis was determined after 2, 5 and 10 hrs of incubation with HA1.83, HA2.27, or MBM-15. Kinetics of cell death of the HLA-A*0201$^+$ ALL-CM (▲) and HLA-A*0205$^+$ ALL-HP (●) cells by the three T cell clones are shown in Figure 6.3A and B, respectively. All three CTL clones showed rapid kinetics of killing HLA-A*0201 positive ALL-CM cells (25% lysis within 2 hrs), suggesting a granule-dependent mechanism of killing. In line with this, expression of FLIP had no effect on lysis of ALL-CM cells by the three CTL clones, whereas blocking experiments with EGTA showed almost complete inhibition of CTL-induced lysis of ALL-CM cells. These data indicate that MHC-restricted CTL-induced cell death of ALL-CM cells is mainly directly PFN or PFN/GrB-mediated.
Kinetics of lysis of ALL-HP cells by MBM-15 (Figure 6.3B) was comparable to ALL-CM cells. This lysis was efficiently inhibited by EGTA, while overexpression of FLIP in the target cell had no effect, again illustrating the involvement of PFN/GrB in MHC-restricted cell death. CTL clones HA2.27 and HA1.83 were not capable of lysing ALL-HP cells in the first 5 hrs of incubation, as also shown in Figure 6.1A. In the time interval from 5 to 10 hrs, up to 30% target cell death was observed. This lysis of ALL-HP cells was completely inhibited by overexpression of FLIP, while no inhibition was observed using EGTA, illustrating that non MHC-restricted lysis was mediated via the death receptor pathway of apoptosis. Noteworthy, the same minor percentages of non MHC-restricted, death-receptor-mediated apoptosis by the three T cell clones could be observed in the HLA-A*0201 positive ALL-CM cells (Figure 6.3A), since EGTA was not able to completely inhibit CTL-induced cell death between 5 and 10 hrs of incubation (dashed lines).

Since caspase-8 activation is essential in death-receptor-mediated apoptosis, we assessed the role of this caspase in HA2.27-induced cell death of ALL-HP and ALL-CM cells using a specific caspase-8 inhibitor (Figure 6.4). As a control, the effect of caspase-8 inhibitor on TRAIL-induced apoptosis was determined. TRAIL-induced apoptosis was blocked in both cell lines by caspase-8 inhibitor (zLETD-FMK). HA2.27-induced cell death of ALL-HP cells but not of ALL-CM cells was efficiently blocked with caspase-8 inhibitor, illustrating the importance of the death-receptor pathway in non MHC-restricted but not in MHC-restricted target cell death.

DISCUSSION

Cytotoxic T cells are considered to selectively kill target cells after recognition of a specific MHC-peptide complex by their TCR. As a consequence of activation via their TCR, the T cells will internalize the TCR complex, [19] and produce IFN-γ. [20;21] Here, we demonstrate another mechanism of target cell killing which is non MHC-restricted.

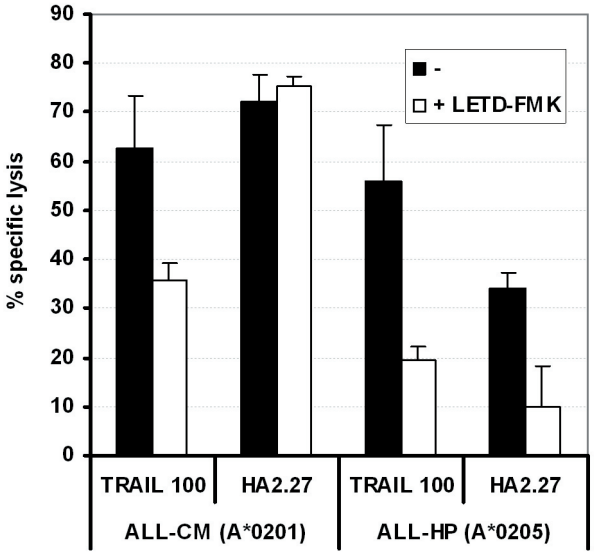

Figure 6.4. The role of caspase-8 in non MHC-restricted target cell death.
ALL-CM and ALL-HP cells were exposed for 20 hrs to 100 ng/mL TRAIL or to the CTL clone HA2.27 at an E/T ratio of 10/1, in presence or absence of the caspase-8 inhibitor z-LETD-FMK (10^{-4} M). Target cell death was determined using ^{51}Cr release assays.

The $CD8^+$ T cells killed these target cells in a non MHC-restricted manner, did not show TCR down regulation, and produced no IFN-γ, indicating that target recognition was TCR-independent. We showed that HLA-peptide specific lysis of target cells was almost completely mediated via granule-mediated cell death, whereas non MHC-restricted target cell death was exclusively mediated via the death-receptor pathway (Figure 6.3).

Arditti *et al*. showed that anti-third-party CTLs were capable of efficiently killing B-CLL cells but not AML blasts in a non MHC-restricted manner via a TCR-independent mechanism of killing. [25] Since we here demonstrate a similar phenomenon in ALL cells and EBV-LCL, CTL-induced non MHC-restricted killing may be specifically occurring in B cells. Although the same group observed an upregulation of CD95/FasR in the B-CLL cells after coculturing with anti-third party CTLs, they also showed that blocking experiments using CD95 antibodies did not inhibit B-CLL-killing by these anti-third-party CTLs. Therefore, they concluded that the killing was not mediated via Fas-induced apoptosis. However, the killing they observed may still have been death-receptor-mediated, since we also did not obtain inhibition of non MHC-restricted target cell death using CD95 blocking antibodies (data not shown). In contrast, complete inhibition was observed when FLIP expression was enhanced in the target

cell, suggesting that other death ligands such as TNF or TRAIL may be involved in the mechanism of non MHC-restricted killing by the CTLs.

In our study, T cells were activated using irradiated feeder cells, PHA and IL-2, which will result in the upregulation of death ligands on the T cell membrane and an increase in expression of intracellular levels of perforin and granzymes. One to two weeks after stimulation these activated T cells were brought into close contact with peptide/MHC disparate target cells (in a 96-wells round-bottom plate) in absence of specific peptide/MHC expressing targets. Since death-receptor-induced apoptosis itself is not directly dependent on TCR/MHC interaction, [22] we hypothesized that all death receptor-expressing target cells may be responsive to these death ligand-expressing T cells. This may explain the lysis of HLA-A*0201 negative target cells by activated HLA-A*0201-restricted T cells shown in Figure 6.1.

In addition to recognition of HLA-A*0201, the HLA-A2-restricted alloreactive CTL clone MBM-15 showed reactivity to the HLA-A*0205 expressing cell line ALL-HP. Lysis of ALL-HP cells by MBM-15 was already observed after 2-5 hrs of incubation (Figures 6.1A and 6.3B), showing similar kinetics of cell death as observed in the HLA-A*0201 expressing cell line ALL-CM. FACS analysis revealed that after stimulation with ALL-HP, the expression of membrane-bound TCR$\alpha\beta$ and CD8 was downregulated, suggesting internalization of the TCR complex by MBM-15. Furthermore, MBM-15-mediated cell death of ALL-HP cells was accompanied by release of IFN-γ (Figure 6.2), which is characteristic of classical TCR-dependent recognition of target cells. [21] In contrast, lysis of ALL-HP cells by the HLA-A*0201-restricted CTL clones HA1.83 and HA2.27 was only observed after 10 hrs of coculture and was not coincided with TCR downregulation and IFN-γ release, which we assume to be non MHC-restricted CTL-induced target cell death.

In this study we demonstrated that non MHC-restricted target cell death was mediated via the death receptor pathway of apoptosis. *In vivo*, this mechanism of cell death has been described to play a role in the regulation of an immune response. [12-15] After clearance of an infection the surplus of activated cytotoxic effector T cells is deleted via a TCR/MHC-peptide-independent death-receptor-mediated apoptosis mechanism [16] to prevent collateral damage caused by a cytokine storm. However, non MHC-restricted target cell death may also result in collateral damage since in presence of specific target cells, $CD8^+$ murine T cells have been reported to kill non MHC-restricted bystander cells via a Fas ligand/Fas-based mechanism. [35;36] We suppose that the destructive effect of this bystander lysis will probably be minimal, because: 1) bystander lysis can only happen temporarily: when all MHC-restricted target cells have been killed, the TCR-MHC specific interaction is lost and the cytotoxic T cells will kill each other via death-receptor-induced apoptosis; 2) only death-receptor expressing bystander cells will be killed.

In conclusion, here we show that CTLs can mediate target cell death via a MHC-restricted and a non MHC-restricted interaction. TCR/MHC-specific interactions between target and effector cell led to PFN/GrB-induced cell death of the target cell, while non MHC-restricted interactions between effector and target cells activated the death receptor pathway in the target cell.

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CHAPTER 7

Summary and General Discussion

SUMMARY

Failure of therapy due to acquired resistance is one of the major problems in the treatment of patients with acute leukemia. Initially, most patients respond well to induction chemotherapy usually consisting of a combination of cytostatic agents including daunorubicin, vincristine, methotrexate, and cytarabine (Ara-C), [1-3] leading to 70-80% complete remissions (CR). However, despite aggressive induction and consolidation therapy, relapses of the disease frequently occur (50-60%) resulting in low overall 5-year survival rates of patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). [4;5] Those patients who relapse more than one year after diagnosis have a good chance to achieve a second remission after administration of the original induction regimen, or at least one of similar intensity. The most effective post-remission therapy, however, is high-dose chemotherapy and total body irradiation followed by allogeneic or autologous stem cell transplantation (SCT), using stem cells from a (partially) HLA-matched donor or from the patient, respectively. SCT can be considered as a rescue procedure to achieve immunologic reconstitution in patients of whom both malignant and normal hematopoiesis were destroyed. The advantage of allogeneic SCT (followed by donor lymphocyte infusions (DLI) at relapse) is that besides causing immunologic reconstitution the donor T cells in the graft may recognize and attack the residual leukemic cells, leading to a graft-versus-leukemia (GVL) effect.
Patients who never achieve a CR or who experience a relapse within 6 months (during treatment) are considered to be resistant to further cytotoxic treatment. Various mechanisms may underlie this resistance, including expression of drug efflux pumps, [6-8] and cell cycle status of the leukemic cell. [9;10] Another cause for the unresponsiveness of leukemic cells to chemotherapy has been shown to be defects in the apoptotic machinery of these leukemic cells. [11-17]

Cytostatic agents (chemotherapy) and donor-derived T lymphocytes (cellular immunotherapy) kill leukemic cells via induction of apoptosis in the target cell. Aberrations in common apoptotic pathways may therefore cause unresponsiveness to both forms of therapy, as has been observed in some patients. Other patients are resistant to chemotherapy but do respond to DLI, indicating that these patients may have defects in chemotherapy-specific pathways. Assessment of potential defects in apoptotic pathways is only possible when the functional apoptotic pathways are unraveled in more detail. Although general therapy-induced apoptotic pathways have been described (Figure 7.1), actual apoptotic mechanisms are mostly much more complicated than shown in this figure. This thesis emphasizes the complexity of the apoptotic pathways that are induced in leukemic cells after treatment with conventional cytostatic drugs or with cytotoxic T cells. Moreover, several controversies that still exist in the field of apoptosis research are highlighted in this thesis, and at least partially unraveled.

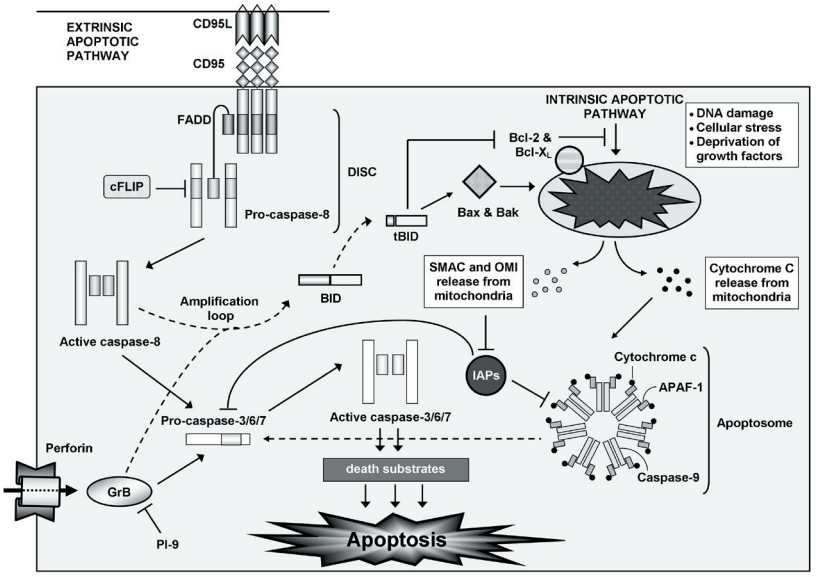

Figure 7.1. Apoptotic pathways induced by chemotherapy or (cellular) immunotherapy (partly adapted from Siegel [18]).

The cell cycle status of leukemic cells affects their sensitivity to chemotherapy, since most chemotherapeutic agents specifically act on proliferating (S or G_2/M phase) or at least activated (G_1) cells, while quiescent (G_0) cells are considered to be unresponsive. Incorporation of Ara-C into DNA, a process specifically occurring during S-phase of the cell cycle, is assumed to be a key event in the mechanism of killing of proliferating leukemic cells. [19] **Chapter 2** describes the unexpected potential of Ara-C to cause apoptosis, characterized by phosphatidyl serine exposure on the cell membrane and caspase activation, in resting (>98% in G_0) primary B cells derived from four previously untreated patients with B-cell chronic lymphocytic leukemia (B-CLL). Cell cycle analysis combined with long-term analysis of cell death using CFSE-based cytotoxicity assays confirmed that these non-proliferating B-CLL cells were killed by Ara-C in G_0 phase of the cell cycle.

Since inhibition of DNA incorporation was not the mechanism of action of Ara-C in G_0-B-CLL cells, other cellular processes must be involved. We demonstrated that increasing concentrations of deoxycytidine (dC) blocked both the formation of Ara-CTP and Ara-C-induced cell death of G_0-B-CLL cells. dC is a structural analogue of Ara-C and the normal metabolite of both RNA and DNA synthesis. In contrast to DNA (repair) synthesis which was hardly detectable, unexpectedly high RNA synthesis was present in G_0-B-CLL cells. This RNA synthesis was blocked by Ara-C for 30%, suggesting that inhibition of RNA synthesis is a potential mechanism of killing resting

G_0-B-CLL cells by Ara-C. B-CLL cells are characterized by high expression levels of B-cell lymphoma-2 (Bcl-2) protein, which causes the prolonged survival of these malignant cells. [20] One of the initiating mechanisms for drug-induced apoptosis in B-CLL cells has been reported to be alteration of the expression levels of pro- or anti-apoptotic proteins (Bcl-2/Bax ratio [21;22]). Therefore, we analyzed whether addition of Ara-C resulted in alterations in Bcl-2, Bax or Mcl-1 protein levels. We showed that the survival protein Mcl-1 was downregulated in response to Ara-C, which may be a consequence of the decreased RNA synthesis. In conclusion, both downregulation of RNA synthesis and Mcl-1 expression appear to contribute to the mechanism of action of Ara-C in G_0-B-CLL cells.

Besides investigating the mechanisms that are involved in chemotherapy-induced apoptosis of resting leukemic cells, it is very important to gain more insight into the apoptotic pathways induced by cytostatic agents in proliferating leukemic cells, since resistance of leukemic cells to chemotherapy-induced apoptosis frequently occurs in patients with acute leukemia. One of the controversial topics in chemotherapy-induced apoptosis is the pathway via which cell death is initiated. Treatment with chemotherapy may lead to specific activation of the mitochondrial pathway, in which cytochrome C release and caspase-9 activation have been reported to play a central role. (Figure 7.1) [23,24] Alternatively or perhaps additionally it may activate the death receptor pathway of apoptosis, in which formation of a Death Inducing Signaling Complex (DISC) and caspase-8 (FLICE) activation are key events. [25] In **Chapter 3** of this thesis, the role of the death receptor pathway in chemotherapy-induced apoptosis of human derived myeloid and lymphoblastic leukemia cell lines is described. The rapid and substantial activation of caspase-8 that we observed after treatment with camptothecin, combined with the effective inhibition of chemotherapy-induced apoptosis by caspase-8 inhibitor, strongly suggested involvement of the death-receptor pathway in apoptosis induced by cytostatic agents. This assumption was examined by introducing retroviral constructs encoding Bcl-2 or FLICE inhibitory protein (FLIP) into the target cells, and subsequently analyzing the sensitivity of these cell lines for various cytostatic drugs. Enhanced Bcl-2 expression was used to specifically block the mitochondrial pathway, whereas FLIP selectively prevented death-receptor-induced apoptosis. No inhibitory effect of elevated expression of FLIP on camptothecin-induced apoptosis or caspase-8 activation was observed, while introduction of Bcl-2 resulted in almost complete inhibition of camptothecin-mediated cell death and caspase-8 activation in all three cell lines studied, indicating that in these cell lines chemotherapy-induced apoptosis is completely dependent on the mitochondrial pathway. Moreover, caspase-8 apparently plays a central role in both death receptor- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia.

Not only controversy existed about the role of the death receptor pathway in chemotherapy-induced apoptosis, the involvement of this pathway in the elimination of leukemic cells by cytotoxic T lymphocytes (CTLs) was also largely unclear. In **Chapter 4** we studied whether in addition to cell death mediated by perforin (PFN) and granzyme B (GrB) death receptor-induced apoptosis contributes to the elimination of human tumor cells by CTLs. In most studies that have been published, CTL-mediated target cell death was only analyzed after 4 hrs of interaction between target and effector cell. Since death-receptor-mediated apoptosis is slow compared to PFN/GrB-induced cell death, in this study we used T cell clones with slow and rapid kinetics of killing derived from a patient with chronic myeloid leukemia (CML), and analyzed cell death in various time intervals. The three CTL clones used in this study all recognized primary CML cells from the patient. Because for these types of studies insufficient leukemic cells can be isolated from the patient, EBV-LCL from the patient were used as target cells in this study. To determine the involvement of the death receptor pathway, a retroviral construct encoding FLIP was introduced into these target cells. A CTL clone capable of killing 50% of the target cells within 2 hrs of incubation primarily acted by release of PFN and GrB (no inhibition by FLIP was observed). In contrast, two CTL clones showing slower target cell killing kinetics partially used the death receptor pathway (~30% inhibition by FLIP). Thus, not only PFN/GrB release but also the death receptor pathway can play a role in the execution mechanism of cytotoxic T cells.

Whereas the anti-apoptotic protein FLIP specifically blocks the death-receptor pathway of apoptosis, proteinase inhibitor-9 (PI-9) has been reported to be a specific inhibitor of GrB-induced cell death, [26-28] and should therefore not affect Fas-induced apoptosis. However, in **Chapter 5**, we demonstrate that PI-9 is less specific than previously expected. Two different cell lines transduced with retroviral constructs encoding FLIP or PI-9 together with their wild type counterparts were exposed for 5 or 24 hrs to Fas apoptosis-inducing antibodies. In both types of cell lines we showed substantial and significant inhibition of Fas-induced apoptosis by enhanced PI-9 expression, although the levels of inhibition were lower than in the FLIP-expressing cell lines. PI-9 did not affect chemotherapy-induced apoptosis, showing that PI-9 interferes with the death receptor pathway but not with the mitochondrial pathway.

Cytotoxic T cells are considered to selectively kill target cells after specific TCR-mediated recognition of target antigen in the context of the appropriate HLA. In **Chapter 6**, we describe another T cell-mediated mechanism of killing target cells which is non MHC-restricted and is independent of TCR-triggering. HLA-A2-restricted CTL clones showed cytolytic activity to HLA-A2-expressing target cells via classical TCR/MHC-dependent interactions resulting in 40-70% lysis after 4 hrs of incubation. As a consequence of this specific TCR triggering, the T cells internalized their

TCRs, and produced interferon (IFN)-γ. This MHC-restricted target cell death was completely inhibited by pre-incubating the T cells with the calcium chelator EGTA, which inhibits all secretion pathways of the CTL, showing that the lysis was mediated by PFN and/or GrB. In addition the same CTL clones exerted non MHC-restricted target cell death (10-30%) after 10 hrs of coculture, which was not accompanied by TCR downregulation and IFN-γ release. This lysis could be specifically blocked by FLIP expression in the target cells, demonstrating death-receptor-induced apoptosis. A similar non MHC-restricted mechanism of killing is used for the removal of the surplus of T cells after clearance of an infection to prevent collateral damage. [29]

The results presented in this thesis emphasize the complexity of the mechanisms involved in apoptosis induction in leukemic cells after treatment with chemotherapy or cellular immunotherapy.

GENERAL DISCUSSION

Background

Defects in the apoptotic machinery of leukemic cells have been shown to cause resistance of these cells to chemotherapy and/or cellular immunotherapy. [11-17] Apoptosis is induced and regulated by a complex network of proteins that are connected via various signal transduction cascades. Although the exact pathways are not precisely known, induction of apoptosis eventually leads to death of the target cell. In the last decades a lot of research has been performed on many aspects of apoptosis by different research groups. General proposed mechanisms of chemo- and immunotherapy-induced apoptosis have been composed from the separate results of these experiments, leading to the simplified model presented in Figure 7.1. One major problem of this model is that it is composed of results of so many different experiments, and therefore a "general" mechanism actually does not exist. In addition, not all mechanisms of apoptosis are completely unraveled and still many controversies exist, which are highlighted in this thesis.

The paradoxic mechanism of action of cytostatic agents like Ara-C

One of the inconsistencies of chemotherapy treatment is the mechanism of action of cytostatic drugs like Ara-C. Like other chemotherapeutic agents Ara-C is supposed to exert its action preferably in cycling cells. [30-32] Incorporation of Ara-C into DNA, a process specifically occurring during S-phase of the cell cycle, and leading to termination of DNA synthesis, is assumed to be a key event in the mechanism of killing of proliferating leukemic cells. [19] In addition, Ara-C inhibits DNA repair by blocking topoisomerase I-mediated DNA religation. [33] The unexpected killing by Ara-C of cells not in proliferation or active phase of the cell cycle, as demonstrated in **chapter 2** of this thesis for G_0-B-CLL cells, revealed that Ara-C also induces apoptosis via other mechanisms of action. Although this observation was not expected, a similar phenomenon is seen in patients with acute leukemia treated with chemotherapy, since not only the rapidly dividing leukemic blasts, but also normal resting B and T lymphocytes are frequently killed by the cytostatic drugs. It is intriguing why these G_0 cells are killed, while in former studies specific deletion of cycling cells was demonstrated. [10;33] In chapter 2, we additionally studied B-CLL cells that were stimulated with CD40L. Culturing of B-CLL cells for 7 days on irradiated mouse fibroblasts expressing human CD40L induced proliferation in these B-CLL cells resulting in the following cell cycle distribution: 15% S/G_2M, 69% G_1, and 16% G_0. These cells were less responsive to Ara-C than primary B-CLL cells, but after 48 to 72 hrs of incubation specific deletion of cells from the S-phase of the cell cycle was observed, suggesting that these CD40 stimulated B-CLL cells behaved more or less like normal proliferating cells. The G_0 cells that were left in this CD40L-stimulated population were unresponsive to Ara-C like other G_0 cells in proliferating cell lines. Apparently, these CD40L-stimulated "G_0" cells are not identical to primary B-CLL cells.

It was demonstrated in chapter 2 that both downregulation of RNA synthesis and Mcl-1 expression appear to contribute to the mechanism of action of Ara-C in G_0-B-CLL cells. Enhanced expression levels of Bcl-2 but perhaps also of Mcl-1 cause the prolonged survival observed in B-CLL cells. [20] Nowadays, clinical studies are ongoing to investigate the potential of Bcl-2 antisense oligonucleotides (Oblimersen) in the treatment of B-CLL. [34] Based on the results of chapter 2, Mcl-1 may be another interesting target to downregulate. This hypothesis has to be evaluated in *in vitro* studies aimed at specific downmodulation of this gene in B-CLL cells, for instance by siRNA or antisense RNA strategies.
The findings described in chapter 2 will probably not directly imply a clinical application for Ara-C in the treatment of B-CLL, especially since combination therapy using a combination of fludarabine and Ara-C was not more effective than treatment with fludarabine alone. [35] However, the unexpected potential of Ara-C, which has been considered to be S-phase specific, to kill G_0-B-CLL cells makes it likely that other nucleotide analogues such as gemcitabine or clofarabine may be effective in killing B-CLL cells as well.

The controversial role of the death receptor pathway in chemotherapy-induced apoptosis

Cytostatic agents have been reported to induce apoptosis via the mitochondrial pathway. [23,24] However, involvement of the death receptor pathway in chemotherapy-induced apoptosis has also been shown by some investigators who reported that activation of the death receptor pathway by chemotherapy occurred via extracellular triggering of death receptors due to drug-induced upregulation of Fas receptor and ligand leading to autocrine or paracrine induction of Fas-mediated apoptosis. [36-40] In the study described in chapter 3, chemotherapy-induced apoptosis in AML and ALL leukemic cell lines was shown to be completely mediated via the mitochondrial pathway. Although AML and ALL cell lines were used in this study instead of primary leukemic cells, we suppose that especially the ALL cell lines resemble the primary ALL cells, and that the results can be extrapolated to the clinical situation. The death receptor pathway was not involved, although one of the key regulators of this pathway, namely caspase-8, likely played an important role in the execution of the leukemic cells by chemotherapy as well. This unexpected involvement of caspase-8 in mitochondria-mediated apoptosis makes it likely that in some former studies in which chemotherapy-induced caspase-8 activation was found incorrect involvement of the death receptor pathway has been concluded.

The group of Fulda postulated that the pathway of chemotherapy-induced apoptosis is cell-type dependent. [41] In so-called type I cells both the death receptor and the mitochondrial pathway were activated upon drug treatment, whereas in type II cells selective induction of the mitochondrial pathway was demonstrated. According to

this theory, AML and ALL cells are categorized as type II cells. Fulda *et al*. also proposed that type II cells are characterized by the fact that Fas-induced apoptosis is enhanced via a mitochondrial bypass (see Figure 7.1). [42] In our studies, however, apoptosis induced by Fas agonistic antibodies or TRAIL was completely mediated by the death receptor pathway, since complete inhibition of Fas-induced apoptosis was found in cells overexpressing FLIP while no inhibition was observed in cells with Bcl-2 overexpression. Apparently, the classification of cells is more complicated than a distinction into type I and II cells as suggested by the group of Fulda.

The impact of common elements involved in different apoptotic cascades

An important finding of this study was that, apparently, caspase-8 plays a central role in both death receptor- and chemotherapy-induced apoptosis of malignant cells from patients with acute leukemia. To investigate this theory in more detail, additional studies should be performed in which caspase-8 expression is downregulated in leukemic cells, for instance via a siRNA-based protocol, and subsequently the effect of this knockdown on sensitivity to chemotherapy is analyzed. If these data are in line with our theory this may have implications for therapy. It is known that caspase-8 is downregulated in certain tumors. [15;43;44] Therefore, therapeutic strategies focusing at modulation and activation of caspase-8 may sensitize drug-resistant malignancies to chemotherapy. However in practice this will be complicated, since caspase-8 is also present in normal healthy cells, which will also be destroyed when caspase-8 is not specifically targeted in malignant cells. On the other hand, regular chemotherapy also destroys normal hematopoiesis which may be restored by allogeneic SCT.

The existence of common important elements in the mitochondrial and the death receptor pathway may also explain the synergistic effect observed using combination therapies. Various studies have reported on increased sensitivity of cancer cells to Fas or TRAIL when patients were simultaneously treated with low dose chemotherapy. [45;46] This cotreatment with chemotherapy may cause upregulation of death receptors, [46] but additionally it may activate common apoptotic elements such as caspase-3 [45;47] or caspase-8, which later on facilitates TRAIL-induced apoptosis.

Cross resistance of Fas/TRAIL resistant cells to anticancer agents which is commonly observed in leukemia patients, can also partly be explained by defects in a common element of both pathways, [48] such as caspase-8. When defects in common elements of the two pathways are detected in a patient with leukemia this may be important in further treatment stratification. Moreover, if CTL-induced target cell death is also largely mediated via the death receptor pathway, defects in for instance caspase-8 may also cause decreased responsiveness to cellular immunotherapy in these patients.

The influence of effector-target interactions on the kinetics and the execution pathway of CTL-induced target cell death

In response to recognition of target antigen by their membrane TCRs, CTLs potentially use two pathways for lysis of target cells. [49;50] Cell lysis occurs either via a rapid PFN/GrB-mediated mechanism of cell death (<90 min) [51], or alternatively, via activation of the death receptor pathway in the target cell [50;52;53] causing relatively slow elimination of this target cell (>4 hrs) [54] as also demonstrated in chapters 4 and 6 of this thesis. It is largely unknown how these pathways are activated. Since cytotoxic T lymphocytes can use secretion of perforin (PFN) and granzyme B (GrB) to kill their target cells, why would they in addition have the potential to induce death receptor-mediated apoptosis? Virus-infected and tumorigenic cells have indeed been reported to be predominantly eliminated via rapid granule-mediated cell death. [55;56] Death receptor-mediated cell death, in contrast, primarily has been described to be involved in eliminating autoreactive T cells and downsize immune responses after infection. [57-60] In chapter 4 another role of the death receptor pathway is described, namely, execution of human target cells by cytotoxic T cells if not all target cells have been destroyed by release of PFN/GrB. In addition the death receptor pathway has also been shown to be involved in CTL-induced non MHC-restricted target cell death (Chapter 6).

Based on the results described in chapters 4 and 6, we hypothesize that the strength of interaction between target and effector cell determines the kinetics of target cell death and perhaps also the pathway of cell death. More specifically, we assume that weak effector-target interactions may be sufficient to induce the death receptor pathway of apoptosis, but cannot or only marginally lead to the release of PFN/GrB. In Chapter 4 was shown that the T cell clone C6-2 slowly killed EBV-LCL derived from a patient with leukemia. The same CTL was previously shown to be capable of rapidly killing monocytes of the same patient, [61] indicating that the execution machinery of the T cell is intact. Since the EBV-LCL could be killed within 4 hrs by another CTL clone (HY-A1), the observed differences in kinetics of killing are also not caused by any defects in one of the apoptotic mechanisms of the target cell. Therefore, more likely they are caused by differences in effector-target interaction, for instance due to variant levels of peptide presentation by the HLA molecules on the different target cells. Probably, the Cw6-restricted CTL clones recognize minor antigens highly expressed on monocytes but poorly expressed on EBV-LCL from the patient resulting in a low avidity interaction between TCR and MHC/peptide complex. The low amounts of PFN that were released are assumed to be a consequence of this weak interaction, as well as the fact that target cell death was at least partially executed via the death receptor pathway (Chapter 4).

The results shown in Chapter 6 also support the hypothesis that low avidity effector-target interactions only lead to death-receptor-induced apoptosis of target cells, while

strong effector-target interactions mainly induce PFN/GrB-induced target cell death. In addition to MHC-restricted target cell death, $CD8^+$ T cells were demonstrated to be capable of killing target cells in a non MHC-restricted manner. For the latter mechanism of lysis the TCR was not involved, implying a low avidity effector-target interaction. This non MHC-restricted target cell death was demonstrated to be completely mediated via activation of the death receptor pathway in the target cell. In contrast, in case of a strong effector-target cell interaction, as involved in TCR-dependent MHC-restricted recognition of target cells, the same cytotoxic T cells used PFN/GrB release as the main execution mechanism to induce rapid target cell death.

Why weak effector-target interactions can lead to activation of the death receptor pathway, but not to the release of PFN and GrB remains largely unknown. A model has been proposed assuming that after ligand binding the TCR complex has to undergo a number of modifications, including clustering of receptors, tyrosine phosphorylation steps and SH2 interactions, before a signal can be transmitted. [62] The requirement for these modifications results in a temporal lag between ligand binding and receptor signaling. At each step of this cascade the MHC/peptide-TCR complex may dissociate making it very unlikely that low-avidity MHC/peptide-TCR complexes can ever assemble the final signaling complex resulting in release of PFN and granzymes. However, a sufficiently high density of low-affinity MHC/peptide complexes can support the early steps of the process and lead to a distinct (partial) signaling pathway resulting in death-receptor-mediated apoptosis.
This model is supported by some murine studies demonstrating that interaction of CTLs with target cells exogenously loaded with single-amino-acid variants of the optimal MHC-binding peptide resulted in Fas-mediated target cell death while PFN/GrB-mediated cell lysis was absent. [63-65] When the same less-than-optimal peptides were endogenously processed, using a virus-based infection model, CTL-mediated cell death was completely absent. In Chapter 6 similar results were described showing that the HLA-A*0205 expressing cell line ALL-HP was not killed within 4 hrs by the HLA-A*0201-restricted T cell clone HA2.27 (Figure 6.1). Unpublished results of our group revealed that exogenous loading of this HLA-A*0205 positive cell line with high concentrations of HA-2 peptide resulted in rapid PFN/GrB-mediated cell death by CTL HA2.27. By artificially offering more specific MHC/peptide complexes to the TCR, the avidity of the MHC/peptide-TCR complex is apparently sufficiently enhanced to overcome a certain threshold of TCR triggering and making PFN/GrB release possible. In conclusion, small differences in avidity of MHC/peptide-TCR complexes can highly affect recognition by that specific TCR and determine which effector mechanisms can be used by the T cell.

From a physiological point of view it is not surprising that weak effector-target interactions could not lead to the release of PFN/GrB. Misdirected effector molecules

would cause a lot of damage to neighboring tissues. In the complex selection mechanism taking place in the thymus to delete potentially self-reactive T cells, it is important, however, that weak interactions can lead to death-receptor-mediated apoptosis.

Overexpression of anti-apoptotic genes as a method to study the role of different apoptotic pathways

Enhancing the expression of FLIP has been demonstrated to be an effective method to block the death receptor pathway in different leukemic cell lines (Chapters 3 and 4 of this thesis). PI-9 is considered to be an efficient and selective inhibitor of Granzyme B. Retroviral introduction of PI-9, FLIP, or both constructs into a target cell would therefore provide an elegant approach to study all effector mechanisms of a CTL. However, in chapter 5 it was demonstrated that overexpression of PI-9 inhibited Fas-induced apoptosis. Apparently, proteins that are supposed to act specifically in a certain pathway may interfere in other pathways, which may lead to incorrect conclusions.

Retroviral transfer of PI-9 can thus not be used to study CTL-induced PFN/GrB-mediated target cell death. Alternative strategies to evaluate the role of PFN and GrB in CTL-mediated cytotoxicity may be measuring PFN/GrB release using flow cytometry (as shown in chapter 4) or performing inhibition experiments using EGTA (chapter 6) or concanamycin A (CMA). The Ca^{2+}-chelator EGTA efficiently inhibits all (Ca^{2+}-dependent) secretion pathways in the T cell, [66] while CMA is an inhibitor of vacuolar type H^{+}-ATPase that inhibits PFN-based cytotoxicity, mostly by accelerated degradation of PFN caused by an increase in the pH of lytic granules. [67]

The inhibitory effect of PI-9 on Fas-induced cell death was much more pronounced in one of the two cell lines studied. We speculate that this may be caused by differential usage of Fas-cell death pathways by the two cell lines studied. In the earlier mentioned type I cells, Fas-induced apoptosis is completely dependent on the death-receptor pathway. In these cells the highest inhibition by PI-9 was observed. In type II cells in contrast, Fas-induced apoptosis is partly mediated via the mitochondrial pathway of apoptosis. Dependent on the site of interference of PI-9 in the apoptotic cascade, the inhibitory effect may differ between type I and II cell lines. This may also explain why this inhibitory effect was not observed by other groups that only studied one or two (type II) cell lines.

Conclusion

In conclusion, the results described in this thesis demonstrate that apoptosis induction in leukemic cells after treatment with chemotherapy or (cellular) immunotherapy is very complex and frequently dependent on the target cell studied, and on the interaction

between target and effector cell. It was shown that G_0 cells derived from patients with B-CLL compared to G_0 cells from patients with acute leukemia responded differently to Ara-C treatment, which may be partially explained by the different mechanisms of action exerted by Ara-C. In this thesis the role of the death receptor pathway in both chemotherapy-induced and CTL-induced apoptosis of leukemic cells was unraveled in more detail, at least in the cell lines that were studied. Considering future strategies in the treatment of leukemia, it will be worthwhile to focus on the investigation and development of agents that restore normal or therapy-induced apoptosis in resistant leukemic cells. These agents should be combined with conventional treatment modalities. For B-CLL, already a number of agents are under investigation which target not only Bcl-2, but also other anti-apoptotic proteins in CLL cells. Other attractive targets are proteins that are common in multiple apoptotic pathways, for instance caspase-8. Defects in caspase-8 or other common caspases like caspase-3 may cause multiple drug resistance and also cross resistance to immunotherapeutic interventions. Modulation or activation of these proteins will sensitize the leukemic cell simultaneously to all these forms of therapy.

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SAMENVATTING

Hematopoiese en leukemie

De normale aanmaak van bloedcellen, hematopoiese genaamd, vindt plaats in het beenmerg (BM). Hier bevinden zich hematopoietische stamcellen (HSC) die via verschillende differentiatiestappen uit kunnen rijpen tot verschillende types bloedcellen, zoals rode bloedcellen (erythrocyten), bloedplaatjes (thrombocyten) en veschillende soorten witte bloedcellen (leukocyten, o.a. lymfocyten en granulocyten). Rode bloedcellen zijn belangrijk voor het zuurstoftransport naar de weefsels, bloedplaatjes zijn betrokken bij de bloedstolling en witte bloedcellen spelen een belangrijke rol in de afweer tegen verschillende ziekteverwekkers.

Tijdens het differentiatieproces van onrijpe voorlopercellen naar rijpe witte bloedcellen kunnen er mutaties optreden, wat uiteindelijk kan leiden tot een ongeremde en ongecontroleerde groei van getransformeerde witte bloedcellen die niet meer verder kunnen uitrijpen. Deze populatie cellen hoopt zich op in het BM totdat op een gegeven moment het BM vol is en de maligne cellen in het bloed terechtkomen. Wanneer dit het geval is, spreekt men van leukemie. Door de ophoping van leukemiecellen in het BM wordt de aanmaak van normale bloedcellen verstoord, wat leidt tot symptomen als moeheid, bleekheid, blauwe plekken, bloedingen en infecties. Er bestaan verschillende vormen van leukemie, afhankelijk van in welke uitrijpingslijn en in welk stadium de transformatie tot leukemiecel heeft plaatsgevonden. De meest bekende vormen van leukemie zijn acute myeloide leukemie (AML), acute lymfatische leukemie (ALL), chronische myeloide leukemie (CML) en chronische lymfatische leukemie (CLL).

Behandeling van leukemie

De behandeling van leukemie hangt erg af van het type leukemie. CLL komt vooral voor bij oudere mensen en heeft een relatief mild verloop. Vaak wordt pas in een later stadium van de ziekte begonnen met therapie, die dan meestal bestaat uit verschillende combinaties van chemotherapie en eventueel Rituximab. Rituximab is een antistof gericht tegen het CD20 molecuul dat specifiek op het membraan van B cellen, dus ook op B-CLL cellen, voorkomt, waardoor alleen B cellen door Rituximab vernietigd worden.

CML is een stamcelziekte die wordt veroorzaakt door een translocatie tussen chromosoom 9 en 22, waardoor een stukje van chromosoom 9 van plaats wisselt met een stukje van chromosoom 22. Zowel op chromosoom 9 als op chromosoom 22 breekt het chromosoom midden in een gen. Op chromosoom 9 is dat het *ABL*-gen en op chromosoom 22 is dat het *BCR*-gen. Door de translocatie onstaat het fusiegen *BCR-ABL* op chromosoom 22, wat leidt tot de vorming van het fusie-eiwit BCR-ABL. Het BCR-ABL eiwit is verantwoordelijk voor de sterke groei en abnormale ontwikkeling van witte bloedcellen. Behandeling van CML in de chronische fase

is tegenwoordig specifiek gericht op remming van de tyrosine kinase activiteit van het BCR-ABL fusie-eiwit door middel van het geneesmiddel Imatinib. Door deze relatief nieuwe aanpak met Imatinib is de overlevingskans van patiënten met CML de laatste jaren flink verbeterd (van 50% naar 90% overleving, 5 jaar na behandeling). Toch reageert een deel van de patiënten niet goed op deze behandeling (o.a. door resistentie tegen Imatinib). Bij het merendeel van deze patiënten gaat de CML uiteindelijk over van een chronische naar een acute fase, de zogenaamde CML in blastencrisis, die dan vervolgens als een acute leukemie behandeld wordt.

De behandeling van acute leukemie richt zich op het zo volledig mogelijk vernietigen van de leukemiecellen, voornamelijk met behulp van chemotherapie en bestraling. De meest effectieve behandeling tegen acute leukemie (bij volwassenen) is hoge dosis chemotherapie en totale lichaamsbestraling gevolgd door allogene of autologe stamceltransplantatie (SCT), waarbij respectievelijk gebruik wordt gemaakt van stamcellen van een (gedeeltelijk) HLA-gematchte donor (vaak een broer of zus) of van de patiënt zelf. SCT is nodig om weer een normale bloedcelaanmaak op gang te brengen in deze patiënten van wie zowel de kwaadaardige als de normale hematopoiese vernietigd zijn (door de chemotherapie en de bestraling). Het voordeel van allogene SCT is dat naast het herstellen van de hematopoiese tevens een gunstig effect op de eventueel resterende leukemiecellen verkregen kan worden, het zogenaamde graft-versus-leukemia (GVL) effect. Dit GVL effect berust op de reactiviteit van de aanwezige T cellen in het transplantaat die nog resterende leukemiecellen van de patiënt kunnen herkennen en vernietigen. Met het doel om nogmaals een GVL effect te verkrijgen, worden soms na de allogene SCT opnieuw lymfocyten van dezelfde donor gegeven, de zogenaamde donor lymfocyten infusies (DLI).

Resistentie

Eén van de belangrijkste problemen in de behandeling van patiënten met acute leukemie is resistentie (aangeboren of verworven) van de leukemiecellen waardoor de therapie niet (meer) aanslaat. In eerste instantie reageren de meeste patiënten goed op inductie chemotherapie die gewoonlijk bestaat uit een combinatie van cytostatica zoals daunorubicine, vincristine, methotrexaat en cytarabine (Ara-C). In 70-80% van de patiënten leidt dit tot een complete remissie (er worden geen leukemiecellen meer gedetecteerd). Ondanks agressieve inductie- en consolidatietherapie treedt in 50-60% van de patiënten een recidief van de ziekte op, waardoor de genezingskans van patiënten met AML of ALL klein is. De patiënten die na meer dan een jaar na diagnose recidiveren hebben een goede kans om in een tweede remissie te komen na behandeling met de originele inductietherapie, of een protocol van vergelijkbare intensiteit.

Patiënten die nooit in complete remissie geraken of al snel een recidief krijgen

reageren meestal niet meer op verdere behandeling met dezelfde cytostatica als ze in eerste instantie hebben gekregen. Deze resistentie kan door verschillende mechanismen veroorzaakt worden, zoals het opreguleren door de leukemiecellen van bepaalde eiwitten, drug efflux pompen genaamd, waardoor de cytostatica weer naar buiten gepompt worden voordat ze de cel vernietigd hebben. Verder is de fase van de celcyclus waarin de leukemiecel zich bevindt op moment van blootstelling aan de chemotherapie belangrijk, omdat cellen in rust minder gevoelig zijn dan cellen die aan het delen zijn. Een andere oorzaak waardoor leukemiecellen resistent kunnen zijn tegen chemotherapie zijn defecten in de eiwitten die betrokken zijn in de cascade die uiteindelijk leidt tot apoptose van de leukemiecel. Dit laatste resistentiemechanisme is de directe aanleiding voor het in dit proefschrift beschreven onderzoek.

Apoptose en morfologie

Apoptose, of geprogrammeerde celdood, is een mechanisme om op een gecontroleerde manier ongewenste cellen uit het lichaam te verwijderen, in tegenstelling tot necrose, waarbij de cel plotseling dood gaat en de celinhoud vrij komt in het omringende weefsel wat leidt tot ongewenste ontstekingsreacties. Apoptose speelt een belangrijke rol tijdens de ontwikkeling van een embryo, maar is ook essentieel voor bijvoorbeeld de opbouw van een goed functionerend immuunsysteem. Als het mechanisme van apoptose ontregeld raakt, wordt de balans tussen celdeling en celdood verstoort, wat kan leiden tot auto-immuunziektes, neuro-degeneratieve aandoeningen en kanker.

Apoptose verloopt via een gereguleerd proces dat via een aantal stappen uiteindelijk leidt tot de dood van de cel. Tijdens het apoptoseproces zijn er verschillende fases te onderscheiden, waarbij de morfologie van de cel constant veranderd. Eerst krimpt de cel en krijgt het chromatine (DNA en eiwitten in de celkern) een compactere structuur. Tijdens deze fase gaat ook de symmetrie van de celmembraan verloren waardoor phosphatidylserine zichtbaar wordt op het celoppervlak. Vervolgens komen er uitstulpingen in het celmembraan waardoor de cel zijn ronde vorm verliest. In de volgende stappen condenseert het chromatine verder en vindt DNA fragmentatie plaats. Uiteindelijk splitst de cel zich in fragmenten die apoptotische lichaampjes worden genoemd. Deze celfragmenten worden door macrofagen opgeruimd zodat er geen ontstekingsreacties ontstaan.

Regulatie van apoptose door caspases

Het gehele apoptotische proces wordt gereguleerd door bepaalde eiwitten, de zogenaamde cysteine-afhankelijke aspartaat-specifieke proteases, ofwel caspases. Caspases hebben zowel een rol in de inductie van apoptose (initiator caspases, zoals caspase-2, -8, -9 en -10) als in de executiefase van apoptose (caspase-3, -6 en -7). Caspases komen in de cel voor als inactieve procaspases. Na activatie wordt door middel van een aantal klievingsstappen een actief caspase gevormd bestaande uit

twee grote en twee kleine domeinen. Actieve caspases kunnen specifiek bepaalde substraten binden en knippen het doormidden na een aspartaat groep. Tot de caspasesubstraten behoren zowel apoptose-regulerende eiwitten als structurele eiwitten.

Intrinsieke en extrinsieke apoptoseroute
Caspases kunnen zowel via een intrinsieke als via een extrinsieke apoptoseroute geactiveerd worden. Deze routes worden schematisch beschreven in Figuur 7.1. In de intrinsieke route, ook wel mitochondriële route genoemd, wordt apoptose van binnen uit de target cel geïnitieerd. Deze route wordt geactiveerd als mitochondriën (celstructuren die zorgen voor de energievoorziening in de cel) doorlaatbaar worden onder invloed van bepaalde stressfactoren, waardoor pro-apoptotische eiwitten zoals cytochroom C in het cytoplasma terecht komen. Cytochroom C bindt vervolgens aan twee eiwitten: Apaf-1 en procaspase-9. In dit complex, een apoptosoom genaamd, wordt procaspase-9 geactiveerd. Caspase-9 kan op zijn beurt allerlei andere caspases activeren, waaronder de effector caspase-3, wat uiteindelijk resulteert in de dood van de cel.
Apoptose kan ook van buiten de target cel geïnduceerd worden (extrinsieke route) door binding van zogenaamde death liganden (bijvoorbeeld Fas ligand) aan death receptoren (bijvoorbeeld de Fas receptor) die aanwezig zijn op de celmembraan van de target cel. Death receptoren bestaan uit een gedeelte dat zich buiten de cel bevindt, een gedeelte dat door de celmembraan steekt en een gedeelte dat zich in de cel bevindt. Door de binding aan de buitenkant van de cel wordt de death-receptor-geïnduceerde apoptose route, kortweg death-receptor route, in de target cel aangezet. De death liganden die voor de activatie van de death receptor route zorgen, bevinden zich o.a. op de celmembraan van cytotoxische T cellen. Na activatie worden drie death receptoren naar elkaar toe getrokken (trimerisatie). Hierdoor kan het eiwit FADD aan het intracellulaire domein van de death receptoren binden en wordt procaspase-8 aangetrokken. Samen vormen deze eiwitten het zogenaamde “death-inducing signaling complex” (DISC). In dit complex wordt procaspase-8 geactiveerd. Actief caspase-8 kan vervolgens of direct caspase-3 activeren, of via een ander eiwit de mitochondriële route activeren. Beide routes leiden uiteindelijk tot apoptose van de target cel.

STUDIES UIT DIT PROEFSCHRIFT

Cytostatica (chemotherapie) en T lymphocyten afkomstig van de donor (cellulaire immunotherapie) maken leukemiecellen dood via inductie van apoptose. Afwijkingen in gemeenschappelijke apoptoseroutes zouden dus ongevoeligheid tegen beide vormen van therapie kunnen veroorzaken, zoals waargenomen wordt in sommige

patiënten. Andere patiënten zijn resistent tegen chemotherapie, maar reageren nog wel op DLI, wat aangeeft dat deze patiënten waarschijnlijk defecten hebben in chemotherapie-specifieke routes. Het is slechts mogelijk om potentiële afwijkingen in apoptoseroutes in patiënten aan te tonen als er meer inzicht verkregen wordt in hoe normale functionele apoptoseroutes eruit zien. Alhoewel er wel algemene therapie-geïnduceerde apoptoseroutes beschreven zijn (Figuur 7.1), zijn de werkelijke apoptoseroutes die door therapie aangezet worden vaak veel complexer dan in dit figuur wordt weergegeven. In dit proefschrift wordt benadrukt hoe complex de apoptoseroutes zijn die in leukemiecellen worden geïnduceerd na behandeling met traditionele cytostatica of met cytotoxische T cellen (cellulaire immunotherapie). Bovendien worden verschillende tegenstrijdigheden die nog steeds bestaan op het gebied van apoptose belicht en verder uitgezocht.

Het paradoxale werkingsmechanisme van Ara-C

Om zichzelf te vermeerderen doorloopt een cel een bepaalde celcyclus, die bestaat uit verschillende fases. In de G_1 ("gap 1") fase vindt cytoplasmagroei plaats en bereidt de cel zich voor om zijn DNA te vermeerderen door bepaalde enzymen aan te maken. In de S ("synthese") fase verdubbelt de cel zijn DNA zodat bij de uiteindelijke deling beide dochtercellen precies hetzelfde DNA mee krijgen. In de G_2 ("gap 2") fase controleert de cel of al het DNA inderdaad netjes gedupliceerd is en bereidt de cel zich voor op een celdeling. In de M ("mitose") fase vindt de feitelijke celdeling plaats waardoor uiteindelijk twee identieke dochtercellen ontstaan. In de beide gap fases bevinden zich belangrijke controlepunten. Op deze momenten wordt besloten of de cel door kan gaan met zijn cyclus of in apoptose moet gaan als het niet verantwoord is om de deling verder door te zetten. Tijdens de G_1 fase bestaat er ook de mogelijkheid voor de cel om in rust te gaan (G_0-fase) en (voorlopig) niet meer door te gaan met prolifereren.

De meeste cytostatica induceren DNA schade en hebben alleen een effect op cellen die actief bezig zijn om hun DNA te vermeerderen en in deling te gaan, terwijl cellen in rust (G_0) beschouwd worden als ongevoelig. Inbouw van Ara-C in het DNA, een proces dat specifiek plaatsvindt tijdens de S-fase van de celcyclus, wordt beschouwd als het belangrijkste werkingsmechanisme van Ara-C om proliferende cellen dood te maken. In **hoofdstuk 2** beschrijven we de onverwachte bevinding van het vermogen van Ara-C om ook apoptose te induceren in rustende (meer dan 98% van de cellen zit in G_0) primaire B cellen afkomstig van vier tot dan toe onbehandelde patiënten met chronische lymfatische B-cel leukemie (B-CLL). Experimenteel werd in deze B-CLL cellen na behandeling met Ara-C zowel expressie van phosphatidyl serine aan de buitenkant van de celmembraan als caspase-activatie, twee belangrijke eigenschappen van apoptose, aangetoond. Met behulp van celcyclus analyse in combinatie met lange termijn analyse van celdood met behulp van CFSE-gebaseerde

cytotoxiciteitsassays konden we bevestigen dat deze niet-prolifererende B-CLL cellen door Ara-C in de G_0-fase van de celcyclus zijn gedood.

Aangezien remming van DNA synthese niet het werkingsmechanisme van Ara-C in deze G_0-B-CLL cellen kan zijn geweest, moeten er andere cellulaire processen betrokken zijn. In deze studie hebben we aangetoond dat oplopende concentraties van deoxycytidine (dC), een structurele analoog van Ara-C en tevens de normale bouwsteen van zowel RNA als DNA synthese, zowel de vorming van Ara-CTP als Ara-C-geïnduceerde celdood van G_0-B-CLL cellen remden. In tegenstelling tot DNA (repair) synthese welke nauwelijks detecteerbaar was, werd een onverwacht hoge RNA synthese gevonden in G_0-B-CLL cellen. Deze RNA synthese werd voor 30% geremd door Ara-C, wat suggereert dat remming van RNA synthese een mogelijk werkingsmechanisme van Ara-C in rustende G_0-B-CLL cellen is. B-CLL cellen worden gekenmerkt door hoge expressie niveaus van het eiwit B-cel lymphoma-2 (Bcl-2), dat een langere overleving van deze maligne cellen veroorzaakt. In eerdere studies is reeds aangetoond dat verandering van de expressie levels van pro-of anti-apoptotische eiwitten (met name de Bcl-2/Bax ratio) drug-geïnduceerde apoptose in B-CLL cellen kan initiëren. Daarom hebben we geanalyseerd of blootstelling aan Ara-C de eiwitniveaus van Bcl-2, Bax of Mcl-1 in B-CLL cellen veranderde. We vonden dat Mcl-1 expressie door Ara-C behandeling werd gedownreguleerd, wat een gevolg van de verminderde RNA synthese in deze cellen zou kunnen zijn. Concluderend kunnen we zeggen dat zowel de remming van RNA synthese als de downregulatie van het anti-apoptotische eiwit Mcl-1 kunnen bijdragen aan het werkingsmechanisme van Ara-C in G_0-B-CLL cellen.

De rol van de death receptor route in chemotherapie-geïnduceerde apoptose
Naast het onderzoeken van de mechanismen die betrokken zijn bij chemotherapie-geïnduceerde apoptose van rustende leukemiecellen, is het erg belangrijk om meer inzicht te verkrijgen in de apoptoseroutes die worden geïnduceerd door cytostatica in prolifererende leukemiecellen, aangezien resistentie van leukemiecellen tegen chemotherapie-geïnduceerde apoptose vaak optreedt in patiënten met acute leukemie. Eén van de tegenstrijdige onderwerpen op het gebied van chemotherapie-geïnduceerde apoptose is via welke route celdood geïnitieerd wordt. Behandeling met chemotherapie kan enerzijds leiden tot specifieke activatie van de mitochondriële route, waarin uitscheiding van cytochroom C en caspase-9 een centrale rol spelen. Een alternatieve of misschien additionele route die tot apoptose leidt en die door chemotherapie aangezet zou kunnen worden is de “death receptor” route, waarin vorming van de DISC en caspase-8 (FLICE) activatie belangrijke gebeurtenissen zijn (zie Figuur 7.1). In **hoofdstuk 3** van dit proefschrift beschrijven we de rol die de death receptor route speelt in chemotherapie-geïnduceerde apoptose van humane cellijnen die gemaakt zijn uit AML of ALL cellen van patiënten. De snelle

en aanzienlijke activatie van caspase-8 die we waarnamen na behandeling met de drug camptothecine, gecombineerd met de effectieve remming van chemotherapie-geïnduceerde apoptose door commerciële caspase-8 remmers, wekten sterk de suggestie dat de death receptor route betrokken is in door cytostatica geïnduceerde apoptose. Deze veronderstelling werd onderzocht door Bcl-2 en FLICE inhibitory protein (FLIP) tot overexpressie te brengen in de cellijnen met behulp van retrovitrale constructen, en vervolgens de gevoeligheid van deze cellijnen voor verschillende cytostatica te testen. Verhoogde expressie van Bcl-2 werd gebruikt om specifiek de mitochondriële route te remmen, terwijl door middel van FLIP expressie juist selectief death receptor-geïnduceerde apoptose kon worden geblokkeerd. FLIP overexpressie gaf geen remmend efect op camptothecine-geïnduceerde apoptose of caspase-8 activatie. Verhoogde Bcl-2 expressie, daarentegen, resulteerde in een bijna complete remming van camptothecine-gemedieerde celdood en caspase-8 activatie in alle drie cellijnen die we bestudeerd hebben. Deze resultaten geven aan dat chemotherapie-geïnduceerde apoptose in deze cellijnen compleet afhankelijk is van de mitochondriële route. Bovendien konden we uit deze studie opmaken dat caspase-8 blijkbaar een centrale rol speelt in zowel death receptor- als chemotherapie-geïnduceerde apoptose van maligne cellen afkomstig van patiënten met acute leukemie.

De rol van de death-receptor route in CTL-geïnduceerde target celdood

Er bestond niet alleen veel controversie over de rol van de death-receptor route in chemotherapie-geïnduceerde celdood, het was ook grotendeels onduidelijk in hoeverre deze route bijdraagt aan de eliminatie van leukemische cellen door cytotoxische T lymfocyten (CTL). In **hoofdstuk 4** hebben we onderzocht of CTL naast het uitscheiden van perforine (PFN) en granzyme B (GrB) tevens gebruik maken van hun death receptors om tumorcellen dood te maken. In de meeste studies die tot nu toe gepubliceerd zijn, werd alleen naar hele snelle CTL-gemedieerde celdood gekeken die binnen 4 uur na interactie tussen de tumor cel en de T cel optreedt. Omdat death-receptor gemedieerde apoptose langzaam verloopt in verhouding tot PFN/GrB geïnduceerde celdood, hebben we in deze studie T celklonen gebruikt die hun target cellen of snel of langzaam om zeep hielpen (snelle en langzame kinetiek van “killen”), en vervolgens gedurende verschillende tijdsintervallen de celdood bestudeerd. Deze CTL klonen waren afkomstig uit een patiënt met CML en herkenden allemaal primaire CML cellen van de patiënt. Omdat voor dit soort studies niet voldoende leukemische cellen uit de patiënt verkregen kunnen worden, werden EBV-getransformeerde B-cellen (EBV-LCL) van de patiënt als target cellen in onze studies gebruikt. Om de rol van de death-receptor route te bestuderen, werd FLIP tot overexpressie gebracht in deze target cellen. Een CTL kloon die 50% van de target cellen doodde binnen 2 uur, bleek hoofdzakelijk te werken door PFN en GrB uit te scheiden. Er werd in dit geval ook geen remming door FLIP gevonden. De twee

T celklonen die een langzamere kinetiek van killen lieten zien, bleken in ieder geval gedeeltelijk hun target cellen via de death-receptor route dood te maken aangezien ongeveer 30% remming met FLIP werd gevonden. Hieruit konden we concluderen dat cytotoxische T cellen niet alleen uitstoot van hun granulae, maar ook de death-receptor route gebruiken om hun target cellen te vernietigen.

PI-9 remt niet alleen GrB- maar ook Fas-geïnduceerde apoptose

Zoals het anti-apoptotische eiwit FLIP specifiek de death-receptor gemedieerde apoptoseroute blokkeert, zo was beschreven dat proteinase inhibitor-9 (PI-9) een specieke remmer is van GrB-geïnduceerde celdood en geen effect zou hebben op Fas-gemedieerde apoptose. Echter, in **hoofdstuk 5** laten we zien dat PI-9 een minder specifieke remmer is dan door andere onderzoekers werd gerapporteerd. Voor onze studie hebben we twee verschillende soorten cellijnen getransduceerd met retrovirale constructen die codeerden voor FLIP of PI-9. Vervolgens werd zowel in de wildtype als in de FLIP- of PI-9 getransduceerde cellijnen de Fas-receptor geactiveerd met behulp van antilichamen die Fas-geïnduceerde apoptose veroorzaken. In beide types cellijnen konden we een aanzienlijke remming van Fas-geïnduceerde apoptose door verhoogde PI-9 expressie aantonen, hoewel de remming minder was dan in de cellijnen die FLIP tot overexpressie brachten. PI-9 expressie had geen effect op chemotherapie-geïnduceerde apoptose, wat aangeeft dat PI-9 interfereert met de death-receptor route maar niet met de mitochondriële route van apoptose. Uit deze resultaten kunnen we concluderen dat het niet mogelijk is om door middel van PI-9 overexpressie specifiek GrB-geïnduceerde apoptose te bestuderen.

TCR-afhankelijke en onafhankelijke target celdood

Cytotoxische T cellen worden verondersteld hun target cellen selectief te doden, nadat ze met hun TCR specifiek een bepaald antigen in de context van het juiste HLA molecuul hebben herkend. In **hoofdstuk 6** beschrijven we een ander mechanisme dat door T cellen gebruikt kan worden om target cellen te vernietigen. Dit mechanisme is niet afhankelijk van het juiste HLA en triggering van de TCR is geen vereiste.
In dit hoofdstuk laten we zien dat HLA-A2 gerestricteerde CTL klonen cytolytische activiteit vertoonden tegen target cellen die HLA-A2 tot expressie brachten, wat resulteerde in 40-70% lysis na 4 uur incubatie. Als gevolg van deze klassieke TCR-MHC/peptide interactie, werd de TCR geactiveerd en vervolgens geïnternaliseerd, en produceerden de T cellen IFN-γ. Deze MHC-gerestricteerde target celdood werd compleet geremd door de T cellen te pre-incuberen met EGTA. EGTA vangt Ca^{2+} weg, waardoor alle Ca^{2+}-afhankelijke processen, dus ook secretieroutes, geremd worden. Deze resultaten laten zien dat TCR/MHC-afhankelijke celdood compleet verloopt via uitscheiding van PFN en/of GrB, aangezien de death-receptor route niet afhankelijk is van calcium.
Naast de klasieke manier om hun target celllen te doden, lieten dezelfde

T celklonen ook nog MHC-onafhankelijke lysis van target cellen zien (10-30%) na 10 uur incubatie. Dit mechanisme van T cel-gemedieerde celdood ging niet gepaard met TCR downregulatie en resulteerde bovendien niet in IFN-γ productie door de T cel. Verder toonden we aan dat deze lysis verliep via de death-receptor route, aangezien FLIP overexpressie een complete remming van deze lysis tot gevolg had. Een vergelijkbaar niet-MHC gerestricteerd eliminatiemechanisme wordt gebruikt om na afloop van een immuunrespons als gevolg van bijvoorbeeld een infectie het overschot aan T cellen op te ruimen, zodat geen bijkomende schade optreedt.

Conclusie

De resultaten die in dit proefschrift beschreven worden, benadrukken hoe complex de mechanismes zijn die geïnduceerd worden in leukemiecellen na behandeling met chemotherapie of cellulaire immunotherapie. Tevens wordt duidelijk dat het type cel dat bestudeerd wordt en de mate van interactie tussen target en effector cel grotendeels bepalen welke apoptoseroute aangezet worden. Zo zagen we bijvoorbeeld dat G_0 cellen die afkomstig waren van een patiënt met B-CLL anders reageerden op behandeling met Ara-C dan G_0 cellen van een patiënt met acute leukemie.
Verder werd in dit proefschrift de rol van de death-receptor route in zowel chemotherapie- als CTL-geïnduceerde apoptose van leukemiecellen verder opgehelderd, tenminste wat betreft de cellijnen die we bestudeerd hebben.

Het is de moeite waard om toekomstige strategieën voor de behandeling van leukemie te richten op de ontwikkeling van nieuwe middelen die de normale of therapie-geïnduceerde apoptose kunnen herstellen in resistente leukemiecellen. Voor de behandeling van B-CLL valt bijvoorbeeld te denken aan remmers van anti-apoptotische eiwitten zoals Bcl-2 en ook Mcl-1, waarvan al een aantal in ontwikkeling zijn. Deze middelen zouden dan gecombineerd moeten worden met conventionele behandelingsmethoden. Andere aantrekkelijke “target”eiwitten zijn eiwitten die betrokken zijn in meerdere apoptoseroutes, zoals bijvoorbeeld caspase-8. Defecten in caspase-8 of in andere gemeenschappelijke caspases zoals caspase-3 zouden resistentie tegen verschillende cytostatica (multiple drug resistance) maar ook resistentie tegen immunotherapie kunnen veroorzaken. Wanneer juist een dergelijk eiwit gemoduleerd of geactiveerd zou kunnen worden, zou de leukemiecel in één keer gevoelig kunnen worden voor al deze vormen van therapie.

LIST OF ABBREVIATIONS

7-AAD	7-amino-actinomycin D
Ab	antibody
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
Apaf-1	apoptosis inducing factor-1
Ara-C	cytosine arabinoside; cytarabine
Ara-CTP	Ara-C triphosphate
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
Bid	BH3-interacting domain death agonist
BM	bone marrow
Camp	camptothecin
CARD	caspase recruitment domain
CFSE	carboxyfluorescein diacetate succinimidyl ester
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CML-AP	CML-accelerated phase
CML-BP	CML blast phase
CML-CP	CML chronic phase
CR	complete remission
CTL	cytotoxic T lymphocyte
Dauno	daunorubicin
dC	deoxycytidine
DD	death domain
DED	death effector domain
DFS	disease free survival
DISC	death inducing signaling complex
DLI	donor lymphocyte infusion
DNA	deoxyribonucleic acid
EBV	Eppstein Barr virus
EGTA	ethylene glycol tetraacetic acid
E/T	effector/target
FADD	Fas-associated death domain
FasL	Fas ligand
FasR	Fas receptor
FLICE	FADD-like IL-1 converting enzyme
FLIP	FLICE-like inhibitory protein
GrB	granzyme B

GVHD	graft versus host disease
GVL	graft versus leukemia
HLA	human leukocyte antigen
HSC	hematopoietic stem cell
IAP	inhibitor of apoptosis
IFN	interferon
Mcl-1	myeloid cell leukemia sequence 1
mHag	minor histocompatibility antigen
MHC	major histocompatibility complex
MRD	minimal residual disease
NGFR	nerve growth factor receptor
NK	natural killer
PFN	perforin
PI	propidium iodide
PI-9	proteinase inhibitor 9
PR	partial response
PS	phosphatidylserine
RNA	ribonucleic acid
siRNA	small interfering RNA
SCT	stem cell transplantation
TCR	T cell receptor
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
z-VAD-FMK	N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone

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CURRICULUM VITAE

Jeltje de Vries werd geboren op 23 januari 1977 in Veenwouden. Na het behalen van haar eindexamen aan het Stedelijk Gymnasium te Leeuwarden in 1995, begon zij datzelfde jaar aan haar studie Bioprocestechnologie aan de Wageningen Universiteit. Na het afronden van haar propaedeuse in 1996, koos zij als afstudeerrichting de cellulair/moleculaire specialisatie. Haar afstudeervakken werden uitgevoerd bij de vakgroep Erfelijkheidsleer van de Wageningen Universiteit (Prof. Dr. C. Heyting) en bij de afdeling Klinische Genetica (Dr. M.M. Mannens en Dr. C.R. Bezzina) van het Amsterdam Medisch Centrum. Vervolgens liep zij stage bij de afdeling Pediatrische Immunologie van het Wilhelmina Kinderziekenhuis te Utrecht onder begeleiding van Dr. Ir. G. Rijkers en Dr. R. Brooimans. In januari 2001 behaalde ze haar doctoraalexamen. Vanaf april 2001 begon zij als AIO haar promotieonderzoek bij de Experimentele Hematologie, afdeling Hematologie (hoofd Prof. Dr. R. Willemze) van het Leids Universitair Medisch Centrum. Het in dit proefschrift beschreven onderzoek werd uitgevoerd onder begeleiding van Dr. R.M.Y. Barge en Prof. Dr. J.H.F. Falkenburg. Vanaf april 2007 is zij werkzaam als Wetenschappelijk Onderzoeker bij de afdeling Immunologie (hoofd Prof. Dr. R. Benner) van het Erasmus MC te Rotterdam.