Research Laboratory of Children's Hospital, University of Ulm

Chairman: Prof. Dr. Klaus-Michael Debatin

Activation of apoptosis pathways by different classes of anticancer drugs

Dissertation for the applying for a Doctor Degree of Medicine (Dr. med.)

Faculty of Medicine, University of Ulm

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2. Berichterstatter: Prof. Dr. Dr. Dr. A. Grünert

Tag der Promotion: 26. 10. 2001
To my family:

Chen Longgui
&
Liu Chang
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Abbreviations

PARP: poly (ADP-ribose) polymerase
PBS: phosphate-buffered saline
PAGE: polyacrylamide gel electrophoresis
zVAD-fmk: benzoyloxy carbonyl-Val-Ala-Asp-fluoromethyl ketone
ROS: reactive oxygen species
ΔΨm: mitochondrial transmembrane potential
DiOC6(3): 3,3’-dihexyloxycarbocyanine iodide
FACS: fluorescence-activated cell-sorting
PT: permeability transition
AIF: Apoptosis-inducing factor
BetA: betulinic acid
BA: bongkrekic acid
BSA: bovine serum albumin
mAb: monoclonal antibody
CD95L: CD95 ligand
Eto: etoposide
Ara-c: cytarabine
Doxo: doxorubicin
4-HCP: 4-hydroxy-cyclophosphamide
MTX: methotrexate
Cyt. C: cytochrome c
DISC: death-inducing signaling complex
FADD: Fas-associated death domain protein
Apaf-1: apoptosis protease-activating factor 1
VDAC: voltage-dependent anion channel(s)
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate-conjugated
PI: propidium iodide
Gy: gray
<table>
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<th>Abbreviation</th>
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<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>RIP</td>
<td>receptor interacting protein</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>octylphenol-polyethyleneglycol ether</td>
</tr>
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<td>EDTA</td>
<td>ethylene diamintetraacetic acid</td>
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1. Introduction

Cells die by two primary processes: A) necrosis, in which the release of intracellular proteases and lysozymes induce an inflammatory response, or B) apoptosis, also known as programmed cell death, where the cell remnants quietly disappear as they are phagocytosed by surrounding cells.

1.1. Apoptosis: definitions and mechanisms

1.1.1. Cell biology of apoptosis

The major physiological mechanism of cell removal is apoptosis - a Greek descriptive term for falling leaves or petals. Apoptosis describes the process by which cells are 'silently' removed under normal conditions when they reach the end of their life span, are damaged, or superfluous. It is a general tissue phenomenon necessary for development and homeostasis: elimination of redundant cells during embrogenesis, cell atrophy upon endocrine withdrawal or loss of essential growth factors or cytokines, tissue remodelling and repair, and removal of cells that have sustained genotoxic damage. Its conserved features reflect its similarly evolutionarily conserved genetic characteristics, from nematode worm to man. Apoptosis is strictly a morphological description and other morphologies of developmental programmed cell death exist (Kerr et al., 1972; Majno et al., 1995; Häcker, 2000; Chinnaiyan et al., 1997).

Apoptotic cells exhibit a characteristic pattern of changes, including cytoplasmic shrinkage, active membrane blebbing, chromatin condensation and, typically, fragmentation into membrane-enclosed vesicles, apoptotic body (Ucker et al., 1992; Kawabat et al., 1999; Wyllie, 1999; Mills et al., 1999). This readily visible transformation is accompanied by a number of biochemical changes. Changes at the cell surface include the externalization of phosphatidylserine and other alterations that promote recognition by phagocytes. Intracellular changes include the degradation of the chromosomal DNA into high-molecular-weight and oligonucleosomal fragments, as well as cleavage of a specific
subset of cellular polypeptides (Ellis et al., 1991; Rotello et al., 1994; Franc et al., 1996; Savill, 1996).

The apoptotic process may be set in motion by: A) genes responding to DNA damage; B) death signals received at the cell membrane (Fas ligand), or C) proteolytic enzymes entering directly into the cell (granzymes). The final events, evidenced by the changes in cell structure and disassembly, are the work of specific proteases (caspases) (Evans, 1993).

1.1.2. Execution of programmed cell death by caspases

Caspases are currently considered as the central executioners of many, if not all, apoptotic pathways (Chinnaiyan et al., 1997; Alnemri, 1997; Kroemer et al., 1998; Budihardjo et al., 1998). Many of the proteolytic cleavages during apoptosis result from the action of a unique family of cysteine-dependent proteases called caspases. The various members of this protease family differ in primary structure and substrate specificity but share several carboxyl side of aspartate residues. First, each caspase cleaves at the carboxyl side of aspartate residues. Second, each active caspase is a synthesized as a zymogen that contains an N-terminal prodomain, a large subunit and a small subunit. Finally, proteolytic cleavage to liberate each caspase involves sequential cleavages at two or more small subunits from one another and from the prodomain. The fact that these activating cleavages occur at sites that could be cleaved by caspases led to the concept that caspase activation might involve either a proteolytic cascade or an autoactivation process (Earnshaw et al., 1999; Nicholson, 1999; Walker et al., 1994; Salvesen et al., 1997).

Of the twelve known human caspases, six (caspases-3, -6, -7, -8, -9, and -10) are definitely involved in apoptosis in various model systems. One current classification scheme divides these apoptotic caspases into two classes, effector (or 'downstream') caspases, which are responsible for most of the cleavages that disassemble the cell, and initiator (or 'upstream') caspases, which initiate the proteolytic cascade (Depraetere et al., 1998; Thornberry et al, 1998).

Caspase-3, -6, and -7 are the major effector caspases characterized to date. Once activated, these enzymes are capable of cleaving the vast majority of polypeptides that undergo
proteolysis in apoptotic cells (Earnshaw et al., 1999; Tewari et al., 1995; Sakahira et al., 1998; Sahara et al., 1999). Interestingly, overexpression of these caspases in mammalian cells is relatively non-toxic, suggesting that these precursors have limited capacity for autoactivation. Instead, effector caspases are usually activated by other proteases.

Caspase-8 and -9 are the major initiator caspases identified to date. Zymogen forms of these enzymes display low but detectable protease activity. This activity increases when the prodomains of these zymogens interact with certain binding partners. Upon activation, caspase-8 and -9 acquire the ability to cleave and activate caspases (Juo et al., 1998; Nagata, 1997).

An increasing number of proteins have been found to be cleaved by caspases, and for some of them an apoptotic function has been proposed. Among different substrates are enzymes involved in genome function, such as the DNA repair enzyme poly (adenosine diphosphate-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK), or regulators of the cell cycle, including retinoblastoma protein, the p53 regulator MDM-2, MEKK, and protein kinase C-δ. Substrates of the nucleus and cytoskeleton include lamins, Gas2, gelsolin, and fodrin. Furthermore, it has been found that DNA cleavage is triggered upon caspase-mediated degradation of the inhibitory subunit of a novel endonuclease, designated caspase-activated DNase.

Current knowledge indicates that individual caspase have distinct substrate specificities, inhibitor profiles, and abilities to process each other. These findings suggest that caspases form a hierarchical network which, similar to the complement system, may function as an amplifier for a given apoptotic stimulus (Garcia-Calvo et al., 1999; Nicholson, 1999; Los et al., 1999).

1.1.3. Two main pathways of apoptosis

One of the best-defined apoptotic pathways is mediated by the death receptor CD95 (APO-1/Fas). Triggering of CD95 by its natural ligand or agonistic antibodies induces the formation of DISC that consists of the adapter protein FADD and FLICE/caspase-8. Complex formation is initiated through homophilic interaction of the death domains
present in the intracellular part of both CD95 and FADD. FADD, in addition, contains a second interaction region called the DED, which couples to caspase-8 as the most proximal element in the caspase cascade. Further downstream, caspase-8 presumably triggers the proteolytic activation of other caspases and cleavage of cellular substrates (Krammer, 1999; Schulze-Osthoff et al., 1998).

Another apoptotic pathway is the mitochondrial pathway. It has been shown that mitochondria play an important role in regulation of apoptosis. An early event in this process is the loss of the mitochondrial transmembrane potential $\Delta \Psi_{m}$, which induce the release of molecules contained in the intermembrane space of the mitochondria to the cytosol. Among the released molecules is cytochrome c that, on entry in the cytosol, induces oligomerization of Apaf-1 (caspase recruitment domain) in the presence of ATP. In turn, oligomerized Apaf-1 binds to cytosolic procaspase-9 in a so-called apoptosome complex and induces processing and activates the downstream caspase cascade. Other molecules released from the mitochondria include several procaspases and the flavoprotein AIF (apoptosis inducing factor) that translocates to the nucleus and triggers caspase-independent nuclear changes. Mitochondrial apoptosis signal is regulated by molecules of the Bcl-2 family, which have been shown to control mitochondrial membrane integrity by interaction with the mitochondrial membranes. The antiapoptotic properties of Bcl-2 and related proteins have been related to their ability to prevent these mitochondrial events, whereas the targeting of BH3 domain-only proteins of the Bcl-2 family such as Bid, Bim or Bad from various parts of the cell to the mitochondria was shown to activate the death process by inducing the mitochondrial release of proapoptotic molecules (Li et al., 1997; Stennicke et al., 1999).

1.2. Cytotoxic anticancer drugs and apoptosis

Anticancer drugs have been shown to target diverse cellular functions in mediating cell death in chemosensitive tumors. Cytotoxic drugs which are currently used for the treatment of malignant diseases such as etoposide, cytarabine, cyclophosphamide, doxorubicin and methotrexate are thought to exert their effects through inhibition of topoisomerase II (etoposide), DNA-polymerase (cytarabine), antagonization of folic acid
(methotrexate), inhibition of DNA-crosslinking (cyclophosphamide) and DNA-intercalation (doxorubicin).

Although the primary intracellular targets of drug action are rather distinct, it has become evident that drug-induced cytotoxicity ultimately converges on a common pathway, causing apoptosis (Debatin, 1999; Kaufmann et al., 2000; Mkin et al., 2000). Cells exposed to anticancer drugs display apoptosis alterations, such as cell shrinkage, chromatin condensation, and internucleosomal DNA fragmentation. A close link between apoptosis and the mechanism of drug action has been demonstrated by the involvement of similar genetic components. Overexpression of Bcl-2 proteins can confer drug resistance in transfected tumor cells. A number of investigations exposed a critical role of the tumor suppressor p53 in apoptosis after drug treatment. p53 requires an upstream DNA damage signal to allow it to function, and the clues as to how this comes about were provided by the observations that p53 is induced by DNA-damaging agents, including γ-irradiation and chemotherapeutic agents. After exposure of the cell to DNA damaged, p53 protein levels are rapidly upregulated by a post-transcriptional mechanism involving both stabilization and possible modification of a latent form of p53. DNA strand breaks are sufficient stimulus for this p53 response and it has been suggested that a single double-strand DNA break per cell is sufficient. The outcome of the activation of p53 is either apoptosis or a cell cycle arrest. But it is clear that the response to activation of p53 is tissue specific; the cellular outcome will also depend upon the balance between pro-apoptotic signaling from p53, and its downstream events, and anti-apoptotic survival signaling provided by various molecules. Finally, it has been recently shown that drug-induced cytotoxicity involves proteases of the caspase family, because specific inhibitors of caspases prevented cell death after treatment with different anticancer agents.

A number of studies have raised the possibility that anticancer drugs trigger apoptosis by inducing the synthesis of CD95-L, which then bind to CD95 receptor and activates the death receptor pathway in an autocrine or paracrine fashion. Drug-induced increases in CD95 mRNA, CD95-L and upregulation of CD95 was observed after treatment of different tumors with cytotoxic drugs such as doxorubicin, cisplatin, methotrexate, cytarabine and etoposide at therapeutic concentrations. Treatment of leukemias or solid tumors, including neuroblastoma, hepatoblastoma, medulloblastoma, colon carcinoma and breast cancer cells with cytotoxic drugs induces CD95-L expression and mediates
autocrine suicide or paracrine cell death following binding to its receptor. Blockade of CD95-L/receptor interaction using antagonistic antibodies to the receptor markedly reduced drug-triggered apoptosis. Thus, production of CD95-L and crosslinking of its cognate receptor are probably involved in drug-mediated cell death. Moreover, CD95 expression was unregulated upon treatment with cytotoxic drugs, which increases sensitivity of physiological apoptotic signals (Friesen et al., 1996; Fulda et al., 1997a, b).

Alterations of mitochondrial functions such as permeability transition (PT) have been found to play a major role in the apoptotic process induced by anticancer drugs (Costantini et al., 2000). Exposure of many cultured hematological cell lines to a cytotoxic anticancer drug can cause mitochondrial dysfunction that include loss of mitochondrial membrane potential ($\Delta \Psi_m$), release of cytochrome c and AIF from the mitochondrial intermembrane space to the cytosol, and the generation of reactive oxygen species. Anticancer drugs also destroy the balance of between proapoptotic and antiapoptotic members of Bcl-2 family, which reduce the stabilization role of the mitochondrial membrane by anti-apoptotic Bcl-2-like proteins.

### 1.3. Aims and summary of the project

Over the past 20 years, anticancer combination therapy using protocols based on clinical experience and empirical data has achieved long term remission and cure in 70-80% of patients with pediatric acute lymphoblastic leukemia. However, the most prevalent of malignancies have proved to be more or less resistant to anticancer drugs. Dose escalation using high-dose chemotherapy may have resulted in a modest improvement in responses but has not constituted a breakthrough. The dose intensity of most chemotherapeutic regimens is limited primarily by the degree of toxicity encountered. Acute toxicities common to many of the anticancer drugs include myelosuppression, nausea and vomiting, alopecia, orointestinal mucositis, liver function test abnormalities, allergic or cutaneous reactions, and local ulceration from subcutaneous drug extravasation.

The primary role of the pediatric oncologist is to orchestrate the administration of complex combination chemotherapy regiments to children. Special care must be taken because the anticancer drugs used in these regimens have the lowest therapeutic index of any class of
drugs and predictably produce significant, even life-threatening toxic reactions at therapeutic doses. However, allowing significant dose reductions or delays in therapy to attenuate these toxicities may compromise dose-intensity and place the patient at an increased risk for disease recurrence. The cancer chemotherapist must carefully balance the risks of toxicities from therapy against the risk of tumor recurrence from inadequate treatment. Unfortunately, the development and clinical usage of cancer chemotherapy remains largely empiric, and the mechanisms of action of most anticancer drugs are nonselective targeting vital macromolecules (e.g. nucleic acids) or metabolic pathways.

To ensure that these drugs are used safely and effectively, an understanding of the mechanism of drug action and time kinetics is crucial. Although extensive studies of the biochemical and molecular pharmacology of drug-target cell interaction have been performed, the precise molecular requirement by which anticancer drugs initiate apoptosis pathways are poorly defined.

It is known that in empirical medicine, these different anticancer drugs have different characteristic concerning specific anti-tumor or anti-leukemic efficacy and side effects on normal tissue. While solid tumors are often treated with cisplatin, treatment of leukemia is based on the use of anthracyclines and antimetabolites. Many drugs also have unique toxicities affecting normal tissues, such as cardiotoxicity associated with anthracyclines, hemorrhagic cystitis associated with cyclophosphamide and ifosfamide, peripheral neuropathy from vincristine, and coagulopathy from L-asparaginase.

We therefore hypothesized that the different clinical used anticancer drugs might induce apoptosis in a drug specific manner. Thus the clinical observed differences could be reflected by different activation of apoptosis signaling pathways.

We therefore investigated induction of apoptosis, activation of caspases and involvement of mitochondrial signaling in the well defined Jurkat cell culture system by five conventional used anticancer drugs: etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, and methotrexate, in order to identify drug specific activation of distinct apoptosis pathways.
In the studies presented, we found some differences in apoptosis induced by these five anticancer drugs: (1) the anticancer drug-induced apoptosis appears in different time kinetics, etoposide and cytarabine were found to be early acting drugs, while 4-hydroxy-cyclophosphamide, doxorubicin, and methotrexate were late acting drugs. (2) Interestingly, higher doses of cytarabine induce less apoptosis, whereas lower doses of cytarabine induce more apoptosis. (3) Etoposide strongly induced caspases activation, compared to cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin and methotrexate. Besides this difference, we also found that all drugs induced apoptosis in a similar manner. Both activation of mitochondrial signaling and caspase activation were essential for execution of programmed cell death (PCD) induced by anticancer drugs.
2. Materials and Methods

2.1. Materials

2.1.1. Reagents and equipment for cell culture

Human Leukemia T-cell line Jurkat American Type Culture Collection
Human Leukemia T-cell line H9 American Type Culture Collection
Human neuroblastoma cell line SHEP American Type Culture Collection
RPMI 1640 medium Life Technologies, Paisley, Scotland
Penicillin-Streptomycin Life Technologies, Paisley, Scotland
L-Glutamine Life Technologies, Paisley, Scotland
Fetal Calf Serum (FCS) Biochrom KG, Berlin, Germany
HEPES- Buffer (1 M) Biochrom, Berlin, Germany
Trypsin/EDTA Biochrom, Berlin, Germany
Trypan Blue Solution (0.4%) Sigma-Aldrich, England
Safety Cabinet Heraeus, Germany
CO₂ Incubator WTC binder, Germany
Inver Microscope Leika, Portugal
Tissue Culture Flask Becton Dickinson Labware, England
Tissue Culture Plate Becton Dickinson Labware, USA
Sterile Syringe Becton Dickinson Labware, Germany
Sterile Pipette Becton Dickinson Labware, USA
Pipetter Bilson, France
Sterile Filter Schleicher & Schnell, Germany

2.1.2. Reagents and equipment for flow cytometric analysis

PBS Biochrom KG, Berlin, Germany
HANKS' Life Technologies, Paisley, Scotland
Annexin V FITC Boehringer Mannheim, Germany
Steofundin B/Braun, Germany
Propidium Iodide Sigma-Aldrich Chemie, Germany
Triton X-100 Sigma-Aldrich Chemie, Germany
Trinatriumcitrate Dihydrate Sigma-Aldrich Chemie, Switzerland
Paraformaldehyde (PFA) Merck, Darmstadt, Germany
β-Mercaptoethanol Merck, Darmstadt, Germany
Protein A Sigma-Aldrich Chemie, USA
z-VAD.fmk (Z-Val-Ala-Asp (Ome)-FMK) Enzyme Systems, San Diego, USA
Ethanol Merck, Darmstadt, Germany
3,3’-Dihexyloxycarbocyanine Iodide Mo Bi Tec, Netherlands
(DiOC₆(3))
Dimethyld Sulfoxide (DMSO) Serva Electrophoresis, Germany
Bovine Serum Albumin (BSA) Boehringer Ingelheim, Germany
Sodium Azide Sigma, USA
Optimized Sheath Fluid Becton Dickinson, Belgium
Flow Cytometry (FACSCalibur) Becton Dickinson, Heiderberg, Germany
Thermobath Sink Elvo Labortechnik, Germany
Vortex Scientific Industries, USA
Centrifuge (Varifuge 3.0R) Heraeus, Germany
Centrifuge Tube (Polypropylene Conical Tube) Becton Dickinson Labware, France

2.1.3. Reagents and equipment for western blot

Tris Base Sigma, USA
Glycin Carl Roth, Karlsruhe, Germany
Glycerol J.T. Baker, Holland
Skim Milk Powder Merck, Darmstadt, Germany
Bromophenol Blue Sigma, USA
Sodium Salt (SDS) Sigma-Aldrich Chemie, Germany
Methanol Merck, Darmstadt, Germany
Tween 20 Gerbu Biotecknik, Germany
Protease Inhibitor Cocktail Sigma, USA
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<th>Chemical/Instrument</th>
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<td>Gerbu Biotechnik, Germany</td>
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<td>EGTA</td>
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<td>Dithiothreitol (DTT)</td>
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<td>BCA Protein Assay Reagent</td>
<td>Pierce, USA</td>
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<td>Sucrose</td>
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<td>Mini Centrifuge</td>
<td>MS Laborgerät, Heidelberg, Germany</td>
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<td>-20°C refrigerator</td>
<td>Liebher, Italy</td>
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<tr>
<td>-80°C refrigerator</td>
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<td>Electrophoresis Cell (Criterion Cell)</td>
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<td>Semi Dry Transfer Cell</td>
<td>Bio-Rad, USA</td>
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<td>Precast Gel</td>
<td>Bio-Rad, USA</td>
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<td>Eppendorf-Netheler-Hinz, Germany</td>
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<td>Merck Eurolab, Germany</td>
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<td>Molecular Weight Marker (Rainbow)</td>
<td>Amersham Pharmacia Biotech, England</td>
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<tr>
<td>Western Blotting Regents (ECL)</td>
<td>Amersham Pharmacia Biotech, England</td>
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<td>X-ray Film Processor (Hyper Processor)</td>
<td>Amersham Life Science, England</td>
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<tr>
<td>UV/Visible Spectrophotometer</td>
<td>Pharmacia Biotech, England</td>
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<tr>
<td>(Ultrspec 2000)</td>
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<tr>
<td>Digital pH Meter (210A)</td>
<td>Orion Research, Boston, USA</td>
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<tr>
<td>Platform Shaker (Ploymax 1040)</td>
<td>Heidolph, Germany</td>
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### 2.1.4. Anticancer drugs
Etoposide
Cytarabine (Ara-c)
4-Hydroxy-cyclophosphamide
Doxorubicin
Methotrexate

Sigma-Aldrich Chemie, Germany
Pfizer, Germany
Asta, Germany
Pharmcia, Italy
Lederle, Germany

2.1.5. Antibodies

Anti-CD95 monoclonal antibody (IgG₃)

Mouse IgG1,κ (MOPC-21)(M9269)
Mouse IgG2b (clone DAK-G09)(X 0944)
(Isotype, Negative Control)
Goat F (ab') 2 Anti-Mouse-IgG2b-FITC
(GAM IgG 2b FITC)
Anti-mouse Cytochrome c
(65981A) (clone 7H8.2C12)
Anti-cytochrome oxidase subunit IV mAb
(COX-IV) (A6431, clone 20E8-C12)
Anti-Caspase-8/Flice mAb
(clone C15, hybridoma supernatant)
Anti-Caspase-3/CPP32 mAb
(C31720)
Anti-PARP mAb
(65196A, clone C2-10)
Anti-Fas Ligand/CD95L mAb (F37720)
Anti-RIP mAb (R41220)
Anti-FADD mAb (F36620)
Anti-Human Bid (AF846)
Anti-human Bcl-2 mAb
(65111A, clone Bcl-2/100)
Anti-β-Actin mAb
(A-5441, clone AC-15)
Anti-Mouse IgG-HRP
(sc-2005, HRP-conjugate)

Sigma-Aldrich, Germany
DAKO, Denmark
Southern Biotechnology Associates
Birmingham, USA
PharMingen, USA
Molecular Probes, Germany
Kindly Provided by Prof. Krammer,
DKFZ Heidelberg, Germany
Transduction Laboratories, Lexington,
KY
PharMingen, USA
Transduction Laboratories
Transduction Laboratories
R&D Systems, England
PharMingen, San Diego, CA
Sigma, USA
Santa Cruz Biotechnology, Germany
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<th>Anti-Rabbit IgG-HRP</th>
<th>Santa Cruz Biotechnology, Germany</th>
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2.2. Methods

2.2.1. Cell culture

Human leukemia T-cell lines Jurkat, H9, and Neuroblastoma (Shep) cell line were obtained from American Type Culture Collection (Rockville, MD) and cultured in 75-cm² tissue culture flasks in RPMI-1640 medium containing 10% heat-inactivated fetal calf serum (FCS), 100 U of penicillin per milliliter, 0.1 mg streptomycin per milliliter, 2 mM glutamine, and 10 mmol/L HEPES. Cells were grown at 37°C in a 5% CO₂ atmosphere and maintained in log phase.

2.2.2. Cell preservation and reconstitution

When being in a best growing phase, cells were spun down and washed 3 time with PBS, resuspended with FCS plus 10% DMSO. Aliquots of 1 x 10⁶ cells were transferred into a cryogenic vial and frozen at -80°C. For long-term storage, the frozen cells were placed in liquid nitrogen. For reconstitution, a face guard and protective gloves and clothing must be worn whenever an vial is removed from liquid nitrogen, because the vial that has been submerged in liquid nitrogen can explode upon removal if it has not been properly sealed and the plastic fragments fly at high force in all directions creating a hazard. The frozen cells were thawed by incubation of cryogenic vials in a covered water bath at 37°C for 1 min and washed with prewarmed medium before resuspension with the corresponding medium.

2.2.3. Cell stimulation

Anti-Apo-1 mAb 1mg/ml were kept as stock solution at -20°C. Etoposide (Eto) was dissolved in Dimethyd Sulfoxide (DMSO) at a concentration of 20 mg/ml, Methotrexate (MTX) dissolved in 0.1 N NaOH at a concentration of 20mg/ml, doxorubicin (Doxo) and cytarabine (Ara-C) dissolved in sterile distilled water at a concentration of 1mg/ml and kept as stock solution at -20°C. 4-hydroxy-cyclophosphamide (4-HCP) was dissolved in sterile distilled water at a concentration of 1mg/ml and kept as stock solution at -80°C. Cyclophosphamide, which is used in patients, is not an appropriate stimulus in vitro,
because this compound must undergo hydroxylation at the 4-carbon position before expressing cytotoxic activity; this reaction is catalyzed by hepatic microsomal enzymes. So 4-hydroxy-cyclophosphamide must be used in vitro to get the same effect as the clinical use.

Prior to stimulation, cells were incubated 24 hours in 75-cm$^2$ cell culture flasks in same medium and then seeded in 24-well plate for stimulation. Cells were stimulated with agonist Anti-Apo-1 (anti-CD95) mAb or etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, and Methotrexate, or irradiated using a $^{137}$Cs source (2 x 415 Ci) at the doses and time points as indicated in the individual figure legends. Control culture were treated with the appropriate amount of DMSO and NaOH, used as solvent for some anticancer drugs and peptide inhibitor. Cells were harvested at the appropriate time points, and then subjected to various processing procedures for the different purpose of analysis.

### 2.2.4. Inhibitor studies

Benzyloxy carbonyl-Val-Ala-Asp fluoromethyl ketone (zVAD-fmk) was dissolved in DMSO at a concentration of 20 mM and kept as solution at -20°C.

For the inhibitor studies, cells cultured as described above were treated with 50 or 100 µM zVAD-fmk prior to the addition of stimulus, or only using zVAD-fmk without stimulus as control.

### 2.2.5. Flow cytometry

#### 2.2.5.1. Analysis of annexin V and PI positive cells

**A. Annexin V and PI staining**

For double labeling procedures, after exposure to the apoptotic stimulus, 5 x 10$^5$ cells were harvested into a 5 ml test tube and washed with annexin V buffer (140 mM NaCl, 2.5 mM CaCl$_2$, 10 mM Hepes, pH 7.4) for 7 min, at 4°C, 1300 rpm. And then the pellets were
resuspended with annexin V working solution (1 µl Annexin V-FITC and 19 µl buffer) and incubated for 15 min, at 4°C in the dark. The cells were washed again with buffer at 1300 rpm for 7 min, at 4°C. Before the measurement, the cells were added 10 µl propidium iodide (PI) work solution (20 µg/ml) 10 µl (the concentration of PI work solution is 1 mg/ml).

B. Flow cytometry

Labeled cells were suspended in 300 µl buffer. Flow cytometric analysis was performed on a FACSCalibur flow cytometry with an excitation wavelength of 488 nm. Data acquisition and analysis were performed by the CellQuest software (Becton Dickinson). 30,000 events were collected for each analysis. Cell debris was excluded by setting appropriate light scatter gates. Photomultiplier voltages were adjusted to have the unlabeled cell population fall in the first decade of fluorescence. Cells labeled with only -FITC or PI were used to adjust the compensation. Annexin V-FITC were detected through the FL1 channel, equipped with a 530-nm (20-nm band pass) filter. PI were detected through the FL2 channel, equipped with a 575-nm (20-nm band pass) filter. The data were acquired and analyzed with CELLQuest software (Becton Dickinson).

2.2.5.2. Quantification of DNA fragmentation

After stimulated, Aliquots 5 x 10^5 cells were washed with PBS without Ca^{++} at 1300 rpm for 7 min, at 4°C. The cell pellets were gently resuspended in 525 µl hypotonic lysis solution (PI 25 µg in 0.1% trinatriumcitrate-dihydrate 250 µl plus 0.1% Triton X-100 250µl) and incubated at 4°C overnight in the dark. This hypotonic lysis solution can break the cell membrane and let PI stain the nuclear, because early during apoptosis the cell membrane is still intact. Cells were analyzed for DNA content using flow cytometry (FACSCalibur) by examining 10,000 events with excitation wavelength of 488 nm. The emission wavelengths were detected through the FL3 channel, equipped with a 650 nm (20-nm band pass) filter. The data were acquired and analyzed with CELLQuest software (Becton Dickinson).

2.2.5.3. Analysis of mitochondria membrane potential ($\Delta \Psi_m$)
To evaluate $\Delta \Psi_m$, the cationic lipophilic fluorochrome 3,3'-dihexyloxy carbocyanine iodide (DiOC$_6$(3)) was used. 5 x $10^5$ cells were placed into a 5 ml test tube, added 3 ml PBS, and centrifuged at 1300 rpm for 7 min, at 4°C. The supernatants were removed by aspiration. The cell pellets were mixed gently with 20 nM DiOC$_6$(3) and incubated for 15 min, at 37°C in the dark. DiOC$_6$(3) was prepared from a 40 µM stock solution in DMSO. This solution was diluted with PBS, pH 7.4, to a 400 nM working solution. Cells were washed once with PBS; cell suspensions were prepared for flow cytometry. The live cells were gated and DiOC$_6$(3) was detected through the FL1 channel. $\Delta \Psi_m$ low cells were those displaying DiOC$_6$(3) fluorescence less than the fluorescence of control cells in the absence of the apoptotic stimulus. The data were acquired and analyzed with CELLQuest software (Becton Dickinson).

2.2.5.4. Quantification of cytoplasmic cytochrome c

5 x $10^5$ cells were placed into a 5 ml test tube, added 3 ml wash solution (HANKS' plus 1% BSA and 0.1% sodium azide) and centrifuged at 1300 rpm for 7 min, at 4°C. The pellets were resuspended and fixed for 20 min, at 4°C with 100 µl 4% paraformaldehyde (PFA), which was freshly prepared. The cells were washed again as described above, resuspended with 50 µl 0.2% saponin in PBS containing 5 µl Mouse IgG1, k (MOPC-21) for the purpose of permeabilization and blocking nonspecific binding and incubated for 5 min, at the room temperature. And then the cells were added with 20 µl first antibody, anti-cytochrome c (1:20 dilution in wash solution) and incubated for 20 min, at 4°C. As a negative control, in parallel cells were added with only 20 µl mouse IgG2b (isotype Ab) (1:20 dilution in wash solution) and incubated as the first antibody. The wash step was repeated. The pellets were suspended with 20 µl second antibody, Goat F(ab')$_2$ Anti-Mouse-IgG2b-FITC (GAM IgG 2b TITC) (1:20 dilution in wash solution) for 20 min, at 4°C. In parallel, cells were added with only second antibody. Wash step was repeated. Pellets were resuspended with 100 µl 4% PFA for flow cytometric analysis. Cytochrome c was detected through the FL1 channel with an excitation wavelength of 488 nm.

2.2.6. Cytosolic and mitochondrial extracts preparation
Approximately $2.5 \times 10^7$ cells were required for each preparation. The cell suspension was transferred into a 50 ml centrifuge tube. The cells were pelleted by centrifugation at 600 x g for 5 min, at 4° C. The cell pellets were washed twice with 20 ml of cold PBS, pH 7.4. It is important to remove all PBS from the cell pellet. The cell pellets were resuspended with 800 µl fractionation buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitor cocktail), and incubated for 20 min, on ice. During this time the tubes were tapped from time to time in order to resuspend the cell pellet.

Cells were then disrupted by 25 strokes with Dounce homogenizer. To establish the optimum conditions for cell homogenization, the trypan blue exclusion assay, which discriminates between broken (stained) cells and intact cells (unstained), can be used. For the trypan blue exclusion test, a 0.4% solution of trypan blue in PBS was diluted 1: 10 with the cell suspension and was examined under the microscope. After dounce homogenization, the cell homogenates were transferred to an Eppendorf tube and nuclei, unbroken cells, and large debris were removed by centrifugation at 800 x g for 10 min, at 4° C. Supernatants containing mitochondria were transferred to a new Eppendorf tube and further centrifuged at 10,000 x g for 25 min, at 4° C. The resulting supernatants were saved as cytosolic extracts at -70° C until further analysis. The mitochondrial pellets were lysed with 100 µl of fractionation buffer. Samples were vortexed from time to time during the 20 min incubation period on ice. This was mitochondrial fraction and could be stored at -70° C until further analysis.

### 2.2.7. Western blot analysis

#### 2.2.7.1. Cell lysis

After incubation with apoptosis-inducing stimuli for indicated length of time, aliquot of $1 \times 10^7$ cells were transferred to 50 ml centrifuge tube and centrifuged at 1300 rpm for 5 min, at 4° C. Pellets were suspended with 2 times cold PBS and centrifuged as described above. Pellets were solubilized in 500 µl lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 7.5, 10% Glycerol, 0.5% Triton X-100, and protease inhibitor cocktail) and incubated for 30 min. Samples were vortexed from time to time during the 30 min
incubation period on ice. After lysis, samples were centrifuged at 14000 rpm for 15 min, at 4°C. The resulting supernatants containing extraction of proteins were stored at -70°C until further analysis.

2.2.7.2. Quantification of protein

Quantification of protein was carried out with the BCA reagent. The reagent combines the well-known reduction of Cu^{2+} by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+}) using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water soluble complex was detected with spectrophotometer at 562 nm. Standard working curve was obtained from the different dilution of BSA.

2.2.7.3. Electrophoreses

A. Loading and running samples

40 µg protein per well were mixed with 5x SDS loading buffer (50 mM Tris-Cl (pH 6.8), 1% SDS, 0.05% bromophenol blue, 5% glycerol, and 10% β-mercaptoethanol) and boiled for 5 min to denature the proteins. Precast gel was mounted in the electrophoresis apparatus and wells were washed immediately with deionized water. Running buffer (25 mM Tris, 250 mM glycine, 0.5% SDS) was added to the top and bottom reservoirs. Any bubble was removed that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Samples were loaded into the bottom of the wells and molecule weight marker was loaded in the same time.

The Electrophoresis Apparatus was connected to an electric power supply. 80 V was applied to the gel and the gel was run until the bromophenol blue reaches the bottom of the resolving gel (about 3 hours). The power supply was turned off.

B. Blotting
The graphite plates of Semi Dry Apparatus were rinsed with distilled water and any bead of liquid was wiped off. Gloves were worn. Six pieces of Gel-Blotting Paper and one piece of Nitrocellulose Membrane were cut to the exact size of the SDS-polyacrylamide gel. If the paper or membrane was larger than the gel, there was a good chance that the overhanging edges of the paper and the filter would touch, causing a short circuit that would prevent the transfer of protein from the gel. One corner of the filter was marked with a soft-lead pencil. The one piece of nitrocellulose membrane and the six piece of paper were soaked in a shallow tray containing a small amount of transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS, 20% methanol). The three sheets of paper and one piece of the nitrocellulose membrane were placed on the bench (the bottom was the anode) one on top of the other so that they were exactly aligned. The glass plates holding the SDS-polyacrylamide gel were removed from the electrophoresis tank and the gel was placed exactly on the top of the nitrocellulose membrane. The final three sheets of paper were placed on the gel. Any air bubbles was squeezed out with a glass pipette. The electrical leads were connected and 120 mA current was applied for a period of 145 min.

2.2.7.4. Immunoblotting

A. Block non-specific binding

The nitrocellulose membrane was taken out of the Semi Dry Transfer Apparatus, soaked into blocking buffer (5% nonfat dried milk, 0.1% Tween 20 and PBS) and incubated for 1 hour at room temperature with gentle agitation on a platform shaker or incubated overnight at 4°C. This step was used for blocking unspecific bindings. After that, the membrane was washed for 10 min with wash buffer (PBS-T) (0.1% Tween and PBS). The wash step was repeated three times.

B. Binding of the primary antibody

Primary antibodies were suitable diluted in 10-15 ml with wash buffer: mouse anti-cytochrome c monoclonal antibody 1:1000; mouse anti-cytochrome oxidase (subunit IV) (COX IV) monoclonal antibody 1:1000; mouse anti-caspase-8/FLICE monoclonal antibody C15 1:5 dilution of hybridoma supernatant; mouse anti-caspase-3/CPP32 monoclonal antibody 1:1000; mouse anti-PARP monoclonal antibody 1:2500; mouse anti-
Fas ligand/CD95L monoclonal antibody 1:1000; mouse ant-RIP monoclonal antibody 1:1000; mouse ant-FADD monoclonal antibody 1:250; rabbit anti-bid polyclonal antibody 1:1000; mouse ant-Bcl-2 monoclonal antibody 1:500; mouse anti-β-actin antibody 1:5000. The membrane was incubated with the primary antibody as described above at room temperature for 1 hour or overnight at 4 °C on a platform shaker with gentle agitation. After that, the membrane was washed for three times with PBS-T as described above.

C. Binding of the secondary antibody

Second antibodies were suitable diluted in 15ml with wash buffer: The horseradish peroxidase-conjugated anti-mouse IgG or horseradish peroxidase-conjugated anti-rabbit IgG 1:5000. The membrane was incubated with the secondary antibody at room temperature for 1 hour on a platform shaker. After that, the membrane was washed for three times as described above.

2.2.7.5. Detection

The ECL detection reagents were take out of bottle, mixed at an equal volume of detection solution 1 with detection solution 2 to give sufficient liquid to cover the membrane and incubated for 1 min, at room temperature. The blots, protein side up, were placed in the film cassette. All the wok should be done as quickly as possible. The film was developed on X-ray Film Processor (Hyper Processor) after an appropriate length of exposure time. Sometimes it may take one or several hours to see the faint cleaved fragments.

2.2.7.6. Reprobing membranes

For protein loading equivalent control, the membrane was stripped and reprobed. The membrane was washed with 20 ml PBS-T for 30 min, at room temperature on a platform shaker and then submerged in 20 ml stripping buffer (0.2 M NaOH) for 10 min, at room temperature. The membrane washed with deionized water for 10 min and with PBS-T for 2 x 15 min. After that, the membrane was undergone blocking, immunoblotting and detection procedure as described above. But if the reprobed protein was β-actin, the blocking step can be skipped.
2.2.8. Statistical analysis

All experiments were performed in triplicate unless otherwise noted; results are expressed as mean ± standard deviation.
3. Results

3.1. Induction of apoptosis by anticancer drugs

3.1.1. Apoptosis induced by treatment of Jurkat cells with etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin and methotrexate

3.1.1.1. Dose and time kinetics for anticancer drug treatment

To investigate whether there are difference of induction of apoptosis by different anticancer drugs in dose and time, Jurkat cells were treated with various doses of anticancer drugs for different time points. All conditions were performed in triplicate. The cells were dual-stained with annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry. Cells without stimuli were used as control.

These results demonstrate that there was a time-dependent fashion in annexin V/PI double positive cells induced by etoposide, cytarabine (Ara-c), 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate as shown in Figure 1. Apoptosis in response to etoposide and cytarabine was early, whereas apoptosis in response to 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate was significantly late. At 12 hours, the observed maximal apoptosis for etoposide at the maximal concentration, 100 µg/ml, was about 50%, for cytarabine at the concentration of 1-10 µg/ml was greater than 35%. In contrast, at same time point, the observed maximal apoptosis for 4-hydroxy-cyclophosphamide, doxorubicin, and methotrexate at their maximal concentration (4-hydroxy-cyclophosphamide 5µg/ml, doxorubicin 1µg/ml, methotrexate 500µg/ml) was no more than 15%. Until 36 hours, the observed apoptosis for 4-hydroxoy-cyclophosphamide at maximal concentration was greater than 50%; until 24 hours, the observed apoptosis for doxorubicin at maximal concentration was still less than 35%.

This data suggested that the effect of etoposide and cytarabine was earlier than 4-hydroxy-cyclophosphamide, doxorubicin and methotrexate. Collectively, these results demonstrate
Figure 1. Different anticancer drugs induced apoptosis in a different time and dose fashion. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto), cytarabine (Ara-c), 4-hydroxy-cyclophosphamide (4-HCP), doxorubicin (Doxo) and methotrexate (MTX) respectively at the indicated time points and doses. Apoptosis was assessed by FACS analysis of annexin V and propidium iodide double staining.
that etoposide and cytarabine were early acting drugs, whereas 4-hydroxy-
cyclophosphamide, doxorubicin and methotrexate were late acting drugs.

The cells underwent dose-dependent apoptosis in response to anticancer drugs, except for
cytarabine. The doses of etoposide, 10-100µg/ml induced a similar amount of apoptosis
(about 50%), at 12 hours. The maximum apoptosis induced by 4-hydroxy-
cyclophosphamide at maximum concentration 5µg/ml at 36 hours was nearly 55%. The
maximum apoptosis induced by doxorubicin at maximum concentration 1µg/ml at 36
hours was 70%. The amount of apoptosis induced by methotrexate at concentration of 1-
500µg/ml at 36 hours was not big different, from 25% to 32%.

Interestingly, we found an exceptional pattern of dose effect relationship for cytarabine.
Higher concentration of cytarabine induced less apoptosis, whereas lower concentration
induced more apoptosis. At 12 hours, e.g. a concentration of 1µg/ml, 5µg/ml or 10µg/ml
produced: about 35% apoptotic cells, whereas a concentration of 100µg/ml and 500µg/ml
produced less than 20% apoptotic cells. The data indicated different mechanisms of
apoptosis induction at low or high concentration.

3.1.1.2. Comparison of different apoptotic signs induced by different
drugs

Since we found differences in time of apoptosis induction, we further investigated that the
different drugs induced different signs of apoptosis. For this, cells were treated with the
drugs in one dose and different time points and apoptosis was detected with forward/side
scatter, annexin V/PI double staining and DNA fragmentation by flow cytometry.

Morphological, biochemical, and molecular changes that occur during apoptosis serve as
specific markers to identify apoptotic cells by cytometry. An early event of apoptosis is
dehydration, which leads to cell shrinkage. This change is reflected by an alteration in the
way the cells scatter the light of the laser beam in a flow cytometer. The intensity of light
scattered by apoptotic cells in a forward direction along the laser beam, which correlates
with cell size, is diminished. Late apoptotic cells or individual apoptotic bodies are
characterized by a low intensity of the forward scatter signal. Chromatin condensation,
Figure 2. Detection of early drug-induced apoptosis by different apoptosis assays. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with epotoside (Eto 30µg/ml) and cytarabine (Ara-c 30µg/ml) respectively at 37 °C in a CO₂ incubator for the indicated time points. (A) Dead cells were observed according to the morphologic and granularity changes on flow cytometry. (B) Hypodiploid cells were detected by flow cytometry after the cell's nuclei were stained with propidium iodide; (C) Annexin V positive cells were measured by flow cytometry after the cells were stained with annexin V. (D) PI positive cells were assessed by flow cytometry after the cells were stained with propidium iodide.
which often is followed by nuclear fragmentation, is another characteristic feature of apoptosis. These changes increase the propensity of the cell to refract light, which may be manifested by a transient increase in the intensity of light scattered at a 90° angle in the direction of the laser beam (side scatter). Analysis of the forward and side light scatter signals of the cells thus provide the means to identify apoptotic cells by flow cytometry on the basis of their properties without measurement of fluorescence.

From the FSC versus SSC dot plot of FACSCarlibur, the population of apoptosis cells was easily distinguished from the population of live cells. The population of apoptosis cells was formed with a low forward scatter (FSC) and high side scatter (SSC) profile, which is a characteristic of apoptotic cells. More apoptotic cells appeared in 12 hours for early drugs, whereas apoptotic cells appeared in 24 hours for late drugs as shown in Figure 2A, 3A. There was no difference in the apoptotic phenotype induced by different drugs in forward/side scatter analysis.

DNA fragmentation is so characteristic an event of apoptosis that it is considered to be a hallmark of this mode of cell death. Initially, DNA is cleaved at the sites of attachment of chromatin loops to the nuclear matrix. Which results in discrete 50- to 300-kb size fragments. Subsequently, although not in every cell type, DNA is cleaved at the internucleosomal sections. As a result, the products of DNA cleavage are discontinuous, nucleosomal and oligonucleosomal DNA fragments of approximately 180 bp and multiples of this size. The analysis of the cellular DNA content of apoptotic cells from which degraded DNA was extracted reveals them as cells with fractional DNA content, represented by the sub-G1 peak on DNA content frequency histograms. This approach is currently the most frequently used in flow cytometry to identify and quantify apoptotic cells.

The DNA fragmentation can be measured by analysis of propidium iodide stained nuclei on flow cytometry. The reduced DNA content of apoptotic nuclei resulted in a unequivocal hypodiploid DNA (sub G1) peak, which was easily discriminable from the narrow peak of cells with normal (diploid) DNA content in red fluorescence channels. The percentage of apoptotic nuclei was quantified. This assay is more sensitive than forward/side scatter assay. In the same time points (except for etoposide in 24 hours), the percentage of apoptosis was detected by this assay was higher than that detected by forward/side scatter
Figure 3. Detection of late drug-induced apoptosis by different apoptosis assays. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml) and methotrexate (MTX 30µg/ml) respectively at 37°C in a CO₂ incubator for the indicated time points. (A) Dead cells were observed according to the morphologic and granularity changes on flow cytometry. (B) Hypodiploid cells were detected by flow cytometry after the cell's nuclei were stained with propidium iodide; (C) Annexin V positive cells were measured by flow cytometry after the cells were stained with annexin V. (D) PI positive cells were assessed by flow cytometry after the cells were stained with propidium iodide.
as shown in Figure 2A, 2B, 3A, 3B. For etoposide in 24 hours, more than 85% cell death (forward/side scatter), in this situation this assay is not sensitive. From the results of doxorubicin in 24 hours, the percentage of dead cells was lower than 70% (forward/side scatter). In this situation, this assay was also more sensitive than forward/side scatter.

In live cells the plasma membrane phospholipids, phosphatidylcholine, and spingomyelin are exposed on the external leaflet of the lipid bilayer while phophatidylserine is almost exclusively on the inner surface. The phospholipid asymmetry leading to exposure of phosphatidylserine on the outside of the plasma membrane occurs early during apoptosis. This change is detected by annexin V-fluorescein isothiocyanate (FITC) conjugate, which preferentially binds to negatively charged phospholipids such as phosphatidylserine. The staining is done in combination with propidium iodide (PI), which is excluded from live and early apoptotic cells but stains DNA and RAN in late apoptotic and necrotic cells, the plasma membranes of which are disrupted. In this experiment system, annexin V staining assay for detecting apoptosis was most sensitive, especially for etoposide as shown in Fig. 2C, 3C. At 12 hours, Annexin V positive cells-induced by etoposide was 80%, which was two times greater than morphologic changes (FSC/SSC) at the same time point.

The duration of apoptosis is relatively short and variable depending on cell type, inducer of apoptosis. Each of the methods presented above has its advantages and suffers limitations. So combination of several apoptosis assay can provide a more definite identification of apoptotic cells.

Among these four assays detecting apoptosis, a similar pattern of anticancer drug-induced apoptosis was found that the number of apoptotic cells increased over the increasing time period. The results further confirm that the etoposide and cytarabine effect was earlier than 4-hydroxy-cyclophosphamide, doxorubicin and methotrexate.

3.1.1.3. **Annexin V single positive cells appear through treatment with early anticancer drugs, but not with late anticancer drugs**

Previous studies showed that after cells were stimulated by anticancer drugs and agonistic anti-CD95-antibodies, through staining cells with annexin V-FITC and PI, it is possible to
Figure 4. Difference of annexin V single positive for early and late drugs. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml), etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml) and methotrexate (MTX 30µg/ml) respectively at 37°C in a CO₂ incubator for the indicated time points. (A) Annexin V positive cells in gate 1 were assessed by flow cytometry after the cells were double stained with annexin V and propidium iodide. (B) Flow cytometry profiles of forward scatter versus side scatter and propidium iodide staining versus annexin V staining.
detect live, nonapoptotic cells (annexin V/PI double negative), early apoptotic cells (annexin V single positive), and late apoptotic or necrotic cells (annexin V/PI double positive) by flow cytometry. But in this experiment system, the annexin V single positive profile of early and late drugs was different. After cells were stained with annexin V and PI, several subpopulation were visualized in the quadrant profile. In the FSC/SSC dot plot, the apoptotic cells were nearly same for anti-CD95 in 3h, 6h, compared with etoposide in 6 hours, 12 hours and 4-hydroxy-cyclophosphamide in 12 hours, 48 hours, whereas in the annexin V versus PI quadrant profile, there was big difference between etoposide and 4-hydroxy-cyclophosphamide in annexin V single positive. Etoposide induces more annexin V single positive (upper left quadrant) like anti-CD95. Especially in the early time points such as in 3 hours (anti-CD95) and in 6 hours (etoposide), annexin V single positive much greater than annexin V/PI double positive (upper right quadrant). In contrast, 4-hydroxy-cyclophosphamide induce much greater annexin V/PI double positive than single positive in 12 hours and 48 hours as shown in Figure 4. This means that PS externalization is an early event in apoptosis induced by anti-CD95 and etoposide, In contrast, in apoptosis induced by late drugs, PS externalization occurs as the same time as disruption of membrane integrity.

3.1.2. Comparison of apoptosis induced by anticancer drugs with apoptosis induced by death receptor signaling and γ-radiation

3.1.2.1. Agonistic anti-CD95 antibody-induced apoptosis

One of the best-defined apoptotic pathways is mediated by the death receptor CD95 (Apo-1/Fas). In order to compare anti-CD95 antibody-induced apoptosis with anticancer drug-induced apoptosis, we investigated the agonist anti-CD95 antibody induce apoptosis using the same approach.

Jurkat cells were treated with the agonist anti-CD95 Ig3 antibody plus protein A as shown in Figure 5 and apoptosis was detected with forward/side scatter, annexin V/PI double staining on flow cytometry FACSCalibur. Agonist anti-CD95 antibody induce apoptosis was more rapidly and efficiently than anticancer drugs. In 6 hours more than 40% cells underwent apoptotic morphologic changes. In this situation, the pattern of membrane
Figure 5. Detection of anti-CD95-induced apoptosis by different apoptosis assays. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) at 37°C in a CO2 incubator for the indicated time points. (A) Dead cells were observed according to the morphologic and granularity changes on flow cytometry. (B) Annexin V positive cells were measured by flow cytometry after the cells were stained with annexin V. (C) PI positive cells were assessed by flow cytometry after the cells were stained with propidium iodide.
Figure 6. Detection of γ-radiation-induced apoptosis by different apoptosis assays.
Jurkat cells were cultured in normal growth medium and then either left untreated (×) or treated with γ-radiation 20 Gy (■), γ-radiation 10 Gy (□), γ-radiation 5 Gy (▲). After irradiation, the cells were incubated at 37°C in a CO₂ incubator for the indicated time points. (A) Dead cells were observed according to the morphologic and granularity changes on flow cytometry. (B) Hypodiploid cells were detected by flow cytometry after the cell's nuclei were stained with propidium iodide; (C) Annexin V positive cells were measured by flow cytometry after the cells were stained with annexin V-FITC. (D) PI positive cells were assessed by flow cytometry after the cells were stained with propidium iodide.
integrity loss was as the same as that of morphologic changes. In these three assays detecting apoptosis, annexin V positive was most sensitive. In 6 hours nearly 100% cells were annexin V positive. It suggested that in anti-CD95 antibody-induced apoptosis, PS externalization was a very early event.

3.1.2.2. \( \gamma \)-radiation-induced apoptosis

\( \gamma \)-radiation is a common procedure to treat cancer patients in the clinic. In the present study, we included the \( \gamma \)-radiation-induced apoptosis to compare with anticancer drug-induced apoptosis.

Jurkat cells were treated with \( \gamma \)-radiation with the dose used in the clinic patients. After irradiation, the cells were incubated at 37°C and 5% CO\(_2\) for different time points as shown in Figure 6. \( \gamma \)-radiation-induced apoptosis was time- and dose-dependent. These apoptotic features were like those induced by late drugs. PS externalization (annexin V positive) appears at the similar time point as morphologic changes, DNA fragmentation and membrane integrity loss. In other words, PS externalization was not an early event in \( \gamma \)-radiation-induced apoptosis.

3.2. Expression of death receptor associated molecules induced by anticancer drugs

Previous studies found that the CD95 system, which is known as a key regulator of the immune system, also mediated drug-induced apoptosis of leukemia. To investigate whether anticancer drug treatment of Jurkat cells stimulate expression of death receptor associated molecules, we detected CD95 ligand (Apo-1/ Fas ligand), FADD (Fas-associated death domain protein) and RIP (receptor interacting protein) by western blot.

Jurkat cells were either left untreated or treated with etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate and anti-CD95 antibody plus protein A respectively as described in Figure 7. At the indicated time points, cells were collected. Protein extracts were prepared and fractionated by SDS-PAGE.
Figure 7. Expression of death receptor associated molecules induced by anticancer drugs and anti-CD95 antibody. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml), methotrexate (MTX 30µg/ml) and anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) respectively at 37°C in a CO₂ incubator for the indicated time points. Total cellular protein 40µg per lane was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with antibodies that recognize mouse anti-Fas ligand monoclonal antibody, mouse anti-FADD monoclonal antibody and mouse anti-RIP monoclonal antibody. Arrows indicate the full-length molecules or specific cleavage fragments. Equal protein loading was confirmed by reprobing the stripped blots with mouse anti-β-actin monoclonal antibody. The results shown are representative of at least three independent experiments.
CD95L, 45kDa, and FADD, 24kDa, and RIP, 74kDa, expression were detected by specific antibody. The results showed anticancer drugs, similar to anti-CD95, upregulate CD95L and FADD expression. For early drugs, at 6 hours CD95L and FADD increased markedly, for anti-CD95 antibody, the increase of the expression appeared as early as 3 hours, for late drugs, the increase appeared at 12 hours. Cleavage of RIP was observed in Figure 7. Among the stimuli, anti-CD95 antibody and etoposide most efficiently cleave RIP in this system. These findings suggest CD95 system take part in anticancer drug-inducing apoptosis in Jurkat cells, at least in part.

3.3. Caspases activation by anticancer drugs

3.3.1. Caspase-8, caspase-3 activation and PARP cleavage

Caspases comprise a family of different cysteine protease that are synthesized as inactive zymogens and converted to an active complex composed of several heterodimeric subunits. To investigate differences in caspase activation by different anticancer drugs, we analyzed activation of caspase-8, caspase-3 and cleavage of caspase substrate PARP. We monitored the processing of procaspases in immunoblot analyses using antibodies specific to individual proteases. Jurkat cells were either left untreated or treated with etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate and anti-CD95 antibody plus protein A respectively as described in Figure 8. At the indicated time points, cells were collected. Protein extracts were prepared and fractionated by SDS-PAGE.

We first investigated the processing of caspase-8, the most proximal caspase during CD-95-mediated apoptosis. Caspase-8 is synthesized as an inactive precursor of 55 kDa, which was detected a double protein band, representing the isoforms porcaspase-8a and porcaspase-8b, and, following formation of a 43-kDa, 41-kDa intermediate cleavage product, processed to a p18 heterodimer.

Treatment of cells with anticancer drugs resulted in the conversion of the inactive 32-kDa caspase-3 precursor to the proteolytically cleaved p17 subunit, indicating that caspase-3 was activated during drug-induced apoptosis. In a detailed time-response assay, we further
Figure 8. Caspase-8, caspase-3 and PARP are cleaved with different kinetics by early and late drugs. Jurkat cells were cultured in normal growth medium and then either left untreated (M, as control) or treated with etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml), methotrexate (MTX 30µg/ml) and anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) respectively for the indicated time points. Total cellular protein 40µg per lane was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with the respective mAb. Arrows indicated the position of full length caspase-8 which exist as two isoforms and it's cleavage fragment p43, p41 and p18, full length caspase-3 and it's cleavage fragment p17, full length PARP and it's cleavage fragment p85. Equal protein loading was confirmed by reprobing the stripped blots with mouse anti-β-actin monoclonal antibody.
measured the cleavage of poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair, which is specifically cleaved by caspase-3 during apoptosis. Figure 8 demonstrates that PARP, a 116-kDa protein, was cleaved into the characteristic 85-kDa fragment in the course of treatment of anticancer drugs and anti-CD95 antibody.

The cleavage pattern of the individual caspase and PARP did not differ between the anticancer drugs and anti-CD95 antibody stimulation. However, corresponding to the different kinetics of apoptosis, Anti-CD95 antibody-induced caspase activation was more rapid and efficient than etoposide and cytarabine; 4-hydroxy-cyclophosphamide-, doxorubicin- and methotrexate-induced caspase activation occurs latest. For etoposide and anti-CD95 antibody-induced caspase-8 cleavage, the active fragment p18 was clearly presented on blot, but for cytarabine and late drugs, although the p43 and p41 intermediate cleavage products were clear on the blot, the p18 was not appear on the blot. The reason could be that the time points are not appropriate for these drugs. For early drug-induced caspase-3 activation, the cleaved fragment p17 was clearly presented on the blot. For late drug-induced caspase-3 activation, the faint band of p17 also can be seen on the blot. Taken together, these data showed all anticancer drugs activated caspases.

3.3.2. The caspase inhibitor zVAD-fmk blocks drug-induced apoptosis

To determine whether anticancer drug-induced apoptosis was dependent upon caspases activation, cells were pretreated with zVAD-fmk, abroad peptide caspase inhibitor, and then exposed to the stimuli, etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate or anti-CD95 antibody, respectively, in the indicated dose and time points as shown in figure 9, 10. In the parallel experiment, cells were treated only with stimuli. Apoptosis of the cells was determined by forward/side scatter, annexin V/PI double staining and quantification of DNA fragmentation on flow cytometry FACSCalibur. Similar to anti-CD95, early drug (etoposide, cytarabine)-induced apoptosis was completely inhibited by zVAD-fmk. Late drug (4-hydroxy-cyclophosphamide, doxorubicin, methotrexate)- induced apoptosis was completely inhibited by zVAD in early time point; whereas in late time point only partially inhibited. The pan-caspase inhibitor zVAD-fmk blocked not only externalization of phosphatidylserine (PS) and DNA
Figure 9. zVAD-fmk inhibits early drug-induced apoptosis. (A) Jurkat cells were cultured in normal growth medium and then either left untreated (M, medium) or treated with etoposide (Eto 30µg/ml) and cytarabine (Ara-c 30µg/ml) in the presence or absence of zVAD-fmk (100µM) respectively at 37°C in a CO₂ incubator for the indicated time points. Dead cells, DNA fragmentation, annexin V and PI positive cells were assessed by flow cytometry as described in Materials and Methods. Data were the mean of triplicates with standard deviation. Similar results were obtained in 3 separated experiments. (B) Representative profiles of DNA fragmentation induced by anticancer early drugs in the presence or absence of zVAD-fmk (100µM) for the indicated time points.
Figure 10. zVAD-fmk inhibits late drug-induced apoptosis. (A) Jurkat cells were cultured in normal growth medium and then either left untreated (medium) or treated with 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml) and methotrexate (MTX 30 µg/ml) in the presence or absence of zVAD-fmk (100µM) respectively at 37°C in a CO₂ incubator for the indicated time points. Dead cells, DNA fragmentation, annexin V and PI positive cells were assessed by flow cytometry. Data were the mean of triplicates with standard deviation. Similar results were obtained in 3 separated experiments. (B) Representative profiles of DNA fragmentation induced by anticancer drugs alone or in the presence of zVAD-fmk (100µM) for the indicated time points.
fragmentation, but also cell morphologic changes and loss of membrane integrity. The different apoptosis assays presented the same patterns. The ability of zVAD-fmk to effectively inhibit caspase function suggested that anticancer drug-induced apoptosis is a caspase-mediated event. This means that in all drugs investigated induction of apoptosis largely depends on activation of caspases. However partial inhibition at late time points indicated an involvement of additional caspase independent death pathways.

3.4. Disturbance of mitochondrial function induced by anticancer drugs

3.4.1. Alterations of mitochondrial transmembrane potential ($\Delta \Psi_m$)

3.4.1.1. Time course of drugs-induced $\Delta \Psi_m$ loss

Mitochondrial alterations are a central coordinating event in apoptosis signaling. In order to investigate difference in apoptosis induction by different drugs, we analyzed induction of mitochondrial membrane changes. One of these membrane changes involves disruption of the inner membrane transmembrane potential ($\Delta \Psi_m$) through the opening of permeability transition pores, known as the mitochondrial permeability transition pore (PT). After pore opening, the normally impermeable inner membrane becomes permeable to ions and solutes, and the negatively charged environment of the matrix is lost. Intermembrane permeabilization can be assessed indirectly, by determining a reduction in the $\Delta \Psi_m$. For this, cells are incubated with lipophilic cationic fluorochromes such as DiOC6(3) (3,3′dihexyloxycarbocyanine iodide), a lipophilic cation, which accumulates in the mitochondria matrix, driven by the electrochemical gradient. According to the Nernst equation, every 61.5-mV increase in $\Delta \Psi_m$ (usually 120–170 mV, negative inside) leads to a 10-fold increase in cation concentration in the mitochondrial matrix. Therefore, the concentration of such cation is two to three logs higher in the matrix than in the cytosol. A reduction in fluorescence intensity in the emission spectrum, as measured by flow cytometry, is then interpreted as an indication of $\Delta \Psi_m$ dissipation.
Figure 11. Anticancer drugs and anti-CD95 antibody cause ΔΨₘ disruption. (A) Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto 30μg/ml), cytarabine (Ara-c 30μg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3μg/ml), doxorubicin (Doxo 0.15μg/ml), methotrexate (MTX 30μg/ml) and anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) respectively at 37°C in a CO₂ incubator for the indicated time points. ΔΨₘ loss was determined using 40 nM DiOC₆(3) on flow cytometry. Data were the mean of triplicates with standard deviations. Similar results were obtained in 3 separated experiments. (B) Representative profiles of ΔΨₘ loss induced by the anticancer drugs.
Figure 12. Effects of zVAD-fmk on ΔΨ<sub>m</sub> alteration induced by anticancer drugs and anti-CD95 antibody. (A) Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml), methotrexate (MTX 30µg/ml) and anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) in the presence or absence of zVAD-fmk respectively at 37°C in a CO2 incubator for the indicated time points. ΔΨ<sub>m</sub> alteration was determined using 40 nM DiOC<sub>6</sub>(3) on flow cytrometry as described in Materials and Methods. Data were the mean of triplicates with standard deviation. Similar results were obtained in 3 separated experiments. (B) Representative profiles of ΔΨ<sub>m</sub> alteration induced by etoposide and 4-hydroxy-cyclophosphamide in presence or absence of zVAD-fmk.
Cells were treated with etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate and anti-CD95 in indicated doses. At the appropriate time point, the cells were stained with DiOC$_6$(3). DiOC$_6$(3) targets to the negative environment of the matrix in metabolically active mitochondria, where it emits intensely in the FL1 channel during apoptosis, dissipation of $\Delta \Psi_m$ leads to leakage of DiOC$_6$(3) from the matrix, which can be measured by flow cytometry a decrease in the fluorescence intensity of DiOC$_6$(3). This is visualized as $\Delta \Psi_m$ low cell subpopulation, which shift to the left in the FACS histogram. In the absence of treatment (medium alone), $\Delta \Psi_m$ fluorescence was apparent ($\Delta \Psi_m$ high cells), indicating retention of the dye in mitochondria and an intact $\Delta \Psi_m$ as shown in Figure 11. Jurkat cells treated with stimuli exhibited a reduction in the retention of DiOC$_6$(3) seen as a shift in the population from DiOC$_6$(3) high cells to DiOC$_6$(3) low cells. This shift indicated a compromise in $\Delta \Psi_m$ integrity. Generally, anti-CD95 antibody-induced $\Delta \Psi_m$ loss was more rapid and efficient. Anti-CD95 antibody-induced $\Delta \Psi_m$ low cells nearly equal to annexin V positive cells in live cells in 3 hours, approximate 45%. In contrast, etoposide-induced $\Delta \Psi_m$ low cells was much higher (47%) than annexin V positive cells (23%) in live cells in 6 hours. These results suggest that maybe etoposide induces breakdown of $\Delta \Psi_m$ in a different mechanism from anti-CD95. On the other hand, it means etoposide-induced $\Delta \Psi_m$ loss is the same early event of apoptosis as externalization of PS.

### 3.4.1.2. Caspase inhibitor abrogated anti-CD95-induced $\Delta \Psi_m$ loss but not drug-mediated $\Delta \Psi_m$ loss

To investigate whether the mitochondria transmembrane potential disruption was caspase-dependent, in a parallel experiment, the cells were pretreated with zVAD-fmk. And then the cells were treated with various stimuli: etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, methotrexate and anti-CD95 antibody in the indicated dose and time points as shown in Figure 12.

The data showed that zVAD-fmk completely abrogated $\Delta \Psi_m$ collapse induced by anti-CD95 from 3 hours to 9 hours. The pan caspase inhibitor zVAD-fmk partially blocked $\Delta \Psi_m$ loss induced by early drugs (etoposide, cytarabine) in 6 hours and 12 hours, but not in 24 hours; whereas zVAD-fmk enhanced $\Delta \Psi_m$ disruption induced by late drugs (4-hydroxy-cyclophosphamide, doxorubicin, methotrexate). Thus, in all drugs analyzed, $\Delta \Psi_m$ reduction
Figure 13. Cytochrome c release from mitochondria into cytosol induced by anticancer drugs and anti-CD95 antibody. (A) Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml), methotrexate (MTX 30µg/ml) or anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) at 37°C in a CO₂ incubator for the indicated time points. Total cellular protein 40µg per lane was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with antibodies that recognize mouse anti-cytochrome c monoclonal antibody. Arrows indicate the position of cytochrome c or β-actin. Equal protein loading was confirmed by reprobing the stripped blots with mouse anti-β-actin monoclonal antibody. The results shown are representative of at least three independent experiments.
Figure 14. Cytochrome c release quantified by flow cytometry. (A) Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml), methotrexate (MTX 30µg/ml) and anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) respectively at 37°C in a CO₂ incubator for the indicated time points. The cells were harvested and stained with anti-cytochrome c. Control cells were stained with isotype-matched antibody or secondary antibody alone. The cytochrome c low cells were quantified by flow cytometry using CellQuest software as described in Materials and Methods. The data were the mean of triplicates with standard deviation. (B) Representative Profiles of cytochrome c release induced by etoposide during the incubation times.
was not caspase dependent, while in anti-CD95 antibody-induced apoptosis, $\Delta \Psi_m$ loss was caspase dependent.

### 3.4.2. Cytochrome c release induced by anticancer drugs

Recent evidence has demonstrated that mitochondria participate in the execution of apoptosis by release of cytochrome c. Binding of cytochrome c to Apaf-1 results in the cleavage of procaspase-9 or other caspase, which in turn activate caspase-3. To directly examine mitochondrial involvement, release of cytochrome c into the cytoplasm was determined.

After Jurkat cells were treated with stimuli, mitochondria fraction was isolated from cytosol. The cytochrome c was detected in mitochondria fraction and cytosol fraction by western blot. Similar to apoptosis pattern, anticancer drugs- and anti-CD95 induced cytochrome c release was time-dependent. Figure 13 Showed cytochrome c increase in cytosol over the course of incubation time; in the same time, cytochrome c decrease gradually in mitochondria. These results suggest $\Delta \Psi_m$ loss induced by anticancer drugs and anti-CD95 lead to cytochrome c translocation from mitochondria into cytosol. In the same time, cytochrome c release was quantified by flow cytrometry as shown in Figure 14. The pattern was similar to the results of Western Blot.

### 3.4.3. Anticancer drugs cleave Bid and Bcl-2

Bid, a member of the Bcl-2 family, was recently identified as a physiological substrate of caspase-8 that is responsible for mitochondrial damage. Similar to anti-CD95, anticancer drugs induced proteolysis of Bid, 24kDa, into the mature p15 Bid fragment as shown in figure 16. Although all cancer drugs induced Bid cleavage, etoposide and anti-CD95 were much more efficient as indicated.

The anti-apoptosis protein Bcl-2 reported to convert to proapoptosis regulator following cleavage by caspase-3. Figure 16 showed the Bcl-2 protein, 26kDa, was cleaved into p21 fragmentation by anti-CD95 and anticancer drugs. The pattern was like Bid cleavage,
Figure 15. Bid and Bcl-2 cleaved by anticancer drugs and anti-CD95 antibody. Jurkat cells were cultured in normal growth medium and then either left untreated (medium, as control) or treated with etoposide (Eto 30µg/ml), cytarabine (Ara-c 30µg/ml), 4-hydroxy-cyclophosphamide (4-HCP 3µg/ml), doxorubicin (Doxo 0.15µg/ml), methotrexate (MTX 30µg/ml) and anti-CD95 antibody (20ng/ml) plus protein A (2.5ng/ml) respectively at 37°C in a CO₂ incubator for the indicated time points. Total cellular protein 40µg per lane was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with rabbit anti-bid polyclonal antibody and mouse anti-Bcl-2 monoclonal antibody. Arrows indicated the position of full length bid, bcl-2 and their cleavage fragments. Equal protein loading was confirmed by reprobing the stripped blots with mouse anti-β-actin monoclonal antibody.
etoposide and anti-CD95 were much more efficient; Bcl-2 was less strongly cleaved by Cytarabine, cyclophosphamide, doxorubicin and methotrexate.
4. Discussion

Etoposide, cytarabine, cyclophosphamide, doxorubicin, and methotrexate are commonly used antileukemic drugs. Despite their generalized use for more than 30 years, their mechanisms of cytotoxicity have been a long-time matter of debate. During the past 20 years, several hypotheses have been formulated, including DNA intercalation/binding, inhibition of topoisomerase II, free-radical generation, and damage to cell membranes. In recent years, it has been found that anticancer drugs induce the intrinsic program of cell death known as apoptosis (Arends et al., 1991; Mesner et al., 1997).

The molecular signaling pathways which are initiated in response chemotherapy-induced cellular damage, and lead to the eventual apoptotic death of the cell, are largely undefined. Although the ability to induce apoptosis has been examined in a variety of human tumor cell types using one or several anticancer drugs, the molecular steps of anticancer drug-induced apoptosis and its determinants have not been comprehensively evaluated in the human acute leukemia cells. The elucidation of these pathways and different activation of apoptosis signaling molecules may reveal promising new targets for anticancer therapies, and should contribute to design a drug's schedule of administration and new combination chemotherapy protocol. The parameters of caspase activation and mitochondrial apoptosis signaling induced by anticancer drugs may be used for monitoring drug efficacy in primary leukemia cells.

4.1. Different drugs induce apoptosis in a different time and dose fashion and represent different feature of apoptosis

Different drugs are used for the treatment of different cancer cells and different drugs produce different side effects in clinic. Our data show that anticancer drugs induced apoptosis in a different time and dose fashion. As a whole, anticancer drugs induced time-
dependent apoptosis, but for each drug, the different pattern was broad. Etoposide- and cytarabine-induced apoptosis appear earlier. At 12 hours, the observed maximal apoptosis for etoposide at the maximal concentration was about 50%, for cytarabine at the concentration of 1-10 µg/ml was more than 35%. In contrast, at same time point, the observed maximal apoptosis for 4-hydroxy-cyclophosphamide, doxorubicin, and methotrexate at their maximal concentration was less than 15%. This data suggested that taking effect of etoposide and cytarabine was earlier than 4-hydroxy-cyclophosphamide, doxorubicin and methotrexate.

Interestingly, higher dose of cytarabine induced less apoptosis, whereas lower dose of cytarabine induced more apoptosis. This just is a phenomenon observed in this experiment system. It is need to observe in clinic. It is also need to investigate the mechanism of producing this phenomenon. If this phenomenon is common, it means the dose escalation approach is no use for cytarabine.

The duration of apoptosis is relatively short and variable depending on cell type, inducer of apoptosis. Each of the methods used in present experiment has its advantages and suffers limitations. So combination of several apoptosis assay can provide a more definite identification of apoptotic cells.

Recent studies have shown that a critical event during apoptosis appears to be the acquisition of plasma membrane changes that allows phagocytes to recognize and engulf these cells before the rupture (Martin et al., 1995; Bino et al., 1999). One of these plasma membrane changes is the translocation of phosphatidylserine to the outer layer, where it becomes exposed to the external surface of the cell. Once on the cell surface, PS can be detected by staining with FITC conjugate of annexin V, a protein with high affinity for PS. Regardless of the apoptotic stimulus, externalization of PS occurs earlier than the nuclear changes. Thus, the FITC conjugated annexin V binding assay of PS detects early phase of apoptosis before the loss of cell membrane integrity (chan et al., 1998; Trauth et al., 2000).

In the present study, the four assays detecting apoptosis demonstrated roughly similar pattern, in which the number of apoptotic cells increased with the increasing coculture time period in presence of anticancer drugs. But in these four assays also differences could be detected. Similar to anti-CD95 antibody, early drugs, especially etoposide, induced
apoptosis more strongly and rapidly in annexin V binding assay than in other assays. In contrast, similar to γ-radiation, late drugs induced apoptosis with a similar pattern in all four assays. After further analysis of the annexin V single positive cells, we can find that similar to anti-CD95 antibody, the number of induced by early acting drugs was much greater than annexin V/PI double positive cells. In contrast, late drugs-induced annexin V single positive cells were much less than annexin V/PI double positive cells. Collectively, early drugs and late drugs induced the translocation of phosphatidylserine from the inner membrane to the outer layer in a different pattern. In other words, the assay's sensitivity was relevant to the apoptotic stimulus. For early drugs, the annexin V binding assay could detect early phase of apoptosis before the loss of cell membrane integrity; for late drugs, externalization of PS was detected at approximately the same time as the loss of cell membrane integrity.

With these different features of anticancer drugs-induced apoptosis, we can more precisely evaluate the different anticancer drugs-induced apoptosis when using different assays.

### 4.2. CD95-associated signaling molecules and anticancer drugs

In 1989 two groups independently isolated mouse-derived antibodies that were cytolytic for various human cell lines (Trauth et al., 1989; Yonehara et al., 1989). The cell surface proteins recognized by the antibodies were designated Apo-1 and Fas, respectively. The antibody to Apo-1 was an immunoglobulin G (IgG3) antibody, whereas the antibody to Fas (anti-Fas) was classified as IgM. Sequencing and cloning of the Apo-1/Fas proteins and cDNAs, respectively, showed that receptor of Apo-1 and Fas were identical. The 5th Workshop on Leukocyte Typing suggested the name CD95 for the receptor. CD95 (Apo-1/Fas) belongs to the subfamily of death receptor, which is part of the TNF-receptor (TNF-R) superfamily. Members of this family are characterized by two to five copies of cysteine-rich extracellular death domain (DD). The DD is essential for transduction of the apoptotic signal.

CD95 is a widely expressed glycosylated cell surface molecule of approximately 45 to 52 kDa (335 amino acids). It is a type I transmembrane receptor and can also occur in several
soluble forms (Itoh et al., 1991; Oehm et al., 1992; Cheng et al., 1994). The human gene for CD95, APT, was localized to chromosome 10q23 and the mouse gene to chromosome 19 (Lichter et al., 1992; Watanabe-Fukunaga et al., 1992). Expression of the CD95 gene and cell surface protein are enhanced by IFN-r and TNF and by activation of lymphocytes (Klas et al., 1993; Leithäuser et al., 1993). Apoptosis can be triggered by agonistic antibodies and by the natural ligand of the receptor, CD95L, expressed in a more restricted way than CD95. CD95L was cloned from the cDNA of a killer cell (PC60-d10S) and show to be a TNF-related type II transmembrane molecule. The mouse and human CD95L genes were mapped to chromosome 1 (Takahashi et al., 1994a,b). Killer cells expressing CD95L were shown to kill target cells in a Ca^{2+}-independent fashion via CD95-CD95L interaction (Anel et al., 1995). In addition, human CD95L overexpressed in COS cells was found in the supernatant and induced apoptosis in a soluble form. Soluble CD95L is found as a trimer and is generated from the transmembrane form by the activity of a metalloprotease (Krammer et al., 1995; Nagata et al., 1995; Peter et al., 1999).

The main death pathway initiated from Fas activation involves a series of death-associated molecules, including FADD (Fas-associated-death domain-containing protein), which is an adaptor protein that is recruited to Fas receptor upon its engagement. FADD then binds to and activates procaspase-8, which is believed to be the first step of a proteolytic cascade that triggers activation of other caspases. RIP (receptor interacting protein) was another containing death domain protein to bind to CD95, which contains an N-terminal region with homology to protein kinases and a C-terminal region containing a cytoplasmic death domain present in both Fas and TNF-R1. These molecules were all cloned in the yeast two hybrid systems with the cytoplasmic part of CD95 used as a bait. The two death domain-proteins FADD and RIP bind to the CD95-death domain directly. Overexpression of FADD and RIP causes apoptosis (Chinnaiyan et al., 1995; Juo et al., 1999; Boldin et al., 1995; Stanger et al., 1995).

Based on the concept of activation-induced death in T cells, the cytotoxicity of anticancer treatment using cytotoxic drugs or γ-radiation has been studied with respect to involvement of CD95 receptor/ligand interaction mostly in cell lines derived from patients with T-cell acute lymphoblastic leukemia (Friesen et al., 1996). Doxorubicin and other cytotoxic drugs used in the chemotherapy of the leukemias were found to induce CD95L expression in cell lines and patient derived leukemia cells. The cell lines used constitutively express CD95
and are sensitive to CD95-mediated apoptosis triggered via anti-Apo-1 antibody or the natural ligand. Drug-induced apoptosis was strongly diminished by blocking CD95 receptor/ligand interaction with an anti-Apo-1 F(ab)_2 fragment or using cell lines in which CD95 was downregulated by prolonged exposure to an ant-CD95 antibody, suggesting that activation of CD95L/receptor interaction critically contributes to drug-induced cell death.

The contribution of doxorubicin-CD95L receptor interaction to cytotoxicity was most pronounced at the lower concentrations of doxorubicin (up to 50-100ng/ml) which may be achieved during therapy in vivo. In doxorubicin resistant cell lines derived from parental sensitive cells no induction of CD95L was found. Induction of CD95L and activation of CD95L/CD95 interaction has also been found with bleomycin in hepatoblastoma cells, 5-fluorouracil (5-FU) in colon carcinoma cells and various chemotherapeutic drugs in medulloblastoma and neuroblastoma cells (Fulda et al., 1997a,b; Houghton et al., 1997; Müller et al., 1997). In addition to CD95L, increased expression of CD95 is induced in cells with a low level of CD95 expression. These findings indicated that activation of death-inducing ligands, such as CD95L and CD95, are part of the cellular responds to cytotoxic treatments that damage DNA, disturb metabolism or affect the mitotic apparatus.

Our results show that, similar to anti-CD95 antibody, anticancer drugs upregulated Fas Ligand and FADD expression and cleave RIP over the incubation time.

The role of CD95-associated signaling pathway in anticancer drugs-induced apoptosis will depend on a more detailed understanding of the mechanisms of Fas-mediated apoptosis. It seems clear that CD95/CD95L and a number of chemotherapeutic agents utilize the caspase cascade as an effector of apoptosis. However, contrasting findings regarding the role of the CD95/CD95L pathway in the response to chemotherapy suggests a heterogeneity in the regulation of this pathway, which needs to be further explored (Micheau et al., 1997; Wesselbor et al., 1999; Findley et al., 1999).

4.3. The central role of caspase in drug-induced apoptosis
Caspases are a family of mammalian cysteine aspartic proteases that play a central role in the death process. The 14 caspases so far identified in mammalian cells, of which 12 human enzymes are known, are synthesized as inactive proenzymes that must be cleaved at key aspartate residues to be activated (Nicholson et al., 1999). X-ray analyses have shown that activated enzymes form a tetramer containing two large and two small subunits (Walker et al., 1994; Rotonda et al., 1996). Based on their structural and functional homologies, mammalian caspases have been classified in two sub-families. Members of the caspase-1 sub-family (caspase-1, -4, -5, -11, -12 and -14) are mainly involved in cytokine maturation and inflammation, though they could contribute to some apoptotic pathways. Members of the caspase-3 sub-family (caspase-2, -3, -6, -7, -8, -9, -10) play a central role in apoptosis. Further subdivision can be made in this latter subfamily, depending on the size of their prodomain.

Because caspases exist as latent zymogens, the question remains as to how the zymogens are activated. Current evidence suggests that activation may proceed by autoactivation, transactivation, or proteolysis by other proteinases (Wolf BB et al., 1999). Adapter molecules link apoptotic sensors such as death receptors and mitochondria to procaspases. To accomplish this, adapters generally contain one domain that couples the adapter to the sensor and another that binds to long prodomain procaspases. These domains include death domains (DDs), death effector domains (DEDs), and caspase recruitment domains (CARDs) (Huang et al., 1996; Eberstadt et al., 1998; Chou et al., 1998).

The central role of caspases in drug-induced apoptosis is suggested by the observation that several procaspases are cleaved in their active fragments during the cell death process. In addition, extracts from drug-treated cells cleave peptide substrates that mimic the sequence specifically recognized by several enzymes of this family. In U937 cells, caspase-3 and caspase-6 appear to play a central role in apoptosis triggered by topoisomerase inhibitors while the various isoforms of caspase-2 modulate their activity (Dubrez et al., 1996; Dubrez et al., 1998; Droin et al., 1998; Sakahira et al., 1999). In addition, data from caspase-9 and Apaf-1 knockout mice have suggested that the generation of a caspase-9-containing apoptosome complex was crucial for drug-induced apoptosis. Caspase-3 is required for some typical hallmarks of apoptosis such as DNA fragmentation and membrane blebbing (Sakahira et al., 1998; Sahara et al., 1999). The other caspases
involved in the cell death process could vary, depending on the cell type and the apoptotic stimulus.

Our results here showed that similar to anti-CD95 antibody, anticancer drugs induce activation of caspases-8, -3. The cleavage pattern of the individual caspases and PARP did not differ between the anticancer drugs and anti-CD95 antibody stimulation. However, corresponding to the different kinetics of apoptosis, Anti-CD95 antibody-induced caspase activation was more rapid and efficient than etoposide and cytarabine; 4-hydroxy-cyclophosphamide-, doxorubicin- and methotrexate-induced caspase activation occurs latest. Collectively, these data show that caspases are involved in drug-mediated apoptosis.

Besides from directly observed drug-induced caspase activation, we also investigated the effect of the broad-spectrum tripeptide caspase inhibitor zVAD-fmk on anticancer drug-induced apoptosis. The results show that zVAD-fmk blocked not only externalization of phosphatidylserine and DNA fragmentation, but also cell morphologic changes and membrane integrity loss. The patterns was similar to zVAD-fmk inhibited the anti-CD95-induced changes of apoptosis in Jurkat cells. These observations provide additional evidence that anticancer drug-induced these changes of apoptosis depend on the function of caspases.

Comparable results have been found in other cell types or with anticancer drugs. Treatment of promyelocytic HL-60 cells with clinically achievable doses of etoposide caused caspase-9 activation and apoptosis. Apoptosis was blocked by cotreatment with zVAD-fmk. Complete inhibition of apoptotic phenotype and cell death by zVAD-fmk was also observed in promonocytic THP.1 cells treated with etoposide, and activation of caspases-2, -3, -6, and -7. Etoposide-induced activation of caspases-3, -7, -8, and -9 in IMR90E1A cells expressing a dominant-negative mutant of caspase-9 was blocked, but cells still died (Perkins et al., 1998; Fearhead et al., 1998).

In line with previous studies, our results demonstrate that induction of apoptosis by anticancer drugs was entirely dependent on the intracellular activation of caspases, because (a) cell death was completely prevented by zVAD-fmk, a broad caspases inhibitor, and (b) proteolytic cleavage of the multiple procaspases to their active enzymes, as well as cleavage of the caspase-specific substrates, could be observed.
4.4. Disturbance of mitochondrial function induced by anticancer drugs

Mitochondria are now recognized as being important in the control of cell survival and death (Green et al., 1989; Gottlieb, 2000; Kroemer et al., 2000; Costantini et al., 2000). The apoptotic changes of mitochondria consist in $\Delta \Psi_m$ loss, transient swelling of the mitochondrial matrix, mechanical rupture of the outer membrane and/or its nonspecific permeabilization by giant protein-permeation pores, and release of soluble intermembrane proteins through the outer membrane.

In addition to a role of the CD95 system in anticancer drug-induced apoptosis, alteration of mitochondrial functions such as PT (permeability transition) have been found to play a major role in the chemotherapeutic agent-induced apoptosis. Mitochondria undergoing PT release apoptogenic proteins such as cytochrome c or AIF from the mitochondrial intermembrane space into the cytosol, where they can activate caspases and endonucleases (Liu et al., 1996; Vier et al., 1999). The connection of mitochondrial PT to activation of the caspase cascade appears to be complex. On one hand, recombinant caspases can induce PT, probably via a direct effect on the PT pore complex. On the other hand, caspases are activated by mitochondrial intermembrane protein, suggesting that caspase can act either upstream or downstream of mitochondria.

Mitochondrial function during apoptosis seems to be controlled by the Bcl-2 family of proteins. Bcl-2 and several of its homologues have been localized to intracellular membranes, including the mitochondrial membrane. Overexpression of the antiapoptotic molecules Bcl-2 and Bcl-X$_L$ has been found to confer resistance to anticancer treatment (Yang et al., 1997; Kluck et al., 1997). Bcl-2 and Bcl-X$_L$ may inhibit apoptosis through the capacity to prevent PT and/or to stabilize the barrier function of the outer mitochondrial membrane. Both Bcl-2 and Bcl-X$_L$ have been shown to prevent opening of the purified PT pore complex reconstituted in liposomes. In addition, it has been suggested that Bcl-2 and Bcl-X$_L$ can bind cytosolic caspases via Apaf-1 to the mitochondrial membrane, thereby preventing their activation. Bcl-X$_L$ has recently been reported to prevent Apaf-1-dependent caspase-9 activation via interaction with Apaf-1.
Betulinic acid, a pentacyclic triterpene, is a novel experimental anticancer drug. It possesses an antitumoral activity in vitro and in vivo in melanoma, neuroectodermal tumors, and glioma cell lines. Fulda et al (Fulda et al., 1998) have shown that betulinic acid induces apoptosis via direct mitochondrial alterations. All of these effects have been observed in intact cells and in cell-free systems. When added to isolated mitochondria, betulinic acid directly induces loss of $\Delta \Psi_m$ in a way that is not affected by the caspase inhibitor zVAD-fmk and yet is inhibited by Bongkrekic acid.

A currently open question is whether the breakdown of $\Delta \Psi_m$ is an 'assassin or accomplice' (Green et al., 1989) since under some circumstances the breakdown of $\Delta \Psi_m$ occurs downstream of caspase activation. Similarly, time course analysis of $\Delta \Psi_m$ breakdown and cytochrome c release revealed contradictory results (Heibei et al., 1999). Whereas in some models the breakdown of $\Delta \Psi_m$ occurred considerably later than cytochrome c release and caspase activation, other showed that $\Delta \Psi_m$ loss and the release of cytochrome c occurred rather simultaneously. Our data reveal that anticancer drugs induce a breakdown of $\Delta \Psi_m$ independently of caspase activation. The time course of caspase activation and the breakdown of $\Delta \Psi_m$ are very similar.

Previous studies suggested that a decline of $\Delta \Psi_m$ may be an early event in apoptosis, including Fas-dependent signaling. In present results, anticancer drug-induced $\Delta \Psi_m$ loss, similar to anti-CD95-induced $\Delta \Psi_m$ loss, occurs at the same time points as PS externalization and DNA fragmentation. Anti-CD95 antibody-induced $\Delta \Psi_m$ loss can be blocked by zVAD-fmk. In contrast, anticancer drug-induced-$\Delta \Psi_m$ loss cannot be blocked by zVAD-fmk. These results suggested that anti-CD95 antibody-induced $\Delta \Psi_m$ loss was via caspase activation; whereas anticancer drug-induced $\Delta \Psi_m$ loss was not via caspase activation. In other words, the direct effect of anticancer drugs on mitochondria leads to mitochondrial transmembrane potential dissipation. These results are fundamentally different from those reported by Gamen et al (Gamen et al., 2000), who used doxorubicin as stimulus in Jurkat cells. Their results showed that doxorubicin induced $\Delta \Psi_m$ loss was prevented by coincubation with zVAD-fmk.

Numerous reports have described the ability of various specific and nonspecific DNA-damaging agents to stimulate the release of mitochondrial cytochrome c. In contrast to death receptor-mediated apoptosis, during which caspase-8 activity is often responsible for
the cleavage of a cytosolic substrate, e.g. Bid, which targets mitochondria triggering the release of cytochrome c, this event is traditionally accepted as caspase-independent in chemical- and/or DNA damage-induced apoptosis.

In the present study, anti-CD95 antibody-induced cytochrome c release occurs at 3 hours; etoposide and cytarabine at 6 hours; while 4-hydroxy-cyclophosphamide, doxorubicin and methatrexate nearly at 12 hours, at the same time of the appearance of cells displaying apoptotic morphologic changes, PS translocation, DNA fragmentation and loss of cell membrane integrity.

Cytochrome c, once released from mitochondria, is believed to form a complex with Apaf-1 and caspase-9. This 'apoptosome' then mediates activation of caspase-3 in an ATP-dependent fashion. We do not have any direct evidence that cytochrome c release during anticancer drug-mediated apoptosis form a complex with Apaf-1 and caspase-9. Nevertheless, such an interaction has been described for Jurkats in response to the CD95 pathway, and so it is not unreasonable to assume that such a process may occur in these cells in response to anticancer drugs. Since anticancer drugs are capable of directly activating caspase-3 in intact cells, one possible role for the Apaf-1/cytochrome c/caspase-9 complex is to amplify the cascade.

Proteins of the Bcl-2 family are major regulators of the mitochondria-initiated caspase activation pathway. The anti-apoptotic members of this family, including Bcl-2 and Bcl-X<sub>L</sub>, preserve mitochondrial integrity and prevent the release of cytochrome c in the presence of apoptotic stimuli (Kluck et al., 1997; Yang et al., 1997). Conversely, the proapoptotic members of this family such as Bad, Bax, Bid, and Bim move from other cellular compartments to mitochondria in response to apoptotic stimuli and promote cytochrome c release (Reed, 1998; Antonsson et al., 2000; Gross A et al., 1999).

Cleavage of Bid was observed in anticancer drug- as well as anti-CD95-mediated apoptosis. Bid is most likely cleaved by low concentrations caspase-8 but can be cleaved by higher concentrations of caspase-3 (Sun et al., 1999). Thus in anticancer drug-induced apoptosis, inhibition of Bid cleavage by zVAD-fmk may be due to inhibition of processing/activity of either caspase-8 at a later stage of the apoptotic process, resulting in cleavage of Bid. The cleaved bid moves to mitochondrial form cytosol, insert in
mitochondrial membrane and promotes release of mitochondrial cytochrome c and other pro-apoptotic proteins such as AIF, SMAC, DIABLO (Du C et al., 2000; Verhagen AM et al., 2000), thereby further amplifying the apoptotic program.

The bcl-2 gene was originally isolated from the t(14;18) chromosomal breakpoint in follicular B-lymphoma cells (Tsujimoto et al., 1985). This gene has been shown to prevent apoptosis induced by growth-factor deprivation in certain haematopoietic cell lines. 26 kDa Bcl-2 protein, the product of bcl-2 gene, is an integral intracellular membrane protein that inhibits programmed cell death induced by multiple insults in a wide variety of cell types. Both biochemical and genetic evidence indicates that Bcl-2 family member can regulate cell death induced by caspases. A number of substrates for the caspase proteases have now been identified, including protein kinases, the retinoblastoma protein, cytoskeletal proteins, and several autoantigens. Cleavage of these proteins by caspase may either activate or inactivate essential functions or produce cleavage products with altered activities. Previous research showed that when bcl-2 was cleaved by capases, the conversion of Bcl-2 became a Bax-like death effector. Other investigators also reported the cleaved Bcl-2 fragment increased the sensitivity to VP-16 (Etoposide). In current studies, Bcl-2 was cleaved in anti-CD95 antibody- and anticancer drug-induced apoptosis, in agreement with previous observations. The anti-apoptosis protein Bcl-2 was converted to proapoptosis regulators following cleavage by caspase-3 (Cheng et al., 1997; Wang et al., 2001).

Besides of death receptor signaling pathway, caspase activation and mitochondrial signaling pathway, there are several other mechanisms involved in drug-induced apoptosis, such as p53 tumor suppressor gene, lipid-dependent signaling pathway and generation of oxygen radicals (Solary E et al., 2000).

Overall, understanding the mechanisms of anticancer drug-induced apoptosis is of principal importance for developing effective strategies in tumor therapy. Our results show that etoposide- and cytarybine-induced apoptosis was early; 4-hydroxy-cyclophosphamide-, doxorubicin-, and methotrexate-induced apoptosis was late. Higher doses of cytarabine induce lower rate of apoptosis than lower doses of cytarabine. In all drugs tested, apoptosis could be inhibited by caspases, suggesting induction of caspase dependent cell death by all drugs. Also mitochondrial transmembrane potential loss and cytochrome c release were induced by all drugs. The different characteristics of anticancer drugs e.g. time of apoptosis.
induction are probably due to events upstream of the common mitochondrial and caspase signaling pathways. Since all tested drugs induced caspase activation and mitochondrial apoptosis signaling, measurement of these parameters can be used for assessment of drug efficacy in primary leukemia cells.
5. Summary

It is known that in empirical medicine, different anticancer drugs have different characteristic concerning specific anti-tumor or anti-leukemic efficacy and side effects on normal tissue. While solid tumors are often treated with cisplatin, treatment of leukemia is based on the use of anthracyclines and antimetabolites. Many drugs also have unique toxicities affecting normal tissues, such as the cardiotoxicity associated with the anthracyclines, the hemorrhagic cystitis associated with the cyclophosphamide and ifosfamide. The peripheral neuropathy from vincristine, and the coagulopathy from L-asparaginase. We therefore hypothesized that the different clinical used anticancer drugs might induce apoptosis in a drug specific manner. Thus the clinical observed difference could be reflected by different activation of apoptosis signaling pathways. The molecular signaling pathways which are initiated in response to anticancer drug-induced cellular damage, and lead to the eventual apoptosis death of the cell, are largely undefined. Previous studies show the caspase family are the critical executioners of apoptosis and anticancer drug-induced apoptosis by activating two major cell-intrinsic pathways, one that begins with ligation of cell surface death receptors, such as CD95, and another that involves mitochondrial release of cytochrome c. We therefore investigated induction of apoptosis, activation of caspases and involvement of mitochondrial signaling in the well defined Jurkat human leukemic T cells by five conventional used anticancer drugs: etoposide, cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin, and methotrexate, in order to identify drug specific activation of distinct apoptosis pathways.

In present studies, we found some difference of apoptosis induced by these five anticancer drugs: (1) the anticancer drug-induced apoptosis appeared in different time kinetics, etoposide and cytarabine were early acting drugs, while 4-hydroxy-cyclophosphamide, doxorubicin, and methotrexate were late acting drugs. (2) Interestingly, higher doses of cytarabine induced less apoptosis, whereas lower doses of cytarabine induced more apoptosis. (3) Similar to CD95, early drug-induced PS externalization was earlier than drug-induced membrane integrity loss, whereas late drug-induced PS externalization and membrane integrity loss occurred in the same time, similar to γ-radiation-induced
apoptosis. (4) Etoposide strongly induced caspases activation, compared to cytarabine, 4-hydroxy-cyclophosphamide, doxorubicin and methotrexate. Besides these differences, we found that all drugs induced apoptosis in a similar manner by all anticancer drugs. All tested drugs can activate caspase-8, caspase-3 and cleave PARP. Furthermore, these five drug-induced apoptosis could be inhibited by the pan-caspase inhibitor zVAD-fmk, suggesting that drugs induce apoptosis in a caspase dependent way. All drugs induce mitochondrial transmembrane potential reduction and all drug-induced $\Delta \Psi_m$ loss can not be blocked by caspase inhibitor zVAD-fmk, indicating that caspases are not required for early mitochondrial changes. All drugs induced cytochrome c translocation from mitochondria into cytosol. This suggests that mitochondrial signaling and caspase activation are commonly activated in programmed cell death induced by cytostatic drugs used for anticancer treatment. Since all tested drugs induced caspase activation and mitochondrial apoptosis signaling, measurement of these parameters can be used for assessment of drug efficacy in primary leukemia cells. The different characteristics of anticancer drugs e.g. time of apoptosis induction are probably due to events upstream of the common mitochondrial and caspase signaling pathways.
6. References


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# Curriculum Vitae

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## Education

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