ALL-TRANS RETINOIC ACID-INDUCED APOPTOSIS IN ACUTE MYELOBLASTIC LEUKEMIA CELLS
With a special emphasis on p53, Bcl-2, and mitochondria

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AIPING ZHENG

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Abstract

All-trans retinoic acid (ATRA) is a derivative of vitamin A. It is able to stimulate neutrophilic differentiation of normal progenitors and acute promyelocytic leukemia (APL) cells. Although ATRA-induced differentiation is not observed in any other acute myeloblastic leukemia (AML) subtypes, ATRA is known to be able to inhibit AML blast cell proliferation. The present in vitro study using AML cell lines representing subtypes other than APL focuses on the following questions: (1) Is the inhibitory effect of ATRA on AML cell growth related to apoptosis of cells? (2) Are the effects of ATRA dependent on two important regulators of apoptosis, p53 and Bcl-2? (3) Do mitochondria have any role in mediating the effects of ATRA? ATRA-induced apoptosis in AML cells was observed by morphology, DNA fragmentation, phosphatidylserine externalization, and poly(ADPribose)/polymerase (PARP) cleavage. It was a slow event, manifested as DNA cleavage after 48 hours exposure and as morphological apoptosis after 72 hours exposure. The AML cells expressed constitutively p53 as determined by immunohistochemistry, Western blotting and flow cytometry. However, no mutation of TP53 was observed in exons 5 to 8 as analysed with a single strand conformation polymorphism technique. As the flow cytometer analysis showed, most of p53 was in a aberrant conformation, which was not changed into a wild type conformation by ATRA. Two of the cell lines were analysed more specifically in relation to Bcl-2 and mitochondrial function: ATRA-induced apoptosis of the cell lines was associated with down-regulation of Bcl-2. Western blotting showed ATRA-induced apoptosis also to be related to the release of cytochrome c from mitochondria into cytosol, resulting in the activation of caspase-3, an apoptotic effector, which was manifested as a cleavage of its substrate PARP. The process was also accompanied by disruption of the mitochondrial membrane potential as determined fluoricytometrically. These results show that ATRA is able to induce apoptosis in AML cells other than APL, and ATRA-induced apoptosis in the AML cells studied is related to the down-regulation of Bcl-2 and the disruption of mitochondrial function, but is independent of the p53 pathway.

Keywords: cell growth, programmed cell death, tretinoin
To Zhiqi
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Abbreviations

AIDS  Acquired immunodeficiency syndrome
AIF  Apoptosis-inducing factor
AML  Acute myeloblastic leukemia
Apaf-1  Apoptosis-activating factor 1
APL  Acute promyelocytic leukemia
ATRA  All-trans retinoic acid
BM  Bone marrow
Cdk  Cyclin-dependent kinase
CRABP  Cellular retinoic acid binding protein
CSF-1  Colony stimulating factor-1
DATP  Deoxy-adenosine triphosphate
DNA  Deoxyribonucleic acid
DNA-PK  DNA-dependent protein kinase
ECL  Enhanced chemiluminescence
ELISA  Enzyme-linked immunosorbent assay
ER  Endoplasmic reticulum
FAB  French-American-British
FCS  Fetal calf serum
FITC  Fluorescein isothiocyanate
GM-CSF  Granulocyte-macrophage colony-stimulating factor
G-CSF  Granulocyte colony-stimulating factor
IL  Interleukin
KD  Kilodalton
Mc  Methycellulose
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>α-MEM</td>
<td>Alpha-minimal essential medium</td>
</tr>
<tr>
<td>MGG</td>
<td>May-Grünwald-Giemsa</td>
</tr>
<tr>
<td>MRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>OU</td>
<td>Oulu University</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly(ADP ribose)polymerase</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCD</td>
<td>Programmed cell death</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PML</td>
<td>Promyelocytic leukemia</td>
</tr>
<tr>
<td>PT</td>
<td>Permeability transition</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoic acid receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>RAR specific regulatory element</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol-binding protein</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoic X receptor</td>
</tr>
<tr>
<td>RXRE</td>
<td>RXR specific regulatory element</td>
</tr>
<tr>
<td>SSCP</td>
<td>Single-strand conformation polymorphism</td>
</tr>
<tr>
<td>TP53</td>
<td>Tumor suppressor gene p53</td>
</tr>
<tr>
<td>ΔΨm</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>Wt</td>
<td>Wild type</td>
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List of original papers

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References
1 Introduction

Apoptosis or programmed cell death (PCD) is a genetically determined cellular program essential for the normal development and maintenance of tissue homeostasis. Excessive cell death may lead to impaired development and degenerative diseases, whereas a lack of apoptotic cell death may lead to the development of cancer (Thompson 1995). Therapeutics that modulate apoptosis provide a new opportunity for the treatment of numerous diseases, including cancer (Granville et al. 1998).

The process of apoptosis is controlled through the expression of an increasing number of genes. Some gene products are activators of apoptosis, whereas others are inhibitors. Among them, there are two well-characterized gene products, p53 (activator) and Bcl-2 (inhibitor) (Evan & Littlewood 1998, Adams & Cory 1998).

The tumor suppressor gene p53 (TP53) is the most frequently mutated gene identified so far in human cancer, resulting in conformationally altered and inactive p53 protein (Hainaut et al. 1997). The wild type (wt) p53 acts as a tumor suppressor gene, whereas mutated p53 behaves as an oncogene. (Gottlieb & Oren 1996, Hainaut & Vähäkangas 1997). In addition there are cancers, in which conformationally altered p53 occurs without gene mutations (Levine 1997). In acute myeloblastic leukemia (AML), mutations in the p53 gene have been found to be very rare, occurring in less than 5% of patients (Fenaux et al. 1992, Schottelius et al. 1994). It is not known whether p53 is inactivated posttranscriptionally in AML. The restoration of p53 function would be very interesting in terms of developing treatments for leukemia.

The bcl-2 proto-oncogene was first identified at the t(14;18) chromosomal translocation present in the majority of follicular non-Hodgkin lymphomas (Tsujimoto et al. 1984). The function of Bcl-2 is to prolong cell survival by preventing the onset of apoptosis. Bcl-2 is expressed in both normal and malignant hematopoietic cells, including normal bone marrow (BM) stem/progenitor cells and acute and chronic myeloid leukemia blast cells. Overexpression of Bcl-2 in AML has been shown to associate with a poor clinical response to chemotherapy (for a review, see Reed 1997).

Recent studies underline the importance of mitochondria in the regulation of apoptosis (Mignotte & Vayssiere 1998, Green & Reed 1998). The efflux of the mitochondrial respiratory chain protein cytochrome c from mitochondria into cytosol seems to play an essential role in the activation of cell death (Liu et al. 1996). It has been speculated that the
prerequisite for this phenomenon would be a perturbation in the mitochondrial membrane function leading to a permeability transition (PT) and a drop in mitochondrial membrane potential ($\Delta\Psi_m$) (Mignotte & Vayssiere 1998).

Retinoids, including all-trans retinoic acid (ATRA), are involved in the growth and differentiation of hematopoietic cells. Nowadays, ATRA is used as the first-line treatment in acute promyelocytic leukemia (APL) worldwide due to its differentiating effect on leukemia cells, which was first described by Breitman and coworkers in 1980 and 1981. However, the clinical use of retinoids in the therapy of other AML subtypes has been scarce, although the inhibitory role of retinoids on the growth of AML cells in vitro was documented in 1982 (Douer & Koeffler 1982). It is not known whether the anti-proliferative effect of retinoids is solely related to the growth arrest of cells or whether retinoids have a capacity to induce apoptosis in cells.
2 Review of the literature

2.1 Hematopoiesis and acute myeloblastic leukemia

2.1.1 Hematopoiesis

Blood cells are derived from the small number of pluripotent hematopoietic stem cells that reside in the BM. They are capable of both self-renewal and commitment generating progenitor cells from which all blood cells differentiate and mature. Because the life span of most blood cells is short, their production rate in the BM is high, even in steady-state conditions, resulting in more than $1 \times 10^{12}$ cells per day in adult humans. In stress conditions, the BM is able to increase its production of blood cells of different lineages over 10-fold. Thus, it is understandable that strict control of the process of cell renewal, commitment, differentiation and maturation is necessary (Bondurant & Koury 1999). The factors triggering renewal and commitment are incompletely understood. It may be either a random process or determined by local environmental signals (Morrison et al. 1995, Bondurant & Koury 1999, Testa & Dexter 1999). The hematopoietic process is regulated by multiple growth factors, specific cytoadhesion proteins and transcriptional factors including other factors functioning in the BM microenvironment (Tenen et al. 1997, Bondurant & Koury 1999). Among them, retinoic acid receptors (RARs) and their ligands play some role in myeloid differentiation (Tenen et al. 1997).

2.1.2 AML

AML is a rapidly progressive malignant hematopoietic disease with a poor prognosis. Although the current induction therapy regimens afford complete remission in 60-80% of the patients with AML (Stone & Mayer 1993, Rowe & Liesweld 1996), only 10-20% of these patients reach a long-term disease-free survival of over 5 years (Hamblin 1995).
About 30-45% of the patients can expect a long remission after intensive post-remission chemotherapy (Rowe et al. 1994, Mayer et al. 1994, Elonen et al. 1998). Furthermore, allogeneic stem cell transplantation during the first remission may result in a 10-year disease-free survival for 45-60% of the patients (Rowe & Liesweld 1996). However, intensive treatment is not possible in all cases due to its toxicity and adverse effects. New less toxic treatment schedules are urgently needed, particularly for elderly patients.

AML has been suggested to arise from a transformed pluripotent hematopoietic stem or progenitor cell, and it is therefore called a clonal disease (Fialkow et al. 1981, Keinanen et al. 1988, McCulloch 1993). AML is characterized by progressive accumulation of relatively immature, poorly functioning myeloid blasts in the BM and peripheral blood (PB). It eventually leads to inhibition of the production and differentiation of cells within the normal hematopoietic compartments (Broxmeyer et al. 1981). AML is diagnosed morphologically, using the criteria proposed by the French-American-British (FAB) Co-operative Group (Bennett et al. 1976, Bennett et al. 1985).

AML cells respond to the same regulatory influences that control normal hemopoiesis (McCulloch et al. 1990). Various hematopoietic growth factors alone or in combination stimulate AML blast cell growth in cultures (Vellenga et al. 1987, Miyauchi et al. 1988, Delwel et al. 1988, Salem et al. 1990). However, some degree of spontaneous leukemic cell proliferation can be observed in a majority of cases (Hunter et al. 1993). This is thought to be mediated by growth factors produced either autocrinely and/or paracrinely (Murohashi et al. 1989, Russell et al. 1992). Although AML blast cells may undergo low-degree aberrant differentiation in a growth factor-supplemented methylcellulose (mc) culture (Salem et al. 1989), they are usually incapable of maturing to terminally differentiated myeloid cells (Santini et al. 1991). The FAB subtype M3 of AML, i.e. acute promyelocytic leukemia (APL), is an exception.

### 2.2 Vitamin A

“Retinoids” is a generic term, which includes both naturally occurring molecules and synthetic compounds showing specific biological activities resembling those of vitamin A (retinol). The term “vitamin A” is used to refer to a naturally occurring fat-soluble vitamin, all-trans retinol (Pinnock & Alderman 1992), which is obtained from the diet as preformed retinoids (retinyl esters) from animal sources and as provitamin carotenoids (including β-carotene) from plant sources. These are converted to retinol in the gut, absorbed, and stored in the liver as retinyl palmitate. Vitamin A plays an important role in the maintenance of normal growth, vision, reproduction, cell membrane integrity, and bone formation. Vitamin A deficiency results in night blindness, inhibition of spermatogenesis, and potential teratogenesis. In animals, vitamin A deficiency has been associated with a higher incidence of cancer and increased susceptibility to chemical carcinogens (Miller 1998).
2.3 ATRA

ATRA (tretinoin) is one of the naturally occurring vitamin A derivatives (Miller 1998). In the plasma, retinol and ATRA are bound tightly to the retinol-binding protein (RBP) and albumin, respectively (Blomhoff et al. 1990). Retinol is the major circulating retinoid in the human body and its plasma levels remain near 2 µmol/L under normal conditions (Blomhoff et al. 1990), while retinoic acid is not stored but is rapidly excreted (Orfanos et al. 1997). The normal plasma concentration of ATRA is 4-14 nmol/L (Blaner & Olson 1994).

ATRA appears to enter cells by simple diffusion. Once within the cell ATRA is bound by cellular retinoic acid binding proteins (CRABP) I, II and albumin (Blomhoff et al. 1990, Lupulescu 1993). The role of CRABPs is not well defined. They might be involved in intracellular retinoid transport (Takase et al. 1986) and retinoid metabolism (Fiorella & Napoli 1994).

The biological effects of retinoic acid are generally mediated through nuclear receptors. Six RAR and retinoic X receptors (RXR) (both with α, β, and γ subtypes) that are members of the steroid-thyroid superfamily of nuclear receptors have been identified (Miller 1998). In most retinoid-inducible genes, transcriptional activation occurs through RAR-RXR heterodimers acting at RAR-specific regulatory elements (RAREs) or RXR-specific regulatory elements (RXREs). The RAR-RXR heterodimers interact in the absence of the ligand with a large ubiquitous nuclear protein (N-CoR), which mediates transcriptional repression through its interaction with other proteins including mSin3A and HDAC (Horlein et al. 1995, Heinzel et al. 1997). A current model explaining RAR activity suggests that the addition of a retinoic acid ligand results in a distinct conformational change in the RAR-RXR complex and recruitment of transcriptional co-activators (Kamei et al. 1996, Chakravarti et al. 1996, Chen et al. 1997). Thus, the addition of the retinoic acid ligand normally converts RAR-RXR from a transcriptional repressor to a transcriptional activator (Collins 1998). ATRA binds to RARs with high affinity and alters gene expression as a consequence of this direct ligand interaction (Allenby et al. 1993). ATRA does not have a binding activity with RXRs (Heyman et al. 1992, Levin et al. 1992). The activated nuclear receptors control the expression of genes that mediate the effects of retinoids, including the regulation of cell differentiation and growth and the induction of apoptosis (Sporn et al. 1994).

2.3.1 ATRA and myeloid differentiation

Multiple myeloid genes are induced during treatment with ATRA, including those for cell surface adhesion molecules, intrinsic host defense systems, extrinsic cytokines, colony-stimulating factor receptors, structural proteins, and enzymes. Some of these are induced with some delay after the ATRA treatment, making them unlike the primary targets of RAR-α. The few identified direct targets of RAR-α tend to be transcription factors themselves, including the RARs (Gudas 1994). For instance, ATRA treatment of fresh APL cells upregulates RAR-α, correlating with the presence of a RARE in the RAR-α promot-
er (Chomienne et al. 1991, Leroy et al. 1991). Therefore, up-regulation of the RAR-α gene could be one way to induce myeloid differentiation by ATRA. Moreover, RAR-α is preferentially expressed in myeloid tissue (Largman et al. 1989). ATRA can also promote terminal differentiation of promyelocytic (HL-60, NB4) and monoblastic (U937) hematopoietic cells (Gillis & Goa 1995). However, ATRA does not induce differentiation in other AML subtypes, although it is able to inhibit the clonal growth of fresh leukemic cells and cell lines from patients with AML (Douer & Koeffler 1982, Wang et al. 1989, Koistinen et al. 1991, Tohda et al. 1992, Sakashita et al. 1993).

It was found recently that ATRA is able to inhibit the growth of a human myeloma cell line, OPM-2 through up-regulation of p21 (WAF1) and consequent dephosphorylation of the retinoblastoma protein (Chen et al. 1999). It has previously been shown that p21 is a direct target of RAR in myeloid cells (Liu et al. 1996).

### 2.3.2 ATRA as a chemotherapeutic agent

ATRA is considered the active form of retinol in all tissues except retina (Chytil 1986, Sporn et al. 1994). ATRA is a very potent promoter of growth and controller of differentiation in many organ systems (Lotan 1980), including hematopoiesis, by inducing differentiation of promyelocytes into mature granulocytes in normal hematopoiesis (Douer & Koeffler 1982) as well as in APL (Breitman et al. 1981, Ferrero et al. 1982). This phenomenon is accompanied by the induction of apoptosis (Martin et al. 1990, Okazawa et al. 1996). Therapy of APL with ATRA is now well established. By mid-1996, at least 3000 patients diagnosed for APL had been treated with ATRA worldwide. Combined data published from Chinese, French, American and Japanese sources indicate a mean complete remission response of 85% (Warrell 1997). APL is characterized by an early block in myeloid maturation and is associated with a t(15;17) chromosomal translocation, in which the RARα gene on chromosome 17 fuses to the promyelocytic leukemia (PML) gene on chromosome 15. The PML/RARα fusion protein appears to be responsible for the failure of promyelocytes to differentiate and explains, at the molecular level, why only the leukemia cells (e.g. APL) expressing this protein are sensitive to ATRA (Gillis & Goa 1995).

Apart from its effects on leukemia, ATRA appears to be a potent radiosensitizer of human breast cancer cells, human and murine melanoma cells, human head and neck tumor cells as well as normal and transformed human skin fibroblasts in vitro (Rutz et al. 1989, Schiller et al. 1994, DeLaney et al. 1996).

### 2.3.3 ATRA as a chemopreventive agent

Retinoids are the most important chemopreventive agents that have reached the stage of clinical trials (Hong & Itri 1994, Hong et al. 1995, Lippman et al. 1995). Over the last couple of decades, numerous investigations have established a strong relationship
between vitamin A and the development of cancer. Vitamin A deficiency in experimental animals has been associated with a higher incidence of cancer and increased susceptibility to chemical carcinogens (Moon et al. 1994). Furthermore, epidemiological studies have indicated that individuals with a lower dietary vitamin A intake are at a higher risk to develop cancer (Hong & Itri 1994). These observations have led to the hypothesis that physiological levels of retinoids protect the organism against the development of pre-malignant and malignant lesions. Therefore, clinical trials focus mostly on individuals at an increased risk for cancer, such as patients with pre-malignant lesions or patients who have been successfully treated for an early-stage carcinoma and have a high risk of developing a recurrence cancer (Evans & Kaye 1999). Experimental models of carcinogenesis have demonstrated the efficacy of pharmacological levels of retinoids in preventing the development of cancer of the skin, oral cavity, lung, mammary gland, prostate, bladder, liver, and pancreas in animals exposed to carcinogenic agents (Moon et al. 1994). ATRA has been shown to suppress the transforming effects of chemical, physical, and viral carcinogens on both murine and human cell cultures (Lotan 1996). Clinical trials have indicated that retinoids may be useful for the prevention of cancers of the upper digestive tract, skin, breast, and ovaries (Hong & Itri 1994, Lippman et al. 1995, DePalo et al. 1995).

2.4 Apoptosis

2.4.1 Definition of apoptosis

Cell death constitutes one of the key events in biology. At least two modes of cell death can be distinguished: apoptosis and necrosis. Apoptosis is a strictly regulated (programmed) device responsible for the systematic removal of superfluous, aged, harmful, abnormal or misplaced cells (Thompson 1995, Kroemer et al. 1995).

Naturally occurring cell death was for the first time mentioned in the literature in 1842, when Carl Vogt described the death of notochordal and cartilaginous cells during development (Vogt 1842). In 1885, the morphological changes of apoptosis were thoroughly described by Flemming and given the name ‘chromatolysis, a term which was widely used for the next 30 years (Flemming 1885). The dwindling interest in cell death at the turn of the century is believed to be due to a language barrier: fewer scientists were fluent in German, the language in which most of the early studies were published. It was only in 1951 that this topic was resumed by Glucksman, who observed the death of individual cells in a larger population during vertebrate development (Glucksman 1951). In 1964, Lockshin and Williams introduced the term “PCD” to describe the developmentally regulated elimination of specific cells during the transformation of larvae into adult moths (Lockshin & Williams 1965). Finally, the word “apoptosis” was introduced in 1972 by Kerr, Currie and Wyllie to describe the common morphological changes that characterize the process of cellular self-destruction (Clarke & Clarke 1995, 1996). The term “PCD” was defined to refer to any of gene-mediated process of cell demise, regardless of the ini-
tiating stimulus. The term “apoptosis” was originally proposed simply to describe the morphological changes associated with cellular self-destruction. Since not all PCD occurs by apoptosis and not all instances of apoptosis require new gene expression (Martz et al. 1989, Schwartz et al. 1993), these two terms have lost their specific meanings, and any process that results in the morphological changes associated with apoptosis is now instinctively also referred to as PCD, regardless of the trigger or absence/presence of new gene expression (Linz 1998).

Apoptosis/PCD serves as the major mechanism for precise regulation of cell numbers (Raff 1992) and as a defense mechanism to remove unwanted and potentially dangerous cells, such as self-reactive lymphocytes (Golstein 1997), cells that have been infected by viruses (Vaux et al. 1994), and tumor cells (Williams 1991). In addition to the beneficial effects of apoptosis, inappropriate activation of cell death may cause or contribute to a variety of diseases, including acquired immunodeficiency syndrome (AIDS) (Ameisen et al. 1995), neurodegenerative diseases, and ischemic strokes (Martinou 1995, Raff et al. 1993). Conversely, a defect in apoptosis activation could be responsible for some autoimmune diseases (Tan 1994), and such defects are also involved in oncogenesis (Bursch et al. 1992, Thompson 1995, Nicholson 1996).

In contrast to the swelling of a necrotic cell that leads to cell membrane rupture and leakage of cell cytoplasm, resulting in an inflammatory reaction in neighboring cells, apoptotic cells rapidly and inconspicuously condense into variable-sized membrane-bound fragments that are phagocytosed by neighboring tissue cells without any associated inflammation. Apoptotic cells can be distinguished based on morphological and biochemical cell changes, membrane blebbing, cell shrinkage, chromatin condensation, cleavage of genomic DNA into 180-200 base pair fragments, fragmentation of cells into membrane-bound apoptotic bodies whose surface expresses potent triggers for phagocytosis (Wyllie 1987, Schwartzman & Cidlowski 1993, Bosman et al. 1996, Wyllie 1998).

All nucleated mammalian cells in both developing and mature organs are able to undergo apoptosis (Ishizaki et al. 1995, Weil et al. 1996) and constitutively express all the protein components required to execute the death program (Jacobson et al. 1994, Weil et al. 1996), which are normally suppressed by (extracellular) survival signals. The specific signaling pathway activated depends on the cell type and on the subcellular element targeted by each type of stress. Various upstream signaling cascades may converge on a common final effector mechanism to disintegrate the dying cell (Kolesnick 1998).

2.4.2 Process of apoptosis

The process of apoptosis can be divided into three fundamental steps: initiation, commitment and execution (Figure 1). Initiation is activated by various death-triggering signals, including chemotherapeutic agents, cell contact, radiation, cytokines, steroid hormones, growth factor deprivation, physical trauma, oncogene expression, viruses, oxidants and agents that alter calcium homeostasis. Commitment consists of biochemical transduction of the death signal. This is followed by the execution stage, in which the activated machinery acts on multiple cellular targets and, finally, the destruction phase of which the
dead or dying cell is broken down (Reed 1997, Mignotte & Vayssiere 1998, Wickremasinghe & Hoffbrand 1999). The commitment and execution stages are still highly conserved. Most of the described apoptotic model systems manifest in two major execution programs downstream of the death signal: the caspase pathway and organelle dysfunction, of which mitochondrial dysfunction is the best characterized subtype (Green & Reed 1998). As the members of the Bcl-2 family of proteins reside upstream of the irreversible cellular damage and focus much of their efforts at the level of mitochondria, they play a pivotal role in determining whether a cell will live or die. Therefore, they have a major role in controlling the cellular commitment to apoptosis in the mitochondria-dependent apoptotic pathway (see section 2.5.2.2, 2.5.3.1, and 2.5.3.2). The Bcl-2-regulated commitment step culminates in the release of cytochrome $c$, consisting an “apoptosome”, by recruiting Apaf-1 and procaspase-9 and by finally activating caspase-9. The activated caspase-9 then processes and activates other caspases to orchestrate the biochemical execution of cells.

![Diagram of apoptosis pathway](image)

**Fig. 1. A model for the apoptosis pathway dependent on mitochondria.** Numerous stimuli may serve as initiators of death signaling. The signaling may converge to a few common molecules that can make the decision between death or life, and the bcl-2 family proteins play a prominent role in this. Once the signaling gets through, caspases are activated and consequently cleave a variety of specific target proteins in the cell, resulting directly or indirectly in the morphological and biochemical characteristics of cells undergoing apoptosis.

The caspases are among the most specific proteases, with an unusual and absolute requirement for cleavage after aspartic acid, which are activated during apoptosis (Hansen & Oren 1997). The mammalian caspase family now comprises at least 13 known members, most of which have been definitively implicated in apoptosis (Alnemri et al. 1996). *In vitro* experiments suggest that some caspases could activate themselves, while some others could activate other caspases, acting as a proteolytic cascade (Nicholson & Thornberry 1997). Caspase-3, 6, and 7 are terminal members of caspase cascades and recognize critical cellular substrates, whose cleavage contributes to the morphological and
functional changes associated with apoptosis (Thornberry & Lazebnik 1998). The caspase-3 substrates are PARP, an enzyme involved in regulation of DNA repair, and gelsolin, a cytoskeletal protein. Caspase-3 activation also results in DNA cleavage via inactivation of an inhibitor of DNA fragmentation factor, the endonuclease responsible for internucleosomal cleavage of chromatin (Wickremasinghe & Hoffbrand 1999). Recent findings showed that caspase-3 has a mitochondrial and cytosolic distribution in nonapoptotic cells. The mitochondrial caspase-3, which is located in the intermembrane space, was shown to be activated by numerous pro-apoptotic stimuli and this activation could be blocked by bcl-2 (Mancini et al. 1998). Once the caspases are activated, the cell is irreversibly committed to cell death (Reed 1997).

Mitochondria-independent apoptotic pathways also exist. For instance, apoptotic signaling initiated by death receptors, oxidative stress or cytotoxic cytokines does not always take place through mitochondrial mechanisms, but by direct activation of the initiator caspases (e.g. caspase 8), which, in turn, leads to the activation of effector caspases (e.g. caspase 3), resulting in cellular disassembly (Ashkenazi & Dixit 1998).

2.5 Regulation of apoptosis

2.5.1 p53

2.5.1.1 Function of p53

The p53 tumor suppressor protein was first described 20 years ago as a protein that binds the large T antigen of the SV40 virus, (Linzer & Levine 1979, Lane & Crawford 1979) and a tumor antigen (Deleo et al. 1979). TP53 encodes a 53 kilodalton (kD) nuclear phosphoprotein, which participates in tumor suppression, cell cycle control, DNA repair, stress responses, cell senescence, genomic stability, and apoptosis (Mowat 1998).

In normal tissue, the p53 protein exists in the cell in a latent inactive form with a short half-life (only 20-30 minutes). Upon exposure to stress signals, however, p53 undergoes a conformational change to its active form with a significant increase in its protein level. The stress signals that activate p53 include exposure to different types of DNA damage (Kastan et al. 1991, Huang et al. 1996), hypoxia (Graeber et al. 1996), ribonucleotide depletion (Linke et al. 1996), heat shock (Ohnishi et al. 1996) and, exposure to nitric oxide (NO) (Forrester et al. 1996). In addition, p53 activity is triggered by a variety of oncogenic proteins, including Myc (Heremeking & Eick 1994), Ras (Serrano et al. 1997), adenovirus E1A (Deea & White 1993), and β-catenin (Damalas et al. 1999).

p53 primarily functions as either a transcriptional transactivator able to activate the expression of target genes containing p53 binding sites (Ko & Prives 1996) or a repressor protein able to repress the expression of certain genes in suitable circumstances, generally ones containing a TATA box in their promoters, but lacking p53 binding sites. These
genes associate with growth control and cell cycle checkpoints (e.g., p21<sub>WAF1/CIP1</sub>, GADD45, WIP1, MDM2, EGFR, PCNA, CyclinD1, CyclinG, TGF<sub>α</sub> and 14-3-3<sub>σ</sub>), DNA repair (GADD45, PCNA, and p21<sub>WAF1/CIP1</sub>) and apoptosis (BAX, BCL-2, BCL-X<sub>I</sub>, Fas, FasL, IGF-BP3, PAG608, and DR5) (Schwartz & Rotter 1998, Amundson et al. 1998). Some of the target genes of p53 involved in cell cycle arrest and apoptosis are illustrated in Figure 2.

**Fig. 2. Schematic diagram showing some target genes of p53 participating in cell cycle arrest and apoptosis.**

Cell growth arrest induced by p53 is essential to allow sufficient time for DNA repair prior to subsequent DNA replication. This response minimizes the accumulation of genetic errors during DNA replication or chromosome segregation, hence maintaining the integrity of the genome (Levine 1997). A major part of p53-mediated growth arrest proceeds through induction of the cyclin-dependent kinase (Cdk) inhibitor p21 (Hansen & Oren 1997). The p21 (<i>WAF1/CIP1/SDI</i>) gene was discovered at about the same time by several groups as a p53-inducible gene (el Deiry et al. 1993) and as an inhibitor of several Cdns essential for driving the cell to enter the S phase (Sherr 1996).

Another important function of p53 is the induction of apoptosis. Overexpression of p53 protein or induction of p53 by DNA-damaging agents increases the expression of the bax gene and decreases the expression of the bcl-2 gene (Miyashita et al. 1994b, Zhan et al. 1994a). p53 DNA-binding sequences have been found in the bax gene promoter (Miyashita & Reed 1995). However, some experiments have suggested that bax is not important for p53-dependent apoptosis in some tissues (Knudson et al. 1995, Brady et al. 1996, McCurrach et al. 1997), while other studies have shown that bax is important for p53-dependent apoptosis in some tissues (Knudson et al. 1995, Brady et al. 1996, McCurrach et al. 1997). Other death-inducing members of the bcl-2 family may be possible targets of p53 transactivation, such as Bak and Bik (Boyd et al. 1995, Chittenden et al. 1995, Kiefer et al. 1995). Another potential tar-
get for p53 transactivation in apoptosis is the Fas/APO-1 receptor, which is up-regulated by p53 (Owen-Schaub et al. 1995). Expression of wt p53 in a colon tumor cell line resulted in increased anti-Fas antibody killing (Tamura et al. 1995). Recently, more targets for p53 involved in apoptosis have been identified. They are KILLER/DR5, the PIG genes, IGF-BP3, and PAG608 (El-Deiry 1998).

The apoptotic activity of p53 can be regulated by a variety of mechanisms. The most common and efficient mechanism for inactivating p53 function is through point mutations in the gene, which result in conformationally altered protein (Symonds et al. 1994). The conformation of p53 may, however, also be changed for reasons other than missense mutations, such as changes in the redox condition, temperature and phosphorylation status of the protein (Hainaut 1995, Steegenga 1996, Levine 1997). DNA damage leading to elevated mdm-2 also leads to p53 inactivation (Linzer & Levine 1979, Bargonetti et al. 1991, Momand et al. 1992). Inactive p53, in turn, loses its capacity to activate mdm-2. Therefore, the mdm2-dependent degradation pathway of p53 does not operate and mutant p53 accumulates in cells (Prives & Hall 1999). p53 activities can be regulated by post-translational modifications and allosteric modulation of its DNA-binding ability (Gottlieb & Oren 1996, Steegenga et al. 1996). Furthermore, cytoplasmic sequestration of p53 can abrogate its DNA growth inhibitory function (Knippschild et al. 1996). The p53 function may be compromised indirectly by proteins acting downstream to p53, Bcl-2 and E1B 19 kD proteins block p53-mediated apoptosis by antagonizing Bax (White 1996).

The p53 tumor suppressor gene is the most frequently mutated gene in human tumors (Vogelstein 1990; Hollstein et al. 1991). Among the different tumor types, p53 mutation frequencies vary from high in lung, colon (over 50%), esophageal, ovarian and pancreatic cancers (40-45%) to moderate in renal and breast cancers (20-30%) and to low in melanomas (10%). p53 mutations are rarely found in Wilm’s tumor or testicular and pituitary cancers (Velculescu & El-Deiry 1996). In addition, the p53 status of a tumor is critical for the therapeutic response, since the inactivation of p53 in cancer has been associated with poor survival, refractory disease and chemoresistancy (Lowe et al. 1993, Soini et al. 1993, Marks et al. 1996, Dive 1997). It is conceivable that attempts to restore the function of p53 in cancer cells are worth development in cancer treatment (Harris 1996). The first in vivo studies on p53 gene therapy in lung cancer have already been published (Fujiwara et al. 1994, Roth et al. 1996). A plausible new form of cancer therapy would be to activate p53 by restoring the wt p53 conformation of cancer cells (Harris 1996, Levine 1997). This would become possible through characterization of the p53 regulatory pathways.

The most precise method for detecting p53 mutations is by direct sequencing. Over 80% of all p53 mutations are found in exons 5 to 8, but sequence analysis of 2 to 11 is required for the detection of all mutations (Casey et al. 1996). An alternative technique is single-strand conformation polymorphism (SSCP), which is based on the altered electrophoretic mobility of DNA sequences containing point mutations. This method was shown to have a selectivity and specificity of 90% in detecting p53 mutations (Velculescu & El-Deiry 1996).

p53 can exist in two different conformations in cells; one with a suppressor effect and one with a promoter effect on cell proliferation (Milner & Medcalf 1991, Ullrich et al. 1992). The different conformations of p53 can react with a number of p53 monoclonal
antibodies, PAb421, PAb1801, PAb240 and PAb1620, and it was suggested that the sup-
pressor form of p53 is PAb1260+/PAb240-, while the promoter form is PAb1620-/ PAb240+ (Milner & Medcalf 1991).

2.5.1.2 p53 abnormalities in AML

p53 mutations are less common in hematological malignancies, including AML, than in
solid tumors, in which p53 represents the most frequently mutated gene (Slingerland et al.
tions in AML is as low as under 5% (Fenaux et al. 1992, Schottelius et al. 1994). On the
other hand, the p53 protein in AML cells adopts the conformation of mutant p53 identified
by the antibody pAb240 (Zhang et al. 1992). One explanation could be that p53 is an
allostearic protein that can exist potentially in two conformations that may be dependent on
the cell cycle. This alteration of p53 conformation in normal dividing myeloid and lym-
phoid cells may be associated with a temporary inability of the protein to halt cells at the
G1 phase, allowing these cells to proliferate (Milner 1984, Milner & Medcalf 1991). In
AML, this “permanent” alteration in conformation could be a mechanism causing prefer-
ential proliferation over differentiation of AML cells (Imamura et al. 1994). Possible
explanation for the p53 inactivity in AML could be that about 50% of AML samples have
elevated expression of mdm-2 (murine double minute) (Bueso-Ramos et al. 1993), which
binds to wt p53 and negatively regulates its transcriptional activation (Zhu et al. 1993, Zhu
et al. 1994, Haber 1997).

2.5.2 Bcl-2

2.5.2.1 Bcl-2 and its homologs

Apart from p53, another well characterized, prominent family of apoptosis regulators is
represented by Bcl-2 and its homologs. bcl-2 is the name given to the B-cell lymphoma/
leukemia-2 gene, which was first discovered due to its involvement in the t(14;18) chromosomal translocation commonly observed in follicular non-Hodgkin’s lymphoma
(Tsujimoto et al. 1984).

The product of the bcl-2 gene is the 25 kD oncoprotein, which has been shown to be
localized predominantly within the mitochondrial membrane (Hockenbery et al. 1990,
Hockenbery et al. 1991) and also in the endoplasmic reticulum and nuclear membranes
(Chen-Levy & Clearly 1990, Monaghan et al. 1992, Jacobson et al. 1993). During emb-
yogenesis, Bcl-2 is widespread, but in adults it is topographically restricted to long-lived
cells and progenitor cells, including all hematopoietic lineages that derive from a renew-
ing stem cell (Hockenbery et al. 1991). In tumors with the t(14;18) translocation, the bcl-2 gene is transposed from chromosome 18, band q21, to the transcriptionally active immunoglobulin heavy-chain sequence on chromosome 14, band q32, resulting in overexpression of bcl-2 mRNA and protein (Tsujimoto et al. 1984). This is the most common translocation found in human lymphoid malignancies, and it is present in 80% of follicular lymphoma cases. However, high levels of Bcl-2 are also found in other hematological malignancies, which lack the t(14;18) translocation (Pezzella et al. 1990, Zutter et al. 1991).

At least 17 homologs of Bcl-2 have been so far described in the human (Reed 1999). They share one or more of the four conserved regions with the Bcl-2 protein, which are known as the Bcl-2 homology domains: BH1 (residues 136-155), BH2 (187-202), BH3 (93-107) and BH4 (10-30) (Reed et al. 1996). They have been functionally divided into two groups: 1) anti-apoptotic group, including Bcl-2, Bcl-XL, Mcl-1, A1/Bfl-1, Bcl-W, Boo/Diva and Nr-13, and 2) pro-apoptotic group, including Bax, Bcl-Xs, Bok/Mtd, Bad, Blk, Bak, Bik/Nbk, Hrk, Bim/Bod, Nip3, Nix/Bnip3 and Bid. Many of them can physically interact with each other, forming a complex network of homodimers and heterodimers. In this way, it is assumed that the ratio of activators to inhibitors of apoptosis in a cell could determine the propensity of the cell to undergo PCD (Korsmeyer 1995, Reed 1997, Gross et al. 1999).

### 2.5.2.2 Function of the bcl-2 family of proteins

Most members of the Bcl-2 family of proteins contain a hydrophobic stretch of amino acids at their carboxy-termini that cause them to insert post-translationally into biologic membranes, primarily the outer mitochondrial membrane, nuclear envelope, and parts of the endoplasmic reticulum (Krajewski et al. 1993, Gonzalez-Garcia et al. 1994, Zha et al. 1996). Unlike the classical oncogene, which functions by increasing the rate of cell proliferation, Bcl-2 acts as a regulator of cell death by prolonging cell survival and inhibiting apoptosis (Vaux et al. 1988; Hockenbery et al. 1990). Although the precise mechanism by which Bcl-2 and its homologs influence apoptosis remains unresolved, it was suggested that these proteins possess at least three general functions: (A) dimerization with other Bcl-2 proteins. For instance, anti-apoptotic proteins, such as Bcl-2, and pro-apoptotic proteins, such as Bax, engage in close combat with each other, and the ratio of bcl-2:bax, thus, serves as a rheostat to determine the susceptibility to apoptosis (Olsvik & Korsmeyer 1994, Korsmeyer 1999). (B) Bcl-2 (Schendel et al. 1997), Bcl-XL (Minn et al. 1997) and Bax (Antonsson et al. 1997, Reed 1997, Schleisinger et al. 1997) can generally form 20-300 pS single channels in a model of planar phospholipid membranes. Under some conditions, the channels become much larger (~2 nS) (Antonsson et al. 1997, Schleisinger et al. 1997). Therefore, it was suggested they might be involved in the regulation of the release of apoptogenic proteins, such as cytochrome c and apoptosis-inducing factor (AIF), from mitochondria into cytosol during apoptosis. However, the pore-forming capabilities of the proteins of the Bcl-2 family have not been proven yet in vivo, leaving this functional aspect of the proteins unresolved. (C) Bcl-2 and some of its anti-
apoptotic homologs, such as Bcl-XL, can bind (or at least co-immunoprecipitate with) a wide variety of other cellular proteins directly or indirectly involved in cell death regulation. These interactions suggest that one of the roles of Bcl-2 is to serve as a site onto which other proteins can dock, thereby altering cellular activities. The docking of proteins onto Bcl-2 may sequester proteins, such as apoptosis-activating factor 1 (Apaf-1) or calcineurin, so that they cannot interact with other proteins in cytosol. Apaf-1 can associate with several death proteases, including caspase-4, 8, 9, in mammalian cells. As a result of this docking, cytosolic cytochrome c might fail to find its functional partner Apaf-1 and to activate pro-caspase-9. In the case of calcineurin, which is a calcium-regulated protein phosphatase, binding to Bcl-2 sequesters this protein at membranes, thus preventing it from dephosphorylating proteins in cytosol (Shibasaki 1997). Although the biologic significance of calcineurin binding to Bcl-2 has not been firmly established, it presumably plays a role in the suppression of apoptosis in the presence of elevated cytosolic calcium. Alternatively, docking with Bcl-2 may localize proteins, such as Raf-1, to various membranes within the cell, so that they can interact with other membrane-associated proteins. For example, the nonphosphorylated Bad, a pro-apoptotic member of the Bcl-2 family, represents the active form bound to membrane-associated Bcl-XL, which would displace Bax, resulting in cell death (Yang et al. 1995). The interaction of Raf-1 with Bcl-2 allows the protein kinase, which is normally found primarily in cytosol, to translocate to mitochondrial membranes and to cause the inactivation of Bad, a proapoptotic member of the Bcl-2 family, by phosphorylation (Wang et al. 1996, Zha et al. 1996).

In addition, Bcl-2 is able to prevent apoptosis by minimizing the formation of reactive oxygen species (ROS) under aerobic conditions (Jacobson & Raff 1995, Shimizu et al. 1995) and by preventing permeability transition (Decaudin et al. 1997).

2.5.2.3 Expression of bcl-2 in normal and abnormal hematopoiesis

The expression of bcl-2 in hematopoietic cells was observed to relate inversely to maturation, i.e. most Bcl-2 exists in early myeloid precursors, including myeloblasts and promyelocytes, but is absent in myelocytes and neutrophils. Monocytes and B-cell precursors express intermediate levels of bcl-2 (Delia et al. 1992, DiGiuseppe et al. 1996). Moreover, the expression of bcl-2 is highly related to CD34-positive cells fractionated from either normal adult or fetal BM samples (Bradbury & Russell 1995, Porwitt-MacDonald et al. 1995). However, very early hematopoietic cells characterized as CD34+ lin–CD38– were found to express bcl-XL but not bcl-2 (Park et al. 1995).

bcl-2 is expressed in about 40% of AML (Reed 1995). By using flow cytometry techniques, Bcl-2 can be detected in up to 90% of AML specimens obtained at diagnosis, although the mean levels of Bcl-2 could be 40-fold in some AML samples compared to some others (Delia et al. 1992, Campos et al. 1993, Bensi et al. 1995, Porwit-MacDonald et al. 1995). Karakas and co-workers analyzed 219 AML BM samples by reverse transcriptase polymerase chain reaction (RT-PCR). They detected mRNA of bcl-2 in 133 of their 156 (84%) patients at diagnosis and in 40 out of 42 (95%) at relapse (Karakas et al. 1998). High bcl-2 expression in AML were correlated with the stem cell marker CD34

The mechanism by which ATRA inhibits blast cell growth in AML is still unclear. However, there is evidence that ATRA is able to down-regulate bcl-2 in AML cells. McCulloch’s group observed, in two AML cell lines, that down-regulation of bcl-2 in mRNA began after two hours of ATRA (10^{-7}M) exposure, while the protein level began to decrease at 36 hours (Hu et al.1995). ATRA was also able to shorten the half-life of Bcl-2, while the growth factor GM-CSF alone or in combination with G-CSF favored bcl-2 expression (Hu et al. 1996). Russell’s group reported the effect of ATRA (10^{-6}) on bcl-2 protein expression based on flow cytometry and blast cells from 25 AML patients, showing down-regulation of bcl-2 in 32% of the cases (Bradbury et al. 1996). Pisani and co-workers also confirmed that ATRA (5×10^{-7}M) down-regulates the expression of bcl-2 in AML blasts (Pisani et al. 1997).

### 2.5.3 Mitochondria

#### 2.5.3.1 Function of mitochondria

Mitochondria are bacterium-sized organelles in eukaryotic cells, and they were first identified by light microscopy in the nineteenth century. It has been thought since 1948 that the only function of mitochondria is to produce energy (ATP) for all eukaryotic cells by combining oxygen with food molecules. In 1989, Lancaster and co-workers showed for the first time that mitochondria are involved in cell death (Lancaster et al. 1989). Now it is clear that mitochondria play an important role in apoptosis for at least three reasons: (A) Most members of the Bcl-2 family, central apoptotic regulators, are located in mitochondrial membrane, and the existence of mitochondria seems necessary for the function of bcl-2 proteins (Newmeyer et al. 1994, Zhu et al. 1996). (B) The mitochondria that undergo permeability transition (PT) liberate a ~50 kD apoptogenic protein, called AIF, which is a protease and capable of directly activating caspase-3 (Susin et al.1996). Another protein that can be released from mitochondria is cytochrome c, which cannot, in contrast to AIF, be able to directly function in the nuclei. (C) Mitochondria can produce a cell-death-signaling reactive oxygen species (ROS), which sometimes turns mitochondria into central executors of apoptosis (Golstein 1997, Kroemer et al. 1997, Reed 1997). Moreover, it has been shown that the mitochondrial membrane potential (∆Ψ_m) may decrease before the fragmentation of DNA (Vayssiere et al.1994, Petit et al. 1995, Zamzami et al. 1995). In certain experimental systems, however, PT may occur more than 4 or 5 hours after the death-inducing signal (Yang et al. 1997, Kluck et al. 1997).
Cytochrome c is a small water-soluble and very stable hemoprotein of 13 kD, including 104 amino acid residues (Skulachev 1998). It is encoded as apo-cytochrome c, translated on cytoplasmic ribosomes, and follows a unique pathway into mitochondria that does not require the signal sequence, electrochemical potential or general protein translocation machinery (Mayer et al. 1995). On entry into the intermembrane space, the apo-protein gains a heme group and becomes the fully folded holo-cytochrome c. This globular, positively charged protein can no longer pass through the outer mitochondrial membrane and is thought to snuggle up to the cytochrome c oxidase complex located in the inner membrane (Mignotte & Vaysiere 1998). It was believed for many years that the function of cytochrome c was only to transport electrons from cytochrome c1 to cytochrome oxidase. Because the transport per se is not coupled to energy transduction, the role of cytochrome c seemed to be restricted to the function of a shuttle connecting two respiratory chain energy transducers, i.e. the bc1 complex (complex III) and cytochrome oxidase (complex IV). In 1996, this classic theory was shaken by Wang and co-workers, who discovered that this protein is somehow involved in apoptosis (Liu et al. 1996). They observed that the addition of deoxy-adenosine triphosphate (dATP) to a cell-free extract of HeLa cells resulted in typical apoptotic changes and that at least two factors would be necessary for the induction of this type of apoptosis. One of them appeared to be cytochrome c. Subsequently, other experiments using both subcellular fractionation methods (cell free systems) and confocal immunofluorescence microscopy (intact cells) demonstrated that, following exposure of the cell to apoptotic stimuli, cytochrome c is rapidly released from mitochondria into cytosol (Kluck et al. 1997, Yang et al. 1997).

How can holo-cytochrome c escape from the outer membrane of mitochondria into cytosol? First, Petit and co-workers (1996) showed a strong correlation between mitochondrial PT and apoptosis. They suggested that PT pore opening is one of the reasons that causes cytochrome c release from mitochondria. Mitochondrial PT is caused by the opening of a large cyclosporin-inhibited conductance channel (~1.5 nS) in the inner membrane of mitochondria, resulting in dissipation of the electrochemical (H+) gradient and osmotic swelling due to the high salt concentration in the mitochondrial matrix. The swelling that follows the PT pore opening causes a rupture of the outer membrane, since the surface area of the inner membrane with its cristae is considerably larger than that of the surrounding outer membrane. The rupture of the outer membrane could result in the escape of proteins from mitochondria, e.g. cytochrome c and AIF. PT pore opening induced by different stimuli, e.g. cyanide, oligomycin, and atracyslide, can be protected by Bcl-2 and Bcl-XL, while Bax can induce a loss of \( \Delta \Psi \)m through a caspase-independent mechanism (Reed 1998). The second model predicts a specific channel located in the outer membrane that allows release of cytochrome c. In this regard, both anti-apoptotic (Bcl-2, Bcl-XL) and pro-apoptotic (Bax) members of the bcl-2 family have been demonstrated to form ion channels in synthetic membranes in vitro (Minn et al. 1997, Schendel et al. 1997, Antonsson et al. 1997, Schlesinger et al. 1997). Moreover, under some in vitro circumstances, Bcl-2 can evidently prevent the formation of Bax channels in liposomes. This supported the finding by Wang’s group. They reported that staurosporine-induced release of cytochrome c into cytosol in HL-60 leukemia cells is blocked by over-
expression of the anti-apoptotic proteins, Bcl-2 and Bcl-X<sub>L</sub> (Yang et al. 1997). However some studies, have suggested that the release of cytochrome <em>c</em> from mitochondria into cytosol occurs prior to the PT pore opening (Kluck et al. 1997, Yang et al. 1997), it is possible that bax creates pores in the outer membrane which are large enough to allow the escape of cytochrome <em>c</em> (Susin et al. 1997). Using a cell-free system (isolated mitochondria), Reed’s group demonstrated that by releasing only a small proportion (~20%) of total cytochrome <em>c</em> initially, bax may favor the induction of a cell death process that involves rapid activation of caspases, causing apoptosis. In contrast, the broad-specific caspase inhibitor zVAD-fmk and the caspase-inhibiting protein X-IAP had no effect on bax-induced release of cytochrome <em>c</em> from mitochondria <em>in vitro</em>, but prevented the subsequent activation of caspases in cytosolic extracts (Jurgensmeier et al. 1998).

Fig. 3. Cytochrome <em>c</em>-mediated cell death. Cytochrome <em>c</em> could be released from mitochondria into cytosol by apoptotic stimuli, e.g. growth factor depletion or DNA damage. Together with its cytosol partner, Apaf-1, it can induce apoptosis through the activation of caspases when dATP is present (1). The activated caspases can feed forward the induction of mitochondrial PT pore opening, resulting in increases cytochrome <em>c</em> release (2). Bcl-2 can prevent this apoptotic signaling by either inhibiting cytochrome <em>c</em> release or docking Apaf-1. On the other hand, cytochrome <em>c</em> release can cause electron transport interruption in mitochondria, which leads to cell death by necrosis (3).

Once liberated from mitochondria into cytosol, cytochrome <em>c</em> could lead to cell death in at least three ways (Figure 3). (A) It can convert procaspase-9 to active caspase-9 together with Apaf-1 and dATP. Active caspase-9 then cleaves pro-caspase-3 into caspase-3, a terminal enzyme involved in apoptosis (Zou et al. 1997, Li et al. 1997, Zou et al. 1999). (B) Active caspases can induce mitochondrial PT pore opening, at least as defined by the loss of ΔΨ<sub>m</sub> (Hirsch et al. 1997, Susin et al. 1997). The mechanisms responsible may entail proteolytic activation of a pro-apoptotic protein such as Bid (Li et al. 1998, Luo et al. 1998), or proteolytic removal of the N-terminal BH4 domains from Bcl-2 and Bcl-X<sub>L</sub>, converting them to killer proteins (Cheng et al. 1997). These observations imply the existence of an amplification mechanism by which cytochrome <em>c</em> release from a few mito-
chondria can induce caspase activation, thereby recruiting more mitochondria into the process. (C) The loss of cytochrome \( c \) from mitochondria can also interrupt electron transport in the inner membrane of mitochondria, since cytochrome \( c \) is responsible for the transfer of electrons from complex III to IV in the respiratory chain. The interruption of electron chain transport has several potential consequences, including generation of ROS, a loss of the electrochemical gradient across the inner membrane and ATP depletion leading to cell death by necrosis (Reed 1998).
3 Purpose of the present study

AML remains incurable in the majority of patients, largely due to resistance to chemotherapy. The important mechanism of action of chemotherapeutics is to induce apoptosis in the target cells. Thus, knowledge of the biological mechanisms of apoptosis in AML cells would provide a clue to finding new treatment strategies. ATRA, a vitamin A derivative, is able to inhibit the growth of leukemic cells. According to preliminary experiments we had a reason to hypothesize that ATRA is able to induce apoptosis in AML. The purpose of the present study was answer to the following questions:

1. What are the general features of ATRA-induced apoptosis in AML cells?
2. What is the status of Bcl-2 and p53, well-known regulators of apoptosis, in these cell lines, and do they participate into mediating the effects of ATRA.
3. What is the role of mitochondria in ATRA-induced apoptosis?
4 Materials and methods

4.1 AML cells

4.1.1 Source of cells

The study was carried out in accordance with the Helsinki Declaration and approved by the Ethical Committee of the Faculty of Medicine, University of Oulu. After informed consent, blood samples from PB and/or BM was drawn at the same time as those for clinical tests before chemotherapy. The AML blast cells that gave rise to the cell lines were obtained from AML patients admitted to the Leukemia Treatment Unit at the Department of Internal Medicine, University of Oulu. The diagnosis of AML was based on May-Grünwald-Giemsa (MGG), Sudan B black, and esterase stainings of bone marrow and blood smears according to the FAB classification criteria (Bennet et al. 1976). The clinical features of the patients and the corresponding nomenclature of the cell lines are given in tables 1 and 2. The patients numbered 1, 2, 3 and 8 represent de novo AML and those numbered 4-7 relapsed and clinically chemoresistant disease. The chemotherapy of the patients was carried out by following the AML-86 treatment protocol of the Finnish Leukaemia Group (Elonen et al. 1998).

4.1.2 Establishment and characterization of AML cell lines

Mononuclear cells (including blast cells) were separated by Ficoll-Metrizoate (Nycomed, Oslo, Norway) density gradient centrifugation. The cells then were cryopreserved at -70°C in the presence of 50% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY, USA), 10% dimethyl sulfoxide (Aldrich-Chemie, Steinheim, Germany) and α-minimal essential medium (α-MEM, Gibco). For the cultures, the cells were quickly thawed, washed twice with α-MEM and cultured at a high cell density of 1-2x10⁶/ml in
α-MEM and 10% FCS in the presence of the following growth factors: 100 U/ml of interleukin-3 (IL-3) and IL-6 (Sandoz, Forschungsinstitut, Vienna, Austria), 100 U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Novartis, Helsinki, Finland), and 40 ng/ml of mast cell growth factor (Immunex Corporation, Seattle, WA, USA). After culturing for 6 to 12 weeks in the above mentioned culture media, the cells were allowed to proliferate in 10% FCS and α-MEM for the next three months. Thereafter the cells were frozen at -70°C. For the experiments the cells were thawed and continuously grown in the presence of 10% FCS and α-MEM in a humid atmosphere at 37°C with 5% CO₂ at a cell density of 3.5×10⁷/ml. Fresh medium was generally changed every 3-4 days, and every other day for the experiments. The cell were in the exponential growth phase when used for experiments. The doubling time of the cell line cells varied from 2.5 to 3.5 days, the mean value being 2.9 ± 0.3 days.

The AML cell lines were numbered from 1 to 8 corresponding to the patients presented in table 1, and they were labelled as Oulu University (OU)-AML cell lines. The OU-AML cell lines 1 to 8 were used in the papers I and II, the cell lines 3, 4, 7 and 8 in paper III, and the cell lines 3 and 7 in paper IV.

Table 1. Characteristics of the patients from whom the OU-AML cell lines originated.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Age/Sex</th>
<th>FAB</th>
<th>Blast cell source</th>
<th>Phase of the disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>OU-AML-1</td>
<td>26/F</td>
<td>M4</td>
<td>BM</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>OU-AML-2</td>
<td>52/F</td>
<td>M2</td>
<td>PB</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>OU-AML-3</td>
<td>48/M</td>
<td>M4</td>
<td>BM</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>OU-AML-4</td>
<td>39/M</td>
<td>M2</td>
<td>BM</td>
<td>Relapse</td>
</tr>
<tr>
<td>OU-AML-5</td>
<td>70/M</td>
<td>M5B</td>
<td>BM</td>
<td>Relapse</td>
</tr>
<tr>
<td>OU-AML-6</td>
<td>47/F</td>
<td>M1</td>
<td>PB</td>
<td>Relapse</td>
</tr>
<tr>
<td>OU-AML-7</td>
<td>63/F</td>
<td>M4</td>
<td>PB</td>
<td>Diagnosis</td>
</tr>
<tr>
<td>OU-AML-8</td>
<td>63/F</td>
<td>M4</td>
<td>BM</td>
<td>Diagnosis</td>
</tr>
</tbody>
</table>

* F, female; M, male; PB, peripheral blood; BM, bone marrow

To detect possible mycoplasma in the cell cultures, a nucleic acid hybridization based technique was used (Gen-Probe Mycoplasma T.C. rapid detection system, Gen-Probe Incorp., San Diego, CA, USA). All the cell lines were free of mycoplasma.

The morphology of the AML cell line cells was estimated from MGG-stained cytospin preparations. It was typical of myeloblasts.

Immunophenotype analyses were carried out by using monoclonal antibodies against differentiation antigens and a FACSort flow cytometer (Becton & Dickinson). The informative immunophenotypes of the cell lines are presented in paper II. None of the cell lines expressed glycophorin A antigen or any of the following cluster and differentiation (CD) antigens: CD3, CD19, CD61 or CD117.

Cell line karyotypes were analysed by a standard Giemsa banding technique. The karyotype was diploid in the cell lines 2 and 3 and hyperdiploid (triploid or tetraploid) in the others. Otherwise, the chromosome patterns were chaotic, containing innumerable changes. However, no structural changes were observed in chromosome 17p in any of the cell lines.
4.2 ATRA

ATRA was purchased from Sigma (Sigma Chemical Company, St Louis, MO, USA). It was prepared just before use in 100% ethanol and diluted into α-MEM at the desired concentration. The final concentration of ethanol was equal to or less than 0.01% in the experiments and not toxic for cells. All experiments involving ATRA were performed in subdued light, and the tubes and culture plates containing ATRA were covered with aluminium foil.

4.3 Cell culture methods

4.3.1 Colony-forming assay

A modified semi-solid methylcellulose (mc) method described by Buick and co-workers (Buick et al. 1977) was used to investigate the effect of ATRA on the formation of clonogenic blast cell colonies. The cells were plated in 96-microwell plates (Greiner, Alphen a/d Rijn, The Netherlands) in 0.1 ml of basic growth medium [10% heat-inactivated FCS in α-MEM] and 0.9% mc (Aldrich-Chemie, Steinheim, Germany) with or without ATRA and incubated in a humidified atmosphere at 37°C with 5% CO₂ at a cell density of 3 × 10³ cell/well. All the cultures were performed in triplicate. Colony formation was observed and classified using an inverted microscope. Colonies from three wells/sample containing more than 20 cells were counted and the mean colony number was calculated.

4.3.2 Suspension culture assay

In the suspension culture, 1 × 10⁶ cells were incubated in the presence or absence of ATRA in 1 ml of basic growth medium in 24-multiwell plates (Becton Dickinson & Company, Lincoln Park, New Jersey, USA) as described previously (Nara & McCulloch 1985). The cell number and viability were determined by vital dye exclusion (0.4% trypan blue, Sigma Chemical Co. Ltd., Irvine, UK) using a standard hemocytometer.
4.4 Estimation of differentiation

4.4.1 Morphology

MGG-stained cytospin preparations and a standard technique developed to evaluate and count granulocyte lineage cells were used to estimate the differentiation and maturation of blast cells (Dacie & Lewis 1984). At least 500 cells were counted.

4.4.2 Nitroblutetrazolium test

$1 \times 10^6$ cells in a 1 ml volume were incubated at 37°C for 25 min in the presence of 0.1% nitroblue tetrazolium and 100 ng of phorbol myristate acetate. After incubation, the cells were cytospinned and the slides stained with MGG. At least 200 cells were counted and scored for the presence of blue-black formazan granules (Chomienne et al. 1986).

4.5 Detection of apoptosis

4.5.1 Morphology

The estimation of apoptosis was based on an evaluation of typical morphological changes (Wyllie 1987) in the cells from MGG-stained cytospin preparations using a light microscope. Apoptosis was also analyzable from some of the immunohistochemistry slides by this method.

4.5.2 Detection of internucleosomal fragmentation of genomic DNA

The genomic DNA of $1 \times 10^6$ cells was extracted, purified, and spectrophotometrically quantified by absorbance at 260 nm. One μg of DNA was labeled at the 3’ ends with Digoxigenin-11-ddUTP (Boehringer-Mannheim GmbH, Mannheim, Germany) using 25 IU of terminal transferase (Boehringer-Mannheim). The labeled DNA samples were loaded onto 2% agarose gels, separated by electrophoresis for 3h at 50V and by Southern blotting on Hybond N+ nitrocellulose membrane. The internucleosomal fragmentation of DIG-labeled DNA was detected by a chemiluminescent substrate for alkaline phosphatase, CSPD (Boehringer-Mannheim). The experiments were done in duplicate.
4.5.3 Externalization of phosphatidylserine

A fluorescein-isothiocyanate-conjugated (FITC) annexin V (Boehringer Mannheim, Manheim, Germany) with propidium iodide was used in flow cytometry to detect apoptotic cells. Annexin V has been shown to interact strongly and specifically with phosphatidylserine residues, and it can be used to detect apoptosis by targeting for the loss of plasma membrane symmetry (van Engeland et al., 1998). For the analyses, the cells were harvested from a suspension culture, washed twice with phosphate-buffered saline (PBS) and incubated in annexin V-Fluos labeling solution (2% Annexin-V-Fluos and 1.0 μg/ml propidium iodide in 10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 5mM CaCl₂) for 15 min at room temperature in dark. After that, the cells were washed twice with Hepes buffer without labeling reagents and analyzed using a FACSort flow cytometer (Becton Dickinson). The experiments were repeated 2 to 3 times.

4.5.4 Cleavage of Poly(ADP ribose)polymerase

The cells harvested from the suspension cultures were first washed twice with PBS. 2x10⁶ cells were directly lysed in 50 μl of Laemmli sample buffer containing a mixture of protease inhibitors (CompleteTM, EDTA-free; Boehringer Mannheim GmbH, Mannheim, Germany). The lysed cells were centrifuged at 2000 rpm for 4 min and the supernatant was collected and stored at -80°C. 15 μl of the cell lysate was applied to 12% SDS-PAGE (Bio-Rad, Hercules, CA, USA) and transferred on to Hypond nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK). After blocking for 30 min with 8% dried fat-free milk in Tris-buffered saline-Tween (TBS-T) (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% Tween-20), the membranes were incubated for 1 hour with the primary antibody, a mouse anti-human monoclonal antibody for PARP (Pharmingen, San Diego, CA, USA). The specific protein-antibody complex was detected by using a secondary antibody, horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Amersham, UK) and an enhanced chemiluminescence (ECL) detection kit (Amersham, UK). The antibody dilutions were 1:10 000. In each membrane, immunoblotting of actin, a house-keeping protein, was used to equalize protein loading.
4.6 Analysis of TP53 mutations and expression

4.6.1 Analysis of TP53 mutations with single strand conformation polymorphism

Exons 5 to 8 of TP53 were amplified individually by polymerase chain reaction (PCR) using two sets of intron primers for each of the exons, the second set being internal to the first one (nested primers) (Lehman et al. 1991). The primers were kindly provided by Dr. Curtis C Harris (Laboratory of Human Carcinogenesis, NIH, NCI, Bethesda, MD 20892). Dynazyme DNA polymerase and the respective buffer (Finnzymes, Espoo, Finland) were used in the PCR with other reagents and under reaction conditions described earlier (Vähäkangas et al. 1992). Negative controls (reaction mixture without the template) were included in each amplification in order to test for contaminations. The size of the amplified DNA was controlled electrophoretically in a 3% NuSieve 3:1 agarose gel (FMC, Finnzymes) with molecular size markers. The amplified DNA was purified by running in a 3% NuSieve 3:1 agarose gel. Bands of appropriate sizes were cut out of the gel and the DNA was eluted with ammonium acetate. Eluted DNA was precipitated with 100% ethanol in a freezer and the precipitated DNA was dissolved into 30 to 50 µl of TE buffer.

For SSCP, a 1:1 mixture of purified DNA and bromophenolblue-formaldehyde stop solution (Sequenase Kit, US Biochemicals) was denatured for 5 min at 100°C, and one µl of the mixture was used for each run (Welsh et al. 1997). The samples were loaded on a 20% homogeneous polyacrylamide gel, and the gel was run on Pharmacia Phastsystem semi-dry electrophoresis equipment with neutral buffer strips (Pharmacia Biotech, Uppsala, Sweden). The gels were stained with the Silver Staining kit (Pharmacia) according to the manufacturer’s instructions. For negative controls, TP53 exons were amplified from wt lymphocyte DNA. For a positive control, lymphocyte DNA was amplified using a mutated 5’ primer (Welsh et al. 1997). The efficiency of the described SSCP method in detecting mutations within TP53 exons 5-8 has been shown to be 98%, i.e. 98% of the known mutations can be detected (Welsh et al. 1997).

4.6.2 Detection of p53

4.6.2.1 Immunohistochemistry

The cells were prepared for immunohistochemistry as described earlier (Soini et al. 1992, Rämö et al. 1995) by fixing in 10% neutral formalin for 2-3 days at room temperature, after which the cells were pelleted by centrifugation. The cell pellet was suspended in melted 2% agarose, and the agarose block was further embedded in paraffin. Four-µm-thick sections were placed on slides and stained for p53 protein by using the routine avi-
4.6.2.2 Western blotting

The cells harvested from the suspension cultures were first washed twice with phosphate-buffered saline (PBS) and then lysed in two ways as follows: for whole-cell extracts, \(2 \times 10^6\) cells were directly lysed in 50 ul of Laemmli sample buffer, and for nuclear and cytoplasmic extracts, \(1 \times 10^7\) cells were lysed in 150 ul of low-salt HEPES buffer (20 mM HEPES, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, 1mM DTT, 0.1% NP40). Both buffers contained the following protease inhibitors: 500 mM PMSF, 2mg/ml aprotinin, 1.4 mg/ml pepstatin A and 1mg/ml antipain. After 10 min on ice, the lysed cells were centrifuged at 2000 rpm for 4 min and the supernatant (containing the whole-cell or cytoplasmic extract) was collected. The pellet from low-salt HEPES buffer was lysed further in 50 ul of HEPES buffer with a high salt content of 500 mM NaCl. The second supernatant (containing the nuclear extract) was collected after shaking the mixture at \(+4^\circ\)C and centrifugation at 14000 rpm for 15 min. The protein contents of the cytoplasmic and nuclear extracts were determined by Bio-Rad protein assay (Bio-Rad Laboratories, USA). 15 ul of cell lysate and 20 \(\mu\)g of cytoplasmic or 40 \(\mu\)g of nuclear extract were applied to 12% SDS-PAGE (Bio-Rad, Hercules, CA, USA) and thereafter to Western blotting. The procedure of Western blotting was done similarly to PARP detection, except that the primary antibody was a mouse anti-human p53, DO7 (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK). OVCAR-3 ovarian carcinoma cell line cells, which express both cytoplasmic and nuclear p53 protein (Rämet et al., 1998), were used in all experiments as positive controls. The p53 protein is present in its denatured form in Western blot analysis. DO7 antibody recognizes both mutated and wt protein. Molecular weight markers were used to make sure that the studied protein was 53 kD in size.

The Western blot signals were quantified using a Molecular Dynamics 300A Computing Densitometer (Sunnyvale, CA, USA). Expression is given as the specific protein-to-actin signal ratio, and the ratio without ATRA exposure is used as a control value.

4.6.2.3 Flow cytometry

When analysed by flow cytometry, the p53 protein remains its native, non-denatured form (Zhu et al. 1993). To study the protein conformation of native p53, the cell lines were investigated flow-cytometrically by using three different monoclonal anti-p53 antibodies. For the analyses, the cells were harvested from the suspension cultures and washed twice with PBS, whereafter they were treated with 70% cold ethanol for 15 min and washed...
twice with PBS. The permeabilized cells were incubated for 30 min at room temperature with one of the mouse anti-human p53 monoclonal antibodies or with a non-specific mouse isotype control. The antibody-treated cells were washed twice with PBS and incubated with fluorescein isothiocyanate-conjugated (FITC) rabbit anti-mouse F(ab)2 fragments (Dakopatts, Glostrup, Denmark) for 30 min. Then the cells were washed twice with PBS, and a total of $10^4$ cells were analyzed using a FACSort flow cytometer (Becton-Dickinson).

The following monoclonal mouse anti-human antibodies were used in the flow cytometer analyses: DO7, Ab3 (clone PAB 240) and Ab5 (clone PAb 1620), the first having been purchased from Novocastra Lab and the others from Oncogene Research Products/Calbiochem (Cambridge, MA, USA). Ab5 recognises only the wt p53, whereas Ab3 detects only a mutated form of the protein. The mutated p53 recognised by Ab3 can be either a protein translated from a mutated p53 gene, or a wt p53 that is in a mutational protein conformation (Zhu et al. 1993). The antibody DO7 recognizes the p53 protein regardless of the conformation.

### 4.7 Analysis of Bcl-2

#### 4.7.1 Western blotting

The cells were harvested and washed twice with ice-cold PBS. Bcl-2 was analyzed using either whole-cell extracts or both mitochondrial and cytosolic fractions. The whole-cell extracts were prepared as described above. The basic methodology for the preparation of mitochondria and cytosol fractions was as described by Yang et al. 1997 (Yang et al. 1997). In brief, the cells were resuspended in five volumes of buffer A (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium-EDTA, 1 mM sodium-EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose and a mixture of protease inhibitors (Complete™, EDTA-free; Boehringer Mannheim). The cells were homogenized with 10 strokes of a Teflon homogenizer, and the homogenates were centrifuged twice at 750 g for 10 min at 4°C. The supernatants were centrifuged at 10 000 g for 15 min at 4°C, and the resulting mitochondria pellets were resuspended in buffer A containing 250 mM sucrose and frozen in multiple samples at –80°C. The supernatants of the 10 000 g spin were further centrifuged at 100 000 g for 1 hour at 4°C, and the resulting supernatants (cytosolic protein) were divided into samples and frozen at –80°C. The protein contents of the mitochondria and cytosol fractions were determined by Bio-Rad protein assay (Bio-Rad Laboratories, USA). 15 µl of cell lysate and 30 µg of cytosolic or mitochondrial protein were applied to 12% SDS-PAGE (Bio-Rad, Hercules, CA, USA). Western blotting was done similarly as for p53 detection, but the primary antibody was a mouse anti-human Bcl-2 (DAKO A/S, Glostrup, Denmark).
4.7.2 Flow cytometry

The expression of bcl-2 was detected as described for p53. The primary antibody was the same as for Western blotting. The results are presented as either cell numbers or values describing the change in mean fluorescence intensity (ΔMFI).

4.7.3 Enzyme-linked immunosorbent assay

Bcl-2 was determined using the Bcl-2 ELISA kit (Calbiochem, Cambridge, MA, USA) according to the manufacturer’s instructions with small modifications. In brief, 1x10^6 cells were washed twice with PBS and resuspended in 200 µl of resuspension buffer (50 mM Tris, containing 5 mM EDTA, 0.2 mM PMSF, 1 l/ml pepstatin, and 0.5 g/ml leupeptin adjusted to pH 7.4). After the addition of 40 µl of antigen extraction agent (provided) the sample was kept on ice for 30 minutes with occasional vortexing and then centrifuged for 5 min. The protein content of the lysate that was formed was determined by Bio-Rad protein assay (Bio-Rad Laboratories, USA), and the lysate was then diluted with sample diluent (provided) down to 100 µl, including 60 µg of protein in the case of OU-AML-3 and 40 µg in case of OU-AML-7. Then 50 µl of the detector antibody (provided) and 50 µl of the diluted sample or the Bcl-2 standard (in duplicate) were added to the microtiter wells (provided) and incubated for two hours at room temperature. Unbound material was then washed out with wash buffer (provided) and 100 µl of diluted horseradish peroxidase-conjugated anti-FITC antibody (provided), was added. After 30 minutes’ incubation in dark the wells were washed. This was repeated once. After that, 100 µl of stop solution was added. The colored reaction product was quantified immediately using a spectrophotometric plate reader (Victor™ 1420 Multilabel Counter, Wallac) at dual wavelengths of 450/595 nm. The standard curve was made using six standards (0, 5.12, 12.8, 32, 80 and 200 U/ml). The Bcl-2 values were then determined by interpolation from the standard curve. The Bcl-2 values of U/ml were transformed to corresponding values per µg of protein.

4.8 Analysis of Bax

Bax was detected from mitochondrial and cytosolic fractions by Western blotting as described for Bcl-2. The primary antibody was a mouse monoclonal antibody against human Bax (Pharmingen, San Diego, CA, USA).
4.9 Detection of cytochrome c

For cytochrome c, the protein was detected in either mitochondrial or cytoplasmic fractions by Western blotting as described for Bcl-2. The primary antibody was a mouse monoclonal antibody against human cytochrome c (Pharmingen, San Diego, CA, USA).

4.10 Analysis of mitochondrial membrane potential

The variation of $\Delta \Psi_m$ during ATRA exposure was studied using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; Molecular Probes, Inc., Eugene, OR, USA) (Cossarizza et al. 1993, Salvioli et al. 1997). After exposure of the cells to ATRA, the cell suspension was adjusted to a density of $0.75 \times 10^6$ cells/ml and incubated in complete medium for 25 min at $37^\circ C$ in a humidified atmosphere of 5% CO$_2$ in dark with 10 $\mu$g/ml JC-1. At the end of the incubation period the cells were washed twice in cold PBS, resuspended in a total volume of 350 $\mu$l and analyzed using a FACSort flow cytometer (Becton-Dickinson).

4.11 Statistical analyses

The results are presented either as mean values of two experiments or the mean ± SD of three or more experiments. The two-tailed paired t-test or the Wilcoxon signed-rank test was used to assess the differences between the different groups of matched controls.
5 Results

5.1 Effect of ATRA on AML cell growth

5.1.1 In methylcellulose (I)

Inhibition of clonogenic blast cell growth was observed in the case of each individual cell line when ATRA was present in mc (figure 3 in paper I) or the cells were exposed to ATRA for 72 hours before plating into mc (figure 5 in paper I). A significant concentration-dependent decrease in colony formation was observed when all the eight AML cell lines are evaluated (figure 4; table 3 in paper I). Moreover, the size of the colonies also decreased in a concentration-dependent manner (figure 4 in paper I).

Fig. 4. Number of colonies in methylcellulose (mc) in the presence of all-trans retinoic acid (ATRA) (A) or after exposure of the cells to ATRA in suspension for 72 hours before plating them into mc (B). The results are mean values (±SD) of eight OU-AML cell lines in two independent experiments. **, P<0.01; ***, P<0.001; NS=not significant.
5.1.2 In suspension (I)

The viability of OU-AML cells in suspension remained over 90% for 48 hours in the presence of various concentrations of ATRA. During 72 hours' exposure to ATRA, the viability and proliferating ability of the cells decreased (figure 5; table 2 in paper I) in a concentration-dependent manner.

5.2 Effect of ATRA on cell differentiation (I)

There was no morphological differentiation observable in any of the AML cell lines studied after the ATRA treatment. The AML cells retained their blast cell morphology. The NBT test revealed no functional signs of differentiation, either. The experiments were made at different concentrations and with different schedules.

Fig. 5. Viability (A) and proliferation rate (B) of cells exposed to ATRA at different concentrations in suspension culture for 72 hours. The results are mean values (±SD) of eight OU-AML cell lines in two independent experiments. **, P<0.01; ***, P<0.001.
5.3 Effect of ATRA on the induction of apoptosis

5.3.1 Morphological changes (I)

After the addition of ATRA to the culture medium for 72 hours, typical concentration-dependent morphological changes of apoptosis (figure 7 in paper I) were observed in MGG-stained slides in all of the eight OU-AML cell lines (figure 6; table 4 in paper I). 1 μM ATRA caused more prominent morphological changes than 0.1 μM ATRA. However, if the exposure was shortened to 48 hours, even 1 μM ATRA failed to cause any detectable morphological changes in the cytoplasm or nucleus of leukemia cells.

![Graph showing percentage of apoptotic cells after addition of ATRA to culture medium for 72 hours](image)

Fig. 6. Percentage of apoptotic cells after addition of all-trans retinoic acid (ATRA) to culture medium for 72 hours. The results are mean values (±SD) of eight OU-AML cell lines.

5.3.2 DNA fragmentation (I)

When the cell lines were exposed to ATRA for 6, 12, or 24 hours and the concentration of ATRA was varied from 0.001 μmol/L to 1.0 μmol/L, no DNA fragmentation was observed in any of the eight OU-AML cell lines. When the exposure to ATRA was continued for 48 hours and ATRA was used as a 0.1 μM and 1.0 μM concentrations, DNA fragmentation was observed in all the AML cell lines. Representative gel electrophoreses are shown in paper I, figure 8.
5.3.3 Phosphatidylserine externalization (III, IV)

Apoptosis was determined fluorocytometrically using annexin V, which recognizes the externalization of phosphatidylserine. In basic growth conditions, the number of apoptotic cells (annexin V-positive/propidium iodide-negative) varied from 2 ± 1% (OU-AML-5) to 7 ± 6% (OU-AML-7) when the culture time was extended to 72 hours (III, table 1). After 72 hours’ exposure to 1.0 µM ATRA, the percentage of apoptotic cells varied from 16 ± 8% (OU-AML-7) to 61 ± 4% (OU-AML-3) (III, table 1). If the exposure to ATRA was continued for 96 hours, the number of annexin V-positive cells increased, as shown in paper IV, figure 2.

5.3.4 Cleavage of PARP (IV)

The expression of PARP was analysed by Western blotting from the whole-cell lysate of OU-AML-3 and OU-AML-7 cells. Full-sized PARP (116 kD) either remained stable or decreased during ATRA incubation. At the same time, however, the cleaved form of PARP, a fragment of 85 kD, gradually increased, being most expressive after 72 and 96 hours exposure of cells to ATRA (IV, figure 3).

5.4 Gene status of TP53 (II)

No mutations in the p53 gene were found in any of the cell lines analysed by PCR-SSCP of amplified exons 5-8. A representative example of SSCP is shown in paper II, figure 2.

5.5 Expression of p53 in basic growth conditions (II)

Expression of p53 was consistently observed in each of the cell lines by immunohistochemistry, although the number of positive cells per AML cell line varied one experiment to another (II, table 3). The expression of p53 was localised in both the cytoplasmic and the nuclear compartments of the cells, which was confirmed by both immunohistochemical staining (II, figure 3) and Western blotting (II, figure 4). P53 was also detected in the native cells which gave rise to the OU-AML cell lines 4, 5 and 8. The amount of p53 was found to be increased after 6 weeks’ culture and to increase further in the course of long-term culture.

Five of the OU-AML cell lines (3, 4, 5, 7 and 8) were analyzed by flow cytometry in order to determine the expression, and particularly conformation of p53. The number of p53-positive cells was over 90% when determined by anti-p53 antibody DO7 (II, table 4). p53 was mostly expressed in a mutational conformation as analysed by the anti-p53 anti-
body Ab3. A negligible number of AML cells, however, contained p53 in a wt conformation when analysed by anti-p53 antibody Ab5, which has been shown to detect only wt conformation (II, table 4).

5.6 Effect of ATRA on the expression of p53 (III)

The effect of ATRA on p53 expression was studied in the OU-AML cell lines 3,4,5,7 and 8. Although the number of p53-positive cells was not accurately analyzable by immunohistochemistry using CM-1 anti-p53 antibody in the presence of ATRA, it was notable that both p53-positive and p53-negative cells showed apoptotic morphology on some slides (III, figure 3).

Using Western blotting, the expression of p53 was shown to decrease in the presence of ATRA in both the cytoplasmic and the nuclear fractions of the studied AML cell lines, when the DO7 anti-p53 antibody was used for the detection of expression (III, figure 4A-4B).

Flow cytometry showed the number of p53-positive cells to be high in each of the cell lines as analyzed by the DO7 antibody the mean value being 98 ± 2%. After exposure of the cells to ATRA for 72 hours, an obvious decrease in the p53 content of the cells was observed, as shown in paper III, table 2, as a percentage change in mean fluorescence intensity.

5.7 Expression of Bcl-2 and Bax (III)

Mitochondrial and cytosolic Bcl-2 were analysed by Western blotting in all the AML cell lines. Bcl-2 was only found in the mitochondrial fractions (all data not shown). The expression of bcl-2 in OU-AML-3 and OU-AML-7 is shown in paper III, figure 7, and in paper IV, figure 3. In case of these cell lines, Bcl-2 was measured by the ELISA technique in basal growth conditions from the whole-cell lysate and found to be $3.4 \pm 0.6$ U/µg of protein in OU-AML-3 and $5.9 \pm 0.1$ U/µg of protein in OU-AML-7 cells. The expression of Bax was studied in OU-AML-3 cells only, and it was observed both in the mitochondrial and the cytosolic fraction in Western blotting (III, figure 7).

5.8 Effect of ATRA on the expression of Bcl-2 and Bax (III, IV)

The expression of Bcl-2 was clearly down-regulated by ATRA in the AML cell lines 3,4,5,7, and 8, when the analysis was done on the whole-cell lysate after 72 hours’ exposure (III, figure 6). Time-dependent down-regulation of mitochondrial Bcl-2 was observed in the OU-AML-3 and -7 cell lines, as analyzed by Western blotting, flow cytometry and ELISA (IV, table 1). ELISA and Western blotting showed a decrease in the
Bcl-2 levels after 12 hours’ incubation of cells with 1.0 µM ATRA. In flow cytometry, down-regulation of Bcl-2 began after 24 hours’ exposure of cells to ATRA. During the incubation of cells with ATRA, Bcl-2 was not found in cytosol in Western blotting. The analyses were repeated twice to three times with comparable results.

The effect of ATRA on Bax expression was studied only in the case of OU-AML-3. There was no increase in Bax expression in Western blotting during ATRA exposure (III, figure 7).

5.9 Effect of ATRA on cytochrome c and its translocation (IV)

A tiny amount of cytochrome c was observed in the cytosolic fraction of OU-AML-3 and OU-AML-7 in basic growth conditions. The most prominent expression of cytochrome c was, however, observed, in the mitochondrial fractions (IV, figure 3). After incubation with 1.0 µM ATRA for 96 hours, cytosolic cytochrome c was induced in both cell lines, up to 8.0-fold in OU-AML-3 and up to 2.7-fold in OU-AML-7. It was obvious that the expression of mitochondrial cytochrome c also increased for up to 72 hours in OU-AML-3 and for up to 96 hours in OU-AML-7 (IV, figure 3).

5.10 Effect of ATRA on mitochondrial membrane potential (IV)

∆Ψm was analyzed by flow cytometry using a JC-1 fluorescent probe in OU-AML-3 and OU-AML-7 cells. A drop in ∆Ψm was observed after 72 hours’ incubation of cells with 1.0 µM ATRA as an increase in monomers and a decrease in J aggregates. The drop was more evident in the OU-AML-3 than the OU-AML-7 cells (IV, figure 4). When the cells were exposed to ATRA for 72 and 96 hours, the percentage of cells able to sustain a high mitochondrial membrane potential decreased to 49% and 27% in the case of OU-AML-3, but only to 88% and 82% in the case of OU-AML-7.
6 Discussion

6.1 Effect of ATRA on AML cell growth and differentiation

In the present study, ATRA was found to suppress the growth of myeloblastic leukemia cells in a time- and dose-dependent manner. This is in congruence with many previous studies (Douer & Koefler 1982, Largman et al. 1989, Wang et al. 1989, Chomienne et al. 1990, Koistinen et al. 1991, Tohda et al. 1992, Nilsson et al. 1995). Moreover, the present study showed that there was no induction of differentiation and maturation of myeloid blast cells by ATRA in AML representing subtypes other than APL. This result is also in agreement with some earlier findings (Lawrence et al. 1987, Largman et al. 1989, Chomienne et al. 1990, Chen et al. 1991).

6.2 Effect of ATRA on the induction of apoptosis in AML cells

Although the present study showed that ATRA is able to suppress the growth of AML cells, it was unclear whether the suppressive effect is related solely to the growth arrest of the cells, or whether ATRA is able to induce programmed cell death. It was demonstrated in the present study that ATRA is able to induce apoptosis in AML cells. The role of ATRA as an inducer of apoptosis might be clinically important.

ATRA at concentrations of 0.1 µM and 1.0 µM was mostly used in the present study, since these concentrations are clinically obtainable and tolerable. At these concentrations, obvious inhibition of cell growth and induction of apoptosis require a relatively long time, 72 hours in these AML cell lines. This could be one explanation why the use of ATRA as a single agent has been disappointing in leukemia therapy (Kramer et al. 1991). Clinically, at the beginning of the therapy, standard ATRA schedules (45-80 mg/m²/day) can be used to maintain a concentration of 1.0 µmol/L and 0.1 µmol/L for about 4 hours only, and during the first week of treatment the concentrations drop sharply (Gillis & Goa 1995). It is difficult to estimate whether the concentrations of ATRA and exposure times used in the in vitro study would be efficient in the clinical situation. Regarding this point,
a combination of ATRA with other agents could be an option to increase the apoptotic effect of ATRA. Some studies have already been done to prove this idea in vitro (Lishner et al. 1989, Tohda et al. 1992) and in vivo (Kramer et al. 1991)

6.3 p53 status and the effect of ATRA on the status

After it was demonstrated that ATRA is able to induce apoptosis in AML cell lines, it was questioned whether the apoptosis is dependent on the p53 pathway. The status of TP53 was examined first and the protein status and the effect of ATRA on it later. The results of immunohistochemistry, Western blotting and flow cytometry showed that p53 was expressed in all of the cell lines, although no mutations in the exons 5-8 of the gene were found as indicated by PCR-SSCP. Since the SSCP method used in the present study, utilizing 2 temperatures for each exon and good temperature control, is able to detect 98% of p53 mutations within the exons 5-8, not much additional data could be expected to be gained by sequencing (Greenblatt et al. 1994, Welsh et al. 1997). The present findings showed that the culture conditions per se do not predispose AML cells to p53 mutations, and mutations are not a requirement for the establishment of AML cell lines, as has previously been suggested (Sugimoto et al. 1992). In addition, it can be proposed that p53 mutations do not play a major role in the leukemogenesis or responses to chemotherapy in patients from whom the cell lines were generated.

It was previously assumed that the overexpression of p53 in tumor cells implicates mutation of the p53 gene generating inactive protein. It has been shown, however, that certain tumor types express high levels of p53 protein in the absence of mutations of the p53 gene (Peng et al. 1993, Hall & Lane 1994, Castren et al. 1998). The present results show that AML also belongs to this category of malignancies.

Most of the p53 protein was recognised by the Ab3 anti-p53 antibody when analyzed by flow cytometry, i.e. it was in a mutational or promoter conformation. Apart from AML cells (Rivas et al. 1992, Zhang et al. 1992, Zhu et al. 1993, 1994), the mutational p53 conformation has also been detected in normal hematopoietic progenitor cells (Rivas et al. 1992, Zhang et al. 1992, Bi et al. 1994). This conformation may represent a condition where wt p53 is in an inactive form and permits cell proliferation instead of acting as a suppressor of the cell cycle. Also, the fast growth of the cell line cells in suspension speaks for the possibility that most of p53 was not functional as a suppressor.

The immunohistochemistry and Western blotting analyses showed that the p53 protein was located both in the nucleus and in the cytoplasm of the cells. According to the literature, wt p53 is mainly located in the nucleus, acting as a transcription factor. However, there are reports suggesting that cytoplasmic p53 represents an inactivated protein, which is logical in a view of the main functions of the p53 protein (Funk et al. 1992). One reason for the accumulation of p53 in the cytoplasm of the studied AML cell lines might be a failure in the translocation of the protein to the nucleus (Moll et al. 1996). Alternatively, an unknown factor in the cytoplasm, contributing to the mutated formation of the genetically wt p53 protein might have captured the p53 in the cytoplasm. Theoretically, it could be a protein inhibitor of some kind, such as a virus protein or mdm-2 (Linzer & Levine
1979, Bargonetti et al. 1991, Momand et al. 1992). There were no major changes in the p53 expression levels in the repeated analyses performed on the cell lines within 72 hours, which speaks for well-balanced production and degradation of the p53 protein in the cells.

The p53 gene is located in chromosome 17p13 (Isobe et al. 1986, Mcbride et al. 1986). In chromosomal analyses, the number of chromosomes in the cell lines was higher than normal, and several chromosomes 17 were frequently seen, which could partly explain the observed expression of the p53 protein.

The present study suggests that p53 has no role in ATRA-induced apoptosis in AML. Firstly, ATRA was able to induce apoptosis in both p53-positive and p53-negative cells, as showed by immunohistochemistry. Moreover, p53 was clearly decreased after ATRA exposure, not increased, which has been considered a sign of its activation (Fritsche et al. 1993). There is evidence to suggest, that an inactive cytoplasmic form of p53 could be activated by translocation to the nucleus (Younish-Rouach et al. 1991). The present results do not, however, support the idea that ATRA could activate p53 by changing the translocation pattern of the protein. Finally, as analysed by flow cytometry, the conformation of p53 was not changed during ATRA exposure, but remained in an aberrant conformation.

The pathways which control cell death during normal development and differentiation are mostly p53-independent (White 1996). It is well known that ATRA regulates normal cell growth and differentiation (Lotan 1980). Thus, apoptosis induced by ATRA does not need to be associated with the p53 pathway at all. This is well in line with the notion that ATRA is able to induce differentiation and apoptosis of HL-60 cells, which lack TP53 (Martin et al. 1990). Nevertheless, a decline in mutational or mutated p53 levels might be advantageous for cancer treatment by decreasing the resistance to both p53-dependent and p53-independent apoptotic events (Lotem & Sachs 1994, Peled et al. 1996).

In AML, mutations of the p53 gene are rare (Fenaux et al. 1992, Schottelius et al. 1994). Based on the present findings and according to the previously published studies (Zhang et al. 1992, Zhu et al. 1993, 1994), it is probable that p53 in AML is inactive due to a change in the protein conformation. Conversion of the inactive p53 protein conformation to an active one might be an interesting option to improve AML treatment. Such restored p53 activity might inhibit AML cell growth or increase the susceptibility of these cells to standard treatments. Changes in the p53 protein conformation have been achieved in embryonal carcinoma cells by exposing them to etoposide (Lutzker & Levine 1996).

### 6.4 Downregulation of bcl-2 by ATRA

It was shown in the present study, by using three different methods, that apoptosis induced by ATRA is related to down-regulation of bcl-2 in AML cells. Although down-regulation of bcl-2 by ATRA has been recognized in AML previously (Hu et al. 1995, Bradbury et al. 1996, Pisani et al. 1997), its possible role in apoptosis has not been veri-
fied. However, Bcl-2 has been shown to inhibit ATRA-induced apoptosis during both neural development (Okazawa et al. 1996) and the differentiation of HL-60 cells (Nau-movski & Cleary 1994, Benito et al. 1995).

The down-regulation of bcl-2 was more prominent in OU-AML-3 than OU-AML-7 cells, and apoptotic events were also more prominent in OU-AML-3 than OU-AML-7 cells. This might be related to the different concentrations of Bcl-2 in the cell lines. As quantified by ELISA, the expression of bcl-2 was 5.9 ± 0.1 U/µg of protein in OU-AML-7, and 3.4 ± 0.6 U/µg of protein in OU-AML-3 in basic growth conditions. Previous studies have shown that a high Bcl-2 content is able to protect cancer cells from the apoptotic effects of drugs and favours chemoresistance (Campos et al. 1994, Keith et al. 1995, Reed 1997b). There are also studies which do not support this idea (Lotem & Sachs 1994, Banker et al. 1997, Ketley et al. 1997, Pisani et al. 1997). Bcl-2 is able to counteract the efflux of cytochrome c from mitochondria to cytosol (Kluck et al. 1997, Yang et al. 1997, Schendel et al. 1997) and thus to inhibit the extension of apoptosis. There is evidence to suggest that Bcl-2 might prevent the drop in $\Delta \Psi_m$ by enhancing the $H^+$ efflux from mitochondria to cytosol (Shimizu et al. 1998).

6.5 Role of mitochondria in ATRA-induced apoptosis

It was shown in the present study that ATRA-induced apoptosis was related to disruption of mitochondrial functions. Changes in mitochondrial functions were manifested as a leakage of cytochrome c to cytosol and a drop in $\Delta \Psi_m$. The cytochrome c efflux to cytosol has been considered an early sign of apoptosis (Liu et al. 1996), related to the drop in $\Delta \Psi_m$ (Scarlett & Murphy 1997, Yang & Cortopassi 1998). This has also been shown to take place independently of any change in $\Delta \Psi_m$ (Kluck et al. 1997, Yang et al. 1997, Bossy-Wetzel et al. 1998). Moreover, apoptosis may occur in some systems without any efflux of cytochrome c to cytosol (Chauhan et al. 1997, Tang et al. 1998). In the present study, during exposure of AML cells to ATRA, cytochrome c efflux was observed before the drop in $\Delta \Psi_m$ and it was accompanied by the first sign of apoptosis, i.e. DNA fragmentation and phosphatidylserine externalization. However, the ability of mitochondria to sustain high cytochrome c levels in their membrane was obvious, since the most extensive leakage of cytochrome c to cytosol was observed at the same time as a clear disruption in $\Delta \Psi_m$ and an activation of caspase-3 were also observed, i.e. after 72-96 hours’ exposure of the cells to ATRA. The drop in $\Delta \Psi_m$ was observed earlier in OU-AML-3 than OU-AML-7 cells, which might relate to the different contents of Bcl-2 in the cell lines.

Cytosolic cytochrome c could be observed in these cell lines during cell growth in the absence of ATRA. This might relate to the slight spontaneous apoptosis observed in basal growth conditions, or it may be unrelated to this. It has been shown previously that a small amount of cytochrome c in cytosol does not necessarily lead to apoptosis, if there are no other apoptosis-activating agents present (Reed 1997a). Bcl-2 is able to dock proteins (e.g. Apaf-1 and Raf-1) to the mitochondrial membrane and thereby inhibit their activation (Mignotte & Vayssiere 1998). In the present study, the Bcl-2 levels of the cell
lines remained high during cell growth in the absence of ATRA (data not shown), and this might protect cells against apoptosis, despite of the presence of cytochrome c in cytosol. In the studied cell lines, bcl-2 was recognized in the mitochondrial but not in the cytosolic fractions, underlining its protective effects on mitochondria.

It is now known that many procaspases and other apoptosis-activating factors, in addition to cytochrome c, are located in the intermembrane space of mitochondria, whose release into cytosol is also needed in addition of the release of cytochrome c, until the caspase system is fully able to be activated (Susin et al. 1999, Slee et al. 1999). The present study indicates that ATRA downregulates bcl-2, resulting in an efflux of cytochrome c into cytosol, decreased mitochondrial membrane potential, and increased disruption of mitochondrial function, which finally leads to the activation of caspases and PARP cleavage.

6.6 A proposed model for ATRA-induced apoptosis in AML

Our data support a model of ATRA-induced apoptosis in AML, beginning with downregulation of bcl-2. Possible downstream mechanisms for the induction of apoptosis could be: (A) Decreased Bcl-2 would result in predominant presence of Bax in the cells, which forms pores on mitochondrial membranes, to allow translocation of cytochrome c, to the cytosol. The release of cytochrome c activates the “apoptosome”, consisting of cytochrome c, Apaf-1, and caspase-9, and results in apoptotic cell destruction. (B) Decreased Bcl-2 would cause mitochondrial permeability to be increased in the cells. Activation of apoptosis would then occur as described in (A). (C) Decreased Bcl-2 would cause a protein kinase, Raf-1, to be dissociated from the mitochondrial membrane, which, therefore, could not inactivate Bad, a pro-apoptotic member of the Bcl-2 family. Bad would thereby be activated, bind to antiapoptotic Bcl-X<sub>L</sub>, and induce cell death as described in (A).
7 Conclusions

- The present study shows that it is possible to induce \textit{in vitro} apoptosis by ATRA in AML.
- The effect of ATRA on cell growth and apoptosis is dependent on the concentration of and exposure time to ATRA.
- The findings support studies, where ATRA is used as a chemotherapeutic agent alone or in combination with other agents \textit{in vitro} and \textit{in vivo} in AML.
- Apoptosis induced by ATRA is associated with down-regulation of bcl-2, a protector of mitochondria.
- ATRA-induced apoptosis in AML is a slow event and might relate to the Bcl-2 content of the cells.
- ATRA-induced apoptosis was related to changes in mitochondrial function, i.e. the cytochrome c efflux into cytosol and the drop in mitochondrial membrane potential.
- The cytochrome c efflux, the fragmentation of DNA and the externalization of phosphatidyl-serine could start before any changes take place in the mitochondrial membrane potential.
- AML cells express high levels of p53 without the presence of mutations at the gene level. The p53 protein is located both in the nucleus and in the cytoplasm being mostly in mutational conformation, which suggests that it is not functional in its usual role as a suppressor of the cell cycle. These AML cells offer a good target for studies aiming at restoration of the normal function of p53.
- It was shown here that ATRA, although it induces arrest in cell growth and apoptosis, is not the molecule which restores the function of p53. It seems unlike that ATRA-induced apoptosis is related to the p53 pathway. Thus, it is more likely that ATRA acts as a physiological agent inducing a developmental apoptotic pathway, rather than being a genotoxic or cell-damaging agent activating a p53-dependent apoptotic pathway.
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