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Apoptosis in the Myelodysplastic Syndromes:
Protective effect of G-CSF

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Für meine Mutter

Und

In Erinnerung an meinen Vater
ABSTRACT

Increased apoptosis of hematopoietic progenitor cells is a hallmark for the myelodysplastic syndromes (MDS), and one of the main reasons for the ineffective hematopoiesis and subsequent cytopenia characteristic for the disease. In particular in low risk MDS, such as refractory anemia (RA) and refractory anemia with ringed sideroblasts (RARS), severe transfusion-dependent anemia leading to constant fatigue and weakness and therefore reduced quality of life often constitutes the main clinical problem. The development of specific treatment strategies for MDS is hampered by the lack of deep pathogenetic understanding of the disease. We have previously shown that treatment with granulocyte colony stimulation factor (G-CSF) and erythropoietin (EPO) reduces the number of apoptotic erythroid bone marrow precursors, and may abolish transfusion need in patients with low-risk MDS. Although our data suggest that G-CSF blockage of erythroid apoptosis occurs at the mitochondrial level, additional studies are required to understand the entire mechanism of G-CSF action. Thus, the present studies were initiated to explore mechanisms for apoptosis and the effects of G-CSF in cell lines and primary MDS progenitors.

Bone marrow cells from RARS cultured in vitro showed increased spontaneous apoptosis and a higher sensitivity to ligation of the Fas death receptor compared to progenitor cells from healthy individuals. Both erythroid and myeloid colony growth was reduced, indicating a defect at the stem cell level. Moreover, RARS cells displayed elevated caspase-3 activity, which could be reduced by the caspase inhibitor DEVD-fmk. Fas-antagonistic antibody fragments did not inhibit apoptosis in RARS cultures. Co-culture with G-CSF reduced caspase activity and generation of reactive oxygen species, while restoring mitochondrial potential and erythroid colony growth. No such effects were observed in normal bone marrow.

In order to further explore the anti-apoptotic effects of G-CSF, the myeloid P39 cell line, originally derived from an MDS patient, was treated with all-trans retinoic acid (ATRA) in the presence of G-CSF. ATRA induced differentiation and subsequent apoptosis. It affected mitochondrial functioning long before any signs of apoptosis appeared. ATRA-induced mitochondrial alterations were characterized by diminished mitochondrial oxygen consumption and decreased calcium uptake by mitochondria, leading to a lower mitochondrial matrix calcium concentration. Interestingly, G-CSF prevented mitochondrial deterioration. It partially restored respiration as well as the capacity of mitochondria to accumulate calcium. Mitochondrial alterations occurred at an early time point in ATRA-treated cells and were later followed by apoptosis, as detected by apoptotic nuclear morphology. G-CSF prevented both the early mitochondrial changes and the later apoptotic manifestations suggesting that mitochondrial dysfunction is an important step for ATRA-induced apoptosis.

Thus, the ability of ATRA and G-CSF to modulate mitochondrial respiration and intracellular calcium control are novel findings which help to give a better insight in their precise molecular mode of action. The restoration of mitochondrial functioning by G-CSF offers a new explanation for its anti-apoptotic function in the treatment of MDS.
LIST OF PUBLICATIONS


II. Hellström-Lindberg E, Schmidt-Mende J, Forsblom AM, Christensson B, Fadeel B and Zhivotovsky B: Apoptosis in refractory anemia with ringed sideroblasts is initiated at the stem cell level and associated with increased activation of caspases. *Br J Haematol* 2001; 112: 714-726


Further Publications, not included in this thesis:


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<tr>
<td>Δψₘ</td>
<td>Mitochondrial transmembrane potential</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide Translocator</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease-activating factor-1</td>
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<tr>
<td>ATG</td>
<td>Anti-thymocyte globulin</td>
</tr>
<tr>
<td>ATO</td>
<td>Arsenic trioxide</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BFU</td>
<td>Burst forming unit</td>
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<tr>
<td>BH</td>
<td>Bcl-2 homology</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CMML</td>
<td>Chronic myelomonocytic leukemia</td>
</tr>
<tr>
<td>DIABLO</td>
<td>Direct IAP binding protein with low pi</td>
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<tr>
<td>DISC</td>
<td>Death-inducing signalin complex</td>
</tr>
<tr>
<td>DLK-1</td>
<td>Delta-like protein-1</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>EPO-R</td>
<td>Erythropoietin receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FAB</td>
<td>French-American-British</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>Fas</td>
<td>Fas receptor</td>
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<tr>
<td>FasL</td>
<td>Fas ligand</td>
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<tr>
<td>G3PDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>GpA</td>
<td>Glycophorin A</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitors of apoptosis proteins</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IPSS</td>
<td>International Prognostic Scoring System</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<tr>
<td>MNC</td>
<td>Mononuclear bone marrow cells</td>
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<tr>
<td>MDS</td>
<td>Myelodysplastic syndromes</td>
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<tr>
<td>MPT</td>
<td>Mitochondrial permeability transition</td>
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<tr>
<td>mtDNA</td>
<td>Mitochondrial DNA</td>
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<tr>
<td>mtF</td>
<td>Mitochondrial ferritin</td>
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<tr>
<td>NBM</td>
<td>Normal bone marrow</td>
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<tr>
<td>PARP</td>
<td>Poly ADP-ribose polymerase</td>
</tr>
<tr>
<td>PMCA</td>
<td>Plasma membrane calcium pump</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>RA</td>
<td>Refractory anemia</td>
</tr>
<tr>
<td>RAEB</td>
<td>Refractory anemia with excess of blasts</td>
</tr>
<tr>
<td>RAEB-t</td>
<td>Refractory anemia with excess of blasts in transformation</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------------------------</td>
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<tr>
<td>RARS</td>
<td>Refractory anemia with ringed sideroblasts</td>
</tr>
<tr>
<td>RCMD</td>
<td>Refractory cytopenia with multilineage dysplasia</td>
</tr>
<tr>
<td>RCMD-RS</td>
<td>Refractory cytopenia with multilineage dysplasia with ringed sideroblasts</td>
</tr>
<tr>
<td>RCR</td>
<td>Respiratory control ratio</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>Smac</td>
<td>Second mitochondrial activator of caspases</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>t-MDS</td>
<td>Therapy-related MDS</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF-receptor member 1A</td>
</tr>
<tr>
<td>TRADD</td>
<td>TNF-receptor-associated death domain</td>
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<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP-biotin nick end labeling</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage-dependent anion channel</td>
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1 GENERAL INTRODUCTION

1.1 APOPTOSIS

In 1972 Kerr, Wyllie and Currie (Kerr et al., 1972) introduced the term apoptosis to describe a morphologically defined form of cell death. Some manifestations of apoptosis had also been described earlier by others (for review: Vaux, 2002, Lockshin & Zakeri, 2001). In 2002, Sydney Brenner, John Sulston and Robert Horvitz awarded the Nobel Prize in Physiology or Medicine for their studies using the nematode Caenorhabditis elegans as model organism to study genetic regulation of organ development and programmed cell death. Based on their work a number of genes involved in programmed cell death were described and their precise function was characterized. During evolution the apoptotic machinery is highly conserved among species and human homologues of the Caenorhabditis elegans genes have been discovered.

Apoptosis is an important mechanism in embryonic development and organogenesis. Furthermore, apoptotic cell death can be the natural endpoint of a life span of a cell and is thereby involved in normal cell turnover of adult tissues. Apoptosis is used as a defense strategy to remove infected (by viruses, bacteria), mutated (tumor) or damaged cells. Thus, evading of apoptosis is one important of several steps in the transformation of a normal cell into a cancer cell (Hanahan & Weinberg, 2000). Apoptosis can be triggered by cell surface death receptors, radiation and cytostatic drugs, which may target DNA, mitochondria or cytoplasmatic proteins. Furthermore, many environmental toxins and cellular stresses can also trigger apoptotic cell death (e.g., oxidative stress, alcohol).

1.1.1 Apoptosis versus necrosis and autophagy

Cell death can be schematically divided into three main different types (Lockshin & Zakeri, 2004): autophagy, apoptosis and necrosis (Figure 1.1).
There is some overlap between these types of cell death, which will be discussed below. Apoptosis is morphologically (Kerr et al., 1972) and biochemically (Hengartner, 2000) described by cell shrinkage, membrane blebbing, activation of DNases and proteases, condensation and fragmentation of DNA into 50 to 300 kb and smaller ca 180 bp fragments as well by the externalization of phosphatidlyserine on the plasma membrane. Finally, condensed apoptotic bodies are formed, which can be engulfed by neighboring or phagocytic cells. Since cell content is not released into the interstitium, apoptosis does not trigger any inflammation response in the tissue.

Necrosis can be divided into primary and secondary necrosis. Primary necrosis is – in contrast to apoptosis - an apparently uncontrolled and mainly pathologic form of cell death. Loss of ATP and ion pump activity lead to swelling of organelles and the entire cell. Nuclear swelling leads to karyolysis. In cells undergoing necrosis, the plasma membrane is destroyed early. Leakage of intracellular proteins into interstitium evokes an inflammatory response. Secondary necrosis occurs after apoptotic cell death, when apoptotic cells fail to be removed by heterophagy.

Apart from the described differences between apoptosis and necrosis, there are a few arguments present against the clear distinction between these two types of cell death: The same toxin can induce apoptosis (low, subnecrotic dose) or primary necrosis at a high dose. The “decision” whether a cell dies by apoptosis or necrosis mainly depends on the severity of the insult to the cell (Fadeel et al., 1999a). Many diseases labeled as necrotic involve also apoptosis (apoplexy, myocardial infarction, anoxia, ischemia-reperfusion damage). Mitochondrial permeability transition (see below) is involved in both apoptosis and necrosis (Crompton, 1999). Furthermore, modulation of cellular ATP levels can shift apoptotic responses to necrosis (low ATP) and vice versa (high ATP) (Nicotera et al., 1998). Classical apoptosis inducers (serum withdrawal, etoposide, glucocorticoids) induce non-apoptotic cell death in the presence of caspase inhibitors such as z-VAD-fmk.

Another described form of cell death is autophagy or autophagic (lysosomal) cell death, which can be recognized by formation of many large double membrane-bound autophagic vacuoles (autophagosomes) in the cytoplasm (Meijer & Codogno, 2004). The lysosomal compartment is expanded. Cell organelles are captured in the autophagosomes, which fuse with the lysosomes. Autophagy is a normal physiological process that does not necessarily lead to cell death. It is involved in routine turnover of cellular proteins and organelles. The autophagic pathway is used for bulk proteolysis, while the ubiquitin pathway is necessary for fine control of protein degradation. During starvation autophagy is used for recycling of materials. There is no clear discrepancy between autophagy and apoptosis. Unused, starving, or hormone-deprived cells can atrophy, removing the bulk of their cytoplasm by autophagy. It is conceivable that the autophagy is a cellular protective mechanism to reduce metabolic demand by the cell to stave off deaths. When autophagy has reached its limit, apoptotic cell death might occur. A close correlation between autophagy and apoptosis is furthermore supported by the observation that lysosomal enzymes are capable to induce apoptosis in cells (for review: Guicciardi et al., 2004).
1.1.2 The extrinsic and intrinsic apoptotic pathway

Apoptotic stimuli can be functionally divided into extrinsic and intrinsic factors, which initiate the apoptotic process via interaction with extra- or intracellular targets (Figure 1.2). The extrinsic pathway engages the activation of death receptors on the cell surface. The intrinsic (metabolic) pathway involves mainly the triggering, regulation and amplification of apoptotic signals by mitochondria, but also by the endoplasmic reticulum (ER), lysosomes and the nucleus.

Upon binding of ligands of the tumor necrosis factor (TNF) family (Sartorius et al., 2001), death receptors such as CD95 (Apo-1/Fas), TNF receptor member 1A (TNFR1), and the TNF-related apoptosis-inducing ligand receptors (TRAIL) DR4 and DR5 aggregate and form membrane bound signaling complexes. These complexes recruit the Fas-associated death domain (FADD) adaptor protein or the TNF receptor-associated death domain (TRADD), respectively. The adaptor proteins bind to procaspase-8, resulting in formation of the so called death inducing signaling complex (DISC). Upon association of procaspase-8 to the DISC complex, it is autocatalytically cleaved and activated. Active caspase-8 is released from the complex as a heterotetramer of two small and two large subunits. It serves as initiator caspase activating other further downstream executioner caspases that cleave cellular death substrates resulting in apoptosis. Additionally, caspase-8 can cleave Bid, a pro apoptotic member of the Bcl-2 protein family, to truncated Bid (tBid). Since Bid is involved in the regulation of intrinsic mitochondrial pathway of apoptosis, its activation by cleavage provides a link between the extrinsic and intrinsic pathway of apoptosis.

The intrinsic pathway involves the regulation of apoptosis via mitochondria and is characterized by the release of mitochondrial intermembrane space proteins including cytochrome c, Apoptosis Inducing Factor (AIF), second mitochondrial activator of caspases (Smac)/ direct IAP Binding protein with low pI (DIABLO), Endonuclease G and Omi/HtrA2 into the cytosol (van Loo et al., 2002). This cell death pathway is controlled by Bcl-2 family proteins. Released cytochrome c together with the cytosolic Apaf-1 forms a complex in the presence of dATP, which is essential for proteolytic processing of procaspase-9. Apaf-1 is not only a simple caspase-9 activator, but it is rather an essential regulatory subunit of the caspase-9 holoenzyme, which is often together with cytochrome c and dATP referred to as the apoptosome. The apoptosome has a seven-fold symmetry, and forms a wheel-like structure. Caspase-9 cleaves and activates procaspase-3. Since caspase-3 is activated in the extrinsic as well as the intrinsic pathways of apoptosis, its activation provides a link between these different mechanisms. Thus, it is assumed that the extrinsic and intrinsic pathway lead to one common execution pathway, which explains that late apoptotic cells display uniform morphology and similar biochemical hallmarks, albeit the fact that apoptosis can be induced by variety of different stimuli.
1.1.3 The caspase family

Caspases are cysteine-rich aspartic-specific proteases, which are activated specifically in apoptotic cells (Fadeel et al., 2000). Caspases are expressed as inactive pro-enzymes in the cells and activation is induced by cleavage of the proenzymes (Cohen, 1997). The active enzyme consists of two small and two large subunits forming a tetramer. Three different mechanisms of caspase activation are described so far (Hengartner, 2000).

1. Induced proximity: Several procaspase-8 zymogens are recruited to the DISC complex upon cell surface death receptor trimerization. In this membrane-bound signaling complex, a high concentration of the procaspase-8 molecules might be sufficient to process and activate each other.

2. Activation mediated by regulatory subunits: Caspase activation can be closely controlled by a regulatory subunit. Unlike other caspases, proteolytic processing of procaspase-9 has only a minor effect in the enzyme’s catalytic activity. The key requirement for caspase-9 activation is its association with the cytosolic protein Apaf-1 in the apoptosome complex as described in detail above.

3. Activation by upstream located caspases: An activated caspase can cleave and activate another caspase in a cascade of reactions.

So far, 14 different caspases are known, which are divided into several groups based on their function in the cell. Although overexpression of all 14 caspases resulted in cell death, not all of them are involved in apoptosis. Thus, caspase-1, -4, -5, -13 and
-14 (group 1) have little or even unknown roles in the apoptotic pathways. Many of them involve in the maturation and production of inflammatory cytokines (pro-inflammatory caspases).

Caspase-2, -8, -9 and -10 (group 2) belong to so called initiator (or apical) caspases. These enzymes are located upstream in the apoptotic pathway and are involved in the activation of other caspases.

Caspase-3, -6, and -7 (group 3) belong to effector caspases, which perform their function downstream in the apoptotic pathway by cleavage of proteins in the cytoskeleton, cytoplasm and the nucleus, such as the poly(ADP-ribose)polymerase (PARP), the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), the structural proteins lamin and α-fodrin as well as the delta isoform of the protein kinase C (PKCδ). The degradation of numerous structural and regulatory proteins during apoptosis is important for morphological and biochemical changes, which can be detected in apoptotic cells like DNA fragmentation, chromatin condensation and membrane blebbing.

1.1.4 The Bcl-2 family

The initiation of apoptosis and its fatal consequence for cells have to be tightly regulated. One important regulation system of apoptosis is represented by the Bcl-2 protein family. This protein family consists of a large group of proteins, which have the ability to induce and enhance as well as inhibit apoptosis (Fadeel et al., 1999b). Bcl-2 family members are recognized through their homology in discrete domains designated BH domains 1 to 4 (Chan & Yu, 2004). A carboxy-terminal hydrophobic transmembrane region (membrane anchor) is found in most family members. The Bcl-2 family proteins can be divided into two groups, namely, inhibitors (Bcl-2, Bcl-XL, Bcl-w, A-1/Bfl-1, Mcl-1) and promoters (Bax, Bak, Bel-Xδ, Bad, Bid, Puma, Noxa) of apoptosis. Generally speaking, family members that act as inhibitors of cell death harbor at least three domains (BH1, BH2, BH4), which are important for protein-protein interaction and the suppression of apoptosis. The BH3 domain is essential for cytotoxic activity in the pro-apoptotic family members. A special group of pro-apoptotic Bcl-2 family members, the so called “BH3-only” members, contain exclusively the BH3 region and are believed to sense cell damage.

Regulation of mitochondrial protein release and ions by influencing the mitochondrial outer membrane permeability is one important regulatory mechanism in controlling apoptosis in cells. Furthermore, it has been shown that Bcl-2 proteins influence endoplasmatic and nuclei calcium regulation (Orrenius et al., 2003). The function of Bcl-2 protein can be regulated in a variety of ways. Transcriptional regulation is for example important for the pro-apoptotic members Noxa and Puma, which can be induced by p53 (Villunger et al., 2003). Cleavage of Bid to truncated Bid (tBid) is known to activate its pro-apoptotic function. Translocation of Bcl-2 family members to mitochondria is often needed for their proper apoptosis-regulating function (tBid, Bax, Bak, Bcl-Xδ). Physical interaction and dimerization with other Bcl-2 family proteins is another way to control their function. Thus, the anti-apoptotic members appear to function, at least in part, by antagonizing pro-apoptotic family members and vice versa. The ability of these proteins to form homo- and heterodimers relates to their different function during apoptosis and the ratio of anti- versus pro-apoptotic proteins may determine a cell’s susceptibility to death signals (Fadeel et al., 1999b).
Bcl-2 and Bcl-X\textsubscript{L} are two anti-apoptotic family members whose function has been intensively studied. Although both proteins are important for cell survival, their function is cell specific. Bcl- X\textsubscript{L} is important for survival of erythroblasts in the bone marrow and its expression is regulated by the renal growth hormone erythropoietin (EPO) (Silva et al., 1999); (Gregory et al., 1999). Bcl-2 was for the first time described in B-cell follicular lymphoma. However, increased expression of these proteins has been detected in several other cancers such as prostate, breast, lung and colorectal cancer and is associated with chemoresistance and radioresistance of some types of malignancies. Bcl-2 is strongly expressed in CD34\textsuperscript{+} hematopoietic stem cells. Upon differentiation, its expression decreases and other anti-apoptotic family members such as Bcl-X\textsubscript{L} and Mcl-1 fulfill their function in the more differentiated hematopoietic progenitor cells (Josefsen et al., 2000).

The pro-apoptotic proteins Bax and Bak have been shown to facilitate cytochrome c release from mitochondria and fulfill an important role in the intrinsic pathway of apoptosis. Conformation changes are essential for their activation. In healthy cells, Bax exist as monomeric protein in the cytoplasm. Upon apoptosis induction, Bax oligomerizes (Tan et al., 1999) and translocates to the mitochondrial outer membrane (Griffiths et al., 1999; Wolter et al., 1997). When Bax is cleaved, its pro-apoptotic properties might increase or decrease, depending on the experimental system (Gao & Dou, 2000; Wood & Newcomb, 2000). Bak, on the other hand, is constitutively expressed in the mitochondrial membrane and can associate with the voltage-dependent anion channel. There is some redundancy in the function of Bcl-2 family proteins. Downregulation of Bcl-2 leads to increased expression of Bcl-X\textsubscript{L} (Tacke et al., 2004). While knock-out of either Bax or Bak in mice do not result in major abnormalities, mice with double knockout of both genes die \textit{in utero} with dramatic developmental defects (Lindsten et al., 2000).

1.2 MITOCHONDRIA AND INTRACELLULAR CALCIUM

Mitochondria play an important role in the aerobic generation of energy for cells. Oxidative phosphorylation is used to produce ATP from breakdown of carbohydrates and fatty acids. Mitochondria are surrounded by a double-membrane system. The inner membrane is highly folded and forms cristae, which extend into the inner matrix compartment of mitochondria. Between the inner and the outer membrane the intermembrane space is located.

Mitochondria are unique among intracellular organelles since they have their own DNA, which encodes tRNA, mRNA and some mitochondrial proteins (13 of at least 70 proteins). Nuclear encoded mitochondrial proteins are synthesized in the cytosol and transported into mitochondria. Thus, the mitochondrial genetic control involves interplay between mitochondrial and nuclear genomes (Smeitink et al., 2001). In the electron (respiratory) chain mitochondria build up an electrochemical gradient, which the cell utilizes to produce ATP. During glycolysis and the citric acid cycle, most of the free energy released by the oxidation of glucose to CO\textsubscript{2} is retained in the reduced coenzymes NADH and FADH\textsubscript{2}. Throughout respiration, electrons are released from NADH and FADH\textsubscript{2} and are transferred via 4 protein complexes (I-IV) and two carriers, coenzyme Q and cytochrome c to O\textsubscript{2}. The electron transport releases energy, which is stored in the form of a proton gradient across the inner mitochondrial membrane. Thus, this free energy is accumulated as an electrical potential (positive charged protons will
be pumped out from the mitochondrial matrix) and a proton concentration gradient. The proton $\text{F}_1\text{F}_0$-ATPase uses this electrochemical gradient to generate ATP. Cytochrome $c$ is found in the intermembrane space and mediates the transport of electrons between complex III (cytochrome $c$ reductase complex) and complex IV (cytochrome $c$ oxidase) (Smeitink et al., 2001).

### 1.2.1 Intracellular calcium regulation

Additional to aerobic ATP production, another important function of mitochondria is the regulation of intracellular calcium homeostasis (Figure 1.3).

Calcium is an intracellular second messenger (excitation of nerve cells, muscle contraction) and the calcium resting concentration inside cells is tightly controlled at 100 to 200 nM compared to high extracellular levels, which range between 1 to 2 mM. This huge concentration difference results in a very large electrochemical gradient for calcium ions across the cell membrane (mM versus nM) (Orrenius et al., 2003). Calcium can enter the cells via transport channels in the plasma membrane, which are voltage-sensitive, store-operated or receptor-mediated. The most common voltage-gated channel in the plasma membrane is the so called L-type channel, which can be blocked by Nifedipine. Since there is a continuous leak flow of calcium into the cells, the plasma membrane contains calcium ATPase-type pumps (PMCA), which translocate calcium back into the interstitium. Once inside the cell, calcium can interact with calcium-binding proteins or become sequestered into the ER (in muscle cells called sarcoplasmic reticulum) or into mitochondria.

Under physiologic conditions, the highest concentration of calcium is found in the ER (mM levels). Sarco-endoplasmatic ATPase-type pumps transport calcium into the ER. Upon receptor stimulation calcium can be released from the ER.

Under pathologic conditions, characterized by highly increased intracellular calcium levels, mitochondria have been found to sequester significant amounts of calcium. Mitochondrial calcium uptake is carried out by a uniporter in the inner mitochondria membrane, which transport calcium into the mitochondrial matrix where it is bound to inorganic phosphate and precipitate as calcium-phosphate. Compared to the ER transport mechanism, the affinity for calcium of the uniporter is low, mitochondrial calcium transport is slow and the amount of the mitochondrial calcium is small under physiological conditions. The electrophoretical transport is dependent on the negative mitochondrial membrane potential. Mitochondria can release calcium through different pathways: reversal of the uniporter, sodium-proton-dependent calcium exchange, or as a consequence of the opening of the mitochondrial permeability transition (MPT) pore (Orrenius et al., 2003).

Increased mitochondrial matrix concentrations can stimulate oxidative phosphorylation and thereby increase ATP production. Furthermore, it can modify the shape of cytosolic calcium pulses (Gunter et al., 2004). The low affinity of the mitochondrial import system is overcome by the proximity of mitochondria to the ER. Since some mitochondria creates “hotspots” close to ER calcium release channels, it is possible that calcium uptake by mitochondria limits calcium-induced desensitization of these ER release channels. Furthermore, by opening of the MPT pore, calcium can activate apoptotic mechanisms (Kroemer et al., 1997).
Figure 1.3. The regulation of intracellular calcium compartmentalization

1.2.2 Mitochondria and apoptosis

Recruitment of mitochondria in the pathway of apoptosis results in the permeabilization of the outer membrane and the release of intermembrane space proteins into the cytosol. This permeabilization can be caused by two different processes: pore formation by pro-apoptotic Bcl-2 family members in the outer mitochondrial membrane, or rupture of the outer membrane following opening of the MPT pore complex.

Upon activation, pro-apoptotic Bcl-2 family members like Bax, Bak and other “BH3-only” proteins translocate to mitochondria, insert into the outer membrane and form a pore in the outer membrane, which is large enough to allow the release of intermembrane space proteins like cytochrome c (Antonsson et al., 2000; Eskes et al., 1998; Eskes et al., 2000). This process can be antagonized by anti-apoptotic proteins like Bcl-2 and Bcl-XL.

In the MPT model (Crompton et al., 1999), rupture of the outer membrane can be indirectly catalyzed by calcium and is a secondary effect following permeabilization of the inner membrane. High matrix concentration of calcium triggers the opening of the MPT pore complex, which is localized at the contact site of outer and inner mitochondrial membrane. After opening, calcium and other low-molecular-mass matrix components (Mr< 1500) can easily leave mitochondria, and water and other ions can
enter the mitochondrial matrix, which results in swelling of mitochondria. Since the surface of the highly folded inner membrane is much greater than that of the outer membrane, mitochondrial swelling can lead to rupture of the outer membrane followed by the release of intermembrane space proteins into the cytosol. The MPT pore is a large protein complex, which consists of the Voltage-Dependent-Anion-Channel (VDAC), the Adenine Nucleotide Translocator (ANT) and mitochondrial cyclophilin D. Besides calcium, additional activators of the MPT pore are oxidative stress, high pyridine nucleotide and thiol oxidation, cytosolic alkalization or low mitochondrial transmembrane potential (Crompton, 2000). The involvement of the MPT pore has been shown in ischemic reperfusion damage of heart and liver cells, as well as in glutamate-induced neurotoxicity. Reperfusion after ischemia is combined with low ATP levels and high cytosolic calcium concentrations leading to calcium overload and pore opening.

There exist some links between Bcl-2 proteins, calcium and MPT induction. Bcl-2 is endogenous and potent inhibitor of MPT induction. The mechanism of this inhibition is still unclear, but it has been shown that Bcl-2 can interact with VDAC, supporting the channel in its normal configuration (Shimizu et al., 1999). Additionally, it has been suggested that Bax interacts with the VDAC-ANT complex (Marzo et al., 1998). Furthermore, Bcl-2 family proteins can influence intracellular calcium homeostasis. Bcl-2 overexpression increases the permeability of the ER membrane for calcium and reduces thereby the filling of the ER calcium store. This is followed by a diminished extent of capacitative calcium entry over the plasma membrane into the cytosol, when calcium is released from the ER. Additionally to Bcl-2, pro-apoptotic Bcl-2 members such as Bax and Bak can influence calcium handling of cells. Overexpression of Bax and Bak leads to increased calcium translocation from the ER to mitochondria followed by cytochrome c release. Furthermore, it has been shown that Bax can facilitate MPT induction in isolated mitochondria (Gogvadze et al., 2001).

1.3 CONCLUSION – APOPTOSIS AND MITOCHONDRIA

Apoptotic cell death is an important physiological process in adult tissues to maintain cellular homeostasis. Deregulation of apoptosis is believed to play a role in degenerative (too much apoptosis) and proliferative diseases (too little apoptosis). Furthermore, tumor cells are often resistant to apoptotic stimuli and may express higher amount of anti-apoptotic Bcl-2 family proteins. Mitochondria are important organelles in the regulation and initiation of apoptosis and different apoptotic pathways might converge into one common execution pathway downstream of mitochondrial-regulated events.
### 1.4 MYELODYSPLASTIC SYNDROMES (MDS)

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal stem cell disorders characterized by ineffective hematopoiesis, mild to severe pancytopenia, dysplastic changes of bone marrow progenitors and a high risk for evolution to acute myeloid leukemia (AML).

Almost every recent journal reporting in the field of the myelodysplastic syndromes might include a sentence similar to the above stated one. This broad definition is widely accepted and agreed upon by scientists working in the field of MDS. Before 1982, when the French-American-British (FAB) classification (Bennett et al., 1982) was proposed as a new tool to diagnose and classify MDS, a variety of different terms like preleukemia or preleukemic syndrome, subacute leukemia, smouldering leukemia (for review: Steensma & Tefferi, 2003) had been used by hematologists restricting the view of MDS to a purely preleukemic condition. This ignored important features of MDS: The distinct biological characteristics and the specific clinical features mainly caused by severe cytopenias. Moreover, progression to overt AML will not occur in the majority of MDS patients.

After 1982, the first international definition of MDS made it possible to diagnose patients according to common criteria and investigate the pathogenetic events in MDS, as well as to initiate international clinical studies with the aim to improve the treatment of and outcome for patients.

#### 1.4.1 Clinical features and diagnosis of MDS

The incidence of *de novo* MDS is 3-4/100 000 / year, increasing with age. Median age ranges between 60 and 70 years in different studies. MDS is rare in children, but exists as a distinct disease also in this group (Niemeyer et al., 2005). In contrast to *de novo* MDS, therapy-related MDS (t-MDS) is not age-related and may occur in as many as 15% of patients within a decade following intensive combined modality treatment for cancer (Pedersen-Bjergaard et al., 2000). The observed incidence of MDS has increased over time, due to recognition of the syndrome by physicians, aging of the population, and maybe due to environmental influence. Death usually occurs secondary to the complications of marrow failure or by progression to AML (Aul et al., 2001).

The typical clinical symptoms of MDS are caused by severe cytopenia. Due to anemia, patients suffer from fatigue and weakness, which often leads to reduced quality of life (Hellstrom-Lindberg et al., 2003; Silverman et al., 2002). Fatigue and dyspnoe may develop over a prolonged period, often exceeding 6 to12 months. Furthermore hypotension, tachycardia, pallor, dyspnoe may occur. If pancytopenia is present, additional complications like bleeding and severe infections are common (Hofmann & Koeffler, 2005).

The diagnosis of MDS is based on cytopenia or anemia in combination with dysplastic features in bone marrow progenitor cells. Since similar symptoms can be caused by vitamin or mineral deficiency (iron, B6, B12, folate) as well as ethanol abuse and drug reactions, such conditions have to be excluded before a diagnosis of MDS can be made. Furthermore, marrow dysplasia can be observed in acute viral infections or chemical toxicity but should be transient in these cases. MDS is sometimes discovered due to abnormal findings at a routine blood examination, and may be asymptomatic at the time of diagnosis.
1.4.2 Classifications

1.4.2.1 FAB

In 1982, a French-American-British working group (Bennett et al., 1976; Bennett et al., 1982) implemented a new classification for MDS based on the number of myeloblasts in bone marrow and blood, the presence or absence of ringed sideroblasts in the bone marrow, and the number of monocytes in the blood. Five subgroups were distinguished: refractory anemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts (RAEB), chronic myelomonocytic leukemia (CMML) and RAEB in transformation (RAEB-T) (Table 1.1).

<table>
<thead>
<tr>
<th>FAB subtype</th>
<th>Blasts in the bone marrow</th>
<th>Blasts in the blood</th>
<th>&gt;15% ringed sideroblasts in the bone marrow</th>
<th>Monocytes &gt;1000/ mm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDS 1, RA</td>
<td>&lt;5%</td>
<td>&lt;1%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MDS 2, RARS</td>
<td>&lt;5%</td>
<td>&lt;1%</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MDS 3, RAEB</td>
<td>5-20%</td>
<td>&lt;5%</td>
<td>+-</td>
<td>-</td>
</tr>
<tr>
<td>MDS 4, CMML</td>
<td>&lt;20%</td>
<td>&lt;5%</td>
<td>+-</td>
<td>+</td>
</tr>
<tr>
<td>MDS 5, RAEB-t</td>
<td>21-30%</td>
<td>&gt;5%</td>
<td>+-</td>
<td>+-</td>
</tr>
</tbody>
</table>

Table 1.1. FAB classification of MDS (Bennett et al., 1982)

1.4.2.2 WHO

The FAB classification based on morphological criteria was recently revised resulting in the World Health Organization (WHO) classification (Jaffe et al., 2001). The WHO classification aims to provide more homogeneous MDS categories, but is still mainly based on common morphological criteria (Table 1.2). The FAB subgroups RA and RARS were redefined to encompass cases with dysplastic features in erythroid lineage only, while two new categories, refractory cytopenia with multilineage dysplasia either without (RCMD) or with ringed sideroblasts (RCMD-RS) were added. The distinction between unilineage and multilineage dysplasia was based on prognostic studies, in which a significant impact on patient survival rates was demonstrated (Aul et al., 1992; Aul et al., 2002). In particular the difference in outcome between RARS and RCMD-RS has been underlined by recent publications (Germing et al., 2000; Howe et al., 2004).

RAEB was divided into RAEB-I and RAEB-II with 5-9% and 10-19% blasts in the bone marrow, respectively. Furthermore, the long clinically recognized 5q-syndrome was included as a separate entity and includes patients with an isolated deletion of the long arm of chromosome 5 (Van den Berghe & Michaux, 1997).

Two FAB subgroups were excluded from the WHO classification: RAEBt was included in the AML classification, and CMML was included in a new category, mixed myeloproliferative/myelodysplastic syndromes.
<table>
<thead>
<tr>
<th>RA</th>
<th>anemia</th>
<th>erythroid dysplasia only</th>
<th>no or rare blasts</th>
<th>&lt; 5% blasts</th>
<th>&lt;15% ringed sideroblasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>RARS</td>
<td>anemia</td>
<td>erythroid dysplasia only</td>
<td>no or rare blasts</td>
<td>&lt; 5% blasts</td>
<td>≥ 15% ringed sideroblasts</td>
</tr>
<tr>
<td>RCMD</td>
<td>cytopenias (bi- or pancytopenia)</td>
<td>dysplasia ≥ 10% of the cells</td>
<td>no or rare blasts</td>
<td>&lt; 5% blasts</td>
<td>&lt;15% ringed sideroblasts</td>
</tr>
<tr>
<td></td>
<td>no Auer rods</td>
<td>of two or more myeloid cell lines</td>
<td>&lt; 15% ringed sideroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;1x10⁹/ l monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCMD-RS</td>
<td>cytopenias (bi- or pancytopenia)</td>
<td>dysplasia ≥ 10% of the cells</td>
<td>no or rare blasts</td>
<td>&lt; 5% blasts</td>
<td>&lt;15% ringed sideroblasts</td>
</tr>
<tr>
<td></td>
<td>no Auer rods</td>
<td>of two or more myeloid cell lines</td>
<td>&lt; 15% ringed sideroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;1x10⁹/ l monocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAEB-I</td>
<td>cytopenias</td>
<td>unilineage or multilineage dysplasia</td>
<td>&lt; 5% blasts</td>
<td>5 - 9% blasts</td>
<td></td>
</tr>
<tr>
<td></td>
<td>no Auer rods</td>
<td>no Auer rods</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAEB-II</td>
<td>cytopenias</td>
<td>unilineage or multilineage dysplasia</td>
<td>5 - 19% blasts</td>
<td>10 - 19% blasts (&gt; 20% = AML)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Auer rods +/-</td>
<td>Auer rods +/-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDS-u</td>
<td>cytopenias</td>
<td>unilineage dysplasia: one myeloid cell line</td>
<td>&lt; 5% blasts</td>
<td>no Auer rods</td>
<td></td>
</tr>
<tr>
<td></td>
<td>no Auer rods</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5q-</td>
<td>anemia</td>
<td>normal or increased megakaryocytes</td>
<td>normal or increased megakaryocytes</td>
<td>with hypolobulated nuclei</td>
<td></td>
</tr>
<tr>
<td></td>
<td>normal or increased platelet count</td>
<td>&lt; 5% blasts</td>
<td>&lt; 5% blasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt; 5% blasts</td>
<td>isolated del 5q abnormality</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2. WHO classification (Jaffé et al., 2001)

1.4.3 Bone marrow morphology

MDS bone marrow biopsies usually show increased cellularity as a consequence of ineffective hematopoiesis and relative hyperproliferation. However, the cellularity may also be normal or - in a few cases - even decreased (hypoplastic MDS). The distinction between severely hypoplastic MDS and aplastic anemia can be difficult, and it is generally considered that there exists an overlap between these two syndromes (Maciejewski et al., 2002). Increased fibrosis can be observed in up to 10% of the cases. Typical cytological features of dysplasia are shown in Table 1.3.

<table>
<thead>
<tr>
<th>Erythropoiesis</th>
<th>Granulopoiesis</th>
<th>Megakaryopoiesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polynuclear erythroblasts</td>
<td>Hypersegmentation</td>
<td>Hypersegmentation</td>
</tr>
<tr>
<td>Ringed sideroblasts</td>
<td>Hyposegmentation</td>
<td>Hyposegmentation</td>
</tr>
<tr>
<td>Megaloblastic maturation</td>
<td>Hypogranulation</td>
<td>Micromegakaryocytes</td>
</tr>
<tr>
<td></td>
<td>(Pseudo-Pelger cells)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1.3. Typical dysplastic changes in the bone marrow from MDS patients
1.4.4 Cytogenetic aberrations

Cytogenetic changes can be observed in 30 to 50% of primary MDS and in up to 80% of secondary (therapy-related) MDS. In other words: around 50% of de novo MDS cases have normal metaphase cytogenetic findings. Large cytogenetic lesions detectable by conventional karyotypic analysis are not likely to be primary events in the pathogenesis of MDS. A monoclonal pattern, as assessed by analysis of the inactivation pattern of various X-linked genes in MDS marrow precursors, may precede the development of an overt cytogenetic lesion by several years (Legare et al., 1997). Although many cytogenetic aberrations found in MDS can also be observed in AML, balanced translocations are rarely observed in MDS. The most frequent chromosomal abnormalities in MDS involve partial or complete deletions of chromosomes 5, 7, 11, 12 and 20 and/or trisomy 8 (Table 1.4.). Multiple or complex aberrations are more frequent in advanced MDS, MDS-AML or therapy-related MDS (t-MDS). Deletion of the long arm of chromosome 5 (5q- syndrome) is the most common chromosomal aberration in MDS (Hofmann & Koeffler, 2005). The 5q- syndrome is characterized by elevated number of hypolobulated megakaryocytes in the bone marrow, thrombocytopenia and macrocytic anemia. Furthermore, it has a low rate of transformation to AML and implies a relatively good patient prognosis.

<table>
<thead>
<tr>
<th>Numerical</th>
<th>Translocations</th>
<th>Deletions</th>
</tr>
</thead>
<tbody>
<tr>
<td>+8 (19%)</td>
<td>inv 3 (7%)</td>
<td>del 5q (27%)</td>
</tr>
<tr>
<td>-7 (15%)</td>
<td>t(1;7) (2%)</td>
<td>del 11q (7%)</td>
</tr>
<tr>
<td>+21 (7%)</td>
<td>t(1;3) (1%)</td>
<td>del 12q (5%)</td>
</tr>
<tr>
<td>-5 (7%)</td>
<td>t(3;3) (1%)</td>
<td>del 20q (5%)</td>
</tr>
<tr>
<td></td>
<td>t(6;9) (&lt;1%)</td>
<td>del 7q (4%)</td>
</tr>
<tr>
<td></td>
<td>t(5;12) (&lt;1%)</td>
<td>del 13q (2%)</td>
</tr>
</tbody>
</table>

Table 1.4. Chromosomal aberrations in MDS patients. The frequency of chromosomal aberration is given in parentheses (Hofmann & Koeffler, 2003; Hofmann et al., 2004b)
1.4.5 Prognosis of MDS

The definition of MDS encompasses a heterogeneous group of disease entities. This makes it difficult for physicians to advise their patients regarding prognosis and treatment. Upon disease diagnosis, the overall survival of patients can differ from several years, or even decades with an indolent clinical course to a few months until death occurs due to bone marrow failure, or transformation to AML. Therefore, different attempts have been made to estimate the prognosis for individual patients. The following variables with a prognostic value for disease outcome have been used in the different prognostic scoring systems:

1. Number and degree of cytopenias
2. Number of myeloblasts in bone marrow or blood
3. Number and type of cytogenetic abnormalities
4. Dysplastic features in non-erythroid cells
5. Lactate dehydrogenase (LDH) serum levels (especially useful in the evaluation of CMML patients)

The first score published was the so-called Bournemouth score, which was based on hemoglobin level, neutrophil and platelet counts and percentage of bone marrow blasts (Worsley et al., 1988). Sanz and coworkers developed and validated a regression model for predicting survival of MDS patients (Sanz et al., 1989). Important factors for survival were blast cells, platelet count and age. The Lille score included for the first time karyotype as prognostic factor in MDS (Morel et al., 1996).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0</th>
<th>0.5</th>
<th>Points</th>
<th>1</th>
<th>1.5</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% blasts in bone marrow</td>
<td>0-4</td>
<td>5-10</td>
<td>-</td>
<td>11-20</td>
<td>21-29</td>
<td></td>
</tr>
<tr>
<td>Number of cytopenias (1)</td>
<td>0-1</td>
<td>2-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Cytogenetic risk group (2)</td>
<td>Low</td>
<td>Intermediate</td>
<td>High</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Risk group</th>
<th>Overall Score</th>
<th>Median survival (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low risk</td>
<td>0</td>
<td>5.7</td>
</tr>
<tr>
<td>Intermediate I risk</td>
<td>0.5-1</td>
<td>3.5</td>
</tr>
<tr>
<td>Intermediate II risk</td>
<td>1.5-2</td>
<td>1.2</td>
</tr>
<tr>
<td>High risk</td>
<td>&gt;2</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 1.5. International prognostic scoring system (IPSS) for myelodysplastic syndromes. (1) Platelets < 100,000/μl, Hb < 10 g/dl, neutrophils < 1800/μl; (2) Low risk: normal, 5q-, 20q-, -Y; High risk: complex aberrations (≥ 3 anomalies), chromosome 7 aberrations; Intermediate risk: all other aberrations

The International Prognostic Scoring System (IPSS), which was established as a method to estimate prognosis of MDS patients in 1997 is currently considered to be the
most reliable prognostic tool (Greenberg et al., 1997). The prognostic variables for disease outcome regarding survival and AML transformation encompass cytogenetic aberrations, the percentage of bone marrow blasts, and the number of cytopenias (Table 1.5).

### 1.4.6 Molecular alterations in MDS

In contrast to e.g. AML, in which large microarray studies have contributed to the understanding of the disease (Haferlach et al., 2005; Kohlmann et al., 2005; Kohlmann et al., 2003), the number of published gene array studies in MDS is limited, as is the number of investigated patients per study. Conclusions must therefore be considered preliminary. The MDS studies have revealed increased expression of the delta-like protein (dlk) in low risk MDS cases compared to controls and AML (Langer et al., 2004; Miyazato et al., 2001). The detailed function of this protein is uncertain, but it has role in cellular growth and differentiation programs (Ohno et al., 2001; Li et al., 2005). The activation of oncogenes like ras (mutations in 10 to 15% of MDS cases) has also been described, but their prognostic role remains debated (Hirai, 2003). The tumor suppressor gene p53 is inactivated in approximately 5-10% of MDS patients, often in association with a 17p deletion (Hirai, 2003). Activation of ras and inactivation of p53 are found more frequently in high risk MDS with complex karyotypes. The FLT3 gene encodes a tyrosine kinase involved in proliferation and differentiation of hematopoietic precursor cells. Mutation in FLT3 gene has been associated with a specific subtype of AML (Yokota et al., 1997), but has only been found in around 5% of MDS cases (Zwierzina et al., 1999).

Epigenetic silencing by DNA methylation is a basic cellular mechanism for regulating gene activity (List et al., 2004). DNA methylation of the cell cyle gene p15, and other genes have been frequently detected in hematological malignancies. p15 promoter methylation was in one study closely associated with a deletion or loss of chromosome arm 7q (Christiansen et al., 2003). The p15 gene methylation was less frequent in low-risk MDS (RA and RARS), than in high-risk MDS cases (RAEB, RAEB-t, CMML and MDS in transformation to AML). Furthermore, methylation status was closely correlated with disease progression in MDS. Thus, hypermethylation of p15 is involved in the pathobiology of MDS and is one of the important late events in the development of MDS. Other genes which expression might be reduced by promotor methylation in MDS and AML are p53 and p73, Rb and cadherin (for review: Kinoshita, 2004).

The Mixed Lineage Leukemia (MLL) gene (also called ALL-1, HRX, and Htrx-1) has been identified in leukemia patients with 11q23 translocations. There is evidence that the MLL gene rearrangement affects early hematopoietic progenitor cells. Since MLL gene arrangements are not involved in the pathogenesis of de novo MDS (Pappa et al., 2004), these arrangements might be more important for the transformation to AML.

The impact of mitochondrial DNA mutations is a matter of controversy. While some groups (Reddy et al., 2002) claim that there exits an increased incidence of mitochondrial cytochrome c-oxidase gene mutations in patients with MDS, Shin and co-workers were neither able to confirm these mutations in sideroblastic anemia, nor any evidence of “hot spots” in cytochrome c oxidase I and II genes (Shin et al., 2003). Their data do not support a major role for mitochondrial genomic instability in
myelodysplasia. Recently, it has been shown that mutations in the mitochondrial matrix chaperone protein HSPA9B can lead to decreased mitochondrial function producing oxidative stress and apoptosis distinctly in hematopoietic cells (Craven et al., 2005). Interestingly, HSPA9B is located on chromosome 5q, which is lost in ca 25% of MDS.

### 1.4.7 Models for the development of MDS and its progression to AML

Fearon and Vogelstein (Fearon & Vogelstein, 1990) have developed the famous so called “Vogelgram” demonstrating the multi-step process of cancer development in the colon (Figure 1.4).

![Figure 1.4. Stepwise model of genetic changes from colon adenoma to invasive colon cancer by Fearon et al. (Fearon & Vogelstein, 1990)](image)

Similar stepwise mechanisms might be taken into consideration as reasons for the clonal development of MDS and the transformation to AML (Figure 1.5), but general and common mutations like in colon cancer, which could explain the alterations in proliferation, differentiation and apoptosis have not yet been found.

Most cases of MDS are idiopathic (de novo MDS) and in these cases the primary initial (genetic) cause for the development of MDS remains unknown. However, several factors, such as anti-neoplastic alkylating agents, ionizing radiation, and benzene, are discussed to be etiological factors for MDS.

A consequence of clonal evolution is usual an increase of blasts indicating that MDS has progressed to AML (Figure 1.5). Parallel, a decrease in apoptosis of stem cells, and maturation can be detected (Parker et al., 2000). Deregulation of tumor suppressor genes, defective DNA repair enzymes, and immunological abnormalities have been suggested as factors triggering loss of differentiation and progression towards acute leukemia.

Pedersen-Bjergaard et al. have suggested a more detailed model for the causality of genetic alterations in the development of MDS and AML. This research group used t-MDS and t-AML and the main different cytogenetic aberrations (involving 7, 7q, 5, 5q and 8) as basis (Pedersen-Bjergaard et al., 2002). According to their model, 7 major different genetic changes (A to G, see Figure 1.6) in different types of MDS and AML can be observed. However, in this model and opposite to the Vogelgram, the first and initial preclinical genetic changes are not known yet.
Alkylating cytostatic treatment can induce the so-called two “alkylator-types” of MDS and AML (Figure 1.6). Either total loss or partial loss of the long arm of chromosome 7 describes one of the alkylator subgroups (subgroup A in Figure 1.6). Silencing of p15 by promoter-methylation and ras mutations can be detected and may mark the disease progression from MDS to AML. Another alkylation t-MDS/ t-AML subgroup is characterized by loss of chromosome 5 or the partial loss of the long arm of this chromosome (5q-, group B in the Figure 1.6). Similar to the Vogelgram model, p53 mutations and complex karyotypes are characteristic for late changes of cancer progression. The prognosis of this subgroup is poor.

Opposite to the 5q loss in t-MDS, the 5q-syndrome (group C) is a special de novo MDS entity. It is characterized by low risk of transformation to AML, makrocytic anemia, thrombocytosis and hyposegmented giant megakaryocytes and relatively good prognosis. The commonly deleted region on 5q seems in these cases to be located more telomeric than in 5q- of t-MDS. Monosomy 7 (subgroup D) is frequently observed in children with MDS, and it is characteristic of juvenile myelomonocytic leukemia.

Topoisomerase II inhibitors (for example anthracyclines) are also leukogemic and induce t-AML rather than t-MDS (subgroup E). Cytogenetic abnormalities include balanced reciprocal translocations involving chromosome bands 11q23, 21q22, and more rarely inversion (16), or t(15;17). These aberrations have been shown to generate fusion between the MLL, AML1, CBFB or RARA genes and various partner genes and the chimeric genes are supposed to act as dominant oncogenes.

A normal karyotype can be observed in 40 to 50% of patients (subgroup F). Internal tandem duplications of genes like MLL and FL3 genes have been demonstrated in a subpopulation of these patients. Trisomy 8 with or without other chromosome aberrations may be present in patients with de novo MDS and AML (subgroup G). Leukemic cells with trisomy 8 showed down-regulation of several apoptosis-related genes and a moderate overexpression of many genes located to

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**Figure 1.5. Clonal stepwise progression from early MDS to AML**

Insult → Alteration I → Alteration II → Transformation

Normal stem cell +/− genetic predisposition

Early MDS
- Apoptosis
- Maturation
- Function
- Patient prognosis
- Proliferation

AML
- Dysplasia, Clonal expansion,
- Number of blasts, Genomic instability and
- Cytogenetic abnormalities,
- Increased expression of anti-apoptotic versus
- pro-apoptotic Bcl-2 family proteins
chromosome 8 as compared to leukemic cells with a normal karyotype or to normal cells. These results suggest a simple gene-dosage effect of the supernumerary chromosome 8 (Virtaneva et al., 2001).

1.4.8 Apoptosis and MDS

Apoptosis is an important non-pathologic regulatory process in normal erythropoiesis (for review: Testa, 2004). Homeostasis of the erythropoietic system requires an appropriate balance between erythroid cell production and red blood cell destruction. During the differentiation process, the cells become progressively sensitive to EPO that controls both the survival and proliferation of erythroid cells. Several proteins important for regulation of cell death play crucial role in erythropoiesis. Thus, Fas/Fas-L interaction is important for the negative regulation of later erythroblasts. Furthermore, caspase activity is required for the process of normal erythroid differentiation. A transient caspase-3 and -7 activation can be observed during the stages of erythroid maturation corresponding to proerythroblasts and basophilic erythroblasts (Zermati et al., 2001).

Since the beginning of 1990, it is generally believed that deregulated and pathologically increased apoptosis of bone marrow precursor cells leads to anemia/pancytopenia in MDS (for review: Mundle, 2001; Parker & Mufti, 2000; Parker &
It is, however, unclear whether apoptosis targets mainly MDS clonal cells or similarly affects normal hematopoietic progenitor cells, since there is so far no reliable biological tumor cell marker for many MDS patients.

Increased cell death in the bone marrow can be an explanation for one of the paradoxes of MDS: The presence of peripheral blood cytopenias despite typically hypercellular bone marrow. Although it is known for more than 10 years that apoptosis is an important pathophysiologic process in MDS, the exact cause-relationship between apoptosis and peripheral blood cytopenia is not clarified. Several investigations have reported a lack of correlation between the degree of apoptosis of progenitor cells in the bone marrow and the degree of peripheral cytopenia (Hellstrom-Lindberg et al., 1997a; Raza et al., 1995; Tsopou et al., 1999).

Furthermore, the reasons for the increased apoptosis are still unclear and discussed. Extrinsic factors on the one hand, or intrinsic/metabolic cellular features of the clone on the other hand might trigger cell death of the myeloid progenitor cells.

1.4.8.1 The extrinsic pathway in MDS

The extrinsic apoptosis pathway is characterized by the initiation of apoptosis through activation of cell surface death receptors. Since MDS is a clonal disease, it can be assumed that the bone marrow microenvironment is changed by or as a response to the abnormal cell clone. Indeed, changed cytokine levels with elevated pro-inflammatory cytokines like IL-3, IL-6, IFN-γ, TGF-β or TNF-α are detected in serum of MDS patients (for review: Rosenfeld & List, 2000). Furthermore, the immune system of the patient might attack the clonal cells. Immunosuppressive therapy has been used successfully in a subset of patients with RA (Molldrem et al., 1997).

It has been described that death receptors like Fas, TNF-α and TRAIL as well as adapter Fas-associated death domain (FADD) are overexpressed and trigger apoptosis in early stage MDS erythroid cells (Claessens et al., 2002; Fontenay-Roupie et al., 1999; Mundle et al., 1999a; Mundle et al., 1999b). Another group has reported that Fas-mediated apoptosis is important in regulation cell death in MDS cells with trisomy 8, but not in cells with other cytogenetic abnormalities (Sloand et al., 2002). Furthermore, blocking of the extrinsic apoptosis pathway by transduction of cells with dominant negative mutant form of FADD led to inhibited caspase activity and cell death and increased BFU-E (Claessens et al., 2005). In contrast, our data suggests that blocking of the Fas receptor by antagonistic antibodies did not rescue RARS cells from apoptosis (Hellstrom-Lindberg et al., 2001).

1.4.8.2 The intrinsic pathway and the importance of mitochondria in the pathology of MDS

Other groups suggest intrinsic signals as the main reason for apoptosis and changed proliferation patterns in MDS (Span et al., 2005). As mentioned above, important organelles in the initiation and amplification of intrinsic apoptotic signals are mitochondria. Metabolic changes resulting in depolarization of mitochondria and release of mitochondrial proteins, such as cytochrome c, can lead to caspase activation and apoptotic cell death. In RARS, the characteristic ringed sideroblasts have extensive iron deposits within the mitochondria matrix. It was for long assumed that these were amorphous iron deposits, but recent studies demonstrate that the iron in ringed
sideroblasts is bound to aberrant mitochondrial ferritin (MtF), which may represent a specific marker of sideroblastic anemia since it is not found in normal erythropoiesis (Cazzola et al., 2003). It is unclear, how this iron accumulation might disturb normal mitochondrial functioning. Furthermore in MDS, mitochondrial DNA mutations (Gattermann, 2000) or mutations of nuclear-encoded mitochondrial proteins (Craven et al., 2005) have been observed.

As shown by our group, the expression of mitochondrial ferritin occurs at a very early stage of erythroid differentiation in RARS and is paralleled by an up-regulation of genes involved in erythroid differentiation (Tehranchi et al., 2005). Again, it is unclear how this might interfere with the mitochondrial function and whether the pathological iron and ferritin distribution might induce apoptosis. Interestingly, spontaneous cytochrome c release can be found in RARS erythroid progenitor cells, and can be sufficiently blocked by G-CSF (Tehranchi et al., 2003). These described mitochondrial alterations might contribute to pathological triggering of apoptosis in RARS.

1.4.8.3 Apoptosis and clonal expansion – a discrepancy?

One dilemma in recent MDS disease models is the discrepancy between increased apoptosis and clonal survival and expansion, and progression to AML. If apoptosis is pathologically increased in clonal MDS cells, this should consequently lead to extinction of the clone and its abnormal precursor cells, but it is obvious that this is not the case. Using the two above described apoptosis models as base, the following hypothetical explanations can be postulated:

Involvement of the extrinsic pathway in the pathogenesis of MDS could be used as model to understand the importance of apoptosis regulation for MDS. In low risk MDS, cells still undergo apoptosis triggered by constant external signals. Over time, the clonal cells develop resistance against these external factors and acquire an increased propensity for uncontrolled proliferation. Thereby, increased cellular survival versus constant external suppression might be one important factor in the development of AML. Interestingly, it has been shown that the ratio of anti-apoptotic proteins like Bcl-2, Bcl-X_L to pro-apoptotic proteins like Bax, Bak is changing when MDS progresses towards AML: An increase of pro- versus anti-apoptotic proteins (Parker et al., 1998; Parker et al., 2000) was observed.

It is more difficult to understand how intrinsically regulated apoptosis might be connected to survival and expansion of the abnormal MDS clone. In this case, intrinsic clonal alterations must include two contradictory effects: on one hand the changes are supposed to initiate cell death, but on the other hand they must provide a growth and survival advantage for the clone. Moreover, induced by intrinsic cellular mechanisms, an increased ratio of cell death to proliferation should logically give rise to stem cell elimination and a hypocellular marrow rather than hypercellularity frequently observed.

This discrepancy might be solved by the hypothesis that the clonal alterations offer growth advantage for early (dividing and proliferating) stem cells, but later upon stimulation of the differentiation program, the same clonal alterations are disadvantageous for the cell, inhibit its maturation and finally trigger its death.

1.4.9 Treatment of MDS

There is no common treatment for MDS. However, a variety of different treatment approaches are used for the different subcategories of the disease (Figure 1.7).
Further development of rational therapies and improvement in the outcome for patients with MDS will eventually be possible only through a comprehensive understanding of the pathophysiology of the disease. Advanced classifications of MDS based on genetic and biological factors, and the identification of new risk factors are needed to better define treatment approaches that could improve quality of life and prolong overall survival. In general, most patients should be encouraged to participate in clinical trials (for review: Cazzola et al., 1998; Faderl & Kantarjian, 2004; Kouides & Bennett, 1999 and Hellstrom-Lindberg, 1999; Hellstrom-Lindberg et al., 2000).

![Figure 1.7. Different treatment approaches in MDS (modified after Kouides & Bennett, 1999)]

A careful and precise diagnosis of MDS is the base for successful treatment (for review: Hofmann & Koeffler, 2005 and List, 2002). Patients should be grouped according to the FAB and WHO classifications, and be given an IPSS score. Furthermore, active supportive care with transfusions and antibiotics should be used when necessary. It might be a difficult task to select the best treatment option for each particular MDS patient. The decision has to be made whether to make an attempt to cure MDS, or whether to aim at improvement of symptoms and hopefully survival. The risk group of the patient (low versus high) is an important factor, which has to be considered. The low risk group incorporates patients with IPSS low and intermediate I, while the high risk group incorporates intermediate II and high risk patients (Faderl & Kantarjian, 2004).
Current treatment approaches can be divided into the following groups:

1.4.9.1 Supportive therapy with transfusions and antibiotic treatment

A high quality supportive care should always be the basic approach for the major clinical symptoms of MDS, i.e. anemia, thrombocytopenia and infections related to neutropenia. Hematopoiesis is not manipulated. Iron overload can occur as side effect of frequent blood transfusions and iron chelating therapy using desferrioxamine is needed for many patients. Iron chelation should usually be started when the amount of red blood cell transfusions exceeds more than 20 to 25 units or the serum ferritin level is higher than 1500 μg/l.

1.4.9.2 Stem cell transplantation

Currently, the only treatment that can cure MDS is allogenic stem cell transplantation. However, this treatment carries a significant risk of treatment-related mortality and morbidity. The treatment outcome is dependent on the selection of patients. Variables that influence the outcome are age, morphology, and cytogenetics. Results are better in younger patients, in patients with low-risk MDS, and in patients who undergo transplantation within one year from diagnosis (Appelbaum & Anderson, 1998; Runde et al., 1998). However, less than 50% of patients are cured by this treatment, due to a high risk both for relapse and transplant-related mortality. The high median age of patients with MDS is the major barrier for this treatment. Autologous stem cell transplantation may produce long-lasting remissions in selected patients, but is limited to patients, who have achieved a complete remission and can undergo stem cell harvest (De Witte et al., 1997). Results for older patients and patients who are in recurrence are still unsatisfactory. Several recent clinical trials have evaluated the effect of reduced intensity conditioning regimen followed by allogenic stem cell transplantation (Chan et al., 2003; Luger & Sacks, 2002; Taussig et al., 2003). Disease free survival was comparable to that in patients, who received conventional regimens, in spite of an older and more fragile patient population.

1.4.9.3 Conventional high-dose (intensive) chemotherapy

High-dose chemotherapy aims to eradicate the myelodysplastic clone and to achieve a polyclonal hematopoiesis (Hofmann et al., 2004a). Favorable factors for chemotherapy are age < 50 years, normal karyotype, an FAB diagnosis of RAEB-t. Although remission rate only are slightly worse than for de novo AML, complete remission duration is short. The vast majority of patients relapse and cure is rare (Hast et al., 2003). Predictive models that may help clinicians to decide whether to choose this type of treatment are warranted. Functional drug resistance and prolonged drug-induced aplasia constitute the major drawbacks of this treatment (for review: Beran, 2000).

1.4.9.4 Low-dose chemotherapy

Low dose chemotherapy with cytosine arabinosid (ara-C) as a non-curative treatment may improve peripheral blood values and reduce blast counts, but randomized studies failed to show a difference in overall survival between treated patients and patients receiving supportive care only (Miller et al., 1992).
1.4.9.5 Growth-factor treatment

EPO may improve anemia in MDS, but patients with RARS respond significantly less often to treatment than other low-risk MDS subgroups as shown in a meta-analysis (Hellstrom-Lindberg, 1995). In RARS, the combination of G-CSF and EPO-treatment may improve hemoglobin levels and decrease the transfusion need without causing major side effects (Hellstrom-Lindberg et al., 1997b; Negrin et al., 1996 and Hellstrom-Lindberg et al., 1998). The Scandinavian-American response score (Hellstrom-Lindberg et al., 1997b) might be used as a tool to select patient groups with higher probability to respond, which has been confirmed by another independent group (Remacha et al., 1999). It has been shown that quality of life is increased in patients responding to growth factor treatment (Hellstrom-Lindberg et al., 2003). The high costs of the therapy are a major disadvantage, as is the fact that more than 50% of low-risk patients are refractory to treatment. Several studies have shown that EPO and G-CSF in combination have synergistical effect on erythropoiesis, especially in WHO-RARS patients (Howe et al., 2004; Jadersten et al., 2005). There is some evidence that also the combination of GM-CSF and EPO may offer good erythroid response rates, but the experience is limited compared to G-CSF + EPO, and GM-CSF has more side effects.

In a 45 months follow up study of 129 patients, which were included in Nordic G-CSF + EPO studies, a median response duration of 23 months was observed. Complete responders showed longer response than partial responders and the response rate was longer in Low/Int-1 classified patients than in higher risk groups. Treatment with growth factors did not increase the risk for leukemic transformation (Jadersten et al., 2005).

1.4.9.6 Immunosuppressive treatment

Immunosuppressive treatment with cyclosporine (Jonasova et al., 1998; Catalano et al., 2000) and antithymocyte globulin (Molldrem et al., 1997) may induce long-lasting responses in hypoplastic RA, which suggests similar pathogenetic mechanisms for hypoplastic RA and aplastic anemia (AA). In addition, the number of CD34+ stem cells is reduced in both diseases (Fuchigami et al., 2000). RARS patients, patients with an increase of marrow blasts, and elderly patients do not seem to respond to immunosuppressive therapy.

1.4.9.7 Novel therapies for MDS

In May 2004, the hypomethylating agent 5-azacytidine was approved by the American Food and Drug Administration (FDA) for the treatment of all subtypes of MDS (Kaminskas et al., 2005). 5-Azacytidine significantly delays the transformation towards leukemia (for review: Silverman, 2001). Repeated courses of low-dose decitabine, another hypomethylating agent, induce cytogenetic remissions in a substantial number of elderly MDS patients with pre-existing chromosomal abnormalities. Patients with 'high-risk' chromosomal abnormalities may particularly benefit from this treatment (Lubbert et al., 2001). The mechanisms of action of these drugs are not really clear, but azacytidine and decitabine seem to exert their action on multiple proliferation-associated genes silenced by hypermethylation in MDS (Sigalotti et al., 2003). Direct cytotoxicity can not be excluded.
The immunmodulatory drug Thalidomide, which has been successfully used in the treatment of multiple myeloma patients, has also been tested in MDS trials, but has troublesome side effects (Musto, 2004; Raza et al., 2001; Strupp et al., 2002). Lenalidomide, a structural thalidomide analogue (Corral et al., 1999), is currently tested in MDS. The drug effects are complex and it has been shown that it decreases TNF-α release and other cytokine levels like (II-1β, II-6 and II-12). Furthermore, it inhibits angiogenesis by the suppression of plasma VEGF levels. Lenalidomide has proven to be extraordinary effective in patients with 5q- syndrome, in which it induced complete erythroid remission and cytogenetic response in more than 2/3 of the patients, compared to 57 and 12 percent in patients with normal or complex karyotype, respectively (List et al., 2005). The authors of this study conclude that lenalidomide has hematological activity in patients with low-risk MDS, also those refractory to EPO, or who are unlikely to benefit from conventional therapy. Thus, lenalidomide might be a new and promising treatment option for low-risk MDS patients and in particular patients with 5q- syndrome.

Arsenic trioxide (ATO) has also been tested in the treatment of MDS. A variety of different drug mechanisms such as induction of apoptosis, cell differentiation, abrogation of proliferation and inhibition of angiogenesis have been suggested as the mechanisms of action. The overall response to arsenic trioxide is about 25% (mostly partial responses). ATO was tolerated well, also by elderly patients (List et al., 2003; Raza et al., 2004; Vey, 2004).

1.5 CONCLUSION - MDS

MDS is heterogeneous group of clonal stem cell disorders clinically characterized by severe cytopenias and a high risk of the transformation to AML. Although it is generally believed that cytopenias are caused by excessive apoptosis of bone marrow progenitor cells, the initiating reasons for increased apoptosis are still under intense investigation and no common mechanisms has yet been found. Intrinsic as well as extrinsic factors might be responsible for the pathological response of MDS bone marrow cells to a variety of different apoptotic stimuli.
2 PRESENT INVESTIGATION

2.1 AIM OF THE STUDY

Apoptosis plays an essential part in the pathogenesis of MDS. It has been shown that the hematopoietic growth factors EPO and G-CSF may increase hemoglobin levels and reduce apoptosis of marrow progenitors. However, initial trigger mechanisms for apoptosis as well as the precise molecular mechanisms behind the antiapoptotic effects of G-CSF remain to be clarified. The present work focused on intrinsically-mediated apoptosis, different techniques to investigate apoptosis, and on mitochondrial function. The experiments were based on the following aims and questions:

Paper I: To establish a new and improved freezing method for primary bone marrow cells. To investigate possible artifacts, which can be induced by storage of bone marrow cells in the freezer.

Paper II: To assess the role of Fas-L/Fas interaction in the increased apoptosis of MDS bone marrow cells. Which enzymes - in particular caspases- are activated in MDS mononuclear cells during apoptosis? Does inhibition of caspases affect cell proliferation and survival of bone marrow progenitor cells?

Paper III: G-CSF is used in combination with EPO as treatment for anemia in a subgroup of MDS patients. How does G-CSF influence cell growth and apoptosis of bone marrow cells in in vitro culture? What are the effects of G-CSF on caspase activation, mitochondrial membrane potential and colony growth?

Paper IV: To assess the cellular and molecular effects behind the anti-apoptotic action of G-CSF in hematopoietic cells. Are mitochondria and their function influenced by G-CSF and does G-CSF interfere with oxidative phosphorylation? Since we used ATRA to induce apoptosis in bone marrow cells, the present work also led to an investigation of the molecular mechanisms of ATRA-induced apoptosis, mainly focusing on how ATRA influences intracellular calcium regulation.
2.2 METHODOLOGY

2.2.1 General comments

The techniques used in this study are described in detail in papers I-IV. Here, materials and methods will be listed and briefly commented upon.

In the first paper, methods were established for the experiments with primary bone marrow cells. The data presented in this thesis was built upon work with cell lines as well as primary bone marrow cell cultures. Since experiments using patient material usually have the disadvantage of a relative shortage of cells and broader result variations, we decided not to work solely with primary bone marrow cell cultures (paper I-III). Hence, we also used the P39 cell line to investigate basic apoptosis mechanisms in bone marrow leukemia cells as well as the anti-apoptotic effects of G-CSF (paper IV).

2.2.2 Bone marrow from patients and healthy volunteers (papers I-III)

Bone marrow needle aspirates (5-10 ml per aspirate) were obtained from the posterior iliaca crest (RARS patients and normal bone marrow donors) or sternum (thoracic surgery patients, normal bone marrow). The aspirate was subjected to Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation (<1,077 g/ml) at 1500 rpm for 30 minutes at room temperature to isolate mononuclear cells (MNC). After washing twice in PBS (GIBCO BRL, Invitrogen AB, Stockholm) at 1500 rpm for 10 minutes the cells were resuspended in RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (GIBCO BRL). Cells were then grown at a concentration of 5 x 10^5 cells/ml in 25 ml tissue culture flasks (TPP, Trasadingen, Switzerland) and incubated at 37°C and 5% CO2 in air for various time periods (4, 24, and 48 hours).

2.2.3 Glycophorin A (GpA) and CD 34+ cell isolation (papers II, III)

The MNC were separated for CD 34 or GpA positivity using the Mini Macs system (Miltenyi Biotec, Bergisch Gladbach, Germany). According to the manufacturer’s instructions, cells were incubated with CD 34 or GpA magnetic-labelled antibodies (20 µl/10^7 cells), respectively. After incubation for 15 minutes at 4°C, cells were washed and separated by a magnetic column and used for further investigation. Prior to the experiments described in this thesis, purity of the obtained cell fractions was tested by Giemsa staining and FACS analysis in 2 patients and 2 controls.

2.2.4 CD 34 colony assay (papers II, III)

Aliquots of CD 34+ cells were incubated in medium RPMI 1640 (GIBCO BRL) supplemented with 10% fetal calf serum overnight with the Fas-agonistic antibody CH-11 (1 µg/ml) in the presence or absence of G-CSF (Neupogen, Amgen, Stockholm, Sweden, 100 ng/ml). The following day 10^4 CD 34+ cells/ml were seeded in triplicate from each position in MethoCult 4434 medium (containing methylcellulose, fetal bovine serum, bovine serum albumin, 2-mercaptoethanol, L-glutamine, stem cell factor, GM-CSF, IL-3, and EPO; StemCell Technologies Inc., Vancouver, Canada) and Falcon petri dishes 10008, 35 x 10mm (Becton Dickinson, Plymouth, UK) and cultured for 14 days at 37°C in 5% humidified air. Erythroid colonies (defined as CFU-E and
BFU-E) and myeloid colonies (defined as CFU-G, CFU-M and CFU-GM) were counted under an inverted microscope and a mean value was calculated for each culture condition.

2.2.5 Cell lines and their cultivation (papers I, IV)

Two cell lines were used in our study. The P39 cell line/Tsugane is a myelomonocytic cell line derived from the peripheral blood of a patient suffering from leukemia following MDS (kindly provided by Prof. Y. Yoshida, Center for South East Asian Studies, Kyoto University, Kyoto, Japan). The Jurkat T-lymphocyte cell line was purchased from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown in RPMI 1640 (Sigma, Stockholm, Sweden) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin (1% in the medium) at 37ºC in a humidified atmosphere containing 5% CO2. Cells were used for the experiments, in exponential growth phase.

2.2.6 Isolation of rat liver mitochondria (paper IV)

The liver of male Sprague-Dawley rats was minced on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5), supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600 x g for 8 min at 4ºC. The supernatant was decanted and recentrifuged at 5,500 x g for 15 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at 5,500 x g for 15 min. The final mitochondrial pellet was resuspended in MSH buffer at a protein concentration of 80-100 mg/ml. The quality of isolated mitochondria was controlled by the calculation of the respiratory control ratio (RCR) of V3 to V4 respiration. In all experiments RCR was > 3.5.

2.2.7 Measurement of mitochondrial oxidative phosphorylation (paper IV)

Isolated rat liver mitochondria or P39 cells (5×10⁶) were resuspended in 1 or 0.3 ml of buffer (150 mM KCl, 5 mM KH₂PO₄, 1 mM MgSO₄, 5 mM Tris, pH 7.4), respectively. Measurement of respiration was performed at constant temperature, 30ºC. Cells were permeabilized with 0.005 % digitonin. Succinate (5 mM) in the presence of rotenone (2 μM), malate + pyruvate (5 mM each) and tetramethylphenylenediamine (TMPD, 0.5 mM) + ascorbate (1 mM) were used as mitochondrial substrates. Changes in the oxygen concentration were monitored with an oxygen electrode (Hansatech Instruments, Norfolk, UK) and analyzed with the OxygraphPlus software (Hansatech Instruments, Norfolk, UK). ADP and carbonyl cyanide m-chlorophenylhydrazone (CCCP), were used in concentrations of 75 μM and 0.2 μM for the experiments with cells and in the concentrations of 200 μM and 1 μM for the experiments with isolated mitochondria, respectively. V4 respiration was measured in mitochondria as the steady state respiration after expenditure of ADP. In cells basal V4 respiration was estimated in the presence of 1 μM atractyloside, which blocks ADP entry into mitochondria.
2.2.8 Mitochondrial calcium accumulation capacity measurements (paper IV)

For measurement of mitochondrial calcium accumulation capacity 1.5x10^6 cells were suspended in 400 μl of buffer (150 mM KCl, 5 mM KH₂PO₄, 5 mM Succinate, 1 mM MgSO₄, 5 mM Tris, pH 7.4). Cells were permeabilized with 0.005% digitonin and 2 μM rotenone was added in order to maintain pyridine nucleotides in a reduced form. Mitochondrial calcium uptake was induced by sequential additions of calcium to the cells. Calcium concentrations changes were registered using a calcium sensitive electrode (Thermo Orion, Beverly, USA) and visualized with a chart recorder.

2.2.9 Mitochondrial calcium concentration (paper IV)

The fluorescent calcium-sensitive dye Rhod-2 AM (2.5 μM, MoBiTec, Göttingen, Germany) was used to assess intramitochondrial calcium. Cells were stained with the dye, washed and re-incubated for 30 minutes in medium without the dye at 37ºC to reduce cytoplasmic background of the dye. The multivalent cation Rhod-2 AM is accumulated by energized mitochondria according to the mitochondrial membrane potential, hydrolyzed, and trapped in the mitochondrial matrix. Measurements of the Rhod-2 AM fluorescence were performed using a plate reader (Labsystems, Stockholm, Sweden) at 544 nm excitation and 590 emission wavelengths. To obtain maximum fluorescence (f max) cells were sequentially lysed by a detergent, Triton X-100. Minimum fluorescence value (f min) was obtained upon addition of the calcium chelator EGTA (5mM, final concentration). Intramitochondrial calcium concentrations were calculated using the formula [Ca]ₘ = kd x (f-fmin)/(fmax-f), where kd (the binding constant) = 570 nM and f = fluorescence. Fluorescence microscopy was used to confirm that the vast majority of Rhod-2 AM fluorescence was associated with mitochondria.

2.2.10 Antibodies and Western blotting (papers I- IV)

Samples were mixed with Laemmli’s loading buffer, boiled for 5 min and subjected to 15% SDS-PAGE at 130 V followed by electroblotting to nitrocellulose for 2 h at 100 V. Membranes were blocked for 1 h with 5% nonfat milk in phosphate-buffered saline at room temperature and subsequently probed overnight with primary antibody. The membranes were rinsed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:10000). Following incubation with secondary antibody membranes were rinsed and bound antibodies were detected using enhanced chemiluminescence according the manufacturer’s instructions. For preparation of cytosolic extracts cells were permeabilized with 0.005 % digitonin and incubated for 5 minutes on ice in a buffer containing 150 mM KCl, 5 mM KH₂PO₄, 1 mM MgSO₄, 5 mM succinate, 5 mM Tris and 1mM EGTA at pH 7.4. Subsequently, whole cell lysates or supernatant and pellet fractions were analyzed by SDS–PAGE followed by western blotting. The primary antibodies we used in the studies were raised against cytochrome c (1:2500, BD Bioscience, San Diego, USA), Bax (1:1000, BD Bioscience, San Diego, USA), Bid (1:1000, Cell Signaling Technology, Beverly, USA), Bel-2 (1:100, Dako, Glostrup, Denmark), G3PDH (1:2000, Nordic BioSite, Täby, Sweden), actin (1:100, Sigma, St. Louis, MO, USA), PARP (1:5000, Biomol, Plymouth, PA, USA), caspase-3
(1:1500, BD Bioscience, San Diego, USA) and caspase-8 (1:1000, BD Bioscience, San Diego, USA).

### 2.2.11 Flow cytometry measurements (papers II-IV)

After incubation with the respective fluorescent dye, fluorescence was measured with a FACScan Flow cytometer (Becton Dickinson, San Jose, CA, USA). Necrotic cells were excluded based on forward and side scatter criteria and data were analyzed using the CellQuest (Becton Dickinson) and the freeware WinMDI software.

#### Mitochondrial transmembrane potential

The mitochondrial transmembrane potential ($\Delta \psi_m$) was measured by the fluorescent cationic dyes 3,3’-dihexyloxycarbocyanine iodide (DiOC$_6$(3), final concentration 20 nM, Molecular Probes, Leiden, The Netherlands) or tetramethylrhodamine ethyl ester (TMRE, final concentration 25 nM, Molecular Probes), which normally accumulate in mitochondria as a direct function of $\Delta \psi_m$. Cells were incubated with dye in phenolred-free medium or PBS for 30 minutes at 37 ºC.

#### Reactive oxygen production

ROS were visualized by the dye dihydroethidium (HE, 4 mM, Molecular Probes), which is oxidized to the fluorescence-emitting substance ethidium in the presence of ROS. Cells were incubated with the dye in PBS for 30 minutes at 37 ºC.

#### Annexin V

Exposure of phosphatidylserine on the cell surface was detected by Annexin V-FITC (APOPTEST, NeXins Research BV, Hoeven, The Netherlands), according to the manufacturer's instructions. Late apoptotic/necrotic cells were defined as cells that were permeable to propidium iodide and early apoptotic cells as Annexin V-positive, propidium iodide-negative cells.

#### Intracellular calcium concentration

Changes in intracellular calcium concentrations were visualized by the dye Fluo-4 AM (5 μM, Molecular Probes, Leiden, The Netherlands).

### 2.2.12 Apoptotic nuclear morphology (paper IV)

Apoptosis was assessed by estimating morphologic features as fragmented nuclei and condensed chromatin in May-Grünwald-Giemsa staining on cytospined slides. The number of apoptotic cells was expressed in percentage of a total number of 200 cells counted per slide.

### 2.2.13 Caspase assay (papers II-IV)

The measurement of DEVD-AMC and IETD-AMC (Peptide Institute, Osaka, Japan) cleavage was performed using a fluorometric assay. One million cells were sedimented and washed once with PBS. After centrifugation cells were resuspended in 25 μl PBS, added to a microtiter plate and combined with the appropriate peptide substrate dissolved in a standard reaction buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol (DTT) and 0.1 % 3-[ (3-cholamidopropyl) dimethylammonio]-1-...
propanesulfonate (CHAPS), pH 7.25). Cleavage of the fluorogenic peptide substrate was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence units were converted into the amount of AMC (pmoles) using a standard curve generated with free AMC. Data from duplicate samples were then analyzed by linear regression.

2.2.14 TUNEL staining (papers II, III)

Induction of apoptosis results in the generation of single-strand DNA breaks. These breaks can be detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nicked labeling (TUNEL) method (Gavrieli et al., 1992). Using a fluorescence microscope with a green and a red filter, increased amount of single-strand breaks lead to an increase of the green signal without any change in the DNA content, measured by the red PI signal. This method requires cell fixation with cross-linking agents, which prevents the extraction of degraded DNA. Thus, cell suspensions were fixed in 4% neutral buffered formalin for 10 minutes. Cytospins were made with 100,000 cells per slide. After centrifugation (500 rpm, 4 minutes) the cytospins were air-dried. For staining the commercial IN Situ Apoptosis Detection Kit (Intergen, Oxford, UK) was used. The cytospins were digested in Equilibration Buffer (for 1 min at room temperature). Dioxigenin-dUTP was catalytically added to DNA by TdT enzyme (incubation 1 hour at 37°C) and visualization of the reaction was done by incubation with anti-dioxigenin antibody conjugated to FITC (30 min at room temperature). Counterstaining was made with propidium iodide (PI, 0.6 µg/ml). One cytospin from each assay treated according to the same procedure but with TdT enzyme exchanged by distilled water, was used as a negative control. The percentage of apoptotic cells (Cells with FITC-positive nuclei were interpreted as apoptotic cells) was based on a differential count of 200 nucleated cells.

2.2.15 Proliferation measurements (papers II, III)

Aliquots containing $10^5$ cells in 200 µl complete medium were grown in triplicates in a 96-wells test plate (TPP, Trasadingen, Switzerland), at 37°C in fully humidified air and 5% CO₂. 1 µCi (20 µl of 50 µCi/ml) 3H-thymidine was added for 24 hours at the time points of 0, 24, 48, and 72 hours after the start of cell culture. Cells were harvested with a Combi Cell Harvester (Skatron, Lier, Norway) on filter paper and added to scintillation fluid (2 ml OptiScint ‘HiSafe’, Wallac Scintillation Products, Turku, Finland). The radioactivity was measured with a liquid scintillation counter (Wallac 1409).

2.2.16 Analysis of differentiation (paper IV)

Differentiation of P39 cells was determined with nitroblue-tetrazolium test (NBT) after stimulation with phorbol-myristate-acetate. Briefly, 1x10⁶ cells were spun down at 240g for 5 minutes. Cells were resuspended in 20% FBS in RPMI pre-warmed to 37°C. The cell suspension was mixed with equal volume of NBT working solution (NBT, 1.5 mg/ml and phorbol-myristate-acetate 400 ng/ml in PBS) pre-warmed to 37°C, and incubated for 30 minutes. Cytospins preparation were air dried, fixed in methanol and counterstained with May Grünwald-Giemsa. The percentage of cell containing dark blue formazan deposits were counted in a total of 200 cells.
2.2.17 ATP measurements (paper IV)

ATP level was measured by a Berthold luminometer (Berthold, Bundoora, Australia) using the commercial available ATP bioluminescence assay kit CLS II (Roche, Bromma, Sweden), according to the manufactures instructions. Briefly, 1x10^6 cells were boiled in 100 μl buffer containing 100 MM Tris, 4 mM EDTA (pH 7.75) for 2 minutes. The samples were centrifuged at 1000 g and ATP level in the supernatants was measured by adding luciferase reagent. ATP concentrations were calculated from a log-log plot of standard curve data.
2.3 RESULTS

2.3.1 Paper I

In the first study we investigated and described the best and easiest way to freeze and store mononuclear bone marrow cells for later protein analysis by Western blot technique. The challenge was to prevent protein degradation. Storage of dry cell pellets in the freezer at -80°C was performed. Opposite to P39 and Jurkat cell line cells, storage in the freezer of MNC induced artificial cleavage of several of the investigated proteins (actin, Bcl-2, caspase-3 and -8). Cleavage of these proteins could be inhibited by the addition of broad-spectrum protease inhibitor (Complete mini, Roche, Bromma, Sweden) to the cells prior to freezing. Furthermore, thawing on ice prevented cleavage of certain proteins. It is reasonable to assume that a higher level of intrinsic proteolytic activity is responsible for the degradation of the apoptosis-related proteins in bone marrow cells.

The results of this study offer new insights that may prevent the misinterpretation of certain laboratory findings. When Western blot investigations were performed in the subsequent studies, addition of the protease inhibitor and thawing on ice were regularly used.

![Figure 2.1. Comparison of protein cleavage patterns in mononuclear bone marrow cells, the P39 cell line and the Jurkat T-cell line. ‘Fresh’ – freshly isolated cells were resuspended in Laemmli’s buffer and loaded onto the gel: (1) cells frozen at -80°C and thawed on ice; (2) cells frozen at -80°C and thawed at room temperature; (3) cells frozen with protease inhibitors at -80°C and thawed on ice; (4) cells frozen with protease inhibitors at -80°C and thawed at room temperature; (5) cells frozen in liquid nitrogen and thawed at room temperature; and (6) cells frozen in liquid nitrogen with protease inhibitors and thawed on ice.](image)

2.3.2 Paper II

In this study, the developed methods in combination with well-established approaches were used to investigate apoptosis in primary bone marrow cultures from RARS patients. The karyotype was normal in all patients but, one who had a 20q- aberration. When treated, patients received either transfusions or growth factor treatment with EPO with or without the addition of G-CSF.

Upon incubation in vitro, cells from patients showed increased spontaneous apoptosis as measured by nuclear apoptotic changes and phosphatidylserine exposure.
Additionally, Fas ligation caused significantly more apoptosis in patient cells compared to normal bone marrow cells. Treatment with antagonistic Fas antibodies did not prevent spontaneous apoptosis of these cells.

Caspase-3-like activity was measured by the cleavage of the substrate DEVD-AMC and the increase of free fluorescent AMC. Immediately after separation there was no significant difference in caspase-3-like activity between RARS and control MNC. After 4 and 24 hours of *in vitro* culture, RARS cells showed significantly more activity than controls. When additional apoptosis was triggered by Fas stimulation, caspase activity increased more in patient cells than in normal control bone marrow cells.

![Figure 2.2](image)

*Figure 2.2. Caspase-3-like activity in bone marrow cells from patients and donor: effects of Fas antibodies and caspase inhibition. Caspase-3-like activity in mononuclear bone marrow cells from patients with RARS (1-9) and healthy controls (c1-c6). The figure shows the effect of incubation with FCS (control culture), f(ab)’2 (Fas-antagonistic antibody fragments), CH-11 (Fas-agonistic antibody) and DEVD-fmk (caspase-3-like inhibitor). The figure shows caspase activation after 4 h of incubation.*

The expression of Fas receptor on RARS cells showed a heterogeneous pattern. Three transfusion-dependent patients showed a relatively high expression, while two patients with stable hemoglobin had lower Fas expression than controls. No clear increase of Fas receptor expression could be observed during short-time *in vitro* culture of MNC. Inhibition of the Fas death receptor pathway by Fas antagonistic antibody fragments did not affect spontaneous apoptosis of cultured RARS or control bone marrow cells. In some normal bone marrow cell cultures Fas ligation induced increased cell
proliferation, while this phenomenon was only observed in one patient culture. This patient also had severe rheumatoid arthritis and had just recovered from pneumonia. There was no correlation between Fas receptor expression and the increase of caspase activity after incubation with Fas agonistic antibodies. The expression of Bcl-2 was low in MNCs from patient and controls suggesting that the number of immature blasts in the MNC population was low.

The overall number of erythroid colonies in RARS patient cultures was significantly decreased (median value of 17 erythroid colony forming units (CFU-E) for RARS and 200 CFU-E for controls). A reduction was observed also in CFU-GM colony growth although 3 of 10 patients had normal white blood count. Fas stimulation reduced erythroid growth even further while Fas receptor blocking peptides did not have any significant influence on erythroid or myeloid colony growth. DEVD-fmk enhanced CFU-E for in four of the RARS cultures, while control cultures remained unaffected.

2.3.3 Paper III

Anemic RARS patients may respond to combined treatment with the growth factors G-CSF and EPO and achieve normalized hemoglobin levels and transfusion independency. G-CSF and EPO show synergistic effects in vivo. Thus, we focused on the anti-apoptotic effect of G-CSF with special interest in apoptosis-related mitochondrial changes. Nine RARS patients were included.

Two of the patients had cytogenetic aberrations (46, XX +22 and 46, XY, del 11 q). Co-culture of MNC from RARS patients with G-CSF significantly reduced caspase-8 and caspase-3 activities as well as the number of cells displaying nuclear changes characteristic for apoptosis. By Western blot, it could also be confirmed that processing of caspase-3 was blocked by G-CSF (Figure 2.3). At later time points G-CSF inhibited a decrease in the mitochondrial transmembrane potential and the generation of reactive oxygen species (ROS). G-CSF also enhanced the erythroid clonogenic capacity of
isolated bone marrow progenitor (CD 34+) cells, in particular in patients with severely decreased erythroid colony growth.

2.3.4 Paper IV

We have previously shown that G-CSF blocks ATRA-induced apoptosis in the MDS cell line P39 (Hassan et al., 1999). Using this cell line as a model, we focused on the molecular mechanisms behind the anti-apoptotic effects of G-CSF. Our results indicated that ATRA reduced mitochondrial respiration. Opposite to ATP levels, which were not changed by ATRA, mitochondrial calcium accumulation capacity was significantly decreased in ATRA-treated cells. This decrease in calcium uptake capacity was an early and upstream event and occurred long before any signs of apoptosis could be detected. Nifedipine as an L-type plasma membrane calcium channel blocker partially blocked ATRA-induced apoptosis suggesting that mitochondrial-caused imbalanced intracellular calcium homeostasis might trigger apoptosis in ATRA-treated cells. This hypothesis was furthermore supported by the finding that thapsigargin, which blocks ER uptake of calcium and thereby releases calcium ions from the ER, even enhanced the apoptotic effects of ATRA. G-CSF increased significantly mitochondrial respiration in ATRA-treated cells. By stabilizing mitochondrial respiration, it even increased mitochondrial calcium accumulation capacity.

In conclusion, both, ATRA and G-CSF, have a significant influence on respiration as well as mitochondrial calcium accumulation. Intracellular calcium deregulation might be a novel and significant mediator of ATRA-induced apoptosis. We believe that results obtained in this study are important for understanding the molecular mechanisms of G-CSF and ATRA action in more detail.
Figure 2.4. Proposed sequence of ATRA-mediated early and late mitochondrial changes. Early ATRA-induced alterations are characterized by decreased mitochondrial respiration and calcium uptake capacity. Late changes are characterized by the activation of Bcl-2 family proteins, such as Bid and Bax, followed by cytochrome c release from mitochondria. Finally, caspases are activated and apoptosis is executed. G-CSF and Nifedipine inhibit apoptosis, but their mode of action is different. G-CSF inhibits early mitochondrial changes, while Nifedipine influences calcium content in the cells.
2.4 DISCUSSION

2.4.1 Apoptosis in MDS

The involvement of apoptosis in the pathogenesis of MDS has been widely accepted. The vast majority of studies reveal a higher degree of apoptotic bone marrow cells from patients compared to control bone marrow. Interestingly, probably due to the use of different apoptosis markers as well as different handling of the bone marrow material, there is a substantial variety in the estimated number of apoptotic marrow cells between different studies (from a few percent to more than 75 percent) (for review: Parker & Mufti, 2004). Studies exploring MDS pathogenesis constitute a challenge. Firstly, there is a lack of reliable early disease markers; secondly MDS incorporates a spectrum of disorders with widely disparate biologies. Furthermore, MDS bone marrow samples show varying degrees of cellular differentiation as well as variable mixtures of normal and malignant progenitor cells (Asano et al., 1994). Although clinical symptoms and pathologic bone marrow morphology are quite similar within the different FAB subgroups of MDS, it has to be assumed that even in these subgroups different pathologic mechanisms might trigger similar disease pathways. Interestingly, the new WHO classification tries to acknowledge recent findings and as a result divides RA and RARS into groups with unilineage and multilineage dysplasia.

According to our studies (papers II, III), bone marrow precursor cells of MDS patients have a higher propensity to undergo apoptosis. After 4 days of in vitro culture, spontaneous apoptosis increases significantly more in the patient cultures than in control cultures. Caspase-3-like and -8 activities are slightly increased directly after cell separation. Co-culture with Fas-agonistic antibodies leads to significantly higher caspase activity in patient cultures compared to control cultures. The same pattern can be observed when phosphatidylserine exposure on the cell surface is investigated. Upon inhibition of caspase-3-like activity, an increase in CFU-E is detected in 4 out of 9 RARS patients.

Restricted to the MDS subtype RARS, it can be concluded that apoptosis regulation in RARS patient cells is significantly altered and that the patient cells have a higher propensity to undergo spontaneous as well as Fas-induced apoptosis. Since apoptosis is an important process in the regulation of erythropoiesis (Testa, 2004), it is most likely that this deregulation of cell death mechanisms in MDS is an important reason for anemia (and cytopenia) in RARS.

2.4.2 Involvement of the extrinsic pathway

As described above, the heterogeneity and lack of early disease markers make it difficult to study the mechanisms of, as well as reasons for cell death in MDS. Consequently, it is complicated to determine whether apoptosis affects also normal hematopoietic cells. Since studies of MDS bone marrow have revealed signs of increased apoptosis in bone marrow stromal cells (Aizawa et al., 1999; Raza et al., 1995), it might be assumed that apoptosis in MDS is triggered by external pathways and affects the abnormal cell clone as well as the normal hematopoietic cells and other surrounding cells such as stromal cells. Interestingly, the genetic abnormality of the cell clone in the 5q- syndrome gives the unique opportunity to follow a disease marker when cells are cultured in vitro. Recent results of our MDS research group indicate that EPO selectively promotes growth of cytogenetically normal cells in the 5q- syndrome.
when this cytokine was added \textit{in vitro}. This again suggests that normal \textit{in vivo} hematopoiesis might be suppressed by extrinsic mechanisms in these patients.

Since the extrinsic Fas signaling is a key regulating system in erythropoiesis (Testa, 2004), we investigated whether blocking of the Fas receptor could increase hematopoiesis in MDS (paper II). It had been suggested that overexpression of the receptor as well as its ligand might trigger apoptosis in MDS marrow cells. We observed a huge variation in FAS expression between patient samples, with no overall significant difference between RARS and normal samples. Fas receptor stimulation results in increased apoptosis in RARS cells compared to the control cultures, but Fas blocking antibody fragments could neither inhibit apoptosis, nor increase general proliferation or erythroid colony growth.

The Fas receptor is constitutively expressed on the surface of erythroblasts. Upregulation can be detected under differentiation towards mature erythroblasts. Thus, the heterogeneous expression pattern of Fas expression between individual patient samples may be explained by a block in differentiation at distinct and different maturation stage and must not be related to pathologic apoptotic mechanisms in MDS. In our study, three transfusion-dependent patients show upregulation of the Fas receptor, which might cause apoptosis of erythroid progenitor cells and subsequent anemia. Since the Fas ligand normally can only be detected in late phases of erythroid differentiation, the fate of late erythroblasts is dependent on the EPO as growth factor, which sufficiently blocks Fas-induced apoptosis in these cells (De Maria et al., 1999). The involvement of Fas in apoptosis of MDS cells has been intensively studied and discussed. While our results with the Fas antagonistic antibody fragment do not reveal any importance of Fas for spontaneous apoptosis in the patient cell cultures, other groups have reported upregulation of Fas and its ligand in MDS (Claessens et al., 2002; Fontenay-Roupie et al., 1999; Gersuk et al., 1998; Gersuk et al., 1996). Since our study focuses on RARS patients, it is difficult to compare the results with other studies, in which the number of RARS patients is often low, or elevated Fas expression is not related to one specific MDS subgroup. Furthermore, upregulation can not be shown in all patients although increased apoptosis can be seen in almost all low risk MDS patients.

Interestingly, Sloand and coworker show that Fas-mediated apoptosis is important for cells with trisomy 8 but not in MDS cells with other cytogenetic abnormalities (Sloand et al., 2002). The group by Dror et al. shows higher apoptosis, increased caspase-3 activation and upregulation of cytochrome c, Apaf-1 and Bax in a patient with RARS, whereas Fas and FADD expression was not altered compared to controls (Dror, 2003).

In conclusion, Fas and FasL interaction is an important regulatory element in erythropoiesis. Deregulation of this control mechanism might be one of the factors for elevated apoptosis in MDS, but our studies do not support a major role for this mechanisms in the erythroid apoptosis of RARS. Thus, it is reasonable to assume that apoptosis is not triggered exclusively by one single mechanism, and that RARS and RA may have quite different reasons for ineffective erythropoiesis. Recent studies from our group supports this conclusion (Tehranchi et al., 2003).

It has also been suggested that other cytokines or intrinsic cellular mechanisms might induce or regulate apoptosis in MDS. For example, elevated level of TRAIL has been found in MDS samples (Campion et al., 2005). In addition, Sawanobori et al.
observed increased expression of the TNF-α receptors TNFR-I and –II (Sawanobori et al., 2003) in RA patient bone marrow cells.

2.4.3 Involvement of the intrinsic (mitochondrial) pathway in the pathogenesis of RARS

RARS erythroblasts show iron accumulation in the mitochondrial matrix. An aberrant mitochondrial ferritin is almost exclusively expressed in RARS erythroblasts (Cazzola et al., 2003). Its expression increases during erythroid maturation (Tehranchi et al., 2005). Additionally, there is a positive correlation between spontaneous apoptosis in erythroblasts and the expression of mitochondrial ferritin. Therefore, mitochondria might trigger and regulate apoptosis in RARS erythroblasts and mitochondrial dysfunction might lead to the observed high degree of spontaneous apoptosis. Furthermore, defect mitochondria could amplify cell death receptor signals and thereby trigger an increased and pathologic response to Fas ligation as shown in papers II and III.

We have demonstrated that mitochondrial events such as loss of the mitochondrial membrane potential as well as generation of ROS are relatively late and secondary events, while activation of caspases occurred fast within a few hours after Fas ligation (paper III). However, using the erythroblast culture method, we observe evidence for spontaneous mitochondrial release of cytochrome c into the cytosol already in very immature erythroid progenitors and throughout maturation (Tehranchi et al., 2003). Furthermore, activity of caspase-9, an initiator caspase downstream of mitochondrial apoptotic signaling, is increased in RARS patient bone marrow cells. This suggests that mitochondria play an important regulative role in apoptosis in RARS.

Mutation of mitochondrial DNA has been proposed as a potential underlying cause for the pathological iron distribution in RARS (Broker et al., 1998; Gattermann, 2000; Gattermann et al., 1993; Hofhaus & Gattermann, 1999). These mutations could impair the electron chain and thereby lead to an inappropriate valence form (Fe^{2+}), which can not be inserted by ferrochelatase into the haem molecule. Recently, mt-DNA mutations were also described in other MDS subtypes (Gattermann et al., 2004). These changes could lead to a complete loss of the energy-dependent iron uptake of the erythroblasts and could explain the absence of ringed sideroblasts in other MDS subgroups. Furthermore, mitochondrial dysfunction could be the reason for genetic instability, thereby facilitating the transforming event as well as further chromosomal changes that initiate subclones and drive the clonal evolution of MDS towards leukemia.

Reddy et al. claim that there exists an increased incidence of mitochondrial cytochrome c-oxidase gene mutation in patients with MDS (Reddy et al., 2002). Shin and coworkers were not able to confirm these mutations in sideroblastic anemia. Their data do not support a major role for genomic instability in myelodysplasia (Shin et al., 2003).

In conclusion, expression of abnormal mitochondrial ferritin and iron accumulation in the mitochondrial matrix might lead to hypersensitization of RARS progenitor cells to apoptotic triggers like the Fas ligand and might explain the observed increased caspase activity as shown in papers II and III. Pathologically increased
apoptosis in RARS might be caused by enhanced mitochondrial amplification of apoptotic signals.

### 2.4.4 Anti-apoptotic effect of G-CSF in MDS

In papers III and IV we investigated the anti-apoptotic effects of the growth factor G-CSF, which, in combination with EPO, is used to treat anemia in a subset of MDS patients. Especially in low risk MDS patients, blocking of death signals by growth factors might be an efficient way to overcome anemia. The synergic effect of EPO and G-CSF is most pronounced in the RARS subgroup (Hellstrom-Lindberg et al., 2003). It has been shown that EPO induces the expression of Bel-XL expression in late erythroblasts. It is well known that Bel-XL has an anti-apoptotic function and decreases apoptosis at late stages of erythropoiesis (Gregory et al., 1999). Opposite to EPO, the anti-apoptotic molecular mechanisms of the G-CSF effects are still under intense investigation and are not well understood.

In paper III, we showed that G-CSF decreases caspase activation, the loss of the mitochondrial membrane potential and ROS production in bone marrow progenitor cells treated with Fas ligand. Rapid G-CSF effects in terms of significantly reduced caspase activity are observed within 4 hours of incubation. Furthermore, we showed that G-CSF inhibited mitochondrial cytochrome c release within 4 hours in erythroblasts cultures (Tehranchi et al., 2003).

Different mechanisms for this inhibitory effect of G-CSF have been suggested. Usually, the anti-apoptotic effects of G-CSF were assessed in neutrophils under in vitro culture conditions. Upregulation of anti-apoptotic Bcl-2 family members like A1, Mcl-1, Bcl-2 or the downregulation of Bax were demonstrated (Carlsson et al., 2004; Chuang et al., 1998; Dibbert et al., 1999; Hamasaki et al., 1998). Another group has reported an increase in cIAP2 expression after G-CSF incubation (Hasegawa et al., 2003).

In order to further explore and understand the molecular mechanisms of the anti-apoptotic effect of G-CSF, we used the myelodysplastic cell line P39 and induce apoptosis in this cell line with ATRA. Upon ATRA incubation, P39 cells underwent differentiation (within the first 2 days) followed by apoptosis (day 3 and day 4). Interestingly, G-CSF was able to block apoptosis without interfering with the differentiation process. Maturation induced by ATRA was linked to decreased mitochondrial respiration combined with a decreased mitochondrial calcium uptake capacity. Furthermore, we showed that increased intracellular calcium levels trigger apoptosis in ATRA-differentiated P39 cells. It has been known that increase in intracellular calcium enhances differentiation (Chapekar et al., 1987; Launay et al., 2003). Additional to the influence of calcium on differentiation, our results indicate that decreased mitochondrial calcium uptake is an important factor for ATRA-induced apoptosis.

Focusing on the anti-apoptotic mechanism of G-CSF in this cell line model, we observed enhanced mitochondrial respiration and calcium uptake capacity upon G-CSF treatment. These results indicate that the anti-apoptotic effects of G-CSF can be linked to mitochondrial functioning (increased oxidative phosphorylation and increased mitochondrial calcium accumulation capacity, Figure 2.5). To our knowledge, this novel mechanism of G-CSF action has not been previously described.
Treatment with EPO and G-CSF shows the best response rates in RARS patients, which have iron deposits in their mitochondria, and it could therefore be speculated that G-CSF also might increase mitochondrial functioning in this particular MDS subtype, and thereby decrease apoptosis. Interestingly, it has been shown that cytochrome $c$ oxidase activity as well as oligomycin-sensitive ATPase activity is lower in neutrophils derived from MDS patients compared to neutrophils derived from control persons. Citrate synthetase activity is similar in both groups indicating that total amount of mitochondria is not changed in the MDS patient group (Aoki, 1980).

Figure 2.5. Possible mechanisms of the anti-apoptotic effect of G-CSF
2.5 CONCLUSIONS

Based on our results, we can conclude:

- Apoptosis is an important feature in the pathogenesis of MDS. Mitochondrial iron overload in RARS might decrease functioning of this organelle and thereby trigger apoptosis.

- Our data, which are mainly based on RARS patient cells do not support involvement of the Fas system in the pathology of MDS. However, we can not exclude that Fas ligation is important for apoptosis in other subtypes of MDS.

- G-CSF shows anti-apoptotic effects in MDS \textit{in vitro} cell cultures. Similar anti-apoptotic effects are most likely to be responsible for the clinically observed response in patients treated with G-CSF in combination with EPO.

- G-CSF is able to influence mitochondrial functioning, which can explain its rapid anti-apoptotic effect. Increased respiration enhances the mitochondrial capacity to accumulate calcium. Since our results were obtained in a cell line, further studies with MDS patient bone marrow cells are required to investigate these processes in more detail.

2.6 FUTURE PERSPECTIVES

For future MDS research, it is essential to gain an improved understanding of the molecular mechanisms, which cause the described key features of MDS; block in differentiation, hyperproliferation, dysplasia and apoptosis. Up to date, the reasons for apoptosis of MDS progenitor cells are not well understood.

During the last years, two new drugs have been tested in clinical trials, 5-azacytidine and the structural thalidomide analog lenalidomide. Because of promising results in these clinical trials, the hypomethylating agent 5-azacytidine is the first drug to be approved by the FDA for the treatment of MDS. Interestingly, it might offer a new treatment option for high risk MDS, whose possibilities for effective treatment at present are relatively limited. Lenalidomide shows promising response rates especially in patients with the 5q- syndrome and new clinical trails are ongoing to validate the preliminary data. For both drugs, the detailed mechanism of action is not yet known.

Growth factor treatment with G-CSF and EPO in combination shows good response in approximately 50% of RARS patients, which demonstrate increased hemoglobin levels and abrogated need for red blood cell transfusions. But again, in detail it is still unknown how this treatment interferes with the pathologically altered erythropoiesis and why RARS patients show the best respond rates. Furthermore, it is unclear why - after a time of successful growth factor treatment - patients may become refractory to this treatment. And it remains to be elucidated whether growth factors are restoring polyclonality by promoting growth of normal cells or whether clonal cells, which are supposed to undergo apoptosis are recovered by this treatment.

Investigation of the mechanisms of action of these novel treatments for MDS offers the possibility to understand more about the regimens, their risk and their benefits. Furthermore, it might be possible to characterize a specific subgroup of
patients, which will benefit most. Additionally, learning about these drugs and their mechanism of action makes it possible to learn more about MDS in general.

In our cell line studies, G-CSF significantly increased mitochondrial functioning and the ability of mitochondria to buffer high cytosolic calcium concentrations. It remains to be clarified whether the same effect of G-CSF can be observed in MDS bone marrow cell cultures and whether mitochondrial control of calcium homeostasis is an important regulatory factor also in the control of erythroblast apoptosis. The erythroblast culture method can be used to observe whether G-CSF influences respiration and mitochondrial calcium content in erythropoietic progenitor cells.
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