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BONE MARROW APOPTOSIS IN MYELODYSPLASTIC SYNDROMES

Dissertation zur Erlangung des Doktorgrades der Medizin

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1. Abstract

1.1. Abstract in English

The bone marrow of patients with myelodysplastic syndromes (MDS) is characterised by an increased number of apoptotic precursor cells leading to ineffective haematopoiesis and cytopenia. In patients with refractory anaemia with ringed sideroblasts (RARS), apoptosis and ineffective erythropoiesis are probably the main mechanisms leading to severe anaemia, since these patients do not have any increase in bone marrow blasts and only a low risk for transformation to acute myeloid leukaemia. Increased Fas ligand/ receptor interaction may be one reason for the high grade of apoptosis in haematopoietic progenitors. Around 50% of the RARS patients respond well to treatment with growth factors, e.g. the combination of granulocyte colony stimulation factor (G-CSF) and erythropoietin (EPO). This treatment can induce long-lasting normalisation of haemoglobin levels and is accompanied by reduced bone marrow apoptosis.

In this work various aspects of apoptosis were studied using mononuclear bone marrow cells isolated from patients with RARS. Fas-agonistic antibodies were used to trigger apoptosis in these cells.

Results: Bone marrow mononuclear cells (MNC) from RARS patients cultured *in vitro* underwent increased spontaneous apoptosis and in addition displayed a higher sensitivity to triggering of apoptosis by Fas ligation. Antibodies antagonistic to Fas did not prevent the intensified spontaneous apoptosis of these cells. Compared to MNC isolated from normal controls, caspase-3 activity was elevated in *in vitro* cultures of RARS cells and could be reduced by co-culture with the caspase-3 inhibitor DEVD-fmk. Co-culture of MNC from RARS patients with G-CSF leaded to a reduction of caspase-8 and caspase-3 activity as well as to a reduction in the number of cells displaying nuclear changes characteristic for apoptosis.

Apoptosis-related mitochondrial changes, characterised by a decrease in the mitochondrial transmembrane potential ($\Delta\psi_m$) and the generation of reactive oxygen species (ROS), were late events compared to the early increase in caspase activity and could be prevented by G-CSF. Especially in patients with severely reduced erythroid colony growth, G-CSF can enhance the clonogenic capacity of isolated bone marrow progenitor (CD 34+) cells.

1.2. Kurzfassung in deutscher Sprache

Das Myelodysplastische Syndrom (MDS) umfasst eine Gruppe von Erkrankungen der hämatopoetischen Stammzellen mit Proliferations- und Differenzierungsstörungen der drei blutbildenden Zellsysteme. Die Apoptose hämatopoetischer Vorläuferzellen im Knochenmark mag eine Ursache für die ineffektive Hämatopoese von MDS Patienten sein und somit zu deren Anämie, Neutro- und Thrombozytopenie führen. Gesteigerte Fas-Rezeptor/ Ligand Interaktion wird als Ursache für die erhöhte Apoptose der Progenitorzellen im Knochenmark der MDS Patienten diskutiert. Als ein Maß für die gesteigerte Apoptose kann die Aktivierung von intrazellulären Proteasen, den Caspasen, angesehen werden. Die refraktäre Anämie mit Ringsideroblasten (RARS) ist definitionsgemäß eine MDS Untergruppe, die durch mehr als 15% Ringsiderblasten im Knochenmark gekennzeichnet ist. In klinischen Studien wurde deutlich, dass viele RARS Patienten auf die kombinierte Behandlung mit den Zytokinen G-CSF und Erythropoietin (EPO) mit einem Anstieg der Erythrozytenzahl und des Hämoglobins im peripheren Blut reagieren. Gleichzeitige histologische Untersuchungen nach erfolgreicher Behandlung zeigen eine verminderte Anzahl apoptotischer Knochenmarkszellen.

In dieser Doktorarbeit werden standardisierte Untersuchungstechniken der Apoptoseforschung eingesetzt, die an die Arbeit mit mononukleären Knochenmarkszellen (MNC) von RARS Patienten angepasst wurden. Die Apoptose wird *in vitro* durch Fas-agonistische Antikörper induziert.

Ergebnisse: Unter *in vitro* Kulturbedingungen ist die spontane Apoptoserate der MNC von RARS Patienten höher als die der MNC von gesunden Probanden. Desweiteren erhöhen agonistische Liganden des Fas-Rezeptors im stärkeren Maße die Apoptoserate von Patientenzellen als von Kontrollkulturen mit normalen Zellen. Bestätigt werden diese Resultate durch eine signifikant erhöhte intrazelluläre Caspase-3 Enzymaktivität bei Patienten. Diese kann durch die Zugabe des Caspase-3-Inhibitors DEVD-fmk zur Zellkultur gesenkt werden. Fas-antagonistische Antikörper hemmen dagegen nicht die erhöhte Caspaseaktivität und die damit verbundene Apoptose der Patientenzellen *in vitro.*

Der Wachstumsfaktor G-CSF kann die durch Fas-agonistische Antikörper induzierte Erhöhung der Caspase-8 und –3 Enzymaktivitäten abschwächen, wenn er simultan mit den Fas-agonistischen Antikörpern zu den Zellkulturen zugefügt wird. Gleichzeitig sinkt die Anzahl

apoptotischer Zellen. Mitochondriale Veränderungen, die durch den Zelltod hervorgerufen und durch den Verlust des mitochondrialen Transmembranpotentials ($\Delta\psi_m$) und die erhöhte intrazelluläre Konzentration von reaktiven Sauerstoffradikalen (ROS) charakterisiert werden, sind späte Ereignisse innerhalb des Ablaufs des programmierten Zelltods von myeloischen MNC der RARS Patienten und können wiederum durch G-CSF verhindert werden.

Knochenmarksprogenitorzellen der MDS Patienten, deren erythroides Koloniewachstum *in vitro* stark vermindert ist, zeigen bei der gleichzeitigen Kultur mit G-CSF einen Anstieg der erythrozytären Kolonien.

2. Abbreviations

$\Delta\psi_m$	mitochondrial transmembrane potential
5-aza	5-azacytidine
AA	aplastic anaemia
ADP	adenosin diphosphate
AIF	apoptosis inducing factor
AMC	aminomethyl coumarin
AML	acute myeloid leukaemia
APS	ammonium persulfate
ara-C	cytosine arabinoside
BFU	burst forming unit
CAPS	cylcohexylamino-propanesulfonic acid
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1- propane sulfonate
CFU	colony forming unit
CMML	chronic myelomonocytic leukemia
CMML-t	chronic myelomonocytic leukemia in transformation
CH-11	name of the cell clone producing Fas-agonistic antibodies
DEVD-AMC	Asp-Glu-Val-Asp aminomethyl coumarin
DEVD-fmk	Asp-Glu-Val-Asp fluoromethyl ketone
$DiOC_6(3)$	3,3´dihexyloxacarbocyanine iodide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dUTP	uridine triphosphate
EPO	erythropoietin
FAB	French-American-British
Fas-L	Fas ligand
Fas	Fas receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
G3PDH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GpA	glycophorin A
HE	dihydroethidium
IETD-AMC	Ile-Glu-Thr-Asp aminomethyl coumarin
INF-α	interferon-α
IPSS	International Prognostic Scoring System
MNC	mononuclear bone marrow cells
MDS	myelodysplastic syndromes
MPT	mitochondrial permeability transition
mt-DNA	mitochondrial DNA
NP-40	octylphenoxy polyethoxy ethanol
PARP	poly(ADP-ribose)polymerase

PBS	phosphate buffered saline
PI	propidium iodide
PKCδ	delta isoform of the protein kinase C
PSA	pure sideroblastic anaemia
RA	refractory anaemia
RAEB	refractory anaemia with excess of blasts
RAEB-t	refractory anaemia with excess of blasts in transformation
RARS	refractory anaemia with ringed sideroblasts
ROS	reactive oxygen species
SDS	sodium dodecyl sulphate
Smac	second mitochondria-derived activator of caspases
STS	staurosporine
TdT	terminal deoxynucleotidyl transferase
TEMED	tetramethylethylendiamine
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor receptor
TUNEL	TdT-mediated dUTP-biotin nick end labelling
Tween 20	polyoxyethylensorbitan monolaurat
VP 16	etoposide

3. Introduction

3.1. Apoptosis as a biological cell death mechanism

3.1.1. Apoptosis versus necrosis *(Table 3.1, Figure 3.1)*

There are at least two mechanisms of cell death: apoptosis and necrosis. Apoptosis can be the natural endpoint of the life span of a cell, or may be caused by an insult to the cell. The ”decision” of the cell to die by necrosis or apoptosis is thought to depend largely on the severity of the insult. Necrosis can be described as a passive process induced by extreme cell damage. Hallmarks for necrosis are nuclear swelling leading to karyolysis, loss of cytoplasmic structure, dysfunction and rupture of different cell organelles and finally cytolysis of the whole cell. Furthermore, the release of necrotic cell enzymes leads to a destruction and inflammation of surrounding cells.

Apoptosis is an active process, also described as cellular suicide and can be induced by a variety of different stimuli. Morphological hallmarks for apoptosis are nuclear pyknosis and fragmentation, membrane blebbing and cytoplasmic shrinkage. Finally, a phagocytic cell will *in vivo* engulf the apoptotic cell or rest of it, the so-called apoptotic body.

The apoptotic process does not affect the surrounding cells of the tissue, which is one reason for the difficulties to detect apoptotic cells in histological examinations. While necrosis can be detected by morphological alterations and disintegration of complete cell groups or a whole tissue leading to inflammation and scarring, apoptotic cells die without these changes in the surrounding tissue. This may furthermore explain the relatively late description of the apoptotic morphology by Kerr, Wyllie and Currie *(Kerr et al., 1972)*.

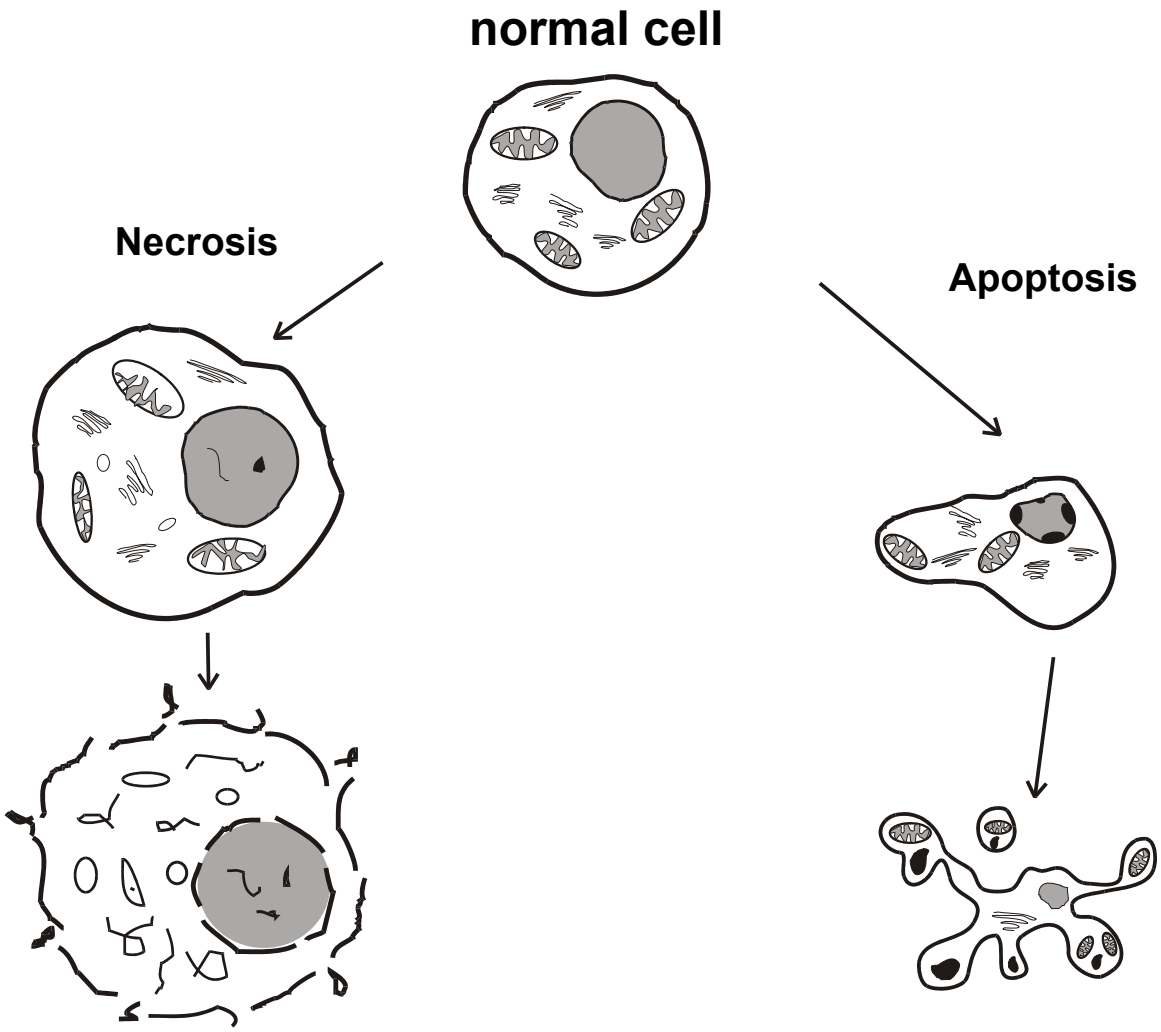


Figure 3.1. Apoptosis versus necrosis

A	Apoptosis	Necrosis
1. Nuclei	Dense condensation and fragmentation	Swelling of the nucleus, unorganised lysis of DNA
2. Cytosolic organelles	Intact (generally)	Disrupted
3. Plasma membrane	Blebbing; maintained integrity	Blebbing, breakdown and lysis
4. Cell volume	Shrinkage	Swelling
5. Inflammatory response	No	Yes

B	Apoptosis	Necrosis
1. Nuclei	Organised, 50- 300 kb and smaller ~180 bp fragments	Unorganised, random DNA degradation
2. Mitochondria	Specific release of intermembrane space proteins	Disruption
3. Enzyme Activation	DNases and proteases	Lysosomal enzyme release
4. Plasma membrane	Phosphatidylserine externalisation	Lysis

Table 3.1. (A) Morphological and (B) biochemical differences between apoptosis and necrosis

3.1.2. The apoptotic pathway *(Figure 3.2)*

Apoptosis proceeds through several phases, including the initiation and the execution phase. The initiation phase may be induced by a variety of stimuli having different intracellular compartments as targets. It is assumed that alternative pathways in the initiation phase sooner or later lead to one common execution pathway. This model explains why late apoptotic cells can display a uniform morphology and similar biochemical hallmarks, albeit the fact that the apoptotic death can be induced by a variety of different stimuli. Among them, death ligands are

described that trigger apoptosis by binding to specific death receptors on the cell surface, such as the Fas receptor (Fas, CD95, APO-1) and the TNF receptors TNF-RI and TNF-RII with their corresponding ligands Fas ligand (Fas-L) and TNF-α *(Krammer et al., 1999).* Fas and TNF receptors belong to the tumour necrosis factor receptor (TNF) superfamily. Other stimuli, such as radiation or cytostatic drugs may target the nucleus (deoxyribonucleic acid (DNA)), mitochondria or cytoplasmatic proteins. Reactive oxygen species (ROS) produced by disturbances of the mitochondrial function or externally added to the cells (hydrogen peroxide) are other examples of inducers of apoptosis. At higher concentrations they can also induce necrosis.

Recently, an important function for mitochondria in the apoptotic pathway has been discovered (for review*: Mignotte et al., 1998).* Although the integrity of mitochondrial morphology and function was assumed to be a hallmark of apoptosis in contrast to necrosis, it was recently shown that mitochondria have a central executioner and/ or regulator function in cells undergoing apoptosis. A drop in the mitochondrial transmembrane potential ($\Delta\psi_m$) and the release of up to 10 different apoptogenic proteins, including cytochrome *c,* second mitochondria-derived activator of caspases (Smac), and apoptosis-inducing factor (AIF) from mitochondria into the cytosol, have been described in cells during apoptosis. Subsequent disruption of the electron transport chain leads to an increased concentration of ROS within the cell (for review: *Kroemer et al., 1997*). The exact mechanism and time order of this mitochondrial protein release and the drop of the transmembrane potential is still a matter of controversy. One model proposes that the mitochondrial permeability transition (MPT) pore localised in the inner mitochondrial membrane plays an important role in this process (for review: *Crompton, 1999).* An opening of this pore during apoptotic cell death leads to swelling of the mitochondrial matrix and drop of potential by depolarisation of the inner membrane. Because the surface area of the highly folded inner membrane is much greater than that of the outer membrane, swelling can lead to rupture of the outer membrane followed by the release of intermembrane space proteins into the cytosol.

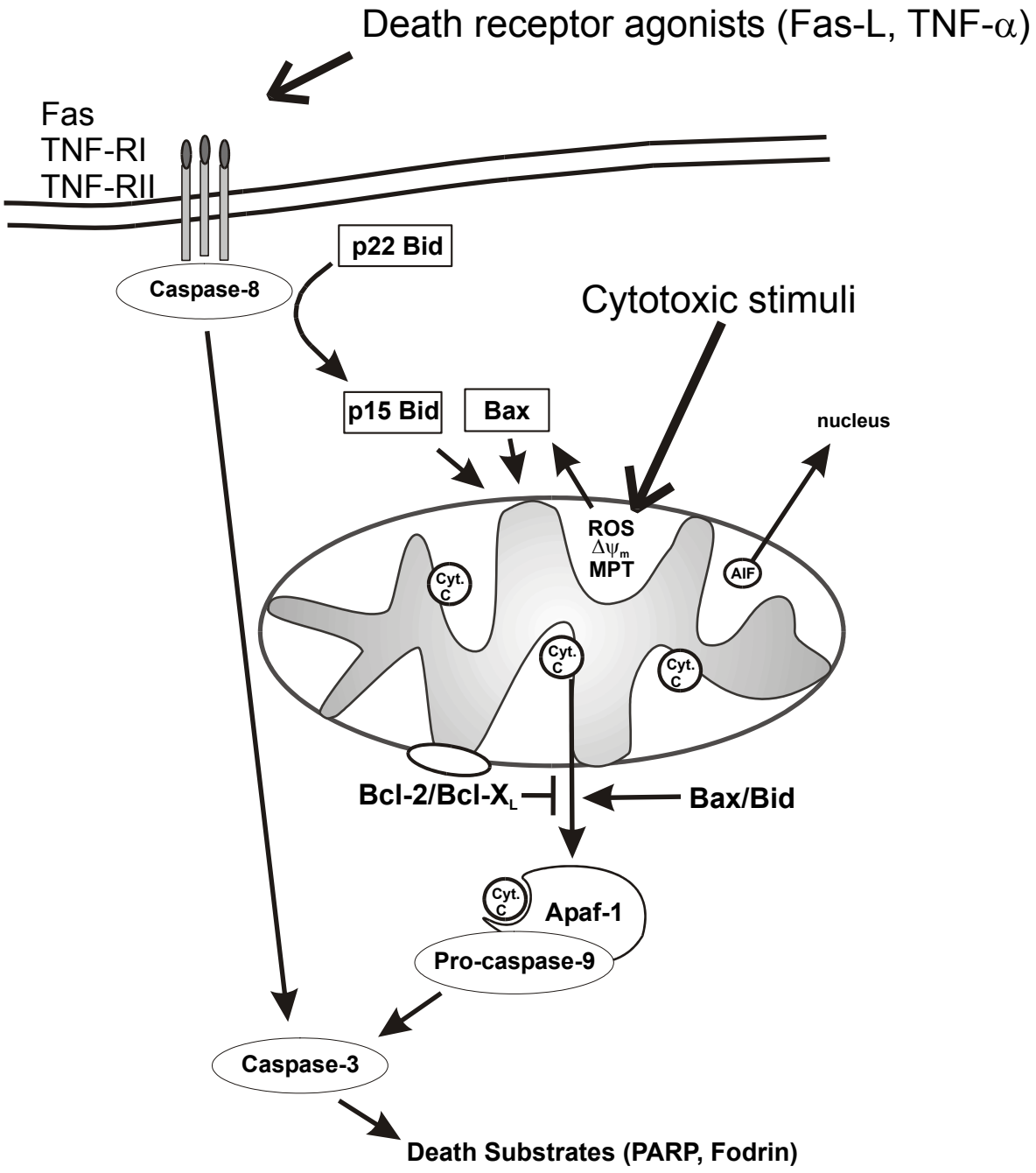


Figure 3.2. The apoptotic pathway inside a cell. *The caspase activation cascade and the influence of some Bcl-2 family members are shown.*

3.1.3. Caspases – proteases with important role in apoptosis *(Figure 3.2, 3.3)*

Caspases (**c**ysteine proteases that cleave after **asp**artic acid) are a group of proteases that are expressed as inactive proenzymes in the cells. Cleavage of the proform is followed by formation of tetramer, which consists of 2 small and 2 large subunits. Only this tetramer represents the active enzyme. The activation of caspases comprises an intracellular cascade of reactions that can be compared with the haemostasis system (*Figure 3.2)*. An activated caspase can autocatalytically activate itself or other caspases. So far, 14 different caspases are known and can be divided into several groups based on their function in the cell (for review: *Cohen, 1997* and *Fadeel et al., 2000*).

Although overexpression of all 14 caspases resulted in cell death, not all of them are involved in apoptosis. Thus, caspases-1, -4, -5, -13, and -14 (group 1) have little or even unknown roles on the apoptotic pathway. Many of them involve in the maturation and production for inflammatory cytokines.

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

Caspases-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 unit 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).

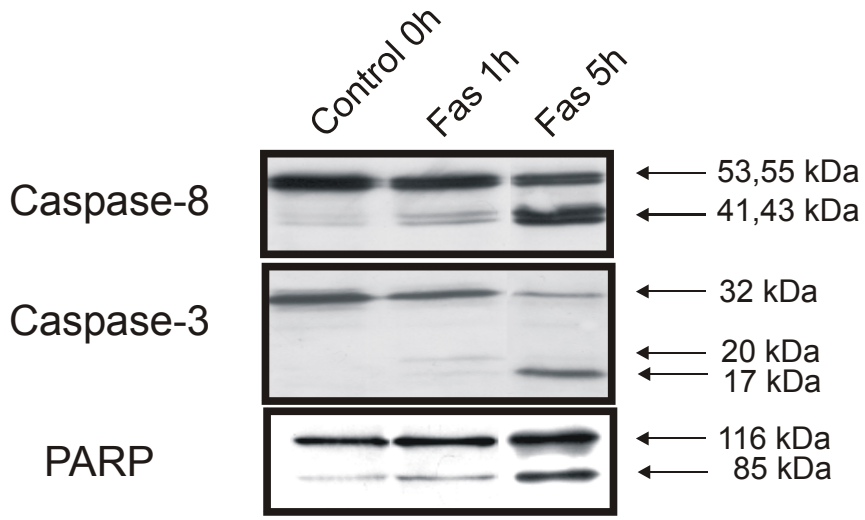


Figure 3.3. Western blot detection of the proform as well as the active cleavage product of caspase-8 and -3 upon apoptosis induction by Fas agonistic antibodies in Jurkat T-cells. The death substrate PARP is cleaved by active caspases.

3.1.4. The Bcl-2 family *(Figure3.2)*

There is a long list of intracellular proteins that have been assigned a pro- (e.g. c-myc, p53, Bax, Bid) or antiapoptotic (e.g. Bcl-2, Bcl-X_L) function. The Bcl-2 protein family consists of proteins with the ability to modify the apoptotic pathway in different ways (acceleration or inhibition) (for review*: Fadeel et al., 1999)*. The ability of these proteins to form homo- and heterodimers relates to their different functions during apoptosis. The ratio of anti- versus pro-apoptotic proteins may determine a cell´s susceptibility to death signals *(Fadeel et al., 1999)*. The best studied anti-apoptotic proteins are Bcl-2 and Bcl-X_L that in different experimental models can block apoptosis by interference on the mitochondrial level. Although both proteins are important for cell survival, their function is cell specific. Thus, Bcl-X_L is important for the survival of erythroblasts in the bone marrow and can be regulated by the renal growth hormone erythropoietin (EPO) *(Gregory et al., 1999)*. The proapoptotic proteins Bid and Bax of the Bcl-2 family have been shown to induce apoptosis also by interacting with the mitochondrial membrane.

3.1.5. The physiological functions of apoptosis

The first references to a type of cell death distinct from necrosis came from developmental studies. Apoptosis plays important role for normal cell turnover during embryogenesis and in adult tissues (for review: *Vaux et al., 1999)*. Furthermore, apoptosis is used as a defence strategy to remove infected (by viruses, bacteria), mutated (tumour) or damaged cells. For example, early death of the host cell is an ideal way to limit viral replication and spread. Auto-reactive lymphocytes are killed by Fas ligation.

3.1.6. Apoptosis in disease *(Figure 3.4)*

Either too little or too much apoptosis has been identified in many human disorders leading to proliferative or degenerative diseases, respectively (for review: *Fadeel et al., 1999)*. Dysregulation of apoptosis can be implicated in autoimmune diseases, viral and bacterial infections, neurodegenerative disorders and cancer. Tumour cells are often resistant to apoptotic stimuli and may express higher amounts of anti-apoptotic proteins from the Bcl-2 family. Replication of a virus depends on the viability of the infected host cell. Therefore, there are many of viral proteins, which inhibit different steps of the apoptotic cascade. These proteins include inhibitors of p53, homologues to the antiapoptotic Bcl-2 family, as well as direct inhibitors of activated caspases.

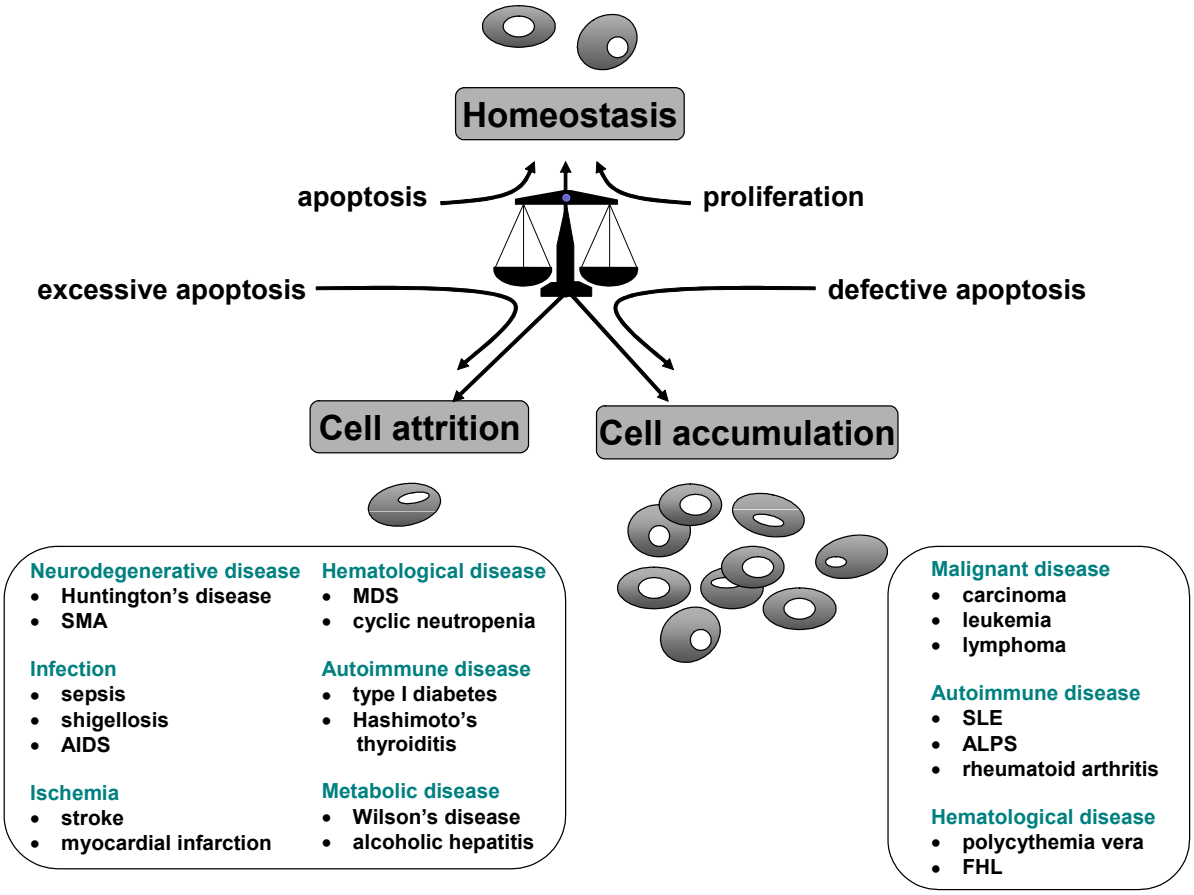


Figure 3.4. Apoptosis in human disease*. Some examples of degenerative and proliferative diseases in which dysregulation of apoptosis is believed to play a role are listed. Specific mutations in apoptosis genes have been identified in a number of these pathologies. ALPS, autoimmune lymphoproliferative syndrome; FHL, familial hemophagocytic lymphohistiocytosis; MDS, myelodysplastic syndromes; SLE, systemic lupus erythematosus; SMA, spinal muscular atrophy (after Robertson et al., 2002)*

3.2. The Myelodysplastic syndromes (MDS)

3.2.1. Clinical features

The myelodysplastic syndromes constitute a group of clonal stem cell disorders characterised by ineffective haematopoiesis and a high risk (approximately one third of all patients) for evolution to acute myeloid leukaemia (AML).

The incidence of MDS is 3- 4/ 100 000/ year, increasing with age and with a median age of around 70 years. Death usually occurs secondary to the complications of marrow failure (infections or bleeding) or progression to acute leukaemia. The typical clinical symptoms of MDS are anaemia or pancytopenia *(Hellström-Lindberg et al., 1999)*. Blood transfusions are frequently needed, which may lead to secondary haemochromatosis.

3.2.2. Classification

MDS can be divided into several subgroups. Depending on the number of blasts in the bone marrow and blood, the number of monocytes in the blood, and the presence or absence of ringed sideroblasts in the bone marrow, the French-American-British (FAB) classification distinguishes five subgroups of MDS: refractory anaemia (RA), RA with ringed sideroblasts (RARS), RA with excess of blasts (RAEB), chronic myelomonocytic leukaemia (CMML) and RAEB in transformation (RAEB-t) *(Table 3.2)*.

FAB subtype	Blasts in the Bone marrow	Blasts in the Blood	>15% ring sideroblasts In the bone marrow	Monocytes >1000/mm^3
MDS 1, RA	< 5%	<1%	-	-
MDS 2, RARS	<5%	<1%	+	-
MDS 3, RAEB	5-20%	<5%	+/-	-
MDS 4, CMML	<20%	<5%	+/-	+
MDS 5, RAEB-t	21-30%	>5%	+/-	+/-

Table 3.2. FAB classification of MDS

Recently, a new classification *(Table 3.3)* has been published by a working group of the World Health Organisation (WHO), defining the 5q- syndrome as separate entity.

Category	Peripheral Blood	Bone Marrow
1a. RA without dysplasia	Blasts < 1%; monocytes < 1000 /mm^3	Blasts < 5%; ringed sideroblasts < 15%
1b. RA with dysplasia	Same + dysgranulocytes and/ or giant platelets	Same + dysgranulocytes and/ or dysmegarkaryocytes
2a. RARS without dysplasia	Blasts < 1%; monocytes < 1000 /mm^3	Blasts < 5%; ringed sideroblasts ≥ 15%
2b. RARS with dysplasia	Same + dysgranulocytes and/ or giant platelets	Same + dysgranulocytes and/ or dysmegarkaryocytes
3a. RAEB-I	Blasts 1-5%; monocytes < 1000 /mm^3	Blasts 5-10%
3b. RAEB-II	Blasts 6-20%; monocytes< 1000/ mm^3	Blasts 11-20%
4.CMML *	Blasts < 1-20%; monocytes > 1000/ mm^3	Blasts 0-20%

Table 3.3. The new WHO classification *(Jaffe et al., 2001)*
** List under other FAB subtypes when white blood cell count < 13 000/ mm^3; otherwise list under myeloproliferative disorders (chronic myeloid leukaemias)*

The International Prognostic Scoring System (IPSS) has been established as a method to estimate prognosis of MDS patients *(Greenberg et al., 1997).* It incorporates variables with a prognostic value for disease outcome, i. e. cytogenetic aberrations, the percentage of bone marrow blasts, and number of cytopenias involved *(Table 3.4)*.

Parameter	POINTS				
	0	**0.5**	**1**	**1.5**	**2**
% blasts in bone marrow	0-4	5-10	-	11-20	21-29
Number of cytopenias[1]	0-1	2-3	-	-	-
Cytogenetic risk group[2]	Low	Intermediate	High	-	-

Risk group	Overall Score	Median Survival
Low risk	0	5.7 years
Intermediate I risk	0.5-1	3.5 years
Intermediate II risk	1.5-2	1.2 years
High risk	>2.5	0.4 years

Table 3.4. International prognostic scoring system (IPSS) for myelodysplastic syndromes.
[1]Platelets <100.000/ µl, Hb<10 g/ dl, neutrophils<1500/ µl;
[2]Low risk: normal, 5q-, 20q-, -Y; High risk: complex aberrations (≥ 3 anomalies), chromosome 7 aberrations; Intermediate risk: all other aberrations

Although all subtypes of MDS show dysplastic changes and ineffective haematopoiesis, it is most likely that these subtypes have partly different pathological mechanisms. This is further supported by huge differences in prognosis and response to treatment. RAEB and RAEB-t have a high risk for transformation rate to AML and are therefore considered to be ”high risk” subtypes compared to RA and RARS, which are considered to be ”low risk” subtypes. Patients often progress from ”low risk” or ”early” into the ”high risk” or ”late” MDS subtypes. The reason for this shift is basically unknown.

3.2.3. Bone marrow morphology

The bone marrow is characterised by dysplastic alterations *(Table 3.5)* in one, two, or three haematopoietic lineages. Bone marrow is often hyperplastic, but normo- and even hypoplasia may occur. Several studies have demonstrated an increased number of apoptotic bone marrow precursors (for review: *Parker et al., 2000)*.

	Erythropoiesis	Granulopoiesis	Megakaryopoiesis
Morphology	Polynuclear blasts Ringsideroblasts Megaloblastic maturation	Hypersegmentation Hyposegmentation Hypogranulation (Pseudo-Pelger cells)	Hypersegmentation Hyposegmentation

Table 3.5. Dysplastic changes in the bone marrow from MDS patients

3.2.4. Pathogenesis

There are two main MDS features: clonal evolution and apoptosis of bone marrow progenitor cells. The genetic damage originates in the haematopoietic stem cell compartment. Involvement of more than one clone is suggested in a study by Schmetzer *et al.* using a combination of cytogenetic and Southern blot techniques *(Schmetzer et al., 2000).* The damage may occur stepwise and may be related to genes controlling proliferation and maturation of the cells. Some models propose genetic damages in the mitochondrial DNA (mt-DNA) *(Gattermann et al., 1997* and for review: *Gattermann, 1999).* Alterations of the immune system may also be a reason for the cytopenias seen in MDS.

3.2.5. Apoptosis and MDS

MDS patients show pancytopenia in spite of high or normal proliferation in the bone marrow. Recent studies show excessive apoptosis of the progenitor cells in the bone marrow as an explanation for this discrepancy *(Raza et al., 1995* and *Hellström-Lindberg et al., 1997),* but the exact mechanism for the high grade of apoptosis still remains unclear. As mentioned above, immunological mechanism may play a role and immunosuppressive therapy has successfully used to treat certain patients with RA *(Molldrem et al., 1997).* Changes in the bone marrow microenvironment leading to high levels of TNF-α and Interferon-γ (INF-γ) could be another reasons for apoptosis *(Mundle et al., 1999)*, since these cytokines can induce the expression of

Fas on bone marrow stem (CD34+ cells) and progenitor cells, which usually do not express this receptor. Transforming growth factor beta and interleukin-1 beta converting enzyme, which are both elevated in MDS patients (*Mundle et al., 1998)* may exert a dual effect by stimulating proliferation of the early CD 34+ MDS progenitors while inducing apoptosis in their progeny. Unstable mitochondrial DNA and disturbances of the respiratory chain with higher levels of ROS may be still other mechanisms that could facilitate spontaneous apoptotic cell death in RARS patients *(Gattermann et al., 1997)*. Progression to AML with decrease in apoptosis and maturation but increase in proliferation might be caused by alterations in the ratio between anti-apoptotic versus pro-apoptotic proteins *(Parker et al., 2000* and *Parker et al, 1998).* Dysregulation of tumour suppressor genes, defective DNA repair enzymes, and immunological abnormalities have also been suggested as factors triggering loss of differentiation and progression towards acute leukaemia.

3.2.6. RARS

This subtype represents approximately 15% of all MDS patients (*Greenberg et al, 1997).* An analysis of 553 primary MDS and 31 secondary MDS cases published by Aul and co-workers reported a frequency of 25% RARS among primary MDS, and 13% of secondary cases (for review: *Aul et al., 1995* and *Aul et al., 1992).* Using new data of their MDS registry, the same group classified 20% (328) out of 1600 MDS patients as belonging to the RARS subtype (*Germing et al., 2000).* According to the definition of RARS, more than 15% of all affected erythroblasts show amorphous iron deposits in their mitochondria, which can be visualised with specific dyes (Berlin blue). In bone marrow smears these mitochondria localise around the nucleus in a ring-like manner, thus giving the name to this MDS subtype. Clustering of mitochondria around the nucleus has also been described in TNF-α induced apoptosis *(De Vos et al., 1998)* and could therefore be a sign for an apoptotic process in these erythroblasts.

The main clinical problem of patients with RARS is severe and often transfusion-dependent anaemia leading to secondary haemochromatosis. In comparison to other subtypes, RARS patients have a longer median survival (29- 71 months) and a lower risk for transformation into acute leukaemia.

Germing *et al. (Germing et al., 2000)* divide the RARS group further into two subtypes based on cytomorphological differences, i.e. into cases with pure (dyserythropoietic) sideroblastic anaemia (PSA) and a true myelodysplastic form (RARS), which is characterised by additional dysplastic changes of granulopoiesis and/ or megakaryopoiesis. They showed that these two types differ considerably in terms of survival and risk to transformation into AML. The same distinction has been made in the proposal for the new WHO classification of neoplastic diseases of the haematopoietic system (*Table 3.3),* (*Jaffe et al., 2001).*

Mutations of mitochondrial DNA has been propose as a potential underlying cause for the pathological iron distribution in RARS. These mutations could impair the electron chain and thereby lead to an inappropriate valence form (Fe^{2+}) which cannot be inserted into the haeme molecule by ferrochelatase *(Gattermann et al., 1997)*. Recently, mt-DNA mutations have also been described in other MDS subtypes (*Gattermann, 1999*). These changes could lead to a complete loss of the energy-dependent iron uptake of the erythroblasts and could explain the absence of ring sideroblasts in these 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 leukaemia.

However, it remains unclear which mechanism is responsible for the mitochondrial iron overload and which functional disturbances are caused by it. There may be a link between the iron overload and the high propensity of the patients´ bone marrow cells to undergo apoptosis.

3.2.7. Treatment of MDS

There exists no common treatment strategy in MDS, but a variety of different treatments are used for the different subcategories *(Figure 3.5).* Classification of yet unclear underlying pathogenic mechanisms may help to better define treatment approaches that improve quality of life and prolong overall survival. Oligoclonality and clonal shifts could provide an explanation for the observed variable treatment responses (for review: *Cazzola et al*., *1998* and *Kouides et al., 1999* and *Hellström-Lindberg, 1999).*

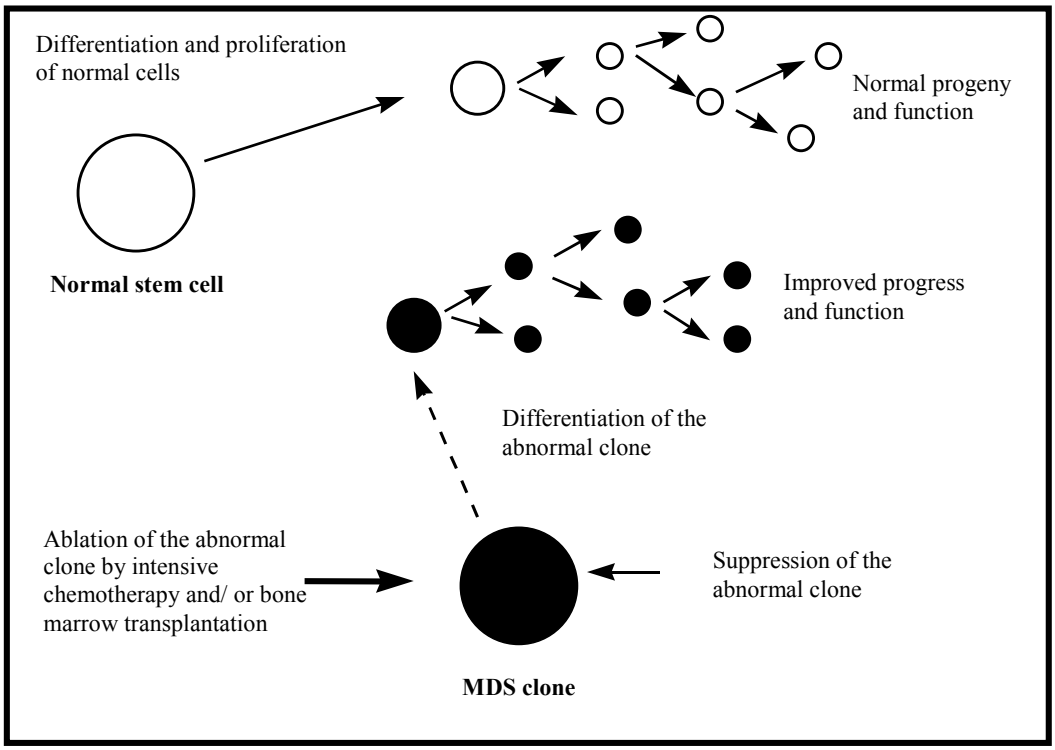


Figure 3.5. Different treatment approaches in MDS *(modified after Kouides et al., 1999)*

Factors that must be considered before selecting a successful and sufficient treatment are: **a)** age of the patient, **b)** MDS subtype and estimated risk for leukaemic progression to AML, **c)** degree of ineffective haematopoiesis and transfusion need, **d)** signs of immune-mediated myelosuppression (for review: *Hellström-Lindberg, 1999).* Current treatment approaches can be divided into the following groups:

1. Supportive therapy with transfusions and antibiotic treatment

This is a treatment for the major clinical symptoms of MDS, i. e. anaemia, thrombocytopenia and infections induced by neutropenia. Haematopoiesis is not manipulated. Iron chelation therapy using desferrioxamine is needed in many patients.

2. *Stem cell transplantation*

Currently, the only treatment that can cure MDS patients is allogenic stem cell transplantation. The treatment outcome is dependent on the selection of patients. Variables that influence the outcome are age, morphology, and cytogenetics. Often, high age of the patient will be the major barrier for this treatment. Autologous stem cell transplantation may produce long-lasting remissions in selected patients, but whether this is a true curative approach remains to be seen *(de Witte T, 2001).*

3. Conventional high-dose chemotherapy

High-dose chemotherapy aims to eradicate the myelodysplastic clone and to achieve a polyclonal haematopoiesis. The vast majority of patients, however, relapses and cure is rare. 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.

4. Low-dose chemotherapy

Low dose chemotherapy with cytosine arabinosid (ara-C) as an non-curative treatment may improve peripheral blood values and reduce blast counts, but randomised studies failed to show a difference in overall survival between treated patients and patients receiving supportive care only *(Miller et al., 1992)*. Other drugs used in low concentrations are Melphalan and the hypomethylating agents 5-azacytidine and decitabine (5-aza-2'-deoxycytidine). 5-Azacytidine significantly delayed the transformation towards leukaemia *(Silvermann et al., 1998)*. Repeated courses of low-dose decitabine induce cytogenetic remissions in a substantial number of elderly MDS patients with pre-existing chromosomal abnormalties. Patients with 'high-risk' chromosomal abnormalities may particularly benefit from this treatment *(Lubbert et al., 2001* and *Wijermans et al., 1997)*. Low-dose chemotherapy may be an effective and cheap treatment and does not cause side effects such as nausea and alopecia and has a moderate treatment-related

mortality, provided patients are selected according predictive models for this treatment (*Hellström-Lindberg et al., 1992 and Hellström-Lindberg et al., 1994*). Suggested functional mechanisms behind positive treatment responders are induced differentiation, but mainly cytotoxic drug effects.

5. Growth-factor treatment

EPO may improve anaemia in MDS, but patients with RARS respond significantly less to treatment than other low-risk MDS subgroups as shown in a meta-analysis *(Hellström-Lindberg, 1995)*. In RARS, the combination of granulocyte colony stimulation-factor (G-CSF) and EPO-treatment may improve haemoglobin levels and decrease the transfusion need without causing major side effects *(Negrin et al., 1996; Hellström-Lindberg, 1997* and *Hellström-Lindberg, 1998)*. The Scandinavian-American response score *(Hellström-Lindberg et al., 1997)* might be used as a tool to select patient groups with probability to respond, and has been confirmed by another independent group *(Remacha et al., 1999)*. High costs of the therapy are the major disadvantage. The studies mentioned above suggest that EPO and G-CSF in combination have synergistical effect on erythropoiesis, especially in RARS patients. 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.

6. Immunosuppressive treatment

Immunosuppressive treatment with cyclosporine *(Jonasova et al., 1998 and Catalano et al., 2000)* and antithymocyte globulin (*Molldrem et al., 1997*) shows the best response in hypoplastic RA, which suggests similar pathogenic mechanisms for RA and aplastic anaemia (AA). Additionally, in both diseases the number of CD34+ stem cells is reduced *(Fuchigami et al., 2000)*. RARS patients do not seem to respond to immunosuppressive therapy.

4. Aims of the study

The importance of apoptosis in MDS is known, but the initiating events and the apoptotic mechanisms are unclear. In this research project, methods used to study apoptosis in cell lines had to be adapted for the work with mononuclear bone marrow cells (MNC) from patients with RARS. These methods included Western blot analysis and caspase enzyme assays. The restriction to only one subtype of MDS was based on the idea that cell populations from a relatively homogenous patient group might provide more significant results and thereby allow us to draw more specific conclusions. A distinct common feature of RARS is the typical pathological iron deposits in the mitochondria.

The experiments were based on the following questions:

- What are the optimal methods to store bone marrow cells for Western blot investigations?
- Which functional influence has Fas-L/ Fas interaction on apoptosis in RARS bone marrow?
- Which enzymes in particular (caspases) are activated in RARS MNC during apoptotic cell death? Does the inhibition of caspase activity affect cell proliferation and survival? Do alterations in the expression of intracellular pro- or anti-apoptotic proteins play a role in the pathophysiology of RARS?
- Could changes within the mitochondria be responsible for the high rate of apoptosis in MNC of RARS patients? What are the relations between mitochondrial iron overload and increased progenitor cell apoptosis in RARS?
- How does G-CSF improve the erythroid growth in RARS bone marrow? Does G-CSF have anti-apoptotic effects?

5. Materials and Methods

5.1. Patients and controls

Thirteen patients with RARS with a mean age of 74 years were included in this study *(Table 5.1).* The project was initiated before the new WHO classification was published. Bone marrow morphology was scored accordingly the FAB classification at the beginning of the study. Bone marrow from two patients was examined twice, i. e. before and after 20 weeks from start of treatment with G-CSF + EPO. Normal bone marrow samples were obtained from patients undergoing thoracic surgery and from healthy volunteers. Informed consent was obtained from both patients and controls, and the study followed the guidelines of the local investigation review board of the Karolinska Institutet.

Patient No	Age (years/ sex)	Duration Of disease (month)	Blood values			Karyotype	Ongoing treatment
			Hb value (g/ L)	WBC (per nL)	Plt count (per nL)		
1	85/ F	12	92	5.7	252	46, XX	Transfusions
2a	81/ F	2	112	5.5	268	46, XX	Transfusions
2b			130	14.3	363		G-CSF + EPO
3a	82/ F	6	95	6.2	336	46, XX	Transfusions
3b			125	24.6	356		G-CSF + EPO
4	62/ F	18	100	6.9	298	46, XX	No treatment
5	85/ F	54	92	5.5	421	46, XX, 20q-	Transfusions
6	59/ M	88	83	1.6	103	46, XY	Transfusions
7	74/ M	154	93	4.7	307	46, XY	No treatment
8	81/ M	64	110	4.4	218	46, XY	No treatment
9	47/ F	4	85	5.2	263	46, XX	Transfusions
10	75/ F	4	76	7.2	166	46, XX	Transfusions
11	79/ M	48	102	6.4	893	46, XY	Hydroxyurea
12	82/ F	24	84	3.8	212	46, XX,+22	Transfusions
13	72/ M	17	109	5.4	212	46, XY, del11q	No treatment

Table 5.1. Clinical characterisation of the RARS patients at the time of all sampling.
Transfusions = regular transfusions of packed red blood cells. Patient 11 has hydroxyurea treatment because of high platelet counts

5.2. Bone marrow samples and suspension cultures

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 5x 10^5 cells/ ml in 25 ml tissue culture flasks (TPP, Trasadingen, Switzerland) and incubated at 37°C and 5% CO_2 in air for various time periods (4 hours, 24 hours, and 48 hours). MNC were cultured in the presence of FCS alone (control culture) or with different agents, e. g, the Fas–antagonistic antibody f(ab)´2 (1 μg/ ml, kindly provided by P. H. Krammer, German Cancer Research Center, Heidelberg, Germany), the Fas-agonistic antibody CH-11 (1 μg/ ml, Medical Biological Laboratories Co. Ltd., Nagoya, Japan) the caspase-3 peptide inhibitor DEVD-fmk (10 μM, Enzyme Systems Products, Livermore, USA), the growth factor G-CSF (Neupogen, 100 ng/ ml, Amgen, Stockholm, Sweden), the cytotoxic drug etoposide (VP 16, 10 μg/ ml, Bristol Myers, Bromma, Sweden) and/ or the antibiotic staurosporine (1 μM STS, Sigma, Stockholm, Sweden).

5.3. CD34 and Glycophorin A (GpA) separation

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 glycophorin A (GpA) magnetic-labelled antibodies (20 μl/ $1x10^7$ 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.

5.4. CD 34 colony assay

Aliquots of CD 34+ cells were incubated in medium RPM 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, rh stem cell factor, rh GM-CSF, rh IL-3, and rh EPO; StemCell Technologies Inc., Vancouver, Canada) and Falcon petri dishes 10008, 35x10mm (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.

5.5. Cell lines and their cultivation

Two cell lines were used as control in this study. The P39 cell line/Tsugane is a myelomonocytic cell line derived from the peripheral blood of a patient suffering from leukaemia 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). The cells were grown in RPMI 1640 (GIBCO BRL) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin and streptomycin (1% in the medium) at 37°C in a humidified atmosphere containing 5% CO_2. In exponential growth phase, cells were used for the experiments.

5.6. Western blot analysis

Storage of the cells for Western blot analysis (results described in chapter 6.1)

Freezing and thawing conditions were tested for the experiments whose results are described in chapter 6.1. MNC, P39 and Jurkat cells (always $1x10^6$ cells) were frozen using the following conditions or combinations of them:

a) Dry cell pellets ($1x10^6$ cells) in 1.5 ml Eppendorf centrifuge tubes (Hamburg, Germany) packed in boxes were frozen in a -80°C freezer and stored in the same freezer.

b) Dry cell pellets ($1x10^6$ cells) in 1.5 ml Eppendorf centrifuge tubes were resuspended in 80 µl PBS containing protease inhibitors (Complete Mini, Roche, Bromma, Sweden; one tabled in 10 ml PBS) before freezing and storage at -80°C.

c) Dry cell pellets ($1x10^6$ cells) in 1.5 ml Eppendorf centrifuge tubes were snap frozen in liquid nitrogen and then stored in a -80°C freezer. The cells were thawed either at room temperature or on ice.

In one experiment we kept the cells overnight in medium at 4°C.

Storage of the cells for Western blot analysis (results described in chapter 6.2- 6.4)

The freezing experiments (chapter 6.1) led to the following storage method of cells for Western blot analysis. Samples of $1x10^6$ MNC were washed in PBS and then resuspended in 80 µl PBS containing broad-spectrum protease inhibitors (Complete Mini, Roche, Bromma, Sweden; one tablet in 10 ml PBS) prior to freezing. Cells were routinely kept frozen at -80°C. After storage samples were thawed on ice prior to further investigations. P39 and Jurkat cells were frozen as a dry cell pellet ($1x10^6$) without protease inhibitors.

Western blot

Dry cell pellets (Jurkat, P39) were resuspended in 80 µl PBS and 20 µl 5x Laemmli´s loading buffer containing 62.5 mM Tris-Cl, pH 6.8, 20% glycerol, 10% SDS, 0.025% bromphenol blue and 5% α-mercaptoethanol. MNC frozen in PBS in the presence of the broad-spectrum protease inhibitor were thawed and mixed with 20 µl loading buffer. All samples were boiled for 5 minutes. Fifty µl of these mixtures were resolved on 15% and 7.5% SDS polyacrylamide gels *(Table 5.2)*. The gels were run at constant 130 V at 4°C until the bromphenol reached the bottom of the resolving gel.

Separating gel			**Running buffer** (10x concentration)	
Gel concentration	**15%**	**7.5%**	Tris base	90 g
			Glycine	432 g
Double distilled water/ ml	2.35	4.85	SDS	30 g
1.5 M Tris-HCl, pH 8.8/ ml	2.5	2.5	Double distilled water	3 l
10% SDS/ µl	100	100		
AA (protogel), 30% stock/ ml	5	2.5	50 ml for one electorphoretic run 10x buffer and 450 ml double distilled water	
10% APS/ µl	50	50		
TEMED/ µl	5	5		

Stacking gel		**Transfer buffer**	
gel concentration	**4%**	0.1 M CAPS, pH 11	100 ml
		Double distilled water	700 ml
Double distilled water/ ml	7.29	Methanol	200 ml
1.0 M Tris-HCl, pH 6.8/ ml	1.25		
10% SDS/ µl	100		
AA (protogel) 30% stock/ ml	1.3		
10% APS/ µl	50		
TEMED/ µl	10		

Table 5.2. Gel preparation, running buffer, and transfer buffer for Western blot analysis

The gels were transblotted to nitrocellulose membranes (Sartorius, Göttingen, Germany) for 2 hours at constant 100 V at 4°C. Ice and stirrer magnet were used to keep a constant temperature in the transfer chamber. The membranes were blocked for 1 hour at room temperature in PBS supplemented with 5% milk and 0.15% Tween and stained with the primary antibodies overnight at 4^{o}C. The following day, after several washings with PBS and PBS containing 0.15% Tween, membranes were incubated for 1 hour with the secondary antibody. After further washes with PBS and PBS containing 0.15% Tween *(Table 5.3)* the membranes

were developed with an ECL kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden), according to the manufacturer's instructions. The luminescence was detected on an X-ray film (Fuji, Düsseldorf, Germany).

	Time	Solution
1. primary antibodies	Overnight	PBS, 0.1% NaN_3
2. washing	10 min	PBS
3. washing	10 min	PBS + 0.15% Tween
4. washing	10 min	PBS
5. secondary antibodies	1 hour	PBS, 2.5% milk
6. washing	10 min	PBS
7. washing	10 min	PBS + 0.15% Tween
8. washing	10 min	PBS

Table 5.3. Incubation and washing times

Antibodies

Primary antibodies were used as indicated in the following table *(Table 5.4)*. The antibodies were diluted in PBS supplemented with 1% BSA and 0.1% NaN_3. Proteins of interest were caspase proenzymes forms (30- 55 kDa) and their activated cleavage products (10- 35 kDa), Bcl-2, poly(ADP-ribose)polymerase (PARP), actin and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as a marker for equal protein amount loaded onto the gel.

Antigen	Antibody (dilution)	Source
Actin	Rabbit IgG (1:100)	Sigma, St. Louis, MO, USA
Bcl-2	Mouse IgG (1:100)	Dako (Glostrup, Denmark)
G3PDH	Rabbit IgG (1:3000)	Trevigen (Gaithersburg, MD, USA)
PARP	Mouse IgG (1:5000)	Biomol (Plymouth, PA, USA)
Pro-caspase-3	Mouse IgG (1:5000)	Gift from Dr. D. W. Nicholson (Merck Frosst Center for Therapeutic Research, Quebec, Canada)
Pro-caspase-8	Mouse IgG (1:20)	Gift from Dr. P. H. Krammer (German Cancer Research Center,Heidelberg, Germany)

Table 5.4. Primary antibodies

As secondary antibodies, peroxidase conjugated goat anti- rabbit IgG and goat anti-mouse IgG from Pierce (Rockford, IL, USA) were used at a concentration of 1:5000 in PBS containing 2.5% milk.

5.7. Caspase enzyme assay

Using the tetrapeptides DEVD and IETD conjugated to aminomethyl coumarin (AMC), a continuous fluorometric assay for caspase-3 and caspase-8 activity was performed. Free aminomethyl coumarin (AMC) is a fluorogenic molecule with a 355 nm excitation and a 460 nm emission wavelength. Caspase-3 like enzymes cleave the molecule after the tetrapeptide sequence DEVD and the increase of the amount of free AMC can be detected by the increase in fluorescence. Caspase-8 cleaves after the sequence IETD. The measurement of DEVD-AMC and IETD-AMC cleavage was performed in a fluorometric assay modified from Nicholson et al. *(Nicholson et al., 1995)*. Briefly, $1x10^6$ cells were taken, washed twice in PBS and frozen as a dry cell pellet at -80^o C. For the assay the cells were kept on ice and resuspended in 50 µl PBS. The appropriate peptide substrate (DEVD-AMC or IETD-AMC, Peptide Institute, Osaka, Japan) was combined in a standard reaction buffer containing 100 mM HEPES, 10% sucrose, 5 mM dithiothreitol (DTT), 10^{-4}% octylphenoxy polyethoxy ethanol [NP-40] and 0.1% 3-[(3-cholamidopropyl) dimethylammonio]-1- propane sulfonate (CHAPS pH 7.25) and added to the cell lysates on a microtiter plate. Cleavage of the fluorogenic peptide substrate was monitored by AMC liberation in a Fluoroscan plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths during a time period of 30 minutes *(Figure 5.1)*. Fluorescence units were converted to pmol of AMC using a standard curve generated with free AMC. Data from duplicate samples were then analysed by linear regression.

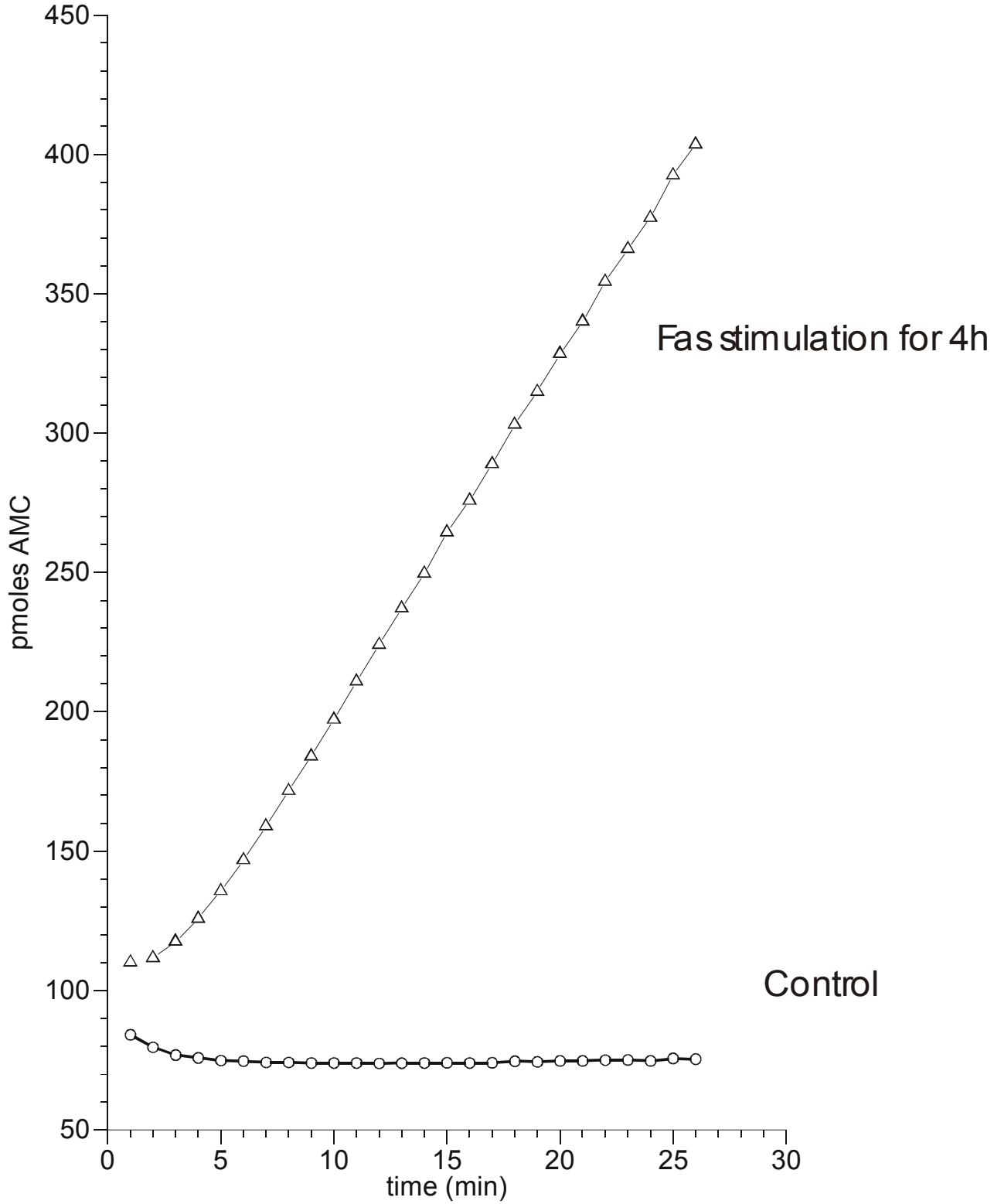


Figure 5.1. Example for caspase activity after 4h Fas ligation of 5x 10^5 MNC compared to untreated cells (control). *The linear part of the curve was used to calculate the slope of the graph indicating the generation of pmoles AMC/ min.*

5.8. TUNEL staining

Induction of apoptosis results in the generation of single-strand DNA breaks. These can be detected using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) method *(Gavrieli et al., 1992).* Using a fluorescence microscope with a green and a red filter, increased 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 visualisation 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.

5.9. Proliferation

Aliquots containing 10^5 cell 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% CO_2. At 0, 24, 48, and 72 hours after the start of cell culture 1 μCi (20 μl of 50 μlCi/ ml) ^{3}H-thymidine was added for 24 hours. Cells were harvested with a Combi Cell Harvester (Skatron, Lier, Norway) on filter paper and added to scintillation fluid (2 ml OptiScint ´HiSafe`, Wallac Scinitillation Products, Turku, Finland). The radioactivity was measured with a liquid scintillation counter (Wallac 1409).

5.10. Measurement of mitochondrial transmembrane potential ($\Delta\psi_m$) and production of reactive oxygen species (ROS) by FACS analysis

The loss of mitochondrial transmembrane potential ($\Delta\psi_m$) was measured using the cationic fluorescent dye 3,3´dihexyloxacarbocyanine iodide ($DiOC_6(3)$, 20 nM as final concentration, Molecular Probes, Leiden, The Netherlands), which accumulates in mitochondria as a direct function of $\Delta\psi_m$. ROS were visualised by the dye dihydroethidium (HE, 4 µM as final concentration, Molecular Probes, Leiden, The Netherlands), which is oxidised to the fluorescence-emitting substance ethidium in the presence of ROS.

Briefly, 5 $x10^5$ cells were collected, spun down and resuspended in 500 µl PBS containing the dyes. After 30 minutes incubation at 37°C in the dark, the cells were stored on ice and analysed on a FACScan Flow cytometer (Becton Dickinson, San Jose, CA, USA). Necrotic cells were excluded based on forward and side scatter criteria and data were calculated using the CellQuest software (Becton Dickinson).

5.11. Statistics

Results are given as mean values ± SD and also as median values + range. For comparison of related and unrelated samples, paired and unpaired two-tailed t-tests, respectively, were used. Significance was presumed if $p< 0.05$. All results were calculated using the computer programme StatView (SAS Institute Inc., Cary, NC, USA).

6. Results and discussion

6.1. Optimising of mononuclear cell freezing for Western blot analysis

These experiments were designed to improve Western blot analysis for frozen mononuclear bone marrow cells. In short-term bone marrow cultures, freezing is an important storage method that immediately stops all biological processes inside cells and allows the comparison of different treatments at different time points. Integrity of the proteins of interest after freezing is of paramount importance for the Western blot analysis. Therefore, the following experiments were done to optimise the conditions for Western blot analysis of MNC from bone marrow of patients and normal controls. All experiments are repeated twice and representative data are shown. Different freezing conditions of MNC led to the appearance of different protein bands in the Western blot analysis while the same variety of freezing conditions did not influence the Western blot results in the cell lines (P39 and Jurkat). *Figure 6.1* (normal MNC) and *Figure 6.2* (MNC isolated from a RARS patient) are representative examples for the detected differences due to modified conditions.

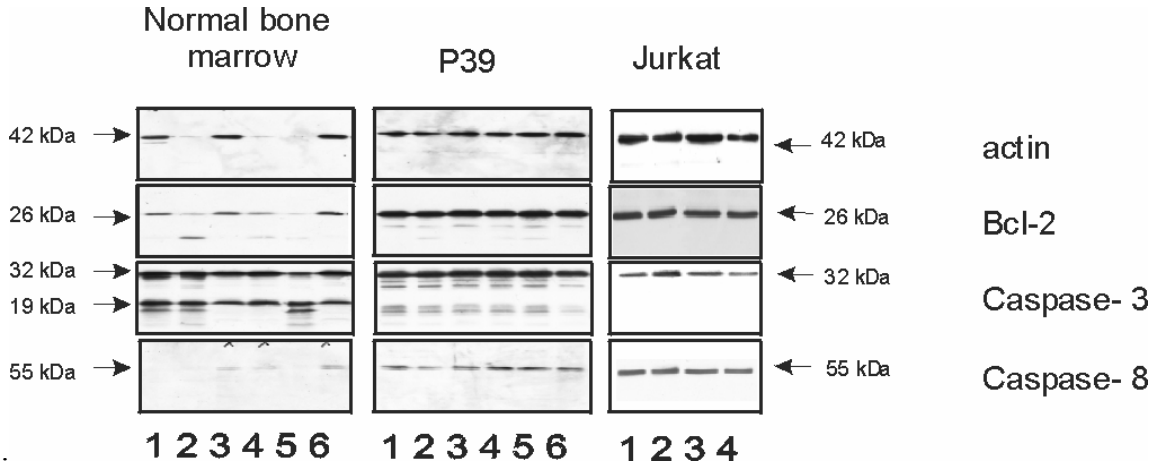


Figure 6.1. Protein cleavage after freezing of mononuclear bone marrow cells isolated from a healthy donor in comparison to the Jurkat and P39 cell lines
Lane 1. Cells frozen at -80°C and thawed on ice
Lane 2. Cells frozen at -80°C and thawed at room temperature
*Lane 3. Cells frozen with **protease inhibitors** at -80°C and thawed on ice*
*Lane 4. Cells frozen with **protease inhibitors** at -80°C and thawed at room temperature*
Lane 5. Cells frozen in liquid nitrogen and thawed at room temperature
*Lane 6. Cells frozen in liquid nitrogen with **protease inhibitors** and thawed on ice*

Freezing of MNC at -80°C without protease inhibitors led to degradation of pro-caspases-3 and –8. Temperature of freezing (at -80°C or in liquid nitrogen) was not a critical step and did not influence the degradation of proteins *(Figure 6.1 lanes 2 and 3 vs. lanes 5 and 6)*. Cleavage of Bcl-2 and Actin was observed when samples were thawed at room temperature, even when they were frozen in presence of broad-spectrum protease inhibitors. In the bone marrow isolated from RARS patients, Actin cleavage could not be blocked totally by the inhibitor and thawing on ice *(Figure 6.2, lane 2)*. *Table 6.1* summarises the influence of the broad-spectrum **protease inhibitor** and **thawing on ice** on the integrity of protein.

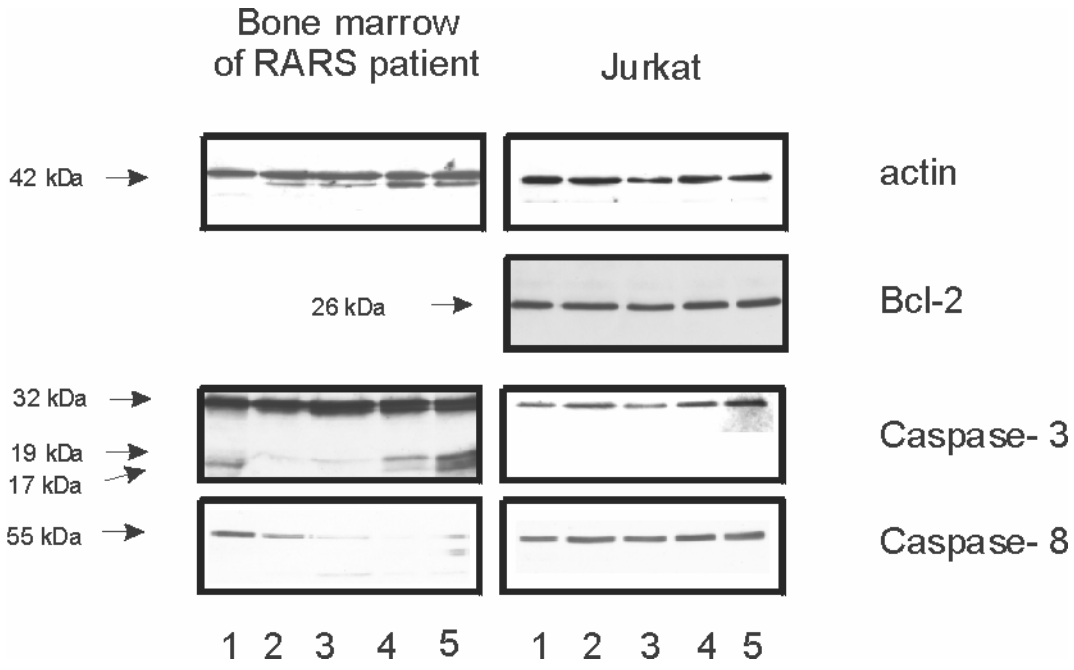


Figure 6.2. Protein cleavage after freezing of mononuclear bone marrow cells isolated from a patient with RARS in comparison to the Jurkat cell line
Lane 1. Cells not frozen, overnight in medium
*Lane 2. Cells frozen with **protease inhibitors** at -80℃ and thawed on ice*
*Lane 3. Cells frozen with **protease inhibitors** at -80℃ and thawed at room temperature*
Lane 4. Cell frozen at -80℃ and thawed on ice
Lane 5. Cells frozen at -80℃ and thawed at room temperature

	Protein of interest	Normal bone marrow *(Figure 6.1)*	RARS bone marrow *(Figure 6.2)*
Adding of protease inhibitor		***Lane 3, 4, 6***	***Lane 2, 3***
	Actin	Only detectable in the positions frozen with the inhibitor	Less cleavage of actin
	Bcl-2	No influence	**Patient cells does not express detectable amount of Bcl-2**
	Caspase-3	Inhibitor prevents cleavage products	Inhibitor prevents cleavage products
	Caspase-8	Only detectable in the positions frozen with the inhibitor	Only detectable in the positions frozen with the inhibitor
Thawing on ice		***Lane 1, 3, 6***	***Lane 2, 4***
	Actin	Only detectable in the positions thawed on ice	No influence
	Bcl-2	Without cleavage in these positions	Patient cells does not express detectable amount of Bcl-2
	Caspase-3	No influence	No influence
	Caspase-8	No influence	No influence

Table 6.1. Influence of the broad-spectrum protease inhibitor and thawing on ice on integrity of proteins measured by Western blot analysis.

These results suggest that adding a broad-spectrum protease inhibitor and thawing on ice was the best method to avoid false results by protein cleavage during storage of MNC. Cleavage of all tested proteins was inhibited and mostly totally blocked. Keeping the cells overnight at 4° C before the analysis was not an advantageous, since an activation of caspase-3 with the appearance of the p17 cleavage product was observed *(Figure 6.2, lane 1)*. Interestingly, addition of the broad-spectrum inhibitor and thawing on ice was not necessary for the tested Jurkat and P39 cell lines. Adding loading buffer that immediately lysed cells and inactivated proteases and induces unfolding of proteins was another possibility that was tested to avoid cleavage by proteolytic activity during the thawing process. However, in this case measurement of the protein concentration in the sample was no longer possible.

During apoptosis different proteins undergo proteolysis leading either to their functional activation or inactivation (for review: *Zhivotovsky et al., 1997)*. Caspases are activated by cleavage during apoptotic cell death. Actin cleavage mediated by these activated caspases has also been shown *(Mashima et al., 1999)*. Recently, cleavage of Actin and Bcl-2 was detected in a leukaemic cell line *(Fadeel et al., 1999* and *Hassan et al., 1999)*. In our experiments, all proteins of interest could also be physiologically cleaved during apoptosis. Therefore, artificial cleavage bands could lead to misinterpretation of the Western blot results.

Spontaneous and induced apoptosis of malignant haematological cells are processes that presently are under intensive investigation. Our results may provide new information that prevents the misinterpretation of certain laboratory findings. Stringent conditions for freezing and thawing are of upmost importance to prevent the occurrence for artificial cleavage bands and the misinterpretation of artefacts as pathophysiological mechanisms of disease. Finally, these experiments have led to the storage method as described in the methodology chapter.

6.2. Bcl-2 expression in RARS bone marrow

6.2.1. Results

Changes in oncoprotein expression during the apoptotic processes and in different subcategories of MDS have been recently discussed *(Parker et al., 2000, Parker et al., 1998* and *Rajapaksa et al., 1996)*. We examined cells isolated from 8 RARS patients and 5 normal controls for the apoptosis-related oncoprotein Bcl-2 by Western blot analysis. As described in the methodology chapter, isolated MNC were cultured with the addition of exclusively FCS, the Fas-agonistic antibody CH-11, the antagonistic f(ab)´2 antibodies, or the caspase inhibitor DEVD-fmk. After incubation for 0 hours, 18 hours and 18+ 24 hours, 1x 10^6 cells were collected from each of the four differently treated mononuclear cell fractions. The cells were investigated for the presence of oncoprotein Bcl-2.

All RARS (8 patients) and controls MNC (5 donors) showed very low amounts of Bcl-2 (Western blots not shown), as compared to the Jurkat and P39 cell lines. Only in samples isolated from a healthy 24-year old male donor, higher levels of Bcl-2 could be detected. No changes were observed after incubation during any of the culture additions (FCS only, FCS + CH-11, FCS + f(ab)´2 antibodies, or FCS + DEVD-fmk).

6.2.2. Discussion

Normal or MNC´s from RARS patients did not express detectable amounts of Bcl-2 protein and no changes were observed during culturing. This is not an unexpected finding as other groups have examined oncoprotein expression of MDS patients in detail. Using CD34+ stem cells, Parker *et al.* showed that ”low-risk” MDS was associated with excessive apoptosis and an increased ratio of pro- (Bax, Bad) versus anti-apoptotic Bcl-2-related proteins (Bcl-2, Bcl-X_L) *(Parker et al., 1998)*. Bincoletto and collaborators found lower amounts of Bcl-2 positive cells in MNC from MDS patients compared to normal controls *(Bincoletto et al., 1998*). Rajapaksa *et al*. have measured the ratio of c-myc and Bcl-2 in different subpopulations of MNC from MDS patients *(Rajapaksa et al., 1996)* and found a significant increase in the c-myc-to-Bcl-2 ratio in ”low risk” MDS compared to “high risk” cases. Disease progression to advanced stages with blast excess and transformation towards AML might be connected with an increase of Bcl-2 expression *(Davis et al., 1998)*. Our study included only MNC from RARS patients with bone marrow blasts < 5%. Therefore we cannot draw any specific conclusions about the importance of Bcl-2 for blast increase and disease progression towards AML. However, it is possible that it would be more relevant to study Bcl-X_L in stem cells and early erythroid progenitors from RARS marrow since this protein has been shown to be more important than Bcl-2 for erythropoiesis (Silva *et al., 1996).*

6.3. Apoptosis in RARS: Influence of Fas-agonistic and antagonistic antibodies on caspase activity and nuclear apoptotic changes

6.3.1. Caspase expression and processing examined by Western blot analysis

Western blot data on analysis of caspases were generated from MNC of 8 RARS patients and 5 healthy donors as well as from GpA+ and GpA- subfractions (2 patients, 2 normal controls). Caspase processing was induced by Fas-agonistic antibodies CH-11, the protein kinase C inhibitor staurosporine (STS) or the topoisomerase II inhibitor etoposide (VP 16).

Caspase-8: In MNC cleavage of procaspase-8 (55 and 53 kDa, 2 isoforms) to the activated forms of the enzyme (43 and 41 kDa) was detectable at every time point under all conditions. Differences in the intensity of the two original bands and their cleavage products could not be related to different treatments. In addition, there was no significant difference between patient MNC and normal bone marrow cells (*Figure 6.3).* In the GpA separated cells, activation of caspase-8 could always be detected in GpA- cell fractions with no significant difference between the patient MNC and normal bone marrow cells *(Figure 6.4 and Figure 6.5).*

Caspase-3: In MNC cleavage of this enzyme from the 32 kDa proform to an approximately 19 kDa cleavage product could be detected in all samples. Differences of the intensity of the original bands and their cleavage product could not be related to the different treatments and time points *(Figure 6.3).* The Western blot data of separated GpA+/- fractions showed differences in caspase-3 expression and processing. In GpA+ cells isolated from a patient, the general expression of both investigated pro-caspases was lower than in the GpA- cells *(Figure 6.4)*. Cells isolated from the healthy donor showed more or less the opposite pattern with increased (pro-caspase-8) or approximately the same pro-caspase-3 expression in GpA+ cells *(Figure 6.5).* GpA- cells showed the cleavage band p19 under all culture conditions. After induction of apoptosis further cleavage bands (p17, p12) became apparent whereas in GpA+ cells these bands could only be detected after very long time of film exposure which indicates the low concentration of active caspase-3 in GpA+ cells. *(Figure 6.4)*

poly(ADP-ribose)polymerase (PARP): PARP as a substrate for caspase-3 was mainly expressed in the GpA+ cells. Cleavage of this enzyme indicating increased caspase-3 activity could be detected upon induction of apoptosis by Fas, etoposide or staurosporine. In one patient this cleavage was visible already after GpA cell separation *(Figure 6.4 and Figure 6.5).*

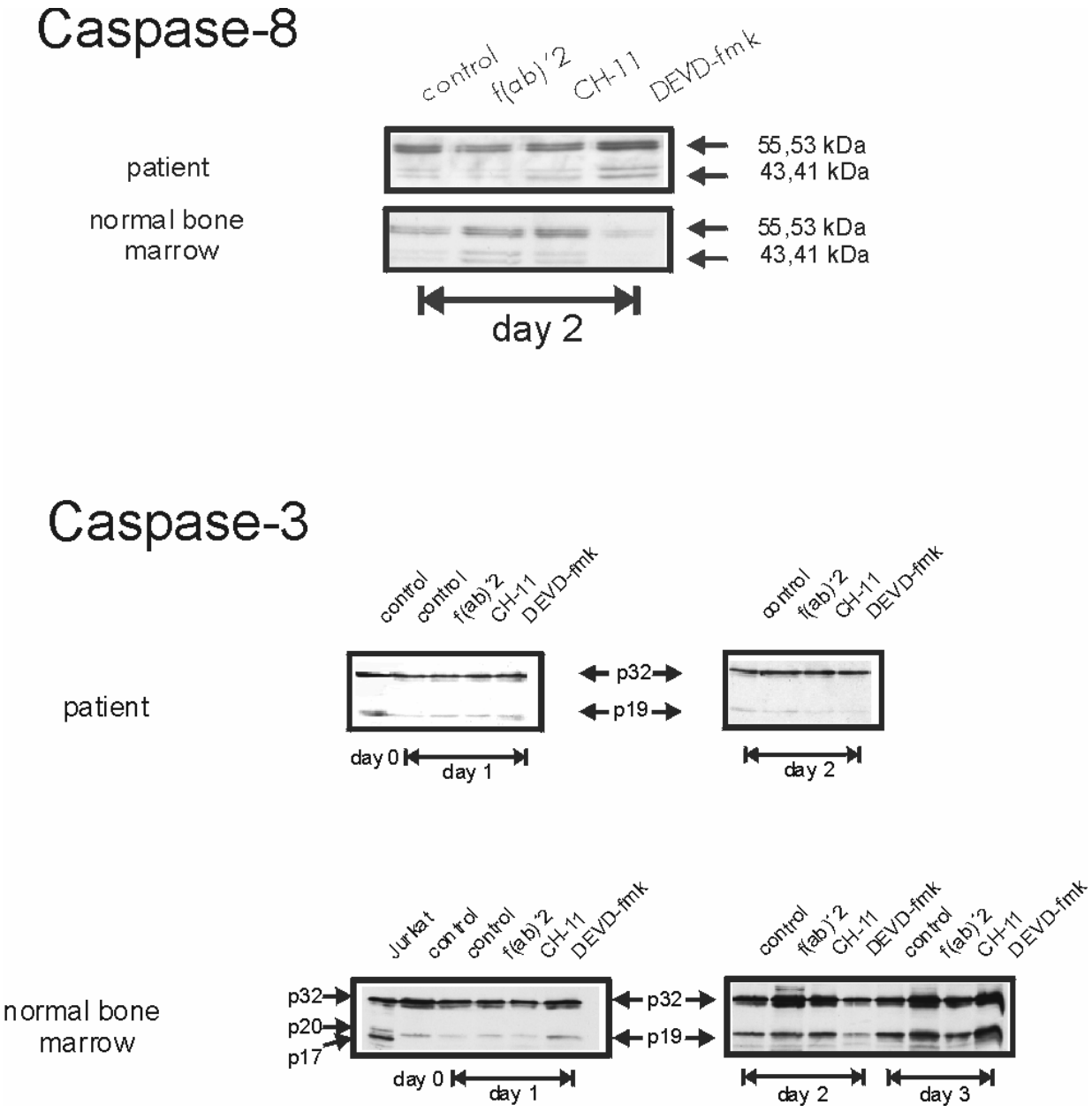


Figure 6.3. Caspase-8 and Caspase-3 processing in MNC isolated from RARS patients and normal controls. *The samples were cultured with FCS only (control), FCS + f(ab)´2, FCS + CH-11 and FCS + DEVD-fmk for 24 hours (day 1), 48 hours and 72 hours (day 2 and 3, respectively)*

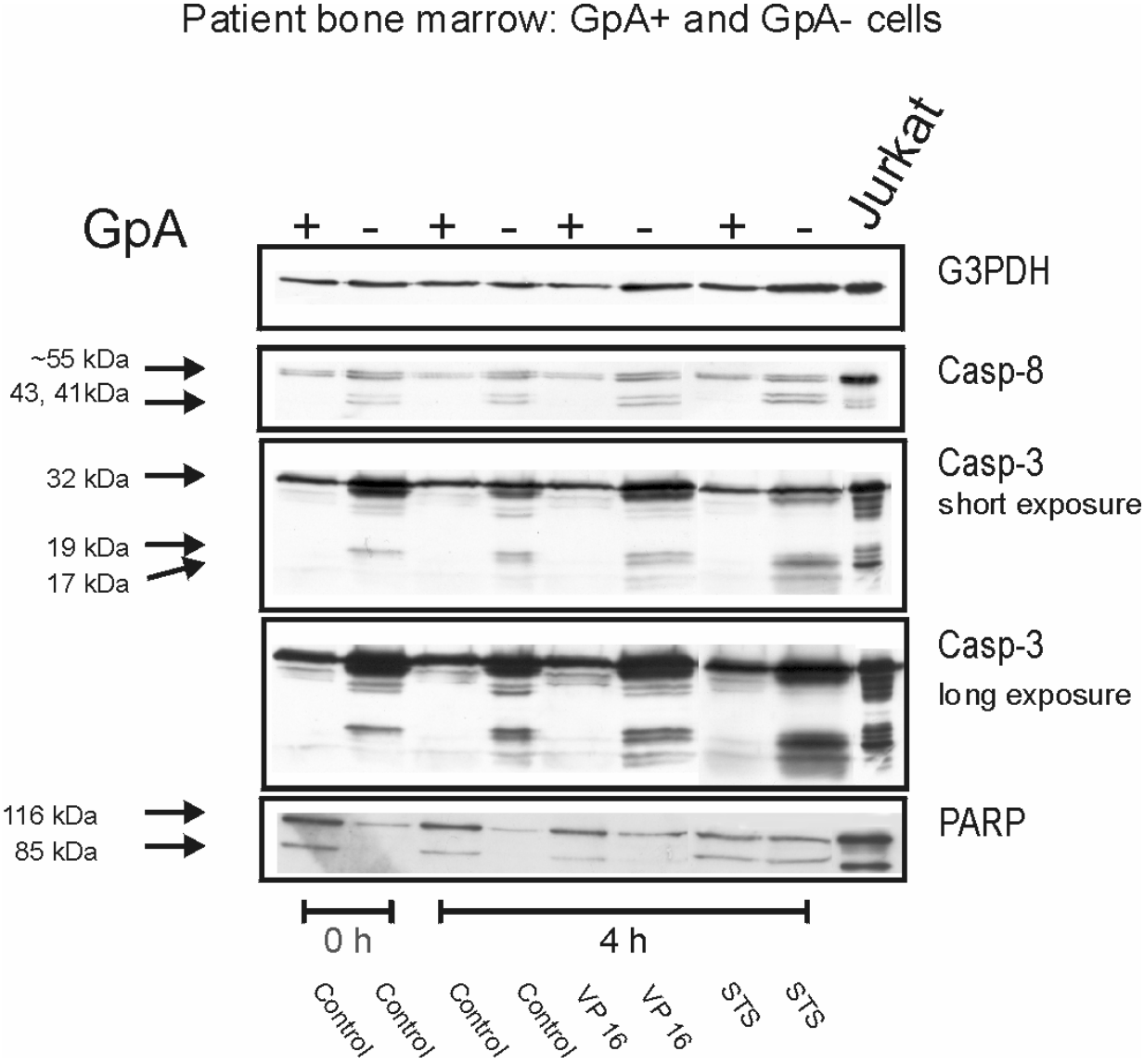


Figure 6.4. Caspase processing in GpA+/- cells isolated from a RARS patient after apoptosis induction. *The glycolysis enzyme G3PDH is shown as a marker for loaded protein amount and cleavage of PARP as an indicator for caspase-3 activity*

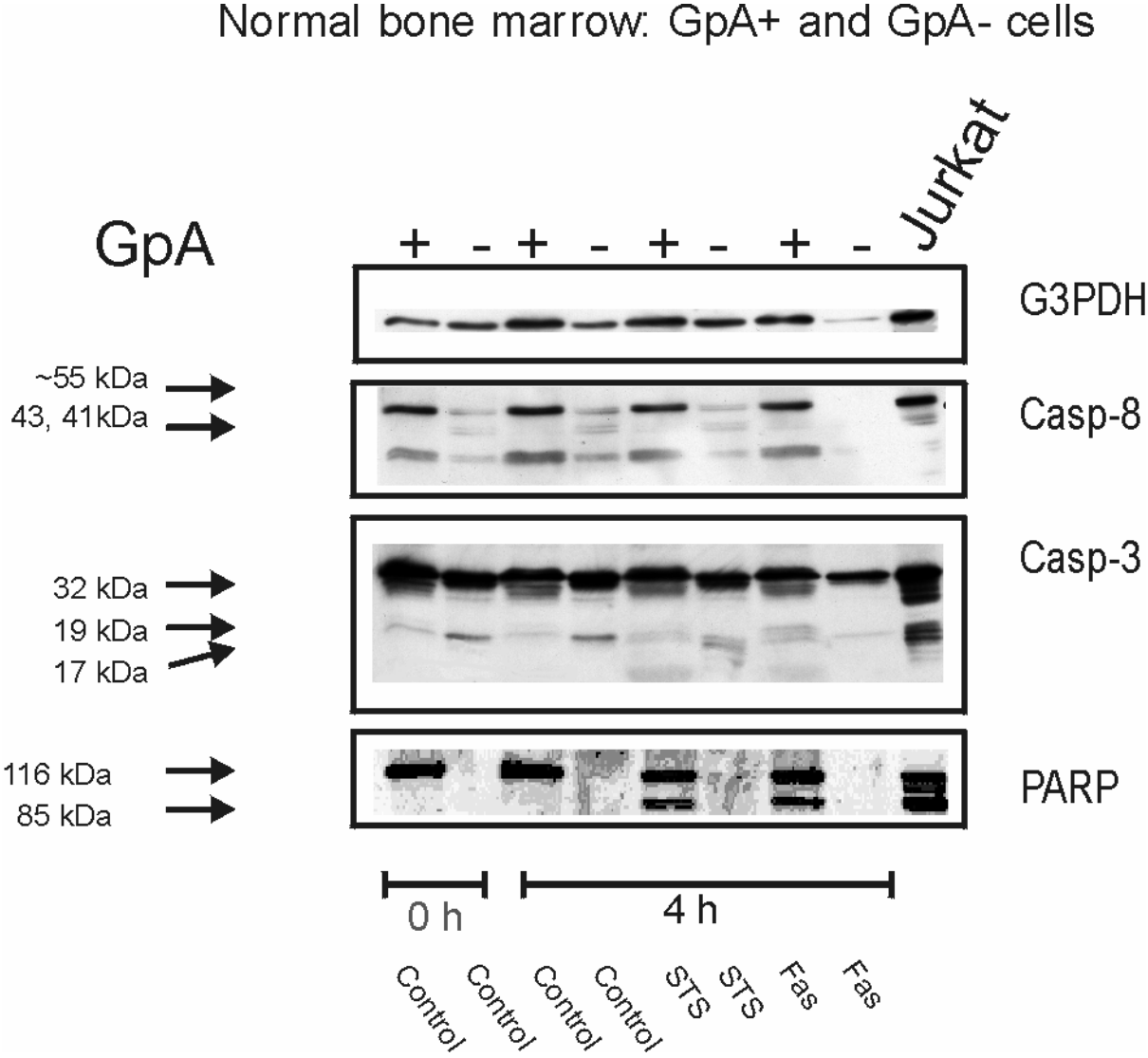


Figure 6.5. Caspase processing in GpA+/- cells (normal bone marrow) after apoptosis induction. *The glycolysis enzyme G3PDH is shown as a marker for loaded protein amount and cleavage of PARP as an indicator for caspase-3 activity*

6.3.2. Caspase enzyme activity

As described in the methodology chapter, MNC isolated from 8 RARS patients and 5 healthy donors were cultured with the addition of exclusively FCS, the Fas-agonistic CH-11, the antagonistic antibodies f(ab)´2 or the caspase inhibitor DEVD-fmk. Cells were harvested after 0 (control), 4, and 24 hours, washed and used for measuring of the caspase-3-like activity (*Figure 6.6)* and, in some cases, caspase-8 activity by cleavage of the fluorochromes DEVD-AMC and IETD-AMC, respectively. Additionally, MNC were first GpA separated and caspase activities were then measured in the two subpopulations of GpA+ and GpA- cells. Patient no 2 and 3 were investigated before (2a, 3a) and after 20 weeks of treatment with G-CSF and EPO (2b, 3b). *Tables 6.2, 6.3 and 6.4* summarise the results, while *Figure 6.7* is a graphic conversion of *Table 6.2*.

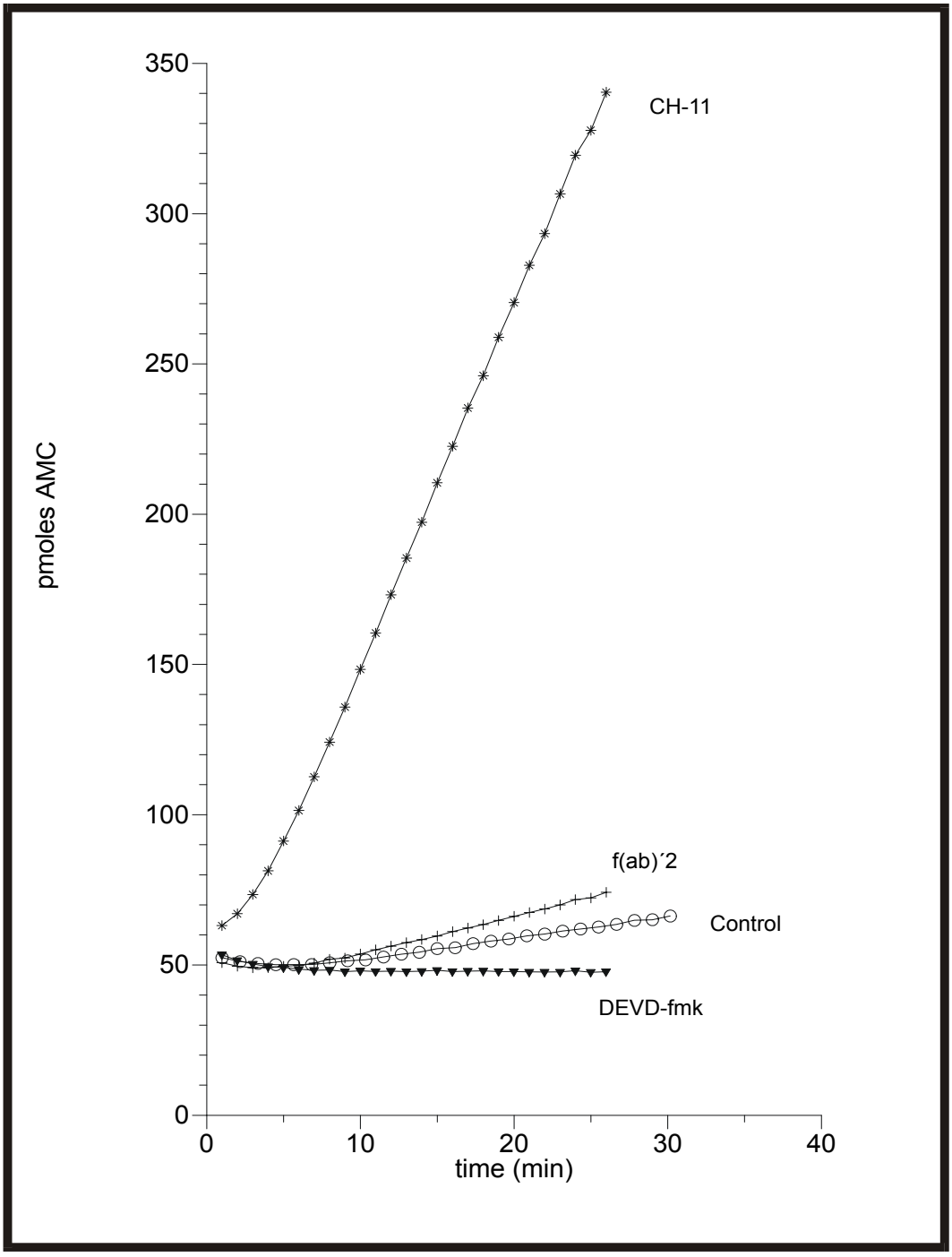


Figure 6.6. Caspase-3-like activity for one RARS patient (no 8) after 4 hours of MNC incubation. *The linear parts of the curves were used to calculate the slopes of the graphs indicating the generation of pmoles free AMC/ min as shown in Table 6.2.*

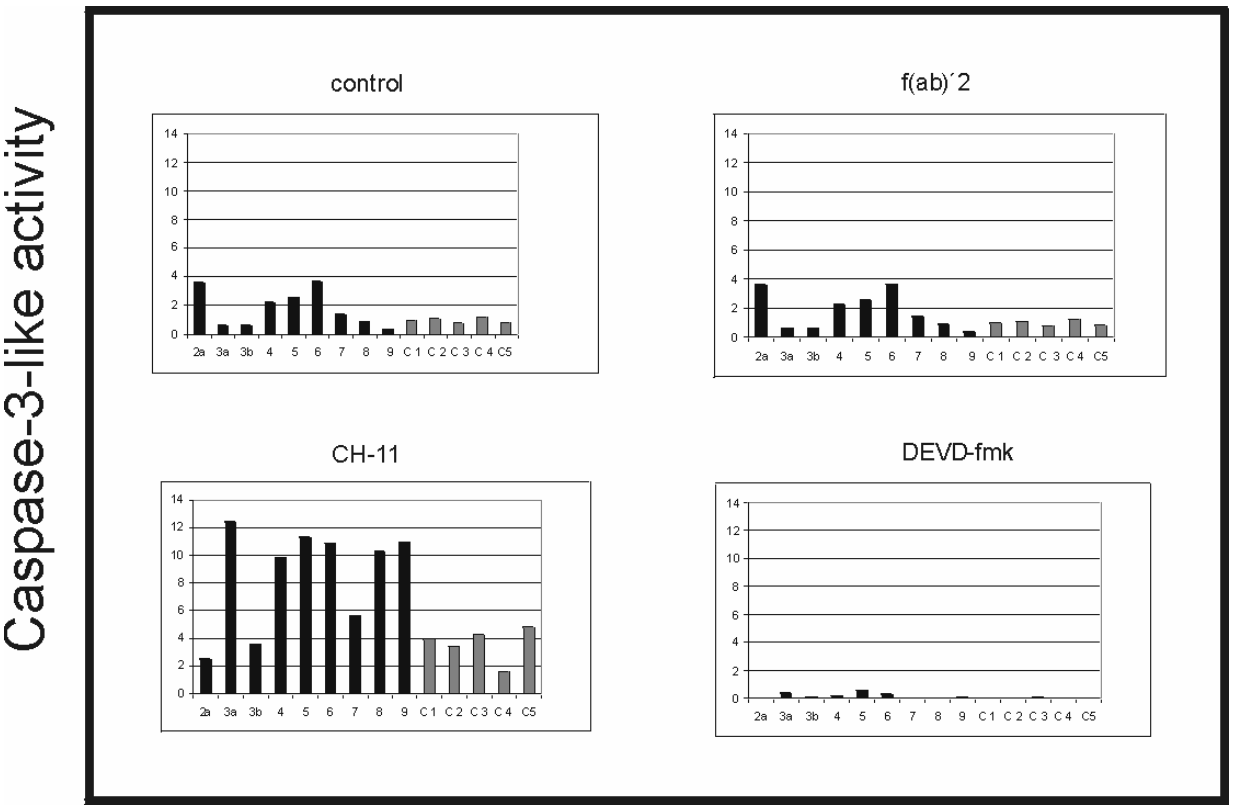


Figure 6.7. Caspase-3-like activity in 8 bone marrow samples isolated from patients (2a-9) and 5 samples isolated from healthy donators (c1-c5) bone marrows in pmoles AMC release/ minute after 4 hours of incubation

RARS MNC exhibited a slightly higher caspase-3-like activity at 4 and 24 hours after incubation as compared to normal controls, but no statistic significance was achieved (p= 0.33 and 0.14, respectively). Fas ligation induced a significantly higher caspase activity in RARS samples compared to the controls, after 4 and 24 hours (p= 0.01 and p= 0.02, respectively). F(ab)´2 fragments antagonistic to the Fas-Receptor had no effect on caspase activity *(Figure 6.7).* DEVD-fmk as a competitive inhibitor reduced significantly caspase activity in all cultures (p= 0.0002).

		Day 0, 0 h	Day 0, 4 h	Day 1, 24 h
RARS 2a	*Control*	1	3	3.1
	f(ab)´2		3.61	3.23
	CH-11		2.53	7.5
	DEVD-fmk		0	1.1
RARS 3a	*Control*	0.44	0.71	1.07
	f(ab)´2		0.62	0.78
	CH-11		12.42	3.89
	DEVD-fmk		0.39	0.07
RARS 3b	*Control*	0.17	0.6	1.17
	f(ab)´2		0.65	1.03
	CH-11		3.62	2.34
	DEVD-fmk		0.11	0.49
RARS 4	*Control*	0.35	2.12	1.47
	f(ab)´2		2.29	3.56
	CH-11		9.84	3.18
	DEVD-fmk		0.2	0.08
RARS 5	*Control*	1.16	2.19	5.7
	f(ab)´2		2.56	3.98
	CH-11		11.37	11.37
	DEVD-fmk		0.56	1.15
RARS 6	*Control*	0.68	3.83	4.08
	f(ab)´2		3.65	2.84
	CH-11		10.9	4.71
	DEVD-fmk		0.33	0.26
RARS 7	*Control*	0.25	1.09	1.16
	f(ab)´2		1.4	1.94
	CH-11		5.66	3.11
	DEVD-fmk		0.07	0.25
RARS 8	*Control*	1.21	0.73	5.39
	f(ab)´2		0.92	3.23
	CH-11		10.32	4.19
	DEVD-fmk		0	0.48
RARS 9	*Control*	0.26	0.54	1.84
	f(ab)´2		0.4	1.48
	CH-11		10.94	4.57
	DEVD-fmk		0.1	0.16
C1	*Control*	0.39	1.04	0.88
	f(ab)´2		0.99	0.82
	CH-11		3.96	1.3
	DEVD-fmk		0.06	0.46
C2	*Control*	0.24	1.31	0.34
	f(ab)´2		1.1	0.25
	CH-11		3.41	0.54
	DEVD-fmk		0.03	0
C3	*Control*	0.23	0.88	1.72
	f(ab)´2		0.77	0.41
	CH-11		4.26	1.29
	DEVD-fmk		0.1	0.43
C6	*Control*	0.97	1.32	1.25
	f(ab)´2		1.22	2.47
	CH-11		1.64	1.1
	DEVD-fmk		0	0.7
C7	*Control*	0.36	0.88	2.51
	f(ab)´2		0.85	2.74
	CH-11		4.84	2.78
	DEVD-fmk		0.04	0.09

Table 6.2. Caspase-3-like activity in pmoles AMC release/ min after 0, 4, and 24 hours incubation of MNC

In this set of experiment, it was possible to measure caspase-8 activity for one patient and one healthy donor *(Table 6.3*). These two cases are too few to allow specific conclusions. However, we started to measure caspase-8 activity routinely in the following set of experiments (see below: chapter 6.4).

		Day 0, 0 h	Day 0, 4 h	Day 1, 24 h
RARS 8	*Control*	0.38	0.46	0.3
	F(ab)´2		0.24	0.19
	CH-11		1.28	0.52
	DEVD-fmk		0.19	0.19
Normal bone marrow 5	*Control*	0.38	0.36	0.41
	F(ab)´2		0.36	0.43
	CH-11		0.67	0.26
	DEVD-fmk		0.18	0.03

Table 6.3. Caspase-8 activity in pmoles AMC release/ min after 0, 4, and 24 hours incubation of MNC

			Day 0, 0 h	Day 0, 4 h	Day 1, 24 h
RARS 8 *	**GpA+**	*CH-11*		4,38	1,43
	GpA-	*CH-11*		8,67	6,12
RARS 9	**GpA+**	*Control*	*0,1*	2,57	0,44
		CH-11		2,86	0,74
	GpA-	*Control*	*1,01*	3,11	1,39
		CH-11		6,9	1,13
Normal Bone marrow 5	**GpA+**	*Control*	0	0.3	0.15
		CH-11		0.3	0.1
	GpA-	*Control*	0.56	2.41	2.25
		CH-11		9.27	3.75

Table 6.4. Caspase-3-like activity in pmoles AMC release/ min after 0, 4, and 24 hours incubation of GpA+ and GpA- cells; * For RARS 8 no control conditions available

When caspase-3-like activity was studied separately in GpA+ and GpA- cells in two patients and one healthy donor, the activity just after cell separation was always higher in the GpA- than in the GpA+ fraction, and the Fas-induced increase was only observed in GpA- cells *(Table 6.4).*

6.3.3. Apoptotic nuclear changes as visualised by TUNEL technique

TUNEL staining (9 patients, 10 controls) was used to estimate the percentage of apoptotic cells after 4 days in culture and directly after separation (Table 6.5 and Figure 6.8).

patients	Day 0	Day 4
1	2.5	21
2a	1	8.5
2b	0.5	14
3a	0.5	5.5
3b	0.5	11
4	2	9
5	0.5	21
6	2	6.5
7	11.5	11.5
8	10.5	8
9	1.5	17.5
controls		
c1	1	14.5
c2	0.5	5.5
c3	2.5	7
c6	2.5	6.6
c8	1	3.5
c9	0.5	4.5
c10	0.5	6.5
c11	1	7
c12	0.5	1
c13	1.5	7

Table 6.5. Percentage of spontaneous apoptotic nuclear changes as measured by TUNEL staining in patients (1-9) and normal controls (c1-c13)

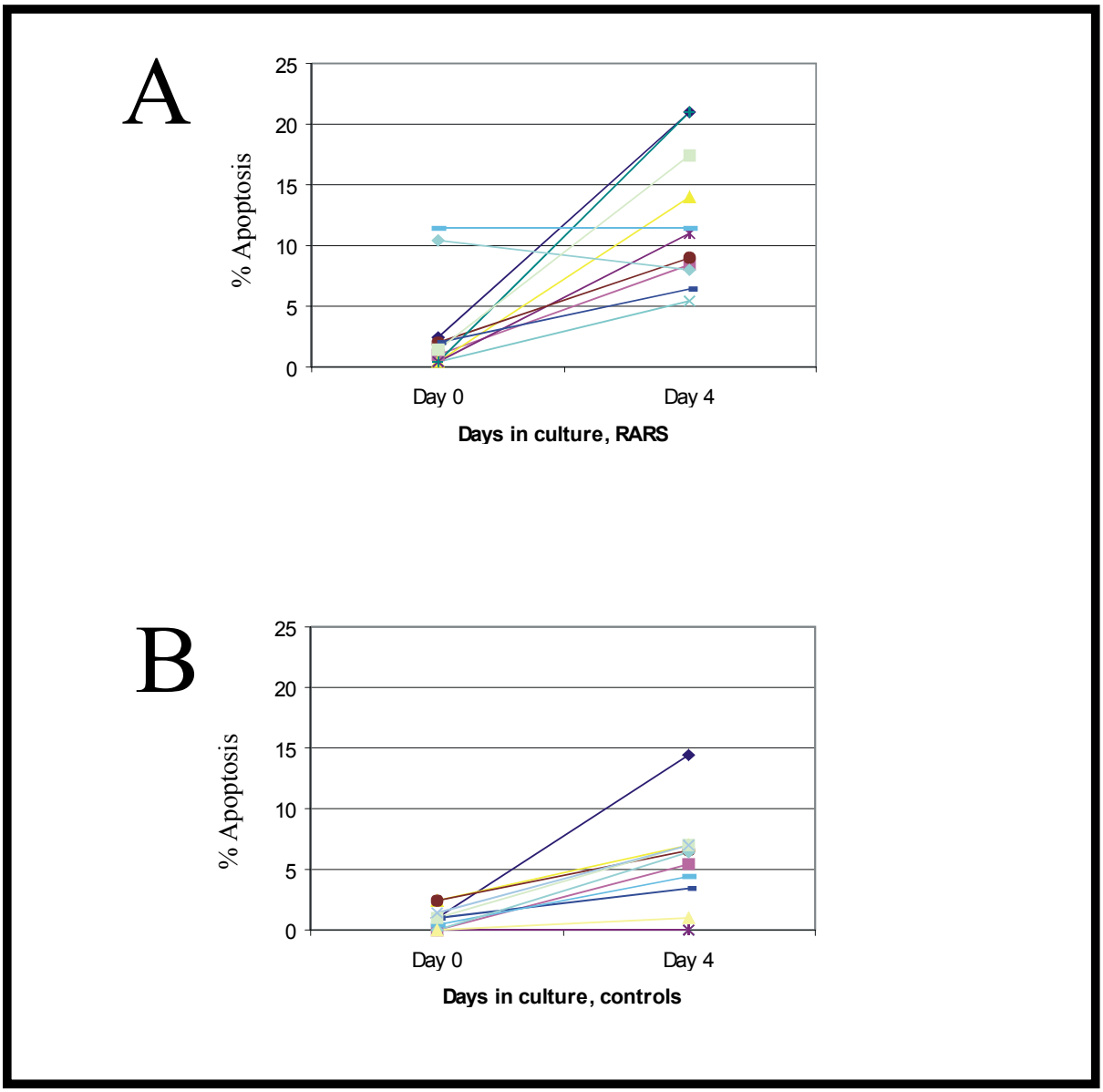
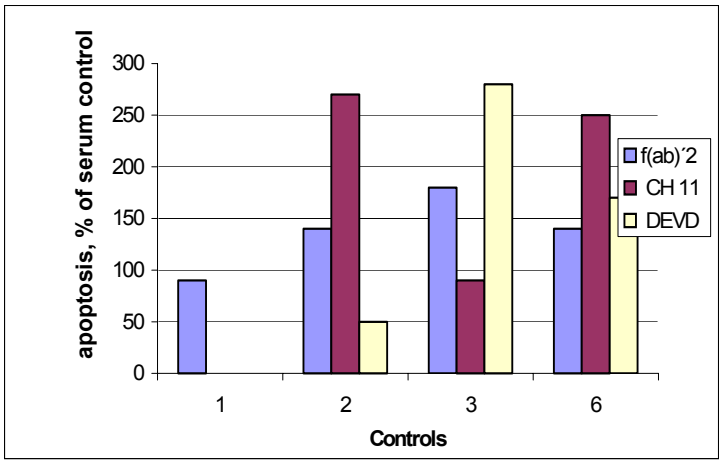


Figure 6.8. Spontaneous apoptosis in cell cultures of patients´ bone marrow cells (A) and normal controls (B) as measured by the TUNEL technique

On day 0, mean TUNEL positivity in RARS cells was somewhat although not significantly higher than in the control cells (3.0 ± 4.0 % vs. 1.2 ± 0.8 %, p= 0.17). The RARS MNC underwent significantly more spontaneous apoptosis in suspension cultures than control MNC. Day 4 TUNEL positivity was 12.14 ± 6.0 % in RARS vs. 6.3 ± 3.5 % in the controls (p=0.01). The effects of different agents on TUNEL positivity in MNC cultures are shown in *Table 6.6* and *Figure 6.9.*

	% apoptosis of control		
patients	**f(ab)´2**	**CH-11**	**DEVD**
1	90	120	90
2a	120	230	50
2b	120	350	160
3a	80	310	110
3b	60	120	80
4	130	280	70
5	•	190	50
6	130	320	70
7	130	270	80
8	110	260	160
9	60	120	30
controls			
c1	90	•	•
c2	140	270	50
c3	180	90	280
c6	140	250	170

Table 6.6. Influence of f(ab)´2, CH-11 and DEVD-fmk after 4 days of incubation. Apoptosis is shown in percentage of control and measured by the TUNEL technique



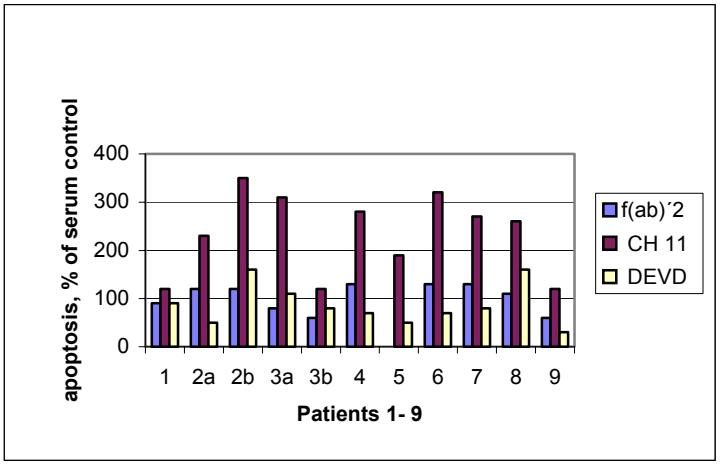


Figure 6.9. Apoptosis given in percent of serum control and measured by the TUNEL technique under cell culturing from patients and controls with the additions f(ab)´2, CH-11 and DEVD-fmk.

Fas-ligation by CH-11 induced apoptosis under all culture conditions (controls 203 ± 99%, RARS 233 ± 85%), but there was no difference between both these groups (p= 0.6). The presence of Fas-blocking fragments f(ab)´2 for 4 days neither induced a significant change in spontaneous apoptosis in the RARS cultures nor in the control cultures (103 ± 28% vs. 138 ± 37%, p= 0.08). A slight difference was observed between RARS and controls in the DEVD-fmk cultures. While the control cultures showed a slight increase in the number of apoptotic cells (167 ± 115%) the RARS cultures showed a decrease (86 ± 42%, p= 0.07 for the difference).

6.3.4. Discussion

Western blot results: The continuous presence of the p19 cleavage product in GpA- cells might be explained by accidental caspase activation during cell separation or by the physiologic role of apoptosis as a control system in haematopoiesis. De Maria and co-workers have shown the importance of the Fas system in erythropoiesis (*De Maria et al., 1999).* While Fas blocks the maturation of erythroblasts to erythrocytes, EPO induces Bcl-X_L in erythroid progenitors and can thereby inhibit apoptosis *(Gregoli et al., 1997).* According to our data, cleavage of the caspase-3 proform in marrow-derived cells generates a p19 intermediary cleavage product that after further cleavage generates the p17 form. This differs from the Jurkat cell line in which an approximately 20 kDa intermediary caspase-3 cleavage product is found. Different caspase isoforms and differences in processing were also observed by other groups (*Faleiro et al., 1997).*

Furthermore, these Western blot results indicated that GpA+ and GpA- cells react differently to apoptotic stimuli. The expression of GpA can be found on mature erythroid precursor cells, while stem cells, myeloid cells, lymphoid cells and early erythroid precursor cells do not express GpA *(Nakahata et al., 1994).* The GpA-cell fraction, in which caspase-3 cleavage and activation could mainly be detected, contained all of these latter bone marrow cells. PARP cleavage in RARS GpA+ cells immediately after cell separation underlined the importance of spontaneous erythroid progenitor cell apoptosis as a cause for the severe anaemia in these patients. We also showed *(Hellström-Lindberg et al., 2001)* that apoptosis in RARS was most likely initiated already at the stem cell level and that it was possible that GpA+ cells were too differentiated to provide informative data about the process of apoptosis in the erythroid lineage. This may explain the reduced procaspase-8 bands in the erythroid GpA+ compartment of RARS cells immediately after cell separation. Because of these differences in protein expression in the different MNC subfractions, ongoing studies in our laboratory attempt to culture relatively homogenous cell populations out of CD 34+ stem cells that can be used for further investigations *(Forsblom et al., 2001).*

Since protein detection by Western blot is qualitative analysis, it is difficult to draw quantitative conclusions from these analyses. Therefore studies using different analytical methods and the analysis of cells at various stages of differentiation are necessary to understand the differences in caspase expression and processing among the different bone marrow cell

fractions.

Caspase enzyme assay and TUNEL results: MNC from RARS patients showed a higher sensitivity to Fas-agonistic antibodies as measured by the enzyme assay and visualised by TUNEL staining, but the f(ab)´2 antagonistic antibodies did not prevent apoptotic events. The general efficiency of f(ab)´2 antibody fragments to block Fas-mediated apoptosis has been observed by other groups *(Dhein et al., 1995* and *Müller et al., 1997)*. In our experiments binding of the f(ab)´2 fragment to the Fas receptor was proven by flow cytometry analysis (data not shown). Thus, f(ab)´2 antibody binds effective to Fas, but fails to inhibit the increased spontaneous apoptosis suggesting that Fas-L/ Fas interaction was not the main reason for increased apoptosis in RARS. Although Fas up-regulation in MDS has been discussed as a cause for the high degree of apoptosis in MDS *(Fontenay-Roupie et al., 1999),* only three of nine RARS patients showed a high Fas expression on GpA+ cells in that study. A higher susceptibility of bone marrow cell from RARS patients to Fas ligation as shown in our experiments may be explained by pathogenic mechanisms located **downstream** of the Fas receptor. Iron-overloaded mitochondria may trigger caspase-3 activation via the mitochondrial pathway. Although ringed sideroblasts are the characteristical hallmark for RARS, it is still unclear whether iron-overloaded mitochondria may increase the sensitivity of erythroid progenitor cells to undergo apoptosis. It is possible that mitochondrial dysfunction leads to the high degree of spontaneous apoptosis and thereby to a pathological response to apoptotic triggers like the Fas-agonistic antibodies.

Results obtained through caspase assay confirmed the Western blot data indicating that active caspase-3 is mainly processed in the GpA- negative cells. Glycophorin A is a surface marker for late erythroid progenitor cells. As shown by Gregoli *et al.* caspases are downregulated under erythroid differentiation and this could be one explanation for the low caspase activity in the GpA+ samples *(Gregoli et al., 1999)*. On the other hand, high haemoglobin concentrations interfered with the fluorescent measurement method. Other analytical methods might therefore be required for more reliable results on the activation of caspases in the GpA sorted cell fractions.

6.4. G-CSF inhibits Fas-triggered apoptosis in RARS bone marrow cells

6.4.1. G-CSF reduces caspase-8 and caspase-3-like enzyme activity

Using the substrate IETD-AMC, initiator caspase-8 activity was measured in the MNC after 4 and 24 hours of incubation. At both time points the activity increased after incubation with Fas agonistic antibodies in patients and controls and was significantly higher in the patients than in the controls ($p= 0.03$ at 4 hours). Addition of G-CSF to the cultures reduced Fas-induced caspase-8 activity in three patients tested (t-test: $p= 0.11$, non-parametric Wilcoxon rank test: $p= 0.07$ at 4 hours), while one patient showed more or less the same activity. In the control cultures no significant effect could be observed *(Figure 6.10)*.

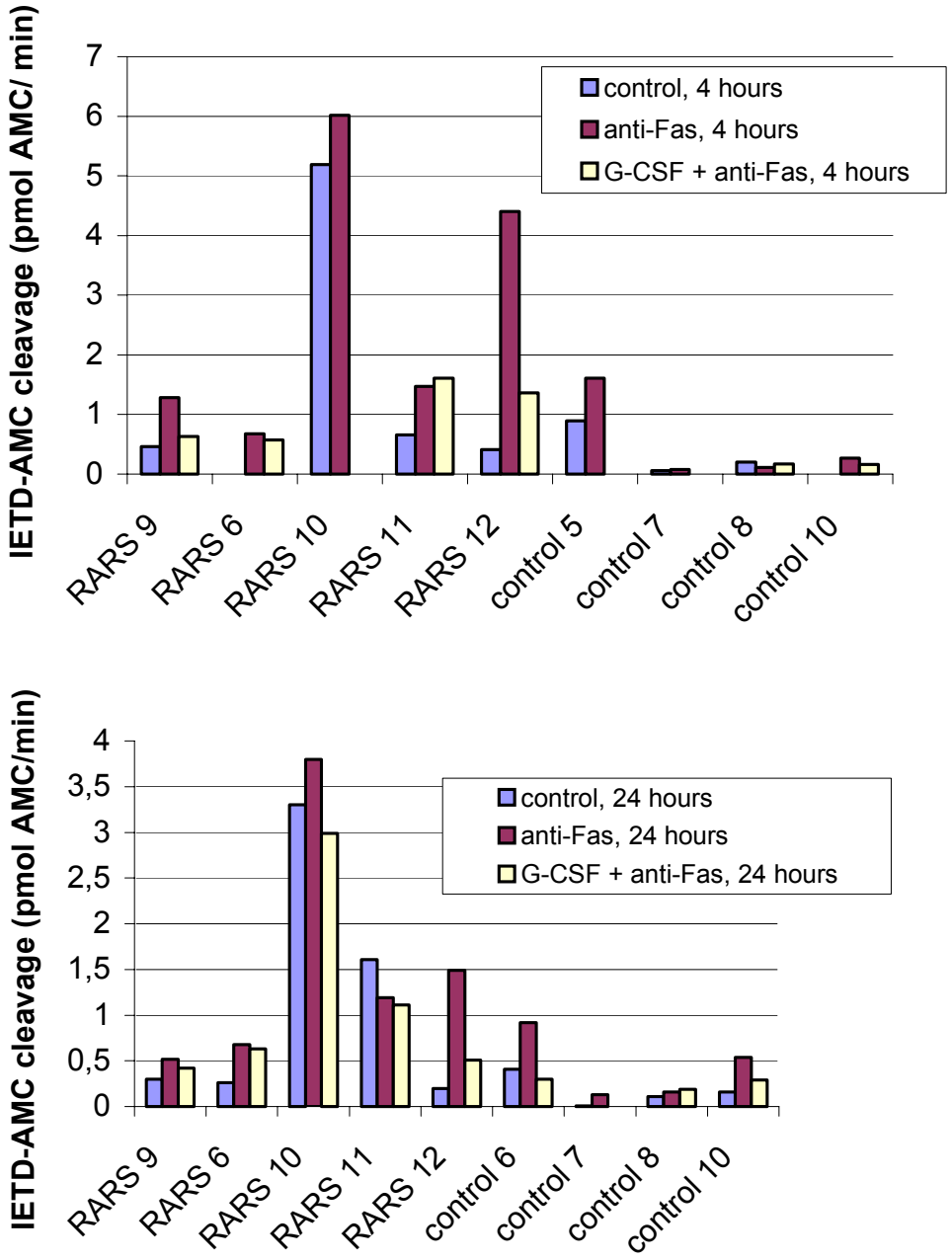


Figure 6.10. Caspase-8 activity in untreated cells and cells incubated with Fas-agonistic antibodies in the presence or absence of G-CSF*. Caspase activity of MNC was determined after 4 and 24 hours by the cleavage of the fluorescent tetrapeptide substrate IETD-AMC as described in Material and Methods.*

DEVD-AMC was used to estimate the effector caspase-3-like activity at 4 and 24 hours of culture. After 4 hours of Fas incubation, the patients´ MNC showed a significantly higher increase in this activity than control MNC (p= 0.0007) *(Figure 6.11).* Addition of G-CSF

significantly reduced caspase-3-like activity in the RARS cultures (p=0.02). The control samples showed a similar tendency. In analogy with the data after 4 hours of culture, an increase in caspase-3 like activity after Fas stimulation and an inhibiting effect of G-CSF addition was seen after 24 hours of culture, although the increase after Fas stimulation was less pronounced *(Figure 6.11)*.

The caspase-3-like activity data could be confirmed by immunoblotting with an anti-caspase-3 antibody that detects the 32 kDa proform as well as the 17 kDa subunit which corresponds to the active form of caspase-3 *(Figure 6.12)*. Addition of Fas antibodies to the cultures resulted in an increase in the amount of the 17 kDa cleavage band. This increase was completely prevented by addition of G-CSF.

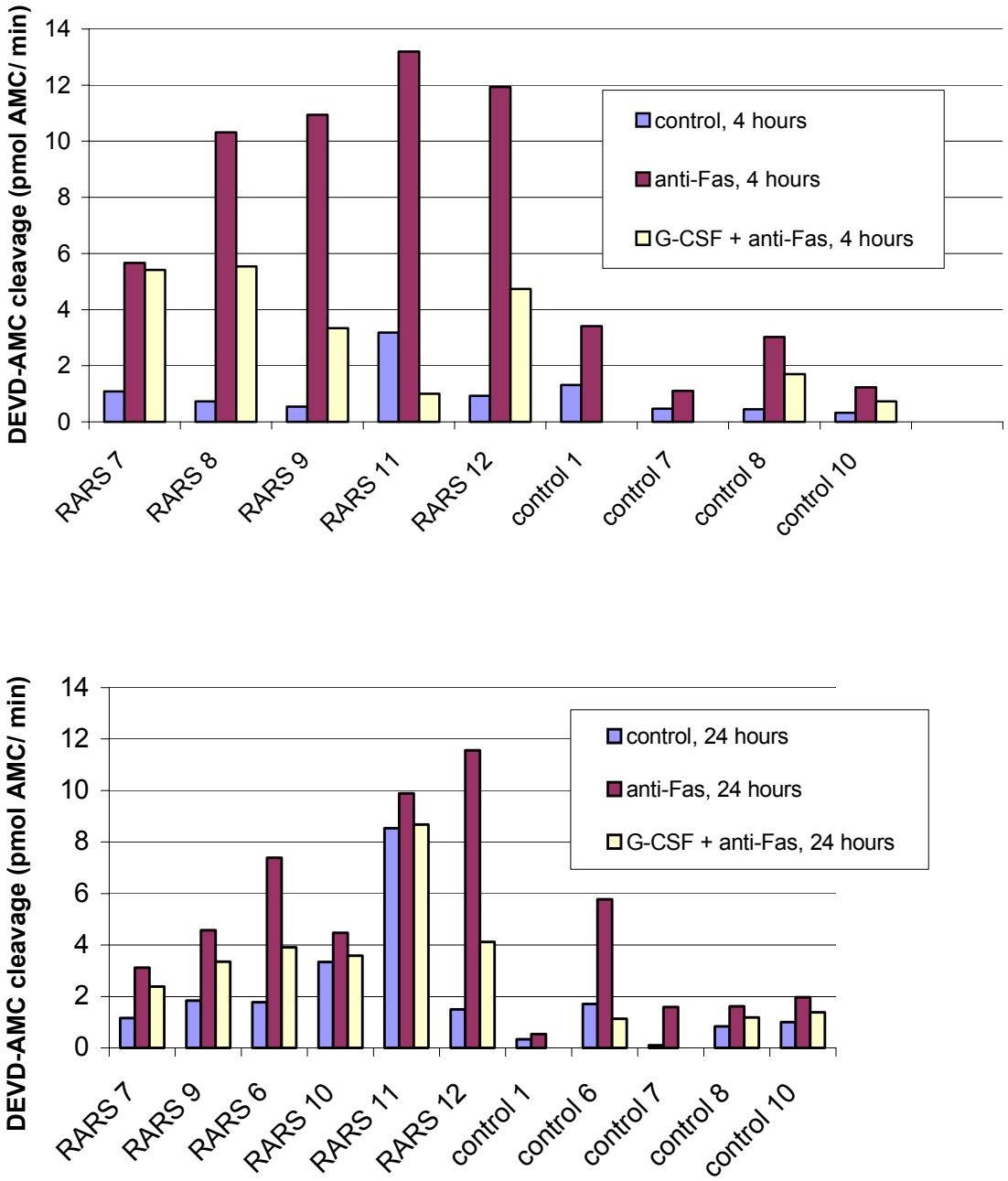


Figure 6.11. Caspase-3-like activity in MNC isolated from patients and controls*. Cells were tested for caspase-3-like activity by the cleavage of the fluorescent tetrapeptide substrate DEVD-AMC after 4 hours and 24 hours.*

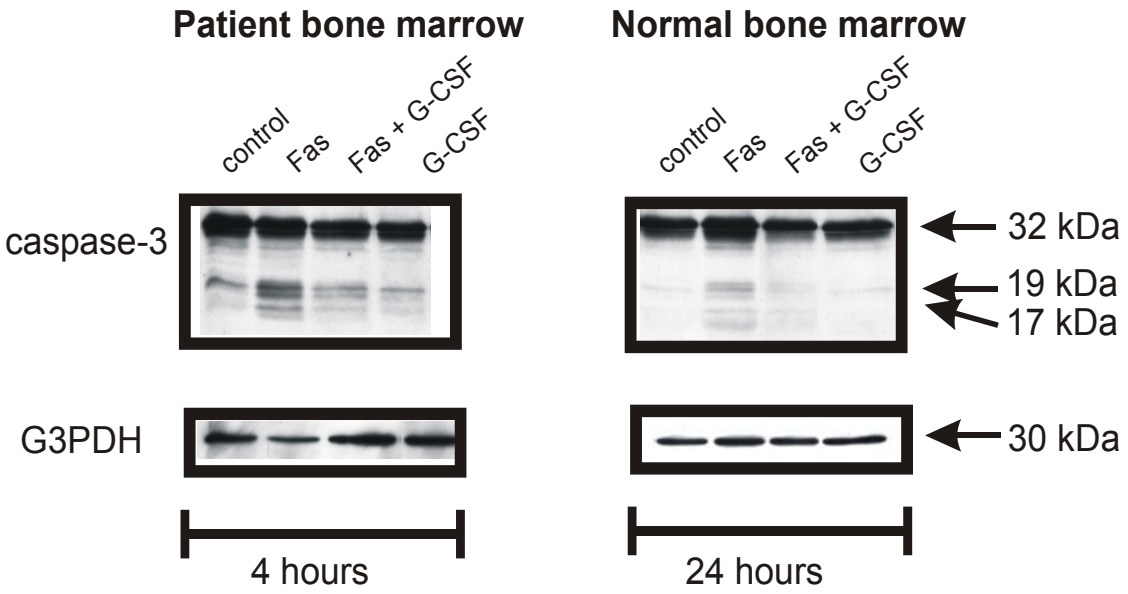


Figure 6.12. Western blot detection of the proform (32 kDa) and the active cleavage product (17kD) of caspase-3 in MNC from one patient and one control*. Patient and donor cells were incubated as indicated for 4 and 24 hours, respectively, and harvested for immunoblotting. Membranes were reprobed with antibodies against G3PDH (30 kDa) as control for equal loading with protein. The data shown are representative of the results obtained with immunoblotting of 3 patient and 2 control cell samples.*

6.4.2. Mitochondrial changes are late events in Fas-induced apoptosis of GpA negative cells and are prevented by G-CSF (2 patients, 2 controls)

Late erythroid, GpA-selected cells were difficult to analyse by flow cytometry due to pronounced heterogeneity. In all cultures of GpA-negative cells a drop of the mitochondrial potential $\Delta\psi_m$ and an increase of ROS was detected, indicative of spontaneous apoptosis of these cells during *in vitro* culture. Prior to culture a mean value of 97% (range 95.5- 98.1) of cells were gated as cells with a high mitochondrial potential and low concentration of intracellular ROS. *Figure 6.13* and *Figure 6.14* show the data obtained from one RARS patient and one normal control, respectively. Cells gated in the right lower quadrant of the dot plot diagrams were interpreted as cells, which retain a high mitochondrial transmembrane potential and do not generate ROS. The percentage of these cells decreased slowly in all tested samples in a time-dependent manner to 96% (range 95.6- 96.4) after 4 hours, 90% (range 84.8- 93.3) after 15 hours and 89% (range 84- 93.61) after 40 hours *in vitro* culture. *Table 6.7* shows this decrease of viable cells for all 4 different culture conditions of cell obtained from one RARS patient. G-CSF addition alone did not influence the loss of transmembrane potential and the increase of ROS at

later time points. Specific conclusions about GpA+ cells could not be drawn because of heterogeneity described above. Therefore it was technically impossible to show the possible functional influence (regarding to apoptotic processes) of the iron overload on the erythroblasts. More studies are required to further elucidate whether the mitochondrial iron overload has an importance influence on apoptosis of erythroid progenitor cells in RARS.

The loss of mitochondrial potential and increase of ROS in GpA- negative cells after Fas ligation were late events in the apoptotic process compared to the early increase of caspase activity discussed above. After 15 hours of incubation a minor decrease of the potential in the Fas treated cultures could be detected *(Figure 6.13, Table 6.7)*. Not until 40 hours of incubation, Fas ligation resulted in a significant drop in potential and an increase of ROS compared to the control position *(Figures 6.13- 6.15, Table 6.7)*. Addition of G-CSF partially prevented the changes caused by death receptor ligation *(Figure 6.15)*.

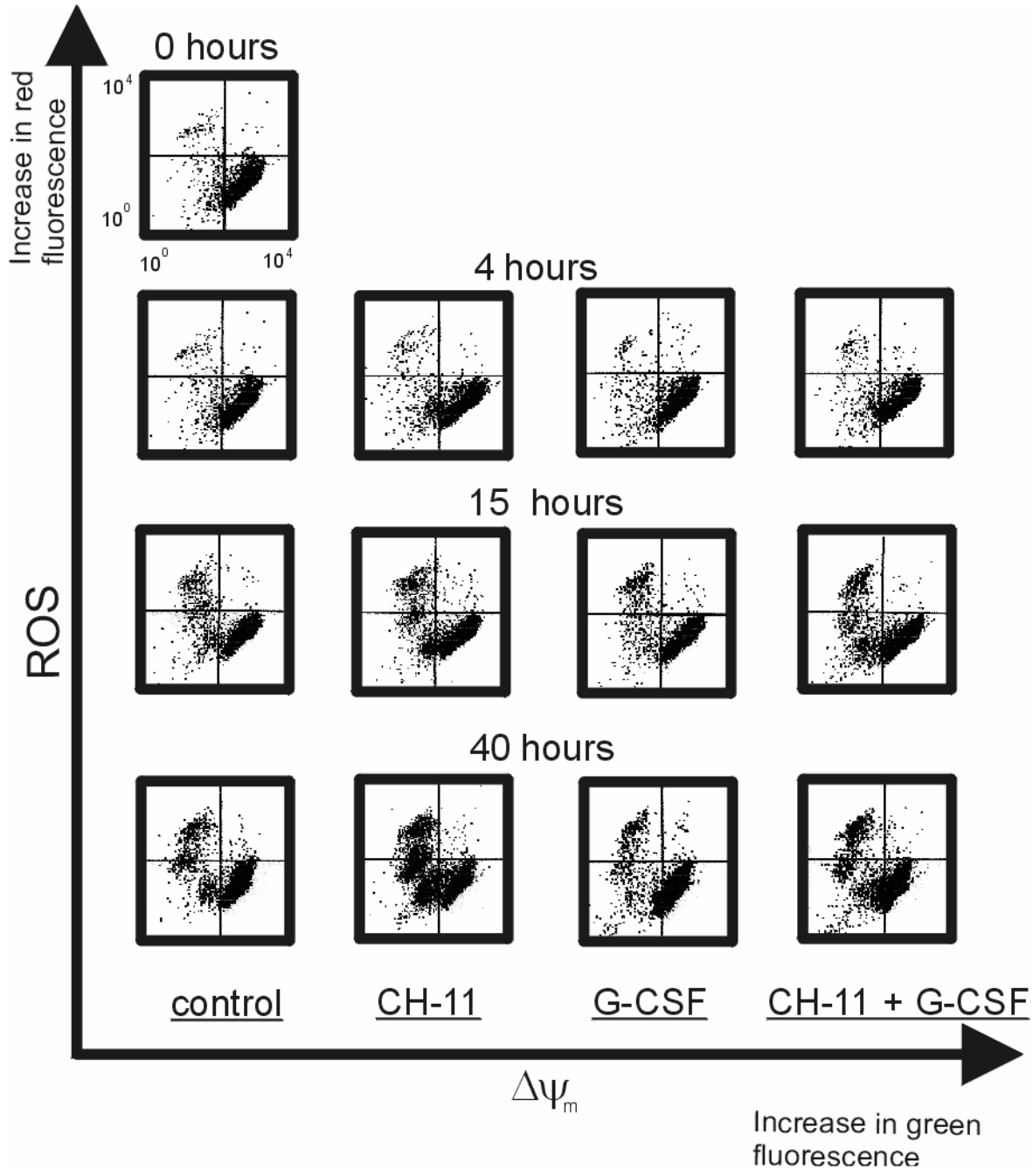


Figure 6.13. Dissipation of the mitochondrial potential and generation of reactive oxygen species in GpA- cells.

Representative data obtained in one RARS patient in which GpA- cells were tested simultaneously for dissipation of mitochondrial transmembrane potential ($\Delta\psi_m$) and production of reactive oxygen species (ROS) as described in Material and Methods.

Time of incubation	% viable cells			
	Control	CH-11	G-CSF	G-CSF + CH-11
0 hours	96			
4 hours	96	95	96	95
15 hours	93	88	93	89
40 hours	89	69	86	81

Table 6.7. Percentage of viable cells (of the same patient as shown in Figure 6.13) defined as cells which retain their $\Delta\psi_m$ and do not generate ROS after 0, 4, 15, and 40 hours incubation with FCS alone (control), CH-11 or G-CSF + CH-11. *These cells are gated in the lower right quadrant of the dot plot diagrams (Figure 6.13).*

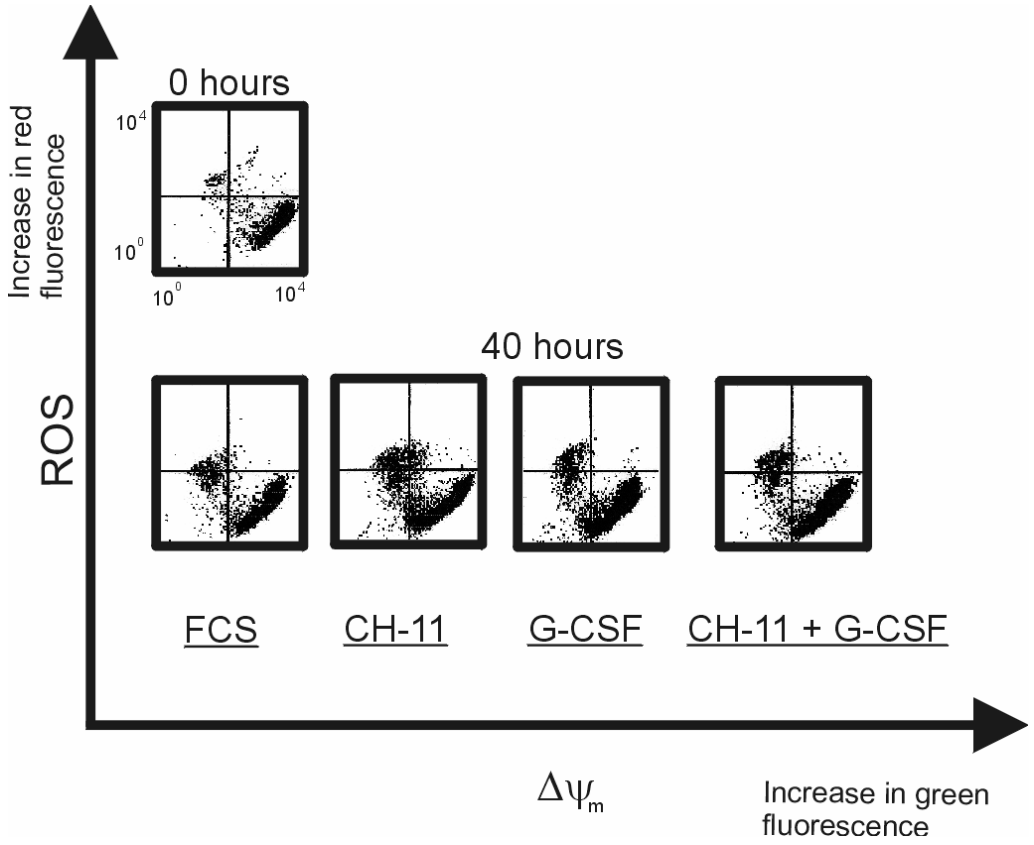


Figure 6.14. Dissipation of the mitochondrial potential and generation of reactive oxygen species in GpA- cells.
Representative data obtained in one normal control in which GpA- cells were tested simultaneously for dissipation of mitochondrial transmembrane potential ($\Delta\psi_m$) and production of reactive oxygen species (ROS).

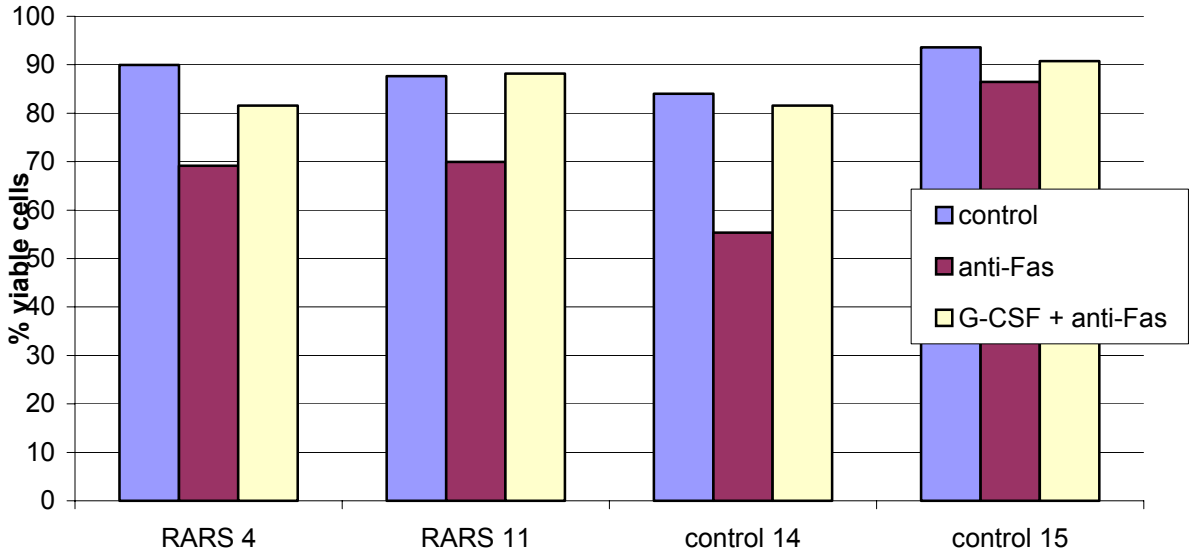


Figure 6.15. Dissipation of the mitochondrial transmembrane potential and generation of reactive oxygen species in GpA- cells.

Percentage of viable cells (2 patients, 2 controls) defined as cells which retain their $\Delta\psi_m$ and do not generate ROS after 40 hours incubation with FCS alone (control), CH-11 or G-CSF + CH-11.

6.4.3. Fas-induced nuclear DNA fragmentation is significantly enhanced in RARS and is reduced by G-CSF

Nuclear DNA fragmentation, as determined by TUNEL positivity, was higher in the patient samples than in the normal controls immediately after cell separation (p= 0.03) (*Figure 6.16*).

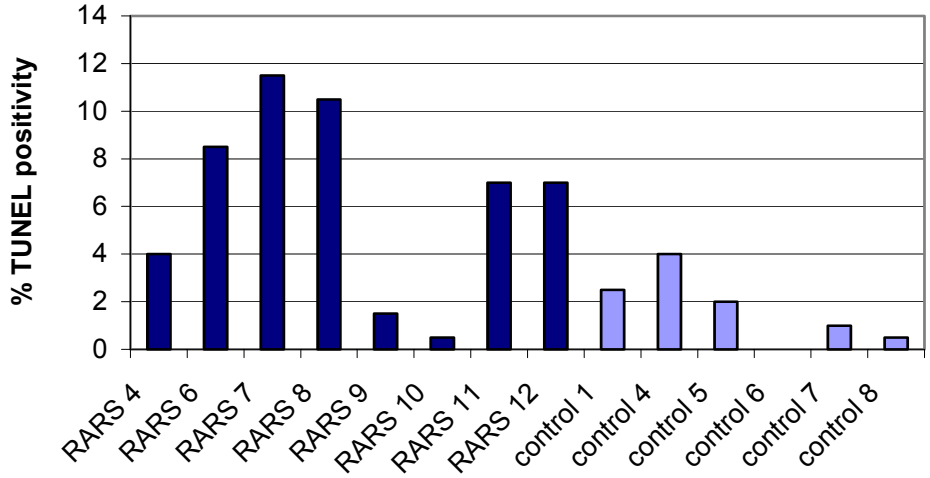


Figure 6.16. TUNEL positivity directly after cell separation*. In control 6 no apoptotic cells were found.*

TUNEL results at day 4 showed significantly more Fas-induced apoptosis in RARS than in normal MNC (p= 0.01, *Figure 6.15).* Mean Fas-induced TUNEL positivity at this time point was 23.8 ± 7.1% for patients and 13.2 ± 5.6% for controls. The addition of G-CSF reduced Fas-induced apoptosis to the same extend in RARS and control samples (p= 0.02 for all samples; p=0.09 for RARS; p= 0.08 for control samples) *(Figure 6.17).* Hence, results obtained with the TUNEL assay confirmed other apoptotic parameters (caspase activation and mitochondrial alterations) as described above.

However, TUNEL positivity in suspension cultures does not become clearly detectable until after several days. At this time point, secondary culture phenomena may present, which may explain the fact that TUNEL is a less specific method.

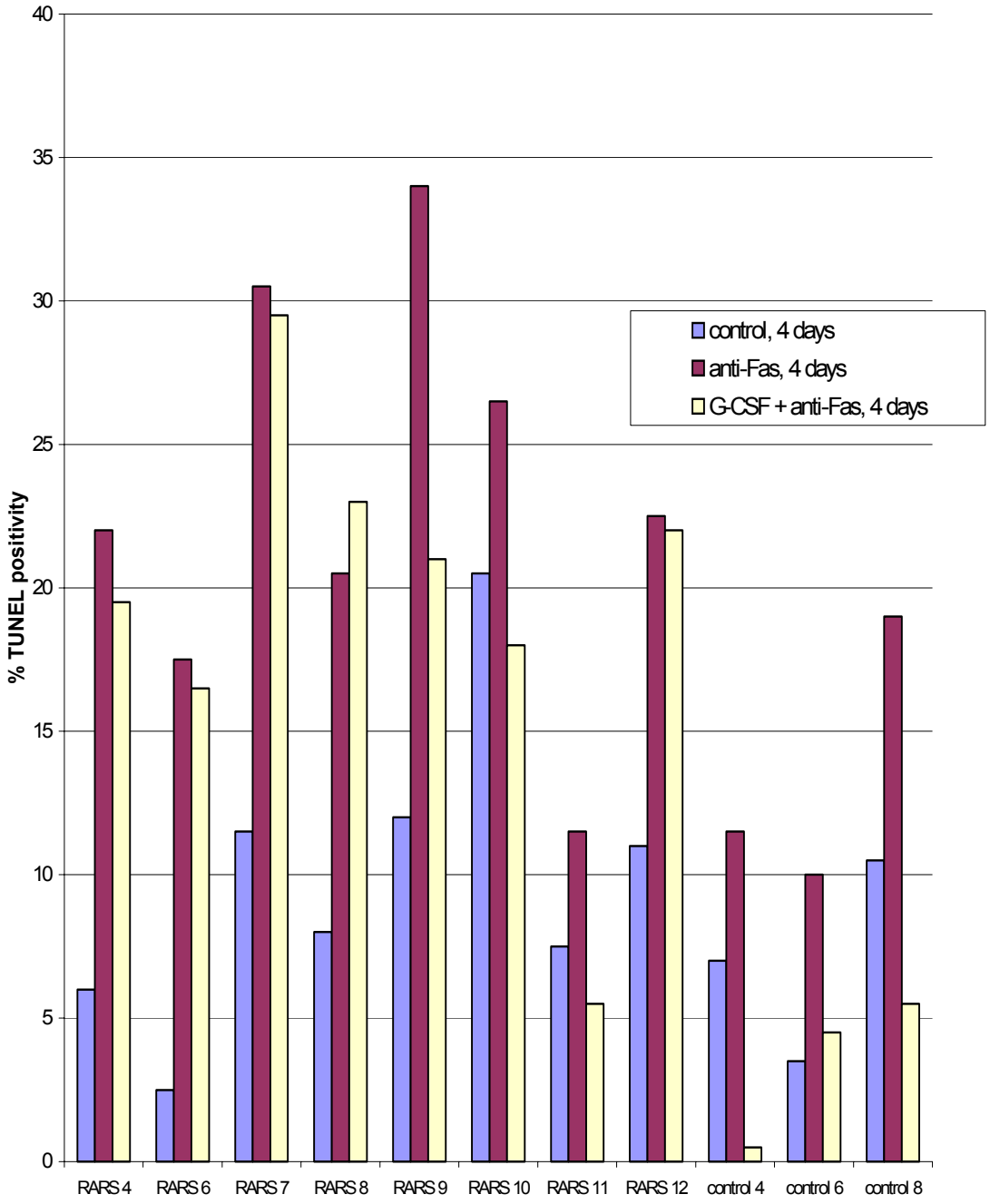


Figure 6.17. Nuclear apoptotic changes in RARS and control bone marrow cells after 4 days in culture*. The percentage of apoptotic cells displaying nuclear DNA fragmentation as determined by TUNEL staining are given for 8 patients and 3 normal controls.*

6.4.4. G-CSF promotes proliferation of Fas-treated RARS and normal donor MNC

In the 24-hours time interval after the start of the cultures, Fas agonistic antibodies reduced cell proliferation in 5 out of 8 patients, whereas no change occurred in 1 and an increase of proliferation in 2 out of 8 patients, as determined by H^3-thymidine incorporation. In 7 out of 8 patients, G-CSF induced proliferation. The combination of Fas agonistic antibodies and G-CSF again induced proliferation in all patients. Fas ligation decreased MNC proliferation in 3 out of 6 controls, while no change occurred in 2 out of 6 and an increase in 1 out of 6 control MNC. The combination of G-CSF and Fas agonistic antibodies induced proliferation in all four control samples. Similar changes were detected at later time points *(Figure 6.18)*.

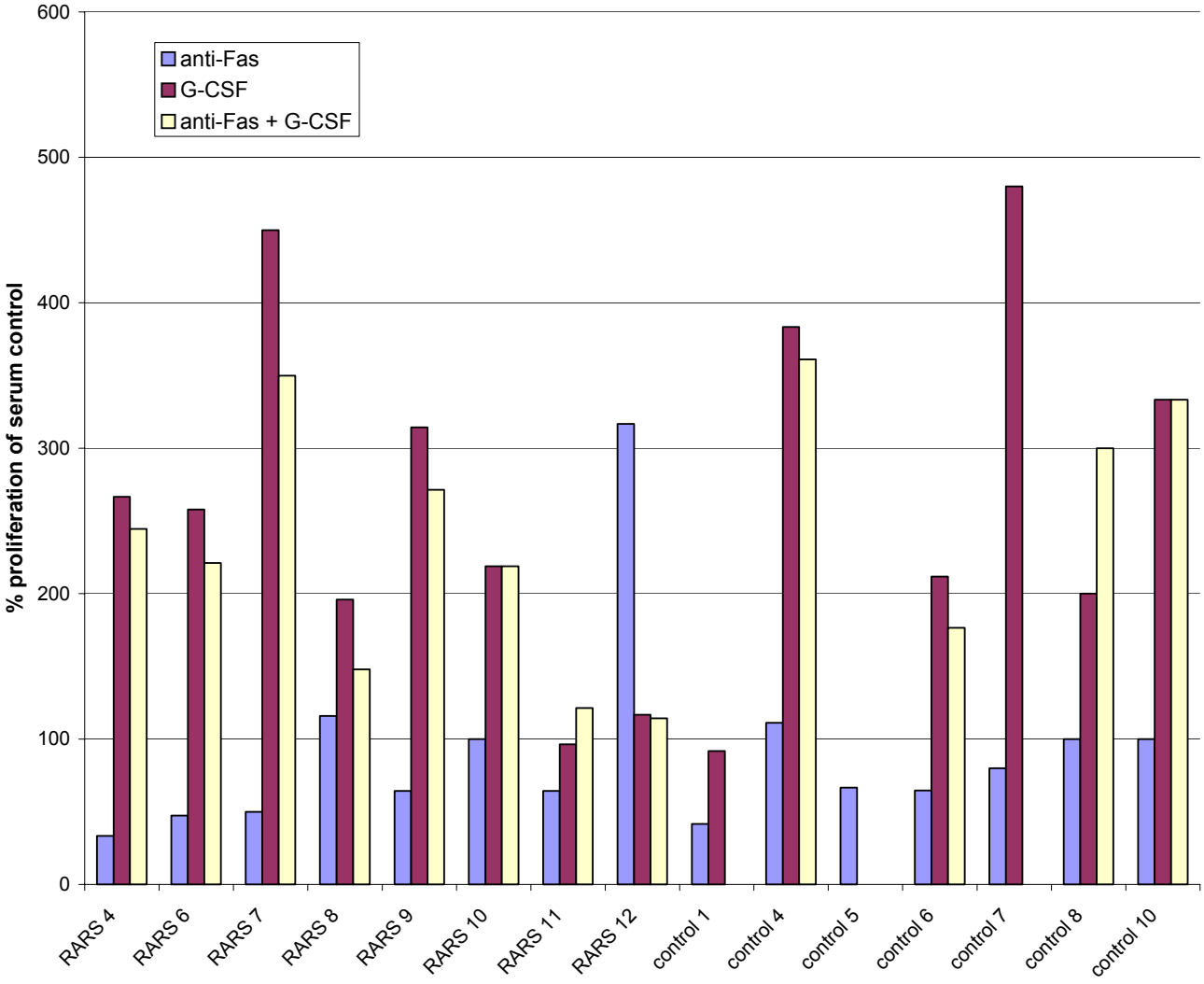


Figure 6.18. Proliferation of RARS and control bone marrow cells.
Proliferation of MNC isolated from patients and controls assessed by ^{3}H-thymidine incorporation. Proliferation of untreated MNC and MNC incubated with Fas-agonistic antibodies in the presence or absence of G-CSF was measured as described in Materials and Methods. The data shown are representative for the 24 hours time interval directly after incubation and are given as percentage of serum control. No G-CSF + anti-Fas data available in control 1 and control 7.

6.4.5. G-CSF improves erythroid colony growth in RARS

Erythroid colony growth of CD 34+ cells was reduced in 6 out of 8 patients compared to normal growth (No statistic calculations were performed, because of small numbers of colony experiments and the large variety of the results). The reduction was severe in five and slight in one *(Table 6.8)*. In 4 out of 5 patients with severely impaired colony growth, G-CSF increased the number of erythroid colonies, while no significant change was observed in the remaining patient. In contrast, in the three patients with moderately reduced or normal erythroid colony growth, exposure to G-CSF reduced the number of colonies. The number of myeloid colonies was reduced in the patients as compared to the controls (56 ± 36.4 vs 140 ± 49.7). Three of eight patients showed a G-CSF induced decrease in CFU-GM, while 5/8 increased. In all four controls G-CSF reduced erythroid colony growth, and myeloid colony growth was enhanced in 3/4 samples.

	Erythroid colonies				*Myeloid colonies*			
Patients	***Control***	***anti-Fas***	***G-CSF***	***G-CSF + anti-Fas***	***Control***	***anti-Fas***	***G-CSF***	***G-CSF + anti-Fas***
RARS 7	8	0	21	Nd	25	4	21	nd
RARS 8	83	130	70	63	106	80	94	40
RARS 9	36	49	59	100	39	22	30	14
RARS 6	44	1	66	63	39	6	78	86
RARS 10	51	21	68	26	101	40	147	93
RARS 4	270	246	180	198	72	58	98	124
RARS 11	171	72	84	59	64	34	79	50
RARS 13	6	8	7	4	2	3	15	16
controls								
control 2	200	210	178	184	200	169	218	192
control 3	140	7	54	59	79	9	10	7
control 12	244	196	132	179	133	68	211	177
control 13	296	214	256	191	146	106	267	202
Mean patients	84 ± 92	66 ± 85	69 ± 52	73 ± 63	56 ± 36	31 ± 28	70 ± 45	60 ± 42
Median patients	47.5 (range 6-270)	35 (0-246)	67 (7-180)	63 (4-198)	51.5 (2-106)	28 (3-80)	78.5 (15-147)	50 (14-124)
Mean Controls	220 ± 66	157 ± 100	155 ± 85	153 ± 63	140 ± 50	88 ± 67	177 ± 114	145 ± 92
Median Controls	222 (range 140-296)	203 (7-214)	155 (54-256)	181.5 (59-191)	139.5 ± (79-200)	87 ± (9-169)	214.5 (10- 267)	184.5 (7-202)

Abbreviations: nd, not determined

Table 6.8. Colony formation in 8 RARS patients and 4 controls

6.4.6. Discussion

Recombinant human granulocyte colony factor (G-CSF) is the drug of choice for treatment of severe congenital and cyclic neutropenia. Up to 95% of these patients will respond to G-CSF treatment with normalisation of blood neutrophil levels (for review: *Welte et al., 1997)*. However, in MDS increased or normalised haemoglobin levels in response to EPO and especially to combined G-CSF and EPO therapy has been demonstrated in a number of clinical studies *(Mantovani et al., 2000; Remacha et al., 1999; Hellström-Lindberg et al., 1998; Hellström-Lindberg et al., 1997* and *Hellström-Lindberg et al., 1993)*. The combined G-CSF and EPO treatment is especially successful in patients with the RARS subtype of MDS. In the present work, addition of G-CSF *in vitro* inhibited caspase activation, the decrease in mitochondrial transmembrane potential $\Delta\psi_m$, and the nuclear apoptotic changes after Fas ligation of RARS and control MNC. In patients with severely impaired erythroid colony growth, G-CSF also increased the number of erythroid colonies grown from CD 34+ cells, opposite to what was observed in normal CD 34+ cells.

Recently, we have shown that the tetrapeptide caspase-3 inhibitor DEVD-fmk (10 μM) reduced apoptosis, increased proliferation and enhanced erythroid colony growth in RARS patient cells *(Hellström-Lindberg et al., 2001)*. It still remains unclear, however, whether the inhibition of caspase activity leads to survival of clonogenic cells or not. Vercammen *et al.* suggested that blocking of apoptosis by z-VAD-fmk might direct the cells towards a necrotic cell death *(Vercammen et al., 1998)*, while Ko *et al*. suggested that caspase inhibition using z-VAD-fmk may rescue cells from apoptosis and maintain their viability and clonogenic potential *(Ko et al., 2000)*. In our experiments, the inhibition of caspase activation by G-CSF blocked Fas-induced apoptosis and the cells maintained their ability to proliferate. Interestingly, G-CSF blocked both caspase-8 and caspase-3-like activity in anti-Fas treated MNC suggesting that it inhibited the caspase cascade at a step prior to the activation of caspase-8. A similar reduction in initiator and effector caspase activity was recently shown for GM-CSF treated mature neutrophils *(William et al., 1999)*.

In addition, we found that the loss of the mitochondrial transmembrane potential $\Delta\psi_m$ and the increase of ROS in GpA-negative cells were rather late events as compared to the activation of caspases. Scaffidi *et al.* have shown that some cell types can undergo Fas-induced apoptosis independently of mitochondrial alterations *(Scaffidi et al. 1999)*. Due to technical difficulties we

were unable to use GpA-positive cells for FACS analysis and cannot at present provide data concerning mitochondrial changes in the actual cell population that displays morphological changes due to iron overloading. This remains to be elucidated in future experiments using more sensitive methods to study the role of the mitochondrial pathway in apoptosis in MDS.

Anti-apoptotic effects of G-CSF in cell line cultures have been previously described *(Hassan et al., 1999).* Philpott *et al*. have shown that G-CSF- mobilised peripheral blood CD 34+ cells are significantly less apoptotic than unstimulated peripheral blood CD 34+ cells *(Philpott et al., 1997)*. A variety of different G-CSF effects on neutrophils such as increased proliferation, adherence, phagocytosis and chemotaxis are known (for review: *Spiekermann et al., 1997)*. Furthermore, it has been shown to block apoptosis and thus prolong survival of peripheral blood neutrophils *(William et al., 1999* and *Colotta et al., 1992*). Recently, Hunter and co-workers have shown that G-CSF like many other cytokines activates the anti-apoptotic PI3-kinase, of which the downstream targets have not yet been discovered *(Hunter et al., 2000).* The same kinase is activated by EPO *(Uddin et al., 2000)*. These observations thus provide a tentative synergistic effect of combined cytokine therapy as compared to the exclusive use of either G-CSF or EPO. Furthermore, EPO may induce the expression of the anti-apoptotic protein Bcl-X_L *(Gregory et al., 1999* and *Silva et al., 1996)* while G-CSF inactivates the pro-apoptotic protein Bad *(Hunter et al., 2000)* and downregulates Bax *(Dibbert et al., 1999).* The work by Takahashi *et al.* confirms that there is no cross-reaction between Fas and the G-CSF receptor that could explain the reduced caspase activation *(Takahashi et al., 1996)*. In addition, our own results indicate that G-CSF does not downregulate Fas expression (data not shown).

7. Conclusion

The results of this thesis summarise our work at the Karolinska Institutet, its proceeding and its problems. The basal aim of this study was to understand more about apoptosis in RARS and its underlying cause(s). Methods used to investigate apoptosis in cell lines had to be adapted for MNC from RARS patients. The first experiments were focused on detection of Bcl-2 expression and caspase processing by Western blot technique. The caspase data were confirmed by a fluorometic enzyme assay. The role of the Fas system in the induction of apoptosis in RARS bone marrow was estimated by addition of Fas-agonistic and -antagonistic antibodies in MNC cultures. In the last part, our interest moved to the effects of the cytokine G-CSF on apoptosis when added to bone marrow cultures.

Bcl-2 expression was decreased in MNC of RARS patients while apoptosis in patient cells was significantly increased as measured by caspase enzyme and the TUNEL assays. Caspase activation was mainly observed in GpA-negative cells while late erythroid progenitor cells, which express GpA, revealed less activity.

Although cells from patients showed an increased sensitivity to Fas-agonistic antibodies, Fas-antagonistic antibodies were not able to block the spontaneous apoptotic process. After Fas ligation of RARS and control MNC, addition of G-CSF *in vitro* inhibited caspase activation, the drop of $\Delta\psi_m$, and nuclear apoptotic morphology. In patients with severely impaired erythroid colony growth, G-CSF also increased the number of erythroid colonies.

Our experiments gave no reason to believe that Fas-L/ Fas interaction was the main pathological mechanism for apoptosis in RARS as discussed by other groups *(Fontenay-Roupie et al., 1999).* A higher susceptibility of bone marrow cell from RARS patients to Fas ligation as shown in our experiments may be explained by pathogenic mechanisms located **downstream** of the Fas receptor. Controversial data about the causes(s) for apoptosis in RARS and difficulties in understanding of the apoptotic process might indicate that there are multifactorial causes that trigger apoptosis in RARS and in MDS, in general. Apoptosis could be a part of the clonal abnormalities inherent to myelodysplasia (by mitochondrial changes?) and simultaneously it might be triggered by other factors like a pathological immune response and altered cytokine levels (INF-α, TNF-α, TNF-β).

Addition of G-CSF *in-vitro* cultures inhibited caspase activation, the decrease in the mitochondrial transmembrane potential and the nuclear apoptotic changes after Fas ligation of RARS and control MNC. G-CSF treatment in combination with EPO may constitute a promising anti-apoptotic and erythrogenic therapy in some RARS patients. Future studies should aim a better characterisation of the patient subgroup, which benefits most from this expensive treatment, and at a better understanding of the apoptotic triggers. Further understanding of the anti-apoptotic effects of G-CSF may result in refined hypotheses about how to improve effective erythropoiesis in MDS.

Many questions remain still open. Investigations using MNC from RARS patients are difficult because of the heterogeneity of this cellular material. E.g. measurement of cell potential and ROS production revealed 3 different populations in the GpA+ cell fraction by forward and sideward FACS scatter analysis (data not shown). Further *in vitro studies* using well-defined bone marrow subpopulations may be a suitable model to characterise cause(s) for apoptosis in MDS and to find specific antiapoptotic treatments. As indicated by the different responses to treatment, the pathological reason of distinct MDS subtypes might differ from each other. Restriction to well defined patient subgroups may help to decrease result heterogeneity.

8. Literature update December 2002

While I was attending my final year of medical studies in Germany, the following articles have been being published focussing on apoptosis and mitochondria in MDS, which somewhat influences my previous conclusions.

Results obtained by the MDS Research group at the Karolinska Institutet (Department of Medicine, Division of Haematology, Huddinge University Hospital and Institute of Environmental Medicine, Division of Toxicology) in Stockholm *(Tehranchi et al., 2002)* indicate that 50% of erythroid progenitor cells derived from MDS "low risk" patients exhibit spontaneous release of cytochrome *c* from mitochondria with ensuing activation of caspase-9, whereas normal erythroid progenitors display neither of these features. Treatment with G-CSF significantly inhibited cytochrome *c* release and suppressed apoptosis, most noticeably in erythroblasts from sideroblastic anaemia patients. Furthermore, inhibition of capase-9 suppressed both spontaneous and Fas-mediated apoptosis of erythroid progenitors in all low-risk MDS cases studied. These results are concordant and further support to the data described in this thesis. However, the reason for the increased cytochrome *c* release in MDS cells and the anti-apoptotic effects of G-CSF are still unclear. Further investigations are needed.

Especially in RARS erythroblasts, Cazzola and co-workers have shown an intronless ferritin gene that encodes a mitochondrial ferritin with ferroxidase activity *(Cazzola et al., 2002).* This mitochondrial ferritin is almost exclusively expressed in ring sideroblasts and may represent a specific marker of sideroblastic anaemia while diffuse cytoplasmic ferritin expression could be found in normal and MDS erythroblasts. Furthermore, there exists a significant relationship between the percentage of mitochondrial ferritin positive erythroblasts and that of ring sideroblasts. Which influence this mitochondrial ferritin overload has on mitochondrial functions and apoptosis is not known.

In contrary to the results described in this thesis, Claessens and co-workers *(Claessens et al., 2002)* found an evidence for Fas-dependent apoptosis in erythroid progenitors from MDS patients. In *in vitro* cultures, a dramatical increase of the Fas-L under culture conditions was detected on MDS erythroblasts as well as a higher expression of the Fas receptor in MDS stem cells than in normal controls. Furthermore, anti-Fas-ligand added to the cultures decreased apoptosis up to 50% in MDS cultures suggesting that Fas and its ligand play a major role on apoptosis in MDS cells. In my opinion, the role of the Fas system in MDS is still unclear. The

differences in the results may be explained by different culture methods. To evaluate the influence of the Fas system on apoptosis in RARS, it has to be investigated whether Fas-antagonistic antibodies are able to block the increased cytochrome *c* release in RARS erythoblasts cultures as Tehranchi *et al.* have shown for G-CSF.

The impact of mitochondrial DNA mutations is a matter of ongoing controversy. While Reddy *et al. (Reddy et al., 2002)* claim that there exist an increased incidence of mitochondrial cytochrome *c*-oxidase gene mutations in patients with MDS, Shin and co-workers were not able to confirm these described mutations in sidoerblastic anaemia, nor "hot spots" in cytochrome *c* oxidase I and II genes *(Shin et al., 2002)*. Their data do not support a major role for mitochondrial genomic instability in myelodysplasia.

Boudard et al. investigated the expression of Bcl-2 family members in MDS *(Boudard et al., 2002)*. In concordance to former results published by other groups *(Parker et al. 2002* and *Parker et al. 1998)*, an increase of Bcl-2 and Bcl-X_l in high risk MDS was found while low-risk MDS cases expressed a higher ratio of Bcl-X_S/ Bcl-X_l.

9. Acknowledgements

I would like to thank especially Associate Professor **Eva Hellström-Lindberg** who initiated my work and supported me from the beginning. This thesis and my work in all their different developmental stages were critically revised by her. Special thanks also to Professor **Boris Zhivotovsky** spending his time to introduce me (as a person who has never worked in a laboratory before) into the scientific field of apoptosis and its research methods. Last but not least, special thanks also to Professor **Arnold Ganser** from the Medizinische Hochschule Hannover who spontaneously gave his support for my work. His careful reviewing of this thesis helped me much to improve quality and content of the text.

Following experimental parts of this thesis were **not** performed by me: CD 34 colony assays, TUNEL studies, and proliferation assays. I would like to thank the co-workers Ann Mari Forsblom and Ramin Tehranchi who mainly performed these experimental parts.

At the haematology laboratory at Huddinge University Hospital I also received much help from Ann Mari Forsblom who never lost control over all different bone marrow cultures and different storage and investigation time points and who was a good teacher for the isolation and culture of primary bone marrow cells. Dr. Bertrand Joseph was very helpful in solving my daily laboratory and computer problems at the Institute of Environmental Medicine and introduced me to the work with the FACS machine. My manuscripts were critically revised by Dr. Afshin Samali, Dr. Joya Chandra, who also organised exotic dinner parties, and Dr. John Robertson who is the original author of Figures 3.1 and 3.2 which were - with some minor changes - shown in the introduction of this thesis. Furthermore, I would like to thank Ramin Tehranchi who finished the last part of the G-CSF experiments while I continued my medical studies at Heidelberg University, Dr. Bengt Fadeel for his assistance in the G-CSF manuscript, and Dr. Vladimir Gogvadze who introduced me to the gym at Karolinska Institutet and the Pizzeria Al Forno in the city. Thanks also to all other ”Blue Group members” at the Institute of Environmental Medicine for a nice working atmosphere and their help.

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Tack så mycket!

Stockholm, Sweden, January 2003

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11. Lebenslauf in deutscher Sprache und Liste der Veröffentlichungen

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Stipendien:

- Forschungsstipendium der Cancer Society in Stockholm (98:112).

- Reise- und Konferenzstipendium für die EuroConference, "*New Cellular Targets of Cytotoxic Mechanisms*", April 2000, San Feliu de Guixols, Spanien

- Reise- und Konferenzstipendium für das Wilsede Meeting "*Modern Trends in Human Leukaemia*", Juni 2000, Wilsede, Deutschland

- PJ- Auslandsstipendium der Vereinten Krankenversicherungs AG

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Wissenschaftliche Veröffentlichungen:

(1) Schmidt-Mende J, Hellström-Lindberg E, Joseph B and Zhivotovsky B: Freezing induces artificial cleavage of apoptosis-related proteins in human bone marrow cells. *J Immunol Methods 2000; 245: 91-94*

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

(3) **Schmidt-Mende J**, Tehranchi R, Forsblom AM, Joseph B, Christensson B, Fadeel B, Zhivotovsky B and Hellström-Lindberg E: Granulocyte colony-stimulating factor inhibits Fas-triggered apoptosis in bone marrow cells isolated from patients with refractory anemia with ringed sideroblasts. *Leukemia 2001; 15: 742-751*.

Posterpräsentationen:

(1) Hellström-Lindberg E, **Schmidt- Mende J**, Backman Johansson C, Forsblom AM, Fadeel B and Zhivotovsky B. Erythroid Apoptosis in Ringsderoblastic anemia (RARS): Influence of Fas antibodies, Caspase inhibition and G-CSF *(poster presented at the conference of the American Scociety of Hematology, December 1999, New Orleans, USA).*

(2) Schmidt-Mende J, Hellström-Lindberg E, Forsblom AM, Fadeel B and Zhivotovsky B. Caspase-3-like acitivity and apoptosis in patients with rinsideroblastic anemia(RARS): Influence of Fas-agonistic and antoagonistic antibodies *(poster presented at the conference "Mechanisms in Toxicity: EuroConference on New Cellular Targets of Cytotoxic Mechanisms", April 2000, San Feliu de Guixols, Spain).*

(3) Tehranchi R, **Schmidt-Mende J**, Forsblom AM, Fadeel B, Zhivotovsky B and Hellström-Lindberg E: Fas-triggered caspase activity is increased in sideroblastic anemia *(poster presented at the MDS conference, June 2001, Stockholm, Sweden).*

Vortrag:

(1) Schmidt-Mende J, Tehranchi R, Forsblom AM, Joseph B, Fadeel B, Zhivotovsky B and Hellström-Lindberg E: G-CSF inhibits Fas-triggered apoptosis in bone marrow cells isolated from patients with refractory anemia with ringed sideroblasts (RARS) (*elected oral presentation at the conference of the American Society of Hematology, December 2000, San Francisco, USA).*

Ich erkläre, dass ich die der Medizinischen Hochschule Hannover zur Promotion eingereichte Dissertation mit dem Titel “Bone marrow apoptosis in myelodysplastic syndromes” im Hämatologielabor des Huddinge Universitetssjukhus (Huddinge Universitätskrankenhaus) und am Institutet för Miljömedicin (Institut für Umweltmedizin), Karolinska Institutet, Stockholm, Schweden unter der Betreuung von Professor Eva Hellström-Lindberg und Professor Boris Zhivotovsky mit der Unterstützung durch Professor Dr. med. Arnold Ganser ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Weiterhin versichere ich , dass ich den beantragten Titel bisher noch nicht erworben habe.

Ergebnisse der Dissertation wurden in folgenden Publikationsorganen *Journal of Immunological Methods, British Journal of Haematology* und *Leukemia* veröffentlicht.

Stockholm, den 11. Januar 2003.