Biology of stem cells and myeloid progenitor cells in myelodysplastic syndromes

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Biology of stem cells and myeloid progenitor cells in myelodysplastic syndromes

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Ter nagedachtenis aan Adriana Wilhelmina (“Mientje”) Liebregts

Contents

Abbreviations

A	apoptosis or programmed cell death
ABC	avidin-biotin/peroxidase complexes
ALIPs	abnormally lokalised immature precursors
AML	acute myeloid leukemia
AnV	Annexin-V
APAAP	alkaline phosphatase antialkaline phosphatase complexes
Ara-C	Cytosine β-D-arabinofuranoside
ATG	antithymocyte globulin
Bad	Bcl-xL/Bcl-2 associated death promoter
Bax	Bcl-2 associated X gene
Bcl-2	B-cell lymphoma/leukemia-2 gene
Bcl-x	a Bcl-x isoform that inhibits PCD
BM	bone marrow
BMMNC	bone marrow mononuclear cells
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAI	caspase-inhibitors
CD	Cluster of Differentiation
CFU-GEMM	colony-forming unit – granulocyte, erythrocyte, monocyte, macrophage
CFU-GM	colony-forming unit – granulocyte, macrophage
c-myc	this proto-oncogene encodes a transcription factor (Myc) that promotes growth, proliferation and apoptosis
Cl	clusters
Cl/Co	cluster/colony ratio
Co	colonies
CPT	camptothecin
CTL	cytotoxic T lymphocyte
DAB	3,3'-diaminobenzidine tetrahydrochloride
ddH_2O	double distilled water
DMSO	dimethylsulfoxide
dUTP	deoxyuridine triphosphate
EGR1	Early Growth Response gene-1
EP	electrophoretic
FAB	French-American-British classification
FADD	Fas associated death domain (= MORT-1)
FAP-1	Fas associated phosphatase-1
Fas	CD95 or APO-1
FasL	Fas ligand
FasR	Fas receptor or Fas

Fc	Fc fragment of immunoglobulin
FC	final concentration
FCM	flow cytometry
FCM-ISEL	modified ISEL technique for FCM
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
(F)ISH	(fluorescence) in situ hybridization
G0	temporarily non-proliferating cells within G0 of cell cyclus (resting phase)
G1	the interval between mitosis (cell division) and S-phase (DNA replication) of the cell cycle
GFs	growth factors
GF	growth fraction
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
G-PBS	glucose-phosphate-buffered saline
Gy	Gray
HR	high-risk
HSC	hematopoietic stem cell(s)
hSCF	human stem cell factor
ICE	IL-1β converting enzyme
IFN	interferon
IH	immunohistochemistry
IL	interleukin
IMDM	Dulbecco's Medium, Iscove's Modification
IPSS	International Prognostic Scoring System
IRF-1	interferon regulatory factor-1
ISEL	in situ end labeling
ISH	in situ hybridization
IUdR	iododeoxyuridine
LI	labeling index or percentage cells in S-phase
LR	low-risk
LTBMC	long-term bone marrow cultures
kbp	kilobase pair
M	Molar
mAb	monoclonal antibodies
MDR1	Multidrug Resistance gene 1
MDS	myelodysplastic syndrome(s)
mFasL	membrane-bound FasL
min.	minutes
MNC	mononuclear cells
NBM	normal bone marrow

N	number (or n), or normality (for solutions)
NK cells	Natural Killer cells
Ntot	total number of aggregates
Nx	number of aggregates at day x
PBS	peripheral blood stem cell or phosphate buffer solution
PBST	peripheral blood stem cell transplantation
PCD	Programmed Cell Death
PE	plating efficiency
PI	propidium iodide
PS	phosphatidylserine
RA / RARS	refractory anemia / RA with ringsideroblasts
RAEB(t)	refractory anemia with excess of blasts (in transformation)
rhu	recombinant human
rpm	revolutions per minute
RPMI	RPMI medium was developed by Moore et. al. at Roswell Park Memorial Institute
sAML	secondary AML after MDS
SC	single cells
SCF	stem cell factor
SCSW	single-cell single-well (assay)
SD	standard deviation
SEM	standard error of mean
sFasL	soluble Fas Ligand
sFas	soluble Fas or FasR
SSC	sodiumchloride-sodiumcitrate solution
TBS	tris buffer solution
TBST	tris buffer solution with Tween 20
Tc	cell cycling time
Td	cell doubling time
TNFα	tumor necrosis factor-α
TNF-R1	tumor necrosis factor receptor 1
Ts	duration of S-phase or DNA synthesis time
TUNEL	terminal deoxynucleotidyl transferase nick-end labeling
VLA	very late antigen
WHO	World Health Organization
XCIP	X chromosome inactivation pattern(s)
Z-VAD-FMK	a cell-permeable, irreversible inhibitor of caspase-1, -3, -4, and -7
Z-DEVD-FMK	a cell-permeable, irreversible inhibitor of caspase-3, -6, -7, -8, and -10.

Chapter 1

Biology of stem and myeloid progenitor cells in myelodyplastic syndromes

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Introduction

The myelodysplastic syndromes (MDS) are hyperproliferative, acquired clonal stem cell disorders, associated with massive intramedullary apoptosis or programmed cell death (PCD). A leukemic phenotype, mainly characterized by an increase of blasts showing differentiation arrest, is gradually observed, as MDS progresses from low-risk (LR-) to high-risk MDS (HR-MDS). Three interacting compartments can be distinguished in MDS bone marrow (BM); the polyclonal, residual normal hematopoiesis, the monoclonal preleukemic compartment, and the blastic leukemic compartment. Within LR-MDS, monoclonal hematopoiesis dominates leukemic blast cell proliferation (<5%), whereas this pattern is reversing during MDS evolution to acute myeloid leukemia (AML). This shift in the balance of proliferation versus apoptosis has to be applied constantly to these three interacting compartments in MDS. For illustration, the following example and nomenclature is used. Stem Cell Factor (SCF) is a major factor to induce differentiation and to mediate the transition from the earliest CD34 negative ($CD34^-$) stem cells to the more differentiated CD34 positive ($CD34^+$) stem cells, whereas IL-6 promotes proliferation and maintains self-renewal of $CD34^+$ stem cells. The balance and interaction between these cytokines may play different roles in normal, monoclonal, and leukemic hematopoiesis *("static profile" of these compartments).* Furthermore, these cytokine levels, their receptor density, and/or their receptor-ligand interaction may change within each hematopoietic compartment as MDS progresses, also changing the balance and interaction of these cytokines between these three pools *("dynamic profile").* Genetic or phenotypic changes of the malignant pool and changing interactions with the environment (stroma or different accessory cells) also cause a complex dynamic change in different interactions (cell-cell, cell-stroma, stroma-stroma) between these three pools as MDS progresses *("complex-dynamic profile").*
This review describes these complex-dynamic profiles of stem cells and progenitors in MDS. Emphasis is put on the origin of monoclonality and its implications in MDS (Part I) and phenotypic-functional studies to understand the biology (balance of proliferation and apoptosis) of MDS in evolution (Part II). Part III of this chapter discusses the FasR/FasL system as one of the most important members of the nerve-growth factor receptor family for carrying apoptosis signal transduction.

Part I. Clonality in MDS

Dysplastic features and an increase of blasts found in hypercellular BM characterize MDS. The International Prognostic Scoring System (IPSS)[1], based

upon the percentage of marrow myeloblasts, cytogenetic characteristics, and the number of cytopenias, defines prognosis and survival of MDS patients with more accuracy than the original French-American-British (FAB) classification. MDS progression to AML is determined by further accumulation of genetic defects in the myelodysplastic clone. Early studies suggested that these aberrant clones may originate in a more committed myeloid stem cell (CFU-GEMM: colony-forming unit–granulocyte, erythrocyte, monocyte) in most patients, evaluated by immunophenotyping and (fluorescence) in situ hybridization or (F)ISH[2-4]. More recent studies proved that cytogenetically aberrant cells could also be detected in the primitive stem cell pool[5,6]. Mehrotra et al.[5] found cytogenetically aberrant cells in a primitive ($CD34^+lin^-$) stem cell compartment. The percentage abnormal cells was not associated with compartment expansion, indicating that these aberrant primitive hematopoietic cells do not show a leukemic phenotype (growth and survival advantage). This primitive compartment with high Multidrug Resistance gen 1 (MDR1) expression accounts for the high relapse rate of MDS patients treated with intensive chemotherapy and autologous BM transplantation.

In general, it is hypothesized that a first hit causes inactivation or deletion of tumor suppressor genes (e.g. Interferon regulatory factor 1 (IRF-1) and Early Growth Response 1 (EGR-1) at 5q31-33 region), DNA repair genes (at 7q22 region) or, although less frequently, activating mutations in a proto-oncogene (e.g. N-ras and its association with chromosome 7 deletions). This first hit will subsequently lead to a "controlled" growth advantage of this "damaged" stem cell and its progeny over the normal pool of stem cells[7]. MDS in preleukemic phase detects monoclonality (in females) by X-chromosome inactivation studies (see below), which may occur before the development of karyotypical abnormalities. The growth advantage of these preleukemic $CD34^+$ cells and its progeny may be caused by an increase of the number of S-phase cells with or without a substantial survival benefit[8]. This genetically altered progenitor cell pool is more prone to additional mutations or deletions ("genomic instability"). S-phase cells are more susceptible to detrimental DNA events because of their status of unpacked and uncoiled DNA and intense DNA synthesis with less DNA repair time. The final behavior of the leukemic clone in MDS is determined by its overall make-up of activated genes. Patients with balanced chromosomal translocations seem more likely to present with overt leukemia than patients with unbalanced chromosomal abnormalities[9,10].

Interestingly, patients with and without abnormal karyotype have no different levels of overall apoptosis[11]. It is therefore tempting to speculate that monoclonality by itself induces immune responses leading to overt apoptosis. This apoptotic process is also conferred to the normal, polyclonal hematopoiesis and stromal tissues as innocent bystanders (see Part II). As polyclonal blood cells are dying intramedullary, relatively more apoptosis-

resistant blood cells with normal or dysplastic appearance in the peripheral blood are found as the progeny of monoclonal hematopoiesis[12]. Furthermore, the percentage cytogenetically aberrant BM blasts is always considerably higher than the BM mature granulocytes indicating a partial maturation arrest (and decreased PCD) of monoclonal hematopoiesis in MDS[13]. Above all, anti-apoptotic therapy in MDS patients sometimes results in disappearance of cytogenetically aberrant clones and resumption of polyclonal hematopoiesis[14]. The role of FasR/FasL in MDS is probably a double-edged sword: a tool of immune surveillance by NK (Natural Killer) cells and/or cytotoxic T cells with enhanced membrane-bound FasL (mFasL) attacking the preleukemic clone(s), which in their turn show decreasing FasR and increasing FasL expression during leukemic progression. This process gradually leads to an escape of the leukemic cells (with high FasL expression) from immunoregulatory cells and probably contributes to progressive PCD of normal and monoclonal preleukemic hematopoiesis with enhanced FasR expression (see part III).
The HUMARA assay which uses a polymorphic gene on the X-chromosome showing a high rate of heterozygosity (>90%) is the most used assay to study X Chromosome Inactivation Patterns (XCIP) to detect monoclonality. The presence of monoclonality is an early feature in MDS. However, differentiation from constitutional excessive Lyonization and acquired skewing associated with increasing age, is a major limitation in interpretation of these assays (total skewing 15-40%). The use of T lymphocytes as control cells and sequential analyses may solve this practical problem. On the other hand, no specific genetic marker is needed for assessment of monoclonality with XCIP. The different XCIP assays used on different sorted subsets of BM and blood in MDS patients have shown monoclonality originating in a primitive ($CD34^+Lin^-/Thy1^+$) or early committed ($CD34^+CD33^+$) stem cell.

Part II. Biological features of $CD34^+$ cells and their myeloid progeny in MDS in evolution

Characteristics of $CD34^+$ cells in MDS

The percentage CD34, CD33, and CD13 positive bone marrow mononuclear cells (BMMNC) increased as patients progressed to HR-MDS, and correlated with shorter survival[15-18]. Also co-expression of CD13 (mean ±90%) was significantly increased in MDS $CD34^+$ cells. This was associated with a predominant outgrowth of colony-forming units–granulocyte, macrophage (CFU-GM), usually showing undifferentiated clusters, as hardly any erythroid aggregates were found[19]. Furthermore, abnormally high ratios of pro- versus anti-apoptotic proteins (c-Myc/Bcl-2 and Bax+Bad/Bcl-2+Bcl-x) were found

within the $CD34^+$ cells of especially LR-MDS patients[20,21]. These ratios reversed in advanced MDS and AML.
The size of the $CD34^+$ pool is rapidly increasing during MDS progression in the majority of patients[8], and a concomitant rise in the number of aberrant blasts occurred, as was detected by flow cytometry (FCM) side scatter and CD45 expression[18]. The morphologically "normal" $CD34^+$ blast cells may contain monoclonal, cytogenetically normal CD34 cells, but this remains to be proven. The presence of circulating $CD34^+$ cells in MDS correlated with leukemic progression, even better than cytogenetics and CFU-GM growth *in vitro*[22]. It may indicate that cell-stroma interactions have changed in HR-MDS, and this may contribute to leukemic evolution.

Proliferation of $CD34^+$ cells and their progeny in MDS

In vivo thymidine analogue (BrdU/IUdR) incorporation studies in MDS patients[23,24] followed by BM immunohistochemistry (IH) have shown an increment of overall proliferation of BMMNC. These studies demonstrated a higher than normal overall myeloid growth fraction (GF: median percentage S-phase cells 25-30%, range 13-49%) with a decreasing trend towards HR-MDS. Furthermore, the total cell cycling times (Tc) increased when RA progressed to RAEB-t (Tc of 37.5 and 56.6 hours, respectively). Within the myeloid compartment, a rapid increment of the percentage of $CD34^+$ cells (from 1.67 to 8.69% from LR- to HR-MDS, respectively) and the percentage $CD34^+$ cells in S-phase (from 0.19 to 0.43%, respectively) was observed during the evolution of MDS[8]. A concomitant rise was also found in the percentage proliferating $CD34^+$ cells within the proliferating myeloid compartment during MDS progression (from 0.35 to 1.44%, respectively). These patterns clearly illustrate a growth advantage within the $CD34^+$ pool, which only partially explains the exponential growth of the size of the $CD34^+$ pool during MDS evolution. Using FCM with Ki-67 (a proliferation marker), Parker et al.[20] found increasing percentages of proliferating (G_1) $CD34^+$ cells (range 10-70%) within the (growing) $CD34^+$ compartment during MDS progression, as the percentage S-phase cells in the $CD34^+$ pool was hardly changing in our study (mean 5-10%)[8]. As the nuclear antigen Ki-67 is not an excellent proliferation marker for myeloid cells and it only distinguishes non-cycling (G_0) from cycling (G_1) cells, the differences between these two studies can be explained by enhanced PCD of $CD34^+$ cells in G_1. Thus, the $CD34^+$ compartment expands as MDS progresses with a tendency to cycle slower than their more mature $CD34^-$ progeny. Furthermore, also the differentiation arrest in leukemic blasts[18], and their progressive survival benefit account for this expansion of $CD34^+$ cells and blasts. It is important to stress that proliferation and differentiation are

progressively uncoupled mechanisms during the evolution of the leukemic clone(s) as MDS progresses.

Proliferation and apoptosis of CD34$^+$ cells and their progeny in MDS: dynamic profiles *in vitro*

In vitro studies with BMMNC of MDS patients have shown decreased colony and increased cluster formation with slower growth kinetics, delayed and disturbed differentiation or arrest at the stage of myelo-monocytic blasts[25], as well as increased apoptosis[26]. This leukemic growth pattern *in vitro* was associated with an increase of blasts and CD34$^+$ BM cells, and correlated with a higher incidence of leukemic transformation with shorter survival[15,27,28]. We studied the profiles of proliferation and PCD (by ISEL) of BMMNC of MDS patients *in vitro*[26] The proliferation, defined by the total number of clusters and colonies, was initially enhanced as compared to normals. But this was rapidly followed by a concomitant increased apoptosis: 75% of clusters and more than 40% of colonies showed more than 50% PCD. In contrast, normal controls showed a median PCD of 50% in clusters and 17% in the colonies. AML patients showed delayed and low colony growth *in vitro*, because of enhanced apoptosis at cluster level (60-80%) compared to a relative low PCD in colonies (20%). Interestingly, some colonies of AML patients showed no apoptosis at all[26].

Single cell assays of CD34$^+$ BM cells in MDS showed a similar biological profile: increased proliferation and apoptosis at cluster level and decreased colony formation showing decreased cell numbers. These colonies showed less overall PCD than normal colonies[29]. As neither stromal interactions nor accessory cell influences are involved in this system, probably (pre)leukemic clones with longer cell cycling time (43.7 hours in MDS versus 33.8 hours in normal colonies) and less PCD can evolve in this system. It implies that the patterns observed are intrinsic properties of MDS progenitor cells. (F)ISH studies have to be performed to distinguish growth patterns of chromosomal aberrant from normal clones.

Long-term bone marrow cultures (LTBMC), analyzing MDS stromal influences on normal CD34$^+$ cells, have shown defective surface coverage and support in promoting proliferation and differentiation[30], leading to increased levels of FasR and apoptosis[31]. In contrast, Deeg et al.[32] found stimulatory effects of MDS stroma on normal CD34$^+$ or MDS CD34$^+$ cells. They suggested that accessory mononuclear (non-stromal) cells or abnormal hematopoietic precursors in the non-adherent marrow fraction provided the inhibitory effects as they produced tumor necrosis factor-α (TNFα) at maximal levels.

LTBMC (with normal stroma) were capable of detecting latent subclones with abnormal karyotypes in the majority of MDS patients with normal cytogenetics.

In some (25%) patients these karyotypes also became apparent *in vivo*.[33]. Furthermore, LTBMC detected profound deficiencies in the number of secondary colony-forming cells and in long-term proliferation of multipotent MDS progenitors, together with disturbed differentiation and stromal interference[30,34,35].

Stroma-free LTBMC with a combination of four growth factors (GFs) showed a normal expansion of MDS progenitor cells with normal or dysplastic differentiation in 50% of cases. Complete unresponsiveness and progressive leukemic growth with 100% immature blasts was found in 30% and 20% of MDS cases, respectively[36]. Furthermore, Novitzky et al.[30] showed that the subgroup of MDS patients with the highest overall BMMNC apoptosis had the best clonogenic growth *in vitro* and showed the best response to anti-TNFα therapy *in vivo*. This shows that the inhibitory and PCD-inducing cytokines and their corresponding cells outweigh their stimulating counterparts in MDS marrows. A high proliferative potential continues to be present in MDS hematopoiesis.

Several mechanisms may explain these altered growth patterns *in vitro*. MDS $CD34^+$ cells with enhanced co-expression of CD13 form predominantly nonerythroid clusters with impaired differentiation[19]. MDS progenitors show a diminished response to granulocyte colony-stimulating factor (G-CSF)[25], and granulocyte-macrophage colony-stimulating factor (GM-CSF)[37], which could be reversed by supersaturating doses in some patients[38]. Stem cell factor (SCF) promotes cluster growth, whereas in combination with other GFs undifferentiated colonies are promoted. SCF may in part be responsible for the growth advantage of MDS clonogenic cells over normal blasts, although no differences in c-kit expression were observed[28,39,40]. Enhanced apoptosis detected in the progeny of BMMNC and $CD34^+$ cells of MDS patients may explain this decreased colony formation. In serum-free cultures and LTBMC, MDS $CD34^+$ cells showed poor or no growth of differentiated colonies, irrespective of their growth type, suggesting the defective support of accessory and stromal cells[31,40]. Influences of stromal and accessory cells are not the only explanation of increased PCD as it was also found in single cell assays of MDS $CD34^+$ cells[29]. Differentiation between cytokine-mediated and/or FasR-FasL mediated PCD is warranted!

Apoptosis of $CD34^+$ cells and their progeny in MDS: dynamic profiles and controversies

Apoptosis can be triggered by a variety of circumstances like growth factor deprivation, receptor interaction like tumor necrosis factor receptor 1 (TNF-R1) and FasR, and cell damage causing molecular and or genomic damage beyond repair. Several quantitative techniques have been developed to

measure apoptosis like immunohistochemistry (IH) or flow cytometry (FCM) by in situ end labeling (ISEL) or TdT-mediated dUTP nick-end labeling (TUNEL). ISEL and TUNEL detect specific DNA fragmentation products developed after the activation of different endonucleases. These techniques proved enhanced intramedullary PCD[41,42], as it was postulated before[43].

Overall apoptosis detected by the ISEL technique on plastic embedded BM biopsies was excessive in MDS. More than 50% of patients showed more than 75% $ISEL^+$ cells[41,44]. In general, less apoptosis was found with TUNEL (mean range 12-46%), especially when BM smears were used[30,45-48]. The detection of PCD by morphology (±3%) underestimates the amount of apoptosis[11]. This phenomenon is explained by the short duration of the apoptotic process. In addition, early apoptotic cells present phosphatidylserine (PS) on their outer membrane, signaling macrophages for engulfment, often before clear apoptotic morphology can be detected. Apoptosis was observed in clusters of marrow cells in BM biopsies and the amount of PCD was positively and significantly correlated with the level and localization of TNFα expression[23]. Both trilineage parenchymal and stromal cells are dying[31,41]. The number of macrophages was clearly increased, showing massive ISEL-positive apoptotic bodies of captured, dead cells[23]. PCD was inversely correlated with leukocyte count[11].

Overall apoptosis of BMMNC measured by TUNEL on BM aspirates (range 20-46%) and BM biopsies (range 47-69%) is high in LR-MDS and decreases during progression of MDS[45,46,48,49]. High rates of overall PCD were correlated with low Bournemouth scores[46] and were significantly correlated with low blast numbers[48]. In contrast, a large number of BM biopsies treated with ISEL showed massive apoptosis (>75%) in most HR-MDS patients. ISEL-positivity ($ISEL^+$) decreased towards intermediate levels (range 33-67%) in LR-MDS, although both high and low ISEL positivity was found in LR-MDS[23,41]. The most likely explanation for these discrepancies in PCD is the difference in used material. More apoptotic cells in MDS marrow aspirates were found in the high-density fraction of mononuclear cells than in the mostly used low-density fraction[50]. A considerable amount of apoptotic cells is damaged and lost during the work-up of marrow aspirates. Differences in the detection of different DNA fragmentation products by ISEL and TUNEL is also a fair explanation[42]. Another explanation may be the heterogeneity of MDS. The apoptotic degree is different in the various compartments ($CD34^+$ vs. $CD34^-$, and leukemic vs. monoclonal vs. polyclonal) in time. These compartments also change in size during MDS progression.

In MDS, various levels of apoptosis within the $CD34^+$ pool were found with different techniques. Massive PCD of $CD34^+$ progenitors was found in LR-MDS, as it decreased towards HR-MDS[20,21]. Parker et al.[20] showed excessive apoptosis (median range 50-60%) by FCM using Annexin-V (AnV), whereas Rajapaksa et al.[21] showed a sub-G_1 peak of 9%, both detected in the $CD34^+$

subset in early MDS. In contrast, TUNEL performed on cytospin preparations of sorted $CD34^+$ cells of MDS patients showed a lower mean PCD of 24%, not significantly different from normals. In general, higher values were found in LR- versus HR-MDS[30]. Increased c-myc/Bcl-2 ratios of $CD34^+$ cells were correlated with enhanced PCD in LR-MDS patients[21]. In addition, pro-apoptotic (Bax+Bad) versus anti-apoptotic (Bcl-2+Bcl-x) ratios were increased in $CD34^+$ cells of patients with LR-MDS. Disease progression was associated with significantly reduced ratios, due to increased Bcl-2 and a reduction in Bad expression[20]. Surprisingly, as these ratios play an eminent role as molecular death switches, they were not associated with apoptosis measured by AnV, whereas they were inversely correlated with IPSS score and cytogenetic risk group. AnV detects PS on the outer membrane of cells. This is probably a marker before the point of "no-return" of PCD, as a fraction of thawed AnV^+CD34^+ cells showed proliferation in single cell assays[51].

Conflicting results were found when $CD34^+$ and $CD34^-$ cell populations were compared with TUNEL by FCM and ISEL by IH[52]. In general, $CD34^-$ cells showed more PCD with TUNEL than $CD34^+$ cells, but this difference was not significant, whereas 56% $CD34^-$ cells versus an occasional $CD34^+$ cell showed $ISEL^+$ in BM biopsies. Different types of nuclear endonucleases found in $CD34^+$ and $CD34^-$ cells causing different DNA fragmentation products may explain this. Above all, ISEL positivity was decreased in blast clusters of advanced MDS and in AML blasts in BM biopsies[41].

The overall balance of these dynamic profiles between increased proliferation and apoptosis results in ineffective hematopoiesis with cytopenias in the peripheral blood. A significant positive correlation was observed between the degree of PCD and proliferation[41]. Also anti-PCD treatment studies[14] clearly showed that PCD and proliferation were correlated phenomena. Parker et al.[20] found the following apoptosis/proliferation ratios in the MDS $CD34^+$ cells: 2.08 (RA/RARS), 1.14 (RAEB), and 1.7 (MDS-AML). Maximum PCD was found in RA/RARS, whereas proliferation peaked in RAEB, and both processes declined towards MDS-AML. “Signal antonymy” is an unique feature for MDS[53]. It means that the cell is dying in S-phase as a result of concomitant engagement into incompatible pathways like proliferation and cell cycle arrest. Signal antonymy in MDS (mean of 54% of S-phase cells) was found in all hematopoietic lineages as well as stromal cells. The explanation for this phenomenon is still unknown.

Complex dynamic profiles in MDS: interactions

Growth factors and cytokines play an important role in the apoptotic processes in MDS. In general, GFs can be considered as survival factors. The end result is determined by the balance between levels of positive and negative

hematopoietic GFs and cytokines and their receptor status. Enhanced expression of TNFα and IFNγ was detected in BM biopsies of MDS patients[23,54]. TNFα expression was significantly correlated with PCD. TNFα-induced cytotoxicity is mediated by reactive oxygen intermediates generated in the mitochondrial respiratory chain. Anti-apoptotic therapy by TNFα-lowering regimens in MDS patients resulted in decreased TNFα levels followed by decreased PCD in BM, and clinical responses in 40-50% of patients[14,30,55]. These results led to Raza's postulation of a dual role of elevated TNFα (and IL-1β) levels in the hematopoiesis of MDS patients: stimulation of proliferation of $CD34^+$ stem and early progenitor cells, but inducing apoptosis in their $CD34^-$ progeny[44]. One contributing link interacting in this paradigm has been found: TNFα (and IFNγ) upregulates FasR/CD95 expression, which is one of the main pathways of introducing cell death signals to cells. Sometimes a decrease or disappearance of cytogenetically aberrant clone(s) was observed during TNFα-lowering therapy[14]. These effects suggest that anti-TNFα treatment may favor normal rather than aberrant hematopoiesis.

Part III. The Fas/FasL system in MDS

Introduction of normal physiology

All members of the nerve growth factor receptor family play dual roles as they can trigger both apoptosis as well as proliferation[56]. One of the members is Fas (CD95, APO-1*),* a 45kDa type I transmembrane glycoprotein. The Fas receptor (FasR, or Fas) is normally expressed on a wide range of mature blood cells (monocytes, neutrophils, NK cells, B and T lymphocytes), and highly expressed on activated lymphocytes. In contrast, Fas is weakly expressed on immature BM cells. $CD34^+/CD38^+$ cells have two-fold higher expression than $CD34^+/CD38^-$ cells[57-59]. The receptor density increases from early $CD34^+$ stem cells to more mature progenitors, and it is particularly upregulated on proliferating myeloid progenitors[60]. Cytokines known to mediate proliferation, maturation, and survival of hematopoiesis facilitate negative growth regulation by the FasR pathway in activated cells[60]. This effect could serve as a negative feedback mechanism by T cells on activated hematopoiesis[61,62]. These findings suggest that the Fas/FasL system plays a role in the homeostasis of hematopoiesis[63]. Furthermore, Fas expression is upregulated in a dose-dependent fashion in IFNγ and TNFα treated marrow $CD34^+$ stem cells and it facilitates FasR-induced PCD[57,58]. The combination of TNFα and IFNγ had a synergistic effect on the induction of Fas expression on progenitors[57]. Activated peripheral blood mononuclear cells were able to produce soluble Fas isoforms[64]. Soluble Fas (sFasR) inhibits apoptosis *in vitro* [65].

Fas Ligand (FasL) is a 40kDa type II transmembrane protein. FasL is predominantly expressed in activated cytotoxic T cells (CTL's), B cells, and NK cells, but it is also expressed on monocytes, neutrophils and tumor cells. Membrane-bound FasL (mFasL) induces PCD by trimerization or cross-linking of the Fas receptor in some Fas-expressing cell lines or memory T cells[66]. It works as a cytotoxic effector molecule of CTL and NK cells, and probably of AML tumor cells. Membrane-bound FasL can be cleaved into a soluble form (sFasL) by a metalloproteinase[67]. Membrane-bound FasL is more potent in promoting PCD than sFasL[68]. The shedding of FasL from the membrane is a mechanism for downregulating its killing activity: sFasL competitively inhibits the killing of T cells by mFasL[68,69]. Above all, Josefsen et al.[59] found that sFasL promoted cell survival of human BM $CD34^+CD38^-$ progenitor cells by suppressing PCD in suspension cultures as well as in single cell assay, whereas PCD was slightly increased in the more mature $CD34^+CD38^+$ cells. These studies demonstrate that the delicate balance between mFasL and sFasL levels represents a (paracrine and/or autocrine) regulator of early hematopoiesis: survival and proliferation promotion by sFasL versus apoptosis induction by mFasL.

Interaction of Fas with its natural ligand (FasL) or with agonistic anti-Fas monoclonal antibodies (like CH11) causes homotrimerization of CD95 and triggers PCD by activation of the FADD/MORT-1 cascade[70]. Concurrent expression of Fas and FasL on the same cell leads to PCD after interaction by membrane folding, although monocyte-derived macrophages could escape from spontaneous or anti-Fas IgM induced apoptosis[71].

Enhanced Fas expression on BM progenitors seems to play a role in ineffective hematopoiesis[57]. They showed that IFNγ and TNFα mediated suppression of colony formation from immature ($CD34^+CD38^-$) and mature ($CD34^+CD38^+$) progenitors was enhanced by FasL without the presence of accessory cells. IFNγ and TNFα cause cell cycle inhibition of hematopoietic cells, upregulate FasR expression on $CD34^+$ cells, and induce ICE expression in these cells which subsequently led to PCD *in vitro* when CH-11 was added[57,58,72]. Above all, tumor cells with FasL expression escape from the T cell-mediated immune surveillance, while they maintain the ability to induce Fas-mediated apoptosis in normal cells, especially in activated lymphocytes[73,74].

The Fas/FasL system in MDS

Immunohistochemical (IH) stained MDS BM sections showed positive staining for Fas (and FasL), whereas BM samples of normals showed no staining[47]. These findings were confirmed by RT-PCR for Fas (and FasL) mRNA[18,47]. Fas^+ cells were found in all cell lineages, including $CD34^+$ cells. Also Lepelley et al.[45] observed increased Fas expression in BM cells (by IH) in about 40% of MDS,

whereas a variable proportion of blasts showed weak Fas expression. Gersuk et al.[18] found increased Fas expression of BMMNC by FCM in MDS. They observed that considerably more $CD34^+$ blasts showed Fas expression in MDS as compared to normal BM (87% vs 25%), but Fas expression intensity on $CD34^+$ cells was negatively correlated to the BM blast number. Leukemic blasts apparently loose Fas expression with progression of MDS[46]. Interestingly, significantly more $CD3^+$ activated T cells with Fas expression were found in MDS BM in comparison to normal BM[18].

Regarding the function of Fas, not all $FasR^+$ BM cells showed TUNEL positivity[47]. Furthermore, MDS BMMNC showed increased caspase-3 mRNA[18,75] with a lower to absent FAP-1 expression, which is an inhibiting modulator of the FasR signal transduction pathway[76]. Bouscary et al.[48] found clearly enhanced apoptosis by TUNEL associated with significantly increased levels of caspase-3 activity and low blast numbers in LR-MDS patients.

Although overall Fas expression on hematopoietic progenitors was increased in MDS, it was not correlated with FAB subtype, the Bournemouth score, apoptosis rates or peripheral cytopenias[45,46]. In contrast, *in vitro* culture studies in MDS have shown decreased clonogenic capacity of CFU-GM and the involvement of enhanced Fas expression on proliferation and PCD[18.45,46,77]. The erythroid lineage seems to be more sensitive for Fas-mediated apoptosis than the myeloid lineage[45,46,78]. Also LTBM cultures with MDS stroma have shown defective support in promoting proliferation and differentiation in combination with increased levels of Fas and apoptosis of these normal progenitors[31]. Higher levels of TNFα and sTNF-R1 were found in marrow plasma of MDS patients as compared to normals[18]. The addition of anti-TNFα mAb or soluble rhuTNFR:Fc to Dexter cultures increased colony numbers[18].

FasL expression in MDS was increased in BM cells of all lineages, irrespective their maturation state, but it was even higher in AML blasts[47,78]. This increase in FasL expression was significantly correlated with FAB subtype, the number of abnormal metaphases, and survival[78]. Furthermore, overall FasL expression in de novo AML was comparable to AML after MDS[78], whereas primary MDS had significantly lower $FasL^+$ aberrant blasts compared to secondary MDS[18]. Gersuk et al.[18] observed variable and increased amounts of FasL on MDS $CD34^+$ blasts in contrast to normal $CD34^+$ cells. FasL expression was inversely associated with TNFα levels and Fas expressing during MDS progression. Furthermore, considerably more BM $FasL^+$ $CD3^+$ cells in MDS (17%) were found as compared with normal BM (2%)[18]. The majority of apoptotic cells by TUNEL were also $FasL^+$ with the exception of macrophages[47]. Macrophages showed considerably more staining for FasL than for Fas. In addition, significantly higher levels of soluble FasL were found in marrow plasma of MDS patients[18]. Soluble FasL seems to be functional in MDS as it inhibited the growth of clonogenic $CD34^+$/HLA-DR^+ progenitors in a dose-dependant way[78].

Similarly, suppression of apoptosis of BM mononuclear cells was observed by treatment with anti-FasL[75].
Overexpression (by RT-PCR and IH) of TNFα was detected more often than overexpression of IFNγ in BMMNC of MDS patients (79% vs. 42%, respectively), in contrast to observations in normal BM[54]. The majority of TNFα and IFNγ producing cells were $CD68^+$ macrophage lineage cells. TNFα and IFNγ upregulate Fas expression in a wide array of hematopoietic cells. A significant correlation was found between TNFα protein levels in marrow plasma and Fas expression on MDS marrow blasts[18] and between TNFα expression (by IH) and the extent of apoptosis in BM biopsies[44].

The following model of immunoregulatory mechanisms in MDS can be postulated from all these observations. Monoclonality develops as one of the first hallmarks in early MDS. The immune system probably detects these aberrant cells and an immune response is triggered. Activated CTL's and NK-cells show increasing expression of mFasL, whereas activated monocytes and macrophages produce increasing amounts of TNFα and IFNγ. Subsequently, upregulation of Fas occurs, especially in the more mature cells. Massive apoptosis develops in both normal and monoclonal compartment by enhanced TNFα levels as well as by increased Fas/FasL interactions. Upregulated Fas-bearing mature and immature normal and stromal BM cells die as innocent bystanders and subsequently proliferation increases to compensate their loss. As particularly proliferating myeloid progenitors have enhanced Fas expression, they also die in increasing numbers (causing signal antonymy). Upregulation of mFasL (subsequently leading to soluble FasL) is a way to defend against attacking FasL-bearing CTL's, NK cells or macrophages. The same happens to the rapidly dividing monoclonal cells, but as a consequence of additional mutations/deletions during high mitotic pressure, these cells acquire a differentiation defect and a survival benefit. Furthermore, these cells are capable of turning down their Fas expression. Alternatively, they develop non-functional truncated Fas splicing variants leading to a survival benefit and consequently a growth advantage. On the other hand, as these blasts maintain enhanced mFasL in order to escape from the triggered immune-surveillance, their enhanced mFasL expression may also contribute to the increased killing of polyclonal hematopoiesis and immunoregulatory cells with increased expression of Fas. During MDS progression, evolution of leukemic clones with decreasing Fas and increasing mFasL turn down their PCD machinery by acquiring additional genetic aberrations. These leukemic clones progressively develop growth advantage at the expense of increasing death of monoclonal preleukemic and normal hematopoiesis.

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Chapter 2

Apparent expansion of CD 34^{+} cells during the evolution of myelodysplastic syndromes to acute myeloid leukemia

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Abstract

Background and objectives

Myelodysplastic syndromes (MDS) are highly proliferative bone marrow (BM) disorders where the primary lesion presumably affects a $CD34^+$ early progenitor or stem cell. We investigated the proliferative characteristics of $CD34^+$ cells *in vivo* of 33 untreated MDS patients (19 RA, 5 RARS, 7 RAEB, 2 RAEBt) and five patients with acute myeloid leukemia after MDS (sAML).

Materials and methods

All patients received a one hour infusion of the thymidine analogue iodo- or bromodeoxyuridine intravenously before a BM aspirate and biopsy was taken. A double-labeling immunohistochemistry technique by monoclonal anti-CD34 and anti-IUdR/BrdU antibodies was developed and performed. By this technique we recognized $CD34^+$ and $CD34^-$ cells actively engaged in DNA synthesis or not.

Results

As MDS evolves a significant increase occurred in the percentage of $CD34^+$ cells of all myeloid cells (mean value: RA/RARS 1.67%, RAEB(t) 8.68%, sAML 23.83%), as well as in the percentage of proliferating $CD34^+$ cells of all myeloid cells (RA/RARS 0.19%, RAEB(t) 0.43% and sAML 3.30%). This was associated with a decreasing trend in the overall myeloid labeling index (LI: RA/RARS 25.8%, RAEB(t) 24.6% and sAML 21.5%). This decrease in overall myeloid LI is due to an exponential increase in the proportion of $CD34^+$ cells of the proliferating compartment during MDS evolution (RA/RARS 0.35%, RAEB(t) 1.44% and sAML 11.98% of all S-phase cells). These $CD34^+$ cells appeared to proliferate more slowly than their more mature $CD34^-$ counterparts, since we found a progressive increment in the mean total cell cycling time (Tc) of all myeloid cells during MDS progression (RA/RARS 39.8, RAEB(t) 45.2 and sAML 65.8 hours).

Conclusions

This study showed that during MDS evolution to sAML the $CD34^+$ compartment develops a growth advantage leading to apparent expansion.

Introduction

In myelodysplastic syndromes (MDS) the initial DNA-altering event probably occurs at the level of the pluripotent stem cell. This early event may cause a change in the cell cycle control mechanism that leads to a growth advantage of this particular clone over their normal counterparts. This clonal expansion causes the frequently found monoclonal hematopoiesis of the nonlymphoid cells in MDS[1-5]. During this process of high proliferative activity secondary events (DNA damaging events or DNA repair mechanism failures) induce a cytogenetically marked subclone, like the frequently found 5q-, monosomy 7, trisomy 8 clones in MDS[6]. This subclone or the following subclones[7-9] are characterized by even more complex cytogenetic abnormalities and are recognized by their immaturity as blasts and/or as abnormally localised immature precursors or ALIPs[10-12]. Eventually these subclones may cause the evolution from RAEB(t) to acute myeloid leukemia (AML) by progressive dedifferentiation and eventually losing their gene-directed programmed cell death (PCD) or apoptosis[13,14].

Over several years more evidence has been found that the high labeling index (LI) in MDS is abrogated by a high apoptotic cell death[9,14-16], which explains the hypercellular bone marrow (BM) with peripheral cytopenias. This increased apoptosis in MDS is visualized by different techniques like high/low molecular weight DNA extraction by electrophoresis, in situ end labeling (ISEL) of DNA, in situ terminal deoxynucleotidyl transferase (TUNEL) assay, and the Annexin V assay by different research groups[17-21]. When RA/RARS develops to RAEB(t) overall apoptosis is high. It slows down during the development of secondary AML (sAML after previous MDS) because of the appearance of a progressive number of ISEL-negative, immature myeloblasts. On the other hand, the high myeloid LI decreases with slowing down of cell cycle times of myeloblasts when evolution occurs to RAEBt and sAML[9,13,15,22]. It is still not proven whether the high proliferation rate (mean overall myeloid LI of 25 to 30%) is a compensatory mechanism for the high apoptosis rate in myelodysplasia[9,23]. However, first results of treating MDS patients with pentoxifylline and ciprofloxacin support this hypothesis: suppression of PCD (by turning down transcription of TNFα) was followed by a decrease in overall myeloid LI[24].

Very little is known regarding the specific proliferative characteristics of CD34+ cells in particular in MDS. By flow cytometry (FCM), a sufficient number of stem cells after selection procedures can usually be obtained, but determining an accurate LI from aspirated and separated cells is frequently proven to be unreliable[25,26]. The best way of determining the accurate LI is obtaining BM biopsies after *in vivo* labeling of S-phase cells[13]. Fortunately, we have been able to develop an method to identify CD34+ cells and to simultaneously examine their proliferative characteristics in BM biopsies. We conducted this

study to achieve a better understanding of their cycling properties during MDS evolution and to study the process of a possible growth advantage of the clone within $CD34^+$ compartment, as more immature myeloblasts occur during MDS progression to sAML.

Materials and methods

Patients

Thirty-three MDS patients (21 males and 12 females, mean (±SD) age 65.8 (±13.8) years) were studied for the proliferative characteristics of $CD34^+$ cells. All MDS patients (FAB classification:19 RA, 5 RARS, 7 RAEB and 2 RAEBt), together with five sAML patients (four males and one female, mean age 52.8 (±10.4) years) and five “normals” (lymphoma patients with uninvolved BM, four males and one female, mean age 56.8 (±22.7) years), who served as controls, were eligible for study after informed consent was obtained. None of the patients had received any therapy (except supportive care) for at least two weeks prior to the one hour infusion of one of the thymidine analogues iodo- or bromodeoxyuridine (IUdR/BrdU) at 100mg/m² intravenously, using a constant rate infusion pump. Each infusion was immediately followed by a BM aspirate and biopsy, which were handled on ice. The infusion protocols were reviewed and approved by the Investigative Review Board of the Rush-Presbyterian-St. Luke’s Medical Centre, National Cancer Institute (NCI) and the Food and Drug Administration. The drugs were supplied by NCI. See Table 2.1 for detailed individual characteristics of all patients and controls.

Single-labeling immunohistochemistry to measure overall myeloid LI

The BM biopsies were labeled by 3D9 (Bioscience Inc, Bethlehem, PA, USA) antibodies against incorporated IUdR/BrdU to detect S-phase cells by showing a brown punctation overlying their nucleï. After counterstaining and taking myeloid morphology into account, an overall myeloid LI could be determined. This procedure was described before in full detail[27] and was also followed for the sAML patients and controls.

Table 2.1 Proliferative characteristics of patients and controls expressed as a percentage of three different compartments: total myeloid cells, total proliferating cells and total CD34$^+$ cells (see Materials and methods section for details).

Serial	Sex	Age	FAB	LI	LICD34	CD34S/S	CD34	CD34S/M	T_s	T_c
1	M	65	RA	20.00	5.62	0.26	0.60	0.034		
2	M	27	RA	28.50	0.00	0.00	0.10	0.000		
3	F	82	RA	13.10					13.40	102.00
4	M	65	RA	32.60	6.25	0.05	0.35	0.022		
5	M	49	RA		0.00	0.00	0.00	0.000		
6	M	68	RA	27.20	9.33	0.62	4.89	0.456	11.70	42.90
7	F	76	RA	24.70	17.71	0.47	0.20	0.035	4.40	17.90
8	M	73	RA	24.90	0.00	0.00	1.00	0.000	8.60	34.50
9	M	64	RA				1.03			
10	M	66	RA	29.20	20.19	0.14	2.88	0.581		
11	M	88	RA	34.20	20.38	1.84	1.21	0.247	13.40	39.30
12	F	83	RA	28.00	12.50	0.04	0.91	0.114	5.40	19.10
13	M	85	RA	27.10	8.39	0.15	1.04	0.087		
14	F	78	RA	20.90	5.86	0.32	3.90	0.229	11.30	54.00
15	F	46	RA	29.90	2.00	0.08	2.89	0.058	7.00	23.40
16	M	63	RA	26.40	3.33	0.05	1.17	0.039	9.90	37.50
17	M	63	RA		23.74	0.66	0.73	0.173		
18	F	83	RA	25.50	4.07	0.37	6.07	0.247	7.30	28.70
19	M	71	RA	20.00	24.26	1.67	3.06	0.742	15.90	77.00
20	M	62	RARS	36.10	25.84	0.31	0.84	0.217	4.60	12.90
21	F	69	RARS		0.00	0.00	0.16	0.000		
22	F	83	RARS		16.67	0.52	3.60	0.600		
23	M	67	RARS	15.40	5.00	0.18	1.71	0.085		
24	M	77	RARS	27.30	0.00	0.00	0.00	0.000	7.70	28.10
25	M	60	RAEB	21.70	2.46	3.12	10.58	0.26	18.40	94.80
26	M	60	RAEB		2.28	0.78	20.41	0.465		
27	M	63	RAEB	27.10	1.65	0.37	4.29	0.071	3.90	14.40
28	F	76	RAEB	24.90	6.35	4.49	11.68	0.742	3.80	14.80
29	M	53	RAEB	34.50	3.57	0.10			4.60	13.30
30	F	40	RAEB	25.20	26.92	2.41	1.94	0.522		
31	F	64	RAEB	15.10	0.00	0.00	0.03	0.000		
32	M	53	RAEBt	24.00	6.49	0.41	10.54	0.684	21.10	88.10
33	F	50	RAEBt	24.20	6.91	1.24	10.00	0.691	11.00	45.60
34	M	52	AML	10.30	0.00	0.00	0.30	0.000	5.80	60.00
35	M	48	AML	32.60	9.81	2.40	7.06	0.693		
36	M	68	AML	19.10	22.61	33.42	55.55	12.56	13.80	72.60
37	M	56	AML	24.70	10.73	15.66	17.94	1.925		
38	F	40	AML	20.90	3.51	8.42	38.31	1.345	13.50	64.70
39	M	31	control	29.20			0.10			
40	M	71	control	25.20	0.00	0.00	0.70	0.000		
41	F	43	control	28.20	0.00	0.00	0.20	0.000		
42	M	51	control		5.71	1.20	0.10	0.006		
43	M	88	control	31.90	0.00	0.00	0.00	0.000		

M=male, F=female, T_s and T_c in hours.

Double-labeling immunohistochemistry CD34/BrdU to measure CD34 cells in S-phase or not

After fixation and decalcification of the BM biopsies, embedding in paraffin was performed. Sections of approximately 6 μm were placed on positively charged Superfrost Plus slides, air-dried and used for immunohistochemistry (IH) at room temperature. After deparaffinization by running through 100% xylene and graded ethanols, rehydration in double distilled water (ddH_2O) was followed by incubation in freshly prepared 3% H_2O_2 for 30 minutes (min.) to block endogenous peroxidase. After rinsing thoroughly in ddH_2O, three washes in respectively 0.15 M Tris Buffer Solution (TBS: 0.15 M sodium chloride in 0.05 M Tris buffer, pH 7.5) and 0.5 M TBS (0.5 M sodium chloride in 0.05 M Tris buffer, pH 7.5) with 0.1% Tween 20 (Sigma)(TBST) was followed by a one hour incubation with a monoclonal mouse anti-CD34 (dilution 1:10) antibody (QBend/10, Biogenex). Again three washes in 0.5 M TBST which was followed by 30 min. incubation with the secondary rabbit-antimouse IgG (1:20) antibody Z259 (Dako, Carpinteria, CA, USA). After three washes in 0.5 M TBST, the tertiary (1:40) antibody D651 (APAAP: alkaline phosphatase antialkaline phosphatase complexes, Dako) was applied for 30 min., followed by washes in 0.5 M TBST. A blue color reaction was developed in the cell membrane and cytoplasm by applying a freshly prepared BCIP/NBT (5-bromo-4-chloro-3-indoxyl phosphate and nitro blue tetrazolium chloride, Dako) with 1 M levamisole (1 μl/ml) for approximately 3 to 10 min. by repeatedly checking the blue color under the microscope. The BCIP/NBT reaction was neutralised by rinsing in ddH_2O. The slides were left overnight in 0.15 M phosphate buffer solution (PBS) (0.15 M sodium chloride in 0.1 M phosphate buffer, pH 7.5) for performing a second IH procedure the next day to detect cells in S-phase.
This procedure was started by applying 1mg/ml nuclease free pronase (Calbiochem, LaJolla, CA, USA) for 45 min. incubation, followed by three washes in 0.15 M PBS. 4N HCl treatment for 20 min. was done to permeabilize cell- and nuclear membrane, which was followed by dip rinsing in ddH2O and three washes in 0.15M PBS and 0.5 M PBST (0.5 M sodium chloride in 0.1 M phosphate buffer with 0.1% Tween 20, pH 7.5) each. The incubation with the primary anti-BrdU/IUdR (1:500) 3D9 containing 1.5% horse serum was stopped after 60 min. with three washes in 0.5 M PBST. The secondary (1:200) biotinylated monoclonal mouse antibody (Vectastain Elite Kit ABC, Vector, Burlingham, CA, USA) in 1.5% horse serum was incubated for 30 min. After washes in 0.5 M PBST, the tertiary (1:50) antibody ABC (avidin-biotin/peroxidase complexes, Vectastain Elite Kit) was also incubated for 30 min. and followed by three washes in 0.5 M PBST. The brown color reaction was developed in the nucleus with 50 mg DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma) in 200 ml 0.05 M Tris buffer, pH 7.5, together with 12-14 μl 30% H_2O_2 for 4 to 10 min., followed by three rinses in dd H_2O.

Postfixation for the BCIP blue color was needed and performed with 2% glutaraldehyde at 4ºC for 20 mins. Dehydration of these paraffin sections was done by graded ethanols. After going through 100% xylene three times 30 seconds, the slides were mounted with xylene-based mounting solution (Permount) using 2 μm thick glass coverslips. No counterstain was used in this double-labeling procedure.

Single-labeling immunohistochemistry to measure the percentage of CD34+ cells of all myeloid cells

The same procedure after deparaffinization was followed as by the double-labeling procedure, but we used DAB/ABC-kit to detect the CD34+ labeled cells (brown cytoplasm and cell membrane) and counterstained the biopsy slides with hematoxylin to differentiate CD34+ cells from the other myeloid cells. No postfixation treatment was needed for these slides.

Morpho-immunohistochemical evaluation of BM biopsies: detection and scoring of CD34+ cells and/or S-phase cells within the myeloid compartment

At least 2000 positively labeled S-phase cells were counted from five or more areas of the single-labeled biopsy for determination of the LI (%). Furthermore, 2000 S-phase labeled myeloid cells were counted by searching for CD34+ fields (aggregates or single cells) in the double-labeling method, whereas to 2000 myeloid cells were counted by searching for CD34+ cells in the single-labeling method. Aggregates of three or more CD34+ cells were seen and scored as a number of single cells. Erythroid and megakaryocytic cells were excluded by morphology. This evaluation procedure was done twice (on different days) by one and the same person, who is an experienced hematomorphologist. These different cells were counted and expressed as a percentage of one of the three different compartments (see Figure 2.1).

Autoradiography for determining Ts (duration of S-phase or DNA doubling time) and calculation of Tc

The bone marrow aspirate samples were double-labeled *in vitro* with tritiated thymidine for the calculation of the duration of S-phase (Ts) by our previously described method[13]. With the use of Ts and LI the total cell cycling time Tc of all myeloid cells can be calculated using the formula described by Wimber and Quastler[28]: $Tc=Ts \times GF/LI$. GF is the growth fraction or the percentage of cells in cycle, which was assumed to be 100%. Ts and Tc are both expressed in hours in Table 2.1.

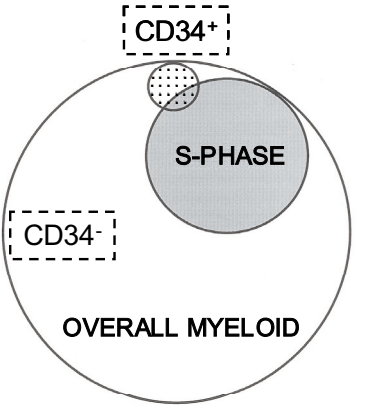

Figure 2.1 Overall myeloid compartment contains $CD34^+$ and $CD34^-$ compartments, both contain a proliferating fraction, taken together as S-phase compartment or LI of overall myeloid compartment.
LI (%):overall myeloid labeling index (LI) or percentage myeloid S-phase cells of all myeloid cells in BM biopsies (grey area); **LICD34 (%):** percentage $CD34^+$ S-phase cells of all $CD34^+$ cells or the labeling index of $CD34^+$ cells (spotted grey area divided by spotted area); **CD34 (%):** percentage $CD34^+$ cells of all myeloid cells (spotted area); **CD34S/S (%):** percentage $CD34^+$ cells in S-phase of all myeloid S-phase cells (spotted grey area divided by grey area); **CD34S/M (%):** percentage $CD34^+$ cells in S-phase of all myeloid cells (%) can be calculated by multiplying CD34 (in %) by LICD34 (in %) divided by 100 (spotted grey area divided by white area).

Statistical analysis

After analyzing the distribution of the different continuous variables in the various subgroups, only LI, Ts, and Tc showed a Gaussian distribution in all the subgroups. Therefore, a distribution-free Wilcoxon-Mann-Whitney test was used to determine statistical significant differences ($P<0.05$) between these skewed-distributed subgroups (Table 2.2). Also the use of median values (with 25-75% interval) is statistically seen more correct to describe these parameters when skewed distributions are involved. Pearson rank correlation tests were used to analyse statistically significant correlations ($P<0.05$) between various sets of two different variables within these subgroups.

Results

After applying the double-labeling IH technique, $CD34^+$ cells showed a blue colored cytoplasm and cell membrane with variable intensity, while the S-phase cells showed brown staining overlying the nucleus. $CD34^+$ cells in S-phase are therefore double-labeled (Figure 2.2). Aspecifically blue stained blood vessel

endothelium and collagen by QBend/10 was not taken into account but served as positive internal control. The majority of cells were non-stained CD34⁻ cells not in S-phase. Sometimes CD34+ aggregates could be found in which all the cells where in S-phase, especially in sAML and the more advanced stages of MDS (Figure 2.3). The mean values (± standard error of mean; SEM) of all determined proliferative parameters of all the groups are shown in Table 2.2 and Figures 2.4 and 2.5. Statistical differences between the various (sub)groups and the level of significance in combination with the median values (with 25-75% interval) of only the skewed-distributed parameters are expressed in Table 2.2.

Table 2.2 Proliferative characteristics of the CD34+, S-phase and overall myeloid compartments in controls, MDS subgroups and sAML.

Variable	Controls n=5	RA/RARS n=24	RAEB(t) n=9	sAML n=5
LI (%)	28.6 ± 1.38	25.8 ± 1.39	24.6 ± 1.91	21.5 ± 3.64
LICD34 (%)	<0.1	9.60 ± 1.91	6.29 ± 2.70	9.33 ± 3.87
		6.10 (2.00–17.71)	3.57 (2.28–6.49)	9.81 (3.51–10.73)
CD34S/S (%)	<0.1	0.35 ± 0.11 (b^3, c^3)	1.44 ± 0.52	11.98 ± 6.00 (e^5)
		0.17 (0.04–0.47)	0.78 (0.37–2.41)	8.42 (2.4–15.66)
CD34 (%)	0.22 ± 0.12	1.67 ± 0.35 (a^2, b^1, c^2)	8.68 ± 2.29 (d^2)	23.83 ± 10.21 (e^2)
	0.1 (0.1–0.2)	1.03 (0.35–2.89)	10.27 (4.29–10.58)	17.94 (7.06–38.31)
CD34S/M (%)	<0.1	0.189 (a^2, b^5, c^3)	0.429 (d^2)	3.304 (e^5)
		0.087 (0.035–0.247)	0.494 (0.260–0.684)	1.345 (0.693–1.93)
Ts (hours)		9.28 ± 1.02	10.5 ± 3.15	11.0 ± 2.62
Tc (hours)		39.8 ± 7.01	45.2 ± 15.5	65.8 ± 3.68

Variables are expressed as mean percentage ± SEM and median (25–75% interval, second line), statistical significant differences between the following subgroups are designated as follows: RA/RARS vs. controls (a), RA/RARS vs. RAEB(t) (b), RA/RARS vs. sAML (c), RAEB(t) vs. controls (d), sAML vs. controls (e), the level of significance (P value) is expressed as a number in superscript (5 means P<0.05 etc).

Labeling index of myeloid cells and CD34+ cells during MDS evolution

A statistically non-significant decrease occurred in the mean overall myeloid LI: RA/RARS 25.8%, RAEB(t) 24.6% and sAML 21.5% (Table 2.2 and Figures 2.4 and 2.5). The controls/lymphoma patients with uninvolved BM had an unexpectedly and unexplained high LI. Subsequently, we determined the size of the CD34+ compartment and the LI of CD34+ cells. A significant increase in the mean percentage of CD34+ cells of all myeloid cells was found with the progression from RA to sAML. The percentages CD34+ cells were 1.67% in RA/RARS, 8.68% in RAEB(t) and 23.83% in sAML, much higher as compared to 0.22% of the controls (Figure 2.4). On the other hand, no significant differences were found in the LI of CD34+ cells during MDS evolution. The mean percentage of proliferating CD34+ cells within the CD34 compartment

was 9.60% in RA/RARS, 6.29% in RAEB(t) and 9.33% in sAML. However, when the proliferating compartment of $CD34^+$ cells as a fraction of all myeloid cells (CD34S/M) was analysed, a significant exponential increase was found during MDS evolution: 0.189% in RA/RARS, 0.429% in RAEB(t) and 3.304% in sAML (Figure 2.4). An exponential increase of proliferating $CD34^+$ cells as a fraction of all myeloid proliferating cells (CD34S/S) was also seen with progression of myelodysplasia to sAML: 0.35% in RA/RARS, 1.44% in RAEB(t) and 11.98% in sAML (Figure 2.4). Sometimes we observed $CD34^+$ aggregates in which all the cells were in S-phase, especially in patients with a high CD34 expression (Figure 2.3).

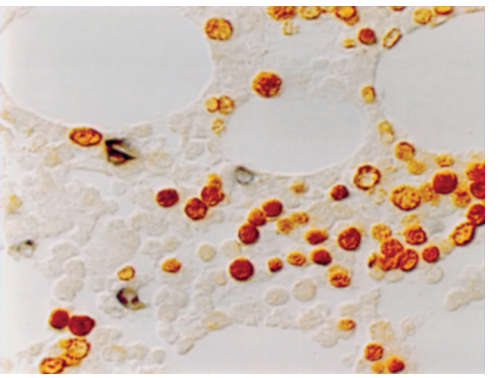

Figure 2.2 Double-labeling immunohistochemistry CD34/BrdU in MDS bone marrow biopsy: $CD34^+$ cells show blue cytoplasm and cell membrane, whereas S-phase cells show a brown nuclear staining. $CD34^+$ cells in S-phase have both features.

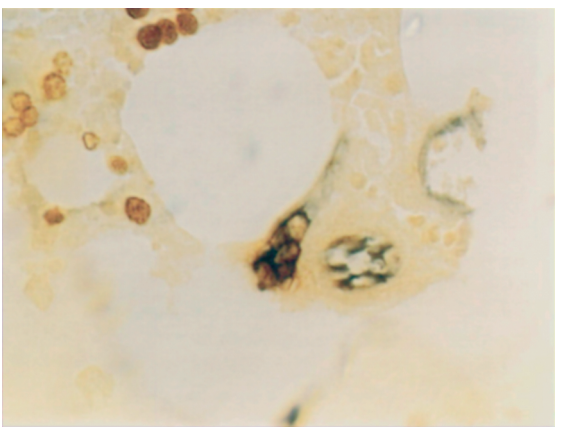

Figure 2.3 Bone marrow biopsy of a patient with RAEBt showing a double-labeled ($CD34^+$/$BrdU^+$) cell aggregate in which all the $CD34^+$ cells are in S-phase.

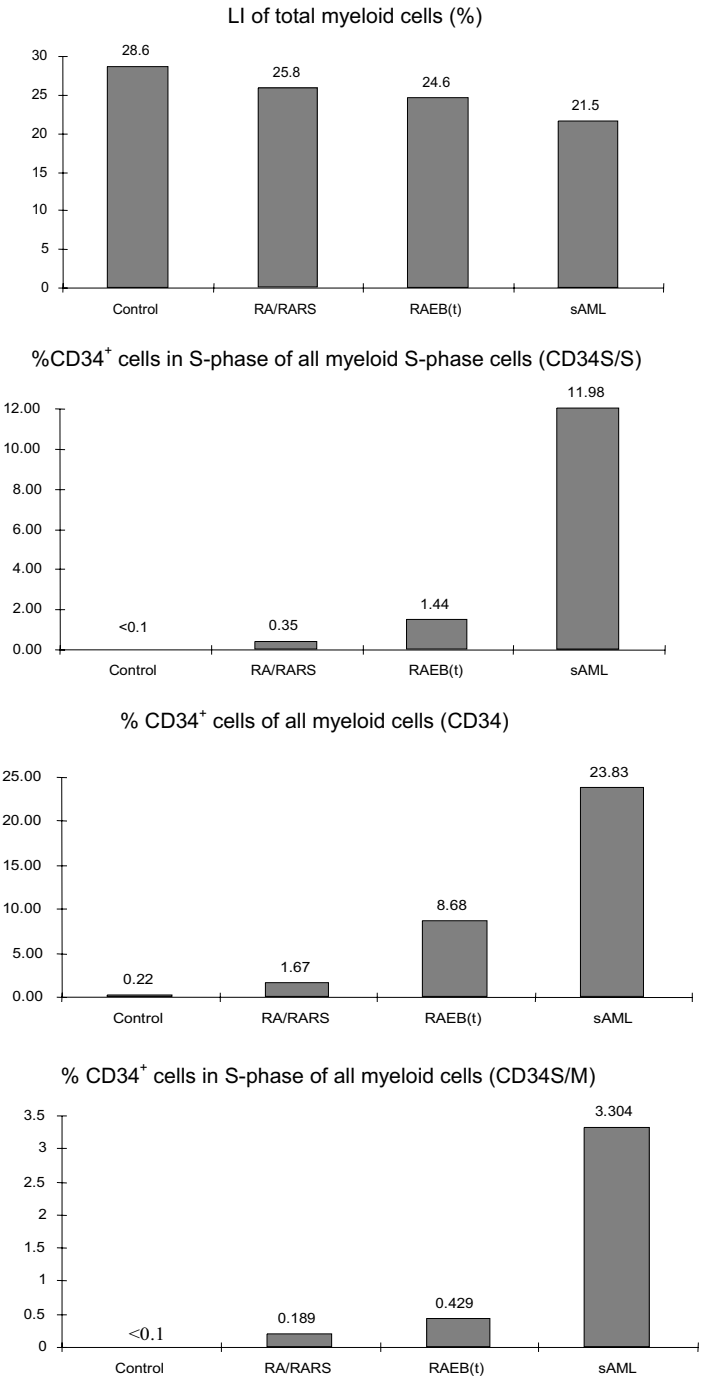

Figure 2.4 Proliferative characteristics of overall myeloid and CD34+cells during MDS evolution.

These data suggest that the $CD34^+$ compartment increases as MDS progresses to sAML. Within this compartment the percentage proliferating $CD34^+$ cells remains the same (LICD34). The absolute number of proliferating $CD34^+$ cells (CD34S/M) increases therefore, whereas a decreasing trend occurs in the size of the overall myeloid proliferating compartment (LI) as MDS evolves to sAML. This means that within this decreasing proliferating overall myeloid compartment, the fraction of proliferating $CD34^+$ cells (CD34S/S) increases at the expense of the proliferating $CD34^-$ fraction during MDS evolution to sAML. The aforementioned changes in the different compartments are depicted together in Figures 2.5a and 2.5b.

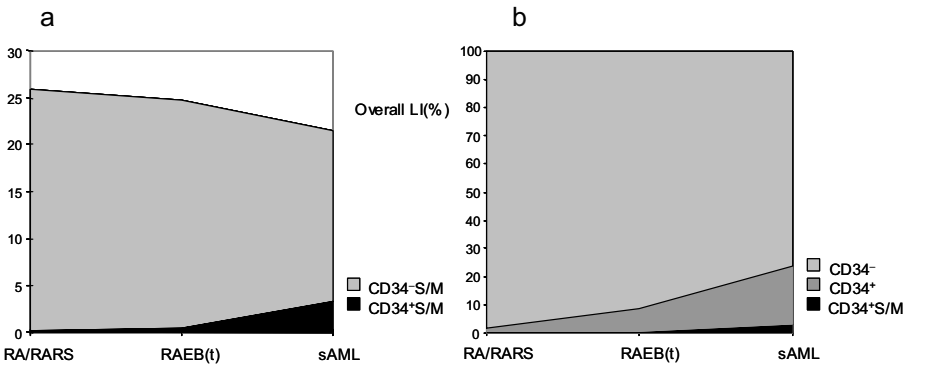

Figure 2.5 (a) Overall myeloid LI (%) divided in % proliferating $CD34^+$ and $CD34^-$ cells of all myeloid cells during MDS progression to sAML. (b) Shift in size of $CD34^+$ and $CD34^-$ compartment (as % of all myeloid cells) during MDS evolution to sAML.

Duration of cell cycle phases

As MDS evolves to sAML, the mean total cell cycling time (Tc) increased progressively, although it just missed statistical significance, whereas no change occurred in the mean duration of S-phase (Ts). The mean Tc and Ts in the different subgroups are respectively 39.8 and 9.28 hours for RA/RARS, 45.2 and 10.5 hours for RAEB(t), and 65.8 and 11.0 hours for sAML (Table 2.2 and Figure 2.6).

Correlations between various proliferative characteristics within the different subgroups

Within the RA/RARS and RAEB(t) subgroups a strong significant correlation was found between Tc and Ts, respectively $r=0.84$ ($P<0.0004$) and $r=0.99$ ($P<0.0004$), which was not found within the sAML group.

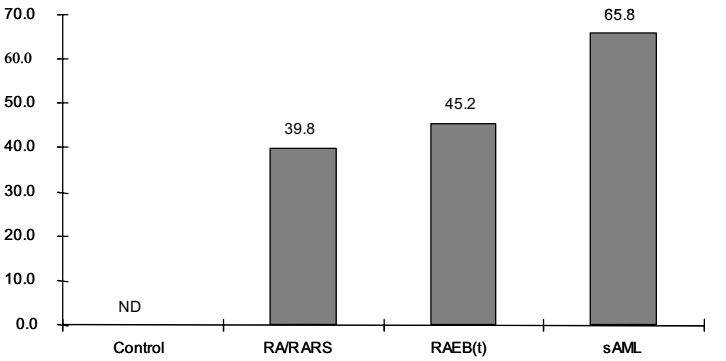

Figure 2.6 Mean total cell cycling time (T_c in hours, on top). ND, not determined.

Within the RA/RARS an increase in the $CD34^+$ fraction of the total myeloid LI leads to a longer Ts and to a longer overall Tc because the percentage proliferating $CD34^+$ cells of all myeloid S-phase cells (CD34S/S) was significantly positive correlated with Tc, Ts and CD34S/M (respectively r=0.59 (P<0.04), r=0.72 (P<0.008) and r=0.59 (P<0.005). Also CD34S/M was significantly correlated with Tc and Ts, respectively r=0.74 (P<0.006) and r=0.70 (P<0.01). The size of the $CD34^+$ compartment depends on the percentage of proliferating $CD34^+$ cells of all myeloid cells: CD34 was significantly correlated with CD34S/M (r=0.64, P<0.002). This means that within the $CD34^+$ compartment of the RA/RARS group the balance between cell proliferation or cycling is favored over apoptosis .

Within the RAEB(t) group and the controls we did not find any of the aforementioned correlations. Regarding the sAML group, similar characteristics were found as in the RA/RARS group: a higher $CD34^+$ fraction within the overall myeloid LI leads to a longer overall Tc and an increment in the percentage of proliferating $CD34^+$ cells of all myeloid cells because the percentage of proliferating $CD34^+$ cells of all S-phase cells (CD34S/S) was positively correlated with Tc (r=0.99, P<0.08) and CD34S/M (r=0.94, P<0.02). Furthermore, the percentage $CD34^+$ cells of all myeloid cells (CD34) was positively correlated with CD34S/S and CD34S/M, respectively r=0.86 (P<0.06) and r=0.83 (P<0.08). Only in sAML a positive correlation was found between LICD34 and CD34S/M (r=0.90, P<0.04). This means that in sAML the balance between CD34 cell proliferation or cycling and apoptosis is even more favored for proliferation than it is the case in RA/RARS.

Discussion

The aim of this study was to get a better insight into the biology of $CD34^+$ cells in relation to the overall myeloid population during the evolution of MDS. Therefore we investigated the evolutionary pattern of proliferation of myeloid cells in general and $CD34^+$ cells in particular by means of IH double-labeling on BM biopsies. In patients with a low CD34 expression the CD34 parameters are a little overrated because of searching for positive fields for CD34 expression. This is not the case in patients with a high CD34 expression because of the fact that almost every field could be scored. Despite some overinterpretation, our values of CD34 (%) are within the same range as observed for the different FAB classifications by a similar study of Soligo et al.[29] using a different method for scoring single-label CD34 immunohistochemistry in BM biopsies of MDS patients.

During MDS progression we observed a significant increment in the percentage of $CD34^+$ cells of all myeloid cells (CD34), which means that the $CD34^+$ compartment enlarges during progression from RA to sAML, which follows a concomitant increase in the percentage of BM blasts by FAB classification. Furthermore, as the overall myeloid LI shows a decreasing tendency from MDS transition to sAML, the percentage proliferating $CD34^+$ cells of all myeloid cells (CD34S/M) increases exponentially at the expense of the proliferating $CD34^-$ compartment. The LICD34 remains the same because the percentage S-phase cells in the increasing $CD34^+$ compartment remains the same, but the absolute number of proliferating $CD34^+$ cells in the $CD34^+$ compartment increases and consequently the absolute number of proliferating $CD34^+$ cells of all myeloid cells. This can be (partially) explained by an exponential increment in the percentage of proliferating $CD34^+$ cells of all myeloid cells in S-phase (CD34S/S). Furthermore, a decreasing number of all myeloid cells in S-phase concurrently with an increase in Tc and no change in Ts during MDS evolution to sAML can be explained by the slower proliferation of the enlarged proliferating $CD34^+$ compartment. Immature $CD34^+$ blasts must cycle slower than their $CD34^-$ counterparts, otherwise a fast $CD34^+$ overgrowth within the BM would occur in every case of MDS, unless apoptosis or a rapid transit time to the $CD34^-$ compartment would prevent this overgrowth. As MDS evolves enhanced differentiation loss and decreased apoptosis occurs in the $CD34^+$ compartment (especially as leukemic blasts are concerned) which further increases the size of this compartment. From previous proliferation studies we already know that during the evolution within MDS and to sAML the overall myeloid LI decreases as Tc prolongs[9,26], but it still has to be proven if (a substantial number of) these increased $CD34^+$ clonogenic blasts with a longer duration of G0/G1-phase cause this phenomenon. Above all, the phenomenon of “signal antonymy” (dying S-phase cells) in MDS was not taken into account

and this characteristic feature of MDS has to be investigated also for its implications for measuring overall Ts and consequently Tc.

Myelodysplasia is primarily characterised by a phase of monoclonality before a (rapid) expansion of leukemic blasts occur. Mehrotra et al.[4] showed in AML that the frequency of cytogenetically aberrant stem cells (CD34$^+$lin$^-$) is uncoupled from compartment size, which means that additional mutation(s) and maturation loss of the blast subpopulation is needed before expansion can occur. Also in MDS these characteristics can be found[30,31]. Furthermore, it was also found by interphase FISH analysis that the percentage of cytogenetically aberrant cells in the CD34$^-$ compartment was higher than in the CD34$^+$ compartment in good prognosis MDS, whereas this percentage of aberrant cells was almost the same in both compartments in poor-prognosis MDS[30]. Dynamic processes of clonal expansion and suppression of normal hematopoiesis and the balance between them are involved in these phenomena. These additional mutations may lead to apparent (and eventually malignant) clonal expansion by several mechanisms: enhanced dedifferentiation or differentiation arrest, increased autonomic proliferation, turning down of apoptosis and eventually decreasing cell cycling times of clonogenic blasts. These mechanisms are clearly incorporated in the FAB classification: the progression of blasts, but even better in the IPSS score. Enhanced dedifferentiation of MDS CD34$^+$ cells was also found in *in vitro* cultures upon growth factors[32-34]. No clear reports can be found investigating the increased autonomic proliferation in MDS. In RAEB(t) and sAML, we previously showed less overall apoptosis, especially in blasts, by using ISEL on BM biopsies[14]. This phenomenon was also recently reported by Bouscary et al.[35] by showing a lower Fas expression on CD34$^+$ cells of patients with advanced stages of MDS and sAML when compared with early MDS stages, which was also associated with less PCD by the TUNEL technique. In our study, we only found a strong positive correlation between LICD34 and CD34S/M in sAML ($r=0.90$, $P<0.04$), probably because apoptosis was found to be completely negative in this clonogenic blast population[14]. Evidence for increased proliferation with slower cell cycling times of CD34$^+$ cells during MDS progression is also reported in this article. The mechanism of eventually decreasing total cell cycling times is seen in the blast subpopulation of ALIP-positive RAEB(t) patients with a very fast evolution to sAML[11,13].

The typical profile of exponential proliferation of CD34$^+$ cells during MDS evolution may be explained by a progressive autonomous proliferation in the CD34$^+$ compartment. Some evidence seen in our biopsies for a possible role of paracrine-induced "signal synchronisation" in CD34$^+$ aggregates, in which all the CD34$^+$ cells were in S-phase, could be used as a morphological substrate for this autonomic proliferation (Figure 2.3). Increasing evidence for autocrine and/or paracrine mechanisms for the explanation of the autonomous growth of

(and anti-apoptotic effects in) AML blasts is found in the literature today[36,37], but no studies in MDS are performed or reported as yet.
Different groups have already shown that the absolute percentage of $CD34^+$ cells/blasts (CD34%) increases during the evolution of MDS[38,39]. The MDS $CD34^+$ cells show clearly promoted proliferative capacity (high cluster/colony ratio in semi-solid medium) with strongly impaired differentiation upon growth factors in *in vitro* cultures[32-34]. TNFα is one of the cytokines which may have a dual role in this process: stimulation of proliferation of presumably early progenitor cells and induction of apoptosis in their more mature progeny[9,14,20,40,41]. TNFα directly stimulates the recruitment and proliferation of very early, primitive progenitors ($CD34^{++}$,$CD38^-$) and induces an increased resistance of the inhibitory effect of TGFβ on these early stem cells[40,42,43]. The immunohistochemically detected high TNFα levels in the BM biopsies of MDS patients are therefore a likely explanation of the increment in the absolute number of $CD34^+$ cells, as well as the proliferating fraction of $CD34^+$ cells when MDS evolves[9,14,41,44]. On the other hand, a positive relationship between the degree of PCD and the level of TNFα in the BM biopsies with a great preponderance of TNFα specifically around more mature ISEL-positive cells could explain the decrease in LI of $CD34^-$ cells, as well as a reduction in size of the $CD34^-$ compartment found in this study. Of course the balance between the various acting cytokines in the BM play the ultimate role in determining the overall proliferation and apoptosis of myeloid cells.
$CD34^+$ overexpression is observed in more than 30% of all MDS patients and CD34 expression is higher (as percentage $CD34^+$ single cells and $CD34^+$ aggregates) in RAEB(t) than in RA/RARS[29,32]. The number of $CD34^+$ aggregates in MDS biopsies are significantly positively correlated with the percentage of BM blasts and ALIPs[45]. A significantly higher frequency of CD34 expression is also found in sAML (or therapy-related AML) when compared with “de novo” AML[46]. In the present study, we showed a continuous process of increasing CD34 expression during the evolution of myelodysplasia to sAML caused by an increasing fraction of $CD34^+$ cells within the total S-phase population. CD34-positivity in BM and the presence of $CD34^+$ cells in circulation in MDS are both correlated with poor overall prognosis and with leukemic transformation[12,29,32,47,48]. Within AML after MDS, this prognostic relevance of CD34 expression has not been demonstrated yet, because no studies on this selected subgroup within AML have been performed. In this study we found a higher CD34 expression in AML after previous MDS as compared with the MDS subgroups, which is the first evidence in literature of a prognostic meaning of CD34 expression in AML-MDS. This poor prognostic meaning of increasing $CD34^+$ aggregates, as well as increasing $CD34^+$ circulating cells in MDS can probably be explained by “signal synchronisation”. This means that growth signalling or initiation in these blast cell aggregates

occurs in a paracrine fashion, causing these cells to go into S-phase at the same time. This phenomenon is possibly responsible for autonomic growth by overruling the need for stromal interactions for proliferation (Figure 2.3). These speculations fit perfectly in the following model: when no or less stromal interaction for growth purposes is needed, less activation of the cytokine-dependent β1-integrins very late antigen (VLA)-4 and VLA-5 on the $CD34^+$ cells can be assumed, which leads to a lower BM fibronectin adhesion and a higher chance of circulating $CD34^+$ cells[49-51]. Above all, recently a lower expression of the cell adhesion molecule L-selectin was found in this primitive $CD34^+$ cell population of MDS patients compared to normals[52]. From a prognostic perspective, it may therefore be very important to apply this double-labeling immunohistochemistry to determine how many $CD34^+$ cells and $CD34^+$ aggregates can be found in the BM biopsies, and especially how many of them are in S-phase.

S-Phase specific agents like cytosine arabinoside (Ara-C) are considered to be most effective in MDS. If chronic cytoreductive therapy (like low-dose Ara-C) is effective enough to kill (because Tc increases with no change in Ts) the aberrant $CD34^+$ stem cell clone(s) will be uncertain. New therapeutic options in the growing population of MDS patients have to be developed. The combination of cytoreductive therapy and biological therapy directed to suppression of proliferation of the aberrant $CD34^+$ clone(s) and suppression of apoptosis of more differentiated BM cells may restore normal polyclonal hematopoiesis in MDS patients.

Conclusions

In summary, as MDS evolves from low-risk to high-risk groups and eventually sAML, we observed an increase in absolute $CD34^+$ cells, as well as an increase in absolute $CD34^+$ cells in S-phase in BM biopsies. We believe that this phenomenon is the result of clonal expansion of genetically altered blast cells with less or no apoptosis and with slower proliferation rates than their more mature $CD34^-$ counterparts in a different microenvironment of cytokines and probably changed stromal interactions. The IH double-labeling technique we described can be used to follow MDS evolution and probably determine prognosis and leukemic transformation with greater accuracy than before and irrespective of their FAB subtype, like Oriani et al.[48] have shown for single-labeling CD34 immunostaining. The different biological processes which lead to changes in size and proliferative capacity of the $CD34^+$ and $CD34^-$ compartments within MDS in evolution have been discussed and are pathophysiologically expressed in Figure 2.7.

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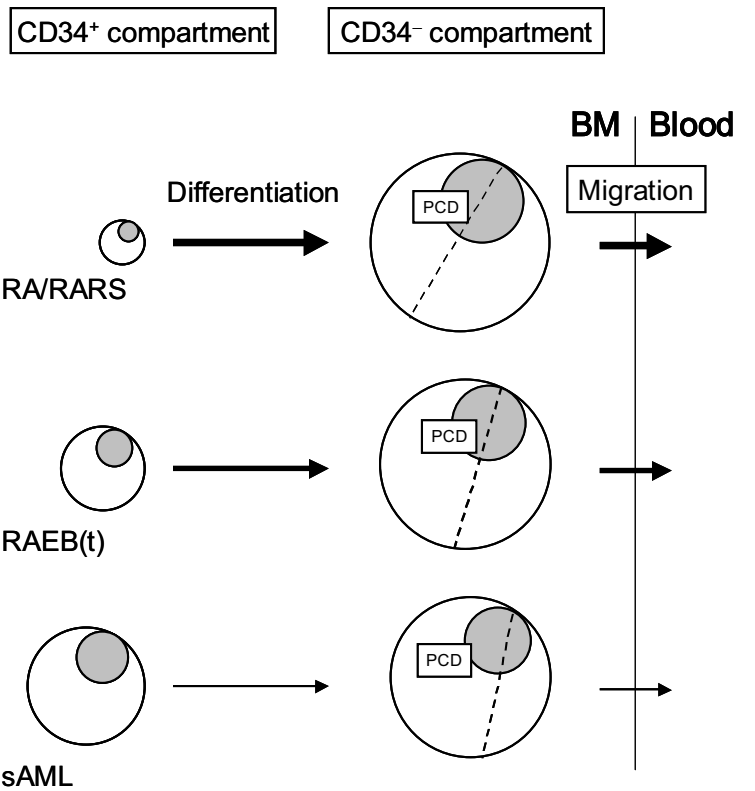

Figure 2.7 Proliferative characterisitcs and size of the $CD34^+$ and $CD34^-$ compartments during MDS evolution to sAML. Gray area, % S-phase cells of all myeloid cells; white area, % cells of all myeloid cells; PCD area, apoptosis of S- and non-S-phase cells in $CD34^-$ compartment.

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Chapter 3

The dynamic process of apoptosis analyzed by flow cytometry using Annexin-V/PI and a modified in situ end labeling technique

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Abstract

Background and objectives

To study the apoptotic process in time we used the following flow cytometric (FCM) techniques: Phosphatidylserine (PS) translocation by Annexin-V(AnV)-FITC, DNA fragmentation by in situ end labeling (ISEL) and propidium iodide (PI) staining. Because PS translocation is assumed to be an early feature of programmed cell death (PCD), we questioned if AnV positivity implies inevitable cell death.

Materials and methods

Apoptosis was induced in Jurkat cells by γ-irradiation, incubation with camptothecin (CPT), or cytosine β-D-arabinofuranoside (Ara-C). At different time intervals, PCD was quantified by AnV/PI and ISEL. To analyze the influence of cell handling procedures on PCD, we applied these three FCM techniques on normal $CD34^+$ bone marrow (BM) stem cells after selection and after a freeze-thaw procedure. Various AnV+/PI– $CD34^+$ fractions were cultured in single-cell single-well (SCSW) assay.

Results

Jurkat cells under three different detrimental conditions showed essentially the same pattern of apoptosis in time. Initially developed AnV+/PI– cells subsequently (within one hour) showed ISEL positivity, after which they turned into AnV+/PI++ cells with even higher levels of ISEL positivity (80-90%). Eventually, they lost some of their PI and ISEL positivity and formed the AnV+/PI+ fraction. Cell handling of $CD34^+$ cells caused high and variable AnV+/PI– fractions (overall range 23-62%). Within total AnV+ and AnV+/PI– populations, only a minority of $CD34^+$ cells showed ISEL positivity (range 4-8% and 0.8-6%, respectively). Different fractions of AnV+/PI– $CD34^+$ cells did have clonogenic capacity in SCSW assay.

Conclusions

PCD of cell suspensions *in vitro* can be followed accurately in time by these three different FCM techniques. PS translocation is rapidly followed (within one hour) by oligo-nucleosomal DNA fragmentation, after which cell (and nuclear) membrane leakage occurs. Detection of PS asymmetry by AnV-FITC is not always associated with (inevitable) apoptosis, as can be concluded from the proliferative capacity of AnV+/PI– $CD34^+$ cells in SCSW assay.

Introduction

Each electrophoretic (EP), immunohistochemical (IH) and flow cytometric (FCM) technique detects only one specific feature of the process of apoptosis. Gel- and pulse-field EP methods detect small oligo-nucleosomal DNA strand breaks (multiples of 180-200 basepairs; "DNA-laddering") and large DNA strand breaks (more than 30-50 kbp), respectively, which are supposed to be relatively late and early features of programmed cell death (PCD). Unfortunately, EP techniques are not applicable for quantification of PCD. In contrast, IH techniques like in situ end labeling (ISEL) and terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) of DNA and FCM methods like Annexin-V (AnV)-fluorescein isothiocyanate (FITC), TUNEL, and propidium iodide (PI) can detect apoptosis of single cells.

AnV detects phosphatidylserine (PS) transposition on the outer plasma membrane, which occurs at a rather early stage of PCD during the so-called "execution phase"[1-6]. This loss of membrane asymmetry develops downstream of the Bcl-2 checkpoint, after the disruption of the mitochondrial transmembrane potential, the release of apoptosis-inducing factor, and the activation of caspases[1]. PS translocation precedes nuclear condensation, loss of membrane integrity (causing PI uptake) and cell shrinkage[6,7]. Whether PS transposition occurs before or after "the point-of-no-return" of PCD is still a matter of debate. ISEL is an IH technique originally described by Wijsman et al.[8] and modified successfully for plastic-embedded BM tissue by Mundle et al.[9]. A mix of four nucleotides with DNA-Polymerase-I is used to detect specific 3'-OH ends of single-strand DNA breaks, which are found after endonuclease activation. Therefore, ISEL is detected in the phase of PCD beyond the point-of-no-return. We modified this ISEL technique and made it applicable for FCM[10,11]. We used it in combination with AnV/PI to study the kinetics of different features of PCD. Furthermore, we questioned if PS translocation under every circumstance means inevitable apoptosis by culturing sorted AnV+/PI– $CD34^+$ cells in a single-cell single-well (SCSW) assay.

Materials and methods

Materials

The T-cell leukemia Jurkat cell line was cultured in RPMI (Gibco, Paisley, UK) with 10% fetal calf serum (FCS; Gibco) and treated in different ways to induce PCD *in vitro.* These cells (0.5×10^6) were either incubated with 4 ml fresh medium containing camptothecin (CPT; 2 μg/ml, Sigma, Zwijndrecht, the Netherlands) or cytosine β-D-arabinofuranoside (Ara-C; 10^{-4} M, Sigma), or they were γ-irradiated with 12.5 Gray (Gy) and subsequently cultured for 7 days.

PCD was measured (in duplicate) by AnV/PI in time and different fractions were sorted and subsequently fixed overnight in freshly prepared 4% paraformaldehyde at 4°C. These cells were stored in 70% ethanol at -20°C for ISEL analysis at a later time.
The BM of three normal donors (D1-D3) was processed by Ficoll density separation (1.077 g/ml, Sigma) to obtain a mononuclear cell fraction. $CD34^+$ progenitors were isolated from this fraction with directly conjugated CD34 antibody-coupled immunomagnetic beads (Dynal, Oslo, Norway). After a wash with glucose-phosphate buffered saline (G-PBS) with bovine serum albumin (BSA), these cells were resuspended in Iscove's medium, supplemented with 10% v/v heat-inactivated FCS and 10% v/v dimethylsulfoxide (DMSO) at a concentration of $0.2\text{-}0.4\times10^6$ cells/ml. Subsequently, the cells were cryopreserved in a temperature-controlled freezer (Kryo 10, Planerbiomed, Sunbury, Middlesex, United Kingdom) and stored in liquid nitrogen at -198°C. Cells were thawed rapidly in a waterbath of 37°C and diluted in FCS, containing 0.2 mg/ml DNAse, 4 M $MgSO_4$ and 15 U/ml heparin. PCD analysis by FCM (AnV/PI and ISEL, in duplicate) was performed immediately after CD34 selection, after thawing, and after 4.5 hours residing in Iscove's medium with FCS in a 5% CO_2 atmosphere at 37°C.

PCD analysis by FCM on an Epics Elite ESP (Beckman Coulter, Hialeah, FL, USA)

For the AnV/PI procedure described in detail in Vermes et al.[3], we always used freshly obtained cells without preceding fixation. After centrifugation, the cells were washed with RPMI medium with 5% FCS. Incubation was performed with AnV-FITC (end-concentration (EC) 1.2 μg/ml, Bender Medsystems/Cordia, Leiden, the Netherlands) with an excess of calcium (2.5 mM) and PI (EC 1.6 μg/ml, Sigma) for 10 minutes (min.). Jurkat cells in medium without cytotoxic treatment served as a negative control. Different AnV/PI fractions were sorted for ISEL by FCM (see also Figure 3.2 for the various compartments): AnV–/PI–, AnV+/PI–, AnV+/PI++, AnV+/PI+.
The modified ISEL technique for FCM (FCM-ISEL) on fixed cells was applied as follows. After washing the cells with PBS-BSA, incubation with SSC (NaCl 0.3 M, sodium citrate 30 mM, pH 7.0) was performed for 20 min. at 78°C, followed by another wash in PBS-BSA. After washing with buffer (Tris HCl 50 mM, $MgCl_2$ 5 mM, β-mercaptoethanol 10 mM, BSA 0.005%, pH 7.5), incubation with DNA-Polymerase-I (20 U/ml, Promega, Madison, WI, USA) together with 11-bio-dUTP (0.5 μM, Sigma), dATP, dCTP, and dGTP (10 μM each, Promega) was carried out at 19°C for 30 min. A wash with PBS-BSA and incubation with streptavidine-Cy5 (1.0 μg/sample) at 4°C for 30 min. was performed. Finally, the cells were washed with PBS-BSA followed by

FCM-ISEL. Incubation of cells without DNA-Polymerase-I served as a negative control. All AnV/PI and ISEL measurements were performed (in duplicate) on $CD34^+$ cells within the life-gate and on Jurkat cells after setting the discriminator to exclude debris by forward and right angle scatter.

Single-Cell Single-Well Assay

Human BM $CD34^+$ cells were stained with AnV-FITC. An autoclone unit sorted single PI^-CD34^+ cells within the life-gate which were bright (AnV++: AnV fluorescence intensity ≥ 3), dull (AnV+: AnV fluorescence intensity 0.5-3) or negative (AnV−: AnV fluorescence intensity <0.5) for AnV in 96-well round-bottom plates (Costar, Corning, NY, USA). Every well was checked for the presence of one single cell by inverted microscopy before culturing. Each well contained 75 μl Iscove's medium (Gibco) supplemented with 2 mM L-glutamine (Flow Laboratories, Zwanenburg, the Netherlands), streptomycin 50mg/ml and penicillin 50 IU/ml (Gibco), 20% v/v FCS, and recombinant growth factors (GFs). This medium was supplemented with granulocyte-colony stimulating factor (20 ng/ml, Amgen, Thousand Oaks, CA, USA), human stem cell factor (25 ng/ml, Amgen), interleukin-3 (50 ng/ml, Sandoz BV, Uden, the Netherlands) and granulocyte macrophage-colony stimulating factor (20 ng/ml, Sandoz). The plates were incubated in a fully humidified, 5% CO_2 incubator at 37°C. The proliferative capacity of these $CD34^+$ cells was assessed by counting the cells in every well at day 14 by an inverted microscope. Proliferative capacity was assessed by enumerating wells with 2 or more cells, more than 50, and more than 500 cells per 96 wells.

Results

The modified ISEL technique works well. FCM-ISEL without adding DNA-Polymerase-I served as a negative control (Figures 3.1A, 3.1B). After adding DNA-Polymerase-I to CPT-treated Jurkat cells, an excellent separation between ISEL+ and ISEL− cells (Figures 3.1C, 3.1D) was found.

Kinetic analysis of CPT-induced apoptosis in Jurkat cells by AnV/PI by FCM

The process of PCD in time during CPT incubation (0-4-22-48-72-169 hours) determined by AnV/PI is depicted in Figure 3.2. Fresh AnV−/PI− cells became AnV+/PI− within 4 hours (mean AnV and PI fluorescence intensities of 27.3 and 0.42, respectively), after which they developed strong PI positivity (AnV+/PI++ within 22 hours: mean AnV and PI fluorescence intensities of 39.1 and 63.1,

respectively). All cells eventually turned into end-stage apoptosis as an AnV+/PI+ population (within 22 hours and more: mean AnV and PI fluorescence intensities of 37.8 and 6.0, respectively). Figure 3.3 shows the time course quantity of these different AnV/PI fractions. The same traverse pattern as depicted in Figure 3.2 was put down in Figure 3.3.

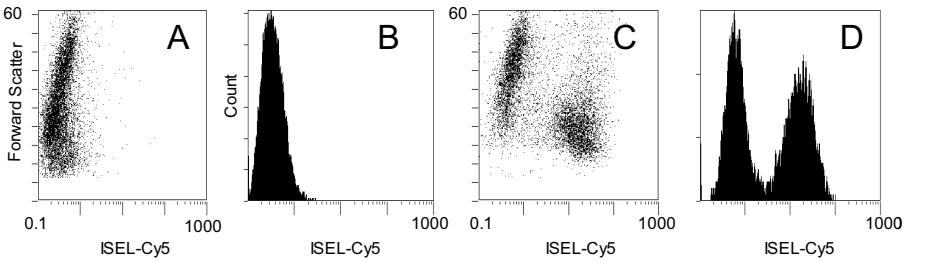

Figure 3.1 Jurkat cells treated with CPT (2 mg/ml) for 50 hours and stained with the FCM-ISEL method. C,D: ISEL+ cells are positioned to the right. A,B: FCM-ISEL without DNA-Polymerase-I (serves as negative control).

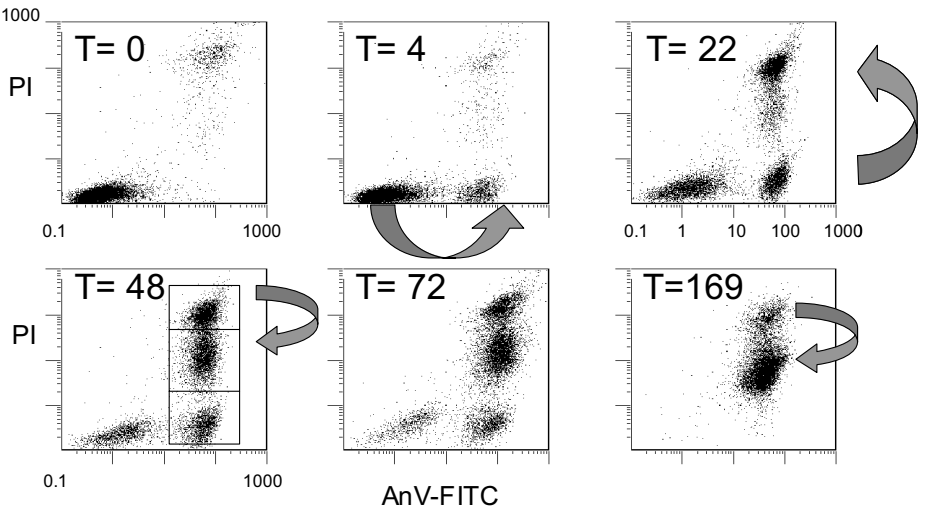

Figure 3.2 The process of apoptosis in time of Jurkat cells during various time periods (T in hours) of CPT incubation (2 mg/ml). Fresh AnV-/PIcells become AnV+/PI- within 4 h and develop strong PI positivity (AnV+/PI++) within 24 h. Subsequently, all these cells turn into an AnV+/PI+ population as the AnV-/PI-, AnV+/PI-, and AnV+/PI++ populations disappear.

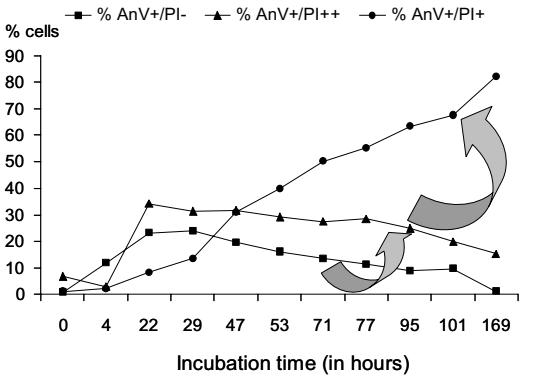

Figure 3.3 Quantification of apoptosis by AnV/PI FCM of Jurkat cells treated with CPT during various incubation periods.

Development in time of AnV/PI and ISEL in CPT-treated Jurkat cells

ISEL was performed on the different AnV/PI fractions on two time points (Figure 3.4). AnV– cells showed no ISEL positivity, whereas ISEL positivity rapidly increased in the AnV+/PI– fraction to almost 70-80%. ISEL positivity ultimately gained 10 to 20% when the AnV+/PI++ fraction was formed, after which it decreased by almost the same magnitude by turning into the end-stage AnV+/PI+ fraction.

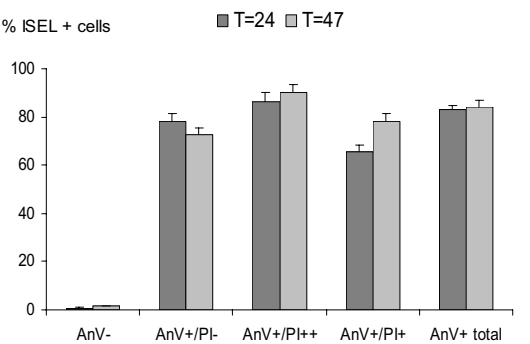

Figure 3.4 Determination of ISEL positivity (mean and SD) of the different AnV/PI fractions of Jurkat cells during 24 and 47 hours of CPT incubation. Dark gray bars: T=24 hours, gray bars: T=47 hours.

If we looked more carefully at the early phase of the PCD process (0-4 hours, Figure 3.5) of CPT-treated Jurkat cells, an evident increment of total AnV

positivity was noted between 2 and 3 hours. This increment of AnV+/PI– cells was rapidly followed by a clear gain of ISEL+ cells (within one hour; between 3 and 4 hours of incubation) with no concomitant rise in PI.

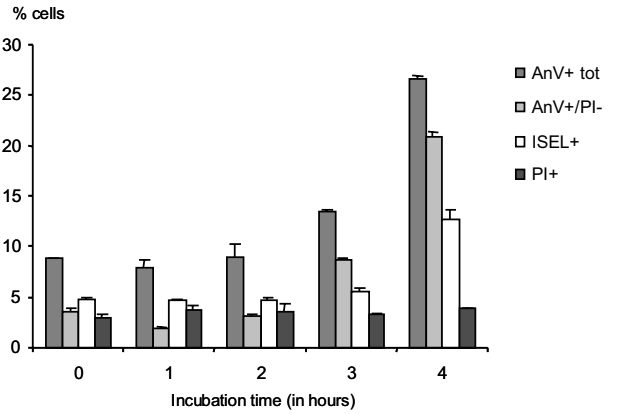

Figure 3.5 Jurkat suspension culture treated with CPT for 4 hours; apoptosis measurements in duplicate, depicted as mean % positive cells (and SD).

PCD development of Jurkat cells treated with three inducers of apoptosis

As can be seen in Figure 3.6, the increment in time of the percentage AnV+ CPT-treated Jurkat cells was followed by a same profile of increment of the percentage ISEL+ and PI+ cells, although the differences were less pronounced as time proceeded. Quite different patterns of PCD of Jurkat cells were found during Ara-C incubation and after γ-irradiation (Figure 3.6); they both showed less PCD and a more gradual increment of apoptosis. But, in general, we always observed the same pattern of phases of PCD in time (AnV+ earlier and higher than ISEL+). The difference between both apoptotic populations was not high (maximum 15-20%). Furthermore, the PI+ fraction remained in the same range as the ISEL+ fraction.

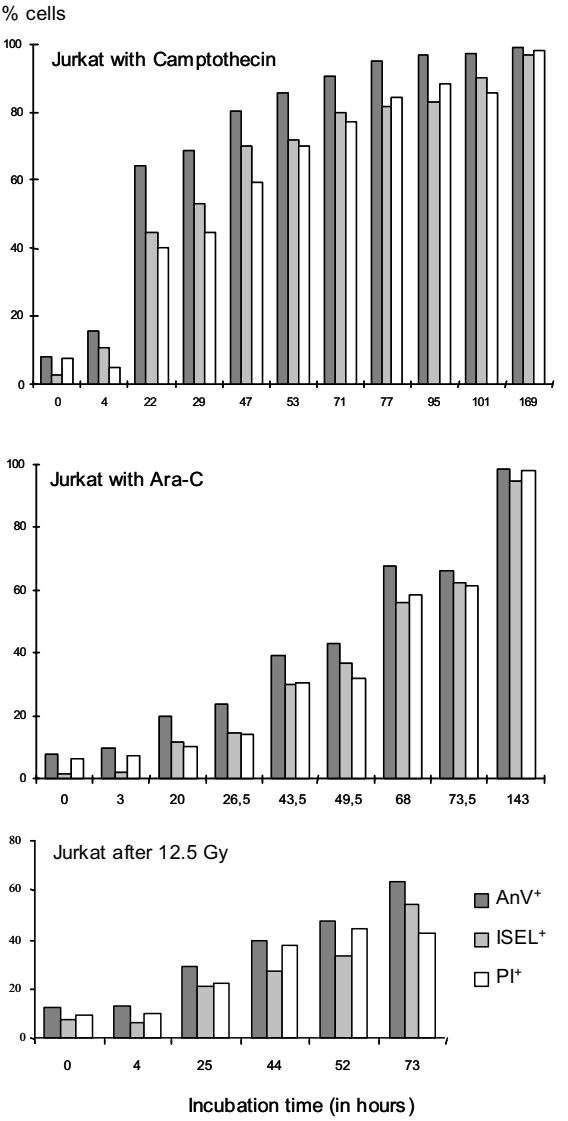

Figure 3.6 The kinetic pattern of apoptosis of Jurkat cells treated with CPT, Ara-C and γ-irradiation is quantified by three FCM techniques; AnV-FITC, ISEL and PI. Dark gray bars: AnV+, gray bars: ISEL+, open bars: PI+.

PCD measurements by FCM of sorted BM CD34$^+$ cells; influence of cell handling

In Table 3.1, the results of D1 of different PCD characteristics (mean % ± SD) are depicted as percentages of the whole CD34$^+$ population within the life-gate after CD34 selection, after viable freezing and thawing, and after 4 hours of incubation in medium after thawing. A variable amount of cell loss is anticipated and was found after each cell handling procedure, as was proven by a gradually increment of the amount of cell debris after each procedure (5.5% to 12.1% to 13.4%, respectively). On the other hand, the percentage of CD34$^+$ cells within the life-gate only showed a slight decrease during these procedures (70.5 to 70.0 to 67%, respectively), representing some decrease in absolute numbers of vital CD34$^+$ cells. An unexpected high amount of total AnV positivity was found (62.2 ± 2.1%) directly after CD34 selection with antibody-coupled beads, of which only a small part showed really features of PCD (ISEL+ of 5.9% versus AnV+/PI+ of 0.35 ± 0.07%). After viable freezing and thawing, the amount of AnV+ CD34$^+$ cells was considerably less but still remained high (39.6 ± 2.5%), whereas only slightly lower mean ISEL+ and AnV+/PI+ levels were found (4.6 ± 0.5% and 0.15 ± 0.07%, respectively). Incubation of the thawed cells in medium for 4.5 hours caused some decrease in total AnV+ (±8%), which was totally attributed by a shift from AnV+/PI- fraction towards the AnV-/PI- population. The amount of AnV+/PI+ hardly changed (0.1%) whereas the amount of ISEL positivity almost doubled (7.6 ± 0.1%). Under all these circumstances, the difference between AnV+/PI- and ISEL+ populations of CD34$^+$ cells remained at least 24% with a maximum of 55%.

Table 3.1 Apoptosis measurements (mean ± SD) of life-gated CD34$^+$ cells after CD34 selection with immunomagnetic beads (D1), and after viable freeze-thawing of CD34$^+$ cells (D1-3), followed by incubation in medium (D1), and plating efficiency in the SCSW assay (D2-D3) with size and number (#) of cell aggregates per 96 wells.

D1 / CD34$^+$	life-gated	AnV-/PI-	AnV+/PI-	AnV+/PI+ mean% ± SD	Total AnV+	ISEL+
After CD34+ selection (in %)		37.8 ± 2.1	61.9 ± 2.1	0.35 ± 0.07	62.2 ± 2.1	5.9
After thawing CD34+ (in %)		60.5 ± 2.5	39.4 ± 2.4	0.15 ± 0.07	39.6 ± 2.5	4.6 ± 0.5
After incubation medium (in%)		68.8	31.1	0.1	31.2	7.6 ± 0.1
D2 / CD34$^+$	Total (%)	**SCSW**	>2 cells	>50 cells	>500 cells	ISEL+ (%)
AnV-/PI-	75.1	**# aggreg.**	18	10	8	0
AnV+/PI-	23.6	AnV+	16	7	4	0.8
		AnV++	14	1	1	4.1
AnV+/PI+	1.3		0	0	0	
D3 / CD34$^+$	Total (%)	**SCSW**	>2 cells	>50 cells	>500 cells	ISEL+ (%)
AnV-/PI-	63.0	**# aggreg.**	27	11	8	0
AnV+/PI-	35.0	AnV+	14	5	4	3.8
		AnV++	8	3	1	5.4
AnV+/PI+	1.5		0	0	0	

Culturing different AnV/PI fractions of thawed CD34$^+$ cells in an SCSW assay

After thawing of the sorted CD34$^+$ cells of donor 2 and 3 (D2, D3), the AnV fractions within the PI- compartment (AnV-, AnV+, AnV++) were used for SCSW assay to detect their proliferation capacity as a golden standard of viability. These AnV fractions were also sorted from the life gate after excluding cell debris. The difference between AnV+/PI- populations of both donors was 10-15% (Table 3.1), which was not correlated with their percentages ISEL+ found within the dull and bright AnV+/PI- CD34$^+$ cells. As AnV-CD34$^+$ cells showed normal growth in SCSW assay, this fraction grew approximately twice as good as their AnV+/PI– counterparts. Within this last group, the dull AnV+ cells grew considerably better than the bright AnV++CD34$^+$ cells. AnV+/PI+ CD34$^+$ cells showed no growth at all, as was expected.

Discussion

In this study we used Jurkat cells to observe the kinetics of three features of PCD by FCM. We also used CD34$^+$ cells to measure apoptosis characteristics after cell handling and to investigate if AnV positivity after cell handling implied inevitable PCD. PS transposition of cells has been recognized as an evolutionary well-saved and ubiquitous marker of early PCD, that is needed for cell engulfment by macrophages before their plasma integrity becomes compromised[5,12]. However, PS translocation is not unique for apoptosis because it was also observed during (secondary) necrosis[13]. Therefore, we used the AnV-FITC assay with PI by time-lapse examination to differentiate among viable (AnV-/PI-), early apoptotic (AnV+/PI-), late apoptotic, and secondary necrotic (AnV+/PI++ or AnV+/PI+) cells[3,4,6,7,13-15]. Furthermore, as we needed a method to distinguish between early and late PCD, we developed a modified ISEL technique for FCM[10,11]. ISEL can be seen as a variant of the TUNEL technique, but TUNEL is used much more frequently in research[16,17]. DNA-Polymerase-I used in ISEL was tested and was able to detect DNA fragments generated by endonuclease activity in Jurkat and human BM CD34$^+$ cells during PCD[18-21]. To study apoptosis in time, we preferred the ISEL technique because it has been shown to be more specific for PCD than for necrosis as compared to TUNEL[20,22,23]. Furthermore, the TUNEL assay is prone to false positive and negative staining[15,24]. ISEL and PI detect specific DNA fragments and loss of cell membrane integrity, respectively, which are features of inevitable PCD, as they occur after each other during the execution phase[1,2,16,25-27]. From our background of research with stem cells, we questioned if detection of AnV (within CD34$^+$ cells) means that the point-of-no-

return of PCD has been passed. This is still a matter of debate, although some evidence in favour of this hypothesis has been gathered[6,28,29].

In line with the above-mentioned considerations, we proved in all Jurkat experiments that membrane PS asymmetry precedes DNA fragmentation and/or membrane perturbation (Figures 3.2, 3.3, and 3.5), as DNA fragmentation precedes cell membrane leakage (Figures 3.4 and 3.5). Others[1,4,6,13,16,17,30-32] have obtained similar results with various techniques (AnV/PI, AnV/PI with TUNEL, AnV/TUNEL, different DNA binding dyes, morphology, comet assay, and laser scanning cytometry), but not in one setup of serial experiments in time combining three PCD-analyzing techniques with three apoptosis-inducing methods. In the experiment of exposing Jurkat cells to CPT, PS expression increased after 2-3 hours (AnV+/PI-) and was followed by ISEL increment by only one hour difference (Figure 3.5). Others also found this relation in time with different techniques[28,32-35] and it emphasizes the discrete line in time to pass the point-of-no-return regarding PCD (of this cell line under these circumstances). On the other hand, Bacsó et al.[32] proved within a CD95-induced Jurkat apoptosis model that virtually all the AnV+/PI– cells, which increased after 2 hours, had apoptotic comets or remnants (as it also detects early 50kb DNA fragments).

After carefully analyzing the process of PCD in time, an interesting pattern was observed. AnV+/PI–/ISEL– viable cells developed into AnV+/PI–/ISEL+ and subsequently AnV+/PI++/ISEL++ cells, representing a more progressive phase of apoptosis with the highest ISEL positivity. These cells turned into AnV+/PI+/ISEL+ cells, which are end-stage apoptotic and/or secondary necrotic cells, presumably characterized by more DNA disintegration, nuclear condensation, and leakage of cell and nuclear membranes causing less PI- and ISEL-positive staining[15]. These results are comparable with the Nicoletti assay[3,36] and with the report of MacNamara et al.[37] who studied HL-60 cells under similar conditions. They determined the PCD of these cells as a sub-G0/G1 peak on DNA histograms with forward and sideward scatter features reflecting cell shrinkage and the presence of apoptotic bodies.

The different PCD patterns of Jurkat cells after γ-irradiation, CPT, or Ara-C were explained by the detrimental action upon different cellular targets and by a different dose-response. One should realize that the death of these cells is necrotic at high levels of insult, whereas PCD is induced at lower levels. For example, γ-irradiation promptly caused single and double DNA strand breaks, some of which were sublethal and could be repaired, some of which were lethal (causing PCD), and some of which were devastating to the cell (causing necrosis). This explains the initial combination of primary necrosis and apoptosis (of especially cells in S-phase[38]), in which the higher amount of necrosis with more PI positivity (higher than ISEL) gradually declined in order to make place for more apoptotic involvement (ISEL>PI). CPT, a DNA-

topoisomerase-I blocker, induces DNA strand breaks of all cells (although DNA-replicating S-phase cells are more sensitive), whereas Ara-C, a pyrimidine antagonist, predominantly effects S-phase cells. Perhaps this could explain a slower increment in PCD by Ara-C as compared to CPT[29,33-35].

High numbers of AnV+/PI- of $CD34^+$ cells within the life-gate occurred after $CD34^+$ selection with immunomagnetic beads (D1: $\pm$ 62%) and they also showed a high variability after rapidly freezing and thawing (mean AnV+/PI- of D1-D3: 33 ± 8%). It is a well-known and accepted phenomenon that a highly variable amount of stem cells is lost after CD34 selection and viable freezing and thawing. Cryopreservation in liquid nitrogen of mononucleated BM cells in 10% DMSO leads to absent trypan blue exclusion in approximately 10-15% cells and to a 25 ± 10% loss of stem cells and colony forming unit-granulocyte macrophages (CFU-GM)[39]. But, the important issue is what are the characteristics of the $CD34^+$ cells within the life-gate regarding cell-viability versus apoptosis after these cell handling procedures? Cryopreservation of hematopoietic stem cells leads to high and variable AnV positivity (range 5-70%), but whether these cells are destined to die remains to be proven and should not be presumed[40]. Membrane alteration or damage could be triggered by the handling during the antibody-coupled immunomagnetic bead selection or induced by controlled freezing (DMSO should prevent crystal formation) and rapidly thawing (DMSO can cause osmotic shock) of these cells[41]. The big difference in percentage between total AnV+ and ISEL+ $CD34^+$ cells (between 20-55%) in combination with an unchangeable low percentage in ISEL+ cells ($\pm$ 5%) after CD34 selection and after thawing argues strongly for a temporary membrane alteration and not for PCD. In our experiment, at least some of these AnV+ cells repaired their membrane activation-alteration and/or damage during incubation in medium (approximately 6-8% in D1), whereas other cells followed their path of PCD as ISEL was increasing. Furthermore, at least 8% (8 of 96 wells) to 15% (14 of 96) of these thawed AnV+/PI- $CD34^+$ cells (AnV++ fraction) had proliferative capacity. On the other hand, these cells represented at least 30% (8 of 27 wells) to 78% (14 of 18 wells) of the normal plating efficiency found within these two controls in this SCSW assay. Normal plating efficiency (using AnV- $CD34^+$ cells) is defined as the number of wells showing proliferative capacity. In contrast, the CFU-GM capacity (to form colonies of more than 40 cells as being granulocytes and macrophages) was strongly and inversely correlated with the AnV intensity of these PI- $CD34^+$ cells. Therefore, AnV should not be used as a marker or as the only marker of apoptosis in experiments with stem cells in which physical membrane activation or alteration may be expected. PCD should be proved by distinct morphological features or by FCM techniques that detect DNA fragmentation products[15,32]. From these experiments, we conclude that in some experimental settings membrane activation or alteration and/or a low apoptotic insult was involved in causing PS

exposition in a commitment phase to apoptosis. This phase of "pre-apoptosis" has been shown to be caspase-independent and reversible if the strength of the stimulus is low and of short duration[26]. A substantial fraction of AnV+/PI– BM $CD34^+$ cells after different cell handling procedures has certainly not passed the point-of-no-return in the process of PCD, as the cells retained proliferative capacity, although to a lower extent. In analogy, cryopreservation and thawing of human spermatozoa[42] were associated with the induction of membrane PS translocation and high post-thaw levels of AnV were found even in the fractions with high sperm motility.

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Chapter 4

Bone marrow mononuclear cells of MDS patients are characterized *in vitro* by hyperproliferation and increased apoptosis independently of stromal interactions

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Abstract

Background and objectives

Enhanced proliferation of MDS progenitors is abrogated by increased programmed cell death (PCD) of their progeny *in vivo*. We investigated whether bone marrow mononuclear cells (BMMNC) of MDS patients also showed enhanced proliferation and apoptosis *in vitro* in comparison with acute myeloid leukemia (AML) and normal BM (NBM).

Design and methods

BMMNC were cultured in agar during 10 days. Proliferation was determined by counting clusters and colonies. Apoptosis was assessed by performing in situ end labeling on these cultures at days 4, 7, and 10.

Results

NBM showed a decrease in the number of clusters in time due to PCD of clusters and due to development of clusters into colonies with low apoptotic level. In MDS patients, about 2-fold more clusters have developed at day 4, and in contrast with NBM, the total number of clusters at day 7 remained high in spite of an increasing percentage of apoptotic clusters (from 52 to 76%) in combination with more colony formation. The number of clusters and colonies showed a sharp decline at day 10 because of persistently high apoptosis at cluster level and increasing PCD in colonies. BMMNC of AML patients showed decreased proliferation with enhanced apoptosis at cluster level in contrast to a relatively low apoptotic level in the colony-forming cells.

Conclusions

Within MDS, increased proliferation is abrogated by enhanced apoptosis, whereas AML showed decreased proliferation with a low level of apoptosis in colony-forming cells. These growth profiles of BMMNC are independent of stromal influences and represent intrinsic features of the MDS progenitors and possibly accessory cell interactions.

Introduction

Myelodysplastic syndromes (MDS) are acquired clonal stem cell disorders characterized by increased overall proliferation and apoptosis, associated with impaired differentiation leading to accumulation of myeloblasts, often with elevated CD34 expression and aberrant immunophenotypes. The overall balance between proliferation and programmed cell death (PCD) within the bone marrow (BM) changes as MDS evolves to acute myeloid leukemia (AML)[1-8]. Monoclonality is the hallmark of MDS and implies a growth advantage of MDS progenitors over normal hematopoiesis. During MDS progression, the clonal myelodysplastic hematopoiesis, characterized by increased proliferation but no survival benefit, gradually transforms into leukemic hematopoiesis, characterized by enhanced cell survival with defective differentiation and a low proliferation rate. This hypothesis is supported by the observation that the overall size of the $CD34^+$ compartment (containing S- and non-S-phase cells) is higher in RAEB(t) when compared with RA/RARS but the percentage $CD34^+$ cells in S-phase remains low in RAEB(t)[4].

Besides the above-mentioned “intrinsic” changes, interactions between the MDS progenitors and the microenvironment ("extrinsic" changes like stromal and/or accessory cell interactions) may also play a role in the increased overall (myeloid) proliferation associated with substantially increased apoptosis[9,10]. Anti-apoptotic therapies in MDS patients (like anti-TNFα therapy, thalidomide, ATG and/or cyclosporine A) have proven to decrease overall PCD and subsequently hyperproliferation within the BM, but the question still remains which intrinsic or extrinsic features are altered and at which maturation level. To clarify these questions we analyzed whether the *in vivo* overall hyperproliferation and increased apoptosis could also be observed *in vitro* by excluding stromal influences. Bone marrow mononuclear cells (BMMNC) of MDS patients were cultured in agar and compared with the “growth profiles” of normal bone marrow (NBM) and AML. The results show that the *in vitro* kinetics of early and late progenitors and their progeny parallels the *in vivo* observations. The role of intrinsic features and possible interactions with non-stromal accessory cells are discussed, on the basis of the *in vitro* profiles of MDS BMMNC presented in this study which are not influenced by stromal interactions.

Materials and methods

Patients

For our studies, we selected de-novo MDS patients (n=5) and AML patients (n=6) with trisomy 8 as sole cytogenetic abnormality. We compared the *in vitro* profiles of both groups with the profiles observed in healthy controls (NBM, n=6). Informed consent was obtained in all cases. The patient characteristics are presented in Table 4.1.

Table 4.1 Individual patient characteristics with mean values in normals.

NBM	Age (yrs)	Gender	FAB	BM blasts (%)	IPSS	GF (%BMMNC)	A%tot	A%-Cl	A%-Co
n=6	37	5M / 1F				0.24	42	54	25
MDS									
1	78	M	RARS	1	0.5	0.10	58	72	36
2	55	M	RAEB	7	1.5	0.33	56	66	41
3	60	F	RAEB	15	2	1.00	62	70	52
4	64	M	RAEB	17	2.5	0.06	40	50	30
5	66	M	RAEBt	26	3	0.58	42	53	26
AML									
1	52	M	M5	32		0.06	40	55	18
2	50	M	M4	38		0.08	44	60	21
3	41	F	M4	48		0.08	48	68	17
4	59	F	M1	76		0.08	51	67	27
5	44	F	M5	95		0.23	41	53	23
6	57	M	M4			0.01	87	100	67

FAB: French American British classification, BM: bone marrow, MNC: mononuclear cells, IPSS: International Prognostic Scoring System, GF: Growth Fraction; % S-phase cells of BMMNC, A%tot: % apoptotic clusters and colonies; overall apoptosis, A%-Clu and A%-Co: % apoptotic clusters and colonies, respectively. All *in vitro* parameters are expressed as mean values of days 4, 7, and 10.

BMMNC collection, cryopreservation, and thawing

BMMNC were obtained from BM aspirates after Ficoll (Sigma, St. Louis, Missouri, USA) density centrifugation (1.077 g/ml). BMMNC were cryo-preserved and stored in liquid nitrogen before thawing[11].

Agar culture conditions and assessment of proliferation

After thawing, BMMNC were resuspended with Iscove's (IMDM, Gibco) supplemented with 20% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories), 0.3% (w/v) bacto-agar (Difco, Detroit, Michigan, USA), and G-CSF (Amgen, Thousand Oaks, CA, USA, final concentration (FC) 20 ng/ml), GM-CSF (Sandoz BV, Uden, the Netherlands, FC 25 ng/ml), IL-3 (Sandoz BV, FC 40 ng/ml), hSCF (Amgen, FC 25 ng/ml). No erythropoietin was used in

order to culture myeloid clusters and colonies. We plated 200,000 cells per 35×10-mm culture dishes (Costar, Cambridge, Massachusetts, USA). After incubation in a fully humidified 37°C atmosphere, the plates were scored with an inverted microscope (Diavert Leitz) on days 4 (8 plates), 7 (6 plates), and 10 (4 plates). The proliferative capacity of BMMNC was defined as the number (N) of clusters (Cl: 10-39 cells) and colonies (Co: ≥40 cells) per culture dish. Ntot is the total number of clusters and colonies together per culture dish. Clusters and colonies were disregarded if they showed clear pycnotic features. After counting, two agar bottoms were cut in halves and citospinned (1500 rpm for 10 minutes) on Superfrost slides, as described in detail before[12]. After drying for 30 min. (minutes), these half-bottoms were put in cold 4% paraformaldehyde in 0.15 M phosphate buffer solution (PBS) for fixation overnight. These slides were stored in 70% ethanol at 4°C until the in situ end labeling (ISEL) or in situ hybridization (ISH) procedures were performed.

ISEL assay adapted for agar cultures on slides

Apoptosis was detected by the ISEL assay[13]. This technique detects oligonucleosomal DNA strand ends of 200-300 base pairs in size using a cocktail of DNA-Polymerase-I and four nucleotides of which only dUTP (11-bio-dUTP, Sigma) is biotinylated. The slides were rinsed in double-distilled water followed by incubation in 0.23% Periodic Acid (Sigma) for 30 min. to block endogenous peroxidase. They were rinsed once in distilled water and then 3 times in 0.15 M PBS (0.15 M sodium chloride in 0.1 M phosphate buffer, pH 7.5) with 0.1% Tween 20 (Sigma) (PBST) for 4 min. each. Subsequently, the slides were immersed in SSC solution (0.3 M sodium chloride, 30 mM sodium citrate, pH 7.0) of 78°C and incubated for 20 min. to enhance the accessibility of DNA for probes. The slides were rinsed in 0.15 M PBST, followed by 3 wash steps for 4 min. each. Incubation with 1 mg/ml Pepsin (Porcine by Serva, Heidelberg, Germany) in 2 M HCl for 20 min. was performed, followed by washes in 0.15 M PBST. Subsequently, the slides were rinsed in Buffer A (50 mM Tris HCl, 5 mM $MgCl_2$, 10 mM 2-β-mercapto-ethanol, 0.005% bovine serum albumin, pH 7.5). The ISEL cocktail containing Buffer A with 20 U/ml DNA-Polymerase-I, 0.01 mM dATP, dCTP, and dGTP (Promega) together with 0.001 mM bio-dUTP was applied to all slides, followed by incubation at 18°C for 2 hours. Afterwards, the slides were rinsed with Buffer A, followed by 3 rinses for 4 min. each, which was followed by 3 wash steps in 0.5M PBS (0.5 M sodium chloride in 0.1 M phosphate buffer, pH 7.5). The secondary mouse anti-biotin (M0743, Dako, Carpinteria, CA, USA) antibody (1:200) was applied for 30 min., followed by 3 washes with 0.5 M PBS. Incubation of the tertiary biotinylated horse anti-mouse (Vectastain Elite kit) antibody (1:200) lasted for 30 min. Finally, ABC solution (avidin-biotin/peroxidase complexes, Vectastain ABC Elite Kit, Vector,

Burlingame, CA, USA) was applied for 30 min. Washes with 0.5 M PBS were followed by 8 min. incubation with 50 mg DAB (3,3'-diaminobenzidine tetrahydro-chloride, Sigma) in 200 ml 0.05 M Tris buffer with 12 µl 30% H_2O_2 (pH 7.5). A brown color reaction occurred only in the nuclei with DNA fragmentation products in situ. Finally, the sections were rinsed in distilled water and were air-dried. The negative control was treated with the ISEL cocktail without DNA-Polymerase-I. Cytospin slides of Jurkat cells treated with and without Camptothecin (2 µg/ml) for 120 hours in suspension cultures were also used as positive (95-100% ISEL positive cells, Figure 4.1A) and negative control (3-5% ISEL positive cells), respectively.

Scoring of apoptosis within the agar cultures on slides

To calculate the percentage of apoptotic cells within each cluster or colony, the cells with a clear brown nucleus (ISEL-positive) and the total number of cells of a cluster or colony were counted (Figure 4.1). Subsequently, apoptosis in clusters and colonies was determined as being <50% and ≥50% ISEL-positive cells. Apoptosis (A) in this study is defined as the number of clusters or colonies showing 50% and more ISEL-positive cells (Figure 4.1B), because apoptosis is overruling proliferation in these aggregates. The degree of apoptosis (A%) is defined as the percentage of clusters or colonies per culture dish showing 50% or more ISEL-positive cells.

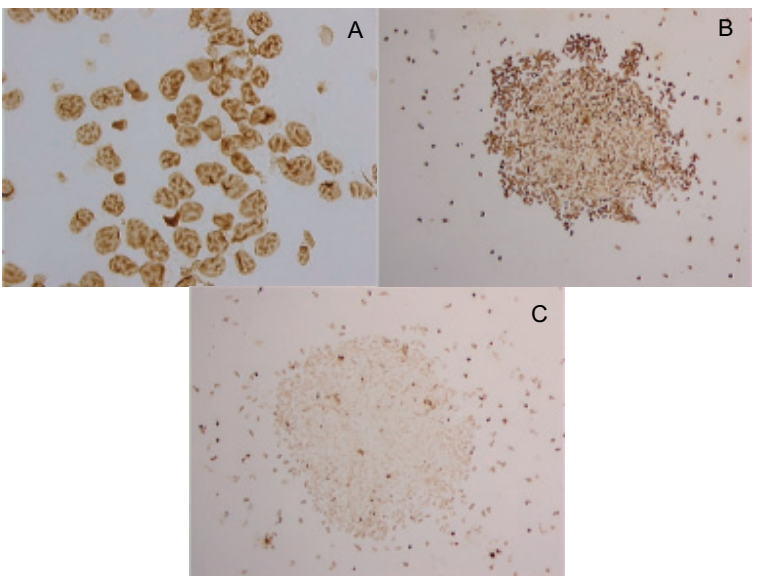

Figure 4.1 Jurkat suspension culture with camptothecin, shown as positive ISEL-control (A), normal colony in agar culture with considerably more than 50% apoptosis (B) versus normal colony with hardly any PCD (C).

ISH studies upon day 10 - agar bottoms

The ISH-procedure performed upon day 10 - agar bottoms paraformaldehyde-fixed slides for detection of chromosome 8 has been described in detail before[12]. Since an aggregate is principally the result of the proliferation of a single progenitor cell and as it always harbors some dying cells breaking down their chromosomes, not all cells within these aggregates have to pick up ISH signals to distinguish between disomic and trisomic 8 aggregates. See Figure 4.2 for examples of this technique. Single cells (SC), clusters and colonies with two or three brown spots within their nuclei were counted on day 10. Aggregates were disregarded and not counted when the ISH signals were not convincingly detectable. Aggregates were counted as more than 75% of viably-looking cells within an aggregate showing ISH signals.

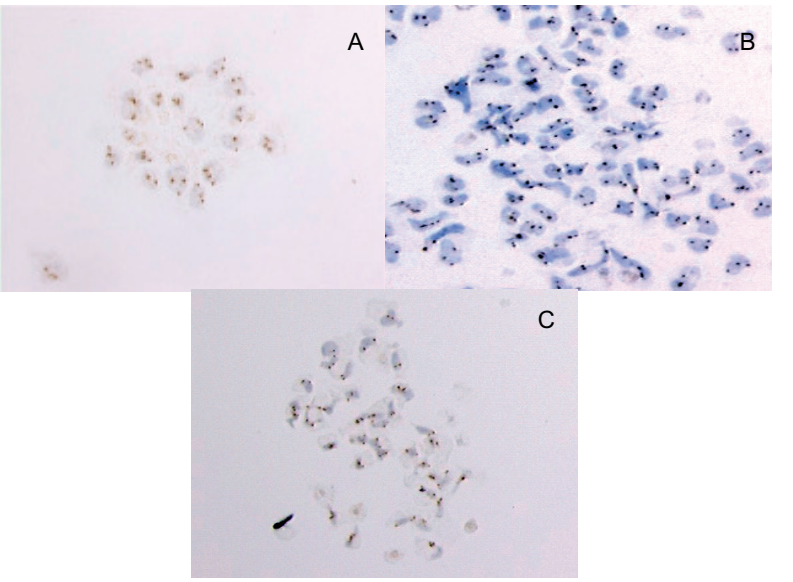

Figure 4.2 ISH procedure with CEP-probe against chromosome 8, performed on agar cultures showing a trisomic cluster (A), a trisomic colony (B), and a disomic cluster (C).

Definitions and statistical analysis

The median numbers (N) of clusters or colonies per patient group were calculated on days 4, 7, and 10. Ntot contains all the counted clusters and colonies per culture dish, regardless of their amount of apoptosis. Cluster/colony ratios (Cl/Co) of days 7 and 10 were determined for each sample and median values were calculated for each group. The median

percentages of apoptotic clusters or colonies defined as having 50% and more ISEL-positivity (A%=A/N×100%) in each study group were also calculated. The overall amount of apoptosis of clusters and colonies together (A%tot) was determined. The growth fraction (GF) or plating efficiency (PE) is defined as the percentage dividing cells of seeded BMMNC and can be calculated by the following formula: GF=Ntot/200.000 cells. Because of the frequently found skewed or non-Gaussian distribution curves of the different variables (especially in the MDS and AML groups), we used medians and applied a distribution-free Wilcoxon Mann-Whitney test to assess statistical differences ($P<0.05$) between the three groups.

Results

During the 3-day intervals between scoring, clusters may either become pycnotic and disappear, or they may continue to proliferate to form larger clusters or colonies followed by differentiation, or they proliferate continuously without differentiation (blast colonies). Furthermore, the progenitors not proliferating at day 0 may enter S-phase during culturing (delayed growth initiation). We deliberately defined clusters as aggregates of 10-39 cells, because it was not feasible to correctly count all small clusters (of 2-9 cells; N2-9), as they occurred frequently in MDS cultures, especially at day 4.

Profiles of proliferation and apoptosis in NBM, MDS, and AML

From day 4 to day 10, normal BM showed a considerable decrease in the number of clusters (from median of 261 to 73) due to apoptosis in 48-53% of the clusters and due to progression into colonies (Table 4.2 visualized in Figure 4.3). The number of colonies and the percentage apoptotic colonies slightly changed from day 7 to day 10 (N=217 to 229 with PCD of 17% to 28%, respectively). These aggregates showed clearly features of cell differentiation (data not shown).

In MDS patients (Table 4.2 and Figure 4.3) considerably more clusters developed (N=342 with 52% PCD on day 4). In contrast to NBM, the total number of clusters remained the same from day 4 to day 7, despite an increase in the percentage of apoptotic clusters (from 52 to 76%). Also more colonies were formed than in NBM (N=280 with 29% PCD vs. N=217 with 28% PCD, respectively). Eventually, the continued high level of apoptosis resulted in a sharp decline (by 58%) of clusters on day 10 (N=140 with 75% PCD). Also the number of colonies clearly decreased (by 55%) to 126 colonies on day 10 because of increasing apoptosis (42%) and a constantly high PCD at cluster level (Figure 4.3). Median Cl/Co ratios in MDS continued to be significantly

higher than normal (0.78 vs. 0.53 on day 7 and 0.97 vs. 0.28 on day 10, respectively).

Table 4.2 Results of *in vitro* proliferation and apoptosis of BMMNC of NBM, MDS, and AML.

Day	NBM (n=6)				MDS (n=5)			AML (n=6)	
	Clusters	SS	Colonies	SS	Clusters	Colonies	SS	Clusters	Colonies
N 4	261 (146-641)	2	0		342 (104-792)			62 (5-180)	
A%	48% (37-67%)	2			52% (31-85%)			79% (70-100%)	
N 7	100 (70-266)		217 (152-450)	2	337 (40-1436)	280 (51-505)	3	73 (7-367)	19 (8-103)
A%	53% (45-72%)		28% (17-36%)		76% (30-84%)	29% (9-61%)		47% (32-100%)	24% (11-100%)
Cl/Co	0.53 (0.33-0.7)	1.2			0.78 (0.55-3.43)			2.96 (0.7-9.64)	
Ntot	309 (238-716)	2			842 (91-1855)			101 (15-470)	
A%tot	34% (27-51%)				41% (25-79%)			44% (26-100%)	
N 10	73 (41-170)		229 (147-571)	2	140 (29-760)	126 (68-531)	3	65 (7-155)	57 (13-129)
A%	51% (35-79%)		17% (9-40%)		75% (44-91%)	42% (20-69%)	3	62% (39-100%)	20% (14-33%)
Cl/Co	0.28 (0.17-0.67)	1.2			0.97 (0.43-5.97)			1.15 (0.54-2.45)	
Ntot	314 (192-741)	2			284 (97-1291)			126 (20-284)	
A%tot	26% (18-45%)	1.2			62% (30-80%)			45% (30-55%)	

In vitro proliferation (Nx: number of aggregates at day x) and apoptosis (A%: % aggregates showing 50% or more apoptosis) of BMNNC: median values (range), Cl/Co: cluster/colony ratio, Ntot and A%tot means overall proliferation and apoptosis, statistical significant differences (SS: P<0.05) by Wilcoxon Mann-Whitney test between the subgroups are designated as follows: 1=NBM vs. MDS, 2=NBM vs. AML, and 3=MDS vs. AML.

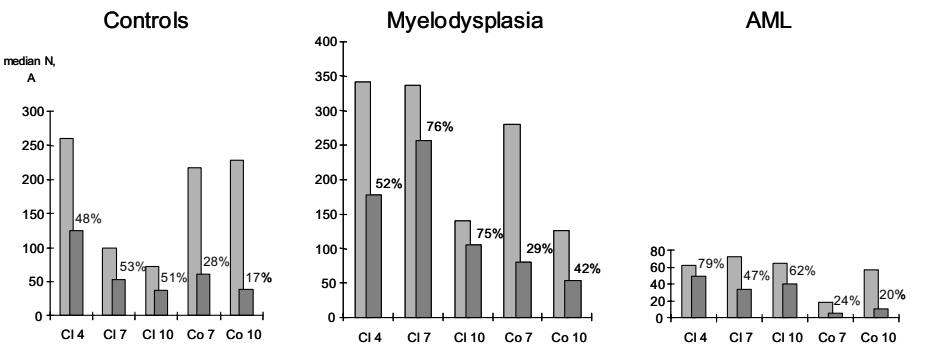

Figure 4.3 The median number of clusters (Cl) and colonies (Co) scored on days 4, 7, 10 (first bar, N=proliferation) in combination with the number (and their percentage) of clusters and colonies which showed 50% or more ISEL positive cells (second bar, A=apoptosis). Apoptosis is overruling proliferation when A% is more than 50%.

This *in vitro* profile in MDS can partially be explained by the initial contribution of numerous small clusters consisting of 2 to 9 cells on day 4 (considerably higher than normal), which dropped dramatically towards day 7. An increasing overall apoptosis from day 7 to 10 (from 41% to 62%) implicated that apoptosis

started to overrule proliferation. Moreover, overall apoptosis on day 10 was significantly higher than in NBM (62% vs. 26%).
In AML proliferation was consistently slower as compared with NBM and MDS, resulting in a low and stable number of clusters (between 62 and 73) from days 4 to 10 (Table 4.2 and Figure 4.3). High percentages of apoptotic clusters throughout the culture period (increasing from 42% to 79% from days 4 to 10), besides a considerably lower contribution of the smaller cluster (N2-9) compartment in time (lower than in normal BM) contributed to the low overall colony formation. A limited number of colonies were formed initially (n=19, PCD 24%), but the numbers increased up to day 10 with consistently low apoptotic involvement and sometimes with blastic appearance (n=57 with PCD 20% at day 10, Table 4.2 and Figure 4.3). These *in vitro* profiles of clusters and colonies caused declining CI/Co ratios from day 7 to 10, which remained significantly higher in AML (2.96-1.15) compared to NBM (0.53-0.28).
Comparing the final size of aggregates, NBM formed the largest colonies (Figure 4.1), whereas AML formed the smallest aggregates, both with a lower degree of apoptosis when compared to MDS.
Regarding overall proliferation (Ntot, Table 4.2) and calculating plating efficiency, normal BMMNC showed an overall GF of 0.24% that declined to 0.16% on day 10. Colony-forming cells represent 0.11%, which is about 70% of the GF at day 10. As compared to NBM, MDS patients showed a GF (although clearly underestimated due to the cluster definition of this study) almost twice as high (0.47%), which hardly changed till day 7, but rapidly decreased (by 70%) to 0.14% on day 10. Furthermore, colony-forming cells represent only 50% of this GF at day 10 (0.06%). In contrast, the GF in AML patients was importantly lower (1/3 of normal: 0.07%), with a markedly decreased colony forming capacity (0.01%). These colony-forming cells in AML increased significantly (0.03%) and represent 60% of GF at day 10.
Overall apoptosis of all aggregates (A%tot, Table 4.2) decreased during follow-up in NBM (from 48 to 26%) and in AML (79 to 45%), whereas it increased in MDS (52 to 62%). In NBM, apoptosis in clusters remained constant whereas it decreased in colonies (48-51% vs. 28 to 17%, respectively). However, median PCD in clusters and colonies increased in MDS (52 to 75% and 29 to 42%, respectively) in contrast to AML (79 to 62% and 24 to 20%, respectively).

ISH of single cells and aggregates compared to *in vitro* data at day 10

The percentages of trisomic 8 cells in the single cells (SC) and aggregates at day 10 of culturing together with their day 10 *in vitro* characteristics are shown in Table 4.3. A correlation could be observed between the percentage BM blasts and the percentage trisomy 8 metaphases in the MDS group (r=0.62), in

contrast to the AML group. Furthermore, a strong positive correlation was observed between the percentage BM blasts and GF in AML (r=0.85), in contrast to MDS (r=0.52). A strong negative correlation between GF and overall apoptosis at day 10 was found in AML (r=-0.75), which was not the case in MDS (r=0.50). The same negative correlation was also found between GF and degree of apoptosis in clusters within AML (r=-0.68). The percentage of trisomy 8 positive clusters and colonies at day 10 was lower than the percentage of +8 metaphases by classical cytogenetics with the exception of two patients (MDS5 and AML5) who showed no clear decrease (Table 4.3). The percentage trisomy 8 positive clusters and/or colonies at day 10 did not differ significantly from the percentage trisomy 8 cultured SC. The percentage trisomy 8 positive clusters and/or colonies at day 10 was higher than 50% in two MDS patients (no. 3 and 5) and in two AML patients (no. 3 and 5), meaning a proliferation advantage of the trisomic clone *in vitro* in 4 of 9 patients (Table 4.3). No clear relationship could be established between the percentage of trisomy 8 positive cells or aggregates after culturing and the degree of apoptosis in all aggregates (A% tot), or in clusters (A%-cl), or in colonies (A%-Co).

Table 4.3 *In vitro* characteristics linked with ISH data of MDS and AML, both obtained at day 10.

	Patients characteristics			*In vitro* characteristics at day 10				ISH data at day 10		
	%blasts	IPSS	%tri8	GF	A%tot	A%-Cl	A%-Co	%SC	%Cl	%Co
NBM n=6				0.19	29.4	56.0	20.8			
MDS										
1	2	0.5	50	0.06	30	49	20	28	25	38
2	7	1.5	4	0.14	80	91	69	14	5	0
3	15	2.0	100	0.65	62	76	42	54	63	33
4	17	2.5	95	0.05	34	44	30	F	F	F
5	26	3.0	80	0.44	70	75	42	80	82	100
AML										
1	32			0.06	41	62	25	38	0	0
2	38		95	0.04	49	61	19	24	100	0
3	48		90	0.07	36	47	14	53	76	20
4	76		70	0.07	51	74	20	14	16	50
5	95		80	0.14	30	39	20	84	98	93
6				0.01	55	100	33	F	F	F

Mean data for normal bone marrow (NBM), F=Failure, IPSS=International Prognostic Scoring System, %tri8=% metaphases with trisomy 8 obtained by cytogenetics, GF=Growth Fraction or % S-phase cells of BMMNC, A%tot=% apoptotic clusters and colonies, A%-Cl=% apoptotic clusters, A%-Co=% apoptotic colonies, %SC=% single cells with trisomy 8, %Cl and %Co=% trisomic clusters and colonies, respectively.

Discussion

This *in vitro* study showed excessive proliferation and apoptosis of BMMNC of MDS patients in concordance with the *in vivo* observations of Raza et al. in BM biopsies. In the hyperproliferative BM, Raza detected massive overall PCD (≥75% ISEL+ cells) of both parenchymal and stromal cells in more than 50% of MDS patients[1,3,9]. We observed that 30-75% of the clusters and colonies of MDS patients without any sign of pycnosis by microscopy consisted of more than 50% ISEL+ cells. This phenomenon is also seen in AML and NBM, although to a lesser extent. It is well known that cells with a normal, viable appearance may already harbor irreversible apoptotic features. Apoptosis in MDS has been considerably underestimated by bright field microscopy in the era before the in situ end labeling (ISEL and TUNEL) techniques[1-3,9,13-15]. The apparent paradox in MDS between hypercellular BM and pancytopenia can be explained by this high level of PCD in MDS BM.

Our study is the first *in vitro* study that simultaneously detected proliferation and apoptosis of BMMNC of MDS and AML patients on different time points. The end-results of *in vitro* culturing of myelodysplastic BMMNC is characterized by increased cluster formation (hyperproliferation) besides decreased colony formation and disturbed differentiation[16], but as our study has shown, both cluster-forming and colony-forming cells went through a phase of hyperproliferation followed by enhanced apoptosis. The increment of blasts and $CD34^+$ cells within MDS correlates with this "leukemic growth pattern" *in vitro*, as these characteristics in MDS were also associated with a higher incidence of leukemic transformation *in vivo*[17-19]. Our study showed a correlation between percentage of blasts and increasing Cl/Co ratios in MDS at day 10 (r=0.81) as well.

The high level of apoptosis corresponds with the pancytopenia in the blood of MDS patients, which in it may provide a proliferation signal, as in our study overall apoptosis was positively correlated with growth fraction at day 10 (r=0.50). Anti-apoptotic therapy induced inhibition of PCD in the BM of MDS patients and improvement of peripheral blood counts was followed by a decrease of the enhanced BM proliferation index[20]. This higher (and underestimated) growth fraction in MDS, which was clearly seen on days 4 and 7 of our study, may initially and partly be caused by a intrinsic limited growth potential of a considerable proportion of late committed progenitor cells. The other and probably increasing component of the growth fraction contains the clonogenic compartment with increased proliferation, slower cell cycling times, and probably higher survival rates. Evidence for this hypothesis is found by double-labeling techniques performed on BM biopsies[1,2] and by the observed increment in size of the $CD34^+$ compartment with more cells in S-phase concomitantly with longer cell cycling times as MDS progressed to AML[4].

Caux et al.[25,26] proved the dual role of increased levels of TNFα[1-3] as an explanation for the combination of hyperproliferation of progenitors and their progeny and increased PCD found in the more differentiated compartment in BM biopsies of MDS patients. These opposite activities may also be linked with the feature of "signal antonymy", cells dying in S-phase, exclusively found in MDS marrows[13]. Within aspirated MDS BM cells, more activated monocytes-macrophages with TNFα-producing capacity are found[2,3,24], and they may contribute as non-stromal accessory cells to the above-mentioned and enduring effects in culture[25,26]. As TNFα also upregulates FasR expression on $CD34^+$ cells and their progeny in a dose-dependent manner, it facilitates apoptosis *in vivo* and in vitro[27,28]. BM biopsies of MDS patients showed enhanced FasR expression on $CD34^+$ cells and their progeny in an increasing fashion towards maturation when compared to normal BM, whereas leukemic blasts loose this expression during progression from MDS[5,29,30]. *In vitro* culture studies in MDS showed a correlation between decreased clonogenic capacity of CFU-GM and enhanced FasR expression[5,30,31]. In addition, within BMs of MDS patients, also more activated lymphocytes and macrophages as accessory cells are found with enhanced FasL expression[5,27] which on their turn may contribute to the enhanced PCD of FasR-bearing cells (by FasR-FasL interaction) in culture. Above all, more leukemic blasts with enhanced FasL and decreased FasR-expression are found during MDS progression to AML[5,29,31] giving rise to PCD of FasR-bearing normal and monoclonal progenitors and their progeny. All these factors may explain the higher proportion of BMMNC of MDS patients initially showing enhanced proliferative capacity (as they were primed *in vivo*) and temporarily (till day 7) overriding the simultaneously enhanced apoptotic impact observed in the progeny of these proliferating cells. But, as PCD-enhancing factors may increase during culturing as mentioned above, the balance is turned and apoptosis subsequently overrules proliferation. It is difficult to determine the degree and impact of non-stromal accessory cells upon proliferation and apoptosis in culture versus the intrinsic growth characteristics of $CD34^+$ cluster-forming and colony-forming cells in BMMNC in MDS. But, we recently performed a study using the same MDS patients as in this study, in which single MDS $CD34^+$ cells were cultured within single wells. We observed a similar increased apoptotic propensity, especially at the cluster-forming cell level[32]. This study provided strong evidence that enhanced apoptosis is an intrinsic feature of MDS progenitor cells.

Trisomy 8 was chosen as the clonal marker in our study of MDS-AML. Trisomy 8 as sole cytogenetic abnormality is found in approximately 5-10% of de novo AML and MDS and is associated with an intermediate prognosis[21]. Trisomy 8 involvement detected by FISH on BM smears is positively correlated with the percentage of trisomic BM blasts in MDS[22]. Comparison of classical cytogenetics with FISH showed that trisomy 8 in MDS and AML had a distinct

proliferative advantage over the disomic population[23]. Although these studies have completely different conditions, we observed a proliferative advantage of the trisomic 8 clone in our study in only 4 of 9 patients (Table 4.3). Nilsson et al.[34] clearly showed the existence of a disomic MDS clone within the stem cell compartment ($CD34^+CD38^-Thy^+$) in MDS patients with trisomy 8. This disomic 8 compartment was functionally impaired showing decreased colony growth in SCSW assay and no long-term-culturing initiated-cells activity or reconstitution in the NOD-SCID transplantation assay. In addition, $CD34^+$ and overall BM cells of MDS patients with trisomy 8 have an increased Fas susceptibility *in vitro* because of increased FasR expression[33], which is concordant with the higher and increasing overall PCD within MDS compared to normals in this study.

BMMNC of AML patients with trisomy 8 showed an *in vitro* pattern of low proliferative capacity with an increased degree of apoptosis in clusters. As the leukemic population has considerably longer total cell cycling times[35] and more delayed growth initiation[18], the number of colonies formed at day 7 is low, but their numbers increased in time with a low degree of apoptosis (20-24%). Probably this "leukemic growth" is becoming more important as characterized by autonomous growth[36] through autocrine-paracrine mechanisms, differentiation arrest and less or eventually no PCD, since we also observed some blastic colony formation with hardly any ISEL-positivity. As stromal interactions are not needed and accessory cells have no influence (as described above), this growth pattern is understandable and fully observed in our culture system.

Finally, regarding the size of the colonies, the largest colonies were clearly observed in NBM because of continuous proliferation with overall low apoptotic insult. Colony forming cells from MDS BMMNC produced more and substantially larger colonies than their AML counterparts as their "primed" proliferative capacity and rate was significantly higher. These characteristics parallel the *in vivo* situation by ^{3}H-thymidine incorporation studies performed on MDS and AML patients, after which BM biopsies were taken and tested by double labeling for S-phase presence and apoptosis[1,2,4,13,35].

We conclude that the overall balance between proliferation and apoptosis of colony-forming cells is in favor of proliferation in normals and AML patients. However, in MDS patients, enhanced proliferation initially overrules PCD, but massive apoptosis ultimately counterbalances the increased proliferation. The *in vivo* enhanced BM proliferation causing hypercellular marrows together with peripheral pancytopenia due to simultaneously high apoptosis are similar to the observations described in our *in vitro* system, despite the absence of stromal influences. Our studies suggest that these "growth" characteristics are caused by growth factors or cytokines produced by non-stromal accessory cells besides intrinsic properties of these progenitors. Our presented *in vitro* system may be used as a model to study several extrinsic factors of influence upon

“programmed” proliferation and Programmed Cell Death within MDS and AML patients, such as anti-TNFα or immune suppressive therapy. As anti-apoptotic therapy in MDS *in vivo* may provide less proliferation pressure upon these monoclonal MDS progenitors, a delay and/or fall in the acquirement of additional clones during MDS progression may alter the natural history of MDS patients.

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Chapter 5

Programmed Cell Death is an intrinsic feature of MDS progenitors, predominantly found in the cluster-forming cells

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Abstract

Background and objectives

Bone marrows (BM) of myelodysplastic syndrome (MDS) patients show increased proliferation and premature programmed cell death (PCD) *in vivo* as well as *in vitro*. We explored the proliferative capacity and apoptotic propensity of $CD34^+$ progenitor cells of MDS patients excluding accessory cell interference.

Materials and methods

$CD34^+CD3^-CD19^-$ cells of five MDS patients and five normal BM (NBM) were sorted as single cells into single wells and were cultured in liquid medium. Wells were evaluated on days 4, 7, 10, and 14. PCD was determined by staining with Annexin-V-FITC. Growth rate and cell doubling time (Td) were calculated for each colony-forming cell.

Results

NBM $CD34^+$ cells formed clusters and colonies and both showed increasing PCD in time, although within colonies the degree of apoptosis was twice as high (about 25%) as compared with clusters at all time points. In MDS increased cluster formation was observed at all evaluation points when compared to NBM, whereas the number of colonies was markedly reduced (1/7 of NBM). These colonies were also smaller, usually smaller than 100 cells. Significantly enhanced levels of PCD of clusters (53-79%) in combination with longer cell doubling times explain this slower formation of smaller colonies. Surprisingly, these colonies showed considerably lower levels of PCD (7-32%) as compared to NBM (1-48%, median values).

Conclusions

In the absence of stromal influences and accessory cells, this study in MDS patients showed intrinsically enhanced proliferation and apoptosis of cluster-forming cells, as the opposite was true for colony-forming cells.

Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by ineffective hematopoiesis, peripheral cytopenias and dysplasia. Hyperproliferation in MDS is abrogated by increased premature programmed cell death (PCD) or apoptosis[1-5]. Increased overall apoptosis in MDS is found in hematopoietic as well as in stromal cells[6,7] and in all MDS subtypes[3,6,8], although some investigators have observed higher apoptotic levels in low-risk (LR-) compared to high-risk (HR-) MDS groups[9,10]. Important factors leading to increased PCD in MDS are 1) increased death-receptor ligands like tumor necrosis factor-α (TNFα) and Fas ligand expression within the bone marrow (BM) microenvironment[2,4,8,11-15]; 2) increased FasR expression on matured myeloid cells as well as on $CD34^+$ cells[9,11,16-18]; and 3) increased numbers of leukemic blasts (with low FasR expression) and $CD3^+$ T cells, both with increased FasL expression within the BM[8,11,16]. Furthermore, some studies have found evidence for increased PCD by changed (intrinsic) mitochondrial characteristics of BM cells of MDS patients[19,20]. Dror clearly demonstrated altered pro-apoptotic changes in the mitochondrial pathway without pro-apoptotic influences of the FasR of the cell membrane in a RARS patient[19].
Increased apoptosis is found especially in the more matured CD34-negative compartment[21]. Several studies also showed enhanced PCD in the $CD34^+$ hematopoietic stem cell (HSC) and progenitor compartment, especially in the LR-MDS group as compared to the HR-MDS patients[21-23]. Accessory cells, besides the intrinsic features of MDS stem cells, may cause this enhanced HSC apoptosis. Despite increased apoptosis of $CD34^+$ cells, this compartment is rapidly increasing in size during MDS progression towards acute myeloid leukemia (AML)[5]. Raza et al. observed decreased in situ end labeling (ISEL) positivity of BM blasts of HR-MDS and AML patients by immunohistochemistry on BM biopsies[6]. By excluding stromal influences and accessory cells, our study explores the intrinsic proliferative capacity and apoptotic propensity of $CD34^+$ HSC and progenitors of MDS patients by liquid culturing of single cells.

Materials and methods

Patients

Cryopreserved BM mononuclear cells (BMMNC) of MDS patients (n=5) and normal BM controls (NBM, n=5) were used for these *in vitro* assays. Only patients with primary MDS with trisomy 8 were used for these studies in order to get a homogeneous group, classified as an intermediate-risk group according to World Health Organization (WHO) classification. The individual patients with their *in vitro* characteristics are presented in Table 5.1: Patient 5 is

ranked as RAEBt according to French-American-British (FAB) classification and as AML according to WHO.

Table 5.1 *In vitro* growth (mean) and apoptosis (median) characteristics of individual MDS patients.

Patients	MDS 1	MDS 2	MDS 3	MDS 4	MDS 5
Diagnosis	RARS	RAEB	RAEB	RAEB	RAEBt
%BM blasts	2%	7%	15%	17%	26%
Trisomy 8 (% metaphases by cytogenetics)	50%	4%	100%	44%	91%
IPSS	**0.5**	**1.5**	**2.0**	**2.5**	**3.0**
Clusters (n/plate; day 4 to 14)	44.8	50.1	37.1	35.5	57.4
Colonies overall (n/plate)	2.5	23.6	1.8	0.12	0.79
Colonies of 100-500 cells (n/plate)	0.23	4	0.37	0.085	0
Colobies >500 cells (n/plate)	0	2.5	0.31	0	0
Cl/Co ratio (day 7 to 14, median)	12.9	1.2	20.8	165.1	49.9
PCD overall (% apoptotic cells/aggregate)	32	14.1	44	88	7.4
PCD of clusters (% apoptotic cells/Cl)	35.7	57	48.6	87	7.3
PCD of colonies (overall)	27.7	8.9	9		18.6
PCD of colonies (40-100 cells)	29.1	24.5	9		18.6
Growth rate to form clusters ≥20 cells (Td)	0.60(40)	0.57 (42.1)	0.40 (60)	*	0.43 (55.8)
No. days to form clusters of 20 cells	6.65	6.92	10.62	*	9.72
Growth rate of CFU-GM (overall, Td)	0.64 (37.5)	0.58 (41.4)	0.47 (51.1)		0.51 (47.1)
No. of days to form colonies (overall)	7.56	8.42	11.00		9.78

IPSS: International Prognostic Scoring System, n: number of aggregates, Cl/Co ratio: cluster/colony ratio, PCD: programmed cell death, growth rate with Td (cell doubling time) in hours between brackets, * hardly any cluster formation of 20 and more cells.

BMMNC collection, cryopreservation, thawing and labeling

BMMNC were isolated (Ficoll-Paque 1.077 g/ml; Pharmacia Biotech, Uppsala, Sweden) and cryopreserved, as described before[24]. After thawing and washing, the cell pellet was resuspended in 100 µl glucose-phosphate-buffered saline (G-PBS) and stained with CD34-PE, CD3-FITC (both ImmunoTech., A Beckman-Coulter Co., Mijdrecht, the Netherlands), and CD19-FITC (Dako A/S, Copenhagen, Denmark) at 4°C for 30 min.[24]. Afterwards, the cells were washed with G-PBS and restored in Iscove's medium with 10% v/v fetal calf serum (FCS) prior to sorting by flow cytometry (FCM).

Single cell sorting of $CD34^{+}CD3^{-}CD19^{-}$ cells from BMMNC

The culture medium consisted of Iscove's, 2 mM glutamine (Flow Laboratories, Irvine, Scotland), streptomycin 50 µg/ml, penicillin 50 IU/ml (Flow Laboratories), supplemented with 20% v/v FCS, 5% w/v bovine serum albumin, and recombinant growth factors; G-CSF (20 ng/ml), hSCF (25 ng/ml; both from Amgen, Thousand Oaks, CA, USA), IL-3 (50 ng/ml), and GM-CSF (20 ng/ml; both from Sandoz BV, Uden, the Netherlands). Before sorting, 10 round-bottom 96-well plates were filled with culture medium, 75 µl per well (Costar #3799, Cambridge, MA, USA).

An Epics Elite flow cytometer, equipped with an autoclone device (Coulter, Miami, FL, USA) was used for single cell sorting. CD34$^+$CD3$^-$CD19$^-$ cells within the life gate were sorted for single-cell single-well (SCSW) assay. These plates were placed in an incubator at 37°C, 5% CO_2 in a fully humidified atmosphere.

SCSW assay to determine proliferation of CD34$^+$/CD3$^-$/CD19$^-$ cells

One day after sorting and using an inverted microscope, a single cell per well was observed in the center of each round-bottom well. The total number of cells per well was counted by inverted microscope at days 4, 7, 10, and 14. The exact number of cells within each well was corrected by counting from stored brightfield images as this inverted microscope was equipped with a bright phase contrast objective and a CDD-camera (Variocam, PCO computer optics, Kellheim, Germany). To estimate the number of cells in large colonies, we matched the areas on a calibration curve, which was made after correlating the area of different-sized colonies with their exact number of cells (data not shown), as described before[25]. Clusters and colonies were defined as aggregates of 2 to 39 cells and a minimum of 40 cells, respectively. Only myeloid clusters and colonies were formed, as no erythropoietin was used. Their morphology with regards to CFU-G, CFU-GM or CFU-M was not scored, as it was not subject of this study. Proliferation is visualized in time in Figure 5.1, as it is defined by the number of clusters and/or colonies per 96-well plate (the sum is called plating efficiency, or PE), and by the size of colonies.

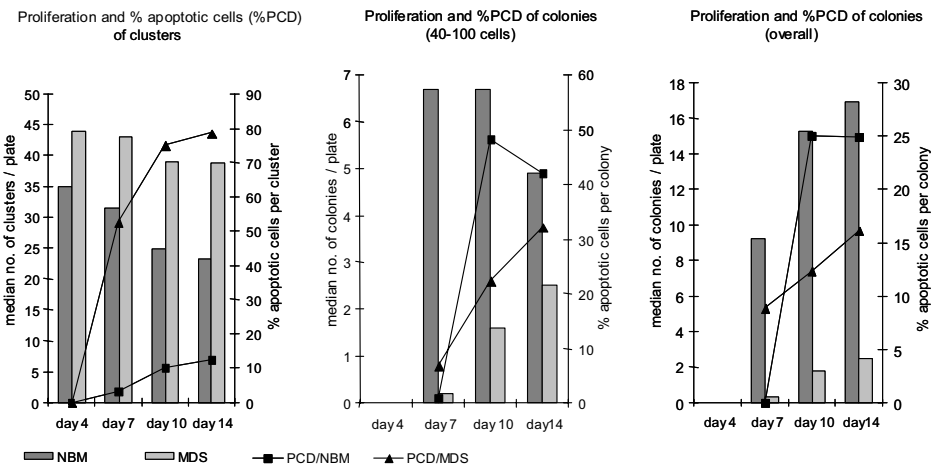

Figure 5.1 *In vitro* profiles of proliferation and apoptosis (%PCD) of CD34$^+$ cells of normals (NBM) and MDS patients during 14 days of culturing, expressed in median values, regarding clusters (0-39 cells), small colonies (40-100 cells), and colonies overall (40 and more cells).

Data analysis of growth profiles

Only wells with clusters (sized ≥20 cells; >4 cell divisions) and colonies at day 14 were used for expressing mean growth curves (Figure 5.2) and for calculating cell doubling times (Td) of cluster-forming cells and colony-forming cells, respectively. Growth rate of these aggregates is defined and expressed as the number of cell population doubling per day from which a Td in hours was calculated[25] .

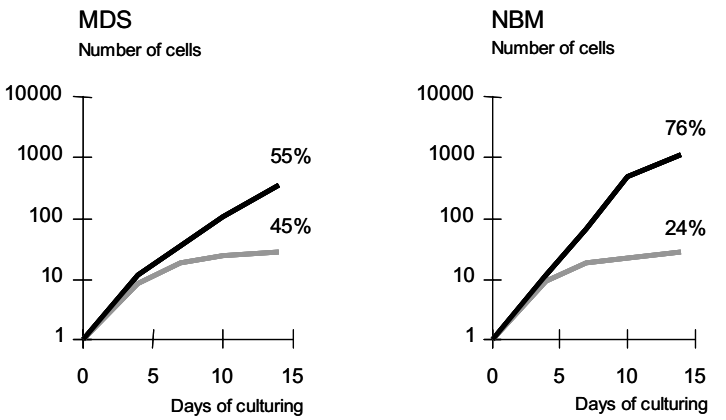

Figure 5.2 Growth curves of $CD34^+$ cells of MDS patients compared with normal BM (NBM) in SCSW assay with their relative amount at day 14 of colony-forming cells (upper line) and cluster-forming cells reaching at least 20 cells (lower line).

Apoptosis detection in SCSW assay with fluorescence microscopy

At each time point, two 96-well plates were evaluated for the number of apoptotic cells within each cluster or colony. An Annexin-V (AnV) solution (end-concentration 1,2 µg/ml) was prepared, containing Iscove's medium, 1.0 M $CaCl_2$ and 5% v/v AnV-FITC (Bender MedSystems, Vienna, Austria). An AnV-FITC solution of 5 µl per well was added; incubation for 30 min. at room temperature was followed by light and fluorescence microscopy imaging (see Figures. 5.3A and 5.3B). After recording brightfield images, the cells were excited with a mercury arc lamp using a 440-490 nm band pass filter for AnV-FITC. Emission was measured with long pass 520 nm and fluorescence images were recorded. Calibration of fluorescence signal detection was performed using flowset fluorospheres (Beckman-Coulter Corporation, Miami, FL, USA). For analysis of the fluorescence images, we used TCL-Image 4.6 software (TNO, Delft, the Netherlands) to objectively define the cut-off level for positive cells in comparison to the background. The mean fluorescence per cell for an apoptotic cell was arbitrarily set to be at least 3 times higher than the mean background fluorescence. The precise method of image analysis was

described before[26]. The exact amount of apoptosis is defined as the percentage AnV+ cells in each cluster or colony.

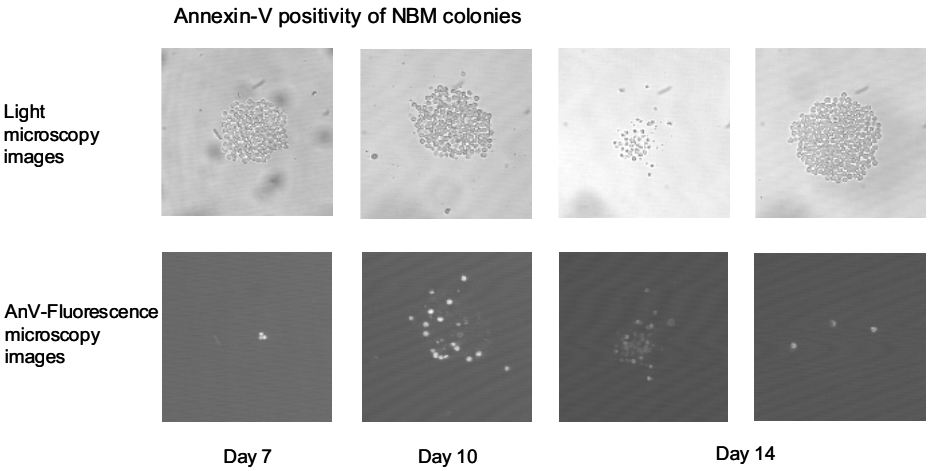

Figure 5.3A Examples of light microscopy with An-V fluorescence microscopy images of aggregates after 7, 10, and 14 days of culturing of NBM CD34+ cells in SCSW assay. See the difference in PCD between colony at day 10 and 14, as the last one has a more blastic appearance. The cluster at day 14 is becoming pycnotic.

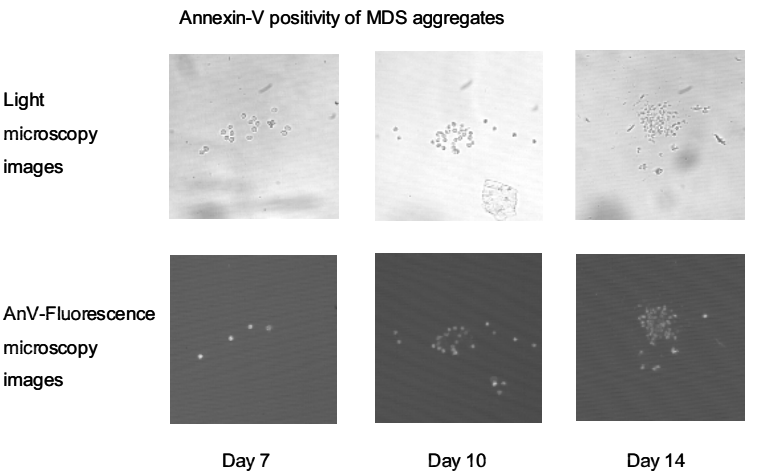

Figure 5.3B Examples of light microscopy with An-V fluorescence microscopy images of aggregates after 7, 10, and 14 days of culturing of MDS BM CD34+ cells in SCSW assay with clearly increased PCD.

Statistics of *in vitro* profiles

To detect statistical differences between both groups regarding proliferation and apoptosis (as both showed a skewed distribution), we used the Mann-Whitney test as appropriate. Different growth and cell death characteristics can be calculated of each patient (see Table 5.1) and normal control. The median values of these parameters of each group (on different time points) are expressed in Table 5.2, whereas the *in vitro* characteristics of each MDS patient was linked with its FAB/WHO classification and International Prognostic Scoring System (IPSS) score[27] and eventually compared in Table 5.1.

Table 5.2 Characteristics of proliferation and apoptosis (median values) in the $CD34^+$ SCSW assay of normals and MDS patients.

Characteristics	Normals / NBM				MDS			
	day 4	day 7	day 10	day 14	day 4	day 7	day 10	day 14
Plating efficiency (ntot/96 wells)	35.0	40.6	40.1	40.1	44.4	46.3	46.8	50.5
Clusters (n/96 wells)	34.9	31.4	24.9	23.1	44.4	43.0	39.1	38.7
Colonies overall (n/96 wells)		9.2	15.3	16.9		0.3	1.8	2.5
Colonies of 40-100 cells (n/96 wells)		6.7	6.7	4.9		0.2	1.6	2.5
Colonies >100 cells (%of PE)		3.0	9.0	12.0		0.0	0.0	0.0
Cl/Co ratio		3.1	1.4	1.1		135.2	20.8	13.4
PCD of clusters (% apoptotic cells)	0.0	4.6	18.7	21.1	0.0	15.1	25.0	48.6
PCD of clusters (% apoptotic cells/Cl)	0.0	3.4	10.1	12.4	0.0	52.8	75.0	78.6
PCD of colonies (overall)		0.0	25.0	24.9		8.9	12.3	16.1
PCD of colonies (40-100 cells)		1.0	48.3	42.0		7.0	22.5	32.1
Growth rate to form clusters >20 cells		0.66		(Td=36.5 hr)		0.50		(Td=48.0 hr)
Number of days to form clusters of 20 cells		7.02				8.47		
Growth rate of CFU-GM (overall)		0.71		(Td=33.8 hr)		0.55		(Td=43.7 hr)
Number of days to form colonies (overall)		8.07				9.19		

n: number of aggregates, ntot: total number of aggregates, Cl/Co ratio: cluster/colony ratio, PCD: programmed cell death or apoptosis, Td: cell doubling time in hours (hr).

Results

Growth and PCD characteristics of normal progenitors

Growth and PCD characteristics of normal progenitors are depicted in Table 5.2 and illustrated in Figures 5.1 and 5.3A.

NBM $CD34^+$ cells showed a maximum number of clusters at day 4 (35 wells per plate) with hardly any apoptotic cells. Subsequently, the number of clusters decreased (from 35 to 23 clusters per plate at day 14), mainly because of on-going formation of colonies (from 9 to 17 per plate at day 14) and, to a lesser extent, because of increasing PCD within the developing clusters (from median 3.4% towards 12.4% apoptotic cells at day 14; see Figure 5.1).

Colony formation rate reached a maximum between days 4 and 7 (Table 5.2), and subsequently the colony number increased slightly from day 10 to 14 despite a concomitant increase of overall PCD (median 0% towards 25% at day 14). This normal growth profile is depicted by the growth curves (n=474) of different cluster-forming and colony-forming CD34$^+$ cells in Figure 5.2: an initial log growth phase with subsequently decreasing growth rates towards finally reaching a plateau. The level of apoptosis within small colonies (40-100 cells) was always significantly higher (ranging from 1% to 48%; Figure 5.1) during the whole culture period as compared with apoptosis in the intermediate and large colonies, as colonies all together showed overall PCD levels from 0% to 25% at day 14. A lower growth potential and earlier differentiation with subsequent apoptosis explains this observation in the smaller colonies. At day 14, the ratio of colonies to larger clusters (≥20 cells) reached 3:1 (Figure 5.2), whereas an overall cluster/colony (Cl/Co) ratio of almost 1 was reached at days 10 and 14 (Table 5.2). At day 14, the relative amount of colonies versus bigger clusters (≥20 cells) versus smaller clusters (<20 cells) are 47%-12%-41% of the plating efficiency, respectively.

Growth and PCD characteristics of MDS progenitors

Growth and PCD characteristics of MDS progenitors are illustrated in Figures 5.1 and 5.3B, and Tables 5.1 (individually) and 5.2 (as a group).

Compared with NBM at all time points, MDS CD34$^+$CD3$^-$CD19$^-$ progenitors formed higher numbers of clusters (median 44-39/plate; Figure 5.1 and Table 5.2) which decreased slowly in time, although high levels of PCD were observed (from 0% towards 79% at day 14; see Figure 5.3B for examples). Furthermore, a consistently and significantly lower level of colony formation was observed in MDS (peak formation rate between days 7 and 10; Figure 5.1). Predominantly colonies of less than 100 cells were formed. At day 14, the ratio of colonies to large clusters (≥20 cells) reached only 1.2:1 (whereas 3:1 in NBM). At day 14, the relative amount of colonies versus bigger clusters (≥20 cells) versus smaller clusters (<20 cells) are 22%: 18%: 60% of the plating efficiency, respectively.

The overall PCD in colonies of MDS patients is also increased in time (from 9 to 16%), but this level was considerably lower compared to NBM (from 0 to 25%). This difference in PCD is even more pronounced (by 10-26% lower) in the smaller colonies (40-100 cells; Figure 5.1 and Table 5.2), which are preferentially formed in MDS. As compared to NBM, the overall plating efficiency of CD34$^+$ cells of MDS patients in SCSW assay increased in time, and was significantly higher at all time points (Table 5.2). Since high cluster formation with very high levels of apoptosis constantly overruled colony formation, it is not surprising that the median Cl/Co ratios of MDS patients

remained far above the normal ratio of 1. The slower formation of clusters and colonies within MDS leads to mean overall Td that is about 10 hours longer as compared to normal controls (Table 5.2). These characteristics are visualized by lower slopes of growth curves (n=411) in Figure 5.2 and a left shift in the distribution of Td in Figure 5.4. This means more proliferating $CD34^+$ cells with longer Td as compared to normal BM. As the initial log growth curves within the first 4 days are similar between normal and MDS progenitors (see similar slopes in Figure 5.2), the longer cell doubling times within MDS patients are due to slower cluster and colony formation after day 4, probably caused by high and increasing apoptosis at cluster level in time.

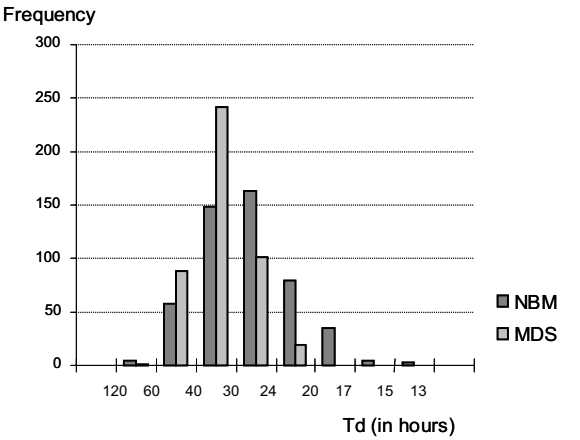

Figure 5.4 Distribution of Td (cell doubling times) of $CD34^+$ cells expressed in fractions of different time periods of normal bone marrow (NBM) and myelodysplastic syndrome (MDS) in SCSW assay.

Relationship between growth and PCD levels according to IPSS score in MDS patients

The five MDS patients in Table 5.1 are ranked from relatively good prognosis, or LR-MDS, to bad prognosis, or HR-MDS, according to FAB or WHO, with less or more than 10% BM blasts, and IPSS score. No correlation was found between percentage BM blasts and percentage trisomy 8 metaphases detected by conventional cytogenetics. Furthermore, no correlations were found between percentage trisomy 8 metaphases or the percentage BM blasts and growth and PCD characteristics within this group. High cluster formation (45-50/plate) with increased PCD (36-57%) and low to normal colony numbers (3-24/plate) with low to normal apoptosis (9-28%) was observed in the LR-MDS patients (# 1 and #2). The RAEB patients (#3 and #4) showed lower formation of clusters with very high levels of apoptosis (49-87%) and therefore

importantly decreased colony numbers (<2/plate) with lower PCD levels. The RAEBt/AML patient (#5) showed the highest numbers of clusters with the lowest apoptosis level (7.4%), in combination with a prolonged doubling time (Td of 47 hours versus 33.8 hours for normals) and subsequently a low number of small colonies characterized by a low apoptotic insult (19%) as compared to normals (42-48%; Table 5.2).

Discussion

The MDS CD34$^+$CD3$^-$CD19$^-$ population (subsequently stated as CD34$^+$ cells) showed a significantly higher overall growth fraction (GF) or plating efficiency (PE), as compared to normal progenitors in this SCSW assay. This increased MDS GF mainly consisted of cluster-forming cells (clusters with <20 and ≥20 cells: respectively 60% and 18%, at day 14). Furthermore, in contrast to normal progenitors, MDS cluster-forming cells showed, although not initially, a slower and lower proliferative potential because of earlier and eminent apoptosis. Also the MDS colony-forming cells, which are a minor fraction (mean 22% of PE at day 14, and median 0.3-2.6% of the progenitors during culturing, whereas 47% and 9.2-16.9%, respectively, in NBM), showed an overall slower and lower proliferative potential leading to small-sized colonies, but with consistently lower intrinsic apoptosis as compared to normal controls. Importantly, these *in vitro* growth and apoptosis characteristics are intrinsic, as they appear without direct influences of stroma or accessory cells. Whether the observation of Dror could account for this enhanced intrinsic PCD beyond the RARS group, as it was especially found in cluster-forming cells in all our MDS patients, merits further research[19,20].

Furthermore, ranking MDS patients towards more advanced FAB/WHO classification, we found a transition from a LR-MDS “growth profile” (with high proliferation and shorter cell-doubling times, lower CI/Co ratios and high apoptosis of clusters and small colonies) towards a more “leukemic growth” profile, with increased CI/Co ratio’s, longer cell doubling times, and much lower apoptosis levels of colony-forming cells as compared to cluster-forming cells. This *in vitro* leukemic growth profile has been associated with increased percentage BM blasts and correlates with a higher propensity to leukemic transformation[28-31]. Furthermore, a cautionary remark is necessary, as we did not use erythropoietin. Erythropoietin has been frequently used in previous studies and its influence upon these *in vitro* “growth characteristics” of the myeloid aggregates is not precisely known. On the other hand, only MDS patients with trisomy 8 (approximately 5-10% of MDS) were chosen in order to obtain the highest possible uniformity in “growth” characteristics. Trisomy 8 clone in MDS has shown to have a distinct proliferative advantage over the

disomic population by comparison of classical cytogenetics with fluorescence in situ hybridization (FISH) of MDS BM[32]. Furthermore, trisomy 8 in MDS is associated with the upregulation of immune/inflammatory genes and downregulation of apoptosis-inhibiting genes of $CD34^+$ cells, which is consistent with the enhanced apoptosis seen in (early) MDS[33]. Finally, trisomy 8 involvement in MDS and AML has distinct survival profiles, clearly showing the leukemic impact during MDS progression[34]. A warning should be made that these growth characteristics of this subpopulation of five MDS patients with trisomy 8 can not be generalized for the whole MDS population.

The association of an increasing S-phase fraction of both the overall myeloid compartment and the $CD34^+$ compartment with a longer total cell cycling time during MDS progression was also observed by double-labeling techniques within BM biopsies[2,4,5]. These studies, combined with our *in vitro* observations, demonstrate the expanded MDS progenitor pool, explaining a higher plating efficiency throughout the SCSW assay. Predominantly clusters with increased PCD are formed from this $CD34^+$ compartment, which implies that most of these $CD34^+$ cells probably belong to the more mature progenitor cell pool with decreased intrinsic proliferative capacity. Or alternatively, their growth potential is abrogated by enhanced intrinsic apoptosis. In analogy with the SCSW assay, long-term bone marrow cultures (LTBMC) of multipotent MDS progenitors with normal stroma but with disturbed stromal interactions have shown similar results like decreased numbers of secondary colony-forming cells with decreased long-term proliferation[35-37]. As MDS progresses, more blasts appear and a subpopulation (or a MDS subclone) with a more leukemic *in vitro* growth profile (slower proliferation with less differentiation and apoptosis) can be observed by the overall delayed and decreased formation of predominantly smaller colonies with decreased PCD in time, as compared to normal BM. Stroma-free LTBMC with MDS progenitors and four growth factors showed similar progressive leukemic growth with immature blasts[38].

Furthermore, as MDS eventually turns into AML, an increase of the MDS clone and the development of subclones are considered, as these clones gradually overcome apoptosis. This study observed a similar profile (Table 5.1) of an increased leukemic growth of some clusters and subsequently small colonies during MDS progression, associated with a lower growth rate and a lower PCD level of colony-forming cells. (F)ISH studies on these clusters and colonies have to be performed to prove this theory. But, in general, the predominance of cluster-forming cells in MDS could be a reflection of the increased progenitor cell pool of which a larger and more mature fraction rapidly divides[5] to overcome the enhanced apoptosis found in the more mature $CD34^-$ progeny[1-4]. Eventually, as MDS progresses to AML, a decreased overall proliferative capacity is the result of increased numbers of normal and/or monoclonal $CD34^+$ and $CD34^-$ cells dying in S-phase (so-called “signal antonymy”). This unique

and frequently found phenomenon in MDS ultimately abrogates the increased proliferative potential of the increased S-phase fraction[1,5]. Also, faster cell divisions of the more mature progenitors leading to earlier (and probably disturbed) maturation and eventually earlier and therefore increased normal cell death could also play a role. Both phenomena are intrinsic features of progenitors, as if their proliferation augmentation was ordered from abroad, as being “the last line of defense within a dying BM with rapid accumulation of AML blasts”.

Colony-forming progenitor cells showed decreased PCD in the SCSW assay, as compared with NBM. This intrinsic feature of more immature MDS colony-forming $CD34^+$ cells definitely accounts for the decreased apoptosis and slower proliferation rate in the SCSW assay. It represents a more leukemic growth pattern within these immature MDS colony-forming cells, probably belonging to a pre-AML subclone, as they are progressively gaining the ability to overcome apoptosis in access of a differentiation arrest on their way to become “real AML blasts”.

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Chapter 6

Caspase-inhibitors decrease Programmed Cell Death of $CD34^+$ cells from MDS patients without restoration of a normal *in vitro* growth pattern

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Abstract

Background and objectives

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by increased proliferation and programmed cell death (PCD). In MDS, we previously showed enhanced apoptosis *in vitro* in the absence of accessory cells. We hypothesize that if this intrinsic apoptosis is inhibited by caspase-inhibitors (CAI: Z-VAD-FMK and Z-DEVD-FMK; single use or in combination), it can restore *in vitro* proliferation of MDS progenitors towards a normal growth profile.

Materials and methods

Single-cell sorted CD34+ cells of MDS patients with trisomy 8 were cultured with and without caspase-inhibitors and the total number and size of clusters (2-39 cells) and colonies (≥40 cells) were counted on days 4, 7, and 10. On days 7 and 10, these aggregates were checked for PCD by Annexin-V-FITC staining.

Results

The declining effect of CAI upon intrinsically enhanced apoptosis was significantly more pronounced in clusters than in colonies, as clusters originally showed higher PCD. In analogy, the effect of CAI on PCD was higher in low-risk (LR) compared to high-risk (HR) MDS, and also colonies showed this effect in the LR-group. The combination of CAI in LR-MDS patients eventually led to lower colony formation with lower PCD of clusters and colonies at day 10. In HR-MDS, CAI induced more clusters at days 4 and 7 with lower PCD at days 7 and 10, whereas colony formation did not change, as it hardly occurred. Furthermore, the size of clusters and colonies did not change significantly with CAI.

Conclusions

CAI decreased apoptosis of *in vitro* cultured $CD34^+$ cells of all MDS patients and led to decreased colony growth in the LR-MDS patients, whereas increased cluster formation with hardly any colony formation was found in the HR-MDS group. The use of CAI in these MDS patients did not restore a normal growth pattern.

Introduction

Myelodysplastic syndromes (MDS) are clonal stem cell disorders characterized by cytopenias due to ineffective haematopoiesis, which is a result of hyperproliferation abrogated by increased apoptosis[1-3]. Increased overall programmed cell death (PCD) in MDS is found in all FAB classification groups[3-5], although the PCD level is higher in low-risk (LR) compared to high-risk (HR) MDS[6,7]. Enhanced apoptosis in myelodysplasia is partly caused by increased death-receptor ligands like TNFα, IL1β, and Fas ligand (FasL) expression within the bone marrow (BM)[2,5,8-13]. Also increased FasR expression on mature myeloid cells as well as on $CD34^+$ cells[6,9,14-16] render these cells more prone for apoptosis. Apoptosis in MDS is also caused by increased numbers of leukemic $CD34^+$ blasts and $CD3^+$ T cells, both with enhanced FasL expression[6,9,14]. Although enhanced PCD is predominantly found in the more mature $CD34^-$ compartment[17], several studies also observed enhanced PCD in the $CD34^+$ hematopoietic stem cells (HSC) and progenitor compartment, especially in the LR-MDS group[17-19].

During MDS progression towards acute myeloid leukemia (AML), the $CD34^+$ cell compartment is increasing[20]. Furthermore, we recently observed that especially the cluster-forming $CD34^+$ cells of MDS patients showed increased PCD in single-cell single-well (SCSW) assay[21]. These *in vitro* observations were done in single progenitors in the absence of stroma or accessory cells, which implies that the apoptotic propensity are intrinsic features of MDS. We hypothesize that caspase-inhibitors (CAI) can reverse this intrinsic enhanced apoptotic propensity and could therefore restore normal growth.

Caspases are cell death proteases which disassembly the cell and its organelles into apoptotic bodies in an orderly fashion. They can be divided in initiators (like caspases 8, 9, and 10) and executioners (caspases 3, 6, and 7). As broad-spectrum, cell-permeable CAI we used Z-VAD-FMK, which has an irreversible inhibitory effect on caspase-1, 3, 4, and 7, versus Z-DEVD-FMK which inhibits caspase-3, 6, 7, 8 and 10. Recent experiments have shown a broader mechanism of action of caspases in the myeloid homeostasis: they are involved in entering the cell cycle and in cytokine-induced proliferation as well[22].

The aim of our study was to explore the effect of these two broad-spectrum CAI and their combination upon 1) the intrinsic increased apoptotic propensity of $CD34^+$ progenitor cells of MDS patients, and 2) the overall balance of proliferation and apoptosis of the $CD34^+$ cells *in vitro* by excluding interference of stromal and accessory cells by culturing in SCSW assays.

Materials and methods

Patients

Cryopreserved BM mononuclear cells (BMMNC) of four MDS patients with trisomy 8 as the sole cytogenetic abnormality (intermediate risk group by cytogenetics) were used as in our previous studies[21]. Table 6.1 and 6.2 show the patient characteristics and the *in vitro* proliferation and apoptosis data.

BMMNC collection, cryopreservation, thawing and labeling

BMMNC were isolated from the interphase after Ficoll-Paque (1.077 g/ml; Pharmacy Biotech, Uppsala, Sweden) density centrifugation and cryopreserved, as described before[23]. After rapidly thawing, these cells were restored in fetal calf serum (FCS) with additives ($MgSO_4$, preserved free heparin, DNAse) to regain cell metabolism before labeling was performed (for concentrations and manufactures see reference 22). BMMNC were washed in glucose-phosphate buffered saline (G-PBS) and centrifuged at 18°C for 10 minutes (min.). The cell pellet was resuspended in 100 μl G-PBS (containing ≤5 million cells/ml) and stained with directly labeled antibodies, as described before[21]. Afterwards, the cells were washed with G-PBS and restored in Iscove's medium with 10% v/v FCS prior to sorting by flow cytometry (FCM).

Single cell sorting of $CD34^+$ cells from BMMNC

Before sorting of $CD34^+$ cells was performed, culture medium was prepared to fill round-bottom 96-wells plates (Costar #3799, Cambridge, MA,USA) with 75 μl per well. The consistence of this culture medium with growth factors (GFs) G-CSF, h-SCF, IL-3 and GM-CSF was described before[21]. Furthermore, the addition of 2 broad-spectrum, cell-permeable, and irreversible inhibitors of caspases (Z-VAD-FMK and Z-DEVD-FMK, Omnilabo Int. B.V., Breda, the Netherlands) were used as single agents (both solutions with final concentration (FC) of 10μM) and in combination (FC 10 μM, each) in order to detect a PCD-lowering effect on the sorted $CD34^+$ cells. An Epics Elite Flow Cytometer, equipped with an autoclone device (Coulter, Miami, FL, USA) was used for single cell sorting. Forward vs. right angle scatter were used to exclude dead cells and debris. $CD34^+CD3^-CD19^-$ cells within the life gate were sorted out of this thawed BMMNC fraction, indicating that lymphocytes (with no proliferation capacity within this assay) were excluded. Eventually, the autoclone unit sorted and seeded one $CD34^+$ cell within one well. Ten 96-well plates were used per patient to perform these SCSW assays in time.

SCSW assay to determine proliferation of $CD34^+$ cells

After sorting, these 96-well plates were immediately placed in an incubator at 37°C, 5% CO_2 in a fully humidified atmosphere. One day after sorting and using an inverted microscope, one single cell per well was observed in more than 98% of these wells. In each well, the total number of cells were counted under a Zeiss (Thornwood, NY, USA) Axiovert 35M inverted microscope at days 4, 7, and 10 by one technician to evaluate the clonogenic capacity of these $CD34^+$ cells. Clusters and colonies were defined as aggregates of 2-39 cells and ≥40 cells, respectively. Proliferation is defined as the number of clusters and colonies per 96-well plate (called plating efficiency; PE), as also the size of clusters and colonies was taken into account. The exact number of cells within each well could be counted using the recorded brightfield images, as described before[24]. Delayed growth initiation means that the plating efficiency at day X+3 is considerably higher (≥10%) than at day X because of delayed initiation of cell division.

Apoptosis detection in SCSW assay with fluorescence microscopy

At days 7 and 10, two 96-well plates of each condition were used for determining the number of apoptotic cells within each cluster or colony, as described before[21]. For analysis of the fluorescence images, we used TCL-Image 4.6 software (TNO, Delft, the Netherlands) to objectively define the cut-off level for positive cells in comparison to the background. The precise method of image analysis was described before[25]. The amount of apoptosis (PCD level) is accurately defined as the percentage Annexin V-$FITC^+$ cells of all cells within each cluster or colony.

Statistics of *in vitro* profiles

To detect statistical differences between the *in vitro* profiles (proliferation with number and size of clusters and colonies versus apoptosis) with and without CAI within one patient, we used different tests. Wilcoxon Mann-Witney test was used because of frequently found skewed distribution of these parameters, and student t-test to detect statistical differences regarding PCD level (% apoptotic cells per aggregate).

Results

Tables 6.1 and 6.2 show the clinical data and the *in vitro* growth and apoptosis characteristics of our four MDS patients with trisomy 8, classified from low-risk (MDS-1 and -2) to high-risk MDS (MDS-3 and -4) according to FAB-WHO with

increasing percentages of BM blasts, $CD34^+$ cells, and subsequently IPSS scores[26].

Table 6.1 Mean *in vitro* growth characteristics of individual MDS patients.

	MDS-1	MDS-2	MDS-3	MDS-4
FAB-WHO classification	MDS-MPS	RA	RAEB-RAEB2	RAEBt-AML
IPSS / %BM blasts	0.5 / 1%	1.0 / 5%	2.0 / 15%	3.0 / 26%
% trisomy 8 by cytogenetics		100%	100%	91%
% $CD34^+$ cells of BMMNC	4%	16%	18%	31%
Cl/Co ratio day 7 and day 10 - CAI	1.5 and 0.5	6.6 and 4.7	24.5	64
Proliferation **clusters**	day 4 - 7 - 10	day 4 - 7 - 10	day 4 - 7 - 10	Day 4 - 7 - 10
number of clusters - CAI	64 - 39 - 21	52 - 46 - 47	33 - 46 - 49	40 - 61 - 64
number of clusters + ZVAD	64 - 27 - 22	51 - 47 - 42	43 - 53 - 47	44 - 67 - 61
number of clusters + DEVD	64 - 32 - 20	46 - 46 - 42	41 - 51 - 47	51 - 66 - 65
number of clusters + combi	59 - 30 - 23	46 - 48 - 51	40 - 48 - 46	47 - 66 - 67
Proliferation **colonies**	day 4 - 7 - 10	day 4 - 7 - 10	day 4 - 7 - 10	Day 4 - 7 - 10
number of colonies - CAI	1 - 26 - 45	0 - 7 - 10	0 - 0 - 2	0 - 0 - 1
number of colonies + ZVAD	0 - 38 - 44	0 - 5 - 6	0 - 0 - 3	0 - 0 - 3
number of colonies + DEVD	0 - 33 - 48	0 - 5 - 6	0 - 0 - 4	0 - 0 - 0
number of colonies + combi	0 - 32 - 37	0 - 3 - 5	0 - 0 - 2	0 - 0 - 1
Results				
changed PE in comparison to -CAI	day4,10:-10%[c]	day4:-10%[d,c] day10:-15%[v,d]	day4:+20-30%[a] day7:+10%[d,v]	day4:+10-30%[a] day7:+10%[a]
delayed growth initiation	no	yes:day7[d], day7-10[c]	yes:day7[b]	yes:day 7[b]
stimulation Co growth day 7	yes:+30%[a]	no:-40%[a]	not present	not present

Cl/Co ratio: cluster/colony ratio, PE: plating efficiency, +/- CAI: with or without caspase-inhibitors; [v]=Z-VAD, [d]=Z-DEVD, [c]=combination of CAI, [a]=all 3 CAI conditions, [b]=All conditions +/- CAI.

The addition of caspase-inhibitors (+CAI; +ZVAD, +DEVD, + combi) in the MPS (myeloproliferative) MDS-1 patient led to significantly more (+20-40%) colony formation at day 7, a difference that was no longer observed at day 10, and it occurred without significant changes in level of apoptosis or the size of the formed aggregates at day 7. At day 10, the combination of CAI ("combi" or "c" in both tables) significantly decreased the level of PCD of both clusters (64% vs. 88%) and colonies (6% vs. 13%, see Table 6.2). Unexpectedly, the lower PCD concurred with almost 20% fewer colonies as compared to control, but without significantly changing the size of these aggregates. This proliferation profile means that there are 2 groups of cluster-forming $CD34^+$ cells: initially fast-growing ones leading to more colonies at day 7 which are stimulated for faster growth by CAI, besides slower-growing ones leading to lower colony formation with less PCD at day 10 by CAI.

Table 6.2 Mean apoptosis of clusters (CI) and colonies (Co) of individual MDS patients, +/- CAI: with or without caspase-inhibitors.

	MDS-1	MDS-2	MDS-3	MDS-4
FAB-WHO	MDS-MPS	RA	RAEB-RAEB2	RAEBt-AML
IPSS / %BM blasts	0.5 / 1%	1.0 / 5%	2.0 / 15%	3.0 / 26%
Apoptosis CI	mean ± CI, median	mean ± CI, median	mean ± CI, median	mean ± CI, median
day 7 - CAI	41% ± 13	26% ± 8, 18%	8% ± 5, 0%	10% ± 7, 0%
day 7 + CAI	42% ± 15	**16% ± 6, 0%[v]**	5% ± 3, 0%[v]	**3% ± 3, 0%**[v]
		9% ± 6, 0%[d]	7% ± 5, 0%[d]	3% ± 3, 0%[d]
		13% ± 7, 0%[c]	5% ± 6, 0%[c]	6% ± 4, 0%[c]
day 10 - CAI	88% ± 9, 100%	26% ± 9, 10%	24% ± 7, 17%	20% ± 8, 0%
day 10 + CAI	81% ± 10, 91%[v]	22% ± 6, 25%[v]	**4% ± 2, 0%[v]**	18% ± 8, 0%[v]
	65% ± 13[d]	22% ± 7, 18%[d]	**11% ± 6, 0%[d]**	19% ± 7, 0%[d]
	64% ± 11[c]	20% ± 6, 18%[c]	**13% ± 6, 2%[c]**	**9% ± 6, 0%[c]**
Apoptosis Co	mean ± CI, median	mean ± CI, median		
day 7 - CAI	4% ± 2, 2%	5% ± 9, 0%	hardly any Co	hardly any Co
day 7 + CAI	3% ± 1, 2%	2% ± 5, 1%	hardly any Co	hardly any Co
day 10 - CAI	13% ± 7, 4%	33% ± 22	hardly any Co	hardly any Co
day 10 + CAI	11% ± 7, 3%[v]	21% ± 9[v]	hardly any Co	hardly any Co
	12% ± 8, 2%[d]	22% ± 22[d]		
	6% ± 4, 1%[c]	**6% ± 5, 7%[c]**		

[v]=Z-VAD, [d]=Z-DEVD, [c]=combination CAI, median is given in case of more than 10% different from mean, CI = 95% confidence interval, bold results mean significantly different from control condition without CAI.

The MDS-2/RA patient showed a typically (LR-)MDS growth pattern with considerably fewer and smaller-sized colonies (<100 cells) and with higher CI/Co ratios than MDS-1 (Table 6.1). The addition of CAI led to a lower PE at day 4 (minus 10% clusters for DEVD and combi) and at day 10 (minus 15% for ZVAD and DEVD), with significantly lower colony formation at day 7 and 10 with all CAI conditions (-30 to 60%). Delayed growth initiation was found for DEVD at day 7 and for the combination of CAI at days 7 and 10 (Table 6.1). Significantly decreased PCD was found within clusters at day 7 for all CAI conditions (9-16%, median 0% vs. 26%, median 18% without CAI), and this translated into colonies with significantly lower PCD at day 10 for the combination of CAI (6% vs. 33% without CAI, Table 6.2). This growth profile with CAI is probably the result of delayed growth initiation and/or slower growth leading to lower cluster formation with lower PCD at day 7, which progresses to lower colony formation with lower apoptosis at day 10.

The MDS patients 3 and 4 are both HR-MDS patients according to FAB and both are showing the highest CI/Co ratios (Table 6.1) with delayed (only at day 10) and hardly any colony formation (≤4). In contrast to LR-MDS patients, these HR-MDS patients showed an increment in clusters of 10-30% and 5-15% at days 4 and 7, respectively, in all CAI treated samples. This difference was

no longer detected at day 10. Furthermore, delayed growth initiation at day 7 (+20-25%) was observed at all conditions (± CAI conditions) in both patients. In MDS-3, a significant decrease of PCD of clusters at day 10 was seen when CAI were added, of which Z-VAD had the most pronounced impact (mean 4% vs. 24% without CAI, Table 6.2). In MDS-4, Z-VAD and the combination of CAI also significantly decreased the level of PCD within clusters at day 7 (3% vs. 10%) and clusters at day 10 (9% vs. 20%), respectively. As hardly any colonies were found, no real conclusions about the influence of CAI upon colonies can be made. Furthermore, no increment in cluster and colony size was observed in association with this PCD lowering effect of CAI in both HR-MDS patients.
In conclusion, both in low and high risk MDS patients, apoptosis was inhibited by CAI. But in LR-MDS, eventually an inhibiting effect on proliferation of colonies was observed, whereas in HR-MDS, more day 4 and 7 clusters were observed, as these differences were no longer seen at day 10.

Discussion

Compared with the proliferation profile of normal BM $CD34^+$ cells in SCSW assay, it has been shown that MDS $CD34^+$ cells initially have a higher growth fraction, leading to the formation of significantly more clusters (with higher PCD) in comparison to colonies (with lower apoptosis), and all together at a slower pace[21]. These studies show “intrinsic” profiles without the influence of accessory cells and stroma. The increasing S-phase fraction within the overall myeloid compartment as within the $CD34^+$ compartment in combination with longer total cell cycling times as MDS progressed, was also observed by double-labeling techniques within BM biopsies[2,8,20]. These studies combined with our similar *in vitro* observations clearly demonstrate this growth advantage of the $CD34^+$ pool of MDS patients causing a higher plating efficiency in SCSW assay. However, it is essential that this so called “growth advantage” of MDS $CD34^+$ cells *in vitro* consist profoundly of cluster-forming units with restricted proliferation capacity and enhanced PCD. It seems likely that these $CD34^+$ cluster-forming cells belong to the more mature HSC pool with decreased intrinsic proliferative capacity. Only a small part of these MDS $CD34^+$ S-phase cells belong to colony-forming units (CFU), which mostly form small colonies of 40-100 cells at a slower pace (longer cell cycling times) and decreased PCD as compared to normal $CD34^+$ cells. Probably these CFU represent the (pre)leukemic clone with or without the cytogenetic marker. As FISH studies upon these small clusters and colonies within these round-bottom wells could not be performed successfully, it still is an assumption.
In this study, we investigated the influence of CAI upon the proliferation and apoptosis of MDS $CD34^+$ progenitors *in vitro*. It can be concluded from this

study that the combination of CAI in LR-MDS patients eventually leads to lower colony formation, while in HR-MDS patients CAI induce increasing numbers of clusters at days 4 and 7, which was no longer observed at day 10. In general, CAI in MDS patients resulted in inhibition of apoptosis of cluster- and colony-forming progenitors. Furthermore, it is obvious from our study that the use of CAI did not restore normal growth in these MDS patients, as it did not stimulate faster (higher numbers) and bigger colony formation.

The inhibition in colony formation in LR-MDS despite the lowering effect of the combination of CAI on apoptosis of clusters and colonies may be due to a growth inhibitory effect of CAI, eg. by slowing down cell cycling and/or going out of cycle. This growth profile by using CAI could resemble stimulation of pre-leukemic growth with longer cell cycling times and lower PCD. This statement has to be proved by FISH studies upon these cultures. On the other hand, in the HR-MDS there was a higher cluster formation with lower PCD by CAI, at least during 7 days. Probably CAI in this HR-MDS group, in which colony-forming $CD34^+$ cells originally show more autonomous growth with decreased apoptosis and longer cell cycling times, led to an acceleration of a leukemic profile leading to more aggregates with substantially more survival advantage. In other words; CAI overruled or restored the decreased intrinsic proliferative potential of cluster-forming progenitor cells in HR-MDS. In analogy: the usage of CAI in vivo in HR-MDS patients should not be used without careful watching, because acceleration towards leukemia could be stimulated.

Furthermore, it has been shown that functional FADD and caspase 8 are needed for apoptosis as well as for cytokine-induced proliferation of hematopoietic progenitor cells[22]. That study showed that the inhibition of hematopoietic colony formation by non-functional FADD and caspase 8 is not primarily due to effects on cell viability but it may rather be due to defects in entering the cell cycle from the G0 state. This probably resembles the delayed growth initiation with CAI found in 3 out of 4 patients at day 7 in our study. Additionally, a higher fraction of proliferating cells going out of cell cycle with more survival advantage during the influence of CAI could also play a role in the LR-MDS patients. In general, it was stated that death receptors have a threshold level of activation for different effects and by this mechanism play a role in myeloid cell homeostasis as a double-edged sword: delayed growth initiation and proliferation (and therefore survival advantage) versus apoptosis.

In conclusion, this study shows that in 4 MDS patients in different stages of the disease, the intrinsic apoptotic features can be inhibited by broad-spectrum caspase inhibitors. However, this reduced apoptosis does not translate into restoration of more colony formation as was seen in normal hematopoiesis.

It is important to stress that this *in vitro* leukemic growth profile in our SCSW assay was observed without cell-cell or cell-stromal interactions, whereas the same leukemic profile was also observed in other culture systems with intact or

disturbed stromal interactions. A reasonable explanation for this phenomenon could be the fact that it is an intrinsically feature of (or “programmed” within) the $CD34^+$ cell, as surrounding growth factors are only conditional for initial proliferation, as subsequently autonomic proliferation with or without production of paracrine growth factors exists. Accessory cells and/or stroma are not that important for actually changing this *in vitro* leukemic profile, but probably act as an accelerator or brake upon the predestined faith of this $CD34^+$ S-phase compartment.

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Summary

Summary, conclusions and future perspectives

The myelodysplastic syndromes (MDS) are highly proliferative bone marrow (BM) disorders characterized by dysplastic features, hyperproliferation, and enhanced intramedullary apoptosis or programmed cell death (PCD). The balance between proliferation and apoptosis in different cell compartments changes in time and plays a role in the progression toward acute myeloid leukaemia (AML). AML blasts proliferate at a lower pace than normal $CD34^+$ cells and they are able to turn down their PCD process. Subsequently, as the proliferation fraction is increasing, PCD is turned off, and eventually the proliferation rate increases dramatically leading to full blown AML. The relation between cell proliferation and apoptosis in MDS, their change in balance during progression towards AML, and the influences of accessory cells were the main topics of research described in this thesis.

In **chapter 1**, we present the current knowledge on the biology of stem and myeloid progenitor cells in MDS. It should be emphasized that the myeloid compartment in MDS contains three different hematopoietic cell populations within a pathological bone marrow (BM) micro-environment: residual polyclonal or normal hematopoiesis, a monoclonal preleukemic population, and a leukemic or blastic hematopoiesis. During MDS evolution, the relative size of these compartments changes; initially the normal hematopoiesis is suppressed, later on during progression to AML the blastic compartment increases. In MDS the initial DNA-altering event (toxic, chemotherapy and/or radiotherapy induced) probably occurs at the level of the pluripotent stem cell. Additional genetic defects lead to growth advantage of the (pre)malignant clone (monoclonality), differentiation arrest and finally suppression of PCD. Accumulation of leukemic blasts increases the apoptotic potential towards residual polyclonal (and monoclonal) hematopoietic cells and stromal cells, as these blasts carry FasL upon their cell membranes inducing apoptosis by Fas-FasL interaction. As differentiating cells express a higher density of FasR on their cell membranes, they are more prone to apoptosis. T-cells with FasL expression act as a double-edged sword; apart from killing leukemic blasts, they also kill the more mature myeloid progenitors and their progeny characterized by an increased FasR expression. The resulting (pan)cytopenia probably stimulates the proliferation of normal (and monoclonal) progenitors. This proliferation pressure in itself increases the chance of acquiring additional (cyto)genetic changes (or mutations) because of decreased DNA repair time. Subclones with additional cytogenetic abnormalities are the result of this process. Eventually apoptosis overrules proliferation within the $CD34^-$ compartment leading to decreased peripheral cell numbers. “Signal antonymy” (cells dying in S-phase) is a frequently observed and unique feature in early

stage MDS. The balance between these different proliferation profiles and apoptosis of the different marrow compartments in MDS, and their change in time during progression to AML, determines the different FAB/WHO-stages and prognostic classifications such as the IPSS (International Prognostic Scoring System) which are based on the percentage of marrow blasts, the number of profound cytopenias, and cytogenetic characteristics.

In **chapter 2,** we investigated the proliferation state of the $CD34^+$ stem and progenitor cell compartment of 33 untreated MDS patients and 5 patients with sAML (with a MDS-prephase) in order to achieve a better understanding of their cycling properties during MDS evolution. All patients received an infusion of the thymidine analogue iodo- or bromodeoxyuridine followed by a BM aspirate and biopsy. A double-labelling immunohistochemistry technique using anti-CD34 and anti-IUdR/BrdU antibodies was developed to discriminate $CD34^+$ and $CD34^-$ cells actively engaged in DNA synthesis or not. As MDS evolves, we observed a significant increase in the percentage of $CD34^+$ cells (mean value: RA/RARS 1.67%, RAEB(t) 8.68%, sAML 23.83%), as well as in the percentage of proliferating $CD34^+$ cells of all myeloid cells (RA/RARS 0.19%, RAEB(t) 0.43% and sAML 3.30%). This increase was associated with a decrease in the overall myeloid labeling index (LI: RA/RARS 25.8%, RAEB(t) 24.6% and sAML 21.5%). This decrease in overall myeloid LI is due to a shift in the ratio of proliferating $CD34^+$ and $CD34^-$ cells, as an exponential increase in the proportion of $CD34^+$ cells of the proliferating compartment during MDS evolution occurred (RA/RARS 0.35%, RAEB(t) 1.44% and sAML 11.98% of all S-phase cells) at the expense of the proliferating $CD34^-$ compartment. As this latter more mature compartment with higher FasR expression decreases during MDS evolution, “signal antonymy” in this compartment may account for this phenomenon. Overall marrow apoptosis is higher in low-risk (LR-) MDS than in high-risk (HR-)MDS because of higher apoptosis in the larger mature $CD34^-$ compartment than in the smaller $CD34^+$ compartment. Furthermore, we observed a progressive increment in the mean total cell cycling time of all myeloid cells during MDS progression (RA/RARS 39.8, RAEB(t) 45.2 and sAML 65.8 hr).
This study showed that during MDS evolution to sAML, the $CD34^+$ compartment develops a growth advantage and, therefore, increases with more cells dividing at a slower pace. Of course, the expansion of this $CD34^+$ compartment might also be due to a differentiation arrest in combination with decreased programmed cell death. The combined proliferation and apoptosis profiles were subject of our subsequent *in vitro* MDS studies.

In **chapter 3** we used cell suspensions to study the different features of the apoptotic process *in vitro* in time by flow cytometric (FCM) techniques. To study

the dynamic process of apoptosis, we used Annexin-V (AnV) for phosphatidylserine (PS) translocation, in situ end labeling (ISEL) for DNA fragmentation, and PI staining for cell membrane leakage. We developed a new flow cytometric ISEL technique, since it was only available as an immunohistochemical technique. Both ISEL and PI techniques detect different phases of PCD. Because PS translocation is assumed to be an early feature of apoptosis, we questioned if AnV positivity also implies inevitable cell death. Therefore, we used suspension cultures of Jurkat cells and induced apoptosis by γ-irradiation, incubation with Camptothecin (CPT), or Cytosine β-D-arabinofuranoside (Ara-C). At different time intervals apoptosis was quantified flow cytometrically by AnV/PI and ISEL. To analyse the consequence of AnV positivity, before or beyond the point of no return of apoptosis, various normal human AnV+/PI– $CD34^+$ fractions were cultured in a single-cell single-well (SCSW) assay.

We observed that all Jurkat cells showed a same pattern of apoptosis in time under these three different apoptosis-inducing conditions. AnV+/PI– cells showed subsequently ISEL positivity, after which they turned into AnV+/PI++ cells with even higher levels of ISEL positivity (80-90%). Eventually, they lost some of their PI and ISEL positivity (due to DNA desintegration). Cell handling after freeze-thawing of normal human $CD34^+$ cells resulted in a highly variable AnV+/PI– fraction (range 23-62%). Within this AnV+ and AnV+/PI– populations only a minority of $CD34^+$ cells showed ISEL positivity (range 4-8% and 0.8-6%, respectively). Furthermore, AnV+/PI– $CD34^+$ cells did have clonogenic capacity in the SCSW assay.

We concluded that PCD *in vitro* can be followed accurately using these three different FCM techniques. PS translocation is rapidly followed by oligo-nucleosomal DNA fragmentation, after which cell (and nuclear) membrane leakage occurs. Detection of PS asymmetry by AnV-FITC *in vitro* may be due to handling of the cells. Furthermore, we showed that Annexin-V positive cells after freezing and thawing could recover and start proliferating again, meaning that PS asymmetry does not always mean that cells have entered a phase of inevitable PCD.

In **chapter 4,** we used an agar culture system to investigate if BM mononuclear cells (BMMNC) of MDS patients also showed enhanced proliferation and apoptosis *in vitro,* with acute myeloid leukemia (AML) and normal BM (NBM) as a reference. BMMNC of de-novo MDS and AML patients with trisomy 8 as the sole cytogenetic marker were used in these studies.

BMMNC were cultured in agar during 10 days. Proliferation was determined by scoring number of clusters (10-39 cells) and colonies (≥40 cells). Their sum is called plating efficiency (PE). Apoptosis was assessed by performing in situ end labeling (ISEL) on these cultures at days 4, 7, and 10. Apoptotic

aggregates are defined as having 50% or more apoptotic or ISEL+ cells per aggregate. The ISEL technique was especially developed for the agar culture system as it detects a PCD feature beyond the point of no return. Furthermore, in situ hybridization (ISH) studies of day 10 - agar bottoms fixed on slides were performed to see if there was a difference in proliferation and apoptosis in time between disomic and trisomic aggregates in MDS and AML patients.

Kinetics in NBM showed a decrease in the number of clusters in time due to increasing PCD (median 50%) of clusters and due to progression to colonies with overall low apoptotic level (median 23%). In MDS patients, about 2-fold more clusters were found at day 4, and in contrast with NBM, the total number of clusters at day 7 remained high in spite of an increasing percentage of apoptotic clusters (from median 52 to 76%) in combination with more colony formation. The observed enhanced PE in MDS is obvious and reflects more cells in S-phase. In contrast to NBM, in MDS we observed numerous small clusters (of 2 to 9 cells; ≤ 3 cell divisions) which contributed to this phenomenon. At day 10, the number of clusters and colonies in MDS showed a sharp decline due to persisting high apoptosis at cluster level (median 75%) and increasing PCD in colonies (median 42%). BMMNC of AML patients showed decreased PE in comparison with normal and MDS; low proliferation with enhanced apoptosis at cluster level (median 62%) and a substantially delayed and reduced number of small colonies which showed relatively low apoptotic insult (median 20%) at day 10.

A strong positive correlation was found between percentage BM blasts and the growth fraction (percentage cells in S-phase) in AML (r=0,85), in contrast to MDS (r=0,52). It means that the S-phase cells *in vitro* in AML represent BM leukemic blasts, as in MDS probably more normal or monoclonal non-blastic progenitors represent the growth fraction. Furthermore, we did not observe a clear proliferative advantage of the trisomic 8 clone in the majority of the patients in this study.

In conclusion**,** we observed increased proliferation in MDS, associated with enhanced apoptosis in time, similar to our observations *in vivo*. AML clusters showed high apoptosis in contrast to a relatively low level of apoptosis in colony-forming cells (CFC). In AML CFC, leukemic transformation resulted in longer cell-cycling times and decreased apoptosis. These growth profiles of BMMNC are independent from stromal influences and represent intrinsic features of progenitor cells themselves, but interactions with non-stromal accessory cells (with or without growth factor production) can not be excluded. Since both proliferation and apoptosis were measured in one culture system, this approach provides insight in the balance of these two different mechanisms. For example, by changing growth factors, and/or ligands, or by inhibition of cell growth receptors, this type of experiments can give insight in the cell proliferation processes: what is an intrinsic (or so called ”programmed”)

cell property, what is the impact of autocrine and paracrine secretion of factors or ligands, and what is the influence of accessory cell interactions on apoptosis and proliferation of MDS (and AML) progenitors?

To rule out the role of accessory cells, we studied in **chapter 5** $CD34^+CD19^-CD3^-$ progenitor cells of 5 MDS patients and 5 normal controls by single cell sorting and culturing in the SCSW assay for 14 days in liquid medium with addition of growth factors. Subsequently, we evaluated the intrinsic proliferative capacity and apoptotic propensity of $CD34^+$ progenitor cells of MDS patients and compared them with normal *in vitro* characteristics.
Cultures were evaluated on days 4, 7, 10, and 14, and cells were counted within each well by inverted light microscopy. PCD was determined by staining with Annexin-V-FITC and the percentage apoptotic AnV+ cells per aggregate was determined by fluorescence microscopy. We used the Annexin-V method instead of ISEL, as the latter technique uses fixed cells and could not be performed within this liquid culture system with low cell numbers. Growth rate and cell doubling time (Td) were calculated for each colony-forming cell after 14 days of culturing.
In normal BM, we observed that CD34+ cells formed clusters and colonies. Both showed increasing PCD in time, although within colonies the degree of apoptosis was twice as high (about 25%) as compared with clusters at all time points. In MDS, increased cluster formation and also increased plating efficiency (PE) was observed at all evaluation points when compared to normal BM, whereas the number of colonies was markedly reduced (1/7 of normal). At day 14 of culturing, the relative amount of colonies compared to larger clusters (≥ 20 cells) and to smaller clusters (< 20 cells) in normal BM was 47% - 12% - 41%, respectively. In MDS these relative amounts were shifted to smaller clusters: 22% - 18% - 60%, respectively. The MDS colonies were also smaller, almost all less than 100 cells. Significantly enhanced levels of PCD in clusters (53-79%) in combination with longer cell doubling times (about 10 hours longer) may explain this reduced number of smaller colonies. Surprisingly, these MDS colonies showed considerably lower levels of PCD in time (7-32%) as compared to normal (1-48%, median values).
This study of CD34+ progenitors in MDS patients showed consistently enhanced plating efficiencies (or an overall higher growth fraction), mainly due a higher number of cluster-forming cells. This was consistent with our findings in the agar culture studies. Furthermore, as compared to normal BM, enhanced *intrinsic* proliferation and apoptosis of MDS cluster-forming $CD34^+$ cells was found, in contrast to the colony-forming $CD34^+$ cells (CFC). Above all, by ranking MDS patients according to the FAB/WHO classification, we found a transition from a LR-MDS growth profile (with high proliferation and shorter cell-doubling times, lower CI/Co ratios and high apoptosis of clusters and colonies)

towards a more “leukemic growth” profile with increased Cl/Co ratio’s, longer cell- doubling times, and subsequently lower apoptosis levels, especially at the level of CFC. Probably these CFC belong to the leukemic clone, in which longer cell-doubling time with decreased or no PCD cause a slow formation of smaller colonies with more blastic morphology.

In **chapter 6** we hypothesized whether caspase-inhibitors (CAI) could block intrinsically enhanced apoptosis of cluster-forming cells in MDS, and by doing so, could restore a normal growth pattern (with higher numbers and larger colonies). Again we used single-cell sorted $CD34^{+}CD3^{-}CD19^{-}$ cells of MDS patients (with trisomy 8) in cultures of 10 days with and without adding CAI. Proliferation and PCD was determined as described in chapter 5.
We observed that the inhibitory effect of CAI on apoptosis was significantly more pronounced in clusters than in colonies. Furthermore, the apoptosis-inhibiting effect of CAI was higher in LR-MDS than in HR-MDS, as also colonies showed this effect in the LR-group. The combination of CAI in LR-MDS patients led to a decrease in colony numbers with lower PCD of these aggregates at day 10. In HR-MDS, CAI induced more clusters at days 4 and 7 with lower PCD at days 7 and 10, whereas the scarce colony formation did not change significantly. Furthermore, the size of clusters and colonies did not change significantly with CAI in both MDS groups.
In general, we conclude that CAI decreased apoptosis of *in vitro* cultured $CD34^{+}$ cells of all MDS patients by inhibiting apoptosis of cluster- and colony-forming progenitors. Interestingly, CAI inhibition of apoptosis in MDS patients certainly did not restore normal growth.
Recent studies have shown a broader mechanism of action of caspases in myeloid homeostasis: they are involved in entering the cell cycle and in cytokine-induced proliferation as well. These mechanisms may play a role when CAI are used *in vitro*: delayed growth initiation was found in 3 out of 4 MDS patients, leading to lower colony formation in LR-MDS patients. On the other hand, in HR-MDS patients CAI may stimulate survival and/or proliferation.

Future perspectives

Modern technologies, like microarrays and microfluidic cards techniques, may provide information which genes are involved in the basic biological processes of MDS (and AML), such as proliferation and apoptosis, the topics of this thesis. Correlation between these molecular and biological pathways is expected to lead to a more precise approach of treatment. Besides chemotherapy, a better rationale for other treatment modalities may be defined,

such as treatment with thalidomide, gemtuzumab ozogamicin, lenalidomide, DNA methyltransferase inhibitors, farnesyl transferase inhibitors, bevacuzimab, and growth factors (erythropoeitin and G-CSF). All these therapies interfere with molecular pathways. The *in vitro* culture systems used in this thesis can be of great value in this approach. Combined analysis of molecular and biological pathways of various cell suspensions under different conditions will give additional insight which genes and proteins are involved in the pathophysiology of MDS. As can be concluded from our *in vivo* and *in vitro* experiments, it seems logically to apply a “missionary approach” within the low-risk MDS group. Suppression of apoptosis may relieve the proliferation pressure. The anti-apoptotic and cytokine modulating therapy seems to work in a minority of LR-MDS patients by suppressing the cytogenetically aberrant clone. As more insight is gained into the different players of the apoptotic pathways, this approach could be fine-tuned and might be more efficient. Subsequently, the “crusader approach” for the remaining preleukemic or leukemic clone might be applied selectively, as the normal stem cell pool is usually damaged and declined in this category of patients.

Today we use an AML-like approach to treat HR-MDS patients, but this approach offers a lower success rate than in primary AML. Recently developed agents may improve relapse-free and overall survival. By *in vitro* testing of these agents (with or without chemotherapy) in the different cell suspensions of these patients, as mentioned above, in combination with molecular and biological analyses, it may lead to a more precise therapeutic rationale. Our *in vitro* culture systems with simultaneous detection of proliferation and apoptosis, as described in this thesis, can therefore be of great value, as they can be used prior to in-vivo testing of newly developed agents.

Samenvatting

Samenvatting, conclusies en toekomstperspectieven

Myelodysplastische syndromen (MDS) zijn beenmerg (BM) aandoeningen, gekenmerkt door verhoogde proliferatie (celdeling), dysplasie (rijpingsstoornis) en verhoogde intramedullaire apoptose of geprogrammeerde celdood (PCD). De balans tussen proliferatie en celdood van de verschillende celtypen in het BM verandert tijdens het ziektebeloop en speelt een cruciale rol in de overgang van MDS naar acute myeloide leukemie (AML). Leukemische blasten prolifereren langzamer dan normale blasten ($CD34^+$ cellen) en tonen verminderde apoptose. Deze vermindering van apoptose gaat uiteindelijk gepaard met een versnelling in proliferatie; beide processen veroorzaken zodoende een accumulatie van leukemische blasten in het BM en bloed wat uiteindelijk een full blown AML tot gevolg heeft. De fysiologische processen van proliferatie en celdood van verschillende celtypen in MDS, hun balans en de verandering hiervan ten tijde van progressie met eventuele invloeden van accessoire cellen, zijn de belangrijkste onderwerpen van onderzoek die in dit proefschrift worden beschreven.

In **hoofdstuk 1** geven we een overzicht weer van de huidige kennis van zaken van de biologie van de stamcellen en voorlopercellen in MDS. We dienen ons te realiseren dat het BM van MDS patiënten is te verdelen in een drietal hematopoietische cel compartimenten te midden van een veranderende micro-omgeving met stromale cellen, zoals oa. vet- en endotheelcellen. Deze drie celpopulaties betreffen residuale polyclonale, normale hematopoiese (HP), monoclonale of preleukemische HP, en (monoclonale) leukemische of blastaire HP. Gedurende de evolutie van MDS naar AML treedt er een verandering op in de relatieve grootte en dus onderlinge verhouding van deze celpopulaties. In het begin wordt de normale HP onderdrukt of gaat dood, terwijl gedurende de overgang van MDS naar AML de blastaire component steeds meer toeneemt. MDS ontstaat waarschijnlijk door een initiële noxe (toxische stof, chemotherapie en/of bestraling) die een DNA-verandering in een pluripotente stamcel teweegbrengt. Vervolgschade leidt tot additionele genetische veranderingen in deze clonale stamcel, hetgeen leidt tot een groeivoordeel, differentiatiestop en uiteindelijk ook verminderde celdood in de leukemische blasten. Als deze blasten accumuleren, initiëren zij onderdrukking (meer apoptose) van de omliggende polyclonale en monoclonale HP en stromale cellen doordat zij via verhoogde FasL expressie op hun celmembraan interactie aangaan met de verhoogde FasR expressie op deze meer uitgerijpte cellen. Bovendien ontstaan gedurende MDS-progressie meer T-cellen met verhoogde FasL expressie die een tweeledig effect sorteren: het (pogen tot) doden van leukemische blasten en via FasL-FasR celdood veroorzaken van de rijpe progenitorcellen en hun nakomelingen als onschuldige omstanders. De

resulterende progressieve pancytopenie geeft waarschijnlijk via een terugkoppelingsmechanisme een verhoogde proliferatiedruk op deze cellen met meer kans op additionele genetische fouten, die niet of slecht gerepareerd worden door een kortere DNA-hersteltijd. Hierdoor ontstaan subklonen met additionele chromosomale afwijkingen. Uiteindelijk overheerst apoptose over proliferatie in het $CD34^-$ compartiment hetgeen tot lagere perifere celaantallen leidt. “Signal antonymy” betekent dat cellen doodgaan ten tijde van de S-fase (= fase van DNA synthese cq. verdubbeling); dit blijkt een karakteristiek, uniek en veelvoorkomend verschijnsel te zijn in MDS. De balans tussen deze verschillende profielen van proliferatie en apoptose van de verschillende celcompartimenten in het BM van MDS patiënten verandert gedurende de overgang van MDS naar AML. Deze progressie wordt weergegeven in de FAB-WHO classificatie en de IPSS: een scoringssysteem gebaseerd op het percentage beenmergblasten, het aantal ernstige cytopenieën en de aard van de cytogenetische afwijkingen.

In **hoofdstuk 2** onderzochten we het proliferatieprofiel van de $CD34^+$ stam- en progenitorcellen van 33 niet-behandelde MDS patiënten en 5 secundaire AML patiënten (na MDS voorstadium) om een beter inzicht te krijgen in het groeivoordeel van $CD34^+$ cellen gedurende de progressie van MDS naar AML. Daartoe kregen alle patiënten een infusie met een thymidineanaloog, gevolgd door een BM-aspiraat en -biopsie. Via immuno-histochemische dubbel-labellingstechnieken konden we $CD34^+$ van $CD34^-$ cellen onderscheiden en het percentage S-fase cellen bepalen. Gedurende MDS evolutie zien we een significante toename van het percentage $CD34^+$ cellen, als ook van het percentage $CD34^+$ cellen in S-fase. Deze toename was geassocieerd met een afname in de myeloide labellingsindex, hetgeen de totale fractie delende cellen beschrijft binnen het totale myeloide ($CD34^+$ en $CD34^-$) compartiment. Dit wordt veroorzaakt door een verschuiving in de verhouding van proliferende $CD34^+$ in S-fase ten koste van een afnemende deling in het CD34- compartiment. Daar dit laatste meer rijpere compartiment een hogere FasR expressie vertoont kan “signal antonymy” hierin een rol spelen. Totale apoptose is in het algemeen in laagrisico (LR-)MDS hoger dan in hoogrisico (HR-)MDS vanwege een groter aandeel van het rijpere $CD34^-$ compartiment in deze LR-groep. Bovendien is het bekend dat onrijpere $CD34^+$ cellen langzamer delen dan de rijpere CD34- nakomelingen. Zodoende is het logisch dat er sprake is van een progressieve toename van de totale celcyclustijd van alle myeloide cellen gedurende de MDS evolutie naar AML (van 40 naar 66 uur), zoals wij in deze studie aantonen.
Concluderend laat deze studie een groeivoordeel zien van het $CD34^+$ compartiment gedurende MDS-evolutie waardoor het totale $CD34^+$ compartiment toeneemt met langzaam delende cellen. Waarschijnlijk zal ook

een toegenomen differentiatiestop en een verminderde apoptose in deze $CD34^+$ cellen een rol spelen, hoewel we dit in onze studie niet hebben onderzocht. De gecombineerde celdood- en celproliferatieprofielen *in vitro* zijn dan ook onderwerp van onze vervolgstudies in MDS.

In **hoofdstuk 3** hebben we de verschillende kenmerken van apoptose in de tijd bestudeerd middels nieuwe flowcytometrische (FCM) technieken op celsuspensies. Om het dynamische profiel van geprogrammeerde celdood te bestuderen, gebruikten we Annexine-V (AnV) om phosphatidylserine (PS)-translokatie van de celmembraan aan te tonen, in situ end labeling (ISEL) techniek om DNA fragmentatieprodukten te detecteren, en PI kleuring voor het vast stellen van celmembraanlekkage. De flowcytometrische ISEL techniek moest ontwikkeld worden, daar deze methode tot nu toe alleen beschikbaar was als een immunohistochemische techniek. ISEL en PI technieken tonen verschillende fasen van irreversibele apoptose aan. PS translokatie toont een vroege fase van celdood aan. Zodoende vroegen wij ons af of dit ook een irreversibele fase van apoptose vertegenwoordigt. Wij gebruikten een Jurkat cellijn voor suspensie cultures en induceerden apoptose middels γ-bestraling, of door middel van incubatie met camptothecine of Ara-C. Na verschillende tijdsintervallen werd apoptose gekwantificeerd middels AnV/PI en ISEL via FCM. Voor het vaststellen of AnV positiviteit een fase van irreversibele celdood vertegenwoordigt, werden verschillende AnV+/PI- humane normale $CD34^+$ cellen na ontdooien gekweekt in een single-cell single-well (SCSW) assay. Deze Jurkat-cellen vertoonden eenzelfde apoptose profiel onder elk van de bovenstaande drie apoptose-inducerende condities: AnV+/PI- cellen vertoonden binnen een uur ook ISEL positiviteit, waarna zij veranderden in AnV+/PI++ cellen met zelfs hogere niveaus van ISEL positiviteit (80-90%). Uiteindelijk verloren al deze cellen enige mate van PI- en ISEL-positiviteit door desintegratie van het DNA. Gecryopreserveerde normale $CD34^+$ cellen toonden na ontdooien een variabele AnV+/PI- fractie (23-62%). Binnen deze AnV+ en AnV+/PI- fracties was sprake van een kleine hoeveelheid cellen die ook ISEL positiviteit vertoonde (resp. 4-8% en 0,8-6%). Bovendien zagen wij ook AnV+/PI- $CD34^+$ cellen die clonogene aktiviteit vertoonden in de SCSW assay, hetgeen impliceert dat PS asymmetrie van de celmembraan nog niet een fase van irreversibele celdood aangeeft.

In **hoofdstuk 4** gebruikten wij de BM mononucleaire cellen (BMMNC) van MDS patiënten in agar cultures om verhoogde mate van proliferatie en apoptose aan te tonen *in vitro*, zoals deze patiënten dit ook hebben laten zien *in vivo*. We gebruikten BM van AML patiënten en normaal BM (NBM) als referentie. BMMNC van de-novo MDS en AML patiënten met trisomie 8 als enige cytogenetische marker (intermediaire risico-groep volgens WHO-FAB) werden

gebruikt in deze studie om de hoogst mogelijke mate van uniformiteit te verkrijgen.
BMMNC werden gekweekt in agar gedurende 10 dagen. Proliferatie werd gedefinieerd als het aantal clusters (10-39 cellen) en kolonies (≥40 cellen) op een bepaalde dag. De som van alle delende cellen, clusters en kolonies wordt "plating efficiency" genoemd (PE). Apoptose werd vastgesteld door middel van een aangepaste ISEL techniek op gefixeerde agar bodems van dag 4, 7 en 10. Apoptotische aggregaten zijn gedefinieerd als hebbende 50% of meer ISEL positieve cellen per aggregaat. In situ hybridisatie (ISH) werd verricht op agarbodems van dag 10 om verschil aan te tonen in proliferatie- en apoptoseprofiel van disome en trisome aggregaten van bovenstaande patiënten.
Agarkweken met NBM lieten een afname in de tijd zien van het aantal clusters door toename van apoptose (mediaan 50%) en overgang van clusters naar kolonies met weinig apoptose (mediaan 23%). In MDS patiënten werden tweemaal zoveel clusters gevormd op dag 4. In tegenstelling tot NBM, bleef het clusteraantal hoog op dag 7 ondanks een forse toename van apoptotische clusters (van mediaan 52 naar 76%) en een groter aantal kolonies dan in NBM. De duidelijk toegenomen PE in MDS gaf aan dat meer cellen in S-fase waren, duidend op een hogere proliferatiefractie dan normaal. Bovendien zagen we in MDS (itt. NBM) de vorming van vele kleine clusters (van 2-9 cellen, ≤3 celverdubbelingen) die ook bijdragen aan dit profiel. Op dag 10 was er sprake van een scherpe daling in aantal clusters en kolonies tgv. persisterende sterk verhoogde apoptose op clusterniveau (mediaan 75%) en toegenomen apoptose op kolonie niveau (mediaan 42%). BMMNC van AML patiënten lieten een duidelijke verlaagde PE zien; lage proliferatie met verhoogde apoptose (mediaan 62%) op cluster niveau en een vertraagde vorming van minder kolonies met relatief lage apoptose (mediaan 20%) op dag 10.
Een sterke positieve correlatie werd gevonden tussen het percentage BM blasten en de groeifractie (% delende cellen) in AML (r=0,85), terwijl dit niet zo duidelijk werd gevonden in MDS (r=0,52). Dit betekent dat de delende cellen in AML voortkomen uit BM leukemische blasten, terwijl dit in MDS veel minder het geval is omdat waarschijnlijk ook normale en monoclonale niet-blastaire progenitors deze groeifractie vertegenwoordigen. In het algemeen kunnen we uit de ISH data concluderen dat er geen proliferatie voordeel van de trisomie 8 kloon werd gevonden in de meerderheid van de patiënten in deze studie.
Concluderend zagen wij in MDS toegenomen proliferatie die uiteindelijk werd gevolgd door verhoogde apoptose conform de *in vivo* bevindingen van een celrijk BM, forse signal antonymy, met cytopenieen in het bloed. In AML-clusters zagen we verhoogde apoptose in tegenstelling tot de relatief lage apoptose van kolonievormende cellen. In AML kolonies resulteerde de leukemische transformatie in langere celcyclustijden met verminderde

apoptose. Deze groeiprofielen van BMMNC zijn onafhankelijk van stromale invloeden en vertegenwoordigen daarom intrinsieke kenmerken van progenitors en mogelijk interacties met niet-stromale accessoire cellen (met of zonder groeifactor productie). Daar wij proliferatie en apoptose tegelijkertijd vaststelden in één kweeksysteem, kregen we inzicht in de balans tussen beiden. Aanvullende studies door bijvoorbeeld andere groeifactoren, liganden, of door toepassing van receptor blokkades, kunnen we via dit kweeksysteem vaststellen wat intrinsiek is (of wel “geprogrammeerd”), en wat de invloed is van autocriene en paracriene secretie van groeifactoren en/of liganden, en wat de invloed is van accessoire celinteracties op apoptose en proliferatie van MDS (en AML) progenitors?

Om de invloed van accessoire cellen in kweken geheel uit te sluiten, bestudeerden wij in **hoofdstuk 5**, $CD34^+CD19^-CD3^-$ progenitors van 5 MDS patiënten en van 5 normale controles door middel van kweken van enkelvoudige cellen. Deze cellen zijn flow-cytometrisch geselecteerd, één cel per celbodem gesorteerd, en vervolgens gekweekt gedurende 14 dagen in vloeibaar medium met toevoeging van groeifactoren. We onderzochten de intrinsieke proliferatieve capaciteit en apoptose van $CD34^+$ progenitorcellen van MDS patiënten en vergeleken deze met de normale *in vitro* profielen.
Op dag 4, 7, 10 en 14 werden deze kweken beoordeeld; binnen elke celbodem werden het aantal cellen geteld door middel van lichtmicroscopie. Apoptose werd vastgesteld door kleuring met Annexin-V-FITC en het percentage apoptotische AnV+ cellen per aggregaat werd vastgesteld door middel van fluorescentie-microscopie. Wij gebruikten de Annexine-V methode in plaats van ISEL, aangezien de laatstgenoemde techniek gebruik maakt van gefixeerde cellen en daarom niet kan worden toegepast op dit vloeibaar kweeksysteem met kleine celaantallen. Groeisnelheid en celverdubbelings-tijden werden berekend voor elke kolonievormende cel na 14 dagen.
In NBM stelden we vast dat $CD34^+$ cellen clusters en kolonies vormden met toename van apoptose in de tijd, waarin de mate van apoptose in kolonies twee keer zo hoog was (ongeveer 25%) in vergelijking met clusters op alle tijdstippen. In MDS vonden we, conform de bevindingen in agarkweken, toegenomen clustervorming evenals toegenomen PE op alle tijdstippen in vergelijking tot NBM, terwijl het aantal kolonies duidelijk was verminderd (1/7 van normaal). Op dag 14 vonden we in NBM met name kolonies (47% van alle aggregaten) vergeleken met grote clusters (≥20 cellen) en kleinere clusters (<20 cellen), respectievelijk 12% en 41%. In MDS waren deze verhoudingen verschoven naar de kleinere clusters: 22% - 18% - 60%, respectievelijk. De MDS kolonies waren ook veel kleiner dan normaal en hadden meestal minder dan 100 cellen. Significant verhoogde mate van apoptose in clusters (53–79%) in combinatie met een langere celverdubbelingstijd (ongeveer 10 uur langer)

verklaren een verminderd aantal en kleinere kolonies. Het was verrassend dat de MDS kolonies duidelijk lagere niveaus van apoptose lieten zien in de tijd (7-32%) in vergelijking tot NBM (1–48%, mediane waarden), en dit verschil (ongeveer 10–26% minder) werd nog duidelijker bij vergelijking van de kleinere kolonies (40–100 cellen) tussen beide groepen.
Deze studie laat een consistente verhoogde PE of groeifractie zien, die met name op rekening komt van clustervormende $CD34^+$ MDS cellen. Dit is in analogie met onze agarkweken. De intrinsiek verhoogde proliferatie en apoptose van clustervormende cellen is duidelijk tegengesteld aan kolonievormende cellen. Indien wij MDS patiënten rangschikken volgens FAB/WHO classificatie, vinden we een overgang van een LR-MDS "groeiprofiel" (met hoge proliferatie en kortere celverdubbelingstijden, lagere Cl/Co ratio's met hogere apoptose van clusters en kolonies) naar een meer leukemisch groeipatroon met verhoogde Cl/Co ratio's, langere celverdubbelingstijden, en vervolgens lagere niveau's van apoptose in met name kolonievormende cellen (CFC). Waarschijnlijk behoren deze CFC tot de leukemische kloon, waarin een langere celverdubbelingstijd met verminderde of geen apoptose een langzamere vorming geeft van kleinere en meer blastaire kolonies.

In **hoofdstuk 6** wilden we de hypothese toetsen of blokkade van deze intrinsiek verhoogde apoptose van clustervormende $CD34^+$ cellen door gebruik van caspase inhibitoren (CAI) op enkelvoudige celniveau een normaal groeipatroon kan herstellen. Wederom gebruikten wij $CD34^+CD3^-CD19^-$ progenitorcellen van MDS patiënten (met trisomie 8) in SCSW culturen gedurende 10 dagen met en zonder toevoeging van CAI. Proliferatie en apoptose werden vastgesteld zoals beschreven in hoofdstuk 5.
We constateerden dat de intrinsiek verhoogde apoptose afnam onder invloed van CAI en dat dit fenomeen sterker was in clusters dan in kolonies. Dit effect van CAI was hoger in LR-MDS dan in HR-MDS, terwijl ook kolonies in de LR-groep dit effect vertoonden. De combinatie van CAI in LR-MDS patiënten leidde tot lagere kolonievorming met lagere apoptose van deze aggregaten op dag 10. CAI induceerde in HR-MDS meer clusters op dag 4 en 7 met lagere apoptose op dag 7 en 10, terwijl het spaarzame aantal kolonies niet duideliik veranderde. Bovendien veranderde de grootte van clusters en kolonies niet significant onder invloed van CAI in beide groepen.
Samenvattend, kunnen we concluderen dat CAI een verlaging van apoptose induceert van *in vitro* gekweekte $CD34^+$ cellen van alle MDS patiënten, zowel van cluster- als van kolonie-vormende progenitors. Echter inhibitie van apoptose leidde tot minder kolonies in LR-MDS, terwijl er meer clusters en nog steeds nauwelijks kolonies werden gevormd in HR-MDS. Dus het gebruik van CAI in MDS patiënten herstelt zeker niet het normale groeipatroon. Recente

experimenten in de literatuur laten een breder werkings-mechanisme zien van caspases in de myeloide homeostase: ze zijn betrokken bij het in cyclus komen en bij cytokine-geïnduceerde proliferatie. Waarschijnlijk spelen deze mechanismen ook een rol als CAI worden gebruikt: vertraagde groei-initiatie werd gevonden in 3 van de 4 MDS patiënten hetgeen leidt tot lagere kolonievorming in LR-MDS patiënten. Anderzijds, in HR-MDS patiënten is het werkingsmechanisme van CAI waarschijnlijk meer stimulatie van overlevingsvoordeel en/of proliferatie daar er meer clustervormende cellen worden gezien.

Toekomstperspectieven

Moderne technologieën, zoals microarrays en microfluidic cards, zullen meer inzicht geven in welke genen betrokken zijn bij basale biologische processen zoals proliferatie en apoptose van MDS (en AML) patiënten, zoals we die getest hebben in dit onderzoek. Koppeling van moleculaire diagnostiek aan deze biologische pathofysiologie zal zeker gaan leiden tot een gerichtere aanpak. Met als gevolg het ontstaan van een betere rationale voor de toepassing van de nieuwere middelen, zoals thalidomide, gemtuzumab ozogamicine, lenalidomide, DNA-methyltransferase-remmers, farnesyl-transferase-remmers, bevacuzimab en groeifactoren (erythropoietine en G-CSF); allen grijpen namelijk in op specifieke moleculaire paden. Met deze aanpak zullen de kweeksystemen, zoals in onze studies ontwikkeld en toegepast, van grote waarde kunnen zijn. Deze kweeksystemen zijn immers uniek voor gecombineerd analyseren van bovenstaande processen; door het toepassen van verschillende condities op verschillende celtypen kan proliferatie en apoptose *in vitro* simultaan bestudeerd worden. De verschillende celtypen kunnen leukemische blasten zijn, maar ook de resterende blasten na toepassing van chemotherapie, of zelfs de leukemische stamcel. Daarnaast kunnen deze cellen op hun oorspronkelijke en vervolgens ook op hun veranderde genetische opmaak met translatie naar eiwitten ten tijde van kweek geanalyseerd worden.

Gedurende de evolutie of progressie van MDS, onstaan uit (pre)leukemische klonen door continue DNA-veranderingen in de tijd, klonen met additionele cytogenetische afwijkingen. Mede op grond van onze bevindingen lijkt het in LR-MDS patiënten rationeel om te starten met een “missionaris” aanpak (van apoptose remming) om de proliferatiedruk te verminderen. De anti-apoptotische aanpak heeft bewezen dat zij in een minderheid van de MDS patiënten de cytogenetisch-aberrante kloon remt. Meer inzicht in de specifieke spelers van al deze apoptotische paden zal deze aanpak verfijnen en breder toepasbaar maken. De volgende stap moet zich richten op de resterende

(pre)leukemische kloon of zelfs stamcel met een meer selectieve “missile” aanpak, daar het normale stamcelcompartiment al reeds is beschadigd en daardoor afgenomen.
In HR-MDS is de aanpak tot op heden vergelijkbaar met die bij AML, maar minder succesvol dan bij primaire AML. Wellicht dat ook hier de combinatie van chemotherapie met nieuwe middelen (liefst volgens een “missile” aanpak) de prognose sterk kan verbeteren. Ook voor dergelijke patiënten kan het *in vitro* testen van nieuwe middelen, met bovenstaande besproken aanpak van analyseren van celsuspensies, eventueel in combinatie met chemotherapie, de prognose verbeteren. De in dit proefschrift beschreven kweeksystemen en simultane analyse van proliferatie en apoptose kunnen van grote waarde zijn voordat deze patiënten aan nieuwe behandelingen worden blootgesteld.

Dankwoord

Dankwoord

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Curriculum vitae

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De schrijver van dit proefschrift werd geboren op 3 april 1963 te Geleen. Na het behalen van Atheneum B diploma (Albert Schweitzer Scholengemeenschap, Geleen) in 1981, startte hij met de studie Geneeskunde aan de Katholieke Universiteit Nijmegen. Hij behaalde het doctoraalexamen in 1986 en het artsexamen in 1989. Ondertussen verrichtte hij onderzoek, onder leiding van dr. Peter Oud, op de afdeling Quantitatieve Microscopie als onderdeel van de afdeling Pathologische Anatomie van het St. Radboud Ziekenhuis (hoofd Prof. dr. G.P. Vooys) naar celkarakteristieken van het mamma- en het cervix-carcinoom in relatie tot hun maligne karakter. Eveneens verrichtte hij onderzoek, onder leiding van dr. Ad Hermus en dr. Ton Bartelink, op de Intensive Care van het St. Radboud Ziekenhuis (hoofd Dr. J. Gimbrère) naar het functioneren van de hypofyse-bijnieras en het vaststellen van de incidentie van relatieve bijnierschorsinsufficientie bij ernstig zieke patiënten. Van 1989 tot begin 1991 volgde hij de opleiding tot Eerste Luitenant-Arts te Hilversum en verrichtte hij onderzoek voor Defensie naar radio-actieve stralingschade van het DNA van leukocyten op de afdeling Hematologie in het St. Radboud Ziekenhuis (initiële opleider Prof. dr. C. Haanen, vervolgens Prof. dr. Theo de Witte). Ten tijde van dit onderzoek ontving hij de “Radboudpluim” voor bijzondere inzet in het kader van patiëntenzorg via de aferese-afdeling, oa. voor intensieve plasmaferese van meningococcensepsis patiënten. Aanvankelijk fungeerde hij vanaf 1991 als AGNIO in het Diakonessen Ziekenhuis te Arnhem (opleider dr. Jan Werre), en vervolgens als AGIO Interne Geneeskunde in het Rijnstate Ziekenhuis te Arnhem (opleider dr. Louis Verschoor) tot halverwege 1995. Vervolgens verrichtte hij een jaar onderzoek op het gebied van myelodysplasie aan het laboratorium van Prof. dr. Azra Raza (Rush Cancer Institute, Chicago, IL, USA), dank zij een subsidie van de Sacha Swarttouw Hijmans Stichting. Dit onderzoek diende als onderbouwing voor het verkrijgen van een AGIKO-schap (subsidie NWO/FMW). Hij voltooide de opleiding Interne Geneeskunde onder leiding van Prof. dr. Jos van der Meer. In 2001 volgde registratie als internist, en in 2003 registratie als hematoloog. Vanaf 2003 werkt B. Span als hematoloog/chef-de-clinique in het Academisch Ziekenhuis te Maastricht.