STUDIES ON ERYTHROPOIESIS IN THE MYELODYSPLASTIC SYNDROME

ONDERZOEK AAN ERYTHROPOIESIS IN HET MYELODYSPLASTISCH SYNDROOM

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF.DR. P.W.C. AKKERMANS M.A.
EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
VRIJDAG 10 NOVEMBER 1995 OM 13.30 UUR

DOOR

BARBARA ANNA MARIA WILHELMINA BACKX

GEBOREN TE ROOSENDAAL EN NISPEN

PROMOTIE-COMMISSIE:

PROMOTOR: Prof. Dr. B. Löwenberg

OVERIGE LEDEN: Prof. Dr. A. Hagenbeek

Prof. Dr. A.M.M.J. Hagemeijer-Hausman

Prof. Dr. M.M.A.C. Langenhuijsen

Offsetdrukkerij Haveka B.V., Alblasserdam

The work described in this thesis was performed at the Department of Hematology, Dr Daniel den Hoed Cancer Center, Rotterdam and was supported by a grant from the Dutch Cancer Society "de Nederlandse Kankerbestrijding". Financial support for the costs of printing was provided by the Dutch Cancer Society.

in dankbare herinnnering aan mijn vader

voor mijn moeder
voor Jos, Karlien en Ivar

CONTENTS

ABBREVIATIONS

AML	acute myeloid leukemia
BFU-E	burst forming unit-erythroid
CFU	colony forming unit
CFU-blast	CFU-blast cells
CFU-Eo	CFU-eosinophil
CFU-G	CFU-granulocyte
CFU-GEMM	CFU-granulocyte-erythroid-macrophage-megakaryocyte
CFU-GM	CFU-granulocyte-macrophage
CFU-M	CFU-macrophage
CMML	chronic myelomonocytic leukemia
CSF	colony stimulating factor
DBA	Diamond-Blackfan anemia
EPO	erythropoietin
FAB	French-American-British cooperative group
FACS	fluorescence activated cell sorting
G-CSF	granulocyte-CSF
GM-CSF	granulocyte macrophage-CSF
HGF	hematopoietic growth factor
IL-*	interleukin-*
KL	kit ligand
M-CSF	macrophage-CSF
MDS	myelodysplastic syndrome
NBM	normal bone marrow
RA	refractory anemia
RAEB	refractory anemia with excess of blasts
RAEB-t	refractory anemia with excess of blasts in transformation
RARS	refractory anemia with ring sideroblasts
TNFα	tumor necrosis factor alpha

CHAPTER ONE

INTRODUCTION

Chapter 1

1.1 The Myelodysplastic syndrome

1.1.1 Classification

A variety of primary hematologic disorders were described, all characterised by ineffective hematopoiesis, peripheral blood cytopenias, and hypercellular bone marrow (Bjorkman, 1956, Rheingold et al., 1963, Linman et al., 1978). Lack of uniform definitions for these disorders, often referred to as smoldering leukemia, preleukemia or refractory anemia, resulted in both difficulties in classification and problems in predicting disease outcome. In an effort to resolve these problems, in 1982 the French-American-British (FAB) Cooperative Study group proposed a new classification system for these bone marrow disorders and termed them myelodysplastic syndromes (MDS)(Bennett et al., 1982). Based on cellular morphology and on the number of blast cells in the peripheral blood and bone marrow, five subtypes of MDS were defined: Refractory anemia (**RA**), refractory anemia with ring sideroblasts (**RARS**), refractory anemia with excess blasts (**RAEB**), refractory anemia with excess blasts in transformation (**RAEB-t**), and chronic myelomonocytic leukemia (**CMML**).

1.1.2 Morphological characteristics

Ineffective hematopoiesis in MDS may involve abnormalities of the erythroid, myeloid and megakaryocytic lineages, for the diagnosis of MDS at least two maturation lineages should be involved:

a. Dyserythropoiesis and anemia

Bone marrow abnormalities of erythroid precursors and peripheral blood red cell abnormalities, both qualitative and quantitative, including presence of ringed sideroblasts, nuclear abnormalities (multinuclearity, abnormal shaped nuclei) and abnormal cytoplasmic features.

b. Dysgranulopoiesis and granulocytopenia

Morphological abnormalities in granulocytes in both bone marrow and peripheral blood.

- abnormalities in nuclear segmentation: hyposegmentation or hypersegmentation with bizarre shapes.
- cytoplasmic abnormalities: agranular or hypogranular cells.

c. Dysmegakaryocytopoiesis and thrombocytopenia

Micromegakaryocytes as well as large mononuclear megakaryocytes and megakaryocytes with multiple small nuclei in bone marrow and presence of giant platelets in the peripheral blood.

FAB subtype	Blast cells (%) Peripheral blood	Bone marrow
Refractory anemia ▸RA◂	≤1	<5
RA with ring sideroblasts ▸RARS◂	≤1	<5 >15% of all nuclear cells: ringed sideroblasts
RA with excess of blasts ▸RAEB◂	<5	5-20
RAEB in transformation ▸RAEB-t◂	≥5 ± Auer rods	20-30 ± Auer rods
Chronic myelomonocytic leukemia ▸CMML◂	<5 >1.10 ^{9}/L monocytes	<20 increase promonocytes

1.1.3 Clonal origin

Different lines of evidence make it likely that MDS is a clonal disorder and several techniques are currently available to study the clonal nature of MDS:
Evidence for clonal hematopoiesis in MDS has been demonstrated by making use of protein polymorphism, e.g., that of the **glucose-6-phosphate dehydrogenase (G6PD) isoenzymes** (Prchal JT et al., 1978, Raskind et al., 1984). This method is based on the fact that women, heterozygous for the X-linked enzyme G6PD, show cellular mosaicism. Whereas in normal tissues a mixture of A and B G6PD enzyme types is found, clonal proliferation in a G6PD heterozygote reveals only one enzyme type (Fialkow et al, 1978). An obvious limitation however of the G6PD analysis is the limited number of heterozygotes available. The presence of **non-random cytogenetic abnormalities** in up to 70% of patients with MDS also has confirmed the clonal nature (Ayraud et al., 1983, Jacobs et al., 1986, Heim et al., 1987, Yunis et al., 1988). More recently, clonality of hematopoietic cells in MDS has been studied at DNA level by using **X-linked restriction fragment length polymorphism (X-RFLP)-methylation analysis**. Analogous to the G6PD studies, this technique is based on differences in X-chromosome inactivation patterns (Vogelstein et al., 1985, Fraser et al., 1987, 1989, Janssen et al., 1989, Tefferi et al., 1990, Abrahamson et al., 1991). Since different combinations of probes can be used, e.g. probes for the phosphoglycerate kinase (PGK) gene and the hypoxanthine phosphoribosyl transferase (HPRT) gene and the M27ß probe, this technique can be applied to the majority of females. In some MDS patients the involvement of specific cell lineages can be demonstrated by detection of somatic **mutations of *ras* oncogenes**, serving as clonal markers (Hirai et al., 1987, Janssen et al., 1987, 1989., Bos, 1989). To study clonality on a limited number of cells, amplification by **polymerase chain reaction (PCR)** can be applied (Janssen et al., 1989, Van Kamp et al., 1992b). Finally, **fluorescent in situ hybridization (FISH)** can be used in MDS patients associated with chromosomal abnormalities, e.g. monosomy 7 or trisomy 8 (Gerritsen et al., 1992, Han et al., 1994).

The results from the accumulating reports however have been conflicting as regards the involvement of the different lineages in the abnormal clone. Whereas some investigators claim that the abnormal clone is capable of myeloid and lymphoid differentiation (Raskind et al., 1984, Janssen et al., 1989, Tefferi et al., 1990), others suggest that clonal hematopoiesis is restricted to cells of myeloid origin (Abrahamson et al., 1991, Culligan et al., 1992, Gerritsen et al., 1992, van Kamp et al., 1992b).

Clonality of hematopoietic tissue is detected in some proportion of normal persons as well. Comparison of suspected monoclonal tissue with analysis of the same tissue in normal populations as well as analysis of uninvolved tissue of the same subject may be important in this respect (Young, 1992).

1.1.4 Clinical picture

Most MDS patients reveal anemia as one of the most prominent abnormalities at the time of diagnosis. Eventually they may become tranfusion dependent. Mild neutropenia and thrombocytopenia are seen in about 50% of the patients. MDS affects patients of all ages, although middle age and elderly patients predominate. MDS occuring in adults aged 50 or less are relatively often familiar cases or appear associated with exposure to potential carcinogens (Foucar et al., 1985, Fenaux et al., 1990). Approximately one third of the patients with MDS show progression to acute myeloid leukemia. Another proportion die of complications related to leukopenia or thrombocytopenia (infections or bleedings). With regard to survival and cause of death, the five morphological subtypes show variable prognosis. Patients with RA and RARS generally have longer median survivals (often more than 5 years) and often die of unrelated diseases. MDS patients with RAEB, and RAEB-t on the other hand show comparatively short survival durations, often less than 1 year, and die of disease complications such as hemorrhage and/or infection or evolution to leukemia (Foucar et al., 1984, Horiike et al., 1988).

Non-random cytogenetic abnormalities are commonly seen in patients with MDS. Acquisition of cytogenetic abnormalities leads to an increased risk of leukemic transformation (Tricot et al., 1984, Horike et al., 1988, Geddes et al., 1990, Ohyashiki et al., 1992). In primary MDS the most frequent abnormalities are **deletions of chromosome 5 (-5/5q-), chromosome 7 (-7/7q-), chromosome 20 (20q-), or the gain of an additional chromosome 8 (8+)**. The presence of specific chromosome abnormalities may correlate with the probability of survival and leukemic transformation. The deletion of the long arm of chromosome 5 (5q-), when present as the sole abnormality, is associated with long-term stable disease and a low rate of leukemic transformation (Van den Berghe et al., 1985, Kerkhofs et al., 1987, Geddes et al., 1990). However, a poor prognosis of survival is seen in patients with 5q- plus other abnormalities (Billström et al., 1988, Wong et al.,

1992). An increasingly unfavourable prognosis is associated with the presence of trisomy 8, -7/del7 and complex chromosomal abnormalities (Yunis at al., 1988, Geddes et al., 1990). The presence of a specific chromosomal abnormality however is not associated with any of the MDS subtypes as defined by the FAB classification (Knapp et al., 1985, Billström et al., 1988, Horike et al., 1988, Jotterand-Bellomo et al., 1990), excepting the 5q- syndrome, characterized by refractory anemia (Van den Berghe et al., 1974).

Specific **point mutations of the *ras*-oncogene** have been found in a wide variety of human malignancies, including MDS (Janssen et al., 1987, Bos et al., 1989, Janssen et al., 1989, Neubauer et al., 1994). The *ras* genes, H-*ras*, K-*ras*, and N-*ras*, encode homologous 21 kD proteins ($p21^{ras}$) that have GTPase activity and have been implicated in the control of cell proliferation. Critical point mutations, e.g. mutations in codons 12, 13 or 61, give rise to mutant gene products that have greatly reduced GTPase activity (Seeburg et al., 1984, Der et al., 1986, Egan et al., 1993). Ras-mutations may be detected at time of diagnosis, or during clinical follow-up, the latter suggesting that Ras-mutations sometimes may be acquired later during the course of the disease (Van Kamp et al., 1992a, Paquette et al., 1993). Controversy exists regarding the clinical implications of Ras-mutations in MDS. Whereas in some studies the presence of Ras-mutations appeared to be associated with progressive disease and poor prognosis (Hirai et al., 1987, Paquette et al., 1993, Horiike et al., 1994), other reports indicated that the presence of Ras-mutations did not adversely affect clinical outcome (Van Kamp et al., 1992a, Neubauer et al., 1994).

The human FMS proto-oncogene encodes the functional receptor for M-CSF (or CSF-1). The FMS gene is the cellular homologue of the v-*fms* gene of the Susan McDonough feline sarcoma virus (Donner et al., 1982). The v-*fms* gene product exhibits ligand independent tyrosine kinase activity and will transform cells in vitro, whereas the normal human FMS gene will not. In vitro gene transfer studies using experimentally mutated versions of c-*fms* have confirmed the importance of mutations at codons 301, 374, and 969 for transforming activity (Roussel et al., 1988, Woolford et al., 1988). The FMS gene is located on chromosome 5q33, a region frequently altered in MDS patients (Jacobs et al., 1986). Potentially activating **point mutations and deletions in the human FMS gene** were described in some patients with myelodysplasia (Ridge et al., 1990, Tobal et al., 1990, Boultwood et al., 1991).

Mutations in the p53 gene, a tumor suppressor gene playing a critical role in cellular proliferation and regulation of the cell cycle, have been suggested to be involved in the

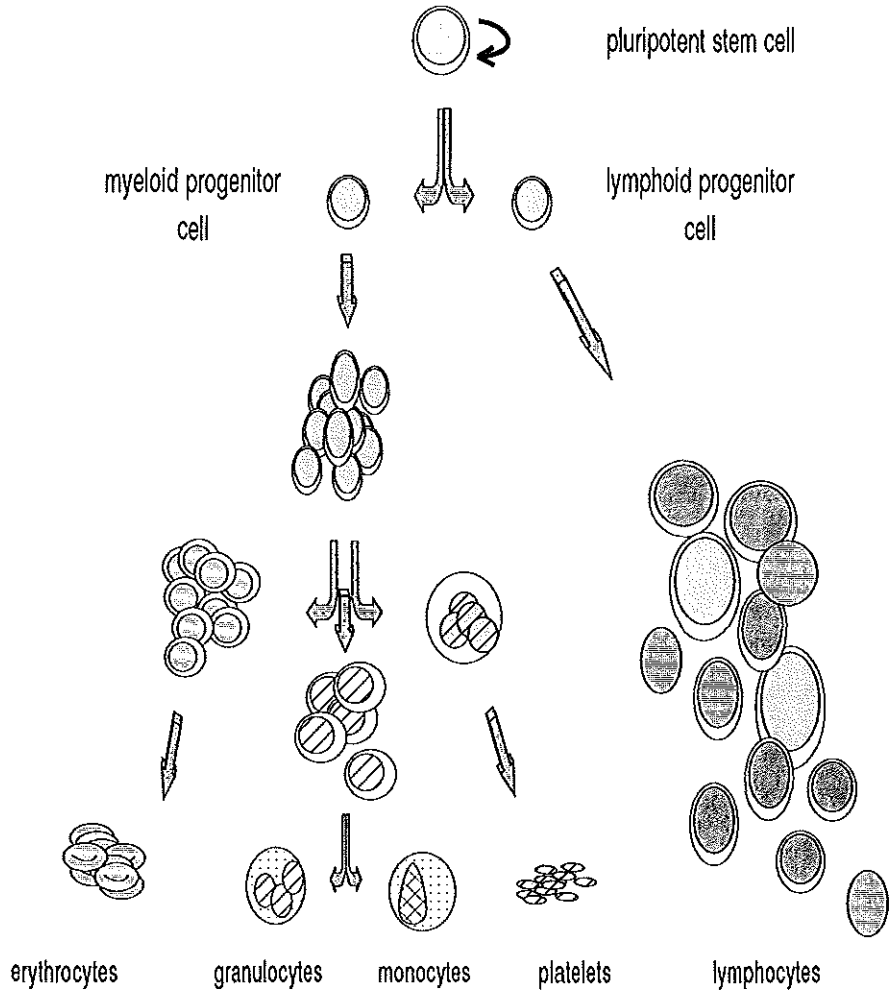

Figure 1 *Schematic representation of the hierarchical organization of normal hematopoiesis.*

pathogenesis of several human malignancies, including a small fraction of patients with MDS (Nigro et al., 1989, Jonveau et al., 1991, Levine et al., 1991, Mori et al., 1992, Sugimoto et al., 1993).

The significance of mutations in the *ras*-FMS-and p53 genes in the development of MDS remains to be resolved.

1.2 Hematopoiesis

1.2.1 Pluripotent stem cells, models of stem cell differentiation

Hematopoiesis, the process of blood cell formation, is a complex proces. A small set of pluripotent stem cells, residing in the bone marrow, possess the ability of self-renewal and generate committed progenitors of various lineages, irreversibly committed to differentiation towards single lineages. This differentiation process results in the formation of functional blood cells, e.g., erythrocytes, granulocytes, monocytes, platelets or lymphocytes, specialized to defend the body against hypoxia, microbial invasion, bleeding and immunological insult (Figure 1).

As many of the blood cells are short lived, the continuous production of blood cells is required to maintain appropiate amounts of these cells. In special situations, such as environmental stress, blood cell production is adjusted to altered needs.

Under steady state-conditions the hematopoietic stem cell is quiescent (G_0). The entry of small numbers of stem cells into cell cycle with subsequent expansion and differentiation is sufficient to sustain steady-state hematopoiesis.

Several models for stem cell commitment have been proposed:

The **hemopoietic inductive microenvironment (HIM) model** proposes that commitment of pluripotent hemopoietic stem cells to monopotent progenitors is determined by a specific inductive microenvironment surrounding the stem cell (lineage-specific anatomical niches) (Trentin, 1970).

In the **stem cell competition model** the control of stem cell differentiation is believed to be regulated by lineage-specific humoral factors such as EPO and colony-stimulating factors (CSF) (Goldwasser, 1975).

A third model implies that lineage selection by the progenitors at the time of cell division is a **stochastic (random) process** (Till et al., 1964, Nakahata et al., 1982a) and that growth factors simply permit the proliferation and development of intrinsically committed cells.

Finally, a **hybrid of the stochastic and inductive model** has been proposed (Just et al., 1991). This hybrid model implies that a ligand-activated receptor alters the balance between proliferation and differentiation in favour of differentiation, but that the type of differentiation is determined by the cell in a stochastic manner.

Distinct in vitro culture systems are used to study the pluripotent hematopoietic stem cell:

- semi-solid cultures: blast cell colony assay (Nakahata et al., 1982b, Nakahata et al., 1982c, Leary et al., 1987) and related assays (Rowley et al., 1987, Brandt et al., 1988).
- long-term bone marrow cultures (LTBMC)(Dexter et al., 1977, Eaves et al., 1988, Sutherland et al., 1990, Lee et al., 1991).

Experimental data arising from these assays do not allow to distinguish whether stem cell commitment follows a stochastic or a regulator induced differentiation process. Analysis of colonies derived from single or paired cells, isolated from blast cell colonies, however favour the concept that lineage selection during stem cell commitment is a random process (Leary et al., 1984, Suda et al., 1984). More recently, Fairbairn (1993) reported that transfection of the FDCP-Mix cell line (a multipotent hemopoietic, Il-3 dependent, cell line) with bcl-2, enabled these FDCP-Mix cells to survive in both serum-containing and serum-deprived conditions and that survival was accompanied by multilineage differentiation. These data support the hypothesis that differentiation is intrinsically determined and that the role of growth factors is enabling rather than inductive.

Hematopoiesis is regulated by a complex network of soluble stimulators and inhibitors,

as well as by cellular interactions in the bone marrow micro environment (stromal cells). Survival, proliferation and differentiation are influenced by a balance between stimulatory and inhibitory signals (Figure 2). Abnormalities in the normal developmental program for the formation of blood cells may result in various types of hematological diseases.

?? In the myelodysplastic syndrome, an abnormality of the hematopoietic stem cell affects normal hematopoiesis, leading to abnormal maturation, eventually resulting in anemia, neutropenia and thrombocytopenia. The questions whether normal numbers of very immature cells (blast cells) are present in MDS marrow and into which distinct lineages these immature marrow cells are able to or do differentiate are addressed in **Chapter 3**.

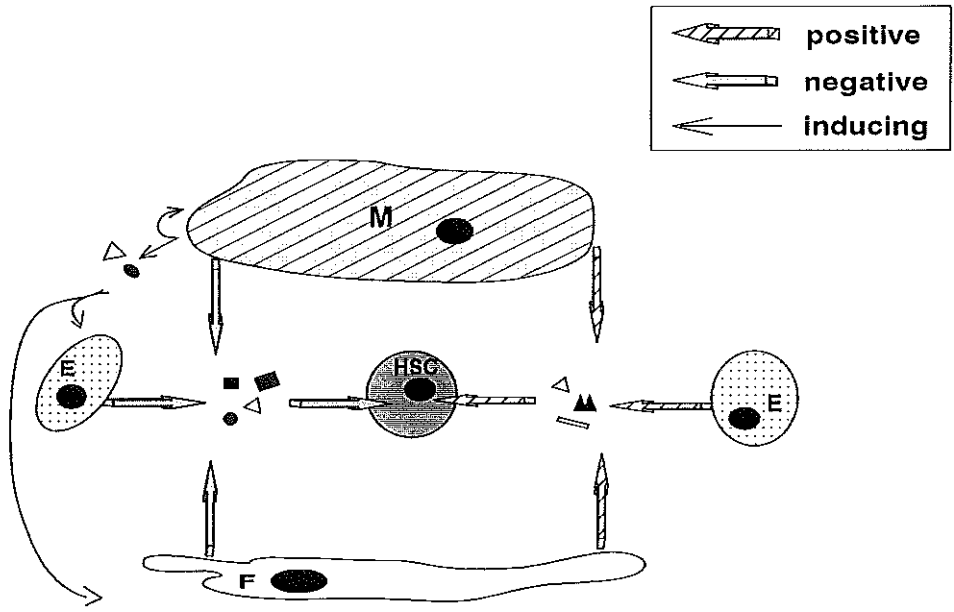

Figure 2 *The interaction of the hematopoietic stem cell (HSC) with the bone marrow microenvironment. Macrophages (M), fibroblasts (F), and endothelial cells (E) produce positive as well as negative growth and differentiation factors (cytokines).*

1.2.2 Hematopoietic growth factors

Hematopoietic growth factors (HGFs) are glycoproteins, acting at all stages of blood cell formation, regulating proliferation, renewal, differentiation and survival (Clark, 1987, Sieff, 1987, Metcalf, 1989, Metcalf, 1993). Most of them are multifunctional molecules, often inducing a range of effects on both hematologic and non-hematologic cells (Dedhar et al., 1988, Berdel et al., 1989, Bussolini et al., 1989). Whereas EPO is produced by the kidney (peritubular cells) and liver (Kupffer cells) (Jacobson et al., 1957, Paul et al., 1984, Koury et al., 1988), other HGFs are mainly produced by monocytes, macrophages, T-lymphocytes, endothelial cells, and fibroblasts (Bagby et al., 1986, Kaushansky et al., 1988, Sieff et al., 1988).

Some HGFs are active on multiple cell lineages, e.g, IL-3, GM-CSF, IL-6, promoting growth and subsequent development of various progenitors (Hapel et al., 1985, Sieff et al., 1985, Metcalf, 1986). Others, like EPO and G-CSF, are more lineage-restricted and induce terminal differentiation (Miyake et al., 1977, Nagata et al., 1986). In addition, the effects of a single factor may be modified by the presence of other factors which can act synergistically or act as antagonists. For example, Kit ligand (KL, also called stem cell factor (SCF), mast cell growth factor (MGF) or steel factor (SLF)) acts synergistically with many other factors (Witte et al., 1990). KL was identified as the ligand deficient in the steel mutant mouse strain (Sl/Sl^d) and inadequate expression of KL was shown to be responsible for the hematopoietic defects noted in the Sl/Sl^d mice. Both membrane bound and soluble forms of KL exist (Anderson et al., 1990).

Tumor necrosis factor alpha (TNFα), a glycoprotein of 17 kD, illustrates an example of a cytokine with a broad spectrum of effects on a large number of different cell types (Carswell et al., 1975, Beutler et al., 1985, Brenner et al., 1988). TNFα was originally characterized by its cytotoxic and cytostatic activity on tumor cells in vitro and in vivo (Old, 1985, Beutler et al., 1987). TNFα is mainly produced by monocytes, and TNFα itself may induce the release of colony stimulating factors from diverse cell types (Krönke et al., 1990, Vilcek et al., 1991). TNFα exists in two forms: a secreted form and and a higher molecular weight cell bound form (Kriegler et al., 1988). Both inhibitory as well as stimulatory effects on in vitro colony growth of hematopoietic progenitor cells are described (Murase et al., 1987a, Caux et al., 1990). The effects of TNFα can be either directly mediated or indirectly mediated by inducing other cells to produce HGFs (Oster et al., 1978, Munker et al., 1986).

?? Investigation of the direct effects of TNF on hematopoietic progenitor cells requires studies with highly enriched progenitor cells, without the interfering effects of accessory cells. In **Chapter 2** the following questions are addressed: How does the effect of TNF on in vitro colony formation of enriched **normal marrow** progenitors relate to the type of growth factor and the different progenitor cell types induced, and how does the effect relate to the TNF concentration?

Isolation of the cDNAs encoding the HGFs enabled the production of sufficient quantities of these proteins to investigate the in vitro effects of these HGFs and several of the HGFs have been or are being tested in a variety of clinical settings to study their in vivo therapeutic potential. Since the myelodysplastic syndrome is characterized by arrested or abnormal differentiation, several treatment protocols are based on induction of maturation. Diverse agents are examined for their ability to promote differentiation of the arrested cells in in vitro studies, and eventually the most promising differentiation promoting agents may be used in clinical trials. Some HGFs, e.g. IL-3, G-CSF, GM-CSF and EPO, are examples of such agents tested in in vivo studies (Ganser et al., 1992).

HGFs exert their biological functions through interactions with specific membrane-spanning receptors, which are present on cells in low numbers. These **HGF-receptors** are not only present on cells of the hemopoietic system and many leukemic cells, but are also found in nonhematopoietic tissue, suggesting a wider role of HGFs outside the context of hematopoiesis (Gearing et al., 1989, Avalos et al., 1990, Jones et al., 1990, Quesenberry et al., 1992). Results from sequence analysis suggest that all HGF-receptors are members of a few large gene families. The EPO-receptor for example is a member of the hematopoietin receptor superfamily (Kaczmarski et al., 1991), c-kit belongs to the receptor tyrosine kinase family (Olsson et al., 1992). Changes in growth factor receptor structure and level of expression may have great impact on the final biological response, and altered receptor function may thus be involved in human hematopoietic disorders (Slamon, 1987, Sherr, 1990). In the myelodysplastic syndromes, characterized by maturational defects of marrow hematopoietic cells, little is known about the mechanism underlying the poor response to HGF stimulation. The impaired growth factor response in MDS could potentially represent a quantitive lack of receptors or could be caused by abnormalities in the HGF-receptors themselves or at a level downstream of the receptors.

?? Ineffective erythropoiesis due to an impaired response to EPO is a prominent abnormality in MDS and KL may restore the in vitro erythroid colony forming response to EPO in a subset of patients (**Chapter 4**, Glinsmann-Gibson et al., 1994,, Piacibello et al., 1994, Soligo et al., 1994). Can this impaired erythroid response in MDS be explained by a quantitative lack of receptors for EPO and KL, respectively EPO-R and c-kit? Does (altered) receptor expression correlate with (impaired) growth factor response? These questions are addressed in **Chapter 5.**

Soluble receptor forms, either formed by proteolytic cleavage of transmembrane receptors (e.g. TNF-R, M-CSF-R) or derived from specific transcripts formed by alternative splice of mRNA (e.g. Il-4-R, Il-7-R), have been identified for many of the HGF-receptors (Downing et al., 1989, Seckinger et al., 1989, Fukunaga et al., 1990). These soluble receptor forms (binding proteins) could function for example as anti-cytokines (negative feed-back regulation), protectors of cytokines (prolongation half-life cytokines) or be involved in signal transduction (binding to signal transducers).

1.2.3 In vitro growth factor response

The development of techniques which allowed the growth of blood progenitors in culture and the cloning of most of the HGFs made a thorough research possible on the role of growth factors in hematopoiesis by in vitro experiments (Pluznik and Sachs, 1965, Bradley and Metcalf, 1966). From these studies it was demonstrated that human bone marrow progenitors require HGFs for survival, growth and differentiation. Several reports describe that in MDS, the in vitro colony forming capacity in response to growth factor stimulation generally is decreased (Mayani et al., 1989, Nagler et al., 1990, Schipperus et al., 1990, Merchav et al., 1991). This impaired colony formation includes the granulocyte-macrophage -erythroid and megakaryocyte lineage (Dan et al., 1993). Some studies report that supersaturating HGF concentrations may improve colony formation (Mayani et al., 1989, Merchav et al., 1991), but this effect most likely is related to an accessory cell effect, since results from studies using purified MDS progenitors, depleted of accessory cells, demonstrate that supra optimal concentrations of HGFs cannot really augment colony formation (Carlo-Stella et al., 1989). Impaired hematopoiesis in MDS

may be related to both abnormalities in growth factor responsiveness of bone marrow progenitor cells as well as abnormalities within the MDS stroma (Merchav et al., 1989). Ohmori et al. (1992) reported that monocyte-derived lipid containing huge macrophages (MDLM) from MDS marrow may be responsible for the suppression of granulopoiesis in patients with MDS. MDLM from MDS marrow and their conditioned medium (MDLM-CM) consistently suppressed the growth of normal CFU-GM colony formation whereas MDLM obtained from normal bone marrow had a CFU-GM enhancing activity. However, Coutinho et al. (1990) could not identify a consistent functional defect of stroma cells in MDS. Several groups tried to correlate FAB subtype with colony growth. In view of the divergent and contradictory results, prediction of FAB subtype by using in vitro clonal growth pattern results seems to be very difficult (Ruutu et al., 1984, May et al., 1985).

The regulatory molecule kit ligand (KL), which has a key role in hematopoiesis, is of special interest since genetic defects involving KL itself or its receptor in mice result in severe anemia (Huang et al., 1990, Witte et al., 1990). In vitro studies with normal human marrow demonstrated that KL by itself has no ability to induce colony formation by human bone marrow progenitors, but in the presence of other HGFs like GM-SCF, IL-3 or EPO, KL improves colony formation dramatically (Bernstein et al., 1991, Broxmeyer et al, 1991, McNiece et al., 1991). In both Diamond Blackfan anemia (DBA), a congenital red blood cell aplasia, and aplastic anemia, characterized by multilineage bone marrow failure, erythroid colony formation of bone marrow cells in response to EPO, like in MDS, is severely decreased (Abkowitz et al., 1991, Bagnara et al., 1991, Olivieri et al., 1991, Wodnar-Filipowicz et al., 1992). Addition of KL to EPO stimulated cultures resulted in a marked increase in eryhroid colony formation in the marrow from patients with DBA and aplastic anemia. These in vitro results suggest that use of KL, either alone or in combination with other factors, may be of potential value in the treatment of these diseases.

?? In most MDS cases erythroid colony formation following stimulation with the erythroid growth factor EPO is markedly reduced. In view of its potential to improve erythroid colony formation in normal marrow, in vitro studies with KL in MDS may disclose whether KL responsiveness in MDS remained intact. Can costimulation with KL enhance EPO induced erythropoiesis in MDS? Does KL affect colony number, size of colonies, or both? Is there a difference between the different MDS subtypes? These questions are addressed in **Chapter 4.**

CHAPTER TWO

POSITIVE AND NEGATIVE EFFECTS OF TUMOR NECROSIS FACTOR ON COLONY GROWTH FROM HIGHLY PURIFIED NORMAL MARROW PROGENITORS

Bianca Backx, Lianne Broeders, Fredrik J Bot, Bob Löwenberg

Leukemia 5:66-70, 1991

Chapter 2

SUMMARY

The effects of recombinant human tumor necrosis factor alpha (TNFα) on colony growth were studied using highly enriched progenitor cells from normal human bone marrow. Supplementation of TNF to culture resulted in a dose-dependent suppression of granulocyte colony-stimulating factor (G-CSF) induced granulocytic colony formation and also erythropoietin (EPO) induced erythroid burst formation. However, the number of erythroid bursts, stimulated by interleukin-3 (IL-3) plus EPO, increased when TNF was added at comparable concentrations. Further, TNF enhanced eosinophilic colony growth induced by IL-3 or granulocytic-macrophage-colony stimulating factor (GM-CSF). In GM-CSF cultures TNF (100-1000 U/mL) also induced granulocytic and macrophage colonies. The addition of neutralizing antibodies against G-CSF, GM-CSF or interleukin-6 (IL-6) to culture did not abrogate the observed effects of TNF, so that stimulation of myeloid colony growth was unlikely to result from the secondary induction of G-CSF or GM-CSF.

Thus TNF exerts favourable effects on hematopoietic progenitors responsive to the more primitive colony stimulating factors (IL-3, GM-CSF) and potent negative effects on precursors reactive to the single lineage G-CSF and EPO. These contrasting effects of TNF suggest that TNF when available to marrow progenitors at similar tissue concentrations, may drive hematopoiesis within the progenitor cell compartment into selected directions.

INTRODUCTION

Tumor necrosis factor alpha (TNFα) is a cytokine that is released by monocytes, macrophages and lymphocytes (Herrmann and Mertelsmann, 1984, Beutler et al., 1985, Nathan, 1987, Thompson et al., 1989). TNF itself may induce the release of colony-stimulating factors from diverse cell types, e.g. granulocyte colony-stimulating factor (G-CSF) from fibroblasts (Koeffler et al., 1987), granulocyte-monocyte colony-stimulating factor (GM-CSF) from fibroblasts and endothelial cells (Koeffler et al., 1987, Koeffler et al., 1988, Munker et al., 1986) and macrophage colony-stimulating factor (M-CSF) from monocytes (Oster et al., 1987). The colony-stimulating factors (CSFs) act as hematopoietic growth factors and control the survival, proliferation and differentiation of hematopoietic progenitor cells *in vitro* (Clark and Kamen , 1987). Therefore in inducing

the production of CSFs, TNF has an important role in the regulation of hematopoiesis.

TNF has been shown to suppress normal erythroid and myeloid colony growth *in vitro* in a dose-dependent fashion (Broxmeyer et al., 1986, Wisniewski et al., 1987). A thorough understanding of the direct effects of TNF on hematopoietic progenitor cells depends on studies with highly purified cell fractions from normal bone marrow in which interfering effects of accessory cells are excluded. In the studies reported here, we investigated the modulating effects of TNF on the *in vitro* colony-forming abilities of highly enriched normal bone marrow progenitors induced by distinct recombinant colony-stimulating factors and with emphasis on the effects of TNF on different progenitor cell types.

MATERIAL AND METHODS

Preparation of cell suspension

Bone marrow samples were aspirated by posterior iliac crest puncture from hematologically normal adults following informed consent. The marrow was collected in Hanks' balanced salt solution (HBBS) with heparin, diluted in HBBS and the mononuclear cells were separated over Ficoll-Isopaque (1.077 g/cm^2; Nycomed, Oslo, Norway) as described previously (Bot et al., 1988).

Enrichment of progenitor cells

Marrow cells were enriched following a two-step cell separation procedure as described previously (Bot et al., 1989a). Cells were depleted of non-clonogenic accessory cells by complement mediated cytolysis using a cocktail of monoclonal antibodies (MoAbs) against the antigens CD14 (B44.1, IgM, monocytes; final dilution 1:1000) (Perussia et al., 1982), CD15 (B4.3, IgM, granulocytic cells; final dilution 1:500) (Van der Reijden et al., 1983) and CD3 (T3, IgG2, final dilution 1:10). This resulted in an average recovery of 50±12% of nucleated cells. Further enrichment was achieved following CD34 labeling (BI3C5, IgG1; Sera-Lab, Crawley Down, UK)(Katz et al., 1985) and fluorescence-activated cell sorting of CD34 positive cells exactly as described previously (Bot et al., 1988). The recovery of the final cell fraction, mainly immature blasts, was 2-5% of the initial number of mononuclear cells. In all experiments cultures stimulated with phytohemagglutinin-leucocyte conditioned medium (PHA-LCM) were run in parallel as

control to assess the recovery of clonogenic cells following sorting, as described previously (Bot et al., 1989b).

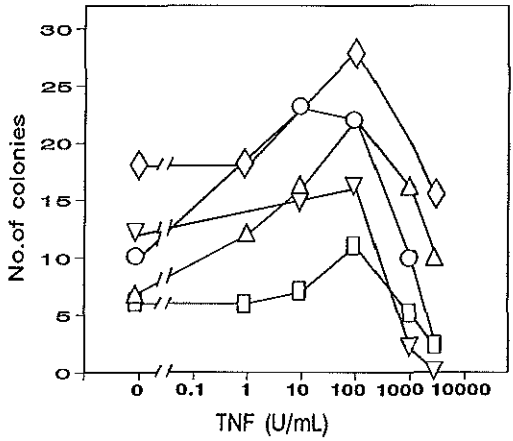

Figure 1 *Positive effect of TNF on IL-3 induced eosinophilic colony growth. Enriched normal bone marrow progenitors (0.5 to 1 × 10^4/mL) were incubated with IL-3 and increasing concentrations of TNF (0-3000 U/mL). Data from five experiments are shown. Number of PHA-LCM induced CFU-Eo: (□) 38; (△) 52; (○) 178; (▽) 100; (◇) 69.*

Recombinant human cytokines and anticytokine antibodies

Recombinant human IL-3, a gift from Dr L. Dorssers and Dr G. Wagemaker (Daniel den Hoed Cancer Center, Rotterdam and Radiobiologic Institute TNO, Rijswijk, The Netherlands) (Dorssers et al., 1987) was used at the optimal concentration of 10 U/mL. Recombinant GM-CSF was donated by Dr J.F.G. Delamarter (Biogen SA, Geneva, Switzerland)(Burgess et al., 1987) and used at the optimal concentration of 1000 U/mL. The sheep anti-human GM-CSF antibody (kindly provided by Dr S. Clark, Genetics Institute, Cambridge, MA, USA) was used at a concentration of 0.25% v/v. Recombinant human G-CSF and the rabbit anti-human G-CSF antibody (gifts from Dr S Gillis, Immunex, Seattle WA, USA) were used at concentrations of 1:10000 and 0.25% v/v respec-

tively. Recombinant human TNFα (specific activity $6x10^7$ U/mg) and the rabbit anti-human TNFα and TNFß antibodies were provided by Dr G.R. Adolf (Boehringer Institute, Vienna, Austria). Anti-TNFα and anti-TNFß were used at concentrations of 1:10000. The concentration of TNFα was as indicated in the figures. Recombinant human erythropoietin (EPO)(Amgen Biologicals, Thousand Oaks, CA, USA) was used at 1 U/mL and the rabbit anti-human IL-6 antibody (a gift from Dr L. Aarden, Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands), was used at a concentration of 0.25% v/v. The optimum stimulatory concentrations had been seperately determined for each growth factor batch (data not shown).

Colony culture assay

Enriched progenitor cells (0.5-1 x 10^4 cells/mL) were cultured in duplicate for 15 days in a mixture of Iscove's modified Dulbecco's medium (IMDM), 1.1% methylcellulose, 30% autologous heparinized plasma, bovine serum albumin (BSA), transferrin, lecithin, sodium selenite, and 2-mercaptoethanol in a humidified atmosphere of 5% CO_2 in air at 37°C as reported previously (Bot et al., 1988), and erythroid, eosinophilic, granulocytic and macrophage colony numbers were scored separately.

RESULTS

TNF enhances IL-3 or GM-CSF induced colony formation in vitro

When highly enriched progenitor cells are plated, IL-3 induces eosinophilic colonies only (Bot et al., 1988). Following the addition of increasing concentrations of TNF to colony culture the number of eosinophilic colonies increase in a dose-dependent manner. Maximum eosinophilic-colony growth (Eo-colony growth) is apparent at 100 U/mL of TNF (Figure 1). Higher concentrations of TNF (1000-3000 U/mL) become inhibitory to Eo-colony growth. Similary GM-CSF induces only eosinophilic colonies from enriched progenitor cells (Bot et al., 1989c). Co-stimulation with TNF results in the formation of greater numbers of Eo-colonies and the appearance of granulocytic colonies and macrophage colonies (Figure 2). The dose-effect relationship of TNF and GM-CSF induced colony growth is identical to that of TNF and IL-3, i.e. with maximal effects at 100 U/mL TNF.

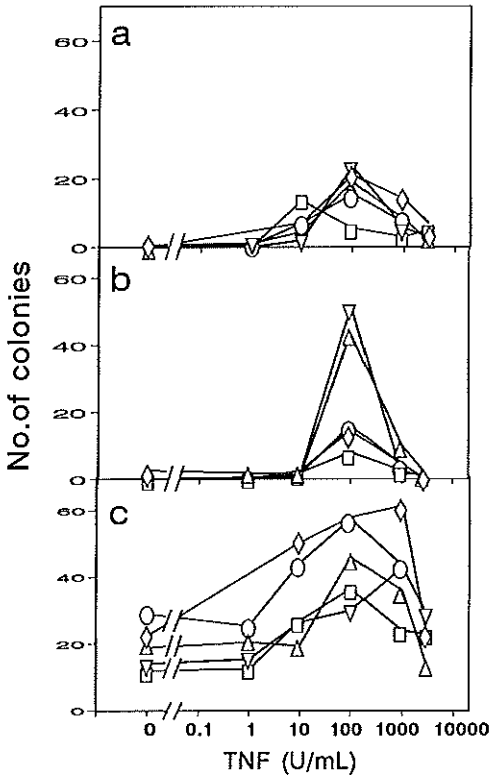

Figure 2 *Positive effect of TNF on GM-CSF induced colonies. Enriched normal bone marrow progenitors (0.5 to 1 × 10^4/mL) were incubated with GM-CSF and increasing concentrations of TNF (0-3000 U/mL) (a) Number of macrophage colonies; (b) Number of granulocytic colonies; (c) Number of eosinophilic colonies. Data from five experiments are shown. Number of PHA-LCM induced CFU-M, CFU-G and CFU-Eo: (□) 19,17 and 52; (▽) 59,40 and 69; (△) 54,33 and 48; (◇) 32,25 and 93; (○) 50,49 and 178.*

TNF inhibits G-CSF or EPO induced colony formation in vitro

Stimulation of enriched bone marrow cells in culture with G-CSF results in the formation of granulocytic colonies. Co-stimulation of G-CSF with TNF inhibits granulocytic colony formation. The negative effect of TNF on G-CSF induced colony growth is dose-dependent and at TNF concentrations of 1000 U/mL granulocytic colony growth is completely suppressed (Figure 3). The addition of TNF also results in a dose-dependent inhibition of erythroid colony formation (BFU-E) induced by EPO alone. However, the

numbers of erythroid bursts formed in response to EPO plus IL-3 increase and only decline at higher TNF concentrations (1000 U/mL) (Figure 4).

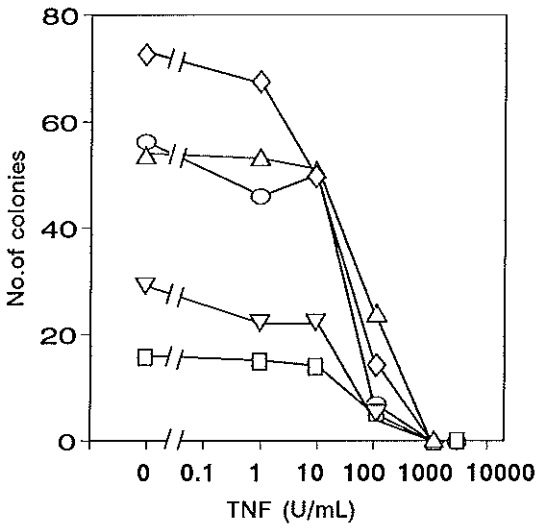

Figure 3 *Negative effect of TNF on G-CSF induced granulocytic colony growth. Enriched normal bone marrow progenitors (0.5 to 1 × 10⁴/mL) were incubated with G-CSF and increasing concentrations of TNF (0-3000 U/mL). Data from five experiments are shown. Number of PHA-LCM induced CFU-G: (□) 17; (▽) 25; (△) 40; (○) 33; (◇) 57.*

Effect of Antibodies against IL-6, GM-CSF, G-CSF, TNFα and TNFß on the TNF response

The results of the above-described experiments indicate that the effects of TNF on the response of enriched bone marrow cells to IL-3 and GM-CSF are synergistic whereas the effects of TNF on the G-CSF and EPO responses are antagonistic. Cultures stimulated with IL-3, GM-CSF, G-CSF, or EPO, plus TNF were performed in the presence of neutralizing antibodies against IL-6, GM-CSF, G-CSF, TNFα, and TNFß. This was carried out to determine whether the effects of TNF are mediated through the induction of secondary IL-6, G-CSF or GM-CSF. None of the modulating TNF effects on colony

growth, i.e. the positive effects of TNF on IL-3 induced colony formation, nor the marked impairment of G-CSF or EPO induced colony growth, were influenced by neutralizing levels of any of the antibodies tested (Table 1). Neither were eosinophilic colony formation or the appearance of CFU-G and CFU-M in GM-CSF plus TNF co-stimulated cultures influenced by the addition of the antibodies (Table 2).

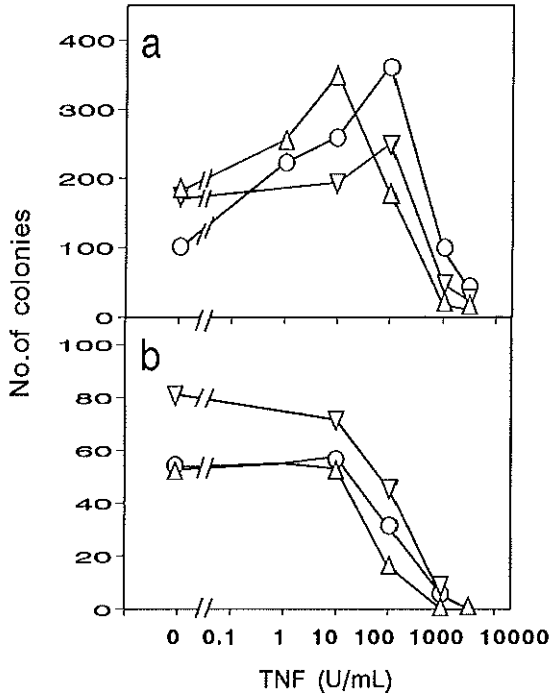

Figure 4 *Effect of TNF on erythroid colony formation. Enriched precursor cells (0,5 to 1 × 10^4/mL) were incubated with (a) EPO plus IL-3 or (b) EPO to which increasing concentrations of TNF were added. Data are results from three experiments. Number of PHA-LCM induced BFU-E: (△) 166; (▽) 200; (○) not carried out.*

Table 1. *Effect of neutralizing antibodies on EPO, IL-3 or G-CSF induced colony formation in the absence or presence of TNF*

Antibody	EPO (BFU-E)		IL-3 (CFU-Eo)		G-CSF (CFU-G)	
	-TNF	+TNF	-TNF	+TNF	-TNF	+TNF
-	30	2	22	60	95	15
αIL-6	27	3	25	53	96	11
αGM-CSF	33	2	29	55	107	12
αG-CSF	34	4	28	60	0	0
αTNF(α+ß)	38	35	21	22	85	94

Purified human hematopoietic progenitors (0.5 to 1x10⁴/ml) were incubated with EPO, IL-3, or G-CSF each with or without TNF (100 U/mL) in the presence of anti-Il-6, anti-G-CSF or anti-GM-CSF. Anti-TNF(α+β) was used as a control. The mean number of colonies per dish of duplicate cultures are shown.

DISCUSSION

The results of these studies demonstrate that TNFα has opposite effects on the *in vitro* proliferative abilities of normal bone marrow progenitors. TNF may suppress the colony-forming capacity of CFU-G and BFU-E in the presence of G-CSF and EPO, respectively. On the other hand TNF may up-regulate the proliferative response of CFU-Eo to IL-3 or GM-CSF. The synergistic and antagonistic effects are apparent at identical TNF concentrations (100-1000 U/mL). These data suggest that TNF, when available to marrow precursors at the same tissue levels, may produce contrasting effects within the progenitor cell compartment and thus modulate hematopoiesis in selected directions.

TNF almost entirely inhibited EPO induced BFU-E from normal human bone marrow progenitors. Inhibition of erythroid burst formation by TNF has been described previously

Table 2. *Effect of neutralizing antibodies on GM-CSF induced colony formation in the absence or presence of TNF*

Antibody	GM-CSF					
	CFU-Eo		CFU-G		CFU-M	
	-TNF	+TNF	-TNF	+TNF	-TNF	+TNF
-	27	60	1	30	0	31
αIL-6	28	78	0	31	0	18
αGM-CSF	0	0	0	0	0	0
αG-CSF	30	73	0	30	0	34
αTNF(α+ß)	24	25	0	0	0	0

Purified human hematopoietic progenitors (0.5 to 1x10^4/mL) were incubated with GM-CSF with or without TNF (100 U/mL) in the presence of anti-IL-6, anti-G-CSF or anti-GM-CSF. Anti-TNF(α+ß) was used as a control. The mean number of colonies per dish of duplicate cultures are shown.

(Broxmeyer et al., 1986, Wisniewski et al., 1987) but a possible effect of accessory cells on those studies has not been excluded. The experiments with TNF and EPO reported here were conducted with highly purified normal marrow progenitors. Remarkably, an increase of erythroid burst formation was seen when 100 U/mL TNF was added to EPO plus IL-3 containing cultures. In comparison, at this same TNF concentration, BFU-E formation generated by EPO alone was inhibited by approximately 50%. Apparently the erythroid progenitors that respond to EPO alone are inhibited by TNF whereas the specific subsets of erythroid progenitors that are inducible by IL-3 plus EPO are stimulated by TNF.

distinction is made between the different types of myeloid colonies. From our results it appears that a positive or negative effect of TNF on colony growth depends on the type of hematopoietic growth factor used, or alternatively, the type of target progenitor stimulated by that factor. IL-3 or GM-CSF induced eosinophilic colony formation is enhanced by TNF whereas TNF inhibits G-CSF induced granulocytic colony formation. Inhibition of human myeloid colony growth by TNF has been described previously in cultures of partially enriched normal bone marrow cells (Broxmeyer et al., 1986, Murase et al., 1987a, Murase et al., 1987b). The fact that we conducted our experiments with highly purified blast cells does not absolutely exclude contaminating cell effects, but makes such effects unlikely.

It might be argued that accessory cell effects would still explain certain of our findings, for instance the observation that in the GM-CSF stimulated cultures higher concentrations of TNF (100 U/mL) resulted in the formation of granulocytes and macrophages. It is known that TNF is a very active inducer of other cytokines and this may have resulted in the production of colony-stimulating factors (Cline and Golde, 1974, Bagby et al., 1983, Koeffler et al., 1987, Oster et al., 1987). However, none of the observed effects could be abolished with antibodies directed against G-CSF, GM-CSF, or IL-6, suggesting that these factors are not involved. The induction of other cytokines by TNF can not be ruled out formally, as other as yet unidentified factors might have been induced by TNF. An antibody against M-CSF was not tested, but in view of the spectrum of activity of M-CSF it is unlikely that the TNF effects could be attributed to M-CSF.

In summary, TNF favours the outgrowth of progenitors that are under control of the more primitive hematopoietic growth factors IL-3 and GM-CSF. At the same time TNF suppresses the proliferative abilities of the progenitors that respond to the late-acting, single-lineage factors G-CSF and EPO. These profound and complex influences on hematopoiesis are probably accomplished through direct cell interactions of TNF with selective progenitor cells.

CHAPTER THREE

BLAST COLONY FORMING CELLS IN MYELODYSPLASTIC SYNDROME: DECREASED POTENTIAL TO GENERATE ERYTHROID PRECURSORS

Bianca Backx, Lianne Broeders, Ivo Touw, Bob Löwenberg

Leukemia 7:75-79, 1993

Chapter 3

SUMMARY

In vitro colony forming abilities of highly purified primitive hematopoietic cells in eight cases of myelodysplastic syndrome (MDS) were studied using the blast cell colony assay. Blast cell colony formation from seven normal bone marrow (NBM) samples was studied in parallel. Blast cell colonies were formed in 7/8 cases of MDS, the numbers not significantly differing from those generated by NBM. In contrast the more mature hematopoietic progenitors (granulocyte-erythroid-macrophage-megakaryocyte colony-forming unit, CFU-GEMM; erythroid burstforming-unit, BFU-E; granulocyte colony-forming unit, CFU-G; eosinophilic colony-forming unit, CFU-Eo) were severely depressed in numbers in MDS marrow. After replating of blast cell colonies in secondary cultures, colonies were obtained in 5/8 MDS cases. A marked difference was evident in the composition of the secondary colonies between MDS and normal marrow. Whereas secondary colonies derived from normal blast cell colonies consisted of about 45% of erythroid cells, MDS blast colonies generated mainly colonies of the granulocytic-monocytic lineage and no erythroid colonies. The normal quantitative level of CFU-blast progenitors in MDS in the context of their impaired ability to generate lineage specific progeny upon secondary plating suggests that the incompetence of maturation of MDS may reside in the CFU-blast progenitor cell being incapable of properly responding to growth factor stimulation.

INTRODUCTION

The myelodysplastic syndromes (MDS) represent a heterogenous group of hematological disorders characterized by abnormal maturation involving one or more of the marrow lineages resulting in anemia, neutropenia and/or thrombocytopenia (Koeffler, 1986, Layton and Mufti, 1986). Several studies concerning the clonality of MDS (Janssen et al., 1989, Tefferi et al., 1990) have provided evidence that MDS are clonal disorders of multilineage hematopoietic precursor cells. In vitro colony formation of MDS bone marrow progenitors is markedly reduced, which is suggestive of a decreased responsiveness of these bone marrow cells to hemopoietins (Mayani et al., 1989, Merchav et al., 1989, Merchav et al., 1991). On the other hand studies on stromal cells derived from MDS have not revealed functional abnormalities of the MDS stroma, suggesting that a

microenvironmental defect is not commonly involved in the pathogenesis of MDS (Coutinho et al., 1990).

The blast cell colony assay (Leary et al., 1987, Leary et al., 1988) identifies immature multilineage progenitor cells in normal bone marrow (NBM) that share with hematopoietic stem cells the self renewal capacity and the ability to develop into multiple hematopoietic lineages (Dexter et al., 1977, Nakahata and Ogawa, 1982b, Nakahata and Ogawa, 1982c, Clark and Kamen, 1987, Rowley et al., 1987). Plating of purified MDS marrow cells revealed essentially normal numbers of blast colonies in most cases. However, upon replating of the blast cell colonies in secondary cultures containing interleukin-3 (IL-3), granulocytic-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and erythropoietin (EPO), it appeared that the blast colony cells derived from MDS, in contrast to those from normal marrow, were unable to give rise to erythroid progeny (BFU-E).

MATERIAL AND METHODS

Patients and preparation of cell suspension

Bone marrow cells were obtained from eight patients with MDS. The patients were classified according to the FAB criteria (Bennett et al., 1982) as refractory anemia (RA, n=1), refractory anemia with excess of blasts (RAEB, n=2), refractory anemia with excess of blasts in transformation (RAEB-t, n=2) and chronic myelomonocytic leukemia (CMML, n=3). Patient characteristics are summarized in Table 1. Normal marrow was obtained from hematologically normal adults following informed consent. The marrow cells were collected in Hanks' balanced salt solution (HBSS) supplemented with heparin. Mononuclear cells were collected after Ficoll-Isopaque density centrifugation as previously described (Bot et al., 1988). Cells were either used fresh or after cryopreservation (Delwel et al., 1987).

Purification of progenitor cells

Marrow cells were enriched by a two-step cell purification procedure as described previously (Bot et al., 1989a). In brief, cells were depleted of accessory cells by complement mediated cytolysis using monoclonal antibodies (MoAbs) against the antigens CD3 [T3, immunoglobulin G2 (IgG2), final dilution 1:10], CD14 (B44.1, IgM, monocytes,

final dilution 1:1000)(Perussia et al., 1982), CD15 (B4.3, IgM, granulocytic cells, final dilution 1:500)(Van der Reijden et al., 1983), CD33 (MY9, IgG2b, final dilution 1:500)(Andrews et al., 1989), and CD_w65 (VIM-2, IgM, final dilution 1:50). The cytolysis procedure resulted in an average recovery of 46±17% and 39±16% of viable MDS and NBM nucleated cells, respectively. For the second purification step, cells were labelled with anti-CD34 MoAb (BI3C5, IgG1, Sera-Lab, Crawley Down, UK)(Katz et al., 1985) and fluorescin-isothiocyanate-conjugated goat-anti-mouse IgG1 (GAM-FITC, Nordic, Tilburg, The Netherlands) and sorted using a fluorescence-activated cell sorter (FACS) as described (Bot et al., 1989a). The recovery of the final fraction of MDS and normal marrow cells was respectively 5±6% and 2±1% of the initial number of mononuclear cells.

Table 1. *Patient characteristics*

Patient	Age(Yr)	Sex	Karyotype	Diagnosis (FAB cytology)
1	73	M	46,xy (63%) 46,xy,18q+(37%)	RA
2	33	F	46,xx,t(3;12)del(7)	RAEB
3	66	M	46,xy	RAEB
4	27	M	46,xy	RAEB-t
5	58	M	46,xy	RAEB-t
6	74	F	46,xx	CMML
7	64	M	46,xy(9%) 46,xy,9q-(91%)	CMML
8	61	F	46,xx	CMML

Table 2. *Colony formation in marrow from MDS patients and normal marrow in the GEMM assay*

Case No.	BFU-E	CFU-GEMM	CFU-G	CFU-M	CFU-GM	CFU-Eo
1	4	0	111	0	0	0
2	0	0	13	8	0	53
3	30	0	15	2	0	9
4	5	0	0	7	284	0
5	91	0	0	8	0	6
6	0	0	10	43	3	0
7	0	0	0	0	0	0
8	14	0	0	0	747	0
NBM (n=6) mean±sd	242±107	10±10	119±63	15±17	3±4	62±51

Results are number of colonies per 10^4 highly enriched cells. NBM, normal bone marrow.

Recombinant human cytokines

Recombinant human IL-3 (Gist Brocades, Delft, The Netherlands) was used at the optimum concentration of 40 ng/mL. Recombinant human interleukin-6 (IL-6) (a gift from Dr L Aarden, Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands) and recombinant human G-CSF (kindly provided by Dr S Gillis, Immunex, Seattle WA, USA) were used at concentrations of 180 ng/mL and 30 ng/mL respectively. Recombinant GM-CSF and recombinant human erythropoietin (EPO) (gifts from Dr S Clark, Genetics Institute, Cambridge MA, USA) were used at optimal concentrations of 90 ng/mL and 1

U/mL, respectively. The optimum stimulatory concentrations had been separately determined for each growth factor batch (data not shown).

Colony assays

CFU-GEMM assay: Purified progenitor cells (0.5-1x10^4 cells/mL) were cultured in duplicate for 15 days in the presence of a combination of growth factors (IL-3, GM-CSF, G-CSF and EPO) in a humidified atmosphere of 5% CO_2 in air at 37°C as described (Bot et al., 1988). Erythroid, eosinophilic, granulocytic, macrophage and mixed colony numbers were scored separately.

CFU-blast cell assay: The method described by Leary and Ogawa (1987) was applied with some modifications. Highly enriched cells (10^4/mL) were first cultured for ten days in a humidified atmosphere of 5% CO_2 in air at 37°C. On day 10 of culture 0.1 mL of IL-3 (final concentration 40 ng/mL) and IL-6 (final concentration 180 ng/mL) was layered over the cultures. Finally, between day 16 and day 24 blast colonies were scored. After counting, colony cells were harvested from the plates for cytological analysis and replating experiments. For the replating experiments individual colonies were suspended in 50 μL of α-medium in a 24-wells plate and cultured in the CFU-GEMM assay. After 14 days of culture, colonies were counted and examined cytologically.

RESULTS

CFU-GM, BFU-E and CFU-blast in MDS and normal marrow

Purified marrow cells from MDS patients and normal donors were plated in the CFU-GEMM assay to assess the various progenitor cells, and the CFU-blast cell assay. The data indicate that growth of various colony types is generally reduced in MDS (Table 2). In 7/8 MDS cases blast cell colonies were formed (Table 3). In the single case (patient #7) without blast colony forming ability, lineage restricted or GEMM colonies were not formed either (Table 2).

The MDS marrow (n=8) contained a mean number of 17 CFU-blast per 10^4 enriched cells. This average frequency was not different from NBM samples (n=7) (Table 3). Also the appearance of blast cell colonies derived from MDS marrow did not differ from those derived from normal marrow (Fig 1).

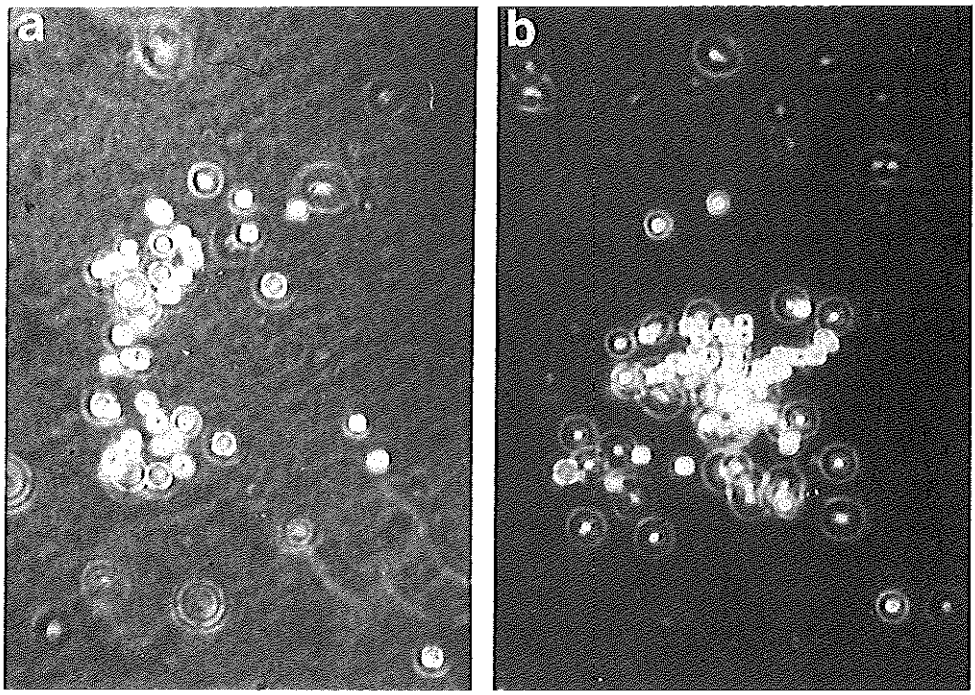

Figure 1 *Blast cell colony derived from normal marrow (a) and from MDS patient #2 (b) (Original magnification ×100).*

Secondary colony formation from blast cell colonies

In 6 cases (except case #3 and the non colony forming case #7) blast colony cells were replated to evaluate the potential of secondary colony formation. In one of these (patient #6) no secondary colonies were formed. In the remaining five cases, the percentage of blast cell colonies giving rise to secondary colonies was comparable to normal marrow results (Table 3). However, a marked difference was apparent as regards the type of secondary colonies generated (Table 4). In none of the five MDS cases BFU-E or CFU-GEMM were formed. In comparison, in normal marrow blast cell colonies produced 45% BFU-E and 3% CFU-GEMM in secondary cultures. The collected data from 645 replated MDS blast colonies revealed that of all 819 secondary colony forming cells generated more than 90% were CFU-GM, and small percentages were CFU-Eo.

No BFU-E appeared in secondary cultures. In contrast, of 301 second generation colonyforming cells (from 196 replates) from normal marrow, 45% were BFU-E, 28%

were CFU-GM, 20% were CFU-Eo, and 3% were CFU-GEMM (Fig 2). Thus MDS blast colony cells did not generate erythroid colony-forming cells under conditions in which normal marrow derived blast cell colonies would give rise to significant proportions of erythroid precursors.

Table 3. *Blast cell colony formation of MDS marrow and secondary plating efficiency of CFU-blasts*

Case No.	mean number CFU-blast/ 10^4 cells	% blast colonies giving rise to secondary colonies *
1	17	17
2	35	45
3	2	-
4	17	46
5	25	29
6	18	0
7	0	-
8	23	44
mean±sd	17±11	30±19
NBM (n=7) mean±sd	18±9	44±22

* *% indicate secondary plating efficiency (data specified in Table 4). NBM, normal bone marrow.*

Table 4. *Secondary colony formation in MDS and NBM*

Case No.	No. primary CFU Blasts	No. sec. colonies	Distribution of secondary colony types (%)*				
			BFU-E	CFU GEMM	CFU GM	CFU Eo	CFU uncl
1	102	41	0	0	90	7	3
2	80	98	0	0	100	0	0
4	98	242	0	0	93	0	7
5	104	66	0	0	100	0	0
6	130	0	0	0	0	0	0
8	127	372	0	0	100	0	0
NBM	196	301	45	3	28	20	4

* *Number of colonies expressed as percentage of total secondary colony formation (sec.CFU). CFU-GM: CFU-G, CFU-M and CFU-GM. CFU-uncl., colonies with unclassified cytology. NBM, normal bone marrow (n=7).*

DISCUSSION

It has previously been shown (Mayani et al., 1989, Merchav et al., 1989, Merchav et al., 1991) and has been confirmed here that the marrow of patients with MDS is frequently defective in generating erythroid and myeloid colonies *in vitro*. This suggests that the committed progenitor cells of these differentiation lineages are reduced in numbers or functionally abnormal. The blast cell colony assay is an *in vitro* assay for more primitive

hematopoietic progenitor cells. While mixed, erythroid, and myeloid colony forming cell numbers in MDS marrow are decreased, the number of CFU-blast per 10^4 progenitor cells in MDS marrow were often found not to be suppressed. In six of eight cases CFU-blast values were normal. However, when CFU-blasts were examined for their ability to propagate precursors along the distinct maturation lines, significant deficiencies became evident.

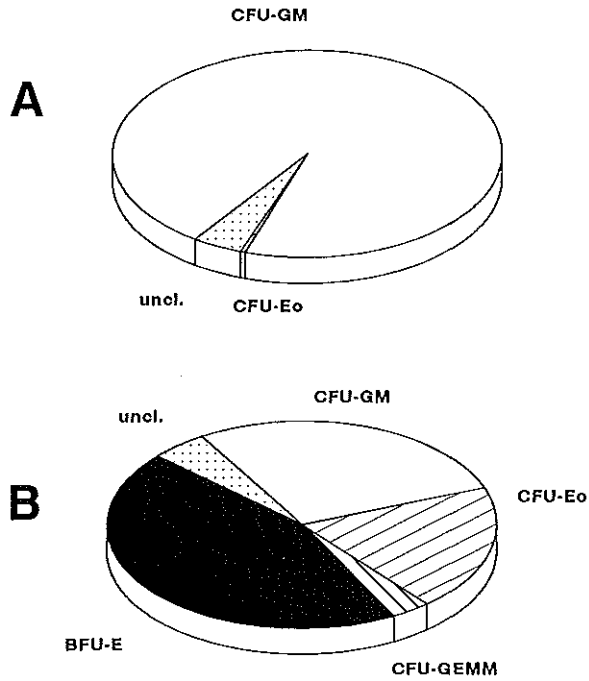

Figure 2 *Composition of blast cell colony derived secondary CFU: individual blast cell colonies were replated, then scored and classified cytologically 14 days after replating. (a) Cytological types of the collected MDS-blast cell colony derived secondary CFU of eight patients are represented (100% = 819 secondary CFU). (b) Cytological score of all secondary colonies derived from NBM-blast cell colonies of seven patients are given (100% = 301 second generation colonies). Uncl: colonies with unclassified cytology; CFU-GM: CFU-M, CFU-G and CFU-GM.*

The inability of CFU-blast to give rise to secondary colony formation in one MDS case (patient #6) is unlikely to be due to the presence of inhibitors, since accessory cells were removed, but most likely the result of an intrinsic abnormality. The frequent normal primary CFU-blast colony formation and the reduced secondary colony growth was apparent in representative MDS cases of different FAB types. Most striking was the absence of secondary BFU-E in cultures derived from MDS blast colonies. In contrast to the significant proportion of BFU-E deriving from normal marrow CFU-blast, the blast colonies of MDS marrow did not generate BFU-E upon replating. Secondary colonies derived from MDS blast colonies were mainly of the CFU-GM type. Although the differential count of marrow smears of the eight MDS patients revealed active erythropoiesis in bone marrow (data not shown) this probably reflects the presence of some additional factors in the marrow (and not present in the *in vitro* system) which can induce characteristically ineffective erythropoiesis.

These results indicate that in MDS the hematopoietic progenitor at the level of the CFU-blast is most prominently defective as regards the ability to give rise to erythroid progeny. Probably the CFU-blasts give rise to daughter cells that are abnormal in their response to EPO and hence do not proliferate and mature normally. These results are in agreement with *in vivo* findings indicating that disturbed erythropoiesis usually can not be explained by an EPO deficiency. EPO levels have generally been demonstrated to be normal or increased (Schouten et al., 1991). Furthermore the response of MDS patients to treatment with EPO, to overcome the anemia, is poor (Merchav et al., 1990, Bowen et al., 1991). However, in infrequent cases anemia in patients with MDS can be improved by high doses of EPO (Stein et al., 1991, Steblet et al., 1990).

The recently cloned c-kit ligand (or stem cell factor or mast cell growth factor), has been demonstrated to enhance the *in vitro* colony formation induced by EPO, G-CSF, GM-CSF or IL-3 (Anderson et al., 1990, Martin et al., 1990, Zsebo et al., 1990, Broxmeyer et al., 1991, McNiece et al., 1991). This factor may be important in improving hematopoiesis in MDS and studies are being performed to test this hypothesis. The observation that reduced mixed, eosinophilic and erythroid colonies were formed from MDS blast cell colonies is probably indicative of an MDS blast cell abnormality that involves more than one maturation lineage. A detailed characterization of disturbed hematopoiesis in MDS may be helpful for defining the cellular abnormalities and designing treatment approaches in individual patients with MDS, e.g. with regard to the ability to restore hematopoietic insufficiencies with hematopoietic growth factors.

CHAPTER FOUR

KIT LIGAND IMPROVES IN VITRO ERYTHROPOIESIS IN MYELODYSPLASTIC SYNDROME

Bianca Backx, Lianne Broeders, Bob Löwenberg

Blood 80:1213-1217, 1992

Chapter 4

SUMMARY

Erythropoiesis in response to erythropoietin (Epo) in myelodysplastic syndrome (MDS) *in vitro* and *in vivo* is severely impaired. We investigated the stimulative effect of c-kit ligand (KL) on the erythroid colony-forming abilities of bone marrow cells from 17 patients with MDS. The effects of normal donor-derived marrow were examined in comparison. Suppression of erythroid colony formation in MDS in response to Epo could not be restored by the addition of interleukin-3 (IL-3) to culture. In culture dishes supplemented with KL, erythroid colony formation was dramatically enhanced, regarding both colony number and size. Colony-forming abilities by MDS progenitors were improved following costimulation with KL, particularly in refractory anemia (RA) and refractory anemia with ring sideroblasts (RARS); however, little enhancement was apparent following KL stimulation of marrow from patients with refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t) and chronic myelomonocytic leukemia (CMML). These results suggest that KL responsiveness of patients with low-risk MDS may still be intact, and that with progression to high-risk MDS, erythroid progenitors lose proliferative reactivity to both KL and Epo stimulation. KL may have a therapeutic role in restoring erythropoiesis in a subset of patients with MDS.

INTRODUCTION

The myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal disorders of hematopoietic pluripotent stem cells characterized by ineffective hematopoiesis resulting in anemia, neutropenia and /or thrombocytopenia (Koeffler, 1986, Layton and Mufti, 1986, Jansen et al., 1989, Tefferi et al., 1990). The *in vitro* erythroid and myeloid colony formation is reduced and the addition of high doses of growth factors can augment colony formation only in infrequent cases of MDS (Mayani et al., 1989, Merchav et al., 1989, Merchav et al., 1991). Clinical trials of MDS patients have shown that the response to treatment with recombinant erythropoietin (Epo) is generally poor (Merchav et al., 1990, Stebler et al., 1990, Bowen et al., 1991, Stein et al., 1991, Van Kamp et al., 1991). Furthermore, Epo levels in the serum of MDS patients are normal or increased (Schouten et al., 1990), indicating that ineffective erythropoiesis is not the result

of a lack of Epo production.

Recently a new growth factor, designated c-kit ligand (KL), mast cell growth factor (MGF), or stem cell factor (SCF) has been isolated and cloned (Anderson et al., 1990, Martin et al., 1990, Zsebo et al., 1990). KL alone has little colony-stimulating activity, but it considerably enhances the *in vitro* colony formation induced by Epo, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), or interleukin-3 (IL-3) (Broxmeyer et al., 1991, McNiece et al., 1991). We report our evaluationevaluated the effects of KL on the *in vitro* colony forming ability of marrow cells from 17 patients with different cytology types of MDS.

MATERIAL AND METHODS

Patients and preparation of cell suspension

Bone marrow cells were obtained from 17 patients with MDS. The patients were classified according to the French-American-British (FAB) criteria (Bennett et al., 1982) as refractory anemia (RA; n=5), refractory anemia with ring sideroblasts (RARS; n=2), refractory anemia with excess of blasts (RAEB; n=4), refractory anemia with excess of blasts in transformation (RAEB-t; n=3) and chronic myelomonocytic leukemia (CMML; n=3). Clinical characteristics are summarized in Table 1. For comparison, marrow was obtained from haematologically normal adults following informed consent. The marrow cells were collected in Hanks' balanced salt solution (HBSS) supplemented with heparin.

Purification of progenitor cells

Mononuclear cells were collected after Ficoll Isopaque density centrifugation as previously described (Bot et al., 1988). Cells were either used fresh or after cryopreservation (Delwel et al., 1987) and then enriched by a two-step cell purification procedure as described previously (Bot et al., 1989).

In brief, cells were depleted of accessory cells by complement mediated cytolysis using monoclonal antibodies (MoAbs) against the antigens CD3 (T3, IgG2, final dilution 1:10), CD14 (B44.1, IgM, monocytes, final dilution 1:1000)(Perussia et al., 1982), and CD15 (B4.3, IgM, granulocytic cells, final dilution 1:500)(Van der Reijden et al., 1983). For the second purification step, cells were labeled with anti-CD34 MoAb (BI3C5, IgG1, Sera-Lab, Crawley Down, UK)(Katz et al., 1985) and fluorescein isothiocyanate (FITC)

Table 1. *Characteristics of patients with MDS*

Patient	Age(Yr)	Sex	Karyotype	FAB subtype
1	75	F	46,XX	RA
2	72	F	46,XX	RA
3	37	M	46,XY	RA
4	18	F	46,XX	RA
5	73	M	46,XY(63%);46,XY,18q+(37%)	RA
6	36	M	46,XY(29%);43,XY,mar5q-,-7,17p+,-18,-21(71%)	RARS
7	38	M	46,XY	RARS
8	33	F	46,XX,t(3,12),del(7)(100%)	RAEB
9	63	M	46,XY	RAEB
10	67	F	46,XX(6%);47,XX, +8(91%);48,XX, +8,-+21(3%)	RAEB
11	52	F	46,XX	RAEB
12	68	M	46,XY(3%);44-48,XY,-4,-16,-18(50%);45-49,XY,-4,-16,-18,5q-,7q-,13q-(47%)	RAEB-t
13	68	M	46,XY(90%),47-48,XY, +8(10%)	RAEB-t
14	27	M	46,XY	RAEB-t
15	64	M	46,XY,(9%),46,XY,9q-(91%)	CMML
16	62	M	46,XY	CMML
17	74	F	46,XX	CMML

-conjugated goat-anti-mouse IgG1 (GAM-FITC, Nordic, Tilburg, The Netherlands) and sorted using a fluorescence-activated cell sorter (FACS) as described (Bot et al., 1989).

Recombinant human cytokines

Recombinant human interleukin-3 (IL-3; Gist Brocades, Delft, The Netherlands) was used at the optimum concentration of 40 ng/mL. Recombinant human KL (MGF, Immunex, Seattle WA, USA) was used at concentrations of 10 to 100 ng/mL. Recombinant human erythropoietin (Epo) (Dr S Clark, Genetics Institute, Cambridge MA, USA) was used at optimal concentration of 1 U/mL. The optimum stimulatory concentrations were determined separately for each growth factor batch (data not shown).

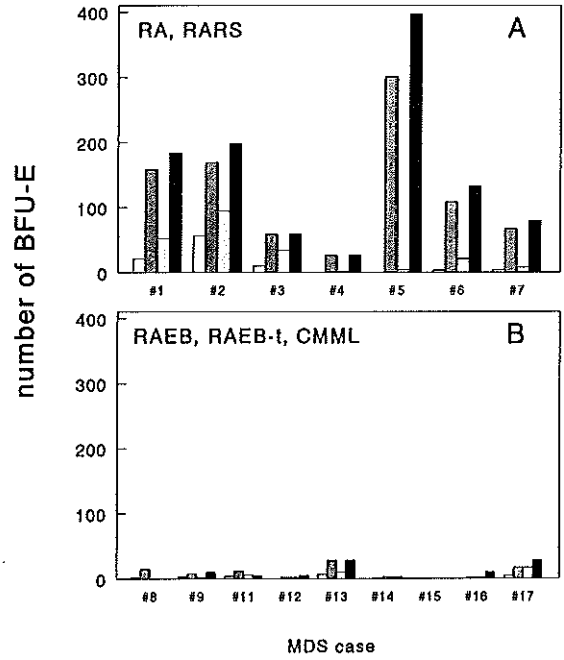

Figure 1 *Effect of KL on erythroid colony formation in MDS: (A) KL (10 ng/mL) enhances the erythroid colony formation in Epo-(1 U/mL) or Epo plus IL-3-(40 ng/mL) induced cultures from RA and RARS marrow. (B) Minimal or no enhancement of erythroid colony growth by KL is observed in marrow from patients with RAEB, RAEB-t, and CMML. Data are expressed as number of erythroid colonies per 10^4 enriched marrow cells. Open bars, Epo; hatched bars, Epo plus KL; dotted bars, Epo plus IL-3; black bars, Epo plus IL-3 plus KL.*

Colony assay

CFU-GEMM assay: Cells after cytolysis with CD3, CD14, and CD15 ($1x10^4$cells/-mL; cases 1-4, 7) or CD34-enriched cells (0.5 to $1x10^4$ cells/mL; cases 5, 6, 8-17), were cultured in duplicate for 15 days in the presence of growth factors (as indicated) in a humidified atmosphere of 5% CO_2 in air at 37°C (20) and erythroid colony numbers were scored as described (Bot et al., 1988).

Determination of colony size

To determine to mean number of erythroid cells per burst, bursts were individually removed by Pasteur pipette from culture dishes. Colonies were collected in phosphate-buffered saline. For each culture condition, three pools of five randomly picked bursts were counted. Results are expressed as the mean of the total number of pooled colony cells per experiment divided by the number of bursts.

Cytogenetic analysis

Marrow cells were cultured in the presence of Epo plus IL-3 or Epo plus IL-3 and KL. After 7 days in culture, the metaphases were harvested according to standard techniques on glass slides (Hagemeyer et al., 1979). The spread metaphases were stained with 10 mg Atebrin (G.T. Gurr) in 100 mL ethanol.

RESULTS

Effect of KL on erythroid colony formation

KL (10 ng/mL) was rarely capable of inducing BFU-E formation in MDS. Following stimulation with KL (with no Epo), scarce erythroid colonies (< 3 burst-forming units-erythroid [BFU-E]/10^4 cells) were formed in only three of 17 cases. KL alone induced BFU-E (31 ± 16 BFU-E/10^4 cells; n=3) from normal marrow. Stimulation with Epo alone gave rise to small numbers of erythroid colonies in MDS (Fig 1). Addition of KL to Epo cultures often considerably enhanced the appearance of erythroid colony numbers in MDS (Fig 1). In normal marrow, KL similarly enhanced Epo-induced erythroid colony formation (Fig 2).

Besides the effect on number of erythroid colonies, KL also favourably influenced the size of erythroid colonies both in MDS and normal marrow (Figs 3 through 5). In the

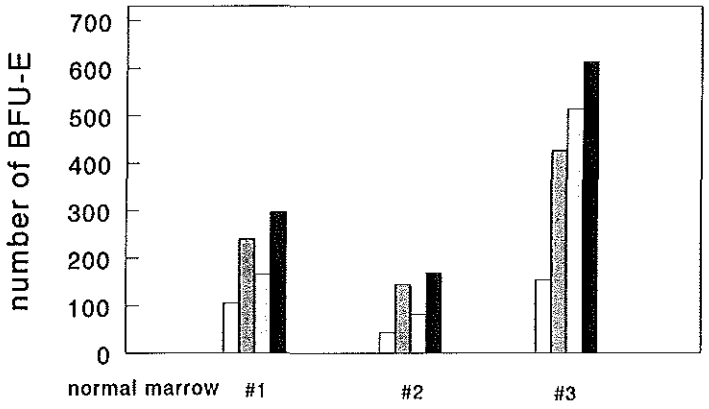

Figure 2 *Effect of KL on erythroid colony formation in normal marrow: Results of three normal marrows are shown. For explanation, see Figure 1. Open bars, Epo; hatched bars, Epo plus KL; dotted bars, Epo plus IL-3; black bars, Epo plus IL-3 plus KL.*

presence of KL and Epo, the mean erythroid colony size increased more than 10-fold as compared with erythroid colony size induced by Epo alone, and more than threefold as compared with size of erythroid colonies induced by Epo plus IL-3. In certain cases of MDS, numbers and size of erythroid colonies following stimulation with the combination of KL and Epo were elevated towards normal.

The positive effect of KL was particularly apparent in patients with RA and RARS (low-risk MDS). The addition of IL-3 to cultures supplemented with KL plus Epo did not further augment the efficiency of erythroid colony growth (number or size). In contrast, in RAEB, RAEB-t, and CMML forms of MDS the addition of KL to Epo-induced or Epo plus IL-3-induced cultures promoted erythroid colony formation minimally. When 10-fold greater concentration of KL (100 ng/mL) were added, identical results were obtained (data not shown).

In patient 5, showing a 18q+ abnormality in 37% of metaphases (Table 1), the majority of erythroid colony formations with and without IL-3 were absolutely KL-dependent. Cells were incubated with KL plus Epo and IL-3 and after 7 days of culture

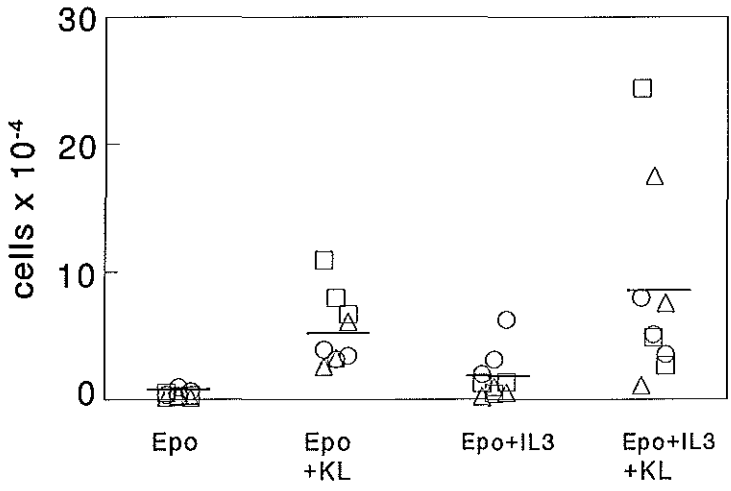

Figure 3 *Effect of KL on size of erythroid colonies in MDS: Erythroid colony size was determined in cultures with Epo (1 U/mL), Epo plus KL (10 ng/mL), Epo plus IL-3 (40 ng/mL), or Epo plus IL-3 plus KL from three cases of MDS (see Material and Methods). On the vertical axes the average number of a pool of five collected colonies is plotted. For each of three cases of MDS, three such estimates were obtained. Horizontal line represents the mean of three estimates of three patients.* △, *Case 1;* ○, *Case 2;* □, *Case 3.*

cytogenetic analysis was performed. Of nine cells examined, five showed an 18 q+ abnormality and four a normal karyotype. Among 11 cells in Epo plus IL-3-induced cultures seven 18q+ cells and four normal cells were identified. These results are consistent with the stimulation of erythroid colonies from the MDS clone.

DISCUSSION

The abilities of *in vitro* erythroid colony formation by marrow of patients with MDS are severely suppressed (Mayani et al., 1989, Merchav et al., 1989, Merchav et al., 1991). The *in vivo* findings would suggest that inappropriate erythropoiesis in these individuals can not be explained by an Epo deficiency, since Epo levels are usually normal or even elevated (Schouten et al., 1991, Jacobs et al., 1989). Furthermore, anemia of the

majority of patients with MDS fails to improve following treatment with Epo (Merchav et al., 1990, Bowen et al., 1991). Our results demonstrate that KL, in combination with Epo, significantly promotes the *in vitro* erythroid colony formation. This effect, an increase in both number and size of colonies, is most likely the consequence of direct stimulation of marrow progenitor cells by KL since the marrow cells were depleted of accessory cells (Bot et al., 1988).

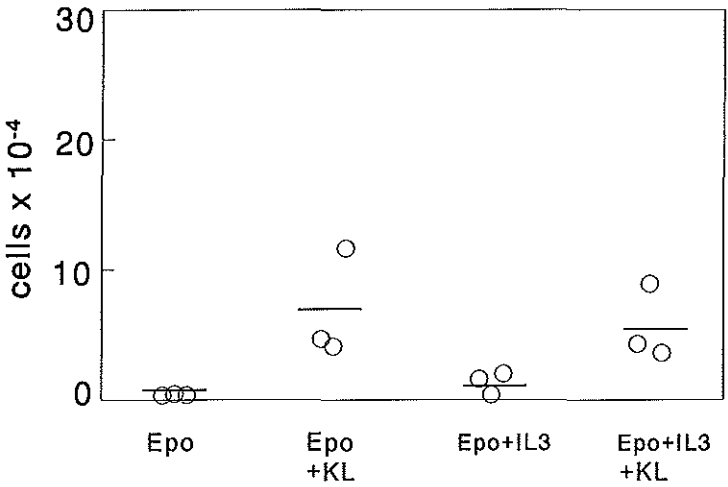

Figure 4 *Effect of KL on size of erythroid colonies in normal marrow: Marrow cultures from one normal donor. For explanation, see Figure 3.*

The effect of KL depends on the cytological type of MDS. Improvement of erythroid growth *in vitro* by KL is especially seen in patients with RA and RARS, and minimal or no responses of erythroid growth in culture to KL are apparent in RAEB, RAEB-t, and CMML cases. The notable increase of BFU-E numbers in the marrow of RA patients, stimulated with KL and Epo, may result in BFU-E numbers comparable to normal marrow values (cases 1 and 2). The low number of BFU-E in RA and RARS is probably not due to a diminished population of erythroid progenitor cells, but to a qualitative inability of these cells to properly respond to stimulation by Epo (Backx et al., 1993).
Thus, in RA and RARS, impaired *in vitro* erythroid colony formation can to a large

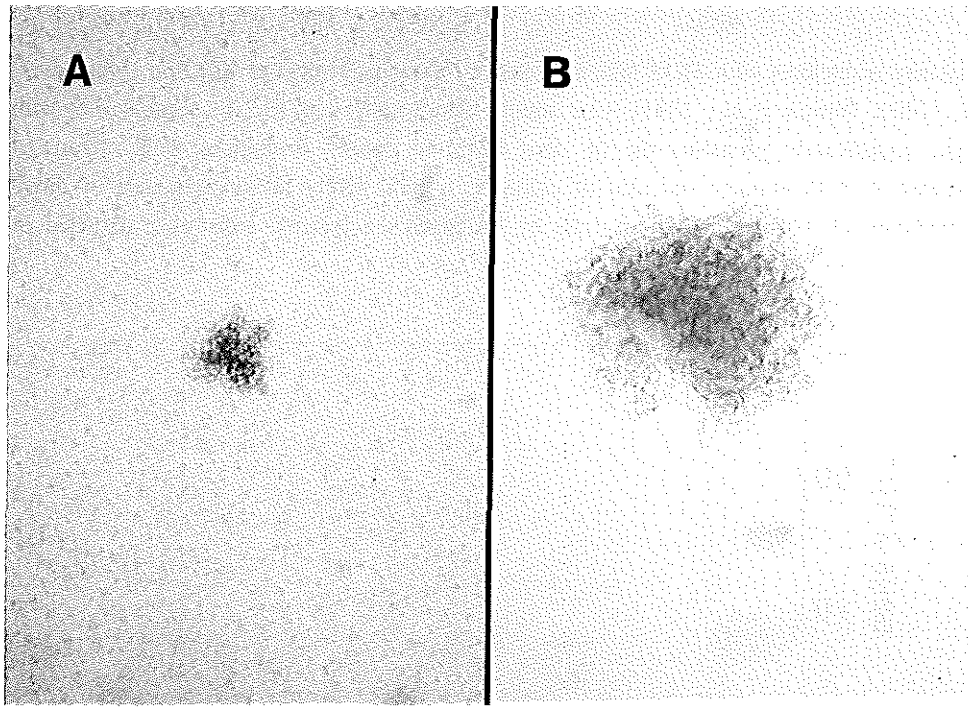

Figure 5 *Erythroid colonies derived from a patient with MDS: Marrow cells (case 2) were stimulated with (A) Epo or (B) Epo plus KL. (Original magnification × 100).*

extent be overcome by costimulation with KL (in combination with Epo). Since the doses of KL used were comparable to concentrations resulting in optimal stimulation of normal marrow, we assume that KL-receptor function in these MDS cases is intact. These *in vitro* data suggest that KL would be a good candidate for use in clinical trials to restore defective erythropoiesis in MDS *in vivo*. The variability of the stimulatory effect of KL among MDS cases potentially provides a means for identifying individuals who would most likely respond to treatment with KL.

In high-risk MDS (e.g., RAEB, RAEB-t, CMML), erythroid progenitor cells that would normally respond to stimulation by KL (in combination with Epo) can no longer be demonstrated. Impaired *in vitro* erythropoiesis in these cases of MDS may thus be caused by progressive loss of responsiveness to KL. As a result, MDS progenitor populations that would respond to Epo, as well as those being inducible by KL, are quantitatively

depressed in high-risk MDS. Whether these progenitors are unable to react properly to KL due to an abnormal functioning KL-receptor or an intrinsic cell defect more down stream to the receptor is presently unclear. Of note is the striking similarity in the response of MDS patients and patients with Diamond-Blackfan anemia (DBA) to KL. In DBA, a selective deficiency of red blood cell precursors exists, resulting in defective erythropoiesis. Analogous to MDS, erythroid progenitors in DBA fail to respond to growth factors like Epo and IL-3 but the addition of KL permits a dramatic *in vitro* increase in both size and number of erythroid colonies (Abkowitz et al., 1991, Bagnara et al., 1991, Olivieri et al., 1991). These results stress the central role of KL in erythropoiesis.

We conclude that in RA and RARS, the population of Epo-responsive target cells in the marrow is severely reduced, but KL-responsive precursors are maintained at normal or near-normal levels. On the other hand in high-risk MDS types (RAEB, RAEB-t, CMML), both Epo-responsive and KL-responsive subsets among the erythroid progenitor cell compartment are greatly reduced. These findings suggest that evolution of MDS and impaired hematopoiesis are associated with progressive loss of Epo and KL responsiveness, probably due to intrinsic inabilities of the cells to respond.

ACKNOWLEDGEMENT

The authors wish to thank Mrs EME Smit (Dept of Cytogenetics, Erasmus University, Rotterdam) for performing cytogenetic analysis.

CHAPTER FIVE

ERYTHROPOIESIS IN MYELODYSPLASTIC SYNDROME: EXPRESSION OF RECEPTORS FOR ERYTHROPOIETIN AND KIT LIGAND

Bianca Backx, Lianne Broeders, Lies H. Hoefsloot, Bert Wognum, Bob Löwenberg

submitted

Chapter 5

SUMMARY

Ineffective erythropoiesis due to an impaired response to erythropoietin (EPO) is a prominent abnormality in myelodysplastic syndromes (MDS). The growth factor kit ligand (KL) may restore the *in vitro* erythroid colony forming response to EPO in a subset of patients. The inability of MDS erythroid progenitors to react properly to EPO and/or KL has not been resolved. We have investigated erythropoietin receptor (EPO-R) and KL receptor (c-kit) expression in 15 cases of MDS by FACS analysis. Percentage of bone marrow cells expressing the EPO-R from patients with MDS were comparable to normal marrow. No apparent correlation was found between number of MDS cells coexpressing the EPO-R and CD34 and impaired erythroid response. C-kit was expressed in most MDS patients, including those not responding to KL in EPO induced cultures. In 9 MDS cases the different splice variants of the EPO-R were analyzed. MDS cells, like normal marrow, expressed the full length EPO-R. These results show that impaired erythroid response in MDS cannot be explained by a quantitative lack of receptors for EPO or KL and that most likely suppression of erythroid response is caused by defective receptor signalling following ligand binding, representing a functional defect within the receptor itself or at a level downstream of the receptor.

INTRODUCTION

The myelodysplastic syndromes (MDS) comprise a group of heterogeneous hematological disorders characterized by ineffective hematopoiesis as one of the characteristic features (Koeffler., 1986, Layton and Mufti., 1986)). Laboratory studies at the time of diagnosis reveal anemia in 90% of these patients. In 1982 the French-American-British (FAB) group classified MDS in five subgroups: refractory anemia (RA), refractory anemia with ring sideroblasts (RARS), refractory anemia with excess of blasts (RAEB), refractory anemia with excess of blasts in transformation (RAEB-t) and chronic myelomonocytic leukemia (CMML) (Bennett et al., 1982). Studies on X-linked restriction fragment length polymorphism, provided evidence to show that MDS is a clonal disorder of the pluripotent hematopoietic stem cell (Janssen et al., 1989, Tefferi et al., 1990). Nonrandom type cytogenetic abnormalities are common (Yunis et al., 1986, Yunis et al., 1988). About 30% of MDS patients show progression to acute nonlymphocytic leukemia.

The *in vitro* colony forming capacity of MDS marrow cells is usually decreased and supra-optimal concentrations of recombinant human growth factors (rhuGF) cannot overcome this deficiency (Mayani et al., 1989, Merchav et al., 1989, Merchav et al., 1991). Various observations make it unlikely that ineffective erythropoiesis is due to an EPO deficiency. First, rhuEPO cannot induce normal erythroid colony formation *in vitro* in MDS (Asano et al., 1994, Merchav et al., 1990, Juvonen et al., 1990). Second, EPO serum levels are generally normal or increased in MDS patients (Schouten et al., 1991, Jacobs et al., 1989). In addition, the anemia in patients with MDS improves following *in vivo* treatment with rhEPO only infrequently (Schouten et al., 1991, Bowen et al., 1991, Stein et al., 1991).

Recently three forms of the EPO receptor (EPO-R) were demonstrated following screening of a cDNA library from an EPO dependent megakaryoblastic celline UT7: the full-length EPO-R (EPO-R-F), the truncated form (EPO-R-T) and a soluble form (EPO-R-S), generated by alternative splicing (Nakamura et al., 1992, Nakamura et al., 1994). Cellular expression correlates with the maturation stage of the cells. Notably, the truncated form of the EPO-R prevails in early-stage progenitor cells. On the other hand, full length EPO-R is predominantly expressed in late stage progenitors. The levels of expression of EPO-R-S in cells of different stages of maturation are not different. Whereas the full length EPO-R is believed to transduce mitogenic signals, the EPO-R-T probably functions as a dominant negative regulator of cell growth (Nakamura et al., 1994).

The regulatory molecule kit ligand (KL), has a key role in erythropoiesis. Genetic defects involving KL itself or its receptor in mice result in severe anemia (Witte et al., 1990, Huang et al., 1990). KL, when incubated with normal human marrow, *in vitro* synergizes with EPO in inducing colony formation (McNiece et al., 1991, Broxmeyer et al., 1991). In Diamond Blackfan anemia (Abkowitz et al., 1991, Bagnara et al., 1991, Olivieri et al., 1991) and aplastic anemia (Wodnar-Filipowicz et al., 1992) KL, in combination with EPO, may also improve erythropoiesis in culture significantly. In a subset of patients with MDS *in vitro* erythropoiesis can be improved by KL and EPO (Backx et al., 1992, Piacibello et al., 1994, Glinsmann-Gibson et al., 1994, Soligo et al., 1994). The mechanism of impaired erythropoiesis has not been clarified and could involve the quantitative lack of EPO or KL receptors on marrow cells, their dysfunction or an abnormality more downstream along the signalling pathway. Here we report data of experiments that were concerned with an analysis on EPO-R- and c-kit-expression in

patients with different subtypes of MDS.

MATERIAL AND METHODS

Patients and preparation of cell suspensions

Bone marrow cells were obtained from 22 patients with MDS. The patients were classified according to the French-American-British (FAB) criteria (Bennett et al., 1982) as refractory anemia (RA;n=9), refractory anemia with ring sideroblasts (RARS;n=2), refractory anemia with excess of blasts (RAEB;n=4), refractory anemia with excess of blasts in transformation (RAEB-t;n=3) and chronic myelomonocytic leukemia (CMML;n=4). Clinical characteristics are summarized in Table 1. For comparison, marrow was obtained from hematologically normal adults following informed consent. The marrow cells were collected in Hanks' balanced salt solution (HBSS) supplemented with heparin. Mononuclear cells were collected after Ficoll Isopaque density centrifugation as previously described (Backx et al., 1993). Cells were either used fresh or after cryopreservation.

Purification of progenitor cells

Marrow cells were enriched by a two-step cell purification procedure as described previously (Bot et al., 1989). In brief, cells were depleted of accessory cells by complement mediated cytolysis using monoclonal antibodies (MoAbs) against the antigens CD3 (T3, IgG2, final dilution 1:10), CD14 (B44.1, IgM, monocytes, final dilution 1:1000) (Perussia et al., 1982), and CD15 (B4.3, IgM, granulocytic cells, final dilution 1:500) (Van der Reijden et al., 1983). For the second purification step, cells were labelled with anti-CD34 MoAb (HPCA-1, IgG1, Becton Dickinson) and FITC-conjugated goat-anti-mouse IgG (GAM-FITC,
Nordic, Tilburg, The Netherlands) and sorted using a fluorescence-activated cell sorter (FACS 440, Becton Dickinson, Mountain View, CA).

Recombinant human cytokines

Recombinant human IL-3 (Gist Brocades, Delft, The Netherlands) was used at the optimum concentration of 40 ng/mL. Recombinant human G-CSF (kindly provided by Dr S Gillis, Immunex, Seattle WA, USA) was used at a concentration of 30 ng/mL.

Table 1. *Characteristics of patients with MDS*

Patient	Age(yr)/Sex	Karyotype	FAB subtype
1	75/F	46,XX	RA
2	72/F	46,XX	RA
3	37/M	46,XY	RA
4	18/F	46,XX	RA
5	80/F	46,XX	RA
6	80/F	46,XX	RA
7	84/F	46,XX	RA
8	73/M	46,XY(63%)/46,XY,18q+ der(18)t(1;18)(q31;q22)(37%)	RA
9	84/M	46,XY	RA
10	36/M	46,XY(12%)/43,XY,der(5)(5pter→ q13::q31→32::?21q11→ter),-7 der(17)t(17;18)(p11;q21),-18, -21(88%)	RARS
11	38/M	46,XY	RARS
12	69/M	46,XY	RAEB
13	33/F	46,XX,t(3;12),(q26;p13), del(7)(q22)	RAEB
14	63/M	46,XY	RAEB
15	52/F	46,XX	RAEB
16	68/M	46,XY(3%)/44-49,X,-Y(47%), -4,5q-,7q-,13q-,+13p+or+i(13q) -16,-18,-20(25%),+1-4mar, +ring(35%)	RAEB-t
17	68/M	46,XY(90%),47-48,XY,+8(10%)	RAEB-t
18	27/M	46,XY	RAEB-t
19	64/M	46,XY,(9%)/46,XY,del(9)(q22q33)	CMML
20	62/M	46,XY	CMML
21	74/F	46,XX	CMML
22	46/M	46,XY	CMML

Recombinant human erythropoietin (EPO) (Dr S Clark, Genetics Institute, Cambridge MA, USA) was used at optimal concentration of 1 U/mL and recombinant human KL (MGF, Immunex, Seattle, WA) was used at a concentration of 10 ng/mL. The optimum stimulatory concentrations were determined for each growth factor batch, using normal marrow specimens.

Colony assay

Cells after cytolysis with CD3, CD14, and CD15 ($1x10^4$ cells/mL; cases #1-#7, #9, #11-#13), or CD34-enriched cells ($0.5\text{-}1x10^4$ cells/ml; cases #8, #10, #14-#22) were cultured in duplicate for 15 days in the presence of growth factors (as indicated) in a humidified atmosphere of 5% CO_2 in air at 37°C and erythroid and granulocytic colony numbers were scored as described (Bot et al., 1988).

Biotin labelling of EPO

Biotin labelling of human recombinant EPO was performed as described previously (Wognum et al., 1990).

Analysis of EPO-R, c-kit and CD34

To specifically detect EPO-R bearing cells, cryopreserved cells were recovered in medium, and $1x10^6$ cells (after cytolysis with CD3, CD14 and CD15) were incubated overnight at 4°C in HBSS containing 2% (vol/vol) fetal calf serum (FCS) and 0.05% (wt/vol) sodium azide (HFN) with biotinylated EPO (bEPO) (1 nmol/L) or, as controls, with unlabelled EPO (1 nmol/L), or with bEPO (1 nmol/L) plus 200 nmol/L unlabelled EPO (to compete for specific binding). After incubation, cells were washed with HBSS containing 0.1% (wt/vol) bovine serum albumin (BSA) and incubated for 50 minutes in HBSS containing 0.1% (wt/vol) BSA and 0.2% (vol/vol) formaldehyde. After fixation, cells were washed and incubated with 1μg/mL of streptavidin-phycoerythrin (SA-PE)(Becton Dickinson) for 30' at 4°C. The fluorescence intensity was amplified by incubating cells with biotinylated anti-streptavidin (b-anti-SA)(Vector Laboratories, Burlingame, CA) for 30' at 4°C, followed by a second incubation with SA-PE (30' at 4°C). Aliquots of bEPO labelled cells were counterstained with either a MoAb against CD34 (HPCA-1, Becton Dickinson) or a MoAB against c-kit (17F11, Immunotech SA, Marseille, France) for 30' at 4°C followed by incubation with GAM-FITC (cells counterstained with GAM-FITC alone were used as control for background fluorescence).

Labelled cells were analyzed by flow cytometry using a FACScan (Becton Dickinson), with linear amplification of the forward and sideward light scatter signals, and logarithmic amplification of the fluorescence signals. Electronic compensation was set on the instrument to reduce spectral overlap between FITC (FL1) and PE (FL2) fluorescence. Specific binding of bEPO was calculated by subtracting the percentage of positively stained cells in samples labelled with bEPO in the presence of 200 fold excess unbiotinylated EPO, from the percentage of positively stained cells in samples labelled with bEPO.

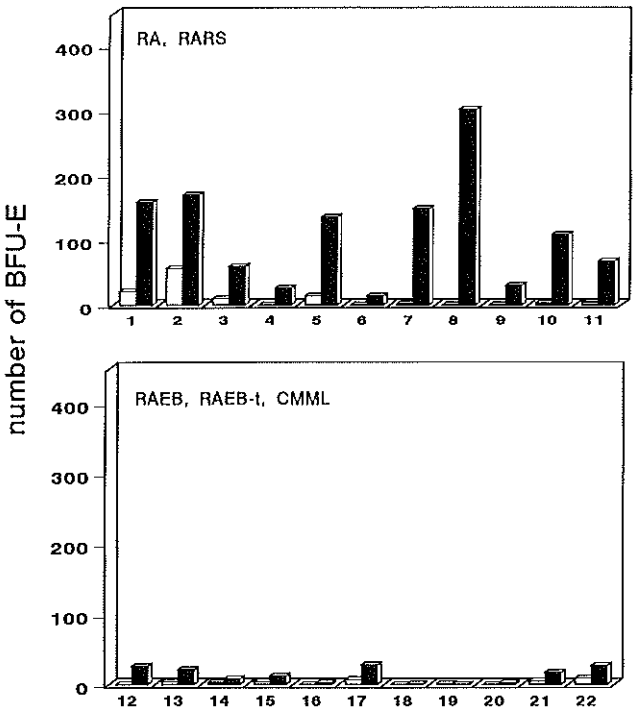

Figure 1 *Effect of KL on erythroid colony formation in MDS. KL (10 ng/mL) enhances erythroid colony formation in EPO (1 U/mL) supplemented cultures from RA and RARS marrow (upper panel). Minimal or no enhancement of erythroid colony growth by KL is seen from marrow of patients with RAEB, RAEB-t and CMML (lower panel). X-axis: data obtained in individuals with MDS (see Table 1). Data on vertical axis are expressed as numbers of erythroid colonies per 10^4 enriched marrow cells. Open bars, EPO; black bars, EPO plus KL.*

Table 2. *Effect KL on granulocytic colony formation*

Patient	G-CSF	G-CSF+KL	(%)	
1	9	20	(222)	-
2	8	15	(188)	-
3	14	37	(264)	+
4	2	4	(200)	-
5	7	15	(214)	-
6	2	8	(400)	-
7	nd	nd		
8	82	155	(189)	+
9	12	34	(283)	+
10	0	24	( )	-
11	4	18	(450)	-
12	62	111	(179)	+
13	41	65	(158)	+
14	0	0	(0)	-
15	21	296	(1410)	+
16	5	2	(40)	-
17	23	62	(269)	+
18	1130	1461	(129)	-
19	0	5	( )	-
20	1	0	(0)	-
21	22	86	(390)	+
22	12	36	(300)	+
N1	61	155	(254)	+
N2	95	281	(296)	+
N3	20	41	(205)	+
N4	133	282	(212)	+

KL (10 ng/mL) was added to G-CSF (30 ng/mL) stimulated cultures from patients with MDS. Results from four normal donors are also shown (N1-N4). Data are expressed as number of granulocytic colonies per 10^4 enriched marrow cells. In parentheses the enhancing effect of KL as percentage of G-CSF induced colony formation; + : Enhancement by KL was considered positive if KL augmented G-CSF induced colony growth by more than 150% and colony numbers exceeded a threshold of 30 colonies per 10^4 cells; nd=not done.

Detection splice variants EPO-R

For detection of splice variants of the EPO-R, reverse transcriptase polymerase chain reaction (RT-PCR) was performed, using the same set of primers as outlined in Nakamura et al. (1992):

ERFR1 sense 5'-TGAGACACCCATGACGTCTCA-3'

ERRV1 antisense 5'-TGTCCAGCACCAGATAGGTA-3'

In short, cryopreserved marrow cells were recovered overnight in culture medium in the presence (to maintain erythroid precursors) or absence of EPO (0.1 U/mL). Total RNA was extracted from patient marrow cells as described (Chomczynski et al., 1987) and used for cDNA preparation using reverse transcriptase (superscript RT)(GIBCO BRL, Breda, The Netherlands) and the antisense primer ERRV1. Amplification was performed on four-tenth of the cDNA reaction mixture with DNA polymerase (Taq DNA polymerase)(Promega) and the above mentioned primers ERFR1 and ERRV1 (30 cycles of denaturing at 94°C, annealing at 61°C, and extension at 72°C)(Perkin Elmer, Cetus). After PCR amplification, samples were run on a 1.5% agarose gel and Southern blot analysis was performed using human EPO-R cDNA as a probe.

RESULTS

Colony formation

EPO did not induce significant erythroid colony formation in culture from the marrow of patients with MDS (mean 7 BFU-E/10^4 cells, range 0-56 BFU-E/10^4 cells, n=22)(Fig 1). In contrast, in normal marrow (n=3), EPO alone induced a mean number of 100 BFU-E/10^4 cells, and KL augmented erythroid colony formation 2-3 fold. In "low risk" MDS cases (RA and RARS, cases #1-11) a marked increase in erythroid colony numbers was apparent when cells were incubated with EPO plus KL (mean 110 BFU-E/10^4 cells, range 14-301 BFU-E/10^4 cells, n=11). However, in most patients with "high risk" MDS (RAEB, RAEB-t and CMML, cases #12-22), KL was unable to augment erythroid colony formation in EPO induced cultures (mean 13 BFU-E/10^4 cells, range 0-28 BFU-E/10^4 cells, n=11)(Fig 1). To investigate whether the unresponsiveness of the marrow cells of the latter "high risk" MDS patients to costimulation with KL is restricted to the EPO responsive cell, KL enhancement of MDS colony formation was examined in addition in G-CSF induced cultures (Table 2). In some MDS cases KL could improve granulocytic

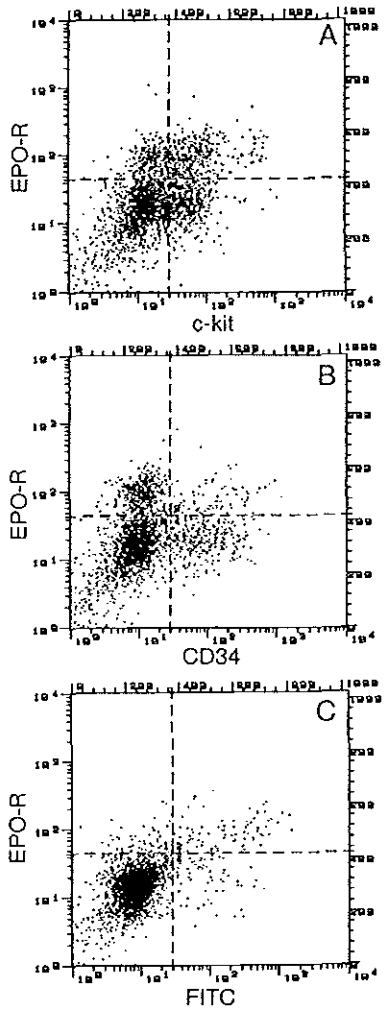

Figure 2 *Flow cytometric analysis of marrow cells in one patient with MDS (case #18).*
(A) Cells were labelled with bEPO (vertical axis) and counterstained with anti-c-kit (horizontal axis). Cells positive for EPO-R (upper quadrants), c-kit positive cells (right quadrants) and cells with dual EPO-R and c-kit positivity (upper right quadrant). (B) Cells were labelled with bEPO (vertical axis) and counterstained with anti-CD34 (horizontal axis). Cells positive for EPO-R (upper quadrants), CD34 positive cells (right quadrants) and cells with dual EPO-R and CD34 positivity (upper right quadrant). (C) Cells labelled with bEpo in the presence of excess unlabelled Epo (vertical axis), and counterstained with GAM-FITC alone (horizontal axis), as control. Dashed lines are plotted arbitrarily to mark background fluorescence.

colony formation whereas no effect of KL on erythroid colony formation was observed (for example cases #15 and #21). In one MDS patient where EPO and EPO plus KL were inactive (case #18), G-CSF alone already gave rise to high colony numbers (1130 CFU-G/10^4 cells). Cells from this MDS patient, when plated at lower cell density (3500 cells/dish in stead of 10^4 cells/dish), were susceptible to enhancement by KL (increase 1.5 fold)(data not shown). In four MDS cases (#14, #16, #19, and #20), KL neither augmented erythroid colony formation nor granulocytic colony formation.

Receptor analysis

EPO-R expression: In all cases of MDS (n=15), EPO-R positive cells could be detected. A representative flow cytogram (MDS case #18) is given in Fig 2. Percentages of EPO-R positive cells, as assessed following labelling with biotinylated EPO, represented 0.5% to 18% of cells in the different MDS marrow cell specimens (Fig 3A). In three MDS patients (cases #7, #18, and #19) although more than 10% of the cells were EPO-R positive, EPO could not induce erythroid colonies from the marrow of these patients (Fig 1). In normal marrow (n=6), percentages of EPO-R positive cells varied between a similar range of values, i.e., between 0.6% and 16% of analyzed cells (Fig 3A).

C-kit expression: In 12/15 MDS specimens c-kit expression was demonstrated (Fig 3A). In most MDS patients the percentages of c-kit positive cells did not exceed 16%. In some MDS cases, e.g. #16 and #19, KL did not enhance colony formation (Fig 1 and Table 2) although c-kit positive cells were clearly detected. In MDS cases #3 and #22, augmenting effects of KL on EPO and G-CSF induced cultures were evident although no c-kit positive cells were detected.

EPO-R and c-kit coexpression: EPO-R labelled cells were counterstained with anti-c-kit so that cells with dual expression of EPO-R and c-kit could be assessed. In all MDS specimens with c-kit positivity, a subpopulation of cells appeared double positive for EPO-R and c-kit. In "low" as well as "high" risk MDS cases percentages of EPO-R positive cells coexpressing c-kit exceeded normal values (Fig 3B).

EPO-R and CD34 coexpression: To investigate whether the reduced response of MDS marrow cells to growth factor stimulation correlated with a shift towards the

immature compartment, CD34 expression was examined (Fig 2B;Fig 4). In most MDS patients the frequencies of CD34 positive cells were comparable or below those observed in normal marrow. Only in one MDS patient (case #16) more than 80% of analyzed cells were CD34 positive (c-kit expression in this patient also exceeded 80%, Fig 3A).

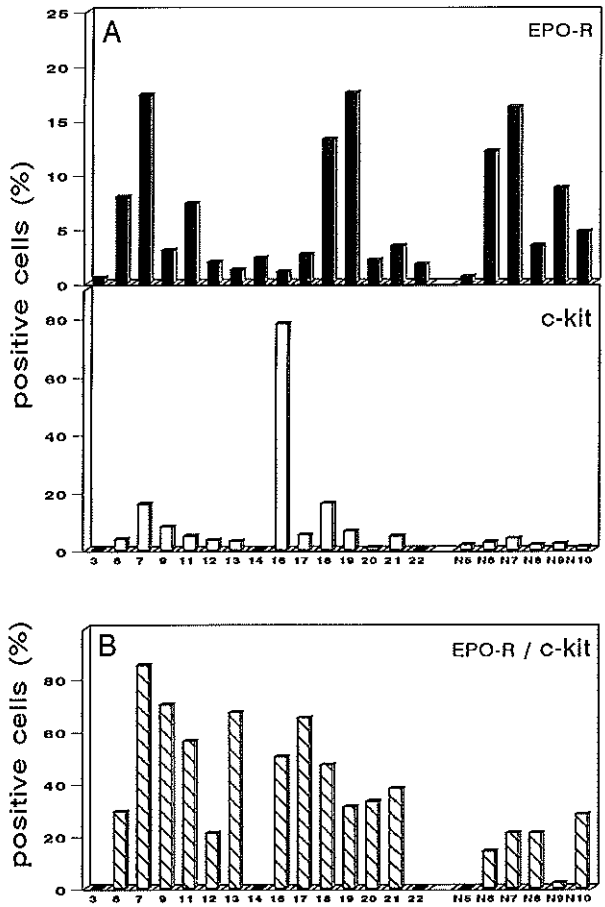

Figure 3 *EPO-R and c-kit expression in MDS and normal marrow. (A) Percentages of marrow cells with EPO-R positive cells (upper panel), or c-kit positive cells (middle panel), are given for individual patients with MDS (see Table 1 for patient characteristics) and six normal subjects (N5-N10).*
Black bars, EPO-R positive cells (%); open bars, c-kit positive cells (%) (B) EPO-R positive cells coexpressing c-kit. EPO-R positive cells were set at 100% and the fractions of EPO-R positive cells coexpressing c-kit are indicated as percentages. Hatched bars, EPO-R positive cells coexpressing c-kit (%).

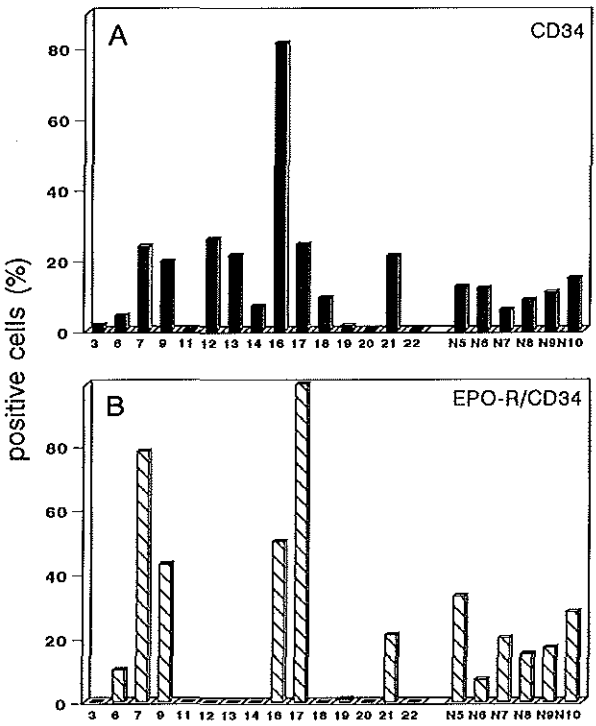

Figure 4 *CD34 expression in MDS and normal marrow. (A) Percentages of marrow cells with CD34 positive cells are given for individuals with MDS (see Table 1 for patient characteristics) and six normal subjects (N5-N10). Black bars, CD34 positive cells (%) (B) EPO-R positive cells coexpressing CD34. EPO-R positive cells were set at 100% (see Fig 3A, upper panel) and the fractions of EPO-R positive cells coexpressing CD34 are indicated as percentages. Hatched bars, EPO-R positive cells coexpressing CD34 (%).*

Cells labelled with bEPO were counterstained with CD34. Percentages of EPO-R positive cells coexpressing CD34, were more variable in MDS as compared to normal marrow (Fig 4B). In some "low risk" MDS cases, none of the EPO-R positive cells coexpressed CD34 whereas in other cases almost all EPO-R positive cells coexpressed CD34 (e.g. cases #3 and #7). The same was apparent in "high risk" MDS, where in certain cases none of the cells coexpressed EPO-R and CD34 at measurable levels and in other cases

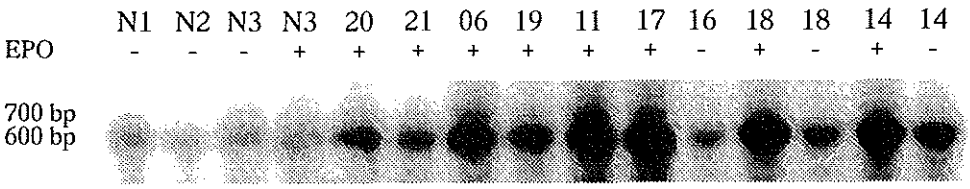

Figure 5 *Expression splice variants in MDS and normal marrow. Marrow cells of 9 MDS patients and 3 normal donors (N1-N3) were incubated for 16 hours in culture medium in the presence (+) or absence (-) of EPO (0.1 U/mL) and then recovered. RNA was isolated and PCR products were run on gel and Southern blot analysis was performed.*
600 bp band representing the full length EPO-R (EPO-R-F), 700 bp band representing the truncated and soluble forms of the EPO-R (resp. EPO-R-T and EPO-R-S) are indicated.

almost all Epo-R positive cells coexpressed CD34 (e.g. cases #13 and #17).

Preincubation: To test whether KL could up- or down regulate EPO-R, marrow cells from patients with MDS (n=15) and normal individuals (n=6) were preincubated for one day in the presence of KL (10 ng/mL). After preincubation of marrow cells with KL, EPO-R expression had not changed. Reciprocally, preincubation with EPO (1 U/mL) for 24 hours did also not affect c-kit expression to a significant level (data not shown).

EPO-R RNA splice variants

To examine the expression of the different EPO-R isoforms in MDS, RNA was isolated from 9 patients with different types of MDS and subjected to RT-PCR. The PCR product was run on gel and Southern blot analysis was performed. In all MDS patients the full length EPO-R (EPO-R-F) was clearly detectable (600 bp band), in some cases also a 700 bp band (representing the EPO-R-T and EPO-R-S) was observed (Fig 5). These results were comparable to those obtained from normal marrow (n=3) (Fig 5).

DISCUSSION

The mechanisms of impaired erythropoiesis in MDS have not been explained. The results presented here show that, although MDS marrow cells are not able to respond properly to EPO, EPO-R are present on MDS cell specimens. These results would indicate that the number of MDS marrow cells expressing the EPO-R do not limit the ability of EPO to induce erythroid colonies from these cells. However, EPO cannot induce erythroid colony formation even in those MDS patients revealing relatively high numbers of cells expressing the EPO-R. Furthermore, the percentages of CD34 positive cells were by and large comparable to normal marrow values (excepting case #16). Percentages of MDS marrow cells coexpressing EPO-R and CD34 however were abnormal, irrespective of the MDS subtype. In some patients a shift of EPO-R positive cells was apparent towards the more mature compartment (EPO-R positive cells were all negative for CD34), in others EPO-R positive cells appeared arrested at the stage of CD34 positivity (the great majority of EPO-R positive cells coexpressed CD34). These results appear indicative of an altered phenotype of EPO-R positive cells in MDS. However, EPO responsiveness appeared independent of the level of coexpression of Epo-R and CD34. In fact there was no apparent correlation between numbers of cells coexpressing EPO-R and CD34, and the (in)ability of EPO to induce erythroid colonies *in vitro*. Therefore it appears that the phenotypic asynchrony of EPO-R and CD34 positive cells in MDS itself does not determine the impaired erythroid response.

Of 9 MDS patients (cases #6,#11,#12,#14,#16-20) a segment of the EPO-R was amplified by RT-PCR to specifically detect the different splice variants. In all patients the 600 bp fragment, corresponding to the full length EPO-R (EPO-R-F), was detected. This EPO-R-F predominates in late progenitors (Nakamura et al., 1992). In some MDS cases, the 700 bp fragments (corresponding with the alternative forms of the EPO-R e.g., the truncated EPO-R and the soluble EPO-R) were also demonstrated. These results indicate that in MDS cells expression of the full length EPO-R is comparable to normal marrow and that the impaired response to EPO in MDS can not be explained by altered expression of the EPO-R-F.

The EPO-R transmits signals for both proliferation and differentiation in erythroid progenitors (Liboi et al., 1993). The results of these investigations do not exclude a structural defect in the EPO-R, leading to altered function (D'Andrea et al., 1991, Quelle

et al., 1991). A structurally abnormal human EPO-R gene involving a 3' end deletion, as described in a dominantly inherited benign human erythrocytosis (De La Chapelle et al., 1993), and the human erythroleukemia cell line TF-1 (Ward et al., 1992), has demonstrated the functional consequences of a genetic defect of the EPO-R. Furthermore, more recently, a mutation in the G-CSF receptor, another member of the cytokine receptor superfamily, has been described (Dong et al., 1993). This G-CSF-R mutant was isolated from a patient with severe congenital neutropenia, and appeared to determine a defective granulocytic maturation response.

KL is a critical regulatory factor in erythropoiesis (Witte et al., 1990, Huang et al., 1990), and may augment EPO induced erythropoiesis in a subset of MDS patients (RA and RARS) (Backx et al., 1992, Piacibello et al., 1994). Receptor data suggest that the inability of cells to respond *in vitro* to KL plus EPO in most "high" risk MDS cases cannot be explained by a lack of cells expressing c-kit and EPO receptors. Furthermore, the inability of KL to promote erythropoiesis in "high risk" cases of MDS, is not (or not always) due to an intrinsic KL pathway defect since KL could augment G-CSF induced colony formation in some cases. This would suggest that the lack of KL enhancement of erythropoiesis resides in the EPO signalling pathway itself rather than in the KL activation cascade. Bone marrow cells of most MDS patients expressed c-kit, as shown by FACS analysis. These results are in agreement with those described by Glinsmann-Gibson et al.(1994), who reported that the heterogeneity in KL responsiveness implies an intrinsic defect in growth regulation not explained by cellular loss of c-kit display. In some MDS cases KL may stimulate colony formation *in vitro*, although no c-kit positive cells were detected. The cells from these patients probably were not really c-kit negative, and express c-kit below detection limit. In one MDS case (#14), where c-kit was not measurable, KL could not improve colony formation (erythroid- nor granulocytic colony formation), suggesting that in this case not only the erythroid but also the granulocytic stimulatory pathway was defective. Further studies would be required to determine whether c-kit is expressed on the membrane at levels below the detection limit or is truly absent.

In Diamond-Blackfan anemia (DBA), a congenital red blood cell aplasia, erythroid progenitors fail to respond to growth factors like EPO and IL-3. Analogous to MDS, the addition of KL permits a significant increase of both the size and number of erythroid colonies *in vitro* (Abkowitz et al., 1991, Bagnara et al., 1991, Olivieri et al., 1991). In two patients with DBA tested, no structural abnormality of c-kit was detected (Abkowitz

et al., 1992). Furthermore, in a study concerned with the involvement of mutations in the Kit-gene, only polymorphisms were detected in a series of 46 patients with MDS (Bowen et al., 1993a). Since both MDS and DBA are clinically heterogenous disorders, abnormalities in the receptor for KL could still exist in certain patients with DBA or MDS.

In one patient with MDS (case #16), CD34 and c-kit expression was demonstrated in more than 80% of cells. Thus, marrow cells of this patient appear to accumulate at a comparatively immature stage (CD34 positive cells) and, similarly as in acute myeloid leukemia (AML), no or hardly any colonies were induced (Griffin and Löwenberg, 1986). In another MDS patient (case #19), scarce colonies were induced in EPO or G-CSF stimulated cultures, although cells showed normal c-kit expression and CD34 expression and relatively high numbers of cells expressing the EPO-R. Most probably an intrinsic defect determines the inability of these cells to respond to both EPO and G-CSF stimulation. Preincubation experiments of MDS marrow cells with either EPO or KL did not result in upregulation of respectively c-kit or EPO-R. Since these results were comparable to results obtained from preincubation experiments with normal marrow, decreased EPO response in MDS apparently is not caused by inhibition of ligand induced upregulation of receptors. Based on the results of the experiments, one may conclude that further studies concerning MDS patients should probably focus on detailed structural and functional analysis of the EPO-R (mutations, deletions) and signalling molecules associating with- or more down stream of the receptor.

CHAPTER SIX

GENERAL DISCUSSION

Chapter 6

6.1 Possible role of TNFα in hematopoietic suppression

TNFα is a pleiotropic cytokine showing to have both inhibitory and stimulatory effects on hematopoietic progenitor cells (**Chapter 2**, Murase et al., 1987a, Caux et al., 1990, Caux et al., 1991). Two TNF receptors (TNF-Rs) have been identified and cloned with molecular masses of 55 kD (p55 TNF-R, TNF-R1) and 75 kD (p75 TNF-R, TNF-R2) (Brockhaus et al., 1990, Loetscher et al., 1990). The two TNF-Rs show no homology between their intracellular domains, suggesting that they use separate signalling pathways (Dembic et al., 1990). From recent reports it has become clear that the two receptors play differential roles in the pleiotropic effects of TNF on hematopoiesis. Both TNF-R1 and TNF-R2 can signal inhibition of primitive progenitor cells such as high proliferative potential colony-forming cells (HPP-CFC) (Rusten et al., 1994a). In contrast, TNF-R1 exclusively mediates the stimulatory effects of TNFα on GM-CSF and IL-3 induced colony formation, as well as TNFα induced inhibition of G-CSF induced colony growth (Rusten et al., 1994b). These results are in agreement with our own (unpublished) data. We found that both the inhibiting effect of TNFα on G-CSF induced colony formation as well as the positive effect of TNFα on GM-CSF or IL-3 induced colony growth were mediated through the TNF-R1. Although the inhibiting effect of TNFα on EPO induced erythropoiesis has been studied extensively (Abboud et al., 1987, Murase et al., 1987a, Wisniewski et al., 1987), recent publications concerning the involvement of the two TNF-Rs in TNFα mediated hematopiesis do not include effects on erythropoiesis. We found (unpublished results) that the negative effect of TNFα on EPO induced erythroid colony formation mainly was mediated through the TNF-R2. Apparently, the TNF-R1 predominantly mediates the (positive as well as negative) effect of TNFα on myeloid colony formation, whereas the TNF-R2 mainly is involved in the inhibiting effect of TNFα on erythroid colony formation.

Recent observations indicate that TNFα is a potent inhibitor of KL-stimulated hematopoiesis (Rusten et al., 1994b). TNFα inhibits the synergistic effect of KL on GM-CSF or IL-3 induced colony formation and completely blocks colony formation stimulated by KL in combination with G-CSF. Furthermore, TNFα downregulates c-kit cell surface expression on $CD34^+$ bone marrow cells. All these effects are predominantly mediated through the TNF-R1 (Khoury et al., 1994, Rusten et al., 1994b). The effect of TNFα on KL plus EPO stimulated erythropoiesis and the involvement of the two TNF-Rs, remains to be resolved. All these studies on normal bone marrow confirm the important and

complex regulatory role of TNF on normal hematopoiesis.

In several disorders the role of TNF also has been studied. For example, TNFα has been associated with the pathogenesis of rheumatoid arthritis and aplastic anemia (Shinohara et al., 1991, Brennan et al., 1992). *In vitro* data suggest that peripheral blood mononuclear cells from patients with rheumatoid arthritis as well as from patients with aplastic anemia, suppress erythropoiesis via the production of TNFα (Shinohara et al., 1991, Katevas et al., 1994). Since ineffective erythropoiesis is one of the most prominent abnormalities in MDS, TNFα, as a potent inhibitor of erythropoiesis, could potentially play a role in impaired erythropoiesis in MDS. Increased release of TNFα could be one of the mechanisms playing a role in impaired erythropoiesis in MDS. Indeed, serum TNFα levels appear to be elevated in MDS patients as compared to normal controls (Symeonidis et al., 1991, Verhoef et al., 1992, Seipelt et al., 1993). However, no differences in TNFα secretion from peripheral blood or bone marrow mononuclear cells are found between myelodysplastic patients and normal controls (Ohmori et al., 1990, Visani et al., 1993). One might hypothesize that increased TNFα serum levels, as observed in MDS, may be due to increased production of TNFα by other than mononuclear cells or to inadequate clearance of TNFα from the circulation. The fact that in standard culture systems (devoid of TNFα) the response of bone marrow cells from patients with MDS to growth factor stimulation still is defective, however would argue for an intrinsic defect in the haematopoietic cell itself and against a role of TNFα in impaired hematopoiesis in MDS. Whether TNFα has a role to contribute remains to be resolved.

6.2 The MDS progenitor cell

Hematopoietic stem cells are characterized by their self renewal capacity and the ability to develop into multiple hematopoietic lineages. One of the assays used to study immature precursor cells is the blast cell colony assay (**Chapter 3**). This assay is based on the fact that very immature multilineage progenitor cells are dormant in cell cycle (G_0) and can survive for a comparatively longer period in the absence of stimuli. Following this interval, the addition of specific growth factors like IL-3 and IL-6, G-CSF, Il-11 or KL, trigger stem cells into active cell cycle (Leary et al., 1992). Blast cell colonies can be induced to differentiate into the distinct lineages following addition of HGFs and generate secondary colonies. As shown in **Chapter 3**, the numbers of blast colonies generated

from the very immature progenitor cells, in most MDS cases appear comparable to those in normal marrow. These results are in agreement with those observed by Soligo et al. (1994). Analysis of the hematopoietic stem cell in long-term bone marrow culture (LTBMC) revealed that the mean number of recovered non-adherent cells from the supernatant of the LTBMCs in MDS were comparable to normal controls. Apparently, decreased *in vitro* colony forming ability in MDS cannot be explained by a substantial decrease in the number of immature progenitor cells since the incidence of immature progenitor cells in MDS are similar to those of normal marrow (**Chapter 3**, Soligo et al., 1994). However, as discussed in **Chapter 3**, MDS blast clonogenic cells are unable to generate erythroid progeny, in contrast to normal marrow from which almost half of the secondary colonies consists of erythroid cells. These results indicate that in MDS the CFU-blast is defective as regards the ability to generate daughter cells that are able to respond properly to EPO. Soligo et al. (1994) only report data as regards the (impaired) ability of the MDS non-adherent cell fraction from LTBMC to induce myeloid progeny.

Sawada et al., (1995) argue that impaired differentiative capacity in MDS could correlate with an altered phenotype of the MDS progenitor cell. They purified $CD34^+$ cells from normal marrow as well as marrow from patients with MDS and analyzed phenotypic subpopulations of these $CD34^+$ cells. MDS $CD34^+$ cells significantly coexpressed $CD13^+$ cells at a higher level compared with normal marrow values. Thus, in MDS, marrow CD34 positive cells are replaced by impaired CD34 positive cells that predominantly express CD13, a marker for the granulomonocytic lineage (erythroid progenitors are CD13-negative), and hardly or none of the MDS progenitor cells induce colonies of the erythroid lineage. This lineage commitment of MDS $CD34^+$ cells towards the nonerythroid lineage as a possible mechanism of impaired erythropoiesis is in conflict with our observations since we show that MDS progenitor cells do express EPO-R (**Chapter 5**). This would suggest that in MDS, marrow cells essentially are capable of responding to EPO and generate erythroid colonies. Although the MDS $CD34^+$ cell reveal phenotypic asynchrony as regards the EPO-R coexpression, the EPO responsiveness appears independent of the level of coexpression of EPO-R and CD34.

6.3 The EPO-R

The mechanism of impaired erythropoiesis in MDS still remains unclear. The

experiments described in **Chapter 5** demonstrate that the inability of MDS progenitor cells to respond properly to EPO cannot be explained by a lack of EPO-receptors since Epo-R expression in MDS was comparable to normal marrow. Furthermore, RT-PCR analysis of the different splice variants of the EPO-R also revealed that MDS cells, like normal marrow, expressed the full-length EPO-R (EPO-R-F). This EPO-R-F is believed to tranduce mitogenic signals (Nakamura et al., 1992 and 1994). Defective erythropoiesis in MDS however could involve a structural defect in the EPO-R (mutations, deletions), defects in the EPO signalling pathway (signalling molecules associating with-or more down stream of the EPO-R), or abnormalities in erythroid specific trancription factors.

The EPO-R, a 507 amino acid membrane spanning protein, is a member of the cytokine receptor superfamily and transmits signals for both proliferation and differentiation in erythroid progenitors (Kaczmarski et al., 1991, Olsson et al., 1992, Liboi et al., 1993). The extracellular domain of the EPO-R shares two distinctive features with other members of the cytokine receptor superfamily, a conserved domain of four cysteines and a tryptophan-serine-X-tryptophan-serine motif just outside the transmembrane region. Examination of the conserved Trp-Ser-X-Trp-Ser motif in the extracellular part of the EpoR by generation of mutant EpoR, revealed that this motif is essential for receptor mediated signal transduction (Chiba et al., 1992). Two discrete domains of the EpoR are identified in the cytoplasmic part. A membrane proximal domain, including two box subdomains (Box 1 and Box 2), required for signal transduction, and a C-terminal domain exerting a negative effect on Epo-induced signal transduction (D'Andrea et al., 1991, Miura et al., 1991, Miura et al., 1993).

The relevance of mutations or deletions of the EPO-R in certain hematopoietic disorders is illustrated by the presence of a mutation in the EPO-R gene, resulting in a truncation of the EPO-R at the negative regulatory C-terminal end, causing a dominantly inherited benign human erythrocytosis (De La Chapelle et al., 1993). Another example of a human condition in which a mutation of the EPO-R may be involved is given in a case of sporadic primary polycythemia (Sokol et al., 1994). In this patient, a mutation in the negative regulatory domain of the EPO-R was found. However, analysis of the functional consequence of this mutation in the EPO-R, revealed that Epo sensitivity of this mutant EPO-R was comparable to wild-type EPO-R implicating that this EPO-R mutation alone apparantly is not sufficient to cause the polycythemia phenotype. Recently, Winkelmann et al. (1995) demonstrated that in TF-1 cells, derived from a human erythroleukemia, abnormal EPO-R expression results from a translocation affecting the EPO-R locus. This

translocation is believed to be involved in the evolution of the erythroleukemia from which TF-1 cells were derived.

These examples of human hematopoietic disorders, in which mutations or deletions of the EPO-R may contribute to the pathogenesisis, are all characterized by erythrocytosis. *In vitro* studies have demonstrated that critical point mutations in the EPO-R, moreover deletions or point mutations within the membrane-proximal region of the cytoplasmic domain, can inactivate receptor function (Miura et al. 1993) and hamper EPO induced erythroid response. Analogous to this *in vitro* data, an abnormal EPO-R, unable to respond properly to EPO stimulation, may be involved in impaired erythropoiesis in MDS.

6.4 EPO-R signal transduction

Various lines of evidence indicate that the EPO-R exists as a multimeric complex. Although homodimerization of the EPO-R is essential for signal transduction of EPO, dimerization alone seems to be insufficient for formation of the complete active EPO-R on the surface of hematopoietic cells (Youssoufian et al., 1993, Takahashi et al. 1995).

Protein tyrosine phosphorylation and de-phosphorylation play an important role in the growth regulation of hematopoiesis (Ihle et al., 1994). Although receptors of the cytokine superfamily (like EPO) lack intrinsic tyrosine phosphorylation activity, ligand binding and receptor activation is rapidly followed by tyrosine phosphorylation of a number of cellular substrates (Ihle et al., 1994). After EPO stimulation, the EPO-R associates with the cytoplasmic protein tyrosine kinase Janus kinase 2 (Jak2) (Witthuhn et al., 1993). Following activation of Jak2, a number of cellular proteins are tyrosine phosphorylated, including GTPase activating protein (GAP), Shc, and the EPO-R itself. Jak2 phosphorylation of the EPO-R creates a binding site for the Src homology 2 (SH-2) domain of hematopoietic cell phosphatase (HCP). HCP plays an essential role in the negative regulation of hematopoiesis (Shultz et al., 1993). The association of HCP with the phosphorylated form of the receptor/Jak2 kinase complex may result in dephosphorylation of substrates and/or associated kinases, thereby down-regulating the response (Yi et al., 1995).

One could hypothesize that abnormalities in the signal transduction pathway, (e.g. HCP- or Jak2 function), resulting in EPO insensitivity, play a role in MDS. For example,

a kinase deficient form of Jak2 appeared to inhibit EPO-induced mitogenesis, indicating that Jak2 kinase activation plays an essential role in mitogenesis (Zhuang et al., 1994). The role of HCP in hematopoiesis is demonstrated by mice, termed motheaten, genetically deficient in HCP. These mice die soon after birth primarily from the accumulation of activated macrophages in the lungs. In addition, a number of hematopoietic abnormalities are apparent, including increased sensitivity to EPO (Van Zant and Shultz, 1989). One may speculate that dysfunction of HCP, other than described in motheaten mice, potentially could result in EPO-insensitivity. Thus, experiments analyzing the signal transduction pathway in MDS are necessary to find out whether (alterations in) signalling molecules play a role in impaired erythropoiesis.

6.5 Erythroid transcription factors

GATA-1, a 413 amino acid polypeptide containing two zinc finger-like DNA-binding domains, is required for normal erythroid differentiation (Chiba et al., 1991, Pevny et al., 1991, Zon et al., 1991, Orkin, 1992, Mouthon et al., 1993). GATA-1 binds to consensus sequences which are present in promoter or enhancer regions of erythroid-specific genes. In early stage of hematopoiesis GATA-1 transactivates the EpoR gene. When Epo specifically binds to its receptor, the receptor mediates a signal which enhances GATA-1 expression. GATA-1 transactivates hemoglobin synthesis-related genes and globin genes in relatively matured erythroid cells. In δ-thalassemia, due to a mutation in the GATA-1 recognition sequence in the promoter region of the δ-globin gene, disturbed GATA-1 binding results in impaired δ-globin expression. This illustrates that dysfunction of GATA-1 binding can affect normal erythroid development (Matsuda et al., 1992, Moi et al., 1992).

Other erythroid specific transcription factors, known to play a role in the regulation of erythropoiesis include the stem cell leukemia gene product (SCL or tal-1), a member of the 'helix-loop-helix' family of transcription factors, and nuclear factor erythroid 2 (NF-E2), a basic-leucin zipper trancription factor (Begley et al., 1989, Orkin et al., 1990, Green et al., 1991, Visvader et al., 1992). A defect in the gene for NF-E2 has been shown to lead to microcytic anemia in mice (Peters et al., 1993). The Evi-1 gene encoding a nuclear, zink finger, DNA binding protein, is normally expressed in the kidney and developing oocytes but not in hematopoietic cells (Morishita et al., 1990).

Inappropiate expression of the Evi-1 gene in myeloid cells has been demonstrated to prohibit terminal differentiation (Morishita et al., 1992). Kreider et al. (1993) have demonstrated that aberrant expression of the Evi-1 gene may impair the normal response of erythroid cells or bone marrow progenitors to EPO, most likely by repressing the transcription of a subset of GATA-1 regulated genes.

These data strongly suggest that transcription factor abnormalities may affect normal erythroid differentiation. Hence, (inappropiate expression of) transcription factors could play a role also in defective erythropoiesis in MDS and this certainly deserves to be the subject of thorough future investigations.

6.6 Kit-ligand and c-kit

Kit-ligand (KL) and c-kit gene loci have been recognized to regulate erythropoiesis in the mouse. The white-spotting (W) locus encodes the transmembrane tyrosine kinase receptor identical with the c-kit proto-oncogene (Chabot et al., 1988, Geissler et al., 1988, Ullrich and Schlessinger, 1990). The so called Steel (Sl) locus (Flanagan and Leder, 1990, Williams et al., 1990, Zsebo et al., 1990) encodes the ligand for c-kit (kit-ligand, KL). In the mouse, mutations or deletions at the W and Sl loci result in microenvironmental hematopoietic abnormalities and a profound macrocytic anemia. The defect in the W locus results in a stem cell abnormality (c-kit lacks or is ineffective). In the Sl mouse abnormalities in the ligand that stimulates c-kit (KL), result in a defective bone marrow microenvironment. As the KL-c-kit complex is critical for growth and development of murine hematopoiesis, investigation of this complex in normal human hematopoiesis as well as in hematopoietic disorders appear of special interest.

KL has been shown to augment colony formation by human hematopoietic stem cells and has strong synergistic activities with hematopoietic growth factors like GM-CSF, G-CSF, IL-3, and EPO to enhance myeloid, and erythroid colony formation (McNiece et al., 1991, Bridell et al., 1992, Ogawa, 1993). Functional examination of c-kit by using antisense oligodeoxynucleotides (oligomers) to suppress c-kit function, revealed that c-kit plays a predominant role in normal erythropoiesis and does not appear to play a critical role in either myeloid or megakaryocytic development, at least at the early progenitor cell level (Ratajczak et al., 1992). Furthermore, using a serum-free culture system it was shown that BFU-E have an absolute requirement for KL to grow and develop (Dai et al.,

1991). KL not only plays a pivotal role in normal erythropoiesis, but also may augment impaired erythropoiesis in several bone marrow failure syndromes like aplastic anemia (AA), and Diamond-Blackfan anemia (DBA) (Olivieri et al., 1991, Alter et al., 1992, Bagnara et al., 1992, Wodnar-Filipowicz et al., 1992). Because DBA patients share several clinical similarities with W and Steel locus mutant mice, DBA might be a candidate for mutations in the gene for c-kit or KL. However, the finding of normal serum levels of KL as well as normal nucleotide sequences for both the KL and c-kit coding regions in DBA patients, as well as the fact that a number of patients with DBA can be cured by bone marrow transplantation (suggesting that the microenvironment is not defective) makes this disorder less likely to be due to a defect involving KL or c-kit (Wiktor-Jedrzejczak et al., 1987, Lenarsky et al., 1988, Abkowitz et al., 1992, Drachtman et al., 1992). As shown in **Chapter 4 and 5**, KL may enhance erythropoiesis in MDS as well. The erythroid enhancing effect of KL was also demonstrated by others (Glinsmann-Gibson et al., 1994, Piacibello et al., 1994, Soligo et al., 1994).

Measurement of KL concentration in serum of 85 MDS patients revealed that mean serum KL concentrations in MDS patients were significantly lower than those in normal subjects. However, for the majority of MDS patients the serum KL concentrations were within the normal range (Bowen et al., 1993b). These results suggest that at most in a proportion of MDS cases impaired hematopoiesis can be explained by very low levels of KL in the circulation. Since slightly lowered concentrations of KL may already be suboptimal for KL dependent hematopoiesis, and the fact that in a number of MDS patients KL serum concentration significantly was reduced, the *in vivo* administration of KL may be of benefit in some MDS patients. Whether the decreased KL serum concentrations in some MDS patients are due to abnormalities in the microenvironment, e.g. abnormal stroma cells revealing defective KL production, or due to an increased removal of KL from the circulation is unclear. Since measurements of KL serum levels only include the secreted form of KL, studies on the expression of the other biological active, membrane bound form of KL are also required to really assess the role of KL in impaired hematopoiesis in MDS.

The inability of KL to improve erythropoiesis in MDS, can unlikely be explained by a complete loss of c-kit bearing cells, since c-kit is expressed also in those cases not responding to KL (**Chapter 5**, Glinsmann-Gibson et al., 1994). Furthermore, the absence of mutations in the c-kit gene in 46 MDS patients, as examined by SSCP-analysis, do not support a role for c-kit in impaired erythropoiesis in MDS (Bowen et al., 1993a).

However, since both DBA and MDS are clinical heterogenous disorders, abnormalities involving c-kit or KL could exist in some patients. Also in patients with acquired or congenital (Fanconi's) aplastic anemia, sharing some clinical features with the anemia of W-defective mice, the role of KL or c-kit in defective hematopoiesis awaits further study. As deficient production of soluble KL may contribute to aplastic anemia (AA) in some patients (Wodnar-Filipowicz et al., 1993), KL may have potential therapeutic value in some AA patients.

REFERENCES

References

REFERENCES

Abboud SL, Gerson SL, Berger NA: The effect of tumor necrosis factor on normal human hematopoietic progenitors. **Cancer** 60:2965, 1987

Abkowitz JL, Sabo KM, Nakamoto B, Blau CA, Martin FH, Zsebo KM, Papayannopoulou T: Diamond-Blackfan anemia: in vitro response of erythroid progenitors to the ligand for c-kit. **Blood** 9:2198, 1991

Abkowitz JL, Broudy VC, Bennett LG, Zsebo KM, Martin FH: Absence of abnormalities of c-kit or its ligand in two patients with Diamond-Blackfan anemia. **Blood** 79:25, 1992

Abrahamson G, Boultwood J, Madden J, Kelly S, Oscier DG, Rack K, Buckle VJ, Wainscoat JS: Clonality of cell populations in refractory anemia using combined approach of gene loss and X-linked restriction fragment length polymorphism-methylation analysis. **Br J Haematol** 79:550, 1991

Alter BP, Knobloch ME, He L, Gillio AP, O'Reilly JO, Reilly LK, Weinberg RS: Effect of stem cell factor on in vitro erythropoiesis in patients with bone marrow failure syndromes. **Blood** 80:3000, 1992

Anderson DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, March CJ, Boswell S, Gimpel SD, Cosman D, Williams DE: Molecular cloning of mast cell growth factor, a hematopoietin that is active in both bound and soluble forms. **Cell** 63:235, 1990

Andrews RG, Singer JW, Bernstein ID: Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 antigens and light scatter properties. **J Exp Med** 169:1721, 1989.

Asano H, Hotta T, Ichihara M, Murate T, Kobayashi M, Saito H: Growth analysis of marrow CD34-positive hematopoietic progenitor cells in patients with myelodysplastic syndrome. **Leukemia** 8:833, 1994

Avalos BR, Gasson JC, Hedvat C, Quan SG, Baldwin GC, Weisbart RH, Williams RE, Golde DW, DiPersio JF: Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. **Blood** 75:851, 1990

Ayraud N, Donzeau M, Raynaud S, Lambert JC: Cytogenetic study of 88 cases of refractory anemia. **Cancer Genet Cytogenet** 8:243, 1983

Backx B, Broeders L, Löwenberg B: Kit-ligand improves erythropoiesis in myelodysplastic syndrome. **Blood** 80:1213, 1992

Backx B, Broeders L, Touw I, and Löwenberg B: Blast colony-forming cells in myelodysplastic syndrome: decreased potential to generate erythroid precursors. **Leukemia** 7:75, 1993

Bagby GC, Dinarello CA, Wallace P, Wagner C, Hefeneider S, McCall E: Interleukin-1 stimulates granulocyte macrophage colony-stimulating factor activity release by vascular endothelial cells. **J Clin Invest** 78:1316, 1986

Bagnara GP, Zauli G, Vitale L, Rosito P, Vecchi V, Paolucci G, Avanzi GC, Ramenghi U, Timeus F, Gabutti V: In vitro growth and regulation of bone marrow enriched CD34+ hematopoietic progenitors in Diamond-Blackfan anemia. **Blood** 9:2203, 1991

Bagnara GP, Strippoli P, Bonsi L, Brizzi MF, Avanzi GC, Timeus F, Ramenghi U, Piaggio G, Tong J, Podesta M, Paolucci G, Pegoraro L, Gabutti V, Bacigalupo A: Effect of stem cell factor on colony growth from acquired and constitutional (Fanconi) aplastic anemia. **Blood** 80:382, 1992

Begley CG, Aplan PD, Denning SM, Haynes BF, Waldmann TA, Kirsch IR: The gene SCL is expressed during early hematopoiesis and encodes a differentiation-related DNA-binding motif. **Proc Natl Acad Sci USA** 86:10128, 1989

Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C, the French-American-British (FAB) co-operative group: Proposals for the classification of the myelodysplastic syndromes. **Br J Haematol** 51:189, 1982

Berdel WE, Danhauser-Riedl S, Steinhauser G, Winton EF: Various human hematopoietic growth factors (interleukin-3, GM-CSF, G-CSF) stimulate the growth of non-hematopoietic tumor cells. **Blood** 73:80, 1989

Bernstein ID, Andrews RG, Zsebo KM: Recombinant human stem cell factor enhances the formation of colonies by CD34+ and CD34+lin- cells, and the generation of colony-forming progeny from

CD34+lin- cells cultured with IL-3, granulocyte colony-stimulating factor. **Blood** 77:2316, 1991

Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YCE, Mathisozn J, Ulevitch R, Cerami A: Identity of tumour necrosis factor and the macrophage-secreted factor cachetin. **Nature** 316:552, 1985

Beutler B, Cerami C: Cachetin: More than a tumor necrosis factor. **N Engl J Med** 316:379, 1987

Billström R, Thiede T, Hansen S, Heim S, Kristoffersson U, Mandahl, Mitelman F: Bone marrow karyotype and prognosis in primary myelodysplastic syndrome. **Eur J Haematol** 41:341, 1988

Bjorkman SE: Chronic refractory anaemia with sideroblastic bone marrow. A study of four cases. **Blood** 11:250, 1956

Bos JL: Ras oncogenes in human cancer: a review. **Cancer Res** 49:4682, 1989

Bot FJ, Dorssers L, Wagemaker G, Löwenberg B: Stimulating spectrum of human recombinant multi-CSF (IL-3) on human marrow precursors: importance of accessory cells. **Blood** 71:1609, 1988

Bot FJ, van Eijk L, Broeders L, Aarden LA, Löwenberg B: Interleukin-6 synergizes with M-CSF in the formation of macrophage colonies from purified human marrow progenitor cells. **Blood** 73:435, 1989

Boultwood J, Rack K, Kelly S, Madden J, Sakaguchi AY, Wang LM, Oscier DG, Buckle VJ, Wainscoat JS: Loss of both CSFR1 (FMS) alleles in patients with myelodysplasia and a chromosome 5 deletion. **Proc Natl Acad Sci USA** 88:6176, 1991

Bowen D, Culligan D, Jacobs A: The treatment in the myelodysplastic syndromes with recombinant human erythropoietin. **Br J Haematol** 77:419, 1991

Bowen DT, Padua RA, Burnett AK, deCastro CM, Kaufman RE: Two new polymorphisms but no mutations of the KIT gene in patients with myelodysplasia at positions corresponding to human FMS and murine W locus mutational hot spots. **Leukemia** 7:1883, 1993a

Bowen D, Yancik S, Bennett L, Culligan D, Resser K: Serum stem cell factor concentration in patients with myelodysplastic syndromes. **Br J Haematol** 85:63, 1993b

Bradley TR, Metcalf D: The growth of mouse bone marrow cells in vitro. **Aust J Exp Biol Med Sci** 44:287, 1966

Brandt J, Baird N, Srour E, Hoffman R: Characterization of a human hematopoietic progenitor cell capable of forming blast cell containing colonies in vitro. **J Clin Invest** 82:1017, 1988

Brennan FM, Maini RN, Feldmann M: TNF alpha, a pivotal role in rheumatoid arthritis? **Br J Rheumatol** 31:293, 1992

Brenner MK: Tumour necrosis factor. **Br J Hematol** 69:149, 1988

Bridell RA, Broudy VC, Bruno E, Brandt JE, Srour EF, Hoffman R: Further phenotypic characterization and isolation of human hematopoietic progenitor cells using a monoclonal antibody to the c-kit receptor. **Blood** 79:3159, 1992

Brockhaus M, Schoenfeld HJ, Schlaeger EJ, Hunziker W, Lesslauer W, Loetscher H: Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. **Proc Natl Acad Sci USA** 87:3127, 1990

Broxmeyer HE, Williams DE, Lu L, Cooper S, Anderson SL, Beyer GS, Hoffman R, Rubin BY: The suppressive influences of human necrosis factor on bone marrow hematopoietic progenitor cells from normal donors and patients with leukemia: synergism of tumor necrosis factor and interferon-gamma. **J Immunol** 136:4487, 1986

Broxmeyer HE, Cooper S, Lu L, Hangoc G, Anderson D, Cosman D, Lyman SD, Williams DE: Effect of murine mast cell growth factor (c-kit proto oncogene ligand) on colony formation by human marrow hematopoietic progenitor cells. **Blood** 77:2142, 1991

Bussolini F, Wang JM, Defilippi P: Granulocyte- and granulocyte-macrophage colony-stimulating factors induce human endothelial cells to migrate and proliferate. **Nature** 337:471, 1989

Carlo-Stella C, Cazzola M, Bergamaschi G, bernasconi P, Dezza L, Invernizzi R, Pedrazzoli P: Growth of human hematopoietic colonies from patients with myelodysplastic syndromes in response to recombinant human granulocyte-macrophage colony-stimulating factor. **Leukemia** 3:363, 1989

Carswell AE, Old LJ, Kassel RL, Green S, Fiore N, Williamson B: An endotoxin-induced serum factor that causes necrosis of tumors. **Proc Natl Acad Sci USA** 72:3666, 1975

Caux C, Sealand S, Favre C, Duvert V, Mannoni P, Banchereau J: Tumor necrosis factor-alpha strongly

References

potentiates interleukin-3 and granulocyte-macrophage colony-stimulating factor-induced proliferation of human CD34+ hematopoietic progenitor cells. **Blood** 75:2293, 1990

Caux C, Favre C, Saeland S, Duvert V, Durand I, Mannoni P, Bancherau J: Potentiation of early hematopoiesis by tumor necrosis factor-α is followed by inhibition of granulopoietic differentiation and proliferation. **Blood** 78:635, 1991

Chabot B, Stephenson DA, Chapman VM, Besmer P, Bernstein A: The proto-oncogene c-kit encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. **Nature** 335:88, 1988

Chiba T, Ikawa Y, Todokoro K: GATA-1 transactivates erythropoietin receptor gene, and erythropoietin receptor-mediated signals enhance GATA-1 gene expression. **Nucl Acids Res** 19:3843, 1991

Chiba T, Amanuma H, Todokoro K: Tryptophan residue of TRP-SER-X-TRP-SER motif in extracellular domains of erythropoietin receptor is essential for signal transduction. **Biochem Biophys Res Comm** 184:485, 1992

Chomczynski P, Sacchi N: Single-step method for RNA isolation by acid guanidinium thiocyanat-phenol-chloroform extraction. **Anal Biochem** 162:156, 1987

Clark SC, Kamen R: The human hematopoietic colony-stimulating factors. **Science** 236:1229, 1987

Coutinho LH, Geary CG, Chang J, Harrison C, Testa NG: Functional studies of bone marrow haemopoietic and stromal cells in the myelodysplastic syndrome. **Br J Hematol** 75:16, 1990

Culligan DJ, Cachia P, Whittaker J, Jacobs A, Padua RA: Clonal lymphocytes are detectable in only some cases of MDS. **Br J Haematol** 81:346, 1992

Dai CH, Krantz SB, Zsebo KM: Human burst-forming units erythoid need direct interaction with stem cell factor for further development. **Blood** 78:2493, 1991

Dan K, An E, Futaka M, Inokuchi K, Gomi S, Yamada T, Ogata K, Tnabe Y, Ohki I, Shinohara T, Nomura T: Megakaryocyte, erythroid and granulocyte-macrophage colony formation in myelodysplastic syndromes. **Acta Haematol** 89:113, 1993

D'Andrea AD, Yoshimura A, Youssoufian H, Zon LI, Koo JW, Lodish HF: The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains. **Mol Cell Biol** 11:1980, 1991

Dedhar S, Gaboury L, Galloway P, Eaves C: Human granulocyte-macrophage colony-stimulating factor is a growth factor active on a variety of cell types of non-hematopoietic origin. **Proc Natl Acad Sci USA** 85:9253, 1988

De La Chapelle A, Träskelin A-L, Juvonen E: Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. **Proc Natl Acad Sci USA** 90:4495, 1993

Delwel R, Dorssers L, Touw I, Wagemaker G, Löwenberg B: Human recombinant multilineage colony stimulating factor (IL-3): stimulator of acute myelocytic leukemia progenitor cells in vitro. **Blood** 70:333, 1987

Dembic Z, Loetscher H, Gubler U, Pan YCE, Lahm HW, Gentz R, Brockhaus M, Lesslauer W: Two human TNF receptors have similar extracellular, but distinct intracellular domain sequences. **Cytokine** 2:231, 1990

Der CJ, Finkel T, Cooper GM: Biological and biochemical properties of human ras genes mutated at codon 61. **Cell** 44:167, 1986

Dexter TM, Allen TC, Lajtha LG: Conditions controlling the proliferation of hematopoietic stem cells in vitro. **J Cell Physiol** 91:335, 1977

Dong F, v Buitenen C, Pouwels K, Hoefsloot LH, Löwenberg B, Touw I: Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. **Mol Cell Biol** 13:7774, 1993

Donner L, Fedele LA, Garon CF, Anderson SJ, Sherr CJ: McDonough feline sarcoma virus: characterization of the molecularly cloned provirus and its feline oncogene (v-fms). **J Virol** 41:489, 1982

Downing JR, Roussel MF, Sherr CJ: Ligand and protein kinase C downmodulate the colony-stimulating factor 1 receptor by independent mechanisms. **Mol Cell Biol** 9:2890, 1989

Drachtman RA, Geissler EN, Alter BP: The SCF and c-kit genes in Diamond-Blackfan anemia. **Blood**

79:2177, 1992
Eaves AC, Eaves CJ: Maintenance and proliferation control of primitive hemopoietic progenitors in long-term cultures of human marrow cells. **Blood Cells** 14:355, 1988
Egan SE, Weinberg RA: The pathway to signal achievement. **Nature** 365:781, 1993
Fairbairn LJ, Cowling GJ, Reipert BM, Dexter TM: Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. **Cell** 74:823, 1993
Fenaux P, Preudhomme C, Estienne MH, Morel P, Lai JL, Jouet JP, Bauters F: De novo myelodysplastic syndromes in adults aged 50 or less. A report on 37 cases. **Leuk Res** 14:1053, 1990
Fialkow PJ, Denham AM, Jacobson RJ, Lowenthal MN: Chronic myelogenous leukemia: Origin of some lymphocytes from leukemic stem cells. **J Clin Invest** 62:815, 1978
Flanagan JG and Leder P: The kit ligand: A cell surface molecule altered in steel mutant fibroblasts. **Cell** 63:185, 1990
Foucar K, Langdon RM, Armitage JO, Olson DB, Carroll TJ: Myelodysplastic syndromes: a clinal and pathological analysis of 109 cases. **Cancer** 56:553, 1985
Fraser NJ, Boyd Y, Brownlee GG, Craig IW: Multiallelic RFLP for M27ß, an anonymous single copy genomic clone at Xp11.3-Xcen (HGM9 provisional no.DXS255). **Nucl Acids Res** 15:9616, 1987
Fraser NJ, Boyd Y, Craig IW: Isolation and characterization of a human variable copy number tandem repeat at Xcenp11.22. **Genomics** 5:144, 1989
Fukunaga R, Seto Y, Mizushima S, Nagata S: Three different mRNAs encoding human granulocyte colony stimulating factor receptor. **Proc Natl Acad Sci USA** 87:8702, 1990
Ganser A, Seipelt G, Eder M, Geissler G, Ottmann O, Hess U, Hoelzer D: Treatment of myelodysplastic syndromes with cytokines and cytotoxic drugs. **Seminars in Oncology** 19:95, 1992
Geddes AD, Bowen DT, Jacobs A: Clonal karyotype abnormalities and clinical progress in the myelodysplastic syndrome. **Br J Haematol** 76:194, 1990
Geissler EN, Ryan MA, Housman DE: The dominant white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. **Cell** 55:185, 1988
Gerritsen WR, Donohue J, Bauman J, Jhanwar SC, Kernan NA, Castro-Malaspina H, O'Reilly RJ, Bourhis JH: Clonal analysis of myelodysplastic syndrome: Monosomy 7 is expressed in the myeloid lineage, but not in the lymphoid lineage as detected by fluorescent in situ hybridisation. **Blood** 80:217, 1992
Glinsmann-Gibson B, Spier C, Baier M, Taetle R, Broudy VC, List AF: Mast cell growth factor (c-kit ligand) restores growth of multipotent progenitors in myelodysplastic syndrome. **Leukemia** 8:827, 1994
Goldwasser E: Erythropoietin and differentiation of red blood cells. **Fed Proc** 34:2285, 1975
Green AR, Salvaris E, Begley CG: Erythroid expression of the 'helix-loop-helix' gene, SCL. **Oncogene** 6:475, 1991
Griffin JD, Löwenberg B: Clonogenic cells in Acute Myeloblastic Leukemia. **Blood** 68:1185, 1986
Hagemeyer A, Smit EME, Bootsma D: Improved identification of chromosomes of leukemic cells in methotrexate treated cells. **Cytogenet Cell Genet** 23:208, 1979
Han K, Lee W, Harris CP, Kim W, Shim S, Meisner LF: Quantifying chromosome changes and lineage involvement in myelodyplastic syndrome (MDS) using fluorescent in situ hybridization (FISH). **Leukemia** 8:81, 1994
Hapel AJ, Fung MC, Johnson RM, Young IG, Johnson G, Metcalf D: Biologic properties of molecularly cloned and expressed murine interleukin-3. **Blood** 65:1453, 1985
Heim S, Mitelman F: Myelodysplastic syndromes. In: Cancer Cytogenetics. Alan R. Liss, New York, p111, 1987
Hirai H, Kobayashi Y, Mano H, Hagiwara K, Maru Y, Omine M, Mizoguchi H, Nishida J, Takaku F: A point mutation at codon 13 of the N-ras oncogene in myelodysplastic syndrome. **Nature** 327:430, 1987
Horiike S, Taniwaki M, Misawa S, Abe T: Chromosome abnormalities and karyotypic evolution in 83 patients with myelodysplastic syndrome and predictive value for prognosis. **Cancer** 61:1129, 1988
Horiike S, Misawa S, Nakai H, Kaneko H, Yokota S, Taniwaki M, Yamane Y, Inazawa J, Abe T, Kashima K: N-ras mutation and karyotypic evolution are closely associated with leukemic transformation in

References

myelodysplastic syndrome. Leukemia 8:1331, 1994

Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, Wellner D, Leder P, Besmer P: The hematopoietic growth factor KL is encoded by the Sl locus and is the ligand of the c-kit receptor. Cell 63:225, 1990

Ihle JN, Witthuhn BA, Quelle FW, Silvennoinen O, Tang B, Yi T: Protein tyrosine phosphorylation in the regulation of hematopoiesis by receptors of the cytokine-receptor superfamily. Blood Cells 20:65, 1994

Jacobs A: Human preleukemia: Do we have a model. Br J Cancer 55:1, 1987

Jacobs A, Janowska-Wieczorek A, Caro J, Bowen DT, Lewis T: Circulating erythropoietin in patients with myelodysplastic syndrome. Br J Haematol 73:36, 1989

Jacobs A: The myelodysplastic syndrome. Eur J Cancer 26:1113, 1990

Jacobs RH, Cornbleet MA, Vardiman JW, Larson RA, LeBeau MM, Rowley JD: Prognostic implications of morphology and karyotype in primary myelodysplastic syndromes. Blood 67:1765, 1986

Jacobson LO, Goldwasser E, Fried W, Plzak L: Role of the kidney in erythropoiesis. Nature 179:633, 1957

Janssen JWG, Steenvoorden ACM, Lyons J, Anger B, Böhlke JU, Bos JL, Seliger H, Bartram CR: Ras gene mutations in acute and chronic myelocytic leukemias, chronic myeloproliferative disorders, and mylodysplastic syndromes. Proc Natl Acad Sci USA 84:9228, 1987

Janssen JWG, Buschle M, Layton M, Drexler HG, Lyons J, van den Berghe H, Heimpel H, Kubanek B, Kleihauer E, Mufti GJ, Bartram CR: Clonal analysis of myelodysplastic syndromes: Evidence of multipotent stem cell origin. Blood 73:248, 1989

Jones SS, D'Andrea AD, Hains LL, Wong GG: Human erythropoietin receptor: cloning, expression and biologic characterization. Blood 76:31, 1990

Jonveaux P, Fenaux P, Quiquandon I, Pignon JM, Laï JL, Loucheux-Lefebre MH, Goossens M, Bauters F, Berger R: Mutations in the p53 gene in myelodyplastic syndromes. Oncogene 6:2243, 1991

Just U, Stocking C, Spooncer E, Dexter TM, Ostertag W: Expression of the GM-CSF gene after retroviral transfer in hematopoietic stem cell lines induces synchronous granulocyte-macrophage differentiation. Cell 64:1163, 1991

Jotterand-Bellomo M, Parlier V, Schmidt PM, Beris Ph: Cytogenetic analysis of 54 cases of myelodysplastic syndrome. Cancer Genet Cytogenet 46:157, 1990

Juvonen E, Ruutu T: Effect of culture conditions on in vitro erythroid colony formation in myelodysplastic syndromes. Exp Haematol 18:1070, 1990

Kaczmarski RS, Mufti GJ: The cytokine superfamily. Blood reviews 5:193, 1991

Katevas P, Andonopoulos AP, Kourakli-Symeonidis A, Manopoulou E, Lafti T, Makri M, Zoumbos NC: Peripheral blood mononuclear cells from patients with rheumatois arthritis suppress erythropoiesis in vitro via the production of tumor necrosis factor alpha. Eur J Haematol 53:26, 1994

Katz FE, Tindle R, Sutherland DR, Greaves MF: Identification of a membrane glycoprotein associated with haematopoietic progenitor cells. Leuk Res 9:191, 1985

Kaushansky K, Lin N, Adamson JW: Interleukin 1 stimulates fibroblasts to sythesize granulocyte-macrophage and granulocyte colony-stimulating factors: mechanisms for the hematopoietic response to inflammation. J Clin Invest 81:92, 1988

Kerkhofs H, Hermans, J, Haak HI, Leeksma CHW: Utility of the FAB classification for myelodysplastic syndromes: Investigation of prognostic factors in 237 cases. Br J Haematol 65:73, 1987

Khoury E, Andre C, Pontvert-Delucq S, Drenou B, Baillou C, Guigon M, Najman A, Lemoine FM: Tumor necrosis factor α (TNFα) downregulates c-kit proto-oncogene product expression in normal and acute myeloid leukemia cd34$^+$ cells via p55 TNFα receptors. Blood 84:2506, 1994

Knapp RH, Dewald GW, Pierre RV: Cytogenetic studies in 174 consecutive patients with preleukemic or myelodysplastic syndromes. Mayo Clin Proc 60:507, 1985

Koeffler HP: Myelodysplastic syndromes. Semin Hematol 23:284, 1986

Koury ST, Bondurant MC, Koury MJ: Localization of erythropoietin synthesizing cells in murine kidneys by in situ hybridization. Blood 71:524, 1988

Kreidler BL, Orkin SH, Ihle JN: Loss of erythopoietin responsiveness in erythroid progenitors due to

expression of the Evi-1 myeloid-transforming gene. **Proc Natl Acad Sci USA** 90:6454, 1993

Kriegler M, Perez C, DeFay K, Albert I, Lu SD: A novel form of TNF/cachetin is a cell surface cytotoxic transmembrane protein. Ramifications of the complex physiology of TNF. **Cell** 53:45, 1988

Krönke M, Schütze S, Scheurich P, Meichle A, Hensel G, Thoma B, Kruppa G, Pfizenmaier K: Tumour necrosis factor signal transduction. Mini review. **Cellular Signalling** 2:1, 1990

Layton DM, Mufti GJ: Myelodysplastic syndromes: their history, evolution and relation to acute myeloid leukemia. **Blut** 53:423, 1986

Leary AG, Ogawa M, Strauss LC, Civin CI: Single cell origin of multilineage colonies in culture: evidence that differentiation of multipotent progenitors and restriction of proliferative potential of monopotent progenitors are stochastic processes. **J Clin Invest** 74:2193, 1984

Leary AG, Ogawa M: Blast cell colony assay for umbilical cord blood and adult bone marrow progenitors. **Blood** 69:953, 1987

Leary AG, Ikebuchi K, Hirai Y, Wong GG, Yang Y, Clark SC, Ogawa M: Synergism between interleukin-6 and interleukin-3 in supporting proliferation of human hematopoietic stem cells: comparison with interleukin-1α. **Blood** 71:1759, 1988

Leary AG, Zeng HQ, Clark SC, Ogawa M: Growth factor requirements for survival in G_0 and entry into the cell cycle of primitive human hemopoietic progenitors. **Proc Natl Acad Sci USA** 89:4013, 1992

Lee KH, Kinashi T, Tohyama K, Tashiro K, Funato N, Hama K, Honjo T: Different stromal cell lines support lineage-selective differentiation of the multipotential bone marrow stem cell clone LyD9. **J Exp Med** 173:1257, 1991

Lenarsky C, Weinberg K, Guinan E, Dukes PP, Barak Y, Ortega J, Siegel S, Williams K, Lazerson J, Weinstein H, Parkman R: Bone marrow transplantation for constitutional pure red cell aplasia. **Blood** 71:226, 1988

Liboi E, Carroll M, D'Andrea A, Mathey-Prevot B: Erythropoietin receptor signals both proliferation and erythroid-specific differentiation. **Proc Natl Acad Sci USA** 90:11351, 1993

Linman JW, Bagby GC: The preleukemic syndrome (hemopoietic dysplasia). **Cancer** 42:854, 1978

Levine AJ, Momand J, Finlay CA: The p53 tumour suppressor gene. **Nature** 351:453, 1991

Loetscher H, Pan YCE, Lahm HW, Gentz R, Brockhaus M, Tabuchi H, Lesslauer W: Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor. **Cell** 61:351, 1990

Martin FH, Suggs SV, Langley KE, Lu HS, Ting J Okino KH, Morris CF, McNiece IK, Jacobsen FW, Mendiaz EA, Birkett NC, Smith KA, Johnson MJ, Parker VP, Flores JC, Patel AC, Fisher EF, Erjavec HO, Herrera CJ, Wypych J, Sachdev RK, Pope JA, Leslie I, Wen D, Lin CH, Cupples RL, Zsebo KM: Primary structure and functional expression of rat and human stem cell factor DNAs. **Cell** 63:203, 1990

Matsuda M, Sakamoto N, Fukumaki Y: δ-Thalassemia caused by disruption of the site fot an erythroid-specific transcription factor, GATA-1, in the δ-globin gene promoter. **Blood** 80:1347, 1992

May SJ, Smith SA, Jacobs A, Williams A, Baily-Wood R: The Myelodysplastic syndrome: analysis of labaratory characteristics in relation to the FAB classification. **Br J Haematol** 59:311, 1985

Mayani H, Baines P, Bowen DT, Jacobs A: In vitro growth of myeloid and erythroid progenitor cells from myelodysplastic patients in response to recombinant human granulocyte-macrophage colony-stimulating factor. **Leukemia** 3:29, 1989

McNiece IK, Langely KE, Zsebo KM: Recombinant human stem cell factor synergizes with GM-CSF, G-CSF, IL-3, and Epo to stimulate human progenitor cells of the myeloid and erythroid lineages. **Exp Hematol** 19:226, 1991

Merchav S, Nagler A, Fleischer-Kurtz G, Tatarsky I: Regulatory abnormalities in the marrow of patients with myelodysplastic syndromes. **Br J Haematol** 73:158, 1989

Merchav S, Nielsen OJ, Rosenbaum H, Sharon R, Brenner B, Tatarsky I, Scigalla P, Wieczorek L: In vitro studies of erythropoietin-dependent regulation of erythropoiesis in myelodysplastic syndromes. **Leukemia** 4:771, 1990

Merchav S, Wagemaker G, Souza LM, Tatarsky I: Impaired response of myelodysplastic marrow progenitors to stimulation with recombinant haemopoietic growth factors. **Leukemia** 5:340, 1991

References

Metcalf D: The molecular biology and functions of the granulocyte macrophage colony-stimulating factors. **Blood** 67:257, 1986

Metcalf D: The molecular control of cell division, differentiation commitment and maturation in hematopoietic cells. **Nature** 339:27, 1989

Metcalf D: Hematopoietic regulators: Redundancy or subtlety ? **Blood** 82:3515, 1993

Miura O, D'Andrea A, Kabat D, Ihle JN: Induction of tyrosine phosphorylation by the erythropoietin receptor correlates with mitogenesis. **Mol Cell Biol** 11:4895, 1991

Miura O, Cleveland JL, Ihle J: Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. **Mol Cell Biol** 13:1788, 1993

Miyake T, Kung CK, Goldwasser E: Purification of human erythropoietin. **J Biol Chem** 252:5558, 1977

Moi P, Loudianos G, Lavinha J, Murra S, Cossu P, Casu R, Oggiano L, Longinotti M, Cao A, Pirastu M: δ-Thalassemia due to a mutation in an erythroid-specific binding protein sequence 3' to the δ-globin gene. **Blood** 79:512, 1992

Mori N, Wada M, Yokota J, Terada M, Okada M, Teramura M, Masuda M, Hoshino T, Oshimi K, Mizoguchi H: Mutations of the p53 tumour suppressor gene in haematologic neoplasms. **Br J Haematol** 81:235, 1992

Morishita KE, Parganas D, Parham T, Matsugi T, Ihle JN: The Evi-1 zink finger myeloid transforming gene is normally expressed in the kidney and developing oocytes. **Oncogene** 5:1419, 1990

Morishita K, Parganas E, Matsugi T, Ihle JN: Expression of the Evi-1 zink finger gene in 32Dcl3 myeloid cells blocks granulocytic differentiation in response to granulocyte colony-stimulating factor. **Mol Cell Biol** 12:183, 1992

Mouthon MA, Bernard O, Mitjavila MT, Romeo PH, Vainchenker W, Mathieu-Mahul D: Expression of tal-1 and GATA-binding proteins during human hematopoiesis. **Blood** 81:647, 1993

Munker R, Gasson J, Ogawa M, Koeffler HP: Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor. **Nature** 323:79, 1986

Murase T, Hotta T, Saito H, Ohno R: Effect of recombinant human tumor necrosis factor on the colony growth of human leukemia progenitor cells and normal hematopoietic progenitor cells. **Blood** 69:467, 1987

Nagata S, Tsuchiya M, Asano S, Kaziro Y, Yamazaki T, Yamamoto O, Hirata Y, Kubota N, Oheda M, Nomura M, Ono M: Molecular cloning and expression of cDNA for human granulocyte colony stimulating factor. **Nature** 319:415, 1986

Nagler A, Ginzton N, Negrin R, Bang D, Donlon T, Greenberg P: Effects of recombinant human granulocyte colony stimulating factor and granulocyte-monocyte colony stimulating factor on in vitro hemopoiesis in the myelodysplastic syndromes. **Leukemia** 4:193, 1990

Nakahata T, Gross AJ, Ogawa M: A stochastic model of self-renewal and commitment to differentiation of the primitive hemopoietic stem cells in culture. **J Cell Physiol** 13:455, 1982a

Nakahata T, Ogawa M: Identification in culture of a class of hemopoietic colony-forming units with extensive capability to self-renew and generate multipotential hemopoietic colonies. **Proc Natl Acad Sci USA** 79:3843, 1982b

Nakahata T, Ogawa M: Hemopoietic colony-forming cells in umbilical cord blood with extensive capability to generate mono-and multipotential hemopoietic progenitors. **J Clin Invest** 70:1324, 1982c

Nakamura Y, Komatsu N, Nakauchi H: A truncated erythropoietin receptor that fails to prevent programmed cell death of erythroid cells. **Science** 257:1138, 1992

Nakamura Y: A truncated erythropoietin receptor and cell death: a reanalysis. **Science** 264:588, 1994

Neubauer A, Greenberg P, Negrin R, Ginzton N, Liu E: Mutations in the ras proto-oncogenes in patients with myelodysplastic syndromes. **Leukemia** 8:638, 1994

Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, Glover T, Collins FS, Weston A, Modali R, Harris CC, Vogelstein B: Mutations in the p53 gene occur in diverse human tumour types. **Nature** 342:705, 1989

Ogawa M: Differentiation and proliferation of hematopoietic stem cells. **Blood** 81:2844, 1993

Ohmori M, Ohmori S, Ueda Y, Tohyama K, Yoshida Y, Uchino H: Myelodysplastic syndrome (MDS)-

associated inhibitory activity on haematopoietic progenitor cells. **Br J Haematol** 74:179, 1990

Ohmori M, Ueda Y, Masutani H, Hirama T, Anzai N, Yoshida Y, Okuma M: Myelodysplastic syndrome (MDS)-associated inhibitory activity on haematopoietic progenitor cells: contribution of monocyte-derived lipid containing huge macrophages. **Br J Haematol** 81:67, 1992

Ohyashiki K, Sasao I, Ohyashiki JH, Murakami T, Tauchi T, Iwabuchi A, Toyama K: Cytogenetic and clinical findings of myelodysplastic syndromes with a poor prognosis. **Cancer** 70:94, 1992

Old LJ: Tumor necrosis factor. **Science** 230:630, 1985

Olivieri NF, Grunberger T, Ben-David Y, Ng J, Williams DE, Lyman S, Anderson DM, Axelrad AA, Correa P, Bernstein A, Freedman MH: Diamond-Blackfan anemia: heterogenous response of hematopoietic progenitor cells in vitro to the protein product of the steel locus. **Blood** 9:2211, 1991

Olison I, Gullberg U, Lantz M, Richter J: The receptors for regulatory molecules of hematopoiesis. **Eur J Haematol** 48:1, 1992

Orkin: Globin gene regulation and switching: circa 1990. **Cell** 63:665, 1990

Orkin SH: GATA-binding transcription factors in hematopoietic cells. **Blood** 80:575, 1992

Oster W, Lindemann A, Horn S, Mertelsmann R, Herrmann F: Tumor necrosis factor (TNF)-alpha but not TNF-beta induces secretion of colony stimulating factor for macrophages (CSF-1) by human monocytes. **Blood** 70:1700, 1987

Paul P, Rothmann SA, McMahon JT, Gordon AS: Erythropoietin secretion by isolated rat Kupffer cells. **Exp Hematol** 12:825, 1984

Paquette RL, Landaw EM, Pierre RV, Kahan J, Leubbert M, Lazcano O, Isaac G, McCormick F, Koeffler HP: N-ras mutations are associated with poor prognosis and increased risk of leukemia in myelodysplastic syndrome. **Blood** 82:590, 1993

Perussia B, Trinchieri G, Lehman D, Jankiewicz J, Lange B, Rovera G: Monoclonal antibodies that detect differentiation antigens on human myelomonocytic cells. **Blood** 59:382, 1982

Peters LL, Andrews NC, Eicher EM, Davidson MB, Orkin SH, Lux SE: Mouse microcytic anemia caused by a defect in the gene encoding the globin enhancer-binding protein NF-E2. **Nature** 362:768, 1993

Pevny L, Simon CM, Robertson E, Klein WH, Tsai SF, D'Agati V, Orkin SH, Costantini F: Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. **Nature** 349:257, 1991

Piacibello W, Sanavio F, Bresso P, Severino A, Carlesso N, Pregno P, Stacchini A, Morelli S, Fubini L, Gallo E, Aglietta M, Ferrero D: Stem cell factor improvement of proliferation and maintenance of hemopoietic progenitors in Myelodysplastic syndromes. **Leukemia** 8:250, 1994

Pluznik DH, Sachs L: The cloning of normal "mast" cells in tissue culture. **J Cell Physiol** 66:319, 1965

Prchal JT, Throckmorton DW, Carroll AJ, Fuson EW, Gams RA, Prchal JF: A common progenitor for human myeloid and lymphoid cells. **Nature** 274:590, 1978

Quelle FW, Wojchowski DM: Localized cytosolic domains of the erythropoietin receptor regulate growth signalling and downmodulate responsiveness to granulocyte-macrophage colony-stimulating factor. **Proc Natl Acad Sci** 88:4801, 1991

Quesenberry PJ, Lowry PA: The colony-stimulating factors. An overview. **Cancer** 70:909, 1992

Raskind WH, Tirumali N, Jacobson R, Singer J, Fialkow PJ: Evidence for a multistep pathogenesis of a Myelodysplastic syndrome. **Blood** 63:1318, 1984

Ratajczak MZ, Luger SM, DeRiel K, Abrahm J, Calabrette B, Gewirtz AM: Role of the kit protooncogene in normal and malignant human hematopoiesis. **Proc Natl Acad Sci USA** 89:1710, 1992

Rheingold JJ, Kaufmann R, Adelson E, Lear A: Smoldering acute leukemia. **New Engl J Med** 268:812, 1963

Ridge SA, Workwood M, Oscier D, Jacobs A, Padua RA: FMS mutations in myelodysplastic, leukemic, and normal subjects. **Proc Natl Acad Sci USA** 87:1377, 1990

Roussel MF, Downing JR, Rettenmier CW, Sherr CJ: A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogen product) activates its transforming potential. **Cell** 55:979, 1988

Rowley SD, Sharkis SJ, Hattenburg C, Sensenbrenner LL: Culture from human bone marrow progenitor

References

cells with an extensive proliferative capacity. **Blood** 69:804, 1987

Rusten LS, Jacobsen FW, Lesslauer W, Loetscher H, Smeland EB, Jacobsen SEW: Bifunctional effects of tumor necrosis factor α (TNFα) on the growth of mature and primitive human hematopoietic progenitor cells: Involvement of p55 and p75 TNF receptors. **Blood** 83:3152, 1994a

Rusten LS, Smeland EB, Jacobsen FW, Lien E, Lesslauer W, Loetscher H, Dubois CM, Jacobsen SEW: Tumor necrosis factor-α inhibits stem cell factor-induced proliferation of human bone marrow progenitor cells in vitro. **J Clin Invest** 94:165, 1994b

Ruutu T, Partanen S, Lintula R, Teerenhovi L, Knuutila S: Erythroid and granulocyte-macrophage colony formation in myelodysplastic syndromes. **Scand J Haematol** 32:395, 1984

Sakamaki K, Wang HM, Miyajima I, Kitamura T, Todokoro K, Harada N, Miyajima A: Ligand-dependent activation of chimeric receptors with the cytoplasmic domian of the interleukin-3 receptor ß subunit (ß113). **J Biol Chem** 268:15833, 1993

Sawada K, Sato N, Notoya A, Tarumi T, Hirayama S, Takano H, Koizumi K, Yasukouchi T, Yamaguchi M, Koike T: Proliferation and differentiation of myelodysplastic $CD34^+$ cells: Phenotypic subpopulations of marrow $CD34^+$ cells. **Blood** 85:194, 1995

Schipperus MR, Sonneveld P, Lindemans J, Vink N, Vlastuin M, Hagemeijer A, Abels J: The combined effects of IL-3, GM-CSF, and G-CSF on the in vitro colony growth of myelodysplastic myeloid progenitor cells. **Leukemia Res** 14:1019, 1990

Schouten HC, Vellenga E, van Rhenen DJ, de Wolf JT, Coppens PJ, Blijham GH: Recombinant human erythropoietin in patients with myelodysplastic syndromes. **Leukemia** 5:432, 1991

Seckinger P, Isaaz S, Dayer JM: Purification and biological characterization of a specific tumor necrosis factor alpha inhibitor. **J Biol Chem** 264:11966, 1989

Seeburg PH, Colby WW, Capon DJ, Goedel DV, Levinson AD: Biological properties of human c-Ha-ras-1 genes mutated at codon 12. **Nature** 312:71, 1984

Seipelt G, Ganser A, Duranceyk H, Maurer A, Ottmann OG, Hoelzer D: Induction of TNF-alpha in patients with myelodysplastic syndrome undergoing treatment with interleukin-3. **Br J Haematol** 84:749, 1993

Sherr CJ: Colony-stimulating factor-1 receptor. **Blood** 75:1, 1990

Shinohara K, Ayame H, Tanaka M, Matsuda M, Ando S, Tajiri M: Increased production of tumor necrosis factor-alpha by peripheral blood mononuclear cells in the patients with aplastic anemia. **Am J Hematol** 37:75, 1991

Shulz LD, Schweitzer PA, Rajan TV, Yi T, Ihle JN, Matthews RJ, Thomas ML, Beier DR: Mutations at the murine motheaten locus are within the hematopoietic cell protein tyrosine phosphatase (Hcph) gene. **Cell** 73:1445, 1993

Sieff CA: Hematopoietic growth factors. **J Clin Invest** 79:1549, 1987

Sieff CA, Niemeyer CM, Mentzer SJ, Faller DV: Interleukin-1, tumor necrosis factor, and the production of colony-stimulating factors by cultured mesenchymal cells. **Blood** 72:1316, 1988

Slamon DJ: Proto-oncogenes and human cancers. **N Engl J Med** 317:955, 1987

Sokol L, Prchal JF, D'Andrea A, Rado TA, Prchal JT: Mutation in the negative regulatory element of the erythropoietin recveptor gene in a case of sporadic primary polycythemia. **Exp Haematol** 22:447, 1994

Soligo D, Servida F, Cortelezzi A, Pedretti D, Uziel L, Morgutti M, Pogliani E, Lamberthenghi Deliliers G: Effects of recombinant human stem cell factor (rh-SCF) on colony formation and long-term bone marrow cultures (LTBMC) in patients with myelodysplastic syndromes. **Eur J Haematol** 52:53, 1994

Stebler C, Tichelli A, Dazzi H, Gratwohl A, Nissen C, Speck B: High-dose recombinant erythropoietin for treatment of anemia in myelodysplastic syndromes and paroxysmal nocturnal hemoglobinuria: a pilot study. **Exp Hematol** 18:1204, 1990

Stein RS, Abels RI, Krantz SB: Pharmacologic doses of recombinant human erythropoietin in the treatment of myelodysplasic syndromes. **Blood** 78:1658, 1991

Suda J, Suda T, Ogawa M: Analysis of differentiation of mouse hemopoietic stem cells in culture by sequential replating of paired progenitors. **Blood** 64:393, 1984

Sugimoto K, Hirano N, Toyoshima H, Chiba S, Mano H, Takaku F, Yazaki Y, Hirai H: Mutations of the

p53 gene in myelodysplastic syndrome (MDS) and MDS derived leukemia. **Blood** 81:3022, 1993

Sutherland HJ, Lansdorp PM, Henkelman DH, Eaves AC, Eaves CJ: Functional characterization of individual human hematopoietic stems cell cultured at limiting dilution on supportive marrow stromal layers. **Proc Natl Acad Sci USA** 87:3584, 1990

Symeonidis A, Kourakli A, Katevas P, Perraki M, Tiniakou M, Matsouka P, Georgoulias V, Zoumbos N: Immune function parameters at diagnosis in patients with myelodyplastic syndromes: Correlation with the FAB classification and prognosis. **Eur J Haematol** 47:277, 1991

Takahashi T, Chiba S, Hirano N, Yazaki Y, Hirai H: Characterization of three erythropoietin (EPO)-binding proteins in various human EPO-responsive cell lines and in cells transfected with human EPO-receptor DNA. **Blood** 85:106, 1995

Tefferi A, Thibodeau SN, Solberg LA: Clonal studies in the myelodysplastic syndrome using X-linked restriction fragment length polymorphism. **Blood** 75:1770, 1990

Till JE, McCulloch EA, Siminovitch L: A stochastic model of stem cell proliferation, based on the growth of spleen colony-forming cells. **Proc Natl Acad Sci USA** 51:29, 1964.

Tobal K, Pagliuca A, Bhatt B, Bailey N, Layton DM, Mufti GJ: Mutation of the human FMS gene (M-CSF receptor) in myelodysplastic syndromes and acute myeloid leukemia. **Leukemia** 4:486, 1990

Trentin JJ: Influence of hematopoietic organ stroma (Hematopoietic inductive microenvironments) on stem cell differentiation. In: Regulation of hematopoiesis (ed. Gordon AS). Appleton-Century-Crofts, New York, p161, 1970

Tricot G, Boogaerts MA, de Wolf-Peeters C, van den Berghe H, Verwilghen RL: The myelodysplastic syndromes: different evolution patterns based on sequential morphological and cytogenetic investigations. **Br J Haematol** 59:659, 1985

Ullrich A, Schlessinger J: Signal transduction by receptors with tyrosine kinase activity. **Cell** 61:203, 1990

Van den Berghe H, Cassiman J, David G, Fryns JP, Michaux JL, Sokal G: Distinct haematological disorder with a deletion of long arm of no. 5 chromosome. **Nature** 251:437, 1974

Van den Berghe H, Vermaelen K, Mecucci C, Barbieri D, Tricot G: The 5q- anomaly. **Cancer Genetics and Cytogenet** 17:189, 1985

Van der Reijden HJ, van Rhenen DJ, Landsdorp PM, van 't Veer MB, Langenhuijsen MMAC, Engelfriet CP, Von Dem Borne AEGKr: A comparison of surface marker analysis and FAB classification in acute myeloid leukemia. **Blood** 61:443, 1983

Van Kamp H, Prinsze-Postema TC, Kluin PM, den Ottolander GJ, Beverstock GC, Willemze R, Fibbe WE: Effect of subcutaneously administered human recombinant erythropoietin on erythropoiesis in patients with myelodysplasia. **Br J Hematol** 78:488, 1991

Van Kamp H, de Pijper C, Verlaan-de Vries M, Bos JL, Leeksma CHW, Kerkhofs H, Willemze R, Fibbe WE, Landegent JE: Longitudinal analysis of point mutations of the N-ras proto-oncogene in patients with myelodysplasia using achieved blood smears. **Blood** 79:1266, 1992a

Van Kamp H, Fibbe WE, Jansen RPM, van der Keur M, de Graaff E, Willemze R, Landegent JE: Clonal involvement of granulocytes and monocytes, but not of T and B lymphocytes and natural killer cells in patients with myelodysplastic syndrome: Analysis by X-linked restriction fragment length polymorphism and polymerase chain reaction of the phosphoglycerate kinase gene. **Blood** 80:1774, 1992b

Van Zant G, Shultz L: Hematopoietic abnormalities of the immunodeficient mouse mutant, viable motheaten (mev). **Exp Haematol** 17:81, 1989

Verhoef GEG, de Schouwer P, Ceuppens JL, van Damme J, Goossens W, Boogaerts MA: Measurement of serum cytokine levels in patients with myelodysplastic syndromes. **Leukemia** 6:1268, 1992

Vilcek J, Lee TH: Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. **J Biol Chem** 266:7313, 1991

Visani G, Zauli G, Tosi P, Fogli M, Cenacchi A, Finelli C, Gamberi B, Ottaviani E, Manfoi S, Tura S: Impairement of GM-CSF production in myelodysplastic syndromes. **Br J Haematol** 84:227, 1993

Visvader JE, Elefanty AG, Strasser A, Adams J: GATA-1 but not SCL induces megakaryocytic differentiation in an early myeloid line. **EMBO J** 11:4557, 1992

Vogelstein B, Fearon ER, Hamilton SR, Feinberg AP: Use of restriction fragment length polymorphism to

References

determine the clonal origin of human tumors. Science 227:642, 1985

Ward JC, Harris KW, Penny LA, Forget BG, Kitamura T, Winkelmann JC: A structurally abnormal erythropoietin receptor gene in a human erythroleukemia cell line. Exp Hematol 20:371, 1992

Wiktor-Jedrzejczak W, Szczylik C, Pojda Z, Siekierzynski M, Kanys J, Klos M, Ratajzak MZ, Pejcz J, Jaskulski D, Gornas P: Success of bone marrow transplantation in congenital Diamond-Blackfan anemia: A case report. Eur J Haematol 38:204, 1987

Williams DE, Eisenman J, Baird A, Rauch C, van Ness K, March CJ, Park LS, Martin U, Mochizuki DY, Boswell HS, Burgess GS, Cosman D, Lyman SD: Identification of a ligand for the c-kit proto-oncogene. Cell 63:167, 1990

Winkelmann JC, Ward J, Mayeux P, Lacombe C, Schimmenti L, Jenkins RB: A translocated erythropoietin receptor gene in a human erythroleukemia cell line (TF-1) expressed an abnormal transcript and a truncated protein. Blood 85:179, 1995

Wisniewski D, Strife A, Atzpodien J, Clarkson BD: Effects of recombinant human tumor necrosis factor on highly enriched progenitor cell populations from normal human bone marrow and peripheral blood and bone marrow from patients with chronic myeloid leukemia. Cancer Res 47:4788, 1987

Witte ON: Steel locus defines new multipotent growth factor. Minireview. Cell 63:5, 1990

Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O, Ihle JN: Jak2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227, 1993

Wodnar-Filipowicz A, Tichelli A, Zsebo KM, Speck B, Nissen C: Stem cell factor stimulates the in vitro growth of bone marrow cells from aplastic anemia patients. Blood 12:3196, 1992

Wodnar-Filipowicz A, Yancik S, Moser Y, Carbonare V, Gratwohl A, Tichelli A, Speck B, Nissen C: Levels of soluble stem cell factor in serum of patients with aplastic anemia. Blood 81:3259, 1993

Wognum AW, Lansdorp PM, Humphries RK, Krystal G: Detection and isolation of the erythropoietin receptor using biotinylated erythropoietin. Blood 76:696, 1990

Wong KF, Chan JKC, Chu YC, Kwong YL: Clonal evolution in primary 5q- syndrome. Cancer 70:100, 1992

Woolford J, Mcauliffe A, Rohrschneider LR: Activation of the feline c-fms proto-oncogene: multiple alterations are required to generate a fully transformed phenotype. Cell 55:965, 1988

Yamamura Y, Kageyama Y, Matuzaki T, Noda M, Ikawa Y: Distinct downstream signaling mechanism between erythropoietin receptor and interleukin-2 receptor. EMBO J 11:4909, 1992

Yi T, Zhang J, Miura O, Ihle JN: Hematopoietic cell phosphatase associates with erythropoietin (EPO) receptor after Epo-induced receptor tyrosine phosphorylation: Identification of potential binding sites. Blood 85:87, 1995

Young NS: The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated. Blood 79:1385, 1992

Youssoufian H, Longmore G, Neumann D, Yoshimura A, Lodish HF: Structure, function and activation of the erythropoietin receptor. Blood 81:2223, 1993

Yunis JJ, Rydell RE, Oken MM, Arnesen MA, Mayer MG, Lobell M: Refined chromosome analysis as an independent prognostic indicator in de novo myelodysplastic syndromes. Blood 67:1721, 1986

Yunis JJ, Lobell M, Arnesen MA, Oken MM, Mayer MG, Rydell RE, Brunning RD: Refined chromosome study helps define prognostic subgroups in most patients with primary myelodysplastic syndrome and acute myelogenous leukaemia. Br J Haematol 68: 189, 1988

Zhuang H, Patel SV, He T, Sonsteby SK, Niu Z, Wojchowski DM: Inhibition of erythropoietin-induced mitogenesis by a kinase-deficient form of Jak2. J Biol Chem 269:21411, 1994

Zon LI, Youssoufian H, Mather C, Lodish HF: Activation of the erythropoietin receptor promoter by transcription factor GATA-1. Proc Natl Acad Sci USA 88:10638, 1991

Zsebo KM, Wypych J, McNiece IK, Lu HS, Smith KA, Karkare SB, Sachdev RK, Yuschenkoff VN, Birkett NC, Williams LR, Satyagal VN, Tung W, Bosselman RA, Mendiaz EA, Langley KE: Identification, purification, and biological characterization of hematopoetic stem cell factor from Buffalo rat liver-conditioned medium. Cell 63:195, 1990

SUMMARY/SAMENVATTING

SUMMARY

Chapter 1 gives a brief overview of the current understanding of normal hematopoiesis. Steady state hematopoiesis is regulated by a network of interactions between positive and negative hematopoietic regulatory proteins. These hematopoietic growth factors, exerting their biological effects through interactions with specific membrane spanning receptors, play a complex, regulatory role in normal hematopoiesis. The identification and cloning of various hematopoietic growth factors (HGFs), enabled the production of large quantities of pure HGFs and made *in vitro* studies possible. Investigation of normal hematopoiesis may elucidate mechanisms involved in disturbed hematopoiesis in hematopoietic malignancies such as the myelodysplastic syndromes. The myelodysplastic syndromes (MDS) represent a heterogenous group of disorders of hematopoietic pluripotent stem cells characterized by ineffective hematopoiesis resulting in anemia, neutropenia and/or thrombocytopenia. Several lines of evidence indicate that MDS is a clonal disorder. Approximately one third of patients with MDS show progression to acute nonlymphocytic leukemia. Most MDS patients show anemia as one of the most prominent abnormalities at the time of diagnosis. The mechanisms leading to ineffective erythropoiesis are still unclear. The *in vitro* growth factor response to erythropoietin (EPO) generally is very poor. A better understanding of EPO unresponsiveness in MDS may potentially add to the development of therapeutic strategies for treatment of anemia in these patients.

Tumor necrosis factor-alpha (TNFα) is a HGF with pleiotropic effects on a variety of cells. These effects can either be mediated directly, e.g. via progenitor cells, or indirectly by inducing the release of HGFs from other cells. In Chapter 2 experiments of TNFα on highly enriched normal bone marrow cells (in order to avoid interference of accessory cells) are presented. We demonstrate that TNFα has opposite effects on the *in vitro* proliferative abilities of normal bone marrow progenitors, depending on the type of stimulus (HGF) and concentration of TNFα used. TNFα may suppress the colony-forming abilities of CFU-G and BFU-E in the presence of G-CSF or EPO. In contrast, TNFα enhances the proliferative response of CFU-Eo to IL-3 or GM-CSF. These results suggest that TNFα favours the outgrowth of progenitors that are under the control of more primitive HGFs, like IL-3 and GM-CSF. At the same time TNFα inhibits the proliferative abilities of the progenitors that respond to the late-acting, single lineage factors G-CSF

and EPO. We assume that the effects of TNFα are accomplished through direct interactions of TNFα with selected target cell progenitors.

The blast cell colony assay, identifying relatively primitive, multilineage progenitor cells, was used to study the proliferative capacities of MDS progenitor cells. In Chapter 3 we demonstrate that highly purified MDS progenitor cells generate normal numbers of blast cell colonies. However, a marked qualitative difference between MDS and normal derived CFU-blast became evident when blast cell colonies were replated in secondary cultures. In MDS, secondary colonies derived from these CFU-blast are unable to generate erythroid progeny. Besides reduced erythroid colony numbers, also reduced mixed and eosinophilic colony numbers are generated from MDS blast colonies. These observations are indicative of an MDS progenitor cell abnormality that involves more than one maturation lineage. The incompetence of maturation of MDS thus may reside in the CFU-blast progenitor cell being incapable of properly responding to growth factor stimulation.

The *in vitro* colony forming capacities of MDS marrow clonogenic cells are frequently impaired. KL enhances the *in vitro* colony formation induced by EPO, G-CSF, GM-CSF or IL-3 in normal marrow considerably.

Chapter 4 describes the effects of KL on EPO induced colony formation in MDS as well as normal marrow. When KL, in combination with EPO, is added to culture, the subnormal erythroid response to EPO is reconstituted to normal levels in a significant proportion of MDS cases. The latter enhancement by KL, regarding both colony number and size, appears restricted to low-risk cytological types of MDS, RA and RARS. Little or no enhancement was apparent following KL stimulation of marrow from patients with RAEB, RAEB-t and CMML (high-risk MDS). These results suggest that KL responsiveness of patients with low-risk MDS may still be intact, and that with progression to high-risk MDS, erythroid progenitors lose proliferative reactivity to both KL and EPO stimulation. This impaired erythroid response to EPO and/or KL stimulation could involve a quantitative lack of EPO or KL receptors on MDS marrow cells, their dysfunction or an abnormality along the signalling pathway.

Chapter 5 deals with the analysis of EPO-R expression and c-kit (KL-receptor) expression in patients with different subtypes of MDS. C-kit is expressed on marrow cells

in most MDS patients, including those not responding to KL in EPO induced cultures. EPO-R are present on all MDS specimens studied. However, EPO cannot induce erythroid colony formation even in those MDS patients revealing relatively high numbers of cells expressing the EPO-R. Analysis of the different splice variants of the EPO-R demonstrate that MDS cells, like normal cells, express the full length EPO-R. Hence, a quantitative lack of EPO-R expression is unlikely to explain the subnormal EPO responsiveness of erythroid progenitor cells in MDS. Results dealing with EPO- and CD34 coexpression reveal an altered phenotype of EPO-R positive cells in MDS. The phenotypic asynchrony of EPO-R and CD34 positive cells in MDS, indicative of abnormal maturation, however, does not correlate with impaired erythroid response. Taken together these results suggest that impaired erythroid response in MDS cannot be explained by a quantitative lack of receptors for EPO or KL.

In Chapter 6 the results are discussed in the perspective of future studies that may be useful to uncover possible mechanisms of impaired erythroid response in MDS.

SAMENVATTING

Het continue proces van bloedcelvorming wordt hematopoiese genoemd. In het beenmerg bevinden zich hematopoietische stamcellen die zich vermenigvuldigen en uitrijpen tot de verschillende soorten functionele bloedcellen, de rode en witte bloedcellen en bloedplaatjes. Dit complexe proces wordt gereguleerd door een aantal moleculen, de zogenaamde hematopoietische groeifactoren. Deze hematopoietische groeifactoren binden aan beenmerg- en bloedcellen via specifieke membraanreceptoren en stimuleren de cellen. Na binding treden er allerlei veranderingen in de cel op, die leiden tot bijvoorbeeld celdeling of uitrijping of tot activering van bepaalde functies van de cel.

Het myelodysplastisch syndroom (MDS) is een verworven aandoening waarbij het proces van bloedcelvorming defect is en de bloedcellen kwantitatief in onvoldoende mate worden aangemaakt en kwalitatief hun functie niet goed kunnen uitoefenen. Uit onderzoek is gebleken dat bij patiënten met MDS de pluripotente hematopoietisch stamcel niet in staat is normaal uit te rijpen tot de verschillende functionele bloedcellen. Het gevolg is dat normale hematopoiese verstoord raakt en patiënten met MDS lijden daardoor onder andere aan een tekort aan functionele rode bloedcellen (anemie), witte bloedcellen (leukopenie) en/of bloedplaatjes (thrombopenie). Gebaseerd op celmorfologie en het aantal onrijpe cellen (blasten) in beenmerg en bloed kunnen MDS patiënten worden onderverdeeld in 5 subklassen: (1) refractaire anemie (**RA**), (2) RA met ring sideroblasten (**RARS**), (3) RA met overmaat blasten (**RAEB**), (4) RAEB in transformatie (**RAEB-t**) en (5) chronische myelomonocytaire leukemie (**CMML**). Bij ongeveer 1/3 deel van de patiënten gaat de ziekte over in een acute myeloide leukemie (AML). Bij patiënten met MDS is de anemie het meest frequente symptoom. De afwijking in de erythropoiese dat tot deze anemie leidt is onopgehelderd. Normaliter worden beenmergcellen onder invloed van de groeifactor erythropoietine (EPO) aangezet tot rode bloedcelvorming. De beenmergcellen van MDS patiënten reageren onvoldoende op EPO. Onderzoek naar het mechanisme van de verstoorde EPO respons beoogt inzicht te geven in de aard van de afwijkingen in de beenmergcellen van patienten met MDS. Dit inzicht kan nuttig zijn voor de behandeling van de anemie.

In hoofdstuk 1 worden de morfologische en klinische aspecten van het myelodysplastisch syndroom besproken. Verder worden de methoden aangeduid waarmee is aangetoond dat MDS een klonale ziekte is. De abnormale bloed -en beenmerg cellen bij

Samenvatting

MDS stammen af van een gemeenschappelijke veranderde hematopoietische stamcel (een kloon). De huidige kennis van de normale hematopoiese wordt beknopt behandeld. Deze kennis is voor een groot deel verworven door het kweken van bloedcellen onder toevoeging van specifieke groeifactoren (*in vitro* kweeksystemen).

Tumor necrosis factor α (TNFα) is een groeifactor die door diverse cellen wordt gemaakt en beenmerg -en bloedcellen kan stimuleren. TNFα kan zowel rechtstreeks als indirect de cellen in het beenmerg stimuleren. In het laatste geval zet TNFα cellen aan tot de produktie van hematopoietische groeifactoren, die op hun beurt de beenmergcellen stimuleren. In hoofdstuk 2 worden de effecten van TNFα op gezuiverde beenmergstamcellen, afkomstig van "gezonde" donoren, besproken. De rijpere bloedcellen, die allerlei factoren afgeven en daardoor de waarneming kunnen beinvloeden, zijn grotendeels verwijderd. Het blijkt dat het effect van TNFα op beenmergcellen afhangt van de gebruikte TNFα concentratie. De koloniegroei onder invloed van EPO uit erythroide voorlopercellen (BFU-E), en de koloniegroei geinduceerd door granulocyt kolonie-stimulerende factor (G-CSF) uit granulocytaire voorlopercellen (CFU-G) worden beiden geremd door TNFα. TNFα stimuleert daarentegen de koloniegroei van eosinophiele voorlopercellen (CFU-Eo) na stimulatie in kweek met interleukine-3 (IL-3) of granulocyt macrofaag kolonie-stimulerende factor (GM-CSF). Deze resultaten tonen aan dat TNFα de groei van voorlopercellen die onder invloed staan van vroeg aangrijpende HGF, zoals IL-3 en GM-CSF, stimuleert. Daarentegen remt TNFα voorlopercellen die afhankelijk zijn van de laat aangrijpende groeifactoren, zoals EPO en G-CSF. TNFα remt of stimuleert dus het effect van groeifactoren op beenmergcellen geheel afhankelijk van het type groeifactor.

De blastcel kolonie assay, een speciaal ontwikkelde kweekmethode om zeer onrijpe voorlopercellen van "gezond" beenmerg (normaal beenmerg=NBM) te bestuderen, kan worden gebruikt om de zeer onrijpe MDS voorloper beenmergcellen (stamcellen) te bestuderen. In hoofdstuk 3 wordt aangetoond dat MDS stamcellen en NBM stamcellen een vergelijkbaar aantal blastcelkolonies induceren. Er is echter een kwalitatief verschil tussen kolonie vorming afkomstig van MDS en die van NBM. Wanneer de blastkolonies namelijk uitgeplaat worden in zogenaamde secondaire kweken blijkt dat de cellen afkomstig van MDS beenmerg niet in staat zijn een nieuwe generatie erythroide cellen te genereren. Dit in tegenstelling tot NBM waaruit in opeenvolgende kweken wel erythroide cellen kunnen

worden opgewekt. Bovendien is de gemengde (erythroide èn myeloide) en eosinophiele koloniegroei bij MDS ook sterk verminderd. Dus bij MDS patienten is de stamcel niet in staat om adequaat op groeifactor stimulatie te reageren en normaal de rijpingsweg af te leggen.

In NBM stimuleert een andere groeifactor "kit-ligand (KL)" de door EPO, G-CSF, GM-CSF en IL-3 geïnduceerde koloniegroei aanzienlijk. In hoofdstuk 4 worden de resultaten besproken van het effect van KL op de door EPO gestimuleerde koloniegroei in MDS beenmerg. De *in vitro* koloniegroei van MDS beenmergcellen is over het algemeen genomen sterk gereduceerd. Het blijkt dat KL in staat is om de verlaagde EPO respons in een aanzienlijk deel van de MDS patiënten (gedeeltelijk) te herstellen. Dit stimulerend effect van KL lijkt echter beperkt tot de groep zogenaamde "low risk" MDS patiënten, RA en RARS. KL heeft weinig tot geen effect op beenmergcellen afkomstig van patiënten met RAEB, RAEB-t en CMML ("high risk" MDS). Deze resultaten suggereren dat beenmergcellen van "low risk" MDS patiënten nog in staat zijn adequaat op KL te reageren. De erythroide voorlopercellen van "high risk" MDS patiënten blijken echter het vermogen verloren te hebben om op KL adequaat te reageren. De verstoorde erythroide respons zou veroorzaakt kunnen worden door het feit dat beenmergcellen van MDS patiënten geen of slecht functionerende receptoren hebben voor EPO en KL, of door een verminderde signaalgeleiding vanuit deze receptoren.

Hoofdstuk 5 beschrijft het onderzoek naar de aanwezigheid van receptoren voor EPO (EPO-receptor, EPO-R) en KL (KL-receptor, c-kit) op MDS beenmergcellen. Receptoren voor KL (c-kit) zijn aanwezig op beenmergcellen van de meeste MDS patiënten, inclusief die patiënten die niet reageren op KL in EPO gestimuleerde kweken. EPO-R zijn aanwezig op beenmergcellen van alle onderzochte MDS patiënten. Maar zelfs bij MDS patiënten waarbij een relatief hoog aantal cellen met EPO-R kan worden aangetoond is EPO niet in staat erythroide koloniegroei te induceren. De verstoorde erythroide response in MDS wordt dus niet veroorzaakt door een afwezigheid van receptoren voor EPO of KL. Analyse van de verschillende splice-varianten van de EPO-R toont aan dat in zowel NBM als MDS de "full-length" EPO-R tot expressie komt. Verder is met behulp van dubbelimmunofluorescentie gekeken naar cellen die zowel de EPO-R als CD34 (antigen, aanwezig op onrijpe voorlopercellen) tot expressie brengen. Uit deze resultaten blijkt dat, hoewel de MDS cellen een gewijzigd fenotype vertonen ten aanzien van EPO-R en CD34

coëxpressie, de verlaagde groeifactorrespons in MDS niet verklaard kan worden door het feit dat CD34 positieve voorlopercellen in het MDS beenmerg geblokkeerd zijn in hun uitrijping en EPO-R negatief blijven.

In hoofdstuk 6 worden de resultaten besproken in de context van de literatuur. De mogelijke rol van TNFα in de pathogenese van verschillende ziekten, waaronder MDS, wordt besproken. Tevens wordt de huidige kennis omtrent KL, zijn receptor c-kit, de EPO-R en daarbij betrokken signaalmoleculen behandeld ten aanzien van de mogelijke mechanismen van verminderde erythroide respons in MDS.

DANKWOORD

Hoewel wetenschappelijk gezien niet het meest opzienbarende onderdeel, vormt het dankwoord toch wel het meest gelezen stukje van het proefschrift. Om (eventuele) teleurstellingen te voorkomen, wil ik daarom beginnen met het bedanken van iedereen die, in wat voor vorm dan ook, hebben meegewerkt aan dit proefschrift. Mocht je je naam (onterecht) niet kunnen terugvinden in dit stukje, troost je met het feit dat mijn dank niet minder groot is (sorry hoor, vergeten is menselijk).

De eerste die ik met name wil noemen is mijn promotor en begeleider Prof. Dr. B. Löwenberg. Bob, ondanks je vaak (te) overvolle agenda wist je toch altijd tijd vrij te maken om in kritisch overleg de voortgang van het onderzoek te bespreken. Bij deze wil ik je bedanken voor de begeleiding de afgelopen jaren.

Prof. Dr. A. Hagenbeek, Prof. Dr. A.M.M.J. Hagemeijer-Hausman en Prof. Dr. M.M.A.C.Langenhuijsen wil ik bedanken voor het kritisch doorlezen van het manuscript.

Lianne, nooit te beroerd om langer door te werken, verdient toch wel een ereplaats. Ik ben je dankbaar voor jouw nimmer aflatende enthousiasme bij het tellen van het zoveelste GEMM'etje. Hoewel de blastkolonies uiteindelijk in onze nachtmerries verschenen meen ik toch te mogen zeggen dat we gelukkig niet voor niets eindeloos hebben zitten replaten (hè, hè, toch nog paranimf). Lianne, bedankt voor de goede en prettige samenwerking.

Mede(lot)kamergenoten (voor korte of langere tijd), Leo, Frans, Freek, Machteld, Valeria (hello chippola), Dong Fan, Inge, alsmede niet-kamergenoten Ivo, Ruud D. en Lies, allemaal bedankt voor de prettige samenwerking en gezelligheid.

Wat is een lab zonder analisten, die naast research, vaak klusjes opknappen waarvan de essentie pas blijkt als ze een keer niet gedaan (kunnen) worden. Hans, Carin, Karin (ik hoop nog vaak "even" met jou te bellen), Marleen, Anita en reeds vertrokken Pauline, Ruud v G., Ton, Thecla, Loes, José, jullie hebben allemaal gezorgd voor een leuke tijd op de Daniel.

Een aantal mensen buiten "Celkweek" wil ik graag even in het zonnetje zetten. Adrie Bot wil ik bedanken voor het feit dat hij altijd opgewekt voor me klaar stond als de FACS toch wel erg raar deed. Adrie ik ben je dankbaar voor het menigmaal verzorgen en weer sorteerklaar maken van je troetelkindje. Verder bedank ik de "jongens van Immuno", afdeling Morfologie van het KCL (Joke en Sipkje), Bert Wognum en de afdeling Cytogenetica (EUR) voor hun hulp. De beschreven experimenten waren niet mogelijk geweest zonder de bereidheid van artsen om "extra" beenmerg te puncteren. Dr. P.

Dankwoord

Wijermans, Dr. K van de Heul, en alle andere niet met name genoemde artsen, alsmede alle anoniem gebleven patiënten , bedankt voor de "beenmergdonaties".

Verder wil ik de afdeling Fotografie bedanken voor het, altijd op tijd, afleveren van "mooie plaatjes". Janine Vrij en Jeanne Vlasveld, ontzettend bedankt voor de keurige verzorging van al het papierwerk.

Tot slot mijn familie. Mijn ouders ben ik enorm dankbaar. Zij hebben voor een thuisbasis gezorgd waar ik altijd met veel plezier naar toe ging. Ik ben hun dankbaar voor hun enorm stimulerende invloed. Helaas heeft "ons pa" de voltooiing van mijn proefschrift niet meer mee mogen maken. Lieve mam, naast het feit dat we ons pa elke dag opnieuw enorm missen, weten we ook dat hij niets liever wilde dan dat ik dit zou afronden. Ik wil dit proefschrift dan ook speciaal aan hem opdragen. Lieve Jos, ook jij hebt me enorm gestimuleerd om toch maar door te zetten. Zullen we samen met onze moppies er nog heel wat leuke jaartjes aan vastknopen?

CURRICULUM VITAE

Bianca Backx werd geboren op 27 april 1964 te Roosendaal. Na het behalen van haar VWO diploma (gymnasium ß) 1982 op het Gertrudis Lyceum te Roosendaal begon zij in datzelfde jaar aan de studie Biologie aan de Rijks Universiteit Utrecht. Tijdens deze studie werd door haar onderzoek verricht bij de vakgroep Moleculaire Celbiologie en bij de vakgroep Hematologie aan het Academisch Ziekenhuis beiden te Utrecht. In 1988 behaalde zij haar doctoraaldiploma Medische Biologie (B5*). Van september 1988 tot april 1993 was zij werkzaam op de afdeling Celkweek van de Daniel den Hoed Kliniek te Rotterdam op een door de Nederlandse Kankerbestrijding gefinancieerd project. Gedurende deze periode werd het in dit proefschrift beschreven onderzoek verricht onder begeleiding van Prof. Dr. B. Löwenberg.