THE MYELODYSPLASTIC SYNDROME:

In vitro growth characteristics of hemopoietic progenitor cells

HET MYELODYSPLASTISCH SYNDROOM:

In vitro groeieigenschappen van hemopoietische voorlopercellen

PROEFSCHRIFT

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Martin Roelof Schipperus
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PROMOTIECOMMISSIE

Promotores: Prof. Dr. J. Abels

                         Prof. Dr. O. Vos

Overige leden: Prof. Dr. B. Löwenberg

                         Dr. R. E. Ploemacher

Voor Marette

Voor Ouren

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CHAPTER 1

GENERAL INTRODUCTION

1.1. HEMOPOIESIS

1.1.1. Pluripotent stem cells

Most of the mature blood cells have a limited lifespan and need to be replaced constantly throughout life. This process of blood cell formation, termed hemopoiesis, is enormous in scale. Some 2×10^{11} erythrocytes and 1.2×10^{11} neutrophils are produced each day. Apart from this large production hematopoiesis has to render cells of eight distinct cell lineages, i.e.: erythrocytes, platelets, neutrophils, eosinophils, basophils, monocytes, B- and T-lymphocytes. It is generally accepted that all mature blood cells, including lymphocytes, originate from common pluripotent hemopoietic stem cells (PHSC) located in the bone marrow in man and also in the spleen in mice. (Till JE & Mc Culloch 1961, Van Bekkum, 1977, Quesenberry et al., 1979; Ogawa et al, 1983)(fig 1). Apart from PHSC and their progeny, precursors of tissue mast cells (Kitamura et al., 1971), osteoclasts (Ash et al., 1980), Kupffer cells (Van Furth, 1980) and dendritic cells (Steinman et al., 1974) are present in the bone marrow. Attempts to purify stem cells have led to the recognition of subpopulations of stem cells with different self-renewal, proliferation and differentiation capacities (Visser et al., 1984, Ploemacher and Brons 1988). The stem cell compartment is heterogeneous with regard to self-renewal and proliferation capacity (Simonovitch, 1963; Worton et al, 1969) and there is a hierarchy of stem cells depending on the number of previous divisions. In later publications evidence came available that after a higher division rate the cells become more committed to differentiation, whereas the stem cells with a low division rate still have the largest proliferative capacity (Rosendaal et al., 1979; Mauch et al, 1980; Hodgson et al, 1984). The self-renewal capacity of the stem cell compartment is extensive and probably does not decline during the normal life-span, but may decrease following extensive stress to the marrow (Mauch et al, 1988).

1.1.2. Models of stem cell renewal and commitment

Several models have been proposed for the mechanism of stem cell self-renewal and commitment. Till and McCulloch (1964) postulated a stochastic model in which the decision of a stem cell to either renew itself, or yield daughter cells committed to differentiation, is governed by probability rules. Trentin (1970) introduced the hemopoietic inductive microenvironment (HIM) model implying that commitment of
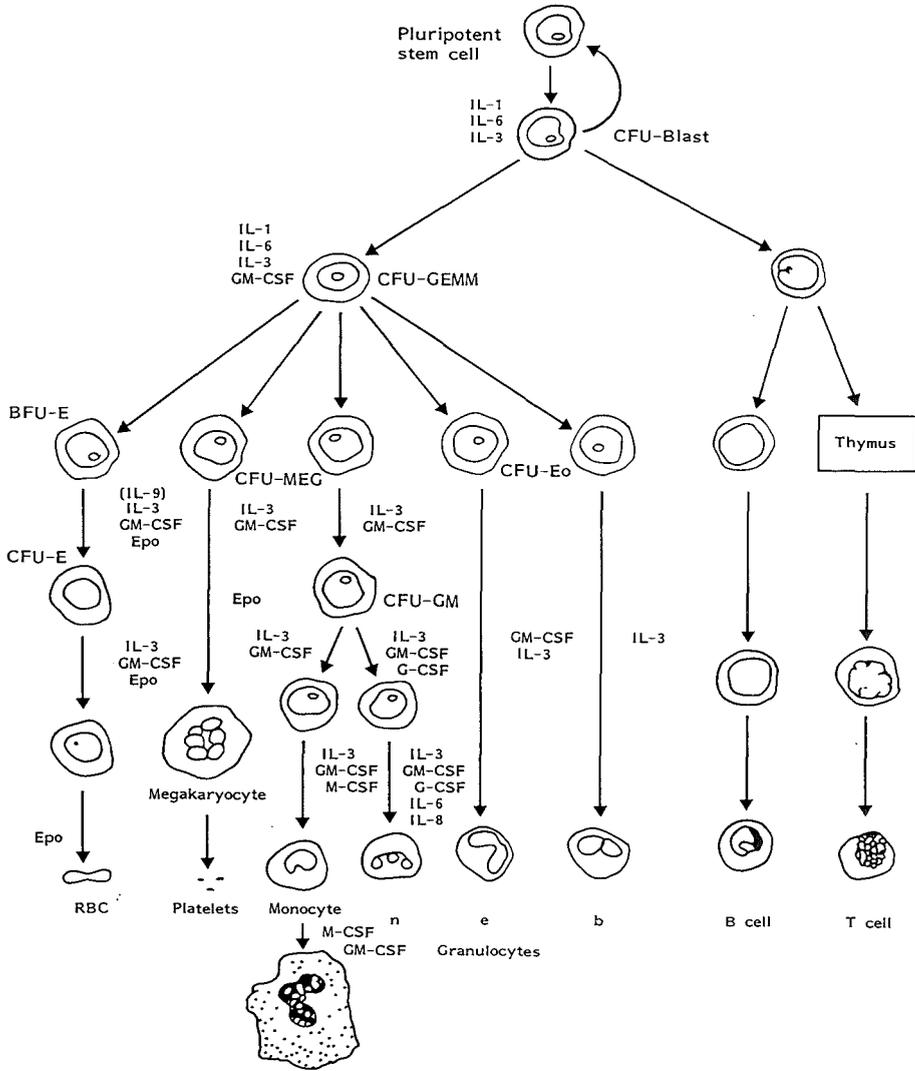
Figure 1. Scheme of normal hemopoiesis and interactions of the colony stimulating factors and interleukins with hemopoietic cells. CFU = colony forming unit; BFU = burst forming unit; IL = interleukin; CSF = colony stimulating factor; Epo = erythropoietin. Adapted from Clark SC and Kamen R. The human hematopoietic colony-stimulating factors. Science 1987; 236: 1229.

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pluripotent hemopoietic stem cells to monopotent progenitors is determined by a specific inductive microenvironment surrounding the stem cell. However, the observations by Magli et al. (1982) on the transient nature of early colonies have presented the need for reinterpretation of the experimental data on which the HIM model was established. In a third model it was proposed that the regulation of differentiation is controlled by humoral factors. The "stem cell competition" model by Van Zant and Goldwasser (1977,1979) is an example of this hypothesis. Their data suggested that humoral factors play an active role in stem cell commitment. However, this model has not gained much support. Therefore, the nature of stem cell commitment remains obscure. Evidence exists indicating that under stress situations, such as bacterial infections or blood loss, mono- and bipotential progenitor cell populations amplify and show higher than normal levels of proliferative activity (Schooly et al, 1965; Lange et al, 1969), whereas the early pluripotent progenitor cells are not triggered into cell cycle (Bruce et al. 1964; Hara et al, 1977). It has been shown that under these conditions humoral factors with stimulatory activity on the proliferation of hematopoietic cells are produced (Watari et al., 1989). The first humoral factors that were reported to stimulate the proliferation of stem cells were ß-adrenergic agents (Byron et al, 1972). More recently it has been shown that the colony stimulating factors, described in more detail below, regulate hemopoiesis not only in vitro but also in vivo (Donahue et al, 1986), indicating that also in vivo stem cells can respond to specific hormonal signals.

1.1.3. In vitro clonogenic assays

The identification of the hemopoietic progenitor cells still depends on in vitro clonogenic assays developed more than twenty years ago. Pluznik and Sachs (1966) and Bradley and Metcalf (1966) discovered independently that suspensions of individual cells from mouse bone marrow could be induced to form colonies of mature granulocytes and macrophages. Each of the colonies consists of one cell clone. In 1970 Pike and Robinson adapted this culture system for the use of human marrow cells. Whereas at first only the growth of granulocyte-macrophage colonies could be induced, improvement of the colony culture system made it possible to grow erythroid and mixed colonies as well. The cell producing a colony is called a colony forming

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unit (CFU). The most primitive in vitro colony forming cell is the CFU-blast (Naka-hata and Ogawa 1982; Suda et al., 1983), which appears to be more primitive than the CFU-GEMM, a pluripotent cell rendering mixed granulocyte-erythrocyte-macrophage-megakaryocyte colonies (Johnson and Metcalf, 1977; Hara et al., 1981; Fauser et al., 1979; Johnson et al, 1980). Recently, using a miniaturized stroma-dependent bone marrow culture assay, various progenitor cell types have been identified in the mouse, which are more primitive than the CFU-GEMM and the CFU-blast. This cobblestone area-forming cell (CAFC) includes primitive precursor cells resposible for long term in vivo repopulation of a depleted hemopoietic system (Ploemacher et al, 1989). The progeny of the CFU-GEMM, the committed progenitor cells produce either granulo-cyte-macrophage colonies (CFU-GM) (Bradley and Metcalf, 1966; Ichikawa et al., 1966; Pike et al., 1970), or colonies of only neutrophilic granulocytes (CFU-G), macrophages (CFU-M), eosinophils (CFU-Eo) (Chervenick et al., 1971; Johnson and Metcalf, 1980), erythroid cells (BFU-E and more mature CFU-E) (Stephenson et al., 1971; Axelrad et al., 1973; Tepperman et al., 1974) or megakaryocytes (CFU-Meg) (Metcalf et al., 1975; Vainchenker et al., 1979) (Fig.1). In most of the culture systems feeder layers were included with leucocytes or cells from an in vitro established cell line. Later it was found that the conditioned medium of the feeder cells contained substances capable to induce colony growth. These substances have become known as the colony stimulating factors.

1.1.4. In vitro clonogenic assays in acute myeloblastic leukemia

Shortly after the development of culture techniques for normal hemopoietic progenitor cells these methods were applied in acute myeloblastic leukemias (Robinson et al., 1971; Moore et al., 1973 and 1974). It was found that despite the apparent homogeneity of most populations of acute myelogenous leukemia (AML) cells, only a small fraction of cells had the capacity to proliferate in vitro. These clonogenic leukemic cells possibly act as stem cells in vivo to maintain the rest of the leukemic cell population. The normal clonogenic assays were often found to be insufficient to induce the formation of AML colonies. The growth of AML clonogenic cells in vitro is characterized by micro- (less than 20 cells) and macro-cluster (20 - 40 cells) formation with defective maturation or persistent blasts within the clusters, a

high cluster colony ratio, singly persisting blasts, or a very low colony formation (<2 colonies/10^5 marrow cells) (Moore et al., 1974). It was concluded that specific clonogenic assays were required to induce more efficient AML colony growth. Exposure of the cells to phytohemaglutinin (PHA) during a 15-hour preincubation in suspension and subsequent culture in soft agar with a leukocyte feeder resulted in the formation of significant numbers of AML clonies of more than 50 cells in the majority of AML patients (Dicke et al., 1976).

In subsequent modifications of the technique, PHA was added directly to cultures containing irradiated leukocytes or cell-line conditioned medium in the agar under-layer and the AML target cells in a liquid overlayer (Löwenberg and Hagemeijer, 1977 and 1980, Schipperus et al., 1988). In these modified cultures it was possible to induce AML colony formation in 80-90% of cases (Swart et al., 1982). The colonies are composed of morphologically identifiable blast cells carrying abberant karyotypes, typical for the AML clone (Löwenberg and Hagemeijer, 1977 and 1980).

1.1.5. Immunological phenotype of clonogenic cells

Hemopoietic cells express many glycoproteins on their surface membrane. Monoclonal antibodies (McAbs) have been raised against many of these glycoprotein antigens. Expression of a set of antigens designates a cell to a particular differentiation stage and McAbs can be used for the identification of the cells wich express the antigens. In order to classify the large number of McAbs against immunologic markers, an international nomenclature has been developed (Bernard et al., 1984; Reinherz et al., 1986; McMichael et al., 1987; Knapp et al., 1989). The various McAbs (and the recognized immunologic markers) are classified in clusters of differentiation (CD). Recently, considerable progress has been made in the identification of normal progenitor cell surface antigens, and different stages of normal progenitor cells can now be recognized by their immunological phenotypes. CFU-GEMMs express HLA-DR, CD33 and CD34. The CFU-GM, CFU-G, CFU-M express CD13, CD15, CDw17, CD31, CDw32, CD33 and CD34 (Van Dongen et al., 1987; Van der Schoot et al., 1989). Studies on the phenotype of the AML-clonogenic cell (AML-CFU) revealed data suggesting that AML-CFU phenotypes vary among different patients. About 1/3 of cases showed phenotypes comparable to CFU-GEMM (or more

primitive) cells, while the other 2/3 expressed phenotypes of committed progenitor cells (Griffin et al., 1986) and therefore representing the neoplastic counterpart of different normal bone marrow progenitor cells. The double expression by one cell of antigens for different lineages can be used as a tumor marker (Smith et al., 1983), although in normal hemopoiesis such double positive cells can also arise in extremely low numbers. Terminal deoxynucleotidyl transferase (TdT) is found on the nuclear membrane of normal precursor B and T cells as well as of their malignant counterparts (i.e. acute lymphoblastic leukemia and some malignant lymphoma cells). However in a majority of AML cases also a subpopulation of the leukemic cells are TdT positive (Bradstock et al., 1981; Jani et al., 1983; Adriaansen et al., 1990). These TdT-positive AML cells may be used to monitor the disease and to detect minimal residual disease (MRD).

1.2. THE HEMOPOIETIC COLONY STIMULATING FACTORS

1.2.1. The effect of colony stimulating factors on progenitor cells

The development of semisolid culture systems necessary to grow bone marrow cells in vitro led to the identification of hemopoietic growth factors (HGF) or hemopoietins; of these erythropoietin (Epo), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), and interleukin-3 (Il-3) have been purified and cloned. At present they can be produced on a large scale through recombinant DNA technology and their biological activities have subsequently been defined (Table 1).

Erythropoietin was the first human hematopoietic growth factor to be identified. The renal origin of erythropoietin was demonstrated by Jacobson (1957). It was originally purified to homogeneity from human urine (Miyake, 1977), and a complementary DNA (cDNA) was later identified and brought to expression in mammalian cells (Jacobs et al., 1985; Lin et al., 1985; Browne et al., 1986). Erythropoietin stimulates the formation of CFU-E (Metcalf and Johnson, 1979), does not support the survival and/or proliferation of BFU-E or any other progenitor cell (Metcalf et al., 1980), although, some effect on the CFU-Meg is reported (Vainchenker et al., 1979; Kawakita et al., 1983; Dessypris et al., 1987; Ishibashi et al., 1987).

Granulocyte colony-stimulating factor (G-CSF) and macrophage colony stimulating factor (M-CSF) are relatively lineage specific hemopoietins which directly support the proliferation of the CFU-G (Nagata et al., 1986; Souza et al., 1986) and CFU-M (Stanley and Guilbert, 1981) respectively. Il-3 and GM-CSF are multipotential hematopoietic growth factors with overlapping but distinct activities (Emerson et al., 1988). Il-3, however, is more effective in stimulating early multipotential progenitors as the CFU-blast and the CFU-GEMM (Leary et al., 1987; Sonada et al., 1988). Il-3 is reported to be more effective than other colony stimulating factors in stimulating long-term maintenance of progenitor cells in vitro (Kobayashi et al., 1989) and acts not by direct stimulation of stem cell self-renewal (Zipori and Lee, 1988) or by triggering early progenitor cells into active cell proliferation, but it is necessary for their continuance of proliferation (Suda et al., 1985). More recent studies indicate that Il-3 stimulates CFU-GEMM, BFU-E, and CFU-Eo directly, whereas the

Table 1. Biological actions of the colony stimulating factors.

Growth Factor	Progenitor cell Target	Mature-Cell Target
Il-3	CFU-blast, CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-Eo, CFU-Meg, CFU-Baso, BFU-E.	Eosinophils, Basophils, monocytes
GM-CSF	CFU-Blast, CFU-GEMM, CFU-GM, CFU-G, CFU-M, CFU-Eo, CFU-Meg.	Granulocytes, Eosinophils, monocytes Basophils
G-CSF	CFU-G	Granulocytes,
M-CSF	CFU-M	Monocytes/-Macrophages
Epo	CFU-E, late BFU-e, CFU-Meg	None

stimulation of CFU-GM, CFU-G and CFU-M requires the presence of monocytes or the addition of GM-CSF (Bot et al., 1989). This suggests that some of the effects of IL-3 are mediated by GM-CSF, produced by accessory cells. Similarly, G-CSF and M-CSF can synergize with GM-CSF in the stimulation of CFU-G and CFU-M respectively (Caracciolo et al., 1987; Leary et al., 1987; Sonada et al., 1988; Ferrero et al., 1989; Namiki and Hara, 1989). These observations are in accordance with the hypothesis of a hierarchy of colony-stimulating factors in hemopoietic development, with the sequence: Il-3, GM-CSF followed by G-CSF, M-CSF or Epo (Metcalf, 1984).

1.2.2. The related interleukins

Most of the interleukins act on lymphoid cells, but some also stimulate, either directly or indirectly, the proliferation and differentiation of other cell types. Il-1 (α and ß) has been shown to regulate the cell growth of fibroblasts (Dukovich et al., 1986), epithelial cells, hemopoietic cells (Sieff et al., 1987, Broudy et al., 1987; Bagby et al., 1986) and lymphoid cells (Tartakovsky et al., 1988; Jelinek et al., 1987; Freedman et al., 1988). Il-2 is a growth and differentiation factor for T-cells (Robb, 1984), Il-4 for resting B-cells (Sanderson et al., 1986), Il-5 for activated B-cells and a growth and

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differentiation factor for eosinophils (Kinashi et al., 1986). Interleukin-6 has been reported to be involved in the induction of immunoglobulin production in activated B cells (Hirano et al., 1985, 1986), induction of Il-2 production, cell growth, and cytotoxic T-cell differentiation of T-cells (Garman et al., 1987, Okado et al., 1988), induction of proliferation of hybridoma/plasmacytomamyeloma cells (Van Snick et al., 1986, 1987; Nordon and Potter 1986; Van Damme et al., 1987; Kawano et al., 1988), stimulation of colony formation of multipotential hemopoietic cells (Ikebuchi et al., 1987), regulation of acute phase response (Andus et al., 1987) and induction of neural differentiation (Satoh et al., 1988). Il-7 is active on immature B cells (Namen et al., 1988). IL-8 is a neutrophil chemotactic factor (Yoshimura et al., 1987; Matsushima et al., 1988). The two most recently defined growth factors are Il-9, which stimules erythroid colony formation in vitro (Yand et al., 1989; Donahue et al., 1989) and Il-10, which is an inhibitor of cytokine synthesis (Moore et al., 1990).

1.2.3. The effect of colony stimulating factors on mature cells

In addition to the effect on progenitor cell proliferation and differentiation, hemopoietic growth factors may act on mature blood cells (Table 1). GM-CSF and G-CSF sustain the viability of neutrophils. They enhance their ability to produce superoxide anions in response to the bacterial peptide f-Met-Leu-Phe, to kill tumor targets through antibody-dependent cellular cytotoxity, and to phagocytize particles (Platzer et al., 1985; Lopez et al., 1986; Klausmann et al., 1988). GM-CSF also regulates the survival and function of eosinophils and macrophages and it is a potent inhibitor of neutrophil migration (Vadas et al., 1983; Gasson et al., 1984; Lopez et al., 1986; Metcalf et al., 1986; Silberstein et al., 1986; Arnaout et al., 1986; Socinski et al.,1988). M-CSF increases the tumoricidal activity of monocytes (Mufson and Ahgajanian, 1987). Il-3 can act as a functional regulator of mature eosinophils, and monocytes (Cannistra et al., 1988; Rothenberg et al., 1988), and is reported to induce basophil histamine release (Haak-Frendscho et al., 1988).

1.2.4. The effect of colony stimulating factors on leukemic and non-hemopoietic cells

Apart from their effect on normal hemopoietic cells the colony-stimulating factors are able to stimulate the proliferation of myeloid and erythroid leukemic cell lines and of myeloid leukemia blast cells in vitro. GM-CSF, G-CSF and Il-3, but less frequently M-CSF, stimulate AML growth in vitro (Hoang et al., 1986; Delwel et al., 1987; Griffin et al., 1986b; Mitjavila et al., 1987; Vellenga et al., 1987; Delwel et al., 1988). The response pattern of cells of different AML cases have been shown to be heterogeneous. Probably different subsets of cells exist in AML with distinct abilities to respond to CSFs (Delwel et al., 1988). Differentiation induction of leukemic blast cells, especially by G-CSF, has also been reported (Souza et al., 1986; Miyauchi et al., 1988). More recently the hemopoietic growth factors have been reported to act also on non-hemopoietic cells. For instance, they may induce human endothelial cells to migrate and proliferate (Bussolino et al, 1989), and may stimulate the growth and/or differentiation of non-hemopoietic tumor cell lines (Ruff et al., 1986; Baldwin et al., 1989; Berdel et al., 1989).

1.2.5. The production of colony stimulating factors.

The colony stimulating factors can be produced by a variety of cells (table 2); Il-3 and GM-CSF by activated T-cells and monocytes (Wong et al., 1985; Otsuka et al., 1988; Sieff et al., 1988; Ernst et al., 1989), whereas fibroblasts, monocytes and endothelial cells can be induced to synthesize GM-CSF and G-CSF (Bagby et al., 1986; Horiguchi et al., 1987; Oster et al., 1987; Yang et al., 1988a; Kaushansky et al., 1988; Vellenga et al., 1988). Fibroblasts and endothelial cells appear to synthesize M-CSF constituti- vely (Sieff et al., 1988). Only erythropoietin and M-CSF are present in the serum in biologically active concentrations (Garcia et al., 1982; Das et al., 1981; Hanamura et al., 1988). G-CSF can be detected transiently following intensive chemotherapy or in disorders associated with neutropenia and in approximately 10% of normal sera (Watari et al., 1989). Il-3 and GM-CSF are not detectable as a circulating molecule. These growth factors seem to act locally in a paracrine fashion. In this respect it is of interesst that GM-CSF has been reported to become compartmentalized in the extracellular matrix of the bone marrow stroma (Gordon et al., 1987; Roberts et al., 1988).

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CYTOKINE CASCADES

MACROPHAGE

IL-1   IL-1   IL-1

FIBROBLAST   T LYMPHOCYTE   ENDOTHELIAL CELL

GM-CSF   IL-2   GM-CSF
G-CSF   IL-3   G-CSF
M-CSF   IL-5   M-CSF
IL-1   IL-6   IL-1
IL-6   IL-6

STEM CELL
PROGENITOR CELL
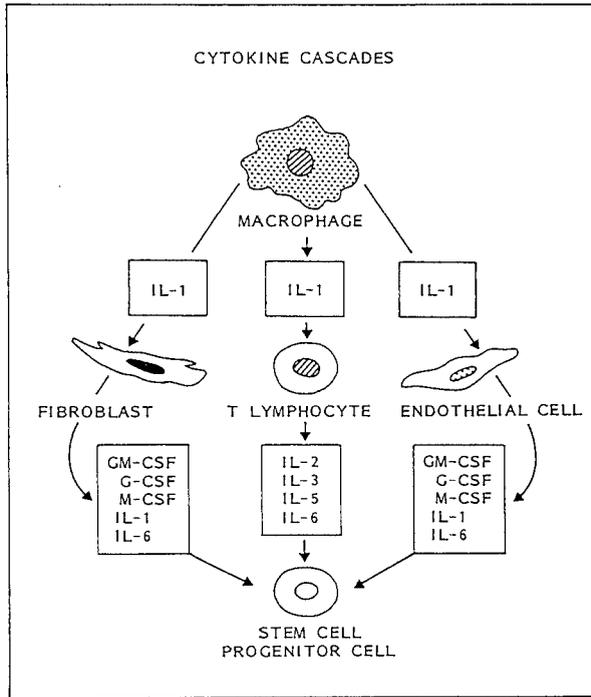
Figure 2. Cytokine cascades involving macrophage production of IL-1, interactions with T cells and stromal cells, and the production of different colony stimulating factors and interleukins that influence bone marrow progenitor cell proliferation and differentiation. According to Moore MAS. Role of interleukin-1 in hemopoiesis. Immunol Res 1989; 8: 165.

1.2.6. Synergistic and additive effects between colony stimulating factors.

Synergism with hemopoietic growth factors was first described by Stanley et al. (1986) for an activity called hemopoietin-1. Hemopoietin-1 has now been demonstrated to be Il-1 (Mochizuki et al., 1987). It has been shown that Il-1 produced in response to inflammatory stress can stimulate the proliferation and differentiation of myeloid progenitor cells. Il-1 is a potential stimulator of the production of G-CSF and GM-CSF by endothelial, stromal and fibroblast cells, of G-CSF, GM-CSF and gamma-interferon by macrophages, and Il-3 and GM-CSF by T cells (Bagby et al., 1986; Bagby, 1987; Sieff, 1987; Broudy et al., 1987; Fibbe et al., 1988) (Fig.2). Furthermore it has been demonstrated that the expression of the genes for G-CSF, GM-CSF, Il-6
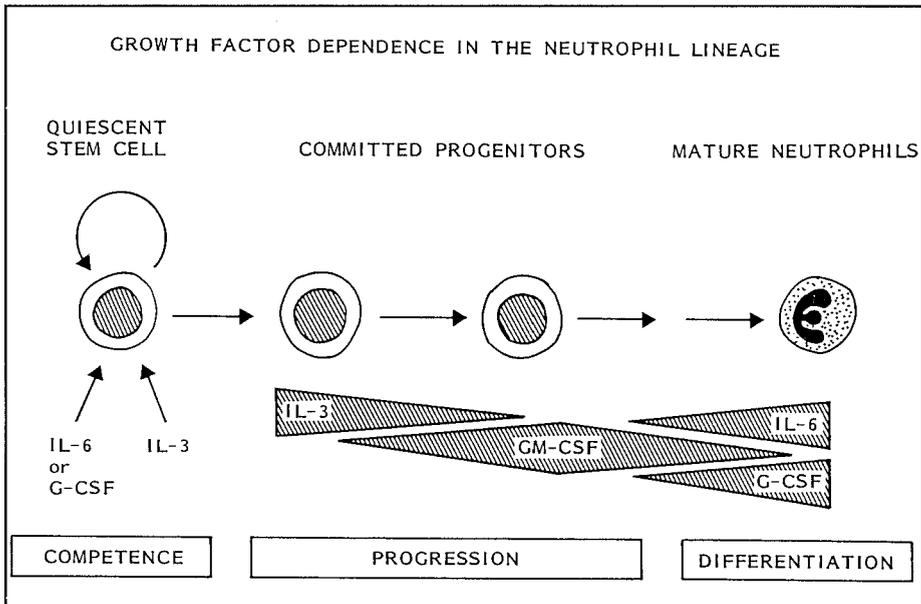
Figure 3. Growth factor dependence in the neutrophil lineage, depicting the target cells for competence, progression and differentiation factors. IL = interleukin; CSF = colony-stimulating factor. (Ikebuchi et al. 1985; Caracciolo et al. 1989).

and Il-1 itself can be induced by Il-1 (Zsebo et al., 1988; Broudy et al., 1987; Sieff et al., 1988; Lee et al., 1987; Kaushansky et al., 1988).

Vice versa, the CSFs are able to induce the elaboration of cytokines, e.g. Il-1 by granulocytes (Lindemann et al., 1988) and mononuclear cells (Sisson and Dinarello, 1988), whereas Il-3 and GM-CSF recruit monocytes to express and secrete G-CSF (Oster et al., 1989).

The action-mechanism of these synergistic effects has not been elucidated yet. One hypothesis postulates that Il-1 induces the expression of hemopoietic growth factor receptors on progenitor cells (Stanley et al., 1986), suggesting a direct effect of Il-1 on progenitor and stem cells. Another possibility is that Il-1 recruits quiescent progenitor cells into cell cycle (Ikebuchi et al., 1987; Leary et al., 1988) resulting in a priming for the action of hemopoietic growth factors. More recently it has been postulated that Il-1 increases the stability of the mRNA of hemopoietic growth factors (Bagby, 1989) produced by accessory cells, suggesting that the effect of Il-1 is indirect through augmenting growth factor production. The action of Il-1 might be even more complex,

since it has been shown that some of the effects of Il-1 are mediated by other factors, for instance Il-6 (Helle et al., 1988). All these data point to the existence of a cytokine network consisting of reciprocal induction of cytokines, transmodulation of cytokine cell surface receptors and synergistic, or additive interactions with cell function. Theoretically the hemopoietic growth factors may be grouped on the basis of these functions. The competence factors prime progenitor cells to make them responsive or more responsive to the activities of other factors. Examples of such factors are Il-1 and Il-6. Subsequently, a progression factor like Il-3 or GM-CSF provides the second signal, after which a lineage specific factor like G-CSF, M-CSF or Epo stimulates terminal differentiation and maturation of the hemopoietic cells. The role of G-CSF and Il-6 in this model is still somewhat controversial, since both cytokines enhance proliferation of primitive stem cells (Ikebuchi et al., 1987; Ikebuchi et al., 1988) and also induce differentiation of committed granulocytic progenitor cells, (Caracciolo et al., 1989), acting therefore both as a competence and a differentiation factor (Fig 3).

1.2.7. Inhibitory effects of cytokines.

All the cytokines described above have growth factor activity. The ability to inhibit cell growth or directly kill cells is limited to τ-interferon (IFNτ) (Raefsky et al., 1985: Mamus et al., 1985), tumor necrosis factor (TNF) (Degliantoni et al., 1985; Broxmeyer et al., 1986), lymphotoxin (LT) (Murphy et al., 1986), and transforming growth factor ß (TGFß) (Espevik et al., 1987; Hino et al., 1988). It was recently appreciated that some of the cytokines (TNFα) (Ishikura et al., 1989), Il-1 (Santoli et al., 1987; Gasparetto et al., 1989) and Il-4 (Rennick et al., 1987) may act as a stimulator in one context, but as an inhibitor in another. This dual action depends either on the presence of other cytokines and the differentiation stage of the target cell or their concentration. Evidence of antagonistic competition between CSFs has been documented by Metcalf (1988). When M-CSF and G-CSF act in combination on bipotential progenitor cells there appears to be an element of competition in the commitment to the formation of granulocytic or macrophage progeny. However, if G-CSF is

Table 2. Main characteristics of the hemopoietic growth factors.

Factor	Molecular Mass (kD)	Chromosomal location	Cellular sources
Il-3	14-28	5q23-31	T-cells, monocytes
GM-CSF	14-35	5q21-32	T-cells, monocytes, fibroblasts, endothelial cells macrophages
G-CSF	18-22	17q11.2-q21	Monocytes, fibroblasts, endothelial cells.
M-CSF	40-90(dimer)	5q33.1	Monocytes, fibroblasts, Endothelial cells.
Il-1α/β	17.5	2q12-21	Monocytes/macrophages, Fibroblasts, Endothelial cells, Smooth muscle cells
Il-6	26	7p21	Fibroblasts, monocytes, T-cells
Epo	34-39	7q11-22	Peritubular cells of the kidney, Kupffer cells.

combined with low concentrations of M-CSF, there is also enhancement of the number and size of granulocytic colonies, demonstrating a delicate balance between antagonism and enhancement.

1.2.8. Biochemistry of the colony stimulating factors.

The CSF's are low molecular weight proteins (< 80 KDa), which are variably glycosylated. Therefore each natural CSF occurs in different molecular weight forms (Clark and Kamen 1987; Sieff et al., 1988) (table 2). For the function of most of the hemopoietic growth factors glycosylation is not essential, but it has been reported that glycosylated GM-CSF is less active in vitro than non-glycosylated GM-CSF (Moonen et al., 1987). Moreover, the biological activity of erythropoietin in vivo is dependent

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on proper glycosylation (Browne et al., 1986). The presence of cysteine residues suggests that disulphide bonds may be structural features of the molecules. The CSF's contain at least four cysteine residues, except Il-3 which contains only two cysteines (Yang et al., 1986). There is no significant sequence homology among these proteins. However, Il-6 was recently reported to show homology with G-CSF in a specific region where four cysteine residues reside. Moreover, the genes of both Il-6 and G-CSF, consisting of four introns and five exons, exhibit similar genomic organization (Hirano et al., 1986). Il-1 has two structurally different forms, α and β, which are products of separate genes on chromosome 2 (Modi et al., 1988). Il-1β is the predominantly secreted form, and Il-1α is predominantly membrane bound. Both Il-1α and β bind to the same receptor (Oppenheim et al., 1986).

It is interesting that in man the genes for Il-3, Il-4, Il-5, GM-CSF, and M-CSF are clustered in the same band on the long arm of chromosome 5 (Yang et al., 1988b; Wong et al., 1985; Pettenati et al., 1987) (table 2). This region has also been shown to contain the proto-oncogen c-fms, encoding the M-CSF receptor , the gene for the endothelial cell growth factor (ECGF) and the gene for the platelet derived growth factor (PDGF) receptor (Le Beau et al., 1986; Sherr et al., 1985). This is especially intriguing since deletions of the long arm of chromosome 5 (5q-) are frequently observed in patients with a myelodysplastic syndrome or acute myeloid leukemia secondary to chemotherapy. The gene for G-CSF has been localized on chromosome 17 (Simmers et al., 1987), whereas the erythropoietin (Law et al., 1986) and Il-6 (Bowcock et al., 1988) gene are being mapped on chromosome 7.

The CSFs have high specific biological activity, stimulating cell proliferation in the $10^{10} - 10^{12}$ M range. Their effects are mediated through a limited number (only a few hundred for each type of CSF) of specific receptors present on cell membranes, yet signalling is achieved by CSFs with low receptor occupancy (Park et al., 1986; Gasson et al., 1986; Dipersio et al., 1988). The receptors comprise an extracellular, transmembrane and intracellular domain. Their binding leads to changes in the intracytoplasmatic domain and activation of a cascade of biochemical responses eventually resulting in stimulation of DNA synthesis. A description of this cascade of responses lies beyond the scope of this thesis.

1.3. THE MYELODYSPLASTIC SYNDROMES

1.3.1. History

The current concept of the myelodysplastic syndrome (MDS) emerges from studies on apparently two different diseases ; refractory anaemia on the one hand and preleukemia on the other. The refractory anemias were recognized in the 1930's, when it became apparent that these anemias were refractory to treatment with all known hematinics. In addition no specific metabolic defects could be identified. Roads and Halsey Barker (1938) described 100 cases of refractory anemia, which was often associated with leukopenia and trombocytopenia.

The observation that acute myeloid leukemia can be preceded by a refractory anemia with also dysplastic features of the bone marrow, was first made at the beginning of this century (Von Leube, 1900; Parkes-Weber, 1904). Hamilton-Paterson (1949) described three patients who presented with refractory anemia and subsequently developed an acute myeloid leukemia, indicating that at least some of the refractory anemias should be considered to be preleukemic. The term 'preleukemia' was introduced by Block et al. in 1953, who observed 12 patients for as long as 27 months prior to the development of acute myelogenous leukemia. In the following years apparently similar conditions were described (Meacham and Weisberger, 1954; Bjorkman, 1956). Prospective studies performed more recently show an evolution pattern from normal hemopoiesis through a clinically recognizable dysplastic syndrome to overt leukemia (Nowell et al., 1986; Todd and Pierre, 1986). Dameshek (1965) was the first to link the refractory anemias to the Di-Guglielmo's erythroleukemia. He proposed that the two were different phases of the same disease and called it the Di-Guglielmo's syndrome. At about the same time a distinct group of refractory anemias was recognized having a prominent population of bone-marrow erythroblasts with perinuclear iron deposits or siderotic granules, the ringed sideroblasts. This subgroup presently known as the acquired idiopathic sideroblastic anemia (AISA) (Björkman, 1956), is usually characterised by a insidious clinical course (Beris et al., 1983). Saarni and Linman (1973) and later Linman and Bagby (1978) developed the concept of 'the preleukemic syndrome', defined by dysplastic features of the megakaryocytes and granulocytes as well as dyserythropoiesis, with no more than 5% blast cells detectable in the bone marrow. Concurrently Dreyfus et al (1970) and

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Dreyfus (1976) described patients with 'refractory anemia with excess of myeloblasts' with identical morphological abnormalities as noted by Linman. In 1982 the French-American-British (FAB) co-operative group (Bennett et al., 1982) proposed the unifying concept of the myelodysplastic syndrome, subdivided in five separate entities:

1. refractory anemia (RA);
2. refractory anemia with ring sideroblasts (RARS)
3. refractory anemia with excess of blasts (RAEB);
4. chronic myelomonocytic leukemia (CMML).
5. refractory anemia with excess of blasts in transformation (RAEBt).

These different groups of the FAB classification are described in more detail below. With cytogenetic analysis of the bone marrow cells and glucose-6-phosphate dehydrogenase isoenzyme studies in female heterozygotes it was possible to demonstrate that MDS is a clonal disease of the pluripotent hemopoietic stem cell (Fialkow et al., 1981; Raskind et al., 1984). Disease progression in MDS may be due to the gradual clonal expansion of the abnormal population with suppression of the normal hemopoiesis (Abkovitch et al., 1984). More recent reports, presenting data on X-linked restriction fragment length polymorphism, provide evidence that MDS is a clonal disorder of the pluripotent stem cell, which clone has totally replaced the normal hemopoiesis (Janssen et al., 1989; Tefferi et al., 1990).

1.3.2. Morphological characteristics of the MDS

In order to describe the morphological features underlying refractory anemia, Lewis and Verwilghen (1972) introduced the term "dyshematopoiesis". Dyshematopoiesis may be observed in one or more hemopoietic cell lines (Table 3):

Dyserythropoiesis:

The presence of ringed sideroblasts, multinuclearity, nuclear fragments, Howell-Jolly bodies and nuclear-cytoplasmic asynchrony with intense cytoplasmic basophilia are morphological features in the bone marrow. In the peripheral blood basophilic stippling and fragmentation of the erythrocytes with moderate anisocytosis and poikilocytosis are usually seen.

Table 3.Qualitative abnormalities in the myelodysplastic syndromes		
Cell lineage	Peripheral blood	Bone marrow
Erythroid	Macrocytes; dimorphic picture; anisopoikilocytosis polychromatic cells; normoblasts (often dyserythropoietic)	Erythroid hyperplasia ring sideroblasts dyserythropoisis Megaloblasts Cytoplasmic vacuolization
Megakaryocytic	Giant platelets; megakaryocyte fragments	Small megakaryocytes with one or two small round nuclei; larger forms with single large ovoid nucleus; polynuclear forms
Granulocytic	Hypo- or agranular neutrophils; speudo-pelger-Huët neutrophils	Promyelocytes with sparse azurophilic hypo- or agranularity of myelocytes, meta-myelocytes and neutrophils
Monocytic	Mature forms sometimes with multiple elongated lobes, some with fine azurophilic granules	Promonocytes sometimes present
Blasts	Usually small mononuclear blasts with scanty agranular (type I) or sparsely granular (type II) blasts.	

Dysgranulopoiesis:

This is characterized by hypogranulation and hyposegementation of the granulocytic cells. Excessive chromatin condensation (pseudo-Pelger-Huët anomaly), but also hypersegmentation with unequally sized lobes can be seen. Additionally persistent basophilia and excess azurophilic of the promyelocytes may be prominent in the bone marrow.

Dysmegakaryocytopoiesis:

Micromegakaryocytes, multiple small hypersegmented nuclei and large mononuclear forms are typical features of the bone marrow. The platelet production may appear normal, increased or decreased. In the peripheral blood abnormal platelets, especially giant forms are often present.

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1.3.3. Classification

Although the FAB entities are defined by arbitrary criteria (Table 4), it is usually possible to classify them according to the following crititeria:

1. Refractory anemia (RA)

The anemia is frequently accompanied by some neutropenia and trombocytopenia. The peripheral blood containes less than 1% blast cells and the bone marrow less than 5%. The bone marrow is normo- to hypercellular. Erythroid hyperplasia with dyserythropoiesis is a common feature. Some degree of dysgranulopoiesis or dys-megakaryopoiesis may be present.

2. Refractory anemia with ringed sideroblasts (RARS) (acquired idiopatic sideroblas-tic anemia, AISA):

The morphological features resemble those of RA, but dysgranulopoiesis and dysmegakaryopoiesis are not as common. The major marrow characteristic is a high number of ringed sideroblast of at least 15% of the nucleated cells.

3. Refractory anemia with excess of blasts (RAEB):

Some degree of peripheral cytopenia is usually seen in all three lineages. Dyseryth-ropoiesis as well as prominent dysgranulopoiesis are present. Blast counts may amount to 5% in the peripheral blood and to 5- 20% in the bone marrow. The bone marrow is normocellular and or often hypercellular, with erythroid and myeloid hyperplasia.

4. Chronic myelomonocytic leukemia (CMML):

The major feature of CMML is the presence of monocytosis in the peripheral blood and the bone marrow. The minimal number of peripheral monocytes required for this MDS entity is $1 \times 10^9/l$. The morphological features of the bone-marrow resemble RAEB in some cases and RA in others.

5. RAEB in transformation (RAEBt):

This MDS category fills in the the gap between RAEB and AML. Bone marrow blast counts amount from 20- 30% and more than 5% circulating blast cells may be present. Auer rods are seen occasionally.

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Table 4.Classification of the myelodysplastic syndrome
─────────────────────────────────────────────────────────────
FAB                   Peripheral                Bone marrow
type                  blood
─────────────────────────────────────────────────────────────
Refractory            < 1% blasts        Dyshemopoiesis   in   one
anemia (RA)                              or more lineages
                                         < 5% blasts

RA with ring-         < 1% blasts        As  RA  with  >15%  ring-
sideroblasts                             sideroblasts
(RARS)

RA with excess        < 5% blasts        As RA with 5-20% blasts
of blasts
(RAEB)

RAEB in trans-        < 5% blasts        As RA with 20-30% blasts
formation             ± Auer rods        ± Auer rods
(RAEBt)

Chronic myelo-        < 5% blasts        As any of the above
monocytic leukemia    > 1 x 10⁹/l        + promonocytes
(CMML)                monocytes
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1.3.4. Clinical features

Usually the patients with a myelodysplastic syndrome are older than 50 years. Most patients present with anemia. Infections or sometimes bleeding may also be the first symptoms. Infection are usually bacterial, respiratory or septicemic. Splenomegaly occurs almost exclusively in CMML.

The natural history of MDS is a transition to overt non-lymphocytic leukemia in approximately half of the patients. However, most patients die of the consequences of marrow failure, i.e., bleedings or infections. Three patterns of evolution based on sequential determinations of bone marrow blasts can be observed in MDS (Tricot et al., 1985 and as illustrated in figure 4: 1) an apparently stable disease with no or a minimal increase of bone marrow blasts 2) initially a morphologically stable disease,

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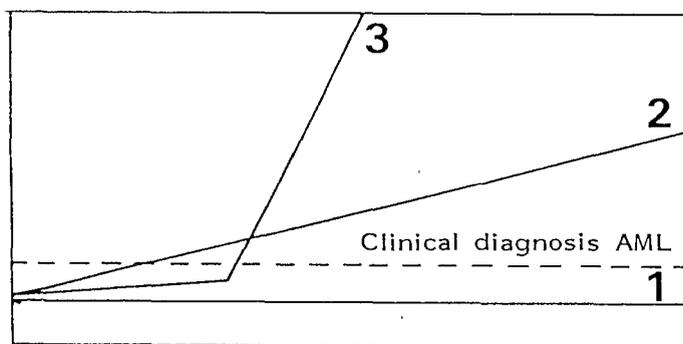
Figure 4. Hypothetical model of evolation patterns in patients with MDS. 1, stable clone, with low proliferative capacities; 2, stable clone, with a more pronounced proliferative capacities; 3, instable clone, with initially a stable disease, but with a high probability of transformation to a clone with a high proliferative capacity. Adapted from Tricot G, et al. 1985.

comparable with pattern 1, but changing into a progressive disease with a rapid increase in blast cells and a rapid transformation to acute leukemia and 3) with a gradual increase in bone marrow blasts. The majority of these patients eventually developed overt myeloid leukemia.

1.3.5. Cytogenetics

Karyotypic abnormalities have been detected in 40-60% of the MDS patients using conventional cytogenetic banding techniques (Jacobs et al., 1986; SIWCL, 1981). Refined high-resolution chromosome analysis has resulted in the detection of non-random cytogenetic abnormalities in 79% of the MDS cases (Yunis et al., 1986; Yunis et al., 1988). The commonest abnormalities in MDS are deletions, rather than reciprocal translocations or inversions. Chromosomal deletions, such as monosomy 5 (-5), 5q-, monosomy 7 (-7), 7q-, -Y, 20q- or trisomy 8 (+8) or complex defects, often including one or more of the above abnormalities mentioned; represent the main chromosomal abnormalities in MDS . These are, however, not specific for MDS because they may also be seen in AML. There is also no apparent correlation between a particular chromosomal abnormality and any of the FAB subtypes of MDS (Knapp et al., 1985; Yunis et al., 1986; Yunis et al., 1988). However, the deletion of

the long arm of chromosome 5 (5q-), when present as a sole defect, is associated with RA or RAEB, female sex, macrocytic anemia, normal or raised platelet counts, and erythroid hyperplasia with mononuclear megakaryocytes in the bone marrow (Kerkhofs et al., 1982; Van den Berghe, 1986). Monosomy 7 (-7) often goes with hypocellular marrow, pancytopenia and defective neutrophil functions (Pederson-Bjergaard et al., 1982). The presence or absence of chromosomal abnormalities has prognostic significance. Certain chromosomal abberations are predictive for an unfavorable prognosis. The 5q- syndrome usually runs a benign clinical course, whereas -7, and 7q- is frequently associated with a rapid progresion and a high probability of leukemic transformation (Anderson and Bagby, 1982; Nowell et al., 1986). The presence of complex cytogenetic abnormalities, an unfavorable prognostic feature, is often found in MDS cases secondary to chemotherapy.

1.3.6. In vitro marrow cultures

The use of in vitro clonogenic or colony assays has provided methods to analyze the hemopoietic progenitor compartments and the hemopoietic colony stimulating factors or cytokines involved in the regulation of cell proliferation and cell differentiation. As described above, it has been shown that MDS is a clonal disease of the hemopoietic stem cell and that the abnormal clone is already fully established in a relatively early stage of the disease. Moreover it was demonstated that both the BFU-E as well as the CFU-GM derived colony cells contain the same cytogenetic abnormality, indicating that they stem from the same abnormal progenitor cell (Abkowitz et al., 1984). Therefore it appears to be likely that the in vitro growth characteristics of MDS bone marrow in clonogenic assays may reflect the functional abnormalities of the hemopoietic progenitor cells in MDS.

The colony forming capacities of all different committed progenitor cells (CFU-GEMM, BFU-E, CFU-E, CFU-GM, CFU-Meg) are usually decreased in MDS patients (Greenberg et al., 1983; Greenberg and Mara, 1979; Chiu and Clark, 1982; Juvonen et al., 1986; Swanson et al., 1986), as in AML patients. A defective maturation of the colony cells is frequently observed (Golde and Cline, 1973; Spitzer et al., 1979; Verma et al., 1979). Generally two growth patterns of the CFU-GM may be discerned: a 'leukemic' and a 'non-leukemic'type (Greenberg and Mara, 1979; Verma

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et al., 1979; Spitzer et al., 1979; Gold et al., 1983). A non-leukemic type of growth is most frequently observed in the RARS (MDS 2) subgroup of MDS, which fits with the observation that RARS is less often accompanied by dysgranulopoiesis (Green- berg and Mara, 1979; Greenberg, 1981). A leukemic type of growth is seen sometimes in RA, but most often in RAEB and RAEBt. In some cases of CMML the marrow culture shows growth of clusters as well as colonies as in CML. A leukemic growth pattern seems to be associated with a higher transformation rate to AML as com- pared with a non-leukemic growth pattern (Verma et al., 1979). The erythroid burst and colony formation in MDS has been investigated less extensively. However, it has become evident that the formation of both BFU-E and CFU-E is defective in a majority of the MDS patients (Hutcheson et al., 1979; Chui and Clark, 1982; Amato and Khan, 1983; Ruutu et al., 1984). Information on the growth of megakaryocyte colonies is even more limited. Juvonen et al. (1989) found a defective CFU-Meg and BFU-E growth in all 10 patients studied, but only four had abnormal CFU-GM growth. Also in long-term marrow cultures the hemopoiesis can rarely be sustained beyond 2-4 weeks, while the cells retain their dysplastic features (Bourbenyi et al., 1987). Attempts to correlate the in vitro growth patterns with the FAB classification have rendered different results. Ruutu et al., (1984) reported a correlation between normal CFU-GM numbers and RARS, whereas May et al., (1985) found no cor- relation between FAB type and colony growth pattern. Oscier et al. (1989) found sig- nificantly higher CFU-GM numbers in CMML than in RAEB and RAEBt in a large series of MDS patients, low or absent BFU-E growth in the majority of the patients with RARS, CMML, RAEB and RAEBt, but normal growth in 10 of the 22 RA patients (Table 5).

Table 5. In vitro colony formation in the myelodysplastic syndromes

Type	CFU-GEMM	CFU-GM	BFU-E	CFU-E	CFU-Meg
RA	N/↓	N/↓	N/↓	N/↓	N/↓
RARS	N	N	N	N	N
RAEB	↓	↓	↓	↓	↓
RAEBt	↓	↓	↓	↓	↓
CMML	N/↑	↑	↓	↓	↓

N, normal; ↑, increased; ↓, decreased numbers of colonies

The nature of the defect leading to abnormal in vitro colony formation of the hemopoietic progenitor cells in MDS is not known. The abnormalities may be either an intrinsic progenitor cell defect, i.e. abnormal growth factor responsiveness, or an environmental one, i.e an abnormal growth factor production. The results obtained with in vitro bone marrow cultures, described above, are not capable to discriminate between these two possibilities, since the marrow cells are not fractionated and therefore contain many accessory cells, which can produce hemopoietic growth factors. Furthermore, all these studies were performed with crude sources of colony stimulating activity. Recently some results have been reported on the response to recombinant hemopoietic growth factors by purified MDS progenitor cells (Carlo-Stella et al., 1989; Schouten et al., 1989), suggesting the presence of an intrinsic progenitor cell abnormality. However, also growth factor production abnormalities by bone marrow accessory cells was recently reported (Merchav et al., 1989).

1.3.7. Prognostic factors

Prognosis in MDS is extremely variable. The FAB classification has some prognostic significance, with a better prognosis for RA and RARS than RAEB and RAEBt. The most important factor in prognosis is the number of blast cells in blood and bone-marrow. (Kerkhofs et al., 1986). The Bournemouth score (Mufti et al., 1985) allocates one point for each of the following features: an Hb concentration $<100g/l$, for neutrophil count $<2.5 \times 10^9/l$ or $> 15 \times 10^9$ in CMML, for a platelet count $<100 \times 10^9$ and for a bone marrow blast count $> 5\%$. Patients with a score of 0 or 1 have the best prognosis with a median survival of 62 months; patients with a score of 2 or 3 have a median survival of 22 months and patients with a score of 4 have a poor prognosis with a median survival of 8 months. A further prognostic factor has been described by Tricot et al., (1984). He designated clusters of myeloblasts abnormally localized in the central marrow as ALIPs (abnormal localization of immature precursors). ALIPs seen in some cases of RA are a independent indicator of a poor prognosis. The importance of karyotype and of the growth pattern of in vitro cultured bone marrow cells for prognosis are described above.

1.3.8. Treatment

Since MDS patients are usually elderly, often only symptomatic treatment can be aplied. Intensive chemotherapy is eligible in patients not older than 50, in which a complete remission rate of 86% can be achieved with conventional chemotherapy (anthracycline and cytosine arabinoside (ARA-C)) or high dose ARA-C (Tricot et al., 1986). In younger patients (<50 years), with HLA-compatible siblings, results of bone-marrow transplatation are encouraging and should be seriously considered as the treatment of choice (Appelbaum et al., 1987; Baines et al., 1988). In older patients some favourable results with low-dose ARA-C have been reported (Chomienne et al., 1987). MDS patients have been treated with G-CSF and GM-CSF (Vadhan-Raj et al.,1987; Antin et al., 1988; Ganser et al., 1989; Negrin et al., 1989). The majority of these patients have shown marked improvement of blood neutrophil counts after G-CSF and GM-CSF treatment but without improvement in platalet counts. In a low proportion of patients improved reticulocyte and hemoglobin levels occurred. Marrow myeloid maturation was better enhanced by G-CSF than by GM-CSF. Caution must be taken with CSF treatment because there is a possibility of accelerating the proliferation of the leukemic clones. However, several investigators recently demontrated that treatment with GM-CSF altered cell growth kinetics (Herrmann et al., 1989) sensitizing leukemic blasts to cytotoxic drugs as shown by enhanced cell-kill in vitro. Studies of cell-kinetics and cytosine-arabinoside metabolism proved that GM-CSF- induced recruitment of quiescent leukemic cells primed them to become more sensitive to cell-cycle specific cytotoxic drugs (Tafuri et al., 1988; Cannistra SA et al., 1988; Andreeff M et al., 1989). Clinical trials with the combination of GM-CSF and ARA-C are presently performed in MDS.

1.4. INTRODUCTION TO THE EXPERIMENTAL WORK

In this thesis studies were performed to characterize the abnormal hemopoiesis in MDS by using immunofluorescence analysis and in vitro colony assays in order to investigate the presence of intrinsic abnormalities of myelodysplastic progenitor cells, i.e. to detect immunophenotypicly abnormal subpopulations and abnormal responsiveness of progenitor cells to the various hemopoietic growth factors.

In chapter 2, six patients with MDS (three with RARS, two with RAEB and one with RAEBt) were studied using double immunofluorescence analysis for the presence of terminal deoxynucleotidyl transferase (TdT) and myeloid-antigen (MM) expression. MM+/TdT+ subpopulations occur in the majority of AMLs and these TdT-positive cells belong to the AML cell-population. The objectives of the experiments described in this chapter were to determine whether MM+/TdT+ cells are detectable in MDS as well and TdT-positivity of myeloid cells is an useful marker for the abnormal granulopoiesis in MDS.

In the following chapters the characteristics of colony formation by low-bouyant density bone marrow cells, depleted of T-cells and adherent cells, were studied. Colony growth patterns were studied in the PHA-clonogenic assay, which is also used for the growth of AML-CFU (Chapter 3). Subsequently the colony formation and growth kinetics of MDS bone marrow myeloid progenitor cells stimulated with recombinant Il-3, GM-CSF and G-CSF were studied and compared with the growth kinetics of normal myeloid progenitor cells and bone marrow cells cultured after exposure to 4-hydroperoxy-cyclophosphamide (4-HC)(Chapter 4). Primitive progenitor cells will be spared during in vitro 4-HC treatment. Therefore, 4-HC exposure will consequently result in a relative enrichement for early progenitor cells. Since the in vitro growth abnormalities of MDS bone marrow may be due to a relative left shift in the progenitor cell compartment, the objective of the 4-HC experiments was to compare the growth kinetics of MDS marrow with those of normal bone marrow relatively enriched for early progenitor cells. In these investigations a semi-solid colony assay was used in order to exclude possible cell-cell interactions and induce clonal growth of stem cell progeny. Since in vitro colony formation of MDS bone marrow stimulated with single CSF was found to be decreased, it was investigated whether combinations of Il-3, GM-CSF and G-CSF could enhance the growth of

MDS myeloid and erythroid progenitor cells, in other words, whether MDS progenitors needed more CSFs for clonal expansion than their normal counterparts (Chapter 5). Finally, the effects of competence factors such as IL-1 and more extensively of Il-6 on the GM-CSF induced colony formation of MDS and normal marrow cells were studied (Chapter 6).

In chapter 7 the presented work is discussed in the context of the literature.

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CHAPTER 2

THE SIGNIFICANCE OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE
POSITIVE SUBPOPULATIONS IN MYELODYSPLASTIC SYNDROMES

M.R. Schipperus, H.J. Adriaansen, K. van Lom, A. Hagemeijer.

Departments of Hematology and Immunology, Academic Hospital Rotterdam and
Cell Biology and Genetics, Erasmus University Rotterdam, PO.box 1738, 3000 DR
Rotterdam, The Netherlands

Abstract

Six patients with a myelodysplastic syndrome (MDS) (three with refractory anemia with ringsideroblasts (RARS), two with RA with excess of blasts (RAEB) and one with RAEB in transformation (RAEBt)) were studied by double immunofluoresence (DIF) analysis for the presence of terminal desoxynucleotidyl transferase (TdT) and myeloid-antigen (MM), such as CD13, CD14, CD15 and CD33, expression. TdT expression was found in 0.1 - 11% of the cells. In four cases (1 RAEBt, 2 RAEB and 1 RARS) 58 - 99% of the TdT+ cells expressed the panmyeloid markers CD13 and/or CD33, whereas the precursor antigen CD34 was present in 26 - 99% of the TdT+ cells. Follow up studies performed in two patients, that evolved into an acute myeloid leukemia (AML), showed in one patient an increase of MM+/TdT+ cells from 11% in RAEBt to 25% in AML-M2. In the other patient the percentage of MM+/TdT+ cells was 0.1% at diagnosis, decreased during remission and increased to 0.2% before relapse RAEB and finally to 35% when AML-M2 emerged. These data indicate that the detection of MM+/TdT+ cells in MDS may facilitate the detection of abnormal myeloid cells and may be useful to monitor these cells during disease progression. No MM+/TdT+ cells were found in 2/3 RARS patients. In these two patients no features of dysgranulopoiesis were found. Moreover the latter two RARS cases had a low Bournemouth score, which indicates a good prognosis. In the other RARS patient 5% MM+/TdT+ cells were detected in parallel with dysgranulopoietic features and a high Bournemouth score, suggesting that DIF staining can discriminate between RARS cases with and without an abnormal granulopoiesis.

Introduction

The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed on the nuclear membrane of normal precursor B and T cells as well as their malignant counterparts, i.e., acute lymphoblastic leukemias and some lymphoblastic lymphomas (1,2). TdT positivity has also been found in acute myeloid leukemia (AML) (3,4,5). This has initially been ascribed to a 'mixed', 'biclonal' or 'biphenotypic' cell population in these leukemias (6). However, the simultaneous demonstration of TdT and Sudan black or myelo-peroxidase positivity in single blast cells proved the existence of 'TdT-positive leukemic myeloblasts' (4,7). Recently, we have demonstrated by double immunofluorescence (DIF) analysis that a TdT-myeloid-antigen double-positive (MM+/TdT+) leukemic subpopulation occurs in the majority of AMLs (8).

The myelodysplastic syndrome (MDS) is a clonal disease of the hemopoietic stem cell characterized by an ineffective hemopoiesis resulting in a cytopenia of one or more cell lineages (9,10). The French-American-British (FAB) co-operative group has defined criteria for the classification of MDS (11), comprising 5 different entities, i.e., refractory anemia (RA), RA with ring-sideroblasts (RARS), RA with excess of blasts (RAEB), chronic myelomonocytic leukemia (CMML) and RAEB in transformation (RAEBt). Up to 40% of MDS cases evolve in an AML during the course of their disease (9). In MDS TdT-positivity has also been cited as an example of mixed-lineage expression (12). However, no DIF analysis has been performed in these cases, and therefore the TdT-positive cells may belong to the myeloid differentiation lineage. We performed DIF analysis for TdT and differentiation markers for the myeloid and lymphoid lineage in six MDS patients (two with RARS, three with RAEB and one with RAEBt) in order to investigate the presence MM+/TdT+ cells. In addition, we applied double marker analysis to monitor the MM+/TdT+ sub-population in two MDS patients during the course of their disease.

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Patients and Methods

Six patients with MDS were investigated; three females and three males, with a median age of 65 year (16-89 y). The clinical and hematological features of the patients are summarized in Table 1. Presenting symptoms were anemia in all cases, infection in two and bleeding in one. The diagnosis MDS was assessed according to the criteria, as defined by the FAB cooperative group (11,13,14), in films of peripheral blood and bone-marrow stained with May-GrÜnwald Giemsa.

Prognostic scores were given to each patient according to the Bounemouth system (15), which allocates one point for each of the following features: an Hb of less than 6.5 mmol/l, for a white blood cell-count of less than 2.5 x 10^9/l, for a platelet count of less than 100 x 10^9/l and for a bone marrow blast count of more than 5%. Patients with a score of 0 or 1 have the best prognosis with a median survival of 62 months, patients with a score of 2 or 3 have a median survival of 22 months and patients with a score of 4 have a poor prognosis with a median survival of 8 months.

Chromosomal analyses were performed at diagnosis and during the course of the disease according to a standard technique (16) and chromosomal abnormalities were described according to the ISCN (17).

Immunological marker analyses were performed as described before (8). The expression of a series of immunological markers was tested, including terminal desoxynucleotidyl transferase (TdT), the B cell markers CD10 (VIL-A1; Dr. W. Knapp, Vienna, Austria) and CD19 (B4; Coulter Clone, Hialeah, FL, USA) the T-cell markers CD2 (T11; Coulter Clone), CD3 (Leu-4; Becton Dickinson, San Jose, CA, USA) and CD7 (3A1; American Type Culture Collection, Rockville, MD, USA), the myeloid markers CD13 (My7; Coulter Clone), CD14 (My4; Coulter Clone), CD15 (VIM-D5; Dr. W. Knapp) and CD33 (My9; Coulter Clone), the HLA-DR antigen (L243; Becton Dickinson), the precursor antigen CD34 (BI-3C5; Seralab, Crawley Down, UK) and glycophorin A (GpA) (VIE-G4; Dr. W. Knapp). The TdT-IF assay was performed by use of rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit (Ig) antiserum (Supertechs, Bethesda, MD, U.S.A). DIF analysis for the differentiation markers CD2, CD7, CD10, CD19, CD13, CD14, CD15, CD33 and CD34 and for TdT were performed as described before (8).

Table 1 Features of patients with MDS at the time of diagnosis

	sex/age	Dx	bone marrow		peripheral blood					bournemouth score
			EB %	MB %	Hb mmol/l	ret %	MCV fl	WBC 10^{-9}	Pl 10^{-9}	
1. M.M.	M/17	RAEBt	62.4	8.8	4.1	40	100	2.9	14	3
2. S.M.	F/16	RAEB	47.6	7.2	3.9	24	111	3.0	36	3
3. J.N.	F/87	RAEB	22.8	13.6	4.1	nd	95	1.6	52	4
4. H.F.	M/76	RARS	42.0	0.4	5.7	9	114	5.7	588	1
5. H.B.	F/65	RARS	57.6	1.2	5.7	10	112	4.8	345	1
6. N.K.	M/89	RARS	40.7	1.7	3.7	nd	89	1.9	59	3
median	65		52.6	4.0	4.1	17	106	3.0	56	

Dx, diagnosis according to the FAB criteria; EB, erythroblasts; MB, myeloblasts; Hb, hemoglobin concentration; ret, reticulocytes; MCV, mean corpuscular volume; WBC, white blood cell-count; Pl, platelet count.

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Results

Patients

According to the FAB criteria patient 1 was classified as RAEBt, patients 2 and 3 as RAEB and patients 4, 5 and 6 as RARS. The median myeloblast cell count was 4.0% (0.4-13.6), erythroblast count 52.6% (22.8-62.4), hemoglobin concentration 4.1 mmol/l, MCV 106 fl, reticulocyte count 17%, white blood cell-count 3.0 x 10^9/l and platelet count 56 x 10^9 /l. Patients 1,2,3 and 6 had a Bournemouth score of 3 or 4, whereas in patients 4 and 5 a score of 1 was found (Table 1).

Patients 1 and 2 progressed to an AML-M2 during their clinical course, within 2 and 15 months respectively. Complete remission was achieved in patient 1 after one course of chemotherapy and in patient 2 after two courses of chemotherapy. Remission duration was 5 and 7 months respectively.

Cytogenetics

Chromosomal analysis was performed at diagnosis in cases 1 and 2. No chromosomal abnormalities were found, in the 32 metaphases investigated in each case. During the course of the disease chromosomal abnormalities could be detected in patient 1, in which the relapse (RAEBt) bone marrow cells contained a previously absent abnormality i.e.: t(1;2), t(7;13) in 20% of the metaphases.

Immunologic marker analysis

The results of the immunological marker analyses, which were performed at the time of diagnosis, are given in Table 2. Low percentages of cells expressing the T-cell markers CD2 and CD3 were found in all cases except in case 3, in which 47% CD2 and 41% CD3-positive cells were detected. In all cases low percentages of the B-cell markers CD10 and CD19 and the monocytic marker CD14 were found. The majority of the mononuclear cells expressed the pan-myeloid markers CD13 and/or CD33. The precursor antigen CD34 was present in <1 - 22% of the cells. Less than 5% CD34-positivity was found in cases 3 and 4 (both RARS), whereas a percentage of above 10% was found in cases 1, 2 and 3 (RAEB and RAEBt cases). Expression of TdT was found in 0.1 - 11% of the cells .

Table 2: **Immunological marker analysis of patients with MDS at the time of diagnosis**

| Patient | Stage | Percentage of MNC and TdT+ cells positive for surface marker | | | | | | | | | | | | |
		TdT	CD2	CD3	CD7	CD10	CD19	CD13	CD14	CD15	CD33	CD34	HLA-DR	GpA
1. M.M.[b]	RAEBt	11	9(0.5)	10	10(5)	<1(0.5)	8(0)	2(39)	2(0)	38(0)	57(97)	22(88)	32(26)	13
2. S.M.	RAEB	0.1	8(1)	6	10(21)	<1(11)	3(0)	4(45)	8(0)	21(1)	79(87)	10(93)	19(73)	29
2. S.M.	AML	29		8			1	67(63)	4	27	80(99)	55		
3. J.N.	RAEB	2.5	47	41	43	<1(6)	6	7	<1	19	37(58)	15(99)	47	2
4. H.F.	RARS	0.1	8	12	11	<1(5)	5	26	2	46	50(9)	4(38)	16	8
5. H.B.	RARS	0.1	18	14	16	<1(53)	3	20	7	48	32(2)	<1(11)	25	<1
6. N.K.	RARS	5	12	12	10	<1(9)	2	32	23	10	48(93)	8(68)	9	2

a. Percentages positivity for surface membrane marker per TdT-positive cells as determined by DIF staining.
b. At end stage disease, diagnosed as AML-M2, 25% $CD33^+$,TdT^+ cells were detected.

Using DIF staining it was found in 4 cases (1-3 and 6) that the majority (58 - 99%) of the TdT+ cells were positive for CD13 and/or CD33, but negative for CD10 (MM+/TdT+) and 68 - 99% of the TdT+ cells were positive for the precursor antigen CD34. However, in cases 4 and 5, in which 0.1% TdT+ cells were detected, only 9.3 and 2.0% of the TdT+ cells were CD33 positive, whereas 11 and 38% of the cells were found to have the CD34+/TdT+ phenotype.
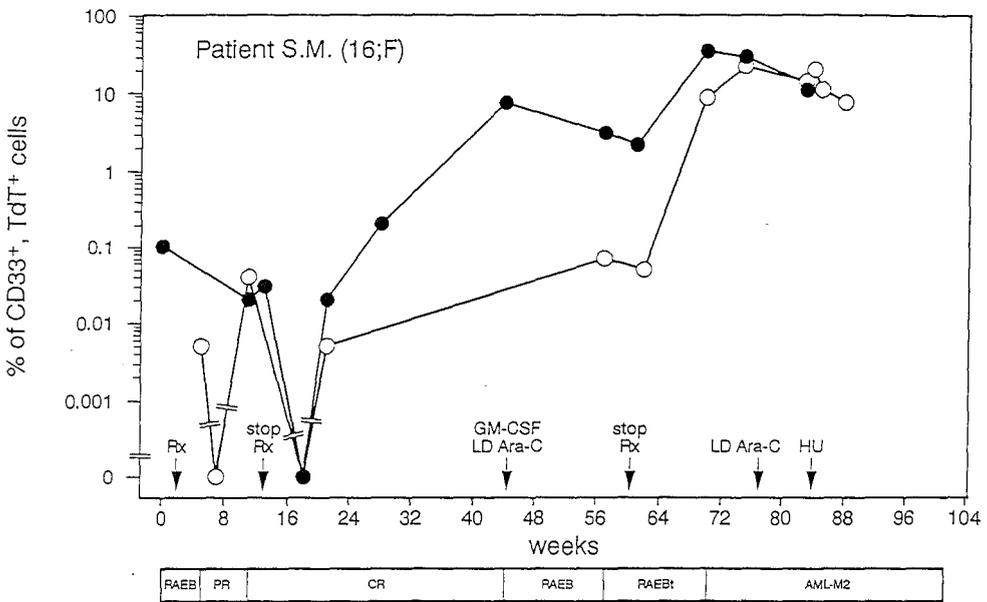
Figure 1: Follow-up of BM samples of patient 2 by use of DIF staining for TdT and the myeloid marker CD33. Clinical phase is based on both clinical observation and cytomorphology of BM (closed circles) and PB samples (open circles). PR = partial remission, CR = complete remission, Re = relapse, Rx = start chemotherapy, Stop Rx = end of chemotherapy. GM-CSF = granulocyte-macrophage colony-stimulating factor, LD Ara-C = low dose cytosine arabinoside, HU = hydroxyurea.

Follow-up studies of two MDS patients

In order to monitor the MM+/TdT+ subpopulation follow-up DIF studies were performed in patients 1 and 2. In patient 1 the CD33+/TdT+ population increased from 11% in the RAEBt to 25% in the AML-M2 stage of the disease. As depicted in

figure 2, in patient 2 the CD33+/TdT+ subpopulation, which made up 0.1% of the cells in the RAEB phase, gradually decreased to below detection at the end of induction therapy when this patient was in complete remission, subsequently CD33+/TdT+ cells became detectable again 6 months before relapse RAEB. This MM+/TdT+ subpopulation rapidly expanded to 35% in the terminal AML-M2 phase.

Discussion

The objective of this study was to determine whether MM+/TdT+ cells are detectable in MDS. We therefore analysed by DIF stainings six MDS cases (one RAEBt, two RAEB and three RARS), and performed follow up studies in two cases, which eventually evolved in an AML. TdT-positivity was found in 0.1 - 11% of the cells. In the two RAEB cases and the RAEBt case the majority of the TdT+ cells were positive for CD13 and/or CD33, which are myeloid surface-antigens that have not been found on normal T or B lymphocytes (18), indicating that the TdT+ cells belong to the myeloid differentiation lineage. Interestingly, CD34 expression was found in 88 - 99% of the TdT+ cells in these three cases. CD34 is expressed by progenitor cells in normal bone marrow as well as immature acute leukemias (19). Therefore, it is likely that the CD34+/TdT+ represent a immature subpopulation within the MDS. It is unlikely that TdT+ cells expressing CD13 and/or CD33 are residual normal elements within the bone marrow, since MM+/TdT+ is detected in normal bone marrows in extreemly low fequency, if detected at all (20,21). Moreover, in patients 1 and 2 MM+/TdT+ cells could be detected in MDS and AML phase of the disease. In patient 1 the percentage of MM+/TdT+ cells increased from 11% in MDS to 25% in AML. In patient 2 the MM+/TdT+ subpopulation was 0.1% at diagnosis, markedly reduced during remission and rapidly increased before relapse RAEB and further increased to 35% in parallel with progression to AML. These data strongly suggest that MM+/TdT+ cells represent an abnormal myeloid subpopulation, which might belong to the leukemic clone and is already detectable in MDS. Furthermore, double marker analysis for a myeloid marker and TdT may be useful to monitor this abnormal subpopulation during disease progression.

In two of the three RARS cases only a minority of the TdT+ cells were found to be

CD33 positive. The percentage MM+/TdT+ cells in these two RARS cases did not exceed the percentages that are found in normal bone marrows (20,21).

This is in agreement with the observation that RARS is less often accompanied by dysgranulopoiesis (22,23). Gatterman et al. (24) have recently distinguished on cytomorphologic grounds between pure sideroblastic anemia (PSA), which is confined to dyserythropoiesis and RARS, which is characterized by additional dysplastic features of granulopoiesis and/or megakaryopoiesis. Both PSA and RARS are diagnosed in the same MDS group (MDS 2 or RARS), but the authors found striking differences in the risk of leukemic transformation and survival. In this respect it is of interest that we found 5% MM+/TdT+ cells in the RARS case with an abnormal granulopoiesis (case 6), suggesting that the presence of MM+/TdT+ cells can discriminate between RARS cases with and without an abnormal granulopoiesis. Remarkebly, in the latter case a high Bournemouth score was found, indicating a worse prognosis than the former two RARS cases with low Bounemouth scores. These preliminary results suggest that the presence of MM+/TdT+ cells may correlate with prognosis. Further studies, however, are required to determine the prognostic significance of MM+/TdT+ cells in MDS.

In conclusion our results demonstrate that MM+/TdT+ subpopulations occur in MDS cases, with an abnormal granulopoiesis and the size of this subpopulation increases in parallel with progression of MDS to AML. These results suggests that MM+/TdT+ cells represent abnormal myeloid cells in MDS. Follow-up studies in two cases demonstrated the feasability to monitor this abnormal subpopulation by DIF staining during the course of the disease.

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CHAPTER 3

IN MYELODYSPLASTIC SYNDROMES PROGRESSION TO LEUKEMIA IS DIRECTLY RELATED TO PHA DEPENDENCY FOR COLONY FORMATION AND INDEPENDENT OF IN VITRO MATURATION CAPACITY

M.R.Schipperus[1], A. Hagemeijer[2], R.E. Ploemacher[2], J. Lindemans[1], J.S.A. Voerman[2], and J. Abels[1]

Institute of Hematology[1] and Department od Cell Biology and Genetics[2], Erasmus University Rotterdam, The Netherlands

SUMMARY

In an agar-liquid double-layer colony assay in which myeloid leukemia colony forming cells require the presence of both the lectin PHA and colony stimulating factor (CSF) for in vitro proliferation, colony formation of bone marrow cells derived from patients with a myelodysplastic syndrome (MDS) was studied.

In five of 14 MDS and all five leukemic transformed MDS cases, colony formation was found to require both PHA and CSF. Three of these five PHA dependent MDS cases progressed to overt leukemia within 1 year, one progressed from RA to RAEB, one patient received AML chemotherapy. PHA-dependent colony formation was associated with higher bone marrow blast counts, but not directly to FAB type or cytogenetic abnormalities. In nine other MDS cases only CSF was required for colony formation. In these PHA-independent cases the course of the disease was stable during the observation time (5-17 months). Two types of colonies were observed in this in vitro system: colonies adherent and colonies nonadherent to the agar under layer. The former consisted of terminally differentiated myeloid cells, and the latter comprised immature cells. This suggests that the percentage adherent colonies formed in vitro may be used as a measure for the maturation defect in MDS. However, no correlation was found between the percentage adherent colonies and progression to leukemia of the MDS cases. Our findings suggest that the dependency on PHA for in vitro colony formation of colony-forming cells in MDS is predictive for the progression to leukemia. However, the in vitro differentiation capacity has no apparent prognostic significance.

INTRODUCTION

The MDS has been subdivided into five clinical entities on morphological criteria by the French-American-British (FAB) cooperative group (1,2). Patients suffering from MDS have a variable clinical course. The high mortality rate results form the complications associated with persistent cytopenias, as well as from leukemic transformation (3).

Prognosis has been associated with the percentage of blast cells in bone marrow and blood (4), cytogenetic abnormalities (5), and in vitro growth patterns of bone marrow cells (6,7).

The formation of granulocyte-macrophage colonies in the Robinson assay system (8) has been reported to be abnormal in fifty to ninety per cent of the MDS patients. The abnormal in vitro colony growth of MDS bone marrow (i.e. absent or severely reduced colony formation, or an increased cluster/colony ratio) resembles that of AML (9-13). An abnormal in vitro growth pattern tends to be correlated with poor prognosis (12,13). In vitro maturation defects are observed both in semi-solid and liquid culture (14,15).

Since colony formation of AML bone marrows in standard semisolid assays is very poor, two types of colony culture assays for AML-CFCs have been developed (16,17). Both in the PHA-leukocyte feeder layer assay and the PHA assay described by Dicke et al. (16) the AML-CFCs were found to be of a low boyant density. In addition, it is suggested that AML cells giving rise to colonies in a PHA assay and the Robinson assay represent distinct leukemic subpopulations (18). Spitzer et al. (19) have studied a small series of oligoblastic leukemia patients at diagnosis with a two-step PHA assay. In a group of 10 patients those with PHA colonies appeared to follow a more rapid clinical course than those without PHA colonies. This finding suggests that the assay detects a leukemic subpopulation with greater proliferative potential than the majority of the leukemia (19).

No reports, that we have knowledge of, describe the characteristics of colony formation of MDS bone marrow in a PHA assay. We used a modified one-step PHA assay to investigate the growth and differentiation of this specific subset of progenitors in MDS and LT-MDS bone marrow and compared this with the morphological classification according the criteria of the FAB group, bone marrow blast counts, the presence of cytogenetic abnormalities and the relation to the clinical course of the disease during the follow-up period.

MATERIAL AND METHODS

Patients
Seventeen patients (ages 17-84 years) with a primary myelodysplastic syndrome, four patients with leukemic transformation of a MDS, five de novo AML patients and seven normal controls were studied at diagnosis. Follow-up culture studies were performed in two MDS cases (24 and 28), and two leukemic transformed MDS cases before and after relapse (31 and 32). Informed consent was obtained according the Helsinki convention. Relevant clinical and hematological data are given in Table 1. The MDS and AML were classified according the FAB nomenclature (1,2). Controls had no hematological malignancy and had normal bone marrow morphology.

Bone marrow cells
Bone marrow of patients was aspirated from the posterior iliac spine as part of diagnostic investigations. Aspirates were collected in glass tubes containing preservative-free heparin. Mononuclear cells with a density less than 1.077 g/ml were prepared by layering the bone marrow cells on Ficoll-isopaque and subsequent centrifugation (20 min, 1000 g). T cell depletion of the mononuclear cells was performed by E-rosette Ficoll separation. Rosettes were prepared by incubating the mononuclear cell suspensions (2 x 10^6) with 1% v/v 2-aminoethylisothioranium bromide treated sheep erythrocytes for 5 min. This mixture was layered on Ficoll (1.077 g/ml) and centrifugated for 20 min, 1000 g. Subsequently, the interphase fraction, now depleted of rosette forming cells, was collected. In addition, adherent cells were removed from the T cell depleted

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Table 1. Clinical, Hematological, and Cytogenetic Data at Time of Study and Results of Colony Assays in Individual Patients

Type of Case	Pt. no.[a]	Sex	Age (yr)	FAB[b]	Bone Marrow		Colony Formation		
					% Blasts	Cytogenetics	+PHA[c]	−PHA[d]	% Adh.[e]
NBM	1	F	78		1.0	ND[f]	528	304	97
	2	F	39		1.0	ND	1271	1302	65
	3	F	26		0.3	ND	99	116	91
	4	M	33		0.5	ND	255	174	90
	5	F	32		0.2	ND	450	432	100
	6	F	32		0.8	ND	337	ND	100
	7	F	71		0.2	ND	671	ND	89
AML	8	M	66	M4	68	n[g]	547	0	3
	9	M	47	M1	81	inv.16(pl3-q22)	3	0	0
	10	M	20	M4	89	t(8;13;17)	86	0	0
	11	M	51	M4	64	n	59	7	8
	12	M	45	M2	72	n	11	4	20
MDS during stable course	13	F	84	RAEB	6.6	ND	140	ND	74
	14	F	79	RAEB	5.2	n	201	ND	79
	15	M	78	CMML	0.4	−y/−y, +8 (10%)	402	ND	98
	16	F	74	RAEBt	24.0	n	202	122	12
	17	M	53	RARS	0.2	n/+8 (3%)	483	453	41
	18	M	65	RARS	5.0	n	84	116	4
	19	M	82	RARS	1.2	ND	142	145	26
	20	F	77	RA	2.4	ND	89	120	97
	21	M	81	RAEB	11.2	n/del(7), del (20)/t(3;17), del(7), del(20)	53	40	8
	22	M	77	RAEB	15.0	n	76	67	25
	23	M	72	RAEB	5.2	n/45 XO (13%)	81	33	54
	24a	M	73	CMML	0.4	n	419	360	87
	24b			CMML	5.8	n	87	20	37
	24c			CMML	1.0	n	407	275	50
MDS with progression	25	M	83	RAEB	20.0	n	123	9	99
	26	M	35	RAEB	20.0	n/−7 (7%)/ +8 (66%)	180	0	26
	27	M	71	RA	5.0	n	117	0	82
	28a	M	37	RAEB	13.0	n	32	2	32
	29	M	17	RAEBt	23.4	n	11	0	79
LT-MDS	30	M	40	M2	42.0	t(8;21)	441	ND	51
	31a	F	38	M5	66.0	n/+8 (10%)	363	9	36
	31b			M2	40.4	n/+8 (4%)	7	0	79
	32a	F	40	M7	50.3	n	60	3	51
	32b			M2	33.0	n	47	0	0
	33	M	78	M2	60.0	complex tetraploid	156	0	41
	28b	M	37	M2	31.6	ND	240	3	80

[a]Pt. no., patient numbers: each number indicates a patient. a, b, and c indicate follow-up culture studies during the course of the disease.
[b]Classifications not defined here are either listed in the text abbreviations footnote or else are standard abbreviations. RAEBt, RAEB in transformation.
[c]Mean number of colonies of three counted dishes per 10^5 plated cells in the presence of 10% GCT-CM and 1% PHA.
[d]Mean number of colonies formed in the presence of 10% GCT-CM.
[e]% Adh., mean percentage of adherent colonies formed in the presence of 10% GCT-CM and 1% PHA.
[f]ND, not determined.
[g]n, normal karyotype 46, XY or 46,XX.

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mononuclear cells by incubating the cell suspensions for 2 hr at 37°C, in a fully humidified atmosphere with 5% CO_2 in air in plastic culture flasks (Costar, 75 cm^2, 1 x 10^6 nucleated cells/ml in culture medium with 10% serum).

Colony assay

Myeloid colonies were grown in a liquid agar double-layer culture. T cell depleted, nonadherent mononuclear bone marrow cells (1 x 10^5) were cultured in a 0.4 ml liquid upper-layer in the presence or absence of 1% (v/v) PHA. The 0.5% agar under- layer contained various concentrations (1 - 20%, but 10% when not specified) of conditioned medium of the giant tumor cell line (Gibco Cat. no. 680-1000) as a source of CSF in a volume of 1 ml in plastic culture dishes (Costar diameter 35 mm). The culture medium consisted of the α-modification of Dulbecco's modified Eagle's MEM, 20% human AB serum (a constant pool) and 10% of a mixture composed of 10% BSA (Fraction 5), egg lecithin (3.75 x 10^{-3} M), Na_2SeO_3 (1.25 x 10^{-5} M), human transferrin (9.62 x 10^{-4} M) in a $FeCl_3$ solution (1.92 x 10^{-3} M) and β-mercaptoethanol (10^{-1} M) in ratios of respectively 75:8:8:8:1. The culture dishes were incubated in a fully humidified atmosphere of 5% CO_2 in air at 37°C for 7 days. Colonies of more than 50 cells were scored using an inverted microscope. The total number of colonies were counted and subsequently colonies adherent to the agar underlayer were identified and counted after removal of the non-adherent colonies with a Pasteur pipet. After counting, the adherent colonies were mass harvested with a Pasteur pipet. Aliquots of single cell suspensions of both non-adherent and adherent colony cell suspensions were used for the preparation of cytospin slides. After metachromatic staining (May-Grünwald Giemsa), the morphological maturation stage of the colony cells was determined. In some cases, intact colonies were plucked for assessment of the morphological maturation stage of single colonies. The presence of T cells in colonies was tested by E-rosette formation.

Cytogenetics

Cytogenetic analyses of patients bone marrow cells were done according to our standard technique (20) as part of the diagnostic investigation. Chromosomes were always identified by banding techniques (R-, Q- and G-bands) and according to the ISCN (21).

RESULTS

Dependency on PHA for colony formation

All 17 MDS bone marrows formed colonies in the presence of PHA and GCT-CM. The mean number of colonies was 176 ± 140 (mean ± 1 SD)/ 1 x 10⁵ cells plated. A representative GCT-CM dose response curve is shown in Figure 1. The 5 LT-MDS cases formed an average of 188 ± 155 colonies under the same culture conditions, whereas the seven normal and the five AML bone marrows formed respectively 516 ± 353 and 141 ± 205 colonies/10⁵ plated cells. Colony cells contained always less than 1% E-rosette forming cells.
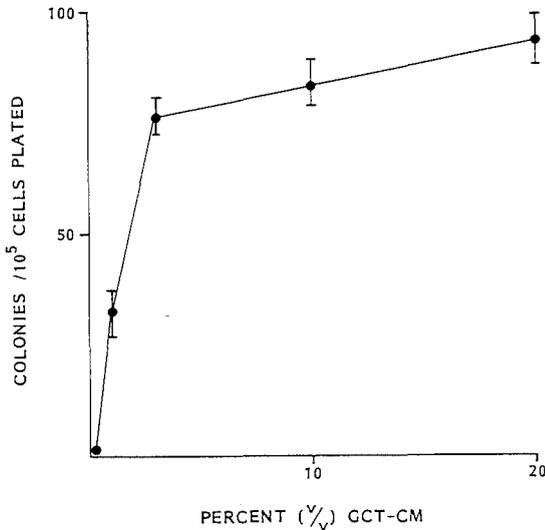
Figure 1. Dose response curve of colony formation stimulated with increasing concentrations of GCT-CM in the presence of 1% PHA. Each point represents the mean of triplicate counts (± 1 SD) of colony culture of bone marrow derived from patient no. 18.

In the absence of PHA, but in the presence of GCT-CM, very few or no colonies were formed in all AML, all LT-MDS cases and five of 14 MDS cases. In seven MDS cases no enhancing effect of PHA on colony formation was observed. In two cases (16 and 23) an increase of colony numbers was found in the presence of PHA, but these cases were not classified as PHA-dependent because a substantial number of colonies (>10) was formed in the absence of PHA.

Follow-up culture studies of individual cases showed no alterations in the growth patterns. Hence, colony formation remained PHA independent (case 24), or was PHA dependent before and after progression to leukemia (cases 28a and 28b), or was PHA dependent on two different time points in the leukemic phase (i.e. before and after relapse) of the disease (cases 31, 32). No cases were observed where the colony formation pattern changed from a PHA independent to a PHA dependent one during the observation time.

Colony adherency

Two types of colonies were observed in this colony assay: those that were adherent to the agar underlayer, and those that were nonadherent. Colonies that were adherent to the agar underlayer consisted of terminally differentiated myeloid cells (Table 2). Invididual adherent colonies were found to contain either macrophages, or a mixture of granulocytes and

Table 2: The average percentages mature cells present in non-adherent and adherent colonies and the granulocyte-macrophage ratio of the mature cells

	NBM (n=4)		MDS (n=8)	
	% maturation[1]	G/M ratio	% maturation	G/M ratio
non-adherent	100	3.3	32.2	0.87
	(99-100)	(2.6-4.3)	(1.3-59.6)	(0-2.0)
adherent	97	0.8	92.4	0.2
	(94-99)	(0.42-1.2)	(83-100)	(0-0.9)

1. *Mature cells are defined as bands, PMN granulocytes or macrophages.*

macrophages. Non-adherent colonies of MDS cultures were predominantly composed of immature cells (Table 2). In the AML group virtually no adherent colonies were found.

The nonadherent colonies consisted of blast cells, whereas in normal bone marrow cultures these colonies contained predominantly mature granulocytes (bands and PMN cells). The differentiation stage of the majority of the non-adherent colony cells in MDS was intermediate to that of NBM and AML (i.e., promonocytes, promyelocytes, meta- and myelocytes). A dose response relation was found between the percentage adherent colonies and the concentration GCT-CM (Table 3). PHA did not have an enhancing effect on the formation of adherent colonies (Table 3). These results indicate that the percentage adherent colonies formed in this assay may be used as a measure of the in vitro maturation of the colony cells.

Table 3:	Percentage adherent colonies formed in the presence of increasing concentrations of GCT-CM			
% PHA	% GCT-CM	Culture[1] 24a	24b	24c
1	1	8	7	17
1	3	23	6	70
1	10	87	37	50
0.1	10	81	nd[2]	nd
0.0	10	74	25	79

1. *colony assays were performed at three different times with bone marrow cells of patient no. 24 (see Table 1).*
2. *nd = not determined.*

In the presence of 10% GCT-CM and 1% PHA, the mean percentage adherent colonies formed in the MDS group was 53% (range 4-99%) and 48% (0-80%) in the LT-MDS, versus 90% (65-100%) in the NBM and 6% (3-20%) in the AML group (Fig. 3). With 1% GCT-CM the mean percentage adherent colonies in the MDS group was 28% (range

1-100%), whereas in the NBM group this figure was 75% (range 49-100%). These results suggest that in MDS both the in vitro maturation capacity and the maturational responsiveness upon the GM-CSF stimulus are reduced.
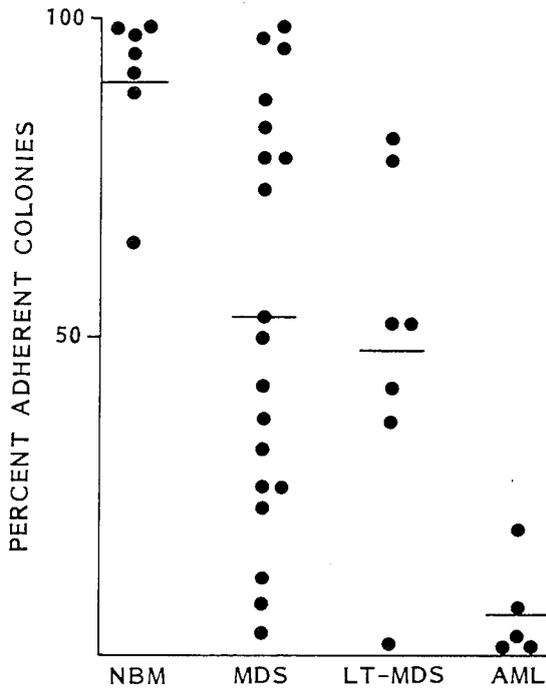
Figure 2. Percentage colonies adherent to the agar under layer in the presence of 10% GCT-CM as a source of GM-CSF and 1% PHA. Horizontal lines indicate the averages of each group.

Relation of colony formation pattern and the evolution to overt leukemia

A positive correlation was found between PHA dependency for colony formation and the progression to overt leukemia in three cases (cases 25, 26 and 28). One of the five MDS cases that had a PHA-dependent colony formation pattern, progressed from RA to RAEB (case 27), and one had originally been classified as M6 (case 29), but was later reclassified as a RAEBt. This last patient received chemotherapy, but did not reach complete remission. The bone marrow morphology after chemotherapy met again the criteria for RAEBt.

In all 14 PHA-independent cases the course of the disease was stable during the observation time of 5-17 months.

PHA-dependent colony formation in MDS cases was related to a higher bone marrow blast count as compared to PHA-independent cases (mean 16.3 versus 7.1). However, no obvious relationship was observed between the FAB type and colony formation pattern. No PHA-dependent cases were present in the RARS or CMML groups. The incidence of progression to leukemia in RA and RAEB varies considerably. In these prognostic heterogenous MDS subgroups, the PHA-dependency for colony formation may separate patients with a better or worse prognosis. Only in one of the PHA dependent MDS cases an abnormality was found during the karyotype analysis at the time of culture (case 26). Compared with NBM, the mean percentage adherent colonies was decreased both in the MDS and the LT-MDS group. Progression to leukemia occured in cases with a high (cases 25, 27 and 29) and a low (cases 26, 28a) percentage adherent colonies. Hence, no apparent correlation exists between the percentage adherent colonies and the progression to leukemia.

DISCUSSION

The presented results indicate that MDS patients may be classified, irrespective of the FAB class, on the basis of the PHA dependency for in vitro colony formation, in a group with a low risk and a group with a high risk for progression to leukemia. PHA dependency correlates with a higher average bone marrow blast count, but not directly with the FAB type or the presence of cytogenetic abnormalities.

The number of blast cells in bone marrow and blood is reported to be an important prognostic parameter (4). In the RA case (no. 27), however, PHA dependent colony formation was found, whereas the bone marrow blast count was low (5%). In this case we observed progression to RAEB, indicating that the PHA dependent in vitro growth pattern precedes the in vivo accumulation of blasts in the bone marrow, rather than being the result of increased numbers of blast cells in the cultured cell suspension.

PHA independent growth with a high percentage adherent colonies was related to a normal growth pattern in the Robinson assay (data not shown). Both PHA independent cases with a low maturation capacity and PHA dependent cases were found to have an abnormal growth pattern in the Robinson assay. This indicates that a further discrimination on the basis of PHA dependency is possible of cases with abnormal growth in the standard colony assay.

The presence of cytogenetic abnormalities is also valued to be an important prognostic parameter in MDS (13). However, we found no direct relationship between karyotype and in vitro growth pattern. This could indicate that growth characteristics of the MDS progenitor cells do not causally relate to the cytogenetic abnormalities found in MDS. The reduced differentiation capacity of MDS CFCs, as measured by the percentage adherent colonies, may reflect the in vivo maturation disorder in MDS. The mean percentages of adherent colonies of the MDS and LT-MDS groups are comparable, whereas this figure is much lower in the AML group. Three of the five PHA-dependent MDS cases (cases 25, 27 and 29) have a high percentage adherent colonies (Table 1). Moreover, sequential studies do not show a consistent decrease in percentage adherent colonies before progression to overt leukemia or during the leukemic phase of MDS (Table 1). It is therefore unlikely that the reduced maturation capacity in MDS is directly related to the progression to leukemia.

Recently, it has been reported that patients with apparently de novo AML with features of trilineage MDS differ from those without associated trilineage MDS (22). Biologically, this newly defined group is comparable to the LT-MDS group described here. It is therefore of interest that we found a higher maturation capacitiy in the LT-MDS group as compared to the AML group, suggesting that also on basis of in vitro growth pattern the former may be separated from the latter.

The implications of our findings for an understanding of MDS and its progression to acute leukemia may be as follows. One possible explanation for the leukemic transformation is the intensification of the maturation block, but our results do not support this, since the differentiation capacity of the CFCs before and after progression to leukemia is comparable. Apparently, more important is the PHA dependency for colony formation. This growth pattern seems to be directly related to the progession to leukemia. It is therefore of interest to investigate the nature of this PHA effect. It has been reported that leukemic blast progenitor cells require the presence of CSF and cell to cell interactions for exponential growth (23). PHA may very well induce cell-cell interactions and subsequently proliferation in the presence of CSF. Preliminary results in our laboratory are in support of this view. However, PHA may be a message on its own or may induce the release of soluble factors by accessory cells. Another question to address is whether this is a a unique "leukemic" feature or whether a similar type of cell exists in normal bone marrow. Normal multipotent stem cells have been described that only respond to M-CSF when concommitantly cultured in the presence of IL-1 (24). IL-1 is reported to exhibit lectin- like specificity (25). PHA may therefore induce similar effects in this culture assay. It is tempting to speculate that the normal biological counterpart of the PHA dependent CFC is the multipotential stem cell.

Our results show that MDS and LT-MDS have a comparable reduced in vitro differentiation capacity. The maturation capacity is not further altered when progression to leukemia occurs. But PHA dependency is clearly related to an impending progression to leukemia. Further studies are required to reveal the mechanism of action of PHA and to determine the prognostic value of PHA dependency for colony formation.

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CHAPTER 4

THE EFFECTS OF IL-3, GM-CSF AND G-CSF ON THE GROWTH KINETICS OF COLONY FORMING CELLS IN MYELODYSPLASTIC SYNDROMES

Martin Schipperus[1], Pieter Sonneveld[1], Jan Lindemans[1], Anne Hagemeijer[2] , Nel Vink[1], Johannes Pegels[3] and Johannes Abels[1]

Department of [1]Hematology· [2]Cell Biology and Genetics· Erasmus University Rotterdam and the [3]St.Franciscus Gasthuis Rotterdam· The Netherlands.

ABSTRACT

In order to obtain more insight into the nature of the abnormal in vitro colony formation in Myelodysplastic syndromes (MDS) we investigated the kinetics of the colony formation of 23 MDS cases in response to recombinant human Il-3 (Il-3), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF) and giant cell tumor cell line conditioned medium (GCT-CM). The kinetics of GCT-CM induced colony formation were comparable to that of G-CSF-induced colony growth, both in MDS and in normal bone marrow cultures. Colony formation was found to be delayed in MDS. The delay in colony formation was most apparent in the GCT-CM responsive progenitor cell compartment. In MDS cases with clinical features of high risk disease this delay was more pronounced as compared to low-risk cases (7 and 3 days respectively, in response to GCT-CM). The delay in colony formation was found to be caused by an increase in the time interval before progenitor cells begun to divide. These results suggest that a prolongation of the time spend in Go of myeloid progenitor cells in MDS may be the cause of the indolent in vitro colony formation observed in this disease.

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INTRODUCTION

The myelodysplastic syndrome is a clonal disorder of the hemopoietic stem cell (1) resulting in cytopenias of one or more cell lineages. In vitro a reduced myeloid and erythroid colony formation is observed (2,3). The reduced colony formation may be due to intrinsic abnormalities of the progenitor cells such as an altered responsiveness for hemopoietic growth factors caused by abnormalities of the growth factor receptors or of the signal transduction system. We (4) and others (5) have previously reported that the responsiveness for hemopoietic growth factors is indeed altered in MDS.

Experiments performed by Francis et al. (6) suggest that mature progenitor cells are more sensitive to hemopoietic growth factors than immature progenitor cells. Suda et al. reported (7) that in mice multipotential stem cells are in G_o for varying periods of time, whereas the doubling time is constant. Furthermore, recent investigations suggest that only the early G_1 phase of the cell cycle is permissive for the stimulation with colony stimulating factors (8). Hence, alterations in the cell cycle status of progenitor cells may result in an alteration in the responsiveness for hemopoietic growth factors.

The aim of the present experiments is to investigate the kinetics of colony formation in MDS in response to recombinant human hematopoietic growth factors. We therefore compared the colony formation kinetics of MDS bone marrow in response to recombinant human interleukin-3 (Il-3), granulocyte-macrophage colony- stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and the conditioned medium of the giant cell tumor cell line (GCT-CM) with normal bone marrow cells (NBM) and NBM cells depleted of committed progenitor cells by in vitro exposure to 4-hydroperoxycyclophosphamide.

MATERIALS AND METHODS

Normal donors. Normal marrow samples were obtained from six healthy volunteers upon informed consent by posterior iliac crest puncture. The marrow was collected in glass tubes containing Hanks/Hepes with preservative-free heparin.

Patients. Seventeen patients with MDS and six patients with leukemic transformation of MDS (LT-MDS) were studied. The MDS cases were classified according the FAB nomenclature (9). Relevant clinical and hematological data are given in Table 1.

On the basis of clinical, cytogenetic and hematological data, patients were divided into a MDS low risk and a MDS high risk group. Criteria for the high risk MDS group were: the presence of more than 10% blast cells in the bone marrow or the presence of blast cells in the periferal blood, complex cytogenetic abnormalities or transformation to overt leukemia during the observation time. The low risk MDS group did not have these features and had a stable clinical course during the observation time.

Preparation of cell suspensions. Bone marrow cells of patients and normal donors were separated over Ficoll-Isopaque (1.077 g/ml, 1,000 g, 20 min); T cell depletion of the mononuclear cells was performed by E-Rosette Ficoll separation. Rosettes were prepared by incubating the mononuclear cell suspension (2×10^6/ml) with an equal volume of 1% (v/v) 2-aminoethylisothiouroniumbromide (Sigma) treated sheep erythrocytes for 5 minutes. Rosettes were centrifuged through Ficoll (1.077 g/ml, 20 min 1,000 g). Subsequently, the interphase fraction was collected and washed in Hanks/Hepes. In addition adherent cells were removed by incubating the cell suspension (1×10^6/ ml in α-Dulbecco's modified Eagle's medium (α-DMEM) with 10% fetal calf serum (FCS)) for 1 hour at 37 ^{0}C, 5% CO_2 in air in a fully humidified atmosphere. Non-adherent cells were collected and washed twice in Hanks/Hepes.

Exposure to 4-hydroperoxycyclophosphamide (4-HC). 1×10^7 Light-density, T-cell depleted, non-adherent cells were incubated with 60 μg/ml 4-HC (Asta-Werke, F.R.G) for 30 minutes at 37°C, washed twice in cold Hanks/Hepes and assayed for the recovery of colony forming cells.

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Table 1. Relevant clinical, haematogical and cytogenetic data of
         patients studied at time of in vitro study

Group	No.	Sex	Age	$D_x{}^a$	% blasts	Cytogenetics
Low risk	1	M	17	RA	2.6	not done
	2	F	78	RA	0.4	n
	3	M	73	CMML	0.4	n[b]
	4	M	68	RARS	0.4	n/hypodiploid(17%)
	5	M	60	CMML	1.2	n/46 XY t(1;19)(p22;q13)(12.5%)
	6	M	73	RARS	0.6	not done
	7	M	77	RAEB	7.6	46 XY, del(11)(q21q24)
	8	M	68	RA	1.5	n
High risk	9	M	18	RAEB	15.6	n
	10	M	78	RAEB	6.8[c]	n
	11	M	65	RAEBt	21.8	n
	12	M	83	RAEBt	20.0	n
	13	F	38	LT-MDS	40.0	n/47 XX,+8(4%)
	14	M	40	LT-MDS	34.2	n/46X,-Y,t(8;21)(20%)
	15	F	75	LT-MDS	30.4	n/complex[d](58%)
	16	F	78	RA→L	4.2	n
	17	M	70	RAEB→L	8.6	n
	18	F	17	RAEB→L	7.2	n
	19	M	68	LT-MDS	77.0	n
	20	M	66	LT-MDS	82.4	n
	21	F	53	RAEB	16.4	n/47 XX,5q-,+21(78%)
	22	M	60	RAEBt	20.0	not done
	23	M	56	LT-MDS	38.4	n/complex[e](62%)

a) RA = Refractory anaemia; RARS = RA with ringsideroblasts, RAEB = RA excess of blasts; RAEBt = RAEB in transformation; CMML = chronic myelomonocytic leukemia; LT-MDS = MDS after evolution to frank leukemia. RA→L or RAEB→L = transformation to overt leukemia occurred in the observation time following bone marrow culture. b) Normal karyotype, i.e., 46,XX or 46,XY c) periferal blood blast count = 5.5% d) 43-45, XX der(2) t(2;17)(p24;q11), -5,del(7)(q21),-13,16q+,-17, 20p-, +1-markers. MDS secondary to intravesical chemotherapy. e) 44,XY,Mar(3),5q-,-7,-12,-16,+dic.Mar-(16p→11::7q21→7pter).

Recombinant human colony-stimulating factors. Recombinant GM-CSF was gene-rously made available by Schering (Kenilworth, NJ) and was used at a concentration of 10ng/ml. Recombinant G-CSF was purchased from Genzyme (Sanbio bv, Uden, The Netherlands) and was used at 100 U/ml. Recombinant Il-3 was a generous gift from Dr G. Wagemaker (Erasmus University, Rotterdam, The Netherlands) and was produced by Gist-brocades (Delft, The Netherlands) and used at a concentration of 10 ng/ml. All applied concentrations of colony stimulating factors were optimal concentrations, as determined by dose-response studies using clonogenic assays on normal light-density, T-cell depleted, non-adherent bone marrow cells.

Giant Cell Tumor-Conditioned Medium (GCT-CM). GCT-CM was prepared by culturing the confluent growing GCT cell line (obtained from the American Type Culture Collection, ATCC TIB 223) for 7 days with α-DMEM with 10% FCS (10 ml per 75 cm^3) and was used as a source of colony stimulating activity at a concentration of 10% v/v.

Colony assay. Cultures were performed in α-DMEM containing 0.9 % methylcellulose (Fluka Methocell MC 4000 mPa's), and supplemented with 20 % FCS, 1% dialyzed bovine serum albumin (BSA), 30 μM egg lecithine, 0.1 μM Sodiumselenite, 7.7 μM fully iron-saturated human transferrin, 100 μM mercaptoethanol. Cultures were performed in 24-wells plates (Costar), 250 μl per well containing 2.5 x 10^4 bone marrow cells. The cultures were incubated at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Colonies of more than 50 cells were counted after 7, 10, 14, 18, and 22 days of culture using an inverted microscope. In selected cases colonies were picked from the well for staining with the May-Grunwald-Giemsa technique and subsequent morphological examination.

Cytogenetics. Cytogenetic analyses of bone marrow cells obtained from patients were done using a standard technique (10), as part of the diagnostic investigation. Chromo-somes were always identified by banding (R-, Q-, G-bands). The karyotypes were reported according to the "International System for Human Cytogenetic Nomenclatu-re" (11). In four cases cytogenetic analyses of the colony cells were performed on pooled colony cells.

Statistics. The Fischer exact test and the two tailed Mann-Whitney test were used to statistically evaluate the differences in frequency distribution of the day of maximal colony formation.
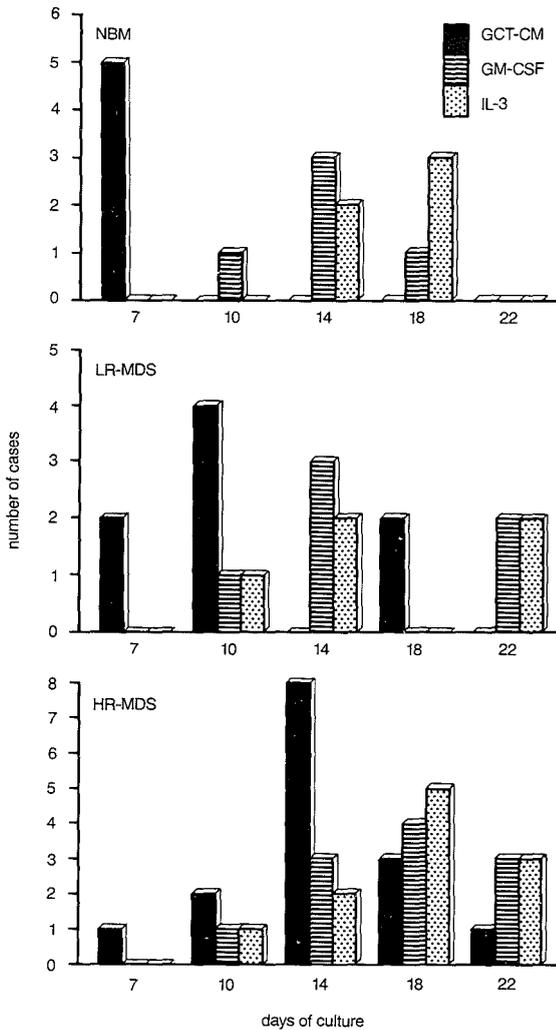
Figure 1. Distribution in time of the number of cases with maximal colony numbers. Values represent the number of cases that were observed to have attained maximum colony numbers in response to GCT-CM, GM-CSF and Il-3 at day 7, 10, 14, 18 and 22

RESULTS

Kinetics of colony formation in response to GCT-CM. In normal bone marrow cultures all cases reached a maximum number of colonies at day 7 (Fig.1). In MDS maximum colony numbers were attained at day 7 of culture in two of the eight (25%) low risk cases and one of the fifteen (6.7%) high risk cases, which were found to be significant differences as compared with the control group (Fisher exact: $p < 0.007$ and $p < 0.001$ respectively). Four of the eight (50%) cases in the MDS group with low risk features were found to have reached maximum numbers of colonies at day 10 and two of the eight (25%) cases on day 18 of culture. In the MDS group with high risk features, 93.3% of the cases had attained the maximum number of colonies on day 10 or later (i.e day 7: 1, day 10: 2 day 14: 8, day 18: 3 and day 22: 1 of the 15 cases) (Fig.1). The differences between the two MDS groups did not reach significance. Colony numbers were comparable to normal bone marrow in the low risk MDS group, but decreased in the high risk MDS group (Table 2). Since GCT-CM was found to stimulate a late progenitor cell and the colonies had a granulocytic appearance, we compared the colony formation kinetics of GCT-CM with that of recombinant G-CSF. An identical growth pattern was observed with either GCT-CM or G-CSF in normal bone marrow and MDS (Table 3).

Kinetics of colony formation in response to GM-CSF. As compared with normal bone marrow, the number of cases that attained a maximum number of colonies at day 14 of culture was decreased in both MDS groups: i.e., from three of the five in NBM to three of the seven in the low risk and three of the 12 in the high risk group. Two of the seven (28.6%) of the low risk and seven of 12 (58%) of the high risk cases reached maximum colony numbers after day 14 of culture in response to GM-CSF (Fig. 1). No statistical significant differences were found between the different groups. Colony numbers formed in response to GM-CSF were identical to normal bone marrow in the low risk MDS group and decreased in the high risk group (table 2).

Kinetics of colony formation in response to Il-3. A low number of myeloid colonies were formed following stimulation with Il-3 in normal bone marrow, low and high risk MDS (Table 2). However, in case 15 and 19 a large number (105 and 196 per 10^5 cells, respectively) of myeloid colonies were formed in response to Il-3, with a

Table 2. Time course of colony formation in response to colony stimulating factors

patient group	Day	Numbers of colonies /10^5 plated cells		
		GCT-CM (10% v/v)	GM-CSF (10 ng/ml)	IL-3 (10ng/ml)
NBM (n=6)	7	114.9 ± 22.3	7.2 ± 3.0	1.3 ± 0.6
	10	66.9 ± 13.9	17.5 ± 3.8	9.1 ± 3.9
	14	32.7 ± 7.5	39.5 ± 6.8	9.4 ± 2.2
	18	31.8 ± 12.7	24.2 ± 8.5	16.3 ± 5.6
	22	8.7 ± 2.4	24.6 ± 6.1	12.1 ± 3.0
Low Risk MDS (n=7)	7	73.0 ± 40.0	12.6 ± 8.8	0.6 ± 0.3
	10	100.8 ± 24.9	31.7 ± 16.5	4.9 ± 1.8
	14	49.9 ± 12.2	40.5 ± 15.5	11.8 ± 5.0
	18	52.7 ± 21.4	26.6 ± 10.0	13.3 ± 6.6
	22	30.8 ± 10.7	26.4 ± 12.0	14.3 ± 8.4
High Risk MDS (n=12)	7	0.7 ± 0.5	0.9 ± 0.5	0.0 ± 0.0
	10	27.3 ± 16.4	3.4 ± 1.6	0.0 ± 0.0
	14	37.8 ± 9.4	15.7 ± 5.1	15.0 ± 8.4
	18	17.3 ± 4.1	17.3 ± 3.9	16.8 ± 6.9
	22	10.5 ± 3.6	15.8 ± 6.5	16.2 ± 8.6

maximum number of colonies on day 14 of culture. No apparent differences in colony formation kinetics between the different groups were observed.

Morphology of the colony cells. From separate cultures the morphology of the colonies on the day of optimal colony formation were assessed. Therefore liquid-nitrogen stored bone marrow of three different normal bone marrows and bone marrow of cases 1, 4, 8, 9, 18, 21 and 22 were thawed and cultured for the number of the days needed for optimal colony growth (as assessed before). Colonies of NBM and low risk MDS cultures (cases 1, 4 and 8) consisted of intermediate and terminally differentiated cells (myelocytes, metamyelocytes, bands, polymorphonuclear cells, monocytes and macrophages). In all cases monocytes and macrophages were only present in GM-CSF stimulated cultures. Basophilic granulocytes were detected in Il-3 stimulated colonies in normal bone marrow and in cases 18 and 22.

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Table 3.
Comparison of the time course of colony formation in
response to GCT-CM (10% v/v) and rhG-CSF (100 U/ml)
expressed as percentage of the maximum number of colonies
formed.
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	NBM[a]			MDS[b]	
Day	GCT-CM	G-CSF		GCT-CM	G-CSF
7	100	100		0	0
10	74	59		84	36
14	41	18		100	100
18	0	0		nd	nd
22	0	0		69	40

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a, one normal control.
b, patient 22.
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Blast cell colonies were only observed in two high risk MDS cases (case 21 and 22). In these cases the colonies formed after stimulation with either GM-CSF, G-CSF or GCT-CM consisted of myeloblasts and promyelocytes (Sudan black positive, α-naphtylacetate-esterase negative).

Cytogenetic analysis of the colony cells. In order to determine whether the colonies were derived from the abnormal MDS clone, cytogenetic analyses were performed on the pooled colony cells of case 4, 7, 21 and 23. No metaphases were obtained in cases 4 and 7. In case 21, 98% of the 50 metaphases contained the same chromosomal abnormality (47 XX, 5q-, +21), as had been found in 78% of the metaphases of the freshly aspirated bone marrow. In case 23 complex cytogenetic abnormalities were found in 62% of the metaphases in the fresh bone marrow, whereas 80% of the 10 metaphases derived from colony cells showed identical complex abnormalities.

Colony growth of NBM cells after exposure to 4HC. Figure 2 shows a representative example of the growth kinetics of normal bone marrow pretreated with 4-HC for depletion of committed progenitors. Colony growth was not observed before day 14 of culture. The time sequence of colony growth in response to respectively GCT-CM, rhGM-CSF and rhIl-3 is identical to nontreated bone marrow, however delayed for 10 to 14 days.
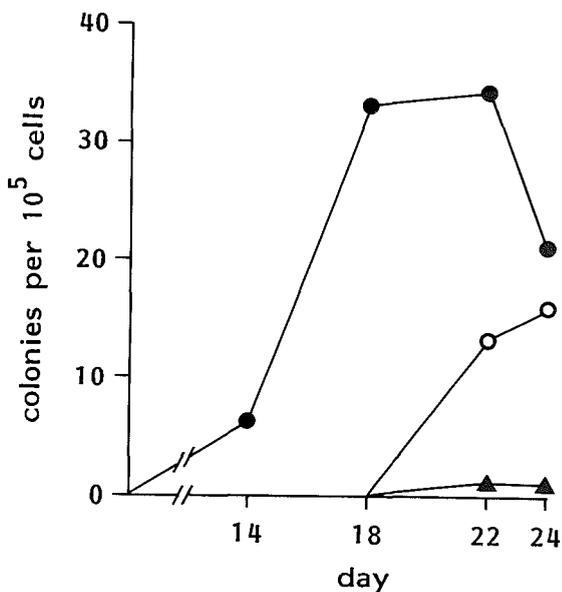
Figure 2. Time course of the colony formation of 4-hydroperoxycyclophosphamide pretreated normal bone marrow: A representative example of the colony formation in response to GCT-CM (closed circles) GM-CSF (open circles) or Il-3 (triangles) of NBM cells after exposure to 4-HC. Symbols represent the number of colonies of triplicate cultures (mean ± 1 SEM).

Identical results were obtained in experiments with bone marrow cells of three other normal volunteers; however the day on which colonies were first observed ranged from day 10-18. After 22 days of culture, cytological characterization of the colonies showed 35 granulocytic colonies following GCT-CM stimulation. After stimulation with GM-CSF, two monocytic colonies and 11 blast-colonies were found. The one colony that was observed following Il-3 stimulation consisted of blast cells (Table 4).

Observation of the initial signs of colony formation. In order to determine whether the delay in colony formation in MDS is caused by either a delay in the initiation of the mitotic cycle of progenitor cells or by a increase in cycling time of the colony cells we performed an additional experiment with one normal control, one low risk MDS (RA, case 2) and two high risk patients (RAEB: case 10 and RAEBt: case 11). Daily inspection of cultures stimulated with GCT-CM showed cluster growth from day 4 onwards in NBM and the low-risk MDS case (Fig.3, upper panels). In the high risk

Table 4. Nature of colony formation by 4HC pretreated
normal bone marrow cells on day 22 of culture

type of colony	number of colonies/ 10^5 plated cells		
	GCT-CM (10% v/v)	GM-CSF (10 ng/ml)	Il-3 (10 ng/ml)
CFU-G	35	0	0
CFU-M	0	2	0
CFU-GM	0	0	0
CFU-blast	0	11	1

cases the first signs of cluster formation was observed on days 6 and 7 (Fig. 3, lower panels). When the number of colonies increased the number of clusters decreased, indicating that clusters rapidly grew to colonies.

DISCUSSION

The data presented here show that in MDS, colony formation of myeloid progenitor cells is delayed during and in parallel with the disease progression. The delay is less pronounced in the low risk MDS group as compared with the high risk MDS group. Differences in the kinetics of colony formation between the low and high risk MDS groups were more apparent following stimulation with GCT-CM than with GM-CSF or Il-3. GCT-CM was found to stimulate primarily the formation of granulocytic colonies. Recombinant G-CSF induced the formation of identical colonies with the same growth kinetics in both normal bone marrow and MDS as GCT-CM, indicating that the effects of GCT-CM may be primarily due to the colony stimulating activity of G-CSF. Since the granulocyte progenitor cell is a relative mature colony forming cell it appears that during progression of MDS the late progenitor cells are more affected than the more early progenitors, such as the CFU-GMs.

Disease progression in MDS may be due to the gradual clonal expansion of the abnormal population, with suppression of normal haemopoiesis.
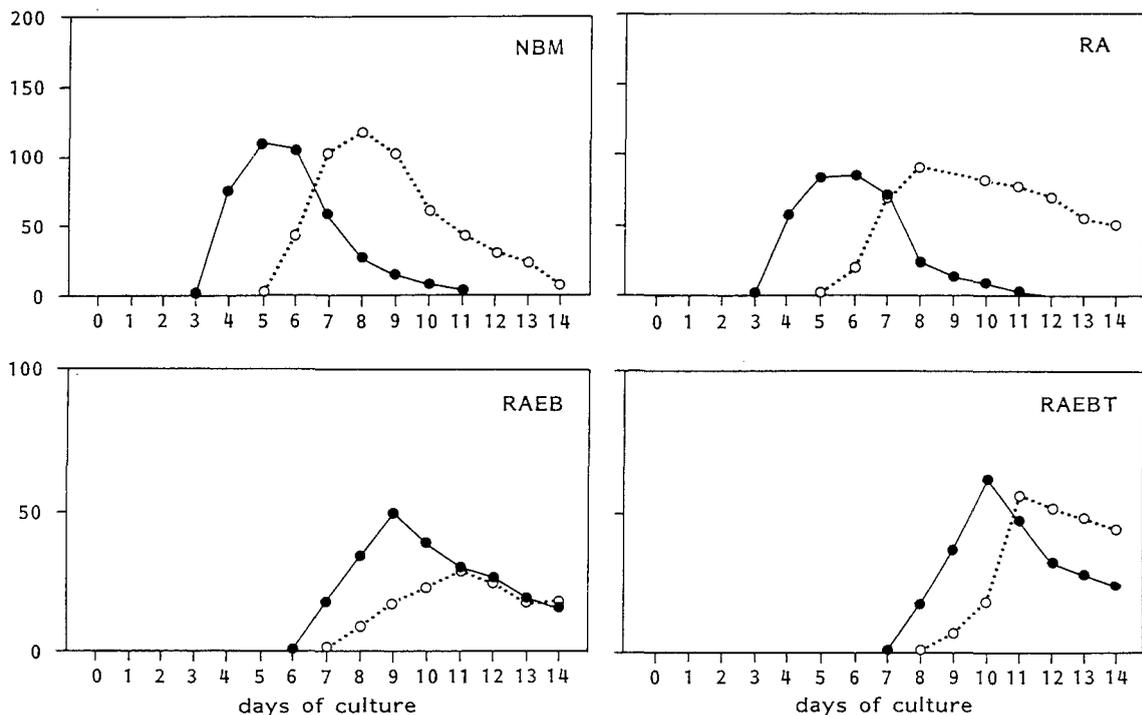
Figure 3. Time course of cluster and colony formation in response to GCT-CM. Cluster (more than 8 cells, closed circles) and colony (more than 50 cells, open circles) formation of one normal bone marrow (upper left panel:NBM), one low risk MDS patient (upper right panel:RA, case 2), and two high risk patients (lower left panel: RAEB, case 10) lower right panel: RAEBt, case 11). Points represent the mean of triplicate cultures.

It has been demonstrated by Abkowitz et al., that when this occurs both erythroid and granulocyte-macrophage colonies appear to be derived from the abnormal clone (12). In our study cytogenetic analyses performed on the colonies of two cases revealed the presence of a chromosomal abnormalities typical for the MDS clone, even in a higher frequency than found in the fresh bone marrow. In these cases a delayed colony formation was observed, indicating that this growth pattern is a characteristic of the abnormal clone. Moreover, recently published results on clonal analysis using recombinant DNA techniques established a clonal origin of the vast majority of the periferal blood and bone marrow cells in almost every MDS patient analyzed (1,13). The suppressed normal hemopoiesis appears to regain its place only after eradication

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of the MDS clone by chemotherapy or differentiation induction therapy (14). Taken together, it seems to be likely that the observed delay in colony formation is specific for the abnormal clone in MDS.

Early progenitor cells are considered to be dormant, i.e., in G_0 of the cell cycle, whereas the more mature progenitor cells are relatively more frequently in active cycle (15). Doubling times of murine committed (late) progenitor cells are reported to be shorter than the doubling times of pluripotent (early) progenitor cells (7). This would imply that the observed delay in colony formation in MDS may be explained by a relative increment of early progenitor cells (left shift) as is also suggested by Haak et al. (16). However, in this study the delay in colony formation was not associated with a relative increase of the GM-CSF responsive compartment, as would be expected in case of a relative enlargement of the earlier compartments. Baines et al. (17) have reported that the colony formation of purified CD34 positive cells in MDS is even decreased as compared to that of CD34 positive normal bone marrow cells. These findings do not support the concept of a left shift in the progenitor cell compartment as an explanation for altered kinetics of colony formation in MDS.

The presence of a functional defect of the progenitor cells may be another explanation. Tsuda et al. (8) have described that only the early G_1 phase of cell cycle is permissive for the stimulation with colony stimulating factors. In this concept an increment of dormant cells in the late progenitor cell compartment would result in a relative decrease of growth factor responsive cells. There is considerable experimental evidence that the majority of the hemopoietic stem cells reside in resting state of cell cycle (7,15). Since it is reported that primitive progenitor cells will be spared during in vitro 4-HC treatment (18,19,20), exposure of bone marrow cells to 4-HC will consequently result in a relative enrichment for non-cycling progenitor cells. Exposure of normal bone marrow cells to 4-HC resulted in the absence of colony formation before day 14 of culture upon stimulation with GCT-CM. In addition, no clusters could be detected before day 7. These findings indicate that all the actively cycling progenitor cells in the bone marrow are depleted by the 4-HC exposure. Maximum numbers of colonies were observed in an identical sequence as in unexposed bone marrow, i.e., first after GCT-CM followed by GM-CSF and Il-3. The delay in colony growth indicates that neither GCT-CM (G-CSF), nor GM-CSF or Il-3 triggers dormant cells

into cell cycle but rather exert their effects after the cells have initiated to cycle. Furthermore, in an additional experiment, daily inspection of untreated cultures showed that in MDS the initial signs of cluster formation were observed later as compared to NBM. In NBM, clusters could already be detected on day 4 of culture, whereas in some MDS cases no sign of growth was observed before day 6 of culture. An increase in colony numbers was accompanied by a decrease in the number of clusters, indicating that clusters rapidly grew to colonies. These results indicate that the observed delay in colony formation in MDS is caused by a greater time-lag before MDS progenitors start to cycle, rather than being the result of a increased cycling time of the colony cells. A prolongation of the time spend in G_0 by MDS progenitor cells, may therefore be the most likely explanation for the observed delay in colony formation.

Recent reports indicate that IL-1 and IL-6 may act as co-stimulants for the growth of normal primitive hemopoietic progenitor cells (22,23) as well as for leukemic blast-cell progenitors (24). Since our results suggest that the growth factors used in this present study are unable to effectively recruit MDS progenitor cells into cell cycle on their own, combinations with co-stimulants are probably needed to accomplish this.

Our findings may have implications for the understanding of the pathogenesis of MDS as well as for the application of growth-factor therapy either alone or in combination with chemotherapy in MDS.

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CHAPTER 5

THE COMBINED EFFECTS OF IL-3, GM-CSF AND G-CSF ON THE IN VITRO GROWTH OF MYELODYSPLASTIC MYELOID PROGENITOR CELLS

Martin R. Schipperus, Pieter Sonneveld, Jan Lindemans, Nel Vink, Margreet Vlastuin, Anne Hagemeijer, Johannes Abels

Department of Hematology and Cell Biology and Genetics, Erasmus University and University Hospital Dijkzigt Rotterdam, The Netherlands

ABSTRACT

The decreased or absent in vitro colony formation in response to single recombinant hematopoietic growth factors has been reported previously. Here we report on the effects of the combination of Interleukin-3 (Il-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF) and the effect of the conditioned medium of the giant tumor cell line (GCT-CM) on the proliferation of myelodysplastic (MDS) marrow myeloid progenitor cells and normal bone marrow (NBM) controls. Colony growth was most effectively sustained by GCT-CM and G-CSF in normal bone marrow (NBM) cultures. GM-CSF and IL-3 were less effective in inducing myeloid granulocytic colony growth, whereas the effects of Il-3 and GM-CSF were found to be approximately additive. The number of NBM granulocytic colonies induced by G-CSF and GCT-CM stimulation were comparable, whereas this granulocyte colony stimulating activity could be neutralized by anti-G-CSF antibodies. In addition GCT-CM was found to contain burst promoting activity, which could be neutralized by anti-Il-3 antibodies. Il-3 did not enhance the G-CSF activity in NBM cultures. No additive effect of stimulation with the combination of Il-3 and GM-CSF was observed in MDS marrow cultures, suggesting that these growth factors act on an identical progenitor cell population in MDS. G-CSF stimulated the growth of significantly lower colony numbers than GCT-CM, in contrast to NBM cultures. The decreased granulocytic colony formation of MDS marrow cells could clearly be enhanced by co-stimulation with Il-3. These results suggest that MDS myeloid progenitor cells require the exposure to both a pluripotent colony stimulating factor, like Il-3, and a lineage specific factor, like G-CSF, for optimal proliferation.

INTRODUCTION

The myelodysplastic syndrome (MDS) is a clonal disorder of the multipotential stem cell (1) characterized by refractory cytopenia and qualitative and quantitative abnormalities of one or more cell lineages in the bone marrow. Up to 40% of the patients with MDS eventually develop an acute myeloblastic leukaemia (AML) (2).

The decreased in vitro colony forming capacities of the MDS bone marrow progenitor cells are extensively described (3-6). In all these studies colony formation was stimulated by crude sources of colony stimulating activity (CSA). The recent availability of recombinant human colony-stimulating factors (CSF) made it possible to investigate the regulation of normal (7,8) and leukaemic haemopoiesis in vitro (9,10). Limited information exists, however, on the responsiveness of MDS colony forming cells for the recombinant CSF. Recently Mayani et al.(11) found subnormal myeloid colony numbers in response to GM-CSF at concentrations shown to be optimal for colony formation in cultures of normal bone marrow, but supersaturating concentrations of GM-CSF were observed to be able to enhance the myeloid colony formation of many MDS marrows. Carlo-Stella et al. (12) have also reported a decreased myeloid colony growth in response to GM-CSF in a majority of the fractionated, accessory cell depleted MDS marrows. However, no effect of high doses of GM-CSF was observed. Since Il-3 and GM-CSF have been reported to recruit monocytes to express and secrete G-CSF (13), the effect of high GM-CSF doses found by Mayani et al. may be indirect.

We have recently found that the conditioned medium of the giant tumor cell line (GCT-CM), produced in our laboratory, stimulates predominantly granulocytic colony formation, whereas the growth of MDS bone marrow derived CFU-G was more effectively sustained than by single recombinant growth factors (14). Since GCT-CM contains various haematopoietic growth factors this effect may be due to additive or synergistic effects of these factors. Although, additive and synergistic effects of combinations of growth factors on leukemic blast cells (15) and normal multipotential haematopoietic progenitors (16) have been reported, their effect on MDS progenitor cells has not been addressed.

The aim of the present study was therefore to compare the colony stimulating activity of GCT-CM with the effects of recombinant human Il-3, GM-CSF and G-CSF, either alone or in combination, on MDS marrow myeloid progenitor cells.

MATERIALS AND METHODS

Normal donors
Normal marrow samples were obtained from five healthy volunteers posterior iliac crest puncture. The marrow was collected in glass tubes containing Hanks/Hepes with preservative-free heparin.

Patients
Seventeen patients with MDS (2 RA, 2 RARS, 6 RAEB, 4 RAEBt and 3 CMML) and 5 patients with leukemic transformation of MDS (LT-MDS) were studied with informed consent. The MDS cases were classified according the FAB nomenclature (17).

Preparation of cell suspensions
Low-density bone marrow cells of patients and normal donors were obtained by Ficoll-Isopaque centrifugation (1.077 g/ml, 1000 g, 20 min). T-cell depletion was performed by rosette formation with 2-aminoisothiouroniumbromide (AET, Sigma) treated sheep erythrocytes and subsequent ficoll separation. In addition adherent cells were removed by incubating the cell suspension (1×10^6/ml in α-DMEM with 10% Fetal Calf Serum (FCS)) for 1 hour at 37°C, 5% CO_2 in a fully humidified atmosphere. Non-adherent cells were collected and washed twice in Hanks/Hepes.

Recombinant human colony stimulating factors and interleukins
Recombinant granulocyte-macrophage colony stimulating factor (rh-GM-CSF) was prepared and generously made available by Schering (Kenilworth, New Yersey, USA) and was used in a concentration of 10 ng/ml. Recombinant granulocyte colony-stimulating factor (G-CSF) was purchased from Genzyme (Boston, MA, USA) and was used at 100 U/ml. The preparation of recombinant Interleukin-3 (Il-3), a generous gift from Dr. G. Wagemaker (Erasmus University Rotterdam) (18) and produced by Gist-Brocades (Rijswijk). Il-3 was used at a concentration of 10 ng/ml. Recombinant human erythropoietin (Boehringer, Mannheim F.R.G.) was used in a

concentration of 2 U/ml. All applied concentrations of colony stimulating factors were optimal concentrations, as determined by dose-response studies using clonogenic assays on normal low-density, T-cell depleted, non-adherent bone marrow cells.

Giant Cell Tumor Conditioned Medium (GCT-CM)

GCT-CM was prepared by culturing the confluent growing cell-line (obtained from the American Type Culture Collection, ATCC TIB 223) for 7 days in α-DMEM with 10% FCS (10ml per 75 cm^3) and was used as a source of colony stimulating activity at a concentration of 10% v/v.

Antibodies directed against G-CSF and Il-3

Polyclonal rabbit anti-human granulocyte-CSF was purchased from Genzyme (Boston, MA, USA). A concentration of 10 ng/ml neutralizes the bioactivity expressed by approximately 100 units of G-CSF. Polyclonal rabbit anti-human interleukin-3 antibody was purchased from Genzyme. Approximately 1 μg neutralizes 200 pg of human Il-3. Neutralization experiments were performed by incubating different concentrations of the antibody with an equal volume of GCT-CM or 100 U/ml G-CSF or 10 ng/ml IL-3 for 1 h at 37°C.

Colony assay

Cultures were performed in the α-modification of Dulbecco's modified Eagles Medium (α-DMEM, Flow) containing 0.9% methylcellulose (Fluka Methocell MC), and supplemented with 20% FCS and 1% dialyzed Bovine Serum Albumin (BSA), 30 μM lecithine, 0.1 μM fully iron-saturated human transferrin, 100 μM mercaptoethanol. Cultures were performed in 24 wells plates (Costar), 250 μl per well containing 2.5×10^4 bone marrow cells. The cultures were incubated at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. Myeloid colonies (more than 50 cells) were counted on day 7 - 10 for the granulocyte colony forming unit (CFU-G),and day 14 - 18 for the granulocyte-macrophage CFU (CFU-GM). Erythroid colonies were counted on day 7 (CFU-E) and erythroid bursts on day 14 (BFU-E). Of some cultures colonies were picked from a well for staining with the May-Grunwald-Giemsa technique and subsequent morphological examination.

Statistical analysis

Data were statistically evaluated with the Wilcoxon's signed-ranks test for two groups.

The effect of αIl-3 and αG-CSF on the activities of GCT-CM

Since GCT-CM was found to stimulate both granulocytic and erythroid colony formation, we investigated as to wether the granulocyte-colony stimulating activity (G-CSA) and the burst promoting activity (BPA) could be neutralized by antibodies directed against G-CSF and Il-3, respectively. Whereas, the G-CSA present in GCT-CM could be neutralized anti-G-CSF antibodies (Fig.1), the BPA present in GCT-CM could be neutralized by anti-Il-3 antibodies (Fig. 2). αIl-3 had no effect on either GCT-CM or GM-CSF induced myeloid colony formation and αG-CSF had no effect on the GCT-CM induced BFU-E formation of normal bone marrow (data not shown).

Effect of GCT-CM, Il-3 and GM-CSF on MDS bone marrow cultures

The effect of stimulation on myeloid colony growth by fractionated, accessory cell depleted, MDS bone marrow with GCT-CM, GM-CSF and Il-3 is shown in Table 2. As in normal bone marrow cultures GCT-CM was found to stimulate predominantly granulocytic colonies. GM-CSF and Il-3 either alone or in combination, were significantly less effective ($p < 0.01$) in inducing myeloid colony formation than GCT-CM. In two cases, however (cases 8 and 17), high numbers of myeloid colonies were formed in response to Il-3 (i.e 105.2 and 196.0 respectively). The effect of stimulation of MDS bone marrow cultures with the combination of GM-CSF and Il-3 was less than additive in thirteen, additive in one and more than additive in two of the sixteen cases.

As described above, in most MDS cases neither GM-CSF nor Il-3 nor their combination were found to be more effective than GCT-CM in stimulating the proliferation of myeloid progenitor cells. However, in case 5 GM-CSF, in cases 8 and 12 Il-3 and in case 18 the combination of Il-3 and GM-CSF induced the formation of more colonies than GCT-CM.

Effects of Il-3 and G-CSF in MDS bone marrow cultures

In contrast to NBM cultures, in MDS marrows G-CSF was less effective in inducing the growth of granulocytic colonies than GCT-CM (Table 3). However, Il-3 could clearly enhance the G-CSF effect in three of the four cases tested. The effect was more than additive in three cases and additive in one. In the former three cases the

Table 2, MDS marrow myeloid colony formation in
response to GCT-CM, GM-CSF, Il-3 and the
combination of GM-CSF with Il-3.

		GCT-CM	GM-CSF	Il-3	GM-CSF + Il-3
1.	RA*	192.0	46.8	14.8	16.0
2.	RA	106.8	5.2	2.8	0.0
3.	RARS	82.8	30.8	34.8	40.0
4.	RARS	316.0	133.2	n.d.[3]	125.2
5.	RAEB	21.2	74.0	44.0	77.2
6.	RAEB	20.0	0.0	0.0	0.0
7.	RAEB	102.8	18.8	n.d.	12.0
8.	RAEB	72.0	16.0	105.2	100.0
9.	RAEB	64.0	42.8	n.d.	46.8
10.	RAEB	132.0	26.8	54.0	45.6
11.	RAEBt	76.0	5.2	9.2	14.4
12.	RAEBt	53.2	65.2	66.0	70.0
13.	RAEBt	94.8	8.0	4.0	9.2
14.	RAEBt	25.2	n.d.	n.d.	n.d.
15.	CMML	14.8	0.0	4.8	4.0
16.	CMML	164.0	82.0	n.d.	44.0
17.	CMML	304.0	138.0	196.0	160.0
18.	LTMDS+	26.0	14.0	18.0	48.0
19.	LTMDS	61.2	38.4	14.8	44.0
20.	LTMDS	14.8	6.8	8.0	13.2
21.	LTMDS	2.8	4.0	1.2	1.2
22.	LTMDS	78.0	14.0	0.0	18.8
23.	LTMDS	15.0	n.d.	n.d	n.d
	Mean	88.7	36.7	31.9	42.4
	SEM	18.3	8.5	11.9	9.5

* MDS-subtype according to the FAB classification.
+ LT-MDS: leukemic transformed MDS.
n.d. not determined

number of colonies formed in response to G-CSF and IL-3 was comparable to the number of colonies formed in response to GCT-CM. In two of these four cases erythroid burst formation was found in response to either GCT-CM or Il-3, although only a few burst were formed in these two cases. No effect of the addition of G-CSF to Il-3 was observed on the erythroid burst formation in MDS bone marrow cultures.

Cytogenetic analysis of the colony cells:

In order to determine whether the colonies were derived from the abnormal MDS clone, cytogenetic analyses were performed on the pooled colony cells of case 3, 9, 10 and 23. No metaphases were obtained in cases 3 and 9. In case 10, 98% of the 50 metaphases contained the same chromosomal abnormality (47 XX, 5q-, +21), as had been found in 78% of the metaphases of the fresh bone marrow. In case 23, a patient with leukaemic stage of a trilineage MDS, complex cytogenetic abnormalities were found in 62% of the metaphases in the fresh bone marrow, whereas 80% of the 10 metaphases derived from colony cells showed identical complex abnormalities.

Table 3, Myeloid colony (CFU-G) and erythroid burst (BFU-E) formation in response to GCT-CM, G-CSF, Il-3 and G-CSF with Il-3 of MDS bone marrow.

	CFU-G*				BFU-E*			
case	10	11	13	14	10	11	13	14
GCT-CM	101.0	69.0	26.8	25.2	0.0	10.7	12.0	0.0
G-CSF	30.0	9.2	12.0	0.0	n.d.	n.d.	n.d.	n.d.
Il-3	28.6	8.4	2.0	5.2	0.0	7.0	3.7	0.0
Il-3 + G-CSF	106.7	18.7	34.8	20.0	0.0	6.7	2.8	0.0

* Each value represent the mean of triplicate cultures performed with low-density, non-adherent, T-cell depleted bone marrow cells.
n.d. not determined.

DISCUSSION

In this study we investigated the response patterns of haematopoietic progenitor cells derived from bone marrow of patients with a myelodysplastic syndrome stimulated with various recombinant human colony stimulating factors and GCT-CM. In particular we addressed the question whether combinations of colony stimulating factors were necessary for optimal proliferation of myeloid progenitor cells in MDS. In normal bone marrow cultures the effect of the combination of GM-CSF and Il-3 rendered more colonies than each factor alone, suggesting that their activities are partially overlapping. This is in agreement with previous data indicating that Il-3 and GM-CSF stimulate overlapping but distinct progenitor cell populations (19,20). As we have also reported previously (14) GCT-CM was found to stimulate primarily the formation of granulocytic colonies. GM-CSF but not GCT-CM was found to stimulate the growth of mixed granulocyte/macrophage colonies. GCT-CM and G-CSF induced comparable numbers of colonies and the colonies were identically granulocytic of nature. Moreover, the granulocytic colony stimulating activity present in GCT-CM was neutralized by antibodies directed against G-CSF. These results indicate that GCT-CM contains G-CSF as a major source of colony stimulating activity. In addition, GCT-CM was found to contain BPA as well, which could be neutralized by anti-IL-3 antibodies, indicating that the BPA in GCT-CM could be assigned to Il-3. The combination of G-CSF and Il-3 did not render more myeloid colonies than G-CSF alone or more erythroid bursts than Il-3 alone, indicating distinct activities of Il-3 and G-CSF on normal bone marrow progenitor cells. Cytogenetic analyses performed on the colonies of two MDS cases revealed the presence of chromosomal abnormalities typical for the MDS clone, even in a higher frequency than found in the fresh bone marrow, indicating that the colonies are derived from the abnormal clone. Apparently the abnormal clone rather than the residual normal haemopoiesis is stimulated by haemopoietic growth factors in MDS. These findings can be explained by the fact that disease progression in MDS is due to the gradual clonal expansion of the abnormal population, with suppression of the normal haemopoiesis, which is demonstrated by the findings of Abkowitz et al.(21), that both erythroid and granulocyte-macrophage colonies appear to be derived from the abnormal clone. More recent reports, presenting data on X-linked restriction fragment length poly-morphisms,

provide evidence that MDS is a clonal disorder of the pluripotent haemopoietic stem cell (1,22), which clone has totally replaced the normal haemopoiesis. Stimulation of the abnormal clone by haemopoietic growth factors is also observed in vivo (23,24). The residual normal haemopoiesis, however, appears to regain its place after eradication of the MDS clone by chemotherapy or differentiation induction therapy (25), and may then be preferentially stimulated by haemopoietic growth factors (26).

In myelodysplastic bone marrow cultures GM-CSF and Il-3 were significantly less effective in inducing myeloid colony formation than GCT-CM. Only in three of the sixteen cases the effect of the combination of Il-3 and GM-CSF was found to be additive or more than additive, whereas in one of those cases more colonies were formed than with GCT-CM. Therefore, our results suggest that the activities of Il-3 and GM-CSF on MDS myeloid progenitor cells are more overlapping than in NBM cultures, which suggests that their target cell populations are largely identical.

Whereas in NBM cultures G-CSF and GCT-CM contained comparable colony stimulating activities, in MDS bone marrow G-CSF did induce a significantly lower number of myeloid colonies than GCT-CM. Since we had previously found that GCT-CM contains Il-3 apart from G-CSF, it appears likely to presume that the combination of these two factors were responsible for the larger number of colonies formed in response to GCT-CM. In three of the four MDS cases tested we could indeed demonstrate a synergistic and in the other case an additive effect of the combination of IL-3 and G-CSF on the growth of myeloid colonies. In the former three cases the effect of co-stimulation with Il-3 and G-CSF was comparable with the effect of GCT-CM, whereas in the latter case GCT-CM was more effective in stimulating MDS progenitor cells. Therefore, it can not be excluded that factors distinct from Il-3 and G-CSF contribute to the potent colony stimulating activity of GCT-CM on MDS bone marrow. Still, it appears that a subset of granulocytic colony forming cells require both Il-3 and G-CSF for optimal colony formation, suggesting that a pluripoietin should be combined with a later-acting factor for optimal effect, which is a characteristic shared by early bone marrow progenitor cells and peripheral blood progenitor cells (27). Some of the effects of Il-3, however, may be indirect, i.e., mediated by residual accessory cells, although all the bone marrows were thoroughly depleted of adherent and T-cells and the dose dependent inhibition of colony formation by αIl-3

suggests a direct action of Il-3. However, to obtain conclusive evidence a larger series of experiments studying the effect the combination of Il-3 and G-CSF and GM-CSF and G-CSF on myeloid colony growth in MDS is required.

We conclude that the activities of Il-3 and GM-CSF on myeloid progenitor cells present in MDS bone-marrows are more overlapping than in NBM cultures. Concomitantly the results obtained in a small series of MDS patients suggest that the majority of MDS progenitor cells are not yet responsive for G-CSF. The synergistic effect of the combination of Il-3 and G-CSF may suggest that these progenitor cells require exposure to a pluripoietin like Il-3 in order to acquire responsiveness for the later-acting factor G-CSF.

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CHAPTER 6

INTERLEUKIN-6 AND INTERLEUKIN-1 ENHANCEMENT OF GM-CSF-DEPEN-
DENT PROLIFERATION OF HAEMATOPOIETIC PROGENITOR CELLS IN
MYELODYSPLASTIC SYNDROMES

Martin Schipperus, Pieter Sonneveld, Jan Lindemans, Kirsten van Lom, Margreet
Vlastuin and Johannes Abels

From the department of Hematology, University Hospital and Erasmus University
Rotterdam, The Netherlands

SUMMARY

Interleukin-1 (Il-1) and Interleukin-6 (Il-6) have been reported to enhance the growth factor dependent colony formation of normal primitive haematopoietic progenitor cells as well as of leukemic blast-cell progenitors. We investigated the effects of Il-1ß and Il-6 in combination with Granulocyte-Macrophage Colony-Stimulating factor (GM-CSF) on the in vitro colony formation of myeloid progenitors from 23 patients with a Myelodysplastic syndrome (MDS). Neither Il-1ß nor Il-6 were found to have colony stimulating activity on their own. In normal bone marrow cultures, either stimulated with optimal or sub-optimal doses of GM-CSF, no enhancing or antagonistic effect of Il-6 or Il-1ß was detected. In a majority of the MDS cases, however, an enhancing effect of Il-6 and Il-1ß in combination with GM-CSF was observed (20 out of 23 and 10 of the 21 cases respectively). In 3 cases of the Il-6 and GM-CSF combination an antagonistic effect was observed as well as in 4 cases of the Il-1ß and GM-CSF combination. A delayed addition of Il-6 to the cultures did not result in an abrogation of the effect, indicating that Il-6 is not required immediately at the initiation of the culture. These results indicate that co-stimulation with IL-6 or IL-1ß is able to augment the GM-CSF activity on MDS myeloid progenitor cells.

INTRODUCTION

The myelodysplastic syndrome (MDS) comprises a group of acquired disorders, which are characterized by an ineffective haemopoiesis resulting in a cytopenia of one or more cell lineages (Koeffler, 1986; Mufti & Galton,1986). Cytogenetic and G-6-PD studies have demonstrated that MDS is a clonal disease of the haemopoietic stem cell (Fialkow et al, 1981; Raskind et al, 1984; Janssen et al, 1989). Its high mortality rate results from the complications associated with persistent cytopenias, as well as from transformation to leukaemia. The abnormal haemopoiesis is reflected in an abnormal in vitro growth pattern of MDS bone marrow (Verma et al, 1979; Partanen et al,1986). Decreased or absent megakaryocyte and erythroid colony formation is a typical finding in MDS (Juvonen et al,1989). An abnormal granulocyte-macrophage colony formation may have prognostic value (Juvonen et al,1989; Haak et al,1986; Schipperus et al,1988). The nature, however, of the aberrant colony formation is poorly understood. The recent cloning of genes of various haemopoietic growth factors (Clark & Kaman, 1987; Sieff,1987), generally referred to as colony stimulating factors (CSF) or interleukins, has made it possible to produce purified CSF in large quantity and to investigate their in vitro and in vivo effects in MDS. The first clinical trials on the effects of recombinant CSF in MDS are being performed already (Vadhan-Raj et al, 1987; Antin et al, 1988; Ganser et al,1989). However, there is still limited information on the effect of recombinant haemopoietic growth factors on MDS in vitro colony formation. Recent reports indicate that the MDS progenitor cells defective response to growth factors is not affected by manipulation of recombinant factor levels or combinations (Baines et al, 1990). Carlo-Stella et al (1989) have reported that GM-CSF (even in high concentrations) is unable to improve the abnormal in-vitro colony formation of bone-marrow cells depleted of accessory cells. However, Mayani et al (1989), using unfractionated bone marrow, found that super-saturating concentrations of GM-CSF could ameliorate the abnormal colony formation. These discordant results may be due to an indirect effect of GM-CSF: the release of additional factors by monocytes and macrophages (Metcalf & Nicola, 1985; Warren & Ralph, 1986), which enhance the GM-CSF dependent colony formation of MDS myeloid progenitor cells. Apart from their activities on the lymphoid lineage, Il-1 and the pleiotropic cytokine Il-6 have been reported to act as co-stimulants for the growth

of normal primitive haematopoietic progenitors (Moore et al,1987; Leary et al,1988) as well as for leukemic blast-cell progenitors (Hoang et al 1988a,b). Il-6 has also been implicated as a co-stimulant in the late stages of neutrophilic differentiation (Caracciolo et al, 1989a, b). The effects of Il-6 and IL-1 on MDS marrow, however, have not been reported so far. The aim of the present study is to investigate whether Il-6 and Il-1ß are able to enhance the in-vitro GM-CSF dependent colony formation of MDS progenitor cells.

MATERIALS AND METHODS

Normal donors. Normal marrow samples were obtained from posterior ilieac crest puncture of five healthy volunteers. The marrow was collected in glass tubes containing Hanks/Hepes with preservative-free heparin.

Patients. 18 patients with MDS (two RA,two RARS, seven RAEB, four RAEBt and three CMML) and five patients with leukaemic transformation of MDS (LT-MDS) were studied upon informed consent. The MDS cases were classified according to the FAB nomenclature (Bennett et al, 1982).

Preparation of cell suspensions. Low-density bone marrow cells of patients and normal donors were obtained by Ficoll-Isopaque centrifugation (1.077 g/ml, 1000 g, 20 min). T-cell depletion was performed by rosette formation with 2-aminoisothiouroniumbromide (AET, Sigma) treated sheep erythrocytes and subsequent ficoll separation. Adherent cells were removed by incubating the cell suspension (1×10^6/ml in α-DMEM with 10% fetal calf serum (FCS)) for 1 hour at 37°C, 5% CO_2 under fully humidified conditions. Non-adherent cells were collected and washed twice in Hanks/Hepes.

Recombinant human CSF and Il. Recombinant GM-CSF was prepared and generously supplied by Schering (Kenilworth, New Jersey, U.S.A) and was used in a concentration of 10 ng/ml. Recombinant human hybridoma growth factor (rh-Il-6) was purified form E.coli (Brakenhoff et al, 1987), a generous gift from Dr L. Aarden (CLB, Amsterdam, The Netherlands). Rh-Il-6 was used in a concentration of 40 U/ml as defined by bioassay (Lansdorp et al, 1986). Recombinant human Interleukin-1 was purchased from Genzyme (Basel,Switzerland) and was used at 10 U/ml as defined by thymocyte assay.

Table 1

Colony formation in response to GM-CSF and the combination of GM-CSF and Il-6 and /or Il-1 in MDS

no	MDS type	GM-CSF	GM-CSF +Il-6	GM-CSF +Il-1	GM-CSF +Il-6 +Il-1
1.	RA	45.0	128.0(2.8)[1]	118.0(2.6)	90.8(2.0)
2.	RA	5.2	6.8(1.3)	13.2(2.5)	12.0(2.3)
3.	RARS	30.8	52.0(1.7)	46.8(1.5)	62.8(2.0)
4.	RARS	133.2	152.0(1.1)	177.2(1.3)	154.8(1.2)
5.	RAEB	74.0	114.0(1.5)	0.0(0.0)	26.0(0.4)
6.	RAEB	0.0	6.0(6.0)	0.0(0.0)	1.2(1.2)
7.	RAEB	18.8	8.0(0.4)	17.2(0.9)	14.8(0.8)
8.	RAEB	16.0	24.0(1.5)	68.0(4.3)	53.2(3.3)
9.	RAEB	42.8	69.2(1.6)	53.2(1.2)	n.d
10.	RAEB	26.8	10.8(0.4)	64.0(2.4)	22.8(0.9)
11.	RAEB	54.0	104.0(1.9)	n.d[2]	n.d
12.	RAEBt	4.0	9.2(2.3)	4.0(1.0)	n.d
13.	RAEBt	65.2	97.2(1.5)	40.0(0.6)	n.d
14.	RAEBt	8.0	16.0(2.0)	8.0(1.0)	37.2(4.7)
15.	RAEBt	38.8	88.0(2.3)	n.d	n.d
16.	CMML	0.0	5.2(5.0)	1.2(1.2)	2.8(2.8)
17.	CMML	38.8	33.2(0.9)	14.0(0.4)	8.0(0.2)
18.	CMML	34.5	61.0(1.8)	35.0(1.0)	44.0(1.3)
19.	LTMDS	14.0	32.0(2.3)	26.6(1.9)	n.d
20.	LTMDS	38.4	40.0(1.0)	76.0(2.0)	66.8(1.7)
21.	LTMDS	6.8	13.2(1.9)	16.0(2.4)	13.2(1.9)
22.	LTMDS	1.2	2.8(2.3)	0.0(0.0)	1.2(1.0)
23.	LTMDS	14.0	32.0(2.3)	14.0(1.0)	n.d
MDS	Mean	30.9	48.0[5](1.6)	37.7(1.2)	38.2(1.2)
	SEM	6.4	9.4	9.7	10.3
NBM					
1.		37.2	46.8(1.3)	36.0(1.0)	38.9(1.0)
2.		58.0	63.4(1.1)	58.6(1.0)	60.1(1.0)
3.		40.6	36.0(0.9)	41.4(1.0)	39.2(1.0)
4.		48.8	45.2(0.9)	45.2(0.9)	43.2(0.9)
5.		45.2	40.0(0.9)	46.8(1.0)	45.2(1.0)
NBM	Mean	46.0	46.1(1.0)	45.6(1.0)	43.2(0.9)
	SEM	3.6	3.9	3.7	2.9

In parenthesis: the relative increase induced by the combination as compared to the GM-CSF effect only. IL-6 - GM-CSF n.d.: not determined.

Colony assay. Cultures were performed in the α-modification of Dulbecco's modified Eagles Medium (α-DMEM, Flow) containing 0.9% methylcellulose (Fluka Methocell MC), and supplemented with 20% FCS and 1% dialyzed Bovine Serum Albumin (BSA), 30 μM lecithine, 0.1 μM fully iron-saturated human transferrin, 100 μM mercaptoethanol. Cultures were performed in 24-well plates (Costar), containing 2.5 x 10^4 bone marrow cells per well (250 μl). The cultures were incubated at 37 °C under fully humidified conditions of 5% CO_2 in air. Colonies (= more than 50 cells) were counted on day 7, 10, 14, 18 and 22. Colonies were taken at random from several cultures and stained according to the May-Grunwald-Giemsa technique for subsequent morphological examination.

Statistical analysis. Statistical evaluation of data was done with Wilcoxon's signed-ranks test for two groups.

RESULTS:

The effect of GM-CSF stimulation on normal and MDS colony formation

No spontaneous colony growth was observed in low-density, non-adherent, T-cell depleted normal or MDS bone marrow cells. Induction of colony formation by GM-CSF is dose dependent. Plateau colony formation was achieved at concentrations of 5 ng/ml rh-GM-CSF in both normal as well as MDS bone marrow cultures. However, colony numbers were decreased in 13/23 (56.5%) MDS cases as compared with normal bone marrow (table 1).

Effect of Il-6 and Il-1ß on colony growth

Il-6 and Il-1ß did not induce colony formation of fractionated normal bone marrow cells, neither singly nor together. As is shown in Fig 1 and Table 1, the addition of Il-6 or Il-1 had no effect on the GM-CSF induced colony formation of normal marrow progenitor cells. However, Il-6 enhanced the GM-CSF effect in 19/ 21 (90.5%) MDS cases, inducing an approximate two-fold increase in colony numbers (p< 0.01). In 3 cases (Table 1: cases 7,10 and 17) colony numbers decreased in the presence of IL-6. In 11/21 (52.4%) cases an average two-fold enhancement of colony growth by Il-1 was observed. Whereas in four cases an inhibition of the colony formation was observed (cases 5,13,17 and 22).

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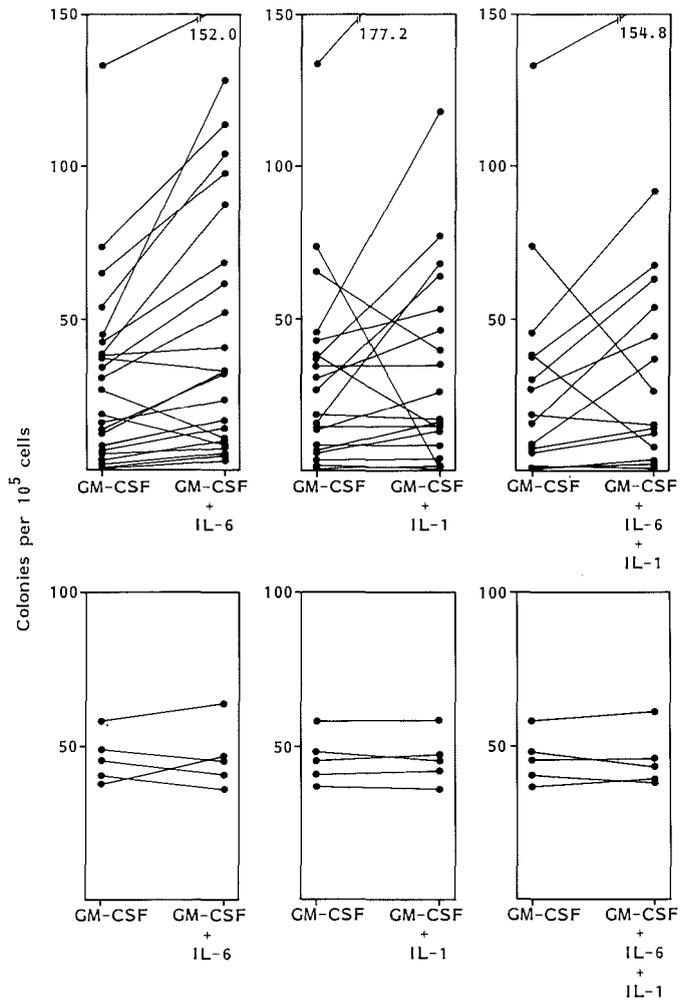
Figure 1: Effect of Il-6, Il-1β and their combination on GM-CSF induced MDS marrow (upper panel) and normal bone marrow (lower panel) colony formation.

In 9/21 cases Il-6 and Il-1 both stimulated the GM-CSF induced colony formation. In case 17 Il-1 and Il-6 both inhibited the colony formation, whereas in four cases opposite effects of Il-1 and Il-6 were found.

Role of accessory cells in the Il-6 enhancement

The effects of Il-6 described above were obtained with T-cell depleted, non-adherent bone marrow cells. The effects of GM-CSF or the GM-CSF - Il-6 combination in
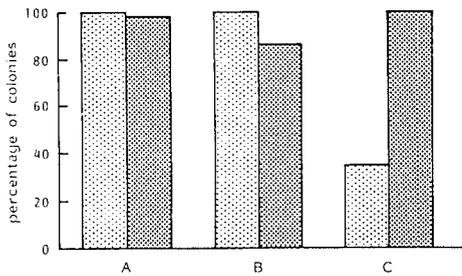
Fig 2. The effects of GM-CSF (light stipple) and the GM-CSF-Il-6 combination (heavy stipple) on low-density (panel A), low-density, adherent cell depleted (panel B), and low-density adherent cell, T-cell depleted bone marrow cells (panel C) of case 1. Data are expressed in percentages of maximal colony growth.

MDS on respectively undepleted, adherent-cell depleted, and T-cell and adherent-cell depleted bone marrow cells are shown in Fig.2. The number of colonies formed by undepleted or adherent cell depleted bone marrow in response to GM-CSF was comparable to those formed by fully depleted bone-marrow cells in response to the combination of GM-CSF - Il-6. This indicates that accessory cells apparently produce Il-6 endogeneously.

Il-6 effect on myeloid progenitor cells in the presence of high and low GM-CSF concentrations

In order to determine whether Il-6 increases progenitor cell responsiveness to GM-CSF, the activity of Il-6 was tested in the presence of high (supersaturating) concentrations of GM-CSF (100 ng/ml). Whereas a further increase of an already saturated GM-CSF dose did not improve the colony growth of MDS marrow, Il-6 had a definite enhancing effect at both GM-CSF concentration levels (Fig 3, left panel). To eliminate the possibility that the IL-6 effect is due to stimulation of endogeneously produced GM-CSF, a control was performed by adding Il-6 to normal bone marrow cultures under conditions of both sub-optimal (1 ng/ml) as well as saturated GM-CSF concentrations (10 ng/ml). No Il-6 effect was observed in in combination with either GM-CSF concentration (Fig 3, right panel).

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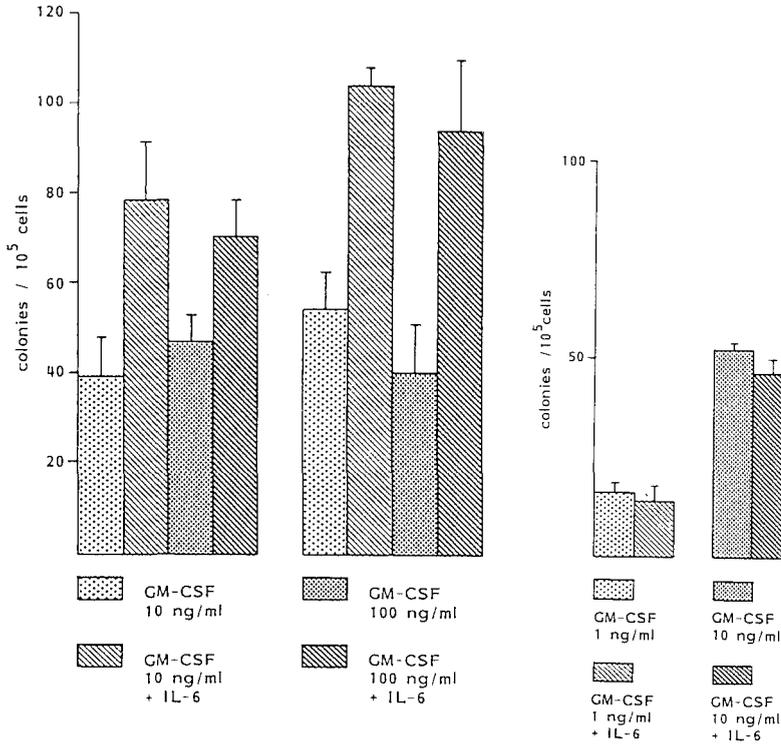
Figure 3: Il-6 effect in the presence of a high (100 ng/ml) dose GM-CSF in MDS (left panel) and a low dose (1 ng/ml) GM-CSF in NBM (right panel) as compared with a normal GM-CSF concentration (10 ng/ml).

Kinetics of GM-CSF or GM-CSF - Il-6 induced colony formation

Time course studies revealed two types of colony growth kinetics in response to GM-CSF: (1) a growth pattern comparable with that of normal bone marrow (Fig 4, panels A and B), or (2) a delayed type of colony growth (panel D). Il-6 was found to augment GM-CSF induced colony numbers (panel B and D). In two cases (6 and 16) colony growth could be observed only in response to the GM-CSF - Il-6 combination (panel C). Il-6 did not affect MDS marrow colony growth kinetics: maximum colony numbers were found at the same day either in the presence or absence of Il-6.

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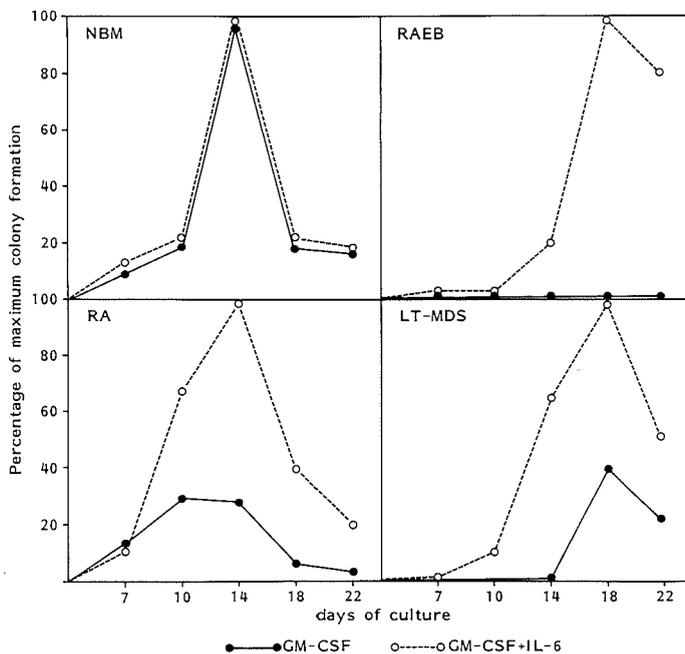
Figure 4: Colony growth kinetics induced by either GM-CSF or the GM-CSF - Il-6 combination in NBM (panel A), Refractory anaemia (RA) (panel B), RA with excess of blasts (RAEB) (panel C) or Leukemic transformed MDS (LT-MDS) (panel D).

The effect of delayed addition of GM-CSF and Il-6

In order to determine whether IL-6 is required at the initiation of the culture, IL-6 was added at consecutively the beginning, at day 1, 2 and 6 of culture. The results indicate that a delayed addition of one to several days did not influence the effect of IL-6. However, a delay in GM-CSF addition of one to several days during continued IL-6 stimulation, resulted in a rapid decline in the number of colonies (Fig 5).

Morphological features of the colony cells

No clear differences in morphology of colony cells formed in response to either GM-CSF or the GM-CSF - Il-6 combination were observed. In case 18 co-stimulation with Il-6 and GM-CSF revealed a 77% increase in colony numbers compared to stimulation with GM-CSF only .

From both groups (GM-CSF - Il-6 as well as GM-CSF stimulation only) five colonies were randomly selected, and differential counts were performed on each of these colonies. In the GM-CSF - IL-6 co-stimulated colonies 14.2% ± 9.8 (M ± SD) of the cells were immature (blasts and promyelocytes), whereas in the GM-CSF stimulated colonies 8.5% ± 3.7 of the cells were immature (table 2). These differences are statistically insignificant.
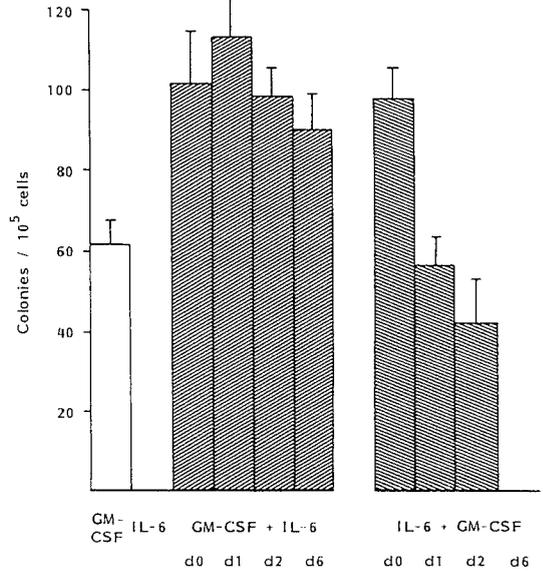
Figure 5. The effect of a delayed addition of either Il-6 or GM-CSF.

Table 2 Differential counts of the colony cells formed in response to either GM-CSF or the GM-CSF – Il-6 combination in MDS (case 18)

	GM-CSF	GM-CSF +Il-6
	% ± SD	% ± SD
blasts/promyelocytes	8.5 ± 3.7	14.2 ± 9.8
Myelocytes	28.5 ± 8.2	28.8 ± 10.5
meta-myelocytes	26.2 ± 6.7	25.0 ± 6.6
bands	10.8 ± 4.0	8.6 ± 1.5
polymorphonuclears	25.3 ± 8.2	23.0 ± 13.2
eosinophils	0.7 ± 0.7	0.4 ± 0.5
monocytes	0.0 ± 0.0	0.0 ± 0.0

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DISCUSSION

In this study we investigated the effects of Il-6 and Il-1 on the GM-CSF stimulated myeloid colony formation by low-density, non-adherent, T-cell depleted bone marrow cells of MDS patients and normal controls. Neither Il-6 nor Il-1 were observed to induce the proliferation of myeloid progenitor cells on their own. Although these results are not concordant with those obtained by Caracciolo et al (1989a,b), who found that Il-6 supported the proliferation of a small number of myeloid colonies, they agree with a number of other reports (Moore et al, 1987; Suda et al, 1988; Bot et al, 1989). Enhancement by Il-6 and Il-1 of GM-CSF induced colony formation was observed in most MDS cases, in contrast to the lack of colony growth enhancement in normal bone marrow. Remarkably, the GM-CSF - IL-6 combination did induce colony formation in two cases in which neither factor alone was able to induce colony growth, indicating that both stimuli are required for proliferation of these progenitor cells. In cases with a reduced response to GM-CSF, neither IL-6, Il-1 nor their combined activity resulted in a rise of the number of colonies into the normal range. This indicates that Il-6 and Il-1 only potentiate the GM-CSF effect, but are not able to normalize a reduced response. Mayani et al (1989) previously reported an enhancing effect by high-dose GM-CSF on myeloid colony formation of unfractionated MDS bone marrow, suggesting a reduced sensitivity of MDS progenitor cells for GM-CSF. These results were not reproduced by more recent data collected by the same group (Baines et al, 1990) or by Carlo-Stella et al (1989). In addition, we did not find a high-dose GM-CSF effect. Since we could demonstrate that accessory cells can substitute for IL-6, the findings of Mayami et al may be due to endogenous release of Il-6 by accessory cells. Our findings also indicate that the Il-6 effect is due to an intrinsic disorder of the colony forming cell rather than to a reduced production of Il-6 by MDS marrow accessory cells.

We found that Il-6 did not affect the colony formation kinetics of MDS marrow. In cases with delayed colony growth kinetics, which we have reported to be a MDS characteristic (Schipperus et al, 1990), Il-6 was not able to shorten the delay in colony growth onset. Moreover, a delayed addition of GM-CSF of one day already totally abrogated the Il-6 effect, whereas the Il-6 effect is not influenced by a delay in administration. Therefore, our results are in agreement of those of Suda et al (1988)

and Caracciolo et al (1989), who found a proliferative Il-6 effect on late granulocyte-macrophage progenitors, already responsive for GM-CSF, instead of acting like a priming factor as has been reported by others (Ikebuchi et al, 1987; Leary et al, 1988; Bot et al, 1989). Of the two groups of colony cells, which were stimulated either by GM-CSF or GM-CSF - IL-6 combination, morphological analysis revealed no significant differences. This indicates that increased colony formation is not accompanied by an increased differentiation of colony cells.

Although in most cases Il-6 and Il-1 augmented the GM-CSF induced colony growth, in 3/21 cases Il-6 antagonized the GM-CSF induced colony formation. This was also observed in four cases with the Il-1 - GM-CSF combination. This dual action of interleukins has been described before, but is still difficult to explain. One possible explanation is that the effects of these interleukins depend on the conditions set by other substances present, i.e other growth factors or still undefined serum factors (Roberts, 1985). In other systems the effects of growth factors have been reported to depend on the developmental (differentiation) stage of the cells (Rennick et al, 1987). However, this does not be exclude the possibility that the production of inhibitors by residual accessory cells is induced in these cases.

We present data indicating that Il-6, and in a lesser extent, Il-1 enhance the GM-CSF induced myeloid colony formation of MDS bone marrow, as previously reported for leukemic myeloid cells and primitive normal haemopoietic progenitor cells. Everson et al, (1989) found that IL-6 and GM-CSF are potential growth factors for chronic myelomonocytic leukemia cells. These recent findings support our results. We observed augmentation of colony formation by Il-6 in all subtypes of MDS, suggesting that this response pattern is present throughout all stages of the disease. Furthermore, our results indicate that the reduced responsiveness for GM-CSF of the late granulocyte-macrophage progenitor cells in MDS may be ameliorated by co-factors like Il-1 or Il-6.

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CHAPTER 7

GENERAL DISCUSSION

MDS classification

The myelodysplastic syndrome (MDS) classified according to the criteria proposed by the FAB-cooperative group encompasses a heterogeneous group of disorders with dyshemopoietic abnormalities in one or more cell lineages, with or without an increase in the percentage of bone marrow blast cells. Although, now generally accepted the classification has been the subject of much controversy for a number of reasons. First, none of the sub-types is wholly exclusive of the others. The RAs with or without an excess of blasts are a relatively homogeneous group. However, their distinction from the acute leukemias, especially erythroleukemia is arbitrary and unclear and perhaps separates unjustly diseases which are biologically an entity. Secondly, whithin one subtype considerable differences in terms of survival and risk of leukemic transformation may occur. In particular this is the case in acquired idiopathic sideroblastic anemia (AISA) or refractory anemia with ringsideroblasts (RARS). Gatterman et al. (1990) have distinguished on cytomorphological grounds between pure sideroblastic anemia (PSA), which is confined to dyserythropoiesis and RARS, which is characterized by additional dysplastic features of granulopoiesis and/or megakaryopoiesis. Both PSA and RARS are diagnosed in the same MDS group (MDS 2 or RARS), but Gatterman and al. found striking differences in the risk of leukemic transformation between PSA and RARS in 94 patients (5 year cumulative rate 1.9 versus 48%). Overall survival was much better in PSA than in RARS (5 year cumulative chance 69% versus 19%). Infections and hemorrhages were frequent causes of death in RARS but not in PSA. The differences in terms of leukemic transformation and survival between cases with dysplastic features in one cell line and cases dysplastic in two or more cell lineages may apply to all MDS subtypes and not only to AISA cases. Saarni and Linman (1973) already made this distinction in their definition of the 'preleukemic syndrome' (PLS). Their criteria included the presence of dysplastic megakaryocytes and granulocytes as well as dyserythropoisis. Using these criteria a syndrome may be identified in which the incidence of acute leukemia ranges up to 45%. More recently Brito-Babapulle et al. (1987) have defined MDS with features of trilineage disease (TMDS). The authors looked for dysplastic features in erythroid, megakaryocytic and granulocytic lineages (i.e. PLS features) in the bone-marrow of AML patients as evidence for pre-existing MDS. TMDS was found in 24

(15%) of the 160 consecutive cases of primary de novo AML, including two of 33 cases of M1, four of 40 cases of M2, none of 18 cases of M3, five of 31 cases of M4, six of 30 cases of M5 and all of six cases of M6. They found that AML cases with TMDS differed from cases without in the occurrence of symptomatic cytopenias but also in lower rates of complete remission. One may conclude, at least in AML-M6 cases, that biologically the leukemia represents the final stage of the malignant clone initiated already in the clinical stage of MDS.

In chapter 2 we present results that support this view. We could demonstrate by double immunofluorescence studies (DIF) analysis that a population of TdT, myeloid-antigen double positive (TdT+/MM+) cells was present in MDS cases with dyshemo-poietic features in more than one lineage (RAEB and RAEBt). The frequency of MM+/TdT+ cells was found to increase rapidly in the leukemic phase of the disease. In one patient the results of follow-up DIF studies showed that the frequency of MM+/TDT+ cells decreased during remission, increased six months before relapse RAEB and finally to 35% in AML. Since these TdT+/MM+ cells are extremely rare in normal bone marrow, they are considered to represent a subpopulation of the leukemic clone in AML (Adriaansen et al., 1990). In MDS MM+/TdT+ cells probably represent the abnormal granulopoiesis, which may have already leukemic features. In this respect it is of interest that a MM+/TdT+ subpopulation was present in a RARS case with an abnormal granulopoiesis, but absent in RARS cases without dysgranulopoiesis. In conclusion, double immunofluorescence analysis may be useful to detect abnormal myeloid cells in MDS and to monitor this subpopulation during disease progression.

In vitro marrow cultures

The colony forming capacities of the various progenitor cells are usually decreased in MDS patients (Greenberg and Mara, 1979; Chui and Clark, 1982; Greenberg et al., 1983; Juvonen et al., 1985). Frequently an abnormal maturation of the colony cells is found (Golde and Cline, 1973; Spitzer et al., 1979; Verma et al., 1979). The main purpose of this study was to reveal the nature of the defect leading to the abnormal in vitro colony formation of the hemopoietic cells in MDS. Since survival, growth and differentiation of hemopoietic cells is dependent on hemopoietic growth factors or

colony stimulating factors we investigated the effects of these factors on the in vitro growth of MDS bone-marrow. The abnormal colony formation may be due to 1) an intrinsic defect of the progenitor cells, i.e. a decreased number of CSF receptors, a lower affinity of the receptors for CSF, or abnormalities in the intracellular CSF-stimulus signal transduction; 2) an environmental defect, i.e. an abnormal growth factor production, either spontaneously or induced, by accessory cells or bone-marrow micro-environment. To eliminate the effects of accessory cells we depleted the bone-marrow cells of T-cells and adherent cells. Although these fractionated bone-marrow cell suspensions may still contain residual accessory cells, no spontaneous colony growth was observed. Moreover Il-1, a potential inducer of the production of hemopoietic growth factors by accessory cells, did not induce colony formation when added as a single factor to the bone-marrow cultures (chapter 6). Therefore we concluded that the bone marrow cells were, at least functionally, depleted of the cells capable of producing CSFs.

In chapter 3 colony formation of normal and MDS bone marrow cells was tested in a liquid-agar double layer colony assay, adapted from the PHA-leukocyte feeder colony assay described by Löwenberg et al. (1980). The feeder layer was replaced by the conditioned medium of the giant tumor cell line (GCT-CM). Colony formation was found in all 7 NBM, 17 MDS and 5 AML cases. Colonies were found to be adherent or non-adherent to the agar underlayer. The adherent colonies consisted of terminally differentiated cells, whereas the non-adherent colonies comprised immature cells. We used the ratio adherent/non-adherent colonies as a measure for the maturation index of the colonies. The maturation index of NBM cases was high and that of AML cases low. MDS and LT-MDS cases had highly variable indices and their mean values were intermediate to that of NBM and AML. No apparent correlation was found between the colony maturation index and FAB type or clinical course of the MDS cases. When PHA was not added to the cultures, no or only a few colonies were observed in AML, LT-MDS and MDS cases with progressive disease. In the absence of PHA, the same number of colonies were found in MDS with a stable clinical course and NBM as in the presence of PHA. We concluded that PHA dependency for colony formation correlated with a higher number of blasts in the bone-marrow and probably with an impending leukemic transformation of the MDS. PHA may either function as: a) a

co-factor for CSF, required for MDS/AML progenitor cell proliferation, b) an inducer of the production of secondary factors necessary for MDS-progenitor proliferation, and c) an inducer of cell-cell contact necessary for the initiation of MDS/AML progenitor-cell proliferation. Since initial cell-cell interaction seems to be a prerequisite for colony formation in the PHA-colony assay (Kluin-Nelemans, thesis 1989, Pulsoni et al., 1989; Reilly et al., 1989) further experiments were performed in semi-solid media in order to exclude the influence of cell-cell interactions.

First the response patterns and growth kinetics of colony-forming cells stimulated with Il-3, GM-CSF, G-CSF and GCT-CM were studied (chapter 4 and 5). Colony formation was found in 23/23 MDS and LT-MDS cases with GCT-CM, in 19/21 (90.5%) with GM-CSF and 15/17 (88%) with GCT-CM stimulation. Colony numbers were below the normal range with GCT-CM stimulation in 9/23 (39%), with GM-CSF in 13/21 (62%) and with IL-3 in 7/17 (41%) of the MDS cases (chapter 5). Therefore, single CSFs appear to stimulate colony formation by MDS bone marrow inadequately. Colony formation was found to be delayed in MDS as compared to NBM cultures (chapter 4). The delay in colony formation was most apparent in GCT-CM stimulated cultures. GCT-CM and G-CSF induced colony formation kinetics were found to be identical both in MDS and NBM cultures. GCT-CM colony formation activity could be neutralized by anti-G-CSF antibodies (chapter 5) indicating that the colony stimulating activity present in GCT-CM can be ascribed to G-CSF. In MDS cases with features of high risk disease (in terms of leukemic transformation and survival), a greater delay than in low risk cases was found. An increased time interval before progenitor cells begun to divide was found to cause the delayed colony formation. This was most apparent in the late (G-CSF responsive) progenitor compartment and progressed in parallel with disease progression. Cytogenetic analysis of the colony cells proved that these cells originated from the abnormal clone (chapter 4 and 5) and therefore we concluded that this growth pattern is a characteristic of the abnormal clone. In normal bone marrow only the early progenitor cells are considered to be in G0 (Lathja, 1979). Our results suggest that in MDS, progenitor cells in the relative late compartment as the CFU-G are more frequently dormant, i.e in G0 of the cell cycle, than their normal counterparts.

A possible explanation for the relative increase of dormant cells in the late progenitor

cell compartment in MDS may be an inadequate stimulation of these cells by single colony stimulating factors. In chapter 5 we therefore studied the effects of combinations of IL-3, GM-CSF and G-CSF on the in vitro growth of myeloid progenitors in MDS. The combination of Il-3 and GM-CSF did not enhance colony numbers above the sum of the colonies formed in the presence of the two factors alone (no synergistic effect). Furthermore, colony numbers formed with the Il-3 - GM-CSF combination rarely exceeded the scores of the dominant factor (no additive effect). These results, which agree with those of Baines et al. (1990), suggest that these factors act on an identical progenitor population in MDS. In contrast, in NBM a partial additive effect of the IL-3 - GM-CSF combination was observed. This is in agreement with previous data indicating that IL-3 and GM-CSF stimulate overlapping, but distinct, progenitor cell populations (Emerson et al., 1988). A synergistic effect of the Il-3 - G-CSF combination was found in MDS but not in NBM. Therefore it appears that MDS myeloid progenitors require both IL-3 and G-CSF for optimal colony formation. We concluded that a pluripotent factor (as IL-3) should be combined with a later acting factor for an optimal colony stimulating effect in MDS. Again, (as the percentage dormant cells) this is a characteristic shared by early bone marrow progenitor cells (Caracciolo et al., 1989a).

Apart from their activities on the lymphoid lineage, Il-1 and the pleiotropic cytokine IL-6 have been reported to act as co-stimulants for the growth of normal, primitive, hemopoietic progenitors (Moore et al.,1987; Leary et al., 1988). Since committed progenitor cells in MDS have many characteristics in common with normal primitive progenitor cells, Il-1 or Il-6 may act as co-stimulants on MDS progenitor cells. In chapter 6 the effects of IL-1 and Il-6 on GM-CSF induced colony formation in NBM and MDS was studied. In normal bone marrow cultures, either stimulated with optimal or suboptimal doses of GM-CSF, no enhancing effect of Il-6 and Il-1ß was detected. However, in the majority of the MDS cases an enhancing effect of IL-6 and IL-1ß on the activity of GM-CSF was observed. No additional effect of a high GM-CSF dose, either in the presence or absence of IL-6 was observed. These results suggest that Il-1 and Il-6 augment the sensitivity of MDS progenitor cells for GM-CSF. Obviously this is not due to the induction of GM-CSF production by MDS progenitor cells or accessory cells, since the enhancing effect of IL-6 was still present

with high doses of GM-CSF. Possibly Il-1 and Il-6 recruit MDS progenitor cells in a stage where they can respond to other factors. However, we found that a delayed administration of Il-6 did not influence its effect. Therefore, our results indicate that IL-6 acts on progenitor cells already responsive for GM-CSF, either by an augmentation the number of GM-CSF receptors on MDS progenitors as has been reported for the Il-1 effect on early progenitors (Bartelmez and Stanley 1985) or by acting in a sequence following GM-CSF as have been reported by Caracciolo (1989b).

How can these additive and synergistic effects of growth-factors on MDS progenitor cells be achieved? One explanation is that one CSF alters the progenitor cells so that they become responsive to stimulation by another, previously inactive CSF. The action mechanism may be, as with Il-1, the enhancement of CSF receptor expression. Alternative explanations may be the induction of rate limiting second messager systems or recruitment of the cells into a CSF responsive phase of cell-cycle. Why this two- or even three-signal system operates on committed MDS progenitors and only on primitive normal progenitor cells is unclear. Whereas all our data indicate that MDS committed progenitor cells are functionally immature and resemble, in that respect, normal pluripotent (early) progenitor cells, one explanation may be that this is primarily due to a maturation defect of the MDS clone, already detectable at the level of the progenitor cell compartment. However, as described in chapter 3, the delay in colony formation of MDS marrow was not associated with a relative increase of the GM-CSF responsive compartment, as would be expected in case of a relative increase of the earlier compartments. Moreover, Baines et al. (1988) have reported that the colony formation of purified CD34-positive cells in MDS is decreased as compared with CD34 normal bone marrow cells. These findings do not support the concept of a left shift in the progenitor cell compartment.

The presence of a functional defect (i.e. an intrinsic disorder) of the progenitor cells in MDS may be another explanation. This intrinsic abnormality makes committed MDS progenitor cells functionally resemble normal early progenitor cells. Hypothetically this may be an adaptive processes of the MDS clone in order to compensate for an absolute or relative stem-cell deficit.

The combined action of IL-6 and GM-CSF or Il-3 and G-CSF recruits both normal stem cells and MDS progenitor cells into cell-cycle. This may be a strong argument

for using these or other comparable combinations of growth factors in future clinical trials for MDS, since these growth factor combinations may effectively induce proliferation of residual normal stem cells and, at the same time, enhance differentiation of the MDS clone. Continuous administration of these factors over longer periods of time are probably needed to initiate a lasting remission. However, since it is unknown whether CSFs can induce irreversible committment, there is a possible risk of stimulation one cell lineage at the cost of another. A more obvious drawback of combination therapy may be a more rapid induction of leukemic transformation as compared with the reported stimulation of proliferation of leukemic blast cells with GM-CSF alone (Herrmann et al., 1989). Well designed clinical trials have to provide us with the answers on these important questions.

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SUMMARY

Blood cell formation results from the continuous proliferation, differentiation and maturation of pluripotent hemopoietic stem cells located in the human bone marrow. In vitro culture assays, developed in the last twenty years, have enabled the identification of the various pluripotent and committed progenitor cells present in human bone marrow by their capacity to form colonies of mature blood cells in vitro. Colony formation dependens on the presence of hemopoietic growth factors in the culture medium, which have become known as the colony stimulating factors (CSFs). At present a number of CSFs can be produced on a large scale through recombinant DNA technology and their biological activities have subsequently been defined. In chapter 1.1 and 1.2 the general principles of hemopoiesis are introduced, i.e., the different models of stem cell renewal and commitment, the various in vitro clonogenic assays for normal as well as for leukemic colony forming cells and the effects of the CSF on progenitor cells and mature blood cells.

The myelodysplastic syndrome (MDS) comprises a group of acquired disorders, which are characterized by an ineffective hemopoiesis resulting in cytopenia of one or more cell lineages. Cytogenetic and G-6-PD studies have demonstrated that MDS is a clonal disease of the hemopoietic stem cell. The results of some studies suggest that normal hemopoiesis is replaced by the abnormal clone already in an early stage of the disease. Up to fourty percent of the MDS patients eventually develop an acute myeloblastic leukemia. The preleukemic nature of the MDS makes this syndrome of particular interest in the study of leukemogenesis. In chapter 1.3 the clinical, morphological and in vitro growth characteristics of the MDS are introduced.

In chapter 2 six patients with MDS (three RARS, two RAEB and one RAEBt) were studied using double immunofluorescence analysis (DIF) for the presence of terminal deoxynucleotidyl transferase (TdT) and myeloid-antigen (MM), such as CD13, CD14, CD15 and CD33 expression. TdT expression was found in 0.1 - 11% of the cells. In four cases (1 RAEBt, 2 RAEB and 1 RARS) 58 - 99% of the TdT+ cells expressed the panmyeloid markers CD13 and/or CD33, whereas the precursor antigen CD34 was present in 68 - 99% of the TdT+ cells. Follow-up studies performed in two patients, that evolved into an acute myeloid leukemia (AML), showed in one patient an increase of MM+/TdT+ cells from 11% in RAEBt to 25% in AML-M2. In the

other patient the percentage of MM+/TdT+ cells was 0.1% at diagnosis, decreased during remission, increased to 0.2% before relapse RAEB and finally to 35% when AML-M2 emerged. These data indicate that MM+TdT+ cells represent abnormal myeloid cells in MDS and their detection may be useful to monitor the abnormal myeloid subpopulation during disease progression. No MM+/TdT+ cells were found in 2/3 RARS patients. In these patients no dysgranulopoietic features were found. In the other RARS case 5% MM+/TdT+ cells were detected along with the presence of an abnormal granulopoiesis, suggesting that DIF staining can discriminate between RARS cases with and without an abnormal granulopoiesis.

The abnormal hemopoiesis in MDS is reflected in an abnormal in vitro growth pattern of MDS bone marrow. Decreased or absent colony formation of erythroid and megakaryocytic progenitor cells is a typical finding. An abnormal granulocyte-macrophage colony growth is often observed. However, the nature of the aberrant colony formation of MDS bone marrow in vitro is poorly understood. The main objective of this thesis was to study the possible defects leading to the abnormal in vitro colony formation of myeloid progenitor cells in MDS. In chapter 3 colony formation of normal (NBM), MDS, MDS in leukemic phase (LT-MDS) and leukemic (AML) bone marrow cells was studied in a modified PHA-leukocyte feeder colony assay, in which the feeder cells were replaced by the conditioned medium of the giant tumor cell line (GCT-CM) as a source of CSFs. It was found that in NBM and MDS marrows of patients with a stable clinical course optimal colony formation was observed with stimulation with GCT-CM alone, whereas LT-MDS, AML and MDS bone marrows of patients that ran a rapid progressive course were dependent on co-stimulation with PHA and GCT-CM for colony formation. Thus, PHA dependency for colony formation may identify MDS cases with a more progressed disease.

In chapter 4 the growth characteristics and kinetics of myeloid NBM and MDS progenitor cells stimulated with recombinant Il-3, GM-CSF and G-CSF were studied. Colony numbers were lower when stimulated with single recombinant CSF than with GCT-CM, although colony formation efficiency was high (at least 88% of cases). Colony formation was found to be delayed in MDS as compared with NBM. The delay in colony formation of the G-CFU was most apparent and in MDS cases with features of high risk disease (i.e., high leukemic transformation risk and short

survival). The delay in colony formation was found to be caused by a increased time interval before progenitor cells initiated division. These results suggest that committed progenitor cells in MDS marrow are relatively more quiescent (in the G0 phase of cell cycle) than NBM progenitor cells and the time spent in Go increases with disease progression.

In chapter 5 the effects on colony formation of the stimulation with combinations of recombinant Il-3, GM-CSF and G-CSF was studied. In NBM cultures an additive effect of the Il-3 - GM-CSF combination was observed, whereas no additive or synergistic effects of these factors were found in MDS. The response to G-CSF was reduced in MDS marrow as compared with NBM. This reduced effect could be ameliorated by Il-3, which acted synergisticly with G-CSF. These results indicate that a reduced colony growth of MDS marrow can be improved by the combined stimulation with a pluripotent and a lineage restricted CSF.

In chapter 6 the effects of Il-1 and Il-6 on the GM-CSF induced proliferation of myeloid progenitor cells in MDS was investigated. In NBM cultures, either stimulated with optimal or sub-optimal doses of GM-CSF, no enhancing effect of IL-6 or Il-1 was observed. However, in a majority of the MDS cases an enhancing effect of Il-1 and Il-6 on GM-CSF induced colony formation was found. This enhancing effect was still present in the presence of high doses of GM-CSF, indicating that enhancement of colony formation was not mediated by the release of additional GM-CSF in the culture. These results suggest that Il-1 and Il-6 augment the sensitivity of progenitor cells for GM-CSF. However, a delayed addition of IL-6 of several days did not abrogate its effect, whereas an delayed administration of GM-CSF resulted in a fall in colony numbers. These results indicate that Il-6 acts on progenitor cells already responsive to GM-CSF. Il-6 may increase the number or affinity of the GM-CSF receptors or act in a sequence following the action of GM-CSF.

In chapter 7 the results of these investigations are discussed in the context of the literature.

SAMENVATTING

De vorming van bloed cellen is het resultaat van de continue proliferatie, differentiatie en uitrijping van pluripotente hemopoietische stamcellen en voorlopercellen, die zich in het menselijk beenmerg bevinden. In hoofdstuk 1.1 en 1.2 worden de algemene principes van de hemopoiese geintroduceerd, waarbij ingegaan wordt op de verschillende bestaande stamcelmodellen, de verschillende in vitro kweeksystemen, die gebruikt worden om normale en leukemische voorlopercellen te kweken en tenslotte de effecten van de CSFs op hemopoietische voorlopercellen en rijpe bloed cellen.

Het myelodysplastische syndroom (MDS) is een verworven aandoening van de hemopoietische stamcel, die resulteert in een ineffectieve hemopoiese. De ineffectiviteit van de hemopoiese komt tot uiting in een cytopenie van een of meerdere cellijnen (anemie, leukopenie en trombopenie). De normale hemopoiese lijkt al in een vroeg stadium van de ziekte geheel vervangen te zijn door de MDS kloon. De MDS-kloon wordt gekenmerkt door een neiging tot maligne ontaarding: ongeveer veertig procent van de MDS patiënten krijgt uiteindelijk een AML. Deze eigenschap maakt het MDS uitermate interessant in het kader van de bestudering van het onstaan van leukemie, omdat meer inzicht in de pathogenese van MDS kan leiden tot een betere kennis van het onstaan van AML. In hoofdstuk 1.3 wordt ingegaan op de klinische, morphologische en in vitro groei eigenschappen van MDS.

In hoofdstuk 2 worden zes MDS patiënten beschreven, waarvan het beenmerg met behulp van dubbelimmunofluorescentie technieken geanalyseerd is. Gezocht werd naar cellen, die zowel het enzym terminale deoxynucleotidyl transferase (TdT) als myeloid-antigenen als CD13, CD14, CD15 en CD33 tot expressie brachten (MM+/TdT+). TdT expressie werd gevonden in 0,1 - 11% van de cellen. Bij vier patiënten (1 RAEBt, 2 RAEB en 1 RARS) bleken 58 - 99% van de TdT+ cellen de panmyeloide-antigenen CD13 en/of CD33 tot expressie te brengen, terwijl het precursor antigeen CD34 in 26 - 99% van de TdT+ cellen aanwezig was. Vervolg studies in twee patiënten lieten een duidelijke toename van de MM+/TdT+ cellen zien van 11% naar 25% en 0,1% naar 35% in respectievelijk de MDS and AML fase. Bij twee van de drie RARS patiënten werden geen MM+/TdT+ cellen gevonden. Deze twee patiënten vertoonden geen afwijkingen in de granulopoiesis. In de andere

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RARS patiënt konden 5% MM+/TdT+ cellen worden aangetoond en deze patiënt had inderdaad een abnormale granulopoiesis. Uit deze gegevens kan geconcludeerd worden dat dubbel-immunofluorescentie analyses de mogelijkheid verschaffen om een abnormale subpopulatie myeloide cellen, aanwezig in MDS beenmerg, te detecteren en te vervolgen gedurende de verschillende fases van de ziekte.

De abnormale hemopoiese bij MDS patiënten vindt zijn weerslag in een abnormaal in vitro groeipatroon van MDS beenmerg. Over het algemeen is de kolonievorming van erythroide, megakaryocytaire en myeloide voorlopercellen afgenomen of afwezig. De oorzaak hiervan is onbekend. Het belangrijkste doel van het onderzoek, beschreven in dit proefschrift, is het achterhalen van afwijkingen in MDS beenmerg, die kunnen leiden tot deze veranderde groeipatronen.

In hoofdstuk 3 worden de groeieigenschappen beschreven van myeloide MDS voorlopercellen in een kweeksysteem, dat door de groep van Löwenberg speciaal ontwikkeld is voor leukemisch beenmerg (PHA-lf-assay). In dit kweeksysteem wordt de proliferatie van voorlopercellen zowel door CSFs als door phytohemagglutinine (PHA) gestimuleerd. Bij normaal beenmerg en beenmerg van MDS patiënten met een stabiel klinisch beloop bleek de koloniegroei optimaal gestimuleerd te worden door CSF alleen. Echter, bij AML, leukemisch getransformeerde MDS, en MDS patiënten met een progressief klinisch beloop, bleek PHA naast CSF onontbeerlijk te zijn voor koloniegroei. Geconcludeerd kan worden dat de afhankelijkheid van PHA voor in vitro koloniegroei een eigenschap is van beenmerg van MDS patiënten met een meer progressief beloop, of een grotere kans op het ontwikkelen van een AML.

In hoofdstuk 4 wordt de groei kinetiek van hemopoietische voorlopercellen in MDS beenmerg, na stimulatie met recombinant interleukine-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF) en granulocyte-CSF (G-CSF) beschreven. In meer dan 88% van de gevallen werd koloniegroei gestimuleerd door een van deze factoren. De kolonie-aantallen waren echter veelal lager dan in kweken gestimuleerd met GCT-CM (welke meerdere groeifactoren bevat). Een vertraagde koloniegroei van MDS beenmerg werd gevonden, welke het meest uitgesproken was bij granulocytaire voorlopercellen (G-CFU). Tevens was de vertraagde groei meer uitgesproken bij MDS patiënten met in het beenmerg meer dan 10 % blasten, complexe cytogenetische afwijkingen, of een gedocumenteerde leukemische ontaarding. De vertraagde

koloniegroei bleek veroorzaakt te worden doordat de voorlopercellen een latere aanvang maakten met de deling. Deze resultaten suggereren dat gecommiteerde voorlopercellen in MDS beenmerg relatief langer in rust zijn (in de G0 fase van de cel cyclus) in vergelijking hun normale tegenvoeters.

In hoofdstuk 5 worden de effecten van stimulatie met combinaties van CSFs op de koloniegroei van MDS beenmerg beschreven. Een duidelijk synergistisch effect van de combinatie Il-3 en G-CSF op MDS myeloide voorlopercellen werd gevonden, terwijl dit effect niet waarneembaar was bij normaal beenmerg. Deze resultaten suggereren dat myeloide voorlopercellen in MDS beenmerg meerdere CSFs nodig hebben voor een optimale stimulatie (waarschijnlijk een progressie factor gecombineerd met een differentiatie factor). Dit is een eigenschap die ook beschreven is voor normale pluripotente voorlopercellen en suggereerd een functionele onrijpheid van myelodysplastische myeloide voorlopercellen.

In hoofdstuk 6 worden de effecten van Il-1 and Il-6 op myeloide voorlopercellen in MDS beenmerg beschreven. Il-1 en Il-6 stimuleren zelf geen koloniegroei, maar kunnen de repons op een tweede CSF vergroten. Bij normale beenmerg kweken werd geen effect van de beide interleukines gezien in combinatie met GM-CSF. Een duidelijk stimulerend effect van Il-1 en Il-6 op de koloniegroei van MDS beenmerg werd echter wel gevonden. Ook in de aanwezigheid van een tienvoudige concentratie GM-CSF was dit stimulerend effect aanwezig. Dit geeft aan dat de stimulatie niet wordt veroorzaakt door een extra productie van GM-CSF. Waarschijnlijk verhogen Il-1 and Il-6 de gevoeligheid van MDS voorlopercellen voor GM-CSF. Identieke resultaten werden verkregen wanneer Il-6 pas na enkele dagen aan de kweken werd toegevoegd, wat impliceert dat Il-6 waarschijnlijk voorlopercellen stimuleert, die al gevoelig zijn voor GM-CSF. Geconcludeerd kan worden dat in MDS, myeloide voorlopercellen verminderd gevoelig zijn voor GM-CSF. Deze verminderde gevoeligheid kan niet verholpen worden met behulp van zeer hoge doseringen GM-CSF, maar wel gedeeltelijk door middel van co-stimulatie met Il-6.

In hoofdstuk 7 worden de resultaten besproken in samenhang met recente literatuur gegevens.

ABBREVIATIONS

AET	2-aminoethylthiouronium bromide hydrobromide
AISA	acquiered idiopathic sideroblastic anemia
ALIP	abnormal localization of immature precursors
AML	acute myeloid leukemia
BFU-E	burst forming unit-erythroid
BPA	burst promoting activity
CAFC	coblestone area forming cell
CD	cluster of differentiation
CFU-blast	colony forming unit-blast cells
CFU-E	colony forming unit-erythroid
CFU-Eo	colony forming unit-eosinophil
CFU-G	colony forming unit-granulocyte
CFU-GEMM	colony forming unit-granulocyte-erythroid-macrophage-megakaryocyte
CFU-GM	colony forming unit-granulocyte-macrophage
CFU-M	colony forming unit-macrophage
CFU-Meg	colony forming unit-megakaryocyte
CM	conditioned medium
CMML	chronic myelomonocytic leukemia
CSF	colony-stimulating factor
ECGF	endothelial cell growth factor
Epo	erythropoietin
FAB	French-American-British cooperative group
FCS	fetal calf serum
GCT-CM	giant cell tumor cell-line conditioned medium
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage-colony stimulating factor
HGF	hemopoietic growth factor
HIM	hemopoietic inductive environment
IL	interleukin
LT	lymphotoxin
LT-MDS	leukemic transformed myelodysplastic syndrome
McAb	monoclonal antibody
M-CSF	macrophage-colony stimulating factor
MDS	myelodysplastic syndrome
MM	myeloid marker
NBM	normal bone marrow
PDGF	platelet derived growth factor
PHA	phytohemagglutinin
PHSC	pluripotent hemopoietic stem cell
PLS	preleukemic syndrome
PSA	pure sideroblastic anemia
RA	refractory anemia
RAEB	refractory anemia with excess of blasts
RAEBt	refractory anemia with excess of blasts in transformation
RARS	refractory anemia with ringsideroblasts
TdT	terminal deoxynucleotidyl transferase
TGF	transforming growth factor
T-MDS	trilineage myelodysplasia
TNF	tumor necrosis factor
4-HC	4-hydroperoxycyclophosphamid

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 6 september 1960 te Den Haag. Na het behalen van het eindexamen Gymnasium ß aan het Haags Montessori Lyceum, studeerde hij geneeskunde aan de Erasmus Universiteit te Rotterdam. Het doctoraal examen behaalde hij op 11 oktober 1983 en het artsexamen op 19 april 1985. Van 1 mei 1985 tot 1 maart 1987 was hij werkzaam als wetenschappelijk medewerker binnen de vakgroep Celbiologie en Genetica, waar een deel van het in dit proefschrift beschreven onderzoek werd verricht. Hierna is hij als tijdelijk wetenschappelijk staflid werkzaam geweest op de afdeling Hematologie van het Academisch Ziekenhuis Rotterdam-Dijkzigt (hoofd: Prof. Dr. J. Abels). Sinds 15 februari 1990 is hij in opleiding tot internist in het St. Elisabeth ziekenhuis te Amersfoort (opleider: Dr. R.A. Geerdink).