GM-CSF, IL-3 AND G-CSF RECEPTORS ON ACUTE MYELOID LEUKEMIA CELLS

FUNCTION, REGULATION OF EXPRESSION, AND LIGAND BINDING CHARACTERISTICS

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FUNCTIE, REGULATIE VAN EXPRESSIE EN BINDINGSEIGENSCHAPPEN

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Voor Mieke, Claudine, Laurens en mijn ouders

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Abbreviations:

aa	amino acids
AIC2A/B	murine GM-CSF/IL-3/IL-5 receptor β subunit
AML	acute myeloid leukemia
CFU	colony-forming unit
CML	chronic myeloid leukemia
CNTF	ciliary neurotropic factor
CRH	cytokine receptor homology (region)
EPO	erythropoietin
FACS	fluorescence activated cell sorting
fMLP	formyl-Met-Leu-Phe
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GH	growth hormone
HGF	hematopoietic growth factor
IL-n	interleukin-n
K_d	dissociation constant
KH97	human GM-CSF/IL-3/IL-5 receptor β subunit
KL	kit-ligand (=MGF=SCF)
LIF	leukemia inhibitory factor
M-CSF	macrophage colony-stimulating factor
MGF	mast cell growth factor
OSM	oncostatin M
PDGF	platelet derived growth factor
PKC	protein kinase C
PRL	prolactin
PTK	protein tyrosine kinase
R	receptor
RA	retinoic acid
RTK	receptor tyrosine kinase
SCF	stem cell factor
TCA	trichloroacetic acid
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TSLP	thymic stroma derived lymphopoietin

CHAPTER 1

INTRODUCTION

1.1 Hematopoiesis

Blood cell formation or hematopoiesis normally occurs in the bone marrow and is a highly dynamic process. On the average, an adult will produce over 10^{11} granulocytes and erythrocytes per day to compensate for normal losses. In periods of stress, such as infection or hemorrhage, the blood cell production may increase by at least tenfold. All blood cells are derived from a population of pluripotent stem cells that are capable of self renewal and can give rise to distinct progenitor cells (1,2) (Fig. 1). Progenitor cells are irreversibly committed to either the erythroid, megakaryocytic, granulocytic, monocytic or lymphoid lineages. Progenitor cells have a great proliferative potential and generate up to 10^5 functional end cells, which are released into the circulation. The maintenance of sufficient terminally differentiated cells is essential for the defense against micro organisms (neutrophils and monocytes) as well as for hemostasis (platelets) and tissue oxygenation (erythrocytes).

Hematopoiesis is controlled by a tightly organized regulatory system in which the bone marrow stroma cells (the so-called micro environment), and the hematopoietic growth factors (HGFs) play crucial roles (Fig. 1). HGFs are produced by the accessory cells such as monocytes, lymphocytes, fibroblasts or endothelial cells, or by nonhematopoietic tissues, for example renal cortex cells, which produce erythropoietin (EPO). The hematopoietic organ maintains its homeostatic condition by virtue of a precisely adjusted balance between positive and negative stimuli delivered by this regulatory system.

The development of clonogenic assays and the molecular cloning of many HGFs acting on myelopoietic progenitors have enabled investigators to study the basic principles of hematopoiesis and hematologic malignancies in vitro (3,4). One of the objectives of these investigations is to obtain insight into the role of the hematopoietic growth factors and their receptors in normal and malignant hematopoiesis, in particular also in acute myeloid leukemia (AML).

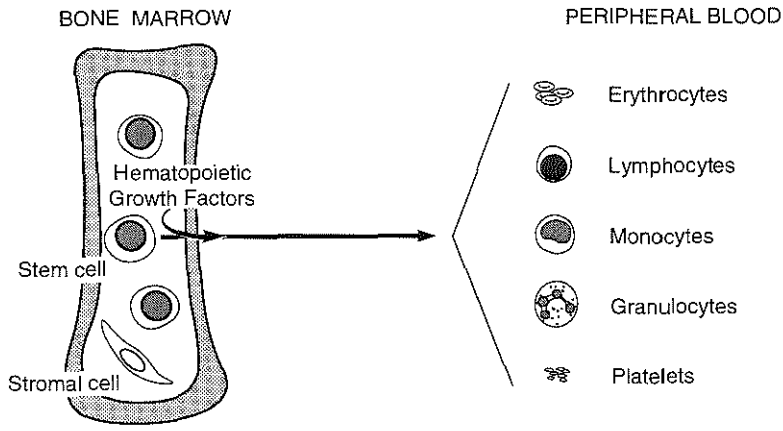

Figure 1. Scheme depicting the different types of blood cells that originate from the bone marrow stem cells.

1.2 Acute myeloid leukemia (AML)

AML is characterized by neoplastic proliferation of immature myeloid cells (mostly blast cells) in the bone marrow and blood (5). Until today, AML remains a highly lethal malignancy. Untreated it will lead to death, due to infections or hemorrhage, usually within a few months after diagnosis. Although the cause of AML remains largely unknown, environmental and occupational factors, such as exposure to chemicals and radiation, may play an important role (6,7).

AML is a clonal disease. All leukemic cells originate from a single ancestor cell, which somehow escapes from the regulatory system that normally controls hematopoiesis. The malignant cells have lost their ability to mature, and start to grow seemingly uncontrolled (8-13). A major consequence of this escape is the infiltration of the bone marrow and suppression of normal hematopoiesis. Subsequently, this leads to the accumulation of immature leukemic cells in the circulation and depletion of functional blood cells. Although the majority of AML cells are blasts, often differentiated cells of the three myelopoietic lineages can also be found (14). This is explained by the fact that the AML clone can arise at different levels of maturation and some cells still have the ability to bypass the maturation block to a limited extent (15-19).

1.3 Hematopoietic growth factors (HGFs)

Many different glycoproteins are involved in the control of hematopoiesis (1,20-24). More than 20 HGF cDNA's have now been cloned and their recombinant products have become available. HGFs are glycosylated proteins ranging in size from 14 to 50 kilodaltons. The glycoprotein moieties are not required for the biological function of HGFs, but may facilitate their transport to various tissues. Certain HGFs, like kit-ligand (KL, also called steel factor (SLF), mast cell growth factor (MGF) or stem cell factor (SCF)), TNF or M-CSF are produced both in a soluble and a nonsoluble, i.e., membrane bound form, as a result of alternative RNA splicing. Cells expressing membrane bound HGF can stimulate target cells only after direct cell contact (25-30). Most of the HGFs are active as single (monomeric) molecules. However, some HGFs form multimers of identical subunits. For example IL-5 and M-CSF are kept in a dimeric form via disulfide bridges, and the biologically active form of tumor necrosis factor (TNF) is a trimer (31). The actions of HGFs are initiated after the interaction of HGF with specific receptors expressed on the cell membrane (see 1.4) followed by intracellular molecular interactions leading to biological responses.

Generally, HGFs play a role in coordinating proliferation, differentiation, survival, and function of hematopoietic cells. Many of the HGFs are pleiotropic in their activities. They are active in more than one hematopoietic differentiation lineage or at different levels of maturation. Furthermore, they are also frequently involved in functional activities including the maintenance of membrane transport mechanisms, phagocytosis and the production and release of other cytokines (32). For example, IL-3 and GM-CSF stimulate the development of various lineages of hematopoietic cells, and act at immature and terminally differentiated cells (1,22,23). G-CSF is more restricted in its actions and is mainly involved in the production and activation of

neutrophilic granulocytes (33). Different HGFs may exert their effects in overlapping target cell populations, often resulting in similar biological activities (1,21,34-38).

A single cytokine, e.g., GM-CSF, can trigger different cellular responses in various cell types. Vice versa, similar biological effects can be induced by different cytokines (IL-3, GM-CSF). These observations raise questions regarding the structure of cytokine receptors and their signaling pathways: (i) Are there different types of receptor structures for GM-CSF? (ii) Can a single receptor bind IL-3 and GM-CSF? The studies presented in this thesis in ***Chapters 7 and 8*** were performed to specifically address these questions.

1.4 HGF control of AML cell growth

Despite the apparent escape from regulation in vivo, most AML blasts have retained their ability to respond to HGFs stimulation in vitro (34,39-44). AML colony growth in semi-solid media fully depends on HGFs, whereas AML cells in suspension culture require HGFs for proliferation, as assayed by ^{3}H-TdR uptake, in approximately 50% of the cases. IL-3, GM-CSF, and G-CSF are the most important HGFs for AML cell proliferation in vitro. Of note is the strikingly parallel proliferative response of different AML cases to GM-CSF and IL-3. From studies in mice carrying mutations in the Sl or W loci it has become apparent that KL and its receptor (c-kit) are essential for normal hematopoiesis. KL also directly stimulates AML blast cell proliferation in 50% to 80% of the AML cases (45-47). Above all, KL is a strong synergistic factor. In combination with IL-3, GM-CSF and G-CSF it has profound enhancing effects on AML blast cell proliferation in vitro (48,49,Chapter 4). The cooperative actions of KL with other HGFs does not result from enhanced expression of HGF receptors (Chapter 4). M-CSF, IL-6 and EPO are to a lesser extent active as exogenous stimulators of AML blast cell DNA synthesis (34,42). In vitro, TNF acts as a modulator of HGF controlled growth of normal hematopoietic and leukemic cells. In combination with IL-3 or GM-CSF, TNF acts synergistically on proliferation and colony formation of AML cells and CD34^{+} cells. On the other hand, TNF inhibits the G-CSF induced proliferation of AML cells (50-52). IL-1 and to a lesser extent also TNF can stimulate AML growth via induction of endogenous HGF release through activation of transcription or stabilization of HGF mRNA in AML cells (53-57). AML cells can become independent of endougenous growth factors through autocrine mechanisms. In approximately 50% of the cases AML cells show significant DNA synthesis in the absence of HGFs (34,54,58-60). In about 40% of the cases this proliferation can be blocked by antibodies against HGFs, including GM-CSF, G-CSF, M-CSF, IL-1 and IL-6.

While HGFs still play an important role in controlling the proliferation of AML progenitor cells in vitro, HGFs do not induce differentiation of primary human leukemia cells in vitro. Only in a limited number of AML cases, in particular those expressing the cytogenetic abnormality t(8;21)(q22;q22) G-CSF can induce neutrophilic maturation (19). Thus, in general AML cells proliferate in response to (endogenously released) HGFs in vitro, but the ability of AML cells to mature is disturbed and cannot be restored by HGFs.

KL stimulates the in vitro proliferation of AML. Particularly, it acts as a synergistic factor in combination with other HGFs including IL-3 GM-CSF, and G-CSF. The expression of KL receptor (encoded by the proto-oncogene c-kit) and the effects of KL on HGF receptors and vice versa were studied in AML ***(Chapter 4)***. The following questions have been addressed: (i) what is the status of KL-receptor in AML, in relationship to the proliferative response? (ii) Does KL upregulate IL-3 or GM-CSF receptors in AML cases that show synergy between KL and IL-3 or GM-CSF, and vice versa, what is the effect of IL-3 and GM-CSF on KL-receptor expression in these AML cases?

In addition to its capacity to induce endogenous HGF production, TNF acts synergistically with IL-3 and GM-CSF in inducing the proliferation of AML cells. In contrast, G-CSF responses are strongly inhibited by TNF. The following questions have been addressed in ***Chapters 5 and 6***: (i) Does TNF upregulate IL-3 or GM-CSF receptors in AML cases that show synergy between TNF and IL-3 or GM-CSF? (ii) Does TNF down modulate G-CSF receptors in AML cases in which the proliferative response to G-CSF is abrogated by TNF?

1.5 Hematopoietic growth factor receptors

Hematopoietic growth factor receptors (HGF-Rs) are transmembrane glycoprotein complexes, often of 2 or more subunits that (i) form a specific ligand recognition site and (ii) have the ability to activate intracellular mechanisms of signal transduction (Fig. 2). It is now generally accepted that di- or multimerization of subunits is required for high affinity binding and signaling (61-68). Cloning of the cDNA's encoding these receptors has been achieved using expression cloning techniques and cross hybridization of cDNA libraries. The cDNAs of many HGF receptors have been isolated, and their structures characterized at the molecular level. The availability of the genetic sequence of these receptors offers the possibility to investigate the structure-function relationship of these proteins and has led to further understanding of the mechanism by which hematopoietic cells communicate with their environment.

1.5.1 Structural versus functional classification of receptors

Based on the nucleotide homology of HGF-R genes, several families of receptors have been identified. By comparing the relative alignment scores of the extracellular domains using computer programs, a distinct subdivision can be made on basis of structural features (69-73). Five receptor categories can be distinguished: (i) The hematopoietin receptor superfamily (Class I cytokine receptors), which is the most recently identified subcategory of membrane receptors, comprises most of the receptors for the HGFs. The structural and functional characteristics of these receptors will be discussed in detail in Chapter 9. (ii) The relatively small interferon (IFN) receptor family (Class II cytokine receptors), comprise type I and II IFN

receptors, and Tissue Factor (TF), which is the receptor for coagulation protease factor VII (70). (**iii**) TNF receptors, FAS, CD40, and nerve growth factor (NGF) binding protein belong to the Class III cytokine receptors. The extracellular domains of these receptors are cysteine rich and share significant homology (74-79). (**iv**) The immunoglobulin superfamily (Class IV cytokine receptors), includes receptors such as the IL-1 receptor, the M-CSF receptor and the platelet derived growth factor (PDGF) receptor (72,80). The extracellular domains of these receptors have an immunoglobulin-like structure with 3 or 5 homologous repeats, analogous to the immunoglobulin constant domain. Apparently, these receptors and the immunoglobulines have evolved distinctively from common ancestral genes. (**v**) Many hormones bind to a group of G-protein coupled receptors such as for example the IL-8 receptor (81-82). These receptors are characterized by a stretch of amino acid residues that span the membrane seven times and are intracellularly coupled to guanine nucleotide binding (G) proteins (83).

An alternative classification of receptors is made on functional characteristics. For example the receptors for KL, CSF-1, PDGF, EGF and insulin, all possess a highly conserved tyrosine kinase catalytic region in their intracellular domains (84-86). Hence, these receptors are also referred to as the group of receptor tyrosine kinases (RTK). The steroid and thyroid hormone receptor super family, a group of nuclear receptors which act as hormone responsive transcription factors (87) have both a ligand and a DNA binding domain. Retinoic acid receptors, which are members of this group are involved in differentiation control of many different tissues including

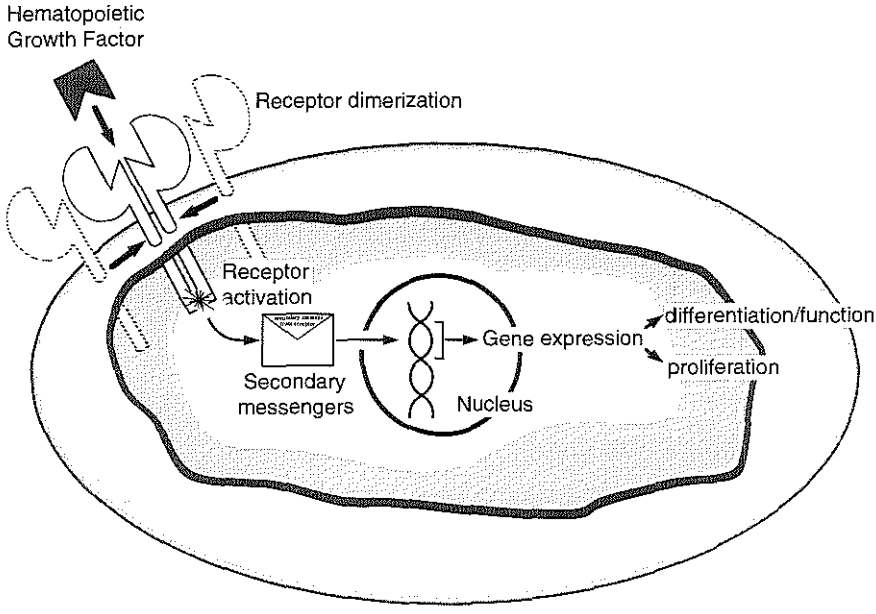

Figure 2. Scheme depicting a hematopoietic growth factor receptor composed of two subunits that is activated by its specific ligand through dimerization. The activated receptor dimer, via secondary messengers, regulates expression of genes that are involved in proliferation, differentiation and function of hematopoietic cells.

myeloid cells (88-90).

1.5.2 IL-3-, GM-CSF-, and G-CSF receptors

IL-3-, GM-CSF-, and G-CSF receptors are members of the cytokine receptor superfamily. Binding studies have revealed that IL-3 and/or GM-CSF receptors are widely expressed on the membranes of human hematopoietic cells, including immature myeloblasts, acute myeloid leukemia cells, several leukemia cell lines, normal monocytes, neutrophils, eosinophils, and basophils (91-101). Although in earlier reports only single affinity class IL-3 and GM-CSF receptors were described, it appeared later that both receptors have high and low affinity binding sites. The dissociation constants of these receptors are in the order of 10-100 pmol/L and 10-100 nmol/L for high and low affinity sites, respectively. In addition, for GM-CSF a third receptor was found with intermediate affinity (102-106). G-CSF receptors are expressed by various cells, from the myeloblast to mature neutrophils, AML cells and leukemic cell lines, but not on erythroid or megakaryocytic cells (107-109). In various hematopoietic cells, single class high affinity G-CSF receptors are apparent with K_d values 100-400 pmol/L. More detailed information about these receptors is provided in Chapter 9.

Immature myeloid cells express GM-CSF receptors with binding characteristics that differ from those expressed on mature myeloid cells (***Chapter 7***). These observations raise two questions: (i) At which stage of maturation are GM-CSF binding features of immature myeloblasts changed into those of terminally differentiated neutrophils? (ii) What is the mechanism responsible for these differences. The experiments presented in ***Chapter 8*** address these questions.

1.6 Hematopoietic growth factor receptors in AML

Oncogenes were first described as retrovirally transduced genes that could induce tumor growth in birds and rodents. These genes later turned out to be activated or modified forms of cellular genes (proto-oncogenes). The study of oncogenic animal retroviruses and the cloning of breakpoints in chromosomal translocations in malignancies, have led to the identification of many proto-oncogenes involved in regulatory systems that control cell growth and cell differentiation. They are involved in the cascade of events of normal cell division: (i) growth factors such as PDGF or KL (110,111); (ii) growth factor receptors such as kit, erbB, fms or mpl (112-115); (iii) signal transducers of growth factor responses such as src, raf or ras (116,117); (iv) transcription factors that mediate growth factor-induced gene expression like fos and jun (116,118).

In vitro studies with AML have shown that AML blast cells can still proliferate, but have essentially lost their ability to show neutrophilic maturation (in response to G-CSF). Furthermore, in 25% of the AML cases, spontaneous proliferation has been demonstrated that is not dependent on autocrine mechanisms. These observations suggest that leukemic outgrowth of immature blast cells may involve abnormal growth factor receptor function. Ensuing the identification of the cellular homologs of v-fms and v-erbB, which encode mutated forms of the receptors for M-CSF and

EGF respectively, it was apparent that there might be an association between leukemia and abnormal receptor function (114,119). Expression of other viral oncogenes that carry altered hematopoietic growth factor receptors such as v-kit or v-mpl (a truncated form of a novel hematopoietic growth factor receptor transduced by the myeloproliferative leukemia virus) have been shown to cause hematological disorders in mice. Artificially introduced point mutations or deletions in cellular genes encoding growth factor receptor genes were able to induce cell transformation. Single point mutations in the extracellular domain of M-CSF receptor resulted in constitutive receptor activation, alleviating the growth factor dependency of murine hematopoietic cell lines and transformed 3T3 fibroblasts. A point mutation in the extracellular domain of the murine EPO receptor has been found to induce erythroleukemia in mice (120,121). There have been few reports of genetic lesions found in receptor genes isolated from patient material. In ten percent of patients with MDS or AML, point mutations of the M-CSF receptor were described (122). However, these mutations were either also found in normal individuals or lacked transforming capacities when expressed in 3T3 fibroblasts.

In addition to structural abnormalities, abnormal transcriptional regulation of a receptor gene can lead to disturbed receptor function. Overexpression may render a cell hyper responsive to the ligand, or even cause transformation. For example overexpression of either EGF receptors or HER2/neu, an EGF receptor-like protein, in 3T3 fibroblasts results in ligand-independent neoplastic transformation (123). The EGF receptor gene is often amplified in breast carcinoma and myc in lung carcinoma (124). Similarly, overexpression of the M-CSF receptor in a murine myeloid cell line renders these cells growth factor independent and tumorigenic (125).

One of the initial goals of this thesis was to document the binding status of GM-CSF, IL-3 and G-CSF receptors on primary AML cells, to investigate whether these receptors may be overexpressed or have an altered ligand binding affinity as compared to normal hematopoietic cells ***(Chapters 2 and 3)***.

CHAPTER 2

IL-3 AND GM-CSF RECEPTORS ON HUMAN AML CELLS AND RELATIONSHIP TO THE PROLIFERATIVE RESPONSE.

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Blood, Vol 74: pp 565-571, 1989

SUMMARY

Interleukin-3 (IL-3) and granulocyte macrophage colony-stimulating factor (GM-CSF) stimulate proliferation of human acute myeloid leukemia (AML) in vitro, although patterns of response among clinical cases are diverse. Whether regulatory abnormalities related to growth factor responses in human AML may establish the outgrowth of the neoplasm remains unclear. We determined receptor numbers and affinity for IL-3 and GM-CSF on human AML cells using human recombinant IL-3 and GM-CSF. In 13 of 15 cases of primary AML high affinity (K_d 26 to 414 pmol/L) receptors for IL-3 were demonstrable on the cells. The average numbers of IL-3 receptors ranged from 21 to 145 receptors per cell. Normal white blood cells showed IL-3 receptors on their surface at similar densities. IL-3 receptor positivity often correlated with GM-CSF receptor positivity of AML, GM-CSF receptors were demonstrated on the cells of 11 of 15 cases although average numbers of GM-CSF receptors were ten times greater. The in vitro response of the cells to exogenous IL-3 or GM-CSF was examined by measuring thymidine uptake. Because IL-3 and GM-CSF were potent inducers of DNA synthesis in vitro, apparently relatively few receptors are required to permit activation of growth. These experiments did not provide evidence for overexpression or increased receptor sensitivity as an explanation for AML growth. In a minority of cases however, the cells were unable to respond to IL-3 (four of 15 cases) or GM-CSF (four of 15 cases) despite normal receptor availability on the cell surface.

INTRODUCTION

IL-3 and GM-CSF belong to the class of CSFs which regulate survival, proliferation and differentiation of hematopoietic progenitor cells in humans (1,2,3,4). Most cases of human AML are dependent on CSF for growth, but some leukemia's appear to proliferate spontaneously as the consequence of autocrine CSF production (5,6,7,8). The growth pattern of AML cells cultured in vitro in the presence of different CSFs shows considerable variation (9,10,11,12). However regulatory abnormalities of growth have not been established in human AML since this diverse growth reactivity may resemble the heterogeneity of response of different stages of normal marrow precursor cells rather than cellular alterations as the result of malignant transformation. CSFs are believed to react with their target cells through membrane-bound receptors (13,14). Little is known about the role that growth factor receptors play in the pathophysiology of human AML. The relationship between growth factor receptor expression and the stimulability of AML cells in vitro has not been investigated. Overexpression of a receptor may have a role in the outgrowth of AML in vivo, but no experimental evidence to support this possibility has yet been provided. One study reported that GM-CSF receptor density on AML cells is not increased; hence overexpression of GM-CSF receptors apparently does not account for a leukemic growth advantage (15). IL-3 is also an effective growth factor for blast cells of AML (9,10,11,12), but no information on IL-3 receptor properties on human AML cells is available. For a better understanding of the mechanisms that lead to proliferation of AML cells in response to IL-3 and GM-CSF, we investigated numbers and affinity of IL-3 and GM-CSF receptors on cells of

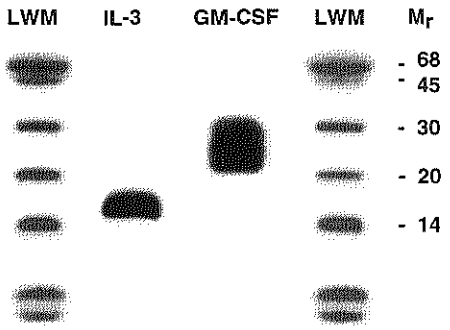

Figure 1. Autoradiography of ^{125}I labeled IL-3 and GM-CSF. Radioiodinated IL-3 and GM-CSF 4×10^5 cpm were electrophoresed in 12% SDS polyacrylamide gel. Autoradiograms were obtained by exposing the gel to Kodak X-Omat AR film at -70°C. Lanes 1 and 4: iodine-labeled mol-wt markers.

patients with AML and correlated these data with the proliferative response of the cells to both molecules.

MATERIALS AND METHODS

Preparation of cells. AML cells were obtained from 15 cases of AML classified according to the criteria of the French-American-British (FAB) committee (16,17): M1 (n=2), M2 (n=3), M4 (n=8) and M5 (n=2). The cells were separated from bone marrow (BM) or peripheral blood (PB) after bovine serum albumin density gradient (BSA) or Ficoll-Isopaque centrifugation (18) and subsequent removal of E-rosette forming cells. The AML cells were cryopreserved in 7.5% dimethyl sulfoxide and 20% inactivated fetal calf serum (FCS). The viability of the cells after thawing was always >95%. Normal BM was obtained by posterior iliac crest puncture from healthy adults and the mononuclear cells were separated over Ficoll-Isopaque density gradient. Eosinophilic cells were separated from the blood of a patient with hypereosinophilia (80×10^9 cells/L) and WBCs from the blood of healthy subjects after sedimentation in 0.1% methyl cellulose. The latter cell preparations were used fresh.

Hematopoietic growth factors. Recombinant IL-3 (Escherichia coli derived) (19) and GM-CSF (CHO cell derived) (20) was prepared and purified at Genetics Institute (Cambridge, MA). GM-CSF used was glycosylated and had a molecular weight (MW) of 20-30 kDa (biological activity 6 to 10×10^{10} U/g). IL-3 was not glycosylated (MW 15 kDa; 2 to 3×10^{10} U/g).

Radioiodination of IL-3 and GM-CSF. Purified recombinant IL-3 and GM-CSF were radiolabeled according to the method described by Bolton and Hunter (21). Bolton-Hunter reagent (BHR) 1 mCi (Amersham laboratories, Amersham, UK) dissolved in benzene was dried under a stream of N_2 in the packing vial at 22°C. The vial was then cooled to 0°C, and 5 μg IL-3 or 5 μg GM-CSF in 20 μl borate buffer (0.1 mol/L, pH 8.2) was applied. The reaction was permitted to proceed for 45 minutes at 0°C, while the vials were agitated every 5 minutes. Labeling was quenched by adding 100 μl of glycine (0.2 mol/L in borate buffer). After 5 minutes on ice, 100 μl of 0.25 % gelatin was added to facilitate recovery of the reactant from the vial. The labeled proteins were separated from the unreacted BHR and the iodinated glycine by chromatography on sepharose columns (GF-5, Pierce Chemical Co., Rockford, IL),

Table 1. Biological activity of radioiodinated IL-3 and GM-CSF

		No. of colonies and clusters		
		Nonlabeled	Mixture	^{125}I-labeled
CSF concentration (pmol/L)		100 %	50/50 %	100 %
IL-3	0.3	0/7	0/6	0/2
	3	6/21	5/28	3/18
	30	14/39	15/41	5/28
	300	11/30	0/0	0/0
GM-CSF	2	2/7	3/5	0/6
	20	12/34	22/38	36/35
	200	31/31	37/43	29/40
	2000	52/41	20/47	0/8

Dose response of titrated concentrations native IL-3, radiolabeled IL-3 and a mixture of native or radiolabeled IL-3 and the same dose response data for GM-CSF. Colonies and clusters induced from 0.5×10^5 Ficoll separated marrow cells in 1 mL cultures were scored at day 14. Values indicate average numbers of colonies (before the "/") and clusters (after the "/") of duplicate cultures.

equilibrated with phosphate buffered saline (PBS) containing 0.1% gelatin and 0.01% Tween 20.

Samples taken from the void volume were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent autoradiography showing a single band for IL-3 at 15 kD and a broader band for GM-CSF at 20-30 kD (Fig. 1). Radiolabeling of IL-3 and GM-CSF using the Bolton and Hunter protocol resulted in preparations that retained their ability to stimulate colony formation by normal BM cells in methyl cellulose (Table 1). The reduced effectiveness in stimulating colony formation by higher concentrations of radiolabeled IL-3 and GM-CSF is most likely attributed to the continuous irradiation by iodine 125 throughout culturing time (14 days). TCA precipitation showed less than 5% nonprecipitable radioactivity for both factors. The maximum binding capacity was estimated at 35-50% for IL-3 and 40-70% for GM-CSF. This given has been used to correct the "Free" cpm in the Scatchard calculations. Specific activity of labeled IL-3 and GM-CSF was determined by self displacement analysis (22) or calculated from the TCA precipitable radioactivity measured in a small sample taken from the reactant (similar results were obtained with both methods, although the selfdisplacement for IL-3 was somewhat more inaccurate as compared with the TCA method). The specific radioactivity was estimated at 8 to 10×10^4 cpm/ng for IL-3 and 4 to 6×10^4 cpm/ng for GM-CSF. The fractions containing the radiolabeled protein were stored at 4°C in 20% glycerol and 0.2% sodium azide and used within four weeks of preparation.

Colony culture assay. The biological activity of radio labeled IL-3 and GM-CSF was determined in normal bone marrow: 0.5×10^5 cells were cultured in a 1 mL mixture of Iscove's modified Dulbecco's medium (IMDM), 0.8% methyl cellulose, 30% FCS, BSA, transferrin, lecithin, sodium-selenite and beta-mercaptoethanol (23). Nonlabeled and radiolabeled IL-3 and GM-CSF were added to the cultures in tenfold increasing concentrations. The experiment also included cultures containing a 50/50% mixture of nonlabeled and radiolabeled CSF in order to establish the negative effects of gamma radiation on colony formation. Clusters > 15 cells and

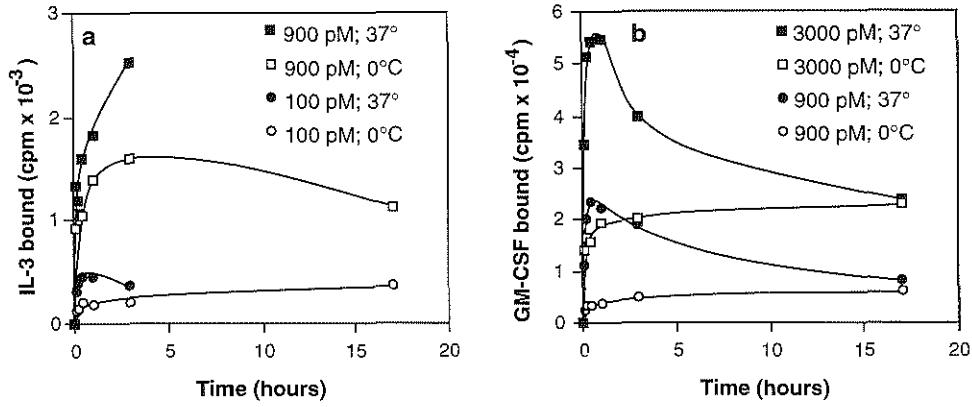

Figure 2. Binding of ^{125}I labeled IL-3 (**a**) and GM-CSF (**b**) to normal WBCs in relation to time. WBCs 4×10^6 were incubated with 100 pmol/L and 950 pmol/L IL-3 or 600 and 3000 pmol/L GM-CSF at 0°C and 37°C. Binding was assessed as a function of time of incubation.

colonies > 50 cells were scored at day 14. Duplicate cultures were established for each point.

Binding of radiolabeled IL-3 and GM-CSF to AML cells. After thawing, AML cells were washed two times in Hanks' balanced salt solution (HBSS). Usually, 2 to 4×10^6 cells were incubated for 1 hour at 37°C in 100 µL α-Minimal Essential Medium (α-MEM)/10% FCS with 10 to 2000 pmol/L radiolabeled IL-3 or GM-CSF with or without 100 nmol/L nonlabeled factor. The cells were then cooled to 0°C, layered over 500 µL calf serum in Eppendorf tubes on ice and centrifuged for 5 minutes at 1000 g. The tubes were frozen in liquid nitrogen, and the tips were cut off for counting in a Packard gamma-counter. Specific binding was defined as the difference between the amount of radioactivity bound in the without unlabeled factor added, and the amount of radioactivity bound with excess unlabeled factor added. Experiments were conducted in duplicate. Receptor numbers and binding affinities were derived by Scatchard analysis (24).

^{3}H-Thymidine incorporation into AML cells. DNA synthesis of AML cells was

Table 2. Binding of ^{125}I-IL-3 and ^{125}I-GM-CSF to WBCs and eosinophils

Cell type		IL-3 Receptors		GM-CSF Receptors	
		Sites/cell	K_d (pmol/L)	Sites/cell	K_d (pmol/L)
WBC	donor 1	137	86	1,579	230
	donor 2	60	53	1,789	392
	donor 3	74	82	1,515	497
	donor 4	134	113	2,091	493
Eosinophils		1,950	494	1,991	636

Receptor numbers (mean per cell) and K_d were derived from binding experiments and subsequent analysis according to Scatchard (described in legend to Fig. 3). WBCs were obtained from different donors.

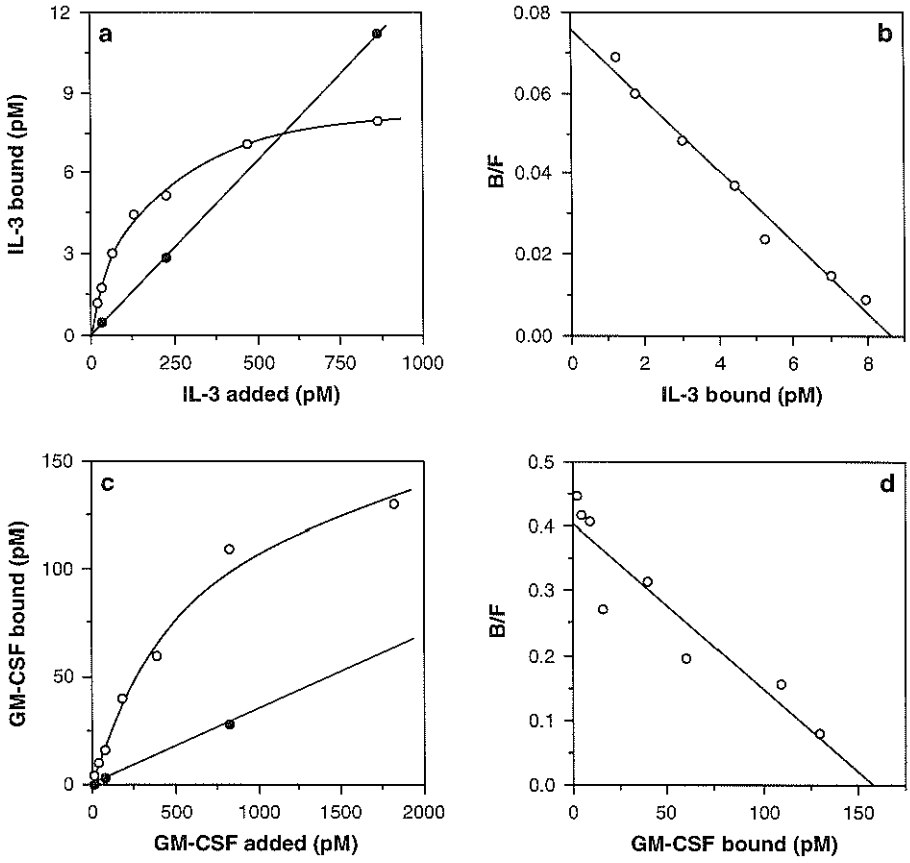

Figure 3. Binding of radiolabeled IL-3 (**a**) and GM-CSF (**c**) to WBCs and Scatchard plots of these data (**b** and **d**). Two to 5 × 10^6 cells and were incubated with increasing amounts of radiolabeled IL-3 or GM-CSF for 1 hour at 37°C. Nonspecific binding was determined in the presence of excess unlabeled factor. Specific binding (open circles) as well as nonspecific binding (closed circles) data are plotted in the left panels. Each point is the mean of two estimates.

measured as described (25). Two × 10^4 cells were cultured for 3 days in 96-well round-bottom microtiter trays in 100 μL serum-free medium, with or without addition of IL-3 or GM-CSF. Four hours before harvesting, 0.1 μCi tritiated thymidine (Amersham) was added. Cells were harvested on nitrocellulose paper using a Titertek Harvester 550 (Flow Laboratories, Isrike, UK). Radioactivity was determined with a scintillation counter (Beckman LS 3800, Fullerton, CA). All experiments were performed in triplicate and data expressed as mean dpm. In each experiment preirradiated cells were used in control cultures to measure background thymidine uptake.

Table 3. Binding of human radioiodinated IL-3 and GM-CSF to their receptors on AML cells

			IL-3 receptors		GM-CSF receptors	
FAB	Case	Blasts† (%)	Sites/cell	K_d (pmol/L)	Sites/cell	K_d (pmol/L)
M1	1	98	56	91	23	64
	2	100	25	467	<Det.	-
M2	3	100	21	326	<Det.	-
	4	ND	<Det.	-	40	157
	5	97	55	176	84	123
M4	6	83	17	183	236	157
	7	89	33	414	561	293
	8	82	21	137	191	162
	9	64	<Det.	-	843	404
	10	85	60	61	245	155
	11	ND	51	65	792	258
	12	ND	38	26	550	197
	13	ND	98	39	783	169
M5	14	67	113	163	949	169
	15	50	145	88	1263	274

Binding assays were performed in duplicate following incubation of 2 to 5 × 10^6 AML cells for 1 hr at 37°C with 8 different concentrations of radiolabeled IL-3 or GM-CSF. Mean receptor numbers per cell and K_d were derived from Scatchard plots (Fig. 4). < Det.: below detection level; ND: not determined; †Blast percentage of AML cells used in binding experiments.

RESULTS

Binding of ^{125}I-IL-3 and ^{125}I-GM-CSF to human WBCs and eosinophils. The binding kinetics of ^{125}I-IL-3 and ^{125}I-GM-CSF to human WBCs reveal that binding is more effective at 37°C than at 0°C (Fig. 2). After 1 hour of exposure at 37°C the amount of cell associated radioactivity began to decline. Specific binding of IL-3 and GM-CSF at 37°C was then determined for WBCs (Fig. 3a and 3b). Scatchard plots indicate relatively low numbers of IL-3 receptors (134 sites per cell) of single class affinity (K_d 113 pmol/L) on WBCs. Eosinophils expressed more receptors (1950 sites per cell) with a K_d of 494 pmol/L. Comparative IL-3 and GM-CSF receptor data for human WBCs of four donors and the patient with eosinophilia are shown in Table 2.

Binding of ^{125}I-IL-3 and ^{125}I-GM-CSF to AML cells. In 13/15 cases, receptors for IL-3 could be demonstrated on AML cells (Fig. 4a and Table 3). The average density of IL-3 receptors varied between 21 and 145 receptors per cell. The K_d ranged from 26 to 414 pmol/L, which is in the same order of magnitude as the K_d of IL-3 receptors of normal WBCs (68 to 113 pmol/L). In 11/15 cases both IL-3 receptors and GM-CSF receptors were found to be expressed on the cells (Table 3). The mean numbers of GM-CSF receptors ranged from 23 to 1263 receptors per cell (K_d 64 to 404 pmol/L). Thus the average numbers of GM-CSF receptors per cell were approximately 10 times greater than those of IL-3 receptors. GM-CSF receptor number on cells of patients with AML of FAB classification M1 or M2 tended to be less than those of patients of the M4 and M5 categories. In contrast IL-3 receptor expression on the cells was not different between AML cases of different FAB subtypes.

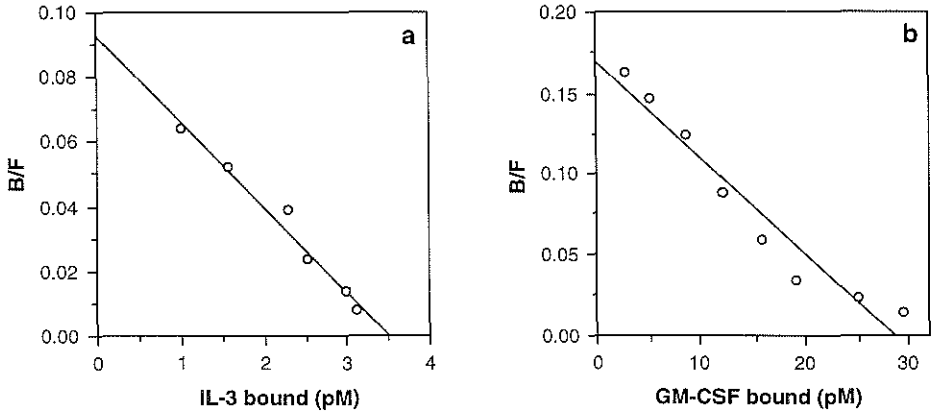

Figure 4. Scatchard plots of IL-3 (**a**) and GM-CSF (**b**) binding experiments on AML cells (case 13). For explanation see the legend to Fig. 3.

Proliferation of AML cells in vitro: Comparison with IL-3 and GM-CSF receptor expression. The stimulative effects of human IL-3 and GM-CSF on DNA synthesis of AML cells in vitro were determined and compared with the growth factor receptor data (Tables 4 and 5). IL-3 was able to increase tritiated thymidine uptake of AML cells in ten of 15 cases (Table 4). In nine of the ten IL-3 responders, receptors for IL-3 could be demonstrated, whereas in one IL-3 responder (case 4) no specific binding of IL-3 was measurable. Probably in the latter case, the cells expressed IL-3 receptors below the sensitivity of the assay. Of the five nonresponders, in four instances IL-3 receptors were demonstrated. In all GM-CSF-responsive cases (9 of 15), GM-CSF receptors were demonstrable on the cells (Table 4). Among the six nonresponders, GM-CSF receptors were not detectable in two cases, whereas significant numbers of GM-CSF receptors were demonstrated on the cells of the four remaining nonresponsive patients.

DISCUSSION

GM-CSF receptors have been demonstrated on AML cells at 44 to 1,074 sites per cell with a K_d of 17 to 290 pmol/L using glycosylated GM-CSF (15) and up to 40 sites per cell (K_d 10 to 68 pmol/L) using nonglycosylated GM-CSF (26). The expression of IL-3 receptors on AML cells has not yet been investigated. The present studies of 15 patients with AML were undertaken to assess IL-3 receptors on AML cells as well as the proliferative response of the cells to IL-3 and to relate these data to GM-CSF receptor expression and stimulability.

High affinity IL-3 receptors are apparent on the cells of most cases of AML (13 of 15). The density of the IL-3 receptors is relatively low (23 to 145 sites per cell). In one of the two IL-3 receptor negative cases (case 9) a proliferative response could be evoked by IL-3, which suggested that these cells carried IL-3 receptors at a density below the sensitivity of the assay. Apparently the number of receptors necessary to elicit a proliferative response in AML cells may be minimal. IL-3 and GM-CSF

Table 4. Relationship between IL-3/GM-CSF induced ^{3}H-TdR uptake and IL-3/GM-CSF receptor positivity of AML cells

		^{3}H-TdR uptake (dpm × 10^{-2})				^{3}H-TdR uptake (dpm × 10^{-2})			
FAB	Case	No IL-3	IL-3 200 U/mL	IL-3 responder	IL-3 receptor	No GM-CSF	GM-CSF 200 U/mL	GM-CSF responder	GM-CSF receptor
M1	1	4.5	15.1	+	+	4.5	10.2	+	+
	2	1.2	1.6	-	+	1.2	1.1	-	-
M2	3	0.7	0.8	-	+	0.7	0.5	-	-
	4	3.6	8.8	-	-	3.6	6.4	-	+
	5	31.0	90.0	+	+	31.0	91.0	+	+
M4	6	19.1	57.5	+	+	19.1	38.9	+	+
	7	11.1	20.2	+	+	11.1	23.3	+	+
	8	39.2	88.9	+	+	39.2	78.4	+	+
	9	14.5	41.5	+	-	14.5	26.3	+	+
	10	11.9	92.0	+	+	11.9	32.2	+	+
	11	53.6	54.6	-	+	53.6	55.2	-	+
	12	90.0	89.4	-	+	90.0	91.3	-	+
	13	80.8	122.4	+	+	80.8	89.0	+	+
M5	14	11.4	26.9	+	+	11.4	13.0	+	+
	15	3.3	17.4	+	+	3.3	6.8	-	+

^{3}H-TdR incorporation by AML cells was determined in triplicate cultures with or without IL-3/GM-CSF. When mean dpm of stimulated cultures were significantly greater than the values of unstimulated control cultures, IL-3 or GM-CSF responses were considered positive (+). IL-3 or GM-CSF receptor positivity based upon data of Table 3.

receptors were often coexpressed on the cells of the same cases of AML. This observation is of interest since apparent similarities exist between the spectrum of stimulative abilities of these two growth factors (27).

Notably, density as well as affinity of the IL-3 receptors of AML cells are in the same order as those of normal human WBC; K_d and mean numbers of IL-3 receptors per cell did not vary as a function of the cytological classification of the AML cells. In contrast, the more immature AML types expressed fewer GM-CSF receptors than those of M4 and M5 cases as well as those of WBCs. Although the limited number of cases do not permit firm conclusions regarding the relationship of receptor density and FAB subtype of AML, GM-CSF receptor density may increases as a function of progressive maturation, a phenomenon previously reported by others as well (15). In the one case with hypereosinophilia, IL-3 receptors were expressed at higher levels (2208 sites per cell). This observation is compatible with autoradiography data in mice indicating that eosinophils express high numbers of IL-3 receptors (28).

Demonstration of IL-3 receptors did not always appear to be predictive of the proliferative response of the cells to IL-3 (Table 4). In five patients, DNA synthesis could not be induced with IL-3. Because in one nonresponder IL-3 receptors were not demonstrated, a true absence of IL-3 receptors may have determined the lack of response. The cells of the other four nonresponders however, expressed average

numbers of IL-3 receptors, comparable to those of the responding cases of AML. Thus in those 4 patients IL-3 was unable of inducing DNA synthesis although IL-3 receptors were available on the cells at normal values. The same phenomenon was observed for GM-CSF, i.e., in four of the six patients whose cells did not respond to GM-CSF (cases 4, 11, 12 and 15), we could demonstrate GM-CSF receptors on the cells. The explanation for this discrepancy between positive growth factor receptor expression and negative response is elusive. These cells may already have been activated by autocrine CSF production, obscuring a measurable IL-3 or GM-CSF response. Indeed, in two nonresponders expressing IL-3 as well as GM-CSF receptors (cases 11 and 12) the cells showed high spontaneous activity. In neither case could the spontaneous proliferation be blocked by neutralizing anti-IL-3 or anti-GM-CSF antibodies. Therefore, these experiments did not provide evidence for endogenous IL-3 or GM-CSF stimulation, although we cannot exclude that growth of these cells due to autocrine stimulation by IL-3/GM-CSF inside the cells had masked exogenous stimulation by IL-3 or GM-CSF. However, in certain other cases IL-3 (cases 2 and 3) and GM-CSF nonresponders (cases 4 and 15) with positive IL-3/GM-CSF receptor expression, the cells did not proliferate spontaneously. Why AML cells of those patients expressed IL-3 or GM-CSF receptors but were unable of responding to these factors is thus unclear. The cells of these patients may have been deficient beyond the level of receptor binding, which would not allow them to elicit a normal proliferative response. Investigations focusing on signal transduction after stimulation with CSFs may be necessary to clarify this question.

CHAPTER 3

GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTORS IN HUMAN ACUTE MYELOID LEUKEMIA

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SUMMARY

The binding of granulocyte colony-stimulating factor (G-CSF) to normal and human acute myeloid leukemia (AML) cells was investigated using radiolabeled recombinant human G-CSF. In all 14 cases of primary AML specific receptors for G-CSF were demonstrated on purified blast cells. The average numbers of G-CSF receptors ranged from very low to 428 receptors per cell (mean). Normal granulocytes showed G-CSF binding sites on their surface at higher densities (703 to 1296 sites per cell). G-CSF receptors appeared of a single affinity type with a dissociation constant (K_d) ranging between 214 to 378 pmol/L for AML blasts and 405 to 648 pmol/L for granulocytes. In 12 of 14 cases including those with relatively low specific binding, G-CSF was a potent inducer of DNA synthesis of blasts in vitro; therefore, apparently relatively few receptors are required to permit activation of AML cell growth. However, in two cases cell cycling was not activated in response to G-CSF despite G-CSF receptor availability. The results show that G-CSF receptors of high affinity are frequently expressed on the blasts of human AML, but their presence may not be a strict indicator of the proliferative responsiveness of the cells to G-CSF.

INTRODUCTION

Colony stimulating factors (CSFs) are essential for the proliferation, differentiation and survival of hematopoietic cells (1,2,3,4). Growth of human acute myeloblastic leukemia (AML) cells in vitro depends also on CSFs but a great variability in response to IL-3, GM-CSF, M-CSF and G-CSF has been observed among different cases (5,6,7,8,9). A major function of G-CSF is its ability to induce neutrophil colonies from normal bone marrow (BM) progenitors (10). G-CSF may also induce maturation of myeloid leukemia (8,10,11). Radiolabeled murine G-CSF binds specifically to receptors on normal and leukemic human myeloid cells and also stimulates functional activities in such cells (12,13,14). Analysis of extended series of AML cases showed that the blasts from a significant proportion of patients are nonresponsive to stimulation by G-CSF (8,9). The inability of the cells to respond to stimulation might be caused by lack of receptors on the membrane or inability of the receptors to transduce a secondary signal into the cells. To gain insight into the action of G-CSF and the mechanisms that lead to proliferation of AML cells in response to this factor, we determined numbers and affinity of G-CSF receptors on blast cells of patients with AML as well as on peripheral blood (PB) granulocytes of healthy individuals and compared these data with the proliferative reactivity of the AML cells to G-CSF in vitro.

MATERIALS AND METHODS

Patients and preparation of AML cells, normal BM cells and granulocytes. AML was diagnosed according to the criteria of the French-American-British (FAB) committee (15,16) (Table 3). BM (all cases except cases 4 and 9) or PB (4 and 9) was taken from the patients at diagnosis before any treatment was administered in all 14 cases. The percentage of blasts in BM specimens before purification is given in Table 3. The leukemic cells were separated from BM or PB after bovine serum albumin

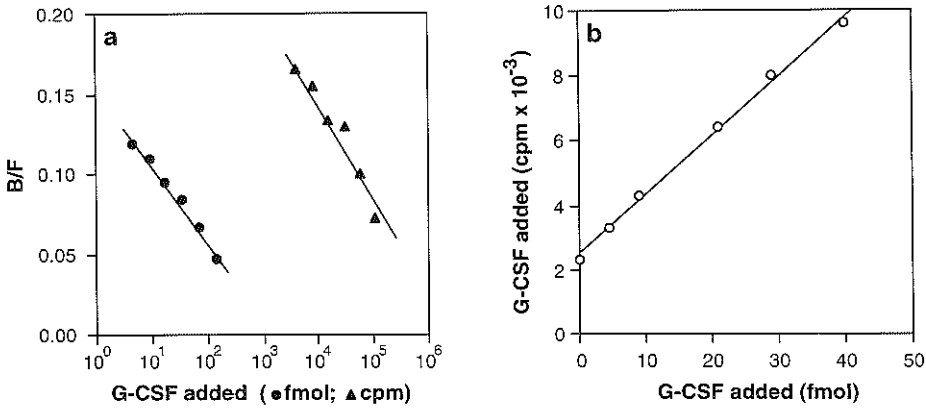

Figure 1. Measurement of specific radioactivity of radiolabeled G-CSF by self displacement. (**a**) Bound/free (B/F) ratio's were determined for titrated concentrations of radiolabeled G-CSF (triangles) versus titration of nonlabeled G-CSF in the presence of 350 pmol/L ^{125}I-G-CSF (circles). Binding of radiolabeled G-CSF paralleled that of radiolabeled G-CSF displaced by nonlabeled G-CSF. (**b**) Total radioactivity added was plotted against the corresponding quantity of unlabeled G-CSF to derive the specific radioactivity. The slope indicates the specific radioactivity of the labeled G-CSF.

density gradient (BSA) or Ficoll-Isopaque centrifugation and subsequent removal of E-rosette forming cells (17,18). In addition, AML cell preparations used in the binding experiments, the proliferative assays, and the colony assay were also depleted from monocytic cells after plastic adherence (19). This resulted in cell preparations with a purity of blasts determined by morphology that was always more than 98%. The viability of the cells after thawing was always more than 95%. Normal BM was obtained by posterior iliac crest puncture from hematologically normal adults and subsequently separated over Ficoll-Isopaque density gradient (20) to isolate the mononuclear cell fraction. White blood cells (WBCs) were separated from the blood of healthy subjects following sedimentation in 0.1% methyl cellulose. Granulocytes were obtained as the sedimented cell fraction following Ficoll-Isopaque centrifugation of WBCs and consisted of 90 to 95% neutrophils, 2 to 6% eosinophils and 3 to 5% monocytes/lymphocytes.

G-CSF and radioiodination. Recombinant human G-CSF (rhG-CSF)(E. Coli derived, MW 18,800 Da) was prepared and purified at Amgen (Thousand Oaks, CA) (10). Purified rhG-CSF was radiolabeled according to the method described by Bolton and Hunter (21). Five hundred μCi of Bolton-Hunter reagent (Amersham laboratories, Amersham, UK) dissolved in benzene was dried down under a stream of N_2 in the packing vial at 22°C. The vial was then cooled down to 0°C and 3 μg rhG-CSF in 15 μl borate buffer (0.1 mol/L, pH 8.2) was applied. The reaction was permitted to proceed for 60 minutes on ice and was quenched by adding 100 μl of glycine (0.2 mol/L in borate buffer). After 5 minutes on ice 100 μl gelatin (0.25 %) was added to aid recovery of the reactant from the vial. The labeled rhG-CSF was separated from the reactant by chromatography on a sepharose column (PIERCE GF5) equilibrated with phosphate buffered saline (PBS) containing 0.1% gelatine and 0.01% Tween 20. Radiolabeling of rhG-CSF resulted in a protein that retained

Table 1. Colony stimulating activity of iodinated G-CSF

G-CSF concentration (pmol/L)	No. of Colonies and Clusters		
	G-CSF 100 %	Mixture 50/50 %	^{125}I-G-CSF 100 %
3	0/0	0/0	0/0
30	5/25	6/39	11/53
300	51/41	78/55	70/48

Colonies and clusters induced by titrated concentrations (3, 30, 300 pmol/L) of native G-CSF, radiolabeled G-CSF and a 50/50% (wt/wt) mixture of G-CSF/^{125}I-G-CSF from 0.5 x 10^5 Ficoll-separated BM cells. Values are the average number of colonies (before the "/") and clusters (after the "/") of duplicate cultures.

biological activity (Table 1), had a specific binding capacity of 50 to 60% and was 85 to 90% trichloroacetic acid insoluble. ^{125}I-G-CSF was stored at 4°C in 20% glycerol and 0.2% sodium azide and used within four weeks of preparation.

Specific radioactivity of ^{125}I-G-CSF. Self displacement (22) was applied to determine the specific activity of G-CSF following labeling (Fig. 1). The parallel displacement curves that were obtained (Fig. 1a) show that the affinities of the radiolabeled and native molecules for the G-CSF receptors of granulocytes were similar and confirm that labeling had not altered the binding characteristics of G-CSF. From the slope of the line (Fig. 1b) the specific radioactivity was estimated at 9.5 $\times$ 10^4 cpm/ng. This specific activity was used to determine the concentration of labeled G-CSF used in the binding experiments.

Binding of labeled G-CSF to AML blasts and granulocytes. To remove any G-CSF that might have remained present on the membrane, the blasts were washed in sodium citrate (pH 4) (23). Before and after this acid wash the cells were washed twice in Hanks' Balanced Salt Solution (HBSS). Two to 4 $\times$ 10^6 cells were incubated for 1 hour at 37°C in 100 µl α-Minimal Essential Medium with 10% FCS in the presence of 10 to 1,200 pmol/L radiolabeled G-CSF with or without excess (i.e., 100 nmol/L) nonlabeled G-CSF. The cells were then cooled down to 0°C, layered over 500 µl calf serum in Eppendorf tubes on ice, and centrifuged for 5 minutes at 1,000 g. The tubes were snapfrozen in liquid nitrogen, and the pellet cut off for counting in a gamma-counter (Packard, Downers Grove, USA). Specific binding was determined as the difference between the amount of radioactivity bound without unlabeled G-CSF and the amount of radioactivity bound with excess unlabeled G-CSF. Experiments were conducted in duplicate. Receptor numbers and binding affinity were derived after analysis according to Scatchard (24). In calculations the maximal binding capacity was used to correct for the free counts.

Colony cultures. To estimate whether the biological activity of radiolabeled G-CSF had been retained normal BM cells (0.5 $\times$ 10^5) were cultured in ca 1 mL mixture of Iscove's modified Dulbecco's medium (IMDM), 0.8% methyl cellulose, 30% FCS, BSA, transferrin, lecithin, sodium-selenite and beta-mercaptoethanol (20). Nonlabeled, labeled and a 50/50% mixture of nonlabeled and labeled G-CSF were added to the cultures in titrated concentrations. Clusters of more than 15 cells and colonies of more than 50 cells were scored at day 14. Each estimate is based on the

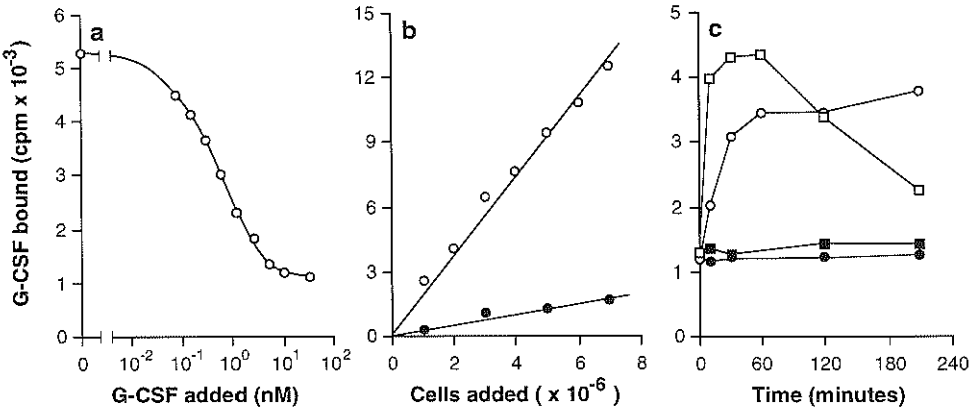

Figure 2. Binding of radioiodinated G-CSF to normal granulocytes. (**a**) Displacement of bound radioiodinated G-CSF (100 pmol/L; 100 μl per tube) by increasing amounts of unlabeled G-CSF. (**b**) relationship between cell number and binding of radioiodinated G-CSF (50 pmol/L; 500 μl per tube) to granulocytes. Increasing numbers of cells were incubated with labeled G-CSF with (closed circles) or without (open circles) excess unlabeled G-CSF. (**c**) Binding of radiolabeled G-CSF to granulocytes as a function of time: 4×10^6 granulocytes were incubated with 300 pmol/L radiolabeled G-CSF at 0 °C (circles) and 37 °C (squares). Total binding (open symbols) as well as nonspecific binding (solid symbols) were assessed at various times of incubation. Values are means of duplicate estimations.

data of duplicate cultures. The effect of G-CSF on AML colony formation was also determined. In these experiments 1×10^5 blasts were plated per dish containing 1 mL of serum-free medium in 0.9% methyl cellulose with and without addition of G-CSF (9). After 14 days clusters of more than 15 cells and colonies of more than 50 cells were scored (Table 3). Each estimate is based on the data of duplicate cultures.

^{3}H-thymidine incorporation into AML blasts. Two $\times 10^4$ blasts were cultured for 72 hours in serum-free medium with or without the G-CSF (9). DNA synthesis of AML blasts was measured as described (9). All experiments were performed in triplicate, data were expressed as mean dpm, and differences of DNA synthesis between stimulated and nonstimulated cultures analyzed for significance ($P>0.95$; Student's *t* test).

RESULTS

Radioiodination of G-CSF. Radiolabeled G-CSF had retained the ability to stimulate colony formation from normal BM cells in methyl cellulose (Table 1). Fig. 2a shows that competition of nonlabeled G-CSF with binding of radiolabeled G-CSF to granulocytes was dose dependent. Both specific and nonspecific binding of radiolabeled G-CSF to granulocytes using excess ligand appeared linear with cell numbers up to 5×10^6 cells and the linear functions extrapolated through zero (Fig. 2b). This was indicative that binding of radiolabeled G-CSF as measured was cell-associated and independent of cell number. Self displacement analysis demonstrated similar affinities of labeled and unlabeled G-CSF for granulocytes (Fig. 1). From these

Table 2. Binding of ^{125}I-G-CSF to granulocytes

Donor	G-CSF receptors	
	Sites per cell	K_d (pmol/L)
1	703	648
2	1,019	405
3	955	492
4	1,066	481
5	1,296	586

Receptor numbers (mean per cell) and K_d were derived from binding experiments and subsequent analysis according to Scatchard (described in legend to Fig. 3). Granulocytes were obtained from 5 different normal donors.

data it was apparent that the ^{125}I-G-CSF preparation permitted quantitative receptor binding studies.

Binding of radiolabeled G-CSF to granulocytes. Binding of ^{125}I-G-CSF to human granulocytes appeared time and temperature dependent (Fig. 2c). G-CSF was more efficiently bound at 37°C than at 0°C. After 1 hour of exposure of the cells to G-CSF at 37°C, the amount of cell associated radioactivity began to decrease. Nonspecific binding (radioactivity bound in the presence of excess unlabeled competitor) was virtually independent of time or temperature. When granulocytes were incubated with increasing concentrations of ^{125}I-G-CSF (20 to 1,250 pmol/L), binding appeared saturable, while nonspecific binding increased linearly with increasing concentrations of labeled rhG-CSF and varied with cell type and cell number from 0.4 to 1.8 % of total input cpm (Fig. 3a). The Scatchard plot of the binding data revealed an average of 1,019 G-CSF sites per cell of high affinity (K_d 405 pmol/L) (Fig. 3a). G-CSF receptor analysis of granulocytes obtained from the blood of 5 different donors showed limited variations as regards receptor densities (703 to 1296 sites per cell) and receptor affinity for G-CSF (K_d 405 to 648 pmol/L) (Table 2).

Binding of radiolabeled G-CSF to AML blasts. Binding of ^{125}I-G-CSF to AML blasts was examined in 14 cases of AML. In all cases specific binding of ^{125}I-G-CSF was measurable (Fig. 3b, Table 3). In five of these cases, binding was too low to permit complete Scatchard analysis. In the other 9 cases Scatchard analysis revealed comparatively low numbers of G-CSF receptors (49 to 428 sites per cell). The apparent dissociation constant ranged between 214 and 373 pmol/L (mean 299 ± 53 pmol/L ± SD), which is similar to the K_d of G-CSF receptors on granulocytes (mean 522 ± 95 pmol/L ± SD). On the average, the cases of M4 cytology expressed greater numbers of G-CSF receptors (mean 274 ± 129 sites per cell ± SD) than did M1 and M2 cases (positive receptor expression but no Scatchard analysis possible in cases 2, 4, 5 and 7; mean 84 ± 31 sites per cell ± SD in the remaining three cases). However, the small number of cases tested do not allow any conclusions regarding the relationship between cytology and receptor expression.

Proliferation of AML blasts in vitro: comparison with receptor data. Although thymidine uptake generally provides a more sensitive assay of proliferative activity of AML cells than the colony assay(9), we also determined the in vitro response of the cells in colony culture. In five cases (cases 7, 10, 11, 12 and 14), a significant

Table 3. Relationship between G-CSF receptor positivity of AML blasts and G-CSF stimulability of ^{3}H-TdR uptake by AML cells

	Case no.	Blast (%)*	G-CSF receptors		Colonies and clusters		^{3}H-TdR uptake (dpm × 10^{-2})		resp.
			Sites/cell	K_d (pM)	No G-CSF	With G-CSF	No G-CSF	With G-CSF	
M1	1	96	108	263	0/0	0/0	9.9	23.5	+
	2	87	§	ND	0/0	0/0	6.9	15.7	+
M2	3	93	94	309	0/0	0/0	1.8	15.7	+
	4	91	§	ND	0/0	0/0	3.6	14.9	+
	5	58	§	ND	0/5	0/20	0.9	4.6	-
	6	87	49	214	0/0	0/0	0.7	1.8	-
	7	86	§	ND	89/ND	399/ND	7.1	25.2	+
M4	8	78	406	284	0/46	0/392	73.2	260.2	+
	9	69	192	373	0/0	0/25	6.7	61.0	+
	10	49	203	378	195/500	296/500	53.7	118.3	+
	11	75	102	276	0/112	436/500	67.4	124.2	+
	12	76	428	274	74/500	500/500	31.0	221.0	+
	13	47	310	324	175/500	195/500	86.4	162.1	+
M5	14	28	§	ND	0/5	5/19	4.5	9.4	+

G-CSF receptor numbers per cell and K_d were derived from Scatchard plot analysis (see Fig. 3). Colony and cluster formation was scored after 2 weeks. Values are the average number of colonies (before "/") and clusters (after "/") per 10^5 cells from duplicate experiments; Colony or cluster numbers higher than 500 are marked 500. Stimulation of ^{3}H-TdR incorporation by AML blasts was determined in cultures with 300 pmol/L G-CSF. When mean dpm of stimulated cultures were significantly greater than the values of unstimulated control cultures, G-CSF responses (last column) were considered positive (+). *percentage of blasts in marrow at diagnosis (see materials and methods). §: specific binding detectable (total binding exceeds nonspecific binding) but complete Scatchard analysis was not performed; in these instances the K_d was not determined (ND).

increase in colony numbers was seen when G-CSF had been added (Table 3). The stimulative effects of rhG-CSF on DNA synthesis of AML cells was determined in serum-free culture and the response of the cells compared with growth factor receptor expression (Table 3). A significant increase of tritiated thymidine uptake was seen in most cases (12 of 14). In 2 cases (cases 5 and 6), DNA synthesis could not be activated although G-CSF receptors were clearly identified on the cells. Receptor density and affinity of the cells from the two nonresponders (case 5, positive specific binding; case 6, 49 sites per cell, K_d 214 pmol/L) were comparable to those of the responding cases.

DISCUSSION

G-CSF receptors were demonstrated on the blast cells of all 14 patients with AML included in this study. In 5 of 14 cases, estimation of G-CSF receptor numbers was not possible owing to low binding, although significant specific binding was evident. In the other nine cases the estimated receptor density ranged between 49 and 428 sites per cell. That the cells from most cases, including 3 of the five patients with low

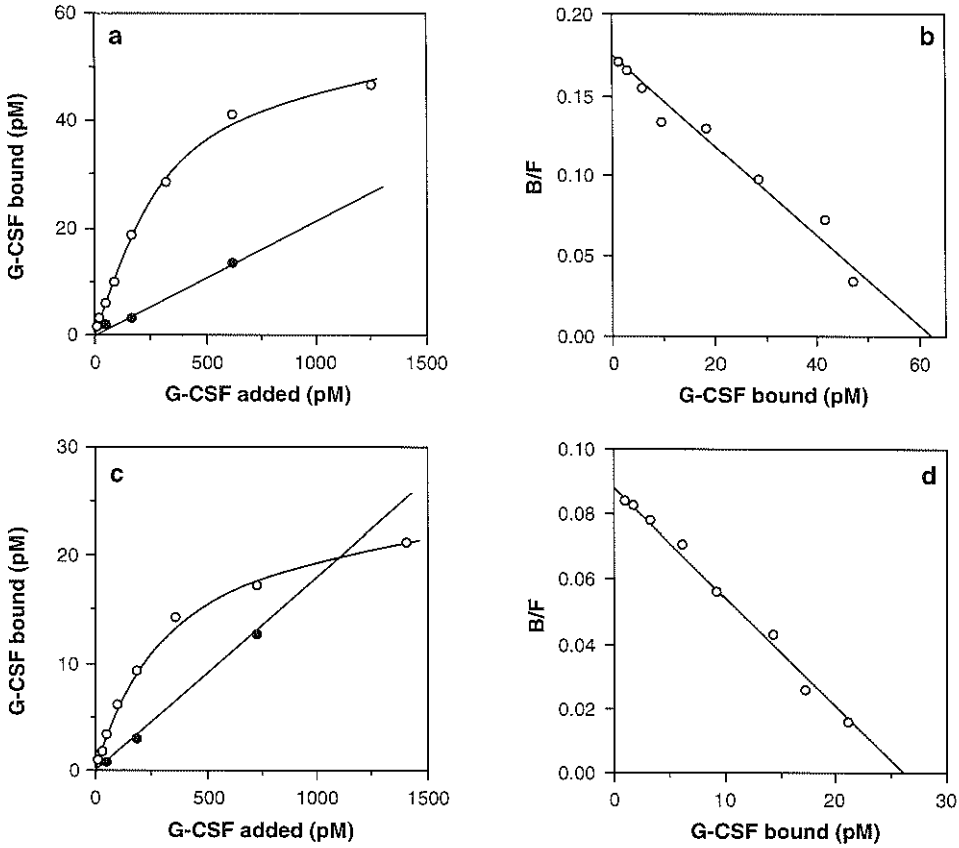

Figure 3. Binding of radiolabeled G-CSF to granulocytes (**a**) and AML blasts (**c**) and Scatchard plots of these data (**b** and **d**). Four × 10^6 granulocytes (donor 2, Table 2) or blasts (AML case 8, Table 3) were incubated with increasing amounts of radiolabeled G-CSF for 1 hour at 37°C. Nonspecific binding was determined in the presence of excess unlabeled G-CSF. Specific binding (open circles) and nonspecific binding (closed circles) data are plotted in the left panels. Each point represents the mean of two estimates. B/F indicates bound/free ratio.

specific binding, showed increased DNA synthesis upon stimulation with G-CSF established that only minimal numbers of receptors per cell are required to evoke a proliferative response to G-CSF. G-CSF receptors demonstrated on the (immature) AML blasts were all of high affinity with a dissociation constant (K_d 212 to 378 pmol/L) which was slightly less than that of G-CSF receptors on granulocytes (K_d 405 to 648 pmol/L). How these properties compare to those of normal immature progenitor cells remains unresolved as long as it is impossible to analyze great numbers of purified marrow precursors. On the other hand, the density of G-CSF receptors on granulocytes (703 to 1296 sites per cell) appeared greater than that on AML blasts. A similar phenomenon (ie, more receptors on granulocytes than on AML blasts), has previously been reported for the GM-CSF receptor (25,26).

We have shown here as well as in a previous study (9) that ^{3}H-TdR uptake provides a more sensitive assay for the mitogenic response of AML cells than the

colony assay. Indeed, in several cases, a significant increase in DNA synthesis was observed, whereas no effect could be demonstrated in the colony assay. Therefore, we have related the receptor data to ^{3}H-TdR uptake as parameter of response (Table 3, last column). G-CSF was able to stimulate DNA synthesis of the blasts from most cases (Table 3). The expression of G-CSF receptors did not unequivocally predict whether these cells would enter active cell cycling in response to G-CSF. In cases 5 and 6, G-CSF was incapable of inducing DNA synthesis, although the blasts of these patients clearly expressed G-CSF receptors on their membranes. The unresponsiveness of AML cells to CSF in spite of receptor availability has also been reported for IL-3 and GM-CSF (26). Why AML cells of some patients express G-CSF receptors, but cannot respond to stimulation by the ligand is unclear. Although in 12 of 14 cases the 72 hour period proved sufficient to detect an increase of DNA synthesis, in the remaining two cases an undetectable response of only a small subpopulation of cells may have occurred. The receptors of the cells of these patients similar to those of granulocytes may be functional for other purposes so that they cannot elicit proliferation. Investigations along the lines of signal transduction may clarify mechanisms that determine the inability of the cells of cases of AML to proliferate in reaction to G-CSF stimulation.

CHAPTER 4

EFFECTS OF KIT LIGAND ON ACUTE MYELOID LEUKEMIA CELLS IN VITRO: EFFECTS OF COMBINATIONS WITH OTHER CYTOKINES.

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SUMMARY

We have investigated the stimulative effects of kit ligand (KL) in primary AML in vitro. KL stimulated DNA synthesis of purified leukemic blasts in 8 of 10 cases and colony formation in 4 cases in serum-free (SF) culture. KL synergized with Interleukin-3 (IL-3; 4 of 10 cases), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF; 3 of 10 cases), Granulocyte-Colony Stimulating Factor (G-CSF; 6 of 10 cases), Macrophage-Colony Stimulating Factor (M-CSF; 1 of 10 cases) and Erythropoietin (EPO; 1 of 10 cases) in its effects on AML cell proliferation when added to culture in combination. Synergistic effects of KL in combination with other CSFs were also seen in the colony assay. Antibodies against GM-CSF, M-CSF, G-CSF and IL-6 did not inhibit the KL response, suggesting that the stimulative effect of KL was not mediated through autocrine release of those cytokines. In contrast, the proliferative response to KL was abrogated in the presence of Tumor Necrosis Factor (TNF; 4 of 10 cases) and Interleukin-4 (IL-4; 2 of 10 cases). FACS analysis with anti c-kit antibodies revealed KL receptor expression in 8 of 9 cases, often in a subpopulation of the cells. Scatchard analysis of KL receptors in two cases indicated the presence of (mean) 1,460 and 41,500 binding sites respectively. In comparison the leukemia cell line MO7e expressed more than 120,000 KL binding sites per cell. AML cells as well as the MO7e cell, expressed one class of high affinity KL receptors (K_d 40 to 160 pmol/L). The KL dose-response curve in the presence of IL-3 or GM-CSF resulted in a higher plateau of DNA synthesis, however no shift in the dose response was apparent. The respective reciprocal dose response relations to GM-CSF, IL-3 or G-CSF were similarly elevated when KL was added. KL did not alter IL-3 and GM-CSF receptor expression, nor did IL-3, GM-CSF, G-CSF, TNF or IL-4 influence KL binding to AML cells. Hence, we conclude that (a) KL directly stimulates AML blast cell proliferation, (b) KL has profound enhancing effects on AML blast cell growth induced by other cytokines, (c) costimulation by dual factors may trigger additional subsets of AML cells that do not respond to the individual factors, (d) synergy between KL and IL-3, GM-CSF or G-CSF and antagonism between KL and TNF or IL-4 are not the consequence of altered sensitivity to HGF stimulation nor changes of the status of membrane receptors.

INTRODUCTION

A variety of hematopoietic growth factors (HGF) are involved in the regulation of normal steady state hematopoiesis (1,2,3,4). The proto-oncogene c-kit, which encodes a membrane receptor in hematopoietic cells, plays an important role in stem cell development (5-10). Kit ligand (KL), steel factor (SLF), mast cell growth factor (MGF), or stem cell factor (SCF) has recently been cloned and identified as the ligand for c-kit (5-10). KL stimulates mast cell growth, but also stimulates proliferation of normal and leukemic progenitor cells (11-14). In addition, enhancing effects of KL have been observed in myelopoiesis, lymphopoiesis and erythropoiesis in combination with other cytokines (15-18).

Like in normal hematopoiesis, the regulation of AML cell growth is under the control of multiple GFs (19-23). In almost all cases the blast cells have retained their ability to respond to HGFs, while in some cases AML cells have become factor independent through autocrine or paracrine mechanisms involving CSFs (24-27). IL-

3, GM-CSF and G-CSF are the most important hematopoietins for in vitro growth of primary AML (19-23). To a lesser extent are M-CSF, IL-6 and EPO active as exogenous stimulators of AML blast cell DNA synthesis (22,23,28). IL-1 and also TNF are able to stimulate AML growth via induction of endogenous HGF release through activation of transcription or stabilization of HGF mRNA in AML cells (29,30).

In order to establish the role of KL in the regulation of AML growth in vitro, we have conducted studies to determine the effects of KL on AML cell proliferation alone and in combination with other cytokines. We have investigated whether synergistic or inhibitory effects of individual cytokines in combination with KL were achieved at the level of KL, IL-3 or GM-CSF receptor modulation.

MATERIALS AND METHODS

Patients and preparation of AML cells. Cells obtained from 10 cases of primary AML were diagnosed according to the criteria of the French-American-British Committee (FAB) (31,32) as M1 (cases 1 to 3), M2 (cases 8 and 10), M4 (cases 4 and 7) and M5 (cases 6 and 9). Bone marrow was obtained after permission. The cells were recovered from the interface following Ficoll-Hypaque density gradient centrifugation, depleted from T cells by E-rosetting and cryopreserved (33,34). After thawing, monocytes were depleted by plastic adherence (23). Cells were cultured in suspension or colony assay in serum-free (SF) medium (23).

Growth factors and neutralizing antibodies. The following purified recombinant growth factor preparations were used: IL-3 (100 U/mL; Gist Brocades, Delft, The Netherlands), GM-CSF and IL-4 (200 U/mL & 100 U/mL; Genetics Institute, Cambridge, MA), M-CSF (2000 U/mL; Cetus Corporation, Emmeryville, CA), G-CSF and KL (10 ng/mL & 25 ng/mL; Immunex Seattle, WA), TNFα (1000 U/mL; Boehringer Institute, Vienna, Austria) and used at the indicated concentrations unless specified otherwise. Polyclonal rabbit anti IL-3, sheep anti GM-CSF (both from Genetics Institute), rabbit anti G-CSF (from Immunex), rabbit anti IL-6 (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and monoclonal anti M-CSF (Cetus) were used in dilutions that completely blocked the effects of optimal concentrations of the corresponding growth factors (35).

^{3}H-Thymidine (^{3}H-TdR) incorporation. DNA synthesis of AML cells was measured in 96 well microtiter plates as described (19,23). The results of triplicate experiments of 2×10^4 cells per well are expressed as means ± standard deviation (SD). Preirradiated (30 Gy) AML cells were always run in parallel to assess background thymidine incorporation. Statistical analysis to determine significant differences ($P<0.05$) to identify additive or synergistic effects, was performed with the Student's *t* test (StatWorks, Cricket Software, Philadelphia, PA).

AML clonogenic assay. AML colony forming units (CFU) were measured in 8 of the 10 cases. In these experiments 1×10^5 cells were plated in 1 mL SF medium that contained 0.9% methyl cellulose, and cultured in humidified 5% CO_2 atmosphere at 37°C in the presence of indicated cytokines (19). All CFUs that contained more than 15 cells were scored. Presented data refer to myeloid colony formation, unless stated

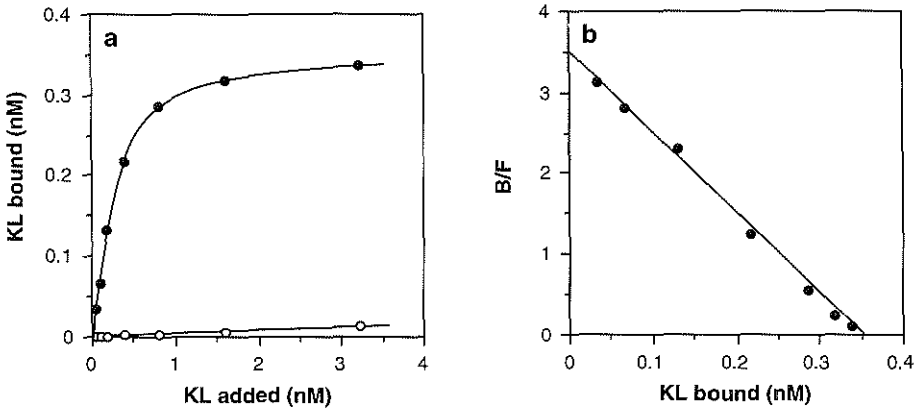

Figure 1. KL receptors on AML cells. AML cells (5×10^5 cells/tube; case 8) were incubated with radiolabeled KL (50-3200 pmol/L) in the absence (closed circles) or presence of excess KL (100 nmol/L; open circles). Inset shows Scatchard transformation of binding data after correction for nonspecific binding. All data represent means of duplicate estimations.

other wise. The values indicate means of duplicate cultures at day 10-14. Colony growth from 1×10^5 cells from AML suspension cultures was assessed in the presence of IL-3, GM-CSF, G-CSF and EPO at day 14.

Fluorescence labeling of the cells. Cells were labeled with the monoclonal antibodies (CD14, CD15, CD33, CD34, CDw65 and anti-c-kit, a kind gift from Dr. H-J Bühring, Univ. of Tübingen, Germany (36)) and with a second step reagent GAM-FITC (Nordic, Tilburg, The Netherlands) as described (37). Fluorescence was measured on a FACS 440 (Becton Dickinson, Mountain View, CA). Cell labeling studies were performed after thawing of the cells and after 7 days of culture in the presence of no factor, KL, IL-3 and G-CSF.

Radiolabeling of cytokines and binding experiments. Purified recombinant GM-CSF, IL-3 and KL were labeled with iodine 125 using the Bolton and Hunter technique as described (38). In brief 0.25×10^6 cells were incubated for 1 hour at 22° C in 100 µL α-Minimal Essential Medium with 1% BSA in the presence of titrated concentrations of radiolabeled KL with or without excess (i.e., 100 nmol/L) nonlabeled KL. GM-CSF and IL-3 receptors were measured in the presence of 400 pmol/L labeled factor. Specific binding was defined as the difference between the amount of radioactivity bound in the absence of nonlabeled factor, and the amount of radioactivity bound in the presence of excess nonlabeled factor. Binding experiments were conducted in triplicate or duplicate (KL Scatchard analysis). Receptor numbers and binding affinities were derived following Scatchard analysis (39), using the ENZfitter computer program (Sigma Chemical co, st. Louis, MO).

Table 1. c-kit expression in AML cells and MO 7e cells

case	FAB	c-kit expression (FACS analysis)	^{125}I-KL binding R (sites/cell)	^{125}I-KL binding K_d (pmol/L)
1	M1	15 %		
2	M1	20 %		
3	M2	5 %		
4	M4	1 %	1,460 ± 30	160 ± 11
5	M4	25 ch.		
6	M5	negative		
7	M4	ND		
8	M2	30 ch.	41,500 ± 1,320	98 ± 3.2
9	M5	2 %		
10	M2	15 ch.		
MO7e			122,000 ± 4,800	41 ± 4.5

c-kit expression on AML cells was determined by FACS analysis and by KL radiolabeling studies. Values indicate the percentage of AML cells contained in the c-kit positive subpopulation, or the peak shift (expressed as the numbers of channels, 17 channels peakshift is equivalent to one log more fluorescence) after c-kit/GAM-FITC labeling. Mean numbers of binding sites per cell and affinity for KL were derived from Scatchard analysis. ND: not determined.

RESULTS

Expression of c-kit on AML cells. KL receptors were studied with anti c-kit antibodies and radiolabeled KL. FACS analysis revealed variable positive expression of c-kit in eight of 9 cases (Table 1). Case 6 did not express detectable levels of c-kit. This case showed a clear response to KL in combination with G-CSF or M-CSF in the clonogenic assay, indicating that this case also expressed c-kit, but below the level of fluorescence detection. The fluorescence histograms revealed in 5 cases a c-kit positive subpopulation of 1-20% of AML cells. In the three remaining cases a peak shift indicated expression of c-kit on the majority of cells. Scatchard analysis of KL binding data of two cases with relatively low c-kit fluorescence (case 4) and high c-kit fluorescence (case 8) revealed the presence of 1,460 and 41,500 binding sites respectively (Table 1, Fig. 1). In comparison, the leukemia cell line MO7e expressed more than 120,000 KL binding sites per cell. The low mean number of KL binding sites on AML cells, as compared to MO7e, probably reflects the fact that only a subpopulation of cells were positive for c-kit. AML cells from cases 4 and 8, as well as the MO7e cell, expressed a single class of high affinity KL receptors (K_d 40-160 pmol/L).

Response of AML blast cells: effect of KL in ^{3}H-TdR and colony assay. The optimal concentration of KL to induce DNA synthesis was tested in two cases. Half maximal effects were reached at 5-6 ng/mL KL and a plateau was reached at 20 ng/mL. Based on these data further experiments were performed with 25 ng/mL. The stimulative effects of KL on the DNA synthesis of ten AML cases were assessed in comparison with nonsupplemented cultures (Table 2). In most cases AML cells show some baseline DNA synthesis as compared to preirradiated controls. In 8 cases, a significant increase of DNA synthesis was observed in culture following stimulation

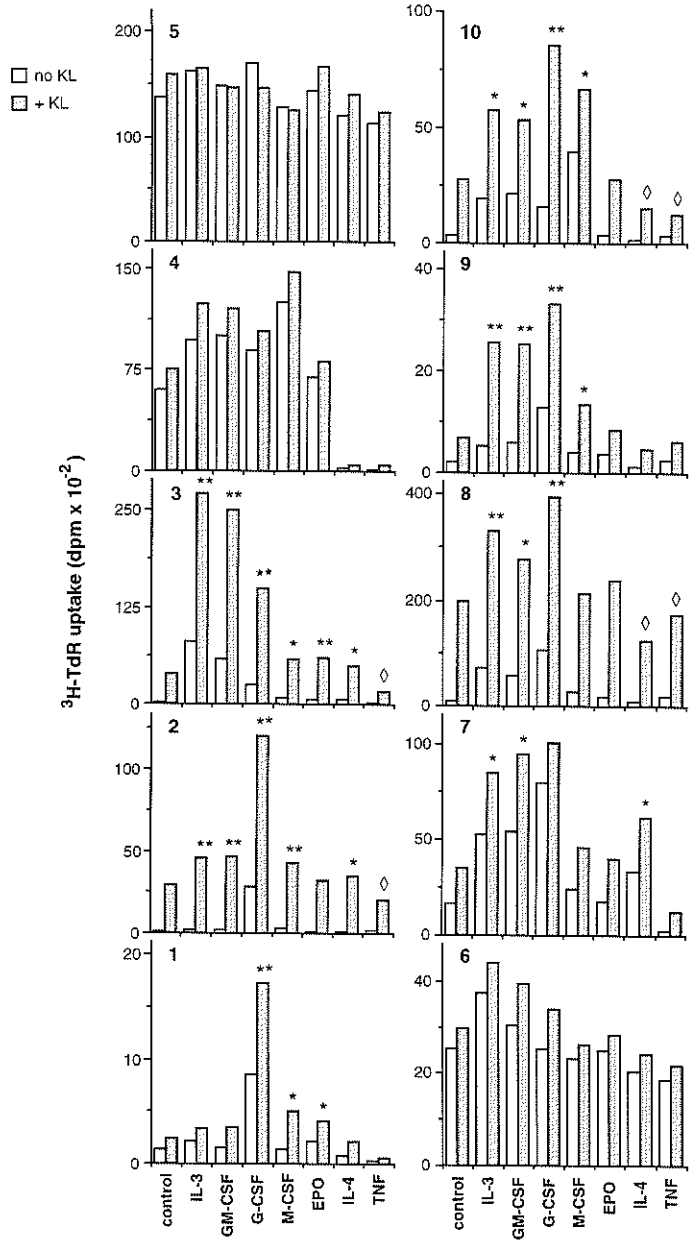

Figure 2. The effects of KL in combination with other cytokines on ^{3}H-TdR uptake by AML cells. ^{3}H-TdR uptake by AML cells from 10 cases was determined in cultures with the individual recombinant factors (IL-3, GM-CSF, M-CSF, EPO, IL-4 and TNFα) or KL in combination with these factors as a measure of proliferation. Values plotted represent the mean of triplicate estimations. * indicates additive action of GFs; ** indicates synergistic action of GFs; ◇ indicates inhibition of KL response. Student's *t* test was used to determine significance of additive, synergistic or inhibitory effects ($P<0.05$).

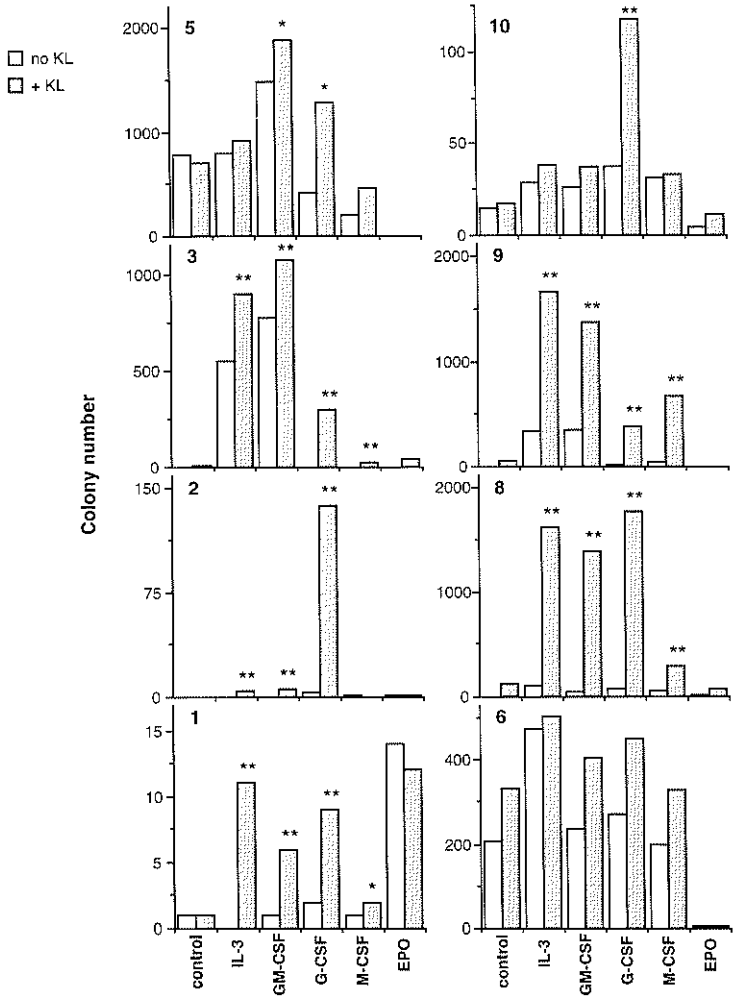

Figure 3. The effects of KL in combination with other cytokines on colony formation from AML blast cells. AML colony formation in culture with KL in the presence of IL-3, GM-CSF, G-CSF, M-CSF or EPO*. Values indicate mean numbers of CFUs (>15 cells) per 1×10^5 cells. * CFU in the presence of EPO indicate erythroid colony growth only; in the absence of EPO, no erythroid colony formation was observed; EPO had no effect on spontaneous or KL induced myeloid colony growth.

with KL. In two cases that exhibited already high spontaneous proliferation (cases 5 and 6), KL did not augment proliferation. Variable spontaneous colony growth (1 to 780 CFU) was measured in 6 cases (Table 2). In the two other cases (2 and 3), spontaneous colony growth was absent. Following the addition of KL to culture in four cases (3, 6, 8 and 9) the numbers of CFU increased.

Effects of anti HGF neutralizing antibodies. KL is a synergistic factor that stimulates AML growth when used as a single factor. Since HGFs are frequently produced by AML cells we have examined the possibility that the effects of KL were

Table 2. Effect of KL in ^{3}H-TdR uptake and colony assay of AML blasts

	^{3}H-TdR uptake (dpm x 10^{-2})			AML-CFU per 10^5 cells	
case	control	KL	irr.	control	KL
1	1.3	2.5	0.2	1	1
2	0.7	29	0.3	0	0
3	2.7	40	0.4	0	11
4	60	75	0.5	nd	nd
5	138	160	1.4	780	710
6	25	30	0.2	210	330
7	16.8	35	0.2	nd	nd
8	7.7	198	0.7	2	117
9	2.1	6.9	0.4	2	54
10	3.3	28	0.3	15	17

^{3}H-TdR uptake and colony formation was determined in the absence (control) or presence of KL. For further explanation see materials and methods. irr. = cultures of preirradiated cells (30 Gy). nd = not determined.

the result of synergy with autocrine HGFs or were obtained through induction of HGFs. A cocktail of blocking antibodies against GM-CSF, G-CSF, M-CSF and IL-6 supplemented to the cultures did not inhibit KL dependent DNA synthesis in six of 8 cases (Table 3). In two cases (3 and 9), moderate suppression (22% and 24%) of KL stimulated proliferation was noted. In case 3 the inhibitory effect was also produced with the selected neutralizing antibodies against GM-CSF and G-CSF and in case 9, with anti GM-CSF and anti IL-6 antibodies.

Response of AML blasts cells: effect of KL in combination with other cytokines. In order to assess the cooperative relationship between KL and other regulatory cytokines, induction of DNA synthesis of AML cells in response to IL-3, GM-CSF, G-CSF, M-CSF, EPO, IL-4 and TNFα was evaluated in the presence and absence of KL (Fig. 2). KL combined with IL-3 or GM-CSF increased the rate of DNA synthesis in most cases as compared to the effects of the single factors alone. In some cases the effect was additive, whereas in others synergy was identified. G-CSF in combination with KL appeared a highly potent mitogenic stimulus of AML cells. Clear synergy was observed in 6 cases. In 5 of these cases (cases 1, 2, 8, 9 and 10), G-CSF plus KL resulted in the highest levels of DNA synthesis as compared to the other KL-cytokine

Table 3. KL stimulated DNA synthesis: effect of anti HGF neutralizing antibodies

	no antibodies added		anti-HGF cocktail	
Case	control	KL	control	KL
1	1.1 ± 0.2	3.3 ± 0.4	0.7 ± 0.2	3.9 ± 1.5
2	2.0 ± 0.1	56 ± 2.3	3.1 ± 0.2	64 ± 3.7
3	0.7 ± 0.1	37.7 ± 0.6	0.7 ± 0.1	**29.0 ± 0.2**
4	43 ± 2.8	45 ± 1.9	38 ± 3.3	50 ± 4.3
7	18.2 ± 0.7	50 ± 2.8	16 ± 1.4	61.0 ± 0.7
8	4.3 ± 0.4	150 ± 15.0	2.4 ± 0.1	114 ± 4.0
9	1.9 ± 0.4	32 ± 1.3	1.2 ± 0.3	**25 ± 1.5**
10	1.4 ± 0.2	9.5 ± 1.1	1.5 ± 0.2	9.8 ± 1.0

Values indicate dpm x 10^{-2} ± SD. Significant inhibition of KL stimulated thymidine uptake is indicated in bold print. control cultures in were performed in the absence of KL anti-HGF cocktail includes: anti-GM-CSF, -G-CSF, -M-CSF and -IL-6 antibodies.

Table 4. KL binding to AML cells after incubation with IL-3, GM-CSF, G-CSF, IL-4 and TNFα

Case	non-incubated	incubated without factor	IL-3	GM-CSF	G-CSF	IL-4	TNF
2	1810 ± 27	1480 ± 11	1330 ± 31	1220 ± 40	1240 ± 37	1236 ± 9	1270 ± 14
3	1480 ± 14	5300 ± 140	3800 ± 190	3790 ± 46	5000 ± 120	3720 ± 14	3430 ± 36
4	148 ± 14	176 ± 2	155 ± 10	180 ± 7	161 ± 7	200 ± 28	100 ± 43
8	2800 ± 73	2110 ± 66	1260 ± 29	1480 ± 21	3100 ± 180	2210 ± 40	2810 ± 48
9	770 ± 15	2680 ± 12	2170 ± 34	2400 ± 30	2030 ± 75	2030 ± 54	850 ± 95
10	1630 ± 36	1160 ± 39	880 ± 49	920 ± 31	845 ± 8	1830 ± 30	1140 ± 99

Cells from 6 AML cases were incubated with no factor IL-3, GM-CSF, G-CSF, IL-4 or TNFα for 18 hours prior to ^{125}I-KL binding studies. Parallel ^{125}I-KL binding experiments were also performed for nonincubated cells. Values refer to mean specific binding ± S.D. of triplicate estimations and are expressed as cpm per 0.25×10^6 cells.

combinations. KL infrequently enhanced proliferative responses to M-CSF and EPO (synergistic effects in cases 2 and 3 respectively).

IL-4 and KL were additive to each other in 3 cases (cases 2, 3 and 7). In contrast, IL-4 inhibited the proliferative response to KL in two cases (cases 8 and 10) to varying degrees. In one other case (case 4), IL-4 inhibited the KL response almost completely, however in this case the spontaneous proliferation was also inhibited by IL-4 alone. TNF suppressed the KL response in 4 cases (cases 2, 3, 8 and 10). In case 4 IL-4 and TNF each inhibited both spontaneous and KL induced proliferative activity.

KL as a single stimulus was not a very efficient inducer of colony growth from AML blast cells (Table 2). However, in combination with IL-3, GM-CSF and G-CSF, KL augmented colony formation essentially in the same cases that also showed synergy in the thymidine uptake assay (cases 1, 2, 3, 8, 9 and case 10 for G-CSF) (Fig. 3). M-CSF in combination with KL enhanced colony formation in 3 cases (cases 3, 8 and 9).

Sensitivity of AML blasts to KL in the presence or absence of IL-3/GM-CSF; sensitivity to IL-3 and GM-CSF in the presence or absence of KL. To investigate the possibility that the sensitivity of the cells to KL or IL-3/GM-CSF/G-CSF was enhanced in the presence of other factors, as an explanation for synergy, KL titration experiments were conducted in the presence or absence of IL-3/GM-CSF/G-CSF, and vice versa IL-3/GM-CSF/G-CSF was titrated in the presence and absence of KL. The KL dose response curves in the absence and presence of IL-3 (Fig. 4a) both reach half maximal effects at identical KL concentrations (i.e. ~6 ng/mL). Identical results were obtained when GM-CSF (Fig. 4c) or G-CSF (data not shown) was supplemented to titrated KL concentrations. The result of the reciprocal experiment indicated that the sensitivity of AML cells to IL-3 did not alter in the presence of KL (ID50s 2-3 U/mL IL-3; Fig. 4b). Similarly, no changes of AML cell sensitivity to stimulation by GM-CSF (Fig. 4d) or G-CSF (data not shown) were seen when both cytokines were titrated in the presence of KL.

Modulation of c-kit by other cytokines and modulation of IL-3 and GM-CSF receptors by KL. In order to verify whether upregulation of receptors for KL, IL-3 or GM-CSF could explain the observed synergistic effects of combined HGFs, we measured the effects of various cytokines on KL receptor expression and vice versa the effect of KL on IL-3 and GM-CSF receptor expression. KL receptors were not

Table 5. IL-3 and GM-CSF binding to AML cells after incubation with KL

case	GM-CSF			IL-3		
	nonincubated cells	incubated without KL	incubated with KL	nonincubated cells	incubated without KL	incubated with KL
3	91 ± 13	214 ± 14	268 ± 18	73 ± 14	70 ± 39	52 ± 17
8	45 ± 34	221 ± 12	242 ± 9	6 ± 34	97 ± 9	64 ± 16
9	47 ± 8	310 ± 28	358 ± 6	12 ± 35	64 ± 12	135 ± 99

^{125}I-GM-CSF and ^{125}I-IL-3 binding results of 3 AML cases. For explanation see Table 4.

upregulated in six of 6 cases, following incubation of the AML cells with IL-3, GM-CSF or G-CSF (Table 4). In fact, IL-3 and GM-CSF slightly suppressed KL receptor expression in three cases (cases 3, 8 and 9). In the AML cases with suppressed responses to KL in the presence of IL-4 or TNFα (Fig. 2), KL receptor expression was not downregulated by the inhibitory cytokines (Table 4). On the other hand GM-CSF or IL-3 binding to AML cells was not influenced by KL in three of 3 cases (Table 5).

Surface markers on AML blast cells before and after culture. Immunophenotyping experiments performed after 7 days of culture in the presence of no factor, MGF, IL-3 or G-CSF, only revealed small differences of maturation. A strongly CD15 positive minor subpopulation appears among the cells from 4 cases after culture in the presence of MGF as compared to nonsupplemented cultures. In two of these cases the shift of CD15 expression was also seen in IL-3 or G-CSF supplemented cultures although less clearly. In two cases MGF induced a minor increase of CDw65 fluorescence as compared to nonsupplemented cultures. In one case the average level of CD33 expression increased after culture with MGF, as a result of the disappearance of a subpopulation with weakly positive CD33 binding.

DISCUSSION

The present study was conducted to gain insight into the role of KL in AML growth. It was found that in eight of 9 cases the AML cells expressed c-kit at sufficiently high levels, for detection with immunofluorescence. Radiobinding experiments revealed that KL receptors on AML cells were all of high affinity, which is in agreement with the KL concentrations required to reach half maximal stimulation of DNA synthesis. The high prevalence of c-kit expression in AML is in agreement with recent work of other groups (13,14) and suggests that KL may play an important role in AML. Indeed the results of activation of DNA synthesis and colony formation demonstrate that KL in addition to IL-3, GM-CSF and G-CSF (19-23), is an important cytokine in regulating AML cell proliferation. KL stimulated DNA synthesis of AML cells in nine of 10 cases. Blocking experiments with antibodies against several HGFs make unlikely that the response to KL resulted from induction of HGF production or is the result of synergy between KL and (minimal) amounts of secreted autocrine HGFs. This suggests that KL is a direct stimulus for AML cells.

Furthermore, the KL induced proliferation of AML cells was significantly augmented by other cytokines. In a number of cases, combination of KL with other factors resulted in clearly additive effects of proliferation, suggesting independence of action due to reactivity of different subsets. In 3 to 6 cases KL acted as a potent

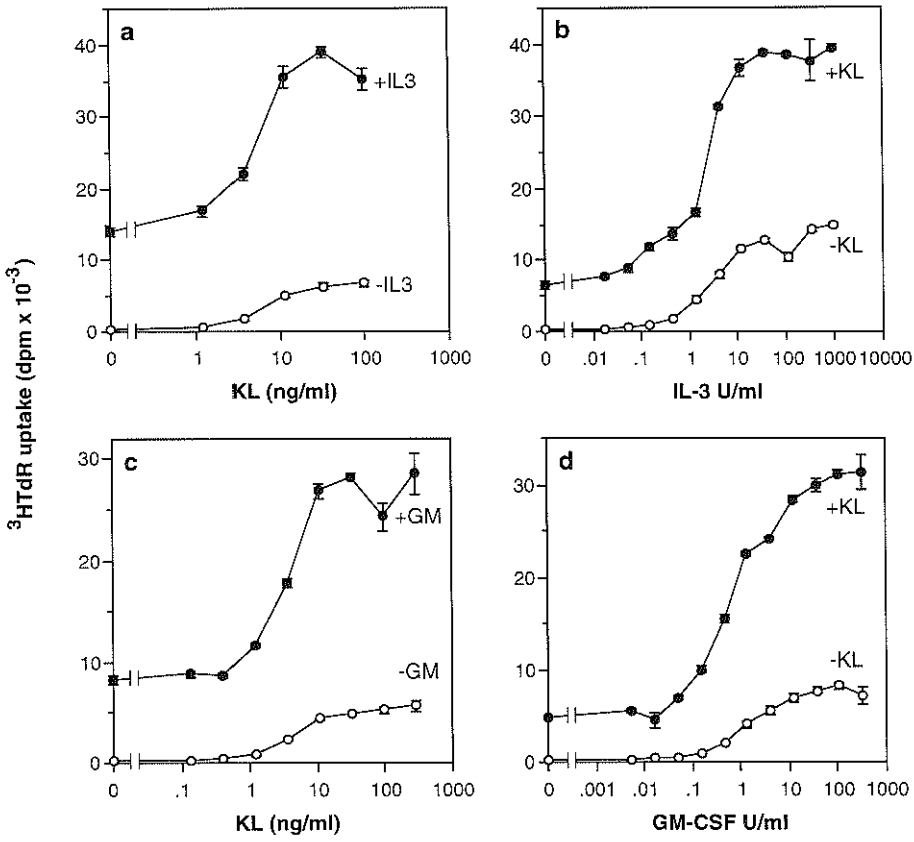

Figure 4. Synergy between KL and IL-3 or GM-CSF. Effects of titrated concentrations of KL in the absence (open circles) or presence (closed circles) of IL-3 (a) or GM-CSF (c) on AML cell thymidine uptake (case 2). The reverse experiments are concerned with the effects of titrated concentrations of IL-3 (b) or GM-CSF (d) in the absence (open circles) or presence (closed circles) of KL on AML cell thymidine uptake (case 2).

synergistic factor in association with IL-3, GM-CSF and G-CSF. The latter observation could be of relevance in AML. KL, which has been described as a stromal factor in vivo (40-43), may promote the outgrowth of leukemic cells in the bone marrow due to its synergistic abilities in combination with other autocrine mechanisms involving CSFs.

The mechanism of synergy between KL and other cytokines remains uncertain. The sensitivity of AML cells to KL stimulation in the presence of IL-3, GM-CSF or G-CSF did not change. Conversely KL did not alter the dose response to GM-CSF, G-CSF or IL-3 either. In addition, radioreceptor studies did not provide any indication for upregulation of KL receptors following incubation with IL-3, GM-CSF or G-CSF. Additionally, the level of GM-CSF or IL-3 receptor expression on the AML cells remained unaltered as well in the presence of KL. Hence, these data demonstrate that synergy between KL and IL-3, GM-CSF or G-CSF is not the result of receptor modulation or the appearance of cells that become receptor positive. Possibly, certain

cells which are a priori KL and IL-3 receptor positive but insensitive to stimulation by one factor, are stimulated by the combination of KL and IL-3. Thus we assume the existence AML cell populations able of responding to optimal concentrations of KL alone and IL-3/GM-CSF/G-CSF alone and subsets that are induced to enter DNA synthesis when IL-3, GM-CSF or G-CSF is combined with KL.

IL-4 exerted enhancing as well as inhibitory effects on KL induced proliferation. Additive effects of KL and IL-4 are probably explained by subpopulations with distinct factor responsiveness and are consistent with the cellular heterogeneity of AML (23). The mechanism responsible for the inhibitory effects of IL-4 remains unexplained. IL-4 has been characterized as T-cell and mast cell derived cytokine that acts on B-cells, T-cells, NK-cells and monocytes (44). Inhibitory effects of IL-4 on the IL-2 response in human B-cells have been related to down regulation of the IL-2 receptor (45,46). However, our receptor data do not suggest a regulatory role for IL-4 of KL receptor expression in AML. The mechanism responsible for TNF inhibition of KL stimulated AML growth also remains unresolved. The inhibitory effects of TNF did not result from downregulation of the KL receptors as was demonstrated to be a possible mechanism for the inhibitory effects of TNF on the G-CSF response in AML (47).

Immunophenotyping did not indicate a clear role of MGF in the regulation of maturation or commitment of AML in vitro. Only minor changes in the pattern of immature and more mature granulocytic/monocytic membrane markers were observed in the presence of MGF. Thus these data do not disclose whether MGF stimulates the survival or self renewal of AML blast cells in vitro

In conclusion, these results indicate that KL, in addition to IL-3, GM-CSF and G-CSF, is an important GF in controlling AML cell proliferation in vitro. Although its synergistic capacities in combination with other CSFs suggest that KL may contribute to the outgrowth of AML cells in vivo, further investigations will be required to provide insight in the role of KL in leukemic cell growth.

CHAPTER 5

TUMOR NECROSIS FACTOR REGULATES THE EXPRESSION OF GM-CSF AND IL-3 RECEPTORS ON HUMAN ACUTE MYELOID LEUKEMIA CELLS

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SUMMARY

Tumor necrosis factor (TNF) acts as a potent enhancer of granulocyte-macrophage colony-stimulating factor (GM-CSF)- and interleukin-3 (IL-3)-induced human acute myeloid leukemia (AML) growth in vitro. We have analyzed the effects of TNFα on the expression of GM-CSF and IL-3 receptors on AML cells. Incubation of blasts from seven patients with AML in serum-free medium with TNF (10^3 U/mL) and subsequent binding studies using ^{125}I-GM-CSF and ^{125}I-IL-3 show that TNF increases the specific binding of GM-CSF (30% to 280%) and IL-3 (40% to 600%) in all cases. From Scatchard plot analysis it appears that TNF upregulates (1) low-affinity GM-CSF binding sites, (2) common high-affinity IL-3/GM-CSF binding sites, and (3) unique (non-GM-CSF binding) IL-3 binding sites. The effect of TNF is dose dependent and is half maximal at a concentration of 100 U/mL, and becomes evident at 18 hours of incubation with TNF at 37°c, but not at 0°C. The GM-CSF dose-response curve of AML-colony-forming units plateaus at a higher level in the presence of TNF, which indicates that additional numbers of cells become responsive to GM-CSF. Incubation of AML blasts with the phorbol ester 12-0-tetradecanoylphorbol-13-acetate or formyl-Met-Leu-Phe (protein kinase C activators) does not influence GM-CSF receptor expression, suggesting that receptor upregulation by TNF is not mediated through activation of protein kinase C. On the other hand, the protein synthesis inhibitor cycloheximide abrogates receptor upregulation induced by TNF. In contrast to these findings in AML, TNF does not upregulate GM-CSF receptor numbers on blood granulocytes or monocytes. We conclude that TNF exerts positive effects on growth factor receptor expression of hematopoietic cells.

INTRODUCTION

Tumor necrosis factor α (TNFα) is a polypeptide with multiple biologic activities, including effects on hematopoiesis (1). Macrophages are the major cellular sources of TNFα (2,3). TNFα may inhibit proliferation of hematopoietic progenitor cells from normal subjects and influence the efficiency of growth of human leukemia (4-7).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) belong to the group of CSFs that regulate proliferation and differentiation of relatively primitive hematopoietic precursor cells (8-11). GM-CSF and IL-3 are potent stimulators of proliferation of human acute myeloid leukemia (AML) in vitro (12-15). TNF acts synergistically with the stimulative effects of GM-CSF (16,17) and IL-3 (17) on AML proliferation in vitro, but may depress G-CSF-mediated AML growth in vitro (17). The mechanism of enhancement of GM-CSF-or IL-3-induced AML cell growth by TNF is unknown.

Here we present studies that were concerned with the question as to whether the TNF effects might be accomplished at the level of membrane receptors. The results of these experiments indicate that TNF upregulates the expression of GM-CSF and IL-3 receptors on AML blasts. These observations may provide a plausible explanation for the synergistic relationship between TNFα and GM-CSF/IL-3 with regard to stimulation of growth in vitro.

MATERIALS AND METHODS

Patients and purification of AML blasts, normal granulocytes, and monocytes. AML blasts were isolated from the peripheral blood and the bone marrow of seven adult untreated AML cases that were classified as M1 (n=3), M4 (n=2), and M5 (n=2) (18,19). Approval was obtained from the Institutional Review Board for these studies. Patients were informed that blood samples were obtained for research purposes, and that their privacy will be protected. AML blasts were separated after Ficoll-Isopaque centrifugation and subsequent removal of E-rosette-forming cells, and then cryopreserved (20). Monocytes were removed from the AML cell preparation by plastic adherence. Morphologic examination of cytospin slides of the final cell suspension showed that more than 98% of the cells were blasts. Granulocytes were obtained from the blood of healthy volunteers after sedimentation in 0.1% methyl cellulose, and subsequent Ficoll-Isopaque centrifugation. The resulting cell fractions consisted of neutrophils (92% to 96%), eosinophils (2% to 5%), and mononuclear cells (3% to 5%). Monocytes (more than 80%) were obtained from the Ficoll-Isopaque interphase of mononuclear cells with subsequent removal of E-rosetting cells.

Incubation of the cells with TNF. Before the binding experiments, AML cells, granulocytes, or monocytes were washed twice in Hank's balanced salt solution (HBSS), then incubated with TNF (10.3 U/mL) in serum-free medium (21) at 37°C for 1, 2, 4, 8, and 18 hours (for AML cells), 18 hours (for monocytes), or 10 to 60 minutes (for granulocytes). The cells were then washed in sodium citrate (pH 4) to remove cell-bound TNF (22). Before and after the latter acid wash, the cells were washed twice in HBSS.

Treatment of AML blasts with fMLP, TPA, and cycloheximide. AML cells (case 7) were incubated in serum-free medium for 18 hours at 37°C without or with TNF (see above). In parallel, blasts were also exposed to protein kinase C (PKC) activators. The phorbol ester 12-0-tetradecanoylphorbol-13-acetate (TPA; Sigma Co, St Louis, MO) was applied at 10 nmol/L, and formyl-Met-Leu-Phe (fMLP; Sigma) was added at 1 μmol/L. Analog incubations were performed with varying concentrations of cycloheximide (0.25, 0.5, and 1 μg/mL). After incubation, the cells were washed in sodium citrate (pH 4). Before and after the acid wash, the cells were washed twice in HBSS and subsequently used in radiolabeling studies.

Radioiodination of GM-CSF and IL-3. Recombinant human GM-CSF (rhGM-CSF, CHO-cell derived; glycosylated, molecular weight [mol wt] 25 K_d) was prepared and purified at Genetics Institute (Cambridge, MA). rhIL-3 (Yersinia derived; nonglycosylated, mol wt 15 K_d) was prepared and purified at Gist Brocades (Delft, The Netherlands). Both proteins were radiolabeled with Bolton and Hunter reagent (Amersham Laboratory, Amersham, UK) (23) as described (24). Specific activities as determined by selfdisplacement analysis (25) were 5 to 7×10^4 cpm/ng for radioiodinated GM-CSF (^{125}I-GM-CSF) and 3 to 6×10^4 cpm/ng for ^{125}I-IL-3. Trichloroacetic acid precipitation showed less than 5% nonprecipitable radioactivity for both factors. The maximum binding capacity was estimated at 90% to 95% for GM-CSF and 55% to 60% for IL-3. In Scatchard calculations the free cpm were corrected for the maximum binding capacity (26).

Table 1. Effect of TNF on Specific Binding of ^{125}I-GM-CSF or ^{125}I-IL-3 to AML Blasts

	^{125}I-GM-CSF binding			^{125}I-IL-3 binding		
case no.	non incubated cells	incubated cells without TNF	incubated cells with TNF	non incubated cells	incubated cells without TNF	incubated cells with TNF
1	242 ± 37	286 ± 85	1,090 ± 85	14 ± 10	11 ± 8	88 ± 23
2	649 ± 72	737 ± 101	1,400 ± 274	283 ± 47	321 ± 55	719 ± 127
3	162 ± 44	237 ± 63	736 ± 245	65 ± 24	82 ± 13	597 ± 62
4	2,876 ± 416	3,816 ± 133	5,864 ± 356	ND	1,577 ± 250	1,752 ± 249
5	2,414 ± 286	3,240 ± 116	4,425 ± 236	1,127 ± 264	1,750 ± 206	3,924 ± 113
6	2,116 ± 235	3,784 ± 135	4,941 ± 100	479 ± 68	518 ± 127	1,054 ± 186
7	817 ± 39	1,586 ± 38	2,714 ± 39	296 ± 149	383 ± 46	551 ± 64

Cells from seven AML cases were incubated with or without TNF for 18 hours before the radiolabeling studies using single concentrations of ^{125}I-GM-CSF or ^{125}I-IL-3. Parallel experiments were performed also for nonincubated cells. Values refer to the mean specific binding ± SD (in cpm) of triplicate estimations. Values were corrected for nonspecific binding. The specific activities for ^{125}I-GM-CSF and ^{125}I-IL-3 were 6.1×10^4 and 4.4×10^4 cpm/ng, respectively. ND: not done.

Binding of radiolabeled GM-CSF and IL-3 to AML blasts, granulocytes, and monocytes. GM-CSF and IL-3 binding to the cells was assessed as described (24). Equilibrium binding conditions for both GM-CSF and IL-3 at 37°C were reached after 1 hour (27). Cells, 1 to 8×10^6, were incubated for 1 hour at 37°C in 100 μL α-minimal essential medium with 1% bovine serum albumin. Initial binding experiments were performed using two concentrations (1 or 2 nmol/L) of ^{125}I-GM-CSF or ^{125}I-IL-3. To allow estimation of both high- and low-affinity GM-CSF receptor numbers and affinities on AML cells, complete binding assays with titrated concentrations of ^{125}I-GM-CSF (20 to 5,000 pmol/L) were performed. Binding experiments with ^{125}I-GM-CSF were also performed in the presence of excess concentrations of nonlabeled IL-3 (see Table 3). Results from these experiments represent low-affinity binding of GM-CSF alone, because IL-3 selectively competes for the high-affinity binding of GM-CSF (28) (the common IL-3/GM-CSF binding site). For a complete IL-3 binding assay, 20 to 2,000 pmol/L ^{125}I-IL-3 was used. Nonspecific binding was determined in parallel incubations in the presence of excess (i.e., 200 nmol/L) unlabeled GM-CSF or IL-3. Specific binding was defined as the difference between the amount of radioactivity bound in the absence of nonlabeled factor and the amount of radioactivity bound in the presence of nonlabeled factor. To distinguish the two different IL-3 binding sites that have been described, (28) excess concentrations of nonlabeled GM-CSF were used in binding experiments performed with ^{125}I-IL-3. The common IL-3/GM-CSF binding sites were defined as sites that bind ^{125}I-IL-3 which are competed for by unlabeled GM-CSF. The unique IL-3 binding sites are those that remain detectable with ^{125}I-IL-3 after competition with GM-CSF (28). All experiments were conducted in duplicate. Samples were counted in a gamma-counter (Packard, Downers Grove, IL). The counting efficiency for ^{125}I was 80%. Receptor numbers and binding affinities were estimated by Scatchard analysis (26). Two affinity receptor analyses were performed with the ENZfitter computer program (Sigma).

AML clonogenic assay. The dose-response curve of GM-CSF was established in the presence and absence of TNF for the clonogenic cells of two AML cases (nos. 4 and 6). In these experiments, 10^5 AML cells were plated in serum-free colony culture

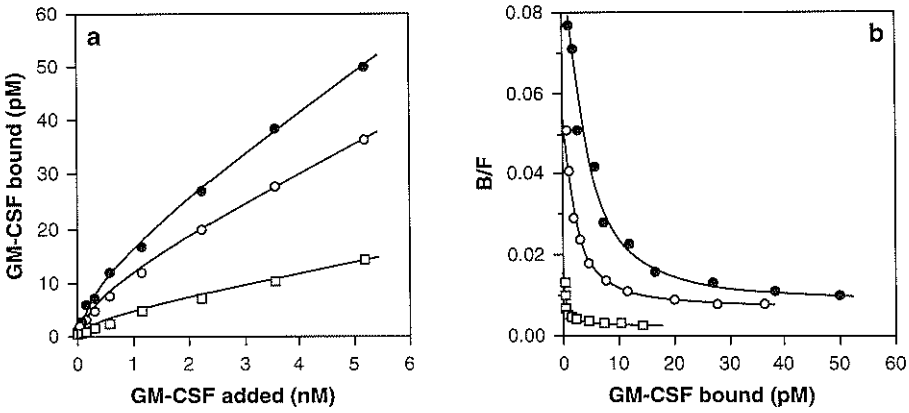

Figure 1. (**a**) Upregulation of ^{125}I-GM-CSF specific binding to AML cells by TNF. Cells (3.8×10^6) of AML case 7 were incubated with (closed circles) or without (open circles) TNF (10^3 U/mL) for 18 hours in serum-free medium and then subjected to radiolabeling studies with titrated concentrations of ^{125}I-GM-CSF. An identical binding assay was performed with non-incubated cells (2.1×10^6) of the same AML case (open squares). Data have been corrected of nonspecific binding. (**b**) Scatchard plots of the data shown in (**a**).

(1 mL) with 0.9% methyl cellulose (17) supplemented with variable concentrations of GM-CSF (0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, 100, 300, and 1,000 U/mL). All aggregates of 10 cells or more were scored after 14 days.

RESULTS

TNF upregulates high- and low-affinity sites of GM-CSF on AML blasts. The AML blasts from seven cases were cultured for 18 hours with TNF (10^3 U/mL). Subsequent incubation of the cells with 1 nmol/L ^{125}I-GM-CSF demonstrated that TNF had augmented specific GM-CSF binding to cells from all seven cases (Table 1) as compared with the cells incubated without TNF or preculture cells. The increase of specific binding ranged from 31% (case 6) to 281% (case 1). In four cases (nos. 4 through 7), AML blasts that were cultured for 18 hours without TNF also expressed greater GM-CSF binding as compared with precultured cells (Table 1). Binding assays with titrated concentrations of ^{125}I-GM-CSF showed that, after TNF exposure, both high-and low-affinity GM-CSF binding sites increased considerably (Table 2, Fig. 1). The TNF-dependent increase of low-affinity GM-CSF receptor numbers on AML blasts was also apparent in separate binding experiments performed after the blockade of high-affinity GM-CSF binding in the presence of an overdose of nonlabeled IL-3 (200 nmol/L) (Table 3) (28). Upregulation of GM-CSF receptors appeared TNF dose dependent with a maximal effect at approximately 300 U/mL (Fig. 2). In separate experiments, AML cells were incubated with TNF for varying time intervals (1, 2, 4, 8 and 18 hours). The increase of GM-CSF binding induced by TNF became expressed after 18 hours (not shown). In control experiments, the effects of TNF on the expression of GM-CSF binding to granulocytes as well as monocytes were investigated. As observed previously, (29) TNF was found to downregulate GM-CSF receptor expression on granulocytes (Table 2). On the other hand, TNF

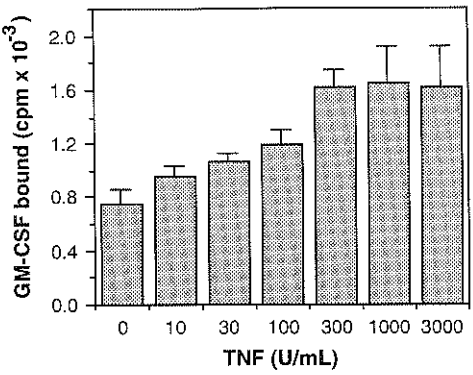

Figure 2. GM-CSF receptor upregulation on AML blasts: Relationship to TNF concentration. Cells (2.2×10^6) of AML case 7 were incubated for 18 hours with varying concentrations of TNF (0, 10, 30 ,100, 300, 1,000, 3,000 U/mL) in serum-free medium. Subsequently, the specific binding of ^{125}I-GM-CSF (using 2 nmol/L ^{125}I-GM-CSF) was determined. Values represent the means of triplicate estimations in cpm ± SD and were corrected for nonspecific binding. The specific activity of ^{125}I-GM-CSF was 5.4×10^4 cpm/ng.

Table 2. Effect of TNF on GM-CSF Receptor Expression (Scatchard analysis)

Cell type		Without TNF		With TNF	
		Sites/cell	Kd (pmol/L)	Sites/cell	Kd (pmol/L)
AML	Case 1	+	+	38 (H)	57
				1200 (L)	13000
	Case 7	190 (H)	18	410 (H)	23
		8100 (L)	4100	16000 (L)	6500
Neutro.	Donor 1	2800	680	1020	580
	Donor 2	1776	520	760	410
Mono	Donor 1	230 (H)	63	200 (H)	46
	Donor 2	165 (H)	87	190 (H)	118

Cells were incubated with or without TNF for 18 hours (AML blasts and monocytes) or for 1 hour (granulocytes), and then subjected to GM-CSF radioreceptor analysis. Receptor numbers (mean per cell) and Kd were derived from Scatchard plot analysis. (+) Indicates that specific binding was detectable but values were too low to permit complete Scatchard analysis. Abbreviations: H, high-affinity binding; L, low-affinity binding.

under identical conditions did not affect GM-CSF receptor expression on monocytes (Table 2).

TNF upregulates high-affinity binding sites of IL-3 on AML blasts. Preliminary binding studies showed that TNF also increased the specific binding of ^{125}I-IL-3 to AML cells in six of seven cases (Table 1). The increase of specific binding of ^{125}I-IL-3 ranged from 44% to 628%. From a complete binding assay with titrated concentrations of ^{125}I-IL-3 with cells from AML cases 1, 3 and 5 (Table 4, Fig. 3), it was evident that IL-3 receptor numbers had increased as compared with the values following the control incubation in the absence of TNF. Cross-competition with excess nonlabeled GM-CSF showed that both the unique IL-3 binding sites as well as the common IL-3/GM-CSF binding sites (28) were upregulated by TNF (Fig. 3). The average numbers of unique IL-3 binding sites increased from 90 sites per cell to 262 sites per cell (Kd 261 pmol/L). The numbers of common IL-3/GM-CSF binding sites calculated by subtraction of unique IL-3 receptor numbers from total IL-3 binding sites increased from 175 sites per cell toward 340 sites per cell.

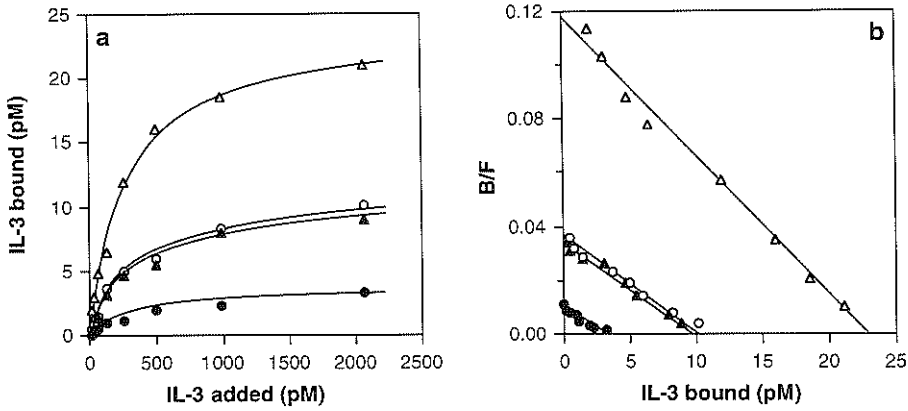

Figure 3. (a) Upregulation of ^{125}I-IL-3 specific binding to AML blasts by TNF. Cells (2.2×10^6) of AML case 5 were incubated with (triangles) or without (circles) TNF (10^3 U/mL) and then subjected to radioreceptor studies using titrated concentrations of ^{125}I-IL-3 in the presence (solid symbols) or absence (open symbols) of 200 nmol/L GM-CSF to discriminate between the common IL-3/GM-CSF and unique high-affinity IL-3 receptors, as outlined in Materials and Methods. (b) Scatchard plots of these binding data. Each point represents the means of duplicate estimations. All data represent specific binding after correction for nonspecific binding.

Table 3. Effect of TNF on Low-Affinity Binding of ^{125}I-GM-CSF to AML Blasts

Case no.	No competitor♦			Competition with IL-3♣		
	nonincubated cells	incubated cells without TNF	incubated cells with TNF	nonincubated cells	incubated cells without TNF	incubated cells with TNF
4	4,000 ± 500	5,600 ± 340	8,700 ± 440	14 ± 10	11 ± 8	88 ± 23
5	1,540 ± 100	1,900 ± 340	3,200 ± 620	1,100 ± 130	1,400 ± 140	2,500 ± 120
6	1,900 ± 170	2,900 ± 190	4,100 ± 170	1,400 ± 130	2,000 ± 240	2,900 ± 320

Cells from AML cases 4, 5, and 6 were incubated without or with TNF for 18 hours before radiolabeling studies using ^{125}I-GM-CSF (1 nmol/L). Comparative experiments were performed with nonincubated cells. Values refer to the means of triplicate estimates of specific binding ± SD (in cpm) in the presence or absence of excess nonlabeled IL-3 (200 nmol/L). Values were corrected for nonspecific binding (20% to 30% of the total binding). The specific activity of ^{125}I-GM-CSF was 5.2×10^4 cpm/ng. ♦Indicates the sum of high- and low-affinity binding of ^{125}I-GM-CSF (without IL-3 competition). ♣Indicates low-affinity binding of ^{125}I-GM-CSF (with IL-3 competition).

Upregulation of GM-CSF receptor expression by TNF is not dependent on PKC activation. Downregulation of the expression of GM-CSF receptors on neutrophils by TNF, as was recently shown (29) is probably mediated through activation of PKC (30). To investigate whether the upregulation of GM-CSF receptors on AML cells is also mediated via activation of PKC, the blasts were exposed to the PKC activators TPA or fMLP. Neither TPA nor fMLP, at concentrations that downregulate GM-CSF receptors on neutrophils, elevated the numbers of GM-CSF receptors on AML blasts (Fig. 4a).

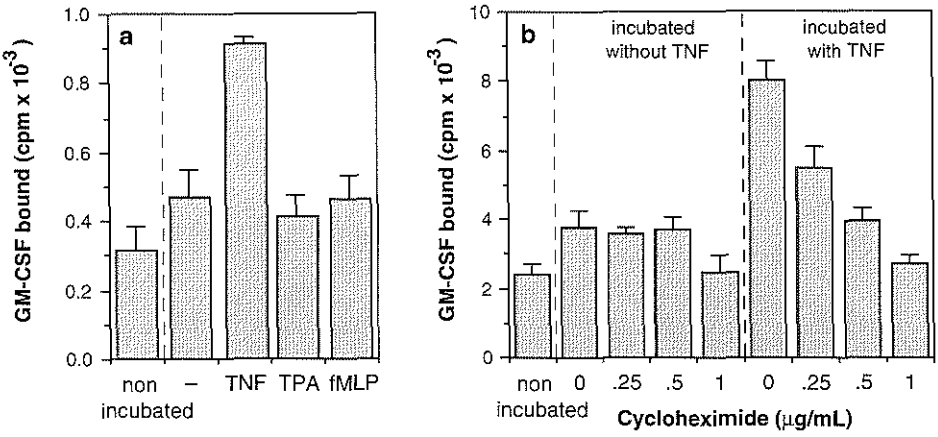

Figure 4. (**a**) GM-CSF receptor upregulation by TNF: Effect of PKC activators. Cells (1.1×10^6) from AML case 7 were incubated in serum-free medium for 18 hours with no supplement (-), TNF (10^3 U/mL), TPA (10 nmol/L), or fMLP (1 μmol/L). Specific binding of ^{125}I-GM-CSF using (1 nmol/L) was subsequently determined. Values represent the means of triplicate estimations in cpm ± SD and were corrected for nonspecific binding. A control experiment was also performed with blasts before incubation (nonincubated). (**b**) GM-CSF receptor upregulation by TNF: Effect of cycloheximide. Cells (7.8×10^6) from AML case 7 were incubated in serum-free medium for 18 hours with increasing concentrations of cycloheximide (0.25, 0.5, 1 μg/mL) in the presence or absence of TNF. Specific binding of ^{125}I-GM-CSF (1 nmol/L) was subsequently determined. Values represent the means of triplicate estimations in cpm ± SD. A control experiment was also performed with nonincubated cells.

GM-CSF receptor upregulation is blocked by cycloheximide. We further examined whether the increase in GM-CSF receptor numbers after incubation of the AML cells with TNF required active protein synthesis. The addition of the protein-synthesis inhibitor cycloheximide at concentrations of 0.25 to 1 μg/mL did not affect GM-CSF binding in the absence of TNF (Fig. 4b). However, at these concentrations, cycloheximide prevented the increase of GM-CSF binding induced by TNF. The spontaneous increase in GM-CSF binding after 18 hours of incubation, which was seen in four cases of AML, was not suppressed by cycloheximide (Fig. 4b).

Altered GM-CSF dose-response relationship of AML colony forming cells as the consequence of TNF addition. Using titrated GM-CSF concentrations, we established the effect of TNF on AML colony formation. The results (Fig. 5) show that there was no change in the sensitivity to GM-CSF as a result of the addition of TNF. TNF did not shift the dose-response curve to the left. Instead, TNF was found to increase the plateau of GM-CSF stimulation, indicating the recruitment of greater cell numbers into proliferation at maximal GM-CSF concentrations.

DISCUSSION

TNF is a regulator of surface expression of hematopoietic growth factor receptors. It downregulates the expression of GM-CSF receptors (29) and G-CSF receptors on granulocytes (30), as well as M-CSF receptors on macrophages (31). In this study, we

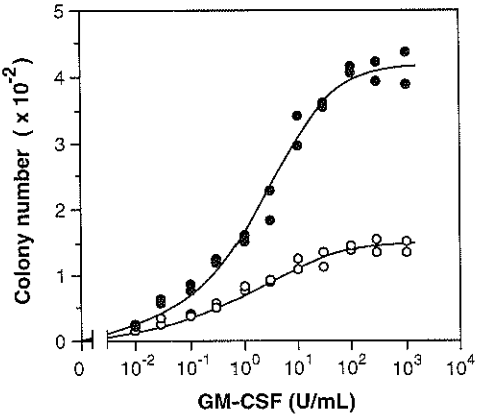

Figure 5. Effect of TNF on GM-CSF-induced colony formation of AML cells: GM-CSF dose response in the presence (closed circles) or absence of TNF (open circles). The data represent colony numbers per 10^5 AML cells (case 4) of duplicate cultures.

investigated the effects of TNF on GM-CSF and IL-3 receptors on AML blasts. It is evident from the results of these experiments (Tables 1 and 2, and Fig. 1) that TNF augments the average level of GM-CSF receptors on AML blasts by approximately twofold. The increase of receptor numbers after TNF exposure is accomplished within approximately 18 hours. Both the high-affinity and low-affinity binding sites of GM-CSF increase on these cells (Tables 2 and 3, Fig. 1). In a recent study, high-affinity IL-3/GM-CSF binding sites, which can bind both IL-3 and GM-CSF, and IL-3-specific high-affinity binding sites have been distinguished on human AML blasts (28). It appears from the results presented here that TNF upregulates both high-affinity receptors, ie, GM-CSF/IL-3 and IL-3 binding sites (Fig. 3 and Table 4). Upregulation of GM-CSF, IL-3, and common GM-CSF/IL-3 receptors is not associated with an apparent change in receptor affinities.

Receptor upregulation by TNF might result in a positive effect of TNF on IL-3- or GM-CSF-mediated AML cell growth. Recent findings have shown that TNF enhances DNA synthesis and colony formation of AML blasts after stimulation with GM-CSF or IL-3 (17). Cells that initially express no or subliminal levels of growth factor receptors may become responsive to IL-3 and GM-CSF after TNF exposure. This would imply that additional subpopulations are triggered to proliferate and that the sensitivity of the cells to GM-CSF or IL-3 has remained constant. The change of the GM-CSF dose-response curve of proliferating AML cells as the consequence of TNF supplementation is in accordance with this conclusion. GM-CSF dose

Table 4. Effect of TNF on IL-3 Receptor Expression

	Without TNF		With TNF	
AML cells	Sites/cell	K_d (pmol/L)	Sites/cell	K_d (pmol/L)
Case 1	+	+	76	157
Case 2	+	+	278	63
Case 3	265	259	602	192

AML cells were incubated with or without TNF for 18 hours. This was followed by IL-3 radioreceptor analysis. Receptor numbers (mean per cell) and Kd were derived from Scatchard plot analysis. (+) Indicates that specific binding was detectable but values were too low to permit complete Scatchard analysis.

dependence of AML-colony-forming units (Fig. 5) or actively DNA synthesizing cells (data not shown) is not shifted to the left in the presence of TNF, being indicative of the same sensitivity of stimulation. In fact the maximal level of stimulation is elevated to a higher plateau. Recently, analogous with the results in human AML, it was observed that IL-3 and TNF or GM-CSF and TNF synergize in stimulating the colony formation of purified normal bone marrow blast cells as well (32). Receptor upregulation could also provide a plausible explanation for IL-3/TNF or GM-CSF/TNF synergistic growth activation of normal hematopoietic progenitors. We assume that in vitro IL-3 and GM-CSF receptor upregulation by TNF may be a feature of leukemic as well as normal hematopoiesis.

The exact mechanism of receptor upregulation by TNF presently remains unresolved. However, the observations that increase of GM-CSF and IL-3 receptors requires an incubation of approximately 18 hours and is inhibited by cycloheximide suggest that the effect of TNF depends on active protein synthesis (Fig. 4b). The negative effects of TNF on the expression of GM-CSF and G-CSF receptors on neutrophils and M-CSF receptors on monocytes appear to be mediated through activation of PKC (29-31). The studies presented here do not support the idea that PKC is similarly involved in receptor upregulation. The PKC activators TPA and fMLP did not influence GM-CSF receptor values (Fig. 4a). The spontaneous increase of GM-CSF receptor numbers in some AML cases in this study is unlikely caused by endogenous TNF production by AML cells (33,34) and subsequent activation of new protein synthesis.

The indirect growth-promoting effects of TNF in hematopoiesis have, until recently, been attributed to the pleiotropic capacity of TNF to induce the release of certain hematopoietic growth factors from endothelial cells, monocytes, or fibroblasts. Based on the investigations presented here, it is evident that the role of TNF in blood cell formation also includes potentially important effects on the expression of growth factor receptors, ie, upregulation of GM-CSF and IL-3 receptors, which may explain the recruitment of cells into the proliferative compartment. These findings, in association with observations indicating G-CSF receptor downregulation by TNF on AML cells and granulocytes (30), demonstrate that TNF acts as a differential regulator of hematopoietic growth factor receptors and expresses the capacity to stimulate versus suppress distinct subsets of proliferating target cells selectively.

CHAPTER 6

TUMOR NECROSIS FACTOR DOWNREGULATES G-CSF RECEPTOR EXPRESSION ON HUMAN AML CELLS AND GRANULOCYTES

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SUMMARY

Tumor necrosis factor (TNF) inhibits granulocyte-colony-stimulating factor (G-CSF)-induced human acute myeloid leukemia (AML) growth in vitro. Incubation of blasts from three patients with AML in serum-free medium with TNF (10^3 U/mL) and subsequent binding studies using ^{125}I-G-CSF reveal that TNF downregulates the numbers of G-CSF receptors by ~70 %. G-CSF receptor numbers on purified blood granulocytes are also downmodulated by TNF. Downregulation of G-CSF receptor expression becomes evident within 10 min after incubation of the cells with TNF at 37°C and is not associated with an apparent change of the dissociation constant (K_d). The TNF effect does not occur at 0°C and cannot be induced by IL-2, IL-6, or GM-CSF. TNF probably exerts its effect through activation of protein kinase C (PKC) as the TNF effect on G-CSF receptor levels can be mimicked by 12-*O*-tetradecanoylphorbol-13-acetate. The PKC inhibitor Straurosporine (Sigma Chemical Co., St. Louis, MO) as well as protease inhibitors can completely prevent G-CSF receptor downmodulation. Thus, it appears TNF may act as a regulator of G-CSF receptor expression in myeloid cells and shut off G-CSF dependent hematopoiesis. The regulatory ability of TNF may explain the antagonism between TNF and G-CSF stimulation.

INTRODUCTION

Tumor necrosis factor α (TNFα) is a nonglycosylated protein with a mol wt of 17,350 (1) synthesized by activated mononuclear phagocytes (2). It has an important role in regulating hematopoiesis. It may induce the release of certain hematopoietic growth factors from diverse cells, e.g., granulocyte macrophage-colony-stimulating factor (GM-CSF) (3) and granulocyte-colony-stimulating factor (G-CSF) (4) from human lung fibroblasts, monocyte colony-stimulating factor (M-CSF) from human monocytes (5), and interleukin-1 from endothelial cells (6).

The role of G-CSF in controlling the survival, proliferation, differentiation, and functional activation of granulocytes and their precursors has been established (7). Further, G-CSF stimulates the proliferation of acute myeloid leukemia (AML) in culture (8-10) and it may also induce maturation of myeloid leukemia (11-13). In previous reports, it has been shown that TNF acts antagonistically with the proliferative effects of G-CSF on AML blasts in vitro (14, 15), and thus appears to be a negative regulator of the G-CSF-induced proliferation of AML cells.

In this study we have addressed the question whether the mechanism of TNF suppression of G-CSF-mediated growth may occur at the level of G-CSF membrane receptors. The results of the experiments reported here show that TNFα downregulates G-CSF receptor expression on AML blasts, suggesting that the antagonistic effect of TNF is accomplished through G-CSF receptor downmodulation. A similar downregulation of G-CSF receptor expression is also evident when blood granulocytes are incubated with TNF. Additional experiments suggest that activation of protein kinase C (PKC) and release of proteases that cleave off the receptors are crucial events in the rapid downregulation of G-CSF receptors.

METHODS

Purification of AML blasts and normal granulocytes. AML cells were isolated from the peripheral blood and the bone marrow of eight adult untreated AML cases that were classified as M1 (n =2), M2 (n =1), M4 (n =2), and M5 (n =3) (16, 17). The AML cells were separated by Ficoll-Isopaque centrifugation and subsequent removal of E-rosette-forming cells (18), and then cryopreserved in 7.5% DMSO and 20% inactivated FCS. The cell preparations were also depleted from monocytic cells by plastic adherence. The viability of the cells after thawing was always >90%. Morphological examination of cytospin slides of the thawed cells after depleting the monocytic fraction revealed that ≥98% of the cells were blasts. Normal peripheral blood granulocytes were obtained from the heparinized blood of healthy volunteers following sedimentation in 0.1% methyl cellulose, and subsequent Ficoll-Isopaque centrifugation. The resultant cell fractions consisted of 92%-96% neutrophils, 2%-5% eosinophils, and 3%-5% monocytes/lymphocytes. The latter cell preparations were used fresh.

Hematopoietic growth factors. Recombinant human GM-CSF (a gift from Dr. S. Clark from Genetics Institute, Cambridge, MA) at 200 U/mL, IL-2 (Cetus Corp., Emeryville, CA) at 50 U/mL, IL-6 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam) at 10^3 U/mL, and TNFa (Dr. Adolf, Boehringer Institute, Vienna, Austria) at 10^3 U/mL were applied for incubation of cells prior to binding studies. These concentrations supported optimal AML cell proliferation under serum-free conditions (10,11,15,19). Recombinant human G-CSF (Amgen Biologicals, Thousand Oaks, CA) was used for radiolabeling purposes.

Treatment of the cells with 12-O-tetradecanoylphorbol-13-acetate (TPA), Staurosporine, and protease inhibitors. To study the mechanism of action of TNF, granulocytes were incubated in serum-free medium (SFM) for 60 min. at 37°C with TPA (Sigma Chemical Co., St. Louis, MO) at 50 ng/mL. The cells were pretreated with the protein kinase C (PKC) inhibitor Staurosporine (Sigma) at 0.1 μM. The cells (1 mL end volume) were also pretreated with 20 μL mixture of protease inhibitors (mixture: 2% gelatine in 0.15 M NaCl, 1 mg/mL Aprotinin, 1 mg/mL Leupeptin, 1 mg/mL Iodoacetamide, 1 mg/mL Bacitracin, and 1 mM PMSF). This pretreatment was applied 30 min before the direct addition of TNF or TPA.

Preincubation of the cells with TNF. Before the binding experiments, the AML cells or granulocytes were washed twice in HBSS, then incubated in SFM (20) for 10 to 60 minutes at 37°C without or with TNF (10^3 U/mL). Finally, the cells were washed twice in HBSS to remove residual TNF.

Binding of radiolabeled G-CSF to AML blasts and granulocytes. Purified rhG-CSF was radiolabeled according to the method of Bolton and Hunter (21) as described (22). Radiolabeling of rhG-CSF resulted in a protein that had a maximal binding capacity of 50 to 60 %, and a specific activity of 20,000 cpm/ng. The cells (4 to 8×10^6 per point) were incubated for 1 hr at 37°C in 100 μL α-minimal essential medium with 10% FCS and with 1 and 2 nmol/L ^{125}I-G-CSF (for preliminary binding estimations) or 20 to 4,000 pmol/L ^{125}I-G-CSF (for the complete binding assay) in the

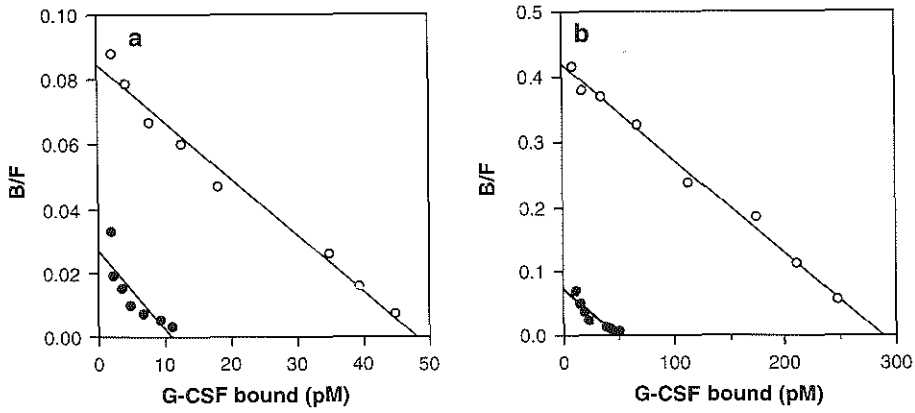

Figure 1. Downregulation of G-CSF receptors by TNF. (**a**) Scatchard plots of ^{125}I-G-CSF binding to AML cells: cells (4.9×10^6) of AML case 1 were pre incubated with (closed circles) or without (open circles) TNF (10^3 U/mL) in serum-free medium for 1 hour, and then subjected to radioreceptor studies with titrated concentrations of ^{125}I-G-CSF as described in Methods. (**b**) Scatchard plots of ^{125}I-G-CSF binding to AML cells: granulocytes (8.8×10^6) were incubated with (closed circles) or without (open circles) TNF (10^3 U/mL) for 1 hour. Data have been corrected for nonspecific binding. Each point represents the mean of duplicate estimations.

presence or absence of excess nonlabeled G-CSF (i.e., 200 nmol/L) exactly as described (22). Specific binding was determined as the difference between the amount of radioactivity bound in the absence, and the amount of radioactivity bound in the presence of nonlabeled G-CSF. Experiments were conducted in duplicate. Receptor numbers and binding affinity were derived following Scatchard analysis (23). In calculations, the maximal binding capacity was used to correct for the free counts.

RESULTS

TNF downregulates the number of high affinity G-CSF receptors on AML blasts. In previous experiments (22) we have demonstrated that AML blasts frequently show low levels of specific binding of ^{125}I-G-CSF, so that in practice it is difficult to assess a suppressive TNF effect on G-CSF receptor expression. The cells from cases 1, 2, and 3 were selected for the complete binding studies because of relatively high G-CSF specific binding as became evident from preliminary ^{125}I-G-CSF binding

Table 1. Effects of TNF on Binding of ^{125}I-G-CSF to AML Blasts

	without TNF		with TNF	
Case no.	Sites/cell	K_d (pmol/L)	Sites/cell	K_d (pmol/L)
1	577 ± 48	546 ± 106	123 ± 37	357 ± 64
2	357 ± 65	472 ± 83	114 ± 28	302 ± 46
3	243 ± 18	386 ± 129	96 ± 21	317 ± 93

Receptor numbers (mean per cell ± SD) and K_d (mean ± SD) were derived from binding experiments and subsequent analysis according to Scatchard. Aspirated bone marrow cells (2 to 5×10^6) were obtained from three cases with AML and used, in duplicate, for each point of analysis.

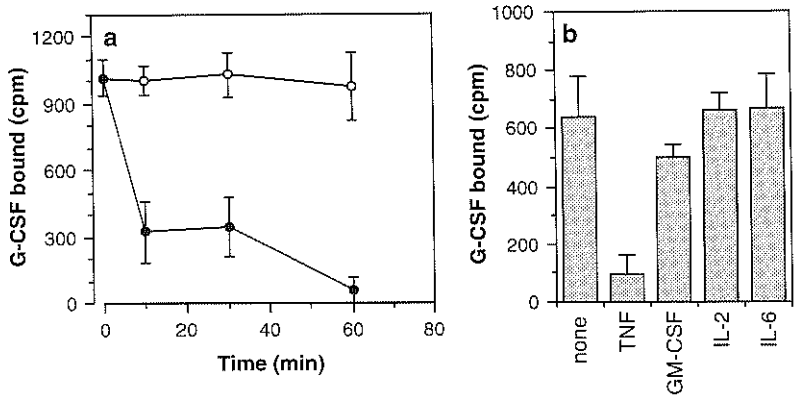

Figure 2. (**a**) Downregulation of G-CSF receptors by TNF as a function of time. Granulocytes (3 × 10^6) were incubated with (closed circles) or without (open circles) TNF (10^3 U/mL) for 0, 10, 30, or 60 minutes. Specific binding of ^{125}I-G-CSF (using 1 nmol/L ^{125}I-G-CSF) was subsequently determined as described in Methods. Values have been corrected for nonspecific binding and represent the means of triplicate estimations in cpm ± SD. (**b**) Downregulation of G-CSF receptors: specific ability of TNF. Granulocytes (2 × 10^6) were incubated with TNF and comparative cytokines, i.e., GM-CSF, IL-2, and IL-6, for 1 hour at 37°C and specific G-CSF binding was assessed (see a).

experiments (data not shown).

Complete binding assays with titrated concentrations of ^{125}I-G-CSF and subsequent Scatchard analysis for cases 1, 2, and 3 revealed that TNF reduced the average numbers of G-CSF receptors on the cells of the three cases by ~70% (Fig. 1a, Table 1). To exclude the possibility that these effect were caused by direct competition between TNF and G-CSF, specific binding of ^{125}I-G-CSF to AML blasts (case 1) was determined in the presence of excess TNF (400 nmol/L) at 0°C for 6 hours. Under these conditions, TNF did not inhibit specific binding of ^{125}I-G-CSF.

TNF down regulates the number of high affinity G-CSF receptors on blood granulocytes. In comparison, we have also determined the effect of TNF on G-CSF receptor expression on granulocytes. Preliminary ^{125}I-G-CSF binding experiments using granulocytes from the blood of four donors demonstrated that preincubation with TNF reduced specific binding of ^{125}I-G-CSF by 70 to 90%. Complete binding experiments (Fig. 1b) revealed that TNF reduced the number of G-CSF receptors on granulocytes from 1,969 ± 186 per cell (K_d 667 ± 109 pmol/L) to 342 ± 47 per cell (K_d 638 ± 76 pmol/L). The downregulatory effect of TNF was already apparent within 10 minutes after addition of TNF to granulocytes (Fig. 2a). This inhibitory effect did not become apparent when the cells were incubated under identical conditions with other recombinant cytokines, i.e., IL-2, IL-6, GM-CSF (Fig. 2b).

G-CSF receptor downregulation dependent on protein kinase C. G-CSF receptor downregulation could also be achieved following exposure of granulocyte to the PKC activator TPA (Fig. 3a). Complete binding experiments (Fig. 3b) using titrated concentrations of ^{125}I-G-CSF revealed that TPA reduced the numbers of G-CSF receptors on granulocytes from 1,332 ± 124 per cell (K_d 614 ± 98 pmol/L) to 487 ± 53

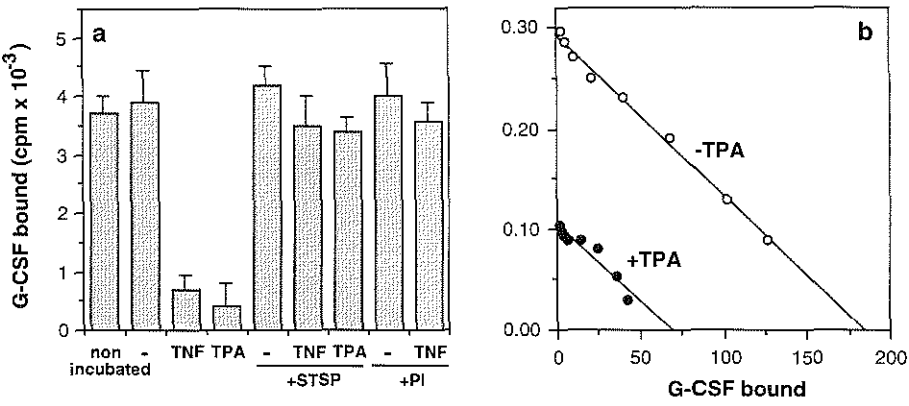

Figure 3. Downregulation of G-CSF receptors: (**a**) effect of TPA, Staurosporine, and protease inhibitors. Granulocytes (5×10^6) were incubated with no supplement (-), Staurosporine (STSP) (0.1 μM), or protease inhibitors (PI) for 30 minutes at 37°C. Then, TNF (10^3 U/mL) or TPA (50 ng/mL) was added for 60 minutes at 37°C. The specific binding of ^{125}I-G-CSF was assessed (see Fig. 2a). A control experiment was also done with non incubated cells. (**b**) downregulation of ^{125}I-G-CSF specific binding to granulocytes by TPA. Granulocytes (8.3×10^6) were incubated with (closed circles) or without (open circles) TPA (50 ng/mL) for 1 hour, and then subjected to radioreceptor studies (see legend to Fig. 1).

per cell (K_d 645 ± 132 pmol/L). To further investigate whether the mechanism of action of TNF might depend on activation of PKC, we have determined the effect of TNF or TPA (PKC activator) on specific binding of ^{125}I-G-CSF to granulocyte following pretreatment with Staurosporine (PKC inhibitor). Staurosporine reversed the inhibitory effect of both TNF and TPA (Fig. 3a). A cocktail of protease inhibitors could also abolish the effect of TNF under the same conditions (Fig. 3a).

DISCUSSION

This study was carried out to investigate the possibility that antagonistic effects between TNF and G-CSF on AML cell proliferation are accomplished at the level of G-CSF membrane receptors. The results reveal that incubation of the cells with TNF decreases G-CSF receptor numbers both on AML blasts and blood granulocytes considerably, i.e., by ~70% of the initial mean receptor number. Suppression of G-CSF receptor binding by TNF could not be attributed to direct cross competition between G-CSF binding and TNF binding because this phenomenon does not occur at 0°C. It is likely that the negative effect of TNF on the level of G-CSF receptor expression may contribute to the antagonistic relationship between the two factors. A reduction of the mean receptor density on the cells may reflect the loss of receptors of a proportion of G-CSF reactive cells and thus these cells may become nonresponsive to G-CSF. The loss of G-CSF stimulability of AML blasts and granulocytes as controlled by TNF may represent an important regulatory function in hematopoiesis (24,25).

G-CSF receptor downregulation by TNF can be characterized by the following features: G-CSF surface receptors disappear relatively rapidly (within 10 minutes), suppression occurs at 37°C, but not at 0°C, maximal suppression is attained at 60

minutes (data not shown), and finally it involves a loss of G-CSF binding sites without an apparent change in receptor affinity. The fact that reduction in G-CSF receptor density by TNF in AML cells and granulocytes is not complete (70%), raises the possibility of functionally different subsets of G-CSF receptors. We have found that TPA can also downregulate G-CSF receptors under the same conditions (Fig. 3a) and that the G-CSF receptors inhibitory effect due to TNF or TPA can be reversed by Staurosporine (PKC inhibitor) or protease inhibitors (Fig. 3a). It is possible that the analogy with the observation of Downing et al (26), who showed that the M-CSF receptor is downmodulated by its ligand as well as by TPA, TNF acts through activation of PKC. PKC, in turn, activates the release of proteases that specifically cleave off the ligand-binding domain from the receptor.

It remains unclear what the physiological significance of receptor downregulation by TNF in AML cells is. However, it is conceivable that a higher concentration of G-CSF is required to excite the same number of receptors after TNF exposure, and thereby rendering the cells less susceptible to G-CSF. The fact that TNF upregulates on the same cell type, receptor for IL-3 and GM-CSF (both early acting factors) (27) suggests that TNF acts as a response modulator. By increasing the susceptibility for IL-3 and GM-CSF and at the same time decreasing the susceptibility for G-CSF, TNF elicits a more immature response pattern to these three CSFs.

The observation that the survival of neutrophils in vitro can be increased by G-CSF (28) is interesting in view of the downregulation of G-CSF receptors on neutrophils by TNF. Possibly TNF acts as a shut-off signal for granulocytes in circulation. However, more research needs to be done to elucidate the biological meaning of this phenomenon.

CHAPTER 7

COMMON BINDING STRUCTURE FOR GM-CSF AND IL-3 ON HUMAN AML CELLS AND MONOCYTES

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SUMMARY

Granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3) control the proliferation of human acute myeloid leukemia (AML) cells in vitro. Previously, we have shown that receptors for GM-CSF and IL-3 are often coexpressed on AML cells. Here we present experiments with purified AML blasts, normal monocytes and granulocytes, that were conducted to analyze the properties of GM-CSF and IL-3 binding proteins in more detail. On AML cells from 8 cases we demonstrate two types of GM-CSF receptors: one with low affinity (dissociation constant {K_d} 5.1 to 24.8 nmol/L) and one with a high affinity (K_d 31 to 104 pmol/L). These AML cells also expressed high affinity receptors for IL-3 (K_d 24 to 104 pmol/L). Cross competition experiments showed that an excess concentration of nonlabeled IL-3 completely prevented the high affinity binding of radiolabeled GM-CSF. This competition occurred at 37°C as well as 4°C. Low affinity GM-CSF binding was not affected by IL-3. Binding of radiolabeled IL-3 could be prevented by nonlabeled GM-CSF. In certain cases, this competition was complete, whereas in others only partial (49 to 77%) reduction of the radiolabeled IL-3 binding was seen. On the basis of these ligand binding features, we propose the existence of three receptor types on AML cells: (i) low affinity GM-CSF receptors that do not bind IL-3, (ii) dual high affinity GM-CSF/IL-3 receptors, and (iii) high affinity IL-3 receptors that do not bind GM-CSF. We could also demonstrate these receptor types on normal monocytes. In addition, a fourth type of receptor was apparent on normal granulocytes (iv), incapable of binding IL-3 and with an intermediate affinity for GM-CSF (~400 pmol/L). Chemical cross-linking showed that GM-CSF and IL-3 both bind to proteins with molecular weight values of 130, 105 and 75, which provides additional evidence for the existence of a common GM-CSF/IL-3 receptor complex.

INTRODUCTION

GM-CSF and IL-3 are hematopoietic growth factors (GF) with multilineage stimulative abilities (1-4) that exert their effects on the cells through specific membrane receptors (5). These factors also induce proliferation of acute myeloid leukemia (AML) cells (6-10). GM-CSF and IL-3 receptors are frequently coexpressed on AML cells (11), and the mitogenic responses among the different cases of AML to GM-CSF and IL-3 often coincide (10). Parallel effects of GM-CSF and IL-3 on colony formation from purified normal human marrow progenitors have also been demonstrated (12,13). These studies suggest that the control of growth and differentiation of hematopoietic cells by IL-3 and GM- CSF is closely interrelated. Recently, evidence has been obtained for the existence of a receptor structure on AML blasts and leukemia cell lines with a high affinity for both GM-CSF and IL-3 (14-16). In the present study, we compared the abilities of AML cells, monocytes and granulocytes to bind GM-CSF and IL-3. We confirm that AML blasts, as well as monocytes express membrane structures that can bind GM-CSF and IL-3 both with an equally high affinity (K_d ~ 60 pmol/L). Preliminary crosslinking experiments indicate that both GM-CSF and IL-3 bind to proteins with Mrs of 130, 105 and 75 kDa, the latter probably being a product of proteolytic cleavage. In addition to this common GM- CSF/IL-3 binding site, three selective GM-CSF or IL-3 binding sites are distinguished, i.e., a low affinity GM-CSF receptor with no affinity for IL-3 (AML

cells and monocytes), a high affinity IL-3 receptor that does not bind GM-CSF (monocytes and some cases of AML) and a receptor with intermediate affinity for GM-CSF that does not bind IL-3 (neutrophils).

MATERIALS AND METHODS

Patients and purification of AML cells and normal cells. Bone marrow (all cases except case 6) or peripheral blood (case 6) was taken from eight patients with AML diagnosed according to the French-American-British (FAB) classification (17,18) (AML case 8: FAB M1; case 2: FAB M2; case 6 FAB M3; cases 1, 3, 4 and 7: FAB M4; case 5: FAB M5). The leukemic cells were separated after bovine serum albumin density gradient (BSA) or Ficoll–Isopaque centrifugation and subsequent removal of E-rosette forming cells (19) and cryopreserved (6). The viability of the cells after thawing was always more than 95%. White blood cells (WBC) were separated from the blood of healthy subjects following sedimentation in 0.1% methyl cellulose. Granulocytes were obtained as the sedimented cell fraction following Ficoll-Isopaque centrifugation of WBC and consisted of 90-95% neutrophils, 2-6% eosinophils and 3-5% monocytes/lymphocytes. Monocytes were obtained from the Ficoll-Isopaque interface of WBC and subsequent removal of E-rosette forming cells and consisted of 70-80% monocytes, 20-30% non E-rosetting lymphocytes and less than 2% granulocytes.

Growth factors and radioiodination. Recombinant human GM-CSF (CHO cell-derived; glycosylated, MW 20-30 kDa) (20) was prepared and purified at Genetics Institute (Cambridge, MA). Recombinant human IL-3 (Yersinia derived; nonglycosylated, MW 15 kDa) was prepared and purified at Gist Brocades (Delft, The Netherlands) (21). Both proteins were radiolabeled with Bolton and Hunter reagent (Amersham Laboratories, Amersham, UK) (22) as described (11). Specific activity of radiolabeled GM-CSF and IL-3 as determined by self displacement analysis (23), was $5\text{-}7 \times 10^4$ cpm/ng for GM-CSF and $8\text{-}10 \times 10^4$ cpm/ng for IL-3. The iodinated preparations retained their ability to stimulate colony formation by normal bone marrow cells in methyl cellulose (11). TCA precipitation showed less than 5% nonprecipitable radioactivity for both factors. The maximum binding capacity was estimated at 40 to 65% for IL-3 and 50 to 95% for GM- CSF. In Scatchard calculations the "free" cpm were corrected for the maximum binding capacity (23).

Binding of labeled GM-CSF and IL-3 to AML blasts, monocytes and granulocytes. GM-CSF and IL-3 binding to the cells was assessed as described (11). Equilibrium binding conditions for both GM-CSF and IL-3 at 37°C and 0°C were reached in 1 and 17 hours respectively (11,15,24). Two to 7×10^6 cells were incubated for 1 hour at 37°C or 18 hours at 4°C in 100 µL α-Minimal Essential Medium with 1% BSA in the presence of 20 to 6,000 pmol/L radiolabeled IL-3 or GM-CSF with or without excess (i.e., 200 nmol/L) nonlabeled factor. Specific binding was defined as the difference between the amount of radioactivity bound in the absence of nonlabeled factor, and the amount of radioactivity bound in the presence of excess nonlabeled factor. Experiments were conducted in duplicate. Receptor numbers and binding affinities were derived following Scatchard analysis and two affinity receptor analysis was performed by the R-Binding program (25,26). Binding characteristics of low affinity

Table 1. Summary of cross competition data in AML and monocytes

		High affinity ^{125}I-GM-CSF binding		High affinity ^{125}I-IL-3 binding	
	case	no competitor	IL-3 competition	no competitor	GM-CSF competition
AML cells	1	217 (31)	0	135 (52)	31 (20)
	2	53 (104)	0	14 (58)	0
	3	61 (57)	0	60 (60)	20 (30)
	4	120 (46)	0	124 (103)	0
	5	275 (70)	0	226 (86)	0
	6	95 (56)	0	121 (36)	0
	7	122 (42)	0	147 (37)	76 (151)
	8	112 (63)	0	234 (24)	0
Monocytes	1	56 (42)	0		ND
	2	348 (106)	0		ND
	3	218 (34)	0	115 (82)	
	4		ND	143 (40)	86 (31)

Values represent mean numbers of binding sites per cell determined from radiolabeling experiments with AML cells (see materials and methods; Fig. 2). K_d values (pmol/L) are indicated in brackets. Low affinity GM-CSF binding was not competed for by nonlabeled IL-3 (see Fig. 2). ND, not determined. , Indicates specific binding detectable but too low for complete Scatchard analysis.

GM-CSF binding sites were assessed by self displacement of 1 nmol/L ^{125}I-GM-CSF, essentially as has been described by Robb et.al. for the low affinity IL-2 receptor (27).

Crosslinking of receptors. Two to 5×10^7 AML cells were incubated with radiolabeled IL-3 or GM-CSF (2 nmol/L) both with and without excess (200 nmol/L) nonlabeled IL-3 or GM-CSF. After 1 hour, the cells were chilled on ice and layered on top of 1 mL fetal calf serum (FCS). After centrifugation for 5 minutes, the cell pellet was rinsed twice in ice-cold HBSS and resuspended in 300 µL PBS. Ethylene glycolbisuccinimidyl succinate (EGS, PIERCE chemical company, Rockford, IL) was then added at a final concentration of 1 mmol/L and incubated at 4°C for 30 minutes. After two acid washes with sodium citrate (pH 4) to remove noncrosslinked but receptor bound ligand (27), the cells were fragmented by repeatedly freezing and thawing in hypotonic phosphate buffered solution (50 mmol/L sodium chloride, 2 mmol/L potassium chloride and 5 mmol/L phosphate buffer). Membrane fragments were isolated after spinning down the nuclei by high speed centrifugation (90 minutes, 60,000 g). In all steps the protease inhibitors, phenyl methylsulphonyl fluoride (PMSF; 2 mmol/L), leupeptin (10 µmol/L), iodoacetamide (2 mmol/L), pepstatin (10 µmol/L) and aprotinin 1 mg/mL were present. Pelleted membrane fragments were boiled in SDS buffer (0.2 mol/L Tris HCl, pH 6.8, 6% SDS. 1% beta-mercaptoethanol, 40% glycerol and bromophenol blue) for 10 minutes and membrane preparations were then analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE, 7.5%) under reducing conditions and subsequent autoradiography.

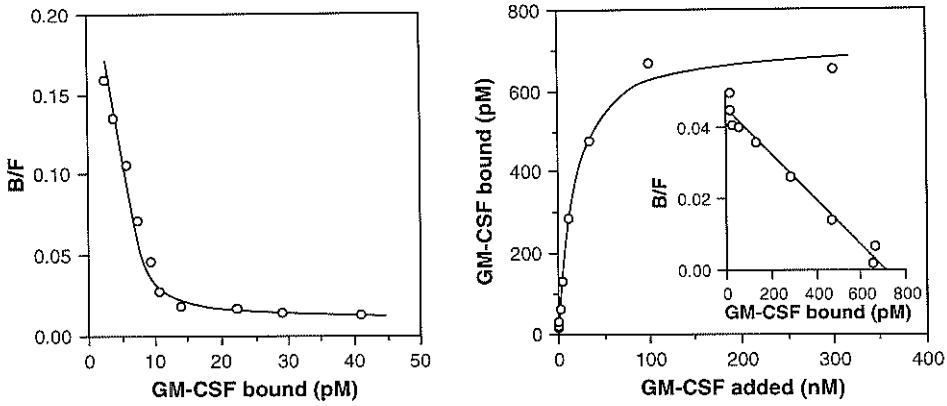

Figure 1. High and low affinity GM-CSF receptors on AML cells. (**a**) Cells (4×10^6) of AML case 7 were incubated with ^{125}I-GM-CSF (30 to 4000 pmol/L) for 18 hours at 4°C. The biphasic Scatchard plot indicates distinct high and low affinity type GM-CSF receptors. (**b**) Binding of GM-CSF to low affinity receptors (AML case 5) was measured in the presence of 1000 pmol/L ^{125}I-GM-CSF and titrated concentrations of nonlabeled GM-CSF (0 to 300 nmol/L). Total binding of GM-CSF was calculated from the ratio labeled/nonlabeled GM-CSF and specific binding of radiolabeled GM-CSF. Subsequent Scatchard analysis of those data revealed 9503 sites per cell, K_d 15.8 nmol/L. Each point represents the mean of two estimations. Nonspecific binding was determined in the presence of excess nonlabeled GM-CSF and was subtracted prior to plotting data.

RESULTS

Binding of radiolabeled GM-CSF and IL-3 to leukemic cells. Binding of titrated concentrations of radiolabeled GM-CSF to AML cells (from 40 to 7,000 pmol/L) consistently produced biphasic Scatchard plots indicating high and low affinity components (Fig. 1a). The average numbers of high affinity GM-CSF receptors among 8 cases was estimated at 132 per cell (distribution 53-275 sites per cell; $K_d \pm$ S.D.: 59 ± 22 pmol/L)(Table 1). Estimations of the numbers and affinity of the low affinity GM-CSF receptors derived from binding experiments with concentrations of radiolabeled GM-CSF up to 7,000 pmol/L showed considerable standard variations, since the estimation of the binding maximum in these calculations could only be reached by extrapolation. In order to assess the low affinity binding component more accurately, titrated concentrations of nonlabeled GM-CSF were added to the cells in the presence of 1 nmol/L radiolabeled GM-CSF. From these experiments total binding of GM-CSF (labeled plus nonlabeled) to the cells could be calculated (27) (Fig. 1b). Subsequent calculations showed saturable binding at GM-CSF concentrations of 33 to 100 nmol/L with a K_d of 5.1 to 24.6 nmol/L (mean ± S.D.: 13.2 ± 6.2 nmol/L). The average numbers of low affinity GM-CSF receptors was 4,688 per cell (range 1,099 to 11,900 sites per cell). In all 8 cases, also receptors for IL-3 could be demonstrated on the AML cells (Table 1). The average density of IL-3 binding sites was estimated at 117 per cell (range 14 to 234) and the average K_d (± S.D.) was estimated at 57 ± 26 pmol/L.

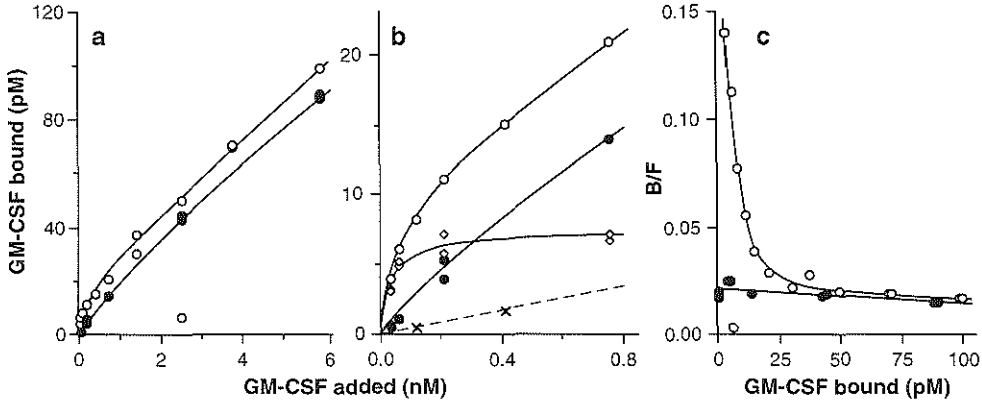

Figure 2. Inhibition of High affinity GM-CSF binding by IL-3 (**a**) AML cells (2×10^6; case 5) were incubated with increasing concentrations of ^{125}I-GM-CSF for 1 hour at 37°C in the absence (open circles) or presence (closed circles) of 200 nM IL-3. The upper curve represents high and low affinity GM-CSF binding, the lower represents low affinity binding of GM-CSF alone. Both curves have been corrected for nonspecific binding. (**b**) The central panel shows in detail (abscissa 0 to 0.8 nM) the same data. Nonspecific binding, determined in the presence of 200 nM nonlabeled GM-CSF (--x--) and high affinity binding (diamonds) determined as the result of GM-CSF binding in the absence of IL-3 minus binding in the presence of IL-3 are shown in addition. The latter curve reaches a plateau at a GM-CSF concentration of 200 pM. (**c**) Scatchard plots of these data showing that IL-3 selectively competes for high affinity GM-CSF binding.

Competition of ^{125}I-GM-CSF binding to AML cells by IL-3. Binding of radiolabeled GM-CSF to AML cells was reduced by an excess concentration of nonlabeled IL-3 (Fig. 2a). The difference between ^{125}I-GM-CSF binding in the absence and presence of nonlabeled IL-3 appeared as a readily saturable component of total binding (Fig. 2b). The Scatchard plot of these data shows that in the presence of IL-3 the steep segment of the biphasic curve disappeared, indicating that IL-3 prevented high affinity ^{125}I-GM-CSF binding, but not low affinity ^{125}I-GM-CSF binding (Fig. 2c). Data from 8 cases of AML are summarized in Table 1. Tumor necrosis factor (TNF) or G-CSF in concentrations up to 100 nmol/L did not affect binding of radiolabeled GM-CSF to AML cells.

Competition of ^{125}I-IL-3 binding to AML cells by GM-CSF. Binding of radiolabeled IL-3 was prevented in the presence of 200 nmol/L GM-CSF in 5 cases

Table 2. GM-CSF receptors on granulocytes

	GM-CSF binding			
	no competitor		after IL-3 competition	
Donor no.	sites/cell	K_d (pmol/L)	sites/cell	K_d (pmol/L)
1	1,949	405	2,090	422
2	2,720	363	2,673	332
3	2,343	469	2,393	444
4	2,155	469	2,393	403

Receptor numbers (mean per cell) and K_d were derived from Scatchard plot analysis (see materials and methods). Granulocytes were obtained from 4 different normal donors.

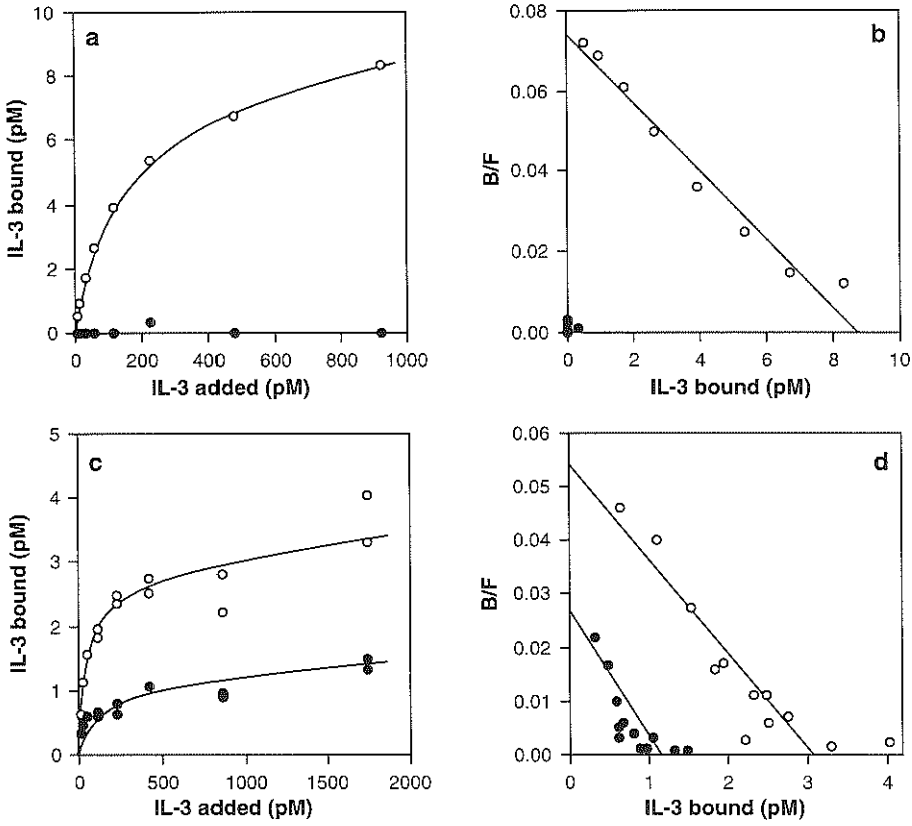

Figure 3. Inhibition of ^{125}I-IL-3 binding to AML by GM-CSF. (a) Binding (AML case 4) of ^{125}I-IL-3 was determined in the absence (open circles) or presence (closed circles) of 200 nmol/L GM-CSF at 4°C. Specific binding of IL-3 was completely blocked by GM-CSF. (b) The same experiment with cells from case 3 showing that GM-CSF reduced IL-3 binding only partially (68%). All data represent specific binding after subtraction of nonspecific binding.

(cases 2, 4, 5, 6 and 8; Fig. 3a, Table 1). In the remaining 3 cases this inhibition was partial (Fig. 3b, Table 1). Scatchard analysis of the IL-3 receptors of the latter three cases revealed that only 23-51% of the initially established numbers of receptors had remained detectable in the presence of excess GM-CSF (Table 1). The affinity of the latter receptors was comparable to the affinity of those that had been estimated in the absence of GM-CSF (Table 1). These results suggest that there are two different high affinity IL-3 binding sites in AML. The first one appears as a dual binding site that can interact with GM-CSF and IL-3, and is detectable in all 8 cases of AML. The second type of IL-3 binding site demonstrated in 3 of the 8 cases does not bind GM-CSF.

Cross competition of ^{125}I-GM-CSF and ^{125}I-IL-3 at 4°C. Cross competition of ^{125}I-IL-3 binding with GM-CSF and, reciprocally, ^{125}I-GM-CSF binding by IL-3 was apparent within 1 hour of incubation at 37°C and 18 hours at 4°C (Fig. 4). The amount of acid resistant radioactivity after 18 hours incubation at 4°C was

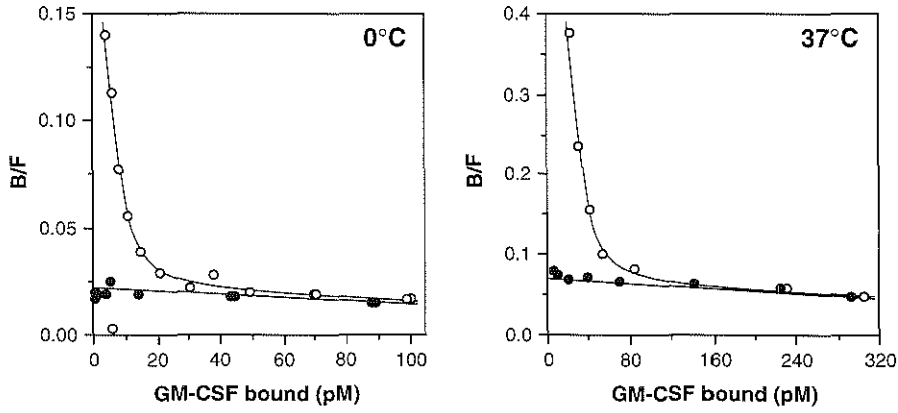

Figure 4. Temperature independence of cross competition of ^{125}I-GM-CSF binding. Scatchard plots derived from binding experiments with radiolabeled GM-CSF performed at 37°C (left panel: AML case 5; 2×10^6 cells per tube; 1 hour incubation time) and at 4°C (right panel: AML case 4, 6×10^6 cells per tube, 18 hours incubation time) in the absence (open circles) or presence (closed circles) of 200 nM IL-3.

approximately 8%, indicating that 92% of the radioactive ligand had remained surface bound under these conditions (results not shown). These data suggest that the cross inhibition of binding for both factors does not require active cell metabolism and therefore resulted from direct interference at the receptor level (rather than down modulation).

Dose dependence of GM-CSF and IL-3 cross inhibition. We then determined the comparative efficiency of GM-CSF and IL-3 as competitors for radiolabeled GM-CSF binding to AML cells, first of patient no. 5 (Fig. 5a). A fixed concentration of radiolabeled GM-CSF (450 pmol/L), deducted from binding curves with and without excess nonlabeled IL-3 to give approximately equal of ^{125}I-GM-CSF binding to high and low affinity binding sites, was displaced by titrated concentrations of nonlabeled GM-CSF or IL-3. Displacement of radiolabeled GM-CSF by IL-3 was dose dependent, but leveled off at a higher plateau than GM-CSF, in accordance with the fact that IL-3 competes for the high affinity GM-CSF binding sites only. From this experiment it was also evident that 200 nmol/L IL-3 (as used in the cross competition experiments) was sufficient to occupy all high affinity dual GM-CSF/IL-3 binding sites. Displacement of radiolabeled IL-3 from AML cells of patient no. 7 by GM-CSF did not equal the displacement by IL-3 (Fig. 5b), illustrating that on these cells the two types of IL-3 receptors were present; i.e., one competed and one not competed for by GM-CSF. The same experiment was performed with AML cells from another patient (case 4) showing complete inhibition of ^{125}I-IL-3 binding by excess GM-CSF (Fig. 5c). As expected, binding of radiolabeled IL-3 was displaced to the same extent by both IL-3 and GM-CSF, confirming that the cells from this AML case indeed only carried the dual GM-CSF/IL-3 binding sites. From the observation that the displacement curves attained by nonlabeled GM-CSF and IL-3 are identical in this experiment, it appears that the common GM-CSF/IL-3 binding structure has equal affinity for both ligands (Fig. 5c).

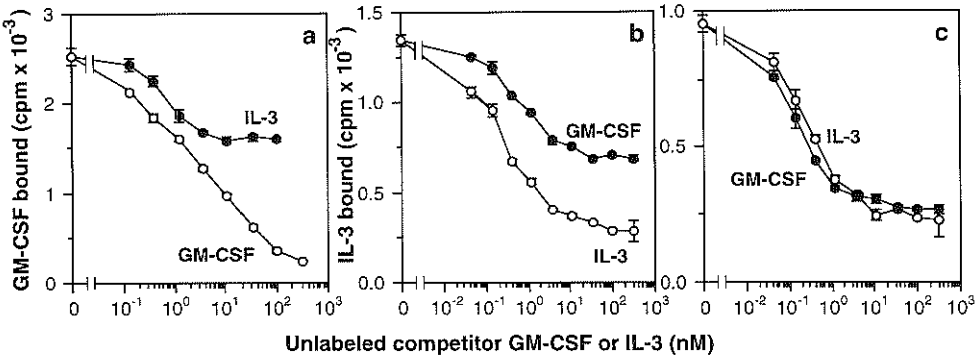

Figure 5. Displacement of radiolabeled GM-CSF or IL-3 from AML cells by homologous and heterologous ligand. (**a**) Cells (4×10^6, AML case 5) were incubated with 450 pmol/L ^{125}I-GM-CSF plus titrated concentrations of either nonlabeled GM-CSF or IL-3 (0 to 300 nmol/L) as competitors for 45 minutes at 37°C. The upper curve shows that IL-3 competed for GM-CSF binding in a dose dependent way. The maximal competition was reached at IL-3 concentration of approximately 5 nmol/L. (**b**) The reciprocal displacement experiment (AML case 7; 7×10^6 cells per tube) using 250 pmol/L ^{125}I-IL-3 showing that IL-3 was displaced by 55% of total specific binding in a dose-dependent manner by GM-CSF. Maximal displacement was reached at a concentration of approximately 5-10 nmol/L for both GM-CSF and IL-3. (**c**) Like (**b**) (AML case 4; 4×10^6 cells per tube), both competitors displaced IL-3 from the cells equally well.

Binding of radiolabeled GM-CSF to normal cells. Binding of radiolabeled GM-CSF to monocytes did not appear saturable at GM-CSF concentrations up to 5 nmol/L and the Scatchard plot was curvilinear (not shown) indicating that, similar to AML, high and low affinity GM-CSF binding was involved. Cross competition with IL-3 showed that the high affinity component of ^{125}I-GM-CSF binding could be inhibited by IL-3. Calculations from three experiments revealed an average number of 207 high affinity binding sites (range 56 to 348) (Table 1) and 4,320 low affinity sites (range 1,087 to 7,892) for GM-CSF on monocytes. Binding of radiolabeled GM-CSF to normal granulocytes was saturable at a GM-CSF concentration of approximately 2 nmol/L and was not affected by the addition of 200 nmol/L IL-3 (Table 2). Thus, the GM-CSF receptor on normal granulocytes appears not capable of binding IL-3. Scatchard analysis of ^{125}I-GM-CSF binding to granulocytes from 4 different donors showed an average of 2,292 binding sites per cell (range 1,949 to 2,729) of single affinity with a K_d of 400 ± 50 pmol/L, which is intermediate between the high and low affinity GM-CSF receptors in AML.

Binding of radiolabeled IL-3 to normal cells. Monocytes expressed 115 to 143 high affinity IL-3 binding sites per cell (K_d ± SD: 61 ± 30 pmol/L), which were also partially inhibited by excess nonlabeled GM-CSF (Table 1). Scatchard analysis revealed 38 to 112 high affinity IL-3 receptors per cell (K_d ± SD: 147 ± 79 pmol/L) on granulocyte samples from three different donors. These samples contained 90-95% neutrophils, 2-6% eosinophils and 3-5% monocytes/lymphocytes. Because eosinophils have been reported to express up to 2,000 IL-3 binding sites per cell (11), it is most likely that the eosinophils in these cell samples had been responsible for IL-3 binding.

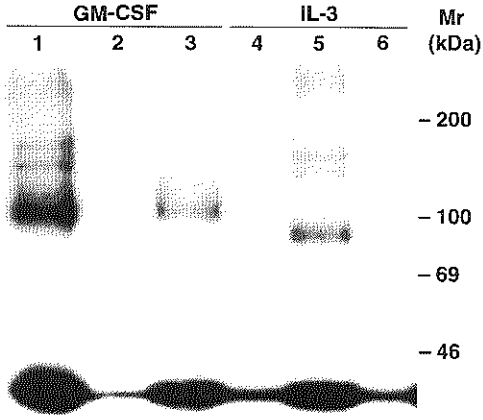

Figure 6. Affinity labeling of the IL-3 and GM-CSF receptors on intact AML cells by chemical crosslinking. ^{125}I-GM-CSF (lane 1-3) or ^{125}I-IL-3 (lane 4-6) were crosslinked to 4×10^7 cells (AML case 5) as described in materials and methods either without nonlabeled factor (lane 1 and 5) or in the presence of excess nonlabeled GM-CSF (lane 2 and 6) or IL-3 (lane 3 and 4) in the initial binding reaction. The crosslinked species were resolved by SDS PAGE. The autoradiograph was obtained by exposing the gel to Kodak X-Omat AR film at -70°C for 4 weeks.

Crosslinking of receptors. Chemical crosslinking was performed to estimate the molecular weights of the GM-CSF and IL-3 binding proteins. Affinity crosslinking using radiolabeled IL-3 and GM-CSF and AML cells is shown in Fig. 6. The autoradiograph demonstrates the migration of three conjugates with apparent MW of 100, 130 and 155 kDa for ^{125}I-GM-CSF (Fig. 6, lane 1). Also, three different conjugates for ^{125}I-IL-3 with MW of 90, 120 and 145 kDa appeared (Fig. 6, lane 5). In 6 separate experiments using cells from different patients the same three bands for GM-CSF as well as IL-3 were observed. In the presence of 100 fold overdose nonlabeled GM-CSF or IL-3 in the initial binding reaction, no bands appeared on the autoradiograph (Fig. 6, lanes 2 and 4). After subtraction of the molecular weights of the ligands (GM-CSF: 25 kDa; IL-3: 15 kDa), GM- CSF and IL-3 binding membrane proteins are estimated to have equal MW of 75, 105 and 130 kDa.

DISCUSSION

In this study we provide evidence for the presence of a binding structure on AML cells and monocytes with high affinity for both IL-3 and GM-CSF. This conclusion has been based upon two sets of experimental findings: (i) Radiolabeled ligand binding showed bidirectional cross competition between GM-CSF and IL-3 binding to AML cells and monocytes. This competition occurred equally well at 37°C and 4°C, indicating that receptor down-modulation was not involved in the observed GM-CSF/IL-3 receptor interaction. (ii) Chemical crosslinking of GM-CSF and IL-3 to AML cells revealed that both factors bind to proteins with similar electrophoretic mobility on SDS polyacrylamide gels with MWs estimated at 70, 105, and 130 kDa. These findings are in agreement with observations recently made by other investigators (14-16) who postulated three subtypes of IL-3 and GM-CSF receptors on human leukemia cell lines and AML cells, one being capable of binding both ligands. We have at present no experimental data that could explain the mechanism of IL-3 and GM-CSF cross competition. The fact that no sequence homology between GM-CSF and IL-3 has been found (28) would be remarkable although not impossible in view of a shared single binding domain on the receptor. Alternatively, the

common GM-CSF/IL-3 binding structure may consist of two separate binding domains which each have high affinity for their respective ligands. Cross competition between the two ligands could then occur through conformational changes upon binding of either ligand preventing binding of the other. It is also possible that the binding domains are located in such proximity that binding of one ligand will preclude binding of the other due to steric hindrance.

We have obtained evidence for the presence of two types of GM-CSF receptors on AML cells and monocytes. In our experiments on human AML with concentrations of radiolabeled GM-CSF up to 6-7 nmol/L, Scatchard plots were in favor of two affinities. When comparable concentrations of radiolabeled GM-CSF were applied to granulocytes, complete saturation was evident (Table 2 and references 11, 24, 29, 30), indicating that the low affinity component that was observed in AML was not inherent to an artifact of the experimental procedure or the result of impaired binding of the ligand due to the labeling procedure. Further evidence for the existence of low affinity receptors for GM-CSF was derived from displacement of radiolabeled GM-CSF by its nonlabeled homologue (Fig. 1b and 5a) and from crosslinking experiments with radiolabeled GM-CSF in the presence of IL-3 (Fig. 6). In earlier reports this distinction of two affinity GM-CSF receptor types has not been made (11,29-31). This discrepancy can be explained by the fact that the initial measurements were performed over only a limited range of free ligand concentrations. Consequently it becomes difficult to distinguish between single affinity versus multiple affinities because of failure to detect a curvature in the Scatchard plot, and one may inadvertently select the wrong model of data fitting. Indeed the existence of high and low affinity GM-CSF receptors is in agreement with later reports on human cell lines, monocytes and AML cells (14,32,33).

Crosslinking of GM-CSF binding membrane proteins revealed three bands of which the lower was markedly broader than the upper two. The fact that omission of protease inhibitors produced an even broader lower band at the expense of the upper two (results not shown) suggested that this band is at least partially the product of proteolysis as has been demonstrated for the IL-3 receptor in mice (34). Since the addition of excess IL-3 in the initial binding reaction with radiolabeled GM-CSF equally reduced the intensity of all three bands, these bands appear all related to the common GM-CSF/IL-3 binding structure (Fig. 6, lane 3). Crosslinking of IL-3 binding proteins revealed components of equal mass.

Granulocytes express GM-CSF receptors that do not bind IL-3 and show an intermediate affinity as compared to the high and low affinity GM-CSF binding sites on AML cells and monocytes. The observation of intermediate affinity GM-CSF binding to granulocytes adds a third distinct GM-CSF receptor type. IL-3 competition experiments did not provide evidence for the presence of this receptor type on AML (Table 2). The physiological relevance of an intermediate affinity GM-CSF receptor could be that neutrophils will be functionally activated by GM-CSF only at relatively high tissue concentrations of GM-CSF; e.g., induced by bacterial infection.

The potential biological role of low affinity GM-CSF binding to AML cells and monocytes remains elusive. Thus far no biological effects have been reported in response to GM-CSF at the nmolar concentration range. Possibly this low affinity GM-CSF binding site represents a precursor of the functional GM-CSF receptor.

The fact that IL-3 and GM-CSF bind to common or closely related structures, raises the possibility that alternative factors trigger the same target cells through a common receptor and perhaps activate the cells via identical intracellular signal pathways. This would explain the marked parallelism of stimulative abilities in normal precursor cells and AML of the two hematopoietins.

CHAPTER 8

GM-CSF Receptors Alter Their Binding Characteristics During Myeloid Maturation Through Upregulation of the Affinity Converting β Subunit (KH97).

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SUMMARY

AML blasts express dual affinity (high and low) GM-CSF binding, and the high affinity GM-CSF binding is counteracted by excess IL-3. Neutrophils express a single class of GM-CSF-R with intermediate affinity that lack IL-3 cross reactivity. Here we demonstrate the differentiation associated changes of GM-CSF binding characteristics in three models representative of different stages of myeloid maturation. We find that high affinity GM-CSF binding is converted into intermediate affinity binding, which still cross reacts with IL-3, beyond the stage of promyelocytes. During terminal maturation towards neutrophils, IL-3 cross reactivity is gradually lost. We sought to determine the mechanism underlying the affinity conversion of the GM-CSF-R. Northern and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of GM-CSF-Rα and β_c (KH97) transcripts did not provide indications for the involvement of GM-CSF-R splice variants in the formation of the intermediate affinity GM-CSF-R complex. In COS-cell transfectants with increasing amounts of β_c in the presence of a fixed number of GM-CSF-Rα chains, the high affinity GM-CSF binding converted into intermediate affinity GM-CSF binding. These results are discussed in view of the concept that increasing expression of β_c subunits may cause alternative oligomerization of the GM-CSF-Rα and β_c subunits resulting in the formation of intermediate rather than high affinity GM-CSF-Rα/β_c complexes.

INTRODUCTION

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) is a hematopoietic growth factor (GF) with multilineage stimulative abilities (1-4), that exerts its effects on hematopoietic cells through specific membrane receptors (5). At the immature stages of myeloid development, GM-CSF regulates proliferation and commitment. In mature neutrophils GM-CSF has a role in functional activation. In immature myeloid cells there is a considerable degree of functional overlap between GM-CSF and interleukin-3 (IL-3) and to some extent also interleukin-5 (IL-5) (6-9).

A common feature of GM-CSF, IL-3 and IL-5 receptors is that the high affinity interaction of these growth factors with their receptors requires the presence of two different receptor subunits, an α and a β subunit. The α subunits are specific for GM-CSF, IL-3 and IL-5 and bind to their respective ligands with low affinity. The affinity converting β subunit, alternatively referred to as β_c or KH97 (10), is shared by the α chains of GM-CSF-R, IL-3-R and IL-5-R in the formation of the respective high affinity receptor complexes (9,11). This explains why GM-CSF can compete with IL-3 and IL-5 in high affinity binding to various human cell types (12-19).

Acute myeloid leukemia (AML) blast cells express both high affinity receptors (K_d ~50 pmol/L) which cross reacts with IL-3 and low affinity GM-CSF binding sites (K_d 5-20 nmol/L) (12-15). In contrast, peripheral blood neutrophils express GM-CSF receptors with a single, intermediate affinity (12-15,18) (K_d ~400 pmol/L), that cannot be competed for by IL-3. No explanation has yet been given for the differences in ligand binding characteristics of GM-CSF-R between AML blasts and neutrophils. Moreover, it has not previously been investigated whether the GM-CSF binding characteristics progressively change during maturation from myeloblasts

towards neutrophils, or whether these alterations occur at the terminal stage of neutrophilic differentiation.

In the present study, we first determined at which stages of maturation the GM-CSF binding features change. For this purpose we used three leukemia models: 1) primary AML cells that mature spontaneously in culture towards myelocytes, 2) chronic phase CML cell samples containing myelocytes, metamyelocytes and band forms and 3) the promyelocytic cell line HL-60 in which retinoic acid (RA) induces neutrophilic differentiation. We subsequently performed a series of experiments to analyze which mechanisms can be held responsible for the GM-CSF-R affinity changes that take place during myeloid maturation.

MATERIALS AND METHODS.

Cells. AML cells from 2 cases of AML, cytologically classified as FAB M1 (22,23) and light density CML chronic phase cells of 3 patients were recovered from the interface of Ficoll-Isopaque centrifugation of WBC from peripheral blood. The cellular composition of these samples is shown in Table 1. None of the patients had received treatment before cell samples were drawn. Normal peripheral blood granulocytes (90-95% pure) were obtained after sedimentation of the blood of healthy subjects in 0.1% methyl cellulose and Ficoll-Isopaque centrifugation (24) as previously described (25). The HL-60 cell line was obtained at passage level 28 (26) from Dr Farzaneh, King Cross College, London UK.

In vitro culture. The HL-60 cells were maintained in RPMI/10% FCS at a density of maximally 5-6 $\times 10^5$ cells/mL. Granulocytic maturation in HL-60 was induced by incubating exponentially growing cells (6×10^5/mL) in RPMI/10% FCS with 1μM all trans retinoic acid (RA, Sigma Chemical CO, St Louis, MO) for 5 or 6 days. After culture with RA the percentage of cells with polymorphic nuclei exceeded 90% (Table 1). The AML cells were cultured in RPMI/10% FCS with 100 pmol/L IL-3 and after 8-20 days harvested for receptor assays. Examination of cytospin smears of cultured AML cells showed that the cells had lost their blastic appearance and had proceeded towards promyelocytic/myelocytic stages of maturation (Table 1).

Radioiodination of growth factors and binding experiments. Recombinant human GM-CSF (glycosylated, chinese hamster ovarium (CHO) cell derived; MW 20-30 kDa) (27) and IL-3 (Escherichia coli derived; MW 15 kDa) (28) were prepared and purified at Genetics Institute (Cambridge, MA). GM-CSF and IL-3 were radiolabeled with Bolton and Hunter reagent (Amersham Laboratories, Amersham, UK) (29) as described (30). Specific activities of radiolabeled GFs as determined by self displacement analysis (31), were 5-7 $\times 10^4$ cpm/ng for GM-CSF and 4-6 $\times 10^4$ cpm/ng for IL-3. Trichloroacetic acid precipitation showed less than 5% nonprecipitable radioactivity for both factors. The maximum binding capacity was estimated at 90% to 95% for GM-CSF and 55% to 60% for IL-3. In Scatchard calculations the "free" cpm were corrected for the maximum binding capacity (31).

GM-CSF and IL-3 receptor analysis were performed as previously described (30). Cross competition experiments were performed with 200 nmol/L heterologous GF (12). Experiments were carried out in duplicate or triplicate. Receptor numbers and

Table 1. Morphology of AML, CML and HL60 cell samples

Cells		myeloblasts	promyelo-cytes	myelocytes	metamyelo-cytes	band forms	segmented	other *
AML #1	d0	67	12	12				9
	d8	12	36	30	7			15
AML #2	d0	58	29					9
	d10	6	28	57				9
CML #1		10	16	16	31	27		
CML #2		8	12	17	32	28		3
CML #3		9	31	30	30			
HL-60			100					
HL-60 + RA					45	33	22	

Numbers indicate percentages of total cells scored. *: indicates other morphologies, i.e., of megakaryocytic, monocytic, and eosinophilic lineages.

binding affinities were derived from Scatchard plot analysis, using the ENZfitter program (Sigma Chemical CO, St Louis, MO).

PCR analysis of GM-CSF-Rα and β transcripts. Total RNA was isolated from the cells after lysis in guanidium thiocyanate containing buffer (32) followed by cesium chloride gradient centrifugation (32). cDNA prepared from these RNAs using reverse transcriptase (MMLV-RT superscript, GIBCO BRL, Gaithersburg MD) were used as templates in the PCR analysis. Primers spanning the extracellular plus transmembrane domain were: 5' TCAGATCTGCACCATGCTTCTCCTG 3' and 5' CCTTTTAAAGAGGAAGCCG 3' corresponding to bp 137 to bp 1193 of the published GM-CSF-Rα sequence (33) and 5' CAGAGCTGACCAGGGAGATGG-TGCT 3' and 5'GTAGATGCCACAGAAGCG 3' corresponding to bp 12 to bp 1426 of the published β_c sequence (10).

Construction of GM-CSF-Rα and β deletion mutants and expression in COS cells. Deletion mutants of both GM-CSF-R subunits, that lacked the complete cytoplasmic part (GM-CSF-RαΔcyt, GM-CSF-RβΔcyt) were obtained by PCR from complete cDNAs, using the primers indicated above. Both truncated receptor cDNA fragments were cloned into the $pCMV_4$ expression plasmid ($pCMV_4$-GM-CSF-RαΔcyt, $pCMV_4$-GM-CSF-RβΔcyt) (34). GM-CSF-Rα cDNA, provided by Dr. N. Gough (33) was cloned into the $pCMV_4$ expression plasmid. The β_c expression plasmid (pSVhuIL-5-Rβ (9)) was provided by Drs. G Plaetinck and J. Tavernier (Roche Research, Gent, Belgium). COS-1 cells were transfected with (complete or truncated) α and β subunits by $CaPO_4$ precipitation (32). The expression of β_c was varied by changing the amount of β_c expression plasmid in the $CaPO_4$ precipitate. Forty-eight hrs post transfection the COS-1 cells were detached and harvested with 1 nmol/L EDTA, gently forced through a 22 gauge needle to obtain a single-cell suspension and used in binding studies.

(^{3}H)-hymidine incorporation into AML cells. DNA synthesis of AML cells was measured as described (6). Two × 10^4 cells were cultured for 3 days in 96-well round-bottom microtiter trays in 100 µL serum-free medium, with or without addition of IL-3 or GM-CSF. Four hours before harvesting, 0.1 µCi titrated thymidine

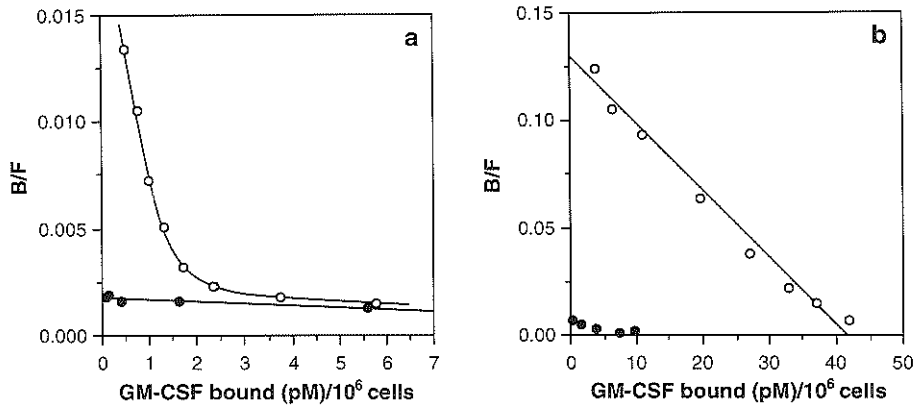

Figure 1. Scatchard plot of GM-CSF receptor-binding in AML cells (case 1) before and after culture. (**a**) AML cells: fresh (6.4×10^6 cells per tube) and (**b**) after 20 days of culture ($.95 \times 10^6$ cells per tube) were incubated with ^{125}I-GM-CSF (40-5000 pmol/L). Binding of GM-CSF was determined as described in Materials and Methods. Scatchard plots of parallel GM-CSF binding experiments that were performed in the absence (open circles) or presence of excess IL-3 (closed circles). Data were corrected for nonspecific binding and normalized for cell numbers (data are expressed as binding per 10^6 cells). Each point represents the mean of two estimations.

(Amersham) was added. Cells were harvested on nitrocellulose paper using a Titertek Harvester 550 (Flow Laboratories, Isrike, UK). Radioactivity was determined with a scintillation counter (Beckman LS 3800, Fullerton, CA). All experiments were performed in triplicate and data expressed as mean dpm.

RESULTS

Comparison of GM-CSF receptor numbers and affinity in noncultured versus cultured AML cells. Binding characteristics of GM-CSF receptors on AML blasts (case 1) before and after culture are shown in Fig. 1 and Table 2. In line with previous observations (12-15,18), the noncultured cells expressed few high affinity GM-CSF receptors (60 sites per cell; K_d 50 pmol/L) and ~30 fold more low affinity GM-CSF receptors (1,900 sites per cell; K_d 20 nmol/L). In the presence of excess (200 nmol/L) nonlabeled IL-3, GM-CSF binding to the high affinity GM-CSF receptors was completely prevented (Fig. 1A). In contrast, after 8-20 days of culture, the cells, then predominantly showing a promyelocytic/myelocytic morphology, had lost the characteristic high affinity GM-CSF binding. In return, intermediate affinity GM-CSF binding sites appeared (Fig. 1B) (K_d=350 pmol/L; R=2,700 sites per cell). Low affinity binding was markedly reduced. Excess IL-3 (200 nmol/L) virtually prevented all GM-CSF binding (81-93 %; Fig. 1B). The remainder of the GM-CSF bound with a low affinity. Theoretically, the presence of low affinity GM-CSF binding after cross competition with IL-3 could be explained by the residual presence of AML blast cells after culture (Table 1), which express excess GM-CSF-Rα chains. However, because the presence of low affinity binding was not evident from the Scatchard analysis without IL-3 competition, it is more likely that the residual low affinity GM-CSF binding is caused by the GM-CSF-Rα subunits dissociated from the intermediate affinity GM-CSF-R complex after IL-3 competition. Comparable GM-CSF binding

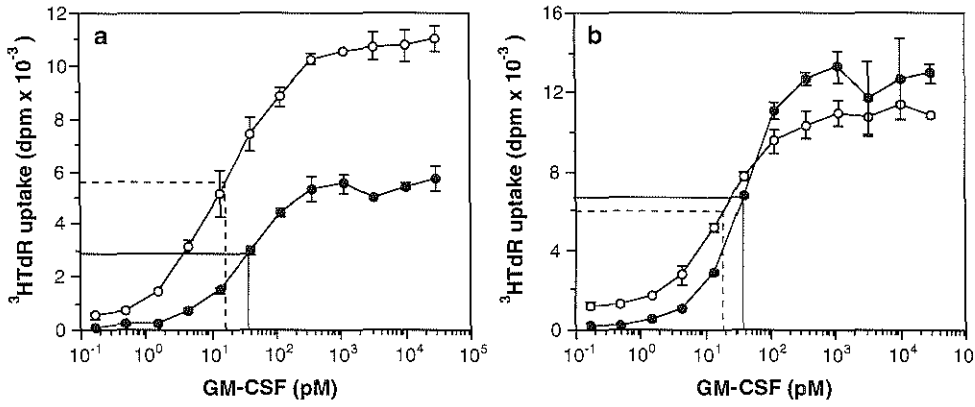

Figure 2. Sensitivity to GM-CSF on fresh and cultured AML cells. DNA synthesis of AML cells (**a** case 2, **b** case 1) that had been cultured for 10 days (closed circles) as well as freshly thawed AML cells (open circles) was determined in the presence of titrated concentrations of GM-CSF. ED50 values for each dose response curve are annotated.

results before and after in vitro culture were obtained in the other AML case (Table 2).

GM-CSF binding to CML cells. The CML chronic phase cells from all three cases exclusively expressed GM-CSF receptors of intermediate affinity type (K_d 330-390 pmol/L), at densities that were somewhat lower than those on normal granulocytes (Table 2). Excess IL-3 competed 20-60% of labeled GM-CSF binding (Table 2). In cases 1 and 2, some low affinity binding appeared to be present after cross competition with IL-3 (Table 2). However in all cases the major portion of IL-3 resistant GM-CSF binding sites expressed the characteristic intermediate affinity.

GM-CSF binding to HL-60 cells before and after treatment with all trans retinoic acid. HL-60 cells expressed 380-470 GM-CSF binding sites per cell of intermediate affinity (K_d 200-370 pmol/L, Table 2). Excess nonlabeled IL-3 competed 30-40% of ^{125}I-GM-CSF binding to HL-60 cells. The K_d value of the GM-CSF binding that remained detectable after IL-3 competition was lower than in the absence of IL-3, suggesting the presence of low affinity binding. After exposure of the HL-60 cells to RA (5-6 days), IL-3 cross reactive GM-CSF binding was completely lost (Table 2). Parallel experiments with radiolabeled IL-3 showed that upon RA treatment the HL-60 cells lost IL-3 binding sites (data not shown).

GM-CSF induces a proliferative response in AML cells expressing intermediate affinity GM-CSF receptors. We measured DNA synthesis in response to GM-CSF in AML cells before (expressing high and low affinity GM-CSF receptors) and after 10 days of culture (when the cells had acquired intermediate affinity GM-CSF receptors). In both cases the cells were still able to respond to GM-CSF after 10 days of culture, indicating that the intermediate affinity GM-CSF receptor is still capable of conducting a mitogenic signal (Fig. 2). The plateau levels of GM-CSF induced DNA synthesis before and after culture did not differ significantly. However, the

Table 2. GM-CSF binding characteristics of AML, CML, HL60 cells and neutrophils

Cells		without IL-3		with IL-3 (200 nmol/L)	
		R	K_d (pmol/L)	R	K_d (pmol/L)
AML #1	d0	60 ± 45	50 ± 16	0	
		1,900 ± 950	20,000 ± 13,000	1,900 ± 780	18,000 ± 7,900
	d8	2,600 ± 82	390 ± 38	ND	
	d20	2,700 ± 50	350 ± 17	720 ± 150	3,000 ± 1,100
AML #2	d0	59 ± 3	44 ± 7	ND	
		1,600 ± 570	30,000 ± 14,000		
	d10	430 ± 14	360 ± 39	♦	
CML #1		610 ± 10	330 ± 20	450 ± 25	610 ± 93
CML #2		540 ± 18	340 ± 30	220 ± 23	980 ± 250
CML #3		520 ± 8	390 ± 16	360 ± 8	410 ± 25
HL60	exp. 1	380 ± 8	370 ± 16	240 ± 9	1,100 ± 71
	exp. 2	470 ± 9	200 ± 9	310 ± 9	120 ± 10
HL60+RA	d5	530 ± 28	250 ± 35	515 ± 59	250 ± 74
	d6	1,230 ± 36	450 ± 33	1,220 ± 46	490 ± 48
Neutrophils (n= 3)		2,100 ± 180	410 ± 53	2,200 ± 160	420 ± 21

Mean numbers of binding sites (R) (mean ± SD) and dissociation constant (K_d)(± SD) were derived from radiolabeling studies with noncultured cells or cells that were cultured during indicated periods (AML and HL-60 cells). Data were analyzed with the program ENZfitter (Elsevier Biosoft). : Binding data were in favor of dual binding characteristics. ♦: Specific binding detectable, but too low for Scatchard analysis. ND: not determined.

dose response curves are indicative of a slight loss of sensitivity to GM-CSF after culture (ED50 before culture: ~20 pmol/L; ED50 after culture: ~40 pmol/L), possibly as a result of the lowered affinity GM-CSF receptor on the cultured cells.

RT-PCR analysis of the extracellular domains of the GM-CSF-Rα and β subunits. By Northern analysis, transcripts for GM-CSF-Rα and β could be detected in AML cells, CML cells, HL-60 cells as well as neutrophils (data not shown). To investigate whether splice variants of the GM-CSF-R extracellular domains would be involved in the formation of intermediate affinity GM-CSF receptors, we analyzed transcripts of the GM-CSF-Rα and β in cells at different stages of myeloid maturation by RT-PCR analysis using PCR primers spanning the extracellular plus transmembrane regions of the α and β chains. We PCR amplified from all cell samples the fragments of the expected lengths of the wild type receptor 1056 nt and 1414 nt respectively, but no alternatively sized fragments (Fig. 3). These data rule out alternative RNA splicing as a mechanism involved in the formation of the intermediate affinity GM-CSF receptors.

Increasing expression of β_c in COS cells results in the conversion from high affinity towards intermediate affinity of the GM-CSF receptor complex. Expression of GM-CSF-Rα and β in principle can reconstitute high affinity GM-CSF receptors in COS cells (10). Truncation of the cytoplasmic part of GM-CSF-Rα and/or β_c does not

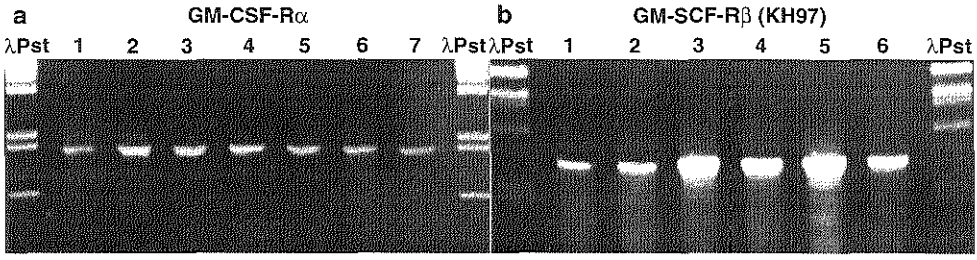

Figure 3. PCR amplification of extracellular domains of GM-CSF-Rα (**a**) and βc (**b**) from AML (lanes a1-a3, b1 and b2); AML day 10 (lane b3); HL-60 (lanes a4 and b5); CML (lane b4) and neutrophils (lanes a5, a6 and b6). λPst indicates Lambda-Pst I markers.

affect the formation of the high affinity GM-CSF receptor complex formed in COS cell transfectants (Fig. 4A). Because immature myeloblasts express few (50 to 100) high affinity GM-CSF receptors, whereas differentiated cells express much higher numbers (2000 to 3000) of intermediate affinity receptors and because Northern analysis indicated that β_c transcripts but not GM-CSF-Rα transcripts increase with progressive maturation, we considered the possibility that increased availability of β_c could be held responsible for the affinity changes during myeloid maturation. To verify this hypothesis, we performed COS-cell transfections in which the GM-CSF-Rα chain expression was held at a constant level and the expression of β_c was increased (Fig. 4B). Indeed we found that increases in the expression of β_c in relation to GM-CSF-Rα affected the affinity of the GM-CSF-Rα/β_c complex in a manner similar to the affinity changes in the leukemia models.

DISCUSSION

The data of this study show that the properties of GM-CSF-R on hematopoietic cells alter with progressive myeloid maturation. During the conversion from myeloblasts towards (pro)myelocytes, the cells lose the characteristic high and low affinity GM-CSF binding to the GM-CSF-Rα/β_c complex and the single GM-CSF-Rα chain, respectively, and acquire intermediate affinity GM-CSF receptors. At this stage of maturation, cross competition by IL-3 for β_c affects the intermediate affinity GM-CSF binding. In the phase during which the cells mature towards terminally differentiated granulocytes, cross competition by IL-3 is gradually lost. This gradual loss of cross competition by IL-3 is caused by the disappearance of IL-3-Rα subunits. In agreement with this notion, RA treated HL-60 and neutrophils were found to lack significant IL-3 binding.

We have attempted to elucidate the events that take place during the first stage of myeloid maturation, leading to the loss of high and low affinity GM-CSF binding and the appearance of intermediate affinity receptors. Two mechanisms have previously been reported to affect the affinity of hemopoietin receptors. Fukunaga et al. (35) have demonstrated that structural alterations of the extracellular domain of the human and murine G-CSF-R, either resulting from naturally occurring alternative RNA splicing or caused by experimentally created deletions, may result in the decreased affinity of the G-CSF-R. We therefore investigated whether GM-CSF-Rα and or β_c splice variants with altered extracellular domains could be

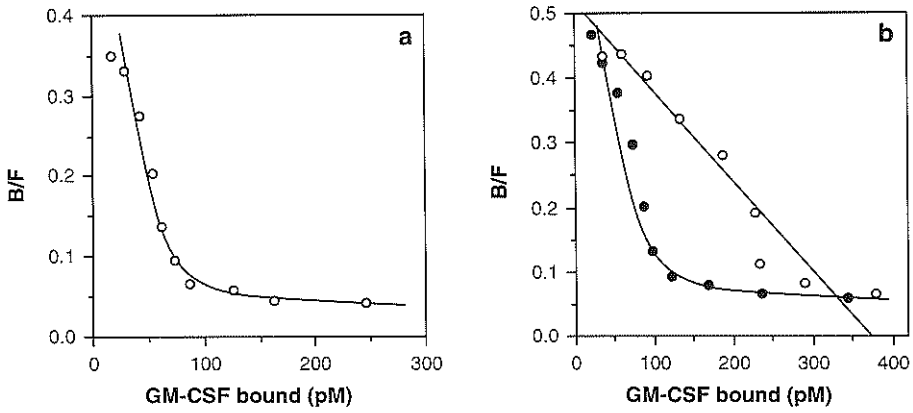

Figure 4. Scatchard plots of GM-CSF receptor-binding in COS transfectants (**a**) GM-CSF-R cytoplasmic deletion mutants expressed in COS cells reconstitute high affinity GM-CSF binding. Scatchard plot of GM-CSF receptor-binding in COS-1 cells cotransfected with GM-CSF-RαΔcyt and GM-CSF-RβΔcyt (K_{d1}=79 pmol/L, R_1=2,100 sites/cells, K_{d2}=18 nmol/L, R_2=25,000 sites/cell). (**b**) The affinity of the GM-CSF receptor complex in COS-cells alters as a result of increased β_c expression. Scatchard plot of GM-CSF binding to COS-1 cells cotransfected with 10 μg GM-CSF-RαΔcyt expression plasmid and 10 μg (closed circles; K_{d1}=83 pmol/L, R_1=2,600 sites/cells, K_{d2}=24 nmol/L, R_2=37,000 sites/cell) or 50 μg (open circles; K_d=680 pmol/L, R=12,000 sites/cells) β_c expression plasmid.

detected in cells expressing the intermediate affinity GM-CSF-R. However, no evidence for the presence of such splice variants in cultured AML cells, HL-60 cells, CML cells and neutrophils was obtained.

The second mechanism that has been shown to influence receptor affinity is the involvement of additional receptor or receptor linked structures. Recently a third receptor chain, involved in the formation and function of the high affinity IL-2 receptor complex has been identified and molecularly cloned (36). COS-cell transfectants expressing human IL-5-Rα and β showed intermediate rather than high affinity IL-5 binding (9). Similarly, IL-6 binds with intermediate affinity to IL-6-R/gp130 COS cell transfectants (37). On this basis, the possible involvement of additional receptor structures in the formation of high affinity IL-5-R as well as the IL-6-R, LIF-R, OSM-R complex has been postulated (9,37). In contrast it has been reported, and here confirmed by us, that COS as well as NIH-3T3 cells (data not shown), cotransfected with GM-CSF-Rα and β_c cDNAs can express high affinity GM-CSF binding (10). Transfection of COS-cells with GM-CSF-Rα and β_c cytoplasmic deletion mutants also resulted in high affinity GM-CSF binding, proving that the intracellular domains are not critical for the formation of high affinity GM-CSF receptors. Thus, no experimental data have been forwarded to indicate that a component additional to the GM-CSF-Rα and β structures is involved in the formation of the high affinity GM-CSF-R complex. On the other hand, our data from COS cell transfection experiments established that the level of β_c expression relative to GM-CSF-Rα directly determines the affinity of the GM-CSF-R complex. Therefore, it appears most likely that during myeloid maturation, GM-CSF-R binding characteristics are altered simply by upregulation of the β_c subunit.

The exact configuration of the high and intermediate affinity GM-CSF-R is still unknown. Kaushansky et al. and Shanafelt et al. have shown that two regions (residues 14-24 and 77-94) in the C-terminus of human GM-CSF are important for biological activity (38,39). In addition the involvement of regions 40-77 and 110-127 in receptor binding has been suggested by Nice et al (40). These results predict that GM-CSF can bind to several distinct binding sites of the GM-CSF-R complex and open the possibility that alternative oligomerization of GM-CSF-Rα and β_c subunits determine the different affinities of the receptor complex. For instance, one could hypothesize that in case of low availability of β_c, a high affinity complex is formed by one α subunit and one β subunit, which could become an intermediate affinity receptor when increased availability of β_c permits $\alpha\beta\beta$ complexes. The latter alternative of receptor complex formation has recently also been proposed for the IL-6-R and LIF-R (41). Further investigations of the interactive domains of GM-CSF and the GM-CSF-R structures are required to solve this issue.

So far, no functional differences between high and intermediate affinity GM-CSF-R have been identified. AML cells expressing the intermediate affinity GM-CSF-R after culture were capable of responding to GM-CSF with only slightly decreased sensitivity as compared with the fresh cells that expressed the high affinity GM-CSF-R. Moreover identical tyrosine phosphorylation patterns were observed after GM-CSF stimulation of MO7e cells, which express high affinity GM-CSF-R and neutrophils (intermediate affinity GM-CSF-R) (42). What then, could be the physiological relevance of alternative oligomerization? Increasing evidence has become available that upon ligand induced di- or oligomerization of receptor subunits, interaction between the intracellular domains of the receptor structures is required for GF receptor activation (35,41,43). Soluble forms of GM-CSF-Rα and IL-5-Rα have been reported to antagonize the activation of the membrane receptors. These observations indicate that the intracellular domains of the GM-CSF-Rα and IL-5-Rα have a role in receptor activation. In contrast, association of a soluble form of the IL-6-Rα (lacking the trans membrane and cytoplasmic parts) with the signal transducing gp130 molecule results in the formation of a functional IL-6-R. Hence it is likely that gp130 subunit dimerization, rather than IL-6-Rα/gp130 dimerization activates the IL-6 signaling process (41). Based on these observations, we now assume that, depending on the availability of β_c subunits, GM-CSF signaling can be mediated through αβ or through αβ and ββ association. Whether these alternative ways of receptor activation have qualitative implications for GM-CSF mediated responses (other than mitogenic activation and tyrosine phosphorylation) or simply represent another example of receptor redundancy (44) remains to be answered. Hence, future investigations will focus on the role of the cytoplasmic domains of the GM-CSF-Rα and β_c subunits in signal transduction activated by the different oligomeric GM-CSF receptor complexes.

CHAPTER 9

THE CYTOKINE RECEPTOR SUPERFAMILY: CURRENT UNDERSTANDING

Introduction

Based on the nucleotide homology of binding domains, several classes of receptors have been identified (Chapter 1). Most HGFs bind to receptors that belong to the hematopoietin receptor superfamily or Class I cytokine receptors. These receptors are integral membrane glycoproteins, with an extracellular N-terminal domain, a single hydrophobic membrane spanning domain, and a C-terminal cytoplasmic domain. In this chapter, the structural and functional properties of the hematopoietin receptor superfamily will be discussed.

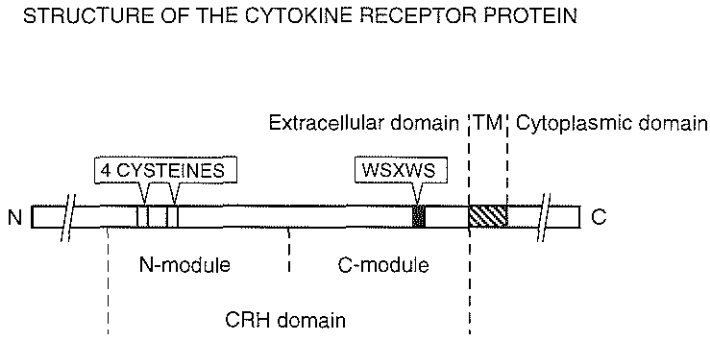

Figure 1. Linear representation of the conserved receptor homology domain of Class I cytokine or hematopoietin receptors. The common motif of these receptors is a ≈210 amino acid region in the extracellular domain that is conserved among all members of the superfamily. Characteristic features are the two sets of conserved cysteine residues in the N-terminal part of the extracellular region, and the WSXWS box in the membrane proximal C-module. TM denotes the transmembrane domain.

9.1 The cytokine receptor homology domain

The cytokine receptor superfamily is defined by a stretch of ≈210 amino acid (aa) residues in the extracellular domain that contains numerous conserved residues and that is essential for ligand binding. This region, referred to as the cytokine or hematopoietin receptor homology (CRH) domain, can be subdivided into two modules of ≈100 aa each (Fig. 1). The N-terminal module shows distinctive conservation of two sets of evenly spaced cysteine residues that form disulfide loops. The membrane proximal (C-terminal) domain contains the highly conserved Trp-Ser-X-Trp-Ser box (WSXWS). Strikingly, both modules express a significant resemblance to fibronectin (FN) type III domains. FN III domains are ≈90 aa modules that are present in adherence molecules expressed at the cell surface (1-3). The similarity of these structural characteristics in the conserved domain suggest that the members of the cytokine receptor family emerged either from a primitive adhesion molecule or from a common ancestral gene. This notion is further stressed by the presence of an additional triple repeat of a FN III domain proximal to the membrane in gp130, the LIF receptor and the G-CSF receptor.

Table 1 . The hematopoietin receptor family

Receptor	EC domain	WSXWS box	Cys	EC*	TM	IC	ref
huIL-2-Rβ	CRH	WSPWS	4	214	25	286	4
huIL-2-Rγ	CRH	WSEWS	4	232	29	86	5
huIL-3-Rα	N-CRH	LSAWS	4	287	20	53	6
muIL-3-Rα	N-CRH	LSSWS	4	315	24	41	7
huIL-4-R	CRH	WSEWS	4	207	24	569	8
muIL-4-R	CRH	WSEWS	4	208	24	553	9,10
huIL-5-Rα	N-CRH	WSEWS	4	315	♦		11
muIL-5-Rα	N-CRH	WGEWS	4	322	22	54	12,13
huIL-6-R	Ig-CRH	WSEWS	4	339	28	82	14
huIL-7-R	Ig-CRH	WSEWS	2	219	25	195	15
huIL-9-R	CRH	WSEWS	4	233	26	231	16
muIL-9-R	CRH	WSEWS	4	233	26	177	16
huGM-CSF-Rα	N-CRH	WSSWS	4	297	27	54	17
muGM-CSF-Rα	N-CRH	WGEWS	4	295	25	38	18
KH97 ($huβ_c$)	$(CRH)_2$	PSKWS/WSEWS	4/4	422	27	432	19
AIC2A ($muβ_{IL3}$)	$(CRH)_2$	PSRWS/WSEWS	4/4	417	26	413	20
AIC2B ($muβ_c$)	$(CRH)_2$	PSRWS/WSKWS	4/4	418	26	430	21
huG-CSF-R	Ig-CRH-$(FN\text{-}III)_3$	WSDWS	4	603	26	183	22,23
muG-CSF-R	Ig-CRH-$(FN\text{-}III)_3$	WSPWS	4	601	24	187	24,25
huEPO-R	CRH	WSAWS	4	225	22	240	26
muEPO-R	CRH	WSAWS	4	225	23	235	27
hu gp130	Ig-CRH-$(FN\text{-}III)_3$	WSDWS	4	597	22	277	28
mu gp130	Ig-CRH-$(FN\text{-}III)_3$	WSDWS	4	595	22	278	29
huLIF-R	$(CRH)_2$-$(FN\text{-}III)_3$	WSDWS/WSKWS	4/2	789	26	238	30
huMPL-R	$(CRH)_2$	WSGWS/WSSWS	4/4	463	22	122	31
huPRL-R	CRH	WSAWS	4	210	24	364	32
huGH-R	CRH	FSEVL	4	246	24	350	33
huCNTF-R	Ig-CRH	WSDWS	4	352			34

CRH, cytokine receptor homology domain; Ig, immunoglobulin-like fold; N, non conserved "cap-region" of ≈80-100 residues characteristic for the α-subunits of IL-3, GM-CSF and IL-5 receptors; FN-III, fibronectin type III domain; Cys denotes the number of conserved cysteines in the CRH domain; EC, TM, IC denote the number of amino acids in the extracellular, transmembrane and intracellular domains respectively; *indicates the number of amino acid residues of the mature protein, i.e., without the signal peptide; hu, human receptor subunit; mu, murine receptor subunit; ♦, only the soluble IL-5-R was cloned , CNTF-R is anchored to the cell membrane by glycosyl phosphatidyl inositol linkage (GPI).

The class I cytokine receptor family does not exclusively contain receptor structures for hematopoietic growth factors and cytokines. Prolactin receptor, growth hormone (GH)-R, and ciliary neurotrophic factor (CNTF)-R also belong to this family (Table 1). In some members of the family, variations of the common motif are apparent. For example LIF receptor, AIC2A, AIC2B, and KH97, have a duplication of the CRH domain (Table 1, Fig. 2). In the latter three receptor molecules this duplication has been accompanied by a replacement of the first residue of the WSXWS motif in the most N-terminally located CRH domain to a tryptophan. Furthermore, the G-CSF, IL-6, CNTF, and IL-7 receptors contain an additional immunoglobulin-like domain, located N-terminal to the CRH domain. LIF receptor, gp130, and G-CSF receptor contain three repeats of a FN type III domain between the CRH and transmembrane domains. Although in several members of the cytokine receptor family the FN-III and Ig domains constitute a substantial part of the extracellular domain, they have not proven to serve any known function. Both the Ig

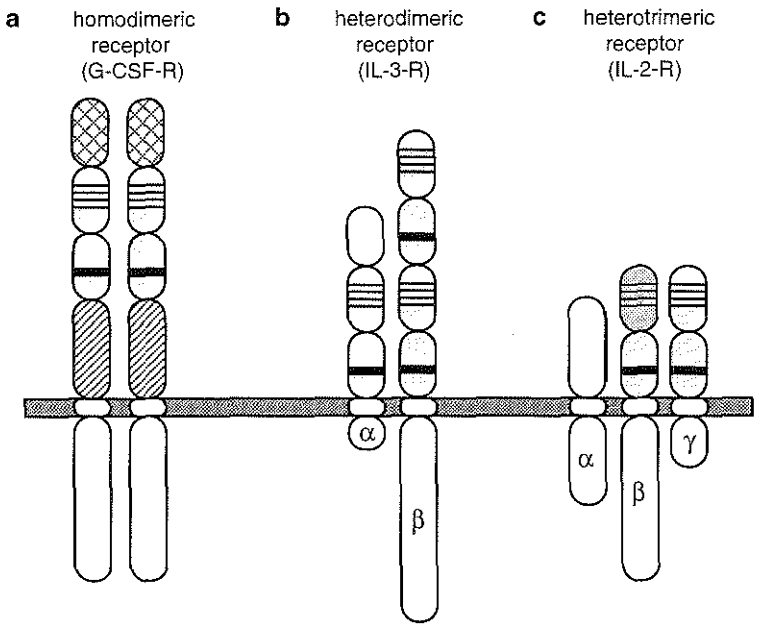

Figure 2. Different dimeric or oligomeric forms of high affinity hematopoietin receptor complexes: (**a**) Cytokine receptors that have high affinity ligand binding by homodimerization, (**b**) heterodimeric cytokine receptors that require two distinct subunits to form a high affinity receptor, (**c**) heterotrimeric high affinity receptor complex. Stippled shading represents CRH domain; hatched area represents triple fibronectin type III domains; crosshatched area represents immunoglobulin-like domains (see Table 1). The G-CSF, IL-3, and IL-2 receptors are shown as examples. IL-2-Rα is not a member of the hematopoietin receptor family.

and FN-III domains in the G-CSF receptor as well as the Ig domain of the IL-6 receptor are not required for ligand binding or signal transduction (35,36).

9.2 HGF receptor splice variants

Splice variants of mRNA have been described for most receptors of the cytokine super family including those of G-CSF, GM-CSF and IL-3. Strikingly, alternative mRNA splicing most often involves exons encoding the transmembrane or intracellular domains. The physiological significance of this phenomenon is still unclear, but expression of different receptors may represent a regulatory mechanism (see also 9.6). Most of the splice variants give rise to soluble receptor forms. Specific transcripts for soluble forms have been identified for the IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, GM-CSF, G-CSF and GH receptors (10,11,16,23,33,37-39). Given the general occurrence of soluble receptor forms, they most likely have important biological functions. They may act antagonistically by forming complexes with secreted HGFs, thus preventing HGFs to interact with their target cells. Alternatively, soluble forms of receptors can also be involved in the formation of functional cellular receptors. This was demonstrated for the soluble forms of IL-6 and CNTF receptors. In the

presence of their respective ligands these receptors associate with the signal transducing gp130, to form high affinity receptor complexes (see below)(28,34).

9.3 Formation of high affinity receptor complexes

High affinity ligand binding involves the association of two or more receptor subunits (di- or oligomerization), a feature which was already shown to be essential for activation of tyrosine kinase receptors (25,39-47).

In certain receptors, two identical subunits form a high affinity (homo)dimeric complex. For instance the high affinity G-CSF and EPO receptors are homodimers (24,42). Although the exact composition of the high affinity IL-4 and IL-7 receptor is not fully clear, it has been suggested that these receptors also form homodimeric complexes (8,48,49) (Fig. 2a). Receptor complex formation may also involve the heterodimerization of two distinct receptor subunits, α and β. For instance, the receptors for human IL-3, GM-CSF, IL-5, IL-6, LIF, CNTF, and IL-11 receptors (2,11,13,19,28,50-53) (Fig. 2b). The α-subunits are primarily cytokine binding proteins and bind their respective ligands with low affinity. The β-subunits are affinity converters, required for the formation of a high affinity binding site and are primarily also responsible for signal transduction. Usually the α-subunits are expressed more abundantly on the cell surface than the β subunits. This explains why receptors of this group also have a low affinity ligand binding component in addition to the high affinity binding site. A third subunit, the γ subunit, for the IL-2 receptor has been cloned. The IL-2-Rγ is required to reconstitute a functional IL-2-Rβ (5), which then can form the functional high affinity IL-2 receptor in association with IL-2-Rα (Fig. 2c). The involvement of a third receptor subunit for high affinity binding has also been suggested, but not proven, for the IL-6, LIF, and OSM receptor system (53). The high affinity CNTF receptor appears to be composed of three subunits, the CNTF-Rα, gp130, and the LIF-R (54,55).

9.4 IL-3, GM-CSF, and IL-5 receptors: a common β subunit involved in the formation of functional receptor complexes

GM-CSF and IL-3 bind to high affinity and low affinity receptors, which are expressed on immature cells and monocytes (Chapter 1). A striking feature of IL-3 and GM-CSF is their cross reactivity with each other's or common receptors on human AML cells, monocytes, eosinophils, and several human myeloid cell lines (56-60,Chapter 7). Cross reactivity between IL-3 or GM-CSF and IL-5 binding has been reported on human eosinophils and basophils (61,62). In contrast to AML cells, mature granulocytes express a single class intermediate affinity GM-CSF receptor, which lacks IL-3 cross reactivity. This finding suggests that the status of the GM-CSF receptor is related to the stage of differentiation. Mechanisms that have been reported to affect the affinity of HGF receptors include glycosylation (EPO-R (27)) or the involvement of additional receptor structures (IL-2-R (5)).

Following the molecular cloning of the GM-CSF receptor (17) it was shown that this receptor binds GM-CSF with the same affinity as the low affinity GM-CSF binding component on AML cells and monocytes. Expression of this receptor

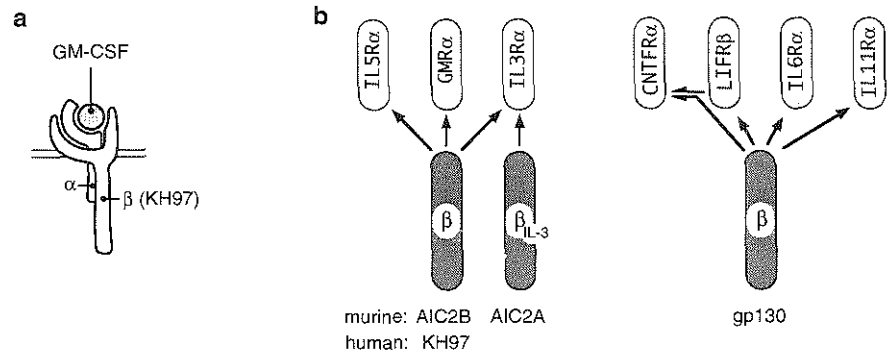

Figure 3. (**a**) The heterodimeric GM-CSF receptor. The high affinity receptor complex is reconstituted by the low affinity GM-CSF receptor (α-subunit) coexpressed with KH97. (**b**) Relationship between IL-3, GM-CSF and IL-5 receptor subunits; CNTF, LIF, IL-6 and IL-11 receptor subunits. The lines indicate possible associations of α and β-subunits.

subunit in factor dependent myeloid cell lines failed to reconstitute a normal functional high affinity GM-CSF receptor. An important step forward came when another member of the hematopoietin family, KH97, was cloned. This protein, which by itself can not bind GM-CSF, was able to convert the low affinity GM-CSF receptor into a biologically functional high affinity GM-CSF receptor (19). In analogy to the IL-2 receptor system, the low affinity GM-CSF receptor was designated the α-subunit (GM-CSF-Rα) and KH97 the β-subunit (Fig. 3a). Subsequent cloning of the IL-3-Rα and IL-5-Rα chains revealed that these subunits utilize KH97 to form high affinity receptors for human IL-3 and IL-5 (6,11,43). The heterodimerization of unique IL-3, GM-CSF and IL-5 α-subunits with a common β-subunit explains how these cytokines can compete for binding to their receptor (Fig. 3b). Reconstitution experiments in 3T3 cells or COS-1 cells show that indeed cross competition can be restored, if the expression of KH97 compared to the α-subunits is low (6). In this situation one ligand can inhibit the (high affinity) binding of another ligand by depleting the availability of the common β-subunit.

In contrast to the human system, two β-subunits have been identified in the mouse: AIC2A and, the highly homologous IL-3 receptor-like protein, AIC2B (20,21). AIC2A was originally identified as the low affinity murine IL-3 receptor and shown to be part of the high affinity IL-3 receptor (63,64). In COS cells, AIC2B did not bind any growth factor at all. Since KH97 was cloned from a cDNA library using AIC2A as a probe, AIC2A and AIC2B were likely to be β subunits in the mouse. The cloning of the murine low affinity IL-3-Rα, GM-CSF-Rα and IL-5-Rα, confirmed that AIC2B was an affinity converter for the murine IL-3, GM-CSF and IL-5 receptors (7,18,44,45). AIC2B, thus appeared to be the murine homologue of the human common β-subunit KH97 (Fig. 3b). AIC2A, which by itself already binds IL-3 with low affinity, appeared to be an unique affinity converter for the IL-3-Rα, thus reconstituting a high affinity IL-3 receptor that predictively lacks cross reactivity with GM-CSF or IL-5 (Fig. 3b).

9.5 Other cytokine receptor systems with common binding structures

LIF, OSM, CNTF, IL-6 and IL-11 are pleiotropic factors with many overlapping biological functions and similar tyrosine phosphorylation patterns in neuronal and hematopoietic cells (30,52,53,65). Like the IL-3, GM-CSF and IL-5 receptor complex these overlapping actions are caused by the shared involvement of a β-subunit, gp130, which was originally identified as the signal transduction and affinity converting component of the IL-6 receptor (28,52,53). Receptor subunits that specifically bind LIF, CNTF, IL-6 and IL-11 with low affinity have been identified, and have been designated LIF-R, CNTF-Rα, IL-6-Rα and IL-11-Rα respectively (Fig. 3c). Heterodimerization of these receptor subunits with gp130 results in the formation of a high affinity receptor complex. Analogous to KH97 and AIC2B, gp130 alone has no affinity for ligand, except OSM which binds to gp130 with low affinity, and has been designated as the β subunit for the LIF, OSM, CNTF, IL-6 and IL-11 receptor systems (52,53,65,66). Although the low affinity LIF-R associates to gp130, similar to CNTF-Rα and IL-6-Rα, it is being referred to as LIF-Rβ, because of its homology to gp130. LIF-Rβ thus acts as an affinity converter subunit for the low affinity OSM receptor, gp130 (53). Formation of the high affinity CNTF receptor complex requires the association of CNTF-Rα with the high affinity LIF receptor complex (gp130 plus LIF-Rβ; Fig. 3) (54,55).

A third group that appears to employ common receptor subunits includes IL-7 and TSLP receptors. Both receptor chains share a high degree of homology, but can only bind their own ligand with low affinity when in a monomeric status. From reconstitution experiments in hemopoietic cell lines it appeared that the high affinity IL-7 receptor complex is a homodimer of IL-7 receptor subunits, whereas high affinity TSLP binding requires the heterodimerization of IL-7 and TSLP receptor subunits (67). The fact that cells from homozygous mice, in which expression of the IL-7 receptor gene was knocked out, do not express TSLP binding suggests that more subunits are involved in the TSLP receptor.

Thus, it appears that the promiscuous use of receptor subunits is a common theme among the members of the hematopoietin receptor superfamily. Although in the past many cross competition binding experiments have not indicated the existence of such receptor molecules, this may be caused by their relative overexpression. Conceivably, additional receptor structures shared by other HGF receptors will be identified in near future.

9.6 G-CSF receptor

G-CSF receptors are single class high affinity receptors (Chapter 1). Cloning of the G-CSF receptor resulted in the identification of only one receptor chain. When expressed in COS-1 cells this structure could transfer high affinity G-CSF binding (25). A functionally active receptor could be established in FDCP-1 cells by a hybrid receptor that contained the extracellular domain of human growth hormone receptor linked to the transmembrane and intracellular domains of the murine G-CSF receptor. Thus, the G-CSF receptor, in analogy to the GH receptor, requires homo- rather than hetero-dimerization for signal transduction (46,47). The structure of the extracellular domain of the cloned G-CSF receptor is a mosaic of hematopoietic receptor, immunoglobulin and fibronectin domains. The transmembrane and

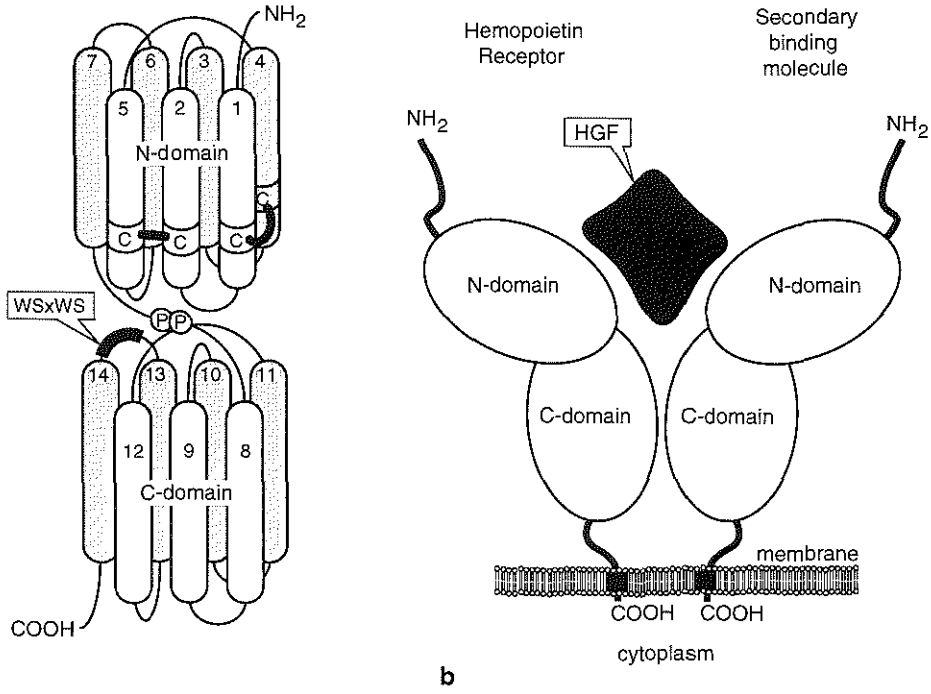

Figure 4. (a) The folding of β strands in the CRH domains. N- and C-domains, each containing 7 antiparallel β-strands folded into barrel shaped structures, are connected via two conserved prolines. (**b**) Configuration of extracellular domain of the hematopoietin receptor based on predictive analysis by Bazan and crystallography of the GH receptor; two CRH domains create a V-shaped ligand binding site. In addition the C-domains of each receptor subunit interact with each other.

cytoplasmic parts are related to the IL-4 receptor, with a sequence homology of ≈50% (25).

Several forms of G-CSF receptor mRNA with different lengths of their cytoplasmic domains can be produced by alternative splicing (22,23). As of yet, the roles of G-CSF receptor variants remain unclear. However, there is evidence that the cytoplasmic domain contains a region with differentiation inducing capacity and a proliferative competence regulatory region (68,69). Although the members of the hematopoietin receptor family have several conserved regions in the extracellular domains, a subgroup also has similar motifs in the cytoplasmic domains and perhaps similar mechanism of signal transduction. The differential expression of variants lacking certain cytoplasmic regions may represent a mechanism to modulate the G-CSF response. The presence of functionally different domains has also been identified in the cytoplasmic part of the EPO receptor. The EPO receptor contains non overlapping positive and negative growth regulatory domains (70).

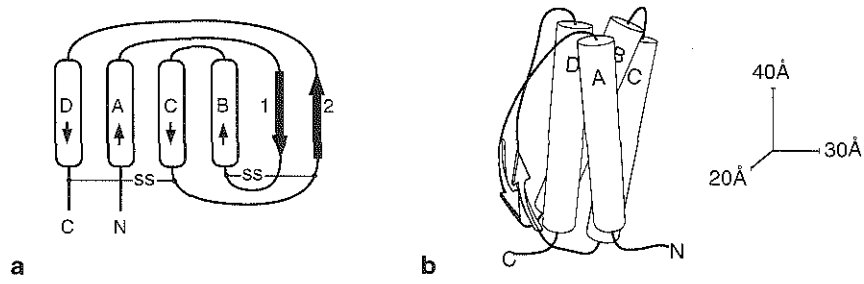

Figure 5. The structure of GM-CSF. (**a**) Diagram showing the secondary structural elements: four α helices (rounded boxes A-D), 2 β strands (arrows 1 and 2), and disulfide bridges (ss). (**b**) Drawing of GM-CSF folding as determined by X-ray crystallography. N and C mark the termini. The axes show approximate dimensions of the GM-CSF molecule. (figure adapted from Diederichs 1991).

9.7 Domains involved in ligand recognition

The extracellular CRH domain consists of two domains (N-domain and C-domain), linked by a small loop containing two proline residues (3). Each domain contains 7 β-strands, folded in two β-sheets of 3 and 4 β-strands respectively, that together form a sandwich (71)(Fig. 4a). The β-sheets of each domain make two barrel shaped structures, that are positioned at an angle (Fig. 4b). The folding of these barrels results in an Ig-like structure. Because HGF receptors are multimeric complexes, presumably two CRH domains cooperate to form a high affinity binding site. Crystallography revealed that GH forces the two receptor molecules in the GH—$(GH\text{-}R)_2$ complex in a symmetrical orientation around an axis perpendicular to the membrane surface. In this complex the residues of the N-domain and C-domain on both receptors that interact with the GH molecule are essentially the same (71). In addition there is a substantial interaction between C-domains of the two receptors, in particular at the base of the C-domain. The specific involvement of the integral CRH domain in ligand binding was demonstrated by mutational analysis of the extracellular domain of the G-CSF receptor (72). These studies further showed that the Ig-like and FN III domains, but not the CRH domains, were dispensable for biological activity. Although both GH and G-CSF receptors are (homodimeric) members of the super family, it still remains unclear how the CRH domains of heteromeric receptors that use an α subunit together with a β subunit that does not bind any ligand, interact to form a high affinity binding site.

The function of the consensus WSXWS box in the CRH domain has recently been studied. Mutational analysis of IL-2, IL-6 and EPO receptors has shown that single residues of the WSXWS motifs of the different receptors are essential for ligand binding (73-75). Initially it was suggested that the WSXWS box could be involved in ligand binding. However, in the GH receptor the WSXWS motif is located away from the ligand binding interface (71), but in close proximity of the hinge region between the two barrel-like domains of the CRH domain (Fig. 4b). Structure-function analysis of the human IL-6 receptor has established the importance of this hinge region for correct protein folding of the CRH domain (75). In this study it was shown that residues important for ligand binding had a tendency to be distributed to the hinge

region. Apparently the WSXWS motif determines how the two barrel structures of the CRH domain are positioned. The residues that are directly involved in ligand recognition are not conserved among different members of the superfamily. This is not unexpected, because each receptor has to interact with it own ligand.

The ligands of the hematopoietin receptors display none, or very little similarities in their amino acid sequences. Nevertheless, a common structural feature of these ligands is a conformation that is rich in α helices, as is evident from predictive modeling of cytokine protein folds and X-ray crystallography. For example GM-CSF, IL-2, IL-3, IL-4, IL-6, G-CSF, EPO, PRL and GH all have four parallel α helices that form a barrel (76-84) (Fig. 5). This "four-α-helix bundle" is a common structural motif in globular proteins and provides the ligand its backbone topology, necessary for biological function (85). Of the four helices, in particular the most N-terminal α helix plays an important role in the high affinity ligand-receptor interaction and biological activity for IL-3, IL-5 and GM-CSF (36,86-89). Minor deletion or single residue substitution in the first α helix completely abrogate high affinity binding and biological activity. Comparison with the other cytokines, that have amino acid homology in the predicted amino terminal α helix, suggests that this region may contain the recognition element of cytokines for the high affinity binding sites on their multi-subunit receptors (88).

9.8 Cytoplasmic domains of HGF receptors

Contrary to the extracellular domains of the hematopoietin receptors, the cytoplasmic domains lack a common motif. The cytoplasmic tails vary substantially in length (41 to 569 aa, Table 1), and share no homology with other known sequences. Nevertheless, certain members of the superfamily share distinct homology of their intracellular domains. The cytoplasmic part of the IL-4 receptor has a similarity to that of the G-CSF receptor (22,25). However, homology between the cytoplasmic region of the G-CSF receptor and gp130, the affinity converter of the IL-6 receptor, is much more pronounced. Three stretches of amino acids (Boxes 1, 2 and 3, Fig. 6) are conserved in these receptor molecules (72,90). These conserved sequences are consequently thought to play a role in signal transduction. Indeed two of these segments (Box 1 and 2) are essential for biological activity of the IL-6 and G-CSF receptors (72,90). The conserved cytoplasmic regions can also be found in other members of the hematopoietin receptors. huIL-2-Rβ, EPO-R, KH97, AIC2A and AIC2B have regions which are highly homologous to Box1 and Box2 of the G-CSF receptor and IL-7-R, MPL and IL-4 have Box1 homologous sequences. For gp130 and the G-CSF receptor it was shown that Box1 and Box2 are essential for mitogenic signaling (68,70,72,90). Deletion mutants that lacked Box1 and Box2 could not mediate IL-6 or G-CSF induced proliferation in the murine IL-3 dependent cell lines BAF3 or FDCP-1. The function of the third conserved domain, Box3, is unclear. On the other hand, it appears that transduction of a HGF stimulus is conferred by other less well confined and non conserved parts of the cytoplasmic domain. For example a single point mutation in the cytoplasmic region of the EPO receptor between Box1 and Box2 completely abrogated its biological activity (91). Most members of the HRSF contain large serine/proline rich stretches that do not overlap with the conserved boxes. The serine rich region of the IL-2-Rβ subunit was shown to be

Box1	
huIL-2-Rβ (249-262)	**K** K V L K C N T **P** D **P** S **K** F
AIC2A (473-486)	Y **R** K W K E K **I** **P** N **P** S **K** **S**
muIL-4-R (235-248)	**K** K I W **W** D Q **I** **P** T **P** A R **S**
huGM-CSF-Rα (350-363)	L **R** I Q R L F P **P** V **P** Q I K
huIL-3-Rα (326-339)	R **R** Y W V M Q R L F **P** R I P
KH97 (473-486)	R **R** K W E E K **I** **P** N **P** S **K** **S**
muIL-5-Rα (326-375)	H L W T R L F P **P** V **P** A P K
huEPO-R (278-291)	K Q K I **W** **P** G **I** **P** S **P** E S E
huIL-7-R (249-262)	**K** P I V **W** **P** S L **P** D H K **K** T
huIL-9-R (298-311)	**K** **R** I F Y Q N V **P** S **P** A M F
hu gp130 (648-661)	**K** K H I **W** **P** N V **P** D **P** S **K** **S**
huG-CSF-R (632-645)	**K** N P L **W** **P** S V **P** D **P** A H **S**
huLIF-R (866-879)	**K** E T F Y **P** D **I** **P** N **P** E N C
MPL (177-190)	R H A L **W** **P** S L **P** D L H R V
Consensus	K R W P I P D P K S
Box2	
huIL-2-Rβ (296-307)	I **S** P **L** **E** V L E R D **K** V
AIC2A (536-546)	**V** **S** P **L** T **I** **E** D P N I
KH97 (535-546)	**V** **S** P **L** T **I** **E** D P - **K** H
huEPO (327-337)	P A S **L** **E** V L S E R C
hu gp130 (691-682)	**V** **S** V V **E** **I** **E** A N D **K** K
huG-CSF-R (672-683)	L T V **L** **E** E D E K K P V
huLIF-R (909-920)	**V** E V **L** **E** T R S A F P K
Consensus	V S L E I E K
Box3	
hu gp130 (772-787)	P S V Q V F S R S E **S** T Q **P** - - **L** **L**
huG-CSF-R (738-753)	P G P G H Y L R C D **S** T Q **P** - - **L** **L**
KH97 (864-814)	G E R P A D V S P T **S** P Q **P** E G **L** **L**
AIC2A (895-813)	G E P R E E V G P A **S** P H **P** E G **L** **L**
Consensus	S P L L

Figure 6. Amino acid sequence of hematopoietin receptors cytoplasmic domains. Three highly conserved segments (amino acid residues are given in parenthesis) are aligned; gaps have been introduced to maximize homology (-).

essential for tyrosine phosphorylation (92) and substitution of positively charged amino acids in the region between Box1 and Box2 of gp130 by serine and glutamine completely abrogated the IL-6 responsiveness in BAF3 cells (90).

Because IL-3, GM-CSF, and IL-6 are pleiotropic factors, conceivably different parts, i.e., for example the Box 3 domain or the "inter-Box" regions might be required for functions other than the growth signal. A common feature of all HGFs that use gp130, G-CSF receptor, or KH97 for signal transduction is the ability to induce myeloid maturation in normal progenitor cells. The particular involvement of Box3 of the G-CSF receptor in granulocytic maturation was implied by the identification of a truncated G-CSF receptor form (68,93). This receptor lacks its C-terminal cytoplasmic domain including Box3, has lost it ability to induce G-CSF dependent terminal granulocytic maturation upon forced expression in L-GM cells.

Homodimerization of the GH, EPO and G-CSF receptors is the first essential event in ligand-induced signal transduction (42,46,47,68,72). The role of homodimerisation of gp130 in IL-6 signaling was recently verified: disulfide-linked homodimerization

of gp130 can be recovered from cell lysates after IL-6 stimulation (94). Unlike the IL-6 receptor, signaling by the CNTF and LIF receptor complexes depends on the heterodimerization of gp130 with the LIF binding protein (LIF-R) (54). These observations suggest that juxtaposition of two identical cytoplasmic domains create a binding site for a second messenger that triggers cell proliferation. In analogy with the formation of the ligand binding site by two CRH domains for the G-CSF receptor, it may be possible that this signaling domain (or multiple signaling domains) is composed of two identical domains (Box1, or Box2, or Box3); one from each receptor subunit. The interaction of two identical domains in the CRH region of the GH receptor was established by X-ray and MRI crystallography of the GH—GH-R complex (71). GH, a nonsymmetrical molecule, binds two copies of the receptor that use essentially the same binding determinants. Furthermore, the two receptor molecules in the GH—$(GH\text{-}R)_2$ complex contact each other via the same residues in the C-terminal domain of each receptor CRH region. This paradigm would predict for the heterodimeric receptor complexes that two homologous cytoplasmic domains associate to form an "active site" upon ligand activation. The two α and β chains of the high affinity GM-CSF receptor each contain a Box1. This would suggest that for the high affinity GM-CSF receptor only the Box1 domains are required for signaling. Indeed it appeared that in KH97 only Box1 is sufficient for growth signal transduction in BAF3 cells and CTLL-2 cells (95). Although these cells cotransfected with the complete GM-CSF-Rα and the KH97 deletion mutant that lacked Box2 and Box3, showed a full proliferative response to GM-CSF, it appeared that the sensitivity to GM-CSF was reduced 1 or 2 log. This suggested that the region between Boxes 1 and 2 or Box2 contains some positive growth regulatory domain. On the other hand the loss of sensitivity could also reflect the effects of extensive truncation on the folding of the KH97 protein. Thus the precise roles of the Box2 and Box3 of KH97 in the IL-3, GM-CSF, and IL-5 receptor complexes are still unclear. In order to study the function and mechanism of action of HGF receptors in normal and abnormal hematopoiesis, investigations should first be concentrated on the delineation of functional domains of the IL-3, GM-CSF, and G-CSF receptors.

9.9 Signal transduction

Relatively little is known about the way HGF receptors can activate signal transducing pathways in their target cell. The cytoplasmic domains of HGF receptors do not contain sequences known to be important in signal transduction, such as tyrosine kinase domains or phosphatases. For other receptor systems these mechanisms have been (partially) dissected. The receptors for steroid hormones can be allosterically modified upon ligand binding, which allows the hormone-receptor complex to bind to its DNA responsive elements in the promoter region of a target gene. The requirement of tyrosine kinase activity for signal transduction has been well established in RTK family (96-98). Tyrosine kinase receptors such as c-kit and M-CSF receptor catalyze the phosphorylation of exogenous substrates as well as tyrosine residues within their own receptor chains. Autophosphorylation appears to be involved in the interaction between activated receptor and intracellular proteins involved in signal transduction such as phospholipase C-γ, PKC, Phosphatidyl inositol 3-kinase, c-raf and GTPase activating protein (96-100). These proteins are

physically associated with cytokine receptors such as EGF, M-CSF and PDGF through a specific (non catalytic) domain, the SH2 domain, which was originally identified as the common motif of many RTKs (96,101). The stimulation of RTKs can rapidly both modify the expression, and activity of transcription factors like fos, jun and myc, which control genes that are important for cell cycling and differentiation (102).

Many HGFs including IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, GM-CSF, G-CSF and EPO receptors have also been demonstrated to induce protein tyrosine phosphorylation in hematopoietic cells (103-113). For example IL-3 and GM-CSF both induce tyrosine phosphorylation of p42 and p44 mitogen activation protein (MAP) kinase and the proto-oncogene product Raf-1 and several other unidentified cytoplasmic substrates (103,112,113). Tyrosine phosphorylation plays a critical role in receptor function. This is demonstrated by mutations in the cytoplasmic domain of EPO and KH97 which concomitantly abrogated mitogenic signaling and tyrosine phosphorylation (91,95,114). KH97 contains two distinct regions in the cytoplasmic domain that are responsible for different signals, both of which seem to require tyrosine kinases. The membrane proximal region is essential for the induction of c-myc and pim-1, the C-terminal region is required for activation of Ras, Raf-1, and MAP kinase as well as induction of c-fos and c-jun (114). IL-2 and IL-3 both regulate the activity of PTKs of the src family in human T-cell lines CTLL-2 and TALL103/2 (115). Phosphorylation of these substrates in response to HGFs is very rapid, but none of these receptors have intrinsic tyrosine kinase activity, suggesting that these receptors must closely associate with secondary tyrosine kinases. Identification of these molecules is important for elucidating the mechanism of HGF signal transduction, and may reveal substrates that mediate distinct HGF functions. As of today there have been only few reports of signaling proteins that associate with activated HGF receptors. Recently, a novel PTK, Jak2 was cloned (116). Using anti AIC2 antibodies Jak2 is coprecipitated from DA3 cell lysates, only after IL-3 stimulation of the cell. These results indicate that Jak2 physically associates with the murine IL-3 receptor and forms a link between receptor activation and signal transduction through protein phosphorylation. Jak2 also associates with the EPO receptor and EPO induced phosphorylation of Jak2 correlates with biological activity (117). Another protein-tyrosine kinase implicated in the signaling pathway triggered by GM-CSF and IL-3 is c-fps/fes. In TF-1 cells, the proto-oncogene product c-fps/fes is tyrosine phosphorylated and activated by stimulation with GM-CSF and IL-3, and becomes associated with KH97 upon GM-CSF stimulation (118).

CHAPTER 10

General Discussion

10.1 IL-3-, GM-CSF, G-CSF and KL receptors in AML

To date, the precise mechanisms that can lead to growth expansion and/or differentiation block of hematopoietic precursor cells have remained largely unidentified. Several mechanisms have been postulated to contribute to unregulated neoplastic growth of AML in vivo, e.g. abnormal growth factor responsiveness. In this thesis the question was addressed whether HGF receptors may be overexpressed or have an altered ligand binding affinity as compared to normal hematopoietic cells. Overexpression of receptors, possibly leading to growth advantage has been postulated to play a role in the myeloproliferative features of AML. Data presented in Chapters 2, 3 and 4 have not shown evidence for overexpression or altered affinities of HGF receptors in clinical AML. Density as well as affinities of IL-3, GM-CSF and G-CSF receptors in AML are of the same magnitude as those of normal hematopoietic cells. The higher average level of KL receptor expression on AML cells is a general feature of RTKs and is in agreement with the expression of e.g. M-CSF in normal hematopoietic cells (1). Although the expression of HGF receptors on AML cells did not always predict whether these cells would enter active cell cycling, it is clear that in most cases the cells have retained the ability to respond to HGF stimulation. Furthermore, no clear correlation has been shown between the numbers of HGF receptors and the ability of the leukemic blast cells to proliferate in vitro in response to exogenous HGFs. However, firm conclusions regarding this relationship cannot be made because of the narrow range of levels of HGF receptor expression and the heterogeneous cellular composition of AML.

A fundamental defect in many cases of AML is the partial or complete loss of the capacity to mature. In vitro, AML cells generally respond to various HGFs including IL-3, GM-CSF, and G-CSF in a proliferative manner, but in contrast to normal progenitor cells, lack the ability to differentiate. One possibility is that although the receptors are still capable of transducing a mitogenic response, the receptors have lost the ability to induce maturation. As demonstrated by artificially induced mutations in receptor genes (Chapter 1), altered receptor function must be considered as one of the potential causes of abnormal hematopoiesis. Although in AML the binding characteristics of HGF receptors are normal, aberrant receptor function may also result from changes in the intracellular domain of a receptor, without affecting the binding characteristics (2). Impaired receptor function is also implied in a subgroup of AML cases. In 14-27% of AML cases, no proliferative response to HGF stimulation was observed despite apparent normal receptor expression (Chapters 2 and 3). In some of these cases, the cells already exhibited high spontaneous growth, which might have obscured the response to the HGF. The nonspontaneously growing cases, however, might for instance carry a defect at the level of signal transduction or transcription regulation of HGF-induced gene expression. Alternatively, these cells may express receptors that are unable to transduce a HGF-triggered mitogenic signal. Of note is the identification of a point mutation in the human G-CSF receptor, that was isolated from a patient with severe congenital neutropenia (SCN) (3). This mutation introduces a stop codon in the cytoplasmic domain of the G-CSF receptor resulting in a truncated receptor form. When expressed in murine myeloid cells, this mutated receptor exhibits normal binding characteristics, and can still transduce a mitogenic signal, but (in contrast to the wild type human G-CSF receptor) fails to induce maturation (3,4). Recently, a

naturally occurring form of the EPO receptor was identified in which part of the cytoplasmic region is deleted by alternative splicing (5). This receptor was able to transduce a mitogenic signal, but in contrast to the wild type receptor, failed to prevent programmed cell death in BAF3 cells. Another functional domain in the EPO receptor was identified, which appears to convey a negative effect on EPO-induced signal transduction. Truncation of this N-terminal 40 aa serine rich domain allowed BAF3 cells to grow maximally in 1/10 the concentration required for growth of cells expressing the wild type receptor (6). The identification of such regulatory domains in the IL-3, GM-CSF or G-CSF receptors and subsequent screening for abnormalities of these domains may reveal a direct relationship between a defective receptor and the observed phenotype of the leukemic cells.

10.2 HGF receptor modulation and regulation of AML growth

Kit ligand (KL) (also called stem cell factor, mast cell growth factor or Steel factor) acts synergistically with several HGFs, including IL-3, GM-CSF, and G-CSF, to induce proliferation of primitive hematopoietic precursor cells and AML blast cells. As shown in Chapter 4 it appears that synergy between KL and IL-3, GM-CSF or G-CSF neither results from induction of receptor expression nor from alterations in sensitivity to HGFs. These findings were confirmed in the human factor dependent cell line MO7e. In these cells GM-CSF do not alter the number or affinity of surface GM-CSF receptors (7,8). Thus the molecular basis for the synergistic activity of KL is probably located further downstream the signaling pathways or involves pathways which are unique. Additional studies have focused on early events of signal transduction, i.e., phosphorylation of cellular substrates following HGF stimulation. These investigations indicate that IL-3, GM-CSF and KL stimulate (tyrosine) phosphorylation of several identical substrates in human myeloid cells, including Raf-1 kinase and p42 mitogen activated protein (MAP) kinase, however, no synergy or additive effects were observed on the level of protein phosphorylation (7,9-11). Also, no additional phosphoproteins were apparent after combined stimulation with KL and other HGFs compared to the individual factor alone. Possibly the synergistic stimulus for cell growth results from unique pathways, triggered by the individual growth factors, which finally can augment proliferation by transcriptional regulation of genes involved in proliferation.

Tumor necrosis factor (TNF) is a regulator of inflammation and cellular immune responses and is an important mediator of immune responses. In AML TNF can enhance proliferation through the induction of HGF production (12). Moreover, TNF acts as a modulator of HGF controlled growth of normal and leukemic cells. TNF acts synergistically with IL-3 and GM-CSF-induced proliferation and colony formation of AML cells and $CD34^+$ cells. Conversely, TNF inhibits the G-CSF induced proliferation of AML cells (13-15). These modulatory effects of TNF are probably the result of transmodulation of hematopoietic growth factor receptor expression (Chapters 5 and 6). In AML, high affinity GM-CSF and IL-3 receptors on AML cells are increased 3 to 6-fold after 18 hr's of exposure to TNF (Chapter 5). Probably enhanced proliferative response related to the modulatory effect of TNF is the result of recruitment of additional subpopulations which become receptor

positive. The exact mechanism of receptor upregulation by TNF is still unclear. In Chapter 5 it was demonstrated that the effect of TNF depends on active protein synthesis, and it has recently been shown that TNF upregulates the expression of the common β receptor subunit mRNA (16). Thus TNF may act through activation of transcription or stabilization of HGF receptor mRNAs in AML cells. TNF has been shown to increase the stability of mRNAs in hematopoietic cells (17,18), but the molecular mechanism responsible for the TNF effects on cytokine receptors needs further elucidation.

Alternatively, it is possible that the modulatory effects of TNF are mediated at the level of transcription of genes that directly relate to cell proliferation. TNF has been shown to activate nuclear transcription factors like NF-κB, AP-1 and NF-jun, which regulate the transcription of jun (19-21). Expression of the c-jun gene in human myeloid leukemia cells is enhanced 4-fold by TNF (22). GM-CSF and IL-3 have also been shown to induce expression of (fos and) jun in myeloid cells (23,24). Immediate early response genes like fos and jun, whose products form a heterodimeric transcription factor complex called AP-1, are both involved in cell cycle progression (25-27). These results indicate that TNF is a pleiotropic factor and that the synergism with other HGFs may involve multiple levels of action.

Interestingly, TNF also exerts negative effects on the proliferative response of AML. In Chapter 6 it was shown that the antagonistic effects between TNF and G-CSF on AML cell proliferation are probably accomplished via transmodulation of G-CSF membrane receptors. TNF downregulates the expression of G-CSF receptors on AML blasts and granulocytes. The effects of TNF on the expression of HGF receptors appear to be mediated through TPA activation of PKC, and could be blocked by protease inhibitors (Chapter 6). Most likely, the mechanism of receptor downmodulation is similar for G-CSF and M-CSF receptors. Activation of PKC by phorbol esters or physiologic inducers of PKC, such as the macrophage activator LPS, induce proteolytic cleavage of the M-CSF receptor in its extracellular domain near the membrane (28). The TNF induced transmodulation of the G-CSF receptor may represent an important regulatory function in G-CSF signaling. The loss of surface receptors will lower the sensitivity to G-CSF. Furthermore, because the ligand binding domain of the receptor released from the cells after proteolytic cleavage is still able to bind its ligand (29), competition of soluble receptors with cell surface receptors may render the AML cells or neutrophils more refractory to G-CSF stimulation. The biological effects of TNF are mediated via two specific membrane receptors, p55 and p75, both of which are expressed in AML (30-33). A recent study using antibodies against both TNF receptors has revealed that all TNF effects in AML, i.e., synergy and antagonism with HGFs, and HGF receptor up and down modulation are mediated via the p55 TNF receptor (33).

10.3 Cross competition between IL-3 and GM-CSF

Experiments dealing with cross competition between GM-CSF and IL-3 in human myeloid cells are presented in Chapter 7 of this thesis. AML cells and monocytes express both high and low affinity GM-CSF receptors. High affinity GM-CSF binding can be competed for by IL-3. Conversely, high affinity IL-3 receptors on AML cells and monocytes can be competed for by GM-CSF. The molecular basis of this

phenomenon lies in the KH97 receptor subunit that is shared by IL-3 and GM-CSF receptors. The role of the GM-CSF-, IL-3-, and IL-5-receptor α chains in signal transduction per se, apart from their role in the formation of the high affinity receptor complex, is still uncertain. Reconstitution experiments have revealed that the high affinity GM-CSF receptor αβ heterodimer, but not the low affinity α receptor is responsible for signal transduction in BAF3 cells (34-37). The sharing of KH97 explains why IL-3, GM-CSF, and to some extent IL-5 have overlapping biological functions (Chapter 1) and induce phosphorylation of a common set of cytoplasmic substrates in myeloid cells (38-41). Apart from these common activities, IL-3, GM-CSF and IL-5 also exhibit biologically distinct activities. IL-5 specifically stimulates human eosinophil function (42). In human mature eosinophils IL-5 blocks apoptosis more efficiently than does IL-3 or GM-CSF (43). In AML t(8;21), IL-5 was able to induce eosinophilic maturation, whereas IL-3 and GM-CSF only promote proliferation in these cells (44). In another study it was shown that in some AML cases, IL-3 induced a high proliferative response, whereas GM-CSF was hardly effective (45). These divergent effects can to some extent be the result of unique cellular distributions of the receptor α subunits. Different cells, which do not coexpress these receptors, may have different response abilities depending on e.g. stage of maturation. However, IL-3, GM-CSF, and to a lesser extent IL-5 receptors are generally coexpressed in myeloid progenitor cells (Chapters 1 and 7). This suggests that the specificity of the cytokine stimulus must result from unique signal pathways that do not involve KH97 alone, but requires the involvement of the unique α subunits. The fact that all three HGFs induce identical tyrosine phosphorylation patterns in myeloid cells (38,46), suggests that α subunits do not differentially activate tyrosine phosphorylation of cytoplasmic substrates. Thus, events other than protein tyrosine phosphorylation may play a role in the determination of the specificity of IL-3, GM-CSF, and IL-5 stimuli. This specificity may result from the interactions of a specific domain in the α subunit and a common domain on KH97. Alternatively, the ligand may determine the alignment in the complex of the α and β subunit, so that each time the same homologous domain of an α subunit is associated with different domains on the β subunit. Investigations focusing on structure and function of the cytoplasmic domains of the α subunits are required to further address these questions.

10.4 Alternative oligomerization of the GM-CSF receptor α and β subunits and consequences for cytoplasmic signaling

IL-3 and GM-CSF have been recognized as pleiotropic factors. They act on immature as well as mature cells of diverse lineages. In Chapter 8, it was shown that the expression of high and low affinity GM-CSF receptors is restricted to the myeloblastic and promyelocytic stage of granulocytic maturation (47). Progression towards the metamyelocyte stage is associated with a transition to intermediate affinity GM-CSF binding, which lack cross competition with IL-3. The existence of an intermediate affinity GM-CSF receptor on mature peripheral blood neutrophils had become apparent from studies described in Chapter 7. COS cell transfections with the human GM-CSF-Rα and KH97 subunits indicate that this conversion is caused by the quantitative difference in expression of KH97 (47). The same phenomenon was

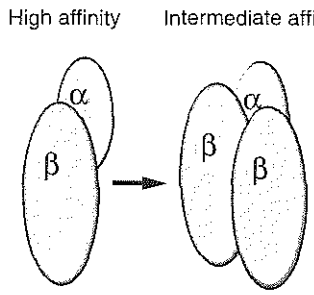

Figure 1. Alternative oligomerization of GM-CSF receptor α and β subunits. Increased expression of the GM-CSF-Rβ permits the formation of receptor complexes that contain two β subunits.

observed for the IL-3 receptor, which converts from a high affinity receptor to intermediate affinity after increased expression of KH97 (48), and it may also explain the discrepancies in affinity that have been found for the native IL-5 receptor expressed human hematopoietic cells and the reconstituted IL-5 receptor in COS cells (37,49). Increased availability of KH97 may lead to the composition of distinct oligomeric complexes of α and β subunits. The exact composition of the high and intermediate affinity GM-CSF receptor complexes are unknown. However, it is conceivable that in a situation of restricted KH97 availability, e.g. in AML cells which express 10 to 50 times more low affinity than high affinity GM-CSF receptors, the high affinity receptor complex comprises one β subunit and one α subunit. Increased levels of KH97 may convert this receptor complex into an intermediate affinity GM-CSF receptor, which, in analogy to the CNTF and IL-6 receptors, comprises two β subunits (Fig. 1). This paradigm has important consequences for the activation of the GM-CSF receptor, in that it offers the GM-CSF receptor alternative ways of secondary signaling, via αβ interaction and via ββ interaction. Since the above model portrays a novel mechanism to modulate a HGF response, it would be attractive to further investigate this hypothesis.

Alternative oligomerization of GM-CSF receptor subunits raises the possibility of homodimeric interaction of two β subunits versus heterodimeric αβ interactions, but the significance of alternative oligomerization for signal transduction is still unknown. Several questions remain to be addressed: (i) Does the intermediate affinity GM-CSF receptor have the ability to induce growth via the interaction of two β subunits (versus α and β in the high affinity complex)? (ii) Does the intermediate affinity GM-CSF receptor mediate a qualitatively distinct signal as compared to the high affinity GM-CSF receptor complex? (iii) Are (signaling) domains on the α and β subunits alternatively involved in the different complexes?

It was shown that homodimerization of G-CSF receptors that lack the cytoplasmic C-terminal region including Box3, fail to induce maturation in L-GM cells (3,4). An attractive hypothesis would be that the intermediate affinity GM-CSF receptor complex, via homodimerization of two KH97 C-terminal domains, is able to induce (granulocytic) maturation of myeloblasts or to induce functional activation in contrast to proliferation. Obviously, in the heterodimeric high affinity GM-CSF receptor, association of two Box3 domains cannot occur, since the α subunit lacks Box3. Accordingly, the only role of the high affinity GM-CSF receptor would be the transduction of a mitogenic signal via the interaction of Box1 of the α subunit with one of the cytoplasmic KH97 domains. The latter hypothesis would imply that both the intermediate affinity GM-CSF receptor as well as the high affinity GM-CSF receptor are able to induce cell growth. It would fit with the observation that AML

blasts, that have matured towards the metamyelocyte stage of differentiation and acquired intermediate affinity receptors, are still able to respond in a proliferative fashion to GM-CSF (Chapter 8). Thus, the level of KH97 expression may play an important modulatory role in adjusting GM-CSF responsiveness.

Boxes 1 of GM-CSF-Rα and KH97 are indispensable for high affinity mitogenic signaling in primary transfectants (50). The ability of GM-CSF to signal via the interaction of two KH97 subunits, would predict that the signaling failure caused by the deletion of Box1 from GM-CSF-Rα, can be overcome by overexpressing KH97 (hence an intermediate affinity GM-CSF receptor). Alternatively, signaling via dimerization of KH97 in the absence of GM-CSF-Rα can be achieved by crosslinking anti KH97 antibodies on BAF3 cells that exclusively express KH97. The qualitative differences of GM-CSF-induced signaling via intermediate or high affinity receptors may be investigated in the L-GM cell line model. Transfection of the GM-CSF receptor subunits in these cells may confirm whether overexpression of KH97 results in GM-CSF-induced maturation.

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SUMMARY & SAMENVATTING

SUMMARY

IL-3, GM-CSF and G-CSF stimulate proliferation of human acute myeloid leukemia in vitro, but patterns of response among clinical cases are diverse. As described in ***Chapters 2 and 3***, numbers and affinity of IL-3, GM-CSF and G-CSF receptors on cells of patients with AML were assessed and correlated with the proliferative response of the cells to IL-3, GM-CSF and G-CSF. In 13 of 15 cases of primary AML high affinity receptors for IL-3 were demonstrable on the cells. The average numbers of IL-3 receptors ranged from 21-145 receptors per cell. Normal white blood cells showed IL-3 receptors on their surface at similar densities. IL-3 receptor positivity often correlated with GM-CSF receptor positivity of AML, GM-CSF receptors were demonstrated on the cells of 11 of 15 cases although average numbers of GM-CSF receptors were 10 times greater. The binding of G-CSF to normal and human AML cells was investigated in a series of 14 cases of primary AML. In all 14 cases specific receptors for G-CSF were demonstrated on purified blast cells. The average numbers of G-CSF receptors ranged from very low (specific binding scarcely detectable) to 428 receptors per cell. Normal granulocytes showed G-CSF binding sites on their surface at higher densities (703 to 1,296 sites/cell). G-CSF receptors appeared of a single affinity type with a dissociation constant (K_d) ranging between 214 to 378 pM for AML blasts and 405 to 648 pM for normal peripheral blood granulocytes. The in vitro response of the cells to exogenous IL-3, GM-CSF or G-CSF was examined by measuring thymidine uptake. IL-3 and GM-CSF were potent inducers of DNA synthesis in vitro. In 12 of 14 cases including those with relatively low specific binding, G-CSF was a potent inducer of DNA synthesis of blasts in vitro; apparently relatively few receptors permit activation of AML cell growth. In a minority of cases however, the cells were unable to respond to IL-3 (4 of 15 cases), GM-CSF (4 of 15 cases) or G-CSF (2 of 14 cases) in spite of normal receptor availability on the cell surface. The inability of the cells to respond to stimulation might be caused by the inability of the receptors to transduce a secondary signal into the cells. The results from these experiments taken together did not provide evidence for overexpression or gross changes in receptor affinity as an explanation for AML growth.

Chapter 4 deals with the role of KL and c-kit in AML cell growth. The proliferative response to KL in relation to c-kit expression was investigated in 10 AML cases. Additionally, the mechanism of synergy of KL in combination with several other HGFs was studied. FACS analysis with anti c-kit antibodies revealed KL receptor expression in 8 of 9 cases, often in a subpopulation of the cells. Scatchard analysis of KL receptors in two AML cases and the cell line MO7e, indicated the presence of (mean) 1,460 to 120,000 binding sites per cell. The same class of high affinity KL receptors (K_d 40 to 160 pmol/L) was detected in all cell samples. KL stimulated DNA synthesis of purified leukemic blasts in 8 of 10 cases and colony formation in 4 cases in serum-free culture. KL synergizes with IL-3 , GM-CSF, but synergizes most with G-CSF. Synergistic effects of KL in combination with other CSFs were also seen in the colony assay. Antibodies against GM-CSF, M-CSF, G-CSF and IL-6 did not inhibit the KL response, suggesting that the stimulative effect of KL was not mediated through autocrine release of those cytokines. In contrast, the proliferative response to KL was abrogated in the presence of TNF (4 of 10 cases) and IL-4 (2 of 10 cases). The KL dose-response curve in the presence of IL-3 or GM-CSF resulted in a higher plateau of DNA synthesis, however no shift in the dose response was apparent. The

respective reciprocal dose response relations to GM-CSF, IL-3 or G-CSF were similarly elevated when KL was added. KL did not alter IL-3 and GM-CSF receptor expression, nor did IL-3, GM-CSF, G-CSF, TNF or IL-4 influence KL binding to AML cells. Apparently the mechanism of synergy lies at other levels of signal transduction.

TNF acts as a potent enhancer of GM-CSF and IL-3-induced human AML growth in vitro. In *Chapter 5* the question was addressed whether the TNF effects might be accomplished at the level of membrane receptors. Incubation of blasts from seven patients with AML in serum-free medium with TNF and subsequent binding studies using radiolabeled IL-3 and GM-CSF, showed that TNF increases the specific binding of GM-CSF (30-280%) and IL-3 (40 to 600%) in all cases. From Scatchard plot analysis it appears that TNF upregulates (1) low affinity GM-CSF binding sites, (2) high affinity common IL-3/GM-CSF binding sites, and (3) high affinity unique (non-GM-CSF binding) IL-3 binding sites. The effect of TNF becomes evident at 18 hours of incubation with TNF at 37 °C, but not at 0 °C. The GM-CSF dose response curve of AML-colony forming units plateaus at a higher level in the presence of TNF, which indicates that additional numbers of cells become responsive to GM-CSF. Incubation of AML blasts with the phorbol ester TPA or fMLP (PKC activators) does not influence GM-CSF receptor expression, suggesting that receptor upregulation by TNF is not mediated through activation of PKC. On the other hand, the protein synthesis inhibitor cycloheximide abrogates receptor upregulation induced by TNF. In contrast to these findings in AML, TNF does not upregulate GM-CSF receptor numbers on blood granulocytes or monocytes.

G-CSF stimulates the proliferation of AML in vitro and it may also induce maturation in some cases of myeloid leukemia. TNF acts antagonistically with the proliferative effects of G-CSF on AML blasts in vitro, and thus appears to be a negative regulator of the G-CSF-induced proliferation of AML cells. In *Chapter 6* the mechanism that may explain the antagonistic effects between TNF and G-CSF was investigated. Incubation of blasts from 3 patients with AML in serum-free medium with TNF, and subsequent binding studies using radiolabeled G-CSF reveal that TNF downregulates the numbers of G-CSF receptors by ± 70%. G-CSF receptor numbers on purified blood granulocytes are also downmodulated by TNF. Downregulation of G-CSF receptor expression becomes evident within 10 minutes after incubation of the cells with TNF at 37 °C and is not associated with an apparent change of the dissociation constant. The TNF effect does not occur at 0 °C and cannot be induced by IL-2, IL-6 or GM-CSF. TNF probably exerts its effect through activation of PKC, as the TNF effect on G-CSF receptor levels can be mimicked by TPA. The PKC inhibitor Staurosporine as well as protease inhibitors completely prevent G-CSF receptor downmodulation. Thus it appears that TNF may act as a regulator of G-CSF receptor expression in myeloid cells and shut off G-CSF dependent hematopoiesis. The regulatory ability of TNF may explain the antagonism between TNF and G-CSF stimulation.

It has been shown that a considerable degree of functional overlap between GM-CSF and IL-3 exists, in particular on immature myeloid cells. In *Chapter 7*, specific experiments were conducted to analyze the properties of GM-CSF and IL-3 binding proteins in more detail. On AML blasts from 8 cases two types of GM-CSF receptors were apparent, i.e., one with low affinity (K_d 5.1 to 24.8 nmol/L) and one with a high affinity (K_d 31 to 104 pmol/L). These AML cells also expressed high affinity

receptors for IL-3 (K_d 24 to 104 pmol/L). Cross competition experiments with AML cells showed that an excess concentration of nonlabeled IL-3 completely prevented the high affinity binding of radiolabeled GM-CSF. Low affinity GM-CSF binding does not show this cross reactivity. Conversely, binding of radiolabeled IL-3 to AML cells could be reduced by nonlabeled GM-CSF. In certain cases, this competition was complete, whereas in others only partial (49 to 77%) reduction of the radiolabeled IL-3 binding was seen. Cross competition occurred equally well at 37 °C and 4 °C, suggesting that the cross reactivity resulted from direct interference at the receptor level (rather than downmodulation). In contrast to AML, normal neutrophils express a single class GM-CSF receptor with intermediate affinity (K_d ~400 pmol/L), that can not be competed for by IL-3. In conclusion, four different receptors for IL-3 and GM-CSF can be postulated: (i) low affinity GM-CSF receptors that do not bind IL-3, (ii) dual high affinity GM-CSF/IL-3 receptors, and (iii) high affinity IL-3 receptors that do not bind GM-CSF. The same receptor types could also demonstrate on normal monocytes, (iv) GM-CSF receptors incapable of binding IL-3 and with an intermediate affinity for GM-CSF (~400 pmol/L). Chemical cross linking revealed that GM-CSF and IL-3 both bind to proteins with Mr values of 130, 105 and 75, which provides additional evidence for the existence of a common GM-CSF/IL-3 receptor complex.

It subsequently became clear that the high affinity GM-CSF receptor is a heterodimeric complex of a specific GM-CSF receptor α subunit and a β subunit (KH97). Cross competition between high affinity GM-CSF and high affinity IL-3 binding is caused by the fact that both high affinity receptors share the common β chain, β_c (KH97). The fact that immature cells express different binding features than do mature neutrophils (Chapter 7), raises the question at which stage of maturation the GM-CSF binding features are converted into those of the terminally differentiated neutrophils and what possible mechanism could underlie these changes. ***Chapter 8*** describes the differentiation associated changes of GM-CSF binding characteristics in three models representing distinct stages of myeloid maturation: 1) primary AML cells that matured spontaneously in culture towards myelocytes, 2) chronic phase CML cell samples that contained myelocytes, metamyelocytes and band forms and 3) the promyelocytic cell line HL-60 in which retinoic acid (RA) induced neutrophilic differentiation. Upon maturation in culture, the primary AML cells lost the characteristic high affinity GM-CSF binding and acquired intermediate affinity GM-CSF receptors. Similar to the high affinity GM-CSF-R of primary AML, these intermediate affinity GM-CSF receptors still showed full cross competition with IL-3. Intermediate affinity GM-CSF receptors that could be competed for by IL-3 were also observed in CML cells and HL-60 cells. Upon RA treatment of the HL-60 cells, IL-3 cross-competition was lost, leaving the intermediate affinity GM-CSF binding typical of peripheral blood neutrophils. Thus, high affinity GM-CSF binding is converted into intermediate affinity binding, which still cross reacts with IL-3, beyond the stage of promyelocytes. Also, during terminal maturation towards neutrophils, IL-3 cross reactivity is gradually lost. Further investigations were conducted to determine the mechanism underlying the affinity conversion of the GM-CSF-R. Northern analysis demonstrated the presence of mRNA encoding the GM-CSF-R α and GM-CSF-R β subunits at every differentiation stage, indicating that the genes of both GM-CSF-R subunits are transcribed throughout neutrophilic maturation. Northern and reverse transcriptase-polymerase

chain reaction analysis of GM-CSF-Rα and β_c (KH97) transcripts did not provide indications for the involvement of GM-CSF-R splice variants in the formation of the intermediate affinity GM-CSF-R complex. Because immature myeloblasts express few high affinity GM-CSF receptors, whereas differentiated cells express much higher numbers of intermediate affinity receptors and because Northern analysis indicated that β_c transcripts but not GM-CSF-Rα transcripts increase with progressive maturation, the possibility that increased availability of β_c caused the affinity changes was investigated. In COS-cell transfectants with increasing amounts of β_c in the presence of a fixed number of GM-CSF-Rα chains, the high affinity GM-CSF binding converted into intermediate affinity GM-CSF binding. Although the exact composition of the high and intermediate affinity GM-CSF receptor complexes still remain unknown, we assume that the affinity shift is caused by increased expression of β_c subunits resulting in alternative oligomerization of the GM-CSF-Rα and β_c subunits.

SAMENVATTING

De aanmaak van bloedcellen (hematopoiese) vindt onder normale omstandigheden plaats in het beenmerg. Hier bevinden zich de hematopoietische stamcellen die door celvermenigvuldiging en uitrijping de verschillende soorten bloedcellen kunnen voortbrengen die uiteindelijk hun taak in het bloed en in diverse andere weefsels moeten vervullen. Dit zeer dynamische proces, verantwoordelijk voor de dagelijkse aanmaak van meer dan 10^{11} leukocyten en erythrocyten, wordt in belangrijke mate gereguleerd door hematopoïetische groeifactoren (HGFs), geproduceerd door o.a. monocyten, endotheelcellen en fibroblasten. Groeifactoren oefenen hun werking op de bloedcellen uit door aan specifieke membraanreceptoren te binden en deze vervolgens te activeren. Bij acute myeloide leukemie (AML) is de aanmaak en uitrijping van leukocyten gestoord. De leukocyten onttrekken zich aan de normale controlemechanismen en verdringen tevens de normale bloedcelaanmaak in het beenmerg. Omdat de hematopoïetische groeifactor-receptoren een cruciale rol spelen in deze controlemechanismen, rijst de vraag welke rol deze receptoren spelen bij de pathogenese van acute myeloide leukemie. De studies die in dit proefschrift worden gepresenteerd behandelen de expressie, specifieke bindingseigenschappen, functie en regulatie van de receptoren voor drie hematopoïetische groeifactoren (granulocyt macrofaag kolonie-stimulerende factor (GM-CSF), interleukine-3 (IL-3) en granulocyt kolonie-stimulerende factor (G-CSF)) bij AML en normale bloedcellen. In ***hoofdstuk 1*** wordt een kort overzicht gegeven over normale bloedcelvorming, AML en hematopoïetische groeifactoren en hun receptoren.

In ***hoofdstuk 2 en 3*** wordt het onderzoek beschreven naar het voorkomen en de karakterisering van IL-3, GM-CSF en G-CSF receptoren op blasten van patienten met AML. Bij 13 van de 15 AML patiënten werden hoog affiene IL-3 receptoren (dissociatie constante (K_d) 26 tot 414 pM) op de leukemische cellen aangetoond. Het gemiddeld aantal IL-3 receptoren per cel varieerde van 21 tot 145. Op leukocyten van gezonde proefpersonen werd een vergelijkbare expressie van IL-3 receptoren met dezelfde affiniteit gemeten. Bij alle 14 AML patiënten die bestudeerd werden bleken G-CSF receptoren op de blasten tot expressie te komen. Gemiddeld varieerde de expressie van de G-CSF receptoren van zeer laag (specifieke binding aantoonbaar) tot gemiddeld 428 receptoren per cel. Op normale granulocyten kwamen hogere receptor aantallen voor (703 tot 1296 G-CSF receptoren per cel). De G-CSF receptoren bleken een enkelvoudige affiniteit te bezitten, met een K_d die varieerde van 214 tot 378 pM op AML blasten en 405 tot 648 pM op normale granulocyten. De expressie van IL-3, GM-CSF en G-CSF receptoren werd vergeleken met de effecten van de groeifactoren op de in vitro proliferatie van de AML cellen. De mate van activering van AML cel proliferatie door IL-3, GM-CSF en G-CSF werd bepaald door de inbouw van radioactief thymidine te meten. IL-3, GM-CSF en G-CSF bleken in de meeste gevallen in staat om DNA synthese van AML cellen in vitro te stimuleren. Klaarblijkelijk zijn slechts weinig receptoren nodig om de AML cellen tot deling aan te zetten. In een aantal gevallen bleken de cellen niet in staat om op IL-3 (4 van 15 gevallen), GM-CSF (4 van 15 gevallen) of G-CSF (2 van de 14 gevallen) te reageren, ondanks de normale expressie van receptoren. Het onvermogen van de cellen om te reageren op de groeifactorprikkel zou verklaard kunen worden door een blokkade in de signaaltransductie van deze receptoren op intracellulair niveau. De resultaten van

deze experimenten geven geen steun aan de hypothese dat overexpressie of een abnormale affiniteit van groeifactorreceptoren een rol spelen bij de aberrante celproliferatie bij AML.

In ***hoofdstuk 4*** wordt ingegaan op de rol van c-kit expressie in AML cellen in relatie met de effecten van de groeifactor kit-ligand (KL) op AML celproliferatie. Tevens is het synergisme van kit-ligand met andere groeifactoren onderzocht. Met behulp van anti c-kit antilichamen en FACS analyse werd KL receptor expressie in 8 van de 9 gevallen waargenomen, vaak alleen in een subpopulatie van de cellen. Scatchard analyses van KL receptoren op twee AML celmonsters en de cellijn MO7e toonden tussen de 1460 en 120.000 KL receptoren aan, in alle gevallen met hoge affiniteit (K_d 40 tot 160 pmol/L). Kit-ligand stimuleerde de DNA synthese van AML cellen in 8 van de 10 patiënten en kolonie groei in 4 gevallen. Kit-ligand bleek synergistisch te werken met IL-3, GM-CSF en met name G-CSF. Daarentegen bleken de effecten van KL op AML proliferatie geremd te kunnen worden door tumor necrosis factor (TNF) (4 van de 10 gevallen) en IL-4 (2 van de 10 gevallen). Antilichamen gericht tegen GM-CSF, M-CSF, G-CSF en IL-6 bleken niet in staat de kit-ligand respons te remmen. Het is derhalve onaannemelijk dat de effecten van KL via autocriene stimulatie van deze groeifactoren tot stand komen. Om het synergisme van KL met andere groeifactoren nader te karakteriseren werden titraties uitgevoerd van KL, al of niet in aanwezigheid van andere groeifactoren en vice versa. In de KL dosis-respons bereikte de mate van DNA synthese een hoger plateau in de aanwezigheid van IL-3 of GM-CSF, echter zonder dat de gevoeligheid voor KL zich wijzigde. Omgekeerd bleken ook de ED_{50} waarden van GM-CSF, IL-3 of G-CSF niet te veranderen in aanwezigheid van KL. Tenslotte werd de mogelijkheid dat het synergisme berust op modulatie van groeifactorreceptoren onderzocht. De expressie van IL-3 en GM-CSF receptoren werd niet beinvloed door KL. De groeifactoren IL-3, GM-CSF, G-CSF, TNF of IL-4 bleken de expressie van kit-ligand receptoren op AML cellen evenmin te beinvloeden. Kennelijk is het mechanisme dat verantwoordelijk is voor het synergisme en antagonisme van kit-ligand met andere groeifactoren niet gelegen op het niveau van de membraan receptoren.

Tumor necrosis factor (TNF) is in staat om de proliferatie van AML cellen gestimuleerd door GM-CSF en IL-3 in vitro synergistisch te verhogen. Onderzoek naar het mechanisme van dit synergisme wordt beschreven in ***hoofdstuk 5***. Er werd onderzocht of deze effecten tot stand komen door modulatie van groeifactorreceptoren. In zeven gevallen van AML bleken de cellen na behandeling met TNF meer IL-3 (40 tot 600%) en GM-CSF (30 tot 280%) te binden. Uit Scatchard analyses bleek dat TNF de expressie verhoogt van (1) de laag affiene GM-CSF receptoren, (2) de gemeenschappelijke hoog affiene IL-3/GM-CSF receptoren alsmede (3) de hoge affieniteits, unieke (niet-GM-CSF bindende) IL-3 receptoren (in hoofdstuk 7 worden de verschillende typen receptoren nader besproken). De verhoogde expressie van deze receptoren treedt op na 18 uur incubatie met TNF bij 37 °C. Bij oplopende concentratie van GM-CSF bleek de koloniegroei van AML blasten een hogere plateauwaarde te bereiken in aanwezigheid van TNF. Incubatie van AML blasten met 12-O-tetradecanoylphorbol-13-acetate (TPA) of formyl-Met-Leu-Phe (fMLP) (beide proteine kinase (PK)-C activerende verbindingen) had geen invloed op de expressie van GM-CSF receptoren. Daarentegen blokkeerden eiwitsynthese-remmers, zoals cycloheximide, de verhoogde expressie van receptoren door TNF wèl. De effecten van TNF op de GM-CSF receptoren komen dus

klaarblijkelijk niet tot stand via de activatie van proteine kinase C, maar wel na activatie van eiwitsynthese. In tegenstelling tot de resultaten bij AML cellen, bleek TNF niet in staat om de expressie van GM-CSF receptoren op granulocyten of monocyten te verhogen. Het effect van TNF op de GM-CSF en IL-3 receptorexpressie is beperkt tot een bepaald rijpingsstadium van myeloide differentiatie.

G-CSF stimuleert de proliferatie van AML in vitro en kan in sommige gevallen ook myeloide uitrijping induceren. TNF blijkt de door G-CSF geinduceerde proliferatie van AML cellen in vitro te remmen. In ***hoofdstuk 6*** wordt ingegaan op de rol van G-CSF receptoren in het antagonisme tussen TNF en G-CSF. Incubatie met TNF van celmonsters van 3 AML patienten bleek het aantal G-CSF receptoren met ± 70% te reduceren. Ditzelfde fenomeen werd ook bij normale granulocyten waargenomen. Het effect van TNF op de G-CSF receptor expressie treedt binnen 10 minuten op bij 37 °C en heeft geen invloed op de affiniteit van de G-CSF receptor. De verlaagde expressie van G-CSF receptoren trad niet op bij 0°C en werd niet met andere cytokinen zoals IL-2, IL-6 of GM-CSF verkregen. Met TPA (PKC activerende verbinding) kon het effect van TNF nagebootst worden, terwijl Staurosporine (PKC remmer) het TNF effect teniet doet. Kennelijk oefent TNF zijn werking op de G-CSF receptor uit via activering van PKC. Het door TNF of TPA geinduceerde effect op de G-CSF receptorexpressie bleek ook met protease remmers geblokkeerd te kunnen worden. Mogelijk komt de reductie van het aantal G-CSF receptoren tot stand doordat het extracellulaire domein enzymatisch van de receptor wordt afgekliefd via een door PKC geactiveerd protease. De resultaten in hoofdstuk 5 en 6 suggereren dat TNF een regulerende rol speelt in de hematopoïese. Via regulatie van IL-3 en GM-CSF receptoren op myeloide cellen kan TNF de proliferative activiteit verhogen. Anderzijds, via regulatie van G-CSF receptoren op myeloide cellen, kan TNF G-CSF afhankelijke hematopoïese blokkeren.

Eerder is aangetoond dat de activiteiten van IL-3 en GM-CSF elkaar voor een groot deel overlappen. Een mogelijke verklaring hiervoor is dat beide groeifactoren aan dezelfde receptor binden. In ***hoofdstuk 7*** wordt nader ingegaan op de specifieke bindings eigenschappen van de receptoren voor IL-3 en GM-CSF. Op AML cellen afkomstig van 8 patiënten bleken twee typen GM-CSF receptoren voor te komen: één met een lage affiniteit (K_d 5,1 tot 24,8 nmol/L) en één met hoge affiniteit (K_d 31 tot 104 pmol/L). Op dezelfde AML cellen komen ook IL-3 receptoren met hoge affiniteit tot expressie (K_d 24 tot 104 pmol/L). Uit kruiscompetitie experimenten met AML cellen bleek dat een grote overmaat niet-radioactief IL-3 de hoog affiene binding van radioactief gemerkt GM-CSF volledig kon blokkeren. De binding van GM-CSF met lage affiniteit werd daarentegen niet door IL-3 beinvloed. Omgekeerd bleek ook dat de binding van radioactief IL-3 door niet-radioactief GM-CSF geremd kon worden. In een aantal gevallen, was deze remming volledig, terwijl bij de overige gevallen van AML slechts gedeeltelijke (49 tot 77%) remming van de radioactief gemerkt IL-3 binding optrad. Het feit dat deze kruiscompetitie zich zowel bij 37°C als bij 4 °C voordoet, sluit receptor transmodulatie uit en geeft aan dat er sprake is van een competitie op het niveau van receptor binding. Neutrofiele granulocyten bleken een geheel andere GM-CSF bindingskarakteristiek te hebben. GM-CSF receptoren op neutrofiele granulocyten vertonen een intermediaire affiniteit voor GM-CSF (K_d ~400 pmol/L) en laten geen kruiscompetitie zien met IL-3. Er zijn dus 4 verschillende receptortypen voor IL-3 en GM-CSF te onderscheiden: (i) laag affiene GM-CSF receptoren die geen IL-3 binden, (ii) gemeenschappelijke hoog affiene IL-3/GM-CSF

receptoren (iii) hoog affiene IL-3 receptoren die geen GM-CSF kunnen binden (deze drie type receptoren werden naast AML cellen ook op monocyten aangetoond) en tenslotte (iv) GM-CSF receptoren met intermediaire affiniteit die geen IL-3 kunnen binden. De betrokkenheid van een gemeenschappelijk GM-CSF/IL-3 receptor(keten) werd verder geillustreerd door chemische crosslinking van radioactief ligand aan de receptor. Zowel GM-CSF als IL-3 bleken aan drie membraan eiwitten met identieke molekuul massa's te binden (130, 105 en 75 kDa).

Uit werk van andere onderzoeksgroepen werd duidelijk dat de hoog affiene GM-CSF receptor bestaat uit een heterodimeer complex van een GM-CSF specifieke α keten en een, door andere receptoren gedeelde, β keten (KH97). De kruisreactiviteit tussen GM-CSF en IL-3 werd hiermee ook verklaard; zowel de IL-3 receptor als de GM-CSF receptor gebruiken beide deze β keten (β_c of KH97). In hoofdstuk 7 is beschreven hoe het expressiepatroon van GM-CSF receptoren op myeloide cellen afhangt van de differentiatiegraad. De vraag rijst in welk rijpingsstadium deze veranderingen plaatsvinden en welk mechanisme hieraan ten grondslag ligt. *Hoofdstuk 8* beschrijft de veranderingen van de GM-CSF receptor expressie in drie modellen die elk opeenvolgende stadia van myeloide uitrijping vertegenwoordigen: 1) AML cellen die in vitro van blasten naar myelocyten uitrijpen, 2) chronische fase CML cellen die verschillende stadia van myelocyten tot segmentvormige granulocyten omvatten, 3) de promyelocyten-cellijn HL-60 waarvan de promyelocyten onder invloed van retinoïdezuur tot functionele granulocyten kunnen uitrijpen. De AML cellen bleken na in vitro uitrijping tot (pro)myelocyten hun kenmerkende hoge en laag affiene GM-CSF receptoren te verliezen om vervolgens intermediair affiene GM-CSF receptoren tot expressie te brengen. Net als de hoog affiene GM-CSF receptoren bleken deze intermediaire receptoren ook IL-3 te binden. Ook bij CML cellen en HL-60 cellen bleek GM-CSF binding met intermediaire affiniteit voor te komen, die kruisreageren. Na inductie met retinoïdezuur van de promyelocytaire HL60 cellen tot uitgerijpte granulocyten bleek de intermediaire GM-CSF receptor ongewijzigd tot expressie te komen, echter, de kruisreactiviteit met IL-3 was verdwenen (overeenkomstig de situatie in normale granulocyten). Voorts wordt in hoofdstuk 8 het mechanisme onderzocht dat verantwoordelijk is voor de veranderde affiniteit van de GM-CSF receptor. Met behulp van Northern analyse konden mRNAs van de GM-CSF-Rα en β_c (KH97) ketens in alle stadia van neutrofiele differentiatie worden aangetoond. Verdere analyse met reverse transcriptase-polymerase kettingreactie van GM-CSF-Rα en β_c mRNAs toonden geen splice-varianten aan. Kennelijk zijn de ongemodificeerde α en β receptorketens betrokken bij de vorming van het intermediair affiene GM-CSF receptor complex. Uit Northern analyse bleek dat het expressie niveau van de β receptorketen mRNA toenam met gelijkblijvend niveau van de α keten bij cellen met een hogere differentiatiegraad. Door in COS cellen met een constant expressie niveau van de GM-CSF-Rα de expressie van β_c te vergroten bleek de GM-CSF binding over te gaan van een hoge naar intermediaire affieniteit. Het lijkt aannemelijk dat de relatieve kwantitatieve expressie van β_c ten opzichte van GM-CSF-Rα de affiniteit in het GM-CSF receptor complex bepaalt.

Hoofdstuk 9 geeft overzicht waarin de verschillende functionele en structurele eigenschappen van de familie van de hematopoietische groeifactorreceptoren en in het bijzonder van de GM-CSF, IL-3 en G-CSF receptoren worden besproken. Aan de orde komen: het voor deze familie kenmerkende CRH domein, het voorkomen en de

rol van splicevarianten van HGF receptoren, de vorming van hoog affiene receptoren, ligandherkenning, het cytoplasmatisch domein en signaal transductie. In de discussie (***hoofdstuk 10***) wordt ingegaan op de rol van IL-3, GM-CSF, G-CSF en KL receptoren in leukemogenese. Het gemeenschappelijk gebruik van één receptorketen door twee receptorsystemen, waarvan de competitie tussen de GM-CSF en IL-3 voor receptor binding het gevolg is, wordt besproken, alsmede de consequentie hiervan t.a.v. de specificiteit versus redundantie van de GM-CSF en IL-3 stimuli. Tenslotte wordt de mogelijke betekenis van alternatieve complexvorming van de GM-CSF receptor β keten voor ligand binding en signaaltransductie bediscussieerd.

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CURRICULUM VITÆ

De schrijver van dit proefschrift werd op 25 mei 1960 te Den Haag geboren. Na het behalen van het diploma atheneum B, aan het Rijnlands Lyceum te Wassenaar in 1978, studeerde hij één jaar aan de Technische Hogeschool van Delft. In 1979 werd met de studie geneeskunde aan de Rijksuniversiteit Leiden aangevangen. Van 1982 tot 1985 was hij als student assistent verbonden aan de afdeling experimentele hematologie van het Radiobiologisch Instituut van TNO te Rijswijk, de afdeling medische informatica van de Rijksuniversiteit te Leiden (hoofd ir. C.L. Tuinstra) en het laboratorium oogheelkunde van het Academisch Ziekenhuis Leiden (hoofd Prof. Dr. J.A. Oosterhuis en Dr. J.A. van Best). Tevens was hij gedurende enkele maanden in 1986 en 1991 werkzaam op de afdeling flow cytometry van het Lawrence Livermore Natl. Laboratory in Californie (hoofd Dr. G.J. van den Engh). Na het behalen van het artsexamen in augustus 1987 was hij werkzaam op de afdeling Celkweek van de Daniel den Hoed Kliniek te Rotterdam (hoofd Prof. Dr. B. Löwenberg). Hier werd het in dit proefschrift beschreven onderzoek verricht. In april 1993 werd begonnen met de opleiding tot patholoog op de afdeling klinische pathologie van het Academisch Ziekenhuis Rotterdam (opleider Prof. Dr. F.T. Bosman).