HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTOR: FUNCTION OF CYTOPLASMIC SUBDOMAINS AND IDENTIFICATION OF RECEPTOR DEFECTS IN CONGENITAL NEUTROPENIA AND ACUTE MYELOID LEUKEMIA

HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTOR: FUNCTION OF CYTOPLASMIC SUBDOMAINS AND IDENTIFICATION OF RECEPTOR DEFECTS IN CONGENITAL NEUTROPENIA AND ACUTE MYELOID LEUKEMIA

HUMANE GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTOR: FUNCTIE VAN CYTOPLASMATISCHE SUBDOMEINEN EN IDENTIFICATIE VAN RECEPTORDEFECTEN IN CONGENITALE NEUTROPENIE EN ACUTE MYELOIDE LEUKEMIE

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM OP GEZAG VAN
DE RECTOR MAGNIFICUS PROF.DR. P.W.C. AKKERMANS M.A.
EN VOLGENS BESLUIT VAN HET COLLEGE VOOR PROMOTIES
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 29 MAART 1995 OM 15.45 UUR

DOOR

FAN DONG

GEBOREN TE SHANGHAI, P.R. CHINA

PROMOTIECOMMISSIE

PROMOTOR:	Prof. Dr. B. Löwenberg
CO-PROMOTOR:	Dr. I.P. Touw
OVERIGE LEDEN:	Prof. Dr. J.A. Grootegoed
	Prof. Dr. J.W. Oosterhuis
	Prof. Dr. A.J.P. Veerman

The work presented in this thesis was performed at the Department of Hematology, Dr. Daniel den Hoed Cancer Center and Institute of Hematology, Erasmus University, Rotterdam, The Netherlands. This work was supported by a grant from the Dutch Cancer Society "de Nederlandse Kankerbestrijding". Financial support from the Dutch Cancer Society for printing this thesis is gratefully acknowledged.

Gedrukt bij Offsetdrukkerij Ridderprint B.V., Ridderkerk

To my wife Yaling
To my daughter Siwen

Contents

Abbreviations:

AML	acute myeloid leukemia
ANC	absolute neutrophil count
bp	base pair
cDNA	complementary DNA
CFU	colony-forming unit
CML	chronic myelogenous leukemia
CNTF	ciliary neurotrophic factor
CRH	cytokine receptor homology
EGF	epidermal growth factor
EPO	erythropoietin
FAB	French-American-British (classification)
FCS	fetal calf serum
FGF	fibroblast growth factor
G-CSF	granulocyte colony-stimulating factor
GH	growth hormone
GM-CSF	granulocyte-macrophage colony-stimulating factor
HGF	hematopoietic growth factor
^{3}H-TdR	tritiated thymidine
IGF	insulin-like growth factor
IL	interleukin
INF	interferon
INS	insulin
JAK	janus kinase or just another kinase
kb	kilobase
Kd	dissociation constant
kDa	kilodolton
KL	kit ligand
LIF	leukemia inhibitory factor
MAP	mitogen activated protein
M-CSF	macrophage colony-stimulating factor
MDS	myelodysplastic syndrome
MPO	myeloperoxidase
NBT	nitroblue tetrazolium
NGF	nerve growth factor
nt	nucleotide
OSM	oncostatin
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PRL	prolactin
PTK	protein tyrosine kinase
R	receptor

RAEB	refractory anemia with excess of blasts
RT	reverse transcriptase
SCN	severe congenital neutropenia
SH	Src homology
SSCP	single strand conformation polymorphism
STAT	signal transduction and activation of transcription
TNF	tumor necrosis factor
TPO	thrombopoietin
WT	wild-type

Chapter 1

INTRODUCTION

1.1 Hematopoiesis

Blood cell formation or hematopoiesis takes place mainly in the bone marrow. A small population of self-renewing pluripotent stem cells, residing in the bone marrow, generate progenitor cells which are committed irreversibly to various hematopoietic lineages. These committed progenitor cells can undergo proliferation, differentiation and terminal maturation to give rise to mature blood cells that include granulocytes, erythrocytes, macrophages, platelets and lymphocytes (Figure 1). Most mature blood cells have only a limited life-span and have to be replenished constantly throughout life to maintain them in proper numbers in the circulation. In an adult of 70 kg weight the steady-state turnover of blood cells per day is estimated to be around 1×10^{12} cells, including 7×10^{11} neutrophilic granulocytes (1). Under stress conditions, such as infections and bleeding, production of blood cells of specific lineages can be enhanced substantially. This remarkable cell renewal process is possible because there exists considerable amplification in the hematopoietic system: a single stem cell is capable of producing more than 10^6 mature blood cells following multiple divisions (2,3). With progressive differentiation, hematopoietic cells gradually lose the capacity for proliferation.

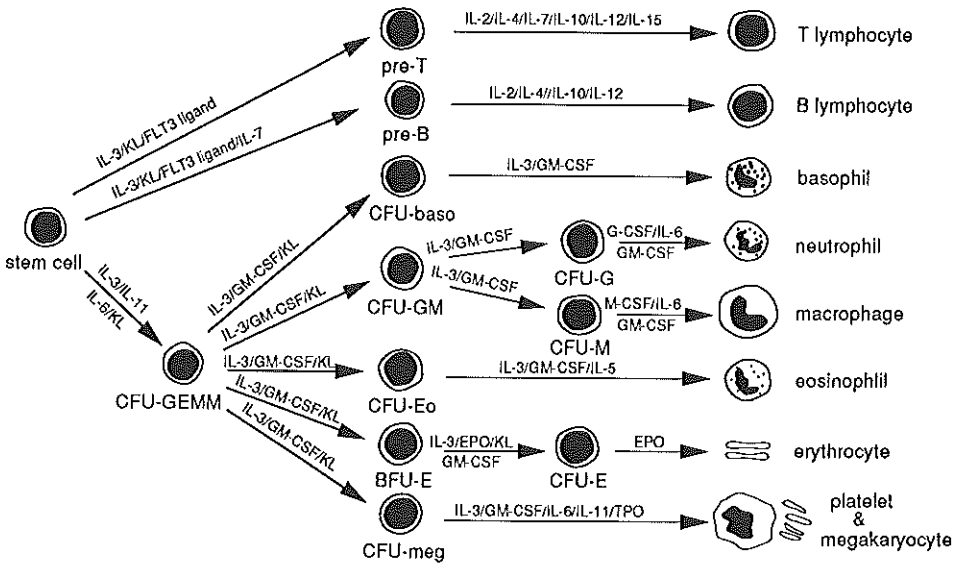

Figure 1. Schematic representation of hematopoiesis. Blood cells of different lineages arise from bone marrow pluripotent stem cells. Hematopoietic progenitor cells at distinct stages of development can be identified by *in vitro* colony culture assays. CFU, colony-forming unit; BFU, burst-forming unit; GEMM, granulocyte-erythroid-monocyte-megakaryocyte; GM, granulocyte-monocyte; E, erythroid; G, granulocyte; M, monocyte; baso, basophil; Eo, eosinophil; Meg, megakaryocyte. Some of the major hematopoietic growth factors (see text below) involved in the regulation of hematopoiesis are also indicated.

Hematopoiesis represents one of the most complex processes for which strict regulatory mechanisms are required. Regulation of hematopoiesis is accomplished essentially by two interacting mechanisms (2,4). Stromal cells in the bone marrow control some aspects of hematopoiesis via direct cell-to-cell contact. In combination with extracellular matrix and a microvascular network, stromal cells also provide a suitable microenvironment for the growth and development of hematopoietic cells. A second regulatory mechanism comprises a network of humoral factors, collectively called hematopoietins or hematopoietic growth factors (HGFs). These factors, which can act locally or circulate in the blood, play a pivotal role in maintaining the homeostasis of hematopoiesis.

1.2 Hematopoietic growth factors (HGFs)

HGFs are glycoprotein hormones involved in the regulation of hematopoiesis. The major cellular sources of HGFs include lymphocytes, monocytes and macrophages, endothelial cells, fibroblasts and stromal cells. Erythropoietin (EPO) is synthesized mainly in the kidney (2). The levels of production of HGFs are normally low, but can be enhanced considerably in response to extracellular stimuli (4). Most HGFs, if not all, are multifunctional and they promote the proliferation, differentiation and survival of hematopoietic cells. In addition, HGFs may activate the functions of mature blood cells. Some HGFs, such as transforming growth factor ß (TGFß) and macrophage inflammatory protein 1α (MIP-α), function primarily as negative regulators and inhibit the proliferation of hematopoietic progenitor cells (3,5). Others, like interleukin- (IL-) 4 and tumor necrosis factor (TNF), may exert both positive and negative effects on the proliferation of hematopoietic cells, depending on the cell lineages and developmental stages (3,6,7).

According to the developmental stages of target cells, HGFs can be grouped into two categories, i.e., the early-acting lineage-nonspecific factors and the late-acting lineage-specific factors (1,2). The first group includes, e.g., IL-1, IL-3, IL-4, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF), kit ligand (KL, also referred to as stem cell factor), IL-11 and IL-12. For instance, IL-3 has been shown to stimulate the growth of early multipotential progenitor cells that can develop into neutrophils, macrophages, eosinophils, basophils, erythrocytes and megakaryocytes (8). Nevertheless, IL-3 does not appear to support the terminal stages of hematopoiesis as IL-3 responsiveness of hematopoietic progenitor cells declines with differentiation and terminal maturation (9,10). In contrast, late-acting HGFs, such as EPO, macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF), IL-5 and thrombopoietin (TPO)(11-13), predominantly affect late stages of hematopoiesis. Most late-acting HGFs are lineage-specific, e.g., EPO regulates erythropoiesis while M-CSF controls the production of macrophages. Late-acting HGFs not only stimulate the proliferation, but also induce the terminal maturation of hematopoietic cells of particular lineages. Such classification, however, is not absolute. For instance, G-CSF also acts on early multipotential progenitor cells (14-16), whereas IL-6 supports the terminal development of certain myeloid cells (17,18).

The genes and complementary DNAs (cDNAs) of most HGFs have been cloned. Studies with recombinant HGFs have established that pleiotropy and apparent redundancy are two

prominent features of HGFs (19,20). Many HGFs, in particular those that act at early stages of hematopoiesis, display overlapping biological activities on a variety of cell types. Conversely, different HGFs can act on the same cell type and mediate similar effects. A third important feature of HGF activities is the functional interplay between these factors (2,4). Two or more factors can interact synergistically or antagonistically in inducing a certain cellular response. Moreover, the production of HGFs in one cell type can be modulated by the actions of other HGFs on that cell type (4).

Some HGFs, such as M-CSF, KL, TNF, IL-1 and FLT3/FLK2 ligand, exist in both secreted and membrane-bound forms (21-27). The physiological role of membrane-bound HGFs in hematopoiesis is unclear. However, it has been suggested that membrane-bound HGFs may exert regulatory effects via direct cell-to-cell contact.

1.3 Regulation of granulopoiesis by G-CSF and other HGFs

Neutrophilic granulocytes are essential components of the host defense system against infections. Like all blood cells, mature granulocytes arise from bone marrow stem cells. In steady state, the majority of stem cells are dormant (1) and only a few of them are activated to produce pluripotent progenitor cells (CFU-GEMM). These early progenitor cells in turn may generate cells increasingly committed to the granulocytic lineage (Figure 1). After a process involving proliferation and maturation, committed granulocytic progenitor cells eventually develop into terminally mature granulocytes.

A number of HGFs, including IL-3, GM-CSF, KL, IL-6 and G-CSF, have been shown to be positive regulators of granulopoiesis and act at different stages of myeloid cell development (4,28). IL-3 and GM-CSF support the proliferation of early myeloid progenitor cells and, to a limited extent, induce the differentiation of these cells (8,29-31). The stimulatory actions of IL-3 and GM-CSF in granulopoiesis have also been demonstrated *in vivo* in both animal models and humans. In addition, GM-CSF modulates the function of mature granulocytes. KL also acts on early progenitor cells, but it appears to function primarily as a synergistic molecule and alone it only induces a modest effect (32-35). Like KL, IL-6 generally synergizes with other HGFs in inducing a proliferative response, but some studies indicate that IL-6 alone is able to promote the growth and maturation of granulocytic progenitor cells, at least in mice (17,18).

G-CSF is unique among the above-mentioned regulators of granulopoiesis in that, apart from stimulating the proliferation, G-CSF strongly induces the terminal maturation of granulocytic progenitor cells. Murine and human G-CSFs were originally identified as a distinct activity that induced the differentiation of a murine myelomonocytic leukemic cell line WEHI-3B$^+$ (36-38). This activity led to its purification from media conditioned by different cell types and subsequent molecular cloning (39,40). Murine and human G-CSFs were found to exert complete two-way biological activity on murine and human cells. In addition to the WEHI-3B$^+$ cells, G-CSF was shown to induce the differentiation of three other murine myeloid cell lines and a human promyelocytic leukemia cell line HL-60 (41-45).

In vitro studies demonstrate that G-CSF supports the proliferation and neutrophilic differentiation of normal hematopoietic progenitor cells (39,40). The half maximal response

to G-CSF is seen at a concentration of 3 pM (46). Although G-CSF *in vitro* is a weaker stimulus for cell proliferation than GM-CSF or IL-3, *in vivo* it elicits a much higher granulocyte count than does GM-CSF or IL-3 (4). Several observations indicate that G-CSF is indispensable for normal granulopoiesis *in vivo*. For instance, dogs injected with human G-CSF developed neutralizing antibodies against human G-CSF, which cross-inhibited canine G-CSF. These dogs with anti-G-CSF antibodies developed prolonged neutropenia (47). Infusion of plasma from a neutropenic dog treated with human G-CSF into a normal dog also produced a neutropenia. Very recently, it was shown that mice lacking G-CSF due to targeted disruption of the G-CSF gene developed congenital chronic neutropenia (48). G-CSF production rises markedly during infections (49,50); however, in G-CSF-deficient mice infection-driven granulopoiesis was severely impaired (48). Collectively, these results indicate that G-CSF has a key role in the regulation of granulopoiesis both in steady state and under stress conditions.

Like many HGFs, G-CSF also promotes cell survival. When deprived of G-CSF, hematopoietic progenitor cells not only cease to proliferate, but also extinguish rapidly by a "self-destruction" mechanism, known as programmed cell death or apoptosis (see below) (51,52). The survival of mature neutrophils is also enhanced by G-CSF, notably at considerable lower concentrations of G-CSF than those required for promoting growth and differentiation of granulocytic progenitors (53). Finally, G-CSF is able to activate certain functions of mature neutrophils, e.g., chemotactic activity (54), production of superoxide, alkaline phosphatase and myeloperoxidase (55-57), and antibody-dependent cell-mediated cytotoxicity against tumor cells (58).

1.4 Cytokine/hematopoietin receptor superfamily

With the molecular cloning of an increasing number of genes encoding various growth factor receptors, it has become clear that these receptors can be grouped into several families on the basis of structural homology (19) (Table 1). These include (i) a superfamily of the cytokine/hematopoietin receptors (59,60); (ii) a relatively small family of the receptors for interferons (INFs) α, ß and γ, and IL-10 as well as tissue factor (TF), which appear to be evolutionarily related to the cytokine/hematopoietin receptors (61-64); (iii) receptors that are structurally related to the TNF receptors (p55 and p75) with characteristic cysteine-rich repeats in the extracellular domain, including the nerve growth factor (NGF) receptor, FAS, CD40, CD27, CD30, OX40 and 4-1BB (65); (iv) the immunoglobulin-like receptor family, of which the IL-1 receptor is a representative (19,60); and (v) receptors that possess intrinsic tyrosine kinase activities in the cytoplasmic domain, including the receptors for M-CSF, KL, FLT3/FLK2, insulin-like growth factor (IGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and insulin (INS) (66). Some receptors, such as c-kit and M-CSF-R, possess features on which basis they can be classified into two different families (Table 1).

The receptors for most HGFs are members of the cytokine/hematopoietin receptor superfamily (59,60), including the receptors for IL-2 to IL-7 (67-72), IL-9 (73), IL-11 (74), GM-CSF (69) and EPO (75), the ß chain of leukemia inhibitory factor receptor (LIF-Rß)

(76), the IL-12 p40 subunit (77) and the receptor for TPO, which is encoded by the *c-mpl* gene (78). This family also includes three receptors whose ligands are not HGFs, i.e., the receptors for growth hormone (GH) (79), prolactin (PRL) (80) and ciliary neurotrophic factor (CNTF) (81). The hematopoietin receptors are anchored on the cell membrane through a single transmembrane domain with the N-termini oriented extracellularly. Characteristic structural features of this family are the presence of four highly conserved cysteine residues and a five-residue motif of Trp-Ser-X-Trp-Ser (WSXWS), all within an approximately 200 amino acid region in the extracellular domain (59,60). This region is referred to as the cytokine receptor homology (CRH) region and is crucial for ligand binding. The CRH region is duplicated in IL-3 receptor ß chain, LIF-Rß and c-mpl (60). The significance of these duplications is unknown. The cytoplasmic domains of hematopoietin receptors are less conserved and do not appear to contain strict consensus sequences indicative of enzymatic activities.

Table 1. Some families of growth factor receptors

Family	Receptor
Cytokine/hematopoietin receptors	IL-2-R, IL-3-R, IL-4-R, IL-5-R, IL-6-R, IL-7-R, IL-9-R, IL-11-R, GM-CSF-R, EPO-R, LIF-R, G-CSF-R, p40 subunit of IL-12, c-mpl, CNTF-R, GH-R, PRL-R
IFN receptors	INF_{α}-R, $INF_{\alpha/ß}$-R, INF_{γ}-R, IL-10-R, TF
TNF receptors	TNF-R1 (p55), TNF-R2 (p75), NGF-R, FAS, CD40, CD27, CD30, OX40, 4-1BB
Immunoglobulin-like receptors	IL-1-R, M-CSF-R, c-kit, FLT3/FLK2, PDGF-Rs, FGF-Rs
Receptors with intrinsic tyrosine kinase activities	M-CSF-R, c-kit, FLT3/FLK2, IGF-R, EGF-R, PDGF-Rs, FGF-Rs, INS-R

An important advance in understanding the structures and functions of the hematopoietin receptors has come from the observations that most of these receptors are multiple-component or multiple-subunit complexes, and that certain components are shared by different receptor systems. For instance, the receptors for IL-3, GM-CSF and IL-5 share the common ß chain (69), whereas the gp130 molecule is shared by the receptors for IL-6, LIF, oncostatin (OSM), CNTF and IL-11 (82-85). The IL-2 receptor complex comprises three subunits, i.e., the α, ß and γ chains. The γ chain of the IL-2 receptor complex is also the component of the receptors for IL-4 and IL-7, and probably IL-13, and therefore is now referred to as γ_c (86-89). Moreover, both the IL-2 receptor ß chain and γ_c chain are part of the receptor complex of a newly cloned cytokine IL-15 (90). Usually, low affinity receptors consist of single subunits, which also determine ligand binding specificity. The common components that associate with the low-affinity subunits are required for converting the low affinity

receptors to high affinity forms. Additionally, common components act as major signaling molecules that couple extracellular ligand binding to the activation of intracellular events of signal transduction. As discussed above, different HGFs may exert similar effects on certain cell types. The apparent functional overlap of many HGFs can be explained, at least in part, by the use of the same signaling molecules in different receptor systems.

1.5 G-CSF Receptor

The receptor for G-CSF (G-CSF-R) is expressed on myeloid progenitor cells and mature neutrophils, but not on cells of other hematopoietic lineages (91,92). Several non-hematopoietic cell types, including endothelial cells, placenta, trophoblastic cells and cell lines derived from small cell lung cancer, have also been reported to express the G-CSF-R (93-95). The function of the G-CSF-R on nonhematopoietic cells is unknown. The numbers of the G-CSF-R on granulocytic progenitor cells increase with neutrophilic differentiation and mature human neutrophils have approximately 500 to 1000 receptors per cell (96,97). Crosslinking experiments indicate that the murine and human G-CSF-Rs have a molecular weight of about 130 to 150 kDa (97-99). Normal human neutrophils express a single class of high affinity G-CSF binding sites with a dissociation constant (Kd) of 200 to 500 pM. Because low concentrations of G-CSF are required for half-maximal response, low occupancy of the G-CSF-R may be sufficient to induce a maximal biologic response. Interestingly, G-CSF-R preparations purified from murine NFS-60 cells demonstrated two binding activities: a high affinity binding (Kd 120 to 360 pM) and a low affinity binding (Kd 2.6 to 4.2 nM). It appeared that the low affinity binding was derived from monomeric G-CSF-R proteins, while the high affinity binding resulted from the formation of the G-CSF-R homodimers (99). Because intact NFS-60 cells displayed G-CSF binding sites of high affinity, these results suggest that the G-CSF-R expressed on intact cells binds G-CSF as homodimeric molecules.

Molecular cloning of the cDNAs encoding the murine and human G-CSF-Rs (100-102) revealed that murine and human G-CSF-Rs are single transmembrane polypeptides of 812 and 813 amino acids, respectively, and share considerable structural similarities (62.5% identity at amino acid level). The extracellular domain of the G-CSF-R contains five subdomains (Figure 2). The CRH region classifies the G-CSF-R as a member of hematopoietin receptor superfamily. In addition, the G-CSF-R contains an immunoglobin-like sequence and three repeats of the fibronectin type III module. Notably, while the majority of the cytokine/hematopoietin receptors form complexes of different receptor subunits (heteromerization), the high affinity G-CSF-R complexes are believed to be homodimers. Like other cytokine/hematopoietin receptors, the cytoplasmic domain of the G-CSF-R lacks kinase activities. Several stretches of amino acids, however, have recently been identified in the G-CSF-R cytoplasmic domain that show limited sequence homology to other members of the hematopoietin receptor superfamily (103). Two stretches of sequences, called "box 1" and "box 2", are situated in the membrane-proximal region of the G-CSF-R (Figure 2). Box 1 and box 2 are also present in several other hematopoietin receptors (103,104). The membrane-distal cytoplasmic region of the G-CSF-R contains a third segment, called "box 3", which is shared only with the IL-6 signal transducer gp130 (71,105). The functions of

these conserved sequences are still largely unknown. However, it has been observed for several hematopoietin receptors that the membrane-proximal cytoplasmic region including box 1 and box 2 is essential for transducing proliferative signals (106-113).

Four different isoforms of the human G-CSF-R encoded by different cDNAs have been described (101,102,114). Analysis of the structure of the human G-CSF-R gene indicates that these isoforms are derived from alternative splicing of RNA transcribed from a single G-CSF-R gene. These G-CSF-R isoforms are all identical in the extracellular domain, but the downstream sequences differ (Figure 2). The G-CSF-R protein with a cytoplasmic domain of 183 amino acids is most homologous to the murine homologue. Two other isoforms have either an altered carboxy-terminus or an insertion of 27 amino acids in the region between box 1 and box 2, and both appear to be abundantly expressed in the placenta. Of particular interest is the identification of a soluble isoform of the G-CSF-R in a leukemic cell line U937 (101). Whether these G-CSF-R isoforms are also expressed in normal granulocytic cells is unclear, and nothing is known regarding their possible physiological role *in vivo*.

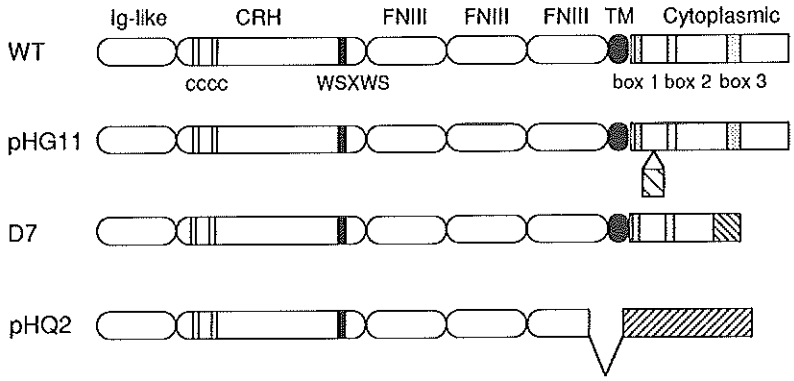

Figure 2. Diagram of the human wild-type (WT) G-CSF-R and its isoforms. The extracellular domain of the G-CSF receptor contains 5 subdomains as indicated. Four conserved cysteine residues and a WSXWS motif are also indicated. Cytoplasmic regions conserved in several members of cytokine/hematopoietin receptor superfamily are shown as boxes 1, 2 and 3. Two G-CSF receptor isoforms contain either an insertion of 27 amino acids in the cytoplasmic domain (pHG11) or an altered carboxy-terminus (D7). Note that the entire transmembrane domain is deleted in pHQ2. The hatched boxes denote sequences distinct from the WT G-CSF-R. Ig-like, immunoglobulin-like; CRH, cytokine receptor homologous; FNIII, fibronectin type III; TM, transmembrane domain.

The human G-CSF-R gene has been mapped to chromosome 1p32-35 (115,116). Analysis of the gene structure revealed that the human G-CSF-R gene contains 17 exons scattered over a locus of 16.5 kb and is present as single copy per haploid genome (117). The G-CSF-R protein is encoded by exons 3-17. Primer extension analysis identified a major and a minor transcription initiation site. The promoter region contains consensus sequences for binding of AP-1, AP-2 and GF-1. Approximately 110 nucleotides upstream of the major transcription

initiation site, there is an element of 18 nucleotides that is homologous to the sequences found in the promoter regions of human myeloperoxidase, neutrophil elastase genes and the murine G-CSF-R gene (118). This element may be important for the tissue-specific expression of these genes in granulocytic cells.

1.6 Mechanisms of G-CSF-R activation and signal transduction

Hematopoietin receptor signaling is initiated through dimerization or oligomerization of receptor complexes (60,119). That receptor dimerization is induced by ligand binding has been convincingly demonstrated in the context of the growth hormone receptor (GH-R), a member of cytokine/hematopoietin receptor superfamily (120,121). It has been shown that each GH molecule has two binding sites that can sequentially bind two GH-R molecules and thereby induce the homodimerization of GH-R chains. Notably, chimeric receptors containing the extracellular domain of the GH-R and intracellular domain of the G-CSF-R also transduce GH-dependent signals (122), indicating that the G-CSF-R works as a homodimer as well. This is consistent with the notion that the G-CSF-R expressed on intact cells binds to G-CSF as homodimeric molecules (99).

G-CSF stimulation results in a wide range of biological responses, including the induction of the expression of early response genes, such as *junB*, c-*fos* and the genes for several acute-phase plasma proteins (123,124). Little is known as to how G-CSF-triggered signals are transduced from the G-CSF-R to the cellular nucleus. Recently, activation of protein tyrosine kinases (PTKs) has been established as a general mechanism of signal transduction that operates in a large variety of receptor systems (19,119,125-127). Activation of the G-CSF-R also results in the rapid phosphorylation of multiple intracellular substrates in various cell systems (128-132). Since the G-CSF-R does not possess intrinsic cytoplasmic kinase activities, it thus appears that certain PTKs associate with the G-CSF-R, either directly or indirectly. It has been suggested that ligand-induced dimerization of the extracellular domains of the hematopoietin receptors results in the association of the cytoplasmic domains, and that this association may be crucial for creating sites for interaction with intracellular signaling molecules or PTKs (119,121).

The molecular identities of the proteins phosphorylated upon G-CSF stimulation are largely unknown. Recently, the JAK (Janus kinase or Just Another Kinase) family PTKs, including JAK1, JAK2, TYK2 and JAK3, have been shown to be tyrosine phosphorylated and activated upon ligand stimulation of several hematopoietin receptors, including the receptors for IL-2 to IL-4, IL-6, IL-7, IL-11, GM-CSF, EPO, LIF, GH and PRL (133-144). It has been suggested that the activation of JAK family PTKs in turn results in tyrosine phosphorylation and activation of a family of latent cytoplasmic transcription factors, called STAT (Signal Transduction and Activation of Transcription) proteins (Figure 3) (145,146). Following their activation, these STAT proteins are assembled into complexes, which then translocate to the nucleus and regulate transcription (147). Because the cytoplasmic domain of the G-CSF-R shares certain structural homology with those hematopoietin receptors capable of mediating the activation of JAK family members, it appears likely that the JAK family PTKs have a role in the signaling cascades of the G-CSF-R as well.

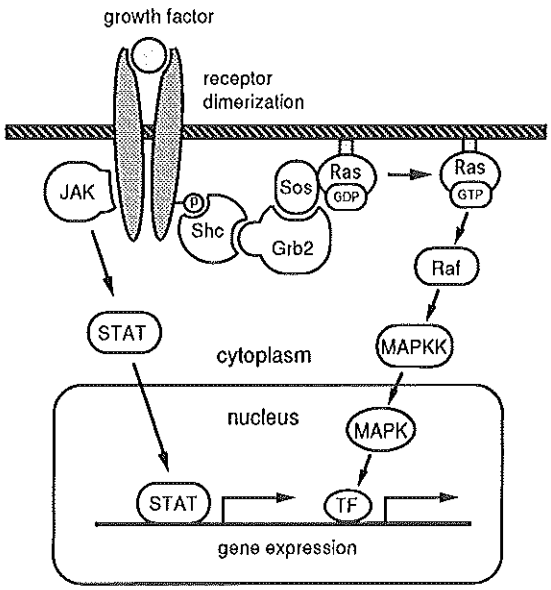

Figure 3. A model for two major signaling pathways of hematopoietin receptors. HGF binding results in receptor dimerization and activation of JAK tyrosine kinases. JAKs may directly activate STAT family transcription factors, which subsequently translocate to the nucleus and regulate gene expression. SH2-adaptor protein Shc may bind to activated receptors and is tyrosine-phosphorylated. Phosphorylated Shc interacts via its SH2 domain with Grb2, which in turn binds to Sos. The Grb2-Sos complex then modulates Ras, leading to activation of the serine/threonine/tyrosine phosphorylation cascade and ultimately induction of transcription. TF, transcription factor.

Members of the Ras family, i.e., $p21^{ras}$ protein, have also been implicated in hematopoietin receptor signal transduction. Activation of $p21^{ras}$ is achieved by converting this molecule from the GDP-bound form to the GTP-bound form, a process that involves interactions with guanine nucleotide exchanger Sos, Src homology 2 (SH2)-adaptor Shc, and the Grb2 molecule (Figure 3) (148). The active $p21^{ras}GTP$ triggers activation of a cascade of downstream protein kinases that include the serine/threonine kinase Raf, tyrosine/threonine kinase MAP (Mitogen Activated Protein) kinase kinase and the serine/threonine kinase MAP kinase, which may eventually lead to activation of gene expression. A number of HGFs including IL-2, IL-3, GM-CSF, KL, M-CSF and EPO have been shown to activate the Ras signaling pathway (149-153). Recently, rapid activation of $p21^{ras}$ and MAP kinase has also been observed following G-CSF stimulation of murine and human hematopoietic cells (154). Notably, signaling through $p21^{ras}$ appeared to be correlated with the G-CSF-induced proliferative response, but not with neutrophilic differentiation.

Finally, activation of the G-CSF-R may trigger other signaling activities, such as activation of Na^+/H^+ exchange (155) and induction of arachidonic acid release from cell

membrane of mature neutrophils, which indicates an early activation of a phospholipase (156). The physiological significance of these observations is unknown.

1.7 Neutropenia

Neutropenia is defined as an absolute neutrophil count (ANC) of 1.5 x 10^9/L or less in the peripheral blood (157). Neutropenia may present as a selective hematocytopenia or as a manifestation of a general pancytopenia. Patients with neutropenia are predisposed to infections, the risk and severity of which directly correlate with the severity and duration of neutropenia. When the ANC falls below 0.2 x 10^9/L, the risk of infection becomes very serious and infection can become life-threatening.

Neutropenia develops as a result of impaired production or increased destruction of neutrophils, or both. Broadly, neutropenia can be grouped into acquired and congenital forms (157). The causes of acquired neutropenia include viral infections (hepatitis, infectious mononucleosis), bacterial infections (tuberculosis, typhoid), autoimmune diseases (systematic lupus erythematosus, rheumatoid arthritis), splenomegaly, tumor infiltration of bone marrow, bone marrow suppression following irradiation or chemotherapy, side effects of drugs, and nutritional deficiencies. Cases in which the causes of neutropenia cannot be identified are referred to as idiopathic neutropenia.

Congenital neutropenia comprises a group of rare but important pediatric diseases (158). Children with congenital neutropenia usually present in infancy or early childhood with fever, infections, and severely reduced ANC. Considerable heterogeneity exists among different cases in terms of clinical presentation, family history, disease progression, and response to therapy. Clinical management of patients with congenital neutropenias remains a challenge as no effective therapeutic interventions have been established that can lead to a cure of these diseases, except for bone marrow transplantation in a few cases and the recent use of G-CSF.

Severe congenital neutropenia (SCN or Kostmann's syndrome) was first described in 1956 as an autosomal recessive disorder in several Swedish families (159). Subsequently, a number of cases were reported in the literature. A family history was not evident in the majority of these cases (160). The most distinctive features of SCN are the occurrence of severe neutropenia in early infancy and a normal marrow cellularity with few myeloid progenitor cells beyond the promyelocyte/myelocyte stage. The onset is marked by the development of frequent episodes of fever, skin infection, pneumonia, other bacterial infections and diarrhea. The peripheral blood ANC is extremely low (below 0.2 x 10^9/L), accompanied sometimes by increased eosinophils and monocytes. The other hematopoietic lineages are generally unaffected. In many cases severe neutropenia results in fatal infections. With improved medical care and frequent antibiotic intervention as well as the recent introduction of G-CSF treatment, survival beyond a few years is now possible, with some patients nowadays reaching adolescence. Unfortunately, upon prolonged survival a few cases of SCN have been reported to develop acute myeloid leukemia (AML) (158,161-163).

It has become evident that SCN is not a uniform entity, but most likely represents a group of heterogeneous disorders with similar clinical manifestations. A definitive description of distinct subtypes, however, is not yet possible because the molecular and cellular mechanisms

of SCN are unknown. In general, no quantitative or qualitative abnormalities in G-CSF production have been observed (164,165). Sera from SCN patients do not appear to contain inhibitory factors or autoantibodies against either G-CSF or granulocytic progenitor cells (166). Although several *in vitro* studies indicate reduced G-CSF responsiveness of bone marrow cells from some patients (167,168), normal-to-increased numbers of the G-CSF-R are detected on neutrophils from SCN patients with normal ligand binding affinity (169). Recently, G-CSF has been widely utilized in the treatment of SCN, and overall favorable responses have been observed (170,171). In contrast, GM-CSF therapy of SCN has not proven successful (172). In view of the increasing comprehension that GM-CSF-induced neutrophilic differentiation requires the action of G-CSF, the differential responses to G-CSF and GM-CSF would indicate specific defects in the G-CSF signaling pathways. Inasmuch as the underlying mechanisms of SCN are heterogeneous, these defects could lie in the cytoplasmic domain of the G-CSF-R or further downstream along the signaling cascades.

1.8 Acute leukemia

Acute leukemia is characterized by an uncontrolled expansion of hematopoietic cells that are arrested at early stages of development. This expansion results in the accumulation of immature nonfunctional leukemic cells in the blood, bone marrow and other tissues, eventually leading to the suppression of normal hematopoiesis. Consequently, patients develop related clinical symptoms and signs such as infections, bleeding and anemia as well as leukemic infiltration of various tissues.

It is now widely accepted that leukemogenesis is a multistep process (173,174). Leukemic cells arise from clonal evolution and expansion of single hematopoietic progenitor cells. The leukemia-initiating cells may initially harbour a single genetic lesion resulting in slight differences in developmental behaviour from its normal counterpart. With the acquisition of additional genetic lesions, the normal program of development in the preleukemic cells becomes increasingly disturbed, which may ultimately lead to full leukemic transformation. Genetic lesions that contribute to leukemogenesis act via two general mechanisms: (i) activation of genes (referred to as oncogenes) whose protein products induce malignant phenotypes, and (ii) inactivation of genes (so-called tumor suppressor genes) whose proteins inhibit leukemic transformation (175). Proteins encoded by these genes are normally involved in the regulation of cell proliferation, differentiation and survival as well as cell cycle progression. On the basis of their roles in signal transduction, these proteins fall into four classes: growth factor, growth factor receptor, cytoplasmic signal transducer and transcription factor (176). Protein products from different groups usually act in collaboration to accomplish an oncogenic transformation.

Leukemic cells apparently escape from the normal regulatory mechanisms of control of cell proliferation, maturation and survival by HGFs. *In vitro* studies have revealed that leukemic cells may exhibit variable degrees of spontaneous proliferative activity (177). This factor-independent proliferation is associated in certain cases with the production of HGFs by leukemic cells, leading to autocrine growth stimulation (177-179). In other cases it may result from the constitutive activation of proliferative signaling molecules, thereby abolishing

the requirement for activation by HGFs (173,174,176,180-182). Nonetheless, only in a minority of cases of leukemia does autonomous proliferation lead to a complete growth factor independence. By and large leukemic cells remain responsive to and dependent on HGFs for proliferation. For instance, IL-3, GM-CSF, G-CSF and M-CSF each can stimulate the proliferation of leukemic cells in 50 to 80% of cases of AML (177). Similar to their effects on normal hematopoietic cells, the various combinations of these HGFs significantly enhance the proliferation of AML cells. However, unlike normal hematopoietic progenitor cells which undergo proliferation and differentiation in response to HGFs, leukemic cells generally show little or no maturation when stimulated with HGFs including G-CSF (177,179). In the majority of AML cases, G-CSF fails to induce significant granulocytic maturation (183-185). Because cell maturation is a process associated with the loss of the proliferative potential, it is conceivable that maturation arrest represents one of the mechanisms whereby leukemic cells bypass the checkpoint of HGF regulation.

Like proliferation and maturation, cell survival is a strictly regulated process. Cells may be actively induced to die under certain conditions, e.g., if they have been produced in excess or because they are harmful, or of no use, to the body (186-188). Active induction of cell death, a process called apoptosis or programmed cell death, is characterized by premature chromatin condensation, cell shrinkage and degradation of nuclear DNA into fragments consisting of multimers of 200 bp. This process is distinct from cell swelling and disruption of plasma membranes seen in necrosis. In addition to abnormalities involving cell proliferation and maturation, uncontrolled survival may also play a role in leukemogenesis and may contribute as much to the clonal expansion of leukemic cells as does proliferation. Apoptosis is an active process that is dependent on the activities of specific gene products (189,190). A number of gene products have been identified as being implicated in the regulation of apoptosis. For instance, Bcl-2 and Bcl-x_l have been shown to inhibit apoptotic process (191-193), whereas p53, Bax, Bcl-x_s, ICE, and IRF-1 may promote this process (193-197). Notably, overexpression of Bcl-2 or inactivation of p53, either of which conceivably may lead to the prolonged cell survival, has been seen in certain cases of hematologic malignancies (198-200). The primary actions of chimeric proteins BCR-ABL and PML-RARα are generally thought to promote cell proliferation and to block cell maturation, respectively; however, recent studies indicate that the two oncoproteins are also capable of suppressing apoptosis (201,202). It appears that deregulation of cell survival represents a general mechanism of leukemogenesis.

Introduction to the experimental work

Signals transduced by the G-CSF-R regulate the proliferation, maturation and survival of myeloid progenitor cells. How these signals are mediated by the G-CSF-R is still poorly understood. It is unknown, for instance, whether the G-CSF-R contains distinct non-overlapping regions in the cytoplasmic domains that are coupled to the different biological effects. A clear insight into the structure/function features of the G-CSF-R not only is important for understanding the signaling mechanism, but may also be useful for clarifying the pathogenesis of certain diseases characterized by defective granulopoiesis, such as

neutropenia and leukemia. Experiments performed in Chapter 2 are aimed at characterizing the functional subdomains in the G-CSF-R cytoplasmic domain. Different G-CSF-R forms with altered or progressively deleted cytoplasmic domains were expressed in several murine hematopoietic cell lines and the proliferation and maturation properties of these transfected cells were examined. In Chapter 3 it is shown that the G-CSF-R is capable of mediating apoptosis signals under certain conditions. The cytoplasmic region responsible for death signaling activity is further defined. The possibility that abnormal G-CSF-R structures may have a role in the etiology of SCN is investigated in Chapter 4. Using single strand conformation polymorphism (SSCP) analysis followed by nucleotide sequencing, the G-CSF-R genes of 6 patients with SCN were analyzed. The presence of an abnormal G-CSF-R gene in different tissues and the *in vitro* colony-forming abilities of bone marrow cells in response to different HGFs were further investigated in one SCN patient. Because patients with SCN show an increased risk of developing AML, the potential role of defective G-CSF-R structures in the progression to leukemia was examined in two SCN patients who had developed AML (Chapter 5). Chapter 6 describes the molecular cloning of a novel G-CSF-R splice variant from granulocytes and the functional characterization of the novel variant. The expression of this G-CSF-R splice variant in leukemic cells was also investigated in a series of patients with AML. In addition, part of the work in Chapter 6 addresses the possible involvement of JAK2 kinase in G-CSF signaling pathways and defines the cytoplasmic region of the G-CSF-R participating in the interaction with JAK2.

References

1. Ogawa M. (1993) Differentiation and proliferation of hematopoietic stem cells. Blood 81: 2844.
2. Hoffbrand AV and Pettit JE. (1993) Essential haematology. Blackwell Scientific Publications, Oxford. 1-11 pp.
3. Moore MAS. (1991) Clinical implications of positive and negative hematopoietic stem cell regulators. Blood 78:1.
4. Metcalf D. (1991) Control of granulocytes and macrophages: molecular, cellular, and clinical aspects. Science 254:529.
5. Plumb M, Graham GJ, Grove M, Reid A, and Pragnell IB. (1991) Molecular aspects of a negative regulator of hematopoiesis. Br. J. Cancer 64:990.
6. Jansen JH, Wientjens GJ, Fibbe WE, Willemze R, and Kluin-Nelemans HC. (1989) Inhibition of human macrophage colony formation by interleukin-4. J. Exp. Med. 170:577.
7. Vellenga E, de Wolf JTHM, Beentjes JAM, Essenlink MT, Smit JW and Halie MR. (1990) Divergent effects of interleukin-4 (IL-4) on the granulocyte colony-stimulating factor and IL-3-supported myeloid colony formation from normal and leukemic bone marrow cells. Blood 75:633.
8. Morris CF, Young IG and Hapel AJ. (1990) Molecular and cellular biology of interleukin-3. In: Colony-stimulating factors. Dexter TM, Garland JM and Testa NG (editors). Marcel Dekker, Inc, New York. 177-214 pp.
9. Koike K, Ihle JN, Ogawa M. (1986) Declining sensitivity to interleukin 3 of murine multipotential hemopoietic progenitors during their development: Application to a culture system that favors blast cell colony formation. J. Clin. Invest. 77:894.
10. Lopez AF, Dyson PG, To LB, Elliott MJ, Milton SE, Russell JA, Juttner CA, Yang Y-C, Clark SC, Vadas MA. (1988) Recombinant human interleukin-3 stimulation of hematopoiesis

in humans: Loss of responsiveness with differentiation in the neutrophilic myeloid series. Blood 72:1797.

11. Lok S, Kaushansky K, Holly RD, Kuijper JL, Lofton-Day CE, Oort PJ, Grant FJ, Heipel MD, Burkhead SK, Kramer JM, Bell LA, Sprecher CA, Blumberg H, Johnson R, Prunkard D, Ching AFT, Mathewes SL, Bailey MC, Forstrom JW, Buddle MM, Osborn SG, Evans SJ, Sheppard PO, Presnell SR, O'Hara PJ, Hagen FS, Roth GJ and Foster DC. (1994) Cloning and expression of murine thrombopoietin cDNA and stimulation of platelet production in vivo. Nature 369:565.
12. Kaushansky K, Lok S, Holly RD, Broudy VC, Lin N, Baily MC, Forstrom JW, Buddle MM, Oort PJ, Hagen FS, Roth GJ, Papayannopoulou T and Foster DC. (1994) Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. Nature 369:568.
13. Bartley TD, Bogenberger J, Hunt P, Li YS, Lu HS, Martin F, Chang MS, Samal B, Nichol JL, Swift S, Johnson MJ, Hsu RY, Parker VP, Suggs S, Skrine JD, Merewether LA, Clogston C, Hsu E, Hokom MM, Hornkohi A, Choi E, Pangelinan M, Sun Y, Mar V, McNinch J, Simonet L, Jacobsen F, Xie C, Shutter J, Chute H, Basu R, Selander L, Trollinger D, Sieu L, Padilla D, Trail G, Elliott G, Izumi R, Covey T, Crouse J, Garcia A, Xu W, Castillo JD, Biron J, Cole S, Hu MCT, Pacifici R, Ponting I, Saris C, Wen D, Yung YP, Lin H and Bosselman RA. (1994) Identification and cloning of a megakaryocyte growth and development factor that is a ligand for the cytokine receptor Mpl. Cell 77:1117.
14. Ikebuchi K, Clark SC, Ihle JM, Souza LM and Ogawa M. (1988) Granulocyte colony-stimulating factor enhances interleukin 3-dependent proliferation of multipotential hemopoietic progenitors. Pro. Natl. Acad. Sci. USA. 85:3445.
15. Bodine DM, Crosier PS and Clark SC. (1991) Effects of hematopoietic growth factors on the survival of primitive stem cells in liquid suspension culture. Blood 78:914.
16. Leary AG, Zeng HQ, Clark SC and Ogawa M. (1992) Growth factor requirements for survival in G_0 and entry into cell cycle of primitive human hemopoietic progenitors. Proc. Natl. Acad. Sci. USA. 89:4013.
17. Suda T, Yamaguchi Y, Suda J, Miura Y, Okano A and Yukio Akiyama. (1988) Effect of interleukin-6 (IL-6) on the differentiation and proliferation of murine and human hemopoietic progenitors. Exp. Hematol. 16:891.
18. Caracciolo D, Clark SC and Rovera G. (1989) Human interleukin-6 supports granulocytic differentiation of hematopoietic progenitor cells and acts synergistically with GM-CSF. Blood 73:666.
19. Miyajima A, Kitamura T, Harada N, Yokota T and Arai K. (1992) Cytokine receptors and signal transduction. Annu. Rev. Immunol. 10:295.
20. Kishimoto T, Taga T and Akira S. (1994) Cytokine signal transduction. Cell 76:253.
21. Stein J, Borzillo GV and Rottenmier CW. (1990) Direct stimulation of cells expressing receptor for macrophage colony-stimulating factor (CSF-1) by a plasma membrane-bound precursor of human CSF-1. Blood 76:1308.
22. Flanagan JG and Leder P. (1990) The kit ligand: a cell surface molecule altered in steel mutant fibroblasts. Cell 63:185.
23. Huang E, Nocka K, Beier DR, Chu TY, Buck J, Lahm HW, Wellner D, Leder P and Besmer P. (1990) The hematopoietic growth factor KL is encoded by the SI locus and is the ligand of the c-kit receptor, the gene product of the W locus. Cell 63:225.
24. Andersom DM, Lyman SD, Baird A, Wignall JM, Eisenman J, Rauch C, March CJ, Boswell HS, Gimpel SD, Cosman D and Williams DE. (1990) Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. Cell 63:235.
25. Kriegler M, Perez C, DeFay K, Albert I, Lu SD. (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramification for the complex physiology of

TNF. Cell 53:45.
26. Kurt-Jones EA, Beller DI, Mizel SB and Unanue ER. (1985) Identification of a membrane-associated IL-1 in macrophages. Proc. Natl. Acad. Sci. USA.82:1204.
27. Hannum C, Culpepper J, Campbell D, McClanahan T, Zurawski S, Bazan JF, Kastelein R, Hudak S, Wagner J, Mattson J, Luh J, Duda G, Martina N, Peterson D, Menon S, Shanafelt A, Muench M, Kelner G, Namikawa R, Rennick D, Roncarolo MG, Zlotnik A, Rosnet O, Dubreuil P, Birnbaum D and Lee F. (1994) Ligand for FLT3/FLK2 receptor tyrosine kinase regulates growth of haematopoietic stem cells and is encoded by variant RNAs. Nature 368:643.
28. Metcalf D. (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature 339:27.
29. Clark SC and Kamen R. (1987) The human hematopoietic colony-stimulating factors. Science 236:1229.
30. Andreeff M and Welte K. (1989) Hematopoietic colony-stimulating factors. Seminars Oncol. 16:211.
31. Gough NM and Nicola NA. (1990) Granulocyte-macrophage colony-stimulating factor. In: Colony-stimulating factors. Dexter TM, Garland JM and Testa NG (editors). Marcel Dekker, Inc, New York and Basel. 111-153 pp.
32. Broxmeyer HE, Cooper S, Lu L, Hangoc G, Anderson D, Cosman D, Lyman SD and Williams DE. (1991) Effect of murine mast cell growth factor (c-kit proto-oncogene ligand) on colony formation by human marrow hematopoietic progenitor cells. Blood 77:2142.
33. Molineux G, Migdalska A, Szmitkowski M, Zsebo K and Dexter TM. (1991) The effects on hematopoiesis of recombinant stem cell factor (ligand for c-kit) administered in vivo to mice either alone or in combination with granulocyte colony-stimulating factor. Blood 78:961.
34. Tsuji K, Zsebo KM and Ogawa M. (1991) Enhancement of murine blast cell colony formation in culture by recombinant rat stem cell factor, ligand for c-kit. Blood 78:1223.
35. Ulich TR, Castillo JD, McNiece IK, Yi ES, Alzona CP, Yin S and Zsebo KM. (1991) Stem cell factor in combination with granulocyte colony-stimulating factor (CSF) or granulocyte-macrophage CSF synergistically increases granulopoiesis in vivo. Blood 78:1954.
36. Burgress AW, Metcalf D. (1980) Characterization of a serum-factor stimulating the differentiation of myelomonocytic leukemic cells. Int. J. Cancer 26:647.
37. Nicola NA, Metcalf D. Matsumoto M and Johnson GR. (1983) Purification of a factor inducing differentiation in murine myelomonocytic leukemia cells: identification as granulocyte colony-stimulating factor (G-CSF). J. Biol. Chem. 258:9017.
38. Welte K, Platzer E, Lu L, Gabrilove J, Levi E, Mertelsmann R, Moore M. (1985) Purification and biological characterization of human pluripotent hematopoietic colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 82:1526.
39. Asano S. (1991) Human granulocyte colony-stimulating factor: its basic aspects and clinical application. Am. J. Pediatr. Hematol. Oncol. 13:400.
40. Demetri GD and Griffin JD. (1991) Granulocyte colony-stimulating factor and its receptor. Blood 78:2791.
41. Tsuda H, Neckers LM and Pluznik DH. (1986) Colony stimulating (CSF)-induced differentiation of murine M1 myeloid leukemic cells is permissive in early G_1 phase. Proc. Natl. Acad. Sci. USA. 83:4317.
42. Tomida M, Yamamoto-Yamaguchi Y, Hozumi M, Okade T and Takaku. (1986) Induction by recombinant human granulocyte colony-stimulating factor of differentiation of mouse myeloid leukemic M1 cells. FEBS Lett. 207:271.
43. Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F, Altmann S, Santoli D and Rovera G. (1987). Cytokine-dependent granulocytic differentiation. Regulation of proliferative and differentiative response in a murine progenitor cell line. J. Immunol. 138:3829.

44. Lee KH, Kinashi T, Tohyama K, Tashiro K, Funato N, Hama K and Honjo T. (1991) Different stromal cell lines support lineage-selective differentiation of the multipotential bone marrow stem cells clone LyD9. J. Exp. Med. 173:1257.
45. Begley CG, Metcalf D and Nicola NA. (1987) Purified colony-stimulating factors (G-CSF and GM-CSF) induce differentiation in human HL60 leukemic cells with suppression of clonogenicity. Int. J. Cancer 39:99.
46. Nicola NA. (1987) Granulocyte colony-stimulating factor and differentiation-induction in myeloid leukemic cells. Int. J. Cell Cloning 5:1.
47. Hammond W, Csiba E, Canin A, Hockman H, Souza L, Layton J amd Dale D. (1987) Chronic neutropenia: a new canine model induced by human granulocyte colony-stimulating factor. J. Clin. Invest. 87:704.
48. Lieschke GJ, Grail D, Hodgson G, Metcalf D, Stanley E, Cheers C, Fowler KJ, Basu S, Zhan YF and Dunn AR. (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophilic mobilization. Blood 84:1737.
49. Kawakami M, Tsutsumi H, Kumakawa T, Abe H, Hirai M, Kurosawa S, Mori M and Fukushima M. (1990) Levels of serum granulocyte colony-stimulating factor in patients with infections. Blood 76:1962.
50. Watari K, Asano S, Shirafuji N, Kodo H, Ozawa K, Takaku F, Kamachi S. (1989) Serum granulocyte colony-stimulating factor levels in healthy volunteers and patients with various disorders as estimated by enzyme immunoassay. Blood 73:117.
51. Allen PD, Bustin SA and Newland AC. (1993) The role of apoptosis (programmed cell death) in haematopoiesis and the immune system. Blood Rev. 7:63.
52. Williams G, Smith CA, Spooncer E, Dexter TM and Taylor DR. (1990) Haematopoietic colony stimulating factors promote cell survival by supporting apoptosis. Nature 343:76.
53. Begley CG, Lopez AF, Nicola NA, Warren DJ, Vadas MA, Sanderson CJ and Metcalf D. (1986) Purified colony-stimulating factors enhance the survival of human neutrophils and eosinohils in in vitro. Blood 68:162.
54. Wang JM, Chen ZG, Colella S, Bonilla MA, Welte K, Bordignon C and Mantovani A. (1988) Chemotactic activity of recombinant human granulocyte colony-stimulating factor. Blood 72:1456.
55. Kitagawa S, Yuo A, Souza LM, et al. (1987) Recomnant human granulocyte colony-stimulating factor enhances superoxide release in human granulocytes stimulated by the chemotactic peptide. Biochem. Biophys. Res. Commun. 144:1143.
56. Sato N, Asano S, Koeffler HP, et al. (1988) Identification of neutrophil alkaline phosphatase-inducing factor in cystic fluid of a squamous cell carcinoma as granulocyte colony-stimulating factor. J. Cell Physiol. 137:272.
57. Morishita K, Tsuchiya M, Asano S, et al. (1987) Chromosomal gene structure of human myeloperoxidase and regulation of its expression by granulocyte colony-stimulating factor. J. Biol. Chem. 262:15208.
58. Lopez AF, Nicola NA, Burgess AW, Metcalf D, Battye FL, Sewell WA and Vadas MA. (1983) Activation of granulocyte cytotoxic function by purified mouse colony-stimulating factor. J. Immunol. 131:2983.
59. Bazan JF. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor ß-chain. Biochem. Biophy. Res. Commun. 164:788.
60. Cosman D. (1993) The hematopoietin receptor superfamily. Cytokine 5:95.
61. Bazan JF. (1990) Haematopoietic receptors and helix cytokines. Immunolology Today 11:350.
62. Bazan JF. (1990) Shared Architecture of hormone binding domains in type I and I interferon receptors. Cell 61:753.

63. Ho ASY, Liu Y, Khan TA, Hsu DH, Bazan JF and Moore KW. (1993) A receptor for interleukin 10 is related to interferon receptors. Proc. Natl. Acad. Sci. USA. 90:11267.
64. Novick D, Cohen B and Rubinstein M. (1994) The human interferon α/ß receptor: Characterization and molecular cloning. Cell 77:391.
65. Smith CA, Farrah T and Goodwin RG. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. Cell 76:959.
66. Ullrich A and Schlessinger J. (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203.
67. Hatakeyama M, Tsudo M, Minamoto S, Kono T, Doi T, Miyata T, Miyasaka M and Taniguchi T. (1989) Interleukin-2 receptor ß chain gene: generation of three receptor forms by cloned human α and ß chain cDNA's. Science 224:552.
68. Takeshita T, Asao H, Ohtani K, Ishii N, Kumaki S, Tanaka N, Munakata H, Nakamura M and Sugamura K. (1992) Cloning of the γ chain of the human IL-2 receptor. Science 257:379.
69. Miyajima A, Mui ALF, Ogorochi T and Sakamaki K. (1993) Receptors for granulocyte-macrophage colony-stimulating factor, interleukin-3, and interleukin-5. Blood. 82:1960.
70. Mosley B, Beckmann MP, March CJ, Idzerda RL, Gimpel SD, VandenBos T, Friend D, Alpert A, Anderson D, Jackson J, Wignall JM, Smith C, Gallis B, Sims JE, Urdal D, Widmer MB, Cosman D and Park LS. (1989) The murine interleukin-4 receptor: molecular cloning and characterization of secreted and membrane bound forms. Cell 59:335.
71. Hibi M, Murakami M, Saito M, Hirano T and Taga T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell 63:1149.
72. Goodwin RG, Friend D, Ziegler SF, Jerzy R, Falk BA, Gimpel S, Cosman D, Dower SK, March CJ, Namen AE and Park LS. (1990) Cloning of the human and murine interleukin-7 receptor: demonstration of a soluble form and homology to a receptor superfamily. Cell 60:941.
73. Renauld JC, Druez C, Kermouni A, Houssiau F, Uyttenhove C, van Roost E and van Snick J. (1992) Expression cloning of the murine and human interleukin 9 receptor cDNAs. Proc. Natl. Acad. Sci. USA. 89:5690.
74. Hilton DJ, Hilton AA, Raicevic A, Rakar S, Harrison-Smith M, Gough NM, Begley CG, Metcalf D, Nicola NA and Wilson TA. (1994) Cloning of a murine IL-11 receptor α-chain; requirement for high affinity binding and signal transduction. EMBO J. 13:4765.
75. Youssoufian H, Longmore G, Neumann D, Yoshimura A and Lodish HF. (1993) Structure, function, and activation of the erythropoietin receptor. Blood 81:2223.
76. Gearing DP, Thut CJ, VandenBos T, Gimpel SD, Delaney PB, King J, Price V, Cosman D and Beckmann MP. (1991) Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J. 10:2839.
77. Gearing DP and Cosman D. (1991) Homology of the p40 subunit of natural killer cell stimulatory factor (NKSF) with the extracellular domain of the interleukin-6 receptor. Cell 66:9.
78. Vigon I, Mornon JP, Cocault L, Mitjavila MT, Tambourin P, Gisselbrecht S and Souyri M. (1992) Molecular cloning and characterization of MPL, the human homolog of the v-mpl oncogene: identification of a member of the hematopoietic growth factor receptor superfamily. Proc. Natl. Acad. Sci. USA. 89:5640.
79. Leung DW, Spencer SA, Cachianes G, Hammonds RG, Collins C, Henzel WJ, Barnard R, Waters MJ and Wood WI. (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature 330:537.
80. Boutin JM, Jolicoeur C, Okamura H, Gagnon J, Edery M, Shirota M, Banville D, Dusanter-Fourt I, Djiane J and Kelly PA. (1988) Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. Cell 53:69.
81. Davis S, Aldrich TH, Valenzuela DM, Wong V, Furth ME, Squinto SP and Yancopoulos

GD. (1991) The receptor for ciliary neurotrophic factor. Science 253:59.
82. Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasher KK, King JA, Gillis S, Mosley B, Ziegler SF and Cosman D. (1992) The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. Science 255:1434.
83. Ip NY, Nye SH, Boulton TG, Davis S, Taga T, Li Y, Birren SJ, Yasukawa K, Kishimoto T, Anderson DJ, Stahl N and Yancopoulos GD. (1992) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell 69:1121.
84. Davis S, Aldrich TH, Stahl N, Pan L, Taga T, Kishimoto T, Ip NY and Yancopoulos GD (1993). LIFß and gp130 as heterodimerizing signal transducer of the tripartite CNTF receptor. Science 260:1805.
85. Yin T, Taga T, Tsang ML, Yasukawa K, Kishimoto T and Yang YC. (1993) Involvement of IL-6 signal transducer gp130 in IL-11-mediated signal transduction. J. Immunol. 151:2555.
86. Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K and Sugamura K. (1993) Sharing of the interleukin-2 (IL-2) receptor γ chain between receptors for IL-2 and IL-4. Science 262:1874.
87. Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X and Leonard WJ. (1993) Interleukin-2 receptor γ chain: a functional component of the interleukin-7 receptor. Science 262:1877.
88. Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friendmann MC, Miyajima A, Puri RK, Paul WE and Leonard WJ. (1993) Interleukin-2 receptor γ chain: a functional component of the interleukin-4 receptor. Science 262:1880.
89. Zurawski SM, Jr FV, Huyghe B and Zurawski G. (1993) Receptor for interleukin-13 and interleukin-4 are complex and share a novel component that functions in signal transduction. EMBO J. 12:2663.
90. Giri JG, Ahdieh M, Eisenman J, Shanebeck K, Grabstein K, Kumaki S, Namen A, Park LS, Cosman D and Anderson D. (1994) Utilization of the ß and γ chains of the IL-2 receptor by the novel cytokine IL-15. EMBO J. 13:2822.
91. Nicola NA and Metcalf D. (1985) Binding of ^{125}I-labeled granulocyte colony-stimulating factor to normal murine hemopoietic cells. J. Cell Physiol. 124:313.
92. Begley CG, Metcalf D and Nicola NA. (1988) Binding characteristics and proliferative action of purified granulocyte-colony stimulating factor (G-CSF) on normal and leukemic human promyelocytes. Exp. Hematol. 16:71.
93. Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJS. Aglietta M, Arese P and Mantovani A. (1989) Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. Nature 337:471.
94. Uzumaki H, Okabe T, Sasaki N, Hagiwara K, Takaku F, Tobita M, Yasukawa K, Ito S and Umezawa. (1989) Identification and characterization of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. Proc. Natl. Acad. Sci, USA. 86:9323.
95. Avalos BR, Gasson JC, Hedvat C, Quan SG, Baldwin GC, Weisbart RH, Williams RE, Golde DW and DiPersio JF. (1990) Human granulocyte colony-stimulating factor: biologic activities and receptor characterization on hematopoietic cells and small cell lung cancer cell lines. Blood 75:851.
96. Budel LM, Touw IP, Delwel R and Löwenberg B. (1989) Granulocyte colony-stimulating factor receptors in human acute myelocytic leukemia. Blood 74:2668.
97. Hanazono Y, Hosoi T, Kuwaki T, Tatsuki S and Miyazono K. (1990) Structural analysis of the receptors for granulocyte colony-stimulating factor on neutrophils. Exp. Hematol. 18:1097.

98. Nicola NA and Peterson L. (1986) Identification of distinct receptors for two hemopoietic growth factors (granulocyte colony-stimulating factor and multipotential colony-stimulating factor) by chemical cross-linking. J. Biol. Chem. 261:12384.
99. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1990) Purification and characterization of the receptor for murine granulocyte colony-stimulating factor. J. Biol. Chem. 265:14008.
100. Fukunaga R, Ishizaka-Ikeda E, Seto Y and Nagata S. (1990) Expression cloning of a receptor for murine granulocyte colony-stimulating factor. Cell 61:341.
101. Fukunaga R, Seto Y, Mizushima S and Nagata S. (1990) Three different mRNAs encoding human granulocyte colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 87:8702.
102. Larsen A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March CJ and Smith CA. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptor, immunoglobulin, and fibronectin domains. J. Exp. Med. 172:1559.
103. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y and Nagata S. (1991) Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. 10:2855.
104. Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T and Kishimoto T. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc. Natl. Acad. Sci. USA. 88:11349.
105. Saito M, Yoshida K, Hibi M, Taga T and Kishimoto T. (1992) Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. J. Immunol. 148:4066.
106. Hatakeyama M, Mori H, Doi T and Taniguchi T. (1989) A restricted cytoplasmic region of IL-2 receptor ß chain is essential for growth signal transduction but not for ligand binding and internalization. Cell 59:837
107. Asao H, Takeshita T, Ishii N, Kumaki S, Nakamura M and Sugamura K. (1993) Reconstitution of functional interleukin 2 receptor complex on fibroblastoid cells: involvement of the cytoplasmic domain of the γ chain in two distinct signaling pathways. Proc. Natl. Acad. Sci. USA. 90:4127.
108. Sakamaki K, Miyajima I, Kitamura T and Miyajima A. (1992) Critical cytoplasmic domains of the common ß subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. EMBO J. 11:3541.
109. Weiss M, Yokoyama C, Shikama Y, Naugle C, Druker B and Sieff CA. (1993) Human granulocyte-macrophage colony-stimulating factor receptor signal transduction requires the proximal cytoplasmic domains of the α and ß subunits. Blood 82:3298.
110. D'Andrea AD, Yoshimura K, Youssoufian H, Zon LI, Koo JW and Lodish HF. (1991) The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains. Mol. Cell. Biol. 11:1980.
111. Quelle DE and Wojchowski DM. (1991) Localized cytosolic domains of the erythropoietin receptor regulate growth signaling and down-modulate responsiveness to granulocyte-macrophage colony-stimulating factor. Proc. Nalt. Acad. Sci. USA. 88:4801.
112. Koettnitz K and kalthoff FS. (1993) Human interleukin-4 receptor signaling requires sequences contained within two cytoplasmic regions. Eur. J. Immunol. 23:988.
113. Keegan AD, Nelms K, White M, Wang LM, Pierce JH and Paul WE. (1994) An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. Cell 76:811.
114. Ziegler SF, Davis T, Schneringer JA, Franklin TL, Tough TW, Teepe M, Larsen A, Williams DE and Smith C. (1991) Alternative forms of the human G-CSF receptor function in growth signal transduction. New Biol. 3:1242.
115. Inazawa J, Fukunaga R, Seto Y, Nakagawa N, Misawa S, Abe T and Nagata S. Assignment of the human granulocyte colony-stimulating factor receptor gene (G-CSFR) to chromosome 1 at region p35-p34.3. Genomics 10:1075.

116. Tweardy DJ, Anderson K, Cannizzaro LA, Steinman RA, Croce CM and Huebner K. (1992) Molecular cloning of cDNAs for the human granulocyte colony-stimulating factor receptor from HL-60 and mapping of the gene to chromosome region 1p32-34. Blood 79:1148.
117. Seto Y, Fukunaga R and Nagata S. (1992) Chromosomal gene organization of the human granulocyte colony-stimulating factor receptor. J. Immunol. 148:259.
118. Ito Y, Seto Y, Brannan CI, Copeland NG, Jenkins NA, Fukunaga R and Nagata S. (1994) Structural analysis of the functional gene and pseudogene encoding the murine granulocyte colony-stimulating factor receptor. Eur. J. Biochem. 220:881.
119. Kishimoto T, Taga T and Akira S. (1994) Cytokine signal transduction. Cell 76:253.
120. Cunningham BC, Ultsch M, de Vos AM, Mulkerrin MG, Clauser KR, Wells JA. (1991) Dimerization of the extracellular domain of the human growth hormone receptor by a single hormone molecule. Science 254:821.
121. de Vos AM, Ultsch M and Kossiakoff AA. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255:306.
122. Ishizaka-Ikeda E, Fukunaga R, Wood WI, Goeddel DV and Nagata S. (1993) Signal transduction mediated by growth hormone receptor and its chimeric molecules with the granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA. 90:123.
123. Adachi K and Saito H. (1992) Induction of junB expression, but not c-jun, by granulocyte colony-stimulating factor or macrophage colony-stimulating factor in the proliferative response of human myeloid leukemia cells. J. Clin. Invest. 89:1657.
124. Ziegler SF, Bird TA, Morella KK, Mosley B, Gearing DP and Baumann H. (1993) Distinct regions of the human granulocyte colony-stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. Mol. Cell. Biol. 13:2384.
125. Ullrich A and Schlessinger J. (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203.
126. Klausner RD and Samelson LE. (1991) T cell antigen receptor activation pathways: the tyrosine kinase connection. Cell 64:875.
127. Weiss A and Littman DR. (1994) Signal transduction by lymphocyte antigen receptors. Cell 76:263.
128. Yamamoto M, Nishimura J, Ideguchi H and Ibayashi H. (1988) Specific phosphorylation of 22-kD proteins by various inducers for granuloid differentiation in myeloid leukemic cells. Leuk. Res. 12:71.
129. Isfort R and Ihle JN. (1990) Multiple hematopoietic growth factors signal through tyrosine phosphorylation. Growth factors 2:213.
130. Evans JPM, Mire-Sluis AR, Hoffbrand AV and Wickremasinghe RG. (1990) Binding of G-CSF, GM-CSF, tumor necrosis factor-α, and γ-interferon to cell surface receptors on human myeloid leukemia cells triggers rapid tyrosine and serine phosphorylation of a 75-Kd protein. Blood 75:88.
131. Akimaru K, Utsumi T, Sato EF, Klostergaard J, Inoue M and Utsumi K. (1992) Role of tyrosyl phosphorylation in neutrophil priming by tumor necrosis factor-α and granulocyte colony stimulating factor. Arc. Biochem. Biophys. 298:703.
132. Linnekin D, Evans G, Michiel D and Farrar WL. (1992) Characterization of a 97-kDa phosphotyrosylprotein regulated by multiple cytokines. J. Biol. Chem. 267:23998.
133. Witthuhn B, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O and Ihle JN. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227.
134. Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN and Carter-Su C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237.
135. Silvennoinen O, Witthuhn BA, Quelle FW, Cleveland JL, Yi T and Ihle JN. (1993) Structure of the murine Jak2 protein-tyrosine kinase and its role in interleukin 3 signal transduction.

Proc. Natl. Acad, Sci. USA. 90:8429.
136. Stahi N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN and Yancopoulos GD. (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 receptor components. Science 263:92.
137. Narazaki M, Witthuhn BA, Yoshida K, Silvennoinen O, Yasukawa K, Ihle JN, Kishimoto T, and Taga T. (1994) Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. Proc. Natl. Acad. Sci. USA. 91:2285.
138. Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, Griffin JD and Ihle JN. (1994) JAK2 associates with the ßc chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol. Cell. Biol. 14:4335.
139. Dusanter-Fourt, Muller O, Ziemiecki A, Mayeux P, Drucker B, Djiane J, Wilks A, Harpur AG, Fischer S and Gisselbrecht S. (1994) Identification of JAK protein tyrosine kinases as signaling molecules for prolactin. Functional analysis of prolactin receptor and prolactin-erythropoietin receptor chimera expressed in lymphoid cells. EMBO J. 13:2583.
140. Yin T, Yasukawa K, Taga T, Kishimoto T and Yang YC. (1994) Identification of a 130-kilodalton tyrosine-phosphorylated protein induced by interleukin-11 as JAK2 tyrosine kinase, which associates with gp130 signal transducer. Exp. Hematol. 22:467.
141. Rane SG and Reddy EP. (1994) JAK3: a novel JAK kinase associated with terminal differentiation of hematopoietic cells. Oncogene 9:2415.
142. Johnston JA, Kawamura M, Kirken RA, Chen YQ, Blake TB, Shibuya K, Ortaldo JR, McVicar DW and O'Shea JJ. (1994) Phosphorylation and activation of the Jak-3 janus kinase in response to interleukin-2. Nature 370:151.
143. Witthuhn BA, Silvennoinen O, Miura O, Lai KS, Cwik C, Liu ET and Ihle JN. (1994) Involvement of the Jak-3 Janus kinase in signalling by interleukins 2 and 4 in lymphoid and myeloid cells. Nature 370:153.
144. Kirken RA, Rui H, Malabarba MG and Farrar WL. (1994) Identification of interleukin-2 receptor-associated tyrosine kinase p116 as novel leukocyte-specific Janus kinase. J. Biol. Chem. 269:19136.
145. Shuai k, Ziemiecki A, Wilks AF, Harpur AG, Sadowski HB, Gilman MZ and Darnell JE. (1993) Polypeptide signalling to the nucleus through tyrosine phosphorylation of JAK and Stat proteins. Nature 336:580.
146. Silvennoinen O, Ihle JN, Schlessinger J and Levy DE. (1993) Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 336:583.
147. Fu XY. (1992) A transcription factor with SH2 and SH3 domains is directly activated by an interferon α-induced cytoplasmic protein tyrosine kinase(s). Cell 70:323.
148. Blenis J. (1993) Signal transduction via the MAP kinases: proceed at your own RSK. Proc. Natl. Acad. Sci. USA. 90:5889.
149. Ravichandran KS and Burakoff SJ. (1994) The adapter protein Shc interacts with the interleukin-2 (IL-2) receptor upon IL-2 stimulation. J. Biol. Chem. 269:1599.
150. Sato N, Sakamaki K, Terada N, Arai K and Miyajima A. (1993) Signal transduction by the high-affinity GM-CSF receptor: two distinct cytoplasmic regions of the common ß subunit responsible for different signaling. EMBO J. 12:4148.
151. Matsuguchi T, Salgia R, Hallek M, Eder M, Druker B, Ernst TJ and Griffin JD. (1994) Shc phosphorylation in myeloid cells is regulated by granulocyte macrophage colony-stimulating factor, interleukin-3, and steel factor and is constitutively increased by p210BCR/ABL. J. Biol. Chem. 269:5016.
152. van der Geer P and Hunter T. (1993) Mutation of Tyr697, a GRB2-binding site, and Tyr721, a PI 3-kinase binding site, abrogates signal transduction by the murine CSF-1 receptor expressed in Rat-2 fibroblasts. EMBO J. 12:5161.

153. Damen JE, Liu L, Cutler RL and Krystal G. (1993) Erythropoietin stimulates the tyrosine phosphorylation of Shc and its association with Grb2 and a 145-Kd tyrosine phosphorylated protein. Blood 82:2296.
154. Bashey A, Healy L and Marshall CJ. (1994) Proliferative but not nonproliferative responses to granulocyte colony-stimulating factor are associated with rapid activation of the $p21^{ras}$/MAP kinase signalling pathway. Blood 83:949.
155. Bussolino F, Wang JM, Turrini F, Alessi D, Ghigo D, Costamagna C, Pescarmona G, Mantovani A and Bosia A. (1989) Stimulation of the Na+/H+ exchange in human endothelial cells activated by granulocyte- and granulocyte-macrophage-colony-stimulating factor. Evidence for a role in proliferation and migration. J. Biol. Chem. 264:18284.
156. Sullivan R, Griffin JD, Simons ER, Schafer AI, Meshulam T, Fredette JP, Maas AK, Gadenne AS, Leavitt JL, Melnick DA. (1987) Effects of recombinant human granulocyte and macrophage colony-stimulating factors on signal transduction pathways in human granulocytes. J. Immunol. 139:3422.
157. Waits W and Johnson DH. (1994) Causes, clinical consequences, and treatment of neutropenia. In: Filgrastin in clinical practice. Morstyn G and Dexter TM (editors). Marcel Dekker, Inc, New York. 51-82 pp.
158. Gillio AP and Gabrilove JL. (1993) Cytokine treatment of inherited bone marrow failure syndromes. Blood 81:1669.
159. Kostmann R. (1956) Infantile genetic agranulocytosis. Acta. Paediatr. Scand. 45 (suppl. 105):1.
160. Dale DC, Davis M and Vincent ME. (1994) Use of filgrastim (r-metHuG-CSF) in severe chronic neutropenia. In: Filgrastin in clinical practice. Morstyn G and Dexter TM (editors). Marcel Dekker, Inc. New York. 83-101 pp.
161. Gilman PA, Jackson DP and Guild HG. (1970) Congenital agranulocytosis: prolonged survival and terminal acute leukemia. Blood 36:576.
162. Rosen RB and Kang SJ. (1979) Congenital agranulocytosis terminating in acute myelomonocytic leukemia. J. Pediatr. 94:406.
163. Wong WY, Williams D, Slovak ML, Charak B, Mazumder A, Snyder D, Powars DR and Brynes RK. (1993) Terminal acute myelogenous leukemia in a patient with congenital agranulocytosis. Am. J. Hematol. 43:133.
164. Pietsch T, Bührer C, Mempel K, Menzel T, Steffens U, Schrader C, Santos F, Zeidler C and Welte K. (1991) Blood mononuclear cells from patients with severe congenital neutropenia are capable of producing granulocyte colony-stimulating factor. Blood 77:1234.
165. Mempel K, Pietsch T, Menzel T, Zeidler C and Welte K. (1991) Increased serum levels of granulocyte colony-stimulating factor in patients with severe congenital neutropenia. Blood 77:1919.
166. Parmley RT, Crist WM, Ragab AH, Boxer LA, Malluh A, Lui VK and Darby CP. (1980) Congenital dysgranulopoietic neutropenia: clinical, serologic, ultrastructural, and in vitro proliferative characteristics. Blood 56:465.
167. Kybayashi M, Yumiba C, Kawaguchi Y, Tanaka Y, Ueda K, Komazawa Y and Okada K. (1990) Abnormal response of myeloid progenitor cells to recombinant human colony-stimulating factors in congenital neutropenia. Blood 75:2143.
168. Hestdal K, Welte K, Lie SO, Keller JR, Ruscetti FW and Abrahamsen TG. (1993) Severe congenital neutropenia: abnormal growth and differentiation of myeloid progenitors to granulocyte colony-stimulating factor (G-CSF) but normal response to G-CSF plus stem cell factor. Blood 82:2991.
169. Kyas U, Pietsch T and Welte K. (1992) Expression of receptors for granulocyte colony-stimulating factor on neutrophils from patients with severe congenital neutropenia and cyclic neutropenia. Blood 79:1144.
170. Bonilla MA, Gillio AP, Ruggeiro M, Kernan NA, Brochstein JA, Abboud M, Fumagalli L,

Vincent M, Gabrilove JL, Welte K, Souza LM and O'Reilly RJ. (1989) Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N. Engl. J. Med. 320:1574.

171. Dale DC, Bonilla MA, Davis MW, Nakanishi AM, Hammond WP, Kurtzberg J, Wang W, Jakubowski A, Winton E, Lalezari P, Robinson W, Glaspy JA, Emerson S, Gabrilove J, Vincent M and Boxer LA. (1993) A randomized controlled phase III trial of recombinant granulocyte colony-stimulating factor (filgrastim) for treatment of severe chronic neutropenia. Blood 81:2496.
172. Welte K, Zeidler C, Reiter A, Müller W, Odenwald E, Souza L and Riehm H. (1990) Differential effects of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in children with severe congenital neutropenia. Blood 75:1056.
173. Hunter T. (1991) Cooperation between oncogenes. Cell 64:249.
174. Sawyers C, Denny CT and Witte ON. (1991) Leukemia and the disruption of normal hematopoiesis. Cell 64:337.
175. Cline MJ. (1994) The molecular basis of leukemia. N. Engl. J. Med. 330:328.
176. Cantley LC, Auger KR, Carpenter C, Duckworth B, Graziani A, Kapeller R and Soltoff S. (1991) Oncogenes and signal transduction. Cell 64:281.
177. Löwenberg B and Touw IP. (1993) Hematopoietic growth factors and their receptors in acute leukemia. Blood 81:281.
178. Cross M and Dexter TM. (1991) Growth factors in development, transformation, and tumorigenesis. Cell 64:271.
179. Löwenberg B and Delwel FR. (1991) The pathology of human acute myeloid leukemia. In: Hematology, basic principles and practice. Hoffman R, Benz Jr. EJ, Shattil SJ, Furrie B and Cohen HJ (editors). Churchill Livingstone, New York. 708-715 pp.
180. Yoshimura A, Longmore G and Lodish HF. (1990) Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. Nature 348:647.
181. Roussel MF, Downing JR, Rettenmier CW and Sherr CJ. (1988) A point mutation in the extracellular domain of the human CSF-1 receptor (c-fms proto-oncogene product) activates its transforming potential. Cell 55:979.
182. Rosenberg N and Krontris TG. (1991) The molecular basis of neoplasia. In: Hematology, basic principles and practice. Hoffman R, Benz Jr. EJ, Shattil SJ, Furrie B and Cohen HJ (editors). Churchill Livingstone, New York. 656-668 pp.
183. Salem M, Delwel R, Mahmoud LA, Clark S, Elbasousy EM and Löwenberg B. (1989) Maturation of human acute myeloid leukaemia in vitro: The response to five recombinant haematopoietic factors in a serum-free system. Br. J. Haematol. 71:363.
184. Santini V, Colombat P, Delwel R, van Gurp R, Touw I and Löwenberg B. (1991) Induction of granulocytic maturation in acute myeloid leukemia by G-CSF and retinoid acids. Leuk. Res. 5:341.
185. Motoji T, Takanashi M, Fuchimoue M, Masuda M, Oshimi K and Mizoguchi H. (1989) Effect of recombinant GM-CSF and recombinant G-CSF on colony formation of blast progenitors in acute myeloblastic leukemia. Exp. Hematol. 17:56.
186. Raff MC. (1992) Social controls on cell survival and cell death. Nature 356:397.
187. Williams GT and Smith CA. (1993) Molecular regulation of apoptosis: genetic controls on cell death. Cell 74:777.
188. Allen PD, Bustin SA and Newland AC. (1993) The role of apoptosis (programmed cell death) in haematopoiesis and the immune system. Blood Rev. 7:63.
189. Schwartz LM and Osborne BA. (1993) Programmed cell death, apoptosis and killer genes. Immunol. Today 14:582.
190. Vaux DL, Haecker G and Strasser A. (1994) An evolutionary perspective on apoptosis. Cell 76:777.

191. Vaux DL, Cory S and Adams JM. (1988) Bcl-2 gene promotes haematopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335:440.
192. Naumovski L and Cleary ML. (1994) Bcl2 inhibits apoptosis associated with terminal differentiation of HL-60 myeloid leukemic cells. Blood 83:2261.
193. Boise LH, González-Garcia M, Postema CE, Ding L, Lindsten T, Turka LA, Mao X, Nuñez G and Thompson CB. (1993) bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell 74:597.
194. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A and Oren M. (1991) Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. Nature 352:345.
195. Oltvai ZN, Milliman CL and Korsmeyer SJ. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74:609.
196. Yuan J, Shaham S, Ledoux S, Ellis HM and Horvitz HR. (1993) The C. elegans cell death gene ced-3 encodes a protein similar to mammalian interleukin-1 beta-converting enzyme. Cell 75:641.
197. Tanaka N, Ishihara M, Kitagawa M, Harada H, Kimura T, Matsuyama T, Lamphier MS, Aizawa S, Mak TW and Taniguchi T. (1994) Cellular commitment to oncogene-induced transformation or apoptosis is dependent on the transcription factor IRF-1. Cell 77:829.
198. Tsujimoto Y, Cossman J, Jaffe E and Croce CM. (1985) Involvement of the Bcl-2 gene in human follicular lymphoma. Science 228:1440.
199. Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM and Dalla-Favera R. (1991) p53 mutations in human lymphoid malignancies: association with Burkitt lymphoma and chronic lymphocytic leukemia. Proc. Natl. Acad. Sci. USA. 88:5413.
200. Fenaux P, Preudhomme C, Quiquandon I, Jonveaux P, Laï JL, Vanrumbeke M, Loucheux-Lefebvre MH, Bauters F, Berger R and Kerckaert JP. (1992) Mutations of the p53 gene in acute myeloid leukemia. Br. J. Haematol. 80:178.
201. McGahon A, Bissonnette R, Schmitt M, Cotter KM, Green DR and Cotter TG. (1994) BCR-ABL maintains resistance of chronic myelogenous leukemia cells to apoptotic cell death. Blood 83:1179.
202. Grignani F, Ferrucci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Grignani F, Peschle C, Nicoletti L and Pelicci PG. (1993) The acute promyelocytic leukemia-specific PML-RARα fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. Cell 74:423.

Chapter 2

DISTINCT CYTOPLASMIC REGIONS OF THE HUMAN G-CSF RECEPTOR INVOLVED IN TRANSDUCTION OF PROLIFERATIVE AND MATURATION SIGNALS

Fan Dong, Carin van Buitenen, Lies H. Hoefsloot,
Bob Löwenberg and Ivo P. Touw

Modified from Mol. Cell. Biol. 13: 7774-7781, 1993

Abstract

The granulocyte colony-stimulating factor receptor (G-CSF-R) transduces signals important for the proliferation and differentiation of myeloid progenitor cells. To identify functionally important regions in the cytoplasmic domain of the G-CSF-R, we compared the actions of the wild-type receptor, two deletion mutants and a natural splice variant in transfectants of the mouse pro-B cell line BAF3 and two myeloid cell lines, 32D and L-GM. A region of 55 amino acids adjacent to the transmembrane domain was found to be sufficient for generating growth signals. The immediate downstream sequence of 30 amino acids substantially enhanced the growth signaling in the three cell lines. In contrast, the carboxy-terminal part of 98 amino acids strongly inhibited the growth signaling in the two myeloid cell lines but not in BAF3 cells. Truncation of this region led to an inability of the G-CSF-R to transduce maturation signals in L-GM cells. An alternative carboxy tail present in a splice variant of the G-CSF-R also inhibited growth signaling, notably in both the myeloid cells and BAF3 cells, but appeared not involved in maturation induction.

Introduction

The formation of blood cells in the bone marrow is controlled by a regulatory network of hematopoietic growth factors (1,2). One of these factors, granulocyte colony-stimulating factor (G-CSF) secreted by macrophages, fibroblasts and endothelial cells, plays an essential role in the regulation of granulopoiesis and the maintenance of neutrophil levels in the peripheral blood (3-5). For instance, G-CSF not only is able to stimulate the proliferation of neutrophilic progenitor cells but also induces maturation of these progenitor cells towards neutrophilic granulocytes.

The murine and human G-CSF-Rs consist of a single polypeptide with a molecular weight of 130 to 150 kDa (6,7). The high affinity interaction between G-CSF and its receptor and subsequent activation of receptor signaling require the formation of homodimeric or oligomeric receptor complexes (6). The molecular cloning of the cDNAs encoding the murine and human G-CSF-Rs revealed that the murine and human G-CSF-Rs contain 812 and 813 amino acids, respectively, with a single transmembrane domain (8-10). The structure of the G-CSF-R shows significant homology to that of the IL-6 signal transducer gp130 (11,12). Expression of the G-CSF-R cDNAs in murine hematopoietic cells leads to formation of high-affinity G-CSF binding sites on the cell surface and renders these cells responsive to G-CSF (13,14). At least four different forms of the human G-CSF-R, resulting from alternative splicing of G-CSF receptor mRNA, have been cloned from human placenta and myeloid leukemia (U937) cells (9,10).

On the basis of structural similarities, the G-CSF-R has been classified as a member of the superfamily of cytokine receptors (15,16). The homologous structures shared by the members of the family consist of four conserved cysteine residues and a WSXWS motif in the extracellular region. The cytoplasmic domain of the family is less conserved, but certain sequence similarities have been reported among the receptors for IL-3 to IL-7, G-CSF, GM-CSF, EPO and the ß chains of the IL-2 and IL-3 receptors (13,17). Several studies have

delineated functionally important regions in the cytoplasmic domain. For instance, a membrane-proximal region of the EPO-R, IL-6 signal transducer gp130, and the ß chains of IL-2 and IL-3 receptors is essential for transmitting growth signals (17-20). In agreement with these findings, it has been shown that the membrane-proximal region of the G-CSF-R is indispensable for growth signal transduction (13,21). The cytoplasmic tail of the EPO-R has been shown to down-modulate the mitogenic response to EPO (18,22). In contrast, the distal cytoplasmic domain of the G-CSF-R has been reported to enhance growth signaling (13).

In this paper, we describe the identification of two cytoplasmic regions of the wild-type (WT) G-CSF-R, located distal to the box 1- and box 2-containing region, one that positively and one that negatively regulates the mitogenic response to G-CSF. We provide evidence showing that a distinct carboxy terminus present in a G-CSF-R splice variant also inhibits growth signaling. Further, we show that the negative regulatory region of the WT G-CSF-R but not of the splice variant is essential for G-CSF-induced neutrophilic maturation. These findings establish that distinct regions of the cytoplasmic domain of the G-CSF-R have a determinative role in G-CSF-induced proliferation and maturation of myeloid progenitor cells.

Materials and methods

Cell lines and culture. The murine pro-B cell line BAF3 (23) was kindly provided by G. Plaetinck (Roche Research Gent, Belgium) and maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and 10 ng of murine IL-3 per ml. Murine myeloid cell lines 32D (24) and L-GM (25) were provided by J. S. Greenberger (University of Massachusetts) and T. Honjo (Kyoto University Faculty of Medicine, Japan). 32D and L-GM cells were maintained in RPMI 1640 medium supplemented with 10% FCS and 10% WEHI-3B cell conditioned medium (WEHI-CM) as a source of murine IL-3.

G-CSF-R expression constructs. For the construction of pLNCX-WT, the WT human G-CSF-R cDNA (10) (pHQ3, kindly provided by S. Nagata and R. Fukunaga) was excised from the pBluescript vector and inserted into the *Hpa*I site of the retroviral expression vector pLNCX (26). For the construction of pLNCX-DC, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed on total RNA isolated from normal granulocytes by using forward primers 5'TGTGATCATCGTGACTCCCTT3' (FW3) and reverse primer 5'CAAGATCTAGTTTACAATACTGAAG3' (RV7), and an 864-bp PCR fragment was subcloned into the pBluescript vector. A 248-bp stretch of the fragment was then cut out with *BsrF*I and *Cla*I and, together with the *Hind*III-*BsrF*I fragment obtained from pLNCX-WT, was inserted into *Hind*III-*Cla*I-cleaved pLNCX vector by triple ligation. Mutant DA was isolated from granulocytes of a patient with congenital neutropenia by RT-PCR using the same set of primers. The construction of the full length DA mutant is essentially same as that described for the DC receptor. Mutant M1 was generated by PCR by introducing a termination codon at amino acid position 686 of the WT G-CSF-R. The PCR fragment was first ligated to the *Hinc*II site of the pBluescript vector and then excised from the plasmid by *Hind*III and *Xho*I digestion, blunt ended, and inserted into the *Hpa*I site of pLNCX.

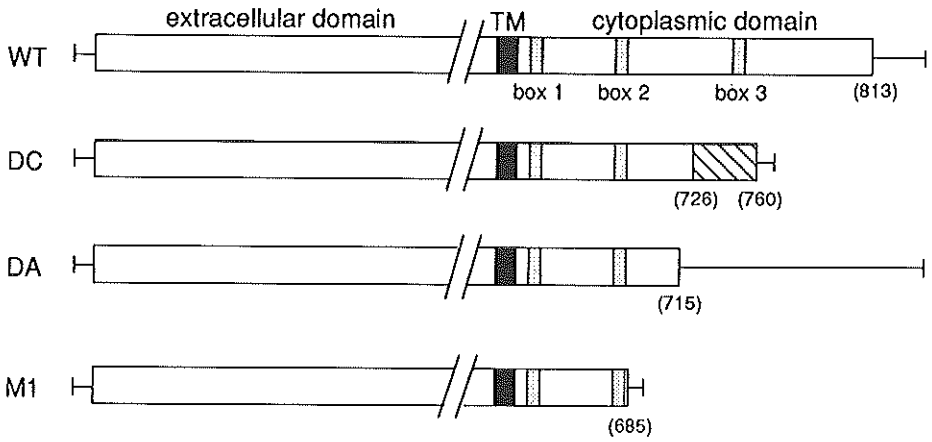

Figure 1. Schematic diagram of the G-CSF receptor cDNAs. Boxes 1, 2 and 3 denote subdomains conserved in some members of the cytokine receptor superfamily. The hatched box indicates distinct cytoplasmic tail of the DC receptor from the corresponding region of the wild-type receptor (see text). Lines indicate noncoding regions. The numbers in parentheses mark amino acid positions. WT, wild type; TM, transmembrane domain.

DNA transfection. The pLNCX expression constructs were linearized by *PvuI* digestion and transfected into BAF3, 32D and L-GM cells by electroporation. Following gene transfer, cells were cultured in IL-3-containing medium for 48 h and then selected in G418 (Gibco-BRL, Breda, The Netherlands) at concentrations of 1.5 (BAF3) or 0.8 (32D and L-GM) mg/ml. G418-resistant clones were expanded and tested for their capacities to specifically bind radiolabeled G-CSF. RT-PCR using primer sets FW3-RV6 (5'GTAGATCTTAGTCATG GGCTTATGG3'), FW3-RV7, and FW3-RV3 (5'TCTCAGGGCACCGGCTTCTTTT3') was performed on the transfectants to test for possible incomplete transfer of sequence encoding the cytoplasmic domains.

G-CSF binding assay. Recombinant human G-CSF (Amgen, Thousand Oaks, CA) was radioiodinated using the method of Bolton and Hunter (27). G-CSF binding characteristics were determined as previous described (28). In brief, cells (10^6) were incubated with serial dilutions of radiolabeled G-CSF ranging from 20 pM to 5 nM at 37°C for 1 h. Incubations in the presence of excess nonlabeled G-CSF were also performed to correct for nonspecific binding. Bound ligand was separated from free ligand by centrifugation (5 min at 1,000 x *g*) through an ice-cold FCS layer. Receptor numbers and binding affinity were calculated as described (28).

DNA synthesis and cell proliferation assays. DNA synthesis was assayed by [^{3}H]-thymidine (^{3}H-TdR) uptake. Cells (10^4) were incubated in triplicate in 100 μl of 10% FCS-RPMI medium supplemented with titrated concentrations of G-CSF or WEHI-CM (20%) in 96-well plates for 24 h. Twelve hours before cell harvest, 1 μCi of ^{3}H-TdR (2 Ci/mM; Amersham, UK) was added to each well. ^{3}H-TdR incorporation was measured by liquid scintillation counting as described previously (29).

Table 1. Binding properties of the G-CSF receptors on cell line transfectants[a]

Cell type	Number of sites/cell	Kd (nM)
BAF/WT	3,030	2.04
BAF/DC	1,500	0.67
BAF/DA	2,560	0.28
BAF/M1	31,410	0.60
32D/WT	1,640	1.86
32D/DC	750	0.68
32D/DA	2,630	0.53
32D/M1	+[b]	ND[c]
L-GM/WT	+	ND
L-GM/DC	+	ND
L-GM/DA	+	ND
L-GM/M1	1290	1.84

[a] parental BAF3, 32D and L-GM cells did not show specific G-CSF binding.
[b] specific binding detectable but too low for an accurate calculation of receptor numbers and affinities.
[c] ND, not determined.

To quantify cell proliferation in suspension culture, cells were incubated in 25-ml culture flasks at an initial density of 0.5 x 10^5/ml (5 ml of culture) in medium supplemented with recombinant human G-CSF at the indicated concentrations or without growth factors. Culture medium was refreshed every 2 to 4 days, and the cell densities were adjusted to approximately 0.5-1 x 10^5/ml. Viable cells were counted on the basis of trypan blue exclusion.

Results

Properties of the G-CSF-R variant and mutants. The structures of the different forms of the human G-CSF-R relevant to this study are summarized in Figure 1. DC represents an RT-PCR product obtained from normal granulocytes that is weakly expressed in comparison with the WT G-CSF-R (data not shown). Nucleotide sequencing revealed that DC is identical to previously described splice variant D7 (9), derived from placenta. From amino acid 727, DC is translated in an altered reading frame and contains 34 carboxy-terminal amino acids distinct from the WT G-CSF-R. DA contains a C-to-T substitution at nucleotide position 2384 that introduces a TAG stop codon resulting in truncation of 98 carboxy-terminal amino acids. M1 contains only 55 amino acids in the cytoplasmic domain that includes the two subdomains called box 1 and box 2 (13,17), with 3 additional amino acids downstream. The different forms of the G-CSF-R were introduced into the murine cell lines BAF3, 32D, and L-GM by electroporation. RT-PCR products derived from the transtectants by using primers that amplify the entire cytoplasmic domains all had the predicted sizes, indicating that no deletions in these regions had occurred during transfection and selection (data not shown).

Table 2. Mitogenic responses of cell line transfectants in ^{3}H-TdR uptake assay after 48 h of culture

Transfectants	dpm (mean ± SD) with:		
	no factor	G-CSF (10 ng/ml)	IL-3 (20% WEHI-CM)
BAF/Neo	33 ± 6	52 ± 20	46,298 ± 463
BAF/WT	35 ± 14	17,845 ± 393	36,833 ± 1,289
BAF/DA	45 ± 6	16,390 ± 344	41,772 ± 1,128
BAF/DC	20 ± 4	5,485 ± 527	19,673 ± 156
BAF/M1	17 ± 5	7,190 ± 360	31,021 ± 434
32D/Neo	33 ± 13	29 ± 20	24,452 ± 196
32D/WT	53 ± 21	954 ± 134	19,780 ± 692
32D/DA	76 ± 47	22,606 ± 633	18,312 ± 549
32D/DC	38 ± 7	1,256 ± 72	14,765 ± 221
32D/M1	149 ± 52	1,309 ± 94	27,554 ± 1,350
L-GM/Neo	40 ± 13	39 ± 8	2,968 ± 297
L-GM/WT	29 ± 7	383 ± 61	3,183 ± 267
L-GM/DA	43 ± 5	5,317 ± 393	3,293 ± 59
L-GM/DC	22 ± 5	441 ± 46	1,566 ± 49
L-GM/M1	22 ± 4	560 ± 50	1,369 ± 178

G-CSF binding properties of BAF3, 32D and L-GM transfectants. The numbers and dissociation constants of the G-CSF-R expressed by the different transfectants were established following binding of ^{125}I-radiolabeled G-CSF (Table 1). While the parental BAF3, 32D, and L-GM cells lacked specific G-CSF binding (data not shown), the different BAF3 transfectants (WT, DC, DA and M1) expressed G-CSF binding sites with affinities ranging from 0.28 to 2.04 nM (Table 1), i.e., comparable to those of the G-CSF-R expressed on human peripheral blood neutrophils (28) and those of the G-CSF-R expressed on cell line transfectants (9,10,14). 32D/WT, 32D/DC, 32D/DA and L-GM/M1 transfectants expressed G-CSF-R with similar average numbers and affinities. On the other hand, 32D/M1, L-GM/WT, L-GM/DC and L-GM/DA transfectants expressed G-CSF-R at detectable but low densities (less than 100 sites per cell), which did not allow an estimation of binding affinities (Table 1). The reasons for these variations in surface expression levels of the G-CSF-R are unknown, but they may relate to differences in protein stability or transportation in the different cell line transfectants.

Mitogenic signaling by different forms of the G-CSF-R. The abilities of different forms of the G-CSF-R to transduce proliferative signals were first examined in ^{3}H-TdR uptake assays. At least three independent clones were examined, giving comparable results. Parental BAF3, 32D and L-GM cells or G418-resistant clones transfected with the vector without G-CSF-R showed no response to G-CSF (Figure 2). G-CSF induced DNA synthesis of BAF3 cells expressing the WT G-CSF-R to a level of approximately 40 to 50% of that induced by the optimal concentration of WEHI-CM (20%) as a source of IL-3 (Table 2 and Figure 2A,

BAF/WT). Deletion of 98 amino acids from the carboxy terminus did not negatively affect G-CSF-induced growth signaling in BAF3 cells (Table 2 and Figure 2A, BAF/DA). However, further deletion of 30 amino acids significantly reduced the G-CSF sensitivity, resulting in a 100-fold increase in the G-CSF concentration (> 100 ng/ml) required to elicit a maximal response (Figure 2A, BAF/M1). BAF3 cells expressing the DC receptor, which contains 11 additional amino acids as compared with the DA receptor, followed by a distinct carboxy tail, also displayed a reduced responsiveness: the magnitude of maximal response was about 30 to 50% of that of BAF/WT (Table 2 and Figure 2A, BAF/DC).

In contrast to the BAF transfectants, 32D cells expressing the WT G-CSF-R exhibited only a weak mitogenic response to G-CSF (Table 2 and Figure 2B, 32D/WT). Similar responses to G-CSF were observed in 32D cells expressing the DC or the M1 receptor (Table 2 and Figure 2B). The 32D/DA cells, however, showed a dramatic increase in responsiveness to G-CSF (Figure 2B, 32D/DA). Additional titration experiments revealed that 32D/DA cells proliferated maximally at 0.1 ng of G-CSF per ml (data not shown).

L-GM cells transfected with the different forms of the G-CSF-R displayed a response pattern similar to that of 32D transfectants (Table 2 and Figure 2C). L-GM cells expressing the WT G-CSF receptor showed a poor proliferative response to G-CSF, and as did L-GM subclones expressing the DC or the M1 receptor. L-GM/DA cells, on the other hand, showed a high rate of DNA synthesis even at low concentrations (0.1 ng/ml) of G-CSF.

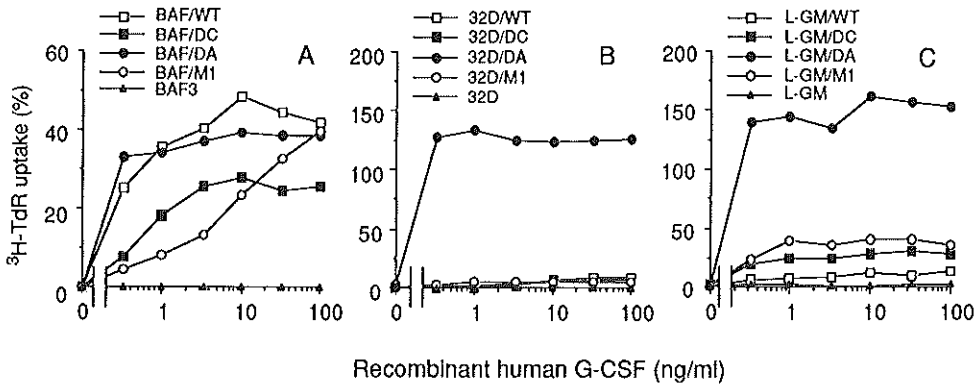

Figure 2. G-CSF responsiveness of BAF3, 32D and L-GM cells expressing different forms of the G-CSF-R. Cell proliferation was measured by the ^{3}H-TdR uptake assay. Data are represented as percentage of maximal response to IL-3 (20% WEHI-CM).

Sustained cell proliferation of transfectants. We next examined whether G-CSF could replace IL-3 as a stimulus for sustained proliferation of transfectants. As shown in Figure 3A and B, BAF/WT and BAF/DA cells became G-CSF dependent and grew equally well in 1 and 100 ng of G-CSF per ml. Although BAF/DC and BAF/M1 cells could also grow continuously at 100 ng of G-CSF per ml with a reduced rate of proliferation (Figure 3B), these cells could not survive at 1 ng of G-CSF per ml (Figure 3A). The response of BAF/M1 cells indicate that the membrane-proximal region of 55 amino acids is sufficient for

generating growth signals. In addition, the data show that a region between amino acids 56 and 85 in the cytoplasmic domain enhances growth signaling. The carboxy-terminal region of the DC receptor, which contains additional 11 amino acids of the WT G-CSF-R and 34 different amino acids in comparison with the DA mutant (Figure 1), exerted a negative effect on growth signaling in BAF3 cells.

The 32D transfectants expressing the WT, DC and M1 receptors died 4 to 6 days after transfer to G-CSF-containing medium (Figure 3C). In contrast, 32D/DA cells proliferated continuously upon stimulation with G-CSF (Figure 3C). Similarly, L-GM/DA cells could be cultured continuously in G-CSF-containing medium, whereas L-GM cells expressing the WT, DC or M1 receptor gradually lost viability (Figure 3D). Thus, the characteristics of the responses observed in 32D and L-GM subclones define a functional subdomain located in the distal carboxy-terminal region of the WT G-CSF-R that appears to suppress the growth signaling in myeloid cells. The altered carboxy terminus of the DC receptor also inhibits G-CSF-induced proliferation of the two myeloid cell lines.

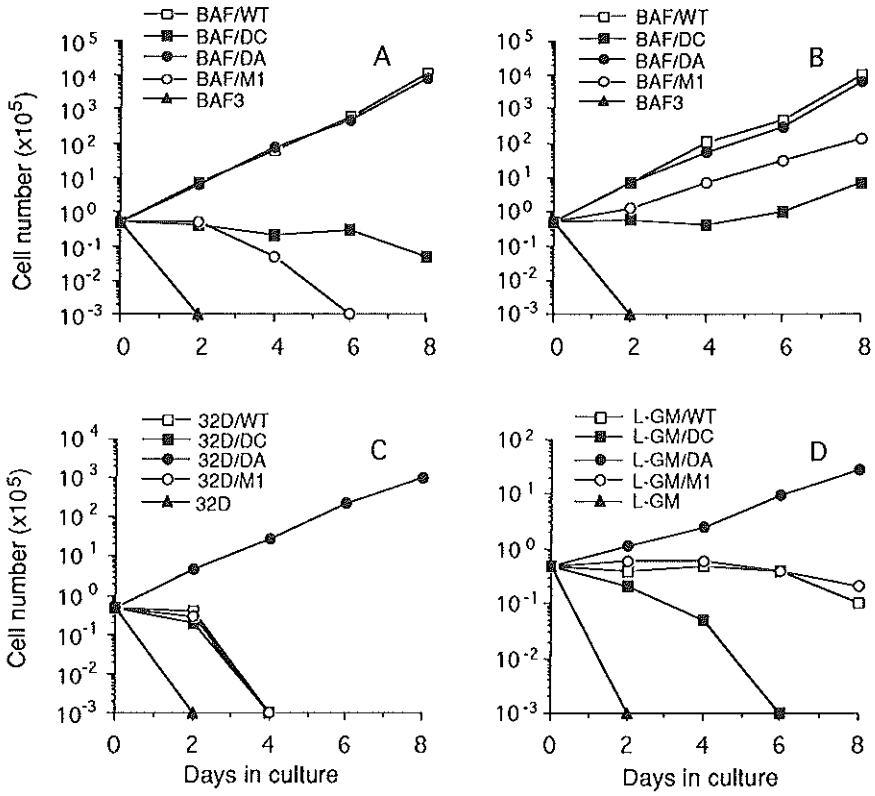

Figure 3. G-CSF-dependent long-term growth of BAF3, 32D and L-GM transfectants. Cells were cultured in G-CSF-containing medium. Viable cell counts were determined at indicated times. BAF3 subclones were cultured in medium containing 1 ng (A) and 100 ng (B) of G-CSF per ml. 32D (C) and L-GM (D) transfectants were cultured in 10 ng of G-CSF per ml except for DA subclones (1 ng of G-CSF per ml).

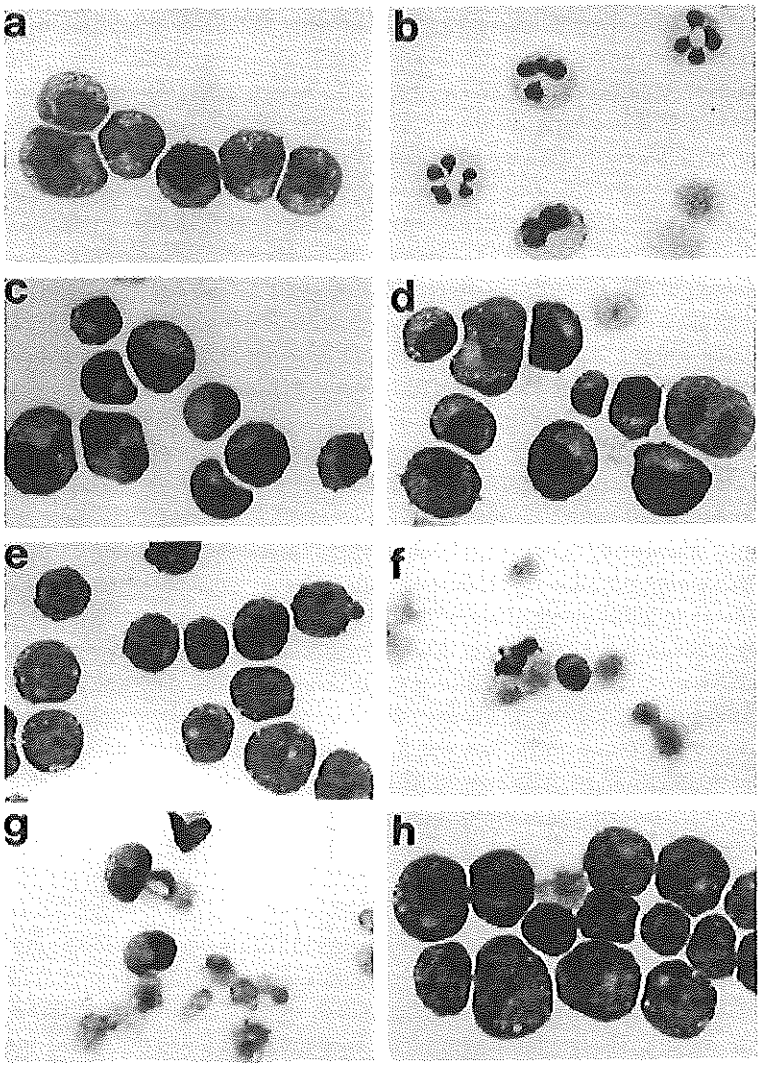

Figure 4. Morphological analysis of 32D and L-GM transfectants. Shown are L-GM (a) and 32D (e) cells maintained in IL-3-containing medium (10% WEHI-CM); L-GM/WT (b), L-GM/DC (c) and L-GM/DA (d) cells cultured in medium containing 10 ng of G-CSF per ml; 32D/WT (f), 32D/DC (g) and 32/DA (h) cells in G-CSF-containing medium. May-Grünwald-Giemsa staining.

Induction of myeloid maturation by different forms of the G-CSF-R. A prominent feature of G-CSF is its capacity to induce neutrophilic differentiation. 32Dcl3 and L-G cells, variant cell lines of 32D and L-GM cells, respectively, have previously been shown to

mature into neutrophilic granulocytes in response to G-CSF (25,30). We therefore examined whether L-GM and 32D cells expressing the different G-CSF-R structures would mature towards neutrophilic granulocytes following G-CSF induction. L-GM/WT cells exhibited immature myeloblastic features when cultured in IL-3-containing medium (Figure 4a). Upon transfer to G-CSF-containing medium, these cells gradually developed into mature neutrophilic granulocytes, showing an enlarged cytoplasm-to-nucleus ratio and lobulated nuclei. On day 9 of G-CSF induction, all cells displayed a morphology characteristic of mature neutrophils (Figure 4b), similar to that of mature L-G cells (25). As shown in Figure 4c and d, L-GM/DC and L-GM/DA cells did not mature towards granulocytes in G-CSF-containing medium. Similar results were obtained with L-GM/M1 transfectants (data not shown). Addition of different concentrations of murine GM-CSF or murine IL-3 to the G-CSF-containing culture medium to prolong cell survival also failed to induce terminal maturation of L-GM/DC or L-GM/M1 cells (data not shown).

32D/WT cells, which were myeloblastic when cultured in IL-3 (Figure 4e), did not mature upon transfer to G-CSF-containing medium. Instead, these cells manifested a phenotype typical of apoptosis (i.e., condensation of nuclei) after culture in G-CSF-containing medium for 4 days (Figure 4f). To exclude the possibility that these cells died of a lack of growth signaling before they could undergo myeloid maturation, cells were grown in G-CSF-containing medium supplemented with different concentrations of WEHI-CM (1, 2.5, 5 and 10%) to allow survival for up to 2 weeks, but still no maturation was noted (data not shown). Similarly, 32D/DC or 32D/M1 subclones were rapidly lost in cultures supplemented with G-CSF and failed to mature (Figure 4g and data not shown). 32D/DA cells also showed no evidence of terminal myeloid maturation when maintained in G-CSF (Figure 4h). These results show that the 32D cells used in this study were incapable of granulocytic maturation in response to G-CSF, suggesting a defect in G-CSF-induced maturation signaling pathway in these cells.

Discussion

In this study, we have delineated distinct functional regions in the cytoplasmic domain of the human G-CSF-R. We show here that a membrane-proximal region of 55 amino acids of the human G-CSF-R is sufficient to generate growth signals, consistent with a recent report by Ziegler *et al* (21). This region contains two subdomains, termed box 1 and box 2, which are well conserved among some members of the cytokine receptor superfamily (13,17). The importance of the box 1- and box 2-containing region for growth signal transduction has been established for several cytokine receptors (17-22,31). It is likely that this region is involved in activation of common or highly related signal transduction pathways. This notion is supported by several receptor transfection studies (17-19,21,22) and by the fact that some of the ligands for these receptors induce similar patterns of tyrosine phosphorylation (32-34).

The most prominent finding of this study is that the carboxy-terminal region of 98 amino acids of the WT G-CSF-R appeared to be involved in neutrophilic maturation. Little is known of the mechanisms by which hematopoietic growth factors control maturation. For instance, it has remained a matter of discussion whether activation of growth factor receptors

truly drives maturation or simply supports the proliferation and survival of cells already programmed to mature along a certain hematopoietic lineage (35-37). With regard to the G-CSF-R, our results with the L-GM transfectants clearly favor the first possibility, because L-GM/WT cells developed into morphologically mature granulocytes when cultured in G-CSF, whereas L-GM/DA cells proliferated and survived in G-CSF without any evidence of maturation. This failure of L-GM/DA cells to mature was thus apparent in spite of their ability to proliferate in response to G-CSF. L-GM/DC and L-GM/M1 cells, which proliferated modestly in response to G-CSF, were also incapable of maturation. Our data therefore indicate that the carboxy-terminal part of the WT G-CSF-R contains a subdomain critical for maturation signaling that is required neither for proliferation nor for survival. A linkage between the carboxy-terminal truncation of the γ_c chain of the receptors for IL-2, IL-4, IL-7 and IL-15, and T cell maturation arrest in patients with X-linked severe combined immunodeficiency has recently been suggested (38). In the G-CSF-R, a subdomain designated box 3 has been identified in this region, which is also present in IL-6 signal transducer gp130 (13). Notably, IL-6 also stimulates granulocytic colony formation by normal mouse marrow cells (39) and was able to induce maturation of M1 leukemic cells (40,41). Moreover, leukemia inhibitory factor (LIF) and oncostatin M (OSM), which share gp130 for signal transduction, have also been reported to induce maturation of M1 cells (42). Thus, it appears likely that the box-3 subdomain may have a direct role in the induction of maturation, possibly through association with a specific cytoplasmic substrate.

The presence of the carboxy-terminal region in the WT G-CSF-R strongly inhibits the mitogenic signaling potential of the G-CSF-R in two myeloid cell lines, 32D and L-GM cells (Figure 2). In contrast, this negative effect on growth signaling was not evident in pro-B BAF3 cells, in which the WT G-CSF-R transduces growth signals as strong as those mediated by the DA mutant. Despite their enhanced sensitivity to G-CSF, 32D/DA and L-GM/DA cells remained fully factor dependent and died within 48 h upon withdrawal of IL-3 or G-CSF (data not shown).

It is still unclear by which mechanism proliferation signaling in myeloid cells is inhibited by the carboxy-terminal region of the WT G-CSF-R. Protein phosphorylation on tyrosine or serine/threonine residues has been shown to be frequently involved in the regulation of receptor functions, both positively and negatively (43,44). One possibility is that the down-regulation of the activity of the G-CSF-R is caused by receptor phosphorylation, as has been demonstrated for the EPO-R (45). It is of note that, in contrast to our results with BAF/WT transfectants, the negative regulatory region of the EPO-R also act in BAF3 cells, suggesting that the carboxy-terminal inhibitory regions of the G-CSF-R and EPO-R act through different mechanisms. Alternatively, this region may contain a subdomain that specifically activates as yet unknown downstream molecules which transmit growth-inhibitory signals. In that case, these molecules would not be active in BAF3 cells. A negative effect of the carboxy-terminal sequence on growth signal transduction has been reported for several other growth factor receptors, including those of the receptor tyrosine kinase family (18,46-48), Thus, negative regulation of proliferation signaling by the carboxy-terminal parts of receptors may be seen as a common feature of different growth factor receptor systems.

We have further shown that a region of 30 amino acids between residues 56 and 85

markedly augments the mitogenic signaling potential of the G-CSF-R. A functionally similar region has been identified in the common ß chain of IL-3, GM-CSF and IL-5 receptors (20). It remains to be established whether this region is actively involved in transduction of mitogenic signals or is important simply for a proper conformation that facilitates signaling from the box-1- and box-2-containing membrane-proximal regions. In this respect, it is relevant that Ziegler *et al* have demonstrated that the region between amino acids 57 and 96 in the cytoplasmic domain of the G-CSF-R is required for the induction of acute-phase protein gene expression in hepatoma cell line transfectants (21). These data appear to support that this region indeed contains a functional subdomain.

Whether the DC splice variant has a physiological role in hematopoiesis is as yet unknown. We have shown that the DC variant is able to transduce growth signals in the three murine cell lines, consistent with a previous report (14). However, the growth signals transduced by the DC receptor is significantly weaker than those transduced by the DA mutant, suggesting that the alternative carboxy tail also acts as a down-modulator of proliferation. Unlike the negative regulatory region of the WT G-CSF-R, the DC tail portion exerts its effect in both lymphoid and myeloid cells, indicating that it must act by a mechanism different from that of the WT G-CSF-R. Because the amino acid sequence in this portion is rather hydrophobic (9), one possibility is that the alternative tail folds towards the cell membrane, thereby hindering the association of growth-signaling molecules with the membrane-proximal region. Because the DC receptor is incompetent for maturation induction, it might act as a negative regulator of maturation at certain stages of myeloid development.

Our findings may have implications for studies dealing with the granulocytic maturation defects in certain hematological diseases, such as neutropenia and acute myeloid leukemia (AML). Conceivably, myeloid progenitor cells expressing a truncated G-CSF-R, lacking the carboxy-terminal negative regulatory region, would proliferate but fail to maturate in response to G-CSF, a phenotype mimicking that of leukemic cells. Thus, a potential linkage between structural abnormalities of the G-CSF-R and AML should be considered. The data presented here should prompt the investigation of such a possibility.

References

1. Clark SC and Kamen R. (1987) The human hematopoietic colony-stimulating factors. Science 236:1229.
2. Metcalf D. (1989) The molecular control of cell division, differentiation commitment and maturation in haemopoietic cells. Nature 339:27.
3. Nicola NA. (1987) Granulocyte colony-stimulating factor and differentiation-induction in myeloid leukemia cells. Int. J. Cell Cloning 5:1.
4. Demetri GD and Griffin JD. (1991) Granulocyte colony-stimulating factor and its receptor. Blood 78:2791.
5. Metcalf D. (1991) Control of granulocytes and macrophages: molecular, cellular, and clinical aspects. Science 254:529.
6. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1990) Purification and characterization of the receptor for murine granulocyte colony-stimulating factor. J. Biol.Chem. 265:14008.
7. Uzumaki H, Okabe T, Sasaki N, Hagiwara K, Takaku F and Itoh S. (1988) Characterization of receptor for granulocyte colony-stimulating factor on human circulating neutrophils. Bio.

Biophys. Res. Commun. 156:1026.
8. Fukunaga R, Ishizaka-Ikeda E, Seto Y and Nagata S. (1990) Expression cloning of a receptor for murine granulocyte colony-stimulating factor. Cell 61:341.
9. Larsen A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March CJ and Smith CA. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptor, immunoglobulin, and fibronectin domains. J. Exp. Med. 172:1559.
10. Fukunaga R, Seto Y, Mizushima S and Nagata S. (1990) Three different mRNAs encoding human granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA. 87:8702.
11. Hibi M, Murakami M, Saito M, Hirano T, Taga T and Kishimoto T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell 63:1149.
12. Saito M, Yoshida K, Hibi M, Taga T and Kishimoto T. (1992) Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. J. Immunol. 148:4066.
13. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y and Nagata S. (1991) Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. 10:2855.
14. Ziegler SF, Davis T, Schneringer JA, Franklin TL, Tough TW, Teepe M, Larsen A, Williams DE and Smith CA. (1991) Alternative forms of the human G-CSF receptor functions in growth signal transduction. New Biol. 3:1242.
15. Bazan JF. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptor, and the P75 IL-2 receptor beta chain. Biochem. Biophys. Res. Commun. 164:788.
16. Cosman D, Lyman SD, Idzerda RL, Beckmann MP, Park LS, Goodwin RG and March CJ. (1990) A new cytokine receptor superfamily. Trends. Biosci. 15:265.
17. Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T and Kishimoto T. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc. Natl. Acad. Sci. USA. 88:11349.
18. D'Andrea AD, Yoshimura A, Youssoufian H, Zon LI, Koo JW and Lodish H. (1991) The cytoplasmic region of the erythropoietin receptor contains nonoverlapping positive and negative growth-regulatory domains. Mol. Cell. Biol. 11:1980.
19. Hatakeyama M, Mori M, Doi T and Taniguchi T. (1989) A restricted cytoplasmic region of IL-2 receptor ß chain is essential for growth signal transduction but not for ligand binding and internalization. Cell 59:837.
20. Sakamaki K, Miyajima I, Kitamura T and Miyajima A. (1992) Critical cytoplasmic domain of the common ß subunit of the human GM-CSF, IL-3 and IL-5 receptors for growth signal transduction and tyrosine phosphorylation. EMBO J. 11:3541.
21. Ziegler SF, Bird TA, Morella KK, Mosley B, Gearing DP and Baumann H. (1993) Distinct regions of the human granulocyte colony-stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. Mol. Cell. Biol. 13:2384
22. Quell DE and Wojchowski DM. (1991) Localized cytosolic domains of the erythropoietin receptor regulate growth signaling and down-modulate responsiveness to granulocyte-macrophage colony-stimulating factor. Proc. Natl. Acad. Sci. USA. 88:4801.
23. Palacios R and Stinmetz M. (1985) IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes in vivo. Cell 41:727.
24. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ and Eckner RJ. (1983) Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil/basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA. 80:2931.
25. Lee KH, Kinashi T, Tohyama K, Tashiro K, Funato N, Hama K and Honjo T. (1991) Different stromal cell lines support lineage-selective differentiation of the multipotential bone marrow stem cell clone LyD9. J. Exp. Med. 173:1257.

26. Miller AD and Rosman GJ. (1989) Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980.
27. Bolton AE and Hunter WM. (1973) The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. Biochem. J. 133:529.
28. Budel LM, Touw IP, Delwel R and Löwenberg B. (1989) Granulocyte colony-stimulating factor receptors in human acute myelocytic leukemia. Blood 74:2668.
29. Salem M, Delwel R, Touw I, Mahmoud L and Löwenberg B. (1988) Human AML colony growth in serum-free culture. Leuk. Res. 12:157.
30. Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F, Altmann S, Santoli D and Rovera G. (1987) Cytokine-dependent granulocytic differentiation: regulation of proliferative and differentiative responses in a murine progenitor cell line. J. Immunol. 138:3829.
31. Miura O, Cleveland JL and Ihle JM. (1993) Inactivation of erythropoietin receptor function by point mutations in a region having homology with other cytokine receptors. Mol. Cell. Biol. 13:1788.
32. Miura O, D'Andrea A, Kabat D and Ihle JN. (1991) Induction of tyrosine phosphorylation by the erythropoietin receptor correlates with mitogenesis. Mol. Cell. Biol. 11:4895.
33. Quelle, FW, Quelle DE and Wojchowski DM. (1991) Interleukin 3, granulocyte-macrophage colony-stimulating factor, and transfected erythropoietin receptors mediate tyrosine phosphorylation of a common cytosolic protein (pp100) in FDC-ER cells. J. Biol. Chem. 267:17055.
34. Evans JPM, Mire-Sluis AR, Hoffbrand AV and Wickremasinghe RG. (1990) Binding of G-CSF, GM-CSF, tumor necrosis factor-α, and γ-interferon to cell surface receptors on human myeloid leukemia cells triggers rapid tyrosine and serine phosphorylation of a 75-Kd protein. Blood 75:88.
35. Just U, Stocking C, Spooncer E, Dexter TM and Ostertag W. (1991) Expression of the GM-CSF gene after retroviral transfer in hematopoietic stem cell lines induces synchronous granulocyte-macrophage differentiation. Cell 64:1163.
36. Miyajima A, Kitamura T, Harada N, Yokota T and Arai K. (1992) Cytokine receptors and signal transduction. Annu. Rev. Immunol. 10:295.
37. Ogawa M. (1993) Differentiation and proliferation of hematopoietic stem cells. Blood 81:2844.
38. Naguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW and Leonard WJ. (1993) Interleukin-2 receptor γ chain mutation results in X-linked combined immunodeficiency in humans. Cell 73:147.
39. Suda T, Yamaguchi Y, Suda J, Miura Y, Okano A and Akiyama Y. (1988) Effect of interleukin 6 (IL-6) on the differentiation and proliferation of murine and human hemopoietic progenitors. Exp. Hematol. 16:891.
40. Hoffman-Liebermann B and Liebermann DA. (1991) Interleukin-6- and leukemia inhibitory factor-induced terminal differentiation of myeloid leukemia cells is blocked at an intermediate stage by constitutive c-myc. Mol. Cell. Biol. 11:2375.
41. Selvakumaran M, Liebermann DA and Hoffman-liebermann B. (1992) Deregulated c-myb disrupts interleukin-6- or leukemia inhibitory factor-induced myeloid differentiation prior to c-myc:role in leukemogenesis. Mol. Cell. Biol. 12:2493.
42. Bruce AG, Hoggatt IH and Rose TM. (1992) Oncostatin M is a differentiation factor for myeloid leukemia cells. J. Immunol. 149:1271.
43. Ullrich A and Schlessinger J. (1990) Signal transduction by receptors with tyrosine kinase activity. Cell 61:203.
44. Klausner RD and Samuelson LE. (1991) T cell antigen receptor activation pathways: the tyrosine kinase connection. Cell 64:875.
45. Yoshimura A and Losish HF. (1992) In vitro phosphorylation of the erythropoietin receptor and an associated protein, pp130. Mol. Cell. Biol. 12:706.

46. Yoshimura A, Longmore G and Lodish HF. (1990) Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity. Nature 348:647.
47. Roussel NF, Dull TJ, Rettenmier CW, Ralph P, Ullrich A and Sherr CW. (1987). Transforming potential of the c-fms proto-oncogene (CSF-1 receptor). Nature. 325:549.
48. Khazaie K, Dull TJ, Graf T, Schlessinger J, Ullrich A, Beug H and Vennström, B. (1988). Truncation of the human EGF receptor leads to differential transforming potentials in primary avian fibroblasts and erythroblasts. EMBO J. 7:3061.

Chapter 3

THE CARBOXY-TERMINAL CYTOPLASMIC REGION OF THE HUMAN G-CSF RECEPTOR MEDIATES APOPTOSIS IN MATURATION-INCOMPETENT MURINE MYELOID CELLS

Fan Dong, Karin Pouwels, Lies H. Hoefsloot,
Henk Rozemuller, Bob Löwenberg and Ivo P. Touw

Submitted for publication

Abstract

G-CSF promotes the survival and proliferation of myeloid progenitors and induces maturation of these cells towards terminally differentiated neutrophils. Using transfectants of the murine IL-3-dependent myeloid cell line 32D that express the human WT G-CSF-R (32D/WT cells), we show here that G-CSF can also exert adverse effects on myeloid cell proliferation and survival. Although initially enhancing IL-3-driven proliferation of 32D/WT cells, G-CSF dramatically inhibited the proliferation of these cells at later stages of culture. The inhibitory effect of G-CSF on the proliferation of 32D/WT cells was not accompanied by progressive neutrophilic maturation. Instead, G-CSF appeared to induce apoptosis in 32D/WT cells. This effect was seen in cultures that contained IL-3 at concentrations that supported long-term proliferation of the cells in the absence of G-CSF. Experiments with 32D cells expressing mutant forms of the G-CSF-R revealed that the death signals were mediated exclusively through the membrane-distal cytoplasmic part of the G-CSF-R.

Introduction

Cell death by apoptosis represents a general mechanism by which unwanted cells are eliminated efficiently from the body (1-3). Apoptosis, like proliferation and differentiation, is a tightly controlled process, which is regulated by extracellular signals. For instance, apoptosis can be induced either by addition of certain factors, such as tumor necrosis factor, or by withdrawal of factors essential for cell survival. Hematopoietic colony-stimulating factors (HGFs) are widely viewed as survival-promoting factors whose deprivation leads to the rapid onset of apoptotic events in hematopoietic cells (4,5). One of these factors, G-CSF, is essential for granulopoiesis (6). In addition to its survival-promoting activity, G-CSF regulates the proliferation and differentiation of myeloid progenitor cells. These activities of G-CSF are mediated by the G-CSF-R that belongs to the superfamily of hematopoietin receptors (7).

The human G-CSF-R consists of a single peptide of 813 amino acids with a single membrane-spanning domain and a cytoplasmic domain of 183 amino acids (8,9). In Chapter 2 it has been shown that the G-CSF-R contains several functional regions in the cytoplasmic domain. A membrane-proximal cytoplasmic region of 55 amino acids is sufficient to generate mitogenic signals, whereas the distal carboxy-terminal region of 98 amino acids of the WT G-CSF-R is involved in maturation signaling and down-modulation of mitogenic response to G-CSF (10). Comparable results have been obtained by others (11,12).

In the present study, we demonstrate that signals transduced by the G-CSF-R have dual and paradoxical effects on the proliferation and survival of the IL-3-dependent murine myeloid cell line 32D in which the human WT G-CSF-R had been introduced and expressed. It is shown that G-CSF initially stimulates the proliferation and survival of these cells. However, upon prolonged culture G-CSF strongly inhibits cell proliferation and further induces apoptosis, even in the presence of optimal concentrations of IL-3. The signals for apoptosis are mediated through the carboxy-terminal cytoplasmic region, i.e., the region that has been shown to be associated with maturation induction (10,11).

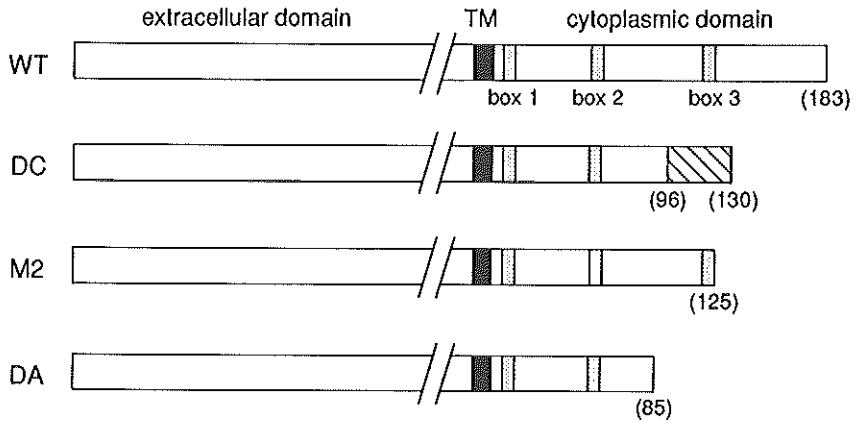

Figure 1. Structures of the different G-CSF-R forms. Boxes 1-3 denote subdomains conserved in several members of the cytokine receptor superfamily. The altered cytoplasmic tail of the DC receptor is shown as a hatched box. The numbers in parentheses indicate amino acid positions. TM, transmembrane domain.

Materials and methods

32D cells and transfectants. Murine 32D cells (13), kindly provided by JS Greenberger (University of Massachussetts), were maintained in RPMI 1640 medium supplemented with 10 % FCS and 10% WEHI-CM as a source of murine IL-3. The 32D parental cells used in this study did not express endogenous G-CSF-R and were nonresponsive to G-CSF. These cells were transfected with the WT and mutant forms of the G-CSF-R subcloned in the retroviral expression vector pLNCX (14) by electroporation. The different G-CSF-R forms relevant to this study are shown in Figure 1. The construction and detailed properties of the WT, DC and DA expression vectors have been described in Chapter 2. For the construction of pLNCX-M2, the WT G-CSF-R cDNA was amplified by polymerase chain reaction using forward primer 5'TGTGATCATCGTGACTCCCTT3' (FW3) and reverse primer 5'AGTCAC GCCAAGAGGGGCTG3' (RV5) that contains an in-frame stop codon. After insertion of the 840-bp PCR fragment in the *Hinc*II site of the pBluescript plasmid, the 3' part of the fragment was cut out from the pBluescript vector with *BsrF*I and *Cla*I. The resultant *BsrF*I-*Cla*I fragment, together with the 5' *Hind*III-*Bsr*FI fragment obtained from pLNCX-WT, was ligated into pLNCX digested with *Hind*III and *Cla*I, thus creating the full-length coding sequence of the M2 mutant. Selection of transfected 32D cells was done in IL-3-containing culture medium supplemented with 0.8 mg of G418 per ml (Gibco-BRL, Breda, The Netherlands). G418-resistant clones were expanded and tested for their capacities to specifically bind radiolabeled G-CSF as described in Chapter 2.

DNA synthesis and cell proliferation assays. DNA synthesis was assayed by ^{3}H-TdR uptake. Cells (10^{4}) were incubated in triplicate in 100 μl of RPMI/FCS medium supplemented with titrated concentrations of WEHI-CM in the presence or absence of 10 ng of

recombinant human G-CSF per ml (Amgen, Thousand Oaks, CA) in 96-well plates. After 24 hours of incubation, 1 μCi of ^{3}H-TdR (2 Ci/mM, Amersham Inc. Amersham, UK) was added to each well. Cells were harvested 12 hours later and radioactivity was measured by liquid scintillation counting as described (15). To quantify cell proliferation in suspension culture, cells were incubated in 25-ml culture flasks (Greiner, Alphen a/d Rijn, The Netherlands) at an initial density of 5 x 10^4/ml in 5 ml of culture medium, supplemented with 10 ng of G-CSF per ml and different concentrations of WEHI-CM as indicated. Culture medium was refreshed every 2 to 4 days and cell densities were readjusted to 5 x 10^4 cells/ml. Viable cells were counted on the basis of trypan blue exclusion.

Morphological analysis. Cell morphology was assessed after spinning of cells on glass slides using a cytocentrifuge. Slides were stained with May-Grünwald-Giemsa reagents prior to microscopic examination. Myeloperoxidase (MPO) staining and nitroblue-tetrazolium (NBT) tests were performed as described (16,17) and at least 400 cells were counted for evaluation.

Analysis of DNA degradation. Genomic DNA was extracted from cells at various stages of culture using the method as described (18), with minor modifications. Briefly, cells (1.5 x 10^7) were washed twice and resuspended in 3 ml of lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM EDTA). Cell lysates were digested by adding 0.2 ml of a proteinase K solution (20 mg/ml) and 0.15 ml of 20% SDS. After overnight incubation at 40°C, 1 ml of saturated NaCl (6 M) was added to the digestion mixtures, shaken vigorously and centrifuged at 1,400 x *g* for 15 min. The supernatants were collected and mixed with 2 volumes of absolute ethanol. The DNA was precipitated by centrifugation at 5,500 x *g* for 30 min. DNA pellets were washed in 70 % ethanol, air-dried and then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Size fractionation of DNA was performed by electrophoresis in a 1% agarose gel containing 0.5 μg of ethidium bromide per ml. 5 μg of DNA were loaded in each lane.

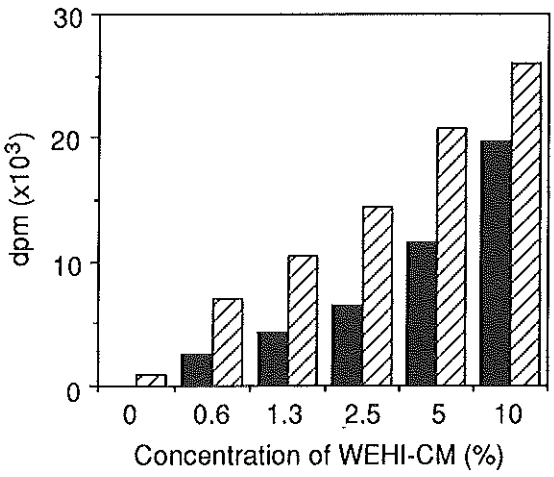

Figure 2. Synergistic effect of G-CSF and IL-3 on the short-term proliferation of 32D/WT cells. Cells were incubated in medium containing increasing concentrations of WEHI-CM in the presence (hatched bars) or absence (black bars) of added G-CSF (10 ng/ml). ^{3}H-TdR incorporation was determined.

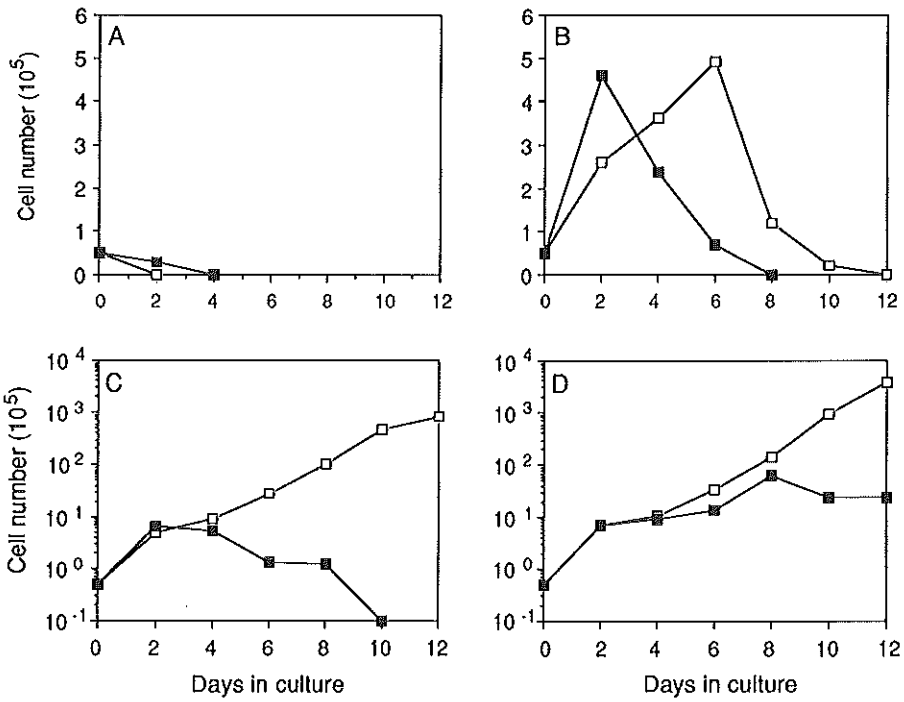

Figure 3. Dual effects of G-CSF on IL-3-induced proliferation of 32D/WT cells. Cells were cultured in medium containing no added (open squares) or 10 ng of G-CSF per ml (closed squares) in the absence (**A**) or presence of 2.5% (**B**), 5% (**C**) or 10% WEHI-CM (**D**). Shown are data from a representative experiment on one 32D/WT clone.

Results

32D cells expressing the WT G-CSF-R show a weak proliferative response to G-CSF. Parental 32D cells or 32D cells transfected with the empty pLNCX vector did not show any response to G-CSF and died overnight in medium containing 10 ng of G-CSF per ml (Chapter 2). G-CSF evoked a weak and transient proliferative response in 32D cells expressing the WT G-CSF-R (32D/WT) in ^{3}H-TdR uptake assays (Figure 2), and the cells died 3 to 6 days after culture in G-CSF-containing medium (Figure 3A). Cytological examination revealed that these G-CSF-treated cells displayed morphologic features of apoptosis without evidence for terminal granulocytic maturation (Chapter 2).

G-CSF initially enhances but subsequently inhibits IL-3-driven proliferation of 32D/WT transfectants. To test whether G-CSF synergized with IL-3 in inducing a proliferative response, 32D/WT cells were cultured in medium containing increasing concentrations of WEHI-CM in the presence or absence of G-CSF (10 ng/ml). Consistent with previous reports (19,20), G-CSF synergized with IL-3 in stimulating the proliferation of

32D/WT cells, as measured in DNA synthesis and cell proliferation assays (Figures 2 and 3). However, these synergistic effects of IL-3 and G-CSF were evident only during the early stages of culture, i.e., the first two days. Thereafter, G-CSF inhibited IL-3-mediated proliferation and survival of 32D/WT cells. 32D/WT cells cultured in 10 ng of G-CSF per ml and 2.5% WEHI-CM initially proliferated at a higher rate than cells cultured in either 10 ng of G-CSF per ml or 2.5% WEHI-CM alone, but they lost viability more rapidly than cells cultured in 2.5% WEHI-CM alone (Figure 3B). Significantly, although 32D/WT cells could grow indefinitely in 5% WEHI-CM, they died within 10 to 14 days in medium containing 5% WEHI-CM and 10 ng of G-CSF per ml (Figure 3C). Four independent clones were analyzed and comparable results were obtained. The negative effect of G-CSF on IL-3-induced proliferation and survival of 32D/WT cells could be overcome partially by increasing the concentration of WEHI-CM in the culture medium (Figure 3D).

Table 1. Cytological features of 32D/WT cells

Growth factor	Morphology	MPO staining	
		% of positive cells	intensity
IL-3 (5% WEHI-CM)	BL,Pro[a]	35	weak[b]
IL-3 + G-CSF[c]	BL,Pro	31	weak

[a] BL, blast; Pro, promyelocyte.
[b] 1-6 deposits per cell.
[c] analyzed 8 days after addition of G-CSF (10 ng/ml).

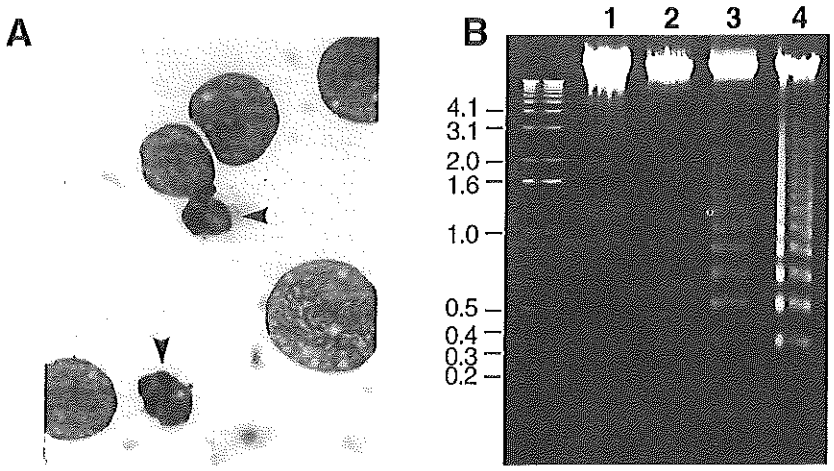

Figure 4. Induction of apoptosis of 32D/WT cells by G-CSF. (A) Morphology of 32D/WT cells cultured in 5% WEHI-CM and 10 ng of G-CSF per ml for 6 days. Arrows indicate apoptotic cells. (B) Electrophoresis of DNA extracted from 32D/WT cells cultured in 10% WEHI-CM (lane 1) and 5% WEHI-CM for 6 days (lane 2), or 5% WEHI-CM and 10 ng of G-CSF per ml for 4 (lane 3) and 6 days (lane 4). The sizes of the marker DNA fragments are indicated on the left in kilobase.

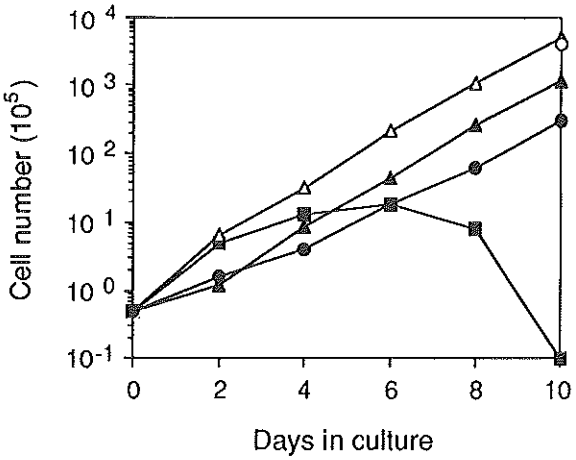

Figure 5. Long-term proliferation of 32D cells expressing the different G-CSF-R forms. Shown are growth curves of 32D cells expressing the WT (closed squares) and DC (open triangles) G-CSF-R in medium containing 5% WEHI-CM and 10 ng of G-CSF per ml, and of 32D cells expressing mutant DA (closed triangles) or M2 (closed circles) in medium containing 10 ng of G-CSF per ml.

32D cells are arrested at early stages of granulocytic maturation. A possible explanation for the growth-inhibitory effects of G-CSF on IL-3-induced proliferation of 32D/WT cells was that the cells matured in response to G-CSF and thereby gradually lost proliferative potential. To assess whether 32D/WT cells underwent granulocytic maturation following exposure to 10 ng of G-CSF per ml and increasing concentrations of IL-3, the morphology of 32D/WT cells cultured in the presence of G-CSF and IL-3 was examined. Morphological changes indicative of terminal granulocytic maturation were not observed, even after 10 to 14 days of culture. The expression of MPO, a marker for early myeloid differentiation (21-23), was then examined. When cultured in IL-3-containing medium, approximately 35% of 32D cells stained weakly positive for MPO activity (Table 1). No significant increase in the quantity of MPO protein were seen when 32D/WT cells was treated with G-CSF (Table 1). The expression of MPO mRNA was not induced by G-CSF stimulation either (data not shown). Moreover, 32D/WT cells cultured in medium containing 10 ng of G-CSF per ml and 5% WEHI-CM for 8 days failed to reduce NBT and did not express Gr-1, a surface antigen expressed on mature granulocytic cells (24) (data not shown). Taken together, these results established that 32D/WT cells lacked the capacity to proceed with terminal granulocytic maturation in response to G-CSF.

G-CSF induces apoptosis in 32D/WT cells. Instead of terminal maturation, 32D/WT cells cultured in IL-3 and G-CSF exhibited morphological features characteristic of apoptosis, e.g., condensation and fragmentation of nuclei and shrinkage of cytoplasm (Figure 4A). In agreement with this, DNA isolated from cells cultured in 10 ng of G-CSF per ml and 5% WEHI-CM yielded a typical ladder of oligonucleosomal-sized bands corresponding to multiples of 200-bp fragments after agarose gel electrophoresis (Figure 4B), indicative of an extensive degradation of DNA at internucleosomal sites. These data demonstrate that the G-CSF-R is capable of transducing signals that result in the premature apoptosis of myeloid cells. Notably, the apoptotic signaling by the G-CSF-R was evident even in the presence of high concentrations of IL-3 that would otherwise be sufficient to support the long-term proliferation and survival of these cells (Figure 3C).

The carboxy-terminal region of the G-CSF-R is responsible for apoptosis signaling. To determine which cytoplasmic region of the G-CSF-R is responsible for G-CSF-induced apoptosis, the growth features of 32D cells transfected with the different G-CSF-R forms (Figure 1) were examined. 32D cells expressing the DC splice variant (32D/DC), in which the 87 carboxy-terminal amino acids of the WT G-CSF-R are replaced by a distinct region of 34 amino acids, showed a weak proliferative response to G-CSF, similar to the response of 32D/WT cells (Chapter 2). However, G-CSF did not suppress the proliferation and survival of 32D/M1 cells supported by either 2.5% or 5% WEHI-CM (Figure 5 and data not shown). It has been shown that this altered carboxy-terminus of the DC variant down-modulates the mitogenic response to G-CSF but lacks the maturation signaling capacity (Chapter 2). Significantly, 32D cells expressing the DA or the M2 mutant, which lack 98 and 58 carboxy-terminal amino acid residues, respectively, continued to proliferate efficiently in medium containing G-CSF alone (Figure 5). No significant differences in the survival and proliferation of 32D/DA and 32D/M2 cell were observed when IL-3 was added to the G-CSF-containing culture (data not shown). Collectively, these results and data presented in Chapter 2 demonstrate that the carboxy-terminal region of the WT G-CSF-R is involved in the induction of both maturation and apoptosis.

Discussion

G-CSF has a prominent role in promoting the survival, proliferation and maturation of myeloid progenitor cells (6). Here, we have shown that G-CSF can also exert opposite effects on myeloid cells. We have demonstrated in the murine IL-3-dependent myeloid 32D cells, transfected with the human G-CSF-R, that although G-CSF initially synergizes with IL-3 in inducing a proliferative response of 32D/WT cells, it suppresses IL-3-dependent long-term proliferation and further induces apoptosis. This inhibitory effect is unlikely due to the down-modulation of IL-3 response by G-CSF because even after prolonged G-CSF exposure, 32D/WT cells proliferated efficiently in IL-3-containing medium upon withdrawal of G-CSF (data not shown).

The 32D cells used in this study were apparently unable to undergo terminal granulocytic maturation in response to G-CSF. This is evident from the analyses of cell morphology and the expression pattern of MPO mRNA and protein as well as from the failure of the cells to express Gr-1 surface antigen and to reduce NBT. In contrast, G-CSF has been demonstrated to induce the terminal granulocytic maturation and survival of murine myeloid 32Dcl3 and L-G cells, which express the endogenous G-CSF-R (23,25), and murine myeloid L-GM cells transfected with the human WT G-CSF-R (Chapter 2). These observations raise the possibility that the induction of cell death by G-CSF is coupled to the inability of the 32D cells to mature towards terminally differentiated neutrophils. In this respect, it is relevant that the maturation- and apoptosis-inducing activities of the WT G-CSF-R are assigned to the same carboxy-terminal cytoplasmic region, making it likely that both maturation and apoptosis signals are mediated by a single functional subdomain within this region. Because IL-3 inhibits G-CSF-induced terminal granulocytic maturation (11,23), it can be envisioned that IL-3 also counteracts the apoptotic effect of G-CSF.

Induction of cell death by G-CSF is not confined to 32D cells. In a preliminary communication, Bessho *et al* reported similar findings with a radiation-induced murine myeloid leukemia cell line (26). Moreover, we have recently found that Brown-Norway rat leukemia LT12 cells (27) transfected with the WT G-CSF-R also become apoptotic in response to G-CSF without evidence of progressive maturation (H. Rozemuller *et al.*, unpublished data). G-CSF fails to induce apoptosis in LT12 cells expressing a G-CSF-R mutant that lacks 128 carboxy-terminal amino acid residues, further confirming that the carboxy-terminal region of the G-CSF-R is responsible for apoptosis induction. Notably, this truncated G-CSF-R is capable of mediating mitogenic signals (M1 mutant, see Chapter 2).

Several lines of evidence suggest that an intrinsic inability to undergo maturation leads to apoptosis when cells are given a 'conflicting' signal to mature. For instance, although G-CSF and EPO induce maturation of 32Dcl3 and 32DEpo1 cells towards granulocytic and erythroid lineages, respectively, these cells die in response to G-CSF and EPO when maturation is blocked by forced expression of the genes encoding helix-loop-helix protein Id, zinc finger protein Evi-1, or cyclin D2 and D3 (28-31). Likewise, interruption of the expression of the *c-fes* gene, whose protein product is important for myeloid maturation (32,33), causes apoptosis of HL-60 cells treated with retinoid acid (34), an inducer of terminal granulocytic maturation of HL-60 cells. Conversely, expression of exogenous wild-type p53 in HL-60 cells results in terminal maturation (35). In M1 leukemic cells, however, exogenous wild-type p53 does not induce maturation but instead apoptosis (36). Significantly, apoptosis of M1 leukemic cells triggered by wild-type p53 is inhibited by IL-6, which induces the terminal maturation of the cells (37).

The idea that apoptosis is associated with maturation failure predicts that the death signaling potential of the G-CSF-R is shared with other hematopoietic growth factor receptors capable of inducing terminal maturation. Indeed, IL-10 has recently been shown to induce apoptosis in maturation-defective B-chronic lymphocytic leukemia (B-CLL) cells, whereas IL-10 normally activates proliferation and maturation of B lymphocytes, including the normal $CD5^+$ counterparts of B-CLL cells (38). Furthermore, it has been demonstrated that IL-6, an inducer of terminal maturation of M1 leukemic cells, caused apoptosis of nonmaturing U937 cells (39). Notably, the carboxy-terminal region of the cytoplasmic domain of the IL-6 signal transducer gp130 shares some structural homology with the G-CSF-R (40).

The molecular mechanisms responsible for apoptosis associated with maturation failure is unknown. It is also unclear whether maturation incompetence leads to apoptosis via the same mechanism or distinct mechanisms are involved in different receptor and cell systems. With respect to G-CSF-induced apoptosis, Northern analysis showed that *p53* and *Bcl-2*, two well-known genes involved in the regulation of cell survival (2,3,41), were lowly expressed in 32D cells and their expressions did not alter during G-CSF-induced apoptosis of 32D/WT (data not shown). Given the expanding list of genes whose primary action is to control cell survival (41,42), it is possible that other gene products than p53 and Bcl-2 may play a role in G-CSF-induced apoptosis. Further studies would be needed to clarify the mechanism by which G-CSF induces apoptosis in 32D/WT cells.

The physiological significance of cell death signaling by the G-CSF-R in the regulation of myelopoiesis is not yet fully clear. One possibility is that the G-CSF-driven mature-or-die

mechanism operates to prevent uncontrolled expansion and survival of maturation-incompetent cells. Alternatively, the onset of apoptosis may represent a mechanism associated with G-CSF-induced terminal granulocytic maturation (43,44), which is activated at premature stages if cells fail to mature. Irrespective of the exact function of the apoptosis- and maturation-inducing region(s) of the G-CSF-R in the regulation of myeloid development, it is evident that the functional disruption of this region will result in a prolonged survival of myeloid cells that are hampered in their maturation abilities, cellular features that are characteristic of AML cells. It will be of importance to investigate whether mutations inactivating the function of the carboxy terminus of the G-CSF-R would occur in AML.

References

1. Raff MC. (1992). Social controls on cell survival and cell death. Nature 356:397.
2. Williams GT and Smith CA. (1993) Molecular regulation of apoptosis: genetic controls on cell death. Cell 74:777.
3. Allen PD, Bustin SA and Newland AC. (1993) The role of apoptosis (programmed cell death) in haemopoiesis and the immune system. Blood Rev. 7:63.
4. Williams GT, Smith CA, Spooncer E, Dexter TM and Taylor DR. (1990) Haemapoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343:76.
5. Koury MJ and Bondurant MC. (1990) Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248:378.
6. Demetri GD and Griffin JD. (1991) Granulocyte colony-stimulating factor and its receptor. Blood 78:2791.
7. Bazan JF. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptor, and the p75 IL-2 receptor beta chain. Biochem. Biophys. Res. Commun. 164:788.
8. Fukunaga R, Seto Y, Mizushima S and Nagata S. (1990) Three different mRNAs encoding human granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA. 87:8702.
9. Larsen A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March CJ and Smith CA. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptor, immunoglobulin, and fibronectin domains. J. Exp. Med. 172:1559.
10. Dong F, van Buitenen C, Pouwels K, Hoefsloot LH, Löwenberg B and Touw IP. (1993) Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. Mol. Cell. Biol. 13:7774.
11. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1993) Growth and differentiation signals mediated by different regions in the cytoplasmic domain of granulocyte colony-stimulating factor receptor. Cell 74:1079.
12. Ziegler SF, Bird TA, Morella KK, Mosley B, Gearing DP and Baumann H. (1993) Distinct regions of the human granulocyte-colony stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. Mol. Cell. Biol. 13:2384.
13. Greenberger JS, Sakakeeny MA, Humphries RK, Eaves CJ and Eckner RJ. (1983) Demonstration of permanent factor-dependent multipotential (erythroid/neutrophil /basophil) hematopoietic progenitor cell lines. Proc. Natl. Acad. Sci. USA. 80:2931.
14. Miller AD and Rosman GJ. (1989) Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980.
15. Salem M, Delwel R, Touw I, Mahmoud L and Löwenberg B. (1988) Human AML colony growth in serum-free culture. Leuk. Res. 12:157.
16. Hayhoe FGJ and Flemans RJ. (1992) Haematological cytology. Wolfe Publishing Ltd. London. 378 pp.

17. Brown BA. (1980) Hematology: principles and procedures (3rd edition). Lea & Febiger, Philadelphia. 183 pp.
18. Miller SA, Dykes DD and Polesky HF. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res. 16:1215.
19. Ikebuchi K, Clark SC, Ihle JN, Souza LM and Ogawa M. (1988) Granulocyte colony-stimulating factor enhances interleukin-3-dependent proliferation of multipotential hemapoietic progenitors. Proc. Natl. Acad. Sci. USA. 85:3445.
20. Takaue Y, Kawano Y, Reading CL, Watanabe T, Abe T, Ninomiya T, Shimizu E, Ogura T, Kuroda Y, Yokobayashi A, Nakahata T, Asano S and Ventura G. (1990) Effects of recombinant human G-CSF, GM-CSF, IL-3 and IL-1α on the growth of purified human peripheral blood progenitors. Blood 76:330.
21. Lübbert M, Herrmann F and Koeffler HP. (1991) Expression and regulation of myeloid-specific genes in normal and leukemic myeloid cells. Blood 77:909.
22. Tobler A, Míller CW, Johnson KR, Selsted ME, Rovera G and Koeffler HP. (1988) Regulation of gene expression of myeloperoxidase during myeloid differentiation. J. Cell. Physiol. 136:215.
23. Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F, Altmann S, Santoli D and Rovera G. (1987) Cytokine-dependent granulocytic differentiation: regulation of proliferative and differentiative responses in a murine progenitor cell line. J. Immunol. 138:3829.
24. Hestdal K, Ruscetti FW, Ihle JM, Jacobsen SEW, Dubois CM, Kopp WC, Longo DL and Keller JR. (1991) Characterization and regulation of RB6-8C5 antigen expression on murine bone marrow cells. J. Immunol. 147:22.
25. Lee KH, Kinashi T, Tohyama K, Tashiro K, Funato N, Hama K and Honjo T. (1991) Different stromal cell lines support lineage-selective differentiation of the multipotential bone marrow stem cell clone LyD9. J. Exp. Med. 173:1257.
26. Bessho M, Yoshida S, Sakate K, Murohashi I, Jinnai I and Hirashima K. (1993) Recombinant human granulocyte colony-stimulating factor suppresses the development of murine myeloid leukemia by inducing apoptosis of leukemic cells. Exp. Hematol. 21:582 (abstr.).
27. Martens ACM, van Bekkum DW and Hagenbeek A. (1990) The BN acute myelocytic leukemia (BNML): a rat model for studying human acute myelocytic leukemia (AML). Leukemia 4:241.
28. Kreider BL, Benezra R, Rovera G and Kadesch T. (1992) Inhibition of myeloid differentiation by the helix-loop-helix protein Id. Science 255:1700.
29. Morishita K, Parganas E, Matsugi T and Ihle JN. (1992) Expression of the Evi-1 zinc finger gene in 32Dcl3 myeloid cells blocks granulocytic differentiation in response to granulocyte colony-stimulating factor. Mol. Cell. Biol. 12:183.
30. Kreider BL, Orkin SH and Ihle JN. (1993) Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. Proc. Natl. Acad. Sci. USA. 90:6454.
31. Kato JY and Sherr CJ. (1993) Inhibition of granulocyte differentiation by G_1 cyclins D2 and D3 but not D1. Proc. Natl. Sci. USA. 90:11513.
32. Smithgall TE, Yu G and Glazer RI. (1988) Identification of the differentiation-associated p93 tyrosine protein kinase of HL-60 leukemia cells as the product of the human c-fes locus and its expression in myelomonocytic cells. J. Biol. Chem. 263:15050.
33. Yu G, Smithgall TE and Glazer RI. (1989) K562 leukemia cells transfected with the human c-fes gene acquire the ability to undergo myeloid differentiation. J. Biol. Chem. 264:10276.
34. Manfredini R, Grande A, Tagliafico E, Barbieri D, Zucchini P, Citro G, Zupi G, Franceschi C, Torelli U and Ferrari S. (1993) Inhibition of c-fes expression by an antisense oligomer causes apoptosis of HL60 cells induced to granulocytic differentiation. J. Exp. Med. 178:381.
35. Soddu S, Blandino G, Citro G, Scardigli R, Piaggio G, Ferber A, Calabretta, B and Sacchi A. (1994) Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells.

Blood 83:2230.
36. Yonish-Rouach E, Resnitzky D, Lotem J, Sachs L, Kimchi A and Oren M. (1991) Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin-6. Nature 352:345.
37. Hoffman-Lieberman B and Liebermann DA. (1991) Interleukin-6- and leukemia inhibitory factor-induced terminal differentiation of myeloid leukemia cells is blocked at an intermediate stage by constitutive c-myc. Mol. Cell. Biol. 11:2375.
38. Fluckiger AC, Durand I and Banchereau J. (1994) Interleukin 10 induces apoptotic cell death of B-chronic lymphocytic leukemia cells. J. Exp. Med. 179:91.
39. Afford SC, Pongracz J, Stockley RA, Crocker J and Burnett D. (1992) The induction by human interleukin-6 of apoptosis in the promonocytic cell line U937 and human neutrophils. J. Biol. Chem. 267:21612.
40. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y and Nagata S. (1991) Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. 10:2855.
41. Vaux DL and Haecker G. (1994) An evolutionary perspective on apoptosis. Cell 76:777.
42. Schwartz, L.M., and B.A. Osborne. 1993. Programmed cell death, apoptosis and killer genes. Immunol. Today 14:582.
43. Martin SJ, Bradley JG and Cotter TG. (1990) HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. Clin. Exp. Immunol. 79:448.
44. Savill JS, Wyllie AH, Henson JE, Walport MJ, Henson PM and Haslett C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. J. Clin. Invest. 83:865.

Chapter 4

IDENTIFICATION OF A NONSENSE MUTATION IN THE G-CSF RECEPTOR IN SEVERE CONGENITAL NEUTROPENIA

Fan Dong, Lies H. Hoefsloot, Anita M. Schelen, Lianne C. A. M. Broeders, Yolande Meijer, Anjo J. P. Veerman, Ivo P. Touw and Bob Löwenberg

Modified from Proc. Natl. Acad. Sci. USA 91: 4480-4484, 1994

Abstract

Severe congenital neutropenia is characterized by profound absolute neutropenia and a maturation arrest of marrow progenitor cells at the promyelocyte-myelocyte stage. Marrow cells from such patients frequently display a reduced responsiveness to G-CSF. G-CSF binds to and activates a specific receptor (G-CSF-R) which transduces signals critical for the proliferation and maturation of granulocytic progenitor cells. Here we report the identification of a somatic point mutation in one allele of the G-CSF-R gene in a patient with SCN. The mutation results in the truncation of 98 carboxy-terminal amino acids of the G-CSF-R that are essential for maturation signaling. We further demonstrate that the mutation was present only in hematopoietic cells of granulocytic lineage. Because the normal allele of the G-CSF-R gene was still expressed in the patient's granulocytes, we suggest that the mutant receptor protein may act in a dominant negative manner to block granulocytic maturation.

Introduction

Severe congenital neutropenia (SCN or Kostmann's syndrome) was first described in 1956 (1). This disorder of granulopoiesis is characterized by severe absolute neutropenia (peripheral blood neutrophil count of 0.2×10^9/L or less) and a maturation arrest of marrow myeloid progenitor cells at the promyelocyte-myelocyte stage. Erythropoiesis and thrombopoiesis are generally normal. Patients with SCN have frequent episodes of severe bacterial infections, usually starting in the first month of life. Affected infants have a poor prognosis and often succumb in the first or second decade despite the improvement in supportive care. Patients also have an increased risk of developing acute leukemia (2). Although an autosomal recessive pattern of inheritance was suggested for SCN, sporadic cases have been reported as well (3-6).

The etiology of SCN remains elusive. No serum inhibitors of marrow cell growth have been detected in patients with SCN (5). The mononuclear cells from these patients were found capable of producing biologically active G-CSF and the serum levels of G-CSF are generally increased (6-7). However, cultured marrow cells from patients with SCN frequently displayed a markedly suppressed responsiveness to G-CSF stimulation (4,8-10). With the clinical availability of recombinant hematopoietic growth factors, it has been observed that most patients respond favorably to *in vivo* administration of G-CSF, as evidenced by a significant increase in circulating neutrophils and a dramatic clinical improvement (3,4,9). In contrast, therapy with GM-CSF is generally ineffective in SCN. These data suggest that a specific defect in G-CSF signal transduction may exist in SCN that can be overcome by providing the patients with pharmacologic dosages of G-CSF.

G-CSF acts primarily in the granulocytic lineage and appears to be the major hematopoietic growth factor involved in the regulation of *in vivo* production of mature neutrophils (11). G-CSF induces the proliferation, differentiation and survival of granulocytic progenitor cells. The diverse effects of G-CSF are mediated through the interaction of G-CSF and the G-CSF-R. The human G-CSF-R cDNA predicts an 813-amino acid peptide with a single

membrane-spanning domain (12,13). Expression of the human G-CSF-R in murine hematopoietic cells resulted in high-affinity binding sites on the cell surface and rendered the cells responsive to G-CSF (14).

The exclusive abnormality of granulopoiesis in SCN prompted us to investigate the G-CSF-R in these patients. We used RT-PCR to amplify the G-CSF-R cDNA of patients with congenital neutropenia and screened for mutations with single strand conformation polymorphism (SSCP) analysis. In one patient, we identified a somatic point mutation which results in the truncation of the carboxy-terminal cytoplasmic region of the G-CSF-R, i.e., a region that has been shown to be involved in the induction of granulocytic maturation. These data indicate that defective G-CSF-R structures may play a role in the development of neutropenia in certain cases of SCN.

Materials and methods

Patients and samples. Patient DA, a 13-year old boy, suffered from severe recurrent infections from 3 days of age onwards. The diagnosis of SCN was established on clinical and laboratory grounds. There was no family history of similar disease. From age 10, the patient started treatment with recombinant human (rh) G-CSF and showed a favorable response with an increase of the neutrophil count from below detectable level to $>10^9$/L.

Bone marrow or blood samples were obtained from patient DA after informed consent from the parents. Marrow progenitor cells were enriched by Ficoll-Isopaque centrifugation and complement-mediated cytolysis of mature T lymphocytes and monocytes following 30 min of incubation with optimal concentrations of monoclonal antibodies against CD3, CD14 and CD15 (15). $CD34^+$ cells were then sorted following CD34 labeling (BI3C5, Sera-Lab, Crawley Down, Sussex, UK). T lymphocytes were isolated from peripheral blood by erythrocyte rosetting of cells from the Ficoll-Isopaque interface. Monocytes were recovered from the latter cell population after plastic adherence in 6-cm Petri dishes (1h at 37°C), and B lymphocytes were collected as non-adherent erythrocyte-rosette-negative mononuclear cells. Granulocytes were obtained from blood as the sedimented cell fraction after Ficoll-Isopaque centrifugation and further depleted of erythrocytes by hypotonic lysis. Erythroid colony cells were picked from *in vitro* cultures supported by EPO and IL-3.

Table 1. Primers used for PCR amplification

Primer	Sequence (5'→3')	Position
FW1	TCGGAAAGGTGAAGTAACTTGTCC	111-134
RV1	TCCATGGGATCAAGACACAG	819-838
FW2	TGCAGGCAGAGAATGCGCTG	777-796
RV2	GAAGATCTCATAGAGCTGAAAG	1657-1678
FW3	TGTGATCATCGTGACTCCCTT	1669-1689
RV6	GTAGATCTTAGTCATGGGTTCATGG	2750-2774
FW5	CCATCACCAAGCTCACAGTG	2244-2263

Underlined nucleotides indicate introduced mismatches. FW, forward; RV, reverse.

Table 2. Comparison of *in vitro* responses of marrow progenitor cells from normal individuals and patient DA to different hematopoietic growth factors

		Colony number			
	Growth factor	G	M	Eo	E
Normals (n=4)	IL-3	0	0-17	15-71	0
	GM-CSF	0	0-36	9-75	0
	G-CSF	44-269	0-35	0	0
	combination*	107-450	18-83	23-61	107-450
Patient DA	IL-3	0	12	37	0
	GM-CSF	0	7	22	0
	G-CSF	7	17	0	0
	combination	78	35	58	108

Numbers indicate colonies per 10^4 $CD34^+$ cells. G, granulocytic; M, macrophage; Eo, eosinophilic; E, erythroid.
* IL-3 + GM-CSF + G-CSF + EPO

Clonogenic assays and recombinant human growth factors. Colony cultures were set up essentially as described (15). Marrow cells were plated in duplicate at 1 x 10^4 per ml ($CD34^+$ cells) or 4 x 10^4 per ml (Ficoll interface) in semisolid medium containing Iscove's modified Dulbecco's medium, 1.1% methylcellulose, 30% FCS, transferrin, lecithin, sodium selenite, and 2-mercaptoethanol with or without growth factors. Colonies were scored after 14 days of incubation at 37°C in humidified 5% CO_2 atmosphere. IL-3 (Gist Brocades, Delft, The Netherlands) was used at 3 ng/ml. GM-CSF and M-CSF (Genetic Institute, Cambridge, MA) were used at 25 and 50 ng/ml. G-CSF and EPO (Amgen, Thousand Oaks) were used at 10 ng/ml and 1 unit/ml, respectively.

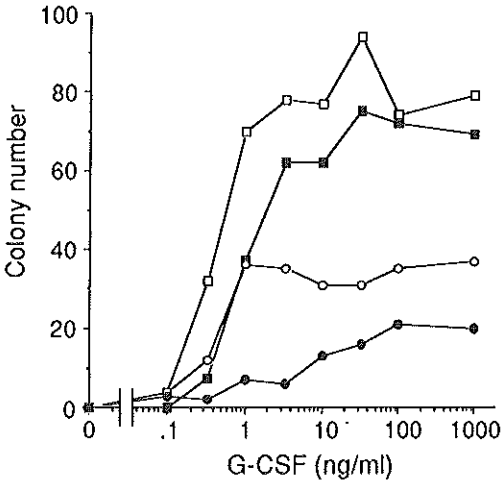

Figure 1. *In vitro* response to G-CSF of marrow cells from patient DA and normal individuals. A reduced sensitivity of marrow cells from patient DA (closed circles) to G-CSF stimulation as compared with the marrow cells from 3 healthy donors (open circles, open and closed squares) was apparent.

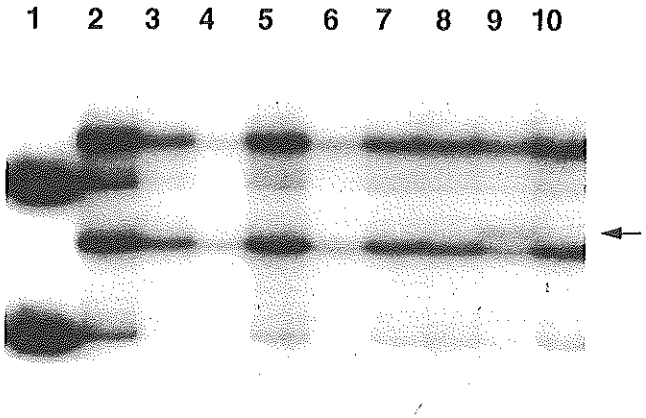

Figure 2 . Screening for mutations in the G-CSF-R by PCR-SSCP analysis. The fragment extending from nt 1669 to 2774 was amplified from WT cDNA (lane 2) and reverse-transcribed cDNA from mRNA extracted from six pediatric patients with congenital neutropenia (lanes 3-6,8,9) and two adult patients with chronic idiopathic neutropenia (lanes 7,10). Part of the autoradiograph of the SSCP gel obtained after *Pvu*II digestion is shown. The position of double strand DNA is shown in lane 1. An abnormally migrating band was seen in lane 9 (arrow).

Amplification of genomic DNA and cDNA by PCR. Genomic DNA was isolated as described (16). Total granulocyte RNA was extracted (17) and first-strand cDNA was synthesized from 1 μg of RNA by using avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) and oligo(dT) or reverse primers. Three sets of primers (Table 1) were designed to amplify the entire coding region of the G-CSF-R cDNA. Part of exon 17 of the G-CSF-R gene was amplified with primers FW5 and RV6. Amplification was performed with Vent DNA polymerase (New England Biolabs) on 1/10th of the cDNA reaction mixture or 0.5 μg of genomic DNA for 35 cycles of 1 min at 94°C, 1.5 min at 51°C and 2 min at 72°C in a thermal cycler (Perkin-Elmer/Cetus).

SSCP analysis. Amplified PCR fragments were purified (Geneclean, Bio 101) after agarose gel separation and 1/100th of the products were subjected to second-round amplification in the presence of [α-^{32}P]-ATP. To increase the sensitivity of SSCP analysis, the labeled products were cleaved into fragments of 50-250 bp by digestion with appropriate restriction enzymes (3 or 4 different enzymes in each case). Samples were then denatured by boiling for 5 minutes in loading buffer (95% [vol/vol] formamide, 20mM EDTA, 0.05% [wt/vol] each of bromophenol blue and xylene cyanol), and loaded on a nondenaturing 8% polyacrylamide gel (acrylamide/methylenebisacrylamide weight ratio, 49:1) containing 10% (vol/vol) glycerol. Electrophoresis at 30 W for 5-8 h was followed by autoradiography.

Subcloning of PCR fragments and DNA sequencing. After agarose gel purification, PCR fragments were ligated into the pBluescript vector (Stratagene). Nucleotide sequences were determined with the dideoxy method using the T7 sequencing kit (Pharmacia).

Results

***In vitro* response to hematopoietic growth factors.** Colony cultures of highly enriched progenitor cells from patient DA were used to evaluate the responsiveness to several hematopoietic growth factors. The numbers of erythroid, macrophage and eosinophilic colonies generated from marrow cells of patient DA in response to EPO, IL-3 and GM-CSF were comparable to normal controls (Table 2). However, G-CSF-induced granulocytic colony formation was significantly impaired. Dose titration experiments with marrow cells recovered from Ficoll-Isopaque interface without further enrichment revealed that the patient's marrow cells not only generated lower numbers of granulocytic colonies but also required higher concentration of G-CSF for maximal colony formation than normal marrow cells (Figure 1). These results showed that the response to G-CSF was exclusively impaired in the patient.

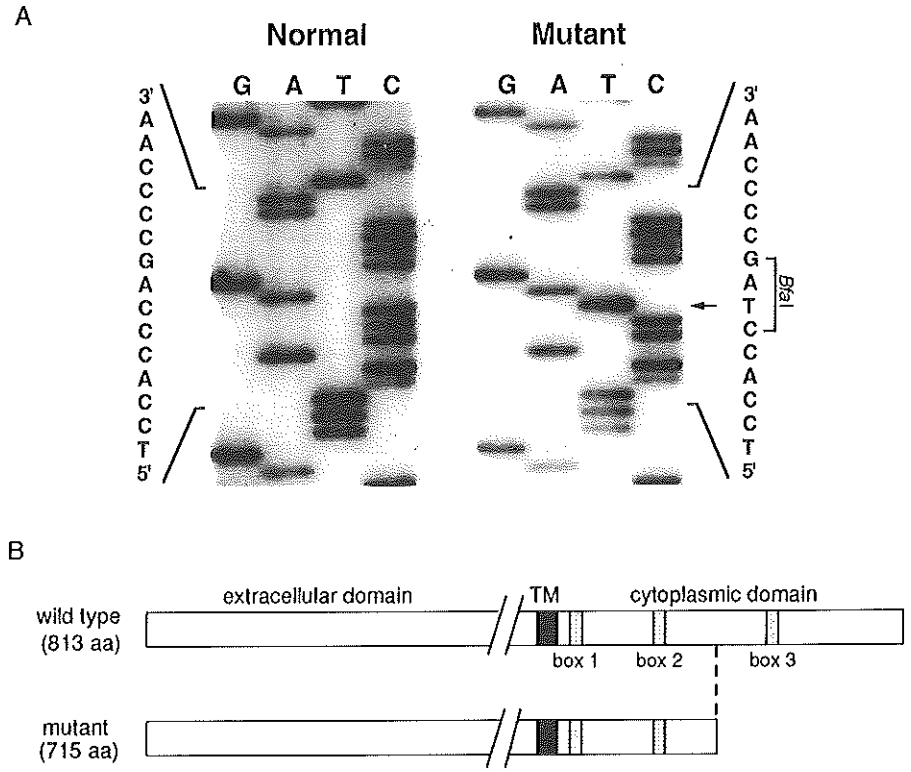

Figure 3. Identification of a point mutation in the G-CSF-R cDNA. **(A)** Partial nucleotide sequences of the normal and mutant G-CSF-R cDNA. PCR fragment amplified with primers FW3 and RV6 (nt 1669-2774) was subcloned into the pBluescript vector. Shown is a portion of the nucleotide sequences spanning the point mutation (C→T) as indicated by the arrow. The restriction site for *Bfa*I created by the mutation is also indicated. **(B)** Structure of the WT and mutant G-CSF-R. Boxes 1-3 denote regions conserved in certain members of the cytokine receptor family. TM, transmembrane domain.

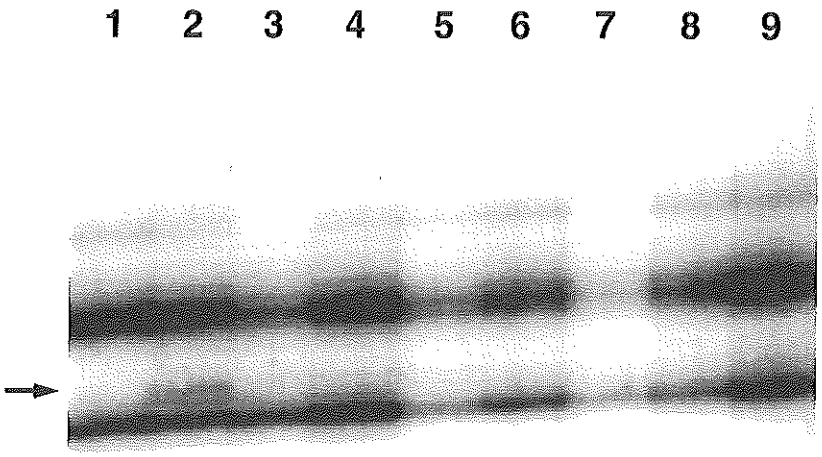

Figure 4. Detection of the point mutation in genomic DNA from different cell populations by PCR-SSCP analysis. Part of exon 17 of the G-CSF-R gene that contains the mutation was amplified. PCR products were digested with *Pvu*II before SSCP analysis. The same abnormally migrating band as that shown in Figure 2 was observed in DNA isolated from patient's granulocytes (lane 2), but not in DNA from monocytes, erythroid colony cells, T or B lymphocytes, and fibroblast cells of the patient (lanes 3 to 7). DNA isolated from the mononuclear cells of the patient's father and mother exhibits a normal SSCP pattern (lanes 8 and 9). Lane 1 shows the SSCP pattern of WT cDNA.

SSCP analysis of G-CSF-R cDNA. The entire coding region of the G-CSF-R cDNA was amplified from total granulocyte RNA of patient DA by using three sets of primers. The resultant PCR fragments were initially separated in agarose gel but no differences in size were detected between the patient's and WT G-CSF-R. To search for small alterations, the PCR fragments were subjected to SSCP analysis following digestion with different restriction enzymes. An abnormally migrating band, in addition to the normally migrating one, was consistently detected when the PCR fragment extending from nt 1669 to 2774 (pHQ3) was digested with *Pvu*II (Figure 2, lane 9). This fragment was then subcloned and the same mobility shift was detected in 9 out of 16 individual clones analyzed (data not shown). An abnormally migrating band was also detected following *Dde*I digestion, and by comparing the patterns of SSCP obtained after *Pvu*II and *Dde*I digestion, the region containing the mutation was narrowed down to nt 2311-2429. No other mutations in the coding region of the G-CSF-R sequence were identified in patient DA or in five other patients with congenital neutropenia and two patients with acquired idiopathic neutropenia (Figure 2).

Identification of a point mutation. Five independent clones were sequenced and a C-to-T transition at nt 2384 (pHQ3) was identified in 3 clones displaying the variant SSCP patterns (Figure 3). The mutation was not present in 2 other clones displaying the normal SSCP pattern. Direct sequencing of PCR products amplified from genomic DNA of the patient's granulocytes confirmed the presence of both the normal and mutant allele (data not shown).

The mutation changes the CAG codon for Gln^{716} to the TAG stop codon (Figure 3). Thus, one of the patient's alleles is predicted to code for a truncated G-CSF-R lacking the 98 carboxy-terminal amino acid residues.

Lineage specificity of the mutation. To investigate whether the mutation was inherited or represented a *de novo* germline or somatic event, a fragment of 530 bp covering the mutation site was amplified from genomic DNA prepared from various cellular sources of patient DA and from blood mononuclear cells of the patient's parents. After *Pvu*II digestion, the amplified fragment was subjected to SSCP analysis. In two independent experiments the mutation was readily detected in patient's granulocytes (Figure 4, lane 2), but not in monocytes, erythroid colony cells, T and B lymphocytes, and skin fibroblasts of patient DA (lanes 3-7). DNA isolated from mononuclear cells of the parents manifested a normal SSCP pattern (lanes 8 and 9). Since the C-to-T transition creates an additional *Bfa*I site (CCAG to CTAG; Figure 3), we also performed *Bfa*I digestion of PCR products to confirm the findings and comparable results were obtained (data not shown).

Discussion

SCN is a rare pediatric disorder and to date about 120 cases have been reported (2). On the basis of the reduced *in vitro* response of marrow progenitor cells to G-CSF, increased serum levels of G-CSF, and differential therapeutic effects of G-CSF and GM-CSF in these patients, a perturbed G-CSF signal transduction has been suggested. Normal or increased numbers of the G-CSF-R are expressed on the neutrophils of SCN patients, with affinities comparable to those on normal neutrophils, suggesting that the aberrant response to G-CSF in SCN is not due to alterations in G-CSF-R expression (18). However, mutations, particularly in the cytoplasmic domain of the G-CSF-R that affect only G-CSF signal transduction, cannot be excluded. Rauprich *et al* (19) have demonstrated that an intracellular protein of approximately 160 kDa is spontaneously phosphorylated in neutrophils from patients with SCN, but not in normal neutrophils. Neutrophils from patients with SCN display an abnormal response to f-Met-Leu-Phe, with decreased superoxide anion production and impaired mobilization of cytosolic calcium (20-22). Nevertheless, the exact nature of molecular defects in SCN has remained unknown.

We describe here the identification of a point mutation in one allele of the G-CSF-R gene in a patient with SCN which results in the deletion of 98 amino acids from the carboxy terminus of the receptor. The mutation was predominantly present in the granulocytic lineage, suggesting that patient DA is mosaic for the mutation. Because the truncated G-CSF-R remains the membrane-proximal cytoplasmic region sufficient for maximal proliferative signaling but lacks the carboxy-terminal maturation-inducing portion (Chapter 2), it is apparent that the underlying defect of the truncated G-CSF-R is its inability to transduce signals for terminal granulocytic maturation. This is consistent with the clinical findings that granulocytic maturation is disturbed in patient DA, and with the fact that marrow cells from the patient displayed an exclusively abnormal response to G-CSF stimulation *in vitro*. Our data strongly imply that the expression of the truncated receptor is the direct cause of neutropenia in the patient.

Two lines of evidence suggest that the truncated receptor was expressed in patient DA, although direct demonstration of the presence of the mutant protein was hampered by the fact that anti-human G-CSF-R antibodies are not yet available. According to SSCP analysis the transcripts of the normal and the mutant G-CSF-R genes were present in patient's granulocytes (Figure 2). Flow cytometric analysis with biotinylated G-CSF and ^{125}I-radiolabeled G-CSF binding assays demonstrated that the surface expressions of the WT and the truncated G-CSF-R were low but comparable in murine BAF3, 32D and L-GM cells (Chapter 2 and data not shown). These data indicate that the mutant G-CSF-R protein is stable and can be expressed on the cell surface. This notion may have important implications for understanding the mechanism whereby the truncated receptor interferes with the normal granulopoiesis. Oligomerization of receptor molecules is required for high affinity binding and subsequent signal transduction (23-27). Thus, the truncated G-CSF-R proteins could form heterodimers with the normal receptor chains, leading to an inability of the G-CSF-R complexes to transduce maturation signals upon G-CSF binding. Such a dominant negative mechanism has been suggested for other receptors as well (28-30). On the other hand, approximately one-fourth of the receptors expressed on the cell surface would still be homodimers of the normal G-CSF-R protein, if both the normal and the truncated receptor were expressed at the same level. Accordingly, it would be expected that the dominant negative effect could be partially overcome by increasing the occupancy of the residual normal G-CSF-R complexes. Indeed, the patient responded favorably to G-CSF therapy (10 μg/kg/day) with a significant increase of peripheral blood neutrophil count.

The predominant presence of the point mutation in granulocytes suggests that progenitor cells mainly committed to the granulocytic lineage were exclusively affected. Because committed progenitor cells are generally thought to be incapable of unlimited self-renewal, the question arises as to how neutropenia persisted in the patient. In this respect, the results presented in Chapter 2 may be informative. Myeloid L-GM and 32D cells expressing the WT G-CSF-R eventually died in G-CSF-containing medium either as a consequence of terminal granulocytic maturation or due to premature apoptosis. In contrast, L-GM and 32D cells transfected with the DA mutant, which lacks 98 carboxy-terminal amino acid residues, proliferated continuously in response to G-CSF. Therefore, it is possible that in the patient, because of the expression of the truncated G-CSF-R, self-renewal of committed granulocytic progenitor cells is favored over differentiation and subsequent cell death, which may lead to the persistence of these progenitor cells. This would imply that, to a certain extent, the abnormal cell population is transformed. Notably, cases of SCN that terminated in AML have been reported (31-33). In addition, our data would imply that G-CSF therapy in certain cases of SCN should be carefully evaluated, because such treatment may lead to an overstimulation of the abnormal cell population. At least three cases of SCN have developed leukemia or myelodysplastic syndrome following G-CSF treatment (2).

It is conceivable that SCN consists of a heterogeneous group of disorders with variable underlying etiology. In fact, six patients with congenital neutropenia were initially screened with SSCP analysis, but an abnormal G-CSF-R was identified in only one patient (Figure 2), suggesting that abnormalities of molecules involved in G-CSF signal transduction downstream of the receptor could also play a role in SCN pathogenesis. However, with more patients

being examined, mutations in other important regions of the G-CSF-R may be identified in SCN. In addition, our results also validate the investigation of the involvement of abnormal G-CSF-R structures in other neutropenias as well as in leukemia. Studies of this kind may help to elucidate the pathogenesis of some hematopoietic disorders and may improve our understanding of G-CSF signal transduction.

References

1. Kostmann R. (1956) Infantile genetic agranulocytosis. Acta. Paedia. Scand. 45:1 (suppl.105).
2. Gillio AP and Gabrilove JL. (1993) cytokine treatment of inherited bone marrow failure syndromes. Blood 81:1669.
3. Bonilla MA, Gillio AP, Ruggeiro M, Kernan NA, Brochstein JA, Abboud M, Fumagalli L, Vincent M, Cabrilove JL, Welte K, Souza LM and O'Reilly RJ. (1989) Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N. Engl. J. Med. 320:1574.
4. Welte K, Zeidler C, Reiter A, Muller W, Odenwald E, Souza L and Riehm H. (1990) Differential effects of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in children with severe congenital neutropenia. Blood 75:1056.
5. Parmley RT, Crist WM, Ragab AH, Boxer LA, Malluh A, Lui VK and Darby CP. (1980) Congenital dysgranulopoietic neutropenia: clinical, serologic, ultrastructural, and in vitro proliferative characteristics. Blood 56:465.
6. Pietsch T, Bührer C, Mempel K, Menzel T, Steffens U, Schrader C, Santos F, Zeidler C and Welte K. (1991) Blood mononuclear cells from patients with severe congenital neutropenia are capable of producing granulocyte colony-stimulating factor. Blood 77:1234.
7. Mempel K, Pietsch T, Menzel T, Zeidler C and Welte K. (1991) Increased serum levels of granulocyte colony-stimulating factor in patients with severe congenital neutropenia. Blood 77:1919.
8. Kobayashi M, Yumiba C, Kawaguchi Y, Tanaka Y, Ueda K, Komazawa Y and Okada K. (1990) Abnormal response of myeloid progenitor cells to recombinant human colony-stimulating factors in congenital neutropenia. Blood 75:2143.
9. Chang J, Craft AW, Reid MM, Coutinho LH and Dexter M. (1990) Lack of response of bone marrow, in vitro, to growth factors in congenital neutropenia. Am. J. Hematol. 35:125.
10. Daghistani D, Jimenez JJ, Toledano SR, Cirocco RE and Yunis AA. (1990) Congenital neutropenia: a case study. Am. J. Pediatr. Hematol. Oncol. 12:210.
11. Demetri GD and Griffin JD. (1991) Granulocyte colony-stimulating factor and its receptor. Blood 78:2791.
12. Larsen A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March CJ and Smith CA. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptors, immunoglobulin, and fibronectin domains J. Exp. Med. 172:1559.
13. Fukunaga R, Seto Y, Mizushima S and Nagata S. (1990) Three different mRNAs encoding human granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA. 87:8702.
14. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y and Nagata S. (1991) Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. 10:2855.
15. Backx B, Broeders L, Bot FJ and Löwenberg B. (1991) Positive and negative effects of tumor necrosis factor on colony growth from highly purified normal marrow progenitors. Leukemia 5:66.
16. Miller SA, Dykes DD and Polesky HF. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res. 16:1215.
17. Chomczynski P and Sacchi N. (1987) Single-step method of RNA isolation by acid

guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156.
18. Kyas U, Pietsch T and Welte K. (1992) Expression of receptors for granulocyte colony-stimulating factor on neutrophils from patients with severe congenital neutropenia and cyclic neutropenia. Blood 79:1144.
19. Rauprich P, Kyas U, Pietsch T, Steffens U and Welte K. (1991) Normal G-CSF receptor expression and altered intracellular protein phosphorylation in neutrophils from patients with severe congenital neutropenia. Blood 78, 105a (abstr., suppl. 1).
20. Roesler J, Emmendörffer A, Elsner J, Zeidler C, Lohmann-Matthes M and Welte K. (1991) Altered function and surface marker expression of neutrophils induced by rhG-CSF treatment in severe congenital neutropenia. Eur. J. Haematol. 46:12.
21. Kurtzberg J and Kilpatrick L. (1992) Defective PMN superoxide anion production in response to FMLP and Con-A: a novel and clinically significant functional abnormality of PMN killing in a patient with congenital neutropenia responsive to G-CSF (Filgrastim). Blood 80, 425a (abstr., suppl. 1).
22. Roesler J, Elsner J, Zaidler C, Lohmann-Matthes M and Welte K. (1992) Impaired mobilization of cytosolic free calcium in neutrophils from patients with severe congenital neutropenia. Blood 80, 95a (abstr., suppl. 1).
23. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1990) Purification and characterization of the receptor for murine granulocyte colony-stimulating factor. J. Biol. Chem. 265:14008.
24. Nicola NA and Metcalf D. (1991) Subunit promiscuity among hemopoietic growth factor receptors. Cell 67:1.
25. Murakami M, Hibi M, Nakagawa N, Nakagawa T, Yasukawa K, Yamanishi K, Taga T and Kishimoto T. (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science 260:1808.
26. Watowich SS, Yoshimura A, Longmore GD, Hilton DJ, Yoshimura Y and Lodish HF. (1992) Homodimerization and constitutive activation of the erythropoietin receptor. Proc. Natl. Acad. Sci. USA. 89:2140.
27. De Vos AM, Ultsch M and Kossiakoff AA. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255:306.
28. Basu A, Raghunath M, Bishayee S and Das M. (1989) Inhibition of tyrosine kinase activity of the epidermal growth factor (EGF) receptor by a truncated receptor that binds to EGF: role for interreceptor interaction in kinase regulation. Mol. Cell. Biol. 9:671.
29. Chatterjee VKK, Nagaya T, Madison LD, Datta S, Rentoumis A and Jameson JL. (1991) Thyroid hormone resistance syndrome: inhibition of normal receptor function by mutant thyroid hormone receptors. J. Clin. Invest. 87:1977.
30. Fleischman RA. (1992) Human piebald trait resulting from a dominant negative mutant allele of the c-kit membrane receptor gene. J. Clin. Invest. 89:1713.
31. Wong WY, Williams D, Slovak ML, Charak B, Mazumder A, Snyder D, Powars DR and Brynes RK. (1993) Terminal acute myelogenous leukemia in a patient with congenital agranulocytosis. Am. J. Hematol. 43:133.
32. Gilman PA, Jackson DP and Guild HG. (1970) Congenital agranulocytosis: prolonged survival and terminal acute leukemia. Blood 36:576.
33. Rosen RB and Kang SJ. (1979) Congenital agranulocytosis terminating in acute myelomonocytic leukemia. J. Pediatr. 94:406.

Chapter 5

MUTATIONS TRUNCATING THE CARBOXY-TERMINAL REGION OF THE G-CSF RECEPTOR IN ACUTE MYELOID LEUKEMIA PRECEDED BY SEVERE CONGENITAL NEUTROPENIA

Fan Dong, Russell K. Brynes, Nicola Tidow,
Karl Welte, Bob Löwenberg, Ivo P. Touw

Submitted for publication

Abstract

Severe congenital neutropenia is characterized by a maturation arrest of myeloid progenitor cells at early stages of development. Some SCN patients have an increased risk of developing AML. G-CSF is the principal growth factor involved in granulopoiesis. Abnormalities in G-CSF signal transduction pathways have been suggested to play a role in the progression to AML in SCN patients. The cytoplasmic domain of the G-CSF-R was amplified by PCR from genomic DNA and reverse-transcribed cDNA of hematopoietic cells obtained from two AML patients with a history of SCN. The PCR products were subjected to nucleotide sequencing. Point mutations at Gln^{718} and Gln^{731}, respectively, of the G-CSF-R were identified in the two patients, leading to the truncation of the carboxy-terminal cytoplasmic region essential for transducing maturation signals. In one patient, the mutation could also be demonstrated during chronic neutropenia prior to conversion to AML. These results indicate that mutations in the G-CSF-R gene interrupting the maturation-signaling function are involved in the pathogenesis of SCN, and are associated with progression to AML in SCN patients.

Introduction

Although originally described as an autosomal recessive entity (1), severe congenital neutropenia (SCN or Kostmann's syndrome) includes a heterogeneous group of disorders with variable inheritance, characterized by early onset of recurrent bacterial infections and severe absolute neutropenia (less than 0.2×10^9 neutrophils/L). Morphological analysis of the bone marrow of SCN patients almost invariably shows a maturation arrest of granulocytic progenitor cells at the promyelocyte/myelocyte stage (2-6). SCN patients have an increased incidence of AML (7-9).

G-CSF is a major hematopoietic growth factor involved in the regulation of granulopoiesis. Several studies have suggested that an abnormal response of granulocytic progenitor cells to G-CSF may play a role in the pathogenesis of SCN. For instance, myeloid progenitor cells from patients with SCN often show a reduced responsiveness to G-CSF *in vitro* (10,11). Pharmacological doses of G-CSF, when applied therapeutically *in vivo*, lead to improvement of neutropenia in the majority of SCN patients (11-13). Defective G-CSF production or serum inhibitors of myeloid cell growth have not been documented in SCN patients so far examined (14-16).

The G-CSF-R is a single polypeptide of 813 amino acids (17,18) that belongs to the hematopoietin receptor superfamily (19). After G-CSF-induced dimerization of receptor chains, the G-CSF-R transduces signals regulating the proliferation, maturation and survival of myeloid progenitor cells. These signals are mediated by distinct cytoplasmic regions of the G-CSF-R. The membrane-proximal region is involved in the transduction of proliferative and survival signals, whereas the distal carboxy-terminal region is responsible for transducing maturation signals and for suppressing G-CSF-R proliferative signaling (20-22). It has been suspected for some time that signaling abnormalities of the G-CSF-R, compromising its maturation-inductive properties, would be involved in the pathogenesis of certain myeloid

disorders characterized by defective granulocytic maturation, such as SCN and AML. In Chapter 4, it is shown that granulocytes from an SCN patient (patient DA) carried a mutation in the G-CSF-R truncating the carboxy-terminal maturation-inducing region (23). Defective G-CSF-R structures have not yet been documented in AML patients. In this paper, we report the identification of point mutations in the G-CSF-R in two AML patients with a history of SCN. Similar to the mutation in patient DA, the mutations in the two AML patients also lead to the truncation of the carboxy-terminal region of the G-CSF-R. Furthermore, we show that the mutation in one patient was already present in the neutropenic phase that preceded AML. These results suggest that mutations disrupting the maturation signaling function of the G-CSF-R may be involved in the development of AML in SCN patients.

Case Reports

Patient BA. The clinical and laboratory features of patient BA have been described elsewhere (9). In brief, the male patient was diagnosed as SCN at the age of 2 years and 9 months. The family history was negative for increased risk of infections. He remained clinically well until age 12, when infections became more frequent and severe. G-CSF therapy was initiated and his peripheral blood absolute neutrophil counts increased up to 6.0 x 10^9/L. Eight months later, while he was on G-CSF therapy, peripheral blood examinations revealed approximately 30% blasts. Repeated bone marrow studies consistently showed a predominance of blasts. Cytogenetic analysis revealed a clonal abnormality in the bone marrow and the karyotype of the leukemic clone was 49, XY, +3, +der(5), t(1;5)(q21;q21), +22. He was diagnosed as AML (FAB-M1) and died 7 months later.

Patient FR. This male patient was diagnosed as SCN in the first year of life when he suffered from severe mastoiditis. The family history was negative for hematological disorders or increased frequency of infections. During the first 20 years of his life, he suffered from frequent episodes of pneumonitis, otitis, tonsillitis, and severe gingivitis. At the age of 18 years, all teeth had to be extracted because of severe tooth abscesses. At the age of 20 years he was enrolled in the phase I/II study of G-CSF (Filgrastim) in Hannover, Germany. At this time point, his bone marrow revealed a maturation arrest of myelopoiesis at the level of promyelocyte/myelocyte with absence of band and segmented neutrophils. There were no signs of myelodysplasia and the cellularity of the bone marrow was normal. He responded well to G-CSF at a dose of 3 μg/kg/day with an increase of neutrophils to above 2 x 10^9/L within the first two weeks of treatment. This neutrophil count could be maintained during the next two years without changing the G-CSF dose. He did not experience severe infections anymore and the quality of life improved dramatically. At the age of 22, two years after G-CSF treatment, a routine bone marrow examination demonstrated monosomy 7 in the myeloid lineage without any signs of dysplasia or leukemia. G-CSF treatment was discontinued immediately, but restarted two month later due to the demand of the patient because of severe stomatitis. Eleven months later, he developed myelodysplasia with thrombocytopenia and anemia (MDS-RAEB). Another 8 months later, the MDS transformed into secondary AML (FAB-M1). Initially, he responded well to anti-leukemic treatment, but experienced an early relapse and died from therapy-resistant leukemia at the age of 26 years.

Materials and methods

PCR amplification. The isolation of genomic DNA from different cellular sources was as described (24). Total RNA was isolated from leukemic cells of patient FR with the single-step isolation method as described by Chomczynsky and Sacchi (25). RNA (1 μg) was reverse-transcribed into cDNA in a 20-μl volume containing 200 units of reverse transcriptase of Moloney murine leukemia virus (Gibco-BRL, Breda, The Netherlands). PCR amplification was done in a 50-μl volume containing 1 μM each primer, 0.2 mM dNTPs, 1 unit of *Taq* polymerase and 1 x standard *Taq* buffer (Promega Cop., Madison, WI). Amplification was done for 35 cycles consisting of 1 min at 94°C, 2 min at 51°C and 2 min at 72°C. The primers used in this study were as follows: FW3, 5'-TGTGATCATCGTGACTCCCTT-3' (forward); FW4, 5'-CTGCTGTTGTTAACCTGCCTC-3' (forward); FW6, 5'-CCAAGAGCAGTTTCCACCCAGGCC-3' (forward); FWI16, 5'-ACCCTTTGTGTTCCACCATG-3' (forward); RV7, 5'-CAAGATCTAGTTTACAATACTGAAG-3' (reverse); RV6, 5'-GTAGATCTTAGTCATGGGCTTATGG-3' (reverse); RV4, 5'-TCTCAGGGGAGATAGTGCCC-3' (reverse). Underlined nucleotides indicate introduced mismatches.

Nucleotide sequencing. After electrophoresis in agarose gels, PCR fragments were purified using Geneclean II kit (Bio 101 Inc., La Jolla, CA). PCR products were either subjected to direct sequencing or sequenced after subcloning in the pBluescript vector (Stratagene Cloning systems, La Jolla, CA). Nucleotide sequencing was performed using the T7 sequencing kit, according to the manufacturer's instructions (Pharmacia P-L Biochemicals Inc., Milwaukee, WI).

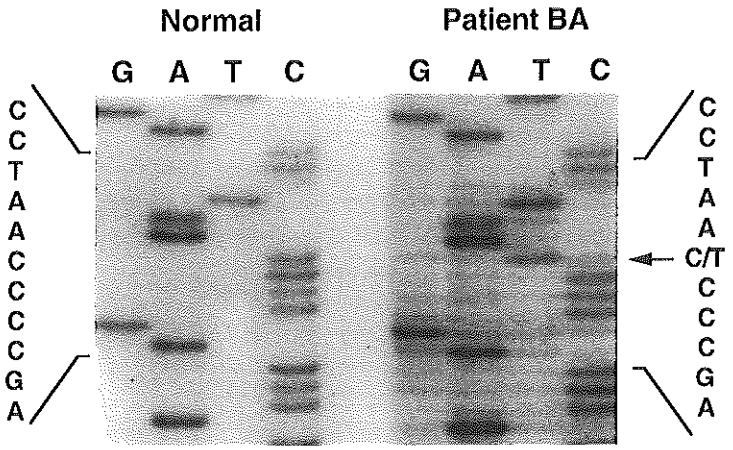

Figure 1. Part of a sequencing gel showing a point mutation in the G-CSF-R of patient BA. Nucleotide sequencing was performed directly on PCR products obtained from bone marrow cells of patient BA and on the WT G-CSF-R cDNA. The arrow indicates the position of the mutation (C→T). Note the presence of both the normal and mutant alleles in the patient.

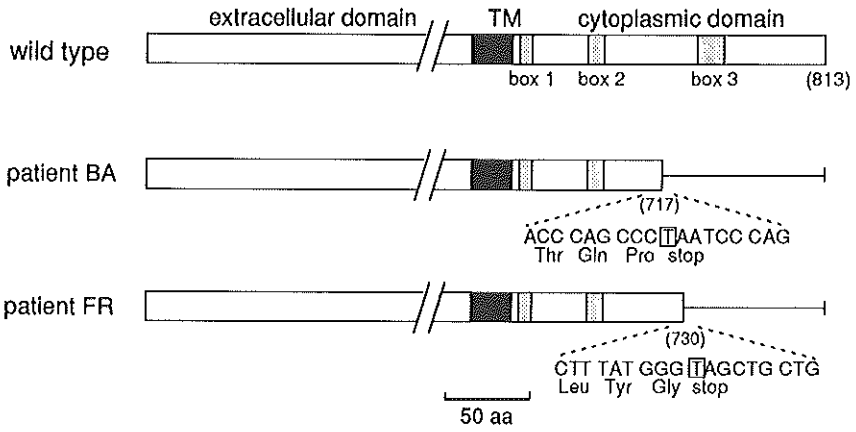

Figure 2. Schematic diagram of the WT and two truncated G-CSF-R structures. Conserved cytoplasmic subdomains are shown as boxes 1-3. Lines indicate carboxy-terminal regions deleted due to point mutations (boxed). Numbers in parentheses indicate amino acid (aa) positions. TM, transmembrane domain.

Restriction analysis of PCR products. PCR products were digested with appropriate restriction enzymes and electrophoresed in agarose gels (FMC Bioproducts, Rockland, ME).

Results

The entire exon 17 (26), which encodes 156 carboxy-terminal amino acids of the cytoplasmic domain, and part of the intron 16 of the G-CSF-R gene were amplified by PCR from genomic DNA isolated from bone marrow cells of patient BA by using primers FWI16 and RV7. The PCR product was subcloned and 18 pooled individual clones were sequenced, which revealed a C-to-T transition at nt 2390 of the G-CSF-R cDNA (17) (data not shown). Direct sequencing of PCR products confirmed the presence of the point mutation (Figure 1). The point mutation changes the CAA codon for Gln^{718} to the TAA stop codon leading to the truncation of 96 carboxy-terminal amino acids of the G-CSF-R cytoplasmic domain including the conserved box-3 segment (Figure 2). A PCR-restriction-analysis-based approach was then utilized to confirm the sequencing data and to examine the ratio of the mutant versus the normal G-CSF-R genes in bone marrow cells of patient BA. A single mismatch was introduced in primer FW6, which creates a *Stu*I restriction site in the PCR product if the point mutation was present in the DNA (Figure 3A). Analysis of 8 individual clones showed that 5 clones contained the mutation (Figure 3B). *Stu*I digestion of the PCR product obtained from bone marrow cells collected at different time points consistently showed that the mutated gene represented a minor proportion in marrow cell-derived DNA (Figure 3C). As leukemic cells comprised approximately 70% of bone marrow cells, this result indicates that the mutation was not constitutional. The mutation was not detected in DNA isolated from the liver and the spleen by *Stu*I digestion (Figure 3C) and nucleotide sequencing (data not shown).

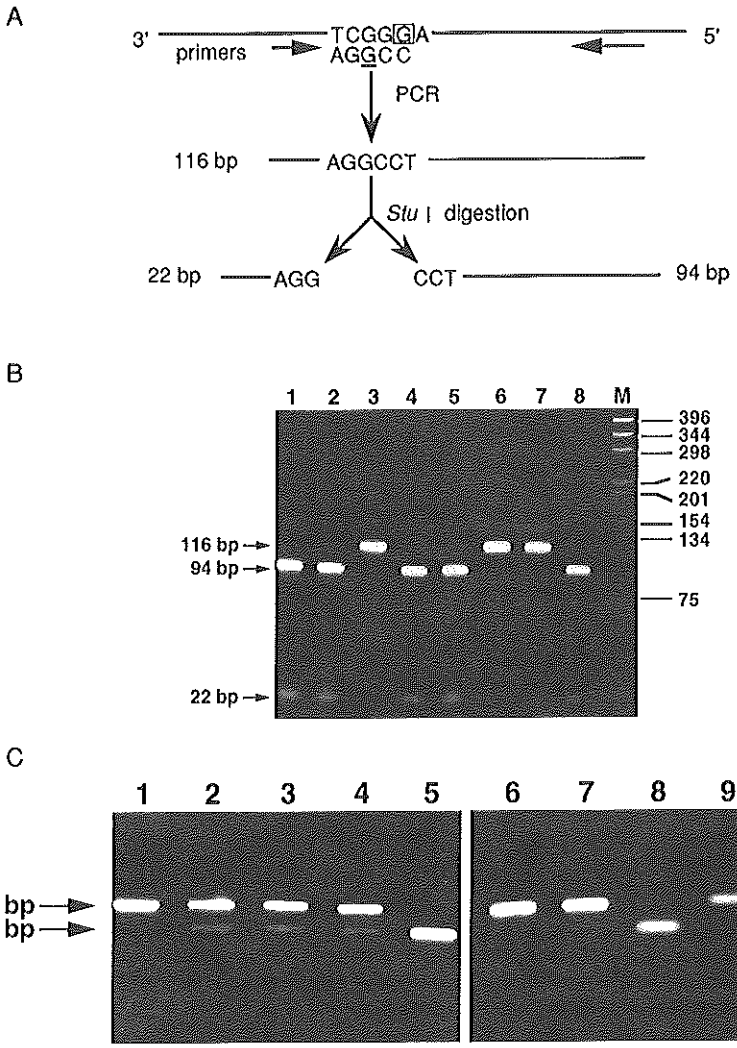

Figure 3. *Stu*I-restriction analysis of PCR product. (A) Scheme of the experimental strategy. A mismatch (underlined) introduced in the forward primer, in combination with the mutation (boxed) in template DNA (in antisense orientation), creates a *Stu*I restriction site in the PCR product. (B) Analysis of individual clones of PCR product obtained from patient BA. 3 μl of PCR reaction mixtures was digested overnight with 8 units of *Stu*I and separated in a 3% NuSieve agarose gel. (C) *Stu*I digestion of PCR product obtained from the WT G-CSF-R cDNA (lanes 1 and 9), from bone marrow cells of patient BA collected on 2/28/90 (lane 2), 12/6/90 (lane 3) and 1/23/91 (lane 4), and from the postmortem liver (lane 6) and the spleen (lane 7) as well as from a single clone of the patient-derived PCR product that carried the point mutation (lanes 5 and 8). Note the relative intensities of the normal and mutant alleles in the patient's bone marrow cells and the absence of mutation in the liver and spleen. The 22-bp fragments are not shown in this figure.

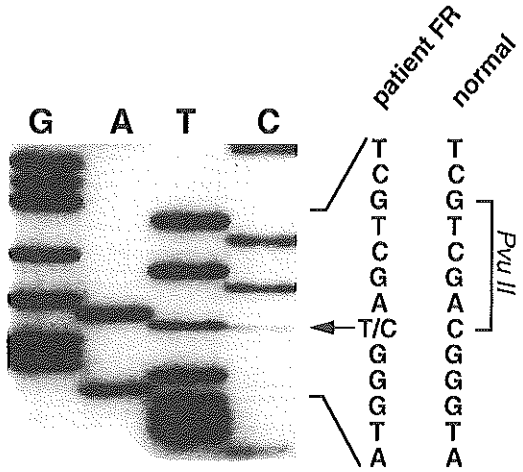

Figure 4. Identification of a point mutation in the cytoplasmic domain of the G-CSF-R in patient FR. RT-PCR product obtained from bone marrow cells of patient FR collected in leukemic phase were subcloned and 16 pooled clones were sequenced. Shown is part of a sequencing gel surrounding the point mutation (C→T), as indicated by the arrow. The *Pvu*II site destroyed by the mutation is indicated. Note that the normal G-CSF-R sequence was also detected.

From leukemic cells of peripheral blood of patient FR, only RNA samples were available. RT-PCR was used to amplify the entire transmembrane and cytoplasmic domains as well as part of the extracellular domain of the G-CSF-R by using primers FW3 and RV7. After subcloning of the PCR product, nucleotide sequencing was performed on 16 pooled individual clones. A C-to-T point mutation was identified at nt 2414 that changes the CAG codon for Gln^{731} to the TAG stop codon (Figure 4). As a result, the 83 carboxy-terminal amino acids of the G-CSF-R are deleted (Figure 2). The mutation destroys a *Pvu*II site in the G-CSF-R cDNA. Digestion of PCR products, amplified with primers FW4 and RV6, with *Pvu*II restriction enzyme revealed the presence of the transcripts of both the normal and mutated G-CSF-R alleles (Figure 5). To investigate whether the point mutation had already been present before patient FR developed AML, DNA was isolated from a bone marrow smear prepared when patient FR was in neutropenic phase prior to the acquisition of monosomy 7. *Pvu*II digestion of PCR product amplified with primers FWI16 and RV6 revealed that, similar to patient BA, only a minor proportion of DNA derived from the bone marrow cells of patient FR contained the mutation (Figure 5). This result would indicate that the mutation had arisen from a *de novo* somatic event in patient FR. As expected, the mutation was also detected by nucleotide sequencing of RT-PCR product derived from neutrophils obtained when patient FR had developed monosomy 7 but had no clinical signs of MDS or AML (data not shown).

Discussion

G-CSF has a key role in the regulation of granulopoiesis (27,28). In addition to its growth- and survival-stimulating activities, G-CSF induces terminal neutrophilic maturation. A point mutation in the G-CSF-R has been identified in an SCN patient, leading to the deletion of a carboxy-terminal region of 98 amino acids involved in maturation induction (patient DA, Chapter 4). The mutation was detected only in hematopoietic cells committed to the granulocytic lineage. As committed progenitor cells are generally believed to have a

limited self-renewal capacity, the lineage-restricted presence of the mutation appeared contradictory to the fact that neutropenia persisted in patient DA. It was assumed that granulocytic progenitor cells of the patient DA had been transformed to a certain extent due to the inability of the truncated G-CSF-R to mediate maturation signals and thereby had acquired preleukemic properties.

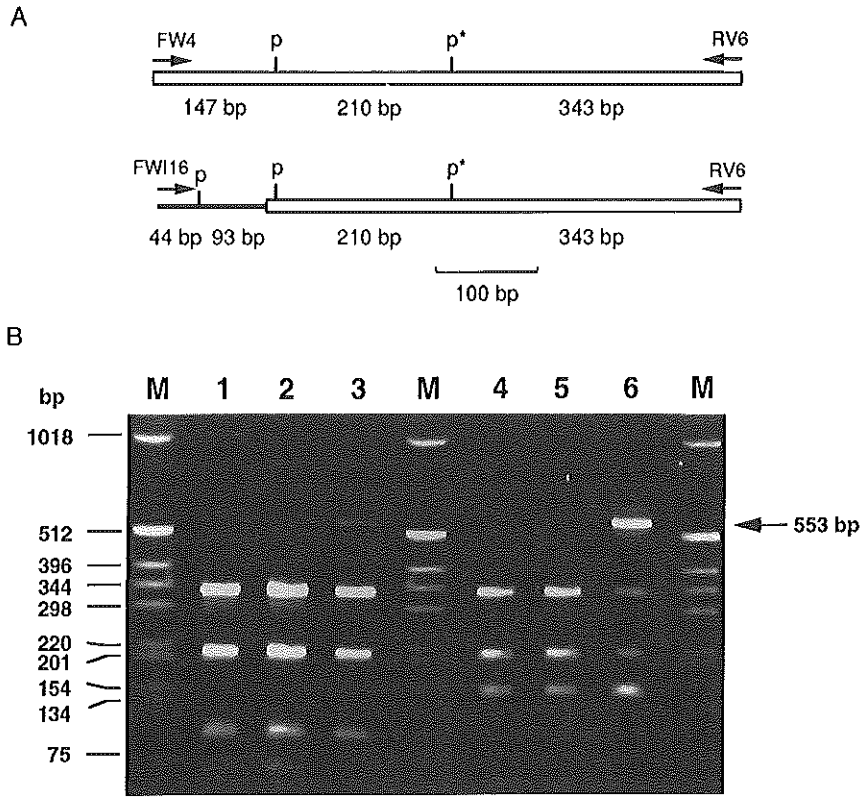

Figure 5. *Pvu*II restriction analysis of PCR products. (A) Schematic representation of PCR products amplified from cDNA (upper portion) and genomic DNA (lower portion). The relative positions of the *Pvu*II restriction sites and the expected sizes of DNA fragments in base pair (bp) after *Pvu*II digestion are indicated. Also indicated are the PCR primers used. Open boxes denote the sequences derived from cDNA and exon 17, whereas intron 16-derived sequence is shown as a bold line. The asterisks indicate the *Pvu*II site eliminated by the point mutation. (B) Detection of the point mutation by *Pvu*II digestion of PCR products. Amplification was performed on genomic DNA prepared from bone marrow cells of normal individuals (lanes 1 and 2) and patient FR in neutropenic phase (lane 3), and on RNA isolated from normal granulocytes of peripheral blood (lanes 4 and 5) and leukemic cells of patient FR (lane 6). 4 μl of PCR products were digested with 16 units of *Pvu*II for 6 hours and separated in a 2% NuSieve agarose gel. Note that the 553-bp fragment (arrow) is only weakly detectable in lane 3, but is evident in lane 6. Partial digestion is excluded, as evident from the complete digestion of the other *Pvu*II sites in the PCR products

Patients with SCN are thought to be predisposed to AML (29). Three SCN patients have been reported to develop AML in the literature (7-9). Of the 185 SCN patients currently registered in the SCN International Disease Registry, nine have progressed to AML or MDS (Welte K., unpublished data). We could obtain materials from two AML patients with a history of SCN. In both cases we found mutations that result in the carboxy-terminal truncation of the G-CSF-R. We established that the mutation in patient FR was already present in neutropenic phase prior to leukemic conversion. On the basis of studies presented in Chapter 2, one can predicts that the truncated G-CSF-R proteins isolated from the two AML patients are defective in maturation signaling, but are capable of transducing strong proliferative signals. These results, together with previous evidence (Chapter 4), suggest that aberrant G-CSF-R structures resulting in abnormal signal transduction contribute to the development of AML. Our data provide the first example that defective structures of the G-CSF-R, a member of the hematopoietin receptor superfamily, are associated with clinical leukemia.

In both patients the mutations were present only in minor proportions of DNA prepared from bone marrow cells. In patient BA, the mutation was absent in spleen and liver tissues. These results establish that the mutations were somatic events. In the two cases described here, the normal G-CSF-R allele was present in the myeloid cells, implying that the truncated G-CSF-R proteins interfere with the normal receptor functions via a dominant negative mechanism. This is entirely consistent with our current understanding of the G-CSF-R activation. It is generally accepted that G-CSF binding results in the dimerization of two G-CSF-R chains, which is an essential step leading to receptor activation (30-32). It thus appears likely that the dominant negative effect may result from the formation of heterodimers between the normal and truncated G-CSF-R chains (33-35).

The underlying mechanisms of SCN are heterogeneous. Not all SCN patients studied thus far were found to have mutations in the G-CSF-R (23,36,37). SCN was originally described as an autosomal recessive disorder by Kostmann in several Swedish families (1). Notably, nucleotide sequencing analysis of the cytoplasmic domain of the G-CSF-R in three SCN patients from these Swedish families failed to detect any mutations (unpublished data). It is possible that post-receptor signaling abnormalities play a role in the pathogenesis of the majority of SCN patients. The cases described here and in Chapter 4 apparently define a subgroup of SCN that is characterized by defective G-CSF-R structures. It appears that this subgroup may represent a preleukemic entity.

G-CSF is now utilized frequently in the treatment of SCN patients (11-13), including the two cases described here. While favorable responses to G-CSF therapy have been observed in most cases, some patients have been reported to develop AML or MDS following G-CSF therapy (29). Notably, our patients also showed transient responses to G-CSF therapy, but leukemic cells expanded and ultimately overgrew the normal hematopoietic cells. Although it remains uncertain whether G-CSF therapy contributes to the progression to AML in SCN patients, our data would argue that caution be taken in administrating G-CSF to certain cases of SCN. It would be important to analyze the G-CSF-R in an extended series of SCN patients. This will allow a critical evaluation of the relationship between defective G-CSF-R structures, progression to AML and the contribution of G-CSF therapy to leukemogenesis.

References

1. Kostmann R. (1956) Infantile genetic agranulocytosis. Acta. Paediatr. Scand. 45:1 (suppl 105).
2. Wriedt K, Kauder E and Mauer AM. (1970) Defective myelopoiesis in congenital neutropenia. N. Engl. J. Med.283:1072.
3. Rodin AE, Haggard ME, Nichols MM and Gustavson LP. (1973) Infantile genetic agranulocytosis: two cases occurring in siblings and one in a distant relative. Am. J. Dis. Child. 126:818.
4. Kostmann R. (1975) Infantile genetic agranulocytosis: a review with presentation of ten new cases. Acta. Paediatr. Scand. 64:362.
5. Amato D, Freedman MH and Saunders EF. (1976) Granulopoiesis in severe congenital neutropenia. Blood 47:531.
6. Kawaguchi Y, Kobayashi M, Tanabe A, Hara M, Nishi Y, Usui T, Nagai S, Nishibayashi Y, Nagao K and Yokoro K. (1985) Granulopoiesis in patients with congenital neutropenia. Am. J. Hematol. 20:223.
7. Gilman PA, Jackson DP and Guild HG. (1970) Congenital agranulocytosis: prolonged survival and terminal acute leukemia. Blood 36:576.
8. Rosen RB and Kang SJ. (1979) Congenital agranulocytosis terminating in acute myelomonocytic leukemia. J. Pediatr. 94:406.
9. Wong WY, Williams D, Slovak ML, Charak B, Mazumder A, Snyder D, Powars DR and Brynes RK. (1993) Terminal acute myelogenous leukemia in a patient with congenital agranulocytosis. Am. J. Hematol. 43:133.
10. Kobayashi M, Yumiba C, Kawaguchi Y, Tanaka Y, Ueda K, Komazawa Y and Okada K. (1990) Abnormal response of myeloid progenitor cells to recombinant human colony-stimulating factors in congenital neutropenia. Blood 75:2143.
11. Welte K, Zeidler C, Reiter A, Muller W, Odenwald E, Souza L and Riehm H. (1990) Differential effects of granulocyte-macrophage colony-stimulating factor and granulocyte colony-stimulating factor in children with severe congenital neutropenia. Blood 75:1056.
12. Bonilla MA, Gillio AP and Ruggeiro M. (1989) Effects of recombinant human granulocyte colony-stimulating factor on neutropenia in patients with congenital agranulocytosis. N. Engl. J. Med. 320:1574.
13. Dale DC, Bonilla MA, Davis MW, Davis MW, Nakanishi AM, Hammond WP, Kurtzberg J, Wang W, Jakubowski A, Winton E, Lalezari P, Robinson W, Glaspy JA, Emerson S, Gabrilove J, Vincent M and Boxer LA. (1993) A randomized controlled phase III trial of recombinant human granulocyte colony-stimulating factor (Filgrastin) for treatment of severe chronic neutropenia. Blood 81:2496.
14. Pietsch, T, Bührer C, Mempel K, Menzel T, Steffens U, Schrader C, Santos F, Zeidler C and Welte K. (1991) Blood mononuclear cells from patients with severe congenital neutropenia are capable of producing granulocyte colony-stimulating factor. Blood 77:1234.
15. Mempel K, Pietsch T, Menzel T, Zeidler C and Welte K. (1991) Increased serum levels of granulocyte colony-stimulating factor in patients with severe congenital neutropenia. Blood 77:1919.
16. Parmley RT, Crist WM, Ragab AH, Boxer LA, Malluh A, Lui VK and Darby CP. (1980) Congenital dysgranulopoietic neutropenia: clinical, serologic, ultrastructural, and in vitro proliferative characteristics. Blood 56:465.
17. Fukunaga R, Seto Y, Mizushima S and Nagata S. (1990) Three different mRNAs encoding human granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA 87:8702.
18. Larsen A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March CJ and Smith CA. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptors, immunoglobulin, and

fibronectin domains. J. Exp. Med. 172:1559.
19. Bazan JF. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor ß-chain. Biochem. Biophys. Res. Commun. 164:788.
20. Dong F, van Buitenen C, Pouwels K, Hoefsloot LH, Löwenberg B and Touw IP. (1993) Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor receptor involved in induction of proliferation and maturation. Mol. Cell. Biol. 13:7774.
21. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1993) Growth and differentiation signals mediated by different regions in the cytoplasmic domain of granulocyte colony-stimulating factor receptor. Cell 74:1079.
22. Ziegler S, Bird TA, Morella KK, Mosley B, Gearing DP and Baumann H. (1993) Distinct regions of the human granulocyte colony-stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. Mol. Cell. Biol. 13:2384.
23. Dong F, Hoefsloot LH, Schelen AM, Broeders LCAM, Meijer Y, Veerman AJP, Touw IP and Löwenberg B. (1994) Identification of a nonsense mutation in the G-CSF receptor in severe congenital neutropenia. Proc. Natl. Acad. Sci. USA 91:4480.
24. Miller SA, Dykes DD and Polesky HF. (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acid Res. 16:1215.
25. Chomczynski P and Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156.
26. Seto Y, Fukunaga R and Nagata S. (1992) Chromosomal gene organization of the human granulocyte colony-stimulating factor receptor. J. Immunol. 148:259.
27. Demetri GD and Griffin JD. (1991) Granulocyte colony-stimulating factor and its receptor. Blood 78:2791.
28. Lieschke GJ, Grail D, Hodgson G, Metcalf D, Stanley E, Cheers C, Fowler KJ, Basu S, Zhan YF and Dunn AR. (1994) Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. Blood 84:1737.
29. Gillio AP and Gabrilove JL. (1993) Cytokine treatment of inherited bone marrow failure syndromes. Blood 81:1669.
30. Fukunaga R, Ishizaka-Ikeda E, and Nagata S. (1990) Purification and characterization of the receptor for murine granulocyte colony-stimulating factor. J. Biol. Chem. 265:14008.
31. de Vos AM, Ultsch M and Kossiakoff AA. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. Science 255:306.
32. Watowich SS, Yoshimura A, Longmore GD, Hilton DJ, Yoshimura Y and Lodish HF. (1992) Homodimerization and constitutive activation of the erythropoietin receptor. Proc. Natl. Acad. Sci. USA 89:22140.
33. Barber DL, DeMartino JC, Showers MO and D'Andrea AD. (1994) A dominant negative erythropoietin (EPO) receptor inhibits EPO-dependent growth and blocks F-gp55-dependent transformation. Mol. Cell. Biol. 14:2257.
34. Kashles O, Yarden Y, Fischer R, Ullrich A and Schlessinger J. (1991) A dominant negative mutation suppresses the function of normal epidermal growth factor receptor by heterodimerization. Mol. Cell. Biol.11:1454.
35. Fleischman RA. (1992) Human piebald trait resulting from a dominant negative mutant allele of the c-kit membrane receptor gene. J. Clin. Invest. 89:1713.
36. Guba SC, Boxer LA and Emerson SG. (1993) G-CSF receptor transmembrane and intracytosolic structure in patients with congenital neutropenia. Blood 82:23a (abstr, suppl 1).
37. Sandoval C, Adams-Graves P, Parganas E, Wang W and Ihle JN. (1993) The cytoplasmic portion of the G-CSF receptor is normal in patients with Kostmann syndrome. Blood 82:185a (abstr, suppl 1).

Chapter 6

A POINT MUTATION IN THE G-CSF RECEPTOR GENE IN A CASE OF ACUTE MYELOID LEUKEMIA RESULTS IN THE OVEREXPRESSION OF A NOVEL G-CSF-R ISOFORM

Fan Dong, Marleen van Paassen, Carin van Buitenen,
Lies H. Hoefsloot, Bob Löwenberg and Ivo P. Touw

Modified from Blood 85: 902-911, 1995

Abstract

A novel human G-CSF-R isoform, designated SD, has been identified in which the distal carboxy-terminal cytoplasmic region, previously shown to be essential for maturation signaling, is substituted by an altered carboxy-terminus. The SD receptor has a high affinity for G-CSF and retains the membrane-proximal cytoplasmic region known to be sufficient for proliferative signaling. Nonetheless, the SD isoform lacks the ability to transduce growth signals in murine BAF3 cells and, in contrast to the WT G-CSF-R, is scarcely capable of activating JAK2 kinase. Expression of the SD receptor was found to be low in normal granulocytes, but was significantly increased in a patient with AML. The leukemic cells of this patient harboured a point mutation in the SD splice donor site of the G-CSF-R gene. These findings provide the first evidence that mutations in the G-CSF-R gene can occur in certain cases of clinical *de novo* AML. The possible contribution of defective G-CSF-R signaling to leukemogenesis is discussed.

Introduction

G-CSF promotes the proliferation, differentiation and survival of progenitor cells which predominantly form granulocytic colonies *in vitro* (1). G-CSF is the major growth factor responsible for the regulation of granulopoiesis and also modulates certain functions of mature granulocytes. In addition, G-CSF appears to have a role in activating primitive pluripotent progenitor or stem cells into cell cycle and inducing their proliferation (2-5). These biological activities of G-CSF have led to the application of G-CSF therapy in a wide variety of clinical conditions (6).

The diverse activities of G-CSF are initiated following binding of G-CSF to the G-CSF-R that belongs to the superfamily of cytokine/hematopoietin receptors (7,8). Although the majority of the family members, including the receptors for IL-2 to IL-7 and GM-CSF, are activated through the formation of heteromeric complexes comprising α, ß and sometimes γ subunits (9-12), the G-CSF-R is thought to form a homo(di)meric complex upon ligand binding (13,14). When expressed in murine hematopoietic cells, the murine and human G-CSF-Rs are able to bind to G-CSF with high affinity and transduce G-CSF-dependent signals for proliferation and maturation (15-17).

Four different forms of the human G-CSF-R, arising from alternative RNA splicing, have been isolated from several cellular sources, one of them being a soluble receptor protein (18,19). The G-CSF-R protein containing a cytoplasmic domain of 183 amino acids shows strongest homology to the murine G-CSF-R and represents the human homologue of the WT G-CSF-R. Although neither type of the G-CSF-R contains consensus motifs in the cytoplasmic domain that would indicate kinase activities, certain sequence similarities have been identified, which are conserved in several members of the cytokine receptor superfamily. Two segments called box 1 and box 2, localized in the membrane-proximal region of the G-CSF-R, are also present in most members of cytokine receptor superfamily (20,21). This membrane-proximal region has been shown to be essential for transmitting growth signals (15,17,20). The carboxy-terminal region of the WT G-CSF-R, which contains

a segment called box 3 that is shared only with IL-6 signal transducer gp130 (22,23), is involved in transduction of maturation signals and in down-modulation of proliferative signaling by the G-CSF-R (15,16).

We report here the molecular cloning of a new G-CSF-R isoform, designated SD, in which the carboxy-terminal cytoplasmic region of the WT G-CSF-R distal to box 2 is replaced by an altered terminus. Although the SD receptor retains the membrane-proximal region including box 1 and box 2 that is essential for proliferative signaling and JAK2 activation, it fails to transduce growth signals in murine BAF3 cells and only marginally induces tyrosine phosphorylation of the JAK2 kinase. The expression of SD receptor is low in normal granulocytes, but is significantly elevated in the leukemic cells of a patient with AML. The AML cells from this patient carried a point mutation in the SD splice donor site of the G-CSF-R gene. To our knowledge, this is the first case of *de novo* AML in which a mutation in the G-CSF-R gene has been identified that may result in the altered signaling properties of the encoded receptor protein.

Materials and methods

AML cells and normal granulocytes. Peripheral blood and bone marrow samples were obtained from normal individuals and patients with AML after informed consent. AML cells and normal granulocytes were isolated as described (24). In brief, leukemic cells were recovered from the interface after Ficoll-Isopaque separation. Subsequently, T cells and monocytes were removed from leukemic cells by erythrocyte-rosetting and adherence to plastic, respectively (24). Normal granulocytes were obtained as sedimented cell fraction after Ficoll-Isopaque centrifugation and further depleted of erythrocytes by hypotonic lysis.

Cell culture. Murine pro-B BAF3 cells were provided by G. Plaetinck (Roche Research, Ghent, Belgium) and were cultured in RPMI 1640 medium supplemented with 10% FCS and 10 ng of murine IL-3 per ml. Subclones of BAF3 cells expressing the different G-CSF-R forms were established as described (Chapter 2) and were maintained in the same culture medium as that for the parental BAF3 cells.

PCR amplification and subcloning. Total RNA was isolated using the guanidinium thiocyanate method (25). RNA (1 μg) was primed with oligo (dT) and reverse-transcribed into cDNA in a 20-μl reaction volume containing 200 units of reverse transcriptase of Moloney murine leukemia virus (MLV) (Gibco-BRL, Breda, The Netherlands). Genomic DNA was prepared as described (26). PCR primers were designed according to the published sequence of the G-CSF-R cDNA (19), and are as follows: FW3, 5'-TGTGATCATCGTG ACTCCCTT-3' (forward, nt 1664 to 1684); FW4, 5'-CTGCTGTTGTTAACCTGCCTC-3' (nt 2070 to 2090); FW6, 5'-CCAAGAGCAGTTTCCACCCAGGCC-3' (nt 2361 to 2384); FWI16, 5'-ACCCTTTGTGTTCCACCAGT-3', (nt -125 to -105, referring to the first nucleotide of exon 17 as +1); RV7, 5'-CAAGATCTAGTTTACAATACTGAAG-3' (reverse, nt 2923 to 2947); RV6, 5'-GTAGATCTTAGTCATGGGCTTATGG-3' (nt 2745 to 2769). Mismatches (underlined) were introduced to create restriction enzyme sites. One-tenth of cDNA reaction mixtures or 1 μg of genomic DNA was used for PCR amplification in a 50-μl volume containing 0.5 μM each primer, 0.2 mM dNTPs, 1 unit of *Taq* polymerase and 1 x

standard *Taq* buffer (Promega Corporation, Madison, WI). PCR amplification was performed for 30 cycles consisting of 1 min at 94°C, 2 min at 51°C and 2 min at 72°C for 30 cycles. After amplification, PCR fragments were size-separated in agarose gels, purified using Geneclean II kit (Bio 101, Inc., La Jolla, CA) and ligated to the *Hinc*II site of the pBluescript vector (Stratagene Cloning Systems, La Jolla, CA).

Plasmid construction and transfection. To reconstitute the full-length coding sequence for the SD receptor, the corresponding PCR fragment (Figure 1B) was subcloned in the pBluescript vector. The 3' portion of the fragment was then cut out from the vector with *BsrF*I and *Cla*I. The resulting fragment was used to replace the 3' sequence of the WT G-CSF-R inserted in the pLNCX expression vector (27). DNA transfection of BAF3 cells was performed by electroporation using 20 μg of expression construct linearized with *Pvu*I. Stably transfected cells were selected by their ability to grow in culture medium containing G418 (Gibco-BRL, Breda, The Netherlands), as previously described (Chapter 2).

Radioactive G-CSF binding. Binding assays with ^{125}I-radiolabeled G-CSF were performed as described (24). BAF/SD cells (1.6×10^6) were incubated with titrated concentrations of ^{125}I-G-CSF (20 pM to 5 nM) in a 100-μl volume at 37°C for 1 h. Cells were sedimented by centrifugation through ice-cold FCS and cell-associated radioactivity was measured. Incubations with excess nonlabeled G-CSF were included to determine nonspecific binding. Data were analyzed as described (24).

^{3}H-thymidine (TdR) incorporation assay. BAF/SD cells or leukemic cells from the patient were washed and incubated in triplicate with various concentrations of indicated growth factors at 10^4 cells/100 μl in 96 microtiter plates. After 24 h (BAF/SD) or 48 h (AML cells) of incubation, 1 μCi of ^{3}H-TdR (2 Ci/mM, Amersham Inc., Amersham, UK) was added to each well, and the cells were incubated for further 12 h before harvesting. ^{3}H-TdR incorporation was measured as described (15).

Immunoprecipitation and immunoblotting. Cells were deprived of growth factors for 14 h and incubated with G-CSF or IL-3 for indicated times. Cells were lysed on ice in lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1mM EDTA, 1% [v/v] Triton X-100, 1 mM PMSF, 100 μM Na_3VO_4, and a cocktail of proteinase inhibitors). Cell lysates were centrifuged at 10,000 x *g* for 30 min and resultant supernatants were incubated on ice for 90 min with rabbit polyclonal anti-JAK2 antiserum (28). Protein A-sepharose beads (Pharmacia LKB, Uppsala, Sweden) were added, incubated on ice for 30 min and washed extensively in lysis buffer. Bound proteins were eluted by boiling in sample buffer (50 mM Tris [pH 6.8], 1% [w/v] SDS, 10% [v/v] glycerol, 1 mM dithiothreitol, and 0.008% [w/v] bromophenol blue) and subjected to 7.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). Filters were blocked by incubation for 30 min in Tris-buffered saline (TBS; 10 mM Tris [pH 7.4], 150 mM NaCl) containing 3% bovine serum albumin, washed extensively in TBS-T (TBS with 0.05% Tween-20) and then incubated with the monoclonal antiphosphotyrosine antibody 4G10 (Upstate Biotechnology Inc., Lake Placid, NY). After washing three times in TBS-T, filters were probed with peroxidase-conjugated rabbit anti-mouse antibodies (DAKO A/S, Denmark). Specific signals were detected by chemiluminescence (DuPont, Hertogenbosch, The Netherlands).

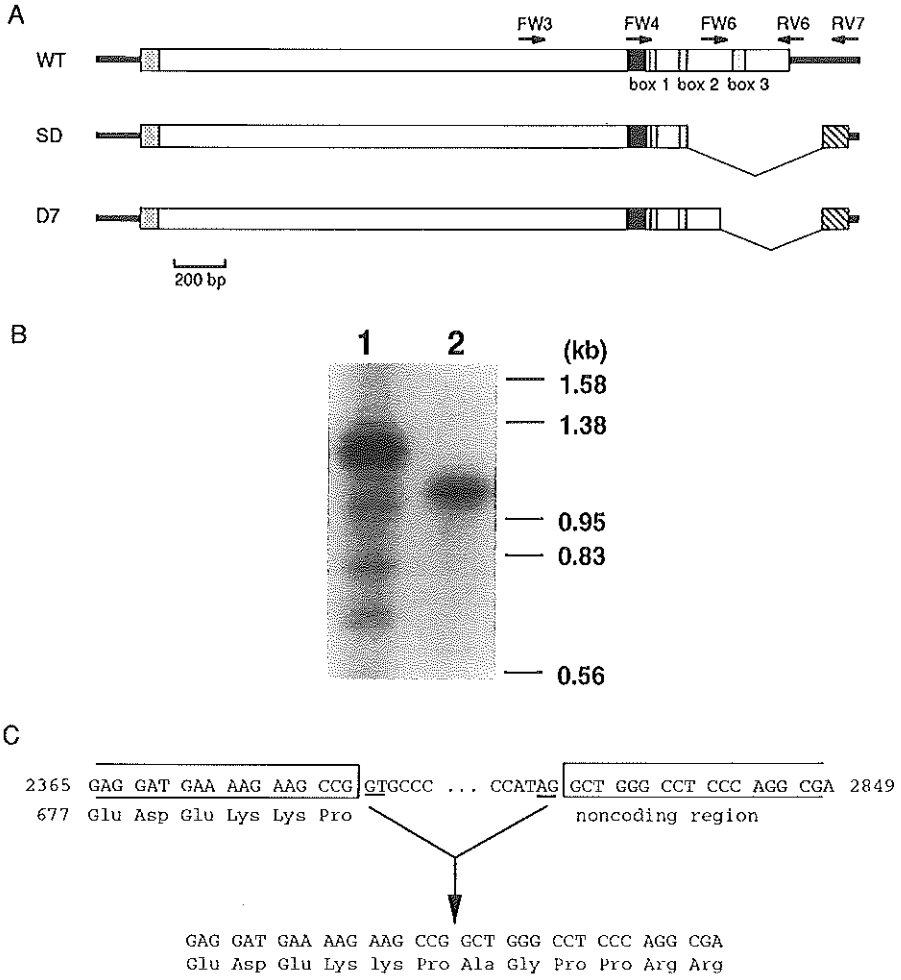

Figure 1. RT-PCR amplification of human G-CSF-R cDNAs. **(A)** Schematic representation of human G-CSF-R cDNAs. The wide bars represent coding regions, of which the solid and stippled regions denote transmembrane domain and signal sequence, respectively. Conserved regions in the cytoplasmic domain are shown as boxes 1-3. Thin solid bars indicate noncoding regions. The sequences lacking in the SD and D7 receptors as a result of alternative splicing are indicated by thin lines. The altered coding regions in the SD and D7 receptors are hatched. Also indicated are the positions of the PCR primers used in this study. **(B)** Southern blot analysis of PCR products. RT-PCR was performed on total RNA isolated from normal granulocytes using primers FW3 and RV7 (lane 1) or FW3 and RV6 (lane 2). 2 μl of PCR products was subjected to electrophoresis in a 0.9% agarose gel, transferred to a nitrocellulose filter and probed with the ^{32}P-labeled human WT G-CSF-R cDNA. **(C)** Sequences flanking the SD deletion boundaries and the corresponding translational reading frame. Numbers indicate nucleotide (upper) and amino acid (low) positions. The consensus nucleotides for RNA splicing are underlined.

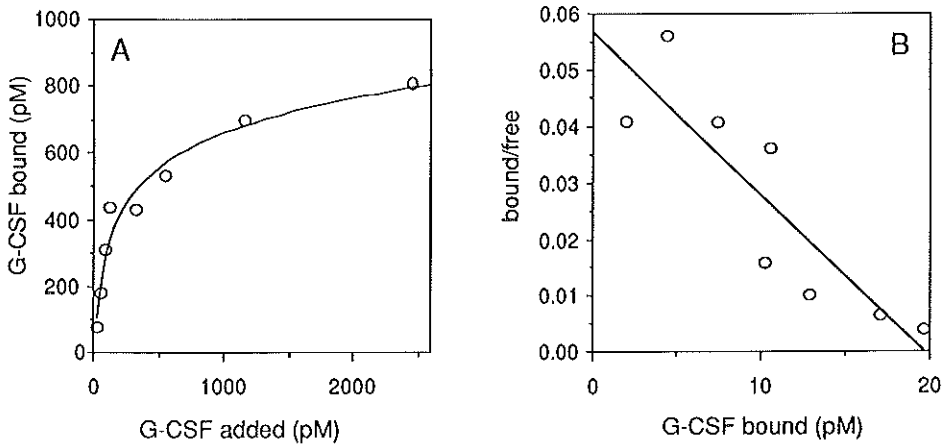

Figure 2. G-CSF binding characteristics of the SD receptors expressed on BAF3 cells. (**A**) Equilibrium binding of ^{125}I-G-CSF to BAF/SD cells. BAF3/SD cells were incubated with titrated concentrations of ^{125}I-G-CSF at 37°C for 1 h. Specific binding was determined after subtraction of non-specific binding. Each point represents the mean of two estimations. (**B**) Scatchard plot of the G-CSF binding data.

Northern blotting. Poly(A)$^+$ RNA was purified on oligo (dT) columns (Pharmacia). 5 μg of total RNA from normal granulocytes or 4 μg of poly(A)$^+$ RNA from leukemic cells was electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde, and then transferred to a nylon filter (Amersham). The filter was hybridized with ^{32}P-labeled DNA probes made with the method of random priming. Final washing of filters were done at 65°C in 0.3 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate).

Results

Cloning of the G-CSF-R cDNAs from human granulocytes. To isolate the human G-CSF-R cDNAs, RT-PCR was performed on total granulocyte RNA of healthy individuals by using primers FW3 and RV7 (Figure 1A). Southern blot analysis of PCR products using the full-length G-CSF-R cDNA as a probe consistently showed 4 bands (Figure 1B). When RT-PCR was performed on RNA isolated from BAF3 cells transfected with human WT G-CSF-R cDNA, or with primers FW3 and RV6 (Figure 1A), only one fragment could be detected (Figure 1B and data not shown). These PCR fragments were subcloned and sequenced. The largest fragment corresponded to the WT G-CSF-R. Unexpectedly, subclones obtained from the two middle-sized fragments all had a nucleotide sequence identical to the previously described splice variant D7 (19). The reason for this phenomenon is unknown. However, similar observations have been made by others (29) and it has been suggested that some PCR products migrate abnormally in agarose gels due to heteroduplex formation (30).

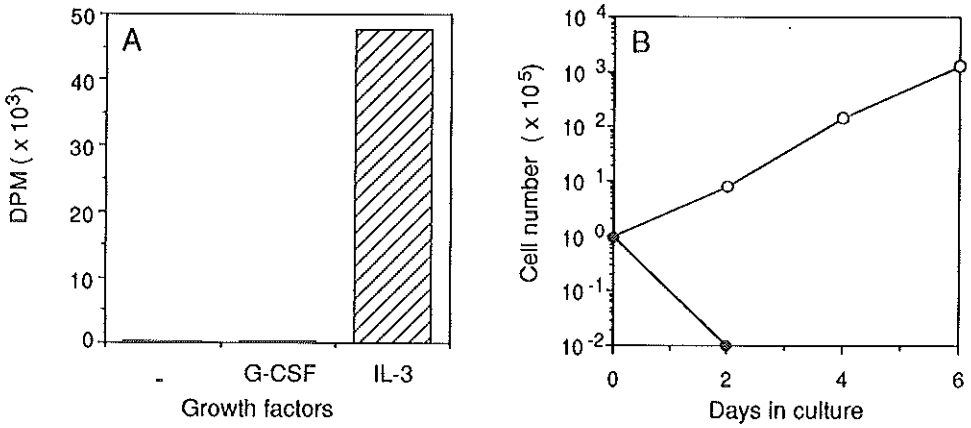

Figure 3. Growth of BAF/SD cells in G-CSF and IL-3. (A) DNA synthesis of BAF/SD cells in response to G-CSF or IL-3 stimulation. Cells were cultured for 24 h in the absence or presence of 100 ng of G-CSF per ml or 10 % of WEHI-CM as a crude source of murine IL-3. Cells were then incubated with ^{3}H-TdR for 12 h and ^{3}H-TdR incorporation was measured. (B) Growth curves of BAF/SD cells in G-CSF or IL-3. Cells were plated initially at 10^5/ml in medium containing 10 ng of IL-3 per ml (open circles) or 100 ng of G-CSF per ml (solid circles).

The smallest PCR fragment had a 549-nucleotide (nt) deletion (nt 2283-2831, Figure 1A) as compared with the WT G-CSF-R cDNA (19). Analysis of nucleotide sequence flanking the deletion boundaries revealed potential RNA splicing donor and acceptor sites (Figure 1C). The splice acceptor site of the SD receptor is shared with splice variant D7 (Figure 1A). The pattern of alternative splicing for SD receptor results in the replacement of the carboxy-terminus of the WT G-CSF-R immediately downstream of box 2 by a carboxy-terminal tail of 34 amino acids identical to that of the D7 splice variant (Figure 1A). Thus, the SD receptor cDNA predicts a truncated G-CSF-R protein lacking the carboxy-terminal region of 130 amino acids of the WT receptor including the box-3 subdomain. The expression of the transcript of this G-CSF-R isoform was detected by RNase protection in granulocytes from 6 normal individuals (data not shown).

Function of the SD receptor in BAF3 transfectants. To study the function of the SD receptor, the full-length cDNA encoding the SD receptor was inserted into the pLNCX retroviral expression vector (27) and transfected into BAF3 cells. After G418 selection, RT-PCR was performed on total RNA extracted from the BAF/SD transfectants to verify the presence of SD transcript. By using primers FW3 and RV7, the 735-bp SD product was readily detected (data not shown). The surface expression and ligand binding characteristics of the SD receptor were examined by radioactive binding assays using ^{125}I-labeled G-CSF. Scatchard analysis revealed that BAF/SD cells expressed a single class of G-CSF binding sites with a dissociation constant (Kd) of 0.34 nM at a mean density of approximately 800 sites per cell (Figure 2). These binding properties were comparable to those of the WT G-

CSF-R expressed on BAF3 cells as well as on other cell types (15,18,19). We next examined whether the SD receptor was able to transduce growth signals in BAF3 cells. This cell line has previously been shown to proliferate in response to G-CSF upon expression of the human G-CSF-R (15). In 5 independent BAF/SD clones, no increase in ^{3}H-TdR incorporation was observed after G-CSF stimulation (Figure 3A). Consistent with this, BAF/SD cells died within 2 days upon transfer from IL-3-containing medium to cultures containing G-CSF alone (Figure 3B).

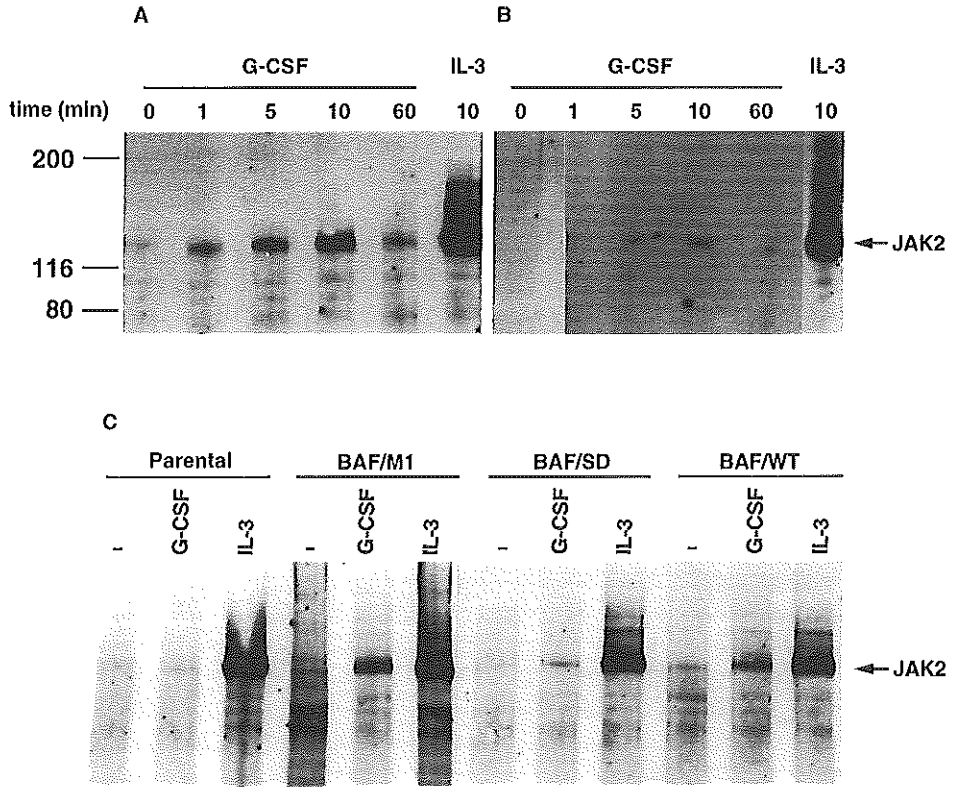

Figure 4. Tyrosine phosphorylation of JAK2 after G-CSF and IL-3 stimulation. (A and B) Kinetics of JAK2 phosphorylation induced by G-CSF stimulation. BAF3 cells expressing the WT G-CSF-R (A) or the SD receptor (B) were incubated with human G-CSF (100 ng/ml) for indicated times. Incubation with murine IL-3 (10 ng/ml) was also included as a positive control. Cell lysates were immunoprecipitated with JAK2 antiserum. The precipitated proteins were subjected to Western blot analysis using the 4G10 antiphosphotyrosine monoclonal antibody. (C) Tyrosine phosphorylation of JAK2 in BAF3 transfectants expressing the different G-CSF-R forms. Cells were incubated for 10 min with G-CSF (1 μg/ml), IL-3 (10 ng/ml) or without factors. All lanes represent lysates from equal amounts of cells.

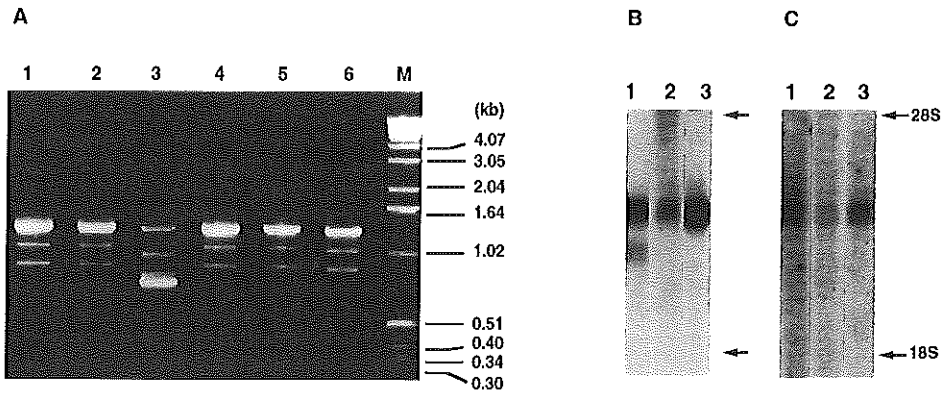

Figure 5. Expression of G-CSF-R transcripts in AML cells. (**A**) RT-PCR analysis of G-CSF-R transcripts in AML cells. RT-PCR was performed with primers FW3 and RV7, which define a fragment of 1284 bp for the WT G-CSF-R, of 865 bp for the D7 receptor, and of 735 bp for the SD receptor. The bands migrating at approximately 1 kb position may represent the heteroduplexes of the WT and D7 DNA fragments (see text). Depicted are results obtained with RNA samples from 6 AML patients (lanes 1 to 6). Note the predominance of the 735-bp fragment in lane 3. (**B** and **C**) Northern blot analysis of G-CSF-R transcripts in normal granulocytes and leukemic cells. 4 μg of Poly(A)$^+$ RNA from leukemic cells (lane 1) and 5 μg of total RNA from granulocytes of two healthy individuals (lanes 2 and 3) were electrophoresed in a 1 % formaldehyde-containing agarose gel, blotted to a nylon filter and probed with the ^{32}P-labeled WT G-CSF-R cDNA (**B**). The same blot was stripped and reprobed with a ^{32}P-labeled PCR fragment defined by primers FW6 and RV6 (**C**).

JAK2 activation by G-CSF in BAF3 transfectants. JAK2, a 130 kDa tyrosine kinase of the Janus kinase family, has recently been implicated as a signaling molecule of several cytokine receptors including the receptors for EPO, IL-3, IL-6, GM-CSF, leukemia inhibitory factor and growth hormone (28,31-35). G-CSF stimulation of BAF3 cells expressing the WT G-CSF-R also resulted in the tyrosine phosphorylation of the JAK2 protein (Figure 4A, BAF/WT). The induction of JAK2 phosphorylation was rapid but transient, occurring as early as 1 min after G-CSF stimulation, peaking at 10 min and declining at 60 min. In contrast, the SD receptor lacking the proliferative signaling capacity only marginally mediated JAK phosphorylation (Figures 4B and C). Notably, the G-CSF-R deletion mutant M1, which contains only 55 amino acid residues in the cytoplasmic region that are sufficient for proliferative signaling (15), was still capable of inducing JAK2 phosphorylation (Figure 4C, BAF/M1). Because the SD receptor contains almost the entire membrane-proximal region preserved in mutant M1, these results suggested that the SD carboxy-terminus negatively influenced JAK2 activation. The data also indicate that induction of JAK2 activation by G-CSF correlates with the growth signalling capacity of the G-CSF-R.

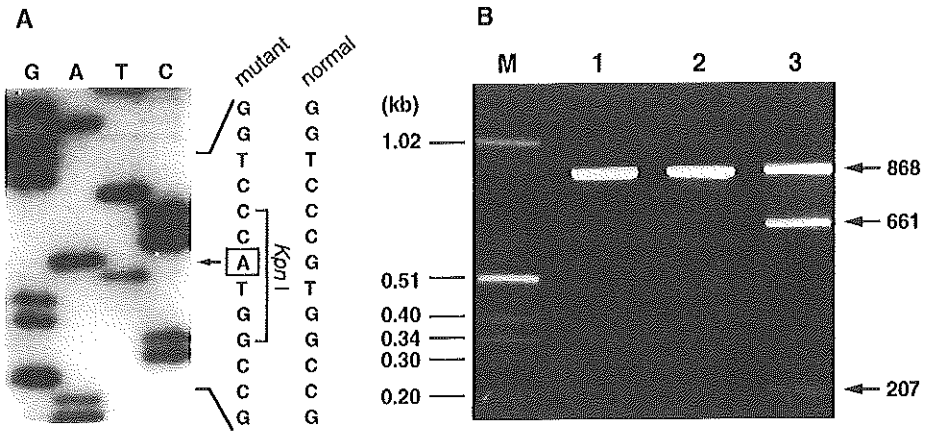

Figure 6. Identification of a point mutation in the splicing donor site of the SD receptor in leukemic cells. (A) part of a sequencing gel surrounding the point mutation. Nucleotide sequencing was performed on PCR product subcloned in pBluescript. The point mutation is boxed. The arrow indicates the position of the mutation in the sequencing gel. The *Kpn*I site created by the point mutation is also indicated. (B) Agarose gel electrophoresis of PCR fragments after *Kpn*I digestion. Using primers FW116 and RV7, the entire exon 17 and part of the intron 16 were amplified from DNA isolated from normal granulocytes (lanes 1 and 2) and leukemic cells (lane 3). 20 μl of PCR products was digested in a 40-μl volume containing 8 units of *Kpn*I for 2 h and then size-separated in a 1 % agarose gel. Partial digestion of PCR fragment was excluded by including a reference DNA containing a *Kpn*I site in the digestion mixtures (data not shown).

Expression of the SD receptor in AML. Results from RT-PCR indicated that human granulocytes predominantly express the transcript of the WT G-CSF-R, whereas transcripts of the D7 and SD receptors are expressed at significantly lower levels. To determine whether this expression pattern may be altered in patients with AML, RNA samples from 70 AML patients were screened for the expression of the G-CSF-R mRNA by RT-PCR using primers FW3 and RV7. In one case, the most abundant PCR fragment consistently ran at a position expected for the SD receptor in 4 independent experiments (Figure 5A). Nucleotide sequencing confirmed that this fragment is identical to the SD cDNA isolated from granulocytes (data not shown). The fragment corresponding to the WT G-CSF-R was still present, though less abundantly, whereas the two bands corresponding to the D7 isoform were not seen on ethidium bromide-stained gels. Similar results were obtained from RT-PCR using primers FW4 and RV7 (Figure 1A), which amplify a fragment of 878 bp for the WT G-CSF-R, and of 329 bp for the SD receptor (data not shown).

Northern blot hybridization was then performed to estimate the relative expression levels of the WT and SD G-CSF-R transcripts. Two different probes were used to distinguish the WT and SD G-CSF-R transcripts. A full-length cDNA encoding the WT G-CSF-R recognizes both the WT and SD transcripts. A PCR fragment of 409 bp, defined by primers FW6 and RV6 (Figure 1A), specifically recognizes the sequence of the WT G-CSF-R lacking in the SD receptor. Using the full-length cDNA as a probe, a band of 3.7 kb corresponding to the

transcript of the WT G-CSF-R was seen in RNA isolated from normal granulocytes as well as from leukemia cells of the patient (Figure 5B). In addition, a smaller transcript of approximately 3.2 kb was detected in RNA from leukemia cells of the patient, but not in RNA samples from normal granulocytes. As expected, this smaller transcript could not be detected when the same blot was reprobed with the 409-bp PCR fragment (Figure 5C). As judged from Northern blot analysis, expression of the SD transcript was slightly less abundant than that of the WT transcript in the AML cells. Although this seems in contrast with the RT-PCR results (Figure 5A), it is likely that the SD transcript was preferentially amplified during PCR due to the size difference between the WT and SD sequences.

Identification of a point mutation in the SD splice donor site in AML cells. RT-PCR results indicated that the enhanced expression of the SD transcript occurred at the expense of the D7 transcript, which shares the splice acceptor site with the SD transcript. This prompted us to investigate whether mutations were present in the region flanking the splice donor site of the SD receptor. The entire exon 17 and part of the intron 16 of the G-CSF-R gene (36) were amplified by PCR using primers FWI16 and RV7 from genomic DNA of the patient's leukemia cells. The 868-bp PCR fragment was subcloned and sequenced. A G-to-A substitution was identified at nt 2285 (19) (Figure 6A), immediately downstream of the 2 consensus nucleotides GT at the SD splice donor site (Figure 1C). No other mutations were found in exon 17. The mutation creates a unique *Kpn*I site. *Kpn*I digestion of PCR product obtained from genomic DNA demonstrated that the AML cells carried both the normal and the mutated alleles of the G-CSF-R gene (Figure 6B). As no somatic cells were available from the patient, we were unable to determine whether the point mutation was constitutional or present only in AML cells.

To exclude that the G-to-A substitution was a polymorphism, DNA samples from 108 control individuals were analyzed either by *Kpn*I digestion or by nucleotide sequencing. In none of these samples could the same nucleotide change be detected. Taken together, these findings strongly argue that the G-to-A mutation resulted in the increased utility of the SD splice donor site. Notably, a minor proportion of the largest RT-PCR fragment (Figure 1B) of the patient could still be cut by *Kpn*I (data not shown), suggesting that not all G-CSF-R RNA precursors carrying the point mutation were spliced into the SD transcript. This result was consistent with Northern analysis which suggested that the SD transcript was somewhat less abundant than WT transcript (Figure 5B). Because the point mutation itself does not change the amino acid (val), the full-length mRNA transcribed from the mutant allele would encode a G-CSF-R protein identical to the WT G-CSF-R.

Growth factor responses of AML cells overexpressing the SD receptor. Because the SD receptor was incapable of transducing mitogenic signals in BAF3 cells, it would be expected that the patient's leukemic cells were less sensitive to G-CSF stimulation. Indeed, leukemic cells from the patient responded poorly to G-CSF in ^{3}H-TdR incorporation assays (Figure 7). A weak mitogenic response was observed only at extremely high concentrations of G-CSF (300 ng/ml), most likely due to the activation of the low numbers of WT G-CSF-R homodimers that may still be formed. The poor G-CSF response of the patient's leukemia cells was in sharp contrast to the responses to IL-3 and GM-CSF, which strongly induce DNA synthesis in the patient's leukemia cells in a dose-dependent manner (Figure 7).

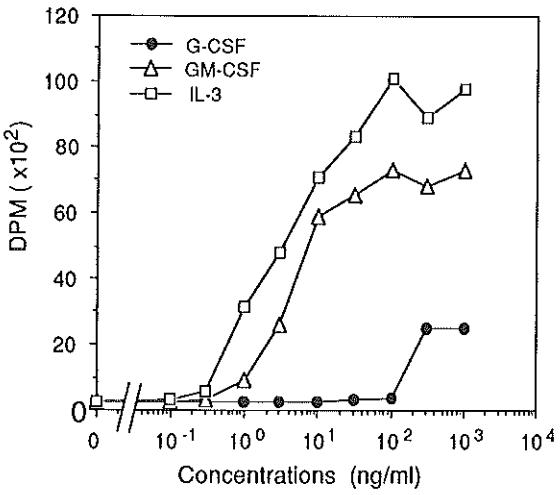

Figure 7. Responsiveness of AML cells overexpressing the SD receptor to different hematopoietic growth factors. Proliferative responses were evaluated in ^{3}H-TdR incorporation assays, as described under Materials and methods.

Discussion

Alternative RNA splicing resulting in the formation of different receptor isoforms has been commonly observed among the members of the hematopoietin/cytokine receptor superfamily (8,37). In general, alternative splicing creates two types of receptor isoforms, i.e., those that differ in the cytoplasmic domain and those that lack the transmembrane domain and therefore encode soluble receptors. The extracellular domain responsible for ligand binding usually remains unchanged. Although their physiological significance is still largely unknown, receptor isoforms may contribute to the complexity of signal transduction or modulate cellular responses to growth factors.

In addition to the wild type form, three other G-CSF-R isoforms have been described. We report here the molecular cloning of a novel G-CSF-R isoform SD from human granulocytes. The biological role of the SD receptor in the regulation of normal granulopoiesis remains speculative. Because the SD receptor is incapable of growth signaling and also lacks the carboxy-terminal region of the WT G-CSF-R essential for maturation induction (15,16), the homodimeric form of the SD receptor could function as a 'sink' for G-CSF. The SD receptor could also form heterodimers with the WT G-CSF-R, thereby interfering with the function of the WT G-CSF-R. In this respect, it is worth noting that an EPO receptor (EPO-R) isoform, also lacking the growth signaling potential, has been shown to inhibit the activity of the WT EPO-R (38), apparently by heterodimerization with the WT EPO-R (39,40). However, in view of its low expression, at least at the RNA level, it is less likely that the SD receptor would exert a major effect on the function of the WT G-CSF-R in granulopoiesis.

We have observed that the expression of the SD receptor was markedly increased in the leukemic cells of an AML patient. Further, we demonstrate this phenomenon to be associated with a G-to-A point mutation at the SD splice donor site. It remains uncertain to which extend the overexpression of the SD receptor had contributed to the development of leukemia

in the patient. The inability of the SD receptor to mediate a strong mitogenic signal and the poor proliferative response of the patient's leukemic cells to G-CSF might even argue against a significant role of overexpressing the SD receptor in leukemogenesis. However, overexpression of the SD receptor would conceivably lead to the increased formation of SD/SD and WT/SD receptor complexes. Notably, the carboxy-terminal cytoplasmic region of the WT G-CSF-R, lacking in the SD receptor, is responsible for maturation signaling (15,16). It is plausible that heterodimerization between the WT and SD receptor molecules would result in the disturbance of maturation signaling by the G-CSF-R. As a result, cells overexpressing SD/SD and SD/WT receptor complexes would fail to mature in response to G-CSF, but accumulate under the influence of other growth factors such as IL-3 and GM-CSF.

Our results provide the first example of aberrant splicing of hematopoietin receptors in clinical AML. However, alternative splicing as a potential oncogenic event has its precedent. Overexpression of splice variants of *WT1* gene that lack growth-inhibitory activity has been suggested as a common mechanism of *WT1* inactivation in Wilms tumor (41) and a point mutation leading to abnormal splicing of the *WT1* transcript has been detected in a patient with Wilms tumor (42).

Finally, data presented here establish that JAK2 is activated by the WT G-CSF-R, but hardly by the SD receptor. The fact that the M1 mutant of the G-CSF-R, which contains only 55 amino acid residues in the cytoplasmic domain, is able to induce JAK2 phosphorylation indicates that interaction with JAK2 is mediated by the membrane-proximal region of the G-CSF-R, similar to the EPO-R, the β_c chain of IL-3 and GM-CSF receptors, and the IL-6 receptor signaling molecule gp130 (28,34,35). It is not clear how the carboxy-terminus of the SD receptor interferes negatively with JAK2 activation and proliferative signaling. One possible explanation is that this region contains a functional subdomain that mediates growth-inhibitory signals. Alternatively, the carboxy-terminus of the SD receptor could simply affect the steric configuration of the membrane-proximal region of the G-CSF-R in a way that hinders the interaction with cytoplasmic growth signaling molecules such as JAK2.

References

1. Demetri GD and Griffin JD. (1991) Granulocyte colony-stimulating factor and its receptor. Blood 78:2791.
2. Moore MAS and Warren DJ. (1987) Synergy of interleukin 1 and granulocyte colony-stimulating factor: in vivo stimulation of stem-cell recovery and hematopoietic regeneration following 5-fluorouracil treatment of mice. Proc. Natl. Acad. Sci. USA 84:7134.
3. Ikebuchi K, Clark SC, Ihle JM, Souza LM and Ogawa M. (1988) Granulocyte colony-stimulating factor enhances interleukin 3-dependent proliferation of multipotential hemapoietic progenitors. Proc. Natl. Acad. Sci. USA 85:2445.
4. Bodine DM, Crosier PS and Clark SC. (1991) Effects of hematopoietic growth factors on the survival of primitive stem cells in liquid suspension culture. Blood 78:914.
5. Leary AG, Zeng HQ, Clark SC and Ogawa M. (1992) Growth factor requirements for survival in G_0 and entry into the cell cycle of primitive human hemapoietic progenitors. Proc. Natl. Acad. Sci. USA 89:4013.
6. Tkatch LS and Tweardy DJ. (1993) Human granulocyte colony-stimulating factor (G-CSF), the premier granulopoietin: biology, clinical utility, and receptor structure and function.

Lymphokine and Cytokine Res. 12:477.
7. Bazan JF. (1989) A novel family of growth factor receptors: a common binding domain in the growth hormone, prolactin, erythropoietin and IL-6 receptors, and the p75 IL-2 receptor ß-chain. Biochem. Biophys. Commun. 164:788.
8. Cosman D. (1993) The hematopoietin receptor superfamily. Cytokine 5:95.
9. Kondo M, Takeshita T, Ishii N, Nakamura M, Watanabe S, Arai K and Suganmura K. (1993) Sharing of the interleukin-2 (IL-2) receptor γ chain between receptors for IL-2 and IL-4. Science 262:1874.
10. Noguchi M, Nakamura Y, Russell SM, Ziegler SF, Tsang M, Cao X and Leonard WJ. (1993) Interleukin-2 receptor γ chain: a functional component of the interleukin-7 receptor. Science 262:1877.
11. Russell SM, Keegan AD, Harada N, Nakamura Y, Noguchi M, Leland P, Friedmann MC, Miyajima A, Puri RK, Paul WE and Leonard WJ. (1993) Interleukin-2 receptor γ chain: a functional component of the interleukin-4 receptor. Science 262:1880.
12. Miyajima A, Kitamura T, Harada N, Yokota T and Arai K. (1992) Cytokine receptors and signal transduction. Annu. Rev. Immunol. 10:295.
13. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1990) Purification and characterization of the receptor for murine granulocyte colony-stimulating factor. J. Biol. Chem. 265:14008.
14. Ishizaka-Ikeda E, Fukunaga R, Wood WI, Goeddel DV and Nagata S. (1993) Signal transduction mediated by growth hormone receptor and its chimeric molecules with the granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA 90:123.
15. Dong F, van Buitenen C, Pouwels K, Hoefsloot LH, Löwenberg B and Touw IP. (1993) Distinct cytoplasmic regions of the human granulocyte colony-stimulating factor involved in induction of proliferation and maturation. Mol. Cell. Biol. 13:7774.
16. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1993) Growth and differentiation signals mediated by the cytoplasmic domain of granulocyte colony-stimulating factor receptor. Cell 74: 1079.
17. Ziegler SF, Bird TA, Morella KK, Mosley B, Gearing DP and Baumann H. (1993) Distinct regions of the human granulocyte-colony-stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. Mol. Cell. Biol. 13:2384.
18. Fukunaga R, Seto Y, Mizushima S and Nagata S. (1990) Three different mRNAs encoding human granulocyte colony-stimulating factor receptor. Proc. Natl. Acad. Sci. USA. 87:8702.
19. Larsen A, Davis T, Curtis BM, Gimpel S, Sims JE, Cosman D, Park L, Sorensen E, March CJ and Smith CA. (1990) Expression cloning of a human granulocyte colony-stimulating factor receptor: a structural mosaic of hematopoietin receptor, immunoglobulin, and fibronectin domains. J. Exp. Med. 172:1559.
20. Fukunaga R, Ishizaka-Ikeda E, Pan CX, Seto Y and Nagata S. (1991) Functional domains of the granulocyte colony-stimulating factor receptor. EMBO J. 10:2855.
21. Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, Taga T and Kishimoto T. (1991) Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. Proc. Natl. Acad. Sci. USA 88:11349.
22. Hibi M, Murakami M, Saito M, Hirano T, Taga T and Kishimoto T. (1990) Molecular cloning and expression of an IL-6 signal transducer, gp130. Cell 63:1149.
23. Saito M, Yoshida K, Hibi M, Taga T and Kishimoto T. (1992) Molecular cloning of a murine IL-6 receptor-associated signal transducer, gp130, and its regulated expression in vivo. J. Immunol. 148:4066.
24. Budel LM, Touw IP, Delwel R and Löwenberg B. (1989) Granulocyte colony-stimulating factor receptors in human acute myelocytic leukemia. Blood 74:2668.
25. Chomczynski P and Sacchi N. (1987) Single-step method for RNA isolation by acid guanidinium thiocyanat-phenol-chloroform extraction. Anal. Biochem. 162:156.
26. Miller SA, Dykes DD and Polesky HF. (1988) A simple salting out procedure for extracting

DNA from human nucleated cells. Nucleic Acid Res. 16:1215.
27. Miller AD and Rosman GJ. (1989) Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980.
28. Witthuhn BA, Quelle FW, Silvennoinen O, Yi T, Tang B, Miura O and Ihle JN. (1993) JAK2 associates with the erythropoietin receptor and is tyrosine phosphorylated and activated following stimulation with erythropoietin. Cell 74:227.
29. Zhang K, Saxon A and Max EE. (1992) Two unusual forms of human immunoglobin E encoded by alternative RNA splicing of ϵ heavy chain membrane exons. J. Exp. Med. 176:233.
30. Zorn AM and Kreig PA. (1991) PCR analysis of alternative splicing pathways: identification of artifacts generated by heteroduplex formation. BioTechniques 11:180.
31. Silvennoinen O, Witthuhn BA, Quelle FW, Cleveland JL, Yi T and Ihle JN. (1993) Structure of the murine JAK2 protein-tyrosine kinase and its role in interleukin 3 signal transduction. Proc. Natl. Acad. Sci. USA 90:8429.
32. Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN and Carter-Su C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell 74:237.
33. Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN and Yancopoulos GD. (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 ß receptor components. Science 262:92.
34. Narazaki M, Witthuhn BA, Yoshida K, Silvennoinen O, Yasukawa K, Ihle JN, Kishimoto T and Taga T. (1994) Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. Proc. Natl. Acad. Sci. USA 91:2285.
35. Quelle FW, Sato N, Witthuhn BA, Inhorn RC, Eder M, Miyajima A, Griffin JD and Ihle JN. (1994) JAK2 associates with the ßc chain of the receptor for granulocyte-macrophage colony-stimulating factor, and its activation requires the membrane-proximal region. Mol. Cell. Biol. 14:4335.
36. Seto Y, Fugunaga R and Nagata S. (1992) Chromosomal gene organization of the human granulocyte colony-stimulating factor receptor. J. Immunol. 148:259.
37. Fernandez-Botran R. (1991) Soluble cytokine receptors: their role in immunoregulation. FASEB. J. 5:2567.
38. Nakamura Y and Nakauchi H. (1992) A truncated erythropoietin receptor and cell death: a reanalysis. Science 264:588.
39. Miura O and Ihle JN. (1993) Dimer- and oligomerization of the erythropoietin receptor by disulfide bond formation and significance of the region near WSXWS motif in intracellular transport. Arch. Biochem. Biophys. 306:200.
40. Barber DL, DeMartino JC, Showers MO and D'Andrea AD. (1994) A dominant negative erytropoietin (EPO) receptor inhibits EPO-dependent growth and blocks F-gp55-dependent transformation. Mol. Cell. Biol. 14:2257.
41. Haber DA, Park S, Maheswaran S, Englert C, Re GG, Hazen-Martin DJ, Sens DA and Garvin AJ. (1993) *WT1*-mediated growth suppression of Wilms tumor cells expressing a *WT1* splicing Variant. Science 262:2057.
42. Schneider S, Wildhardt G, Ludwig R and Royer-Pokora B. (1993) Exon skipping due to a mutation in a donor splice site in the *WT-1* gene is associated with Wilm's tumor and severe genital malformations. Hum. Genet. 91:599.

Chapter 7

GENERAL DISCUSSION

7.1 Functional cytoplasmic regions of the G-CSF-R and multiple signaling pathways

G-CSF exhibits diverse activities in granulopoiesis, regulating the proliferation, differentiation and survival of myeloid progenitor cells. The studies presented in this thesis (Chapter 2) establish that these biologic activities are mediated by distinct cytoplasmic regions of the G-CSF-R (Figure 1). Because the G-CSF-R contains no known motifs in the cytoplasmic domain that would suggest kinase activities, signaling from the G-CSF-R must be achieved through the interactions of its cytoplasmic domain with intracellular kinases or signaling molecules. The presence of discrete functional regions in the G-CSF-R cytoplasmic domain would suggest the existence of multiple signaling pathways that are coupled to distinct biologic events.

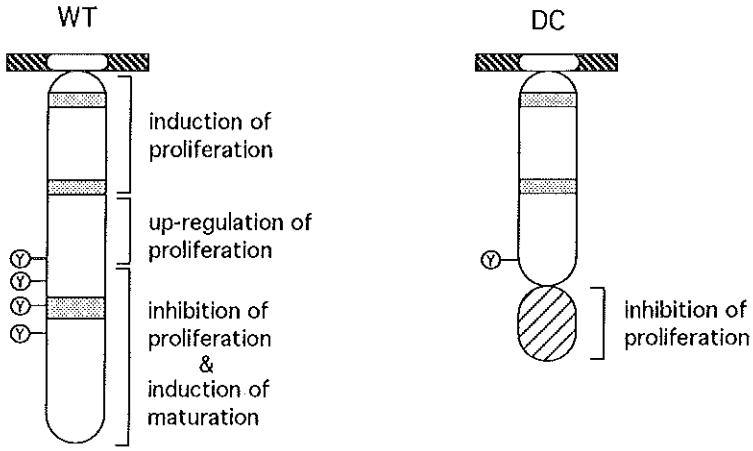

Figure 1. Functional cytoplasmic regions of the G-CSF receptors. The cytoplasmic domain of the WT G-CSF-R is functionally divided into three subdomains, as indicated. The altered carboxy-terminus (hatched box) of the DC receptor is identical to that of the SD receptor (Chapter 6), both created by alternative RNA splicing. Tyrosine residues (Y) present in the cytoplasmic domains of the G-CSF receptors are also indicated.

7.1.1 Proliferative signaling

In Chapter 6, it is shown that JAK2 is rapidly tyrosine-phosphorylated upon stimulation with G-CSF. Apparently, interaction with JAK2 is mediated by the membrane-proximal region of the G-CSF-R. The M1 receptor contains only 55 amino acid residues in the cytoplasmic domain and is fully capable of inducing JAK2 phosphorylation. The membrane-proximal region of the G-CSF-R contains two relatively conserved sequences, i.e., the boxes 1 and 2, that are also present in several other members of the cytokine/hematopoietin receptor superfamily (1). Tyrosine phosphorylation and activation of JAK2 and other

members of the JAK family have also been observed following stimulation with EPO, IL-2 to IL-7, IL-11, GM-CSF, LIF, OSM, and CNTF (2,3).

The JAK family members are likely to be the critical components in the proliferative signaling pathways of the cytokine/hematopoietin receptor superfamily members. It has been shown that a kinase-deficient form of JAK2 inhibits EPO-dependent proliferation in murine DA-1 cells transfected with EPO-R (4). Activation of the JAK signaling pathways may involve multiple JAK family members (2). For instance, both JAK1 and TYK2 are essential in IFN-α/ß receptor signaling, whereas signaling triggered by the IFN-γ receptor involves the activation of JAK1 and JAK2 (5). Similarly, JAK1, JAK2 and TYK2 are activated in response to IL-6, LIF, OSM and CNTF whose receptors share the signaling molecule gp130 (6-8). More recently, JAK1 and JAK3 were found to be selectively associated with the ß and γ_c chains of the IL-2-R, respectively, and activated following IL-2 stimulation (9,10). In addition to JAK2, it has been shown recently that G-CSF stimulation results in the activation of JAK1 (11). Although not examined yet, it is likely that JAK1 activation is also mediated by the membrane-proximal region of the G-CSF-R.

The region of 30 amino acids of the G-CSF-R distal to the box-1 and box-2 motifs appears to contain a second functional subdomain. Although this region *per se* is not required for proliferative signaling, its presence strongly enhances the mitogenic stimulus (Chapter 2). Activation of the G-CSF-R, ectopically expressed in hepatoma cells, results in the induction of acute-phase plasma protein genes and activation of an IL-6-response element (12,13). These two activities of G-CSF require the enhancing region of the G-CSF-R. Interestingly, a functionally similar region has also been identified in the cytoplasmic domains of gp130 and LIF-Rß chain (14). Comparison of the relevant cytoplasmic regions of the G-CSF-R, gp130 and LIF-Rß has revealed a subdomain that shows certain homology among the three receptor proteins. This subdomain consists of a central tyrosine residue in a short stretch of amino acids that are conserved in the G-CSF-R, gp130 and LIF-Rß chain (14). Notably, substitution of this conserved tyrosine in the LIF-Rß chain for phenylalanine or alanine results in markedly decreased signaling capacity. This subdomain may represent the target for an as yet unidentified intracellular kinase or signaling molecule. In this respect, it is noteworthy that activation of $p21^{ras}$ signaling requires the cytoplasmic region of 100 amino acids of the G-CSF-R (15), as compared with the region of 55 amino acids required for JAK2 activation. Because JAK signaling is independent of the functioning of $p21^{ras}$ (16), it appears that the cytoplasmic region between amino acids 56 and 100 including this subdomain plays a role in the activation of $p21^{ras}$. Additional experiments will be needed to determine whether activation of $p21^{ras}$ is indeed mediated by this subdomain.

7.1.2 Maturation signaling

Little is known about the role of HGFs in the regulation of maturation. Two models have been proposed to explain the mechanism controlling cell maturation. The stochastic theory assumes that the ability to mature is an intrinsic feature of hematopoietic cells that is independent of the actions of HGFs. According to this hypothesis, HGFs would simply support cell proliferation and survival that are required for the execution of maturation

programs (17,18). Support for this theory comes from the observation that murine FDC-Pmix cells, transfected with Bcl-2 gene, can mature terminally into different hematopoietic lineages in the absence of any HGFs, suggesting that Bcl-2-mediated survival permits the progress of spontaneous maturation in this cell line (19). The instructive hypothesis, on the other hand, proposes that maturation is actively induced by HGFs (20). The fact that G-CSF, EPO and M-CSF are able to induce the maturation of several murine hematopoietic cell lines, which maintain immature phenotypes when cultured in IL-3, lends support to the latter theory (21-25). However, because G-CSF, EPO and M-CSF also promote cell proliferation and survival, it could be argued that maturation in these situations is the consequence of the survival-promoting rather than the direct maturation-inducing effects of these factors. Proof of an active role of HGFs in maturation induction requires the delineation of distinct cytoplasmic domains in the hematopoietin receptors that specifically transmit maturation signals.

The demonstration that the carboxy-terminal cytoplasmic region of the WT G-CSF-R is specifically involved in transducing maturation signals but not proliferative signals provides such evidence. These results establish that G-CSF indeed acts in an instructive manner to induce terminal granulocytic maturation. Induction of maturation through the carboxy-terminal region of the WT G-CSF-R is observed not only in murine myeloid L-GM cells, but also in murine myeloid FDC-P1 cells (26). Notably, the same maturation-inducing region is not functional in murine hematopoietic pro-B BAF3 cells, suggesting that signaling for granulocytic maturation by the G-CSF-R requires the appropriate intracellular environment or genetic program of the responding cells. This may represent the requirement for the proper intracellular signaling machinery or the ultimate targets at the transcription level.

How signals leading to terminal granulocytic maturation are transduced by the G-CSF-R is poorly understood. It appears that the maturation signals are mediated primarily by the most carboxy-terminal region of 58 amino acids because truncation of this region almost completely inactivates the G-CSF-R for maturation signaling (26). However, full maturation signaling requires the carboxy-terminal region of 121 amino acids that includes most of the enhancing domain, suggesting that multiple intracellular molecules are involved in the transduction of maturation signals. Recently, it has been shown that G-CSF stimulation leads to the rapid activation of the tyrosine kinases Lyn and Syk (27). Lyn appears to be constitutively associated with the G-CSF-R cytoplasmic domain, whereas Syk is recruited into the G-CSF-R/Lyn complex following activation of the G-CSF-R. In addition, transcription factor $p80^{rel}$ also becomes tyrosinephosphorylated in response to G-CSF stimulation, and the phosphorylated $p80^{rel}$ displays an increased DNA binding activity (28). Whether Lyn/Syk and $p80^{rel}$ have a role in G-CSF-triggered maturation signaling needs further studies.

Aside from maturation signaling, the distal carboxy-terminal portion of the WT G-CSF-R also suppresses proliferative signaling mediated by the membrane-proximal region (Chapter 2). It is presently unknown whether induction of maturation and suppression of proliferation are mediated by a single subdomain or whether two disparate mechanisms are involved. In addition to the box-3 motif, the carboxy-terminal region of the G-CSF-R contains 3 out of the 4 tyrosine residues (Tyr^{704}, Tyr^{729}, Tyr^{744} and Tyr^{764}) present in the cytoplasmic domain (Figure 1). G-CSF stimulation has been shown to induce rapid tyrosine phosphorylation of its receptor (11,29). Phosphorylated tyrosine may create sites for binding of tyrosine kinases

via the SH2 domain. In this respect, of particular interest is Tyr^{764} which, in combination with the flanking amino acid residues, forms a potential recognition site for the binding of the SH2 domain of Src homology 3 (SH3) binding protein 2 (3BP-2) (30). 3PB2 is a protein purified by its ability to specifically bind to the SH3 domain of nuclear tyrosine kinase c-ABL (31). Notably, c-ABL negatively regulates cell growth (32) and disruption of c-ABL function due to the formation of BCR-ABL fusion oncoprotein is associated with chronic myelogenous leukemia (CML), a disease characterized by clonal expansion of myeloid cells. Truncation of the 58 carboxy-terminal amino acids of the WT G-CSF-R including Tyr^{764} results in significantly enhanced potential for proliferative signaling (unpublished data). It remains to be determined whether such a signaling cascade indeed exists and is responsible for the growth-inhibitory signaling by the carboxy-terminal region of the WT G-CSF-R.

7.1.3 Regulation of cell survival

A common feature of HGFs is their ability to support both cell proliferation and survival. Numerous studies have demonstrated that the presence of HGFs is a prerequisite for the growth and development of normal hematopoietic cells. Withdrawal of HGFs results in a growth arrest, followed rapidly by apoptosis (33,34). Because the availabilities of HGFs are limited *in vivo*, it is generally assumed that hematopoietic progenitor cells compete for HGFs for survival and the availabilities of HGFs determine the cell numbers (35). In some instances, proliferation can be uncoupled from survival. For example, G-CSF stimulates the proliferation and survival of myeloid progenitor cells but it only supports the survival of mature nondividing granulocytes (36). Although this may be related to the differentiation stage, a single factor may also induce alternative proliferation/survival or only survival, dependent on the concentration of the factor (37). Whether distinct signaling pathways exist that lead to survival and proliferation, respectively, is still unknown.

The signals mediated by the carboxy-terminal maturation-inducing region of the G-CSF-R do not always lead to granulocytic maturation in myeloid cells, but instead may also induce apoptosis (Chapter 3). Induction of maturation and apoptosis is observed selectively in myeloid cells, but not in lymphoid cells. Similar to maturation signaling, induction of apoptosis could be assigned to the carboxy-terminal region of 58 amino acids of the G-CSF-R (Chapter 3). It is thus possible, though not yet proven, that the signals for maturation and apoptosis are in fact identical and are mediated by a single subdomain within this region. A conversion from maturation signaling to apoptosis signaling would likely be determined by whether or not the cells are able to execute the maturation program. Apoptosis would occur as a consequence of maturation failure. This notion is supported by a recent observation that IL-6 could override TGF-ß1-driven apoptosis of M1 leukemic cells by inducing terminal maturation (38). In contrast, constitutive expression of exogenous c-Myc or c-Myb blocked IL-6-induced maturation of M1 leukemic cells, and thus also abrogated the protective effect of IL-6 on TGF-ß1-mediated apoptosis. The death-signaling capacity of the G-CSF-R is apparently in contrast to the well-established role of G-CSF in promoting survival. This would imply that myeloid cells can be driven to undergo apoptosis via two distinct mechanisms, i.e., a lack of survival signals due to HGF deprivation and a failure to mature

in response to HGFs. Thus, on one hand, hematopoietic cells may survive only in the presence of HGFs. On the other hand, they may not simply survive without undergoing maturation in response to HGFs. These two mechanisms may represent two important functional aspects of HGF activities in normal hematopoiesis. The biological significance of such regulatory mechanisms is also obvious: they force hematopoietic cells to compete for limited amounts of HGFs for survival and maturation, thus selecting cells that are fittest for normal development; at the same time they together comprise two distinct checkpoints of preventing cells defective in their behaviour of growth and development from uncontrolled expansion.

7.2 Defective G-CSF receptor in SCN: insight into the regulatory mechanisms of granulopoiesis

The identification of distinct functional subdomains in the G-CSF-R cytoplasmic domain has implications for elucidating the pathological mechanisms of certain hematological disorders characterized by defective granulopoiesis. It has long been suggested that aberrant structures of the cytokine/hematopoietin receptors may have a role in human diseases. This speculation has recently been proven correct by several studies. For instance, a mutation in the EPO-R leading to the truncation of the carboxy-terminal negative-regulatory region has been genetically linked to familial erythrocytosis (39). Truncation of the γ_c chain of the receptors for IL-2, IL-4, IL-7 and IL-15 has been reported to be associated with X-linked severe combined immunodeficiency (40). In addition, mutations in the GH-R resulting in abnormal ligand binding, surface expression and RNA splicing have been described in patients with Laron dwarfism (41-44). Adding to this list, results presented in Chapter 4 demonstrate that granulocytes from a patient with SCN (patient DA) carried a point mutation in the G-CSF-R, leading to the truncation of the carboxy-terminal cytoplasmic region essential for maturation signaling. This finding provides the first *in vivo* evidence that signals transduced by the G-CSF-R are indispensable for the normal development of myeloid progenitor cells. Because SCN is characterized by a maturation arrest of myeloid progenitor cells, the data also indicate that the primary role of G-CSF in granulopoiesis is to induce the terminal maturation of myeloid progenitor cells, and further support the notion that the carboxy-terminal region of the G-CSF-R is involved in maturation induction. Moreover, the fact that the normal allele of the G-CSF-R gene was still expressed in the patient strongly suggests that the mutant G-CSF-R protein acted in a dominant negative fashion to interfere with the function of the WT G-CSF-R (Figure 2).

Because the point mutation was not present in other hematopoietic lineages, it can be concluded that the mutation had not occurred at the level of primitive multipotential progenitor or stem cells, but at the stage of committed granulocytic progenitor cells. The question then arises why the normal stem cells did not give rise to significant levels of mature granulocytes. Apparently, the affected granulocytic progenitor cells of the patient were not only hampered in their maturation ability due to the expression of the truncated G-CSF-R, but also interfered with the development of normal progenitor cells. It remains unclear how these committed progenitor cells expressing the truncated G-CSF-R would

inhibit normal granulopoiesis. One possibility is that a hierarchial regulatory mechanism exists in the granulopoietic system, in which the development of early myeloid progenitor cells is determined, at least in part, by the quantity of their progeny, a form of feedback regulatory mechanism (45). Thus, the sustained survival of late progenitor cells blocked in their development may exert a negative effect on the commitment and differentiation of early progenitor cells. Alternatively, it is possible that the abnormal cell population, which presumably had a growth and survival advantage over the normal progenitor cells, may compete for the limited amounts of HGFs and/or the appropriate microenvironment essential for development. It is noteworthy that suppression of normal hematopoiesis by abnormal cell populations also appears to be the feature of other hematological diseases such as myelodysplastic syndrome (MDS). Like MDS, SCN caused by defective G-CSF-R may represent a preleukemic condition.

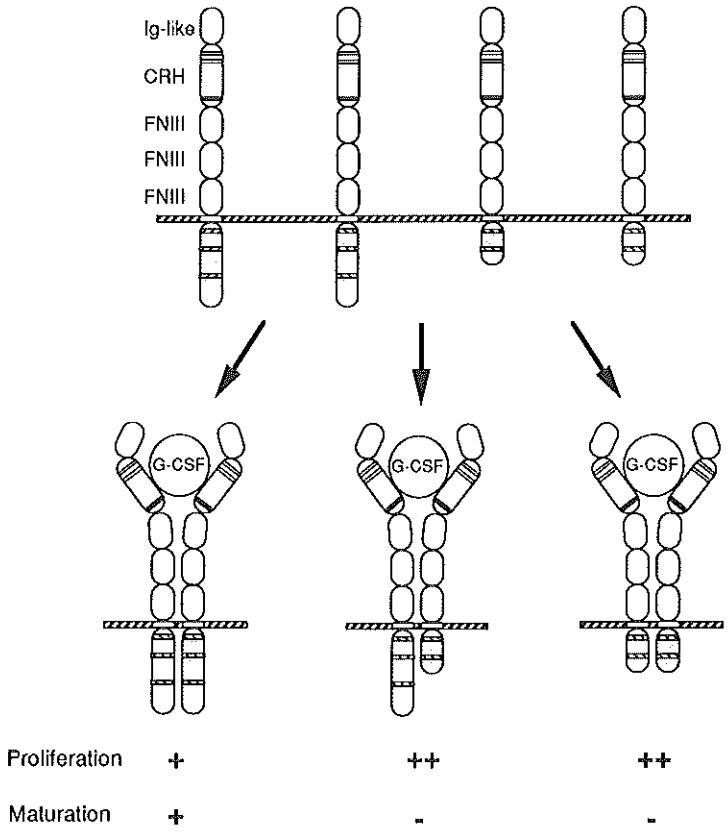

Figure 2. A model for the dominant negative effect of a truncated G-CSF-R on the maturation-inducing function of the WT G-CSF-R. The G-CSF-R deletion mutant may abrogate the maturation signaling activity of the WT G-CSF-R by forming a heterodimer with the WT G-CSF-R.

Table 1. SCN patients analyzed for mutations in the transmembrane and cytoplasmic domains of the G-CSF-R

Patient	Sex/ age	Family history	Nucleotide change	Coding change	Chromosomal abnormality	Disease progression
AH[a]	F/11	-	-	-	ND[d]	-
BA[b]	M/13	-	CAA→TAA	Gln→stop (codon 717)	49,+3, +der(5), t(1;5), +22	AML (FAB-M1)
BJ[a]	F/13	-	-	-	ND	-
BL[b]	F/17	+	-	-	ND	-
BR[a]	M/2	-	-	-	ND	-
BS[b]	M/3	-	-	-	ND	-
DA[c]	M/13	-	CAG→TAG	Gln→stop (codon 715)	-	-
DK[c]	F/11	-	-	-	-	-
FR[b]	M/26	-	CAG→TAG	Gln→stop (codon 730)	45,-7	MDS, AML (FAB-M1)
HA[a]	F/9	-	-	-	ND	-
HD[c]	M/13	-	-	-	-	-
HL[a]	M/11	-	-	-	ND	-
HR[a]	M/5	+	-	-	ND	-
KM[b]	M/2	-	-	-	ND	-
MN[b]	M/18	-	CAA→TAA	Gln→stop (codon 717)	45,-7	-
			CAG→TAG	Gln→stop (codon 719)		
NM[b]	F/10	+	-	-	ND	-
SW[a]	F/28	+	-	-	ND	-
WP[b]	M/9	+	-	-	ND	-

[a] analyzed by SSCP
[b] analyzed by nucleotide sequencing
[c] analyzed by both SSCP and nucleotide sequencing
[d] ND, not determined

7.3 Defective G-CSF receptors in AML: a novel mechanism of leukemogenesis

An increasing number of oncogenes have been recognized to play a role in the development of human leukemias, when their normal functions are disrupted or when they are inappropriately expressed (46,47). In characterization of these oncogenes, it turned out that most of them encode proteins that function either as intracellular signaling molecules or as transcription factors, and play a role in the regulation of cell proliferation, differentiation and survival. For instance, BCR-ABL fusion protein, which arises as a consequence of the

t(9;22) that occurs in approximately 90% of CML, leads to the constitutive activation of tyrosine kinase ABL (48,49). Mutations in $p21^{ras}$ have been detected in about 30% of AML patients (50,51). Several nonrandom chromosomal abnormalities associated with specific subtypes of leukemias have been demonstrated to involve transcription factors, including the E2A/PBX fusion protein of the t(1;19), the PML/RARα fusion protein of the t(15;17), and the AML1/MTG8(ETO) fusion protein of the t(8;21) (52-57). Abnormalities in the hematopoietin receptors resulting in deregulated proliferation and/or differentiation of hematopoietic progenitor cells have not yet been associated with clinical hematological malignancies.

Despite this apparent failure in the past to demonstrate the involvement of abnormal hematopoietin receptors in human leukemias, it has been demonstrated that defective hematopoietin receptors can contribute to the development of leukemias in animals. A mutation (R129C) in the extracellular domain of the murine EPO-R has been shown to cause constitutive homodimerization and activation of the EPO-R mutant protein (58). Introduction of this EPO-R mutant into mice via retroviral infection results in the development of erythroleukemia in the recipient mice (59). Similarly, a duplication of 37 amino acids in the extracellular domain of the human common ß chain (hßc) of the IL-3, GM-CSF and IL-5 receptors confers a growth-factor independence to murine IL-3-dependent myeloid FDC-P1 transfectants (60). FDC-P1 transfectants expressing this hßc mutant were tumorigenic when injected into syngeneic mice, whereas parental FDC-P1 cells or cells expressing the wild-type hßc protein were not. It is also interesting that a human erythroleukemia cell line, TF-1, has been shown to contain a deletion at the 3' end of the EPO-R gene which encodes the negative regulatory carboxy-terminus of the receptor protein (61).

With respect to the potential linkage between abnormal hematopoietin receptors and human leukemias, it is important that the truncated G-CSF-R in patient DA was expressed exclusively in committed granulocytic progenitor cells (Chapter 4). This would imply that the patient's granulocytic progenitors, in addition to being arrested at early stages of maturation as a consequence of expressing the truncated G-CSF-R, had acquired the capacities for sustained self-renewal and survival, raising the possibility that G-CSF-R mutants with carboxy-terminal deletions are transforming. Because a few cases of SCN have been reported to develop AML upon prolonged survival, the question arises whether these patients express abnormal G-CSF-R proteins. In Chapter 5, it is shown that leukemic cells from two SCN patients who terminated in AML also carried mutations in the G-CSF-R that result in the truncation of the carboxy-terminal maturation-inducing region. These results provide the first evidence that abnormal hematopoietin receptor structures are associated with human leukemia. Apparently, the sole expression of a truncated G-CSF-R is insufficient for full leukemic transformation. Leukemic cells from both SCN patients who developed AML had acquired additional genetic alterations involving chromosomal abnormalities. However, deletion of the carboxy-terminal maturation-inducing region of the G-CSF-R may represent an initiating genetic lesion, i.e., a first step in the leukemogenic process.

More recently, two point mutations, both C-to-T substitution, were identified at nt 2390 and 2396 of the G-CSF-R cDNA in an SCN patient (patient MN, Table 1) who had developed monosomy 7 but had no evidence of MDS (unpublished results). Both mutations

truncate the carboxy-terminal region of 96 and 94 amino acids, respectively. Nucleotide sequencing of cloned PCR products demonstrated that the two mutations were present in different clones, indicating that the two mutations exited either on two distinct alleles of the G-CSF-R gene of one cell population or in two different cell clones in the patient. Significantly, both mutations were not present in DNA derived from EB virus-transformed B lymphocytes of the patient (unpublished data). As yet, a total of 18 SCN patients have been analyzed for mutations in the transmembrane and cytoplasmic domains of the G-CSF-R by using either nucleotide sequencing and/or SSCP analyses in our laboratory. Four patients were demonstrated to have mutations in the G-CSF-R, all leading to the carboxy-terminal truncation of the G-CSF-R. Because some cases were analyzed only by SSCP analysis, which in theory could miss certain mutations, it cannot be excluded that the frequency of G-CSF-R mutations in SCN may be higher.

Although mutations in the G-CSF-R have been identified in two AML patients who initially presented as SCN, in at least one case the mutation was shown to be somatic, similar to those detected in patients DA and MN. Assuming that the mutations had been acquired in the late period of life, these patients would have then presented as acquired neutropenia instead of SCN. Thus, it is plausible that mutations in the G-CSF-R could also occur in patients with acquired neutropenia. Clinically, such patients would be diagnosed as idiopathic neutropenia, and some of them would probably progress to AML. In view of this, it may be worthwhile to examine the G-CSF-R in adult patients with AML, particularly in those with a history of acquired neutropenia or MDS accompanied by significant neutropenia.

Finally, it should be pointed out that the studies described in Chapters 4 and 5 mainly concern the cytoplasmic domain of the G-CSF-R. As discussed above, mutations in the extracellular domain leading to the constitutive activation of hematopoietin receptors could also be leukemogenic. Whether similar mutations would exist in the extracellular domain of the G-CSF-R and would be involved in certain cases of human AML remains enigmatic at the moment. On the other hand, it appears that inactivation of the G-CSF-R may play a part in AML as well. In Chapter 6, it is shown that leukemic cells from an AML patient overexpressed a G-CSF-R splice variant that possesses no biological activity. Conceivably, myeloid cells expressing a nonfunctional G-CSF-R would lack the capacity to mature in response to G-CSF, but may survive and even proliferate under the influence of other HGFs that are available *in vivo*.

References

1. Cosman D. (1993) The hematopoietin receptor superfamily. Cytokine 5:95.
2. Ilhe JN, Witthuhn BA, Quelle FW, Yamamoto K, Thierfelder WE, Kreider B and Silvennoinen O. (1994) Signalling by the cytokine receptor superfamily: JAKs and STATs. TIBS 19:222.
3. Yin T, Yasukawa K, Taga T, Kishimoto T and Yang YC. (1994) Identification of a 130-kilodalton tyrosine-phosphorylated protein induced by interleukin-11 as JAK2 tyrosine kinase, which associates with gp130 signal transducer. Exp. Hematol. 22:467.
4. Zhuang H, Patel SV, He T, Sonsteby SK, Niu Z and Wojchowski DM. (1994) Inhibition of erythropoietin-induced mitogenesis by a kinase-deficient forms of Jak2. J. Biol. Chem. 269:21411.

5. Darnell Jr. JE, Kerr IM and Stark GR. (1994) Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signalling proteins. Science 264:1415.
6. Stahl N, Boulton TG, Farruggella T, Ip NY, Davis S, Witthuhn BA, Quelle FW, Silvennoinen O, Barbieri G, Pellegrini S, Ihle JN and Yancopoulos GD. (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 ß receptor components. Science 263:92.
7. Lütticken C, Wegenka UM, Yuan J, Buschmann J, Schindler C, Ziemiecki A, Harpur AG, Wilks AF, Yasukawa K, Taga T, Kishimoto T, Barbieri G, Pellegrini S, Sendtner M, Heinrich PC and Horn F. (1994) Association of transcription factor APRF and protein kinase JAK1 with the interleukin-6 signal transducer gp130. Science 263:89.
8. Narazaki M, Witthuhn BA, Yoshida K, Silvennoinen O, Yasukawa K, Ihle JN, Kishimoto T and Taga T. (1994) Activation of JAK2 kinase mediated by the interleukin 6 signal transducer gp130. Proc. Natl. Acad. Sci. USA. 91:2285.
9. Russell SM, Johnston JA, Noguchi M, Kawamura M, Bacon CM, Friedmann M, Berg M, McVicar DW, Witthuhn BA, Silvennoinen O, Goldman AS, Schmalstieg FC, Ihle JN, O'Shea JJ and Leonard WJ (1994) Interaction of IL-2Rß and γ chains with Jak1 and Jak3: implications for XSCID and XCID. Science 266:1042.
10 Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minani Y, Liu ZJ, Oishi I, Sivennoinen O, Witthuhn BA, Ihle JN and Taniguchi T. (1994) Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. Science 266:1045.
11. Nicholson SE, Oates AC, Harpur AG, Ziemiecki A, Wilks AF and Layton JE. (1994) Tyrosine kinase JAK1 is associated with the granulocyte-colony-stimulating factor receptor and both become tyrosine-phosphorylated after receptor activation. Proc. Natl. Acad. Sci. USA. 91:2985.
12. Ziegler S, Bird TA, Morella KK, Mosley B, Gearing DP and Baumann H. (1993) Distinct regions of the human granulocyte-colony-stimulating factor receptor cytoplasmic domain are required for proliferation and gene induction. Mol. Cell. Biol. 13:2384.
13. Baumann H, Gearing D and Ziegler SF. (1994) Signalling by the cytoplasmic domain of hematopoietin receptors involves two distinguishable mechanisms in hepatic cells. J. Biol. Chem. 269:16297.
14. Baumann H, Symes AJ, Comeau MR, Morella KK, Wang Y, Friend D, Ziegler SF, Fink JS and Gearing DP. (1994) Multiple regions within the cytoplasmic domains of the leukemia inhibitory factor receptor and gp130 cooperate in signal transduction in hepatic and neuronal cells. Mol. Cell. Biol. 14:138.
15. Bashey A, Healy L and Marshall CJ. (1994) Proliferative but not nonproliferative responses to granulocyte colony-stimulating factor are associated with rapid activation of the $p21^{ras}$/MAP kinase signalling pathway. Blood 83:949.
16. Silvennoinen O, Schindler C, Schlessinger J and Levy DE. (1994) Ras-independent growth factor signalling by transcription factor tyrosine phosphorylation. Science 261:1736.
17. Till JE, McCulloch EA and Siminovitch L. (1964) A stochastic model of stem cell proliferation based on the growth of spleen colony forming cells. Proc. Natl. Acad. Sci. USA 51:29.
18. Korn AP, Henkelman RM, Ottensmeyer FP and Till JE. (1973) Investigation of a stochastic model of haemopoiesis. Exp. Hematol. 1:362.
19. Fairbairn LJ, Cowling GJ, Reiper BM and Dexter TM. (1993) Suppression of apoptosis allows differentiation and development of a multipotent hemopoietic cell line in the absence of added growth factors. Cell 74:823.
20. Curry JL and Trentin JJ. (1967) Haemopoietic spleen colony-stimulating factors. Science 236:1229.
21. Valtieri M, Tweardy DJ, Caracciolo D, Johnson K, Mavilio F, Altmann S, Santoli D and Rovera G. (1987) Cytokine-dependent granulocytic differentiation. Regulation of proliferative

and differentiative response in a murine progenitor cell line. J. Immunol. 138:3829.
22. Lee KH, Kinashi T, Tohyama K, Tashiro K, Funato N, Hama K and Honjo T. (1991) Different stromal cell lines support lineage-selective differentiation of the multipotential bone marrow stem cells clone LyD9. J. Exp. Med. 173:1257.
23. Liboi E, Carroll M, D'Andrea AD and Mathey-Prevot B. (1993) Erythropoietin receptor signals both proliferation and erythroid-specific differentiation. Proc. Nalt. Acad. Sci. USA. 90:11351.
24. Chiba T, Nagata Y, Kishi A, Sakamaki K, Miyajima A, Yamamoto M, Engel JD and Todokoro K. (1993) Induction of erythroid-specific gene expression in lymphoid cells. Proc. Natl. Acad. Sci. USA. 90:11593.
25. Borzillo GV, Ashmun RA and Sherr CJ. (1990) Macrophage lineage switching of murine early pre-B lymphoid cells expressing transduced fms genes. Mol. Cell. Biol. 10:2703.
26. Fukunaga R, Ishizaka-Ikeda E and Nagata S. (1993) Growth and differentiation signals mediated by different regions in the cytoplasmic domain of granulocyte colony-stimulating factor receptor. Cell 74:1079.
27. Corey SJ, Burkhardt AL, Bolen JB, Geahlen RL, Tkatch LS and Tweardy DJ. (1994) Granulocyte colony-stimulating factor receptor signalling involves the formation of a three-component complex Lyn and Syk protein-tyrosine kinases. Proc. Natl. Acad. Sci. USA. 91:4683.
28. Druker BJ, Neumann M, Okuda K, Franza BR Jr, and Griffin JD. (1994) rel is rapid tyrosine-phosphorylated following granulocyte-colony stimulating factor treatment of human neutrophils. J. Biol. Chem. 269:5387.
29. Pan C-X, Fukunaga R, Yonehara S and Nagata S. (1993) Unidirectional cross-phosphorylation between the granulocyte colony-stimulating factor and interleukin 3 receptors. J. Biol. Chem. 268:25818.
30. Songyang Z, Shoelson SE, McGland J, Olivier P, Pawson T, Bustelo XR, Barbacid M, Sabe H, Hanafusa H, Yi T, Ren R, Baltimore D, Ratnofsky S, Feldman RA and Cantley LC. (1994) Specific motifs recognized by the SH2 domains of Csk, 3BP2, fps/fes, GRG-2, HCP, SHC, Syk, and Vav. Mol. Cell. Biol. 14:2777.
31. Ren R, Mayer BJ, Cicchetti P and Baltimore D. (1993) Identification of a ten-amino acid proline-rich SH3 binding site. Science 259:1157.
32 Sawyers CL, McLaughlin J, Goga A, Havlik M and Witte O. (1994) The nuclear tyrosine kinase c-Abl negatively regulates cell growth. Cell 77:121.
33. Williams GT, Smith CA, Spooncer E, Dexter TM and Taylor DR. (1990) Haemopoietic colony stimulating factors promote cell survival by suppressing apoptosis. Nature 343:76.
34. Koury MJ and Bondurant MC. (1990) Erythropoietin retards DNA breakdown and prevents programmed death in erythroid progenitor cells. Science 248:378.
35. Raff MC. (1992) Social controls on cell survival and cell death. Nature 256:397.
36. Begley CG, Lopez AF, Nicola NA, Warren DJ, Vadas MA, Sanderson CJ and Metcalf D. (1986) Purified colony-stimulating factors enhance the survival of human neutrophils and eosinophils in in vitro. Blood 68:162.
37. Cross M and Dexter TM. (1991) Growth factors in development, transformation, and tumorigenesis. Cell 64:271.
38. Selvakumaran M, Reed JC, Liebermann D and Hoffman B. (1994) Progression of the myeloid differentiation program is dominant to transforming growth factor-ß-induced apoptosis of M1 myeloid leukemic cells. Blood 84:1036.
39. de la Chapelle A, Träskelin AL and Juvonen E. (1993) Truncated erythropoietin receptor causes dominantly inherited benign human erythrocytosis. Proc. Natl. Acad. Sci. USA. 90:4495.
40. Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW and Leonard WJ. (1993) Interleukin-2 receptor γ chain mutation results in X-linked severe

combined immunodeficiency in humans. Cell 73:147.
41. Amselem S, Duquesnoy P, Attree O, Novelli G, Bousnina S, Postel-Vinay MC and Goossens M. (1989) Laron dwarfism and mutations of the growth hormone-receptor gene. N. Engl. J. Med. 321:989.
42. Amselem S, Sobrier ML, Duquesnoy P, Rappaport R, Postel-Vinay MC. (1991) Recurrent nonsense mutations in the growth hormone receptor from patients with Laron dwarfism. J. Clin. Invest. 87:1098.
43. Berg-MA, Guevara-Aguirre J, Rosenbloom AL, Rosenfeld RG and Francke U. (1992) Mutation creating a new splice site in the growth hormone receptor genes of 37 Ecuadorean patients with Laron syndrome. Hum. Mutat. 1:24.
44. Duquesnoy P, Sobrier ML, Amselem S and Goossens M. (1991) Defective membrane expression of human growth hormone (GH) receptor causes Laron-type GH insensitivity syndrome. Proc. Nalt. Acad. Sci. USA. 88:10272.
45. Prothero J. (1980) Control of stem cell proliferation: a density-dependent commitment model. J. Theor. Biol. 84:725.
46. Sawyers C, Denny CT and Witte ON. (1991) Leukemia and the disruption of normal hematopoiesis. Cell 64:337.
47. Cleary ML. (1991) Oncogenic conversion of transcription factors by chromosomal translocations. Cell 66:619.
48. Pendergast AM, Muller AJ, Havlik MH, Maru Y and Witte ON. (1991) BCR sequences essential for transformation by the BCR-ABL oncogene bind to the ABL SH2 regulatory domain in a non-phosphotyrosine-dependent-dependent manner. Cell 66:161.
49. Muller AJ, Young JC, Pendergast AM, Pondel M, Landau NR, Littman DR and Witte ON. (1991) BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. Mol. Cell. Biol. 11:1785.
50. Bos JL, Toksoz D, Marshall CJ, Verlaan-de Vries M, Veeneman GH, van der Eb AJ, van Boom JH, Janssen JWG and Steenvoorden ACM. (1985) Amino acid substitution at codon 13 of the N-ras oncogene in human acute myeloid leukemia. Nature 315:726.
51. Needleman SW, Kraus MH, Srivastava SK, Levine PH and Aaronson SA. (1986) High frequency of N-ras activation in acute myelogenous leukemia. Blood 67:753.
52. Nourse J, Mellentin JD, Galili N, Wilkinson J, Stanbridge E, Smith SD and Cleary ML. (1990) Chromosomal translocation t(1;19) results in synthesis of a homeobox fusion mRNA that codes for a potential chimeric transcription factor. Cell 60:535.
53. Kakizuka A, Miller WH Jr, Umesono K, Warrell RP Jr, Frankel SR, Murty VVVS, Dmitrovsky E and Evans RM. (1991) Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RARα with a novel putative transcription factor, PML. Cell 66:663.
54. Goddard AD, Borrow J, Freemont PS and Solomon E. (1991) Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. Science 254:1371.
55. Miyoshi H, Shimizu K, Kozu T, Maseki N, Kaneko Y and Ohki M. (1991) t(8;21) breakpoints on chromosome 21 in acute myeloid leukemia are clustered within a limited region of a single gene, AML1. Proc. Natl. Acad. Sci. USA. 88:10431.
56. Erickson P, Gao J, Chang KS, Look T, Whisenant E, Raimondi S, Lasher R, Trujillo J, Rowley J and Drabkin H. (1992) Identification of breakpoints in t(8;21) acute myelogenous leukemia and isolation of a fusion transcript, AML/ETO, with similarity to Drosophila segmentation gene, runt. Blood 80:1825.
57. Miyoshi H, Kozu T, Shimizu K, Enomoto K, Maseki N, Kaneko Y, Kamada N and Ohki M. (1993) The t(8;21) translocation in acute myeloid leukemia results in production of an AML-MTG8 fusion transcript. EMBO J. 12:2715.
58. Yoshimura A, Longmore G and Lodish HF. (1990) Point mutation in the exoplasmic domain of the erythropoietin receptor resulting in hormone-independent activation and tumorigenicity.

Nature 348:647.
59. Longmore GD and Lodish HF. (1991) An activating mutation in the murine erythropoietin receptor induces erythroleukemia in mice: a cytokine receptor superfamily oncogene. Cell 67:1089.
60. D'Andrea R, Rayner J, Moretti P, Lopez A, Goodall GJ, Gonda TJ and Vadas M. (1994) A mutation of the common receptor subunit for interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor, and IL-5 that leads to ligand independence and tumorigenicity. Blood 83:2802.
61. Ward JC, Harris KW, Penny LA, Forget BG, and Kitamura T. (1992) A structurally abnormal erythropoietin receptor gene in a human erythroleukemia cell line. Exp. Hematol. 20:371

SUMMARY

Granulopoiesis is regulated by a network of hematopoietic growth factors (HGFs). G-CSF plays an essential role in the regulation of this process. Apart from being a regulator of cell proliferation and survival, G-CSF is the most powerful inducer of terminal granulocytic maturation of myeloid progenitor cells. The human receptor for G-CSF (G-CSF-R) is a single membrane-spanning polypeptide with a cytoplasmic domain of 183 amino acids. Following its activation, the G-CSF-R mediates signals leading to the diverse cellular responses. In **Chapter 1**, an overview is given of the current understanding of hematopoiesis, the activities of HGFs in the regulation of hematopoiesis, and the functions of the HGF receptors, the G-CSF-R in particular. The clinical features and recent insights into the pathogenesis of neutropenias and acute myeloid leukemia (AML) relevant to the work in this thesis are also introduced. In **Chapter 2**, it is established that the cytoplasmic domain of the G-CSF-R contains distinct functional subdomains that are coupled to specific receptor signaling activities. The membrane-proximal region of 55 amino acids is sufficient for transducing proliferative signals. The further downstream sequence of 30 amino acids significantly enhances mitogenic signaling. In contrast, the distal carboxy-terminal region of the wild-type (WT) G-CSF-R inhibits mitogenic signaling and is essential for mediating signals for terminal granulocytic maturation. It is further shown that the altered carboxy-terminal region of a G-CSF-R splice variant DC also inhibits proliferative signaling, but does not appear to have a role in maturation induction.

In **Chapter 3**, it is shown that under certain conditions G-CSF actively induces apoptosis (programmed cell death) of myeloid cells. G-CSF initially stimulated the proliferation and survival of murine myeloid 32D cells transfected with the WT G-CSF-R (32D/WT). Upon prolonged culture, however, G-CSF inhibited cell proliferation and further induced apoptosis but not terminal granulocytic maturation. Induction of apoptosis of 32D/WT cells by G-CSF was observed even in the presence of optimal concentrations of IL-3 that would otherwise support the long-term proliferation of 32D cells. Examination of the growth patterns of 32D cells expressing the different G-CSF-R structure revealed that the death signals were mediated exclusively by the carboxy-terminal region of the WT G-CSF-R, but not of the DC splice variant. Because maturation and apoptosis are both mediated by the carboxy-terminal region of the G-CSF-R, it is suggested that a single subdomain within this carboxy-terminus would be responsible for transducing signals for granulocytic maturation and apoptosis. Because the 32D cells used in this study were apparently defective in granulocytic maturation, it is hypothesized that apoptosis of 32D/WT cells induced by G-CSF was associated with the inability of the cells to undergo terminal granulocytic maturation.

In **Chapter 4**, the potential involvement of abnormal G-CSF-R structures in the pathogenesis of clinical neutropenias, severe congenital neutropenia (SCN or Kostmann's syndrome) in particular, is investigated. SCN is characterized by a maturation arrest of myeloid progenitor cells at early stages of development. Patients with SCN are at an increased risk of developing AML. The G-CSF-R genes of six patients with SCN and two patients with acquired neutropenia were analyzed by using single strand conformation polymorphism (SSCP) and nucleotide sequencing analyses. A point mutation was identified in one SCN patient, which introduces a premature stop codon for protein translation resulting in the truncation of 98 carboxy-terminal amino acids of the G-CSF-R protein. The normal

allele of the G-CSF-R gene was still expressed in the patient. *In vitro* colony cultures revealed that marrow cells from the patient displayed a considerably reduced responsiveness to G-CSF, whereas responses to IL-3, GM-CSF and EPO were normal. Moreover, it is shown that the point mutation was present exclusively in cells of granulocytic lineage but not in other hematopoietic lineages of the patient, nor in peripheral blood mononuclear cells of the patient's parents, thus establishing that the mutation had been acquired rather than inherited.

In **Chapter 5**, nucleotide sequencing of the G-CSF-R gene was performed in two patients who initially presented as SCN but terminated in AML. In both cases, point mutations were identified in the cytoplasmic domain that introduce premature stop codons leading to the truncation of the carboxy-terminal part of 96 and 85 amino acids, respectively. In one patient, the mutation appeared to be present only in leukemic cells, but not in the liver and spleen tissues, indicating that the mutation had arisen as a somatic event. In another patient, the mutation was detected in bone marrow cells collected when the patient was at neutropenic stage. These data suggest that defective G-CSF-R structures with impaired maturation signaling capacity may contribute to leukemogenesis. Similar to the SCN patient described in chapter 4, the normal allele of the G-CSF-R gene was still present in the two cases, indicating that the truncated G-CSF-Rs act in a dominant negative manner to interfere with the function of the WT G-CSF-R. It is proposed that SCN patients resulting from defective G-CSF-Rs define a subgroup of congenital neutropenias that may represents a preleukemic entity.

Chapter 6 describes the molecular cloning of a novel G-CSF-R splice variant, designated SD, in which the carboxy-terminal part of 137 amino acids of the WT G-CSF-R is replaced by an altered sequence of 34 amino acids identical to the splice variant DC. Although the SD receptor retains the membrane-proximal cytoplasmic region known to be sufficient for proliferative signaling, it did not transduce growth signals in murine hematopoietic cells. The expression of the SD receptor is low in normal granulocytes, but was increased considerably in the leukemic cells of an AML patient. Examination of the G-CSF-R gene of the patient's leukemic cells revealed a point mutation at the SD splice donor site. The normal allele of the G-CSF-R gene was also expressed in the patient. It is thus hypothesized that the SD receptor may also exert a dominant negative effect on the function of the WT G-CSF-R, presumably by forming heterodimers with the WT G-CSF-R. Such SD/WT receptor heterodimers may fail to transduce maturation signals and thereby may play a role in the development of leukemia in this patient.

The potential involvement of JAK2 kinase in the signaling pathway of the G-CSF-R is also investigated in Chapter 6. G-CSF stimulation of murine pro-B BAF3 cells transfected with the WT G-CSF-R resulted in the rapid tyrosine-phosphorylation of the JAK2 proteins. A G-CSF-R deletion mutant (M1), containing only 55 amino acid residues in the cytoplasmic domain, was fully capable of mediating JAK2 activation, indicating that the membrane-proximal cytoplasmic region of the G-CSF-R is involved in the interaction with JAK2. In contrast, although the SD receptor retains this membrane-proximal region required for JAK2 activation, it was unable to activate JAK2. Thus, induction of JAK2 activation correlates with the ability of the G-CSF-R to transduce proliferative signals. These data suggest that JAK2

may be one of the critical components in the proliferative signaling pathway of the G-CSF-R.

Finally, in **Chapter 7** the significance of the findings described in this thesis for the understanding of the function of the G-CSF-R, and the role of G-CSF-R defects in the development of acute myeloid leukemia are being discussed.

SAMENVATTING

De productie van neutrofiele granulocyten (granulopoiese) wordt gereguleerd door een netwerk van hematopoietische groeifactoren (HGFs). Granulocyte colony stimulating factor (G-CSF) speelt een essentiële rol in dit proces. G-CSF stimuleert de proliferatie en overleving van de granulocytaire voorlopercellen en induceert de uitrijping naar terminaal uitgedifferentieerde granulocyten. De humane receptor voor G-CSF (G-CSF-R) is een transmembraan eiwit met een cytoplasmatisch domein bestaande uit 183 aminozuren. Na activering geeft G-CSF-R signalen door, die leiden tot de verschillende cellulaire responsen.

Hoofdstuk 1 geeft een overzicht van de huidige inzichten in de functie van hematopoietische groeifactor receptoren, in het bijzonder de receptor voor G-CSF. Tevens worden in dit hoofdstuk ingegaan op de klinische kenmerken van de aandoeningen neutropenie en acute myeloide leukemie (AML), voor zover relevant voor dit proefschrift.

In **Hoofdstuk 2** worden experimenten beschreven die aantonen dat in het cytoplasmatisch domein van humane G-CSF-R (183 aminozuren) afzonderlijke subdomeinen met specifieke functies kunnen worden herkend. Het 55 aminozuren omvattende membraan-proximaal subdomein is essentiëel voor de doorgeleiding van proliferatieve signalen. Een aansluitende, C-terminaal gelegen sequentie van 30 aminozuren versterkt de proliferatieve signaal functie. Het daarop volgende ongeveer 100 aminozuren bevattende membraan-distale cytoplasmatische subdomein van de 'wildtype' (WT) G-CSF-R remt de proliferatieve signaalfunctie en is essentiëel voor de door G-CSF geinduceerde granulocytaire uitrijping. Verder wordt in dit hoofdstuk beschreven dat de alternatieve C-terminus van een G-CSF-R splice variant (DC) eveneens de proliferatieve signaalfunctie remt, maar niet in staat is om maturatie-signalen door te geven.

In **Hoofdstuk 3** wordt uiteengezet hoe G-CSF, onder bepaalde condities, in staat is om in myeloide cellen geprogrammeerde celdood (apoptosis) te induceren. G-CSF stimuleert aanvankelijk de proliferatie en overleving van 32D cellen getransfecteerd met WT G-CSF-R (32D/WT). Daarentegen blijkt G-CSF vier dagen na de start van de kweek de proliferatie te remmen en geprogrammeerde celdood te induceren, zonder tekenen van granulocytaire uitrijping. Inductie van apoptosis in 32D/WT cellen door G-CSF blijkt ook op te kunnen treden in aanwezigheid van optimale concentraties IL-3, die op zichzelf de lange termijn proliferatie van 32D cellen kunnen onderhouden. Experimenten met 32D transfectanten met gemodificeerde G-CSF-R structuren lieten zien dat de celdood inducerende signalen uitsluitend worden afgegeven door het C-terminale maturatie domein van de WT G-CSF-R. Aangenomen wordt dat de door G-CSF geinduceerde apoptosis in 32D/WT cellen optreedt vanwege het onvermogen van deze cellen om terminaal uit te rijpen tot neutrofiele granulocyten.

Hoofdstuk 4 handelt over de mogelijke betrokkenheid van afwijkende G-CSF-R structuren in de pathogenese van ernstige aangeboren neutropenie. Deze ziekte wordt o.a. gekenmerkt door een rijpings-blokkade in de myeloide voorloper cellen. Patienten met een ernstige aangeboren neutropenie hebben een verhoogde kans AML te krijgen. In 6 patienten met aangeboren neutropenie en 2 patienten met verkregen neutropenie werd het G-CSF-R gen onderzocht op mogelijke afwijkingen met behulp van SSCP (single strand conformational polymorphism) en nucleotide sequentie-analyse. In één patient met

aangeboren neutropenie werd een puntmutatie in het G-CSF-R gen gevonden. Deze mutatie resulteert in de vorming van een getrunceerd G-CSF-R eiwit, waarin 98 C-terminale aminozuren ontbreken. Ook het normale G-CSF-R allel kwam in deze patient tot expressie. De beenmerg cellen van deze patient vertoonden een sterk verlaagde respons op G-CSF in *in vitro* kolonie kweken. Daarentegen was de kolonievorming onder invloed van GM-CSF, IL-3 en EPO normaal. De G-CSF-R puntmutatie bleek uitsluitend aanwezig te zijn in de cellen van de granulocytaire reeks en kon niet worden aangetoond in de bloedcellen van de ouders van de patient. Hieruit blijkt dat de mutatie niet familiair was overgedragen.

In **Hoofdstuk 5** worden de resultaten gepresenteerd van G-CSF-R nucleotide sequentie onderzoek bij twee patienten met ernstige aangeboren neutropenie, die op latere leeftijd AML ontwikkelden. In de AML cellen van beide patienten werden puntmutaties in het G-CSF-R gen gevonden, die resulteerden in de truncering van respectievelijk 96 en 85 C-terminale aminozuren van het cytoplasmatisch domein van de receptor. Van één van deze patienten was materiaal van lever en milt beschikbaar. Aangezien de G-CSF-R mutatie in geen van deze weefsels werd aangetroffen moet worden aangenomen dat er sprake is van een somatisch defect. Bij de tweede patient kon worden vastgesteld dat de mutatie reeds in de fase van neutropenie aanwezig was. Deze resultaten maken aannemelijk dat defecten in het G-CSF-R gen, resulterend in de vorming van G-CSF-R structuren die de maturatie-signaalfunctie missen, kunnen bijdragen aan de ontwikkeling van AML. Mede op basis van deze gegevens kan ernstige aangeboren neutropenie, geassocieerd met afwijkingen in de G-CSF-R, worden gekenmerkt als een preleukemisch ziektebeeld.

In **Hoofdstuk 6** wordt de klonering van een nieuwe G-CSF-R splice variant (SD) beschreven. In SD zijn 137 aminozuren van de C-terminus van WT G-CSF-R vervangen door de alternatieve C-terminus van 34 aminozuren, die ook voorkomt in splice variant DC. De SD receptor komt in normale granulocyten slechts laag tot expressie. Daarentegen werd in de leukemische blasten van een AML patient een sterk verhoogde expressie van de SD variant waargenomen. Dit bleek geassocieerd te zijn met een puntmutatie in het G-CSF-R gen op de 'splice donor' positie van SD. De SD receptor bevat het complete membraan-proximale cytoplasmatische subdomein van 55 aminozuren dat nodig is voor de proliferatieve signaalfunctie. Desalniettemin bleek de SD variant, getransfecteerd in muize hematopoietische cellijnen, niet in staat proliferatie signalen door te geven. Vervolgens werd de betrokkenheid van de z.g.n. JAK kinases in de signaalweg van G-CSF-R onderzocht. Activering van WT G-CSF-R, getransfecteerd in muize pro-B (BAF3) cellen, leidde tot een snelle tyrosine-phosphorylering van JAK2 eiwitten. Ook de activering van de C-terminale deletie-mutant M1, die de 55 membraan-proximale aminozuren van het G-CSF-R cytoplasmatisch domein bezit, resulteerde in de tyrosine-fosforylering van JAK2. Daarentegen bleek splice-variant SD niet of nauwelijks in staat JAK2 te activeren. Dit wijst erop dat de alternatieve C-terminus van 34 aminozuren zowel de proliferatieve signaalfunctie als JAK2 activatie blokkeert.

Tenslotte wordt in **Hoofdstuk 7** de betekenis van de bevindingen, beschreven in dit proefschrift, voor het inzicht in de functie van de G-CSF-R en voor de rol die G-CSF-R defecten kunnen spelen in de ontwikkeling van acute myeloide leukemie bediscussiëerd.

ACKNOWLEDGMENTS

Without the help and generous contribution of many, it would not have been possible for me to complete the studies and prepare this thesis.

I express my deep gratitude to Prof. Bob Löwenberg, my supervisor, for offering me the opportunity to carry out studies in the cell culture laboratory, and for his critical guidance, constant support and generosity.

I thank Prof. J. Anton Grootegoed and Prof. J. Wolter Oosterhuis for reading this thesis and valuable suggestions; Prof. Anjo J. P. Veerman for reading the thesis and for collaboration on the study presented in Chapter 4.

I am grateful to Ivo P. Touw, my co-supervisor and friend, for his enthusiastic support, great care of my scientific researches, and assistance in the preparation of this thesis. "I appreciate the many inspiring discussions with you during the last 4 years, from which I have benefited a lot."

I sincerely thank my colleague Lies Hoefsloot who introduced me into the field of molecular biology, and has always been of great help in my work. "I learnt a lot from the many 'short courses' that you gave to me, which I enjoyed so much. I am also very pleased to have you as my paranymph."

I also wish to thank Ruud Delwel, my colleague and friend, who was the first person I worked with upon arrival in the Netherlands. "Although our collaboration was short, your support and encouragement have been persistent. From the short period of time of working with you, I got the general idea about the laboratory work. This experience had a considerable influence on my later work."

My career as a laboratory investigator was started in the cell culture lab, almost from scratch. My colleagues in cell culture lab have assisted me in various aspects. In particular, I would like to thank Anita Schelen for her important contribution to the experimental work and for being my paranymph; Carin van Buitenen, Marleen van Paassen, and Karin van Gool-Pouwels for their large contribution; Lianne Broeders and Hans Hoogerbrugge for participation in some experiments and for excellent technical assistance. Thanks should also go to Leo Budel, Bianca Backx, Frans Smiers, Dorien van der Plas, John de Koning, Valeria Santini, Inge de Greef for their help, advices and useful discussions. "I very much appreciate your being my colleagues".

I further wish to thank my colleagues at the institute of Hematology, Erasmus University for their help, encouragement and interest in my work. Among many, I would like to mention Rob Ploemacher, Anton Martens, Peter Valk, Henk Rozemuller, Dimitri Breems, Ellen Blokland, Samantha Hol, Yolanda Vankan. "I love the pleasant atmosphere you created, and I have had a great time here thanks to you".

I express my gratitude to Janine Vrij, Jeanne Vlasveld-de Groot, and Ineke de Poorter for their excellent secretarial assistance.

Mirjam Bosman has helped me with the preparation of some figures. I thank her for her time and efforts.

In addition, I am indebted to Dr. Shigekazu Nagata and Dr. Rikiro Fukunaga at the Osaka Biosciece Institute for providing us with the human G-CSF receptor cDNA; Dr.

Tasuku Honjo at Kyoto University Faculty of Medicine, Japan for murine L-GM cell line; Dr. Karl Welte and Dr. Nicola Tidow at Medical School Hannover, Germany and Dr. Russell K. Brynes at City of Hope National Medical Center, Duarte, California, USA for cooperation on the study presented in Chapter 5; Dr. Bruce A. Witthuhn and Dr. James N. Ihle for JAK2 antibodies.

The financial support from the World Health Organization for my first year of stay and training here in the Netherlands is gratefully acknowledged.

Finally, special thanks must go to my wife Yaling for her understanding, encouragement, and good care of me and my daughter who has always been bringing me much happiness.

CURRICULUM VITAE

Fan Dong was born on September 12, 1958 in Shanghai, P.R China. He studied medicine from 1978 to 1982 at Suzhou Medical College in P.R. China. Upon graduation, he became a resident at the First Academic Hospital of Suzhou Medical College. In 1985 he started a postgraduate study at the same college, majoring in Hematology under the supervision of Prof. Zhang Guiru. After completion of his postgraduate study 3 years later, he became an attending physician and lecturer at the Department of Hematology, the First Academic Hospital of Suzhou Medical College. In November of 1990, he arrived in the Netherlands and started his training in cell biology and molecular biology at the cell culture laboratory of the Dr. Daniel den Hoed Cancer Center, initially sponsored by the World Health Organization and late by a grant from the Dutch Cancer Society. At the same time he started working on his Ph.D. thesis under the supervision of Prof. Bob Löwenberg.