Flow cytometric analysis of growth factor receptor expression on hemopoietic progenitors

Flowcytometrische analyse
van groeifaktor receptor expressie
op hemopoietische voorlopercellen

PROEFSCHRIFT

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Theories come and go,
the frog stays

François Jacob,
Institut Pasteur, Paris, France

aan Robbert

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ABBREVIATIONS

AA	amino acids
B:P	biotin:protein (ratio)
BFU-E	erythroid burst-forming unit
BM	bone marrow
BSA	bovine serum albumin
cDNA	complementary DNA
CFU-C	colony-forming unit in culture
CFU-E	erythroid colony-forming unit
CFU-GM	granulocyte/ macrophage colony-forming unit
CFU-S	spleen colony-forming unit
CML	chronic myeloid leukemia
D:P	DIG:protein (ratio)
DIG	digoxigenin
DMSO	dimethyl sulfoxide
EPO	erythropoietin
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FDC-P1	factor-dependent cell line - clone P1
FISH	fluorescent in situ hybridization
FITC	fluorescein isothiocyanate
FLS	forward light scatter
GAM	goat anti-mouse antibodies
GARa	goat anti-rat antibodies
GF	growth factor
GF-R	growth factor receptor
GM-CSF	granulocyte/ macrophage colony-stimulating factor
G-CSF	granulocyte colony-stimulating factor
H58	Hoechst 33258
HH	Hanks' Hepes buffered salt solution
HSA	HH/ serum/ azide
HSC	hemopoietic stem cell

IL-	interleukin-
IP	immunoprecipitation
LTRA	long-term repopulating ability
M-CSF	macrophage colony-stimulating factor
mAb	monoclonal antibody
NHS	*N*-hydroxy succinimide
PB	peripheral blood
PBS	phosphate-buffered saline solution
PCR	polymerase chain reaction
PE	PhycoErythrin
PerCP	peridinin chlorophyll protein
PHA	phytohemagglutinin
PI	propidium iodide
PLS	perpendicular light scatter
PMUE	pregnant mouse uterus extract
PO	peroxidase
PSA	PBS/ serum/ azide
R	receptor
RELACS	Rijswijk experimental light activated cell sorter
Rh123	Rhodamine 123
RT-PCR	reverse transcriptase polymerase chain reaction
SCF	stem cell factor (= KL, Kit ligand / MGF, mast cell growth factor / SF, Steel factor)
SCID	severe combined immunodeficiency
STRA	short-term repopulating ability
TBS	Tris-buffered saline solution
TPO	thrombopoietin
WGA	wheat germ agglutinin

CHAPTER 1

General introduction

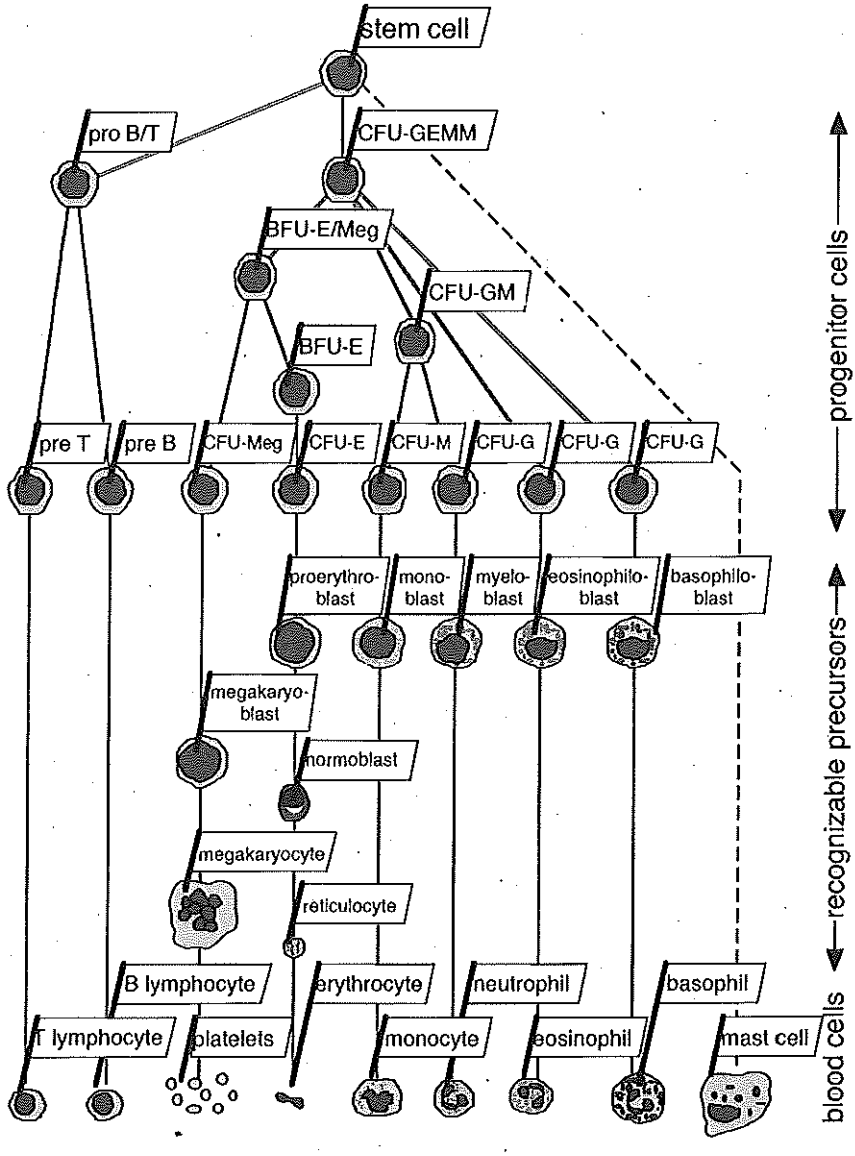
1.1 Hemopoiesis

Blood cells fulfill a number of important functions, including O_2 and CO_2 transport (erythrocytes), blood clotting (platelets), specific immunity and antibody production (lymphocytes), and nonspecific defense against pathogens (monocytes/ macrophages and granulocytes). Most blood cells have a limited life span, varying from several hours to several months. Replacement of these cells requires a daily production of approximately 2 x 10^{11} red and 1.5 x 10^{11} white blood cells in an average human adult. Under changed environmental conditions (e.g., low atmospheric oxygen tension), or pathologic conditions, such as blood loss, tissue damage, or infections, this production capacity may be increased at least 10-fold. The process of blood cell formation, hemopoiesis, takes place mainly in the bone marrow (BM) in adult mammals. In fetal hemopoiesis, liver and spleen also play an important role. There is residual hemopoiesis in the spleen of adult mice.

All blood cells are derived from a small pool of pluripotent hemopoietic stem cells (HSC) [McCulloch, 1983] [Metcalf, 1989]. Most of these are kept in a quiescent state, only a small subset of HSC is actively proliferating at any moment [Becker et al., 1965] [Moore, 1991]. This population is responsible for the formation of daughter cells, which after progressive cell divisions gradually lose their multipotent differentiation potential and acquire the characteristic phenotypic and functional properties of the individual blood cell lineages (**figure 1.1**). These committed progenitors undergo terminal differentiation into mature blood cells, which are released into the circulation.

The mechanism by which the HSC population is maintained and develops into mature blood cells is still incompletely understood. It is possible that blood cell formation is initiated by clonal expansion of activated HSC with asymmetrical cell divisions, resulting in simultaneous formation of new HSC that maintain the stem cell pool as well as committed descendants that eventually differentiate into mature blood cells [Holtzer et al., 1972]. Alternatively, analogous to oocyte development, there might be a limited pool of HSC, which are activated successively and undergo terminal differentiation into mature blood cells [Rosendaal et al., 1979]. According to this theory, the daughter cells are not identical to the parent cell, indicating that 'self renewal' of HSC does not take place. In agreement with such a hypothesis, a decrease in the mean telomere length during proliferation of immature hemopoietic cells into more differentiated precursors has been observed [Vaziri et al., 1994] [Lansdorp, 1995]. Supported by experimental data that demonstrated limits to the self renewal capacity of the stem cell compartment after stress to the hemopoietic system [Mauch et al., 1988], this would make depletion of the HSC compartment conceivable. However, since the daughter cells that result from the first divisions of a HSC may still be pluripotent, the HSC compartment could be effectively replenished when part of these cells return to a quiescent state.

figure 1.1
Schematic representation of hemopoietic stem cell differentiation.



Several models for the mechanism that regulates differentiation of HSC into the different blood cell lineages have been proposed. Commitment to differentiation may be the result of a stochastic process [Till et al., 1964] [Nakahata et al., 1982] [Tsuji and Nakahata, 1989]. According to this hypothesis, commitment to a specific lineage is determined by intrinsic properties of the cell itself and cannot be influenced by external factors. Alternatively, cells may be directed to enter a particular lineage by specific microenvironmental stimuli and/ or lineage-specific hemopoietic growth factors [Curry and Trentin, 1967] [Van Zant and Goldwasser, 1977]. According to this model, the direction HSC differentiation will take is determined by the relative concentration of stimulatory and inhibitory signals. However, although in the stochastic model HSC commitment is an intrinsic property of the HSC itself, whether or not these cells will proliferate or differentiate is still dependent on the availability in the microenvironment of the appropriate GFs and other stimuli to which those cells can respond. Therefore, these models are not necessarily mutually exclusive.

1.2 Hemopoietic growth factors (GFs)

Growth factors (GFs) play an important role in the maintenance of viability [Iscove, 1977] [Wagemaker et al., 1979] [Tushinski et al., 1982] [Williams and Broxmeyer, 1988] [Williams et al., 1990] [Koury and Bondurant, 1990] [Brandt et al., 1994] [Keller et al., 1995] and the stimulation of proliferation and differentiation of cells (reviewed by [Metcalf, 1989] [Moore, 1991]). Hemopoietic GFs are produced locally by stromal cells or hemopoietic cells in the hemopoietic organs, by mature blood cells, by endothelial cells, and by specialized cells in various organs, such as lungs (granulocyte/ macrophage colony stimulating factor, GM-CSF), brain (interleukin-3, IL-3) pancreas (IL-6), kidneys and liver (erythropoietin, EPO). A short summary of the cells that produce hemopoietic GFs is given in **table 1.1**. It should be noted, that some of the data on GF production were based on activities in various biological assays and that production has not in all studies been confirmed by neutralization with GF-specific antibodies or by detection of GF RNA expression in homogeneous cell populations.

Initially, GFs were purified from various natural sources, such as urine (EPO [Miyake et al., 1977] and M-CSF [Motoyoshi et al., 1978]), murine lung cells (G-CSF [Nicola et al., 1983]), or human tonsil cells (IL-2 [Robb and Smith, 1981]), or from GF-producing cell lines (e.g. stimulated Jurkat cells for IL-2 [Gillis et al., 1982], and WEHI-3 cells for IL-3 [Ihle et al., 1982]). Large quantities of recombinant GFs have become available for research and clinical purposes since the molecular cloning of the GF genes and the production of complementary DNA (cDNA), and the development of techniques that enabled expression and large scale production of recombinant GFs in bacteria, yeast, and mammalian cells.

Many of the hemopoietic GFs, which are all glycoproteins, share the same basic architecture, a four α helix bundle. This has been demonstrated for IL-2 [Brandhuber et al., 1987] [Bazan, 1992], IL-3 [Feng et al., 1995], IL-4 [Powers et al., 1992] [Walter et al., 1992b] [Powers et al., 1993], IL-5 [Walter et al., 1992b], IL-6 [Van Dam et al., 1993], GM-CSF [Diederichs et al., 1991] [Walter et al., 1992a], G-CSF [Zink et al., 1992] [Hill et al., 1993] [Werner et al., 1994] [Zink et al., 1994], M-CSF [Pandit et al., 1992], SCF [Bazan, 1991] [Matous et al., 1996], EPO [Krantz, 1991], and thrombopoietin (TPO) [Gurney et al., 1995]. Some properties of these GFs are discussed below.

table 1.1.a. GF production by different cells (overview).

	produced by					
	Stroma[a]	Lymphocytes	Monocytes	Granulocytes	Endothelium	other cells[b]
IL-1	+	–	+	–	+	–
IL-2	–	+	–	–	–	–
IL-3	–	+	–	+	–	+
IL-4	–	+	–	+	–	–
IL-5	–	+	–	–	–	–
IL-6	+	+	+	–	+	+
IL-11	+	–	–	–	+	+
GM-CSF	+	+	+	+	+	+
G-CSF	+	–	+	–	+	–
M-CSF	–	+	+	–	+	–
SCF	+	–	–	–	–	–
EPO	–	–	–	–	–	+
TPO	+	–	–	–	–	+

a 'stroma' is used to indicate fibroblastoid cells
b cells in brain (IL-3 and IL-11), pancreas (IL-6), lungs (GM-CSF and IL-11), skin (IL-11), liver and kidneys (EPO and TPO)

IL- = interleukin-
G(M)-CSF = granulocyte (macrophage)-colony stimulating factor
SCF = stem cell factor
EPO = erythropoietin
TPO = thrombopoietin

literature cited: see second part of table

table 1.1.b. GF production by different cells (references).

GF	produced by	references
IL-1	stromal cells	[Le et al., 1987]
	monocytes	[Dinarello et al., 1984]
	endothelial cells	[Libby et al., 1986] [Wagner et al., 1985]
IL-2	lymphocytes	[Cantrell et al., 1988] [Bommhardt et al., 1992] [June et al., 1989] [Gillis and Watson, 1980] [Gillis et al., 1980] [McGuire and Rothenberg, 1987] [Mochizuki et al., 1980b] [Mochizuki et al., 1980a] [Prystowsky et al., 1982]
IL-3	lymphocytes	[Davignon et al., 1988] [Greenberger et al., 1985] [Oster et al., 1989] [Prystowsky et al., 1982] [Wimperis et al., 1989]
	granulocytes	[Kita et al., 1991]
	brain cells	[Farrar et al., 1989]
IL-4	lymphocytes	[Fernandez-Botran et al., 1986] [Ho et al., 1987] [Lichtman et al., 1987]
	granulocytes	[Brunner et al., 1993]
IL-5	lymphocytes	[Enokihara et al., 1989]
IL-6	stromal cells	[Yang et al., 1988]
	lymphocytes	[Horii et al., 1988] [Smeland et al., 1989]
	monocytes	[Gauldie et al., 1987] [Navarro et al., 1989] [Tosato and Jones, 1990]
	endothelial cells	[Jirik et al., 1989] [Sironi et al., 1989]
	pancreas cells	[Campbell et al., 1989]
IL-11	stromal cells	[Du et al., 1994] [Paul et al., 1990]
	endothelial cells	[Elias et al., 1994]
	other cells	[Du and Williams, 1997]
GM-CSF	stromal cells	[Bagby, 1987] [Broudy et al., 1986b] [Lee et al., 1987] [Yang et al., 1988]
	lymphocytes	[Davignon et al., 1988] [Oster et al., 1989] [Prystowsky et al., 1983] [Wimperis et al., 1989]
	monocytes	[Wimperis et al., 1989]
	granulocytes	[Kita et al., 1991]
	endothelial cells	[Bagby et al., 1986] [Broudy et al., 1986a] [Broudy et al., 1987] [Seelentag et al., 1987] [Segal et al., 1987] [Sieff et al., 1987] [Zsebo et al., 1988]
	lung cells	[Kato and Schleimer, 1994] [Sparrow et al., 1985]
G-CSF	stromal cells	[Ogawa et al., 1996] [Yang et al., 1988]
	monocytes	[Oster et al., 1989]
	endothelial cells	[Broudy et al., 1987] [Seelentag et al., 1987] [Zsebo et al., 1988]
M-CSF	lymphocytes	[Pistoia et al., 1987]
	monocytes	[Horiguchi et al., 1986] [Oster et al., 1989]
	endothelial cells	[Seelentag et al., 1987]
SCF	stromal cells	[Boswell et al., 1990] [Nocka et al., 1990]
EPO	kidney cells	[Beru et al., 1986] [Bondurant and Koury, 1986] [Caro and Erslev, 1984] [Erslev et al., 1980] [Koury et al., 1988] [Lacombe et al., 1988] [Schuster et al., 1987] [Schuster et al., 1989]
	liver cells	[Bondurant and Koury, 1986] [Erslev et al., 1980] [Fried, 1972]
TPO	kidney cells	[Cohen Solal et al., 1996] [Sungaran et al., 1997]
	liver cells	[Cohen Solal et al., 1996] [Sungaran et al., 1997]
	stromal cells	[Nagahisa et al., 1996] [Sungaran et al., 1997]

Interleukin-2

IL-2 is produced by activated T lymphocytes (**table 1.1**). Human IL-2 cDNA encodes a polypeptide of 153 amino acids (AA), the N-terminal 20 AA of which are cleaved off in the secretion process, resulting in a mature IL-2 molecule with a molecular weight of 15 kD [Taniguchi et al., 1983]. The sequences of human and murine IL-2 have 64% homology and point to structural differences in the amino-terminal region of the molecule [Brandhuber et al., 1987]. Murine IL-2 exerts some biological activity on human cells [Zurawski et al., 1986], and human IL-2 shows activity on murine cells [Rosenberg et al., 1984]. *N*-glycosylation sites (Asn-X-Ser or Asn-X-Thr) are absent from the reported amino acid sequence of human IL-2 [Taniguchi et al., 1983]. However, *O*-linked carbohydrates might be responsible for the molecular heterogeneity of the protein [Robb and Smith, 1981]. One disulfide bridge is present between 2 cysteine residues and links two of the four α helices of the protein [Bazan, 1992]. Different regions of the IL-2 molecule interact with the three subunits (see section 1.3) of the IL-2 receptor [Bazan, 1992] [Zurawski et al., 1993].

Interleukin-3

IL-3, also known as multi-CSF, is produced by lymphocytes and granulocytes, and expression of IL-3 mRNA in mouse brain cells has also been demonstrated (**table 1.1**). Murine IL-3 is a glycosylated protein with a molecular weight of approximately 28 kD [Ihle et al., 1982]. Of the 166-AA precursor protein, 26 AA are removed to yield mature murine IL-3 [Fung et al., 1984]. Glycosylation at the 4 potential *N*-glycosylation sites of murine IL-3 is not required for its biological activity in vitro or in vivo, since recombinant bacterial IL-3 as well as a chemically synthesized form of nonglycosylated IL-3 are biologically active [Kindler et al., 1986] [Ziltener et al., 1994]. Human IL-3 consists of 133 AA, with 2 *N*-linked glycosylation sites. Two of the 4 cysteine residues that are present in murine IL-3 are also found in human IL-3 [Dorssers et al., 1987]. In both species, the disulfide bridge between these cysteines is required for protein folding and biological activity [Dorssers et al., 1991]. IL-3 acts in a species-specific manner, e.g., rat IL-3 does not induce proliferation of murine BM cells, v.v. [Cohen et al., 1987]. Human IL-3 is much less effective than rhesus monkey (Macaca mulatta) IL-3 in inducing proliferation of rhesus monkey cells, whereas rhesus monkey IL-3 stimulates human hemopoietic cells with similar efficiency as rhesus monkey cells [Burger et al., 1990]. Chimpanzee (Pan troglodytes) IL-3 can also stimulate human hemopoietic cells, whereas IL-3 from tamarin (Sanguinus oedipus) or marmoset (Callithrix jacchus) cannot [Burger et al., 1994a] [Dorssers et al., 1994]. These differences are reflected in the large heterogeneity between the sequences of different primate IL-3 sequences, and this suggests rapid evolutionary changes of the IL-3 gene during primate evolution [Burger et al., 1994b].

Interleukin-4

IL-4, originally termed B cell stimulatory factor-1, is produced by T lymphocytes and granulocytes (**table 1.1**). Mouse and human IL-4 have been reported to contain 140 and 153 AA, respectively [Lee et al., 1986] [Yokota et al., 1986]. Mature human IL-4 consists of 128 AA as a result of post-translational processing and, like murine IL-4, contains 6 cysteine residues and multiple glycosylation sites [Paul and Ohara, 1987] [Solari et al., 1989] [Le et al., 1991]. The in vitro biological activities from glycosylated and unglycosylated human IL-4 are similar [Solari et al., 1989]. Two of the 3 disulfide bridges that are found in human IL-4 are also present in similar locations in the murine molecule. The different positions of the cysteine residues that are linked by the third disulfide bridge result in structural differences between human and murine IL-4 molecules [Walter et al., 1992b]. Therefore, it is not surprising that IL-4 does not cross-react between these species.

Interleukin-5

IL-5 is produced by T lymphocytes (**table 1.1**). Human IL-5 contains 115 AA. IL-5 forms homodimers that are linked by two intermolecular disulfide bonds. The crystal structure reveals that the structural fold of each domain shows similarity to that of IL-2, IL-4, GM-CSF and M-CSF [Milburn et al., 1993]. A particular property of IL-5 is that it forms dimers, in which each 4-helix domain contains the C-terminal helix of one chain and the N-terminal 3 helices of the other chain [Milburn et al., 1993].

Interleukin-6

IL-6, also known under different names, which include B cell stimulatory factor-2, interferon-β_2, and hybridoma/ plasmacytoma GF, is produced by stroma cells, lymphocytes, and monocytes. In addition, IL-6 secretion by endothelial cells and pancreas cells has also been reported (**table 1.1**). Human IL-6 is a 21-kD glycoprotein containing 184 AA with 4 cysteine residues and 2 potential *N*-linked glycosylation sites [Hirano et al., 1986] [Gauldie et al., 1987]. *N*-linked glycosylation sites are not found in murine and rat IL-6, which are probably only *O*-glycosylated. Human IL-6 stimulates both human and murine cells; murine IL-6 is only active on murine cells [Coulie et al., 1989]. Using various chimeric IL-6 proteins, which have been constructed by introducing different domains of the murine molecule into human IL-6, it has been shown that the domain that contains the first α helix [Bazan, 1990] does not determine species specificity of IL-6 [Van Dam et al., 1993].

GM-CSF

GM-CSF is produced by stroma cells, lymphocytes, monocytes, granulocytes, endothelial cells, and lung cells (**table 1.1**). The gene that encodes GM-CSF is located at mouse chromosome 11 or its human analog human chromosome 5, and is closely linked with the IL-3 gene [Barlow et al., 1987] [Yang et al., 1988a]. These genes might

originate from one gene by gene duplication, especially since they are structurally similar, and share overlapping functions. Murine GM-CSF is a protein of 23 kD [Gough et al., 1984]. Human GM-CSF has a relative molecular mass of 20–23 kD and consists of 144 AA, 17 of which are removed from the precursor protein. GM-CSF contains 2 potential *N*-linked glycosylation sites [Kaushansky et al., 1986] and is highly glycosylated. The two glycosylation sites in murine GM-CSF are located at different positions from those in the human molecule [Walter et al., 1992a]. In vitro, deglycosylated human GM-CSF showed increased affinity for its receptor and increased biological activity [Moonen et al., 1987] [Chiba et al., 1990]. The disulfide bridges in the 4-helix GM-CSF molecule are both located at the same end of the molecule [Diederichs et al., 1991]. Human and murine GM-CSF act species-specific. Studies with chimeric human-murine GM-CSF or with neutralizing monoclonal antibodies have shown the importance of the third α helix for biological activity [Kanakura et al., 1991] [Diederichs et al., 1991].

G-CSF

G-CSF is produced by stroma cells, monocytes, and endothelial cells (**table 1.1**). Murine G-CSF has a molecular weight of 24–25 kD [Nicola et al., 1983]. Human G-CSF is a 19-kD protein that contains 174 AA [Nomura et al., 1986]. The *O*-linked glycoside that is present in human G-CSF is not needed for binding to the receptor [Oh-eda et al., 1990]. The 4 cysteine residues that are present in human G-CSF are required for biological activity [Lu et al., 1992]. The 2 disulfide bonds that are formed by these residues are located at opposite ends of the molecule [Hill et al., 1993].

M-CSF

M-CSF is produced by lymphocytes, monocytes, and endothelial cells (**table 1.1**). Human M-CSF has a molecular weight of 65–70 kD [Pistoia et al., 1987]. As a result of alternative splicing three forms of M-CSF exist, consisting of 256, 438, or 554 AA [Pandit et al., 1992]. The COOH-terminus, which contains a transmembrane region, is not required for in vitro activity [Kawasaki and Ladner, 1990]. Soluble as well as cell-surface forms of M-CSF exist [Rettenmier et al., 1987] [Stein et al., 1990], the former of which are generated by proteolytic cleavage of membrane-bound glycoproteins. Biologically active soluble M-CSF is a homodimer [Stanley et al., 1983], consisting of two 4-helix bundles, which contain 3 intramolecular disulfide bridges in each monomer, and one interchain disulfide bond [Pandit et al., 1992]. Human M-CSF stimulates both human and murine cells [Takahashi et al., 1994].

SCF

SCF is produced by stroma cells (**table 1.1**). Similar to M-CSF, SCF is also found in cell surface and soluble forms [Anderson et al., 1990] [Flanagan and Leder, 1990]. Soluble SCF consists of the first 164 or 165 AA of the extracellular domain of the transmembrane

form, and exists in solution as a noncovalently linked homodimer [Martin et al., 1990]. SCF contains *N*-linked as well as *O*-linked glycosylation sites, and 4 cysteine residues [Zsebo et al., 1990b], which are important for the three-dimensional structure of the GF. These cysteines are conserved among SCF, M-CSF, and Flt3 ligand, the receptors of which also share structural similarities (see section **1.3**). As SCF, M-CSF, and Flt3 ligand also have similar intron/ exon structures, these GFs may be derived from a common ancestral gene, although their AA sequences are ≈90% different [Lyman et al., 1994]. Based on its sequence homology with M-CSF, the positions of 4 α helices of SCF have been predicted [Bazan, 1991]. Studies using human/ murine chimeric cDNA constructs, neutralizing monoclonal antibodies, and murine SCF that had been mutated with corresponding residues from M-CSF point to the importance of the first, third, and fourth helix of SCF for its biological activity [Matous et al., 1996]. SCF is partly species specific, as rat SCF supports growth of murine and human cells, but human SCF hardly stimulates murine cells [Martin et al., 1990].

EPO

EPO is produced by cells from kidney and liver (**table 1.1**). Under conditions where the partial O_2 pressure is low, such as at high altitude, the EPO production is enhanced, providing a feedback mechanism for the regulation of red blood cell production. Human EPO is a 30–34-kD glycoprotein of 166 AA [Miyake et al., 1977] [Jacobs et al., 1985] [Lin et al., 1985]. It contains 4 cysteine residues, which are important for protein folding and biological activity [Wang et al., 1985a] [Lai et al., 1986]. EPO contains 3 *N*-linked and 1 *O*-linked glycosylation sites. Glycosylation of EPO is not necessary for its biological activity in vitro, but essential in vivo [Schooley and Mahlmann, 1971] [Dordal et al., 1985] [Tsuda et al., 1990] [Wasley et al., 1991]. EPO shows a high degree of AA conservation between different mammalian species. Murine EPO is active on human erythroid precursor cells and vice versa, and the same is true for EPO of other species, e.g., sheep [Tepperman et al., 1974] [Shoemaker and Mitsock, 1986] .

TPO

TPO is produced by cells from the kidney and the liver and by stromal cells (**table 1.1**). The protein consists of a highly conserved amino-terminal domain that is related to EPO and a unique carboxy-terminal domain [Bartley et al., 1994] [De Sauvage et al., 1994] [Lok et al., 1994]. The mature form of human TPO contains 332 AA: 153 AA in the N-terminal part and 179 AA in the C-terminal domain [Gurney et al., 1995]. Similar to EPO, TPO contains 4 cysteines. Three of the 4 cysteines present in EPO are conserved in TPO, which suggests that the EPO homology domain may assume a 4-helix bundle configuration [De Sauvage et al., 1994] [Gurney et al., 1995]. A variant form of TPO, in which alternative splicing results in a 4 AA deletion in the EPO homology domain of pig, mouse, and human TPO, is not biologically active [Gurney et al., 1995]. The 6 (human

TPO) or 7 (murine TPO) potential *N*-linked glycosylation sites are located in the C-terminal domain of TPO [Bartley et al., 1994] [De Sauvage et al., 1994] [Lok et al., 1994]. This domain is probably highly glycosylated, as the predicted molecular weight for TPO is 38 kD, whereas immunoprecipitation of human TPO shows a protein of 68–85 kD [Gurney et al., 1995]. In vitro culture experiments have shown that this C-terminal domain of TPO is not required for binding and activation of its receptor [De Sauvage et al., 1994]. This region may act to stabilize and increase the half-life of circulating TPO, and might be cleaved off the full-length TPO protein to generate the 18–25 kD and 28–32 kD species of TPO [Bartley et al., 1994] [De Sauvage et al., 1994]. TPO is not strictly species specific: porcine and murine TPO stimulate human receptors, and human TPO is active on the murine receptor, although less than murine TPO [De Sauvage et al., 1994] [Gurney et al., 1995]. Plasma TPO levels appear to be regulated primarily by TPO binding to the c-Mpl receptor on platelets and thereby by the platelet mass [Fielder et al., 1996].

1.3 Hemopoietic GF receptors

Hemopoietic GF receptors belong in majority to two different families based on homologous sequences of the genes [Olsson et al., 1992]. The tyrosine kinase receptor family consists of cell-surface receptors that possess an intrinsic, ligand-sensitive, protein tyrosine kinase activity [Yarden and Ullrich, 1988] [Ullrich and Schlessinger, 1990]. The receptors of this family are classified into three subclasses, based on distinct structural characteristics (**figure 1.2**). Monomeric forms of tyrosine kinase subclass I receptors, which include the epidermal growth factor (EGF) receptor [Ullrich et al., 1984], contain an

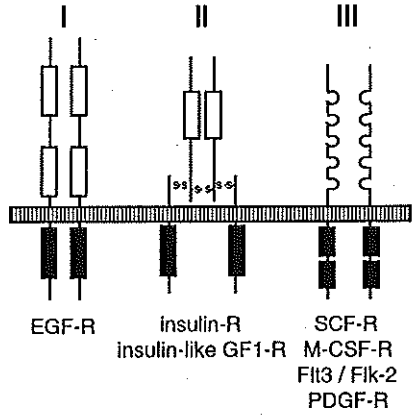


figure 1.2
Schematic representation of tyrosine kinase receptors of different subclasses.
Boxes represent tyrosine kinase regions (black) of the cytoplasmic domains, or cysteine-rich regions (white) within the extracellular domains. Circles represent disulfide-bonded loops.

intracellular tyrosine kinase domain, and two cysteine-rich repeat sequences in the extracellular domain. Similar cysteine-rich sequences and tyrosine kinase domains are found in the subclass II receptors for insulin and insulin-like growth factor I [Ebina et al., 1985] [Ullrich et al., 1986], which consist of disulfide-linked heterotetrameric αβ structures. The third subclass of the tyrosine kinase receptor family is characterized by a bisected intracellular tyrosine kinase domain, and five immunoglobulin-like repeats in the extracellular domain containing 10 conserved cysteine residues and specific flanking sequences [Hanks et al., 1988]. This subclass contains the receptors for SCF [Chabot et al., 1988] and M-CSF [Rettenmier et al., 1985] [Rothwell and Rohrschneider, 1987] [Yeung et al., 1987], the receptor Flt3, also referred to as Flk-2 or STK-1 [Matthews et al., 1991] [Rosnet et al., 1991] [Small et al., 1994], and the receptor for platelet derived growth factor (PDGF) [Yarden et al., 1986] [Gronwald et al., 1988].

The hem(at)opoietin or cytokine receptor superfamily [Cosman et al., 1990] contains most other hemopoietic GF receptors, including the receptors for IL-2 [Sharon et al., 1986] [Hatakeyama et al., 1989] [Takeshita et al., 1992], IL-3 [Itoh et al., 1990], IL-4 [Idzerda et al., 1990], IL-5 [Takaki et al., 1990], IL-6 [Yamasaki et al., 1988], IL-7 [Goodwin et al., 1990], IL-9 [Renauld et al., 1992] [Chang et al., 1994], GM-CSF [Gearing et al., 1989], G-CSF [Fukunaga et al., 1990], EPO [D'Andrea et al., 1989] [Jones et al., 1990], thrombopoietin (TPO) [Vigon et al., 1992], IL-11 [Cherel et al., 1995], and leukemia inhibitory factor (LIF) [Gearing et al., 1992b], and the common receptor units that are mentioned in the next paragraphs. The hemopoietin receptors do not contain intrinsic tyrosine kinase domains and are heterogeneous with respect to the structure of the intracellular domains, which range in length from 54 (GM-CSF receptor) to 568 amino acids (IL-4 receptor). The extracellular domains of these receptors (**figure 1.3**) are identified by four highly conserved cysteine residues (except

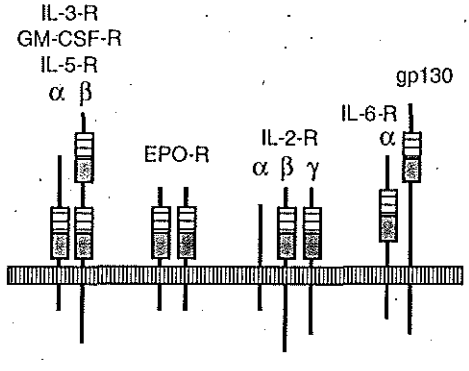


figure 1.3
Schematic representation of examples of hemopoietin receptors.
Boxes represent regions where conserved cysteine residues (striped) or WSXWS residues (shaded) are located within the extracellular domain. Note that the cytoplasmic domains and some parts of the extracellular domains, represented by the rod-like structures, differ considerably.

the IL-7 receptor which has only two) and a five-residue motif of Trp-Ser-X-Trp-Ser (WSXWS), all located within a region of approximately 210 amino acids, which for most receptors is positioned just outside the membrane-spanning domain [Bazan, 1989] [Cosman, 1993].

GF receptor subunits

Most hemopoietic GF receptors function as multi-subunit complexes (**figure 1.2** and 1.3). High affinity class III tyrosine kinase receptors [Ullrich and Schlessinger, 1990] as well as some of the hemopoietin receptors, in particular the receptors for EPO [Ohashi et al., 1994] [Watowich et al., 1994], TPO [Alexander et al., 1995] and G-CSF [Hiraoka et al., 1994] [Horan et al., 1996], are homodimers of single receptor chains. For other receptors the situation is more complex, as high affinity ligand binding and signal transduction require the association of two or more different receptor chains in heterodimeric or hetero-oligomeric complexes. Some of these receptor subunits are used by different receptor systems. For example, the IL-6 receptor consists of a low affinity α subunit and a signal transducing β subunit, gp130 [Taga et al., 1989] [Hibi et al., 1990]. This β subunit is also used by the receptors for IL-11 [Yin et al., 1993] [Fourcin et al., 1994] [Hilton et al., 1994] [Nandurkar et al., 1996], leukemia inhibitory factor (LIF) [Gearing et al., 1992a], oncostatin M [Gearing et al., 1992a], ciliary neurotrophic factor (CNTF) [Ip et al., 1992], and cardiotrophin-1 [Pennica et al., 1995]. This receptor subunit plays an important role in hemopoiesis, as is demonstrated by the severe hemopoietic defects in mutant mouse strains that are deficient for gp130 [Yoshida et al., 1996].

A similar situation as described for these receptors exist for at least two other groups of cytokine receptors. Functional high-affinity receptors for IL-3, GM-CSF, and IL-5 each contain a unique α subunit and a common β subunit, β_c, which is shared by these receptors [Kitamura et al., 1991] [Tavernier et al., 1991] [Sakamaki et al., 1992] [Miyajima et al., 1993]. In addition to the β_c chain, mice have a second, closely homologous β chain, β_{IL-3}, which can replace β_c in the high-affinity receptor for IL-3, but not in the receptors for IL-5 or GM-CSF [Hara and Miyajima, 1992]. Such a second, IL-3 receptor-specific subunit has as yet not been described for other species, and does not seem to occur in primates such as humans.

Receptors for IL-2 consist of an α chain (Tac [Leonard et al., 1984] [Nikaido et al., 1984]), a β chain [Sharon et al., 1986] that is shared with IL-15 [Giri et al., 1994], and a common γ chain [Takeshita et al., 1992] that is shared with the receptors for IL-4 [Kondo et al., 1993], IL-7 [Kawahara et al., 1994], IL-9 [Kimura et al., 1995], and IL-15 [Giri et al., 1994]. Low affinity IL-2 receptors contain only the α chain, which is not necessary for signal transduction. Unlike the other subunits the α chain does not belong to the hemopoietin-receptor family and is not expressed constitutively on resting lymphocytes [Lai et al., 1991]. The β and γ chains do not bind IL-2 individually, but intermediate affinity receptors consist of

heterodimers of β and γ chains [Takeshita et al., 1992]. A 'pseudo-high' affinity IL-2 receptor is formed by interaction of α and β chains [Nakarai et al., 1994], which can only be internalized when combined with the γ subunit. The trimeric high affinity IL-2 receptor has a higher association rate and a lower dissociation rate than the αβ complex [Matsuoka et al., 1993]. Heterodimerization of the β and γ chain is required for signaling of IL-2 [Nakamura et al., 1994] [Nelson et al., 1994] and IL-15 [Giri et al., 1994]. The physiological importance of γ chain is illustrated by severe immunological defects in severe combined immunodeficiency (SCID) patients, who lack a functional gene for this receptor subunit [Matthews et al., 1995] [Pepper et al., 1995] [Leonard, 1996].

The target cell specificity of these GF receptors is primarily determined by the expression of the different α subunits. On cells that coexpress different α chains, responsiveness to the different ligands is influenced by competition for available β subunits. This is dependent on the relative concentration of each receptor subunit, and the affinity of the resulting GF-receptor complex.

1.4 Biological activities of hemopoietic GFs

Analysis of the range of biological activities exerted by individual GFs in vitro and in vivo is essential to identify the differentiation stages and lineages on which GFs act and to establish their physiological role in hemopoiesis. Clonal assays have been developed for analysis of the in vitro proliferation potential of BM cells after plating in semi-solid medium supplemented with GFs [Bradley and Metcalf, 1966] [Guilbert and Iscove, 1976] [Iscove et al., 1980] [Kubota et al., 1983] [Merchav and Wagemaker, 1984] [Eliason and Odartchenko, 1985]. These assays can be used to examine GF actions on unfractionated BM cells as well as on purified HSC and progenitor populations, and are, therefore, of great assistance to identify the differentiation stages and lineages on which individual GFs can act, and to examine interactions between different GFs on defined cell populations.

GF actions on purified human HSC and progenitor cell populations can also be studied in vivo, by transplantation into immunodeficient mice. This allows examination of the effects of different GFs on transplantable stem cells and their progeny in vivo. The GFs can be administered to the cells by regularly injecting the mice with human GFs, or by employing immunodeficient mice carrying human cytokine transgenes. Very few studies have yet been published on GF stimulation of severe combined immunodeficient (SCID) mice transplanted with purified human cells. SCID mice injected with or expressing human IL-3, GM-CSF and SCF show an improved level and duration of engraftment of human BM and cord blood cells [Lapidot et al., 1992] [Bock et al., 1995], but such studies have only been done with unfractionated BM and not with purified HSC or progenitor

cell populations, thus making it difficult to distinguish GF actions on HSC from actions on more mature progenitors.

Effects of GF administration or GF gene overexpression in vivo

Data on the in vivo activities from (pre)clinical studies in which GFs were administered to laboratory animals and human patients, combined with studies using transgenic animals with enforced overexpression of a GF gene, have provided insight into the biological actions of GFs in vivo, their therapeutic usefulness in the clinic as well as adverse side-effects. E.g., in vivo administration of IL-3 to rhesus monkeys resulted in an increase of BM progenitor cell numbers, followed by enhanced production of granulocytes, monocytes, erythrocytes and platelets [Mayer et al., 1989] [Wagemaker et al., 1990]. Formation and activation of basophils after injection of IL-3 led to histamine release and adverse reactions, in particular skin rash, urticaria, and joint swelling [Van Gils et al., 1993] [Van Gils et al., 1995]. In sublethally irradiated mice, IL-3 induced significant recovery of T and B cell populations [Doria et al., 1993], suggesting a role for IL-3 in lymphopoiesis. Possible applications for IL-3 have been shown in the treatment of neutropenia and thrombocytopenia that result from chemotherapy [Ganser et al., 1990] [Ganser, 1993], potentially in combination with other GFs such as GM-CSF [Stahl et al., 1992]. However, recent results with G-CSF and the newly discovered GF thrombopoietin (TPO) suggest that a combination of these GFs will be more effective for these purposes [Neelis et al., in press].

Administration of GM-CSF as a single GF to mice or rhesus monkeys predominantly resulted in increased numbers of macrophages and granulocytes [Metcalf et al., 1987b] [Donahue et al., 1988] [Wielenga, 1990] [Metcalf, 1991]. As injection of GM-CSF has also been shown to enhance the number of myeloid progenitor cells in the peripheral blood [Siena et al., 1989], this GF can be used to mobilize progenitor cells for transplantation purposes [Kessinger et al., 1995]. However, adverse side effects might occur if the malignant cells themselves are stimulated by GM-CSF [Dedhar et al., 1988]. Another possible therapeutic role for GM-CSF is stimulation of immune responses against tumor cells by promoting antigen-presenting cells and antibody-dependent cell cytotoxicity by macrophages [Charak et al., 1993]. It has been shown that genetically modified tumor cells expressing GM-CSF can induce immune responses against pre-existing tumors in mice, and cure the animals from their disease. Similar results have been obtained with other GFs, including IL-6, suggesting potential application of these GFs for immunotherapy against cancer (reviewed by [Gilboa, 1996]).

IL-6 transgenic mice exhibited plasma cell abnormalities and an increased number of granulocyte and monocyte precursors as well as mature granulocytes and megakaryocytes [Hirano et al., 1990] [Kitamura et al., 1995]. Administration of IL-6 to nonhuman primates (cynomolgus monkeys (Macaca fascicularis) and marmosets (Callithrix jacchus)

predominantly resulted in increased peripheral blood thrombocyte counts [Zeidler et al., 1992] [Ryffel et al., 1994]. In addition, thrombocyte counts of irradiated monkeys that were injected with IL-6 did not drop as low as those of control monkeys, and the duration of thrombocytopenia was shorter [Zeidler et al., 1992]. This selective effect on thrombocytopoiesis, combined with the finding that relatively high doses were well tolerated in marmosets [Ryffel et al., 1994], indicates that IL-6 might be useful for the treatment of thrombocytopenic patients. However, the therapeutic usefulness of IL-6 the is limited, as it induces fever and other acute phase reactions in human patients [Weber et al., 1993] [Van Gameren et al., 1994] [D'Hondt et al., 1995] [Schrezenmeier et al., 1995]. Results of a phase I trial of IL-11, which has similar effects on thrombopoiesis [Yonemura et al., 1993], showed no fever after administration of this GF to breast cancer patients [Gordon et al., 1996]. This means that IL-11 has less adverse effects in human patients than IL-6. However, the possible application of IL-11 to alleviate thrombocytopenia has, at least conceptually, become less likely due to the availability of TPO.

A potential role for SCF in alleviation of thrombocytopenia was suggested by elevation of platelet levels after administration of SCF to mice [Chow et al., 1993]. In addition, stimulation of human erythroid colony formation by SCF promised a possible use of this GF to treat anemia in patients with aplastic anemia [Amano et al., 1993]. Moreover, injection of SCF causes an increase of the absolute number of human or murine HSC [Bodine et al., 1993] [Tong et al., 1993], and SCF is very effective for mobilization of stem cells into peripheral blood, especially in combination with G-CSF [Briddell et al., 1993]. However, a serious adverse effect of SCF administration to mice or primates is the expansion and activation of mast cells [Wershil et al., 1992] [Galli et al., 1993]. This also occurred in breast carcinoma patients receiving SCF [Costa et al., 1996]. The acute hypersensitivity-like reaction that results from mast cell activation severely limits the usefulness of SCF as an in vivo therapeutic agent.

Severe adverse effects have not been associated with the clinical uses of EPO or G-CSF. In contrast to the pleiotropic effects of other GFs on various hemopoietic lineages, EPO is a specific regulator of erythropoiesis. EPO administration to hemodialysis patients has been shown to counteract the anemia of renal failure [Winearls et al., 1986] [Eschbach et al., 1987], for which it is now widely used. Injection with recombinant human G-CSF stimulated granulopoiesis in normal hamsters and mice [Cohen et al., 1987], and promoted mobilization of HSC and progenitor cells from BM marrow to blood in mice following treatment with cytotoxic drugs [Neben et al., 1993]. Patients who were treated with G-CSF in a phase I/II clinical trial showed an increase in circulating progenitor cell numbers [Dührsen et al., 1988]. These in vivo effects as well as the absence of significant adverse reactions have permitted the clinical use of G-CSF to treat neutropenic patients and to mobilize HSC and progenitor cells into the circulation for autologous (and allogeneic) transplantation in patients that suffer from malignancies [Morstyn et al., 1994].

Effects of GF (receptor) deficiency in vivo

Analysis of the phenotype of mouse strains that, either naturally or through homologous recombination, lack functional genes for GFs or their receptors, provides another means to examine the physiological functions and target cell range of GFs. Well-known examples of such mouse strains are those that lack functional genes for SCF or its receptor, resulting of mutations of the Steel (*Sl*) [Copeland et al., 1990] [Flanagan and Leder, 1990] [Huang et al., 1990] [Zsebo et al., 1990a] or the white spotting (*W*) locus [Chabot et al., 1988] [Geissler et al., 1988], respectively. The reduced numbers of progenitors and mature blood cells in these mice demonstrate that this GF/ receptor system is indispensable for stem cell outgrowth. However, stem cells are present in the hemopoietic organs of *W/W* embryos, although at reduced numbers, suggesting that Kit is less crucial for the production and survival of stem cells in vivo [Ikuta and Weissman, 1992].

Osteopetrotic (op/op) mice, which do not synthesize biologically active M-CSF as a result of an inactivating mutation, exhibit a severe deficiency in the macrophage lineage [Yoshida et al., 1990]. This defect is progressively corrected when the mice age, which suggests substitution of M-CSF by other GFs. "Knockout" studies have shown, that GM-CSF is not the GF responsible for this correction [Nilsson et al., 1995].

The role of IL-6 has been studied using mice in which either the gene for IL-6 or the gene for the signal transducing subunit of the IL-6 receptor, i.e. gp130 (see section 1.3), has been inactivated by homologous recombination [Bluethmann et al., 1994] [Yoshida et al., 1996]. IL-6 knockout mice develop normally. These mice are very sensitive to infections, which has been attributed to a crucial role for IL-6 in the bactericidal function of macrophages and the T-cell dependent antibody response [Kishimoto, 1994] [Kopf et al., 1994]. Homozygous gp130 mutant mice die before birth. Apart from heart abnormalities, which might be attributed to functional inactivation of cardiotrophin-1, which is one or the other GFs that utilize gp130 (see section 1.3), these embryos have greatly reduced numbers of hemopoietic progenitors in the liver and T cells in the thymus, and approximately 20% of the embryos show impaired erythropoiesis [Yoshida et al., 1996]. Although it is clear that the combination of IL-6/ IL-6 receptor/ gp130 plays an important role in the development of primitive hemopoietic progenitor cells [Sui et al., 1995], the phenotypic differences between IL-6 and gp130 knockout mice demonstrate that other GFs that use gp130 for signal transduction also have important functions in hemopoiesis.

Mice that do not express TPO or its receptor, c-Mpl, develop normally but show decreased platelet counts, whereas other blood cell counts are normal [Gurney et al., 1994] [Alexander et al., 1996] [Carver-Moore et al., 1996]. These results demonstrate that the major physiological role of TPO is platelet production. However, these mice also have reduced numbers of multipotential as well as committed progenitors of multiple lineages in BM, spleen, and peripheral blood [Alexander et al., 1996] [Carver-Moore et al., 1996]. TPO administration to TPO-deficient mice and control mice significantly increased the number

of myeloid, erythroid, and mixed progenitors [Carver-Moore et al., 1996]. This indicates a function for TPO and c-Mpl in the production of primitive pluripotent progenitor cells as well as progenitor cells committed to non-megakaryocytic lineages.

Genetically engineered mice that lack functional genes for GM-CSF or the shared β_c chain of the IL-3, GM-CSF and IL-5 receptors (see section **1.3**) have relatively mild hemopoietic defects [Dranoff and Mulligan, 1994] [Robb et al., 1995]. These mice exhibit lung pathology, probably as a result of a deficiency in lung macrophages, whereas mice that lack the unique IL-3 receptor β chain, which has been found only in mice (see section **1.3**), are normal [Nishinakamura et al., 1995] [Nicola et al., 1996]. Mice that lack β_c and IL-3, and thus have no functional IL-3/ GM-CSF/ IL-5 system, are still viable and have similar defects as the β_c knockout mice [Nishinakamura et al., 1996]. These results demonstrate that IL-3, GM-CSF, and IL-5 are not essential for normal steady state hemopoiesis in vivo. Although this may be true, important physiological functions of these GFs and their receptors in the regulation of stem cell development cannot be excluded, as hemopoietic cell proliferation and differentiation involves the overlapping actions of multiple cytokines. In view of this GF redundancy, it is not possible to obtain an accurate insight into the target cell range and physiological functions of GFs on the basis of the phenotype of knockout mice.

Target cell specificity of hemopoietic GFs

The in vitro and in vivo studies have demonstrated that most GFs display a wide range of biological effects on different cell types, including HSC, committed progenitors at various stages of differentiation, and mature blood cells. A summary of the target cell range of various GFs in hemopoiesis, as derived mainly from functional studies, is shown in **table 1.2.**

In addition to hemopoietic cells, many GFs are also involved in the development and functional activation of nonhemopoietic cell types. These include endothelial cells [Broudy et al., 1987] [Segal et al., 1987] [Sironi et al., 1989] [Zsebo et al., 1988] and stromal fibroblasts [Lee et al., 1987] [Yang et al., 1988b], which produce various GFs in response to IL-1. Other nonhemopoietic target cells for hemopoietic GFs include skin fibroblasts [Katz et al., 1989], pancreatic islet cells [Campbell et al., 1989] and hepatocytes [Nesbitt and Fuller, 1992] (all responsive to IL-6), osteoblasts (stimulated by IL-6 [Kitamura et al., 1995] and GM-CSF [Evans et al., 1989]), brain cells [Manova et al., 1992], melanocytes [Matsui et al., 1990], germ cells [Manova et al., 1993], endometrium and placental tissues [Kauma et al., 1996] (all stimulated by SCF).

table 1.2.a. Target cells for GFs based on biological actions (overview).

	IL-							-CSF			SCF	EPO	TPO
	1	2	3	4	5	6	11	GM	G	M			
Blast/ multilineage progenitors	s/p	–	s/p	p	–	s/p	p	s/p	p	–	s/p	–	p
Monocytic progenitors	p	–	p/d	p[1]	–	p	p	p/d	–	p/d	p	–	p/d
Myeloid progenitors	p	–	p/d	p/d	p	p	p/d/m	p/d	p/d	–	p	–	p/d
Erythroid progenitors	–	–	p	p	–	p	p	p	–	–	p	s/p/d	p/d
Monocytes/ macrophages	g	g	p	(g)[2]	–	d	g/(g)[3]	a	–	s/p/g	–	–	–
Granulocytes:													
neutrophils	–	–	–	a	–	–	–	a	a	–	–	–	–
basophils	a	–	a	–	–	–	–	–	–	–	–	–	–
eosinophils	–	–	–	–	a	–	–	a	–	–	–	–	–
Lymphocytes:													
T	a	p/d	–	p	–	p	g	–	–	–	–	–	–
B	a	a/p	d	a/p	p	m	–	–	–	–	–	–	–

– = no influence reported

The characters **s, p, d, a, m,** and **g** are used to describe the influence that is exerted on the cells (as defined in the respective studies that are cited):

- s = survival
- p = proliferation
- d = differentiation
- a = activation
- m = maturation (final)
- g = stimulation (or abrogation[2,3]) of growth factor production by monocytes/ macrophages or lymphocytes

1 also reported: inhibition of macrophage colony formation by IL-4
2 β interferon synthesis is abrogated by IL-4
3 macrophage inflammatory cytokine production is down-regulated by IL-11

literature cited: see second part of table

table 1.2.b. Target cells for GFs based on biological actions (references).
A. Progenitors.

progenitors	GFs		references
Blast/ Multilineage	IL-1	-	[Ikebuchi et al., 1988b] [Lardon et al., 1994] [Stanley et al., 1986]
	IL-3	-	[Bot et al., 1988] [Garland and Crompton, 1983] [Ihle et al., 1983] [Ikebuchi et al., 1987] [Ikebuchi et al., 1988a] [Leary et al., 1988] [Monette and Sigounas, 1988a] [Monette and Sigounas, 1988b] [Ottmann et al., 1989] [Saeland et al., 1988] [Spivak et al., 1985] [Suda et al., 1985]
	IL-4	-	[Keller et al., 1994] [Kishi et al., 1989] [Migliaccio et al., 1989/1990]
	IL-6	-	[Ikebuchi et al., 1987] [Ikebuchi et al., 1988b] [Koike et al., 1988] [Lardon et al., 1994] [Leary et al., 1988]
	IL-11	-	[Du and Williams, 1997] [Hirayama et al., 1992] [Holyoake et al., 1996]
	GM-CSF	-	[Metcalf et al., 1980] [Metcalf et al., 1986c] [Sieff et al., 1985] [Strife et al., 1987]
	G-CSF	-	[Ikebuchi et al., 1988a] [Ikebuchi et al., 1988b] [Suda et al., 1987]
	SCF	-	[Han, 1992] [Lardon et al., 1994] [Migliaccio et al., 1991c]
	TPO	-	[Ku et al., 1996] [Sitnicka et al., 1996]
Monocytic/ Myeloid	IL-1	-	[Gasparetto et al., 1989]
	IL-3	-	[Bot et al., 1988] [Ema et al., 1990] [McNiece et al., 1989] [Metcalf et al., 1987a] [Migliaccio et al., 1988a] [Migliaccio et al., 1988b] [Migliaccio et al., 1991b] [Monette and Sigounas, 1988a] [Monette and Sigounas, 1988b] [Ottmann et al., 1989] [Quesenberry et al., 1985] [Sonoda et al., 1988]
	IL-4	-	[Jansen et al., 1989] [Kajitani et al., 1989] [Kishi et al., 1989]
	IL-5	-	[Clutterbuck and Sanderson, 1988] [Clutterbuck et al., 1989] [Dvorak et al., 1989] [Ema et al., 1990]
	IL-6	-	[Bot et al., 1989] [Koike et al., 1988] [Caracciolo et al., 1989b] [Hoang et al., 1988]
	IL-11	-	[Du and Williams, 1997] [Musashi et al., 1991]
	GM-CSF	-	[Johnson and Burgess, 1978] [Kaushansky et al., 1986] [Caracciolo et al., 1989b] [McNiece et al., 1989] [Merchav and Wagemaker, 1984] [Metcalf and Merchav, 1982] [Metcalf et al., 1986b] [Metcalf et al., 1986c] [Sieff et al., 1985] [Sonoda et al., 1988] [Strife et al., 1987] [Tomonaga et al., 1986] [Ventura et al., 1990]
	G-CSF	-	[Avalos et al., 1990] [Begley et al., 1988] [Caracciolo et al., 1989a] [Cohen et al., 1987] [De Haan et al., 1994] [Demetri and Griffin, 1991] [Ema et al., 1990] [Ieki et al., 1990] [McNiece et al., 1989] [McNiece et al., 1991] [Metcalf and Nicola, 1983] [Migliaccio et al., 1989] [Strife et al., 1987] [Suda et al., 1987] [Tamura et al., 1987]
	M-CSF	-	[Bicknell et al., 1988] [Bot et al., 1989] [Guilbert and Stanley, 1980]
	SCF	-	[De Haan et al., 1994] [Liesveld et al., 1995] [McNiece et al., 1991] [Migliaccio et al., 1991c]
	TPO	-	[Alexander et al., 1996] [Carver-Moore et al., 1996]
Erythroid	IL-3	-	[Bot et al., 1988] [McNiece et al., 1991] [Migliaccio et al., 1988a] [Migliaccio et al., 1988b] [Monette and Sigounas, 1988a] [Monette and Sigounas, 1988b] [Ottmann et al., 1989] [Saeland et al., 1988] [Sonoda et al., 1988] [Wright Goodman et al., 1985]
	IL-4	-	[Kishi et al., 1989] [Peschel et al., 1987]
	IL-6	-	[Ulich et al., 1991]
	IL-11	-	[Du and Williams, 1997] [Quesniaux et al., 1992]
	GM-CSF	-	[Donahue et al., 1985] [Kaushansky et al., 1986] [McNiece et al., 1991] [Metcalf et al., 1980] [Metcalf et al., 1986a] [Metcalf et al., 1986c] [Migliaccio et al., 1988a] [Ottmann et al., 1989] [Sieff et al., 1985] [Sonoda et al., 1988] [Strife et al., 1987]
	SCF	-	[Dai et al., 1991] [De Haan et al., 1994] [McNiece et al., 1991] [Sui et al., 1996] [Migliaccio et al., 1991c]
	EPO	-	[Iscove, 1977] [De Haan et al., 1994] [Goldwasser et al., 1974] & [Gregory, 1976] & [Haga and Falkanger, 1979] & [Iscove, 1977] [McNiece et al., 1991] [Migliaccio et al., 1988a] [Migliaccio et al., 1988b] [Stephenson et al., 1971] [Tepperman et al., 1974] [Ventura et al., 1990] [Wagemaker et al., 1977] [Wagemaker et al., 1979]
	TPO	-	[Alexander et al., 1996] [Carver-Moore et al., 1996]

B. More mature cells.

cells	GFs	references
Monocytes/ Macrophages	IL-1	[Ruppert and Peters, 1991] [Tosato and Jones, 1990]
	IL-2	[Numerof et al., 1988]
	IL-3	[Chen and Clark, 1986] [Whetton et al., 1986]
	IL-4	[Nickolaus and Zawatzky, 1994] [Novak et al., 1990] [Ogawa et al., 1991] [Sampson et al., 1991] [Zlotnik et al., 1987]
	IL-6	[Ruppert and Peters, 1991]
	IL-11	[Trepicchio et al., 1996]
	GM-CSF	[Dranoff and Mulligan, 1994] [Hamilton et al., 1980] [Handman and Burgess, 1979] [Johnson and Burgess, 1978] [Wang et al., 1985b]
	M-CSF	[Becker et al., 1987] [Chen and Clark, 1986] [Guilbert and Stanley, 1980] [Hamilton et al., 1980] [Stanley et al., 1983] [Tushinski and Stanley, 1983] [Tushinski et al., 1982] [Tushinski and Stanley, 1985] [Warren and Ralph, 1986] [Whetton et al., 1986]
Granulocytes	IL-1	[Subramanian and Bray, 1987]
	IL-3	[Brunner et al., 1993] [MacDonald et al., 1989] [Mayer et al., 1989] [Valent et al., 1989]
	IL-4	[Bober et al., 1995]
	IL-5	[Clutterbuck and Sanderson, 1988] [Enokihara et al., 1988]
	GM-CSF	[Kaufman et al., 1989] [Dranoff and Mulligan, 1994] [Lopez et al., 1983] [Mayer et al., 1987] [Metcalf et al., 1986a] [Schaafsma et al., 1989] [Stanley and Burgess, 1983]
	G-CSF	[Avalos et al., 1990] [Begley et al., 1988] [Burgess and Metcalf, 1980] [Demetri and Griffin, 1991] [Lopez et al., 1983] [Nicola and Metcalf, 1985]
Lymphocytes	IL-1	[Emilie et al., 1988] [Falkoff et al., 1983] [Lipsky et al., 1983] [Maizel et al., 1981] [Tosato et al., 1990]
	IL-2	[Croft and Swain, 1991] [Farrar et al., 1980] [Gillis and Watson, 1980] [Gillis et al., 1980] [Lattime et al., 1983] [Mochizuki et al., 1980b] [Mochizuki et al., 1980a] [Mookerjee et al., 1989] [Tigges et al., 1989] [Weiss, 1989]
	IL-3	[Djeu et al., 1983] [Hapel et al., 1981] [Ihle et al., 1981] [Lattime et al., 1983] [Tadmori et al., 1989]
	IL-4	[Croft and Swain, 1991] [Defrance et al., 1989] [Elenström and Severinson, 1989] [Fernandez-Botran et al., 1986] [Fernandez-Botran et al., 1989] [Lee et al., 1986] [O'Garra et al., 1986] [Ohara and Paul, 1987] [Smeland et al., 1989] [Yokota et al., 1986]
	IL-5	[Harada et al., 1987] [Migita et al., 1990]
	IL-6	[Croft and Swain, 1991] [Emilie et al., 1988] [Helle et al., 1989] [Hirano et al., 1986] [Tosato et al., 1990]
	IL-11	[Anderson et al., 1992]

IL- = interleukin-
G(M)-CSF = granulocyte (macrophage)-colony stimulating factor
SCF = stem cell factor
EPO = erythropoietin
TPO = thrombopoietin

Hemopoietic effects of GFs can be the result of direct stimulation of HSC and progenitors, but also of indirect mechanisms, e.g., through stimulating the release of secondary cytokines from the hemopoietic cells themselves and from nonhemopoietic sources [Broudy et al., 1987] [Hagiwara et al., 1987] [Fibbe et al., 1988] [Numerof et al., 1988] [Helle et al., 1989] [Hültner et al., 1989] [Schaafsma et al., 1989] [Sironi et al., 1989] [Smeland et al., 1989] [Bot et al., 1990] [Tosato and Jones, 1990] [Migliaccio et al., 1991b], or by transmodulating cell

surface receptors for other GFs [Von Hoegen et al., 1989] [Sato et al., 1993] [Hanazono et al., 1995]. This aspect of GF actions makes it difficult to identify the target cells of an individual GF on the basis of its biological actions alone, in particular when heterogeneous cell populations such as unfractionated BM are used. Studies of GF target cell specificity are less obscured by indirect actions via mature cells or nonhemopoietic cells when purified progenitors are used. However, the production of differentiated blood cells usually requires the actions of multiple GFs, and may involve the activity of inhibitory cytokines as well as direct interactions between different cells. Various GFs may act simultaneously on the same cell type and provide synergistic, additive or antagonistic stimuli [Ikebuchi et al., 1987] [Ikebuchi et al., 1988b] [McNiece et al., 1988] [Migliaccio et al., 1988b] [Warren and Moore, 1988] [Bot et al., 1989] [McNiece et al., 1991] [Okada et al., 1992] [De Haan et al., 1994] [Lardon et al., 1994], or they may act sequentially on cells in successive differentiation stages. Therefore, as the endpoints of most biological assays involve the proliferation of the target cells and the production of mature blood cells, it is not possible to distinguish between GF actions on the purified target cells themselves and on their progeny that is produced in culture.

To understand at what differentiation stages an individual GF acts, how it stimulates the survival and/ or outgrowth of the cells, and how different GFs interact on HSC and on more differentiated cells of various hemopoietic lineages it is necessary to identify the cell types that can be directly stimulated by these GFs. Analysis of GF receptor expression on HSC and of changes in GF receptor expression during differentiation of different blood cell lineages provides the most straightforward means to examine the target cell range and thus the possible spectrum of biological activities of GF within the hemopoietic system.

1.5 Examination of GF receptor expression on hemopoietic cells

Different techniques can be used to examine GF receptor expression. The structure of receptor proteins is usually examined by immunoprecipitation of antibody/ receptor- or GF/ receptor-complexes. Similar approaches have been used to study phosphorylation of the receptor after GF stimulation, and to identify interactions with intracellular signaling molecules [Pawson and Gish, 1992] [Budel et al., 1995] [Ihle, 1995].

Receptor RNA expression can be detected using various techniques, including reverse transcription polymerase chain reaction (RT-PCR) (reviewed by [Bell, 1989]) and RNA blotting combined with receptor-specific probes. To gather information about gene expression in a specific subset of hemopoietic cells, it is important that a pure, homogeneous cell population is used, as this technique is sensitive enough to pick up a signal from very low numbers of cells, which may include contaminating cells unrelated

to the cells studied. In addition, this approach does not provide a decisive answer about the cell surface expression of receptor proteins, because of the possibility of discrepancies between RNA levels and protein expression [Migliaccio et al., 1991a] [Hara and Miyajima, 1994].

Quantitative binding experiments, using radioactively labeled GFs, provide information about the average number of receptors per cell, receptor binding kinetics and affinity for the GF. These techniques can also be used to study modulation of receptor levels as a result of GF binding [Fukamachi et al., 1987] [Elbaz et al., 1991a] [Elbaz et al., 1991b] and to examine competition between different GFs [Lopez et al., 1989] [Budel et al., 1990] [Lopez et al., 1991], and have been essential for the identification of receptor subunits that are shared between different GFs. However, such binding studies are not suitable to examine heterogeneous cell populations, as the resulting data represent an average for the whole population. Purified cell populations can be used, but only if sufficient cell numbers can be isolated. The requirement for large cell numbers precludes the use of quantitative binding studies to analyze receptor expression on rare subsets of immature hemopoietic cells. Although it is possible to deduce information about subsets of rare cells by comparing receptor numbers on unfractionated cell populations with those of a cell fraction from which the immature cells have been depleted [Testa et al., 1993], it is doubtful whether the data obtained by such an indirect approach are meaningful, if accurate.

Radioactively labeled GFs are also used for in situ autoradiography, to analyze receptor distribution on cells that can be recognized morphologically. Since multipotent cells comprise a low percentage of the total population of hemopoietic cells, and probably show little morphological heterogeneity, this technique is less suitable for analysis of receptor expression on HSC and immature progenitors. Autoradiography can provide additional information about receptor localization on the membrane or inside the cell, especially when it is combined with electron microscopy [Carpentier et al., 1981]. Electron microscopic analysis of receptor localization can also be performed after cell staining with ferritin- or gold-labeled GF molecules [Haigler et al., 1979] [McKanna et al., 1979] [Boonstra et al., 1985].

Binding of fluorescently labeled GFs or anti-receptor antibodies to cells can be examined by fluorescence microscopy [Dunn et al., 1986] [Carpentier et al., 1987]. A more refined technique, employing confocal scanning laser microscopy (CSLM), might provide additional information about the location of the receptors on or inside the cells. Similar to in situ autoradiography or electron microscopy, fluorescence microscopy has the disadvantage that it is most suitable to examine homogeneous cell populations.

Heterogeneous cell populations can be identified by flow cytometry. Since HSC and progenitors make up only approximately 1% of the nucleated BM cells, and the frequency of HSC in BM has been estimated to be in the range of 1–2 per $1x10^5$ [Harrison et al., 1988]

[Van der Loo et al., 1994], cell samples for flow cytometry are often pre-enriched on basis of differences in physical properties, such as size or density, between immature and more mature cells [Visser and Van Bekkum, 1990]. In the flow cytometer, different subsets of blood or BM can be analyzed after staining with antibodies against various cell surface markers. Additional criteria such as cell size and structure, which are revealed by flow cytometry as the light scatter properties of the cells, can be employed to differentiate between BM subsets [Visser et al., 1980]. Moreover, affinity for the lectin wheat germ agglutinin (WGA) or retention of Rhodamine-123 (Rh123) [Bertoncello et al., 1985] [Ploemacher and Brons, 1988] are often used to purify immature hemopoietic cells [Visser and Bol, 1982] [Ploemacher et al., 1987] [Bauman et al., 1988]. The DNA stain Hoechst 33342 can also be employed to distinguish different subsets of BM cells [Baines and Visser, 1983]. Antigens such as CD34, which is specific for immature cells, are widely used to identify, isolate and characterize primitive hemopoietic cells from human, monkey, or, more recently, murine origin [Civin et al., 1984] [Krause et al., 1994]. Other examples of antigens that are frequently used for positive selection of multipotent cells are Sca-1, which is expressed on immature hemopoietic cell from mice of the Ly6A/E haplotype [Spangrude et al., 1988], or Thy-1, which is expressed on immature murine, rat, and human BM cells [Müller-Sieburg et al., 1986] [McCarthy et al., 1987] [Craig et al., 1993]. Negative selection after staining with antibodies that bind specifically to activated or committed cells is also possible. Examples of these antigens are B220 and CD19, which are present on B lymphocytes [Müller-Sieburg et al., 1986] [Loken et al., 1987a], CD33, which is most strongly expressed on myelomonocytic cells [Andrews et al., 1989], CD38, which is expressed on cells that are committed to the erythroid, myeloid, and lymphoid lineages [Terstappen et al., 1991], the transferrin receptor CD71, which is highly expressed on activated and erythroid cells [Loken et al., 1987b], and the class II histocompatibility antigen HLA-DR [Sutherland et al., 1989]. Combination of positive selection for immature cells and negative selection of mature cells allows identification and isolation of different hemopoietic cell subsets that differ in their degree of differentiation and commitment to different lineages. By combining antibodies to these different cell surface markers with anti-receptor antibodies or labeled GFs, it is possible to identify receptor expression on different hemopoietic subsets and to monitor changes in receptor expression patterns during commitment and lineage-specific differentiation. Using this approach it is also possible to isolate (different subsets of) receptor expressing cells for morphological analysis, in vitro culture, or transplantation purposes, and thus to functionally characterize different hemopoietic cell subsets separated on the basis of their receptor phenotype.

1.6 Objectives and outline of this thesis

The purpose of this study was the development and application of methodology to examine receptor expression on hemopoietic cells by flow cytometry. This method was to be used to detect GF receptors on HSC, and to examine GF receptor expression patterns during lineage commitment and differentiation of various blood cell precursors from HSC to mature blood cells. One of the main objectives was to examine receptor expression in relation to other phenotypic parameters, and to isolate viable cells on the basis of receptor phenotype for morphological and functional analysis. As outlined in section **1.5**, flow cytometry using either receptor-specific antibodies or labeled GF molecules is the method of choice to achieve these purposes, as this enables identification of receptor-expressing cells within heterogeneous cell populations (such as unfractionated BM) and identification of qualitative differences in receptor density on different cells within these populations as well as isolation of receptor-expressing cells by FACS.

We focused on methodology to detect GF receptor expression on the basis of the ability of receptor-positive cells to bind ligand, since cell staining based on binding of ligand is a better predictor for the ability of cells to bind and respond to GFs than methods that employ receptor-specific antibodies. Binding of ligand is indicative of expression of functional high affinity receptors, and specificity of binding can be checked in competition experiments using a combination of labeled and non-labeled ligand. In contrast, anti-receptor antibodies recognize individual subunits of multi-subunit GF receptor complexes and may also bind to nonfunctional receptor isoforms. Moreover, antibodies often show variable levels of binding to non-receptor proteins such as F_C receptors, which is difficult to eliminate or distinguish from specific binding to the receptors.

As GF labeling reagent we chose biotin, which has a high affinity for the egg white protein avidin or its bacterial equivalent streptavidin ($K_d = 10^{-15}$ M) [Guesdon et al., 1979] [Hnatowich et al., 1987] [Wilchek and Bayer, 1988]. The feasibility of preparing and using biotin-labeled GFs to assess receptor expression by flow cytometry was examined in the studies described in **chapter 2**. For these studies, the recombinant hemopoietic GFs IL-2, IL-3, IL-6, GM-CSF, and SCF were biotinylated with retention of their biological activity. We demonstrated that these biotinylated GFs can be used to stain receptor-positive cell lines, peripheral blood lymphocytes, and BM cells when combined with fluorescently labeled (strept)avidin. After amplification of the fluorescence signal, it was possible to detect cells with receptor levels as low as 100 molecules per cell.

In **chapter 3**, we compared various methods and biotinylation reagents to label GFs with biotin for flow cytometric analysis of receptor expression. Using IL-2 as a model for hemopoietic GFs, the most suitable biotin derivatives were selected. Optimal biotin-to-protein ratios and reaction conditions were established to prepare biologically active

biotinylated GFs and achieve maximal specific staining and highest fluorescence intensity on receptor-expressing target cells.

In the study described in **chapter 4**, murine BM hemopoietic cells were isolated on the basis of Kit expression, using flow cytometry after cell staining with anti-Kit antibodies or biotinylated SCF. A higher percentage of BM cells reacted with anti-Kit than with biotin-SCF, suggesting differences in target cell specificity between SCF and anti-Kit. Functional characteristics of Kit-expressing hemopoietic cells were examined, and the presence of short- and long-term repopulating cells in the Kit^+ BM fraction was demonstrated using a quantitative mouse transplantation model.

The experiments described in **chapter 5** were designed to examine the differential staining between biotin-SCF and anti-Kit antibodies that was found on BM cells in the study described in **chapter 4**. Results of these two staining methods for Kit^+ cells were compared on an SCF growing subline and an IL-3 growing subline of the murine cell line FDC-P1. In an attempt to elucidate the molecular basis for observed differences between ligand binding and antibody reactivity of the two sublines, Kit molecules were isolated using immunoprecipitation, and RNA expression of different functional domains of the *c-kit* gene was studied using RT-PCR.

Staining with biotinylated GFs can be combined with analysis of other cell surface markers. Biotinylated human SCF and antibodies against CD34 were used in **chapter 6** for immunophenotyping, isolation, and functional analysis of different subsets of Kit-expressing hemopoietic cells in rhesus monkey BM. After cell sorting based on expression of CD34 and Kit, we showed that myeloid and erythroid progenitors can be distinguished and isolated on the basis of differences in Kit expression levels.

The feasibility to simultaneously stain functional receptors for different GFs was examined in the study described in **chapter 7**. For this purpose, we combined biotin-SCF with digoxigenin (DIG) labeled preparations of EPO, IL-6, and GM-CSF, and with antibodies against other cell surface markers. This technique was applied to analyze patterns of coexpression of Kit and the receptors for these GFs on immature progenitors and differentiating erythroid and myelomonocytic precursors.

In **chapter 8**, the differential expression of the receptors for IL-3, IL-6, GM-CSF, and SCF on subsets of BM cells is reviewed.

In the final chapter, **chapter 9**, the results of our study are summarized and discussed. Limitations and possibilities of the methodology are reviewed, and possible approaches for further research are outlined, aimed at examination of the presence and functional significance of low level receptor expression on HSC and immature progenitors.

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CHAPTER 2

A sensitive method to detect cell surface receptors using biotinylated growth factors

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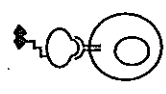

2.1 ABSTRACT

In this study, the feasibility of biotin-labeled growth factors (GFs) to analyze the expression of GF receptors on hemopoietic cells was examined. The recombinant hemopoietic GFs interleukin-2 (IL-2), IL-3, IL-6, granulocyte/ macrophage-colony stimulating factor (GM-CSF), and stem cell factor (SCF) were biotinylated. Examination of the ability of the labeled GFs to stimulate proliferation of GF-dependent cell lines showed that the biotin-GFs remained biologically active. Cells were stained with biotinylated GFs and (strept)avidin-FITC. The fluorescence signal was amplified with alternate layers of biotinylated anti-(strept)avidin and (strept)avidin-FITC, and the samples were analyzed using a flow cytometer. With this highly sensitive method, cells with 100 cell surface receptor molecules were detectable.

2.2 INTRODUCTION

Hemopoietic stem and progenitor cells have low numbers of growth factor (GF) receptors on their surface. After binding to these receptors, hemopoietic GFs regulate the survival, proliferation and differentiation of the primitive hemopoietic cells. The exact relationship between the expression of certain GF receptors and the ability of the cells to differentiate is still not clear. To study this, cells must be sorted based on the presence of GF receptors, and then cultured.

Most receptor studies are performed using either radiolabeled GFs [Foxwell et al., 1988b] [Nicola et al., 1988] [Park et al., 1989] or antibodies against the receptor [Weil Hillman et al., 1990]. With radiolabeled GFs, only functionally intact receptors are labeled. However, ^{125}I-labeled GFs cannot be used to sort cells for culture experiments. Cells, stained with antibodies against the receptors, can be double-stained for other markers and sorted in a fluorescence activated cell sorter (FACS). However, use of anti-receptor antibodies can cause problems if the stained and sorted cells are used for in vivo assays. Antibody stained cells may be eliminated by phagocytosis induced by the F_c parts of the antibodies or by complement-mediated lysis, resulting in selective removal of the receptor expressing cells by the immune system of the recipient animals [Bauman et al., 1985].

In this paper we describe a universally applicable technique for cell staining involving biotinylated GFs, combining the advantages of radiolabeled GFs and antibodies. The recombinant murine hemopoietic GFs interleukin 2 (IL-2), IL-3, IL-6, granulocyte/ macrophage-colony stimulating factor (GM-CSF), and stem cell factor (SCF) were biotinylated. These biotin-GFs retained the capacity to induce proliferation of GF-dependent cells. Several studies on the use of biotin-GFs to detect receptor-expressing cells have been published. In these studies cells with high receptor densities

(10^4–10^5 receptors per cell) were used [Foxwell et al., 1988a] [Yamasaki et al., 1988] [Newman et al., 1989] [Peters and Norback, 1990]. The fluorescence signal of cells with low receptor numbers has to be amplified for analysis in a flow cytometer [Wognum et al., 1992]. We incubated the cells with biotin-GFs and fluorescently labeled (strept)avidin. After amplification of the fluorescence signal with alternate layers of biotinylated anti-(strept)avidin and fluorescently labeled (strept)avidin, it was possible to analyze cells with 100 cell surface receptors in a flow cytometer.

2.3 MATERIALS AND METHODS

Biotinylation of growth factors

Murine recombinant IL-3 (rIL-3) and rGM-CSF (gifts of Behringwerke Aktiengesellschaft, Marburg, Germany), human rIL-2 (Biogen, Geneva, Switzerland), human rIL-6 and rat rSCF (both from Amgen, Cambridge, UK) were biotinylated using biotin-*N*-hydroxy succinimide ester (Pierce, Rockford IL, USA). Different amounts of NHS-biotin in dimethyl sulfoxide (DMSO) were added to solutions of GFs in 0.2 M carbonate-bicarbonate buffer pH 8.5, to obtain various molar biotin:protein (B:P) ratios between 4 and 100 during the reaction. As controls, samples were mock-biotinylated (at B:P 0) by just adding DMSO without biotin. After 3 h of incubation on ice, the samples were dialyzed overnight against PBS to remove the remaining free biotin molecules from the samples.

The protein concentration after biotinylation was determined using the BCA Micro Protein Assay (Pierce). The presence of biotinylated GF was checked by spotting 1 µl of stepwise dilutions of the samples directly on a nitrocellulose membrane, followed by immuno-enzymatic staining of the membrane with streptavidin-alkaline phosphatase and 4-Nitro Blue Tetrazoliumchloride 5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT BCIP) development.

The biotinylated GFs were stored at 4°C in the presence of 0.02% (wt/vol) sodium azide.

Cell lines and biological activity assay

Before adding sodium azide, the biological activity of the biotinylated GFs was determined using GF-dependent cell lines (CTLL-2 for IL-2, T1165 for IL-6, DA-1, 32D, or FDC-P1 for IL-3, and FDC-P1 for GM-CSF and SCF). Cells were grown in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 0.1 mM β-mercapto ethanol, 2 mM L-glutamine, and the appropriate GF. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

To determine the ability of the biotinylated GFs to stimulate cell proliferation, 10^4 cells per well were cultured for 1 day in 96 well microtiter plates in 200 µl RPMI medium containing different amounts of either biotinylated GFs or unmodified GFs. [^{3}H]thymidine (0.25 µCi per well) was added after 24 h. After 16–18 h of thymidine incorporation the cells were harvested, the radioactivity was measured in a liquid scintillation counter, and expressed as counts per min (cpm).

Immunocytochemical staining and flow cytometry

Cells were stained in 50 µl PBS/ Serum/ Azide (PSA): PBS containing 5% (vol/vol) fetal or newborn calf serum and 0.02% (wt/vol) sodium azide. First, 10^6 cells per sample were incubated in 50 µl PSA with biotin-GF (1–100 nM) for 60 min on ice or at room temperature (resulting in similar levels of fluorescence). Next, the samples were stained for 15–30 min on ice, depending on the desired fluorescence wavelength either with avidin-FITC (1:300 vol/vol; Vector Laboratories, Burlingame CA, USA) or streptavidin-BODIPY (1:100 vol/vol; Molecular Probes, Eugene OR, USA), or with streptavidin-PhycoErythrin (1:5 vol/vol; Becton Dickinson, San Jose CA, USA; or 1:200–1:1000; Molecular Probes). After each incubation the samples were washed in PSA. The fluorescence signal was amplified by incubating the cells for 15 min on ice with alternate layers of biotinylated goat-anti-avidin or goat-anti-streptavidin (both 1:100 vol/vol; Vector) and fluorescently labeled (strept)avidin. Specificity of binding of the biotin-GF samples was determined by incubating the cells with biotin-GF in the presence of a 100-fold molar excess of unbiotinylated GF. As a negative control, only PSA was used in the first incubation.

Samples were analyzed using a RELACS (Rijswijk Experimental Light Activated Cell Sorter, TNO, Rijswijk, The Netherlands) flow cytometer. Cells were illuminated with the 488-nm line of an argon ion laser. Green FITC or BODIPY fluorescence was measured through a 530-nm/ 30-nm bandpass filter, orange PE fluorescence was measured through a 575-nm/ 10-nm bandpass filter.

2.4 RESULTS AND DISCUSSION

Biological activity

In staining experiments it is important that the biotinylated GFs bind to the receptors. To determine this, we compared the abilities of biotin-GFs and native GFs to stimulate proliferation of GF-dependent cell lines. On DA-1 cells, IL-3 was shown to have retained full growth promoting activity after biotinylation at B:P ratios of 20 (**figure 2.1** A) or less. After biotinylation at B:P 50, approximately 50% of the growth promoting

ability was recovered, whereas at B:P 100 biotin-IL-3 lost almost all biological activity (not shown).

Biotin-IL-6 also retained most of its growth promoting activity at B:P 20 (**figure 2.1 B**). Similar results were obtained on CTLL-2 cells with biotin-IL-2 (see **chapter 3**), and on FDC-P1 cells with biotinylated SCF (see **chapter 5**) or GM-CSF (not shown).

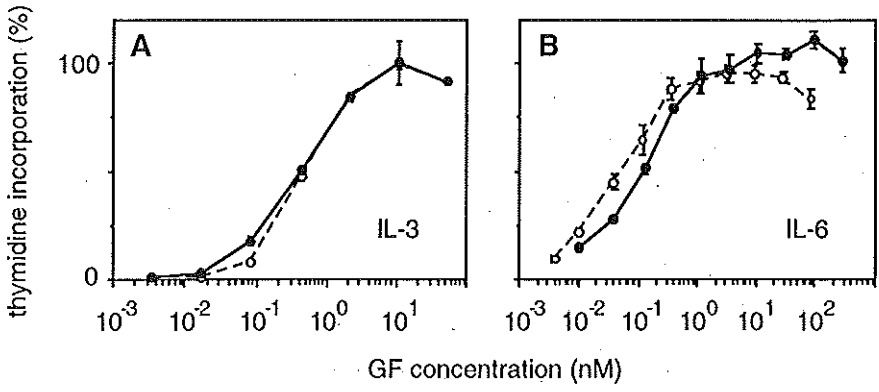

figure 2.1
Biological activity of (**A**) biotin-IL-3 and (**B**) biotin-IL-6.
Incorporation of [^{3}H]thymidine into DNA of GF-dependent cells (**A** DA-1 cells, **B** T1165 cells) is shown as a function of the concentration of native GF (open circles) and biotin-GF (B:P 20, closed circles).

Fluorescence amplification

Cells were incubated with biotin-GF and fluorescently labeled avidin or streptavidin, and the effect of fluorescence amplification with alternate layers of biotinylated anti-(strept)avidin and fluorescently labeled (strept)avidin (depicted schematically in **figure 2.2**) was studied using a flow cytometer. As shown for avidin-FITC in **figure 2.3**, with

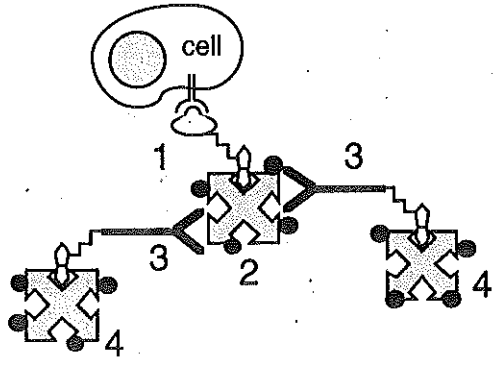

figure 2.2
Schematic drawing of cell staining with biotinylated GFs (**1**) and fluorescently labeled (strept)avidin (**2**), and subsequent amplification with biotinylated anti-(strept)avidin (**3**) and fluorescently labeled (strept)avidin (**4**).

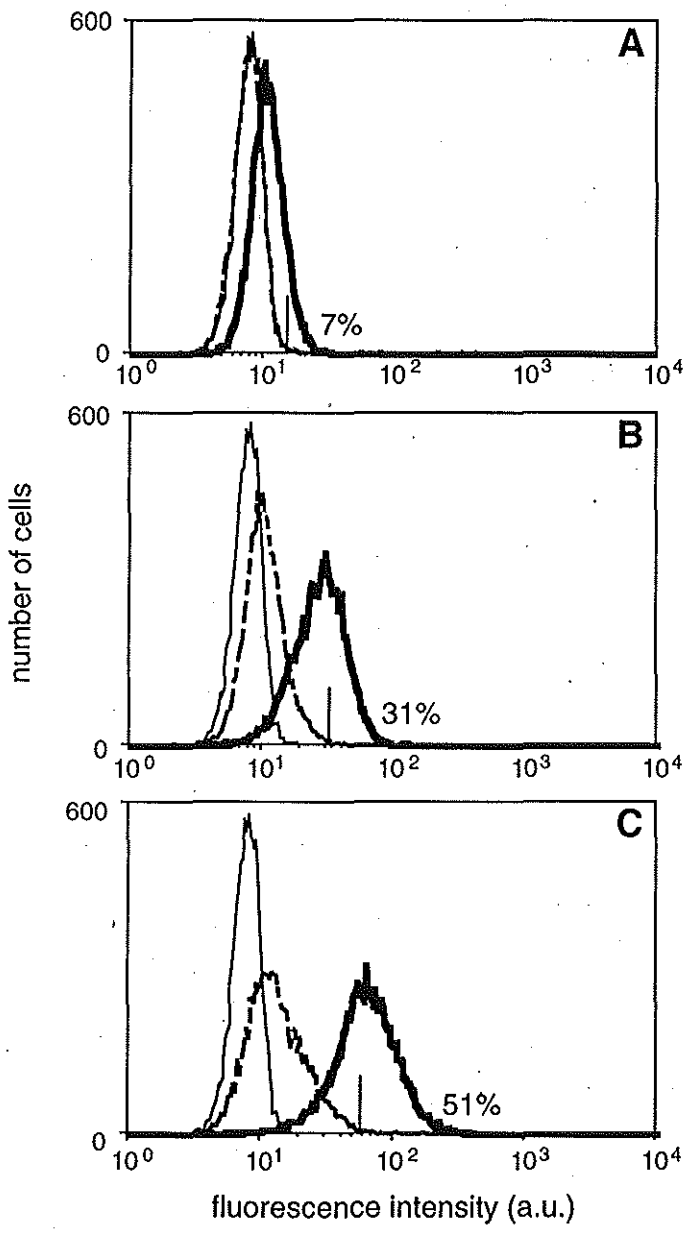

figure 2.3
IL-3 receptor expression on DA-1 cells.
Fluorescence histograms are shown of DA-1 cells, incubated with biotinylated IL-3 (bold solid lines). Cells were stained with avidin-FITC, and fluorescence signals were 1x (**A**), 2x (**B**), or 3x (**C**) amplified with biotinylated anti-avidin and avidin-FITC. Control cells were incubated without biotinylated IL-3 (broken lines). Thin solid lines indicate unstained cells. Markers were set on the basis of fluorescence of control cells to indicate the percentages of IL-3 receptor-positive cells.

each amplification step the difference in fluorescence intensity between negative and positive cells was larger, even if there was an increase in noise (background signal) along with the increase in signal. When a threshold was set for cell sorting experiments at the maximal background fluorescence, about 1% of DA-1 cells were positive without fluorescence amplification. The respective percentages of true positive cells after 1, 2 or 3 amplifications were 7, 31 and 51, as indicated in **figure 2.3 A, B,** and **C,** respectively. These results show that alternate layers of biotinylated anti-avidin and avidin-FITC can be used to achieve a better separation between positive and negative cells after staining with biotin-GF and avidin-FITC.

The sensitivity of the staining method was studied on FDC-P1 cells. These cells have approximately 100 GM-CSF receptors per cell, as shown by equilibrium binding with ^{125}I-GM-CSF and Scatchard analysis (L. Budel, personal communication). Cells were stained with biotinylated GM-CSF and avidin-FITC, and the fluorescence signal was amplified twice. A very weak fluorescence signal was obtained (**figure 2.4**). The specificity of the staining was studied by incubation with biotin-GF in the presence of a 100-fold molar excess of the unbiotinylated GF. This resulted in a fluorescence signal that was approximately equal to the background signal of cells without biotin-GF, as shown in **figure 2.4**, which demonstrated that the staining of FDC-P1 cells with biotin-GM-CSF was specific. Therefore, these results show that cells with 100 cell surface receptors can be stained for analysis using a flow cytometer. However, these results

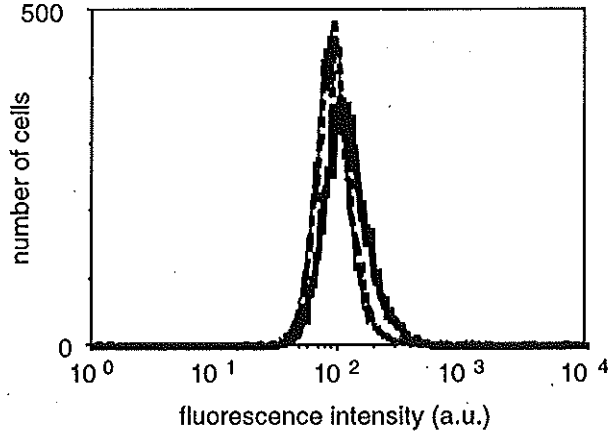

figure 2.4
GM-CSF receptor expression on FDC-P1 cells.
Cells were stained with biotin-GM-CSF and avidin-FITC, 2x amplified with biotinylated anti-avidin and avidin-FITC (bold solid line). Control cells were stained without biotin-GM-CSF (thin solid line), or with biotin-GM-CSF in the presence of a 100-fold molar excess of native GM-CSF (broken line).

were obtained using homogeneous cell suspensions; cells with low receptor numbers may remain undetected in heterogeneous cell suspensions, such as BM, especially when these cells are present at low frequencies.

Since the start of this study several papers on the preparation and use of biotin-GF for detection of receptor-expressing cells have been published [Foxwell et al., 1988a] [Yamasaki et al., 1988] [Newman et al., 1989]. In these studies use was made of cells with high receptor densities (10^4– 10^5 per cell). With our method it is possible to analyze cells with 100 or more cell surface receptors. Other applications of biotin-GF include the study of receptors with different affinities for a GF, like the IL-2 receptor [Weil Hillman et al., 1990], and binding to the same receptor by different GFs such as IL-3 and GM-CSF. For example, competition between biotinylated IL-3 and unbiotinylated GM-CSF vice versa can be used in the study of the receptor or subunit that can bind both IL-3 and GM-CSF [Park et al., 1989] [Kitamura et al., 1991]. Additionally, since occupied GF receptors are rapidly internalized into the cells [Nicola et al., 1988], it may be possible to target streptavidin-conjugated immunotoxins into the cells, using biotin-GF instead of biotinylated antibodies [Meyer et al., 1991].

2.5 ACKNOWLEDGMENTS

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CHAPTER 3

Biotinylation of Interleukin-2 (IL-2) for flow cytometric analysis of IL-2 receptor expression: comparison of different methods

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3.1 ABSTRACT

The main prerequisites for the use of biotinylated ligands to study the expression of growth factor receptors on heterogeneous cell populations, such as peripheral blood or bone marrow, by flow cytometric methods, are that the biotinylated ligand retains its binding ability and that binding of the biotinylated ligand to the receptor does not inhibit the subsequent interaction of biotin with fluorescently tagged avidin or streptavidin. Using Interleukin-2 (IL-2), we compared the usefulness of various biotinylation reagents, NHS-Biotin, S-NHS-Biotin, S-NHS-LC-Biotin, DBB and Photobiotin, and developed optimal biotinylation conditions for the preparation of biologically active biotin-labeled IL-2 and the detection of IL-2 receptor expressing cells by flow cytometry. As determined by spot blot analysis, biotinylation of IL-2 was most efficient at the highest biotin-to-protein (B:P) ratio used. At a B:P ratio of 100, most of the biological activity of IL-2 was retained when S-NHS-LC-Biotin was used. In contrast, most of the biological activity of IL-2 samples that were labeled with NHS-Biotin or Photobiotin was lost under these conditions. Biotin-labeled IL-2 preparations were tested in order to detect IL-2 receptors on IL-2 dependent CTLL-2 cells by flow cytometry after sequential staining with the biotinylated IL-2 and fluorescence tagged streptavidin. A high B:P ratio generally resulted in a high specific fluorescence intensity of the cells, particularly when S-NHS-LC-Biotin was used as the biotinylation reagent. Biotin-IL-2 could also be used to detect IL-2 receptors expressed by lymphocytes in peripheral blood and bone marrow. Comparison of staining of lymphocytes with biotinylated IL-2 and an antibody against the IL-2 receptor α chain demonstrated that only a subset of the cells that showed a strong fluorescence signal after staining with biotinylated IL-2 expressed high numbers of the IL-2 receptor α chain. This is in agreement with the expression of functional IL-2 receptors on resting T cells and NK cells which do not express the α chain. After stimulation with PHA, virtually all lymphocytes expressed the α chain, whereas only part of these cells showed a strong fluorescence signal after staining with biotin-IL-2, while the rest of the cells had very low numbers of IL-2 binding sites. Our results demonstrate that, in addition to staining individual receptor subunits with antibodies, staining with biotinylated IL-2 is a useful indicator of functional IL-2 receptor expression.

3.2 INTRODUCTION

Growth factors control the growth, differentiation and maturation of hemopoietic cells as well as, in many cases, the functional activity of mature end cells, by binding to specific cell surface receptors. To examine the target cell specificity of growth factors (GF) and the presence of GF receptors, in vitro culture systems are used to study

biological responses [Sonoda et al., 1988] [Warren et al., 1989] [Ishida et al., 1991], as well as direct methods to study receptor expression. Direct receptor labeling studies can be performed using radiolabeled GF [Foxwell et al., 1988a] [Nicola et al., 1988] [Park et al., 1989] with the advantage that only functionally intact GF receptors are labeled. However, because ^{125}I-labeled GF are usually quantified as mean number of receptors per cell, these studies are of limited use for heterogeneous cell populations. Receptor expression on subsets of cells in heterogeneous populations can be studied using a fluorescence activated cell sorter (FACS) when cells are labeled with antibodies against the receptors [Weil-Hillman et al., 1990], and double-stained for other markers. However, many receptors consist of several different subunits, and the expression of more than one subunit is often required to generate functional, high affinity receptors [Takeshita et al., 1992b] [Ishii et al., 1994] [Nakamura et al., 1994] [Nelson et al., 1994]. In addition, some receptors share subunits, e.g. the high affinity receptors for human IL-3, GM-CSF and IL-5 [Kitamura et al., 1991] [Tavernier et al., 1991], and the high affinity receptors for IL-2, IL-4 and IL-7 [Kondo et al., 1993] [Noguchi et al., 1993] [Russell et al., 1993] [Kawahara et al., 1994]. Since antibodies usually recognize only one of the constituents of a receptor complex, antibody binding may not always be indicative of expression of functional receptors.

Instead of antibodies or radiolabeled GF, biotinylated GF may be used [Foxwell et al., 1988b] [Yamasaki et al., 1988] [Newman et al., 1989] [Peters and Norback, 1990] [Wognum et al., 1990] to combine the advantages of both other methods. Biotin is a small molecule of 244 Daltons, which can easily be coupled to proteins such as GF. The strong interaction between biotin and the egg white protein avidin, or the bacterial protein streptavidin, can be used to detect binding of biotinylated GF to target cells [Wilchek and Bayer, 1988]. By such a method, functionally intact receptors are labeled and the cells can be analyzed by flow cytometry and sorted using a FACS. The efficiency of this approach depends on the efficiency of the biotinylation reaction and on its effect on the binding affinity and, thus, on the biological activity.

Many different kinds of biotinyl derivatives are available. For biotinylation of proteins, the N-hydroxy succinimide ester of biotin (NHS-Biotin) or its water soluble analog N-hydroxysulfo-succinimide biotin (S-NHS-Biotin), both of which bind primarily to lysine residues under alkaline conditions, are possibly the most often used [Newman et al., 1989] [Pieri and Barritault, 1991]. Variants with an extended spacer arm (S-NHS-Long Chain-, S-NHS-LC-Biotin) can be used to reduce the effect of steric hindrance [Hnatowich et al., 1987]. If a sufficient number of lysine residues is not available, or when labeling of lysine causes changes of the biological activity of the protein, other classes of reactive biotin derivatives can be used to biotinylate other functional groups. Tyrosines or histidines can be labeled with *p*-Diazobenzoyl Biocytin (DBB) [Wilchek et al., 1986]. Sulfhydryls can be biotinylated with *N*-iodoacetyl-*N'*-biotinyl-hexanediamine, or with *N*-[6-(biotinamido)hexyl]-3-(2-pyridyldithio) propionamide (Iodoacetyl-Biotin

and Biotin-HPDP, respectively). An advantage of Biotin-HPDP is, that the S-S bridge which is formed by the biotinylation reaction can be cleaved to remove the biotin [Shimkus et al., 1985], which may be useful to recover biotinylated proteins from avidin affinity columns. Cleaving off biotins from proteins is also possible when sugar residues on glycoproteins are biotinylated using biotin hydrazide [O'Shannessy et al., 1984] [Wognum et al., 1990]. Another biotinylation method uses Photobiotin, a photo-activatible analog of biotin [Forster et al., 1985] [Lacey and Grant, 1987] which forms stable (presumably covalent) linkages when illuminated.

In this study, we have compared the effect of conjugation with different biotin derivatives on the biological activity as well as on the receptor binding ability and resulting labeling intensity of IL-2 and examined the application of biotinylated IL-2 to detect receptor expression on subsets of peripheral blood and bone marrow cells.

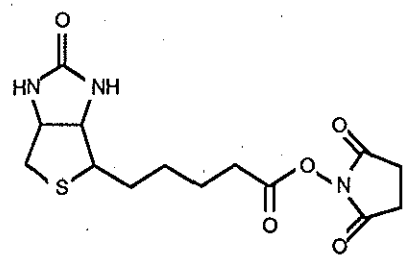
3.3 MATERIALS AND METHODS

Biotinylation

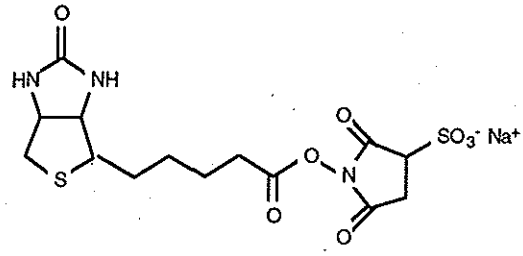
E. Coli derived human recombinant IL-2 (specific activity 2.8 x 10^6 U/mg; Biogen, Geneva, Switzerland) was biotinylated using different biotin derivatives (**figure 3.1**). NHS-Biotin, the water soluble S-NHS-Biotin and its extended spacer arm analog S-NHS-LC-Biotin (all from Pierce, Rockford IL, USA) were dissolved in dimethyl sulfoxide (DMSO) or in 0.1 M carbonate-bicarbonate buffer pH 8.5. DBB precursor (Calbiochem, San Diego CA, USA) was dissolved in DMSO, and prepared according to the manufacturer's instructions. IL-2 was diluted in 0.1 M carbonate-bicarbonate buffer pH 8.5 (for the different NHS-Biotins) or in 0.05 M borate or carbonate buffer pH 9.0 (for DBB). Biotin was added to the GF solution to obtain a molar biotin:protein (B:P) ratio of 100, 20, or 4 during the reaction. As controls, samples were mock-biotinylated (at B:P 0) by just adding DMSO or buffer without biotin. The biotinylation reactions were allowed to take place for 1.5 h (DBB) or 3 h (NHS-Biotin) at room temperature, or for 3 h on ice (S-NHS-Biotins). Photoactivatible biotin (Photobiotin, stock solution of 1 mg/ml; Vector Laboratories, Burlingame CA, USA) was used, according to the manufacturer's instructions at B:P reaction ratios of 10:1, 1:1 and 1:10 (wt/wt; corresponding to molar B:P ratios of approximately 250, 25 and 2.5, respectively).

figure 3.1.
Structure of the different biotinyl derivatives that were used in this study.
(**A**) NHS-Biotin, (**B**) S-NHS-Biotin, (**C**) S-NHS-LC-Biotin, (**D**) DBB and (**E**) Photobiotin.

A



B



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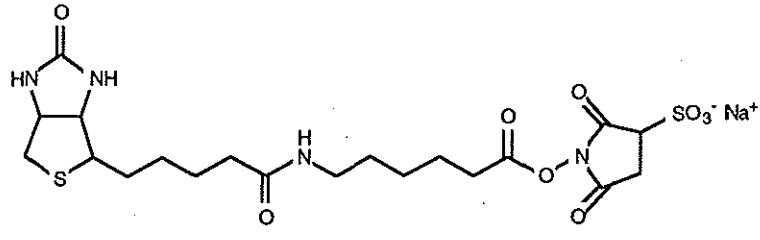
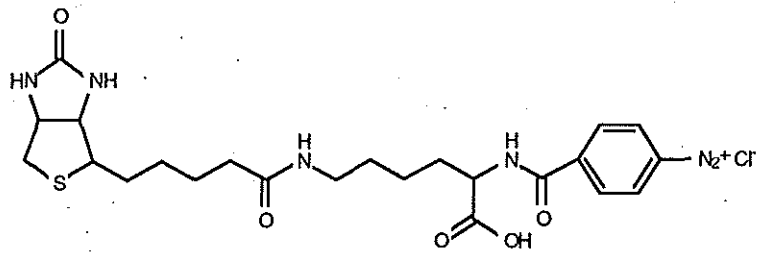
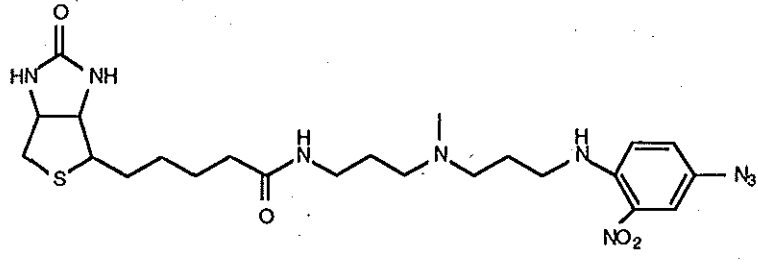


D



E



These reaction mixtures and a control without Photobiotin were illuminated with UV light for 30 min on ice using a mercury arc lamp. The reaction was stopped by adding an equal volume of 0.1 M Tris pH 9.0. The remaining free biotin molecules in the samples were removed either by size exclusion chromatography, using a PD-10 column containing Sephadex G-25 (Pharmacia, Uppsala, Sweden), or by extensive dialysis at 4°C. More protein material was lost during size exclusion chromatography than during dialysis (up to 50% and 15%, respectively). Dialysis of small volumes was achieved by floating a low-protein-binding filter unit with the sample (10,000 NMWL filter unit, Cat. No. UFC3 LGL00, Millipore, Bedford MA, USA) on PBS containing 0.04% Tween-20 and 0.02% (wt/vol) sodium azide. The biotinylated GF were stored at 4°C in the presence of 0.02% (wt/vol) sodium azide. The protein concentration after biotinylation was determined with the BCA Micro Protein Assay (Pierce). The presence of biotinylated IL-2 was determined by spotting 1 µl of stepwise dilutions (1/2) of the samples directly on a nitrocellulose membrane, followed by immuno-enzymatic staining of the membrane with streptavidin-alkaline phosphatase and 4-Nitro Blue Tetrazoliumchloride 5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT BCIP) development.

Cell line and biological activity assays

Cells from the IL-2 dependent mouse cytotoxic T cell line CTLL-2 were grown in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, and 20 U/ml of IL-2. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. To determine the biological activity of the biotinylated GF, 10^4 cells per well of 96 well microtiter plates were cultured in 200 µl RPMI medium containing serial dilutions of either biotin-IL-2 or unmodified IL-2. After 24 h, 0.25 µCi ^{3}H-thymidine was added to each well. The cells were harvested after 16-18 h of thymidine incorporation, and the radioactivity was measured in a liquid scintillation counter. Alternatively, in a non-radioactive assay the number of nucleated cells per well was determined by staining the nuclei with propidium iodide (PI) and measuring the fluorescence intensity. For this purpose, the cells were cultured for 40-48 h, after which 0.5% (vol/vol) Triton X-100 and 0.002% (wt/vol) PI were added to the wells. After approximately 15 min the microtiter plates were read in a Leitz Diavert inverted microfluorometer using green excitation by a halogen lamp.

Mouse bone marrow cells

Bone marrow from 7 week old BCBA (C57BL/LiARij x CBA/BrARij)F1 mice was harvested by flushing femora and tibiae with 1 ml of ice-cold Hanks' Balanced Salt Solution (Laboratoires Eurobio, Paris, France) buffered at pH 6.9 with 10 mM Hepes,

(Merck) (HH, osmolarity 300-305 mOsm/kg). A single cell suspension was obtained by filtration through a six-layer nylon sieve.

Human peripheral blood cells

Peripheral blood cells from normal human volunteers were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine and 90 µg/ml phytohemagglutinin (PHA, 1% of stock solution, Wellcome Foundation Ltd., Dartford, England).

Immunocytochemical staining and flow cytometry

Cells were stained in 50 µl PBS/Serum/Azide (PSA, human cells) or HH/Serum/Azide (HSA, mouse cells): PBS or HH, containing 5% (vol/vol) fetal or newborn calf serum and 0.02% (wt/vol) sodium azide. Cells (10^6 per sample) were incubated with biotin-IL-2 (14-140 nM) in a volume of 50 µl for 60 min at room temperature. Similar results were obtained by incubation on ice. The samples were then stained for 15-30 min on ice, using either avidin-FITC (1:300 vol/vol, Vector Laboratories, Burlingame CA, USA), streptavidin-BODIPY (1:100 vol/vol, Molecular Probes), or streptavidin-PhycoErythrin (streptavidin-PE, 1:5 vol/vol, Becton Dickinson, San Jose CA, USA; or 1:200 vol/vol, Molecular Probes, Eugene OR, USA). After each incubation the samples were washed in PSA or HSA. Low intensity fluorescence signals were amplified by incubating the cells for 15-30 min on ice with alternate layers of biotinylated goat anti-(strept)avidin (both 1:100 vol/vol, Vector) and fluorescence labeled (strept)avidin. Control cells were incubated only with the fluorescence labeled (strept)avidin and the amplifying layers of biotinylated anti-(strept)avidin and fluorescence labeled (strept)avidin. Specificity of binding of the different biotin-IL-2 samples was determined by incubating the cells with biotin-IL-2 in the presence of a 100x molar excess of unbiotinylated IL-2.

For staining with anti-IL-2 receptor α chain antibody, mouse cells were incubated with the antibody 5A2 [Moreau et al., 1987] (undiluted culture supernatant of hybridoma cells, kindly provided by Dr. Thèze, Département d'Immunologie, Institut Pasteur, Paris, France) for 30 min on ice. Human cells were incubated with the rat-anti-TAC antibody 36A1.2 [Olive et al., 1986], followed by staining with FITC-conjugated goat anti-rat antibodies (GARa-FITC; 1:100 vol/vol, TAGO, Burlingame CA, USA). In double-staining experiments, these antibodies were added during amplification of the streptavidin-PE signal: the anti-TAC antibody was added to the cells together with biotinylated goat anti-streptavidin, followed by incubation with GARa-FITC and streptavidin-PE. In some experiments a combination of streptavidin-BODIPY and GARa-PE (1:200 vol/vol, TAGO) was used.

To analyze the expression of IL-2 receptors on subsets of human peripheral blood lymphocytes, cells were double-stained with mouse-anti-CD4 antibodies (1:20 vol/vol, Becton Dickinson) and either anti-TAC or biotin-IL-2, combined with PE-conjugated goat anti-mouse antibodies (GAM-PE; 1:100 vol/vol, Sigma Chemical Company, St. Louis MO, USA) and either GARa-FITC or streptavidin-BODIPY.

Samples were analyzed using a flow cytometer (RELACS: Rijswijk Experimental Light Activated Cell Sorter, ITRI-TNO, Rijswijk, The Netherlands). Cells were illuminated with the 488-nm line of an argon ion laser. Green FITC or BODIPY fluorescence was measured through a 530-nm/ 30-nm bandpass filter (Becton Dickinson, Mountain View CA, USA). Orange PE fluorescence was measured through a 577-nm/ 10-nm bandpass filter.

3.4 RESULTS

Biotinylation of IL-2

IL-2 was biotinylated using either NHS-Biotin, S-NHS-Biotin, S-NHS-LC-Biotin, DBB or Photobiotin (**figure 3.1**). The degree of biotinylation of the different samples was studied by spotting 1 µl of stepwise dilutions of biotin-IL-2 on a nitrocellulose filter and comparing the intensity of the spots following incubation with streptavidin-alkaline phosphatase and NBT BCIP development. The various biotin-IL-2 samples

table 3.1
Spot-blot analysis of the different biotin-IL-2 samples.

	minimal detectable amount (ng) of biotin-IL-2 per µl [a]							
	NHS	S-NHS		S-NHS-LC		DBB	Photo	
	DMSO	DMSO	buffer	DMSO	buffer			
B:P [b] 0	>100	>100	>100	>100	>100	>100	B:P 0	>100
B:P 4	5	20	50	10	10	90	B:P 2.5	>100
B:P 20	1	5	10	1	1	10	B:P 25	40
B:P 100	<1	<1	<1	<1	<1	<1	B:P 250	<1

[a] The minimal detectable amount (ng) of biotin-IL-2 per µl was determined by spotting 1 µl of stepwise dilutions of the biotin-IL-2 samples on nitrocellulose, followed by immuno-enzymatic staining with streptavidin-alkaline phosphatase and NBT BCIP development.
[b] B:P = biotin:protein ratio.

contained different amounts of biotin, as shown by the amounts minimally needed to give a visible stain on the nitrocellulose membrane (**table 3.1**). As expected, the highest degree of biotinylation was achieved at the highest B:P reaction ratios, whereas the B:P 0 controls were not visible on the membranes. The water-soluble S-NHS-LC-Biotin appeared to label with similar efficiency after dissolving in DMSO or in buffer. S-NHS-Biotin labeled slightly more efficiently after dissolving in DMSO than in buffer. NHS-Biotin and S-NHS-LC-Biotin gave the best biotinylation efficiency, followed by S-NHS-Biotin and DBB, which were 4-10 fold less efficient. The lowest degree of biotinylation occurred when IL-2 was labeled with the Photobiotin reagent (**table 3.1**).

Biological activity of biotinylated IL-2 preparations

Using the IL-2 dependent mouse cytotoxic T cell line CTLL-2, the B:P ratio was found to affect the biological activity of biotin-IL-2 (**table 3.2**). At the lowest B:P ratio used in the biotinylation reaction, biological activity was not lost as a result of biotinylation. Higher B:P ratios had a marked negative effect on the biological activity of some biotinyl derivatives (**table 3.2**). Loss of biological activity was highest when IL-2 was biotinylated with NHS-Biotin or Photobiotin. When used at B:P 100, biotinylation with DBB also led to a large loss of biological activity. The use of the water-soluble NHS-Biotins dissolved in DMSO resulted in twofold larger losses of biological activity than when these reagents were dissolved in buffer. The highest recovery of biological activity (≥ 70-80% at B:P 100) was achieved with S-NHS-LC-Biotin and S-NHS-Biotin (**table 3.2**).

table 3.2
Biological activity of the different biotin-IL-2 samples.

	recovery of biological activity [a]							
	NHS	S-NHS		S-NHS-LC		DBB		Photo
	DMSO	DMSO	buffer	DMSO	buffer			
B:P [b] 0	100	100	100	100	100	100	B:P 0	100
B:P 4	100	100	100	100	100	100	B:P 2.5	100
B:P 20	33	90	85	90	100	80	B:P 25	33
B:P 100	5	33	72	40	80	20	B:P 250	7

[a] IL-2 dependent CTLL-2 cells were grown in the presence of different amounts of biotin-IL-2. The amount of biotin-IL-2 needed per well for 50% maximal stimulation of the cells was calculated for the different samples. Results are expressed as % biological activity, as compared to unbiotinylated IL-2 (100%).

[b] B:P = biotin:protein ratio.

Cell staining

Immunocytochemical staining properties of the different biotin-IL-2 samples were compared using flow cytometry. **figure 3.2** shows an example of the fluorescence histograms, resulting from the incubation of CTLL-2 cells with biotin-IL-2 made by biotinylation with S-NHS-LC-Biotin at different B:P ratios. The mean fluorescence intensities of cells, labeled with the different biotin-IL-2 preparations are presented in **table 3.3.** IL-2 labeled with the NHS-Biotins gave the highest fluorescence intensities: with the extra long spacer arm between the protein and the actual biotin molecule (S-NHS-LC-Biotin) the fluorescence signal was even higher than with the normal spacer arm (NHS-Biotin and S-NHS-Biotin). Dissolving the water soluble S-NHS-Biotin or S-NHS-LC-Biotin in DMSO before biotinylating the IL-2 resulted in higher fluorescence intensities of stained cells than dissolving in buffer. In comparison with the various NHS-Biotins, biotinylation with DBB resulted in a lower fluorescence signal. The fluorescence signal resulting from Photobiotin-IL-2 was almost indistinguishable from the background fluorescence of the cells (**table 3.3**). For most biotinyl derivatives, the preparations with the highest B:P ratios resulted in the highest fluorescence intensities. Only with the standard NHS-Biotin was an impaired staining of the B:P 100 sample (relative to the B:P 20) consistently observed (**table 3.3**). For all biotin-IL-2 samples, the fluorescence signal of cells incubated in the presence of a 100x molar excess

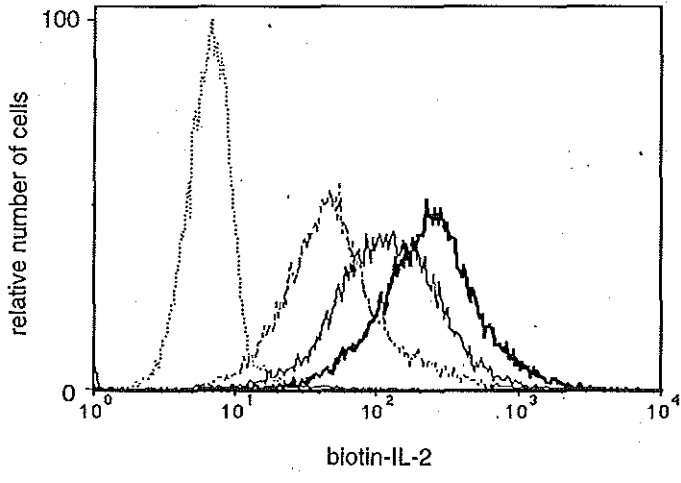


figure 3.2.
Fluorescence histograms of CTLL-2 cells, incubated with different biotin-IL-2 samples and Streptavidin-PE. The fluorescence signal was amplified by two cycles of sequential incubations with biotinylated anti-Streptavidin antibodies and Streptavidin-PE. The cells were labeled with biotin-IL-2, biotinylated with S-NHS-LC-Biotin that was dissolved in DMSO; from right to left are B:P 100, B:P 20 and B:P 4, and the B:P 0 control.

of unbiotinylated IL-2 was similar to that of control cells incubated without biotin-IL-2, which indicated specific binding of biotin-IL-2 to the cells. This is illustrated in **figure 3.3** for CTLL-2 cells, incubated with biotin-IL-2 prepared with NHS-Biotin.

table 3.3
Fluorescence intensity of CTLL-2 cells, stained with the different biotin-IL-2 samples.

	mean fluorescence intensity (arbitrary units) [a]							
	NHS	S-NHS		S-NHS-LC		DBB		Photo
	DMSO	DMSO	buffer	DMSO	buffer			
B:P [b] 0	7	7	8	7	8	7	B:P 0	7
B:P 4	41	20	9	73	28	13	B:P 2.5	18
B:P 20	157	48	32	164	116	59	B:P 25	9
B:P 100	100	164	122	316	288	99	B:P 250	24

[a] CTLL-2 cells were incubated with the different biotin-IL-2 samples and streptavidin-PE, amplified with biotin-anti-streptavidin and streptavidin-PE.
[b] B:P = biotin:protein ratio.

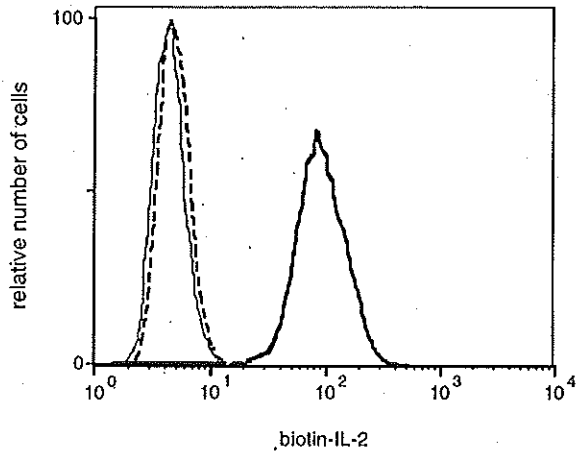


figure 3.3
Fluorescence histograms of CTLL-2 cells, incubated with biotin-IL-2 (thick solid line), in the presence of a 100x molar excess of unbiotinylated IL-2 (broken line), and fluorescence labeled streptavidin, or stained only with fluorescence labeled streptavidin without biotin-IL-2 (thin solid line). The fluorescence signal was amplified using biotinylated anti-streptavidin antibodies and a second incubation with fluorescence labeled streptavidin.

When IL-2 receptors on lymphocytes from peripheral blood or bone marrow were stained, a difference was found between incubation with biotin-IL-2 and with antibodies against the α chain of the IL-2 receptor. The majority of unstimulated lymphocytes from mouse bone marrow as well as human peripheral blood were biotin-IL-2 positive (**figure 3.4 A** and **C**, respectively), whereas a much smaller fraction expressed the IL-2 receptor α chain at a level above background fluorescence (**figure 3.4 B** and **D**). Double-staining experiments revealed that the α chain positive cells were all biotin-IL-2 positive (data not shown). After stimulation of human peripheral blood

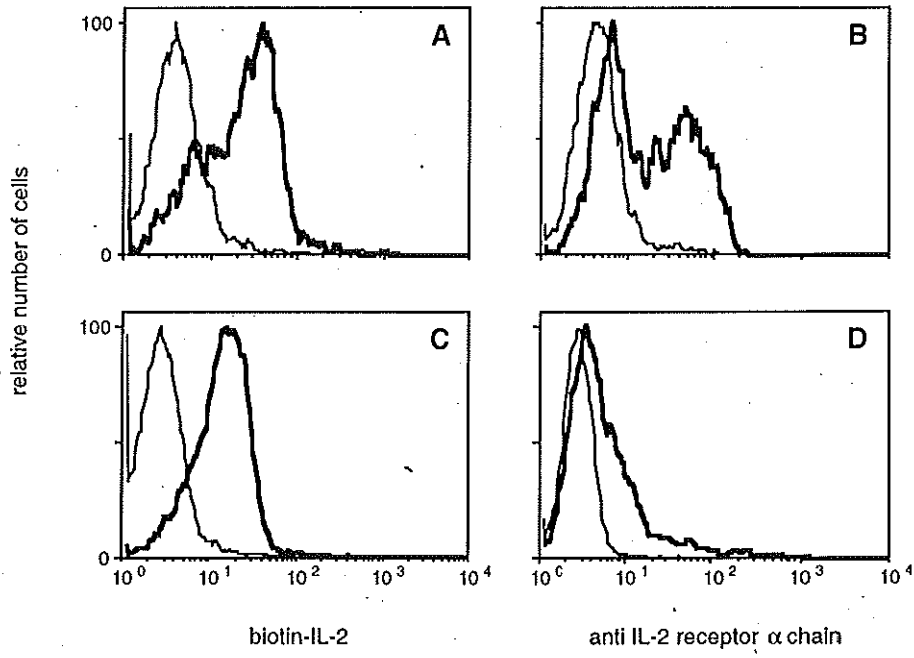


figure 3.4
Fluorescence histograms of mouse bone marrow lymphocytes (**A** and **B**) and human peripheral blood lymphocytes (**C** and **D**), stained with biotin-IL-2 and streptavidin-PE, and amplified (**A** and **C**), or stained with rat-anti-IL-2 receptor α chain antibodies and FITC conjugated goat anti-rat antibodies (**B** and **D**). Thick lines represent stained cells, thin lines unstained control cells.

lymphocytes (PBL) with PHA virtually all cells became α chain-positive (**figure 3.5 B**), whereas a much smaller fraction of the cells stained strongly with biotin-IL-2 (**figure 3.5 A**). Most of the cells that showed a strong fluorescence signal after staining with biotin-IL-2 or anti-TAC were CD4 positive. The proportion of CD4 negative cells that expressed high levels of IL-2 receptor was much lower. The remaining cells were either IL-2 receptor negative or expressed IL-2 receptors at low levels (**figure 3.5**).

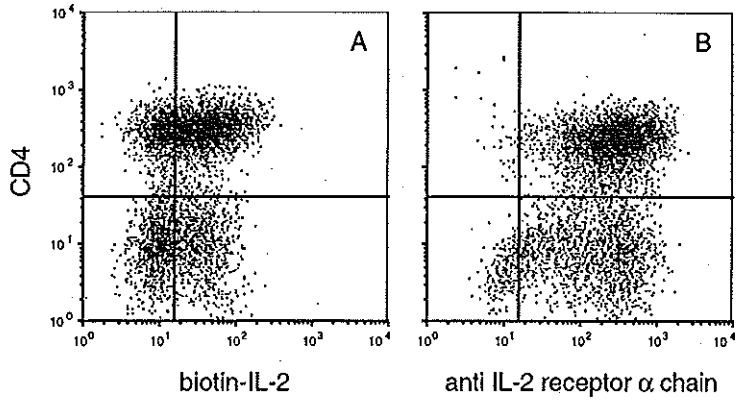


figure 3.5
Dot plots of human peripheral blood lymphocytes that were stimulated with PHA for 3 days, and stained with mouse-anti-CD4 antibodies in the first step, in combination with either biotin-IL-2 (**A**) or rat-anti-IL-2 receptor α chain antibodies (**B**). In the second step, cells were stained with PE-conjugated goat anti-mouse antibodies in combination with either Streptavidin-BODIPY (**A**) or FITC-conjugated goat anti-rat antibodies (**B**). Quadrants were set on the basis of background fluorescence of unstained cells to discriminate between brightly stained cells and cells with low or no fluorescence signal.

3.5 DISCUSSION

This study has evaluated the biotinylation of IL-2 using five different biotin derivatives. The data presented extends previous observations concerning the use of GF labeled with (mostly NHS-) biotin [Foxwell et al., 1988b] [Yamasaki et al., 1988] [Taki et al., 1989] [Peters and Norback, 1990] [Pieri and Barritault, 1991] [De Jong et al., 1992] [Wognum et al., 1993], by comparing the results of labeling with NHS-Biotin, S-NHS-Biotin, S-NHS-LC-Biotin, DBB and Photobiotin. The highest biotinylation efficiency, retention of biological activity and the highest specific staining of IL-2 receptor expressing cells was achieved using S-NHS-LC-Biotin. The results with S-NHS-LC-Biotin could be explained by a more efficient biotinylation, because the extended spacer arm permits the protein to reach the lysine groups, or by a more efficient interaction of the biotinylated IL-2 with the streptavidin conjugates.

Most biotin-IL-2 samples showed reduced activity in stimulating the growth of the IL-2 dependent cell line CTLL-2 when high B:P ratios were used. We cannot exclude the possibility that part of the biological activity of the biotin-IL-2 samples is due to residual unmodified IL-2 molecules. Attempts to separate biotin-IL-2 from IL-2 on an avidin-agarose column were not successful because IL-2 bound nonspecifically to the column. The decreased biological activity of samples with a high B:P ratio is probably the result of impaired receptor binding, caused by conformational changes of the biotin-

IL-2 or by steric hindrance when the biotin molecules are close to the receptor binding site(s) of IL-2. It is also possible that these biotinylated IL-2 molecules can bind to the cells but fail to stimulate cell proliferation. The IL-2 receptor consists of different subunits [Takeshita et al., 1992a], with different IL-2 binding characteristics [Lowenthal and Greene, 1987]. A low affinity IL-2 receptor contains only the 55-kD α chain [Leonard et al., 1984] [Nikaido et al., 1984]. A heterodimeric IL-2 receptor with intermediate affinity for IL-2 consists of the 70- to 75-kD β chain [Sharon et al., 1986] [Baccarini et al., 1989] and the 64-kD γ chain [Takeshita et al., 1992a] [Takeshita et al., 1992b]. A "pseudo-high" affinity IL-2 receptor is formed by interaction of α and β chains [Nakarai et al., 1994], which can only be internalized when combined with the γ subunit [Takeshita et al., 1992a], thereby forming the trimeric high affinity IL-2 receptor which has a higher association rate and a lower dissociation rate than the αβ complex [Matsuoka et al., 1993]. Heterodimerization of the β and γ chain is required for signaling [Nakamura et al., 1994] [Nelson et al., 1994]. The decreased biological activity of samples with a high B:P ratio might thus be caused by steric hindrance, which impedes receptor binding and/ or the formation of receptor complexes that are required for cell signaling or internalization.

Despite the partial loss of biological activity, the biotinylated IL-2 samples prepared at higher B:P ratios generally yielded better fluorescence signals. It is possible that at higher B:P ratios, a higher proportion of biotinylated IL-2 molecules have sufficient numbers of biotin molecules facing away from the receptor binding sites to permit binding of (strept)avidin molecules to receptor bound biotin-IL-2. Only the samples with high B:P ratios that lost almost all biological activity (NHS-Biotin and Photobiotin) showed low fluorescence intensities.

The weak performance of DBB compared to the different NHS-Biotins may be explained from the amino acid composition of IL-2: NHS-Biotins mainly react with lysine residues, of which there are 11 in IL-2, while there are only 3 histidines and 4 tyrosines [Taniguchi et al., 1983] which are necessary for DBB binding. Direct measurement of the number of biotin molecules present on the IL-2, e.g. with 2-(4'-hydroxy-phenylazo)benzoic acid (HABA) [Baxter, 1964], was not attempted, because the procedure requires a large amount of biotinylated protein.

Our experiments show that biotinylated IL-2 can be used for cell staining experiments. When IL-2 receptors on unstimulated lymphocytes from mouse bone marrow or human peripheral blood were labeled, only part of the biotin-IL-2 positive cells expressed high levels of the IL-2 receptor α chain (**figure 3.4**). This is in agreement with the finding that only intermediate affinity IL-2 receptors are present on the surface of resting T cells and NK cells [Dukovich et al., 1987], while the IL-2 receptor α chain is induced only after activation of the cells [Hamblin, 1985] [Lai et al., 1991]. After stimulation of PBL with PHA, virtually all cells stained brightly with anti-TAC, particularly in the CD4 positive subset (**figure 3.5 B**). However, only about two thirds

of these cells showed a high fluorescence signal after incubation with biotin-IL-2 (**figure 3.5 A**). The cells with a low binding capacity for biotin-IL-2 may have a limiting number of IL-2 receptor β and/or γ chains to form high affinity receptors with the α chains. Biotin-IL-2 binding thus appears to be a better marker for the expression of functional IL-2 receptor complexes than binding of anti-TAC. A possible advantage of staining with biotinylated GF for in vivo studies with isolated receptor expressing cells is that avidin-tagged cells are not selectively removed by the immune system of the recipient animals. In contrast, antibody stained cells may be eliminated by phagocytosis induced by the F_C parts of the antibodies or by complement-mediated lysis [Bauman et al., 1985]. As shown in previous studies for other cytokines [Wognum et al., 1990] [De Jong et al., 1992] [Wognum et al., 1993], flow cytometric analysis of receptor expression using biotinylated GF is very sensitive: cells expressing as few as 100 cell surface receptors can be detected. Therefore, biotinylated GF provide a useful tool for investigating the distribution of functional GF receptors, e.g. on subsets of early hemopoietic cells in bone marrow.

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CHAPTER 4

Purification of repopulating cells based on binding of biotinylated Kit ligand

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4.1 ABSTRACT

To characterize Kit expressing mouse bone marrow (BM) cells, and to determine their contribution to short- and long-term repopulation of the hemopoietic system of irradiated recipients, we have purified Kit$^+$ BM cells by flow cytometry. A high level of Kit expression was detectable on 1–2% of BM cells after staining with biotinylated stem cell factor (SCF) or with anti-Kit antibodies (ACK-2). Compared to unfractionated BM, the Kit$^+$ fractions were enriched for immature hemopoietic cells, as shown by morphological differentiation, in vitro culture, and spleen colony formation. Enrichment of colony-forming cells was higher in biotin-SCF$^+$ than ACK-2$^+$ fractions. Colony-forming cells were not found in the Kit$^-$ subsets. To study the hemopoietic repopulation capacity of the Kit$^+$ and Kit$^-$ cells, serial dilutions of the sorted fractions were transplanted into irradiated mice, and peripheral blood of these recipients was monitored regularly for the presence of donor-derived cells during a 1 year period. Nucleated blood cell repopulation by male donor cells in female recipients was assessed using a Y-chromosome specific DNA probe; erythroid repopulation by normal donor cells in W/W^V recipients was examined flow cytometrically by measuring the forward light scatter of donor- and host-type erythrocytes. A 25- to 100-fold enrichment of long-term repopulating ability in the sorted Kit$^+$ fractions showed that Kit$^+$ cells are capable of reconstitution of circulating erythrocytes and nucleated blood cells after BM transplantation. Transient repopulation of the red blood cell lineage was observed after transplantation of Kit$^-$ cells. Detection of donor-derived nucleated cells 1 year after transplantation showed that Kit$^+$ cells contributed to donor-type repopulation of BM, spleen, and thymus. Our data demonstrate that isolation of BM cells on the basis of Kit expression is a useful addition to the methods that are commonly applied in stem cell enrichment protocols.

4.2 INTRODUCTION

The transmembrane tyrosine kinase receptor Kit (CD117) [Chabot et al., 1988] and its ligand, stem cell factor (SCF), also known as Kit ligand, mast cell growth factor, or Steel factor [Anderson et al., 1990] [Huang et al., 1990] [Martin et al., 1990] [Zsebo et al., 1990a] [Zsebo et al., 1990b] are essential for normal hemopoietic cell development in vivo. This is demonstrated by severe hemopoietic defects which result from mutations at the dominant *white spotting (W)* locus, which encodes for Kit [Chabot et al., 1988] [Geissler et al., 1988], and at the *Steel (Sl)* locus, which encodes for SCF [Copeland et al., 1990] [Flanagan and Leder, 1990] [Huang et al., 1990] [Zsebo et al., 1990a]. The important role of Kit and SCF in supporting early hemopoiesis is also demonstrated by the disappearance of almost all

hemopoietic progenitor cells from the bone marrow (BM) of mice injected with neutralizing anti-Kit antibodies [Ogawa et al., 1991].

Soluble SCF, consisting of the N-terminal extracellular domain of the protein, synergizes with other hemopoietic growth factors to stimulate in vitro proliferation and differentiation of immature hemopoietic cells as well as monomyeloid, erythroid, and lymphoid progenitors [Broxmeyer et al., 1991] [De Vries et al., 1991] [McNiece et al., 1991a] [McNiece et al., 1991b] [Metcalf and Nicola, 1991] [Migliaccio et al., 1991] [Tsuji et al., 1991] [Williams et al., 1992] [Morrissey et al., 1994]. In vivo administration of soluble SCF causes an expansion of both immature and differentiated hemopoietic progenitor cells in BM [Tong et al., 1993]. Following injection of SCF, mobilized progenitors appear in the circulation [Andrews et al., 1992] [Bodine et al., 1993], and there is an increase in the number of mature cells of multiple lineages [Molineux et al., 1991] [Ulich et al., 1991] [Chow et al., 1993].

Kit expression was shown on less than 10% of adult BM cells in primates and rodents using anti-Kit antibodies. Results of transplantation studies of different BM fractions showed, that Kit^+ cells were able to repopulate recipient mice [[Okada et al., 1991] [Ikuta and Weissman, 1992] [Okada et al., 1992] [Orlic et al., 1993]. In these experiments, only a small proportion of the Kit^+ cells was selected as a result of the cell isolation procedures, which included counterflow elutriation, depletion of cells that express lineage markers, and purification of WGA^+ or $Sca\text{-}1^+$ cells. Little information is available on the quantitative and qualitative contribution of the Kit^+ population to short- and long-term hemopoietic repopulation of irradiated recipients. It is also not known if, and to what extent, repopulating cells are present in the Kit^- fraction. Therefore, in the present study, mouse BM cells were sorted solely on the basis of Kit expression. To ensure labeling of functional receptors, biotinylated SCF was used for cell staining in parallel with anti-Kit antibodies. To obtain an accurate estimate of the enrichment of immature hemopoietic cells in the Kit^+ BM subset as compared to unfractionated and Kit^- BM cells, the purified Kit^+ cells were assayed for their ability to reconstitute multilineage hemopoiesis after transplantation into irradiated recipients.

4.3 MATERIALS AND METHODS

Mice and BM cell preparation

Mice were bred under specific pathogen-free condition (TNO, Rijswijk, The Netherlands). BM donor mice were 7 weeks old male BCBA (C57BL/LiARij x CBA/BrARij)F1 or 27–29 weeks old male BD2-+/+ (C57BL-W/+ x DBA-2-W^V/+)F1 mice. BM was obtained by flushing excised femora and tibiae with Hepes-buffered (10 mM, pH 6.8-7.1) Hanks' balanced salt solution (without phenol red; Gibco BRL,

Paisley, UK) with an osmolarity of 300–305 mOsm/kg (Hanks' Hepes, abbreviated as HH). Single cell suspensions were obtained by filtration through a six-layer nylon sieve and kept on melting ice until use.

Recipients were 12–20 weeks old BCBA or 21–57 weeks old BD2-W/W^{V} mice, irradiated by use of a ^{137}Cs gamma-cell-20-small-animal irradiator (Atomic Energy of Canada, Ottawa, Canada). Lethally irradiated male BCBA recipients (8.5 Gy total body irradiation) were used for spleen colony assays (CFU-S day 8 and day 12). Lethally or sublethally irradiated female BCBA or W/W^{V} recipients (8.5 Gy or 7 Gy total body irradiation for BCBA, or 3 Gy total body irradiation for W/W^{V}) were used for long-term repopulating ability (LTRA) assays. In the first weeks after BM transplantation, the mice were maintained in laminar flow housing.

Biotinylation of SCF

E. coli-expressed recombinant rat stem cell factor (SCF, kindly provided by AMGEN Ltd., Cambridge, UK) was biotinylated using biotin-*N*-hydroxy succinimide ester (NHS-Biotin, Pierce, Rockford IL, USA) as described previously for IL-2 [De Jong et al., 1992]. Briefly, NHS-Biotin dissolved in dimethyl sulfoxide (DMSO) was added to aliquots of SCF in 0.1 M carbonate-bicarbonate buffer pH 8.5 at molar biotin:protein (B:P) reaction ratios of 10:1, 30:1, and 100:1. A control sample was mock-biotinylated by adding DMSO only. After 3 h incubation at room temperature in the dark, biotin-SCF molecules were separated from the remaining free biotin molecules by extensive dialysis against PBS containing 0.04% Tween-20 and 0.02% (wt/vol) sodium azide, at 4°C (10 000 NMWL filter unit, Cat. No. UFC3 LGL00, Millipore, Bedford MA, USA). Biotin-SCF was stored at –20°C.

The ability of biotin-SCF to stimulate cell proliferation was assayed using a subclone of the factor-dependent cell line FDC-P1 that responded to SCF. The biotin-SCF sample prepared at a B:P ratio of 30:1 retained its biological activity and this preparation was used in further experiments.

Immunocytochemical staining

Mouse BM cells were incubated in 50 µl HH, containing 5% (vol/vol) fetal calf serum and 0.02% (wt/vol) sodium azide (HH/Serum/Azide, abbreviated as HSA). Incubation with the anti-Kit antibody ACK-2 [Nishikawa et al., 1991] (provided by Dr. K. Rajewski, Genetics Institute, Cologne, Germany) (final concentration 38 nM) was performed for 30 min on ice. The samples were then stained with PhycoErythrin- or FITC-conjugated goat anti-rat antibodies (GARa-PE 1:300, GARa-FITC 1:100 vol/vol, both from TAGO, Burlingame CA, USA) for 30 min on ice. After each incubation the samples were washed in HSA. As a negative control, only HSA was used in the first incubation step.

Alternatively, cells were incubated with biotin-SCF (10 nM) for 60 min at room temperature or on ice. The cells were then stained with PE- or BODIPY- conjugated streptavidin (Str-PE or Str-BODIPY, both 3.3 µg/ml final concentration; Molecular Probes, Eugene OR, USA) for 30 min on ice. Specificity of binding of the biotin-SCF samples was determined by incubating the cells with biotin-SCF in the presence of a 100-fold molar excess of unbiotinylated SCF or in the presence of ACK-2, which blocks SCF binding to Kit. As a negative control, only HSA was used in the first incubation step.

To distinguish between living and dead cells Hoechst 33258 (2-(20-(4-hydroxyphenyl)-6-benzimidazole)-6-(1-methyl-4-piperazyl) benzimidazole) (H58, final concentration 1 µg/ml; Molecular Probes) was added to the samples. In some of the experiments, cells sorted on the basis of Kit expression were stained with Rhodamine 123 (Rh123, Eastman Kodak, Rochester NY, USA) as described [Bertoncello et al., 1985].

Flow cytometry

Stained cells were analyzed and sorted using the two-laser RELACS II flow cytometer (Rijswijk experimental light activated cell sorter, TNO, Rijswijk, The Netherlands). Cells were illuminated with the 488-nm line of an argon ion laser (Coherent Innova 90, Palo Alto CA, USA) and UV light (351/364 nm) from an argon ion laser (Spectra Physics, Series 2000, Mountain View CA, USA) at 100 mW laser power. Orange PE fluorescence was measured through a 577-nm longpass filter (Becton Dickinson, Mountain View CA, USA). Green FITC, BODIPY, or Rh123 fluorescence was measured through a 530/30-nm bandpass filter (Becton Dickinson). H58 fluorescence was measured through a KV408 cut-off filter plus a 450-nm shortpass filter. Dead cells stained with H58 were gated out by the FACS at the time of cell sorting. Pulse width (time of flight) of the triggering signal (forward light scatter) was routinely measured to support exclusion of cell clumps.

In vitro colony assay

Sorted populations were assayed for their content of in vitro colony-forming units (CFU-C) by colony formation in agar. Semisolid agar culture medium consisted of 0.3% (wt/vol) agar (Bacto agar, Difco Laboratories) in supplemented Dulbecco's Modified Eagle Medium (Gibco 042 2501H), containing 3 mM L-glutamine, and 20% (vol/vol) of a mixture of 2 volumes of horse serum and 1 volume of fetal calf serum (FCS). Aliquots of 1 ml containing $2x10^4$ unfractionated BM cells or Kit^- cells, or 250–1000 Kit^+ cells, were plated in 35-mm petri dishes (Falcon 1008) in triplo. Cultures were stimulated with recombinant murine IL-3 (1000 Units/ml; Behringwerke AG, Marburg, Germany), recombinant murine GM-CSF (100 U/ml; Behringwerke AG), recombinant rat SCF (100 ng/ml; AMGEN Ltd.), and pregnant mouse uterus extract

(PMUE; 12 µl/ml) [Bradley et al., 1971]. The cultures were maintained in a humidified atmosphere of 10% CO_2 in air for 7 days, after which the colonies were counted.

Spleen colony assay

Unseparated BM cells or Kit^- cells ($2x10^4$), or Kit^+ cells (250–1000) were injected into each of 7 to 10 lethally irradiated mice per experimental group via the tail vein. The spleens were removed on days 8 or 12 after transplantation and fixed in Telleyesniczky's solution (70% ethanol : acetic acid : formalin; 20:1:1). Macroscopically visible spleen colonies were counted.

Repopulation capacity assays

Serial dilutions of cells from male donor mice were injected into lethally or sublethally irradiated female mice. Three recipients were used per experimental group. Between 3 weeks and 1 year after BM transplantation, tail vein blood samples were collected every 2–4 weeks and monitored for the presence of donor-type cells.

Red blood cell chimerism of BD2 recipients was examined by measuring the differential forward light scatter of small, normal, +/+ red blood cells and large, macrocytic, W/W^v red blood cells, using a FACScan flow cytometer (Becton Dickinson, San Jose CA, USA), similar to the method described previously [Van den Bos et al., 1992]. Blood cells were suspended in a hypotonic saline solution (0.75% wt/vol NaCl). Percentages of donor-type red blood cells were determined by setting a marker based on control cells from +/+ and W/W^v mice.

Blood smears were made on microscope slides to study nucleated blood cell chimerism. Slides were air dried overnight, fixed in a mixture of 3 volumes 100% ethanol and 1 volume acetic acid, and washed in ethanol. After baking at 70°C for 3 h, the slides were stored at -20°C under nitrogen gas until analysis for presence of nucleated donor cells by fluorescent in situ hybridization (FISH).

One year after transplantation, mice were sacrificed and femora, spleen and thymus were removed. Cell suspensions were made in a 0.9% (wt/vol) NaCl solution, fixed with a mixture of methanol and acetic acid (3:1), resuspended in a small volume (approximately 100 µl) of methanol-acetic acid, and dropped onto microscope slides. The slides were air dried and stored at -20°C under nitrogen gas until FISH was performed.

Fluorescent in situ hybridization (FISH)

The mouse Y-chromosome specific DNA probe p17-M34/2 [Singh et al., 1994] (Dr. L. Singh, Center for Cellular and Molecular Biology, Hyderabad, India) was labeled with biotin-16-dUTP (Sigma Chemical Co., St. Louis MO, USA) by nick translation. FISH was performed similar to the method described previously [Van Dekken et al., 1989]. Cells were permeabilized in 0.1 M HCl containing 0.05% (vol/vol) Triton X-100 (7.5 min,

37°C) and fixed in a phosphate-buffered saline solution (PBS) containing 1% (vol/vol) formalin (15 min, room temperature). The slides were dehydrated in an ethanol series (70%, 83%, 96%) and air dried. Chromosomes were denatured in 70% (vol/vol) deionized formamide (Merck, Darmstadt, Germany) in 2xSSC for 5 min. at 70°C (1xSSC is 0.15 M NaCl, 0.015 M sodium citrate). The slides were dehydrated in an ethanol series and air dried. The probe (2.5 µg/ml) was denatured for 5 min. at 70°C in the hybridization mixture, which contained 0.5 mg/ml herring sperm DNA, 0.1% (vol/vol) Tween-20, 10% (wt/vol) dextran sulfate, and 50% deionized formamide in 2xSSC, pH 7. Under glass coverslips, hybridization was allowed to take place overnight at 37°C in a humidified atmosphere. Subsequently, the slides were washed three times for 3 min in 50% formamide / 2xSSC followed by two times for 5 min in 2xSSC, both at 45°C (or at room temperature), and immersed in 4xSSC / 0.1% (vol/vol) Triton X-100 at room temperature. The slides were incubated for 20 min at 37°C with fluorescein-labeled avidin DCS (2 µg/ml; avidin-FITC, Vector Laboratories, Burlingame CA, USA) in 4xSSC containing 5% non-fat dry milk (Protifar, Nutricia, Zoetermeer, The Netherlands) and 0.02% NaN_3. After washing the slides twice in 4xSSC / 0.1% (vol/vol) Triton X-100, the fluorescence intensity was amplified using subsequent layers of biotinylated goat anti-avidin antibodies (5 µg/ml, Vector Laboratories) and avidin-FITC [Pinkel et al., 1986]. For microscopic observation the cells were stained for total DNA with propidium iodide (0.5 µg/ml) and DAPI (0.1 µg/ml) in an antifade solution (50 g/l 1,4-diazobicyclo-[2.2.2] octane (B) in a solution of 90:10 (vol/vol) glycerol / 0.1 M Tris-HCl, pH 8). A random sample of at least 100 complete nuclei per slide were counted and scored for probe-related FITC fluorescence, and the percentage of Y chromosome positive cells was determined.

Statistics

Data of in vitro and in vivo colony assays are expressed as average number of colonies per 10^4 cells plated or injected. Standard errors were obtained by taking the square root of the absolute number of colonies counted, assuming that colony counts are Poisson distributed [Blackett, 1974] [Iscove, 1977].

Differences between colony data of different BM fractions were analyzed using the Mann-Whitney test.

4.4 RESULTS

Kit expression on mouse BM cells

The expression of Kit on the cell surface of BM cells was determined using either biotin-SCF or anti-Kit antibodies (ACK-2). A distinct cluster of Kit^+ cells was found

after staining with biotin-SCF and fluorescently labeled streptavidin (**figure 4.1 A**). Staining with biotin-SCF was specific, as shown by incubation of cells with biotin-SCF in the presence of either a 100-fold molar excess of unlabeled SCF or the blocking anti-Kit antibody ACK-2 (**figure 4.1 B**). The fluorescence signal of these control cells was similar to that of cells incubated without biotin-SCF.

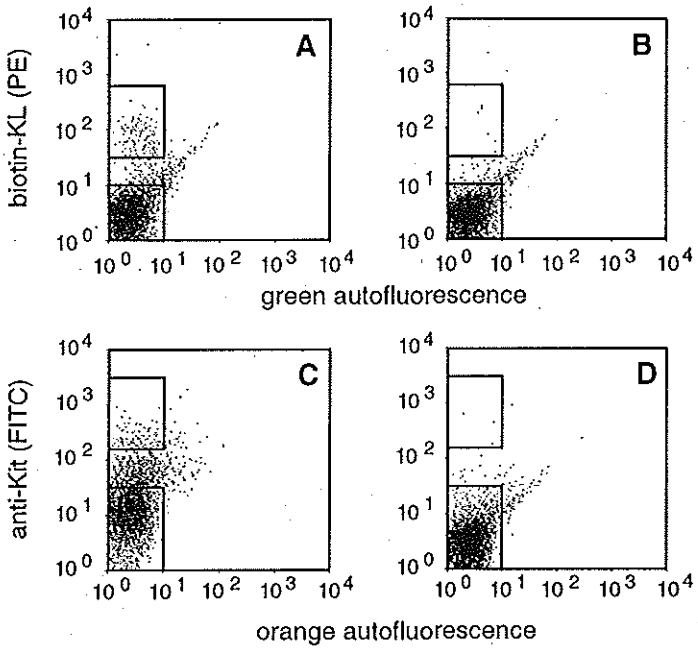

figure 4.1
Kit expression on mouse BM cells.
Cells were stained with biotin-SCF and streptavidin-PE (**A**), or with ACK-2 and FITC-conjugated goat anti-rat antibodies (GARa-FITC) (**C**). Control samples were incubated with biotin-SCF in the presence of ACK-2 before staining with streptavidin-PE (**B**), or only with GARa-FITC (**D**). Indicated are the windows that were used to sort Kit+ and Kit− cells. Green FITC fluorescence and autofluorescence was measured through a 530/30-nm bandpass filter, orange PE fluorescence and autofluorescence through a 577-nm longpass filter.

Staining of BM cells with ACK-2 and fluorescence-tagged second layer antibodies resulted in a different fluorescence distribution than staining with biotin-SCF (**figure 4.1 C**). As also shown by others [Okada et al., 1991] [Orlic et al., 1994], ACK-2 staining resulted in a weak fluorescence signal on a large proportion of the cells, and a bright signal on a small subset of BM cells. This ACK-2$^+$ subset contained a similar percentage of cells (1–2%) as was found in the cluster of biotin-SCF$^+$ cells.

As indicated in **figure 4.1**, the Kit$^-$ fractions as well as the brightest Kit$^+$ cells were sorted in the flow cytometer. The Kit$^+$ cells were negative or low positive for lineage

markers specific for granulocytes, monocytes, macrophages, erythroid and lymphoid cells (15-1.1, 10-2.2 [De Vries, 1988], 45D8 [Bauman et al., 1988], B220, and GM1.2 [Bauman et al., 1986]) (not shown). The majority of the Kit$^+$ cells showed a light scatter pattern of large blast cells. Additionally, part of the ACK-2$^+$ cells displayed a lymphocyte-like scatter pattern (not shown). Cells with this phenotype were not found in the biotin-SCF$^+$ fraction. These results indicated that the Kit+ populations identified with biotin-SCF or anti-Kit are qualitatively different. Unfortunately, double-staining of BM cells with biotin-SCF and ACK-2 to directly compare their target cell specificity was not possible because ACK-2 interfered with binding of biotin-SCF to the cells (as shown in **figure 4.1 B**).

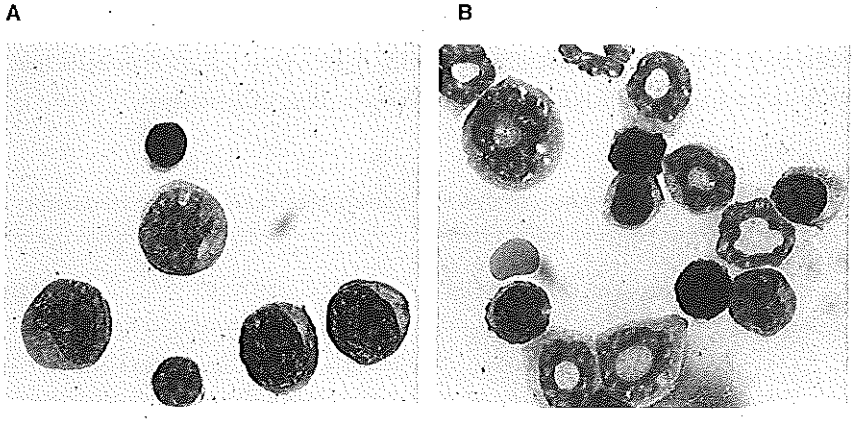

figure 4.2
Morphology of mouse bone marrow cells purified on basis of Kit expression.
May-Grünwald Giemsa stained cytospin preparations of isolated Kit+ (A) and Kit− (B) BM cells. These cells are representative of the fractions obtained by flow cytometry after staining with either biotin-SCF or ACK-2. The Kit+ subsets contained many blasts, and small cells that resemble lymphocytes. The percentage of mature granulocytes and monocytes in the Kit− fractions was 10–fold higher than in the Kit+ subsets. (Original magnification 100x objective.)

Morphological differentiation of Kit positive and negative subpopulations of BM

Purified Kit$^+$ and Kit$^-$ cells differed in morphology (**figure 4.2**). Differential counts of all nucleated cells showed a striking difference in the monomyeloid compartment between Kit$^+$ and Kit$^-$ cells. ACK-2$^+$ as well as biotin-SCF$^+$ fractions contained a 3- to 5-fold more immature myeloid and monocytic cells than unfractionated BM (13–19% and 4%, respectively). In agreement with previous reports [Orlic et al., 1994], many of the cells in the Kit$^+$ fractions (especially the ACK-2$^+$ fraction) morphologically resembled lymphocytes. This lymphocyte-like subset may also contain very immature,

undifferentiated progenitors and erythroid cells, which are difficult to distinguish morphologically from lymphocytes. This implies that the actual percentage of immature Kit$^+$ cells may be higher than the 13–19% that were scored as immature monomyeloid cells.

Mature myeloid and monocytic cells were rare in the Kit$^+$ fractions (3–4%), but comprised 30-40% of the cells in the Kit$^-$ subsets and in unfractionated BM, which is within normal ranges [Hoang et al., 1983] [Bertoncello et al., 1986] [Ploemacher et al., 1993].

In vitro and in vivo colony forming potential of Kit positive BM cells

In vitro culture showed that the sorted Kit$^+$ cells were 20- to 70-fold enriched for colony forming units in culture (CFU-C) as compared to unfractionated BM (**table 4.1**). One in every 5–6 biotin-SCF$^+$ cells, or 1 in every 13–18 ACK-2$^+$ cells formed a colony in vitro. Therefore, Kit$^+$ fractions that were sorted after cell staining with biotin-SCF contained CFU-C at significantly (2–3-fold) higher frequencies than cells that were sorted after staining with ACK-2 (9 independent sorting experiments).

table 4.1
Distribution of in vitro and spleen colony forming potential on the basis of Kit expression.

Population	% [a]	CFU-C [b]		CFU-S d8 [c]		CFU-S d12 [c]	
		Incidence per 10^4 cells	Enrichment Factor	Incidence per 10^4 cells	Enrichment Factor	Incidence per 10^4 cells	Enrichment Factor
control BM	100%	27.5 ± 6.4	1	4.2 ± 0.1	1	5.1 ± 1.4	1
ACK-2$^+$	0.91 ± 0.30%	672.2 ± 109.2 *	24.5	110.4 ± 15.4 *	26.3	136.5 ± 33.1 *	26.8
biotin-SCF$^+$	0.97 ± 0.36%	1838.9 ± 152.6 *	67.0	224.5 ± 27.2 *	58.5	209.0 ± 25.6 *	41.0
ACK-2$^-$	83.51 ± 2.74%	1.5 ± 0.1	0.05	ND [d]		ND	
biotin-SCF$^-$	95.95 ± 3.56%	2.9 ± 0.3	0.11	ND		ND	

[a] Mean (± S.E.M.) of 3 different experiments are shown.
[b] Colony-forming units in culture. In each experiment, the sorted cells were cultured in three replicate dishes.
[c] The sorted cells were injected into groups of 7-10 lethally irradiated (8.5 Gy) recipients per fraction in each experiment. The spleens were removed on days 8 or 12 after transplantation and fixed in Telleyesniczky's solution. Data were corrected for the number of endogenous colonies observed in the control irradiated groups.
[d] ND, not determined. In one experiment, CFU-S were determined using the biotin-SCF$^-$ fraction, which resulted in 0.0 CFU-S d8 and 0.3 ± 0.8 CFU-S d12 per 10^4 cells injected.
* Differences between biotin-SCF+ and ACK-2+ subsets were significant ($P < 0.001$, Mann-Whitney test).

To examine the spleen colony forming potential of the sorted fractions, the cells were injected into lethally irradiated mice, and CFU-S were counted at day 8 or 12 after transplantation. As shown in **table 4.1**, the Kit$^+$ sorted fractions were approximately 25- to 60-fold enriched for CFU-S compared to unfractionated BM. The subsets that were sorted using biotin-SCF contained 1.5- to 2-fold as many spleen colony forming cells as the ACK-2$^+$ fractions.

These differences in colony formation between the Kit$^+$ and Kit$^-$ subsets were not due to stimulation of the cells by biotin-SCF or inhibition by ACK-2, since differences between colony numbers obtained from unfractionated BM after incubation with buffer, biotin-SCF or ACK-2 were not significant (not shown). When forward light scatter criteria were used to separate ACK-2$^+$ cells on the basis of size, the majority of the CFU-S were recovered in the fraction of large blast-like cells, and similar results were obtained for in vitro colony-forming cells (not shown). Recovery of colony-forming cells ranged from 15–40% for ACK-2$^+$ subsets, and from 22–96% for biotin-SCF$^+$ fractions.

Repopulating capacity of Kit positive and negative BM cells

To assess the short- and long-term repopulating ability (STRA and LTRA, respectively) of Kit$^+$ and Kit$^-$ BM cells, sorted cells of male donor mice were transplanted into female recipients. In addition to normal mice, transplantation experiments were performed using W/W^v mice that received sorted fractions of +/+ BM cells. The presence of donor cells in the peripheral blood (PB) of the recipients was monitored regularly during a one year period. Red blood cell chimerism was determined by measuring the percentage of normal-sized erythrocytes in the PB of the W/W^v recipients, which originally suffered from macrocytic anemia (**figure 4.3**). Nucleated blood cell chimerism was assessed using fluorescent in situ hybridization (FISH) to determine the percentage of Y-chromosome-positive nucleated PB cells in the female recipients. As shown in **figure 4.4**, stable chimeras with high percentages of donor-type erythrocytes and nucleated blood cells were found in the mice that had been transplanted with $1x10^5$ unfractionated BM cells. This is consistent with the estimated frequency of at least 1–2 stem cells per $1x10^5$ BM cells [Harrison et al., 1988] [Van der Loo et al., 1994]. Transplantation of 10^3 Kit$^+$ (biotin-SCF$^+$ or ACK-2$^+$) cells resulted in high percentages of donor-type PB cells in 6 out of 7 recipients in this experiment. Repopulation by ACK-2$^+$ cells (not shown) was similar to that of biotin-SCF$^+$ cells. In general, repopulation of erythrocytes occurred faster than that of nucleated blood cells, and higher percentages of donor-type erythrocytes than nucleated blood cells were found. Transplantation of 10^5 Kit$^-$ cells resulted in short-term repopulation of red blood cells, returning to the macrocytic host-type after 10–15 weeks in all mice. Little or no

nucleated blood cell chimerism was observed in these mice during the period that the PB was monitored, which was between 3 weeks and 1 year after transplantation.

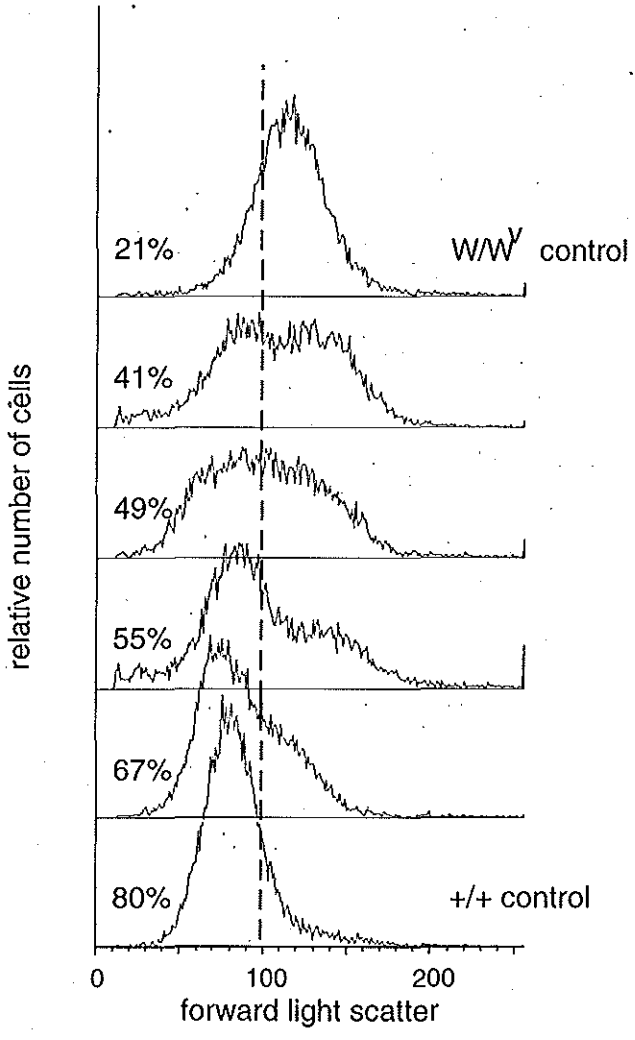

figure 4.3
Frequency distributions of forward light scatter of erythrocytes of W/W^v, +/+, and chimeric mice.
Forward light scatter characteristics of PB erythrocytes of macrocytic W/W^v (top histogram) or normal +/+ (bottom histogram) control mice are shown as a measure of cell size. To illustrate various degrees of chimerism, histograms of 4 W/W^v recipients, 3–4 weeks after transplantation of different sorted fractions of +/+ BM cells are shown. The broken line indicates the marker that is set on basis of forward light scatter characteristics of cells from control mice, to discriminate between normal and macrocytic erythrocytes and to calculate the percentages of normal-sized erythrocytes (shown on the left of the histograms). Because the histograms of W/W^v ("host") and +/+ ("donor") erythrocytes overlap, chimerism percentages range between 20 and 80%.

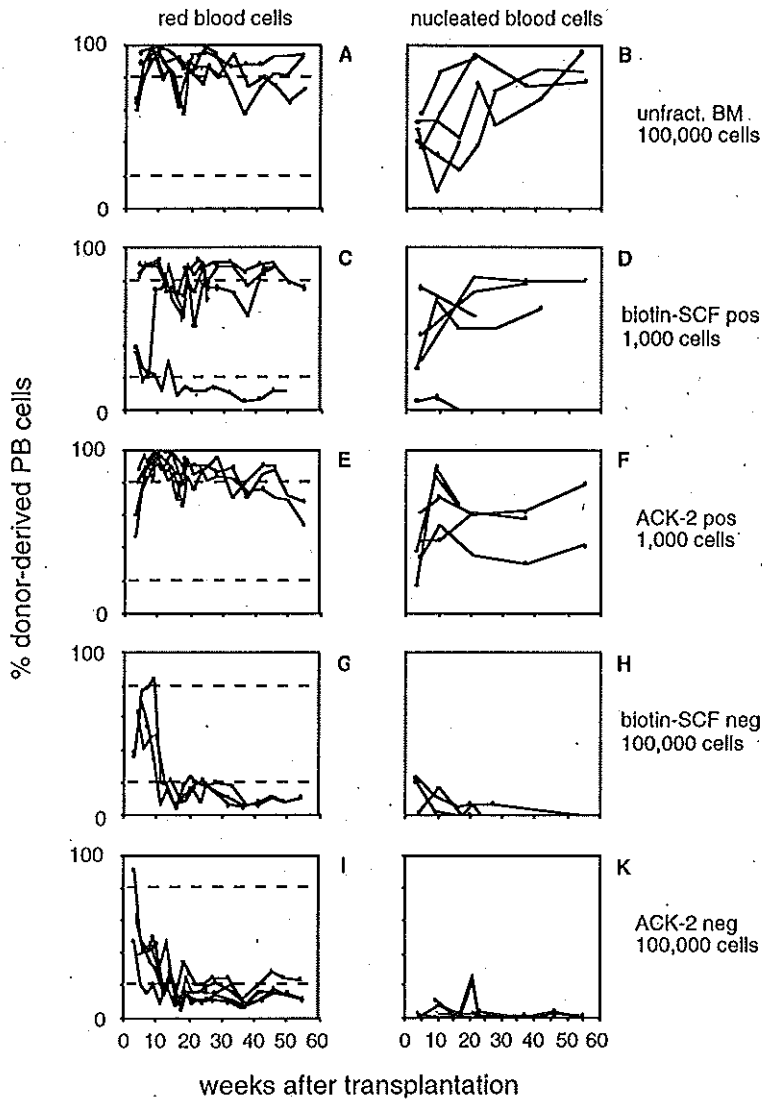

figure 4.4
Time course of erythrocyte and nucleated blood cell chimerism in female W/W^{V} recipients after transplantation with sorted fractions of male +/+ donor BM.
Lines represent data of individual mice. For each recipient, erythrocyte (left panels) as well as nucleated (right panels) blood cell chimerism is shown. Transplanted cells were (**A** and **B**) 100,000 BM cells, (**C** and **D**) 1,000 Kit+ cells stained with biotin-SCF, (**E** and **F**) 1,000 Kit+ cells stained with ACK-2, (**G** and **H**) 100,000 Kit^{-} cells stained with biotin-SCF, and (**I** and **K**) 100,000 Kit^{-} cells stained with ACK-2. Pooled data from 2 independent sorting experiments are shown. Percentages of donor-type PB cells were determined using both FISH (left panels) and flow cytometry (right panels). Broken lines in the left panels indicate the 20–80% range of red blood cell chimerism as described in the legend to **figure 4.3**.

To determine the enrichment of LTRA in the Kit$^+$ fractions compared to Kit$^-$ and unfractionated BM, serial dilutions of sorted cells were transplanted. The results of one of these experiments are depicted in **figure 4.5** and show the percentages of donor-type nucleated blood cells one year after transplantation. Similar results were obtained at earlier time points. To estimate the enrichment of immature cells in the sorted fractions as compared to unfractionated BM, we calculated the cell numbers of the different fractions that were needed to reach 50% donor-derived nucleated blood cells. FISH data from 4 different sorting experiments showed stable chimeras containing 50% donor-type nucleated PB cells after transplantation of 50,000–100,000 unfractionated BM cells or 500–2,000 Kit$^+$ cells (**figure 4.5** and **table 4.2**). This corresponds to a 25–100-fold enrichment for LTRA in the Kit$^+$ fraction. Repopulation by biotin-SCF$^+$ fractions was similar to that of ACK-2$^+$ cells. Enrichment factors based on erythrocyte repopulation could not be calculated, since the majority of mice transplanted with unfractionated or Kit$^+$ BM cells became stable chimeras with high percentages of donor-type red blood cells, even when low cell numbers were used (**table 4.2**). Similar to the nucleated blood cells, the red blood cells of mice transplanted with Kit$^-$ BM cells remained mostly of host-type (**table 4.2** and **figure 4.5**), which demonstrated that the Kit$^-$ fractions were depleted of LTRA cells. Hemopoietic repopulation in mice that had received total BM, sorted after staining with biotin-SCF or ACK-2, was similar to that after transplantation of unstained BM (not shown). This indicated that the staining procedures did not influence the repopulation capacity.

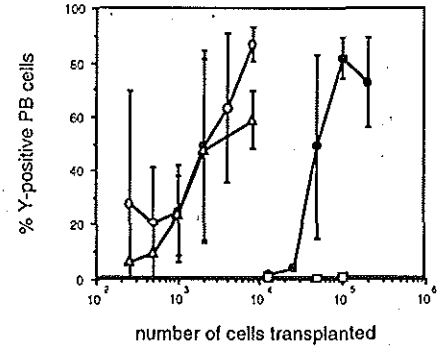

figure 4.5
Nucleated blood cell chimerism in PB of female recipients, one year after transplantation with different numbers of sorted BM cells of male donor mice.
BCBA mice were transplanted with unfractionated BM cells (closed circles), or with sorted Kit+ after incubation with biotin-SCF (open circles) or ACK-2 (triangles). Very few mice transplanted with biotin-SCF$^-$ and ACK-2$^-$ fractions were alive after 1 year, therefore data from surviving mice were pooled (open squares). Percentage of donor-type nucleated cells was determined by FISH. Each dot represents the mean percentage of 2 or 3 recipients; error bars indicate the SD.

table 4.2
Repopulation of nucleated and red blood cells after transplantation of serial dilutions of sorted BM cells.

	# cells injected x 10^3	biotin-SCF+ BM	# cells injected x 10^3	unfractionated BM	biotin-SCF$^-$ BM
nucleated cells [a]	0.25	0 / 5 [c]	12.5	2 / 8 [c]	0 / 5 [c]
	0.5	2 / 5	25	2 / 7	0 / 2
	1	5 / 8	50	3 / 6	0 / 1
	2	4 / 8	100	5 / 7	0 / 3
	4	5 / 6	>200 [d]	6 / 6	3 / 4
	8	6 / 7			
red cells [b]	0.25	1 / 2	12.5	5 / 5	0 / 5
	0.5	2 / 2	25	3 / 4	1 / 2
	1	4 / 5	50	3 / 3	0 / 1
	2	5 / 6	100	4 / 4	0 / 3
	4	3 / 3	>200 [d]	4 / 4	4 / 4
	8	4 / 4			

[a] nucleated blood cell repopulation ability of the indicated fractions was assayed by FISH with a Y-chromosome specific DNA probe, 5 months after transplantation of male donor cells into female recipients.
[b] red blood cell repopulation ability was assayed by FACS analysis of PB samples 5 months after transplantation of +/+ donor cells into W/W^v recipients.
[c] number of mice with more than 50% Y-positive nucleated PB cells, or more than 50% donor-type erythrocytes, respectively, per number of mice assessed. Data from 3 independent experiments were pooled. Similar results were obtained at other timepoints, up to one year posttransplantation.
[d] 200,000–800,000 cells per mouse

Comparison of the long-term repopulation of PB, BM, spleen, and thymus

To investigate the effect of transplantation of the sorted fractions on the nucleated blood cell chimerism in the major hemopoietic organs, the recipients were sacrificed one year after transplantation, and the percentages of Y-chromosome positive nucleated cells in BM, spleen and thymus were determined. The results of one of these experiments are shown in **table 4.3**. A large variation was observed for the percentages of donor-type nucleated cells in PB, BM, spleen and thymus of individual mice, which is in agreement with previously reported differences in the contribution of transplanted cells to various lineages [Lemischka et al., 1986] [Jordan and Lemischka, 1990]. Despite this variation, these results show that Kit$^+$ cells contributed to donor-type repopulation in each of these hemopoietic organs. Pooled data of sorted Kit$^+$ and unfractionated BM cells from 3 different experiments (**figure 4.6**) showed a high correlation between donor-type nucleated cell numbers in PB and BM ($r = 0.88$). As may have been expected, the correlation with spleen and thymus donor-type cells and those in BM is progressively lower, respectively ($r = 0.69$ and 0.50).

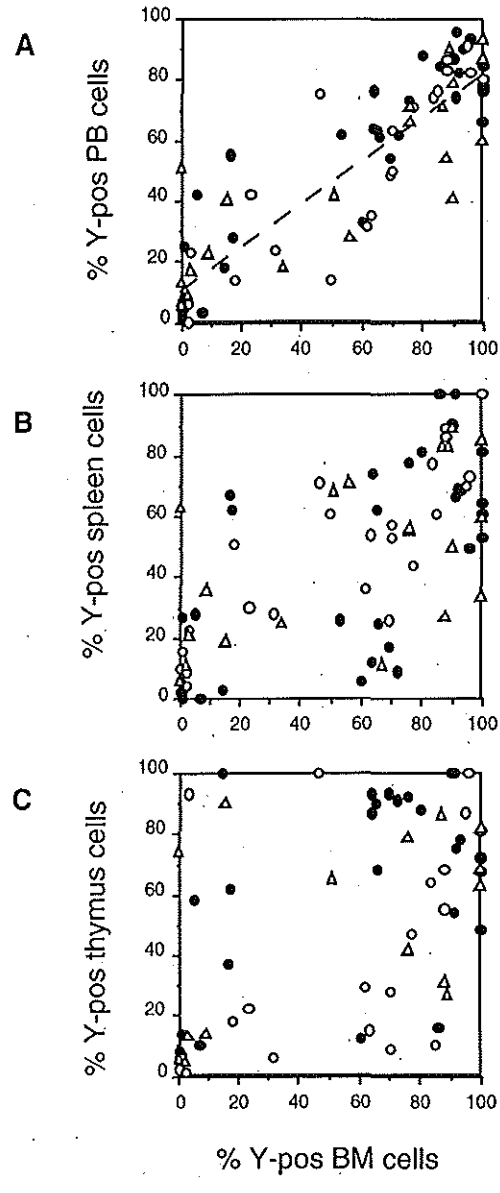

figure 4.6
Correlation between nucleated blood cell chimerism in BM and PB (**A**), spleen (**B**) and thymus (**C**) one year after transplantation of different BM subsets.
Male donor BM cells were injected into female recipient mice. Percentage of donor-type nucleated cells was determined using FISH. Each dot represents data of one mouse. Transplanted cells were unfractionated BM (closed circles; n=32), biotin-SCF+ (open circles; n=23), and ACK-2+ (triangles; n=21) cells. Pooled data are shown from 4 independent experiments, half of which involved BCBA donors and recipients, the other half BD2-+/+ donors and BD2-W/W^{v} recipients. Regression line is indicated in (**A**).

table 4.3
Percentages of donor-type nucleated cells in peripheral blood and hemopoietic organs of 22 individual chimeric mice, 12 months after transplantation.

Population [a]	# cells injected [a] x 10^3	PB	BM	spleen	thymus
biotin-SCF+	0.5	6	1	16	6
	0.5	35	63	54	15
	1	7	0	10	2
	1	23	3	23	93
	1	42	23	30	22
	2	24	31	28	6
	2	74	83	77	64
unfract. BM	25	5	1	2	0
	25	3	0	6	6
	50	25	1	27	14
	50	73	76	78	92
	100	76	64	74	87
	100	87	90	90	100
	200	55	16	67	37
	200	88	80	81	88
	200	74	91	66	100

[a] Per mouse, the indicated number of unfractionated BM cells or biotin-SCF+ cells was transplanted. One year after transplantation, the percentages of Y-chromosome-positive cells in the different organs were determined using FISH.

4.5 DISCUSSION

The aim of this study was to characterize mouse BM cells that were selected solely on the basis of Kit expression, and to determine their contribution to short- and long-time repopulation of the hemopoietic system of irradiated recipients. In order to purify cells that express functional receptors, we stained the cells with biotinylated SCF and compared this method with cell staining using the anti-Kit antibody ACK-2 [Nishikawa et al., 1991].

Sorted Kit$^+$ populations were enriched for immature myeloid and erythroid cells, as shown by morphology, CFU-C, and CFU-S day 8 and 12. Compared to unfractionated BM, the Kit$^+$ fractions were 20–70-fold enriched for clonogenic cells. This enrichment was achieved by using a single selection parameter. Similar enrichment values for CFU-S have been obtained after sorting of Kit$^+$ cells that were negative for a cocktail of markers with specificity for granulocytes, monocytes, macrophages, erythroid and lymphoid cells [Okada et al., 1991]. These markers, which are commonly employed in stem cell purification protocols, are not expressed on Kit$^+$ cells. Therefore, cell staining based on Kit expression is a useful alternative for the selection of lineage-negative cells

to obtain BM fractions that are enriched for immature cells. Since the Kit^+ fractions in the different experiments comprised 1–2% of the nucleated BM cells, 50- to 100-fold enrichment is the maximal value that can be obtained with Kit expression as a single sorting parameter.

Since data on the kinetics of hemopoietic repopulation after transplantation of Kit^+ cells were not available, the present study included periodical monitoring of erythrocyte and nucleated blood cell repopulation of recipient mice during a 1 year period. Nucleated blood cell chimerism was measured by counting Y-chromosome-positive cells. Although this method involves manual counting of the cells, it has the advantage that actual ratios of donor- and host-type cells are measured. These values are more accurate than other parameters for repopulation capacity, such as the percentage of cells with donor-type Ly5 antigen, which is not expressed on erythroid cells [Okada et al., 1991], or the relative quantity of donor and recipient hemoglobin types [Orlic et al., 1993], which only provides a relative measure for chimerism and is not single-cell based. In addition, our study is the first in which serial dilutions of Kit^+, Kit^-, and unfractionated BM cells were transplanted to estimate the enrichment of immature cells in the sorted fractions.

Results of the transplantation experiments demonstrated that the Kit^+ fractions were 25- to 100-fold enriched for long-term repopulating cells as compared to unfractionated BM. In addition, the Kit expressing subsets contained short-term repopulating cells, since lethally irradiated recipients that were transplanted with Kit^+ cells survived the initial cytopenic phase, and showed high percentages of donor-type PB cells within a few weeks after transplantation. In the Kit^- subsets we only found high percentages of short-term repopulating cells for the red blood cell lineage. However, it is possible that we missed the short-term repopulation of white blood cells, which have a much shorter life span than red blood cells, and are likely to have disappeared before the first timepoint examined.

Repopulation with donor-type red blood cells reached stable levels sooner than nucleated blood cell repopulation. After transplantation with the appropriate cell numbers, stable chimeras in which the red cell lineage was completely repopulated by donor-derived erythrocytes were formed within 10 weeks. The percentage of donor-type nucleated blood cells of these same mice ranged from 20 to 100%, depending on the number of cells that were transplanted, and reached stable levels at variable time points, ranging from 10 to 30 weeks after transplantation. Higher and more rapid repopulation by donor-type red blood cells as compared to nonerythroid cells is a common finding in transplantation experiments in mice with defects in erythropoiesis, and indicates a selective advantage of the production of normal over defective erythrocytes [Barker et al., 1988] [Nakano et al., 1989] [Barker et al., 1991] [Van den Bos et al., 1993] [Van den Bos et al., 1994] [Van der Loo et al., 1994].

Similar to what we have recently shown for human SCF on rhesus monkey BM [De Jong et al., 1995], incubation of mouse BM cells with biotinylated SCF results in staining of immature hemopoietic cells as well as differentiating progenitors. Selection of Kit^+ cells after incubation with biotin-SCF yielded fractions with significantly (2- to 3-fold) higher frequencies of clonogenic cells than obtained after staining with ACK-2. It is not certain whether this dissimilarity also occurs at the level of LTRA cells, as a factor 2–3 difference is too small to result in distinctly deviating S-curves showing donor-type nucleated blood cell repopulation (**figure 4.5**). Since no effect of cell staining with biotin-SCF or ACK-2 on colony formation or repopulation capacity was found, the difference between the fractions may be caused by a different composition of the biotin-SCF^+ and the ACK-2^+ populations. This might be reflected in the distinct staining patterns of biotin-SCF and ACK-2 on BM (**figure 4.1**), and the different light scatter [Bertoncello et al., 1985] and Rh123 retention properties of the biotin-SCF^+ and ACK-2^+ fractions [Visser et al., 1993]. Moreover, it is possible that part of the ACK-2^+ cells represent a Kit^- fraction with ACK-2 bound to F_c receptors. Additionally, the percentage of cells that were scored as lymphoid based on morphological criteria was significantly higher in the ACK-2^+ than in the biotin-SCF^+ fraction (not shown). Similar to previous reports [Okada et al., 1991] we have not detected high frequencies of cells expressing lymphocyte markers in the ACK-2^+ BM subset. It is possible that the markers used were not directed against the lymphocyte subset that binds ACK-2. An alternative possibility is that ACK-2 binds to a subset of immature lymphocyte precursors that do not yet express these markers, as has been reported for anti-Kit antibodies on murine and human cells [Matsuzaki et al., 1993] [deCastro et al., 1994]. If this is the case, it would be interesting to determine whether these cells express an isoform of Kit that is recognized by ACK-2, but does not bind SCF.

In primate BM immature and more differentiated progenitors have been shown to differ in their level of Kit expression [De Jong et al., 1995]. It has also been suggested that the Kit^{low} fraction contains the most primitive, dormant progenitors [Katayama et al., 1993] [Ogawa et al., 1994]. For this reason it will be of interest further to dissect BM subsets on the basis of Kit expression levels, combined with additional parameters such as expression of Sca-1 [Ikuta and Weissman, 1992] [Okada et al., 1992], CD34 [Krause et al., 1994], or Flk-2 [Visser et al., 1993] [Orlic et al., 1994], and differences in staining levels of Hoechst 33342 [Baines and Visser, 1983], wheat germ agglutinin (WGA) [Ploemacher et al., 1993], or Rh123 [Ploemacher and Brons, 1988] [Ploemacher and Brons, 1989] to separate long-term repopulating cells from those responsible for short-term reconstitution of individual cell lineages.

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CHAPTER 5

FDC-P1 cells as a model to examine differential binding of stem cell factor (SCF) and anti-Kit antibodies to hemopoietic cells

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5.1 ABSTRACT

Murine bone marrow cells show differential staining with the anti-Kit antibody ACK-2 and with biotinylated stem cell factor (SCF): a higher percentage of the cells reacts with ACK-2 than with biotin-SCF. To examine this difference, Kit expression was studied on two sublines of the murine FDC-P1 cell line, which respond to interleukin-3 (IL-3) and SCF, respectively. Although ACK-2 bound to cells from the IL-3 responsive FDC-P1(IL-3) subline, which demonstrated that Kit was expressed, SCF did not induce detectable proliferation of these cells. Moreover, no or very low fluorescence signals were found using FACS analysis after incubation of FDC-P1(IL-3) cells with biotin-SCF. In contrast, FDC-P1(SCF) cells showed specific staining with ACK-2 as well as biotin-SCF, resulting in fluorescence signals that were approximately 3-fold (for ACK-2) or 40-fold (for biotin-SCF) higher than those of the FDC-P1(IL-3) cells. Since the differential binding of biotin-SCF and anti-Kit could be caused by differences in receptor structure, we studied *c-kit* RNA expression in FDC-P1(IL-3) and FDC-P1(SCF) cells by reverse transcriptase polymerase chain reaction (RT-PCR). We examined expression of the regions of the *c-kit* gene that encode for the SCF binding-site, the putative dimerization site of Kit monomers, and the KitA isoform that is the result of alternative splicing. Qualitative differences were not found between the sublines, but the FDC-P1(SCF) expressed 5–9-fold more *c-kit* RNA than the FDC-P1(IL-3) cells. Further examination of Kit proteins by immunoprecipitation resulted in detection of cell surface expression of the 125–135 kD as well as the 160–165 kD form of Kit on both FDC-P1 sublines. Similar to the *c-kit* RNA expression, a higher expression of Kit proteins was found on the FDC-P1(SCF) than the FDC-P1(IL-3) cells. Taken together, our RT-PCR and immunoprecipitation results show only quantitative differences between FDC-P1(IL-3) and FDC-P1(SCF) cells. The discrepancy between staining with anti-Kit antibodies and biotin-SCF may be caused by the requirement for a minimum number of Kit monomers present on the cell surface to enable efficient dimerization of Kit molecules to form high affinity SCF receptors.

5.2 INTRODUCTION

Stem cell factor (SCF), also known as Kit ligand, mast cell growth factor, or Steel factor, is a growth factor that acts on immature hemopoietic cells [Anderson et al., 1990] [Huang et al., 1990] [Martin et al., 1990] [Zsebo et al., 1990a] [Zsebo et al., 1990b]. SCF exists in transmembrane and soluble forms [Anderson et al., 1990]. The receptor for SCF, Kit (CD117), is encoded by the *c-kit* gene. Kit is a transmembrane glycoprotein, a member of the tyrosine kinase receptor family [Yarden and Ullrich, 1988] that includes the receptors for macrophage-colony stimulating factor (M-CSF) and platelet derived growth factor [Chabot

et al., 1988]: These receptors have five extracellular immunoglobulin-like domains and a bisected intracellular tyrosine kinase domain [Hanks et al., 1988] [Majumder et al., 1988].

Expression of Kit has been found on 1–4% of bone marrow (BM) cells [Lerner et al., 1991] [Papayannopoulou et al., 1991] [De Jong et al., 1995] [De Jong et al., 1996] (**chapter 4** and **chapter 6**). A nonfunctional *c-kit* gene product, caused by a mutation at the *W* (*dominant white spotting*) locus, which encodes for Kit [Chabot et al., 1988] [Geissler et al., 1988], results in defective hemopoiesis in mice [Hayashi et al., 1991]. Transplantation studies showed that the most immature BM cells express Kit, as Kit^+ cells were able to repopulate recipient mice, whereas Kit^- cells were not [Okada et al., 1991] [Ikuta and Weissman, 1992] [Okada et al., 1992] [Orlic et al., 1993] [De Jong et al., 1996] (**chapter 4**). Moreover, following injection of mice with neutralizing anti-Kit antibodies, almost all progenitor cells disappeared from the BM [Ogawa et al., 1991]. These results, combined with the in vivo and in vitro effects of SCF on hemopoietic progenitor cells [Broxmeyer et al., 1991] [McNiece et al., 1991] [Metcalf and Nicola, 1991] [Andrews et al., 1992] [Bodine et al., 1993], demonstrate the important roles of Kit and SCF in the development of hemopoietic cells.

In previous studies we observed qualitative and quantitative differences between Kit^+ mouse BM cells stained with biotin-SCF, and Kit^+ cells stained with the anti-Kit antibody ACK-2 [Visser et al., 1993] [De Jong et al., 1996] (**chapter 4**). A larger fraction of BM cells stained with ACK-2 than with biotin-SCF. Similar staining differences between biotin-SCF and anti-Kit antibodies were found for human BM cells [Wognum et al., 1996]. These differences do not appear to be caused by a higher sensitivity of anti-Kit than biotin-SCF staining, as the fluorescence signals of cells stained with biotin-SCF were comparable to those of cells stained with anti-Kit.

Because of the low frequency of Kit^+ cells in BM, it is difficult to isolate sufficient numbers of Kit^+ cells to examine the differential binding of anti-Kit and biotin-SCF. In this study we examined this difference using the murine early myeloid factor-dependent cell line FDC-P1 [Dexter et al., 1980] [Welham and Schrader, 1991]. FDC-P1 cells are dependent on IL-3 or GM-CSF for growth [Hapel et al., 1984], and although SCF synergizes with these growth factors to induce proliferation of the cells, FDC-P1 cells that are maintained with IL-3 or GM-CSF do not respond to soluble SCF alone [Caruana et al., 1993]. However, as shown by others [Caruana et al., 1993] as well as in this report, the cells can be adapted to grow in SCF. Here we present results that show differences between Kit expression on the original FDC-P1(IL-3) cell line and an SCF responsive subline, FDC-P1(SCF). Examination of the cell surface expression of Kit on these cells demonstrated that, although the cells of both sublines showed similar reactivity with anti-Kit antibodies, there were large differences with respect to the ability to bind biotinylated SCF. To further examine this phenomenon, we compared expression of *c-kit* RNA and Kit proteins in both sublines.

5.3 MATERIALS AND METHODS

Bone marrow cell preparation and WGA staining

Mouse BM was obtained by flushing excised femora and tibiae from 7 weeks old BCBA (C57BL/LiARij x CBA/BrARij)F1 mice (TNO, Rijswijk, The Netherlands) with Hepes-buffered (10 mM, pH 6.8–7.1) Hanks Balanced Salt Solution (without phenol red; Gibco BRL, Paisley, Scotland) with an osmolarity of 300-305 mOsm/kg (Hanks' Hepes). Single cell suspensions were obtained by filtration through a six-layer nylon sieve and kept on melting ice until use.

Cells were incubated for 10–40 min on ice in Hanks Hepes containing 0.5 µg wheat germ agglutinin labeled with fluorescein isothiocyanate (WGA-FITC; Polysciences, Warrington PA, USA) per $60x10^6$ cells. The cells were then washed in Hanks Hepes.

Cell line

Cells from the interleukin-3 (IL-3) dependent FDC-P1 cell line, FDC-P1(IL-3) cells, were grown in RPMI 1640 supplemented with 10% (vol/vol) heat inactivated fetal calf serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, and 10% (vol/vol) conditioned medium of the IL-3 producing WEHI-3B cell line. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. A subline was established by adding SCF (final concentration 10 ng/ml of E. coli-expressed recombinant rat SCF, kindly provided by AMGEN Ltd., Cambridge, UK) to the culture medium, and gradually decreasing the amount of WEHI-conditioned medium with each passage, until no WEHI-conditioned medium was present. Cells from this subline, referred to as FDC-P1(SCF), were then cultured in medium containing 10 ng/ml rat SCF (AMGEN) or Chinese hamster ovary (CHO) murine SCF (kindly provided by Genetics Institute, Cambridge MA, USA).

Biological activity assay

To determine the ability of IL-3 and SCF to stimulate proliferation of FDC-P1(IL-3) and FDC-P1(SCF) cells, 10^4 cells per well of 96-well microtiter plates (Falcon 3072; Becton Dickinson Labware, Lincoln Park NJ, USA) were cultured in 200 µl culture medium containing serial dilutions of growth factor. Cells were cultured for 3 days, after which the cell content of each well was determined by staining the nuclei with propidium iodide (PI) and measuring the fluorescence intensity. For this purpose, 0.5% (vol/vol) Triton X-100 and 0.002% (wt/vol) PI were added to the wells. The microtiter plates were read in a Leitz Diavert inverted microfluorometer using green excitation by a halogen lamp. Alternatively, a commercial proliferation kit (CellTiter 96 AQueous non-radioactive cell proliferation assay, Promega, Madison WI, USA) was used to determine the viable cell content of each well similar to the colorimetric assay described by Mosmann [Mosmann, 1983], but yielding a soluble reaction product. A mixture of 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine methosulphate (PMS) was added to the wells according to the manufacturer's instructions. After 4-18 hours the quantity of formazan, formed by conversion of MTS by dehydrogenase enzymes in metabolically active cells, was measured as the amount of 450 nm absorbance in a Bio-Rad model 450 microplate reader (Bio-Rad, Hercules CA, USA).

labeling of SCF with biotin

SCF was biotinylated using biotin-*N*-hydroxy succinimide ester (NHS-Biotin, Pierce, Rockford IL, USA; or Sigma, St. Louis MO, USA) as described previously (**chapter 4** [De Jong et al., 1996]). The protein concentration after biotinylation was determined using the BCA Micro Protein Assay (Pierce). The presence of biotinylated SCF was checked by spotting 1 µl of stepwise dilutions of the samples directly on a nitrocellulose membrane, followed by immuno-enzymatic staining of the membrane with streptavidin-alkaline phosphatase and 4-Nitro Blue Tetrazoliumchloride 5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT BCIP) development. The ability of biotin-SCF to stimulate proliferation of FDC-P1(SCF) cells was examined. Moreover, to test the efficiency of the biotinylation, biotin-SCF was adsorbed onto streptavidin-agarose beads (Sigma), and the amount of biological activity that remained in the supernatant was determined. Biotin-SCF was stored at –20°C or –80°C in the presence of 0.02% (wt/vol) sodium azide.

Immunocytochemical staining and flow cytometry

Cells (10^6 per sample) were stained with anti-Kit antibodies or with biotin-SCF in 50 µl Hanks Hepes, containing 5% (vol/vol) fetal or newborn calf serum and 0.02% (wt/vol) sodium azide (Hanks' Hepes/Serum/Azide, abbreviated as HSA). Incubation with the anti-Kit antibody ACK-2 [Nishikawa et al., 1991] (final concentration 5.7 µg/ml) (kindly provided by Dr. K. Rajewski, Genetics Institute, Cologne, Germany) was performed for 30 min on ice. The samples were then stained with PhycoErythrin- or FITC-conjugated goat anti-rat mAb (GARa-PE 1:200 (vol/vol), GARa-FITC 1:100 (vol/vol), both from TAGO, Burlingame CA, USA) for 30 min on ice. After each incubation the samples were washed in HSA. As a negative control, only HSA was used in the first incubation.

Alternatively, cells were incubated with biotin-SCF (10 nM) for 60 min at room temperature. Similar results were obtained by overnight incubation on ice. Subsequently, the cells were stained with PE- or BODIPY- conjugated streptavidin (Str-PE or Str-BODIPY, both 3.3 µg/ml final concentration; Molecular Probes, Eugene OR, USA) for 15–30 min on ice. After each incubation the samples were washed in HSA. Specificity of binding of the biotin-SCF samples was determined by incubating the cells with biotin-SCF in the presence of a 100-fold molar excess of unbiotinylated SCF or in the presence

of ACK-2, which is a blocking antibody [Nishikawa et al., 1991]. As a negative control, only HSA was used in the first incubation.

Samples were analyzed using a FACScan (Becton Dickinson, San Jose CA, USA) or RELACS (Rijswijk Experimental Light Activated Cell Sorter, TNO, Rijswijk, The Netherlands) flow cytometer. Cells were illuminated with the 488-nm line of an argon ion laser. Green FITC or BODIPY fluorescence was measured through a 530-nm/ 30-nm bandpass filter, orange PE fluorescence was measured through a 575-nm/ 26-nm or a 585-nm/ 42-nm bandpass filter.

RNA isolation

RNA was isolated by extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture [Chomczynski and Sacchi, 1987]. Briefly, $4x10^6$ cells were lysed by adding 2.5 ml RNA lysis buffer (4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7.0, 10 mM β-mercaptoethanol, 0.5% sodium lauroylsarcosinate) and 12.5 µg 5S ribosomal RNA from E. coli (Boehringer Mannheim, Mannheim, Germany) as carrier, and mixing vigorously. A control sample without cells was treated the same. After 5 min incubation at room temperature, 125 µl 2 M sodium acetate pH 4, 2.5 ml of water-saturated phenol, and 500 µl chloroform were added and mixed sequentially. Samples were incubated for 10 min on ice, then spinned for 5 min at 5000 rpm at 4°C. The water phase (top layer) was transferred to another tube, and 2 volumes of 100% ethanol were added. Samples were aliquotted and stored at -80°C.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was precipitated with ethanol, and samples containing RNA from $0.5–2.0x10^6$ cells were dissolved in diethyl pyrocarbonate (DEPC) treated water (15 µl). RNA was pretreated for 7 min at 95°C, and chilled on ice. cDNA was synthesized from RNA in 100 µl PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 9.0) containing (final concentrations) 1 mM dNTPs (Pharmacia Biotech, Uppsala, Sweden), 5 µM random hexamers (Promega, Madison WI, USA), 2 U/µl ribonuclease inhibitor (RNasin, Promega) and 10 U/ml Moloney Murine Leukemia Virus reverse transcriptase (MLV-RT, Promega) for 1 hour at 37°C. RT reaction was stopped and RNA/cDNA hybrids were denatured by heating the samples for 10 min at 95°C and quickly chilling on ice. RT samples were kept at -20°C.

Custom synthesized *c-kit* DNA primers were made by Pharmacia Biotech (Roosendaal, the Netherlands) (**table 5.1**). The location of the primers was selected in such a way that the PCR product of the primersets spanned introns. Primerset **A** was designed to yield PCR products that include exons 3, 4, and 5, which encode for the second and third extracellular immunoglobulin-like protein domains [Gokkel et al., 1992]. The PCR product of primerset **B** included exons 6–9 which encode for the fourth and fifth protein domains [Gokkel et al., 1992]. Using the same sense primer as in primerset **B**

and an antisense primer that is specific for KitA [Hayashi et al., 1991], we checked the presence of this splice-variant of Kit (primerset C). As a control we used primers for human β-actin [Ponte et al., 1984] that also react with mouse β-actin (primerset D). The primers were purified on a Sep-Pak C18 column (Millipore, Bedford MA, USA) according to the manufacturer's instructions, and freeze dried. The primersets were dissolved in water to a final concentration of 10 μM.

table 5.1
DNA Primers that were used in the polymerase chain reaction

primer	set [a]	location [b]	strand	sequence (5'–3')
EUR-MJ01	A	409 – 430	sense	CAAAGAAGACAGCGACGCGCTG
EUR-MJ02	A	837 – 859	antisense	GCGTCTCCTGGCGTTCATAATTG
EUR-MJ03	B/C	1064 – 1085	sense	CCCGAGCACCAGCAGTGGATAT
EUR-MJ05	B	1580 – 1601	antisense	GCAGCGGCGTGAACAGAGTGTG
EUR-MJ04	C	1566 – 1578 [c]	antisense	GCCTGGATTTGCTctttgttgttac
EUR-MB6	D	741 – 760	sense	CTGGACTTCGAGCAAGAGAT
EUR-MB7	D	1152 – 1171	antisense	TCGTCATACTCCTGCTTGCT

[a] primersets **A**, **B**, and **C** are specific for *c-kit*, primerset **D** for mouse β-actin.
[b] nucleotide numbers according to Qiu et al. [Qiu et al., 1988] for *c-kit* and Tokunaga et al. [Tokunaga et al., 1986] for mouse β-actin.
[c] this primer includes the 12 additional bases, indicated with small letters, that are present in the KitA [Reith et al., 1991] [Gokkel et al., 1992] and not in the Kit isoform.

Non-radioactive PCR was carried out in a total reaction volume of 100 μl (containing 5 μl RT sample) in PCR buffer containing primers at 0.5 μM, dNTPs at 100 μM, and 2.5 U Taq polymerase (Pharmacia Biotech). Starting with 1 min at 94°C, PCR was carried out for 30 cycles in a Perkin Elmer DNA Thermal Cycler (Perkin Elmer Cetus, Überlingen, Germany). Each cycle consisted of denaturation at 94°C for 1 min, annealing at 62°C for 1 min, and extension for 2 min at 72°C. Amplified DNA was detected in 1.5–2% (wt/vol) agarose gels using ethidium bromide (0.5 μg/ml).

Radioactive PCR was carried out similarly, with 2 μCi (74 kBq) [α-^{32}P]ATP (Amersham, Buckinghamshire, England) per sample, but using the RT samples 1:10 diluted compared to the non-radioactive PCR. Amplified DNA was then electrophoresed in 5% (wt/vol) acrylamide/ urea gels. Gels were dried under vacuum at 80°C for 60 min

in a gel dryer (Bio-Rad Laboratories, Hercules CA, USA), and exposed to a blue sensitive CEA Medical X-ray screen (Cea Corp., Strängnäs, Sweden) for 4 h at room temperature. Additionally, the amount of radioactivity in the different bands was measured in a Phosphorimager using ImageQuant software (Molecular Dynamics, Sunnyvale CA, USA). Titration of the number of PCR cycles showed that amplification was still within the linear range after 20 cycles, so this number of cycles was used for semi-quantitative analysis of PCR products.

Biotinylation of cell surface proteins and immunoprecipitation

Cells were biotinylated with biotin-*N*-hydroxy succinimide ester (NHS-Biotin, Sigma) similar to the method as described by Cole et al. [Cole et al., 1987]. Briefly, cells were washed 3x with ice-cold PBS and once with 50 mM borate buffer pH 8.3 (the osmolarity of the buffers was kept at 300 mOsm/l to prevent cell lysis). A suspension of biotin was added to the cells (150 µg biotin to $20x10^6$ cells), and biotinylation was allowed to take place for 30 min at room temperature in the dark. The cells were then washed twice with 50 mM Tris, pH 8.3, 150 mM NaCl to remove and inactivate the remaining NHS-Biotin. After washing twice with ice-cold PBS, the cells were resuspended in 0.2 ml ice-cold PBS in eppendorf tubes, to which 0.8 ml of ice-cold lysis buffer was added. Lysis buffer consisted of PBS containing 0.625% (vol/vol) NP-40 (ICN Biomedicals, Aurora OH, USA), and the protease inhibitors aprotinin (20 µg/ml), leupeptin (20 µg/ml), and Pefabloc (1 mM) (all from Boehringer Mannheim). Under occasional vortexing the samples were incubated on ice for 45 min. Extracts were cleared by centrifugation in an Eppendorf centrifuge for 10 min at 4°C at maximum speed. Supernatant was collected and stored at -80°C.

After preclearing of the lysates with pre-washed protein G beads (Gamma bind G Sepharose beads, Pharmacia Biotech; or ImmunoPure Immobilized Protein G, Pierce, Rockford IL, USA), Kit was immunoprecipitated by incubation for 2 hours or overnight at 4°C, under rocking, with protein G beads to which ACK-2 had been bound. Samples were centrifuged in the microfuge and pellets were washed three times in 1 ml PBS containing 0.5% (vol/vol) NP-40, 1 mg/ml bovine serum albumin (BSA), and protease inhibitors as described above. Pellets were then washed once in 1 ml of this buffer without BSA, and resuspended in 2x sample buffer for SDS-PAGE (1x sample buffer consists of 10 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 1 mM EDTA, 5% β-mercaptoethanol and 0.001% bromophenol blue). Bound proteins were eluted from the beads by boiling for 5 min, and beads were removed by centrifugation of the samples in the Eppendorf centrifuge at maximum speed and 4°C for 2 min through 0.45 µm filters (Millipore).

SDS-PAGE and Western blot analysis

Samples were electrophoresed by SDS-PAGE (stacking gel 4%; separating gel 7.5% containing 5 µl NP-40 per 80 ml) in the mini-PROTEAN II system (Bio-Rad Laboratories). Proteins were blotted onto nitrocellulose filters (Schleicher and Schuell, Dassel, Germany) in 2 hours using an electrical field in a Trans-Blot cell (Bio-Rad Laboratories). Blots were rinsed in Tris-buffered saline solution (TBS; containing 10 mM Tris, 150 mM NaCl, pH 7.4), and blocked overnight in TBS containing 0.05% (vol/vol) Tween-20. Incubation with streptavidin-peroxidase (Str-PO conjugate, Boehringer Mannheim) was performed in TBS containing 0.02% (vol/vol) Tween-20 for 1 h, after which the blots were washed 4 times in TBS-Tween and twice in TBS. The membranes were incubated with chemiluminescence reagent (Dupont NEN Research Products, Boston MA, USA; or Boehringer Mannheim) for 1 min at room temperature, carefully dried with a tissue, put under an overhead sheet in a film cassette, and exposed to REFLECTION™ autoradiography film (Dupont NEN) for 1–120 min.

5.4 RESULTS

Kit expression on murine BM cells

Staining of mouse BM cells with the anti-Kit antibody ACK-2 or with biotin-SCF showed a difference between the percentage of ACK-2$^+$ cells and biotin-SCF$^+$ cells, as is also described in **chapter 4** [De Jong et al., 1996]. This discrepancy is clearly shown in the WGA$^+$ fraction (**figure 5.1**), which consisted of one third of the BM cells and contained all Kit$^+$ cells. Whereas 1.21 ± 0.29% (average ± SD of 7 different experiments) of nucleated mouse BM cells stained specifically with biotin-SCF, the percentage of ACK-2$^+$ cells (2.10 ± 0.49%) was significantly higher (paired t-test; $p < 0.01$). These differences were not caused by a higher sensitivity of anti-Kit than biotin-SCF staining, as the fluorescence signals of cells stained with biotin-SCF were comparable to those of cells stained with anti-Kit. After incubation of BM cells with biotin-SCF in the presence of an excess of unbiotinylated SCF the fluorescence signal of the cells was comparable to that of unstained control cells, which demonstrated that staining with biotin-SCF was specific (not shown). Moreover, as has also been reported previously for SCF binding [Nishikawa et al., 1991], binding of biotin-SCF to the cells was blocked by ACK-2 (not shown), which demonstrated that biotin-SCF bound to the same receptors as ACK-2.

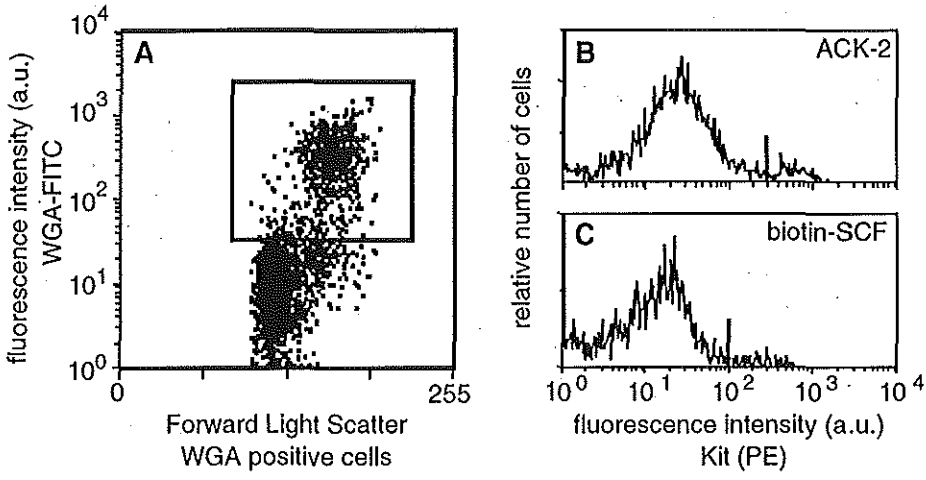

figure 5.1
Kit expression on WGA-positive mouse bone marrow cells.
Cells were double-stained with WGA-FITC and either ACK-2 and PE-conjugated anti-rat antibodies, or biotin-SCF and PE-conjugated streptavidin. Kit expression was analyzed inside a window based on WGA-FITC fluorescence (indicated in **A**). Histograms represent PE fluorescence (in arbitrary units) of WGA-positive cells, incubated with ACK-2 (**B**) or biotin-SCF (**C**). Vertical markers indicate the threshold above which the cells were designated Kit-positive.

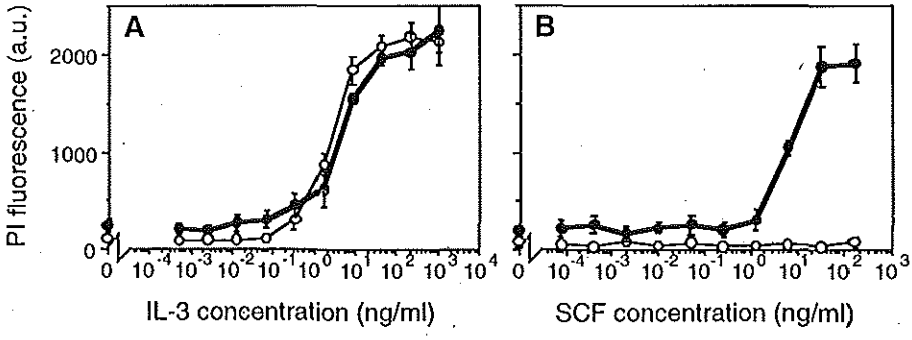

figure 5.2
Biological activity of IL-3 and SCF on different sublines of the FDC-P1 cell line.
Cells were incubated with serial dilutions of IL-3 (**A**) or SCF (**B**) for 3 days in 96-well microtiter plates, after which the propidium iodide (PI) fluorescence of the nuclei was measured as an indication of the cell content of each well. Open circles represent FDC-P1(IL-3) cells, closed circles are FDC-P1(SCF) cells. Each circle represents the mean PI fluorescence (in arbitrary units) of 4 different wells. Standard deviations are indicated.

Induction of FDC-P1 cell proliferation by SCF

To further examine Kit expression, we used an SCF responsive subline from the IL-3 dependent cell line FDC-P1(IL-3), which was raised by gradual weaning of FDC-P1(IL-3) cells from IL-3 to SCF containing medium. The ability of IL-3 to stimulate proliferation of this FDC-P1(SCF) subline was comparable to the effect of IL-3 on the original cell line (**figure 5.2 A**). Whereas the FDC-P1(IL-3) cells did not show detectable proliferation in the presence of SCF (up to 156 ng/ml), SCF induced proliferation of the FDC-P1(SCF) subline in a dose-dependent manner at concentrations higher than 1 ng/ml (**figure 5.2 B**).

Cell surface expression of Kit on FDC-P1 cells

An explanation for the apparent lack of SCF responsiveness of the FDC-P1(IL-3) cells might be absence of Kit on the cell surface of the majority of these cells. Alternatively, it is possible that the FDC-P1(IL-3) cells express an SCF receptor with an impaired ligand binding or activation capacity. To distinguish between these possibilities, FDC-P1(IL-3) and FDC-P1(SCF) cells were stained with ACK-2 or biotin-SCF. ACK-2 bound to FDC-P1(IL-3) as well as FDC-P1(SCF) cells (**figure 5.3**). The average Kit expression on FDC-P1(SCF) cells was approximately 3-fold higher than on FDC-P1(IL-3) cells as deduced from the differences in fluorescence intensities (**table 5.2**). Biotin-SCF was barely detectable on FDC-P1(IL-3) cells, but showed strong binding to FDC-P1(SCF) cells, resulting in a fluorescence intensity of FDC-P1(SCF) cells that was approximately 40-fold stronger than that of the FDC-P1(IL-3) cells (**figure 5.3** and **table 5.2**).

table 5.2
Kit expression on the FDC-P1 sublines.

	mean fluor. intensity		fluor.	mean fluor. intensity		fluor.
subline	ACK-2 [a]	control [b]	index [c]	b-SCF [a]	control [b]	index [c]
FDC-P1(IL-3)	47.8	10.5	**3.6**	9.4	7.1	**0.3**
FDC-P1(SCF)	86.8	6.8	**11.8**	73.0	5.4	**12.4**

[a] Cells were incubated with ACK-2 and GARa-FITC or with biotin-SCF and Str-PE.
[b] Control cells were incubated only with GARa-FITC or Str-PE.
Data represent results of one of 20 experiments giving similar results.
[c] fluorescence index is calculated by the following formula:

$$\text{fluorescence index} = \frac{\text{mean fluorescence test sample} - \text{mean fluorescence control}}{\text{mean fluorescence control}}$$

Biotin-SCF binding to the cells was not limited by low concentrations of the labeled ligand, as cell staining was performed at 10 nM biotin-SCF, resulting in presentation of approximately $6x10^5$ ligand molecules per cell. The discrepancy between ACK-2 and biotin-SCF staining was not caused by impaired binding of biotinylated SCF molecules to Kit, since the biological activity of biotin-SCF was similar to that of unbiotinylated SCF in proliferation assays using FDC-P1(SCF) cells (not shown). Staining with biotin-SCF was specific and due to binding to Kit, as shown by incubation with biotin-SCF in the presence of an excess of unlabeled SCF (**figure 5.3**) or in the presence of ACK-2. These results indicate that, although Kit protein is apparently expressed on both sublines, the majority of the FDC-P1(IL-3) cells binds SCF very weakly or not at all.

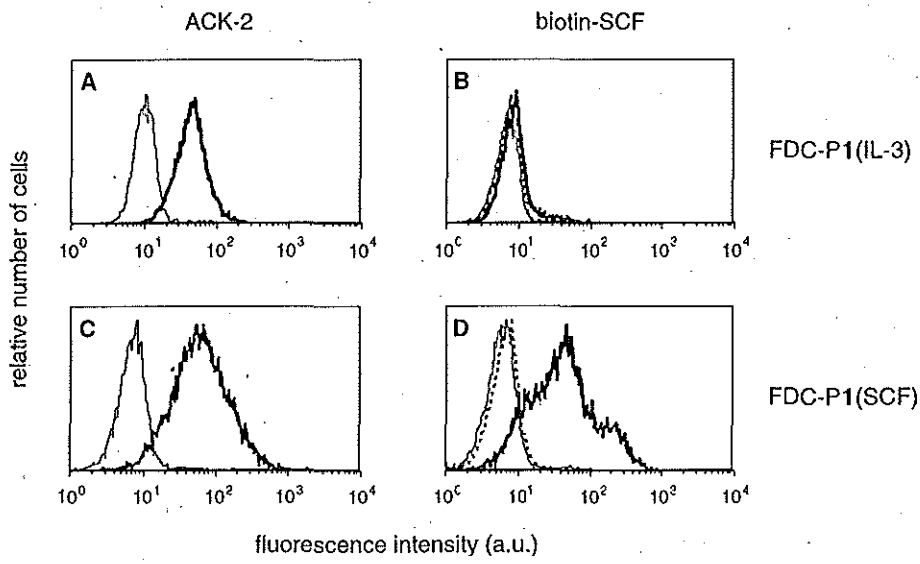

figure 5.3
Kit expression on different sublines of the FDC-P1 cell line.
FDC-P1(IL-3) (**A** and **B**) and FDC-P1(SCF) (**C** and **D**) cells were stained with anti-Kit antibodies or biotinylated SCF. Fluorescence histograms of cells, incubated with anti-Kit antibodies (bold lines) are shown in the left panels, histograms of biotin-SCF stained cells (bold lines) in the right panels. Controls include cells that were only stained with fluorescence conjugated second layer antibodies or streptavidin (thin solid lines), and cells that were incubated with biotin-SCF in the presence of a 100-fold molar excess of unbiotinylated SCF (broken lines).

RNA expression of c-kit

To examine whether the differential binding of biotin-SCF and anti-Kit could be caused by variations in structure of the *c-kit* gene product, e.g. as a result of alternative splicing, we studied *c-kit* RNA expression in FDC-P1(IL-3) and FDC-P1(SCF) cells. Using RT-PCR and specific primers (**table 5.1**), expression of different domains in the extracellular part of Kit was examined, as shown schematically in **figure 5.4 A**. We

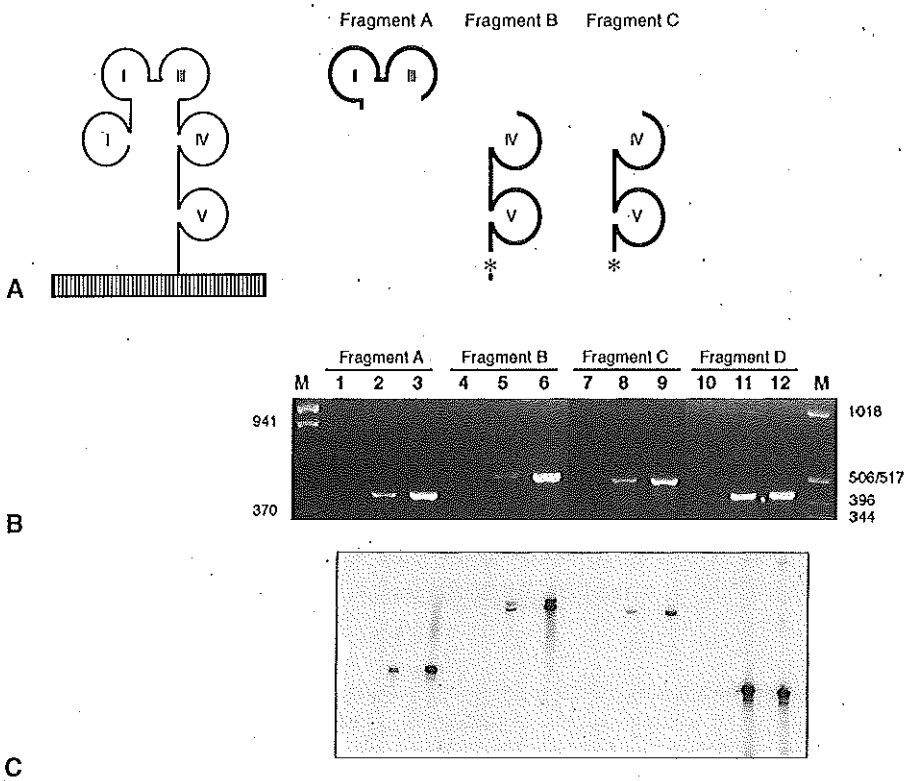

figure 5.4
PCR analysis of *c-kit* RNA expression in different sublines of the FDC-P1 cell line. Total RNA was isolated from each subline, reverse transcribed and PCR-amplified using specific primers for different parts of *c-kit* (primersets A, B and C) or b actin (primerset D). **A** shows a schematic representation of the extracellular domain of Kit in which the five immunoglobulin-like domains [Gokkel et al., 1992] are indicated by Roman numerals. Bold lines represent the parts of the protein of which RNA expression is studied using the different primersets as indicated. The position of the four additional amino acids of the KitA splice variant is indicated with *.
B and **C** show the results of analysis of the non-radioactive and radioactive RT-PCR products on 1.5% agarose gel and 5% acrylamide/ urea gel, respectively. As indicated in table 5.1, primerset A–D yield fragment sizes of 451, 537, 515 and 431 bp, respectively. **Lanes 1, 4, 7, 10** PCR controls; **lanes 2, 5, 8, 11** FDC-P1(IL-3); **lanes 3, 6, 9, 12** FDC-P1(SCF). M = size markers (bp).

checked for deviations in RNA expression of Kit protein domains that are essential for binding of SCF (using primerset A) and for dimerization of Kit molecules (primerset B) [Gokkel et al., 1992] [Blechman et al., 1995]. In both sublines of FDC-P1 a specific PCR product of the appropriate size was detected with each of these primersets, using non-radioactive as well as radioactive PCR methods (**figure 5.4 B** and **C**, respectively).

In the region that was amplified using the primerset for the dimerization site also lies the 12 bp sequence that is characteristic for KitA, an isoform of Kit that contains 4 extra

amino acids within the extracellular domain as a result of alternative splicing [Hayashi et al., 1991] [Reith et al., 1991]. We used a KitA-specific primer (primerset **C**) to examine if expression of this splice variant of the receptor might play a role in the difference between the sublines. In addition to the standard non-radioactive PCR procedure (**figure 5.4 B**), radioactive PCR followed by analysis of the PCR products on acrylamide/ urea gels (**figure 5.4 C**) was used to distinguish between the Kit and KitA bands that result from PCR with primerset **B**. Using these methods, we found that Kit as well as KitA isoforms of the receptor were present in both sublines.

Although the level of expression of β-actin RNA (primerset **D**) was similar in the FDC-P1(IL-3) and FDC-P1(SCF) sublines, results of RT-PCR with the *c-kit* primersets indicated that *c-kit* RNA expression was higher in the FDC-P1(SCF) than in the FDC-P1(IL-3) subline. The amounts of radioactivity in the different bands after radioactive PCR (**figure 5.4 C**) were quantitated using a Phosphorimager. Comparison of the different bands showed that the expression of *c-kit* RNA in FDC-P1(SCF) cells was 5–9-fold higher than in FDC-P1(IL-3) cells (**table 5.3**). Similar Kit:KitA ratios were found in the FDC-P1(IL-3) as in the FDC-P1(SCF) subline (**table 5.4**).

table 5.3
Differences between amounts of PCR product in FDC-P1(IL-3) and FDC-P1(SCF) cells.

		relative amount of radioactivity (%) [a]		
	primerset	FDC-P1(IL-3)	FDC-P1(SCF)	-fold difference [b]
exp. 1	**A**	4.2	29.8	7.1
	B	6.1	49.1	8.0
	C	1.7	9.6	5.6
exp. 2	**A**	5.6	41.8	7.5
	B	5.7	50.0	8.8
	C	2.0	12.4	6.2

[a] Data represent relative amount of radioactivity (see **figure 5.4 C**) measured in a Phosphorimager, after radioactive PCR using different primersets. Amount of radioactivity of PCR products of primersets **A**, **B** and **C** is expressed relative to the internal control (primerset **D**), which was set at 100%. Results from two different experiments are shown.

[b] Differences between the FDC-P1 sublines were calculated by dividing radioactivity of PCR products from FDC-P1(SCF) cells by those from FDC-P1(IL-3) cells.

table 5.4
Kit:KitA ratios of FDC-P1(IL-3) and FDC-P1(SCF) cells.

		relative amount of radioactivity (%) [a]		
	cells	Kit	KitA	Kit:KitA ratio
exp. 1	FDC-P1(IL-3)	4.1	2.3	1.8
	FDC-P1(SCF)	34.2	15.3	2.2
exp. 2	FDC-P1(IL-3)	3.8	2.1	1.8
	FDC-P1(SCF)	38.2	13.8	2.8

[a] Radioactivity was measured in a Phosphorimager. Data represent relative amounts of radioactivity in the two bands that result of PCR with primerset **B** (see **figure 5.4 C**), relative to the internal control (primerset **D**), which was set at 100%. Results from two different experiments are shown.

Isolation of Kit protein

To assess possible differences in the molecular mass of the SCF receptor protein between the FDC-P1(IL-3) and FDC-P1(SCF) cells, Kit was isolated after biotinylation of the cell surface proteins. Lysates from biotinylated cells were immunoprecipitated with ACK-2, separated by SDS gel electrophoresis, and transferred to nitrocellulose. Staining of the blots showed two specific bands in both sublines (**figure 5.5**). Similar as previously reported in murine cells [Nocka et al., 1989] [Nocka et al., 1990] [Tan et al., 1990], these bands represent proteins of approximately 125–135 kD and 160–165 kD. It is possible that these represent differentially glycosylated forms of the protein [Majumder et al., 1988] [Nocka et al., 1989]. In some of the experiments there was also a faint band visible that represented a protein of approximately 105–110 kD (**figure 5.5 B**), which is comparable to the unglycosylated form of Kit [Nocka et al., 1989].

These results indicated that the sizes of Kit proteins in both sublines were similar. As we used equal numbers of FDC-P1(IL-3) and FDC-P1(SCF) cells at the start of each experiment, the amount of protein that was precipitated from the FDC-P1(SCF) cells appeared to exceed that of the FDC-P1(IL-3) cells. This is in agreement with the higher RNA expression in FDC-P1(SCF) than FDC-P1(IL-3) cells.

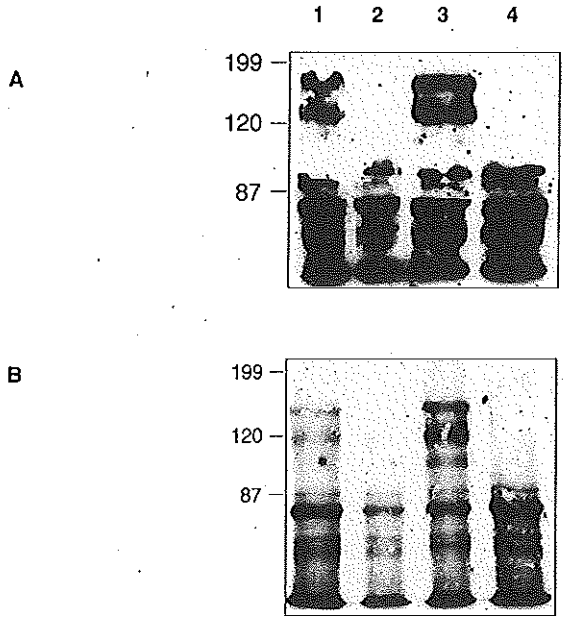

figure 5.5
Analysis of Kit proteins on cells of the FDC-P1 sublines.
A and **B** represent two independent experiments. In each experiment, equal numbers of cells from each subline were surface-labeled with NHS-biotin. Cell lysates were immunoprecipitated with anti-Kit antibody (**lanes 1** and **3**), separated by SDS-PAGE and blotted onto nitrocellulose membranes. Biotinylated proteins were detected using streptavidin peroxidase and chemiluminescence. Immunoprecipitation controls (**lanes 2** and **4**) were treated the same, but no ACK-2 was added.
Lanes 1 and **2** FDC-P1(IL-3), **lanes 3** and **4** FDC-P1(SCF). Molecular weight markers (kD) are shown at the left.

5.5 DISCUSSION

Kit is expressed on the majority of the FDC-P1(IL-3) cells, as detected by staining with the anti-Kit antibody ACK-2 (**figure 5.2**) [Jacobsen et al., 1995]. However, as demonstrated in this study, the cells show no or very low binding of biotin-SCF. Taking into account the detection limit of this method, these results would indicate an expression of less than 50–100 receptors per cell [De Jong et al., 1992] [Wognum et al., 1993] [Wognum et al., 1994]. The deficiency of receptors is in accordance with the inability of SCF to induce proliferation of FDC-P1(IL-3) cells. In contrast, cells of the FDC-P1(SCF) subline do respond to SCF. These cells express higher levels of Kit RNA (5–9x), protein, and anti-Kit (≈3x) binding sites on their surface than the FDC-P1(IL-3) cells. However, biotin-SCF binding resulted in a 40-fold higher signal in FDC-P1(SCF) as compared to FDC-P1(IL-3) cells. This difference is much greater than can be explained by the difference in

Kit expression, which suggests that SCF binding sites are not equivalent to Kit protein, or that not all Kit molecules are able to bind SCF.

To examine if impaired SCF binding was caused by a defective ligand binding site in FDC-P1(IL-3) cells, we used RT-PCR to examine the RNA expression of the *c-kit* region that encodes for the extracellular part of the Kit protein containing this binding site [Blechman et al., 1993] [Lev et al., 1993]. Additionally, to check whether the differences between the FDC-P1 sublines might be caused by a physical inability of the Kit molecules to form receptor dimers, which is essential for high affinity binding of SCF and for signal transduction [Blume-Jensen et al., 1991] [Lev et al., 1992] [Blechman et al., 1995], we studied the expression of RNA that encodes for the part of the Kit protein that contains the putative dimerization site [Gokkel et al., 1992] [Blechman et al., 1995]. Using primersets that include either the exons that encode for the second and third, or the exons that encode for the fourth and fifth immunoglobulin-like domains, we found PCR products of the expected sizes in both FDC-P1 sublines. This demonstrated that RNA for the binding site and RNA for the dimerization site were expressed in both sublines, although there was a 5–9-fold lower expression in the FDC-P1(IL-3) cells than in the FDC-P1(SCF) cells.

RT-PCR was also used to check the presence of the KitA isoform of the receptor. The Kit and KitA isoforms, which are conserved between human and mouse, are coexpressed in normal as well as leukemic cells [Reith et al., 1991] [Crosier et al., 1993] [Piao et al., 1994]. Although the biological role of KitA is still unclear, it is possible that the insert of four extra amino acids immediately upstream of the transmembrane domain [Reith et al., 1991] changes the conformation of Kit and/or its affinity for SCF. Since expression of Kit as well as KitA was detected in both FDC-P1 sublines, with no significant difference between the Kit:KitA ratios of the FDC-P1(SCF) and FDC-P1(IL-3) cells, KitA probably does not play an important role in the difference between the sublines.

To further examine the Kit protein itself, we used immunoprecipitation. Employing this method, simultaneous expression of human or mouse Kit proteins of approximately 120–130 and 145–165 kD has been reported [Nocka et al., 1989] [Blume-Jensen et al., 1991] [Reith et al., 1991], probably representing differentially glycosylated forms of the protein [Majumder et al., 1988] [Nocka et al., 1989]. However, cross-linking studies using ^{125}I-SCF have shown only a complex consisting of the labeled SCF linked to the large Kit protein on the cell surface [Pietsch et al., 1992] [Turner et al., 1992] [Broudy et al., 1993]. Therefore, it could be possible that the smaller form of Kit is not present at the cell surface, like the 125 kD mutant Kit in W/W mice [Nocka et al., 1990], or that it is incapable of binding SCF. Since the latter possibility might provide the explanation for the differences between the FDC-P1(IL-3) and FDC-P1(SCF) cells, we examined the Kit proteins of the two sublines. Using immunoprecipitation with anti-Kit antibodies after labeling of the cell surface proteins with biotin, we found two different species of Kit protein, of approximately 125–135 and 160–165 kD on both sublines. The FDC-P1(SCF) cells

appeared to contain a larger amount of Kit protein than the FDC-P1(IL-3) cells, but the two forms of Kit protein seemed to be present at similar ratios in both sublines. Since only cell surface proteins are detectable using this method, this means that both forms of the Kit protein are expressed on the cell surface of both FDC-P1 sublines. Whether or not the smaller form of the protein is capable of binding SCF remains to be established.

Taken together, no qualitative differences in *c-kit* RNA and Kit protein expression were detected, suggesting that the differences between the FDC-P1(IL-3) and FDC-P1(SCF) cells are caused by quantitative differences of Kit expression on the cell surface of the FDC-P1 sublines. Since expression of *c-kit* RNA as well as cell surface Kit protein was higher in FDC-P1(SCF) than FDC-P1(IL-3) cells, it is possible that the number of SCF-binding Kit molecules on the cell surface of FDC-P1(IL-3) cells is below a certain threshold necessary for efficient dimerization. As a consequence, no high affinity SCF receptors are formed on the FDC-P1(IL-3) cells so that no binding of biotin-SCF to the FDC-P1(IL-3) cells is detectable. This would also explain the inability of soluble SCF to induce proliferation of these cells. High local concentrations of membrane-bound SCF may facilitate Kit dimerization more efficiently than soluble SCF, which might explain why membrane-bound SCF can stimulate proliferation of FDC-P1(IL-3) cells whereas soluble SCF cannot [Caruana et al., 1993]. Additional information about the affinity for soluble ligand and the number of receptors per cell might be obtained by Scatchard analysis [Scatchard, 1949] after binding of ^{125}I-SCF, although it is difficult to obtain accurate measurements on cells that express very few receptors or receptors with a very low affinity.

It will be of interest to further resolve the apparent discrepancy between Kit protein expression and ligand binding, especially since we have found similar differences between cell staining with biotin-SCF and anti-Kit antibodies on mouse (**chapter 4** [De Jong et al., 1996], [Visser et al., 1993], and this study) and human BM cells [Wognum et al., 1996]. The availability of the FDC-P1(IL-3) and FDC-P1(SCF) sublines may prove to be useful in this regard. For instance, the role of the high and low molecular weight forms of Kit in the ability of the FDC-P1 cells to bind SCF can be examined by using digoxigenin-labeled SCF (see also **chapter 7**) for immunoprecipitation of Kit on biotinylated cells. Detailed analysis of the two forms of Kit might also elucidate the influence of differential glycosylation on ligand binding. Apart from direct information on SCF binding, immunoprecipitation of Kit after chemical crosslinking of labeled SCF to the cells might give better insight into the stoichiometry of SCF/Kit complexes. Moreover, such studies might provide information on the effects of interactions of Kit with other growth factor receptors, e.g. the EPO receptor [Wu et al., 1995], or about other, as yet unidentified receptor components that are important for SCF receptor function, like the 200 kD protein that has been reported to associate with Kit [Linnekin et al., 1995].

Above all, these data demonstrate that receptor expression data based on binding studies with anti-receptor antibodies have to be interpreted with caution, as antibody binding may not always predict the ability of the cells to bind and respond to the physiologically relevant ligand.

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CHAPTER 6

Separation of myeloid and erythroid progenitors based on expression of CD34 and c-kit

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6.1 ABSTRACT

In this paper a novel approach is described to physically separate erythroid progenitors from monocyte and granulocyte progenitors, based on the expression of CD34 and Kit. Using biotin-labeled human stem cell factor (SCF) and flow cytometry, Kit was detectable on 2-3% of the nucleated cells in rhesus monkey bone marrow. Combination of biotin-SCF with CD34 monoclonal antibodies showed that Kit was expressed on subsets of $CD34^{low}$ and $CD34^{pos}$ cells. Our data clearly demonstrate that $CD34^{pos}$ cells are more heterogeneous with respect to Kit expression than observed in studies using Kit mAb. A small cluster, approximately 7% of the $CD34^{pos}$ cells, expressed CD34 at submaximal levels, and stained brightly with biotinylated SCF. This $CD34^{pos}$/ kit^{hi} fraction contained predominantly erythroid progenitors (BFU-E). The majority of the granulocytic and monocytic progenitors (CFU-GM) were $CD34^{pos}$/ kit^{med}. Some BFU-E were also detected in the $CD34^{pos}$/ kit^{med} and $CD34^{low}$/ kit^{pos} fractions at low frequency. In the latter subset, most erythroid colony forming units (CFU-E) were recovered. Using three-color flow cytometry, we analyzed expression of Kit in relation to that of CD34 and the class II major histocompatibility antigen, RhLA-DR. The most immature bone marrow cells that can be identified in vitro, i.e. $CD34^{pos}$/ RhLA-DR^{low} cells, were kit^{med}. The $CD34^{pos}$/ kit^{hi} and $CD34^{pos}$/ kit^{neg} subsets predominantly contained the more mature RhLA-DR^{bright} cells. Our results demonstrate that erythroid precursors express c-kit at much higher levels than monomyeloid precursors and pluripotent progenitors. The difference in expression levels of CD34 and c-kit can be exploited to isolate BFU-E populations that are virtually devoid of non-erythroid cells.

6.2 INTRODUCTION

The *c-kit* gene encodes a type I transmembrane glycoprotein and is a member of the tyrosine kinase receptor family [Chabot et al., 1988]. The ligand of Kit exists in both transmembrane and soluble forms and is variously known as stem cell factor (SCF), mast cell growth factor, Steel factor, and Kit ligand [Anderson et al., 1990] [Huang et al., 1990] [Martin et al., 1990] [Zsebo et al., 1990a] [Zsebo et al., 1990b]. Kit (CD117) and its ligand are important for normal hemopoietic cell development in vivo as demonstrated by the severity of hemopoietic defects in W/W mutant mice, which do not have functional *c-kit* [Chabot et al., 1988] [Geissler et al., 1988], and in Steel mutant mice, which lack active SCF [Copeland et al., 1990] [Flanagan and Leder, 1990] [Huang et al., 1990] [Zsebo et al., 1990a].

The molecular cloning of human and rodent SCF has permitted detailed studies into the functional properties of this cytokine. The soluble form of SCF, which consists of the N-terminal extracellular domain of the full-length transmembrane protein, is a poor

stimulator of hemopoietic cells in vitro on its own. However, in the presence of other hemopoietic growth factors (GFs), such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3) and erythropoietin (EPO), SCF enhances proliferation and differentiation of immature hemopoietic cells as well as monomyeloid and erythroid progenitors [Bernstein et al., 1991] [Broxmeyer et al., 1991a] [Broxmeyer et al., 1991b] [Carow et al., 1991] [De Vries et al., 1991] [McNiece et al., 1991a] [Metcalf and Nicola, 1991] [Migliaccio et al., 1991] [Tsuji et al., 1991] [Brandt et al., 1992] [Heyworth et al., 1992] [Migliaccio et al., 1992] [Williams et al., 1992] [Abboud et al., 1994] [Steen et al., 1994]. In addition, SCF affects the development of early lymphoid cells in the presence of IL-7, but is inactive at later stages of T and B cell development [Martin et al., 1990] [McNiece et al., 1991b] [Katayama et al., 1993] [Morrissey et al., 1994]. In vivo administration of SCF leads to a dose-dependent expansion of both primitive and differentiated progenitor cells in the bone marrow (BM) of mice, nonhuman primates or human patients. As a result, mobilized progenitors appear in the peripheral blood. At the same time, there is an increase in the number of mature cells of multiple lineages [Molineux et al., 1991] [Ulich et al., 1991] [Bodine et al., 1992] [Hoffman et al., 1993] [McNiece et al., 1993] [Tong et al., 1993] [Andrews et al., 1994]. The combined in vitro and in vivo activities of SCF indicate that it acts on primitive progenitors as well as more differentiated lineage-committed cells.

Studies with monoclonal antibodies (mAb) against Kit have provided information about the cellular distribution of *c-kit* on murine and human cells. In man Kit is expressed on a large fraction of BM cells that express CD34, an antigen specific for early hemopoietic cells, including stem cells, multipotent and lineage-committed progenitor cells [Civin et al., 1984]. The $CD34^{pos}$/ kit^{pos} fraction includes cells that coexpress surface proteins characteristic for differentiating progenitors, e.g., CD33, CD38, CD71 and HLA-DR, but also developmentally immature cells that lack or express only low levels of these antigens [Ashman et al., 1991] [Papayannopoulou et al., 1991] [Broudy et al., 1992] [Strobl et al., 1992] [Gunji et al., 1993] [Yamaguchi et al., 1993] [Simmons et al., 1994]. In keeping with this, the $CD34^{pos}$/ kit^{pos} subset contains committed monomyeloid and erythroid progenitors (CFU-GM and BFU-E, respectively) as well as cells that produce clonogenic progeny in long-term cultures in the presence of cytokines or stromal feeder cells [Briddell et al., 1992] [Gunji et al., 1993] [Simmons et al., 1994]. Such cells were undetectable or present at very low frequencies in the $CD34^{pos}$/ kit^{neg} fraction. Kit has also been detected on small subsets of $CD34^{pos}$ BM cells that coexpress B- (CD10, CD19) or T- (CD2, CD7) lymphoid-specific antigens, but the majority of B and T cell precursors appears to be kit^{neg} [Ashman et al., 1991] [Strobl et al., 1992] [Simmons et al., 1994].

BFU-E and CFU-E differ in their responsiveness to stimulation by SCF, or inhibition by SCF mAb [Papayannopoulou et al., 1991] [Broudy et al., 1992], indicating a declining role of *c-kit* during late stages of erythroid differentiation. In line with this, a decreasing Kit expression during differentiation of granulocytic, monocytic and erythroid cells was

shown by staining BM cells or cultured BFU-E progeny with ^{125}I-labeled SCF [Metcalf and Nicola, 1991] [Broudy et al., 1992]. However, the exact pattern of Kit expression during especially the early stages of differentiation remains unclear. This may partially be due to the differences in techniques and reagents used to detect and isolate Kit-expressing cells. The ability to distinguish kit^{neg}, kit^{low} and kit^{hi} cells is influenced by the binding affinity of the Kit mAb. In addition, some Kit mAb inhibit outgrowth of SCF responsive cells [Lerner et al., 1991] [Broudy et al., 1992] [Gunji et al., 1993] [Liesveld et al., 1995], which may impede functional characterization of purified kit^{pos} subsets. Detection methods based on binding of the ligand itself provide more reliable information about the capacity of cells to bind and respond to a specific ligand than the use of receptor mAb. Biotin-labeled growth factors have been used extensively to study the expression of functional growth factor receptors on BM cells [Foxwell et al., 1988] [Newman et al., 1989] [Taki et al., 1989] [Peters and Norback, 1990] [Wognum et al., 1990] [Pieri and Barritault, 1991] [De Jong et al., 1992] [Wognum et al., 1992] [Wognum et al., 1993] [Wognum et al., 1994] [De Jong et al., 1995] [Wognum et al., 1995]. In the present study we have biotinylated recombinant human SCF and examined the distribution of functional receptors for SCF on rhesus monkey BM cells. With this method we show that erythroid progenitors can be distinguished and physically separated from immature multipotent cells and committed monocyte and granulocyte progenitors on the basis of CD34 and Kit expression.

6.3 MATERIALS AND METHODS

Biotinylation

Recombinant human SCF (a gift of Dr. S. Gillis, Immunex, Seattle WA, USA) [Anderson et al., 1990] was biotinylated using biotin-*N*-hydroxy succinimide ester (NHS-Biotin, Pierce, Rockford IL, USA) as described previously for other growth factors [De Jong et al., 1992] [Wognum et al., 1993] [Wognum et al., 1994] [Wognum et al., 1995]. Briefly, NHS-Biotin dissolved in dimethyl sulfoxide (DMSO) was added to 10 ng aliquots of SCF in 0.1 M carbonate-bicarbonate buffer pH 8.4 containing 0.02% (vol/vol) Tween-20, at molar Biotin:Protein (B:P) ratios of 10:1, 100:1 or 300:1. A control sample was incubated with DMSO without biotin. After 3h incubation at room temperature in the dark, biotin-SCF molecules were separated from the remaining free biotin molecules in the samples by size exclusion chromatography on a 1 ml Sephadex G-25 column (Pharmacia, Uppsala, Sweden), equilibrated in phosphate buffered saline, containing 0.02% (wt/vol) Tween-20. To test the efficiency of the biotinylation, biotin-SCF was adsorbed onto streptavidin-agarose beads (Sigma, St. Louis MO, USA), and the amount of biological activity that remained in the supernatant was determined. Biotin-SCF was stored at –80°C in the presence of 0.02% (wt/vol) sodium azide.

Biological activity assay

The biological activity of biotin-SCF was measured in a proliferation assay using cells from the human factor-dependent megakaryocyte cell line MO7e [Avanzi et al., 1988] [Hendrie et al., 1991]. Cells were grown in αMEM supplemented with 10% (vol/vol) fetal calf serum, 0.05 mM β-mercapto ethanol, 5 ng/ml human IL-3 and 10% (vol/vol) conditioned medium of the 5637 cell line. Cultures were maintained at 37°C in a humidified atmosphere of 10% CO_2 in air. To determine the biological activity of biotin-SCF, $5x10^4$ cells per well of 96-well microtiter plates (Falcon 3072; Becton Dickinson Labware, Lincoln Park NJ, USA) were cultured in 200 µl medium containing serial dilutions of growth factor. The cells were cultured for 40-48 hours, after which 0.25 µCi ^{3}H-Thymidine was added to each well. The cells were harvested after 16-18 hours of thymidine incorporation, and the radioactivity was measured in a liquid scintillation counter. At a B:P ratio of 10:1 SCF retained all of its biological activity, while the biotinylation efficiency was >99% as determined by adsorption onto streptavidin-agarose beads, so this preparation was used for further experiments.

Low density BM cell preparation

BM aspirates from young adult rhesus monkeys (Macaca mulatta) from the TNO Primate Center, Rijswijk, The Netherlands, were collected in Hanks' Hepes buffered salt solution (HH) with heparin and DNAse. The buffy coat fraction was collected after centrifuging the cells for 15 minutes at 2500 rpm at room temperature. Low density cells were obtained by centrifugation for 30 minutes at 2000 rpm at room temperature over a discontinuous bovine serum albumin (BSA) density gradient [Dicke et al., 1970] consisting of 25%, 23%, 22%, 21% and 17% (wt/vol) BSA in 0.2 M Tris-buffer / Phosphate buffer pH 7.2. Fractions were collected and washed in HH. Erythrocytes were lysed using 10 mM potassium bicarbonate, 155 mM ammonium chloride, pH 7.4, containing 0.1 mM EDTA.

Immunocytochemical staining and flow cytometry

Cells were stained overnight on ice with biotin-SCF (1 nM) in HH, containing 2% (vol/vol) fetal calf serum, 2% (vol/vol) rhesus monkey serum, 0.05% (wt/vol) sodium azide, and DNAse (0.5 mg/ml). Similar results were obtained by incubation for 2 h on ice. Specificity of binding of the biotin-SCF samples was determined by incubating the cells with biotin-SCF in the presence of either a 100-fold molar excess of unbiotinylated SCF or the blocking Kit antibody SR-1 (ascites 1:200 dilution; kindly provided by Dr. V. Broudy, University of Washington, Seattle WA, USA). The cells were incubated for 30 minutes on ice with streptavidin-PhycoErythrin (Streptavidin-PE, 1:150 vol/vol; Molecular Probes, Eugene OR, USA). After each incubation the samples were washed in HH with fetal calf serum and azide. Fluorescence signals were amplified by incubating the cells for 30 minutes on ice with biotinylated PE mAb and Streptavidin-

PE, as described earlier [Wognum et al., 1992]. During the last streptavidin-PE incubation, cells were double-stained with a CD34 mAb (antibody 566, kindly provided by Dr. T. Egeland, The National Hospital, Oslo, Norway) that was labeled with Fluorescein IsoThioCyanate (FITC; Sigma) using standard procedures. For three color analysis, cells were also incubated with a peridinin chlorophyll protein (PerCP) labeled antibody against the human Class II histocompatibility antigen HLA-DR (Becton Dickinson, Mountain View CA, USA) that cross-reacts with rhesus monkey RhLA-DR antigens. To study the expression of the transferrin receptor, double-staining experiments with biotin-SCF and a FITC-conjugated CD71 mAb (Becton Dickinson) were performed. Sorted cells with low CD34 expression were incubated with FITC-labeled CD71 mAb as well.

Samples were analyzed using a FACScan, or sorted using a FACS Vantage (Becton Dickinson, San Jose CA, USA). Cells were illuminated with the 488-nm line of an argon ion laser. Green FITC fluorescence was measured through a 530-nm/ 30-nm bandpass filter. Orange PE fluorescence was measured through a 575-nm/ 26-nm or a 585-nm/ 42-nm bandpass filter. Red PerCP fluorescence was measured through a 650-nm longpass filter. Cells were analyzed in a light scatter window as indicated in **figure 6.2 C** to include cells with intermediate to high forward light scatter (FLS) and low to intermediate perpendicular light scatter (PLS) properties, and to exclude granulocytes, dead cells and cellular debris.

In vitro culture in semisolid medium

Sorted populations were assayed for their content of colony-forming units-granulocyte/ macrophage (CFU-GM), colony-forming units-erythroid (CFU-E) and burst-forming units-erythroid (BFU-E) by in vitro colony formation in semisolid methyl cellulose culture medium. In 35 mm petri dishes (Becton-Dickinson), unsorted cells were plated at 50,000 per dish, sorted subsets were plated at 10,000 per dish (for cells from the light scatter window as shown in **figure 6.2 C**) or at 500-1,000 per dish (for subsets of $CD34^{pos}$ cells) in 1 ml methyl cellulose medium as described [Wognum et al., 1994]. Methyl cellulose cultures included the following components: 0.8% (wt/vol) methyl cellulose in α-MEM (Gibco, Gaithersburg MD, USA); 5% (vol/vol) fetal calf serum (FCS), 1.5% (wt/vol) BSA, 10 µg/ml insulin, 0.6 mg/ml human transferrin, 15 µM linoleic acid, 15 µM cholesterol, 0.1 mM β-mercapto ethanol, 0.1 µM sodium selenite, 1 mg/ml nucleosides, 100 units/ml penicillin and 50 µg/ml streptomycin. Recombinant rhesus monkey interleukin-3 (IL-3) was used at a final concentration of 30 ng/ml, human recombinant granulocyte/ macrophage-colony stimulating factor (GM-CSF; Behring, Germany) at 5 ng/ml, human SCF at 200 ng/ml, G-CSF at 100 ng/ml and human recombinant erythropoietin (EPO; Behring, Germany) at 4 U/ml. CFU-GM cultures were grown in the presence of IL-3, GM-CSF and SCF, with or without adding

G-CSF. BFU-E cultures were grown in the presence of EPO, SCF and 0.2 mM bovine hemin. CFU-E cultures were grown in the presence of EPO and 0.14 mM hemin. Cultures were maintained in a humidified atmosphere of 10% CO_2 in air. Colonies were counted at day 4 (CFU-E) and day 11 or 12 (CFU-GM and BFU-E). Data of duplicate dishes are expressed as average number of colonies per 1000 cells plated. Standard errors were obtained by taking the square root of the absolute number of colonies counted, assuming that crude colony counts are Poisson distributed [Blackett, 1974] [Iscove, 1977]. Incubation of the cells with biotin-SCF did not stimulate colony formation, since no difference in colony numbers was seen between cells that had, and cells that had not been incubated with biotin-SCF.

In vitro culture in liquid medium

For liquid suspension cultures an automated single-cell deposit unit (Becton Dickinson) was used to sort individual cells directly into separate wells of 96-well microtiter plates (Falcon 3072; Becton Dickinson Labware). In different experiments, either 1 or 1000 cells were sorted into 200 µl culture medium per well. Cells were grown in α-MEM without GFs or supplemented with various combinations of IL-3 (30 ng/ml), GM-CSF (5 ng/ml), SCF (200 ng/ml) and EPO (4 U/ml) (final concentrations). In cultures started with 1 cell per well, half of the culture medium was replaced every week. Cells were expanded before reaching confluency by transfer to 1 ml medium in 24 well culture plates (Costar, Cambridge MA). Phenotypic analysis was performed by staining with antibodies against CD34, CD71, CD11b and HLA-DR, followed by FACScan analysis. In cultures started with 1000 cells per well, separate cultures were used for ^{3}H-thymidine incorporation after 3, 5 or 7 days of culture, and for phenotypic and morphological analysis of cells produced after 7 days of culture.

6.4 RESULTS

Biotin-SCF staining of MO7e cells and rhesus monkey BM cells

Cytochemical staining properties of biotin-SCF on MO7e cells and on rhesus monkey BM cells were studied using flow cytometry. All cells of the SCF responsive cell line MO7e and a fraction of the BM cells were stained brightly with biotin-SCF in combination with PE-conjugated streptavidin (**figure 6.1** A and B). Because the number of kit^{pos} cells in unfractionated BM was very low (2-3% of the nucleated cells, corresponding to 7% of the cells inside a light scatter window as indicated in **figure 6.2**), Kit expression was also studied on cells inside a window based on high CD34 expression (**figure 6.1** C). Within the CD34^{pos} subset 30% of the cells were kit^{pos}. The fluorescence signal of MO7e as well as BM cells, incubated with biotin-SCF in the

presence of either unlabeled SCF or the blocking anti-Kit antibody SR-1 [Broudy et al., 1992], was almost identical to that of control cells incubated without biotin-SCF. This indicated that binding of biotin-SCF to these cells was specific and due to binding to Kit.

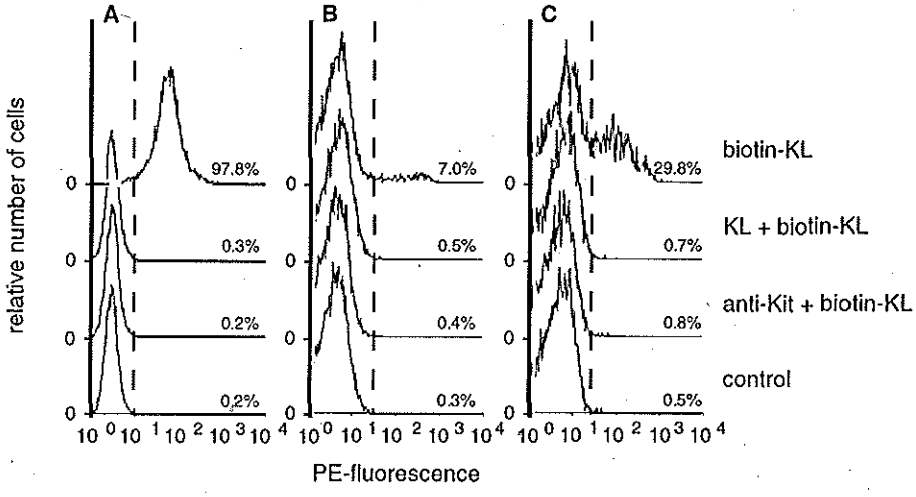

figure 6.1
Kit expression and specificity of biotin-KL staining on MO7e and rhesus monkey BM cells. Cells were sequentially stained with biotin-KL and streptavidin-PE. Rhesus monkey BM cells were double-stained with FITC-labeled CD34 mAb and analyzed inside a light scatter window as indicated in **figure 6.2.**
(**A**) MO7e cells; (**B**) rhesus monkey BM cells (**C**) $CD34^{pos}$ rhesus monkey BM cells. Histograms represent (from top to bottom): cells, incubated with biotin-KL; in the presence of a 100-fold molar excess of unlabeled KL; in the presence of the SR-1 anti-Kit antibody; control cells incubated without biotin-KL. Markers were set on the basis of background fluorescence of unstained cells to indicate the percentages of kit^{pos} cells and to show the specificity of the binding of biotin-KL to the cells.

Distribution of c-kit on rhesus monkey BM cells

Because of the low frequency of kit^{pos} cells in unfractionated BM (**figure 6.1**), the relation between CD34 and Kit expression was studied on low density BM cells (**figure 6.2**). Kit expression was detectable on 15.2 ± 6.1% (3 different experiments) of the low density BM cells inside the light scatter window as indicated in **figure 6.2**. Combination of biotin-SCF with CD34 mAb showed that 30-50% of these Kit positive cells were CD34 positive. The $CD34^{pos}$ cells were heterogeneous with respect to Kit expression. A small subset, containing 7% of the $CD34^{pos}$ cells, expressed high levels of *c-kit* (region 1 in **figure 6.2** A). CD34 expression on these $CD34^{pos}$/ kit^{hi} cells was lower than on another subset, that expressed Kit at intermediate levels ($CD34^{pos}$/ kit^{med}; region 2), which indicated that the latter population included the more immature cells. This kit^{med}

fraction contained more than 60% of the CD34^{pos} cells. The remainder of CD34^{pos} cells had no detectable Kit expression (CD34^{pos}/ kit^{neg}; region 3). Finally, a subset of cells with low CD34 expression expressed *c-kit* in a range from low to high. Most cells in this region, in particular those with high Kit expression, showed a small shift in fluorescence intensity after staining with the CD34 mAb, as compared to cells stained with isotype-control mAb. Therefore, this subset was designated CD34^{low} (CD34^{low}/ kit^{pos}; region 4).

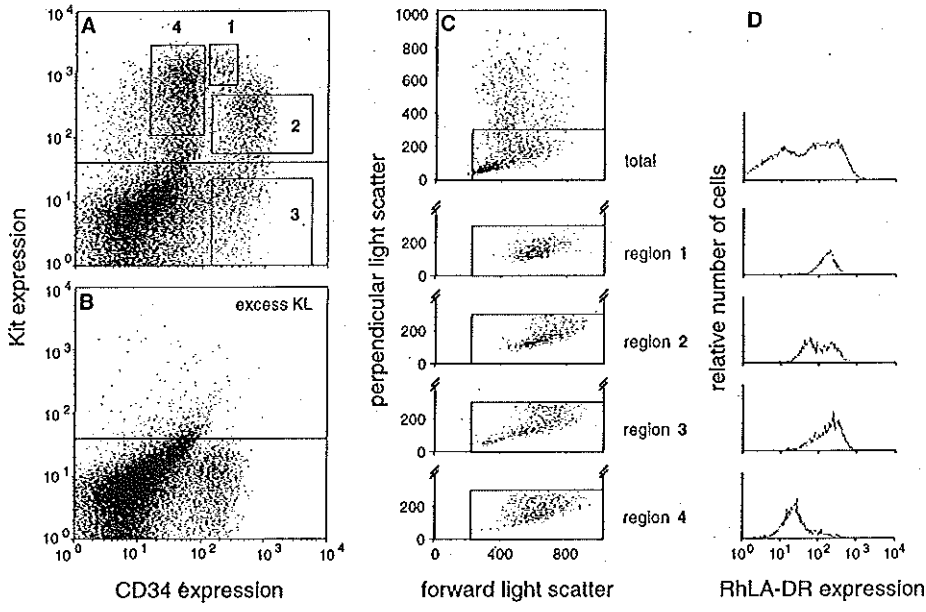

figure 6.2
Expression of CD34 and Kit on rhesus monkey BM cells, and light scatter properties and RhLA-DR expression of different subsets.
Low density rhesus monkey BM cells, stained with biotin-KL and streptavidin-PE and counter-stained with FITC-labeled CD34 mAb and PerCP-labeled HLA-DR mAb after amplification of the fluorescence signal, were analyzed by flow cytometry.
(A) Dot plot of Kit versus CD34 expression. The rectangular boxes indicate the windows used to sort cells on the basis of CD34 and Kit expression levels.
(B) Non-specific binding of biotin-KL was evaluated in the presence of a 100-fold molar excess of unlabeled KL. The horizontal line is set on the basis of background fluorescence of unstained cells to discriminate between brightly stained cells and cells with low or no Kit signal.
(C) Perpendicular versus forward light scatter (PLS versus FLS) of low density cells, and of cells in the regions 1-4 as identified in **A**.
Indicated is the scatter window that was used in the sorting experiments, to include cells with intermediate to high FLS and low to intermediate PLS properties, and to exclude granulocytes, dead cells and cellular debris.
(D) RhLA-DR fluorescence histograms of cells in the same regions as in **C**.

As shown in **figure 6.2 C**, $CD34^{pos}/kit^{hi}$ and $CD34^{pos}/kit^{med}$ cells (regions 1 and 2) displayed light scatter properties characteristic of immature, blast-like cells, i.e., intermediate to high forward light scatter and low perpendicular light scatter. $CD34^{low}/kit^{pos}$ and $CD34^{pos}/kit^{neg}$ cells (regions 3 and 4) were more heterogeneous with respect to light scatter and also contained cells with relatively high perpendicular light scatter. Additionally, small cells with low forward light scatter were found in the $CD34^{pos}/kit^{neg}$ fraction (region 3).

Analysis of RhLA-DR expression on the different subsets (**figure 6.2 D**) indicated that most of the $CD34^{pos}/kit^{hi}$ and $CD34^{pos}/kit^{neg}$ cells (regions 1 and 3) were RhLA-DR^{bright}, whereas the $CD34^{pos}/kit^{med}$ cluster (region 2) contained RhLA-DR^{bright} and RhLA-DR^{dull} cells at almost equal frequencies. The presence of RhLA-DR^{dull} cells in region 2, in combination with the high CD34 expression of this subset, indicated that very immature cells express Kit at low to intermediate levels. RhLA-DR expression was also low on the $CD34^{low}/kit^{hi}$ cells, but these represent relatively mature cells, since CD34 levels on these cells were very low.

Colony forming potential of different fractions of rhesus monkey BM

We assayed the functional abilities of the subsets, discussed in the previous section, in standard colony assays in semisolid culture media. The results of two such experiments are shown in **table 6.1**. Most CFU-GM were found in the $CD34^{pos}/kit^{med}$ fraction, which was 20-30x enriched in CFU-GM as compared to the low density cells. A much lower proportion of CFU-GM was recovered in the $CD34^{pos}/kit^{neg}$ subset and less than 1% of the CFU-GM were found in the $CD34^{pos}/kit^{hi}$ fraction.

The $CD34^{pos}/kit^{hi}$ fraction contained at least 30-fold more BFU-E than CFU-GM. As shown in **table 6.1**, 200-400 in every 1000 $CD34^{pos}/kit^{hi}$ cells developed into a BFU-E colony, compared to 1-3 in every 1000 low density cells, demonstrating an enrichment of 100- to 200-fold. Some BFU-E were detected in the $CD34^{pos}/kit^{med}$ and the $CD34^{low}/kit^{pos}$ fractions, but at 20- to 50-fold lower frequencies than in the $CD34^{pos}/kit^{hi}$ fraction. The erythroid origin of kit^{hi} cells was confirmed by double-staining of cells with biotin-SCF and CD71 mAb, which showed that kit^{hi} cells expressed high levels of the transferrin receptor (**figure 6.3**).

The number of CFU-E colonies recovered after sorting was very low (**table 6.1**). Most of the CFU-E were present in the $CD34^{low}/kit^{pos}$ fraction, suggesting that this fraction contained differentiating erythroid cells. In agreement with this, erythroblasts were the predominant cell type identified in cytocentrifuge preparations from this fraction (not shown). Moreover, restaining of sorted $CD34^{low}/kit^{pos}$ cells with CD71 mAb showed high CD71 expression on these cells (**figure 6.4**). However, on account of the low CFU-E recovery we cannot exclude the possibility that CFU-E were present in other subsets as well.

table 6.1
Colony formation in vitro of different sorted fractions of low density rhesus monkey BM cells.

		sorted fraction [a]	%	CFU-GM per 10^3 cells	CFU-GM recovery [b]	BFU-E per 10^3 cells	BFU-E recovery [b]	CFU-E per 10^3 cells	CFU-E recovery [b]
A	1	$CD34^{pos}/kit^{hi}$	0.33%	14.5 ± 2.7	0.5%	419.3 ± 36.9	43.2%	48.8 ± 3.1	0.5%
	2	$CD34^{pos}/kit^{med}$	3.89%	181.5 ± 9.5	75.9%	21.0 ± 3.2	25.5%	0.0 ± 0.0	0.0%
	3	$CD34^{pos}/kit^{neg}$	2.06%	16.0 ± 2.8	3.5%	0.0 ± 0.0	0.0%	0.0 ± 0.0	0.0%
	4	$CD34^{low}/kit^{pos}$	3.70%	0.0 ± 0.0	0.0%	18.0 ± 3.0	20.8%	133.3 ± 5.2	16.5%
		low density	100%	9.3 ± 0.2	100%	3.2 ± 0.3	100%	29.9 ± 0.5	100%
B	1	$CD34^{pos}/kit^{hi}$	0.45%	0.0 ± 0.0	0.0%	188.0 ± 13.7	76.9%	39.0 ± 11.3	6.8%
	2	$CD34^{pos}/kit^{med}$	3.30%	101.5 ± 7.1	93.0%	3.5 ± 1.3	10.5%	0.0 ± 0.0	0.0%
	3	$CD34^{pos}/kit^{neg}$	2.65%	13.5 ± 2.6	9.9%	0.0 ± 0.0	0.0%	0.0 ± 0.0	0.0%
	4	$CD34^{low}/kit^{pos}$	5.88%	0.0 ± 0.0	0.0%	0.5 ± 0.5	2.7%	13.0 ± 4.6	29.4%
		scatter fraction	100%	3.6 ± 0.4	100%	1.1 ± 0.4	100%	2.6 ± 0.7	100%

[a] Cells inside a light scatter window as shown in **figure 6.2 C** were sorted on the basis of CD34 and Kit expression in regions as shown in **figure 6.2 A**.
Results of two independent sorting experiments (A and B) are shown. Data of duplicate dishes are expressed as average number of colonies per 10^3 cells plated, ± standard errors (the square root of the absolute number of colonies counted, see Materials and Methods).

[b] Recovery was calculated relative to the unsorted low density fraction (experiment A), or relative to the fraction sorted inside a light scatter window as shown in **figure 6.2C** (experiment B).

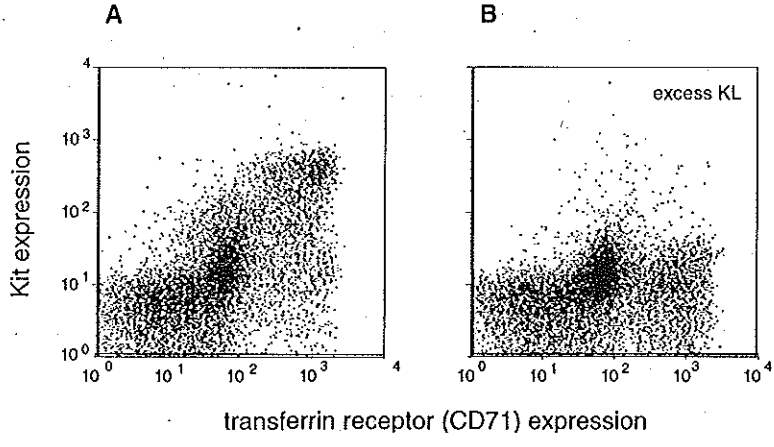

figure 6.3
Expression of CD71 and Kit on low density rhesus monkey BM cells.
Cells were stained with biotin-KL, streptavidin-PE and FITC-labeled CD71 mAb.
(A) Dot plot of Kit versus CD71 expression of cells inside the scatter window as indicated in **figure 6.2 C.**
(B) Non-specific binding of biotin-KL was evaluated in the presence of a 100-fold molar excess of unlabeled KL.

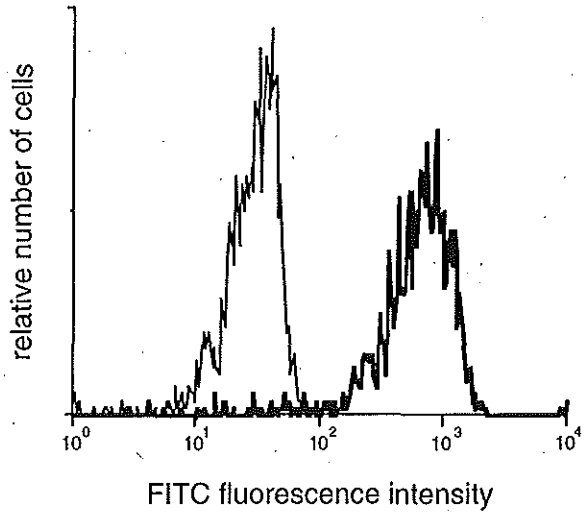

figure 6.4
CD71 expression on sorted CD34^{low}/ kit^{pos} rhesus monkey BM cells.
Low density BM cells, stained with biotin-KL, streptavidin-PE and FITC-labeled CD34 mAb, were sorted in a window similar to region 4 in **figure 6.2 A.** Subsequently, half of the sample was incubated with buffer only (thin line), and the other half with FITC-labeled CD71 mAb (thick line), and reanalyzed.

Growth factor responses in liquid culture

To study the effect of different growth factors on the short term proliferation and differentiation of the various subsets, 1000 cells per well were sorted into liquid medium containing different (combinations of) cytokines. The highest proliferation was found in the wells with cells from the CD34^{pos}/ kit^{med} and CD34^{pos}/ kit^{hi} fractions, when cultured in the presence of SCF + IL-3 + GM-CSF. FACS analysis of the different fractions after 7 days of culture showed mainly erythroid (CD71^{pos}/ CD11b^{neg}/ RhLA-DR^{neg}) cells in the CD34^{pos}/ kit^{hi} fractions, and granulocytic (CD71^{low}/ CD11b^{pos}/ RhLA-DR^{neg}) and monocytic (CD71^{low}/ CD11b^{pos}/ RhLA-DR^{pos}) cells in the CD34^{pos}/ kit^{med} fractions. Examples of CD71 and RhLA-DR expression on cultured erythroid cells and on cultured granulocyte and monocyte precursors are shown in **figure 6.5 A** and **B**, respectively. In agreement with these findings, cytospin preparations showed cells from the erythroid lineage in the CD34^{low}/ kit^{pos} and CD34^{pos}/ kit^{hi} cell cultures, and monomyeloid cells in the CD34^{pos}/ kit^{med} and CD34^{pos}/ kit^{neg} cell cultures (not shown).

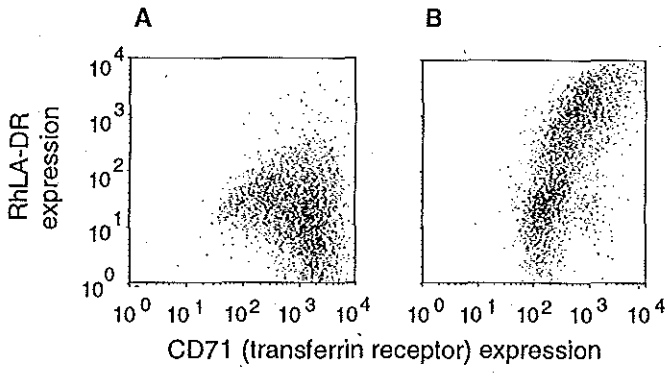

figure 6.5
Phenotypic analysis of cultured fractions of rhesus monkey BM cells.
Low density BM cells were stained with biotin-KL, streptavidin-PE and FITC-labeled CD34 mAb. 1000 cells per well were sorted on the basis of CD34 and Kit expression, as indicated by the regions in **figure 6.2 A.** Sorted cells were cultured in medium in the presence of KL + IL-3 + GM-CSF. After 7 days of culture the cells were incubated with FITC-labeled CD71 mAb and PE-labeled HLA-DR mAb and analyzed by flow cytometry. Shown are cultures that were started with $CD34^{pos}/\ kit^{hi}$ (A) or $CD34^{pos}/\ kit^{med}$ (B) cells.

To further characterize the long-term differentiation potential of the various fractions, individual cells were sorted directly into separate wells of 96-well microtiter plates, and cultured in the presence of IL-3, GM-CSF and SCF for a period of 4 weeks. No significant difference was seen between cultures with and without EPO (not shown). 90% of the wells with $CD34^{pos}/\ kit^{hi}$ and 81% with $CD34^{pos}/\ kit^{med}$ cells contained proliferating cells, whereas only 33% of the $CD34^{pos}/\ kit^{neg}$ cells showed proliferation (**table 6.2**). There was a large difference between the fractions with respect to the amount of cells produced in the wells and to the nature of these clones. In general, the kit^{hi} clones proliferated faster and were exhausted sooner than the kit^{med} clones, which kept on growing up to 4 weeks after sorting. The number of cells in the kit^{neg} clones stayed very low. Microscopic inspection of the wells showed erythroid cells in the kit^{hi} clones, and granulocytic and monocytic cells in the kit^{med} and kit^{neg} clones. About 25% of the wells with kit^{hi} cells (**table 6.2**) proliferated sufficiently to perform a FACS phenotyping experiment, resulting in 46 erythroid ($CD71^{pos}$) and 1 granulocytic ($RhLA\text{-}DR^{neg}/\ CD11b^{pos}$) clone. Only 6 out of 192 wells with kit^{med} cells contained enough cells to be analyzed in this way, resulting in 4 granulocytic ($RhLA\text{-}DR^{neg}/\ CD11b^{pos}$) and 2 monocytic ($RhLA\text{-}DR^{pos}/\ CD11b^{pos}$) clones.

table 6.2
Proliferation of different subsets of $CD34^{pos}$ low density rhesus monkey BM cells.

	fraction	number of wells (from a total of 192)				
		proliferating [a]	expanded [b]	phenotype [c]		
				E	G	M
1	$CD34^{pos}/kit^{hi}$	173	130	46	1	0
2	$CD34^{pos}/kit^{med}$	155	51	0	4	2
3	$CD34^{pos}/kit^{neg}$	64	0			

a Cells were incubated with biotin-KL and CD34 mAb, and sorted one cell per well into liquid medium containing IL-3, GM-CSF and KL with or without EPO. Wells in which proliferation occurred were counted.
b Before reaching confluency, clones with extensive proliferation were expanded into 1 ml cultures.
c Expanded clones that contained sufficient numbers of cells were stained with antibodies against CD71, CD11b and HLA-DR.
E: erythroid ($CD71^{pos}/CD11b^{neg}/RhLA\text{-}DR^{pos}$),
G: granulocytic ($CD71^{low}/CD11b^{pos}/RhLA\text{-}DR^{neg}$),
M: monocytic ($CD71^{low}/CD11b^{pos}/RhLA\text{-}DR^{pos}$).

6.5 DISCUSSION

Kit expression on murine [Okada et al., 1991] [Ikuta and Weissman, 1992] [Okada et al., 1992] [Ogawa et al., 1994] and human [Ashman et al., 1991] [Lerner et al., 1991] [Papayannopoulou et al., 1991] [Briddell et al., 1992] [Broudy et al., 1992] [Gunji et al., 1993] [Simmons et al., 1994] BM cells has been studied extensively, using Kit specific mAb. However, the exact expression pattern of Kit during especially the early stages of hemopoietic cell differentiation is still not clear. Differences between published results may partially be explained by the use of different mAb, because the ability to distinguish kit^{hi}, kit^{med}, and kit^{low} cells is influenced by the binding affinity of the Kit mAb. Moreover, as Kit mAb can inhibit outgrowth of kit^{pos} cells [Lerner et al., 1991] [Broudy et al., 1992] [Gunji et al., 1993] [Liesveld et al., 1995], it is possible that the kit^{pos} cells do not develop optimally in culture if high-affinity mAb are used for sorting. This would lead to a serious underestimation of the number of colony-forming cells in the kit^{pos} subset. Cell staining methods based on the ligand itself cause no such inhibition. In addition, such methods by definition provide more reliable information about the capacity of the cells to bind and respond to a specific ligand than staining with mAb against the receptor. In this study we have used biotinylated SCF to examine the expression of Kit on subsets of low density rhesus

monkey BM cells. Kit was detectable on 2-3% of the nucleated cells, similar to frequencies previously obtained for human BM, using Kit mAb [Ashman et al., 1991] [Lerner et al., 1991]. Double-staining with biotin-SCF and CD34 mAb showed Kit expression on subsets of $CD34^{pos}$ as well as $CD34^{low}$ rhesus monkey BM cells.

A small fraction of $CD34^{pos}$ cells with a high Kit expression was detected, consisting almost exclusively of BFU-E. In line with this, $CD34^{pos}$/ kit^{hi} cells produced erythroblasts in liquid suspension cultures, and the kit^{hi} cells expressed high levels of the transferrin receptor. Some BFU-E were also found in the kit^{med} population. This might reflect insufficient separation of this subset and the kit^{hi} cells, although the kit^{hi} cells are quite a distinct cluster. It is also possible that these kit^{med} cells represent a separate population, e.g. one that is more immature than the kit^{hi} BFU-E, since the kit^{hi} cells express CD34 at a lower level than the kit^{med} population. Heterogeneity within the BFU-E population has also been observed by Simmons et al. [Simmons et al., 1994], who detected a small $CD34^{pos}$/ kit^{neg} BFU-E fraction in sorting experiments using Kit mAb YB5.B8. Using another mAb, NU-c-kit, Gunji et al. [Gunji et al., 1993] separated $CD34^{pos}$ BM cells into kit^{hi}, kit^{low} and kit^{neg} subsets. In contrast to our results, these authors found the highest BFU-E frequencies in the kit^{low} subset, whereas BFU-E frequencies in the kit^{hi} subset were very low [Gunji et al., 1993]. Unfortunately, no information was provided about the overall BFU-E recovery in the sorted fractions, so it cannot be ruled out that colony formation by kit^{hi} cells was underestimated. This might be in accordance with results reported by Broudy et al. [Broudy et al., 1992], who showed that outgrowth of a small subset of human BFU-E is not inhibited by the Kit antibody SR-1. Such inhibition apparently does not occur with cells stained with biotinylated SCF, as demonstrated by the high recovery of BFU-E and CFU-GM after sorting (**table 6.1**).

Most CFU-E were present in the $CD34^{low}$/ kit^{pos} fraction. This population was almost completely erythroid, as demonstrated by high CD71 expression. These results indicate that BFU-E as well as CFU-E display Kit, which correlates well with the insufficient erythropoiesis and the occurrence of macrocytic anemia in W/W mutant mice, which do not have functional *c-kit*. Also in accordance with these results, Papayannopoulou et al. [Papayannopoulou et al., 1991] and Ashman et al. [Ashman et al., 1991] have shown that BM cells that had been isolated via either immune adherence to the SR-1 antibody, or immune rosetting using the YB5.B8 antibody, were highly enriched for erythroid cells. Binding studies with ^{125}I-SCF on cultured BFU-E progeny showed that proerythroblasts labeled much more densely than erythroblasts [Broudy et al., 1992]. Although Kit is present on both BFU-E and CFU-E, SCF is necessary only for BFU-E outgrowth, but not for CFU-E and later erythroid cells [Dai et al., 1991] [Broudy et al., 1992]. Taken together, the data suggest that Kit expression reaches its maximum at the BFU-E stage and gradually declines during terminal erythroid differentiation. This pattern of

Kit expression appears similar to that of CD71 [Loken et al., 1987] and the EPO receptor [Wickrema et al., 1992] [Wognum et al., 1992].

The CD34^{pos}/ kit^{neg} fraction contained virtually no colony-forming cells. Although the presence of very immature cells in this fraction cannot be ruled out completely, most of the cells in this subset are more mature, as based on the relatively low CD34 and high RhLA-DR expression, characteristic for activated and differentiating cells. These CD34^{pos}/ kit^{neg} cells appear to represent primarily monocyte and granulocyte precursors. In addition, part of the CD34^{pos}/ kit^{neg} fraction has low forward and perpendicular light scatter properties, and probably consists of B-lymphocyte precursor cells. In previous studies, Kit has been detected on only small subsets of CD34^{pos}/ CD10^{pos} and CD34^{pos}/ CD19^{pos} B-lymphocyte precursors [Simmons et al., 1994]. This is consistent with an involvement of SCF in early, but not later stages of B cell development [McNiece et al., 1991a] [Nishikawa et al., 1991] [Ogawa et al., 1991] [Ulich et al., 1991].

Part of the CD34^{pos}/ kit^{med} cells showed high CD34 and low RhLA-DR expression, a phenotype that has previously been associated with the most immature cells that can be identified in human BM [Brandt et al., 1988] [Sutherland et al., 1991] [Briddell et al., 1992]. Recently we have shown that the CD34^{pos}/ DR^{dull} rhesus monkey BM subset contains multipotential progenitors with high proliferative capacity [Wognum et al., 1995]. Preliminary results from transplantation experiments indicate that the cells that can reconstitute lethally irradiated rhesus monkeys are also present in this subset (unpublished results). In accordance with the expression of Kit on immature rhesus monkey BM cells, murine bone marrow cells expressing Kit were enriched for hemopoietic stem cells [Okada et al., 1991] [Ikuta and Weissman, 1992] [Orlic et al., 1993]. These results support the conclusion that Kit is already expressed at low to intermediate levels at a very early stage of hemopoiesis. Further studies focusing on subsets of CD34^{pos}/ kit^{med}/ RhLA-DR^{dull} rhesus monkey BM cells will be useful to establish the importance of individual hemopoietic growth factors during stem cell proliferation and differentiation, and to provide candidate stem cell fractions for transplantation studies.

In summary, our data are consistent with a model in which immature, multipotent progenitors are CD34^{pos}/ kit^{med}. Along the monomyeloid lineage, these cells differentiate into CD34^{pos}/ kit^{med} CFU-GM. Kit expression declines after the CFU-GM stage, and the cells lose CD34 expression. Along the erythroid lineage, CD34^{pos}/ kit^{med} progenitors differentiate to kit^{hi} BFU-E, which gradually lose CD34 when they differentiate into CD34^{low}/ kit^{hi} CFU-E. This is followed by a gradual disappearance of Kit expression during terminal differentiation into mature red blood cells. The ability to distinguish the CD34^{pos}/ kit^{hi} population provides a method to obtain highly enriched BFU-E populations that are devoid of non-erythroid cells.

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CHAPTER 7

Coexpression of Kit and the receptors for erythropoietin, interleukin-6 and granulocyte/ macrophage-colony stimulating factor on hemopoietic cells

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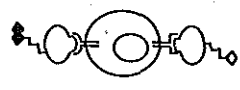
Stem Cells (in press)



7.1 ABSTRACT

The detection of functional growth factor (GF) receptors on subpopulations of hemopoietic cells may provide a further dissection of immature cell subsets. Since little information is available about coexpression of different GF receptors at the level of single hemopoietic cells, we studied the feasibility of simultaneous cell staining with a combination of biotin- and digoxigenin-labeled GFs for flow cytometric detection of functional receptors. Using this methodology, coexpression of Kit and receptors for erythropoietin (EPO), interleukin-6 (IL-6), and granulocyte/ macrophage colony-stimulating factor (GM-CSF) on hemopoietic cells was studied by triple-staining of rhesus monkey bone marrow (BM) cells with labeled GFs and antibodies against other cell surface markers. Most of the immature, $CD34^{++}$ cells were Kit^{+} but did not display detectable levels of EPO-receptors (EPO-R) or GM-CSF-R. Approximately 60% of these $CD34^{++}$/ Kit^{+} cells coexpressed the IL-6-R, demonstrating that immature cells are heterogeneous with respect to IL-6-R expression. Maturation of monomyeloid progenitors, as demonstrated by decreasing CD34 and increasing CD11b expression, is accompanied by a decline of Kit and an increase in GM-CSF-R expression, in such a way that Kit^{+}/ GM-CSF-R^{+} cells are hardly detectable. IL-6-R expression is maintained or even increased during monomyeloid differentiation. IL-6-R and GM-CSF-R were not identified on most $CD71^{++}$ cells, which indicated that these receptors are probably not expressed during erythroid differentiation. Together with previous results, our data show that both Kit and CD71 are upregulated with erythroid commitment of immature progenitors. Upon further differentiation, Kit^{+}/ EPO-R^{-} cells lose CD34 and acquire EPO-R. Maturing erythroid cells eventually lose CD71 and Kit expression but retain the EPO-R. In conclusion, this approach enables further characterization of the specificity of GFs for different bone marrow subpopulations. Apart from insight into the differentiation stages on which individual GFs may act, information about receptor coexpression may be used to identify individual cells that can respond to multiple GFs, and allows further characterization of the regulation of lineage-specific differentiation.

7.2 INTRODUCTION

Survival, growth and differentiation of hemopoietic cells depend on binding of growth factors (GFs) to specific cell surface receptors. Studies with monoclonal antibodies (mAbs) against GF receptors (GF-R) or with labeled GF molecules have enabled analysis of the distribution of GF-R on hemopoietic cells by means of multiparameter flow cytometry [Wognum et al., 1996]. Such approaches enable analysis of receptor expression in relation to the presence of other cell surface antigens.

To detect receptor-expressing cells, the use of labeled GFs is preferable to that of anti-receptor mAbs, because mAbs recognize only one epitope of a receptor, whereas high affinity GF-R for many of the GFs active in hemopoiesis are complexes of more than one subunit [Dukovich et al., 1987] [Hayashida et al., 1990] [Hibi et al., 1990]. Moreover, some of these subunits can be shared by receptors for different GFs [Kitamura et al., 1991] [Tavernier et al., 1991] [Kondo et al., 1993] [Noguchi et al., 1993] [Russell et al., 1993]. Therefore, detection of receptor-expressing cells based on binding of the physiological ligand itself is more indicative of the presence of functional receptors than binding of antibodies.

Previously we have used biotin-labeled GFs to study expression of one GF-R in relation to other parameters such as the presence of markers for early hemopoietic cells, in particular CD34 [Civin et al., 1984] [Andrews et al., 1989], and markers that identify activated or differentiating cells, e.g. CD11b for monocytes and granulocytes, and CD71 for erythroid cells [Newman et al., 1982] [Loken et al., 1987]. This approach has provided information on the expression of receptors for interleukin-3 (IL-3), IL-6, granulocyte/ macrophage colony stimulating factor (GM-CSF), stem cell factor (SCF), and erythropoietin (EPO) on normal hemopoietic cells, and allowed us to compare the receptor phenotype of immature normal cells with that of their malignant counterparts in patients with chronic myeloid leukemia (CML) [Wognum et al., 1992] [Wognum et al., 1993] [Wognum et al., 1994] [De Jong et al., 1995] [Wognum et al., 1995] [De Jong et al., 1996].

The possibility to simultaneously detect two or more GF-R on the same cells could help explain functional and physiological interactions between these GFs on hemopoietic cells. This would also be helpful to further delineate differential expression of these GF-Rs during differentiation of stem cells into mature cells of distinct lineages.

As an alternative to biotinylation, GFs can be labeled with the 391 D molecule digoxigenin (DIG) [Martin et al., 1990] [Gleizes et al., 1994], which can be detected using anti-DIG antibodies. This approach has recently been used to identify binding and internalization of DIG-labeled basic fibroblast growth factor by target cells [Gleizes et al., 1994]. In the present study we have examined the feasibility to directly study the coexpression of two GF-R on hemopoietic cell subsets by staining rhesus monkey BM cells with combinations of biotin- and DIG-labeled GFs. By combining these labeled GF molecules with mAbs against CD34, CD11b, and CD71, we have examined receptor coexpression patterns during development of stem cells to mature blood cells of myelomonocytic and erythroid lineages to demonstrate that such an approach is feasible and yields results that are consistent with existing functional data. For these studies, we used rhesus monkey BM cells, since extensive data on single receptor staining were already available [Wognum et al., 1993] [Wognum et al., 1994] [De Jong et al., 1995] [Wognum et al., 1995].

7.3 MATERIALS AND METHODS

Labeling of GFs with digoxigenin or biotin

Recombinant human SCF (a gift of Dr. S. Gillis, Immunex, Seattle WA, USA) [Anderson et al., 1990] was labeled with digoxigenin (DIG) using Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (NHS-DIG, Boehringer Mannheim, Mannheim, Germany) under similar conditions as described previously for SCF labeling with NHS-Biotin [De Jong et al., 1995]. Briefly, NHS-DIG dissolved in dimethyl sulfoxide (DMSO) was added to 5 µg aliquots of SCF in 0.05 M phosphate buffered saline (PBS) pH 8.5 containing 0.02% (vol/vol) Tween-20, at molar DIG:Protein (D:P) ratios of 10:1, 50:1 or 100:1. A control sample was incubated with DMSO without DIG (D:P 0). After 3 h incubation at room temperature in the dark, DIG-SCF molecules were separated from the remaining free DIG molecules in the samples by size exclusion chromatography on a 1 ml Sephadex G-25 column (Pharmacia, Uppsala, Sweden), equilibrated in PBS pH 7.4, containing 0.02% (wt/vol) Tween-20.

Human glycosylated recombinant IL-6 (kindly provided by Ares-Serono, Geneva, Switzerland) and human recombinant GM-CSF (a gift from Genetics Institute, Cambridge MA, USA) were biotinylated using biotin-*N*-hydroxy-succinimide ester (NHS-Biotin, Sigma, St. Louis MO, USA), under conditions similar to the NHS-DIG labeling of SCF and described previously [Wognum et al., 1993] [Wognum et al., 1994].

Human recombinant EPO (supplied by Behringwerke Aktiengesellschaft, Marburg, Germany) was biotinylated using biotin-aminocaproyl-hydrazide (Calbiochem, La Jolla CA, USA) as described previously [Wognum et al., 1990].

Labeled GFs were stored at –80°C in the presence of 0.02% (wt/vol) sodium azide.

Cell lines and biological activity assay

Before adding sodium azide, the ability of the labeled GF preparations to stimulate cell proliferation was assayed using the appropriate GF dependent cell lines, as described [Wognum et al., 1993] [Wognum et al., 1994] [De Jong et al., 1995]. Briefly, the proliferative response of the human factor-dependent cell line MO7e was used to determine the activity of SCF or GM-CSF [Brizzi et al., 1990] [Hendrie et al., 1991], the human multifactor-responsive myeloid cell line TF-1 was used for EPO [Kitamura et al., 1989], and the IL-6 dependent murine plasmacytoma cell line T1165 for IL-6 [Le et al., 1988]. Cells (1–5 x 10^4 per well of 96-wells flat bottom microtiter plates) were cultured in 200 µl culture medium containing serial dilutions of GF. After 48 hours, 0.25 µCi ^{3}H-Thymidine was added to each well. The cells were harvested after 16–18 hours of thymidine incorporation, and the radioactivity was measured in a liquid scintillation counter. Alternatively, to determine the viable cell content of each well, a commercial

proliferation kit (CellTiter 96 AQueous non-radioactive cell proliferation assay, Promega, Madison WI, USA) was used, based on the colorimetric assay described by Mosmann [Mosmann, 1983]. Therefore, the cells were cultured for 3 days, after which a mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine methosulphate (PMS) was added to the wells according to the manufacturer's instructions. After 4-18 hours the quantity of formazan, formed by conversion of MTS by dehydrogenase enzymes in metabolically active cells, was measured as the amount of 450 nm absorbance in a Bio-Rad model 450 microplate reader (Bio-Rad, Hercules CA, USA).

Low density BM cell preparation

Young adult rhesus monkeys (Macaca mulatta) from the TNO Primate Center, Rijswijk, The Netherlands, were used as BM donors. BM was collected by piercing the head of the humeral shaft with a pediatric spinal needle, or by extrusion of the cells in a tissue press from the cleansed bones of a monkey that was sacrificed for collection of BM for various experiments and transplantation purposes [Van Bekkum and De Vries, 1967]. All animal experiments were performed in accordance with national ethical regulations. BM was collected in Hanks' Hepes buffered salt solution (HH) with heparin and DNAse (0.5 mg/ml). A buffy coat fraction was obtained by centrifuging the cells for 15 min at 2500 rpm at room temperature. To enrich for progenitor cells, buffy coat cells were subjected to centrifugation for 30 min at 2000 rpm at room temperature over a discontinuous bovine serum albumin (BSA) density gradient [Dicke et al., 1970] consisting of 25%, 23%, 22%, 21% and 17% (wt/vol) BSA in 0.2 M Tris-buffer / Phosphate buffer pH 7.2. Fractions were collected and washed in HH. Erythrocytes were lysed using 10 mM potassium bicarbonate, 155 mM ammonium chloride, pH 7.4, containing 0.1 mM EDTA.

Immunocytochemical staining

Cells were incubated overnight on ice with DIG-SCF and either biotin-EPO, biotin-IL-6 or biotin-GM-CSF in HH, containing 2% (vol/vol) FCS, 0.05% (wt/vol) sodium azide, and DNAse (0.5 mg/ml). Specificity of binding of the labeled GF samples was determined by incubating the cells in the presence of a 100-fold molar excess of unlabeled GF. After each incubation the samples were washed in HH with FCS and azide. The cells were stained for 30 min on ice with mouse anti-DIG antibodies (Boehringer Mannheim), followed by a mixture of Fluorescein IsoThioCyanate- or PhycoErythrin-labeled goat anti-mouse antibodies (GAM-FITC, Dako A/S, Glostrup, Denmark; GAM-PE, Sigma, St. Louis MO, USA) and PhycoErythrin- or TriColor-labeled streptavidin (Streptavidin-PE, Molecular Probes, Eugene OR, USA; streptavidin-TriColor, Caltag, San Francisco CA, USA). Indirect staining of the DIG-SCF gave better fluorescence signals than direct staining methods using FITC-labeled

sheep anti-DIG antibodies (Boehringer Mannheim). Attempts to amplify the FITC-labeled sheep anti-DIG signal using FITC-labeled rabbit anti-sheep antibodies (Nordic Immunological Laboratories B.V., Tilburg, The Netherlands) resulted in high background. Fluorescence signals of biotin-GF labeled cells were amplified by incubating the cells for 30 min on ice with biotinylated anti-streptavidin antibodies and fluorescently tagged streptavidin, as described earlier [De Jong et al., 1992]. For three color analysis, cells were stained in a final incubation step with fluorescently tagged antibodies against CD34, CD71, or CD11b. This was done by resuspending the cells in 50 µl HH with FCS and azide containing 4% (vol/vol) normal rhesus serum and 10% (vol/vol) normal mouse serum. After 15 min incubation on ice, 50 µl of the respective antibodies diluted in HH with FCS and azide was added, and the cells were incubated for another 30 min. We used an anti-CD34 antibody (antibody 566, kindly provided by Dr. T. Egeland, Institute of Transplantation Immunology, Rikshospitalet, Oslo, Norway) that was labeled with FITC (Sigma) using standard procedures. CD71-FITC and CD11b-PE mAbs were from Becton Dickinson (Mountain View CA, USA).

To distinguish between living and dead cells, in some of the experiments propidium iodide (PI, final concentration 0.6 µg/ml; Molecular Probes) was added prior to analysis to samples that were stained with FITC and PE, or Hoechst 33258 (H58, final concentration 1 µg/ml; Molecular Probes) was added to samples that were also stained with TriColor.

Flow cytometry

Samples were analyzed using a FACScan or a FACS Vantage flow cytometer (Becton Dickinson, San Jose CA, USA). Cells were illuminated with the 488-nm line of an argon ion laser (Coherent Innova 90, Palo Alto CA, USA). Green FITC fluorescence was measured through a 530-nm/ 30-nm bandpass filter, orange PE fluorescence through a 575-nm/ 26-nm or a 585-nm/ 42-nm bandpass filter, and red TriColor or PI fluorescence was measured through a 620-nm or a 650-nm longpass filter. H58 fluorescence was measured through a KV408 cut-off filter using UV light (351/364 nm) from the second argon ion laser (Spectra Physics, Series 2000, Mountain View CA, USA) of the FACS Vantage. Data of 10,000–20,000 cells per sample were stored in list mode using Lysys software (Becton Dickinson) on a HP340 computer. Data were analyzed using Spyro data analysis software developed by R.R. Jonker (TNO Delft, The Netherlands) on an Apple Macintosh computer. Viable H58- or PI-negative cells were analyzed in a light scatter window as indicated in **figure 6.3 A** to include cells with intermediate to high forward light scatter (FLS) and low to intermediate perpendicular light scatter (PLS) properties, and to exclude granulocytes, dead cells and cellular debris.

Statistics

Results are given as means ± standard deviation if appropriate. Standard deviations were calculated on the assumption of a normal distribution.

7.4 RESULTS

Biological activity and cell staining

SCF was labeled with DIG at molar DIG:Protein (D:P) ratios of 10:1, 50:1 or 100:1. The ability of these DIG-SCF preparations to stimulate cell proliferation was assayed using the human factor-dependent megakaryocyte cell line MO7e [Brizzi et al., 1990] [Hendrie et al., 1991], which expresses 35,000–120,000 high affinity cell surface SCF receptors per cell [Budel et al., 1993] [Turner et al., 1995]. As shown in **figure 7.1**, SCF retained all of its biological activity at a D:P ratio of 10:1. At D:P 50, approximately 67% of the biological activity of SCF remained, whereas at D:P 100 most of the biological activity was lost. All three preparations of DIG-SCF bound to MO7e cells, as was examined by flow cytometry (**figure 7.2**). Binding of DIG-SCF was specific, since

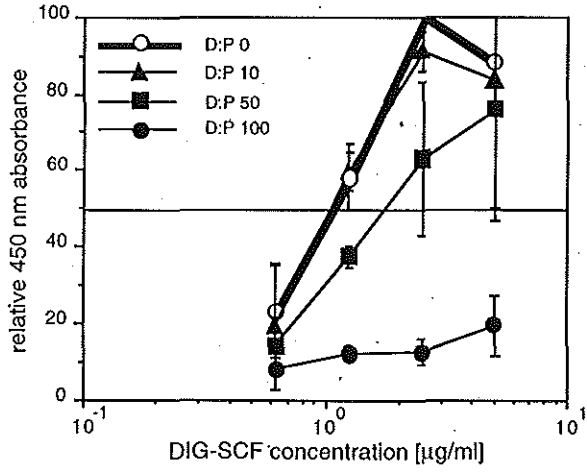


figure 7.1
Biological activity of DIG-SCF on MO7e cells.
DIG-SCF was prepared at different DIG:Protein (D:P) ratios. A control sample (D:P 0) was mock-labeled without adding DIG. MO7e cells were incubated with serial dilutions of DIG-SCF for 3 days in 96-well microtiter plates, after which a mixture of MTS and PMS was added to the wells (see Materials and Methods). The amount of 450 nm absorbance of each well was measured, indicating the amount of metabolically active cells. Open circles represent mock-labeled control SCF; triangles, squares, and closed circles represent DIG-SCF at D:P 10, 50, and 100, respectively. Each data point represents the mean value of 3 different wells. Standard deviations are indicated. Dotted horizontal line indicates 50% maximal stimulation of the cells.

the fluorescence signal of cells incubated with DIG-SCF in the presence of an excess of unlabeled SCF was almost identical to that of control cells incubated without DIG-SCF (**figure 7.2**). Despite the partial loss of biological activity, the DIG-SCF sample prepared at a D:P ratio of 50 yielded a stronger fluorescence signal than the other samples. This preparation was used in further experiments.

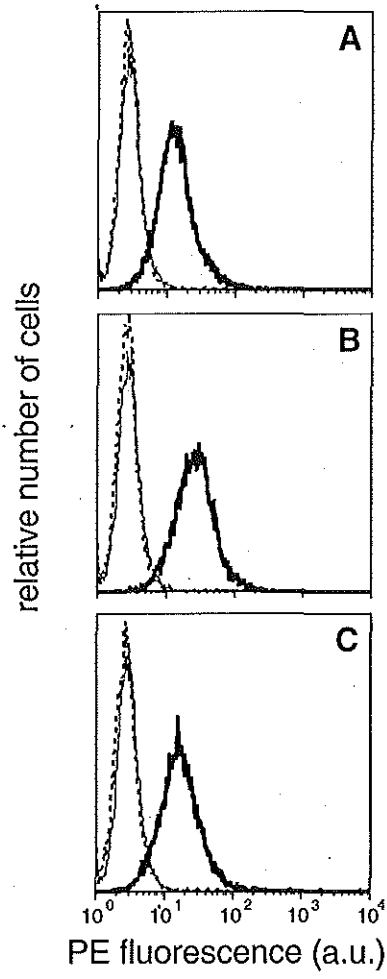


figure 7.2
Fluorescence histogram of Kit expression on MO7e cells.
MO7e cells were sequentially stained with DIG-SCF, anti-DIG mouse antibodies and PE-conjugated anti-mouse goat antibodies (thick solid lines). Control cells were incubated in the presence of a 100-fold molar excess of unlabeled SCF (broken lines), or stained only with anti-DIG mouse antibodies and PE-conjugated anti-mouse goat antibodies without DIG-SCF (thin solid lines).
(**A**) DIG-SCF labeled at a DIG:Protein (D:P) ratio of 10:1,
(**B**) DIG-SCF labeled at a D:P ratio of 50:1,
(**C**) DIG-SCF labeled at a D:P ratio of 100:1.

Correlation of Kit expression and EPO-, IL-6-, and GM-CSF-R expression on BM cells

The availability of DIG-SCF enabled us to examine coexpression of Kit and receptors for other growth factors, using biotinylated preparations of these growth factors. Cell surface expression of Kit on low density rhesus monkey BM cells was studied in relation to the expression of EPO-, IL-6- and GM-CSF-R, respectively, using DIG-labeled SCF in combination with biotinylated preparations of each of these GFs. In **figure 7.3** the results of one of five separate experiments are shown. Staining with the labeled GFs was specific, as demonstrated by incubation with DIG-SCF and biotin-GF in the presence of a 100-fold molar excess of the relevant GF.

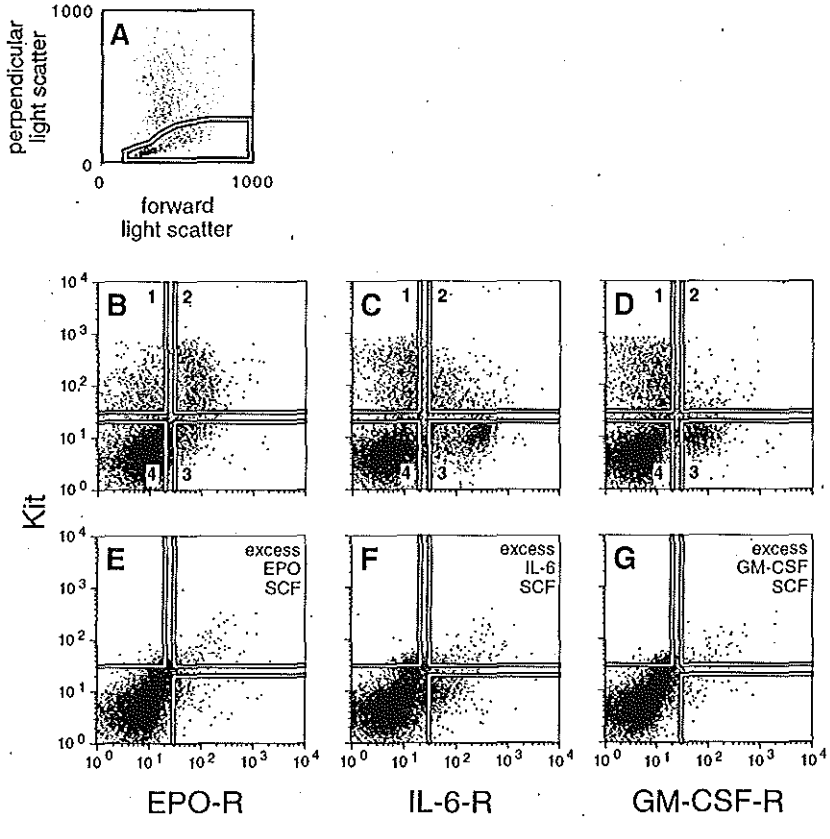


figure 7.3
Light scatter properties and GF-R expression of low density rhesus monkey BM cells.
(A) Perpendicular.versus forward light scatter. The scatter window that includes cells with low to high forward and low to intermediate perpendicular light scatter properties was used to exclude granulocytes, dead cells, and cellular debris.
(B, C, D) Coexpression of Kit and receptors for EPO, IL-6, or GM-CSF, respectively. Cells were stained with DIG-SCF combined with biotinylated EPO, IL-6, or GM-CSF. The windows used to analyze the subsets coexpressing the different GF-R are indicated.
(E, F, G) Control cells were incubated in the presence of a 100-fold molar excess of the appropriate unlabeled GF.

Combination of DIG-labeled SCF with biotinylated EPO (**figure 7.3 B**) showed that more than half of the Kit$^+$ rhesus monkey BM cells also expressed the EPO-R. This Kit$^+$/ EPO-R$^+$ fraction contained approximately 11% of the low density cells (**table 7.1**) and included cells with high as well as cells with low or intermediate Kit expression. A subset of EPO-R$^+$ cells did not express Kit at detectable levels. This population represented approximately 6% of the BM cells and appeared to be contiguous to the Kit^{low}/ EPO-R$^+$ subset. A separate cluster of Kit$^+$ cells did not express the EPO-R, and contained ≈ 9% of the low density BM cells. Most of these cells showed intermediate Kit expression.

Staining with DIG-SCF and biotin-IL-6 (**figure 7.3 C**) indicated that most cells with high Kit expression were IL-6-R$^-$, whereas most cells with high IL-6-R expression did not express Kit. The Kit$^+$/ IL-6-R$^-$ subset contained approximately 10% of the low density BM cells, the Kit$^-$/ IL-6-R$^+$ fraction represented ≈ 22% of the cells (**table 7.1**). A small population, containing approximately 7% of the cells, expressed both receptors, but mostly at intermediate levels.

table 7.1
Distribution of low density rhesus monkey BM cells over different fractions, based on expression of growth factor receptors.

	fraction [a]	% [b]
1	Kit$^+$/ EPO-R$^-$	8.5 ± 1.9
2	Kit$^+$/ EPO-R$^+$	10.6 ± 5.3
3	Kit$^-$/ EPO-R$^+$	5.5 ± 2.5
4	Kit$^-$/ EPO-R$^-$	49.8 ± 4.8
1	Kit$^+$/ IL-6-R$^-$	10.2 ± 1.3
2	Kit$^+$/ IL-6-R$^+$	6.8 ± 0.4
3	Kit$^-$/ IL-6-R$^+$	22.2 ± 0.6
4	Kit$^-$/ IL-6-R$^-$	38.7 ± 0.8
1	Kit$^+$/ GM-CSF-R$^-$	18.4 ± 4.7
2	Kit$^+$/ GM-CSF-R$^+$	ND [c]
3	Kit$^-$/ GM-CSF-R$^+$	11.3 ± 1.3
4	Kit$^-$/ GM-CSF-R$^-$	41.7 ± 3.9

[a] Cells were stained with DIG-SCF and biotinylated EPO, IL-6, or GM-CSF.
[b] Data represent the distribution of low density BM cells over the receptor-expressing regions as indicated in **figure 7.3 B-D** (mean ± SD of 5 separate samples), analyzed inside a light scatter window as shown in **figure 7.3 A**. Note that percentages do not add up to 100% since events outside the 4 receptor-expression windows were left out of the analysis.
[c] ND = not detectable.

Similar to the distribution of Kit and the IL-6-R, analysis of Kit and GM-CSF-R expression (**figure 7.3 D**) showed a subset of Kit$^+$/ GM-CSF-R$^-$ cells, which comprised approximately 11% of the cells, as well as a cluster of Kit$^-$/ GM-CSF-R$^+$ cells, which represented approximately 6% of the low density cells (**table 7.1**). Different from Kit and the IL-6-R, very few, if any, cells expressing both Kit and GM-CSF-R were found.

Phenotypic characterization of BM subsets based on expression of Kit and EPO-R

To examine the nature of the different subsets of GF-R expressing cells, phenotypic analysis of low density rhesus monkey BM cells was performed after triple-staining with labeled GFs and mAbs against different cell surface markers (**figures 7.4, 7.5, 7.6** and **table 7.2**). Consistent with previous findings [Wognum et al., 1992], very few rhesus monkey BM cells that expressed CD34 at high levels, characteristic for immature hemopoietic cells, were EPO-R$^+$ (**figure 7.4 A** and **table 7.2**). Most of the

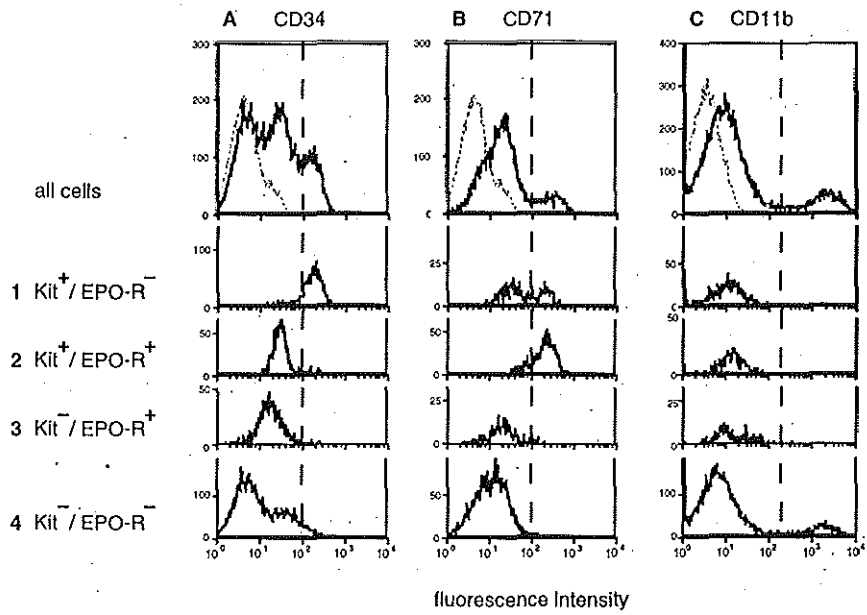


figure 7.4
Expression of CD34, CD71, and CD11b on different fractions of low density rhesus monkey BM cells, based on expression of Kit and the EPO-R.
Cells were stained with labeled GFs and (**A**) anti-CD34, (**B**) anti-CD71, or (**C**) anti-CD11b mAbs, and analyzed inside a light scatter window as shown in **figure 7.3 A**, in the regions that are indicated in **figure 7.3 B**. From top to bottom are shown: all cells; (**1**) Kit+/EPO-R− cells; (**2**) Kit+/EPO-R+ cells; (**3**) Kit−/EPO-R+ cells; (**4**) Kit−/EPO-R− cells. Dotted histograms in the upper panels show background fluorescence of control cells. Vertical broken lines indicate the threshold values that were used to discriminate between cells with low or no fluorescence signal and brightly stained cells. Results are representative for 3 independently performed experiments.

table 7.2
Distribution of low density rhesus monkey BM cells over different fractions, based on expression of growth factor receptors and other cell surface markers.

	fraction [a]	CD34^{++} % [b]		CD71^{++} % [b]		CD11b^{+} % [b]	
1	Kit^{+}/ EPO-R^{-}	8.7	(56.0)	2.5	(14.6)	0.2	(1.9)
2	Kit^{+}/ EPO-R^{+}	0.7	(4.2)	13.2	(78.9)	0.1	(1.0)
3	Kit^{-}/ EPO-R^{+}	0.2	(1.4)	0.3	(1.5)	0.4	(3.2)
4	Kit^{-}/ EPO-R^{-}	2.5	(16.2)	0.4	(2.3)	6.0	(46.3)
1	Kit^{+}/ IL-6-R^{-}	2.8	(20.7)	7.4	(58.9)	0.0	(0.2)
2	Kit^{+}/ IL-6-R^{+}	4.4	(33.4)	1.8	(14.1)	0.8	(4.7)
3	Kit^{-}/ IL-6-R^{+}	2.5	(18.8)	0.2	(1.4)	12.5	(75.2)
4	Kit^{-}/ IL-6-R^{-}	1.2	(9.3)	0.5	(3.7)	0.2	(1.3)
1	Kit^{+}/ GM-CSF-R^{-}	4.1	(53.3)	8.1	(66.7)	0.0	(0.6)
2	Kit^{+}/ GM-CSF-R^{+}	ND [c]		ND [c]		ND [c]	
3	Kit^{-}/ GM-CSF-R^{+}	0.4	(5.0)	0.1	(0.7)	6.3	(37.3)
4	Kit^{-}/ GM-CSF-R^{-}	1.2	(16.5)	0.4	(3.4)	2.0	(11.8)

[a] Cells were stained with labeled growth factors and anti-CD34, anti-CD71 or anti-CD11b mAbs. Data represent results of 1 experiment, which was representative of 3 independently performed experiments. Cells were analyzed inside a light scatter window as shown in **figure 7.3 A**, in the receptor-expressing regions as indicated in **figure 7.3 B-D**.

[b] Data represent the percentages of BM cells that were found in the receptor-expression windows and expressed CD34, CD71, or CD11b, respectively. Between brackets are the same data, relative to the total number of cells expressing these markers (normalized to 100%; note that percentages do not add up to 100% since events outside the 4 receptor-expression windows were left out of the analysis). For example, 8.7% of the BM cells were CD34^{++}/ Kit^{+}/ EPO-R^{-}, which means that 56.0% of all CD34^{++} cells were found inside the Kit^{+}/ EPO-R^{-} region.

[c] ND = not detectable.

CD34^{++} cells were Kit^{+}/ EPO-R^{-} (**figure 7.4 A**). The majority of the Kit^{+}/ EPO-R^{+} cells showed low CD34 expression, demonstrating that these cells were more differentiated than the CD34^{++}/ Kit^{+}/ EPO-R^{-} cells. The average level of CD34 expression on the Kit^{-}/ EPO-R^{+} cells was even lower, indicating that these cells were more mature than the Kit^{+}/ EPO-R^{+} cells. Consistent with earlier results, showing Kit^{+} as well as Kit^{-} cells within the CD34^{++} subset of rhesus monkey BM [De Jong et al., 1995], some CD34^{++} cells were also found in the Kit^{-}/ EPO-R^{-} subset.

Almost all erythroid cells, identified by high CD71 expression [Newman et al., 1982] [Loken et al., 1987], were Kit^{+} (**figure 7.4 B**) [De Jong et al., 1995], and most of these (≈ 80%) expressed the EPO-R (**table 7.2**). These CD71^{++} cells comprised one third of the cells in the Kit^{+}/ EPO-R^{-} fraction and the majority of cells in the Kit^{+}/ EPO-R^{+} fraction. Most of the Kit^{-}/ EPO-R^{+} cells were CD71^{-}. These CD71^{-}/ Kit^{-}/ EPO-R^{+}

cells displayed the low forward and perpendicular light scatter pattern that is characteristic of normoblasts (not shown), which are known to have lost CD71 expression [Loken et al., 1987]. The combined data are consistent with a model in which Kit expression on erythroid progenitors precedes expression of the transferrin receptor and the EPO-R. The data also demonstrate that during terminal erythroid maturation the EPO-R is retained until a later differentiation stage than both Kit and the transferrin receptor.

Analysis of expression of CD11b, which is a marker for differentiating precursors of the granulocyte and monocyte lineages, showed that most of these cells were Kit^- and EPO-R^- (**figure 7.4 C** and **table 7.2**). This is consistent with the absence of the EPO-R on myelomonocytic cells and the disappearance of Kit during later stages of myeloid differentiation (see below).

Phenotypic characterization of BM subsets based on expression of Kit and IL-6-R

In parallel with the examination of Kit and EPO-R expression on hemopoietic subsets, we also studied coexpression of Kit with the IL-6-R and GM-CSF-R, respectively. The immature rhesus monkey BM cells were heterogeneous with respect to IL-6-R expression, since high CD34 expression was found on Kit^+/ IL-6-R^+ as well as Kit^+/ IL-6-R^- cells (**figure 7.5 A** and **table 7.2**). Average CD34 expression was higher on Kit^+/ IL-6-R^+ than on Kit^+/ IL-6-R^- cells, suggesting that on average the Kit^+/ IL-6-R^+ cells were more immature than the Kit^+/ IL-6-R^- cells.

Of the approximately 20–25% $CD34^{++}$ cells that did not express Kit, approximately two thirds were IL-6-R^+ (**figure 7.5 A** and **table 7.2**), which suggests that there are also immature cells that express the IL-6-R but not Kit. Almost all $CD11b^+$ cells were also found in the Kit^-/ IL-6-R^+ subset (**figure 7.5 C**), which is consistent with the expression of IL-6-R during differentiation of monocytes and granulocytes [Wognum et al., 1993]. Therefore, the $CD34^{++}$/ Kit^-/ IL-6-R^+ cells may represent differentiating myelomonocytic progenitors that have lost Kit expression. The $CD11b^-$ cells in the Kit^-/ IL-6-R^+ population probably represent mature T-cells, which have been shown to express IL-6-R at high levels [Taga et al., 1987] [Coulie et al., 1989] [Wognum et al., 1993].

Most of the Kit^+ cells with low CD34 expression, which include erythroid colony-forming cells (CFU-E) and (pro)erythroblasts [De Jong et al., 1995], were found in the IL-6-R^- fraction (**figure 7.5 A**). In agreement with this, IL-6-R were not expressed on the majority of the Kit^+/ $CD71^{++}$ erythroid cells (**figure 7.5 B** and **table 7.2**). However, the presence of approximately 14% of the $CD71^{++}$ cells in the Kit^+/ IL-6-R^+ fraction indicated that the IL-6-R may be present on a subset of erythroid cells.

In conclusion, these results are consistent with a model in which Kit and IL-6-R are coexpressed on a subset of progenitors which includes the most immature cells

identified by high CD34 levels. During maturation of erythroid cells Kit expression increases and IL-6-R expression decreases. In contrast, myeloid differentiation is accompanied by a decrease in Kit expression and an increase in IL-6-R expression.

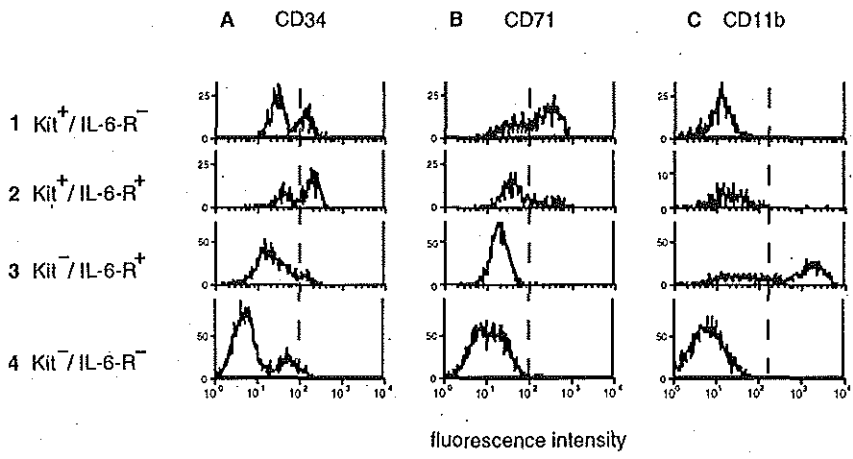


figure 7.5
Expression of CD34, CD71, and CD11b on different fractions of low density rhesus monkey BM cells, based on expression of Kit and the IL-6-R.
Cells were incubated with DIG-SCF combined with biotinylated IL-6 and the same antibodies as in **figure 7.4**. Cells were analyzed inside a light scatter window as shown in **figure 7.3 A**, in the regions that are indicated in **figure 7.3 C**.
(1) Kit+/IL-6-R− cells; (2) Kit+/IL-6-R+ cells; (3) Kit−/IL-6-R+ cells; (4) Kit−/IL-6-R− cells.

Phenotypic characterization of BM subsets based on expression of Kit and GM-CSF-R

As shown in **figure 7.3**, Kit+ and GM-CSF-R+ rhesus monkey BM populations showed little overlap. Kit+/ GM-CSF-R− populations included primitive cells with highest CD34 expression and erythroid cells with high CD71 expression (**figure 7.6 A** and **B**). The Kit−/ GM-CSF-R+ population, on the other hand, contained most of the CD11b+ cells (**figure 7.6 C** and **table 7.2**), which is consistent with the expression of GM-CSF-R on monocytic and granulocytic cells [Walker-and Burgess, 1985] [Wognum et al., 1994]. As shown in **figure 7.6 C**, this GM-CSF-R+ population covered a broad range of CD11b expression, which indicates that GM-CSF-R are expressed from immature ($CD11b^{low}$) to mature ($CD11b^{bright}$) monomyeloid cells. Additionally, some CD11b+/ Kit− cells that did not express detectable amounts of the GM-CSF-R were identified (**figure 7.6 C**), which may represent a subset of NK cells or their precursors. These combined results are consistent with a model in which immature Kit+/ CD11b− cells

lose Kit expression (and acquire GM-CSF-R) during monomyeloid differentiation and develop into CD11b$^+$ cells that express high levels of GM-CSF-R.

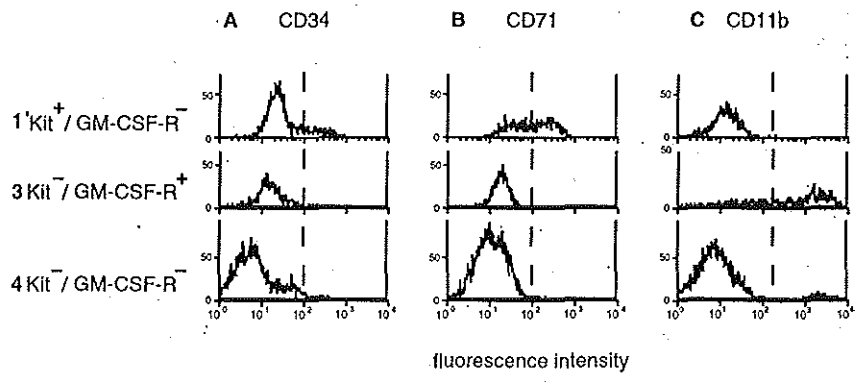


figure 7.6
Expression of CD34, CD71, and CD11b on different fractions of low density rhesus monkey BM cells, based on expression of Kit and the GM-CSF-R.
Cells were incubated with DIG-SCF combined with biotinylated GM-CSF and the same antibodies as in figure 7.4. Cells were analyzed inside a light scatter window as shown in figure 7.3 A, in the regions that are indicated in figure 7.3 D.
(1) Kit+/GM-CSF-R− cells; (3) Kit+/GM-CSF-R+ cells; (4) Kit−/GM-CSF-R− cells (note that the Kit+/GM-CSF-R+ fraction (2) contained too few cells for analysis).

DISCUSSION

In this paper we show that DIG-SCF and biotinylated GFs can be used to study coexpression of Kit and other GF-R on hemopoietic cells. As expected from previous studies using biotin [De Jong et al., 1995], SCF could be labeled efficiently with DIG, with retention of most of its ability to stimulate cell proliferation. By combining DIG-SCF with biotinylated GFs and mAbs against differentiation markers, we were able to examine coexpression of Kit and receptors for EPO, IL-6, and GM-CSF on rhesus monkey BM cells at different stages of differentiation of various blood cell lineages. This allowed a better definition of the subpopulation structure of the immature hemopoietic cell compartment. A schematic representation of changes in GF-R expression during erythroid and monomyeloid differentiation is shown in **figure 7.7**.

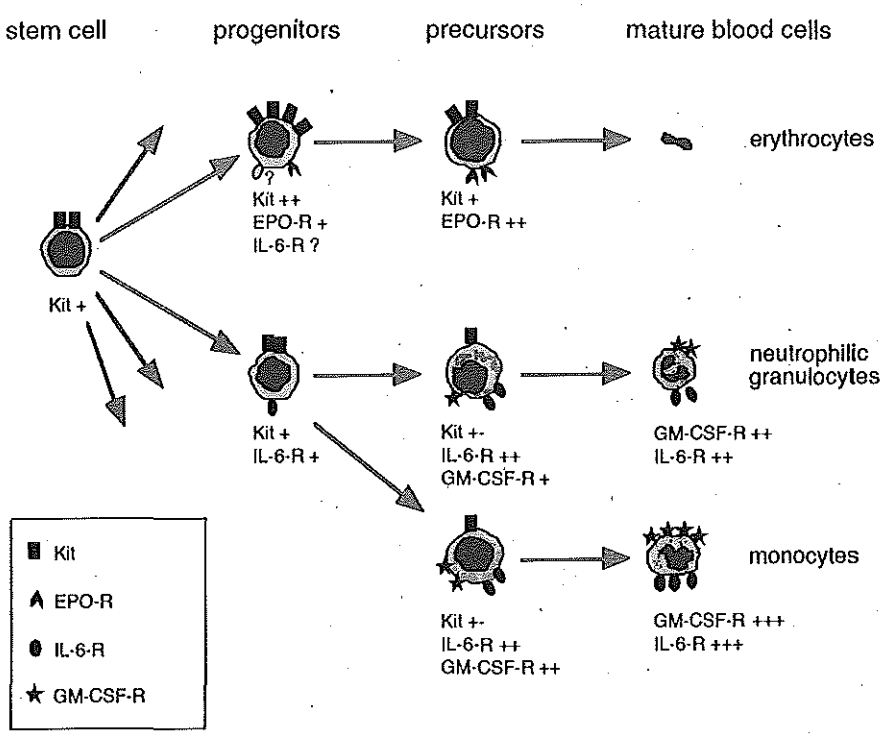


figure 7.7
Schematic representation of erythroid and monomyeloid differentiation, displaying receptor expression for SCF, EPO, IL-6, and GM-CSF.

The majority of the immature, $CD34^{++}$ rhesus monkey BM cells were Kit^{+}. This is in agreement with previous studies using anti-Kit antibodies on human cells [Ashman et al., 1991] [Papayannopoulou et al., 1991] [Briddell et al., 1992] [Gunji et al., 1993] [Simmons et al., 1994] and biotin-SCF on rhesus monkey BM cells [De Jong et al., 1995]. In these reports, using antibodies against CD34, CD33, CD38, and HLA-DR, Kit expression was demonstrated on the most immature subsets of $CD34^{++}$ cells that can be detected in vitro [Brandt et al., 1988] [Sutherland et al., 1989] [Terstappen et al., 1991], which probably contain pluripotent hemopoietic stem cells relevant to hemopoietic reconstitution [Wagemaker et al., 1995]. In line with this, cells with long-term repopulating ability were also found in the Kit^{+} fraction of murine BM [Ogawa et al., 1991] [Okada et al., 1991] [Orlic et al., 1993] [De Jong et al., 1996].

GM-CSF-R were absent from or expressed at a very low level on immature cells. This is in agreement with previous studies using purified $CD34^{+}$ rhesus monkey BM cells, showing no GM-CSF-R on erythroid and myeloid progenitors with light scatter characteristics that were similar to those we used for analysis [Wognum et al., 1994].

Similar findings were recently reported by McKinstry et al., who found little or no binding of radiolabeled GM-CSF to purified mouse BM subsets enriched for hemopoietic stem cells [McKinstry et al., 1997]. Taken together, these results suggest that GM-CSF-R are not expressed on hemopoietic stem cells, or only at extremely low levels.

Within the population of $CD34^{++}/Kit^{+}$ immature rhesus monkey BM cells, IL-6-R^{+} as well as IL-6-R^{-} cells were found, consistent with the previously demonstrated heterogeneity of immature hemopoietic cells with respect to IL-6-R expression [Wognum et al., 1993]. The average CD34 expression of the $CD34^{++}/Kit^{+}/$ IL-6-R^{-} cells is lower than that of the $CD34^{++}/Kit^{+}/$ IL-6-R^{+} cells, which might indicate that the IL-6-R^{+} cells are more immature. Previous three-color immunofluorescence experiments showed that IL-6-R are expressed on a large subset of very immature rhesus monkey or human cells with high CD34 and low HLA-DR and CD38 expression [Wagemaker et al., 1995] [Wognum et al., 1996]. In situ autoradiographic studies and reverse transcriptase polymerase chain reaction (RT-PCR) also showed binding of IL-6 and expression of mRNA for IL-6-R and its partner gp130 on highly enriched fractions of murine BM stem cells and progenitor cells [McKinstry et al., 1997][49, 50]. However, as some cells with a very high CD34 expression are also present in the $Kit^{+}/$ IL-6-R^{-} subset (**figure 7.5**), it is possible that the most immature cells lack the IL-6-R. This is supported by the finding that the majority of the long-term culture-initiating cells (LTC-IC) and mixed colony-forming cells have been found in the $CD34^{+}/$ IL-6-R^{-} fractions of human BM and cord blood [Tajima et al., 1996]. This population did, however, express gp130 and could respond to IL-6 in the presence of soluble IL-6-R [Tajima et al., 1996].

Maturation of progenitors into monomyeloid cells is accompanied by a loss of Kit expression prior to acquisition of the CD11b antigen (**figures 7.4–7.6**). The decline of Kit expression occurs after the progenitor cell stage, since cells that form in vitro colonies (CFU-GM) were previously shown to be $CD34^{++}/Kit^{+}$ [De Jong et al., 1995]. Expression of the IL-6-R increases during monomyeloid differentiation, as most $CD11b^{+}$ cells were IL-6-R^{+}, and high levels of IL-6-R expression have been identified on monocytes, macrophages, and granulocytes, and on the precursors of these cell (**figure 7.5**) [Bauer et al., 1989] [Munck Petersen et al., 1990] [Wognum et al., 1993] [Tajima et al., 1996] [McKinstry et al., 1997]. As GM-CSF-R were present on the majority of the $CD11b^{+}$ cells but not on most of the immature, $CD34^{++}$ cells (**figure 7.6**), expression of GM-CSF-R also increased during differentiation into mature granulocytes and monocytes. Similar results have been derived from binding studies using ^{125}I-GM-CSF or biotin-GM-CSF on cell lines and murine and rhesus monkey BM cells [Heyworth et al., 1991] [Wognum et al., 1994] [McKinstry et al., 1997]. A small cluster of $CD11b^{+}$ cells was found in the $Kit^{-}/$ GM-CSF-R^{-} fraction, and may represent natural killer (NK) cells [Werfel et al., 1991]. $CD11b^{-}$ cells with the scatter pattern of lymphocytes were found in the subsets

that were Kit$^-$, EPO-R$^-$ and GM-CSF-R$^-$. A fraction of these cells expressed the IL-6-R (**figure 7.5**), as is in agreement with the presence of IL-6-R on T lymphocytes and absence on resting B lymphocytes [Taga et al., 1987] [Coulie et al., 1989] [Wognum et al., 1993].

Differentiation into CD71^{++} erythroid cells is accompanied by a gradual loss of CD34 expression of the immature CD34^{++}/ Kit$^+$ cells during the transition of erythroid burst-forming units (BFU-E), which are CD34$^+$ and express very high Kit levels, to CFU-E, which have been found in the CD34^{low}/ Kit$^+$ fraction [De Jong et al., 1995]. Simultaneously, EPO-R, which is not detectable on most CD34^{++} cells, is upregulated during erythroid differentiation, reaching maximal levels on CD71^{++}/ Kit$^+$/ EPO-R$^+$ CFU-E and pro-erythroblasts [Broudy et al., 1991] [Wognum et al., 1992]. Expression of EPO-R on a subset of rhesus monkey BM cells that did not express Kit or CD71, and showed the light scatter pattern characteristic of normoblasts, indicates that the EPO-R is retained on maturing erythroid cells even after the loss of the transferrin receptor and Kit. The expression of IL-6-R on erythroid cells is less clear. Most of the CD71^{++} rhesus monkey BM cells were IL-6-R$^-$, which demonstrated that the IL-6-R is not expressed on the majority of the erythroid cells. Moreover, most erythroid colony-forming cells have been found in the CD34$^+$/ IL-6-R$^-$ fraction of human BM and cord blood [Tajima et al., 1996]. A small subset of CD71^{++} cells was identified in the Kit$^+$/ IL-6-R$^+$ fraction, which may indicate that some erythroid cells expressed the IL-6-R. This would be in line with the reported erythropoietic effect of IL-6, as demonstrated by an increase in BM normoblasts induced by in vivo administration of IL-6 in rats [Ulich et al., 1991]. Alternatively, these cells might also represent activated non-erythroid cells that are Kit$^+$/ IL-6-R$^+$, as CD71 is also upregulated on activated cells of other lineages [Larrick and Cresswell, 1979] [Newman et al., 1982].

In conclusion, GFs that are labeled with DIG provide a useful extension of the existing cell staining methods to detect receptor expressing cells by flow cytometry. It is relatively easy to construct biotin- or DIG-labeled GFs, which retain the ability to bind to receptor expressing cells. By combining different labeled GFs with mAbs against cell surface markers, it is possible to distinguish small subpopulations of bone marrow cells which coexpress various high affinity GF-R simultaneously. This approach is particularly useful to study changes in receptor expression patterns during differentiation of immature hemopoietic cells, and to explain the stimulatory and inhibitory interactions of hemopoietic GFs on phenotypically defined hemopoietic cells. However, it is more difficult to exclude a direct action on a given cell type, because the detection limit of the method is approximately 50-100 receptor molecules per cell [De Jong et al., 1992] [Wognum et al., 1993] [Wognum et al., 1994], whereas expression of only a few receptors can be sufficient for a biological response [Wognum et al., 1994] [Wognum et al., 1995] [Wognum et al., 1996]. It is likely that neither radioactive labeling nor fluorochromes will yield even lower detection levels, which means that detection of such very low

receptor levels can as yet only be achieved by sensitive RT-PCR methods at the single cell level.

Keeping these limitations in mind, simultaneous cell staining with DIG-GF and biotin-GF can be used to identify individual cells responding to multiple GFs. Since two GF-R can engage in physical interactions when coexpressed on the same cell, as has recently been demonstrated for Kit and EPO-R on transfected murine cell lines [Wu et al., 1995], the ability to detect the binding of different GFs to the same target cell can be used to examine whether similar interactions can take place on primary hemopoietic cells. Moreover, it provides an alternative approach to examine sharing of receptor subunits between different GF-R on rare hemopoietic cells, which are difficult to isolate in sufficient numbers to perform competition experiments using radioactively labeled GFs. A combination of various labeled GFs with a panel of cell surface markers will allow further characterization of stem cells and progenitor cell subsets on the basis of GF-R expression.

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CHAPTER 8

Differential expression of receptors for hemopoietic growth factors on subsets of CD34+ hemopoietic cells

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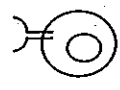
Leukemia and Lymphoma 24: 11-25 (1996)



8.1 ABSTRACT

The production of peripheral blood cells is regulated by hemopoietic growth factors (GFs) which promote the survival of stem cells and stimulate the proliferation and maturation of progenitors as well as effector functions of mature blood cell subsets. The actions of hemopoietic GFs are determined by the cellular distribution of receptors for these GFs within the hemopoietic tissues and by the functional program that receptor-expressing cells can execute after GF stimulation. Identification of stem cells and their progeny and delineation of the GF receptor (GF-R) phenotype of these cells will establish target cell range and functions of individual GFs in hemopoiesis. Cells with specific GF-R can be detected and isolated by flow cytometric methods, e.g., by staining with biotinylated ligand and fluorescently-tagged streptavidin, and classified on the basis of expression of the CD34 antigen and other markers that distinguish immature progenitors from more differentiated cells. Using this approach, distinct expression patterns have been identified for the receptors for interleukin-3 (IL-3), IL-6, granulocyte/ macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) on subsets of $CD34^+$ and $CD34^-$ cells in bone marrow (BM). Expression of the IL-3 receptor (IL-3-R), IL-6-R and GM-CSF-R appears to be very low on the most immature subsets of $CD34^+$ cells, but increases progressively during successive stages of, in particular, myelomonocytic differentiation. In contrast, the receptor for SCF, i.e., Kit, is highly expressed on very immature $CD34^{bright}$/ $HLA\text{-}DR^{dull}$ cells, which include stem cells. Kit levels decline during myelomonocytic and B-lymphoid differentiation of $CD34^+$ cells whereas they increase to maximal levels during early stages of erythropoiesis. The heterogeneity in receptor expression, together with other immunophenotypic characteristics, allows for the identification of distinct progenitor cell subsets and differentiation stages within the $CD34^+$ cell compartment. By selecting appropriate phenotypic criteria it will be possible to further dissect the stem cell compartment and eventually establish the, possibly heterogeneous, hemopoietic GF-R phenotype of pluripotent stem cells.

8.2 INTRODUCTION

The continuous production of peripheral blood cells is maintained by a co-ordinated process of self renewal, proliferation and differentiation of a small pool of hemopoietic cells in the bone marrow (BM) and other hemopoietic organs. Immature hemopoietic cells comprise only ~1% of nucleated BM cells and are identified by expression of the CD34 cell surface antigen [Civin et al., 1984]. The $CD34^+$ population is heterogeneous and contains all cells that are needed for stable, long-term multilineage hemopoiesis and those that are required for short-term production of functional blood cells of individual

lineages [Strauss et al., 1986] [Berenson et al., 1988] [Andrews et al., 1992]. Immature, multipotent progenitors and more differentiated lineage-committed precursors within the $CD34^+$ population can be distinguished on the basis of a variety of phenotypic and functional parameters. These include DNA content, fluorescent dye uptake, and differential expression of cell-surface antigens that are associated with activation status or lineage commitment. Examples of these antigens are HLA-DR, CD38, and Thy-1, which are differentially expressed on immature and more differentiated $CD34^+$ cells, CD71, which is highly expressed on activated and erythroid cells, CD33, which is most strongly expressed on myelomonocytic cells, and CD19, which is present on B-lymphocyte progenitors [Loken et al., 1987b] [Loken et al., 1987a] [Andrews et al., 1989] [Sutherland et al., 1989] [Terstappen et al., 1991] [Craig et al., 1993].

Receptors for hemopoietic GFs provide additional, but less commonly used markers to distinguish $CD34^+$ subpopulations, because they can be differentially expressed on immature progenitors and on differentiating cells of specific lineages. The ability of individual GFs to mediate stem cell survival and promote the outgrowth of specific lineages is determined by the expression of receptors for these GFs on different hemopoietic subsets and by the intrinsic developmental program receptor-expressing cells can execute after being stimulated. By analysis of GF-R expression on $CD34^+$ cells it is possible to identify the differentiation stages and lineages on which individual GFs can act and to further define the possible functions of individual GFs in regulating hemopoiesis at the level of single hemopoietic cells. Analysis of the co-expression of GF-R and changes in receptor expression in response to hemopoietic GF stimulation is important to clarify the complex patterns of redundancy, cooperation and antagonism between different GFs that act on hemopoietic cells

The purpose of this review is to discuss the cellular distribution of GF-R on hemopoietic cells, with emphasis on the receptors for IL-3, IL-6, GM-CSF, and stem cell factor (SCF) on subsets of $CD34^+$ cells.

8.3 DETECTION OF RECEPTORS ON HEMOPOIETIC CELLS

methodological aspects

A variety of techniques has been used, in different studies, to detect GF-R expression on hemopoietic and mature blood cell populations (**table 8.1**). Quantitative binding studies with radiolabeled GFs have provided insight into binding kinetics, affinity and number of receptors on cell lines, leukemic blasts and mature blood cells [Budel et al., 1989] [Park et al., 1989] [Broudy et al., 1992b]. This approach has also enabled analysis of receptor structure and interactions between different GFs, such as

competitive binding to shared receptor subunits and modulation of receptor levels [Lopez et al., 1989] [Elbaz et al., 1991] [Lopez et al., 1991] [Budel et al., 1993].

table 8.1
Methods for detection of receptor expression on hemopoietic cells.

Method	major applications
1. Radiolabeled ligand binding	Quantitative analysis of ligand/receptor interactions in homogeneous cell populations (cell lines, purified cells). Information on receptor structure and on interactions between different ligands (competition, receptor modulation).
2. In situ autoradiography with radiolabeled ligand	Analysis of receptor distribution on morphologically recognizable cells.
3. RT-PCR	Detection of receptor gene expression in small numbers of purified cells.
4. Flow cytometry (anti-receptor antibodies or labeled ligand)	Analysis of receptor distribution within heterogeneous cell populations; comparison with other immuno-phenotypic characteristics; isolation of cells on the basis of receptor expression.

Quantitative binding with radiolabeled ligand requires large cell numbers and can only be used indirectly to measure receptor expression on rare cells, e.g., by correcting receptor numbers on unfractionated hemopoietic cells for those measured on a cell fraction from which $CD34^+$ cells have been depleted [Testa et al., 1993]. Such studies have demonstrated that receptors for IL-3, IL-6, GM-CSF and erythropoietin (EPO) are expressed on $CD34^+$ cells and that expression levels are rapidly modulated in response to GF stimulation. However, this approach does not provide insight into the expression of these receptors on different subsets and differentiation stages within the $CD34^+$ population or enable comparison with morphology or other phenotypic characteristics. In situ autoradiography with radiolabeled ligands is useful to examine receptor distribution in populations of morphologically recognizable hemopoietic precursors and mature blood cells [Sawada et al., 1988] [Broudy et al., 1992b], but is less suitable for analysis of $CD34^+$ subsets, which show little morphological heterogeneity.

Analysis of receptor mRNA expression by RT-PCR methods can provide information on receptor expression on small numbers of purified hemopoietic cells. This approach is very sensitive and can even be used on single cells. However, stringent purification of target cells is required to get reliable results. In addition, the expression of receptor mRNA is not necessarily predictive of the presence of functional receptor

proteins. Discrepancies between mRNA and protein expression levels can occur as a result of variations in gene transcription rates, RNA processing and stability, and mRNA translation efficiency.

Flow cytometric methods that employ receptor-specific monoclonal antibodies or fluorescently tagged GF molecules as probes provide direct information on receptor protein expression and are suitable to detect receptor-expressing cells within heterogeneous populations. These approaches also require few cells and allow simultaneous analysis of other phenotypic characteristics such as expression of CD34 and other cell-surface antigens. In addition, it is possible to isolate hemopoietic cells on the basis of their receptor phenotype for functional characterization.

To obtain relevant information on the ability of cells to respond to a certain GF it is important that receptors are detected that are capable to bind cognate ligands at physiological concentrations. Most functional, high-affinity receptor complexes consist of homodimers [Lev et al., 1992] [Blechman et al., 1995] [Blechman and Yarden, 1995], heterodimers [Hayashida et al., 1990] [Itoh et al., 1990] [Kitamura et al., 1991], or larger complexes [Ward et al., 1994] [Paonessa et al., 1995] of individual receptor chains. Anti-receptor antibodies usually recognize individual receptor subunits and do not distinguish between cells that express functional receptors and cells that only express individual subunits or non ligand-binding isoforms. Hence, variations between the ligand binding ability of different target cell populations, caused by differential expression of individual subunits or isoforms, can remain unnoticed in binding experiments with antibodies.

Flow cytometric detection of hemopoietic GF-R expression using labeled GF molecules has been used with a variety of ligand/ receptor systems, e.g., IL-2, IL-3, IL-6, GM-CSF, G-CSF, SCF and EPO [Shimoda et al., 1992] [Wognum et al., 1992] [Wognum et al., 1993] [Wognum et al., 1994] [De Jong et al., 1995a] [De Jong et al., 1995b] [Wognum et al., 1995]. Most often the GFs are covalently modified with biotin and used in combination with fluorochrome-conjugated streptavidin, but other approaches, e.g., direct conjugation of GFs with fluorochromes or use of digoxigenin-conjugated GFs, in combination with fluorescently conjugated antibodies have been used as well [Gleizes et al., 1994]. Specificity of staining is usually assessed by competition with excess unlabeled ligand or anti-receptor antibody. The high detection sensitivity allows analysis of low receptor levels. For example, IL-6-Rs have been detected on $CD8^+$ T-lymphocytes, which express fewer than 100 high-affinity IL-6-Rs per cell [Taga et al., 1987] [Munck Petersen et al., 1990] [Wognum et al., 1993]. Similarly, GM-CSF receptors have been identified on myelomonocytic cell lines, such as HL60, which express 50-150 GM-CSF receptors per cell [Gasson et al., 1986] [Park et al., 1989] [Wognum et al., 1994].

Receptor expression on immature hemopoietic cells

Information on the receptor phenotype of rhesus monkey BM and on gradual changes in the expression levels during differentiation has been obtained by isolation and culture of receptor-expressing BM cells and by comparing receptor distribution with the expression of CD34 and RhLA-DR using multiparameter flow cytometry. The composition of different subsets of hemopoietic cells in rhesus monkey BM, identified by CD34 and RhLA-DR expression, is presented in **table 8.2**. Examples of the expression of receptors for GM-CSF, IL-3, IL-6 and SCF in relation to CD34 and DR expression on mononuclear BM cells and on purified $CD34^+$ cells are shown in **figure 8.1** and **8.2**, respectively.

The $CD34^{bright}$ population can arbitrarily be divided into DR^{bright} and DR^{dull} cells which, respectively, represent ~0.5% and 0.1-0.2% of mononuclear BM cells. The small DR^{dull} population contains committed progenitor cells and more primitive, multipotent cells that display high proliferative capacity, self renewal, and the ability to generate clonogenic cells for extended periods in hemopoietic GF- or stromal cell-

table 8.2
Phenotype of rhesus monkey bone marrow cells identified on the basis of CD34 and RhLA-DR expression.

region[1]	Fraction	%[2]	Phenotype[3]
1	$CD34^-/DR^{bright}$	5.0-18.8	(immature and) mature B-lymphocytes; (monocyte precursors; activated T-lymphocytes)
2	$CD34^-/DR^{dull}$	9.0-37.4	resting T-lymphocytes; erythroblasts
3	$CD34^{dull}/DR^{bright}$	0.8-2.7	Pre-B-lymphocytes; monocyte and granulocyte precursors; (erythroid progenitors: CFU-E and late BFU-E)
4	$CD34^{dull}/DR^{dull}$	1.5-3.0	erythroblasts; (granulocyte precursors)
5	$CD34^{bright}/DR^{bright}$	0.3-0.8	committed progenitors and precursor cells of all major lineages
6	$CD34^{bright}/DR^{dull}$	0.1-0.2	committed and immature progenitors; (repopulating stem cells)

1 As shown in **figure 8.1 A.**
2 Frequency relative to mononuclear bone marrow cells; range of 4 separate bone marrow samples.
3 Phenotype of major cell types detected in the respective subsets. Between brackets: minor cell types, making up < 5% of the cells in each specific region.

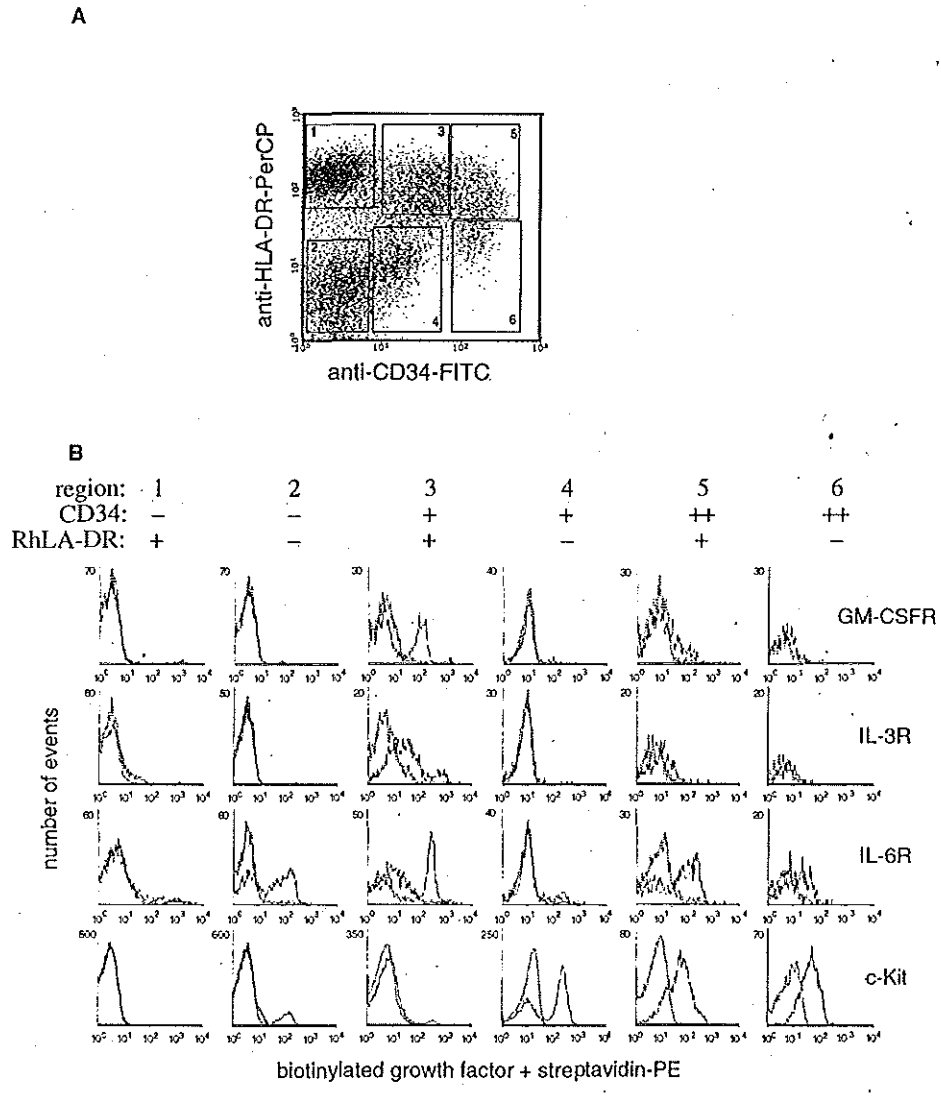


figure 8.1
Hemopoietic growth factor receptor distribution on different subsets of rhesus monkey bone marrow cells.
Low density bone marrow cells were stained first with biotinylated growth factors and then with phycoerythrin-conjugated streptavidin. Cells were counterstained with anti-CD34-FITC and anti-HLA-DR-PerCP and analyzed by three-color flow cytometry.
A. Relation between CD34 and RhLA-DR expression for cells gated in a light scatter window to exclude mature granulocytes, red blood cells, debris and aggregates from the analysis. The rectangular boxes indicate the electronic windows set to analyze the cellular composition (**table 8.2**) and receptor expression profile (**B**) of the different subpopulations that could be identified.
B. GM-CSF-R, IL-3-R, IL-6-R and Kit expression in the six distinct cell populations identified on the basis of CD34 and DR expression, as indicated in A. Solid lines: cells stained with the respective biotinylated growth factors. Dotted lines: negative controls, i.e., cells stained with biotinylated growth factor in the presence of 100-fold excess unlabeled growth factor.

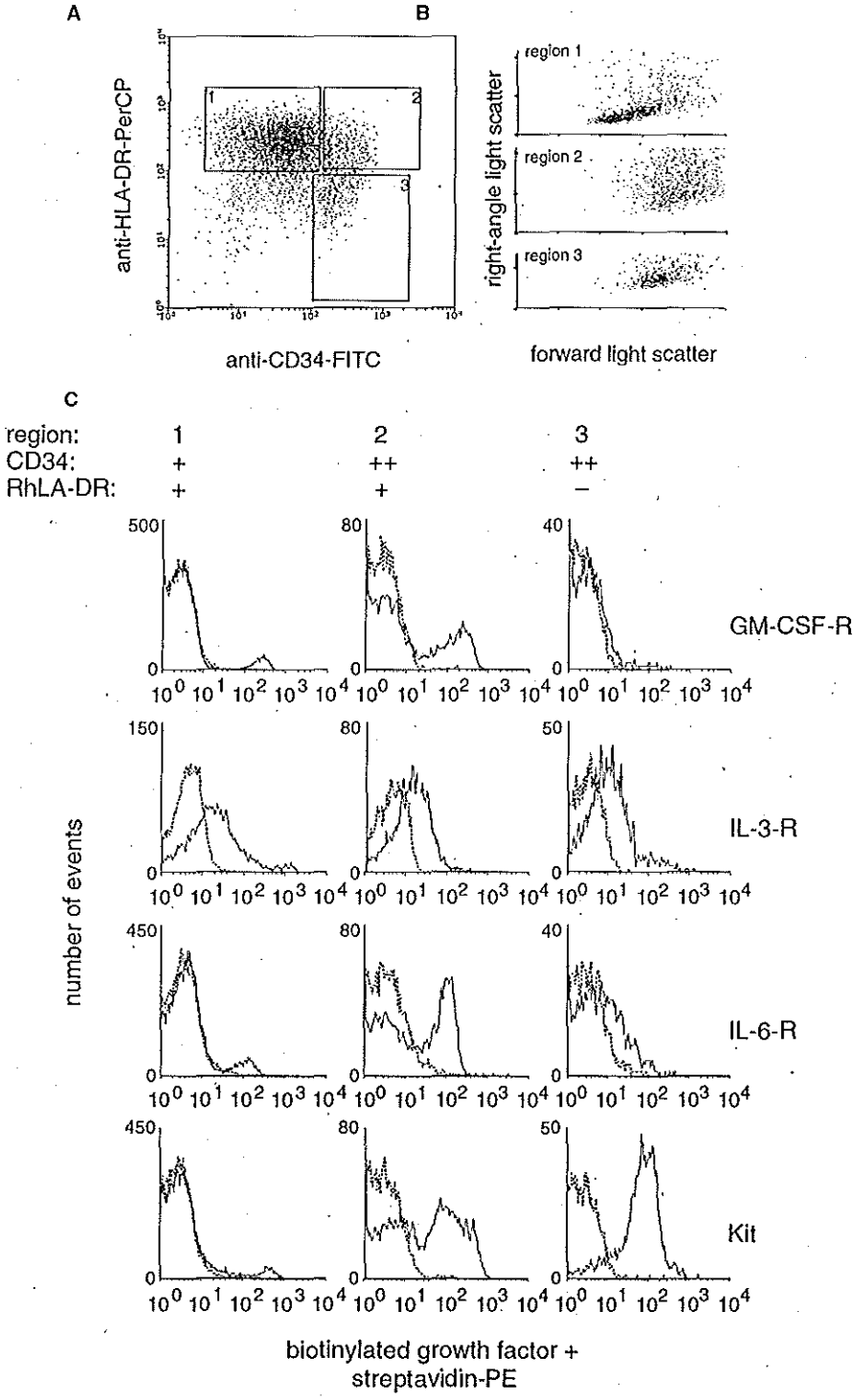
supported in vitro cultures [Brandt et al., 1988] [Sutherland et al., 1989] [Leary et al., 1992] [Wognum et al., 1995]. It is assumed that the populations identified by such in vitro growth behavior contain the cells responsible for rapid hemopoietic reconstitution as well as those that sustain long-term multilineage hemopoiesis in vivo. Although formal proof of this hypothesis will require transplantation studies with allogeneic or genetically marked cells, results of syngeneic transplantation experiments with limited numbers of flow sorted autologous BM cells in rhesus monkeys indicate that rapid and stable multilineage regeneration is mediated by $CD34^{bright}/DR^{dull}$ cells, but not by $CD34^{bright}/DR^{bright}$ cells [Neelis et al; manuscript in preparation].

As shown in **figure 8.2**, Kit as well as the receptors for IL-3 and IL-6 can be detected on $CD34^{bright}/DR^{dull}$ cells, which indicates that these receptors are present on immature hemopoietic cells. In contrast, the GM-CSF-R is barely detectable in this subset, which may indicate that GM-CSF-R expression on immature cells is much lower than that of the other receptors or that it is not expressed on most DR^{dull} cells.

Distribution of the receptors for GM-CSF, IL-3, and IL-6 in the myelomonocytic and erythroid lineages

Differentiation of immature, $CD34^{bright}/DR^{dull}$ cells is accompanied by increased receptor expression. This is particularly evident for the GM-CSF receptor (**figure 8.2**). The highest levels of receptor expression for IL-3, IL-6, and GM-CSF are found on cells with low CD34 and high DR expression, which consist of immature precursors for neutrophils, basophils and monocytes (**figure 8.1**) [Wognum et al., 1994] [Wognum et al., 1995]. Upon further differentiation, maturing neutrophils and monocytes continue to express the IL-6 and GM-CSF receptors, but lose the IL-3-R, at least in rhesus monkeys. Results from radiolabeled IL-3 binding experiments on purified monocytes suggest that the IL-3-R remains expressed at low levels on mature human monocytes

figure 8.2
Hemopoietic growth factor distribution on purified $CD34^+$ cells.
$CD34^+$ rhesus monkey bone marrow cells were affinity-purified using anti-CD34-coated immunomagnetic beads [Egeland and Gaudernack, 1994]. The isolated cells were stained for growth factor receptor, CD34 and RhLA-DR expression and analyzed by flow cytometry.
A. Relation between CD34 and RhLA-DR expression of the isolated $CD34^+$ bone marrow cells. The rectangular boxes identify the three subpopulations which could be identified and that were examined for light scatter (**B**) and receptor expression patterns (**C**). Note that regions 1, 2 and 3 have similar CD34 and DR expression as those in regions 3, 5 and 6 in **figure 8.1** and represent comparable cell populations.
B. Light scatter of the cells identified in the three different regions shown in A. Cells with very low forward and right angle light scatter typical for lymphoid cells were found in region 1; region 2 contained cells with relatively high forward and diffuse right-angle light scatter; whereas most cells in region 3 displayed intermediate forward and low right-angle light scatter.
C. Differential GM-CSF-R, IL-3-R, IL-6-R and Kit expression on each of the 3 $CD34^+$ cell subsets identified in **A**. Broken lines represent control cells (stained in the presence of 100-fold excess unlabeled ligand).


A
B
anti-HLA-DR-PerCP
anti-CD34-FITC
region 1
region 2
region 3
right-angle light scatter
forward light scatter
C
region: 1 2 3
CD34: + ++ ++
RhLA-DR: + + −
GM-CSF-R
IL-3-R
IL-6-R
Kit
number of events
biotinylated growth factor +
streptavidin-PE

[Elliott et al., 1989] [Budel et al., 1990] [Elliott et al., 1992]. It is possible that such results are caused by contamination of monocyte preparations with other cells, e.g., basophils, as we have never found evidence for the presence of IL-3-Rs on monocytes in binding studies with biotinylated IL-3 [Van Gils et al., 1995] [Wognum et al., 1995]. Maturing basophil precursors, which are present in significant quantities in BM and blood of rhesus monkeys during the course of in vivo IL-3 treatment, maintain the IL-3-R at high levels and the GM-CSF receptor at low levels, but lose the IL-6-R [Van Gils et al., 1995]. The IL-3, IL-6, and GM-CSF receptors are thus predominantly expressed on differentiating and mature cells of the myelomonocytic series, but show distinct distribution patterns on precursors and mature cells of individual lineages.

It has been known for some years that early stages of erythropoiesis are regulated by other GFs than EPO [Wagemaker et al., 1979] [Iscove et al., 1982]. It is now clear that SCF is the major physiologic regulator of early erythroid development (see below). IL-3 and GM-CSF have also been implicated as stimulators of early erythropoiesis [Goodman et al., 1985] [Sieff et al., 1985]. Receptors for these cytokines are not detectable on erythroid colony-forming cells or on cells that express erythroid antigens, such as CD71 [Wognum et al., 1994] [Wognum et al., 1995]. This may indicate that IL-3 and GM-CSF require very low receptor levels to stimulate erythroid progenitors directly or that they act indirectly, e.g., via stimulation of GF production by accessory cells. IL-6-Rs can be identified on a small subset of $CD71^{bright}$ BM cells that also express Kit (de Jong et al, submitted). This suggests that IL-6 activities in erythropoiesis may be exerted through direct stimulation of erythroid cells [Ulich et al., 1991].

Kit expression on myelomonocytic and erythroid cells

Variable results have been reported for Kit distribution on cells of different lineages and differentiation stages [Ashman et al., 1991] [Papayannopoulou et al., 1991] [Broudy et al., 1992a] [Strobl et al., 1992] [Gunji et al., 1993] [Reisbach et al., 1993] [Simmons et al., 1994]. These differences may be caused, in part, by differences between the target cell populations, variations in the binding affinity of anti-Kit antibodies used in different studies, and by the ability of some anti-Kit antibodies to inhibit proliferation of Kit^+ cells, which may result in underestimating the clonogenic cell content of sorted Kit^+ subsets. Nevertheless, the conclusion from most antibody studies is that Kit is predominantly associated with immature and erythroid cells, and less with differentiating cells of monomyeloid and lymphoid lineages. Our results with biotinylated SCF are consistent with this view and indicate that most myelomonocytic progenitors, i.e., granulocyte/macrophage colony forming units (CFU-GM), are present in the population of the ~30-50% of $CD34^{bright}$ cells that express Kit at intermediate levels similar to the levels found on more immature, $CD34^{bright}/DR^{dull}$ cells. Kit gradually disappears during granulocyte and monocyte differentiation in parallel with the loss of CD34 [De Jong et al.,

1995b]. A fraction (10-25%) of immature erythroid cells, i.e., erythroid burst forming units (BFU-E), express Kit at similar levels as CFU-GM, but most BFU-E can be isolated in a distinct population that expresses Kit at very high levels and contains few, if any, non-erythroid progenitors [De Jong et al., 1995b]. Direct analysis of Kit and EPO receptor co-expression on BM cells, by combining biotinylated EPO with digoxigenin-labeled SCF shows that Kit expression gradually declines after the CFU-E and pro-erythroblast stages, but is detectable until the normoblast stage [De Jong et al., in press].

Receptor expression on B-lymphoid cells

Distinct receptor expression patterns have also been observed for the B-lymphocyte series. IL-3-Rs are expressed at various stages of B-lymphocyte differentiation (**figure 8.1**). These include $CD34^{dull}$ pre-B-lymphocytes, which express DR, CD10 and CD19, and $CD34^-$ immature B-lymphocytes, which express IgM and low levels of CD20 (**figure 8.3**) [Sato et al., 1993] [Wognum et al., 1995]. $CD20^{bright}$ mature B-cells are receptor-negative (**figure 8.3**). The presence of IL-3-Rs on the B-cell lineage is consistent with the reported activities of this cytokine in regulating B-lymphopoiesis in vitro and in vivo [Palacios et al., 1984] [Tadmori et al., 1989] [Wörmann et al., 1989] [Cockayne et al., 1994].

IL-6-Rs and GM-CSF-Rs have not been identified on B-cell precursors or on mature B-lymphocytes [Coulie et al., 1989] [Wognum et al., 1993]. However, IL-6 has been shown to be essential for the development and continued growth of B cell neoplasms and to be involved in the differentiation of normal B-cells into Ig-secreting plasma cells [Hirano et al., 1986] [Kawano et al., 1988] [Hilbert et al., 1995]. This suggests that IL-6-R may become expressed after B-cell activation. SCF is active at early stages of B-lymphopoiesis, but not at later stages and this is reflected by expression of Kit on only a small fraction of $CD34^+$/ $CD19^+$ BM cells, as determined in binding studies with anti-Kit antibodies [Simmons et al., 1994].

Receptor expression on stem cells

$CD34^{bright}$/ DR^{dull} BM cells constitute a small cell population that is heterogeneous and still a factor 10^2–10^3 remote from a pure stem cell population. The presence of IL-3, IL-6 and SCF receptor expressing cells in the $CD34^{bright}$/ DR^{dull} subset (**figure 8.2**) indicates that these receptors are expressed on immature cells, but does not provide definitive evidence for expression on pluripotent stem cells.

Kit is detectable on the majority of immature $CD34^{bright}$/ DR^{dull} cells in human and rhesus monkey BM (**figure 8.2**) and on $CD34^{bright}$/ $CD38^-$ human BM cells, which represent a comparable immature cell population [Briddell et al., 1992]. For this reason, it is likely that Kit is expressed on pluripotent stem cells. Direct evidence for the presence of Kit on repopulating stem cells comes from transplantation studies in mice, as limited numbers of sorted Kit^+ BM cells can reconstitute short-term and long-term multilineage hemopoiesis in recipient mice, whereas much higher numbers of Kit^- cells

cannot [Ikuta and Weissman, 1992] [Okada et al., 1992] [Orlic et al., 1993] (**chapter 4**). Similar transplantation studies with sorted cells are not possible for the IL-3 and IL-6 receptors since expression levels on immature cells are much lower than that of Kit and insufficient for reliable separation of receptor-positive and receptor-negative cells.

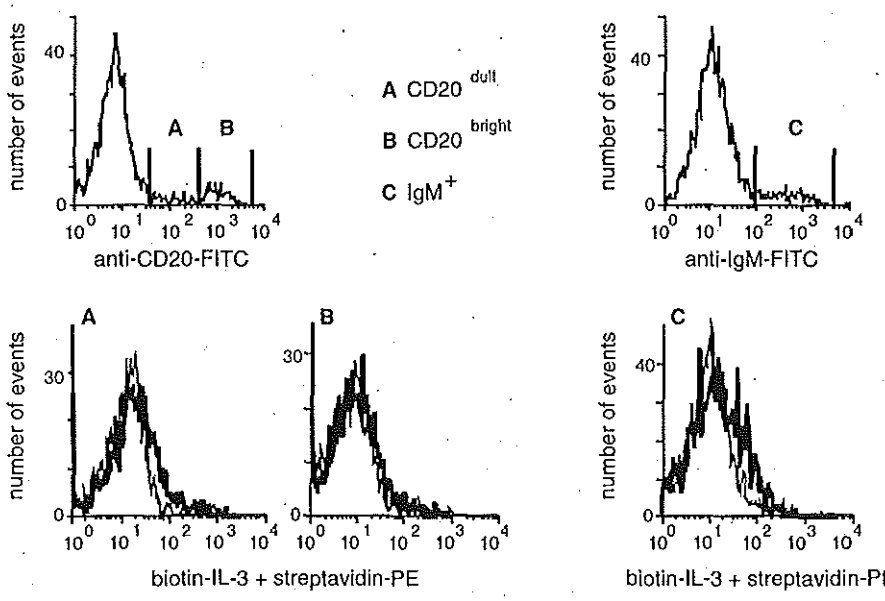


figure 8.3
The IL-3 receptor is expressed at low levels on $CD20^{dull}$/ IgM^+ immature B-cells but not on $CD20^{bright}$ mature B-cells.
Parallel bone marrow samples were stained for IL-3-R expression, using biotinylated IL-3 and streptavidin-PE, counterstained with anti-CD20-FITC and anti-IgM-FITC, respectively, and analyzed by two-color flow cytometry. Note that $CD20^{dull}$ cells (**A**) and a fraction of IgM^+ cells (**C**) show specific staining with biotinylated IL-3, whereas specific staining is not detectable on $CD20^{bright}$ cells (**B**).

Information on the importance of these cytokine/ receptor pairs for early stages of hemopoiesis has been obtained from receptor mRNA expression studies and from studies with mice in which GF- or GF-R-genes were inactivated by homologous recombination. A highly selected fraction of immature hemopoietic cells, prepared by elimination of dividing cells by 5-Fluoro-Uracil treatment, has been shown to express RNA for Kit and for both chains of the IL-6-R, but to be negative for the α chain of the GM-CSF-R and the shared β chain of the IL-3-R, IL-5-R and GM-CSF-R [Berardi et al., 1995]. These data confirm the flow cytometry data that show that Kit and the IL-6-R are expressed on very immature cells, whereas the GM-CSF-R becomes expressed at later stages. These results also suggest that the IL-3-R, although detectable on $CD34^{bright}$/ DR^{dull} cells, may not be expressed on the most primitive cells within this population.

In agreement with receptor expression data, gene knockout mice that lack functional IL-6 or the gp130 signal transducing subunit have reduced numbers of immature hemopoietic cells [Kishimoto et al., 1995] [Kopf et al., 1995]. Thus it appears that IL-6 is important for early hemopoiesis in vivo, although it remains to be established to what extent the activities of IL-6 on stem cells and immature progenitors can be taken over by other cytokines that utilize gp130 for signal transduction, e.g., IL-11, oncostatin-M or Leukemia Inhibitory Factor.

In contrast to IL-6 deficient mice, hemopoiesis is relatively normal in homozygous mutant mice that either lack functional GM-CSF or the shared β chain of the IL-3, IL-5 and GM-CSF receptors [Dranoff and Mulligan, 1994] [Nishinakamura et al., 1995]. This confirms that GM-CSF is nonessential for early hemopoiesis, at least under normal steady-state conditions. Appraisal of the role of IL-3 in early hemopoiesis in vivo will require inactivation of the genes for IL-3 itself, for the IL-3-R α chain, or the genes for both the shared and unique IL-3-R β chains that are co-expressed in murine cells [Hara and Miyajima, 1992], as mice that lack only the gene for the unique IL-3-R β chain are normal [Nishinakamura et al., 1995].

In summary (see **table 8.3**); the combined data indicate that Kit is expressed on stem cells and remains expressed at detectable levels after lineage commitment and during early stages of differentiation after which Kit levels diverge upon further maturation of erythroid, B-lymphoid, and myelomonocytic lineages. In addition to myelomonocytic cells and mature T-lymphocytes, the IL-6-R appears to be expressed on stem cells, but at low levels. The expression of the IL-3-R and GM-CSF-R on stem cells is uncertain. The IL-3-R becomes detectable at earlier differentiation stages than the GM-CSF-R, as the IL-3-R can be detected on $CD34^{bright}/DR^{dull}$ cells, whereas the GM-CSF-R is barely detectable on these cells. Whether or not the IL-3-R expressing $CD34^{bright}/DR^{dull}$ cells include stem cells or consist of progenitors committed to myelomonocytic or B-lymphoid differentiation remains to be established.

Receptor expression on malignant cells

Comparison of the receptor phenotype of normal hemopoietic cells with that of leukemia cells may help to identify the cells that initiate and maintain the malignant clone and may provide information on the GF regulation of leukemic cells. IL-3 and GM-CSF receptors as well as Kit have been identified on acute myeloid leukemia (AML) blasts by equilibrium binding of radiolabeled GFs [Budel et al., 1989] [Broudy et al., 1992b] [Pietsch et al., 1992]. However, the average number of receptors did not correlate with the ability of AML cells to respond to these GFs as measured in short term assays that measure 3H-thymidine incorporation or colony formation. Results of multiparameter FACS studies with anti-receptor antibodies [Ashman et al., 1988] [Bühring et al., 1991] [Reuss-Borst et al., 1994], or with biotinylated ligand (**figure 8.4**) have indicated

heterogeneity within AML populations with respect to the expression of Kit and CD34. This heterogeneity can explain the discrepancies between GF responses and average receptor levels, as only specific subsets of AML cells may have the ability to grow in vitro and the receptor phenotype of these subsets may not correspond with that of the AML population as a whole.

table 8.3
Overview of receptor expression on hemopoietic cells and mature blood cell subsets.

receptor	cell type	expression level	
GM-CSF-R	committed progenitors	≤50	
	myelomonocytic precursors	10^2-$4x10^3$	
	monocytes	~$4x10^3$	
	neutrophilic granulocytes	$5x10^2$-$1x10^3$	
	basophilic granulocytes	~10^2	
IL-3-R	immature progenitors	≤50	
	committed progenitors	~10^2	
	myelomonocytic precursors	10^2-$5x10^3$	
	B-lymphocyte precursors	10^2-$5x10^2$	
	basophilic granulocytes	$1x10^3$-$2x10^3$	a
IL-6-R	stem cells/immature progenitors	≤50	
	progenitors	50-10^2	
	myelomonocytic precursors	10^2-10^3	
	monocytes	10^3	
	neutrophilic granulocytes	~$5x10^2$	
	$CD4^+$ T-lymphocytes	~$4x10^2$	
	$CD8^+$ T-lymphocytes	~$1x10^2$	
Kit	stem cells/immature progenitors	~10^3	
	myelomonocytic progenitors (CFU-GM)	~10^3	
	myelomonocytic precursors	<10^2-10^3	
	erythroid progenitors (BFU-E)	10^3-$6x10^3$	b
	erythroid precursors	10^2-$6x10^3$	b

Receptor numbers are estimated on the basis of the sensitivity of the flow cytometric receptor detection method (~50 receptors per cell), the relative fluorescence intensity of different subsets and by comparison with receptor numbers on purified cell populations obtained in equilibrium binding experiments with radiolabeled growth factors. Only cell subsets on which receptors have been identified are indicated.

a High-affinity sites; basophils also express 15,000-30,000 low affinity IL-3-R; (see [Van Gils et al., 1995])

b Kit expression gradually increased during BFU-E differentiation and declines after the CFU-E/ pro-erythroblast stage.

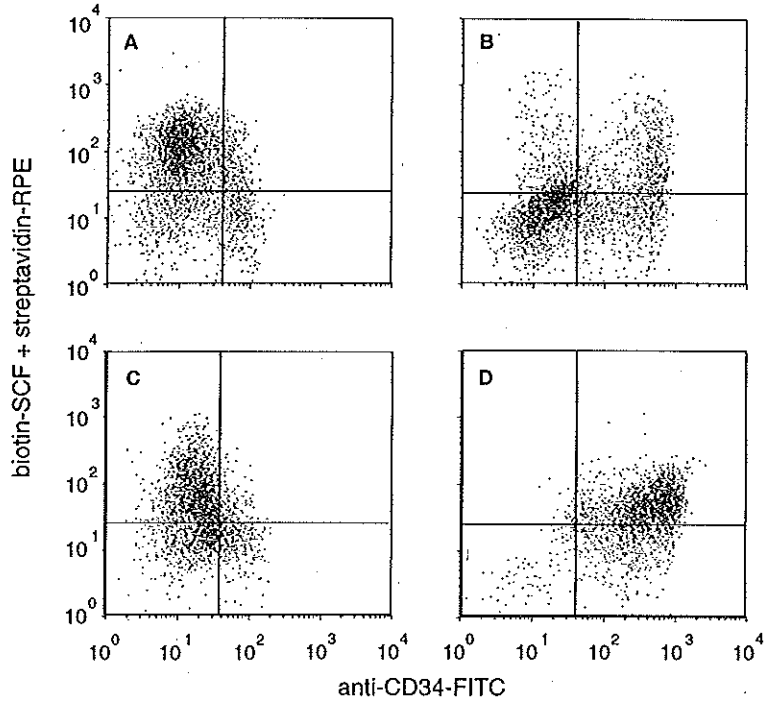


figure 8.4
Heterogeneity of CD34 and Kit expression on AML cells. Bone marrow cells from four patients with AML were stained for Kit expression (using biotinylated SCF and streptavidin-PE) and for CD34 expression (using anti-CD34-FITC) and analyzed on the FACS. The quadrants indicate the limits of CD34 and Kit expression based on the fluorescence of specificity controls (cells stained with biotinylated SCF in the presence of excess unlabeled SCF and cells stained with a FITC-labeled isotype control antibody). Note that each AML sample is heterogeneous with respect to Kit and CD34 expression and that the four AML cases show non-identical cellular distribution of these antigens.

Evidence for functional heterogeneity of AML cells has been obtained in cell culture and SCID mouse transplantation studies with purified AML subsets [Lapidot et al., 1994] [Terpstra et al., 1996]. The results indicate that only specific subsets can initiate leukemia in SCID mice and that in vivo leukemogenic ability of different subsets does not correlate with the ability for short-term growth in vitro [Lapidot et al., 1994] [Terpstra et al., 1996]. In one study, only $CD34^+/ CD38^-$ AML cells could develop in transplanted SCID mice. This suggests that the immunophenotype of leukemia-initiating AML cells is identical to that of the most immature hemopoietic cells that can be identified in normal BM [Lapidot et al., 1994]. However, in another AML case, SCID repopulating cells were present in $CD34^+$ and $CD34^-$ subsets [Terpstra et al., 1996]. This indicates differences between the phenotypes of leukemic stem cells from different AML cases and heterogeneity within the leukemic stem cell pool of individual cases. The apparent

lack of a common phenotype of AML stem cells probably reflects the diversity of target cells and target genes for leukemic transformation in AML and this will complicate the analysis of the GF-R phenotype and GF regulation of the AML subsets with in vivo leukemogenic ability.

Chronic myeloid leukemia (CML) is a more homogeneous disease than AML as it is caused by a specific chromosomal translocation between the *abl* proto-oncogene and the *bcr* locus on chromosomes 9 and 22, respectively, in a pluripotent stem cell or early, multipotent progenitor [Fialkow et al., 1977] [De Klein et al., 1982] [Groffen et al., 1984]. The leukemic stem cell in CML is believed to phenotypically resemble its normal counterparts and to express high levels of CD34. As yet, little is known about the hemopoietic GF-R phenotype of immature CML subsets. Because Kit is expressed on normal stem cells and is involved in regulating early stages of normal hemopoiesis it is of interest to examine the role of the Kit and its ligand in the growth of early CML cells. It is unknown whether *bcr/abl* expression can alter Kit expression and SCF responsiveness in CML cells. Nevertheless, immature CML cells show impaired outgrowth when cultured on SCF deficient stromal cells [Agarwal et al., 1995], and this reduced SCF responsiveness may be related to reduced Kit expression levels on immature, $CD34^{bright}$/ $CD38^-$ CML cells [Wognum et al., 1995]. Alterations in receptor gene expression as a result of $p210^{bcr/abl}$ action may also be involved in the increased expression of the IL-2R α chain and of the EPO receptor on immature CML cells, which are usually not detectable on most normal $CD34^+$ cells [Visani et al., 1987] [Wognum et al., 1992].

Further comparison of GF-R expression on normal hemopoietic cells and those of cells at comparable stages of leukemic cell differentiation will improve our knowledge of the phenotype and GF regulation of leukemic cell survival and proliferation. In addition to a better understanding of the pathophysiology of leukemia this information will be relevant to the design of improved therapeutic strategies aimed at selective elimination of leukemic stem cells.

8.4 Concluding remarks

The heterogeneous composition of the $CD34^+$ cell compartment with respect to immunophenotype and receptor expression profile allows distinction between progenitor cells of individual lineages and at different stages of differentiation. By selecting appropriate immunophenotypic criteria and by combining in vitro stem cell assays with analysis of the repopulating ability of purified cells, e.g. by transplantation into SCID mice or lethally irradiated rhesus monkeys, it will be possible to further dissect the stem cell compartment and eventually focus on the pluripotent stem cell. It

remains to be seen if a single phenotype and receptor profile can be associated with the pluripotent stem cell or that the stem cell constitutes a heterogeneous population itself. Heterogeneity in the relative expression levels of GF-R on stem cells, either as a result of stochastic processes or induced by external stimuli, can confer flexibility on the responsiveness of stem cells to different hemopoietic stimuli. This ensures that sufficient stem cells are recruited in response to any given set of stimuli to meet blood cell demands, while sufficient stem cells remain unresponsive and quiescent. Such a mechanism would be crucial to prevent depletion of the stem cell pool during emergency hemopoiesis.

The details and significance of these mechanisms in regulating the balance between quiescence and activation, self renewal and lineage commitment of pluripotent stem cells are still far from clear. Elucidation of receptor (co-)expression on the pluripotent stem cell and its immediate progeny and of changes in the expression levels of different GF-R on these immature cells in response to GFs and inhibitors will be pivotal to a better understanding of stem cell regulation. In this respect it will also be important to further delineate the expression of the STK-1/Flt3/Flk-2 receptor tyrosine kinase [Small et al., 1994] and the TPO receptor, Mpl [Debili et al., 1995], on stem cells and on progenitors and to examine the modulating effects of soluble GF-R subunits, which may act either as co-stimulators or inhibitors of cell growth (for a recent review see [Heaney and Golde, 1996]) Finally, studies into the GF regulation of stem cells and immature progenitors will be important to select suitable target cell populations and transduction conditions for gene marking and gene therapy of stem cells and for the rational use of GFs to support hemopoietic recovery in myelosuppressed patients.

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8.6 LITERATURE CITED

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CHAPTER 9

Summary and discussion

9.1 Purpose and methodology

Growth factors (GFs) play an important role in the maintenance of viability and the stimulation of proliferation and differentiation of hemopoietic cells. Despite the increasing insight into GF actions in vivo and in various culture systems, little is known about the specific target cells for each individual GF and the mechanisms by which GFs, individually and in cooperation with each other, stimulate hemopoiesis. The interaction of a GF with its target cell is mediated by cell membrane receptors specific for the particular GF. Therefore, analysis of GF-receptor (GF-R) expression on hemopoietic stem cells (HSC) and of changes in receptor expression during differentiation of blood cell lineages provides a direct means to assess the target cell range and thus the possible spectrum of biological activities of GFs within the hemopoietic system.

To obtain relevant information on the ability of cells to respond to a certain GF, it is important that cells are detected that express the functional receptors that are able to bind GFs. In this thesis, the development and application of methodology to examine cell surface receptor protein expression on hemopoietic cells is described. The principal method used in this study is based on sequential staining of cells with biotinylated GF molecules and fluorescently labeled (strept)avidin, followed by analysis of receptor-expressing cells using flow cytometry. This allows simultaneous detection of receptors and other cell surface markers, and is suitable to examine receptor expression on subsets of heterogeneous cell populations such as bone marrow (BM). Moreover, this approach can be used to isolate viable hemopoietic cells on the basis of their receptor expression for functional characterization.

The feasibility of preparing and using biotinylated GFs to study GF-R expression was examined using interleukin-2 (IL-2), IL-3, IL-6, granulocyte/ macrophage colony-stimulating factor (GM-CSF), and stem cell factor (SCF) (**chapter 2**). As GF-R expression on immature hemopoietic cells can be very low, we applied methodology to increase the fluorescence signals of cells stained with biotinylated GFs and fluorescently labeled (strept)avidin. Fluorescence amplification by incubation with alternate layers of biotinylated anti-(strept)avidin antibodies and fluorescently labeled (strept)avidin resulted in a detection sensitivity lower than 100 molecules per cell, as estimated from a cell line with known receptor numbers (**chapter 2**). However, these results were obtained using homogeneous cell suspensions; cells with low receptor numbers may remain undetected in heterogeneous cell suspensions, such as BM, especially when these cells are present at low frequencies.

Using IL-2 as a model system for hemopoietic GFs, different methods and reagents to label GFs with biotin were compared, and the most suitable biotin derivatives were selected (**chapter 3**). These experiments showed that high biotinylation efficiency,

retention of biological activity, and specific staining of receptor-expressing cells can be achieved by GF labeling with N-hydroxy-succinimide (NHS) biotin, potentially with an extended spacer arm. Other biotinylation reagents, such as *p*-Diazobenzoyl Biocytin Precursor (DBB) or photoactivatible biotin, performed less well on IL-2. Optimal biotinylation conditions are dependent on the type of GF, which determines the possibilities for modification of amino acid or sugar residues, and the biotin-to-protein (B:P) ratio that yields the highest degree of conjugation and maximal retention of biological activity. With NHS-biotin we achieved good results on IL-2 as well as all other GFs that were labeled in this study (e.g. **chapter 2**), and was used in the experiments described in this thesis.

9.2 Kit expression studies on murine cells

To characterize hemopoietic cells that express the SCF-R, Kit, murine BM cells were stained either with biotinylated SCF or with anti-Kit antibodies, after which Kit^+ and Kit^- cells were sorted using flow cytometry (**chapter 4**). In vitro and in vivo analysis of these cells showed that Kit is expressed on immature cells at various differentiation stages. The Kit^+ BM fraction contains HSC with long- as well as cells with short-term repopulating ability, cells that form colonies in spleen and cells that form colonies in culture (CFU-S and CFU-C (CFU-GM + BFU-E), respectively), and morphologically recognizable immature myeloid and erythroid cells. In contrast, in the Kit^- BM subsets, very few CFU-C and CFU-S are found, and hardly any long-term repopulating cells.

Compared to unfractionated BM, the biotin-SCF^+ mouse BM subset is 40- to 70-fold enriched for clonogenic cells (**chapter 4**). Since the Kit^+ subset comprises 1–2% of the nucleated BM cells, higher enrichment values than 50- to 100-fold cannot be expected using Kit expression as a single sorting parameter, and additional markers are needed for further enrichment of immature cells. Negative selection against expression of various lineage markers, which is used by some groups in combination with positive selection for Kit expression [Okada et al., 1991], does not result in further enrichment for clonogenic cells, as these markers are not expressed on Kit^+ cells. A possible candidate for further enrichment of Kit^+ fractions for immature cells is Rh123 retention, as we have shown that Kit^+ cells are heterogeneous with respect to Rh123 retention [Visser et al., 1993], and it is known that the $Rh123^{dull}$ fraction contains the HSC, whereas more differentiated progenitors are found in the $Rh123^{bright}$ population [Ploemacher and Brons, 1988]. A disadvantage of Rh123 is that it is technically difficult to achieve sufficient fluorescence compensation between the Rh123 and the Kit-PhycoErythrin (PE) fluorescence signals on the flow cytometer, and two separate sorts are required to purify $Kit^+/Rh123^{dull}$ cells. In this respect the use of other fluorochromes, such as the vital

DNA stain Hoechst 33342, may be of interest to separate primitive progenitors from more mature cells within the Kit$^+$ population [Baines and Visser, 1983] [Goodell et al., 1996].

Within the Kit$^+$ fraction, differences were found between the cells that bound biotin-SCF and the cells that reacted with anti-Kit antibodies (ACK-2). A higher percentage of BM cells could be stained with ACK-2 than with biotin-SCF (**chapter 4**). Selection of Kit$^+$ cells after incubation with biotin-SCF yielded fractions with 2- to 3-fold higher frequencies of clonogenic cells than obtained after staining with ACK-2. An effect of cell staining with biotin-SCF or ACK-2 on colony formation or repopulating capacity was not found, which excludes the possibility that some progenitors were inactivated or stimulated by these reagents. It is possible that these differences reflect a different target cell specificity of biotin-SCF and ACK-2, as the biotin-SCF$^+$ and ACK-2$^+$ fractions display different morphology, light scatter, and Rhodamine 123 (Rh123) retention properties [Visser et al., 1993].

We have attempted to elucidate the molecular basis for the differential cell staining with biotin-SCF and anti-Kit, using sublines of the IL-3 dependent murine cell line FDC-P1 (**chapter 5**). Although ACK-2 binds to parental FDC-P1 cells, which demonstrates Kit protein expression, no or very low fluorescence signals were found after incubation of these cells with biotin-SCF, and SCF did not induce detectable proliferation of the cells. In contrast, after adaptation of FDC-P1 cells to grow in medium supplemented with SCF, a subline was selected that showed specific staining with ACK-2 as well as biotin-SCF. These results indicate that ACK-2 binding (and thus Kit$^+$) cells may be heterogeneous with respect to ligand binding capacity. Amplification of various regions of *c-kit* RNA, using the reverse transcriptase polymerase chain reaction (RT-PCR), did not provide an indication of structural alterations in domains known to be involved in ligand binding or receptor dimerization on the subline that showed Kit expression without binding SCF. Isolation of Kit molecules using immunoprecipitation also did not reveal evidence for qualitative differences in expression of the Kit proteins on the cell surface between the FDC-P1 subline that binds SCF and the subline that does not. Although these results do not exclude the possible occurrence of structural differences that are not detectable with the approaches used, our RT-PCR and immunoprecipitation results suggest that only quantitative differences exist between the FDC-P1 sublines. As the differences in Kit expression are too small to explain the large differences in biotin-SCF binding between the sublines, it was postulated that this discrepancy may be caused by the requirement for a minimum number of Kit monomers to enable efficient dimerization of Kit molecules to form high affinity SCF-R.

9.3 Kit expression studies on rhesus monkey cells

To examine expression of the SCF-R on hemopoietic cells from primate origin, we double-stained rhesus monkey BM cells with biotin-SCF and an antibody against CD34, a marker that is specific for immature cells [Civin et al., 1984] [Krause et al., 1994]. Although recent murine transplantation studies suggest that some HSC in this species may be $CD34^-$ [Osawa et al., 1996], results of autologous and allogeneic transplantation studies with purified simian $CD34^+$ cells [Berenson et al., 1988] [Wielenga, 1990] and evidence from transplantations with purified $CD34^+$ cells in human patients [Berenson et al., 1991] [Kawashima et al., 1996] [Yabe et al., 1996] have demonstrated that all the necessary cells for short-term reconstitution and long-term multilineage hemopoiesis can be found within the $CD34^+$ population.

The in vitro colony-forming potential of $CD34^+$ rhesus monkey BM cells expressing Kit at high, intermediate, or low (undetectable) levels was analyzed (**chapter 6**). These experiments showed, that the majority of the granulocyte/ macrophage colony-forming units (CFU-GM) reside in a fraction of $CD34^+$/ Kit^{med} cells (**figure 9.1**). This subset also contains the cells with highest CD34 expression and low levels of the class II histocompatibility antigen RhLA-DR (the rhesus monkey equivalent of HLA-DR), a

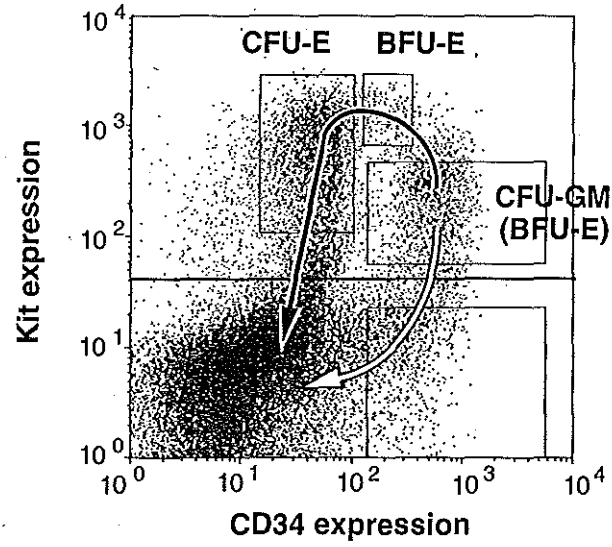

figure 9.1
Changes in CD34 and Kit expression during erythroid (black arrows) and monomyeloid (white arrows) differentiation of rhesus monkey BM cells.

phenotype that is associated with the most primitive cells that can be detected in vitro, e.g. in long-term BM cultures, and that has recently been shown to contain the cells needed for rapid hemopoietic reconstitution in irradiated rhesus monkeys [Neelis et al., manuscript in preparation]. Similar to our results in mice, these data strongly suggest that Kit is expressed, although at low levels, on repopulating HSC in primates. Additional studies, e.g. transplantation with allogeneic or genetically marked rhesus monkey BM cells will be needed to definitively establish whether all cells required for rapid reconstitution as well as stable long-term hemopoiesis express Kit.

During monomyeloid differentiation the immature, $CD34^{+}$/ Kit^{med} rhesus monkey BM cells gradually lose both Kit and CD34 (**figure 9.1**). During erythroid differentiation, Kit expression is initially upregulated. Some erythroid burst-forming units (BFU-E) express Kit at a relatively low level, but most BFU-E are found in a distinct population that expresses very high Kit levels and contains few, if any, non-erythroid progenitors. Upon further differentiation into erythroid colony-forming units (CFU-E), CD34 expression declines, and Kit is gradually lost from the cell surface.

In contrast to our results on rhesus monkey BM, most BFU-E in human BM were found in the Kit^{low} subset, whereas few BFU-E were Kit^{hi} [Gunji et al., 1993] [Simmons et al., 1994]. This might reflect a difference between Kit expression on human and rhesus monkey erythroid cells. In several double-staining experiments with biotin-SCF and anti-CD34, we have found that clusters of CD34- and Kit-expressing subsets in human BM were less distinct than in rhesus monkey BM. This suggests that Kit distribution on human progenitors may indeed be different from that on rhesus monkey progenitors. Another possible explanation for the observed differences is the methodology used to detect Kit expression. All studies of Kit expression on human cells have been performed with anti-Kit antibodies, which may show different target cell specificity to primate BM subsets than biotin-SCF, similar to what we reported for murine BM (**chapter 4**). Indeed we found that a larger fraction of human BM cells binds anti-Kit antibodies than biotin-SCF [Wognum et al., 1996], although the reason for these differences is still unresolved. Cell staining with anti-Kit antibodies can cause inhibition of outgrowth of Kit^{+} cells, which results in underestimation of colony formation by the Kit^{+} cells after staining with anti-Kit [Lerner et al., 1991] [Broudy et al., 1992] [Gunji et al., 1993] [Liesveld et al., 1995]. Neutralization of Kit^{+} cells does not occur after binding of biotin-SCF (**chapter 4** and **chapter 6**). Comparison of in vitro clonogenic ability of the different Kit-expressing subsets of human and rhesus monkey BM after staining with biotin-SCF and with anti-Kit is needed to elucidate these differences, and establish the Kit expression profile for human cells.

9.4 Changes in coexpression of GF-R during differentiation

To define the functional interactions of different GFs on hemopoietic cells it is of interest to examine the patterns of coexpression of these receptors on BM subsets. For simultaneous examination of the expression of receptors for two different GFs on the basis of ligand binding, we developed a method that combines cell staining with biotinylated GFs and digoxigenin- (DIG-) labeled GFs (**chapter 7**). Using this method together with antibodies against other cell surface markers, patterns of coexpression of receptors for SCF and either erythropoietin (EPO), IL-6, or granulocyte/ macrophage colony stimulating factor (GM-CSF) on immature progenitors and on differentiating erythroid and myelomonocytic precursors in rhesus monkey BM were analyzed.

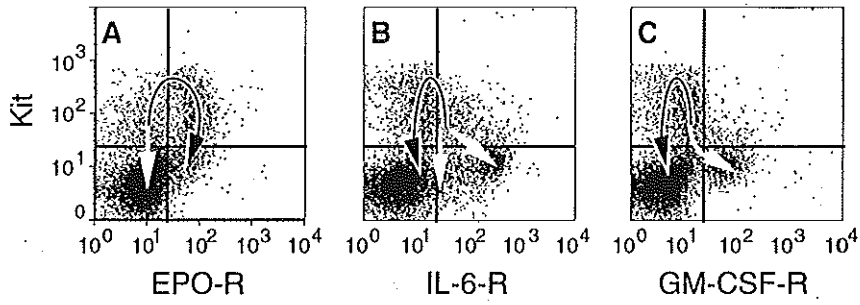

figure 9.2.
Changes in coexpression patterns of Kit and the receptors for EPO, IL-6, and GM-CSF, respectively, during differentiation of monomyeloid (white arrows) and erythroid cells (black arrows).

Simultaneous expression of Kit and EPO-R was not found on the $CD34^+$ cells (**chapter 7**). The absence of EPO-R from HSC is in agreement with the notion that early stages of erythropoiesis are regulated by other GFs than EPO [Iscove, 1977] [Wagemaker et al., 1977] [Wagemaker et al., 1979]. With erythroid commitment of immature progenitors, Kit is upregulated, and the cells then acquire EPO-R (**figure 9.2**). After the erythroid colony-forming unit (CFU-E) and pro-erythroblast stages, Kit expression gradually declines, but remains detectable up to the normoblast stage. EPO-R expression is retained on maturing erythroid cells even after these cells lose CD71 and Kit. These results indicate that SCF is a more important regulator of early erythroid development than EPO, which plays a role on more mature erythroid cells. The coexpression of Kit and the EPO-R may be crucial for the balance between proliferation and differentiation of erythroid cells, as it has recently been shown that tyrosine phosphorylation of the EPO-R by Kit is essential for EPO-induced proliferation but not for differentiation of erythroid precursors [Wu et al., 1997]. Thus, the loss of Kit late in

erythroid development may result in the cessation of EPO-induced mitogenesis and promote the final maturation of erythroblasts.

Part of the $CD34^+/Kit^+$ cells coexpress the IL-6-R (**chapter 7**), and IL-6-R can be detected on $CD34^{bright}/DR^{dull}$ cells (**chapter 8**). As the heterogeneous $CD34^{bright}/DR^{dull}$ BM subset is still a factor 10^2–10^3 remote from a pure stem cell population, we do not know whether IL-6-R are expressed on HSC. Transplantation studies with sorted cells, similar to those described for Kit (**chapter 4**), are not possible for IL-6-R, because the expression level of this receptor on immature cells is not sufficiently high for a reliable separation of receptor-positive and receptor-negative cells. RNA for the IL-6-R as well as RNA for the gp130 signal transduction subunit have been detected in the human immature hemopoietic cell fraction [Berardi et al., 1995]. In addition, gene knockout mice that lack functional IL-6 or gp130 have reduced numbers of immature hemopoietic cells [Kishimoto et al., 1995] [Kopf et al., 1995]. Thus it appears that IL-6-R are expressed at HSC, although at low levels. However, it is also possible that the majority of the HSC express only the gp130 subunit and not the IL-6-R itself, implying that the cells cannot be stimulated by IL-6 alone, but only by a combination of IL-6 and soluble IL-6-R. In agreement with this, most of the long-term culture-initiating cells (LTC-IC) have been found in the $CD34^+$/IL-6-R^- fractions of human BM and cord blood [Tajima et al., 1996].

During granulocyte and monocyte differentiation, Kit gradually disappears, whereas IL-6-R are upregulated (**figure 9.2**). Maturing monocytes and neutrophilic granulocytes express IL-6-R, basophils do not [Wognum et al., 1993] [Van Gils et al., 1995]. During erythroid differentiation, IL-6-R expression is lost on the majority of the cells, although a small subset of erythroid cells may remain IL-6-R^+, as IL-6-R can be identified on a small subset of $CD71^+/Kit^+$ BM cells (**chapter 7**).

Coexpression of Kit and GM-CSF-R was not found on $CD34^+$ cells (**chapter 7**), and no GM-CSF-R were detectable on $CD34^{bright}/DR^{dull}$ cells (**chapter 8**). This implies that the GM-CSF-R is not expressed on HSC, or that its expression level is below the detection limit of 50–100 receptors per cell. GM-CSF-R are also absent from the erythroid lineage, suggesting that the erythroid-burst promoting activity previously attributed to GM-CSF [Metcalf et al., 1980] might be mediated by indirect mechanisms. During monomyeloid differentiation, GM-CSF-R expression levels are upregulated (**figure 2**). RNA for the murine GM-CSF-R β subunit was found in a subset that contains committed progenitor cells, but not in the subset that includes HSC [Visser et al., 1993], which supports the lack of GM-CSF-R on HSC. Moreover, RNA for the shared IL-3/ IL-5/ GM-CSF-R β subunit or the GM-CSF-R α subunit was not found in a highly enriched immature hemopoietic cell fraction of human BM [Berardi et al., 1995]. Further evidence that GM-CSF is dispensable for early stages of hemopoiesis is provided by reports of relatively normal hemopoiesis in homozygous mutant mice that either lack

functional GM-CSF or the shared IL-3/ IL-5/ GM-CSF-R β subunit [Dranoff and Mulligan, 1994] [Nishinakamura et al., 1995].

These studies have shown that it is possible to examine receptor coexpression on cells in different hemopoietic subsets, including immature $CD34^+$ cells. Due to technical limitations we have not been able to directly examine receptor coexpression on the most primitive, $CD34^+$/ $RhLA\text{-}DR^{dull}$ cells. The availability of novel fluorochromes that can be combined in multi-color flow cytometry, as well as the development of advanced multi-laser flow cytometers now enable further examination of receptor coexpression patterns on different BM subsets by ≥ 4-color flow cytometry, e.g. after staining of rhesus monkey BM with biotin- and DIG-labeled GFs and with antibodies against CD34 and RhLA-DR.

9.5 Possibilities and limitations of staining with labeled GFs

Many high affinity GF-R consist of complexes of several subunits, some of which are shared with receptors for other GFs (see **chapter 1**). Anti-receptor antibodies usually recognize individual receptor chains, and are very useful to detect differences in expression of receptor subunits between cell types, and to examine the relationship between structure and function of receptor subunits. If only antibodies against one of the subunits are used, differences between cells that express functional receptors and cells that only express non ligand-binding receptor isoforms can remain unnoticed in binding experiments. Therefore, to detect cells that are able to bind GFs, staining with labeled hemopoietic GF molecules is preferable to staining with anti-receptor antibodies.

The high detection sensitivity of the biotin-GF method allows analysis of cells with low receptor levels. We have estimated on the basis of studies with cell lines and blood cell subsets, of which receptor numbers are known from equilibrium binding studies, that the detection sensitivity of our method is in the range of 50–100 molecules per cell. However, cells with less than 50 receptors may still be GF responsive [Budel et al., 1989]. As it is likely that radioactive labeling will not yield even lower detection levels, and notwithstanding the possibility that novel fluorochromes with superior fluorescence properties or improved fluorescence amplification methods may be developed in the near future, it will be necessary to adopt other methods to detect such very low receptor levels. An example of a very sensitive method to examine receptor expression at the RNA level is RT-PCR. To use this method in the study of GF-R expression on rare cells, high demands must be made on the stringency of the purification of the cells, to exclude signals from contaminating cells.

Expression of receptors that are internalized upon binding of GFs can be demonstrated indirectly, using GF-toxins or biotinylated GFs in combination with streptavidin-toxin conjugates. Internalization of the toxins results in elimination of GF-R-expressing cells, including those with very low receptor expression, and this can be detected by functional characterization of the surviving cells [Siegall et al., 1990] [Kreitman et al., 1992] [Chan et al., 1995] [Chan et al., 1996] [Rozemuller et al., 1996]. Although in principle this technique only requires internalization of 1 toxin molecule, the actual number of cell surface receptors required for effective killing is not known. Recent results indicate, that CFU-GM are not eliminated by incubation with GM-CSF-toxin [Terpstra et al., in press]. Although these data suggest that GM-CSF-R expression on CFU-GM is lower than on AML blasts, it is thus not yet resolved whether these cells are GM-CSF-R$^-$, or express receptor numbers that are too low for the cells to be sensitive to the toxin.

GF-R expression does not necessarily predict GF responsiveness

The assumption that the presence of GF-R on a cell predicts whether that cell can respond to GF stimulation may not always be correct. It is possible that cells that are receptor-negative do respond to GF stimulation, if a complex between the GF and a soluble receptor subunit can bind to a second receptor subunit on the cell. This has been demonstrated for hemopoietic cells that express gp130, but are IL-6-R negative, which can be activated by a complex of IL-6 and soluble IL-6-R [Taga et al., 1989] [Mullberg et al., 1992] [Sui et al., 1996] [Tajima et al., 1996]. On the other hand, when antagonizing soluble receptor subunits are present that neutralize GFs, GF-R positive cells might not be stimulated by these GFs. E.g., the soluble human GM-CSF-R α subunit can antagonize the activity of GM-CSF by competing for GF binding with the high affinity transmembrane GM-CSF-R complex [Brown et al., 1995].

It is also possible that cells that are able to bind GFs do not respond to GF stimulation, due to a lack of (parts of) molecules that are essential for signal transduction but not for ligand binding, such as certain receptor subunits. That truncation or alteration of the intracellular domain of a receptor subunit can cause profound effects on signal transduction and biological responses without altering ligand binding characteristics, has been shown for the G-CSF-R in neutropenic patients [Dong et al., 1993] [Dong et al., 1994] and for the IL-2/ IL-4/ IL-7-R γ subunit in patients with X-chromosome-linked severe combined immunodeficiency (SCID) [Noguchi et al., 1993].

Cells that respond to GF stimulation can be identified by detection of signal transduction events, which may include changes in the intracellular calcium concentration, and the activation of specific kinase pathways. These can be identified by intracellular staining of cells with antibodies that recognize phosphorylated epitopes on specific signaling intermediates. Activation of a limited number of receptor molecules can lead to the recruitment and activation of a cascade of signaling molecules and thus

to amplification of the signal. Therefore, if it becomes possible to detect signal transduction in small populations of phenotypically defined hemopoietic cells, this approach may be useful to detect receptor levels that are too low for direct detection by flow cytometry. In addition to providing a new approach to examine the GF-R phenotype and GF responsiveness of HSC and immature progenitors, analysis of signal transduction events may be useful to obtain essential information about the GF regulation of HSC survival, proliferation, and differentiation, and on functional interactions between GF-R on the level of individual HSC.

9.6 Assessment of GF-R expression on hemopoietic precursors: possible implications and applicability

Elucidation of GF-R expression on immature hemopoietic cells is particularly useful for understanding the GF regulation of HSC and progenitors. This knowledge is important to select suitable target cells and transduction conditions for gene marking and gene therapy. In addition, the feasibility of GF stimulation for in vitro amplification of HSC for transplantation purposes depends on the expression of GF-R on the target cells and the type of biological response induced by these GFs. By analogy, the choice of GFs to support hemopoietic recovery in myelosuppressed patients might be a function of GF-R expression on various types of hemopoietic progenitors. Moreover, knowledge of receptor expression on malignant cells as compared to their normal counterparts may enable the development of strategies to selectively eliminate malignant stem cells.

With exception of Kit, it is still not established which GF-R are already expressed on HSC or which are expressed as a result of activation of quiescent stem cells. Analysis of the distribution of other receptors on immature hemopoietic cells than those examined in this thesis, such as Flt3, also referred to as Flk-2 or STK-1 [Small et al., 1994], and Mpl, the receptor for thrombopoietin (TPO) [Vigon et al., 1992], might supplement information about the GF requirement of HSC.

The non-proliferating state of HSC might either be a passive process involving absence of positive signals or an active process involving suppressive negative regulators [Moore, 1991]. Therefore, analysis of receptors for inhibitory GFs, such as transforming growth factor β (TGFβ), macrophage inflammatory protein 1α (MIP1α), tumor necrosis factor α (TNFα), or interferon, will also be important for a better understanding of the regulation of immature hemopoietic cells [Klimpel et al., 1982] [Ploemacher et al., 1993] [Rusten et al., 1994] [Van Ranst et al., 1996].

Analysis of GF-R expression on HSC using labeled GFs is seriously limited by the expression of low receptor numbers on these cells, and by the lack of suitable phenotypic markers for the immature cell compartment. To distinguish quiescent from

activated stem cells within immature cell compartment, cell cycle parameters such as DNA content or expression of cyclins and other essential proteins involved in cell cycle regulation might be examined, provided that these approaches can be used in a multiparameter setting in combination with analysis of cell surface antigen and GF-R expression.

The GF-R phenotype of pluripotent stem cells may not be uniform. GF-R heterogeneity may form the basis of different possible outcomes of GF stimulation: quiescence, self renewal, lineage commitment and apoptosis. Changes in relative expression levels of different GF-R on HSC, in combination with the relative levels of different GFs in the microenvironment, may determine which fraction of HSC will be activated and become committed to specific differentiation pathways or remain quiescent. Heterogeneity in GF-R expression also provides a possible safeguard against depletion of HSC pool or differentiation into only a limited set of blood cells at the expense of other lineages, because it can be expected that a heterogeneous stem cell population shows differential responses to different activating stimuli.

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Samenvatting
(Summary in Dutch)

1 Bloedcelvorming

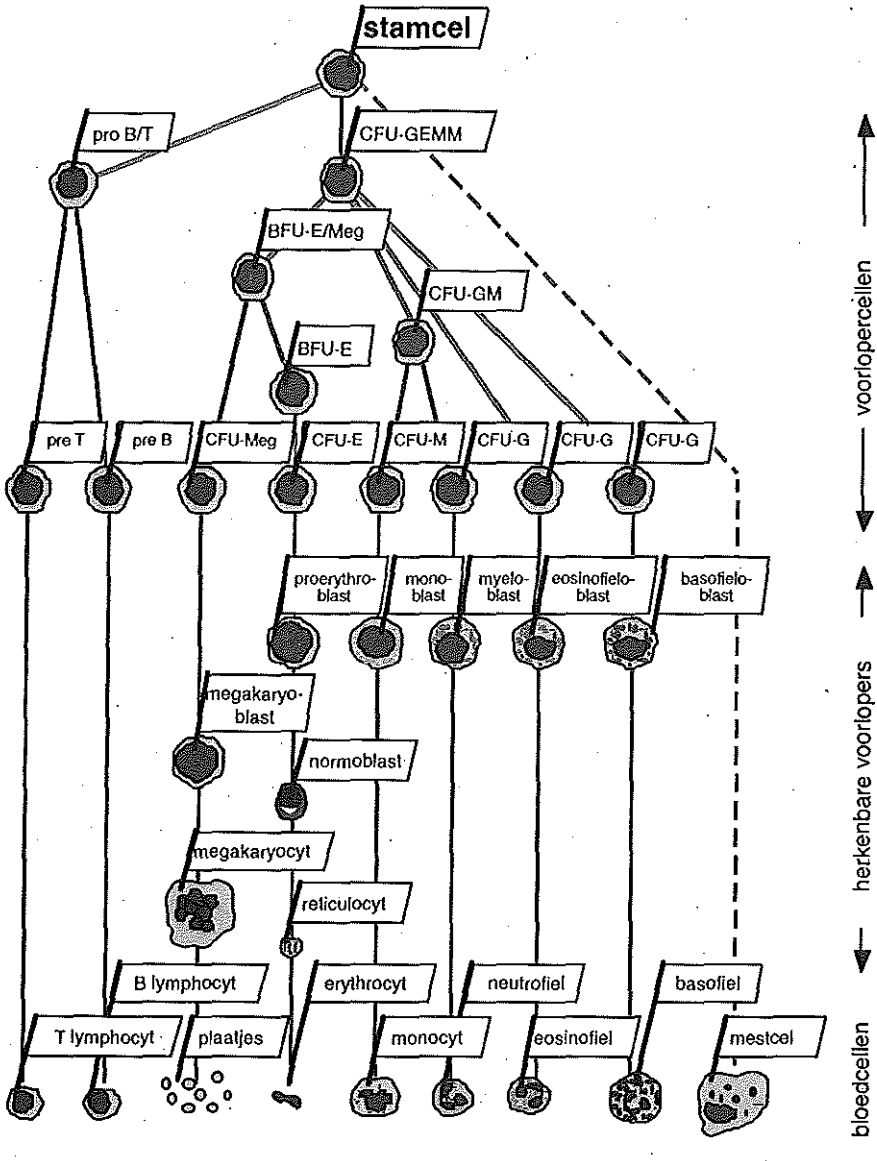
Bloedcellen vervullen een aantal belangrijke functies, waaronder O_2 en CO_2 transport (rode bloedcellen of erythrocyten), bloedstolling (bloedplaatjes), specifieke immuniteit en produktie van antilichamen (lymphocyten), en aspecifieke afweer tegen ziekteverwekkers (monocyten/ macrofagen en granulocyten). De meeste bloedcellen hebben een beperkte levensduur, variërend van enkele uren tot enkele maanden. Voor het vervangen van deze cellen heeft een gemiddelde volwassene een dagelijkse aanmaak van 2×10^{11} rode en $1,5 \times 10^{11}$ witte bloedcellen nodig. Deze produktie-capaciteit kan onder bepaalde omstandigheden (bijvoorbeeld een lage zuurstofspanning) of door oorzaken als bloedverlies, weefselbeschadiging of infecties, zeker 10-maal verhoogd worden. Het proces van bloedcelvorming, hemopoiese, vindt in volwassen zoogdieren voornamelijk plaats in het beenmerg (BM). In de foetale hemopoiese spelen lever en milt een belangrijke rol; in volwassen muizen vindt ook bloedcelvorming plaats in de milt.

Alle bloedcellen zijn afstammelingen van een klein aantal pluripotente hemopoietische stamcellen (HSC). De meeste HSC zijn in rust; slechts een klein deel ervan is actief. Deze populatie is verantwoordelijk voor de vorming van dochtercellen, die na verscheidene celdelingen hun mogelijkheid tot multipotente differentiatie verliezen en de karakteristieke fenotypische en functionele eigenschappen van de verschillende bloedcellijnen verkrijgen (**figuur 1**). Deze voorlopercellen ontwikkelen zich verder tot rijpe bloedcellen die in de bloedsomloop terecht komen.

Groeifaktoren spelen een belangrijke rol in het stimuleren van overleving, deling en uitrijping van hemopoietische cellen. Hoewel er steeds meer bekend wordt over de werking van groeifaktoren in vivo en in allerlei in vitro kweeksystemen, is er nog relatief weinig kennis over de manieren waarop groeifaktoren, alleen en samen met andere, de bloedcelvorming stimuleren. Stamcellen zijn zo zeldzaam, dat het vrijwel niet mogelijk is om deze in grote hoeveelheden te isoleren voor in vitro studies. Bovendien is het moeilijk om in dergelijke studies onderscheid te maken tussen de direkte effekten van een groeifaktor op een voorlopercel en de werking op diens nakomelingen. Ook is het onmogelijk om indirekte effekten op de celgroei waarbij andere groeifaktoren betrokken zijn uit te sluiten. Daarom is in het hier beschreven onderzoek gekozen voor een meer direkte benadering om te bestuderen op welke cellen van het hemopoietische systeem bepaalde groeifaktoren invloed kunnen uitoefenen. Deze aanpak is gebaseerd op het feit dat een groeifaktor reageert met een cel door binding aan celoppervlakte-receptoren die specifiek zijn voor deze groeifaktor. Door gebruik te maken van deze specifieke binding is een methode ontwikkeld om te analyseren of de betreffende receptoren tot expressie komen op hemopoietische voorlopercellen. Deze methode is toegepast bij het bestuderen van de veranderingen in

receptor-expressie tijdens de uitrijping van stam- en voorlopercellen tot volwassen bloedcellen.

figuur 1
Schematische voorstelling van hemopoietische stamceldifferentiatie.



2 Doel en methodologie

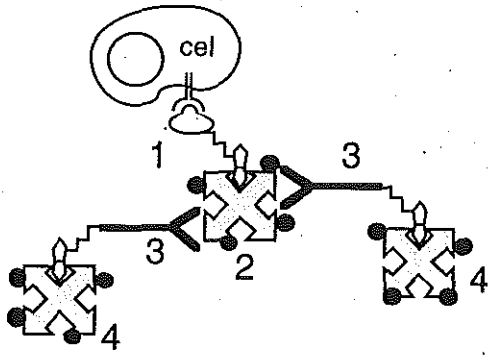
Veel receptoren met hoge affiniteit voor een groeifaktor bestaan uit complexen van verschillende eiwit-molekulen. Sommige van die receptor-ketens maken deel uit van de receptoren van verschillende groeifaktoren (zie **hoofdstuk 1**). Antilichamen tegen receptoren herkennen meestal individuele receptor-onderdelen en kunnen worden toegepast om verschillen in expressie hiervan te ontdekken, en om de relatie tussen struktuur en functie van de receptor-ketens te bestuderen. In bindings-experimenten waarin alleen antilichamen tegen één van de receptor-onderdelen worden gebruikt, worden soms verschillen niet ontdekt tussen cellen die functionele (= groeifaktor bindende) receptoren tot expressie brengen, en cellen die slechts delen van receptoren op het celoppervlak hebben. Voor de detectie van cellen die daadwerkelijk in staat zijn om groeifaktoren te binden verdient het dus de voorkeur groeifaktor-molekulen te gebruiken in plaats van anti-receptor antilichamen.

Voor het aantonen van celoppervlakte-receptoren is in de hier beschreven studies gebruik gemaakt van groeifaktoren waaraan biotine-molekulen gekoppeld zijn. Deze kunnen in een flowcytometer worden aangetoond na binding van (strept)avidine-molekulen met daaraan gekoppelde fluorescerende molekulen (**figuur 2**). Door de kleuring met groeifaktoren te combineren met antilichamen tegen andere molekulen op het celoppervlak is het mogelijk om receptor-expressie op cellen in heterogene populaties, zoals BM, te bestuderen. De cellen ondervinden geen schade van de kleuring, zodat deze aanpak ook gebruikt kan worden om, voor functionele studies, levende hemopoietische cellen te isoleren op basis van receptor-expressie.

De uitvoerbaarheid van het maken en gebruiken van gebiotinyleerde groeifaktoren om receptor-expressie te bestuderen is onderzocht voor verschillende groeifaktoren: interleukine-2 (IL-2), IL-3, IL-6, granulocyt/ macrofaag kolonie-stimulerende faktor ("colony-stimulating factor", oftewel GM-CSF) en stamcelfaktor (SCF) (**hoofdstuk 2**). Op het celoppervlak van vroege hemopoietische cellen zijn soms zeer weinig receptoren aanwezig. Daarom werden methodes ontwikkeld om de fluorescentie-signalen te versterken van cellen die met gebiotinyleerde groeifaktoren en fluorescerende (strept)avidine-molekulen gekleurd waren. Na versterking van het signaal door incubatie met afwisselende lagen van gebiotinyleerde antilichamen tegen (strept)avidine en fluorescerende (strept)avidine-molekulen (**figuur 2**), was het mogelijk cellen met minder dan 100 receptoren per cel te detecteren (**hoofdstuk 2**).

Met IL-2 als modelsysteem voor hemopoietische groeifaktoren werden verschillende methodes en reagentia om biotine-molekulen aan groeifaktoren te koppelen vergeleken, en de meest geschikte biotinepreparaten uitgezocht (**hoofdstuk 3**). Uit deze experimenten bleek dat groeifaktor-koppeling aan *N*-hydroxy-succinimide (NHS) biotine zeer doeltreffend was voor de mate waarin IL-2 gebiotinyleerd werd, het behoud

van biologische activiteit van de groeifaktor en de specifieke kleuring van cellen met receptoren op het celoppervlak. Andere biotinepreparaten, zoals *p*-Diabenzoyl Biocytine Precursor (DBB), of biotine die met behulp van licht activeerbaar is, gaven minder goede resultaten.



figuur 2
Schematische voorstelling van celkleuring met gebiotinyleerde groeifaktoren (1) en fluorescerende (strept)avidinemolekulen (2), en versterking met gebiotinyleerde anti-(strept)avidine antilichamen (3) en een tweede laag van fluorescerende (strept)avidinemolekulen (4).

3 Kit expressie op cellen van de muis

Om te bepalen op welke hemopoietische cellen de SCF receptor (Kit) tot expressie komt werden BM cellen van de muis gekleurd met gebiotinyleerd SCF of met antilichamen tegen Kit, waarna Kit^+ en Kit^- cellen werden gesorteerd met behulp van flowcytometrie (**hoofdstuk 4**). Uit celkweek- en transplantatie-experimenten met deze cellen bleek dat Kit tot expressie komt op hemopoietische voorlopercellen in verschillende stadia van ontwikkeling. Onder de Kit^+ BM cellen bevinden zich HSC die in staat zijn tot stabiele lange termijn repopulatie van getransplanteerde muizen, en voorlopercellen die alleen gedurende een korte periode na transplantatie nieuwe bloedcellen kunnen vormen. Bovendien bevinden zich in de Kit^+ fractie cellen die kolonies kunnen vormen in de milt van getransplanteerde muizen ("colony forming units in spleen", oftewel CFU-S), cellen die kolonies vormen vormen in kweeksystemen ("colony forming units in culture", CFU-C), en morfologisch herkenbare vroege myeloïde en erythroïde cellen. In de Kit^- BM populaties daarentegen werden vrijwel alleen rijpere cellen aangetroffen.

Binnen de Kit^+ BM populatie werden verschillen gevonden tussen de cellen die biotine-SCF binden en de cellen die met het anti-Kit antilichaam ACK-2 reageren. Het percentage BM cellen dat kan worden gekleurd met biotine-SCF is kleiner dan met ACK-2 (**hoofdstuk 4**). In de biotine-SCF^+ fracties is de frequentie van kolonievormende cellen echter 2- tot 3-maal zo hoog als in de $ACK\text{-}2^+$ fracties. Dit

verschil wordt niet veroorzaakt door stimulatie of inactivering van voorlopercellen door biotine-SCF of ACK-2, aangezien er geen effect van celkleuring met deze reagentia op kolonievorming of repopulatie werd waargenomen. Het is mogelijk dat de celpopulaties waaraan biotine-SCF bindt en die waaraan ACK-2 bindt niet identiek zijn, te meer daar de biotine-SCF$^+$ en ACK-2$^+$ fracties ook verschillen vertonen in morfologie, lichtverstrooiing en Rhodamine 123 kleuring.

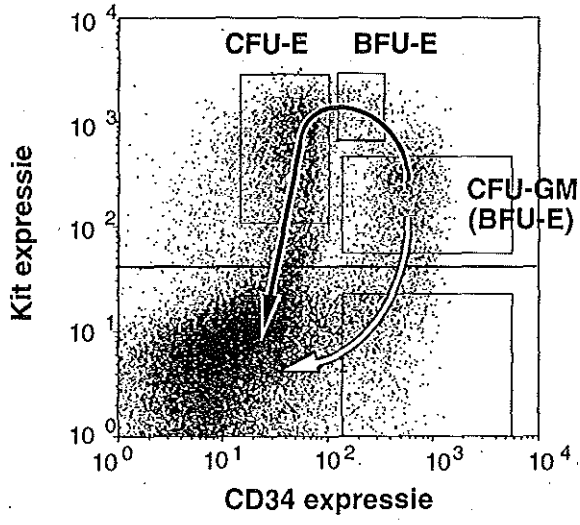
De molekulaire basis voor de verschillende celkleuring met biotine-SCF en ACK-2 werd verder onderzocht met behulp van sublijnen van de IL-3 afhankelijke muizencellijn FDC-P1 (**hoofdstuk 5**). Hoewel ACK-2 bindt aan de oorspronkelijke FDC-P1 cellen, die dus Kit tot expressie brengen, binden deze cellen weinig of geen biotine-SCF. Bovendien veroorzaakt SCF geen detecteerbare deling van deze cellen. Echter, na gewenning van FDC-P1 cellen aan het groeien in medium met SCF, ontstaat een sublijn die specifiek kleurt met zowel ACK-2 als biotine-SCF. Deze resultaten wijzen erop dat ACK-2 bindende (en dus Kit$^+$) cellen heterogeen kunnen zijn wat betreft hun mogelijkheid om SCF te binden. We hebben echter met diverse technieken geen kwalitatieve verschillen in de struktuur van de Kit-molekulen kunnen aantonen op het celoppervlak van de FDC-P1 sublijnen. Hoewel dit niet uitsluit dat er verschillen kunnen zijn in struktuur, die niet aantoonbaar zijn met de gebruikte methodes, suggereren deze resultaten dat de verschillen tussen de FDC-P1 sublijnen van kwantitatieve aard zijn. De verschillen in Kit expressie zijn echter te klein om het grote verschil in biotine-SCF binding tussen de sublijnen te verklaren. We veronderstellen daarom dat ook een verschil in affiniteit van Kit voor SCF een rol speelt. Mogelijk is er een minimum aantal Kit monomeren nodig om efficiënte dimerisatie van Kit molekulen mogelijk te maken, zodat receptoren met een hoge affiniteit voor SCF gevormd kunnen worden. Cellen met lagere receptor-aantallen kunnen wel ACK-2 binden, maar vormen geen hoog-affiene receptoren die SCF kunnen binden.

4 Kit expressie op beenmerg cellen van de rhesusaap

Voor het bestuderen van expressie van de SCF receptor op vroege hemopoietische cellen, en veranderingen tijdens de uitrijping van verschillende typen voorlopercellen, werden BM cellen van de rhesusaap dubbelgekleurd met biotine-SCF en een antilichaam tegen CD34. CD34 is een merker die specifiek is voor vroege bloedvormende cellen. In vitro kolonievorming werd onderzocht van CD34$^+$ cellen die Kit sterk, middelmatig of zwak tot expressie brachten (**hoofdstuk 6**). Deze experimenten tonen aan dat de meerderheid van de cellen die granulocyt/ macrofaag kolonies vormen (de zogenaamde CFU-GM) zich bevinden in een fractie van CD34$^+$

cellen met middelmatige Kit expressie (**figuur 3**). Tijdens de verdere differentiatie verliezen deze cellen geleidelijk zowel Kit als CD34 van het celoppervlak.

Tijdens de erythroïde differentiatie wordt Kit expressie aanvankelijk hoger. Sommige vroege erythroïde cellen (de "erythroid burst forming units", BFU-E) brengen Kit tot expressie op een relatief laag niveau, maar de meeste BFU-E worden gevonden in een aparte populatie met een zeer hoge Kit expressie (**figuur 3**). Tijdens de verdere differentiatie tot latere eythroïde kolonievormers ("erythroid colony forming units", CFU-E) neemt de CD34 expressie af en verdwijnt Kit geleidelijk van het celoppervlak.

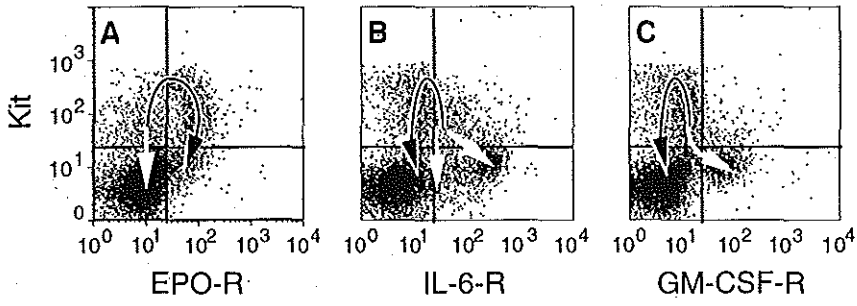


figuur 3
Veranderingen in expressie van Kit en CD34 tijdens de differentiatie van BM cellen van een rhesusaap tot erythroïde (zwarte pijlen) en monomyeloïde cellen (witte pijlen).

5 Veranderingen in receptor co-expressie tijdens de differentiatie

Om de functionele interacties van verschillende groeifaktoren op hemopoietische cellen te bepalen, is het nuttig om de co-expressie patronen van deze receptoren op BM te bestuderen. Hiertoe werd een methode ontwikkeld waarin celkleuring met gebiotinyleerde groeifaktoren gecombineerd wordt met andere groeifaktoren, waaraan digoxigenine (DIG) molekulen gekoppeld zijn (**hoofdstuk 7**). Met behulp van deze methode samen met antilichamen tegen andere celoppervlakte-merkers werd co-expressie van receptoren voor SCF en respectievelijk erythropoietine (EPO), IL-6, en GM-CSF op cellen in BM van rhesusapen geanalyseerd. De resultaten van deze

experimenten zijn samengevat in **figuur 4**, waarin de co-expressie patronen van deze receptoren op vroege voorlopers en op differentiërende erythroïde en myeloïde voorlopercellen getoond worden. De expressie van de receptoren voor IL-3, IL-6, GM-CSF, en SCF op verschillende BM cellen is besproken in **hoofdstuk 8**.



figuur 4
Veranderingen in co-expressie patronen van Kit en de receptoren voor respectievelijk EPO, IL-6 en GM-CSF tijdens de differentiatie van monomyeloïde (witte pijlen) en erythroïde cellen (zwarte pijlen).

6 Conclusies en suggesties voor verder onderzoek

Onderzoek van receptor-expressie op vroege hemopoietische cellen is nodig om de regulatie van HSC en voorlopercellen door groeifaktoren te begrijpen. Deze kennis is belangrijk voor specifieke therapeutische toepassingen van groeifaktoren, zoals de ex vivo expansie van stam- en voorlopercellen voor transplantatie-doeleinden en gentherapie. Kennis hierover kan ook gebruikt worden bij de keuze van groeifaktoren die toegediend worden om de hemopoiese te stimuleren van patienten van wie de bloedcelvorming is onderdrukt, bijvoorbeeld als gevolg van chemotherapie of bestraling. Daarnaast kan kennis van receptor-expressie op leukemie-cellen, vergeleken met hun normale tegenhangers, inzicht geven in de abnormale groeiregulatie van maligne hemopoietische cellen en een bijdrage leveren aan het ontwikkelen van methodes om deze cellen selectief te elimineren.

Met uitzondering van Kit is nog niet bekend welke groeifaktor-receptoren tot expressie komen op HSC en welke tot expressie gebracht worden na activeren van rustende stamcellen. Analyse van andere receptoren dan die onderzocht zijn in dit proefschrift, zoals bijvoorbeeld Flt3/ Flk-2 en de TPO receptor, c-Mpl, kan informatie toevoegen over de groeifaktoren die nodig zijn voor HSC. Ook onderzoek van receptoren van negatieve regulatoren kan in dit opzicht belangrijk zijn, aangezien

dergelijke remmende "groei"faktoren belangrijke funkties kunnen hebben in het in rust-toestand houden van stamcellen.

Analyse van receptor-expressie op HSC wordt bemoeilijkt door het lage receptor-aantal dat deze cellen tot expressie brengen, en de afwezigheid van bruikbare merkers om stamcellen van andere primitieve voorlopercellen te onderscheiden. Om rustende en geactiveerde stamcellen van elkaar te onderscheiden kan wellicht gebruik gemaakt worden van celcyclus parameters zoals DNA gehalte, of expressie van cyclines en andere eiwitten die essentieel zijn voor de regulatie van de celcyclus.

Dit onderzoek heeft niet geleid tot identificatie van het receptor-fenotype van repopulerende stamcellen. Het is mogelijk, dat dit fenotype niet voor alle HSC hetzelfde is. Heterogeniteit in receptor-expressie zou de basis kunnen vormen van verschillende effekten van groeifaktor stimulatie, zoals vorming van dochtercellen die identiek zijn aan de oorspronkelijke stamcel, differentiatie tot rijpere cellen, celdood door apoptose, of juist rust. Veranderingen in het relatieve expressie-niveau van verschillende receptoren op stamcellen kunnen, samen met de beschikbaarheid van verschillende groeifaktoren, bepalen welk deel van de HSC geactiveerd wordt en welk deel in rust blijft. Heterogeniteit van receptor-expressie kan ook dienen als beveiliging tegen uitputting van de stamcelvoorraad als gevolg van een sterke of langdurige stimulus, of tegen differentiatie van stamcellen tot slechts één type bloedcellen ten koste van andere typen, aangezien het waarschijnlijk is dat een heterogene stamcelpopulatie op verschillende manieren zal reageren op verschillende stimuli.

CURRICULUM VITAE

Marg de Jong werd op 27 december 1962 geboren te Rotterdam. In 1981 behaalde zij het Gymnasium β diploma aan het Gymnasium Erasmianum te Rotterdam. In datzelfde jaar begon zij met de studie Biologie aan de Rijksuniversiteit te Utrecht, waar het propaedeutisch examen werd behaald in 1982 en het kandidaatsexamen in 1984. Tijdens de doctoraalstudie deed zij onderzoek naar de struktuur van de testis van Ischnura elegans (Odonata) bij de vakgroep Cytogenetica onder leiding van professor dr J.M. van Brink. Tevens werd onderzoek verricht naar het effekt van stress op de lokalisatie van het heat shock protein hsp84, onder leiding van dr P.M.P. van Bergen en Henegouwen en dr W.A.M. Linnemans van de projektgroep Elektronen Microscopische Struktuur Analyse, vakgroep Moleculaire Celbiologie. Bij deze vakgroep verrichtte zij ook een literatuurstudie naar de internalisatie van de Epidermal Growth Factor receptor. Na het behalen van de eerstegraads onderwijsbevoegdheid onder leiding van drs C.P. Koetsier en R.W. Janzen verbonden aan de afdeling Didaktiek van de Biologie, was zij tijdens het schooljaar 1987/'88 als biologiedocente werkzaam bij het Gooi's Lyceum te Bussum. In augustus 1988 legde zij het doctoraalexamen oude stijl Biologie af. In 1989 werd de bevoegdheid behaald voor het verrichten van dierexperimenteel onderzoek conform artikel 9 van de Wet op Dierproeven.

In oktober 1988 begon zij bij TNO in Rijswijk onder leiding van dr J.G.J. Bauman met het in dit proefschrift beschreven onderzoek, en wel bij het Radiobiologisch Instituut (directeur: prof. dr D.W. van Bekkum) en het Instituut Radiobiologie van de Erasmus Universiteit Rotterdam, in de groep van dr J.W.M. Visser. Dit onderzoek werd in januari 1993 voortgezet aan de Erasmus Universiteit Rotterdam bij dr A.W. Wognum bij het Instituut Hematologie (prof. dr B Löwenberg), in de groep van dr G. Wagemaker.

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DANKWOORD

Zoals de meeste proefschriften is ook dit exemplaar tot stand gekomen door de inzet van en de samenwerking met vele personen. Tijdens mijn promotie-onderzoek ben ik werkzaam geweest op twee verschillende instellingen: TNO in Rijswijk en de Erasmus Universiteit te Rotterdam. Van binnen (en buiten) deze instellingen zijn vele mensen direkt of indirekt betrokken geweest bij de totstandkoming van dit boekje. Iedereen die hieraan heeft bijgedragen ben ik zeer erkentelijk; zonder daarmee anderen tekort te willen doen wil ik een aantal mensen hier met name noemen.

Tijdens het eerste gedeelte van het onderzoek, dat op TNO in Rijswijk is uitgevoerd in het Radiobiologisch Instituut (later het Instituut voor Radiobiologie en Immunologie, nog later opgegaan in het Medisch Biologisch Laboratorium) van Professor van Bekkum, heb ik veel geleerd over flowcytometrie onder leiding van Jan Bauman en Jan Visser. Daarbij werd ik terzijde gestaan door Henk Rozemuller, soms ook door Mia Hogeweg-Platenburg en Esther Donders. Jan Keij dacht mee over nachtelijke experimenten waarbij meer lenzen, draden en versterkers nodig waren dan in het hele gebouw te vinden waren. Ad Groenewegen was altijd bereid om de touwtjes van de FACS en RELACS weer op de goede manier aan elkaar te knopen. Richard Jonker zorgde voor de computer-programmatuur waarmee we alle resultaten weer konden verwerken. Aan de algehele verwarring werd met veel plezier bijgedragen door mijn "tweelingbroertje" Ruud Hulspas die samen met Pieter-Jaap Krijtenburg de CSLM onveilig en de omgeving donker maakte. En als het allemaal te laat dreigde te worden, kwam Jan Hendrikx aanfietsen met een piepschuim doos achterop met daarin een driegangendiner. Toen we met de in situ hybridisatie experimenten begonnen profiteerden we, af en toe met hulp van Cecilia Tan, van de expertise en materialen van Jan Bayer. En dan heb ik het nog niet eens gehad over het contact met "de andere groepen", zoals "de Beenmergkamer" van Ton Hagenbeek en Anton Martens, waar zelfs de computers dialogen voerden, en ik altijd met vragen over foto-biotine bij Ger Arkesteijn terecht kon, en Kees de Groot net dat artikeltje voor me had liggen dat ik gemist had in de strijd om bij te blijven in de literatuur. Bij "de groep Wagemaker" mochten we met de werkbesprekingen meedraaien en kregen we af en toe een flesje IL-2 toegeschoven.

Na de verhuizing naar de afdeling Hematologie van de Erasmus Universiteit Rotterdam ging ik zelf bij "de Wagemakers" horen. Daar kwam ik, samen met Hannie Busking-van der Lelij, terecht op een projekt van mijn co-promotor Bert Wognum. Bij de lange termijn repopulatie experimenten kreeg ik veel hulp van Trui Visser en vooral ook van Dorinde Kieboom-Pluimes. Met de eindeloze hoeveelheden in situ glaasjes hielp Wati Dimjati. Ook Astrid Borsboom liet zich af en toe inschakelen. Toen we de kleuringen op rhesus beenmerg gingen loslaten reisden we aanvankelijk naar Hilco

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marg.