Regulation of the Differentiation of WEHI-3B D$^+$ Leukemia Cells by Granulocyte Colony-stimulating Factor Receptor

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Abstract. To investigate the role of the G-CSF receptor (G-CSFR) in mediating the action of G-CSF, WEHI-3B D$^+$ murine myelomonocytic leukemia cells were transfected with a plasmid containing the murine G-CSFR gene. Overexpression of G-CSFR in transfected clones was demonstrated by northern blotting, binding of [^{125}I]rhG-CSF and cross-linking experiments. A high level of expression of the G-CSFR did not promote or suppress cellular proliferation or initiate differentiation; however, exposure of transfected cells to G-CSF in suspension culture caused a large percentage of the population to enter a differentiation pathway, as determined by two markers of the mature state, the ability of cells to reduce nitroblue tetrazolium (NBT) and to express the differentiation antigen Mac-1 (CD11b) on the cell surface. Thus, upon treatment with 10 ng/ml of G-CSF, 60% or more of transfected cells exhibited NBT positivity; whereas, in contrast, nontransfected cells exhibited only 6% NBT positivity in response to G-CSF. An eightfold increase in Mac-1 expression over that of the parental line was also observed in transfected cells exposed to G-CSF. The growth rate of the transfected clones was decreased by exposure to G-CSF, presumably due to terminal differentiation. The findings suggest that the predominant function of G-CSF and its receptor in WEHI-3B D$^+$ cells is to mediate differentiation and that the level of the G-CSFR portion of the signal transduction mechanism in this malignant cell line is important for a response to the maturation inducing function of the cytokine.

GRANULOCYTE colony-stimulating factor (G-CSF)[1] regulates the proliferation and differentiation of granulocytic progenitor cells, and stimulates functional activities and survival of mature granulocytes (see review references 18, 22, 23, 39, 40). Administration of G-CSF causes a rapid increase in peripheral blood neutrophils; therefore, the cytokine has been used in clinical trials with patients who are myelosuppressed as a result of cancer chemotherapy, irradiation therapy, or bone marrow transplantation (20, 31). Colombo et al. (8) have shown that introduction of the G-CSF gene into colon adenocarcinoma C-26 cells suppresses the tumorigenicity of these cells through the recruitment and targeting of neutrophilic granulocytes to the G-CSF-releasing cells. G-CSF also inhibits the metastatic spread of hematogenous and nonhematogenous tumors in mice (15).

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1. *Abbreviations used in this paper*: G-CSFR, G-CSF receptor; GAPD, glyceraldehyde-3-phosphate dehydrogenase; GM-CSF, granulocyte macrophage colony-stimulating factor; IL, interleukin; NBT, nitroblue tetrazolium; RA, retinoic acid; rhG-CSF, recombinant human granulocyte colony-stimulating factor.

G-CSF receptor (G-CSFR) is expressed predominantly in normal progenitor and mature neutrophils and in various myeloid leukemia cells (1, 24–26, 28). Some nonhematopoietic cells, such as human endothelial, placental and carcinoma cells, have also been shown to express the G-CSFR (3, 6, 33). The murine receptor has been purified from NFS60 cells and has a mol wt of 100,000–130,000 (10, 11).

WEHI-3B D$^+$ murine myelomonocytic leukemia cells express a low level of G-CSF receptors as determined by northern hybridization (11) and [^{125}I]G-CSF binding (25) experiments. Semisolid cultures of WEHI-3B D$^+$ cells formed granulocytic colonies in the presence of G-CSF; suppression of the clonogenicity of these cells was accompanied by morphological changes in the colonies (5, 16). Similar effects were also observed with M1 and HL-60 leukemia cells (2, 32). These findings suggest that G-CSF is an inducer of the differentiation of these leukemia cells. Some investigators, however, have recently reported that G-CSF does not induce the differentiation of WEHI-3B D$^+$ (4) and HL-60 (36) cells, but is required for the survival of mature progeny (4). Because only one receptor has been found that specifically binds G-CSF, it is assumed that the biological effects of the cytokine are mediated through the interaction of G-CSF with the receptor. For this reason, we have investigated the role of the G-CSFR in regulating the growth and differentiation

of WEHI-3B D+ leukemia cells produced by G-CSF. The level of G-CSFR expression in WEHI-3B D+ cells was elevated by transfection with an expression vector containing the G-CSFR gene and the effects of overexpression of the G-CSFR on cellular proliferation and differentiation were determined. Although parental WEHI-3B D+ cells responded poorly if at all to the inducing activity of G-CSF, the expression of excess levels of the G-CSFR in cells transfected with this gene resulted in the attainment of the mature phenotype in a large percentage of cells after exposure to the cytokine.

Materials and Methods

Cell Culture and Differentiation

WEHI-3B D+ leukemia cells and clones derived therefrom were maintained in suspension culture in McCoy's 5A modified medium supplemented with 15% FBS (GIBCO Laboratories, Grand Island, NY) at 37°C in a humidified atmosphere of 95% air/5% CO_2. Exponentially growing cells were treated with inducers of differentiation in fresh culture medium to initiate maturation. Cell numbers were determined daily using a particle counter (Model ZM, Coulter Electronics, Inc., Hialeah, FL) connected to a channelyzer model 256 (Coulter Electronics, Inc.).

Clonal growth and differentiation of WEHI-3B D+ cells were determined using soft agar culture. Briefly, 250 cells were suspended in 3 ml of 0.3% agar in McCoy's 5A medium containing 20% FBS with or without G-CSF and cultured for 6 d. Classification of the differentiation status of colonies was accomplished by the methodology of Metcalf (16) using an inverted microscope.

The ability of cells to reduce nitroblue tetrazolium (NBT) was used as a functional marker of differentiation. One × 10^6 cells were collected by centrifugation at 300 *g* for 5 min and resuspended in 1 ml of FBS-free medium containing 0.1% NBT and 2 μM 12-O-tetradecanoylphorbol 13-acetate. The suspension was incubated at 37°C for 30 min with shaking and the percentage of cells with blue-black formazan deposits was determined on 200 consecutive cells.

The level of the differentiation marker Mac-1 (CD11b) antigen on the cell surface was also analyzed by indirect immunofluorescent staining and flow cytometry. One × 10^6 cells were collected by centrifugation at 300 *g* for 5 min, washed once with PBS, and then incubated with 100 μl of rat anti-Mac-1 monoclonal antibody at a final concentration of 10 μg/ml in PBS containing 0.1% BSA on ice for 30 min. Unbound antibody was removed by washing cells twice with PBS containing 0.1% BSA. Cells were then stained with 100 μl of affinity-purified, phycoerythrin-conjugated goat anti-rat IgG antibody at a final concentration of 25 μg/ml in PBS containing 0.1% BSA on ice for 30 min. Cells were then washed twice, and the amount of bound antibody was quantified using a FACS IV flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA). Excitation of the fluorochrome was achieved at 488 nm with a Spectra-Physics 2025 argon ion laser (Spectra-Physics, Mountain View, CA). The phycoerythrin emission was collected through a 575/22 nm land pass filter (Omega Optical, Brattleboro, VT). Twenty thousand cells were observed for each sample.

Construction of Expression Plasmids Containing the G-CSFR Gene

Because the cDNA of the G-CSFR gene in the plasmid pBLJ17 (kindly provided by Dr. Shigekagu Nagata, Osaka Bioscience Institute, Japan) has no polyadenylation signal, the G-CSFR gene with its natural start and stop codons was released from the plasmid with XbaI digestion and subcloned into the XbaI site of pRc/RSV (Invitrogen, San Diego, CA). The BamHI fragment containing the G-CSFR cDNA plus the bovine growth hormone polyadenylation signal was cut out with the BamHI restriction enzyme from the resulting plasmid pRGR2, and then cloned into the BamHI site of the vector p75/15v, which was derived from p75/15 (a gift from Dr. Ulrich Rüther, European Molecular Biology Laboratory, Germany) by removing the *c-fos* DNA fragment. The final resulting plasmid pGR7-2 contained the bacterial *neo* gene with an SV40 promoter, which conferred resistance to the antibiotic G-418 in eukaryotic cells; the human metallothionein promoter, under which the expression of the G-CSFR gene may be stimulated by the treatment of cells with cadmium; and part of the polyoma virus early region sequence, including the origin of replication, the promoter, the enhancer, and 40% of the amino terminal of the large T antigen coding sequence, which has been shown to be sufficient for immortalization of murine fibroblasts (29).

Transfection and Single Cell Cloning

Exponentially growing cells were washed with PBS, and resuspended in PBS at a density of 1 × 10^7 cells/ml. Eight × 10^6 cells were incubated with 15 μg of linearized plasmid DNA for 10 min and transfected by electroporation using a Bio-Rad Gene Pulser™ (BioRad Laboratories, Hercules, CA) apparatus at 625 V/cm. 10 min later, cells were seeded in McCoy's 5A modified medium supplemented with 15% FBS. G-418 was added at a concentration of 400 μg/ml after 16 h. Transfected cells were enriched by G-418 selection for two weeks. Single cells were then cloned using a FACS IV flow cytometer which placed single cells in each well of 96-well plates and the resulting single cell clones were expanded in multiwell plates.

Northern Hybridization

Total cellular RNA was isolated from 2 × 10^6 cells using the guanidine thiocyanate/acid phenol method described by Chomczynski and Sacchi (7), separated by 1% agarose gel electrophoresis, transferred onto GeneScreen membranes (New England Nuclear/DuPont, Boston, MA) and hybridized with [α-^{32}P]dCTP-labeled cDNA probes.

Radiolabeling of Recombinant Human G-CSF (rhG-CSF)

Mutant rhG-CSF, Tyr1,3-rhG-CSF (donated by Kirin Brewery Co. Ltd., Japan) (37), in which threonine-1 and leucine-3 were replaced with tyrosine residues, was radioiodinated to 2-6 × 10^4 cpm/ng of protein using IODO-GEN (Pierce Chemical Co., Rockford, IL). 5 μg of Tyr1,3-rhG-CSF was incubated with 500 μCi of $Na^{125}I$ in 300 μl of PBS on ice for 15 min in a glass tube coated with 10 μg of IODO-GEN. The iodinated Tyr1,3-rhG-CSF was separated from unbound $Na^{125}I$ by gel filtration on a PD-10 column equilibrated with HBSS containing 0.2% BSA and 0.02% Tween-20.

Binding of [^{125}I]rhG-CSF to Cells

For each assay, 2 × 10^6 cells were collected, washed with binding medium (HBSS containing 20 mM Hepes, pH 7.4, and 10% FBS), resuspended in binding medium and incubated with various concentrations of [^{125}I]rhG-CSF in the absence or presence of a 100-fold excess of unlabeled rhG-CSF in a total volume of 100 μl at 15°C for 3 h. The cell suspension was then layered on 300 μl of FBS in a microcentrifuge tube and centrifuged at 500 *g* for 2 min at 4°C. The supernatant was removed by aspiration and the tube was cut off just above the cell pellet. The radioactivity of the pellet was quantified using an LKB Compugamma Counter (Pharmacia/LKB Biotechnology, Inc., Piscataway, NJ). Specific binding was obtained by subtracting the radioactivity observed in the presence of unlabeled rhG-CSF from the radioactivity obtained in the absence of unlabeled rhG-CSF.

Chemical Cross-linking of [^{125}I]rhG-CSF to Its Receptor

Binding of [^{125}I]rhG-CSF to cells was carried out as described above. Briefly, 10^7 cells were incubated with 4 nM [^{125}I]rhG-CSF in the absence or presence of a 100-fold concentration of unlabeled rhG-CSF in a total of 300 μl of binding medium. After incubation, the complex of [^{125}I]rhG-CSF and its receptor was cross-linked by disuccinimidyl suberate and disuccinimidyl tartarate and a cell extract was obtained by the methodology of Fugunaka et al. (10). The proteins present in 10 μl of the extract were separated on a 5–20% gradient polyacrylamide gel in the presence of SDS. The gel was then dried and exposed to x-ray film for 2–4 d at −80°C with intensifying screens.

Results

Expression of G-CSFR mRNA in Transfected Clones

Exponentially growing WEHI-3B D+ cells were transfected with linearized pGR7-2 by electroporation. The vector p75/15v, which was the same as pGR7-2 but devoid of the G-CSFR and the bovine growth hormone polyadenylation

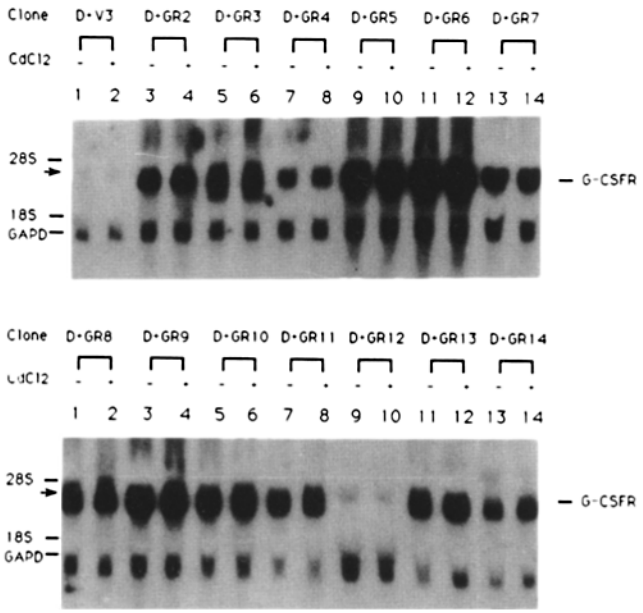

Figure 1. Expression of G-CSFR mRNA in WEHI-3B D⁺ clones transfected with pGR7-2. Total cellular RNA from cells grown in the absence or presence of 50 μM $CdCl_2$ was isolated and hybridized with ^{32}P-radiolabeled probes for the G-CSFR and glyceraldehyde phosphate-3-dehydrogenase (*GAPD*) genes. The position of the endogenous G-CSFR mRNA is indicated by an arrow. D+V3 and D+GR2-14 represent WEHI-3B D⁺ clones transfected with the control plasmid and pGR7-2, respectively.

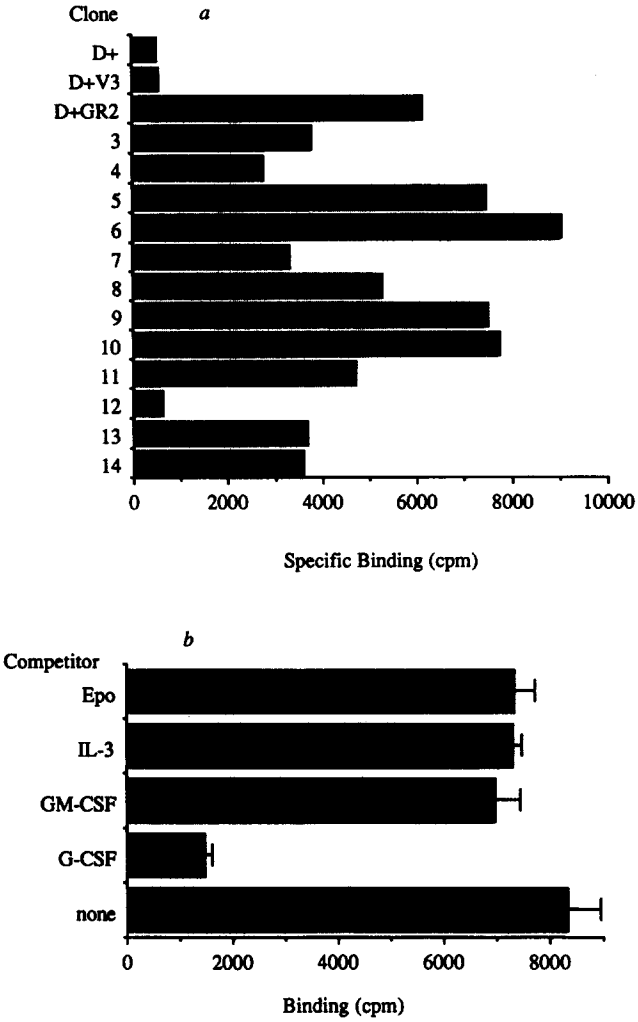

Figure 2. Specific binding of [^{125}I]rhG-CSF to G-CSFR transfected clones. (*a*) Comparison of the specific rhG-CSF binding capacity of WEHI-3B D⁺ clones transfected with pGR7-2. Two × 10^6 cells were incubated with 1 nM of [^{125}I]rhG-CSF for 3 h at 15°C in the absence or presence of excess cold rhG-CSF. The specific binding of [^{125}I]rhG-CSF was obtained by subtracting nonspecifically bound radioactivity from the total amount bound. Data are the average of duplicate determinations and the experiment was repeated three times with similar results being obtained. (*b*) Competition between [^{125}I]rhG-CSF binding and other cytokines. Two × 10^6 D+GR6 cells were incubated with [^{125}I]rhG-CSF in the absence or presence of cold rhG-CSF (100 ng), recombinant murine GM-CSF (100 ng), IL-3 (500 U), or erythropoietin (400 U) for 3 h at 15°C. Each value represents the average of triplicate determinations ± SD.

signal, was employed in parallel as a control. Transfected cells were enriched by selection with 400 μg/ml of G-418 and single cell clones were obtained by flow cytometry. The G-418 resistant clones were expanded and 13 pGR7-2 transfected clones, designated D+GR2-14, were randomly selected for characterization. The expression of G-CSFR mRNA was examined in these clones by northern hybridization. Total cellular RNA was isolated from each clone, separated on formaldehyde agarose gels, transferred to GeneScreen membranes and hybridized with a G-CSFR cDNA probe (Fig. 1). Human glyceraldehyde-3-phosphate dehydrogenase (GAPD) cDNA obtained from the American Type Culture Collection (Rockville, MD) was used as a control. The levels of G-CSFR mRNA differed in the various clones, but all of these cell lines, except D+GR12, showed an increased level of G-CSFR mRNA compared to the control D+V3, which represents WEHI-3B D⁺ cells transfected with the control plasmid p75/15v. Endogenous G-CSFR mRNA was barely detectable in the D+V3 clone and required prolonged exposure. As expected, the size of the exogenous G-CSFR mRNA was slightly smaller than that of the endogenous G-CSFR mRNA. Because the human metallothionein promoter is leaky and the treatment of these clones with cadmium under the experimental conditions employed did not increase the levels of G-CSFR mRNA, subsequent binding experiments were carried out in the absence of cadmium.

Specific Binding of [^{125}I]rhG-CSF to Transfected Clones

To determine whether exogenously expressed G-CSFR mRNA is translated into a functional G-CSFR protein, and to examine the relationship between the level of the G-CSFR and cellular proliferation and differentiation, the specific [^{125}I]rhG-CSF binding capacities of the transfected clones were determined (Fig. 2 *a*). Two × 10^6 cells of each clone were incubated with 1 nM [^{125}I]rhG-CSF in the absence or presence of a 100-fold excess of cold rhG-CSF and the specific binding was calculated. Vector transfected cells (D+V3) exhibited the same level of G-CSFR expression as the parental WEHI-3B D⁺ cells. In contrast, pGR7-2 transfected clones exhibited a 5- to 16-fold increase in [^{125}I]rhG-CSF specific binding, except for clone 12, which had an

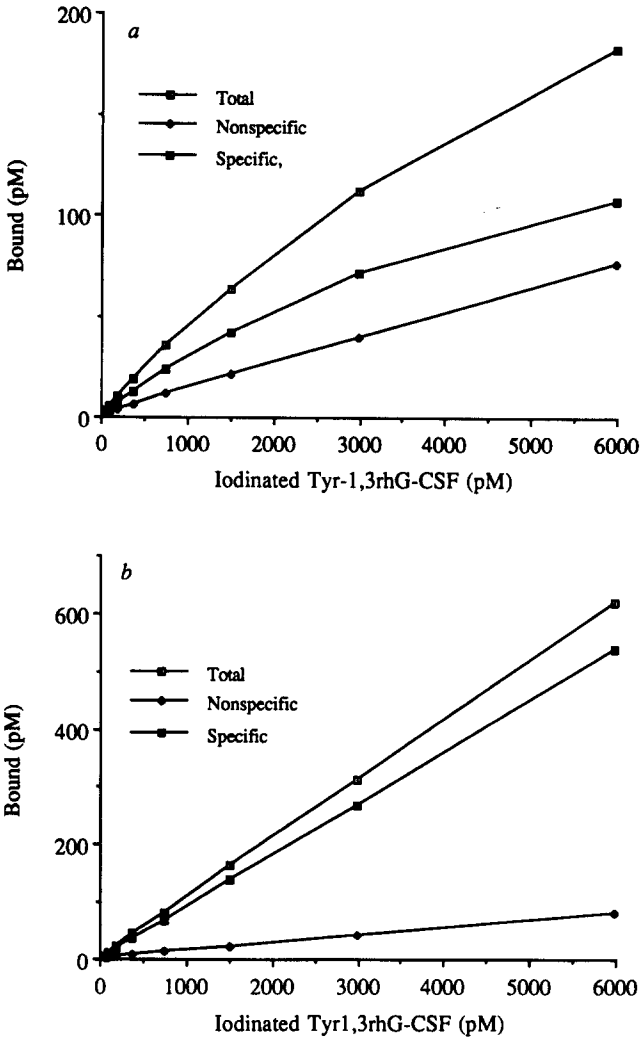

Figure 3. Binding characteristics of [^{125}I]rhG-CSF to WEHI-3B D$^+$ (*a*) and D+GR6 cells (*b*). Cells were incubated with various concentrations of labeled ligands in the absence or presence of a 100-fold excess of unlabeled rhG-CSF for 3 h at 15°C. Each value is the mean of triplicate determinations.

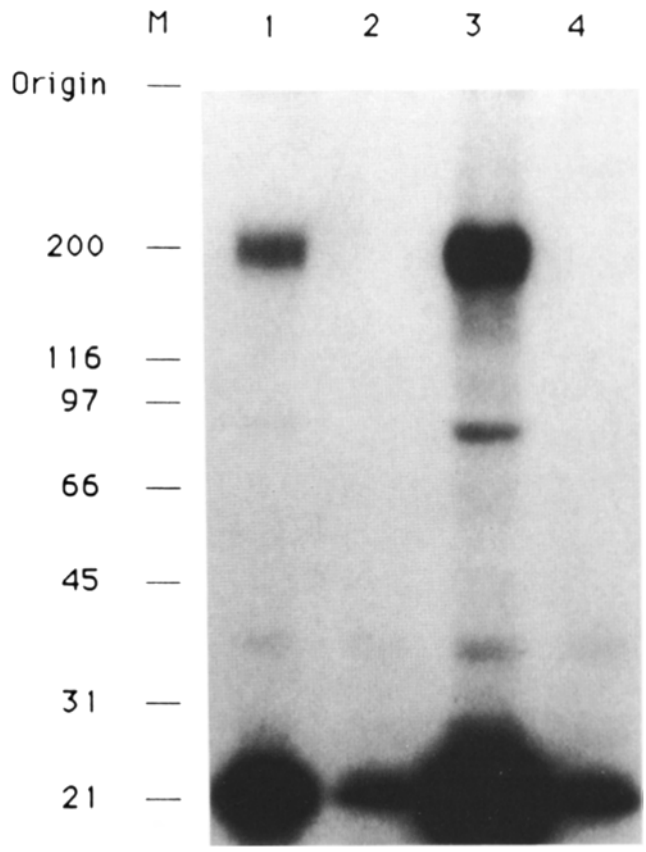

Figure 4. Chemical cross-linking of G-CSF to its receptor in WEHI-3B D$^+$ (lanes *1* and *2*) and D+GR6 (lanes *3* and *4*) cells. Cells were incubated with 4 nM [^{125}I]rhG-CSF in the absence (lanes *1* and *3*) or the presence (lanes *2* and *4*) of excess cold rhG-CSF at 4°C for 4 h. The complex of [^{125}I]rhG-CSF and the G-CSF receptor cross-linked by disuccinimidyl suberate and disuccinimidyl tartarate was analyzed on a 5–20% gradient polyacrylamide gel in the presence of SDS. Molecular standards are shown in kD.

mRNA level and an [^{125}I]rhG-CSF binding capacity essentially equivalent to that of D+V3 cells. In general, the level of specific binding of G-CSF approximated the increase in G-CSFR mRNA in the various clones. The binding of the rhG-CSF to transfected cells appeared to be specific, since neither recombinant murine GM-CSF, IL-3, or erythropoietin were significantly competitive with [^{125}I]rhG-CSF, whereas unlabeled rhG-CSF was an effective competitor (Fig. 2 *b*). To further characterize the binding of [^{125}I]rhG-CSF to the G-CSFR transfected clones, cells were incubated with [^{125}I]rhG-CSF at various concentrations with or without cold rhG-CSF. Fig. 3 illustrates the binding characteristics of [^{125}I]rhG-CSF to WEHI-3B D$^+$ parental and clone D+GR6 cells. Partial saturation of [^{125}I]rhG-CSF binding was observed in WEHI-3B D$^+$ cells but not in D+GR6 cells even at a concentration of 6,000 pM. This is probably due to the high levels of expression of the G-CSFR, because both clones showed essentially the same level of nonspecific binding of the cytokine. Scatchard analysis revealed that WEHI-3B D$^+$ cells have ~6,000 binding sites per cell for Tyr1,3-rhG-CSF, with a dissociation constant of 5.2 nM. Because saturation was not achieved with D+GR6 cells, the number of binding sites per cell and the dissociation constant could not be determined by this method. However, when D+GR6 cells were incubated with 3 nM of [^{125}I]rhG-CSF in the presence of 5–128 nM unlabeled rhG-CSF in a displacement assay, Scatchard analysis showed ~44,000 binding sites per D+GR6 cell, with a dissociation constant (6.0 nM) similar to that of parental WEHI-3B D$^+$ cells.

Chemical Cross-linking of [^{125}I]rhG-CSF to the G-CSFR

The level of expression and the size of the G-CSFR molecules in WEHI-3B D$^+$ cells and in D+GR clones were also determined by chemical cross-linking to ascertain whether the exogenous G-CSFR mRNA was translated into a full-length protein, thereby allowing a more effective assessment of the role of the G-CSFR in the proliferation and differentiation of these cells. Clone D+GR6 and WEHI-3B D$^+$ cells were incubated with [^{125}I]rhG-CSF and the bound [^{125}I]rhG-CSF was cross-linked to its receptor by disuccinimidyl suberate and disuccinimidyl tartarate. The [^{125}I]rhG-CSF-receptor complex was analyzed on a 5–20% gradient polyacrylamide gel in the presence of SDS (Fig. 4). In parental

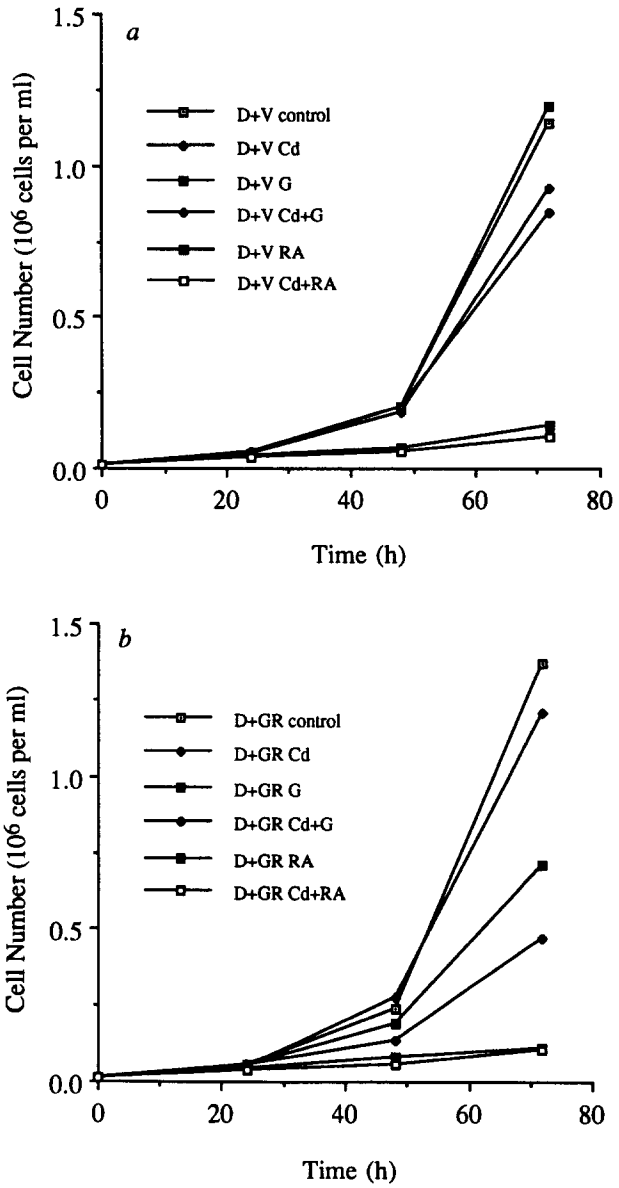

Figure 5. Effects of the overexpression of the G-CSFR on cellular proliferation. Cells were seeded at 1.3 × 10^4 cells/ml and growth was monitored daily for 3 d in the presence of various agents. *D+V*, vector transfected cells (*a*); *D+GR*, pGR7-2 transfected cells (*b*); *Cd*, 50 μM cadmium chloride; *G*, 10 ng/ml of rhG-CSF; *RA*, 7 μM.

cells, two major bands were observed, one with a mol wt of 20,000, which corresponds to free radioiodinated G-CSF, and the other with a mol wt of 195,000, which corresponds to the G-CSF-receptor complex. After subtraction of the mol wt of the ligand, the size of the G-CSFR in parental cells was estimated to be 175 kD. Two minor bands were also seen when the gel was exposed for a longer period of time; a 38-kD band, which probably represents the dimeric form of [^{125}I]rhG-CSF, and an 83-kD band whose identity is unknown. D+GR6 cells showed the same band pattern as that of parental WEHI-3B D$^+$ cells, confirming that the entire G-CSFR protein was synthesized from the exogenous G-CSFR gene in transfected cells. The level of the G-CSFR, however, was ~10-fold higher in the D+GR6 clone than in parental cells as determined by densitometry. This finding

*Table I. Clonal differentiation of WEHI-3B D$^+$ and D+GR6 Cells Induced by rhG-CSF**

Cell line	rhG-CSF	UD	PD	D
			%	
WEHI-3B D$^+$	–	90(142)	8(12)	2(3)
	+	44(49)	30(33)	26(29)
D+GR6	–	92(150)	6(9)	2(4)
	+	0(0)	0(0)	100(37)

* 250 cells per plate were incubated in soft agar medium with or without 10 ng/ml of rhG-CSF for 6 d. The number of colonies were counted and classified as a percentage of total colonies as described in Materials and Methods. The values in parentheses represent the mean colony number of three plates. UD, undifferentiated; PD, partially differentiated; D, differentiated.

is consistent with the increased level of G-CSFR mRNA present in this clone.

Effects of G-CSFR Expression on Cellular Growth and Differentiation

Because northern hybridization, [^{125}I]rhG-CSF binding, and chemical cross-linking experiments have shown that a full-length functional G-CSFR is expressed from the exogenous G-CSFR gene in transfected clones, it was appropriate to evaluate the effects of the overexpression of the G-CSFR gene on cellular proliferation and differentiation. WEHI-3B D$^+$ cells were transfected with either pGR7-2 or the control vector, selected with G-418, then treated with 50 μM cadmium chloride, 10 ng/ml of rhG-CSF, 7 μM retinoic acid (RA), or combinations thereof, and their effects on cellular growth were measured (Fig. 5). Untreated pGR7-2 transfected cells (D+GR) had a growth rate similar to that of vector transfected control cells (D+V), indicating that overexpression of the G-CSFR itself does not promote the proliferation of these cells. Cadmium treatment decreased the growth rate slightly, presumably as a result of cytotoxicity produced by the metal ion. Treatment of D+V cells with rhG-CSF did not result in a decrease in the growth rate; however, when D+GR cells were exposed to rhG-CSF, only 52% of the rate of growth of untreated cells was observed. The combination of G-CSF and cadmium decreased cellular replication of D+GR cells to 34% of that of untreated cells (68% inhibition of growth), whereas this treatment resulted in only 26% inhibition of growth in D+V cells. RA, employed as a control, markedly depressed the growth of both D+V and D+GR cells to a similar degree.

The effects of G-CSF on the colony morphology of WEHI-3B D$^+$ cells have been studied in semisolid culture (5, 16). The methodology employed in these investigations was used to evaluate the effects of the overexpression of the G-CSFR on the colony morphology of WEHI-3B D$^+$ cells. In this system (16), colonies were classified as "undifferentiated" if they consisted of a tight aggregate of cells without outlying elements, or as "partially differentiated" if they possessed a peripheral halo of loosely dispersed cells around the central aggregate. Differentiated colonies were composed entirely of a loosely dispersed collection of cells. In the absence of G-CSF, ~90% of WEHI-3B D$^+$ or D+GR6 colonies were undifferentiated (Table I). Exposure to 10 ng/ml of rhG-CSF caused approximately 26% of WEHI-3B D$^+$ colonies to exhibit a differentiated phenotype, while all of the D+GR6 colonies were fully differentiated. Fewer colonies were

*Table II. Differentiation of WEHI-3B D⁺ Parental, Vector Transfected (D+V), and rhG-CSFR Transfected (D+GR) Cells Determined by NBT Positivity**

Cell line	Treatment‡	NBT positivity
		%§
WEHI-3B D⁺	None	0.5 ± 0.5
	Cd	1.0 ± 0.5
	rhG-CSF	4.5 ± 2.5
	Cd+rhG+CSF	6.0 ± 3.0
	RA	49.0 ± 4.0
D+V	None	2.1 ± 1.6
	Cd	4.8 ± 2.1
	rhG-CSF	6.3 ± 4.6
	Cd+rhG-CSF	12.8 ± 3.8
	RA	52.8 ± 1.3
D+GR	None	1.8 ± 0.9
	Cd	4.0 ± 1.5
	rhG-CSF	60.0 ± 12.7
	Cd+rhG-CSF	72.4 ± 8.4
	RA	62.0 ± 3.0

* Differentiation was assessed after cells were exposed to various treatments for 3 d.
‡ Cd, 50 μM $CdCl_2$; rhG-CSF, 10 ng/ml of rhG-CSF; Cd+rhG-CSF, 50 μM $CdCl_2$ plus 10 ng/ml of rhG-CSF; RA, 7 μM.
§ Each value is the average of three independent experiments ± SD.

formed from WEHI-3B D⁺ (71% of untreated cells) and many fewer from D+GR6 cells (only 23% of untreated cells) when exposed to rhG-CSF, even though about the same number of colonies were formed from both cell lines in the absence of rhG-CSF. This finding demonstrates that overexpression of the G-CSFR resulted in a decrease in cell growth in response to the cytokine. This is consistent with the decrease in the growth rate of D+GR cells exposed to G-CSF in suspension culture (Fig. 5).

Considering the high local cell density in the developing colonies in soft agar culture, which allows for cell communication as a short-range signal through extensive cell to cell contact, the differentiation observed might not be induced by G-CSF, but might be due to a spontaneous event caused by an autocrine mechanism which occurs at high cell density as suggested by some investigators (4). To determine whether the increased differentiation of the transfected cells in soft agar culture was directly due to the overexpression of the G-CSFR, the effects of the overexpression of the receptor was determined at the lower cell density that exists in suspension culture, with differentiation being assayed in a relatively short period of time (i.e., 3 d instead of the 6 d used in the soft agar culture) using two different markers of the mature state, the ability of cells to reduce NBT and the level of Mac-1 expression. WEHI-3B D⁺ cells were transfected with pGR7-2 and selected with G-418, and the entire population of transfected cells (D+GR) was exposed to rhG-CSF at 10 ng/ml. Both parental cells and those transfected with the p75/15v vector alone were used as controls; neither of these controls exhibited significant spontaneous differentiation (Table II). The pGR7-2 transfected cells (D+GR) exhibited similar behavior in the absence of rhG-CSF. These findings indicate that overexpression of the G-CSFR itself is not sufficient to initiate maturation, and contrasts with the c-Jun protein, where overexpression in WEHI-3B D⁺ cells triggers the differentiation process (13). When G-CSFR transfected cells were exposed to 10 ng/ml of G-CSF, however, 60% or more of the D+GR cells exhibited a differentiated phenotype. In contrast, treatment of vector transfected cells or parental cells with the cytokine did not result in a major commitment to a differentiation pathway. RA induced essentially the same degree of differentiation in all instances, indicating that all of the cell lines employed had the inherent ability to differentiate and that the overexpression of the G-CSFR is necessary for the G-CSF induced differentiation of D+GR cells.

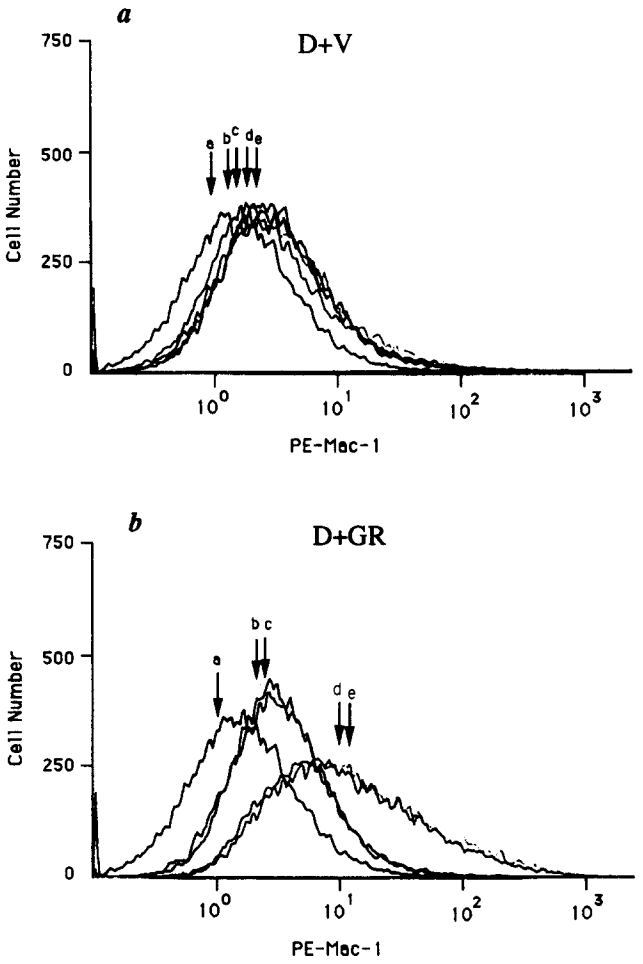

Figure 6. Expression of the differentiation antigen Mac-1 on the surface membrane of *D+V* (*a*) and *D+GR* (*b*) cells. Cells were treated with 10 ng/ml of rhG-CSF for 3 d and stained with monoclonal anti-Mac-1 and phycoerythrin-conjugated goat anti-rat IgG antibody. The level of Mac-1 expression was determined by flow cytometry. (*a*) Untreated WEHI-3B D⁺; (*b*) control; (*c*) 50 μM cadmium chloride; (*d*) 10 ng/ml of rhG-CSF; (*e*) 50 μM cadmium chloride and 10 ng/ml of rhG-CSF.

Immunofluorescent staining of the cell surface antigen Mac-1 was employed as a second marker of the mature state to further confirm the differentiation status of D+GR cells induced by rhG-CSF. After a 3-d exposure of these cells to rhG-CSF, cells were stained with monoclonal anti-Mac-1 antibody and phycoerythrin-conjugated goat anti-rat IgG antibody, and the level of Mac-1 was determined by flow cytometry (Fig. 6). A broad Mac-1 positive peak appeared when D+GR cells were treated with rhG-CSF or cadmium plus rhG-CSF, whereas no such peak was observed in D+V vec-

Table III. Differentiation of WEHI-3B D⁺ Clones Transfected with the G-CSF Receptor Gene‡*

Clone	Untreated	Cd	NBT positivity rhG-CSF	Cd+rhG-CSF
			%	
D+V3	0.7 ± 0.2	1.8 ± 1.2	0.5 ± 0.4	1.3 ± 0.8
D+GR2	0.8 ± 0.2	3.7 ± 4.5	42.9 ± 15.7	43.5 ± 12.8
3	0.7 ± 0.2	1.5 ± 2.1	29.8 ± 4.6	45.2 ± 21.5
4	0.7 ± 0.2	1.3 ± 0.2	60.9 ± 14.2	77.3 ± 6.6
5	2.3 ± 2.3	9.2 ± 5.7	72.3 ± 9.1	62.0 ± 18.0
6	0.8 ± 0.3	4.8 ± 0.8	51.3 ± 4.5	56.2 ± 12.9
7	0.3 ± 0.5	0.5 ± 0.4	46.2 ± 15.9	51.5 ± 15.8
8	0.8 ± 0.2	1.7 ± 0.5	63.8 ± 14.9	59.5 ± 18.9
9	0.2 ± 0.2	2.7 ± 3.1	27.0 ± 0.8	34.7 ± 3.7
10	1.8 ± 0.6	2.6 ± 2.8	54.5 ± 17.2	54.7 ± 7.9
11	0.8 ± 0.8	0.3 ± 0.5	9.3 ± 1.9	21.2 ± 4.6
12	2.8 ± 3.3	5.7 ± 4.5	4.0 ± 2.0	5.5 ± 3.9
13	0.5 ± 0.4	0.8 ± 0.3	70.0 ± 5.0	59.7 ± 15.2
14	8.5 ± 1.5	3.2 ± 2.1	59.8 ± 12.3	59.7 ± 21.5

* Cells were treated for 3 d with 50 μM $CdCl_2$, 10 ng/ml of rhG-CSF, or the combination thereof, and the percentage of NBT positive cells was determined as described in Materials and Methods.
‡ Each value is the average of three independent experiments ± SD.

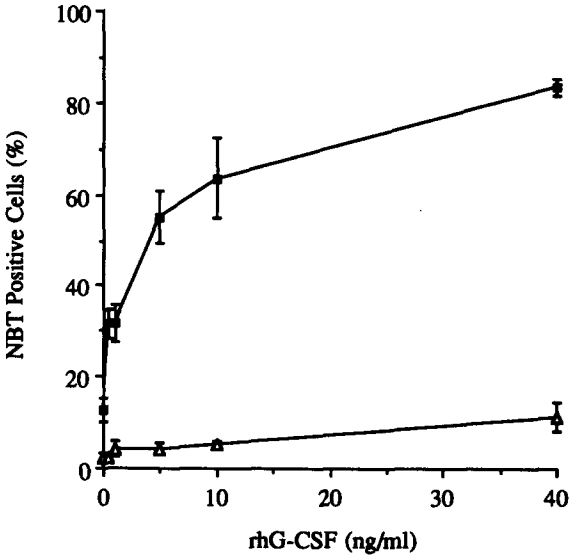

Figure 7. Concentration-dependent differentiation of D+GR6 cells (■) induced by rhG-CSF. Cells were exposed to various concentrations of the cytokine for 3 d and the percentage of NBT positive cells was determined as described in Materials and Methods. D+V3 vector containing cells (△) were used as the control. Each point represents the average of three values ± SD.

tor control cells treated in an analogous manner. Quantification of the relative fluorescence intensity of the cells demonstrated that rhG-CSF or rhG-CSF plus cadmium treated D+GR cells increased Mac-1 expression by 7.9- and 9.5-fold, respectively, over parental WEHI-3B D⁺ cells. No cell adhesion accompanied the increase in Mac-1 expression, suggesting that cells were undergoing granulocytic maturation.

Because (*a*) WEHI-3B D⁺ cells, which have a relatively low number of G-CSFR and a relatively poor response to the differentiation inducing properties of G-CSF, and (*b*) the overexpression of the G-CSFR converted the cells to highly responsive ones, we further investigated the relationship between cell differentiation and the level of G-CSFR expression in all of the selected D+GR clones. The 13 single cell clones were treated with cadmium, G-CSF, or their combination for 3 d, and their capacity to reduce NBT was measured and is shown in Table III. Except for D+GR12, all of the clones which had at least a fivefold increase in the expression of the G-CSFR (Fig. 2) exhibited a significant degree of differentiation in response to G-CSF, confirming that the increased expression of the receptor results in an enhancement in the ability of cells to enter the differentiation pathway. However, no strict relationship existed between the degree of differentiation and the absolute level of the G-CSFR. Clone D+GR12, which had a low concentration of G-CSFR mRNA and a low level of [^{125}I]rhG-CSF binding, was not induced to differentiate by G-CSF. Thus, this clone was similar to parental cells in all of the aspects studied except for its resistance to G-418.

The sensitivities of D+V3 and D+GR6 cells to the differentiation inducing capacity of various concentrations of rhG-CSF were determined in suspension culture and the results are shown in Fig. 7. A concentration-dependent increase in the percentage of differentiated D+GR6 cells was obtained, with more than 80% of the cellular population exhibiting a differentiated phenotype at the highest concentration of rhG-CSF employed, whereas only 12% of D+V3 vector transfected cells expressed NBT positivity at the highest level of rhG-CSF tested. The concentration-dependent induction of differentiation in the clone expressing a high level of G-CSFR demonstrated that the differentiation-inducing effect is G-CSF specific.

IL-3 is known to support the growth of 32D C13 cells and to antagonize the differentiation inducing effects of G-CSF in this cell line (35). Because WEHI-3B D⁺ cells constitutively secrete IL-3 (12), we conducted an experiment designed to provide indirect evidence as to whether the differentiation of D+GR cells was achieved through inhibition of the secretion of IL-3 by the G-CSF signal transduction pathway. The assumption was made that if IL-3 antagonized the effects of G-CSF, supplementation of the medium by IL-3 would suppress G-CSF induced differentiation. However, no such interference with the maturation produced by G-CSF in D+GR6 cells was observed when a concentration of IL-3 up to 100 U/ml was employed (Table IV).

Discussion

G-CSF has been reported to be an inducer of the differentiation of WEHI-3B D⁺ cells, assayed by culture in semisolid agar (5, 27). Under these conditions, granulocytic colonies were formed and the clonogenic activity of the cells was greatly reduced by G-CSF. In contrast, Böhmer and Burgess (4) have reported that G-CSF is not an inducer of the differentiation of WEHI-3B D⁺ cells, but is required for the survival of mature progeny in suspension culture at low cell density. We have also found that G-CSF is not an effective initiator of the maturation of WEHI-3B D⁺ cells, and only a small percentage of cells exhibited a differentiated phenotype when exposed to a concentration of the cytokine as high as 50 ng/ml in suspension culture (14). To gain a clear understanding of the effects of G-CSF, we have examined the role of its receptor in the maturation process. This was accom-

*Table IV. The Lack of Effect of IL-3 on the Differentiation of D+GR6 Induced by rhG-CSF**

G-CSF	IL-3	NBT positivity
(*ng/ml*)	(*U/ml*)	%‡
0	0	0.3 ± 0.3
10	0	43.5 ± 6.7
10	1	47.2 ± 1.3
10	5	43.0 ± 2.2
10	20	42.5 ± 4.8
10	100	51.0 ± 8.8

* D+GR6 cells were treated for 3 d with 10 ng/ml of rhG-CSF in the absence or presence of various concentrations of IL-3.
‡ Each value represents the mean ± SD of three separate flasks.

plished by the introduction of the G-CSFR gene into WEHI-3B D+ cells and evaluation of the effects of the overexpression of the receptor in both semisolid and suspension culture systems. In soft agar culture, 10 ng/ml of rhG-CSF produced a differentiated morphology in all of the colonies formed by the cloning of D+GR6 cells, while only 26% of parental cells were differentiated under the same conditions. In an analogous manner, in suspension culture, more than 60% of the cellular population exhibited a mature phenotype, even at relatively low concentrations of G-CSF in G-CSFR transfected cells, whereas about 6% differentiation was seen in the vector transfected cells under identical conditions. Vector transfected control cells showed a very slight increase in NBT positivity over that of parental cells when exposed to G-CSF. It is unlikely that this increase is due to a specific selection of high G-CSFR expressing cells by G-418, but is probably due to the presence of residual nontransfected dying cells after G-418 selection.

A decrease in the growth rate accompanied the differentiated state. The decrease in growth was seen as early as 36 h after exposure to G-CSF at a relatively low cell density (1×10^5 cells/ml); thus, no extensive cell-to-cell contact and no loss of the proliferation-stimulating activity of the FBS presumably occurred. Therefore, it was assumed that the differentiation and the decrease in growth rate were directly the result of the interaction between G-CSF and its receptor. These findings confirm the role of G-CSF and its receptor in the initiation of the differentiation process, and because excess receptor was required to initiate maturation, the findings appear to provide an example of a deficiency in the receptor portion of the signal transduction mechanism in this malignant cell line that interfered with the capacity of the cytokine to function as a regulator of growth and differentiation.

When the differentiation inducing effects of G-CSF on D+GR cells were compared to those of RA, the percentage of differentiated cells was found to be similar. However, the total number of cells exhibiting the differentiated phenotype was much greater with the cytokine, because when D+GR cells were exposed to RA or rhG-CSF, the cell density was much lower with the retinoid, indicating that RA is a stronger inhibitor of growth than G-CSF. Thus, the major role of G-CSF in this system appears to be the induction of differentiation.

The dissociation constant for G-CSF and the number of binding sites for the cytokine on each WEHI-3B D+ cell observed in the present study does not correspond to that of an earlier report (25). It is conceivable that these differences are due to the sources of the G-CSF that were utilized in the binding experiments. Murine native G-CSF was used in the early study, while a mutant recombinant human G-CSF (Tyr1,3-rhG-CSF) was employed in the present experiments. The mutation in this recombinant G-CSF does not affect the specificity of G-CSF for its receptor, and the cytokine still retains its biological activity after radioiodination (37). We have found, however, that a relatively high nonspecific binding of Tyr1,3-rhG-CSF to WEHI-3B D+ cells occurs under the experimental conditions employed, which may contribute to the higher value that was obtained for the dissociation constant and the number of binding sites per cell. The G-CSFR transfected cells have the same level of nonspecific binding as the parental cells, but much higher specific binding. Considering the high specific binding and the results from the northern hybridization and cross-linking experiments, the values for the dissociation constant and the number of G-CSFRs present in parental WEHI-3B D+ cells, does not affect the overall interpretation of the effects of the overexpression of the G-CSFR on the differentiation and proliferation of WEHI-3B D+ cells.

Proliferation and differentiation are often opposing processes. G-CSF can both stimulate growth and induce differentiation, depending upon the cell line. With WEHI-3B D+, M1, 32D C13 and HL-60 cells, the predominant effect of G-CSF may be to induce maturation (5, 17, 32), whereas in NFS60, AML-193 and OCI/AML1a cells, the major effect of the cytokine appears to be stimulation of growth, at least initially (21, 30, 38). Overexpression of the G-CSFR gene in the IL-3 dependent murine myeloid cell line FDC-P1 and the pro-B cell line BAF-B03 enhances the proliferation of these cells in response to G-CSF, whereas the cytokine does not stimulate growth in the IL-2 dependent T cell line CTLL-2 when G-CSFR is overexpressed (9). We have used the G-CSFR cDNA originally cloned from NFS60 cells and have obtained a different effect (i.e., differentiation) in WEHI-3B D+ cells, confirming that the same protein, which mediates the stimulation of growth in NFS60 cells, induces differentiation in WEHI-3B D+ cells. These findings indicate that the signal transduced in these two cell lines must differ after occupancy of the receptor. In this regard, it is worth noting that the *c-myb* and *evi-1* genes, which may play important roles in differentiation, have been rearranged in NFS60 cells but not in WEHI-3B D+ cells (19), and that a greater degree of differentiation of AML-193 cells is obtained when G-CSF is used in combination with RA than with RA alone, although G-CSF itself sustains the growth of this cell line (34).

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