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 [125I]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) 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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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 [125I]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% CO₂. 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 channelizer 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 WEHI-3B D⁺ leukemia cells and clones derived therefrom were main-

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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 CdCl₂ was isolated and hybridized with ³²P-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.

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 [¹²⁵I]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 [¹²⁵I]rhG-CSF binding capacities of the transfected clones were determined (Fig. 2 a). Two × 10⁶ cells of each clone were incubated with 1 nM [¹²⁵I]rhG-CSF for 3 h at 15°C in the absence or presence of excess cold rhG-CSF. The specific binding of [¹²⁵I]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 [¹²⁵I]rhG-CSF binding and other cytokines. Two × 10⁶ D+GR6 cells were incubated with [¹²⁵I]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.
mRNA level and an [125I]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 no significant competitive binding occurred 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

Figure 3. Binding characteristics of [125I]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.

Chemical Cross-linking of [125I]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 [125I]rhG-CSF and the bound [125I]rhG-CSF was cross-linked to its receptor by disuccinimidyl suberate and disuccinimidyl tartrate. The [125I]rhG-CSF-receptor complex was analyzed on a 5–20% gradient polyacrylamide gel in the presence of SDS. Molecular standards are shown in kD.

Tyrl,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 [125I]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.

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 [125I]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 [125I]rhG-CSF and the G-CSF receptor cross-linked by disuccinimidyl suberate and disuccinimidyl tartrate was analyzed on a 5–20% gradient polyacrylamide gel in the presence of SDS. Molecular standards are shown in kD.
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 [125I]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 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, [125I]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

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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.
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 |
|-----------|-----------|----------------|
|           |           | % w            |
| 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 ± 6.4  |
|           | RA        | 62.0 ± 3.0     |

* Differentiation was assessed after cells were exposed to various treatments for 3 d.
† Cd, 50 μM CdCl₂; rhG-CSF, 10 ng/ml of rhG-CSF; Cd + rhG-CSF, 50 μM CdCl₂ 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 (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 pGRT-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 any significant spontaneous differentiation (Table II). The pGRT-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.

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 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.

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. 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.
adhesion accompanied the increase in Mac-1 expression, D+GR cells increased Mac-1 expression by 7.9- and 9.5-fold, respectively, over parental WEHI-3B D+ cells. No cell

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

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differentiation inducing properties of G-CSF, and (b) the

ty when exposed to a concentration of the cytokine as high

tion of differentiation in the clone expressing a high level

generated the effects of 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 constituti-

tively secrete IL-3 (12), we conducted an experiment de-
signed to provide indirect evidence as to whether the
differentiation of D+GR cells was achieved through inhibi-
tion of the secretion of IL-3 by the G-CSF signal transduc-
tion pathway. The assumption was made that if IL-3 an-
tagonized the differentiation inducing effects of G-CSF,
supplementation of the medium by IL-3 would suppress G-CSF induced differentiation. However, no such interference with the maturation pro-
duced by G-CSF in D+GR cells was observed when a con-
centration of IL-3 up to 100 U/ml was employed (Table IV).

Discussion

G-CSF has been reported to be an inducer of the differenti-
ation of WEHI-3B D+ cells, assayed by culture in semi-
solid 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 pheno-
type when exposed to a concentration of the cytokine as high
as 50 ng/ml in suspension culture (14). To gain a clear un-
derstanding of the effects of G-CSF, we have examined the role
of its receptor in the maturation process. This was accom-

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

| Clone | Untreated | Cd | rhG-CSF | Cd + rhG-CSF |
|-------|-----------|----|---------|-------------|
|       | NBT positivity | % |         |             |
|       |           |    |         |             |
| 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 ± 0.5 | 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 | 7.0 ± 5.0 | 59.7 ± 15.2 |
| 14    | 3.5 ± 1.5 | 3.2 ± 2.1 | 59.8 ± 12.3 | 59.7 ± 21.5 |

* Cells were treated for 3 d with 50 μM CdCl2, 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.
Table IV. The Lack of Effect of IL-3 on the Differentiation of D+GR6 Induced by rhG-CSF*

| G-CSF (ng/ml) | IL-3 (U/ml) | NBT positivity % ± SD |
|--------------|------------|----------------------|
| 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 days 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.

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