Human Colony-Stimulating Factor 1 (CSF-1) Receptor Confers CSF-1 Responsiveness to Interleukin-3–Dependent 32DCL3 Mouse Myeloid Cells and Abrogates Differentiation in Response to Granulocyte CSF

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Interleukin-3 (IL-3)–dependent mouse myeloid 32DCl3 cells differentiate to neutrophils in response to granulocyte colony-stimulating factor (G-CSF). Introduction of the human c-fms gene, which encodes the receptor for CSF-1, into 32DCl3 cells gave rise to variants that were able to proliferate in medium containing either murine IL-3 or human recombinant CSF-1, but were unable to differentiate to granulocytes in response to G-CSF. Unlike parental 32DCl3 cells, CSF-1–responsive derivatives expressed non-specific esterase when grown in CSF-1, but did not exhibit many other morphologic, immunologic, or functional properties of mononuclear phagocyte differentiation, or express murine CSF-1 receptors. Accelerated turnover of the human CSF-1 receptor was observed in response to CSF-1 and phorbol esters, but not after stimulation with IL-3 or bacterial lipopolysaccharide. Although both CSF-1 and IL-3 induced tyrosine phosphorylation of heterologous substrates in the dually responsive cells, differences in the patterns of substrate phosphorylation were observed in response to the two hematopoietins. We conclude that expression of the human CSF-1 receptor in 32DCl3 cells not only induces CSF-1 responsiveness, but alters its phenotype in a way that prohibits granulocyte differentiation.

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for 3 weeks in complete medium containing IL-3 and 800 µg/mL G418. The medium was then supplemented with heavy metals (1×10⁻⁷ mol/L ZnSO₄ and 5×10⁻⁷ mol/L CdSO₄), and selection was continued in the presence of IL-3 and G418 for 2 more weeks.

Equal aliquots of cells from G418-resistant cultures electroporated with the c-fms gene in sense orientation were pooled, and cells expressing human CSF-1R were selected by fluorescence-activated cell sorting using a monoclonal antibody (MoAb) to the human receptor. The sorted CSF-1R-positive population (designated 32D[R+]) was maintained in medium containing IL-3. In parallel, cells from each independent culture were grown in complete medium supplemented with 2,000 U/mL (0.9 nmol/L) of human recombinant CSF-1. Seven cultures yielding CSF-1-positive cells were obtained, each of which (designated 32D[R+]/C, C1.2, and C1.3) were characterized in detail.

**RESULTS**

**Transduction of human CSF-1R into IL-3-responsive 32DC13 cells.** The mouse myeloid cell line, 32DC13, is dependent on IL-3 for proliferation and survival, but terminally differentiates into mature granulocytes when IL-3 is removed from the culture medium and is replaced by G-CSF. To determine whether 32DC13 cells could be reprogrammed to proliferate in CSF-1 and whether stimulation by CSF-1 would affect their pattern of differentiation, we introduced the human c-fms gene encoding CSF-1R into these cells and tested their response to different hematopoietins.

Human c-fms cDNA, cloned into an inducible expression vector containing a metal-responsive enhancer fused to the β-globin promoter, was electroporated into 32DC13 cells with pSV2neo, a plasmid that confers resistance to the antibiotic G418. The electroporated cells were then divided at 3×10⁶ cells per culture into a 24-well plate and grown for 3 weeks in medium containing IL-3 and G418. Twenty-one G418-resistant cultures were obtained and segregated for 2 additional weeks in medium containing IL-3 and heavy metals to induce expression of human CSF-1R. Equal aliquots from each of the 21 cultures were harvested and pooled, and CSF-1 receptor-positive cells (here designated 32D[R+]) were purified by fluorescence-activated cell sorting (FACS) with an MoAb directed to an extracellular epitope of human CSF-1R. These cells were continuously maintained in medium containing IL-3. Under these conditions, 32D[R+] cells expressed moderate levels of human CSF-1R, even in the absence of heavy metals (Fig 1), and receptor expression was not increased further when heavy metals were added to the medium.

In parallel, cells from the 21 individual G418-resistant cultures were transferred into medium containing human recombinant CSF-1 and heavy metals, yielding seven cultures capable of continuous growth in CSF-1. By contrast, 24 cultures of 32DC13 cells electroporated with a plasmid containing the c-fms gene in an anti-sense orientation together with pSV2neo yielded 11 G418-resistant cultures, none of which were able to grow in human CSF-1. Thus, CSF-1 responsiveness appeared to depend on expression of the transduced c-fms gene.

Of the seven CSF-1-responsive cultures, three grew very slowly, whereas one showed a factor-independent phenotype and proliferated in the absence of either CSF-1 or IL-3. The remaining three cultures (designated 32D[R+]/C1.1, C1.2, and C1.3) were dependent on either human CSF-1 or murine CSF-1R.
IL-3 for survival and proliferation, but died in the absence of either factor. When these CSF-1-responsive cell lines were analyzed by FACS for expression of human CSF-1R, they expressed significantly higher levels of the receptor than pooled 32D[R+] cells grown in IL-3 (Fig 1). These levels of CSF-1R are equivalent to those detected on murine BAC1.2F5 macrophages (about 1 x 10^5 CSF-1 binding sites/cell). Although the experimental protocol prohibited an estimation of their frequency, the results suggested that only those cells that expressed high levels of human CSF-1R selectively grew out in response to CSF-1. Cells from the one factor-independent culture also exhibited very high levels of CSF-1R expression (data not shown), consistent with previous results suggesting that overexpression of CSF-1R in immature myeloid cells may contribute to a factor-independent phenotype.16

Growth and differentiation of CSF-1-responsive cells. Subsequent experiments were performed with the three CSF-1-responsive cell lines and with single cell subclones of each. Because similar data were obtained with each of these cell lines, only representative data with 32D[R+]C1.1 cells are shown below. Figure 2 shows growth curves under different conditions for parental 32DC13 cells (A) and for a CSF-1-responsive clone (B). The parental cells proliferated only in the presence of IL-3. When transferred to medium containing human recombinant CSF-1, their viability declined rapidly, and no CSF-1-responsive or factor-independent variants arose from cultures containing more than 5 x 10^6 cells. In contrast, 32D[R+]C1.1 cells grew equally well in response to murine IL-3, human CSF-1, or in medium containing both growth factors, but died in the absence of either. Although human CSF-1 can bind with high affinity to both human and mouse CSF-1R, murine CSF-1 does not bind to human CSF-1R or induce the growth of cells bearing the human receptor.21,22 As expected, murine CSF-1 did not support the growth or survival of 32D[R+]C1.1 cells, implying that they did not express murine CSF-1R (see below) and that their growth in response to human CSF-1 was mediated by the transduced c-fms gene product.

When washed and transferred to medium containing G-CSF, 32DC13 cells eventually stopped growing (Fig 2B) and differentiated to granulocytes. Figure 3 contrasts the morphology of the parental cells maintained in IL-3 (A) with those cultured for 10 days in G-CSF (B) and illustrates the conversion of immature myeloid cells to more mature granulocytes containing banded or segmented nuclei.14 In contrast, the survival of CSF-1-responsive 32D[R+]C1.1 cells was not supported by G-CSF (Fig 2B). Although these cells could be maintained for a somewhat longer period of time in medium containing G-CSF than in unsupplemented medium, no viable cells remained after 10 days of G-CSF treatment. Moreover, no morphologic evidence of granulocytic differentiation was observed in G-CSF-treated 32D[R+]C1.1, C1.2, or C1.3 cultures maintained during this period, nor did G-CSF inhibit their growth in response to either IL-3 or CSF-1 (data not shown).

Because the CSF-1-responsive cell lines were unable to differentiate to granulocytes in response to G-CSF, we considered the possibility that CSF-1 induced an alternative genetic program leading toward monocytic maturation. Therefore, the cells were assayed for production of nonspecific esterases as markers of monocytic differentiation. The term nonspecific esterase is reserved for enzymes capable of hydrolyzing simple esters of N-free alcohols and organic acids. For those enzymes that use a-naphthyl butyrate as a substrate, the reaction products are generally confined to those detected on parental 32DC13 cells (negative data

Fig 2. Growth of parental 32DC13 (A) and CSF-1-responsive 32D[R+]C1.1 cells (B) in different media. Cells seeded at the indicated densities in T25 flasks were grown in unsupplemented Iscove's complete medium (■) or in media containing IL-3 (□), CSF-1 (△), G-CSF (□), or IL-3 plus CSF-1 (□). Exponentially proliferating cultures were counted, depopulated, and fed every 2 days, and the total cells were estimated from the growth of the remaining population.
REPROGRAMMING THE CSF-1 RESPONSE

not shown). Treatment of these cells with other potential inducing agents including GM-CSF, LPS, TPA, PGE₂, LTB₄, 1,25-dihydroxyvitamin D₃, or 5-azacytidine did not induce monocytic maturation.

32D[R⁺]C1.1 cells do not express murine CSF-1R. The most typical marker of maturation in the mononuclear phagocyte lineage is expression of CSF-1R itself. The inability of murine CSF-1 to support the proliferation of 32D[R⁺]C1.1, C1.2, and C1.3 cells suggested that they did not express murine CSF-1R. To confirm these results, 32DC13, 32D[R⁺], and 32D[R⁺]C1.1 cells were metabolically labeled with [³⁵S]methionine, and human CSF-1R was immunoprecipitated from detergent lysates using a MoAb that does not crossreact with murine CSF-1R. The cleared lysates were then reacted with a polyclonal rabbit antiserum raised to a human c-fms polypeptide that crossreacts with the murine receptor. As a positive control, lysates of metabolically labeled murine BAC1.2F5 macrophages, which express high levels of the mouse CSF-1 receptor, were analyzed in parallel. All immunoprecipitates were denatured and separated on polyacrylamide gels containing sodium dodecyl sulfate, and receptors were detected by autoradiography of the dried slab gels. Under these labeling conditions, both the immature intracellular form (about 130 Kd) and mature cell surface form (about 165 Kd) of CSF-1R are detected; the two forms differ solely in their composition of asparagine-linked oligosaccharide chains.

Figure 4 (lanes 1 and 5) shows that parental 32DC13 cells did not express CSF-1R. As expected from FACS analysis (Fig 1), 32D[R⁺] cells expressed significantly lower levels of human CSF-1R (lane 2) than 32D[R⁺]C1.1 cells (lane 3), whereas both cell lines failed to express murine CSF-1R (lanes 6 and 7). The MoAb to human CSF-1R does not react with the murine receptor expressed in BAC1.2F5 cells (lane 4), but the latter species was readily detected with the polyclonal rabbit antiserum (lane 8). Again, by these criteria, the levels of human CSF-1R synthesis in 32D[R⁺]C1.1 cells were similar to those observed for the murine receptor in BAC1.2F5 macrophages. However, the molecular mass of human CSF-1R in 32DC13 cell derivatives was greater than that of the murine receptor in BAC1.2F5 cells, probably reflecting cell-specific differences in the processing of oligosaccharide chains. Similar results were previously observed for v-fms gene products expressed in murine myeloid and macrophage cell lines, respectively.

Human CSF-1R is not transmodulated by IL-3. After ligand binding, CSF-1R is rapidly internalized and degraded in lysosomes (receptor downmodulation). Activators of protein kinase C, such as the phorbol ester TPA, also induce accelerated receptor turnover by inducing a protease that cleaves CSF-1R near its transmembrane segment; this process (receptor transmodulation) results in the release of the receptor ligand-binding domain from the cell and concomitant internalization of the 50-Kd tyrosine kinase domain that is degraded intracellularly. Treatment of macrophages with bacterial lipopolysaccharide induces transmodulation of CSF-1R through the same mechanism. Because IL-3 has been reported to induce a loss of CSF-1 binding sites from mouse bone marrow cells, we attempted to determine whether it would similarly induce CSF-1R turnover in 32D[R⁺]C1.1 cells that expressed both classes of receptors. 32D[R⁺]C1.1 and BAC1.2F5 cells labeled with...
Similarly, murine and human (lane 7) and TPA (lane 8) showed accelerated turnover in myeloid cells (lane 5). A protein of 140 Kd corresponded in molecular weight as the appearance of major phosphotyrosine-containing bands were detected in each lanes 1, 2, 4, and 7). A protein of 140 Kd corresponded in mass to the previously reported IL-3 binding protein, a second polypeptide of 95 Kd showed an even greater signal intensity. In contrast, CSF-1 stimulation induced tyrosine phosphorylation of CSF-1R (about 165 Kd) as well as the appearance of 110-, 95-, and 85-Kd phosphoproteins (lane 8). We also observed a phosphoprotein of the same molecular weight as CSF-1R in starved 32D[R+]C1.1 cells (lane 6); this background level of receptor tyrosine phosphorylation was even higher in the factor-independent variant (data not shown), suggesting that high levels of receptor expression are accom
Similarly, introduction of the c-erbB proto-oncogene encoding the epidermal growth factor (EGF) receptor into 32DC13 or FDC-P1 cells rendered them responsive to EGF. 38,41 Although transduction of the CSF-1 and EGF receptor genes into mouse myeloid cell lines could, in some cases, induce ligand-dependent shifts toward more mature phenotypes, 39,40 the cells did not terminally differentiate. Moreover, insertion of constitutive oncogene coded tyrosine kinases, such as v-erbB, 42 v-abl, 43-45 v-src, 46 or v-fms, 47 into these same cell lines abrogated their IL-3 dependence but did not induce differentiation and, in the case of 32DC13 cells, inhibited granulocyte maturation in response to G-CSF. 44 The growth of 32D[R+]C1.1, C1.2, and C1.3 cells in CSF-1 induced nonspecific esterase activity and rendered the cells unable to differentiate in response to G-CSF. Induction of esterase activity was not readily reversible, since recultivation of the cells for 10 days in IL-3 did not lead to a detectable diminution of enzymatic activity. One interpretation is that CSF-1 induced partial differentiation toward monocytes, leading concomitantly to a loss of G-CSF responsiveness. However, the cells did not express other functional or phenotypic markers of monocytic differentiation, including murine CSF-1R itself, nor could they be induced to mature within the monocyte lineage by GM-CSF, LPS, PGE2, LTβR, TPA, vitamin D3, or 5-azacytidine. Thus, although 32DC13 cells remain competent to differentiate to granulocytes, they may be inherently unable to complete the alternative differentiation program characteristic of normal mononuclear phagocytes.

Normal IL-3–responsive bone marrow progenitors are committed to differentiate, and although more mature myeloid elements arising from them can be isolated, they cannot be maintained in culture. 4 In contrast, the process of establishing factor-dependent cell lines may select for the constitutive expression of transcriptional regulatory factors that inhibit receptor-mediated signals for differentiation. Mouse leukemic cells containing retroviraly activated c-myb 48 and Evi-1 49 genes represent examples of immortalized lines that remain IL-3–dependent but are unable to differentiate. The latter proto-oncogenes encode nuclear DNA-binding proteins that are likely to regulate gene expression. Evi-1 is not normally expressed in hematopoietic cells, 42 but its activation in myeloid cells interrupts their differentiation program without abrogating their growth factor dependence. We would predict that introduction of c-fms into such cells might render them CSF-1 responsive without inducing complete monocytic maturation. An additional caveat in interpreting our experiments is that human CSF-1R may not function appropriately in mouse myeloid cells; eg, FDC-P1 cells that expressed human CSF-1R showed no evidence of monocytic differentiation, 18 whereas the murine c-fms gene induced monocytic features when the cells were propagated in CSF-1. 19

After introduction of the human c-fms gene with pSV2neo, the levels of human CSF-1R expressed in a mixed population of receptor-positive 32D[R+] cells were relatively low, and only 7 of 21 individual G418-resistant cultures ultimately gave rise to cells that grew in CSF-1. In contrast, 32D[R+]C1.1, C1.2, and C1.3 cells, selected for growth in

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**FIG 6. Identification of phosphotyrosine-containing proteins in IL-3 or CSF-1 stimulated cells.** 32DC13 cells (lanes 1 and 2), 32D[R+] cells (lanes 3 through 5), and 32D[R+]C1.1 cells (lanes 6 through 8) were cultured in factor-free medium for 18 hours and either left unstimulated (lanes 1, 3, and 6) or incubated for 10 minutes with 250 U/ml murine IL-3 (lanes 2, 4, and 7) or with 2 nmol/L human recombinant CSF-1 (lanes 5 and 8) before lysis. Proteins were separated on denaturing polyacrylamide gels, transferred to nitrocellulose, and blotted with an antibody to phosphotyrosine followed by 125I-labeled *Staphylococcus aureus* protein A. 44 The positions of known molecular weight standards are indicated in kilodaltons in the left margin. Tyrosine phosphorylated substrates discussed in the text are denoted by arrowheads in each lane.
medium containing human recombinant CSF-1, expressed significantly more CSF-1R than cells passaged in IL-3, as judged both by FACS analysis and by biosynthetic labeling studies. Thus, only those transformants expressing high levels of human CSF-1R appeared to have had a proliferative advantage when the cells were shifted to medium containing CSF-1. In FDC-P1 cells, high levels of human CSF-1R expression were also associated with the emergence of factor-independent variants, which depended on CSF-1R-mediated signals for cell growth. In contrast, 32D[R+]C1.1, C1.2, and C1.3 cells remained factor-dependent throughout months of continuous passage in culture, as did single-cell subclones derived from them, and only one factor-independent cell line was obtained. Based on the elevated basal levels of receptor tyrosine phosphorylation observed in these cells, we presume that CSF-1R kinase activity can provide certain variants with a selective growth advantage, even in the absence of ligand.

Because 32D[R+]C1.1 cells remained dually responsive to IL-3 and CSF-1, we were able to examine whether IL-3 treatment transmodulated the CSF-1R receptor by inducing its accelerated degradation. We considered this possibility because IL-3 treatment at 37°C can induce the rapid loss of CSF-1 binding sites from normal mouse bone marrow cells by an as yet ill-defined mechanism. Although phorbol esters and other inducers of protein kinase C can transmodulate CSF-1R by activating a protease that cleaves the receptor near its membrane-spanning segment, IL-3 treatment had no effect on CSF-1R turnover in 32D[R+]C1.1 cells. Reciprocally, insertion of the constitutively active v-fms oncogene into FDC-P1 cells, although able to confer factor-independence, did not affect the number or affinity of IL-3 binding sites expressed at the cell surface. TPA transmodulated CSF-1R in both 32D[R+]C1.1 and BAC1.2F5 cells, but LPS induced CSF-1R turnover only in BAC1.2F5 macrophages. This implies that LPS does not mediate its effects by directly activating protein kinase C, but rather, its active moiety (lipid A) may bind to another receptor that is differentially expressed in these two cell types.

Although the mechanisms of signal transduction by the IL-3 receptor remain unclear, IL-3 has been shown to bind to a 140-Kd cell surface glycoprotein and to stimulate its phosphorylation on tyrosine residues. After stimulation of 32D[R+]C1.1 cells with IL-3, immunoblotting analyses performed with antibodies to phosphotyrosine showed the presence of a 140-Kd substrate as well as a second major tyrosine phosphorylated species of 95 Kd. One or both of these proteins might well represent components of the IL-3 receptor or, alternatively, substrates of an IL-3 receptor-associated tyrosine kinase. CSF-1 treatment of the same cells induced the appearance of different tyrosine phosphorylated substrates, including human CSF-1R itself. However, like IL-3, CSF-1 induced tyrosine phosphorylation of a 95-Kd protein. Apart from their molecular masses, we have no further biochemical evidence that the 95-Kd phosphoproteins observed in response to IL-3 and CSF-1 represent the identical polypeptide. Therefore, although direct transmodulation of CSF-1R by IL-3 was not observed, our results do not preclude "crosstalk" between the two receptor signaling pathways.

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