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The Zinc Finger Transcription Factor Egr-1 Potentiates Macrophage Differentiation of Hematopoietic Cells

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Previously we have shown that the zinc finger transcription factor Egr-1 is essential for and restricts differentiation of hematopoietic cells along the macrophage lineage, raising the possibility that *Egr*-1 actually plays a deterministic role in governing the development of hematopoietic precursor cells along the monocytic lineage. To test this hypothesis, we have taken advantage of interleukin-3-dependent 32Dcl3 hematopoietic precursor cells which, in addition to undergoing granulocytic differentiation in response to granulocyte colony-stimulating factor, were found to be induced for limited proliferation, but not differentiation, by granulocyte-macrophage colony-stimulating factor. It was shown that ectopic expression of *Egr*-1 blocked granulocyte colony-stimulating factor-induced terminal granulocytic differentiation, consistent with previous findings. In addition, ectopic expression of *Egr*-1 endowed 32Dcl3 cells with the ability to be induced by granulocyte-macrophage colony-stimulating factor for terminal differentiation exclusively along the macrophage lineage. Thus, evidence that Egr-1 potentiates terminal macrophage differentiation has been obtained, suggesting that *Egr*-1 plays a deterministic role in governing the development of hematopoietic cells along the macrophage lineage.

A hierarchy of hematopoietic progenitor cells in the bone marrow proliferate and terminally differentiate along multiple, distinct cell lineages, whereby the short life span and limited proliferative capability of most mature blood cell types dictate continuous regeneration. Hematopoiesis is a profound example of cell homeostasis which is regulated throughout life, including the proliferation and differentiation of myeloid precursor cells into granulocytes and macrophages (17, 22, 24). Clearly, a variety of control mechanisms are needed to maintain steady-state levels of mature blood cells, as well as to stimulate the rapid production of specific cell types as needed. To achieve this requires the participation of many factors, including positive and negative regulators of growth and differentiation, which determine survival, growth stimulation, differentiation, functional activation, and programmed cell death (apoptosis) (23, 26).

To identify genes that may play a role in the regulation of hematopoietic cell differentiation, we have isolated cDNA clones of myeloid differentiation primary response (*MyD*) genes, activated in the absence of de novo protein synthesis, in HL-60 and M1 cells following induction for macrophage or granulocyte differentiation (14, 16, 20). In the course of this work, the gene encoding the zinc finger transcription factor Egr-1 (Krox24, NGIF-A, or Zif268/Tis8) has been identified as a myeloid differentiation primary response gene, specifically induced upon HL-60 macrophage differentiation. *Egr*-1 was initially identified as a growth response gene in cultured fibroblasts (4, 8, 11, 28) and subsequently shown to be induced in response to B-cell maturation as well as during differentiation of nerve, bone, and myeloid cells (2, 18, 27, 29, 30). *Egr*-1 was found by us to be a macrophage differentiation primary response gene which is essential for and restricts differentiation of hematopoietic cells along the macrophage lineage (20).

These observations raised the possibility that Egr-1 actually potentiates the development of hematopoietic precursor cells along the monocytic lineage. In the present work this hypothesis was tested with the hematopoietic progenitor cell line 32Dcl3, which requires interleukin-3 (IL-3) for growth, undergoes limited proliferation and terminal granulocytic differentiation upon removal of IL-3 and addition of granulocyte colony-stimulating factor (G-CSF), and also exhibits limited proliferative capability, but no differentiation, in response to granulocyte-macrophage colony-stimulating factor (GM-CSF). We have shown that ectopic expression of *Egr*-1 in 32Dcl3 clones blocked G-CSF-induced granulocytic differentiation and endowed these cells with the ability to be induced by GM-CSF for terminal differentiation exclusively along the macrophage lineage. Thus, ectopic expression of *Egr*-1 altered the differentiation potential of these cells, allowing terminal monocytic differentiation. These results suggest that *Egr*-1 plays a deterministic role in monocytic differentiation.

MATERIALS AND METHODS

Cells and cell culture. 32Dcl3(G) cells, obtained from Giovanni Rovera (Wistar Institute), were cultured in RPMI 1640 (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 10% WEHI-3B conditioned medium (CM) (source of IL-3) (31) and are referred to as 32Dcl3. To isolate clones of 32Dcl3 cells which were responsive to GM-CSF, 32Dcl3 cells were seeded in tissue culture plates (10^3 cells per 50-mm-diameter culture plate) in a thin layer (1.7 ml) of soft agar (0.33%) on top of a base (5 ml) of hard agar (0.5%) in RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, with the hard agar containing various concentrations of purified recombinant murine GM-CSF (2 to 20 ng/ml; a gift from Amgen, Inc.), essentially as previously described (5, 12). Each day, plates were examined microscopically for the development of GM-CSF-responsive colonies. Following 4 to 8 days of incubation in a humidified atmosphere at 37°C with 10% CO_2, one to five colonies per plate were usually scored at GM-CSF concentrations of ≥10 ng/ml; most of these colonies were observed to degenerate following longer incubation

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times (>12 days). To obtain clones of 32Dcl3 cells that respond to GM-CSF, clusters (≤25 cells) of 32Dcl3 cells were isolated from the soft agar with sterile Pasteur pipettes, following 5 to 6 days of incubation in the presence of 10 ng of GM-CSF per ml, by dispersing the agar clot in 1 ml of RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 10% WEHI-3B conditioned medium (13). Following expansion of each clone in the presence of IL-3, cells from each clone were tested for their ability to proliferate and/or differentiate in culture in the presence of purified recombinant G-CSF (10 ng/ml) (a gift from Amgen, Inc.) or GM-CSF (10 ng/ml) (a gift from Amgen, Inc.). 32Dcl3 clones GM-2, GM-3, GM-5, GM-6, GM-7, GM-9, and GM-16 all behaved similarly to each other as well as to parental 32Dcl3 with regard to growth and differentiation, as well as Egr-1 expression, following treatment with either G-CSF or GM-CSF, although each cytokine elicited a different response (see Results). To remove IL-3, cells were washed twice in complete medium in the absence of IL-3; then either G-CSF or GM-CSF was added. Cells were seeded at a density of 0.2×10^6/ml to be maintained in IL-3 and were seeded at a density of 0.5×10^6/ml when either G-CSF or CM-CSF was added to the culture medium. The culture medium, including appropriate cytokines, was changed every 48 h. With G-CSF, following 48 h the cells were usually reseeded at a concentration of 0.5×10^6/ml to avoid overgrowth. Viable cell numbers were determined by trypan blue dye exclusion with counting in a hemocytometer. In all cases, experiments were repeated at least three times.

Assay for differentiation. Morphological differentiation was determined by counting at least 300 cells on May-Grunwald-Giemsa-stained cytospin smears and scoring the proportion of immature blast cells, cells at the intermediate granulocyte or monocyte stage of differentiation, and mature granulocytes or macrophages (20). The nonspecific esterase (NSE) assay was carried out by staining cells on plates (19). Results of all experiments represent the means from at least three independent determinations with standard deviations up to 15% (e.g., 11% = 11% ± 1.6%).

General recombinant DNA techniques, expression vectors, and DNA probes. Plasmid preparations, restriction enzyme digestions, DNA fragment preparations, and agarose gel electrophoresis were as described before (6, 25). Probes for the murine *Egr*-1, β-actin, lysozyme, and ferritin genes were the same as used previously (6, 15, 20, 25); the murine myeloperoxidase gene was kindly provided by G. Rovera (Wistar). DNA for probes was labeled by random priming (RadPrime DNA labeling; catalog number 18428-011; GIBCO-BRL) to a specific activity equal to or greater than 10^9 cpm/μg. Genomic DNA extraction and Southern blot analysis were done as described previously (14, 20).

RNA extraction, Northern blotting, and hybridization. RNA was extracted by the method of Chomczynski and Sacchi (3) with guanidinium thiocyanate. Total RNA (10 μg per lane; the presence of equal amounts of RNA in each lane was confirmed by equal intensity of ethidium bromide staining of rRNA bands) was electrophoresed on 1% agarose formaldehyde gels. Northern (RNA) blots with Duralon-UV membranes (Stratagene) were prepared and UV cross-linked (Stratalinker; Stratagene) prior to baking for 2 h. Blots were hybridized in a mixture of 50% deionized formamide, 10% dextran sulfate, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), and 100 μg of sheared salmon sperm DNA per ml at 42°C with 10^6 cpm of probe per ml for 12 to 16 h. Blots were washed at room temperature twice for 5 min each time in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and at 60°C twice for 30 min each time in 0.1× SSC–1% SDS and were exposed to X-ray film at −80°C for 48 to 72 h. Stripping blots of probe to rehybridize was done as described previously (14).

G-CSFR and GM-CSFR expression analysis by PCR. To increase the sensitivity of detection of transcripts encoding G-CSF receptors (G-CSFR) and GM-CSF receptors (GM-CSFR), reverse transcription (RT-PCR) was carried out with aliquots of RNA essentially as described previously (1). Briefly, 3 μg of total RNA, extracted by the method of Chomczynski and Sacchi (3), was reverse transcribed with the GIBCO-BRL Superscript preamplification system (catalog number 180-89-011), according to the manufacturer's instructions, in a final volume of 21 μl with oligo(dT) as a primer. For PCR, 2 μl of cDNA was taken from each RT reaction volume and samples were diluted to 50 μl with 10× BMB, yielding 0.1 mM deoxynucleoside triphosphates, 0.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), and 50 mM KCl (1× BMB); then a 0.1 μM concentration of each primer and 5 U of *Taq* DNA polymerase (BMB) were added. Samples were covered with 50 μl of mineral oil, heated at 94°C for 5 min, and subjected to PCR in a Perkin-Elmer thermal cycler for 15 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 62°C, and 2 min of polymerization at 72°C; finally, 5 min of polymerization was done at 72°C. The primers used were selected with the aid of the program PCRPLAN (PCGENE; Intelligenetics). For the GM-CSFR transcripts, the 24-mers corresponded to bases 253 to 276 and 1158 to 1134 of the α subunit of the murine GM-CSFR gene. For the G-CSFR transcripts, the primers corresponded to bases 307 to 326 and 1217 to 1240 of the murine G-CSFR gene. To monitor for efficiency and reproducibility of PCR amplification, β-actin transcripts were assessed with murine β-actin amplimers (Clontech). After extraction with $CHCl_3$, 40 μl of products was electrophoresed on 1% agarose gel, blotted, and hybridized with the murine GM-CSFR α-subunit probe (800-bp *Not*I-*Pst*I fragment excised from pBluescript SK [clone 71, a gift from L. S. Parks, Immunex]) (21), containing the coding region of the murine GM-CSFR α-subunit cDNA and including the amplified PCR region; the murine G-CSFR probe (3.2-kb *Xho*I cDNA insert in pBluescript [a gift from J. Ihle]); or a β-actin probe (catalog number 9800-1; within the amplified PCR region; Clontech). Control samples not reverse transcribed were used to monitor for possible contamination with genomic DNA.

Establishment of 32Dcl3 cell lines that ectopically express an *Egr*-1 transgene. To establish 32Dcl3 cell lines that ectopically express an *Egr*-1 transgene, both parental and clone GM-7 32Dcl3 cells were transfected via electroporation with plasmid pAC.Egr-1S or pHbAPr-1-neo (as a control) (20). A pulse (600 V, 25 μF; Bio-Rad Gene Pulser) was delivered to a 0.8-ml suspension containing 5.2×10^6 cells and 50 μg of linearized plasmid DNA. The cells were appropriately diluted and after 48 h were seeded at 5×10^4/ml in growth media containing G418 (geneticin, 400 μg/ml; GIBCO) and IL-3 (10% WEHI-3B conditioned medium), and 1-ml aliquots were dispensed into 24-well trays. After 3 to 4 weeks, cultures from wells containing surviving cells were expanded in the presence of G418 and IL-3. Transfectants were maintained in growth media including 200 μg of G418 plus IL-3. Transfectants analyzed at both the molecular and genetic levels were clones 32Dneo1, 32Dneo4, 32DEgr1.2, 32DEgr1.5, 32DEgr1.16, and 32DEgr1.19, which were derived from parental 32Dcl3, and clones 32Dneo7, 32DNeo9, 32DEgr1.6, 32DEgr1.10, 32DEgr1.14, and 32DEgr1.25, which were derived from 32Dcl3 clone GM-7.

RESULTS

Both G-CSF and GM-CSF stimulated the proliferation of IL-3-dependent, hematopoietic precursor 32Dcl3 cells (Fig. 1A), where the proliferative response of the cells to GM-CSF was observed to take place after a brief lag period which coincided with a loss of viability. A gradual cessation of proliferation between 6 and 8 days was observed as a response to treatment by either of these cytokines. May-Grunwald-Giemsa-stained cytospin smears of cells at various times following treatment with the different cytokines were analyzed to determine if any differentiation occurred. Immature blast cells are characterized by scant cytoplasm and round or oval nuclei, granulocyte intermediates are characterized by dented but not lobulated nuclei, and mature granulocyte cells are characterized by banded and lobulated nuclei. In the case of G-CSF-stimulated cells, limited proliferation was correlated with terminal differentiation of the cells into neutrophilic granulocytes (Fig. 1B). For cells treated with GM-CSF, the loss in the proliferative response was not accompanied by cell differentiation (Fig. 1B), indicating that the proliferative response of the 32Dcl3 cells to GM-CSF was limited and occurred in the absence of cell differentiation. In addition to the loss in viability immediately following treatment with GM-CSF, loss of viability of GM-CSF-treated cells, as determined by trypan blue exclusion, started to be noticed after 10 days, with no viable cells detected following 16 days.

To determine if the response of the 32Dcl3 cells to GM-CSF was indicative of a heterogeneous population of cells, clonal populations of 32Dcl3, which proliferated in the presence of GM-CSF, were isolated. As described in detail in Materials and Methods, cells were seeded in agar containing various concentrations of GM-CSF. Following 4 to 8 days, colonies were detected with GM-CSF concentrations of ≥10 ng/ml; most of these colonies were observed to degenerate following longer incubation times. Therefore, to obtain clones, clusters (≤25 cells) of 32Dcl3 cells were isolated and expanded in the presence of IL-3. Each clone was tested for its ability to proliferate and/or differentiate in culture in the presence of G-CSF or GM-CSF. All clones (GM-2, GM-3, GM-5, GM-6, GM-9, and GM-16) behaved similarly to the starting population of 32Dcl3 cells, demonstrating that the response of the initial population of cells to GM-CSF was not due to its being a heterogeneous population.

In this work all experiments were carried out using the initial starting population of 32Dcl3 cells as well as the GM-7 clone.

Expression of endogenous *Egr*-1 and establishment of 32Dcl3 clones ectopically expressing *Egr*-1. It has been previously shown that *Egr*-1 is a myeloid differentiation primary response gene which is activated upon induction of monocytic, but not

FIG. 1. Growth and differentiation characteristics of 32Dcl3 cells following stimulation with G-CSF or GM-CSF. (A) Growth kinetics in culture medium supplemented with either IL-3 (10% WEHI-3B conditioned medium), G-CSF (10 ng/ml), or GM-CSF (10 ng/ml). Cells were seeded as indicated, and viable cell numbers were determined by trypan blue dye exclusion with counting in a hemocytometer. (B) Photomicrographs (magnification, ×400) of 32Dcl3 cells in the presence of IL-3, following 9 days of stimulation with G-CSF and following 10 days of stimulation with GM-CSF. Shown are representative photomicrographs of May-Grunwald-Giemsa-stained cytospin smears, prepared as previously described (20). Clones of 32Dcl3 cells which respond to GM-CSF were isolated as described in Materials and Methods and tested for their response to G-CSF and GM-CSF in liquid culture. Similar results were obtained for parental 32Dcl3 cells, as well as GM-CSF-responsive 32Dcl3 clones GM-2, GM-3, GM-5, GM-6, GM-7, GM-9, and GM-16. All values represent the means from three independent experiments.

granulocytic, differentiation, independent of de novo protein synthesis (20). To ascertain if the 32Dcl3 cell line is an appropriate model system to test our hypothesis that *Egr*-1 plays a deterministic role in monocytic differentiation, *Egr*-1 expression was assessed following treatment with either G-CSF or GM-CSF by Northern blot analysis. As can be seen in Fig. 2A, *Egr*-1 transcripts were not detectable in cells cultured in the presence of either IL-3 (zero time point) or GM-CSF. However, consistent with what was previously observed for *Egr*-1 expression in normal murine myeloid precursor-enriched bone marrow cells induced for granulocytic differentiation with G-CSF (20), *Egr*-1 transcripts were detected at late times following G-CSF stimulation of 32Dcl3 cells for granulocytic differentiation (Fig. 2A).

One means to better understand the role that *Egr*-1 plays in the control of blood cell differentiation is to examine the consequences of ectopic expression of *Egr*-1 on the differentiation potential of 32Dcl3 cells following stimulation with either G-CSF, which induces granulocytic differentiation, or GM-CSF, which induces limited proliferation in the absence of cell differentiation (Fig. 1). 32Dcl3 cell lines constitutively expressing an *Egr*-1 transgene (32DEgr1) have been established via electroporation with the vector pAC.Egr-1S, with the coding region of *Egr*-1 under control of the human β-actin promoter (Fig. 2B, C, and D). Southern blot analysis of each of the clones (data shown for clones 32DEgr1.6 and 32DEgr1.25) confirmed that each was an independent clone by distinct integration sites of the *Egr*-1 transgene (Fig. 2B). The lack of *Egr*-1 transcripts in untreated parental and 32Dneo cell lines and the presence of *Egr*-1 transgene transcripts in four different transfectants, which was not regulated by either G-CSF or GM-CSF, are shown in Fig. 2C and D.

All of the experiments described below were carried out at least three times with clones 32DEgr1.2, 32DEgr1.5, 32DEgr 1.6, 32DEgr1.25, 32Dneo1, and 32Dneo7, as well as with parental 32Dcl3 (both the starting population and clone GM-7). In addition, clones 32DEgr1.10, 32DEgr1.14, Egr1.16, Egr1.19, 32Dneo4, and 32Dneo9 were employed for cell biology experiments.

Effect of ectopic *Egr*-1 expression on G-CSF-stimulated 32Dcl3 cells. The effect of ectopic expression of *Egr*-1 on G-CSF-mediated growth and granulocytic differentiation was determined. In contrast to parental 32Dcl3 and 32Dneo clones, 32DEgr1 cell lines underwent limited proliferation in the presence of G-CSF, with many dead cells present the first 2 days following treatment (Fig. 3A). By 10 days following treatment, no dead cells were observed and the cell population consisted of predominantly intermediate-stage granulocytes (Table 1), with very few mature cells. This is in contrast to parental 32Dcl3 and 32Dneo clones, in which over 80% of the cells were

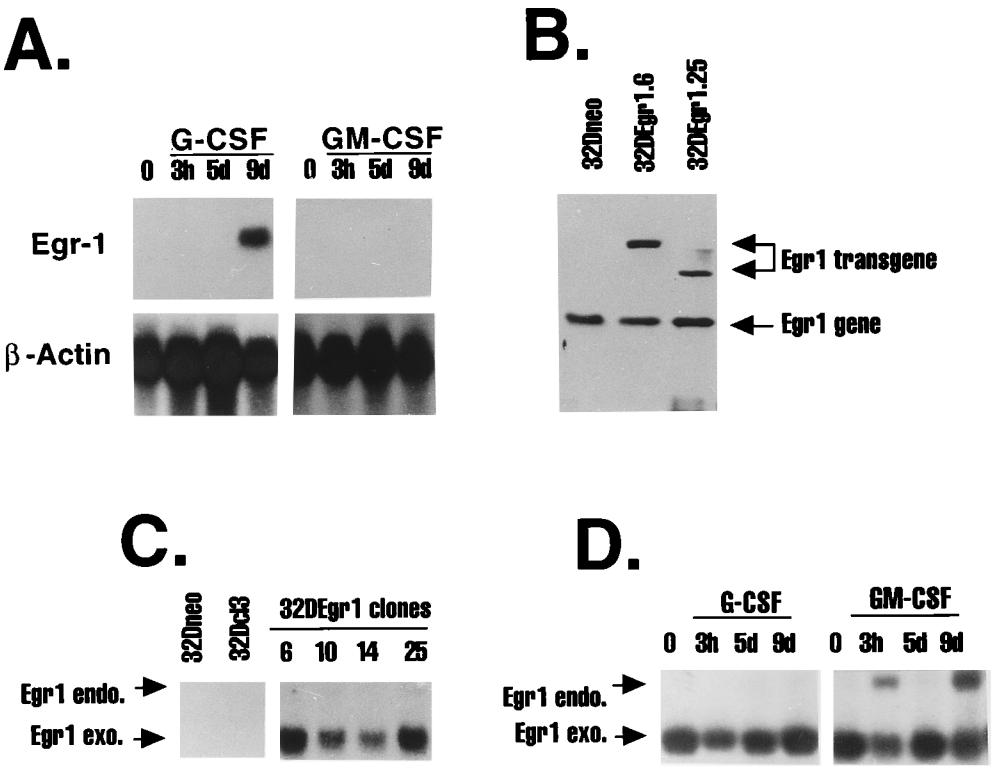

FIG. 2. (A) Analysis of endogenous *Egr*-1 expression in 32Dcl3 cells before and after stimulation with either G-CSF or GM-CSF; (B, C, and D) establishment of 32Dcl3 cell lines that ectopically express an *Egr*-1 transgene. (A) Endogenous *Egr*-1 expression was analyzed by hybridization of a murine *Egr*-1 probe to RNA blots, using total RNA (10 μg per lane) extracted from 32Dcl3 cells before and after stimulation with G-CSF (10 ng/ml) or GM-CSF (10 ng/ml) at the indicated times. The same Northern blots were stripped and reprobed with β-actin to demonstrate that equal amounts of RNA were present in each lane. (B) Southern blot analysis of genomic DNA from 32Dneo and two different 32DEgr1 clones. Genomic DNA (10 μg) was digested with *Eco*RI (null cutter for pAC.Egr-1S, the transfecting plasmid), resolved on a 1% agarose gel, transferred to GeneScreen Plus (NEN), and hybridized to a murine *Egr*-1 DNA probe. (C) Expression of *Egr*-1 in four different 32DEgr1 clones. Total RNA was extracted from the parental 32Dcl3, 32Dneo, and 32Degr1 clones, and *Egr*-1 expression was determined by Northern blot analysis. Transcripts from endogenous (endo.) and exogenous (exo.) *Egr*-1 genes are indicated. (D) Northern blot analysis of *Egr*-1 expression in the 32DEgr1.6 cell line following treatment with G-CSF (10 ng/ml) or GM-CSF (10 ng/ml) for the indicated times.

mature granulocytes. Since eight distinct 32DEgr1 clones exhibited the same response to G-CSF, it can be concluded that the observed pattern of survival, proliferation, and limited differentiation reflects the behavior of a clonal population of cells.

The limited response to G-CSF of the Egr-1-expressing clones of 32Dcl3 could be due to the suppression of the expression of G-CSFR. In the 32Dcl3, 32Dneo, and 32DEgr1 cell lines, G-CSFR transcripts were expressed at comparable levels in all the clones examined (Fig. 3C), and the level was upregulated following stimulation with G-CSF (data not shown). Thus, the Egr-1 block to terminal granulocytic differentiation is not related to alterations in the level of G-CSFR expression. In addition, in all these cell lines myeloperoxidase transcripts were induced to the same extent by G-CSF. However, no endogenous *Egr*-1 transcripts were induced in 32DEgr1 cell lines by G-CSF, whereas G-CSF treatment of parental and 32Dneo cell lines resulted in induction of *Egr*-1 transcripts, detected 9 days following treatment (compare Fig. 2A and D). Taken together, these data demonstrated that 32DEgr1 cells exhibited a restricted response to G-CSF, undergoing limited differentiation but failing to terminally differentiate.

These observations extended our previously reported findings that ectopic expression of *Egr*-1 blocks dimethyl sulfoxide-induced granulocytic differentiation of human myeloblastic leukemia HL60 cells (20) to show that it also blocked terminal granulocytic differentiation induced by physiological factors.

Effect of ectopic *Egr*-1 expression on GM-CSF-stimulated 32Dcl3 cells. Parental 32Dcl3 cells did not differentiate following stimulation with GM-CSF; however, there was limited proliferation, and the cells remained viable for 10 days (Fig. 1). Given that *Egr*-1 appears to be necessary for and restricts differentiation to the monocytic lineage (20), the question of whether *Egr*-1 can play a role in promoting monocytic differentiation can be asked. With this in mind, the effect of GM-CSF treatment on 32DEgr1 cells was determined.

As seen in Fig. 4, all GM-CSF-treated 32D cell lines examined exhibited limited proliferation. In contrast to parental 32Dcl3 and 32Dneo cell lines, 32DEgr1 cell lines underwent macrophage differentiation (Table 2 and Fig. 4). This was evident from the characteristic macrophage morphology of 32DEgr1 cells (>50% by 6 days; >86% by 10 days). The cells first became adherent and later flattened and spread out on the surface of the culture dish, acquiring a macrophage-like morphology (Fig. 4B). As seen by May-Grunwald-Giemsa staining, immature blast cells are characterized by scant cytoplasm and round or oval nuclei; cells at intermediate monocyte stages of differentiation are flattened, with a larger cytoplasm-to-nucleus ratio, irregularly shaped nuclei, and few interspersed or no vacuoles; and mature macrophage-like cells are flattened and spread out cells interspersed with numerous vacuoles (Fig. 4B). No evidence of granulocytic differentiation was observed in 32DEgr1 cells treated with GM-CSF. As noted previously, neither the parental 32Dcl3 nor 32Dneo cells underwent mor-

FIG. 3. Analysis of the growth and differentiation characteristics of the parental 32Dcl3, 32Dneo, and 32DEgr1 cell lines in response to G-CSF (10 ng/ml). (A) Growth kinetics following treatment with G-CSF. Viable cell numbers were determined by trypan blue exclusion with counting in a hemocytometer. (B) Photomicrographs of 32Dneo7 and 32DEgr1.6 cells stained with May-Grunwald-Giemsa stain (magnification, ×400) in the presence of IL-3 and following stimulation for 9 days with G-CSF. The photomicrographs of cells stained with May-Grunwald-Giemsa stain were taken from cytospin smears. 32DEgr1.2, 32DEgr 1.5, 32DEgr1.10, 32DEgr1.14, 32DEgr1.16, 32DEgr1.19, and 32DEgr1.25 cells gave results similar to those of 32DEgr1.6; 32Dneo1, 32Dneo4, 32Dneo9, and parental cells gave results similar to those of 32Dneo7. (C) RT-PCR analysis of the level of transcripts encoding G-CSFR in 32DEgr1 cells compared with that in 32Dneo cells. RT-PCR was performed, and PCR products were visualized and quantitated as described in Materials and Methods. (D) Analysis of the expression of myeloperoxidase (MPO) mRNA in 32DEgr1.6 cells compared with that in 32Dneo7 cells, before and after stimulation with G-CSF. Northern blots of total RNA (10 μg per lane) were prepared as described in Materials and Methods and hybridized with probes specific to myeloperoxidase. These are representative experiments, where similar results were obtained three times.

phological differentiation to monocytes following stimulation with GM-CSF (Fig. 1 and 4 and Table 2).

In addition to morphology, we assayed terminal differentiation markers, including the terminal macrophage-specific marker NSE (Table 2), and expression of lysozyme and ferritin light chains (Fig. 5A). When stimulated with GM-CSF, after 10 days 92 to 100% of the 32DEgr1 cells stained positive for NSE, whereas 5%, or fewer, of the parental and 32Dneo cells stained for NSE (Table 2). Interestingly, ectopic expression of *Egr*-1 resulted in elevated expression of NSE in the absence of morphological differentiation in cells unstimulated with IL-3.

Analysis of the expression of lysozyme and ferritin light-chain mRNAs, molecular markers of macrophage differentiation of M1 myeloid leukemic cells (15, 25), further corroborated the finding that 32DEgr1 cells underwent macrophage differentiation following stimulation with GM-CSF. Expression of lysozyme was not detected in unstimulated or GM-CSF-treated 32Dneo cells (Fig. 5A), whereas in the 32DEgr1 cells lysozyme mRNA was induced to high levels by 5 days and persisted at the same level up to 10 days following stimulation with GM-CSF (Fig. 5A). Low basal-level expression of ferritin mRNA was detected in the uninduced 32Dneo cells, with a slight increase at late times following stimulation with GM-CSF (Fig. 5A). In contrast, in the 32DEgr1 cells expression of ferritin mRNA reproducibly was observed to be elevated in the unstimulated cells compared to that in 32Dneo cells (analogous to NSE expression [Table 2]) and increased up to ~15-fold following stimulation with GM-CSF. Finally, endogenous *Egr*-1 transcripts were induced at both early and late times following treatment of 32DEgr1 cells with GM-CSF (Fig. 2D), similar to what was observed during differentiation of myeloid precursor-enriched bone marrow and M1 myeloid cells (20), whereas no GM-CSF-induced *Egr*-1 transcripts were detected in parental or 32Dneo cells (Fig. 2A).

To ascertain if the ability of the 32DEgr1 cells to undergo GM-CSF-induced macrophage differentiation is due to up-regulation of GM-CSFR, the expression of GM-CSFR in 32Dneo and 32DEgr1 transfectants was determined. No difference was observed in the low basal level of transcripts encoding GM-CSFR in unstimulated 32Dneo cells and 32DEgr1 cells (Fig. 5B). However, transcripts encoding GM-CSFR were induced and continued to be expressed at higher levels in 32DEgr1 cells than in 32Dneo cells following stimulation with GM-CSF (Fig. 5B), consistent with the observed macrophage differentiation of these cells.

Taken together, these data demonstrate that ectopic expression of *Egr*-1 actually endowed the 32Dcl3 cells with the capability to be induced by GM-CSF for terminal differentiation exclusively along the macrophage lineage (Fig. 4B), suggesting a deterministic role for *Egr*-1 in macrophage differentiation.

Effect of G-CSF priming on GM-CSF responsiveness of parental and *Egr*-1-expressing 32Dcl3 cells. It has been shown that priming 32Dcl3 cells with G-CSF induces GM-CSFR; when these cells are then stimulated with GM-CSF, they undergo differentiation along both the granulocytic and monocytic lineages and also maintain themselves as a self-renewing population (7). To increase our understanding of the role of *Egr*-1 as a regulator of blood cell development, the effect of ectopic expression of *Egr*-1 on G-CSF-primed 32Dcl3 cells was ascertained.

As reported, G-CSF-primed 32Dcl3 cells treated with GM-

TABLE 1. Differentiation characteristics following stimulation with G-CSF in 32Dneo versus 32DEgr1 clones

Cell line	Granulocytic cell type (%)[a]		
	Blast	Intermediate	Mature
32Dneo1	2	14	84
32Dneo7	2	16	82
32DEgr1.2	24	73	3
32DEgr1.5	23	72	5
32DEgr1.6	27	71	2
32DEgr1.25	26	65	9

[a] Cell morphology was determined following 9 days of stimulation with G-CSF. Morphological differentiation was determined by counting at least 300 cells on May-Grunwald-Giemsa-stained cytospin smears and scoring the proportion of immature blast cells, cells at intermediate stages of differentiation, and mature myeloid cells. Results of all experiments represent the means of at least three independent determinations, with standard deviations up to 15% (e.g., 14% = 14% ± 2.1%).

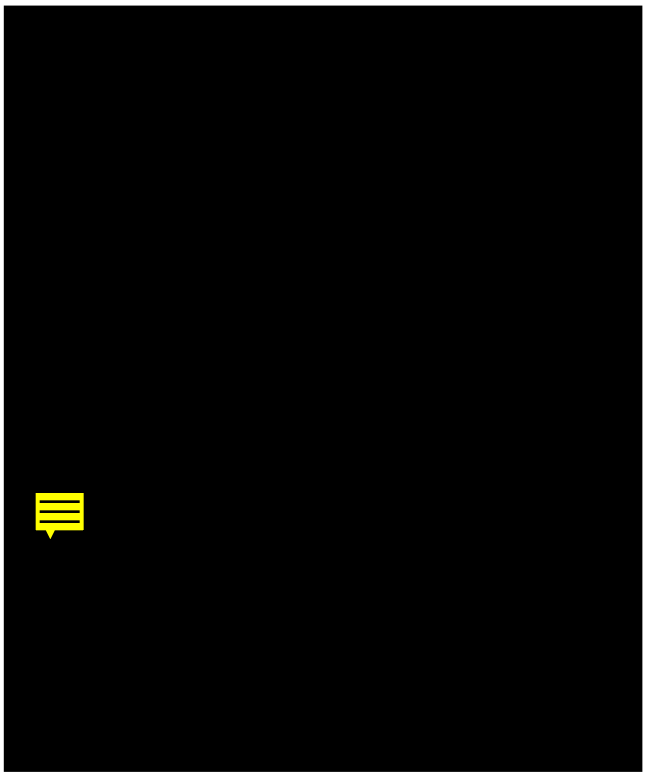

FIG. 4. Analysis of the growth and differentiation characteristics of the parental 32Dcl3, 32Dneo, and 32DEgr1 cell lines in response to GM-CSF (10 ng/ml). (A) Growth kinetics following treatment with GM-CSF. Viable cell numbers were determined by trypan blue exclusion with counting in a hemocytometer. (B) Photomicrographs of 32Dneo7 and 32DEgr1.6 cells stained with May-Grunwald-Giemsa stain (magnification, ×400) or in culture (magnification, ×90) in the presence of IL-3, following stimulation for 9 days with G-CSF or for 10 days with GM-CSF. The photomicrographs of cells stained with May-Grunwald-Giemsa stain were taken from cytospin smears, whereas the photomicrographs of cells in culture were taken after rinsing the tissue culture plates with RPMI 1640 medium (three times), so that only cells which remained attached to the surface of the tissue culture plate are shown. It should be noted that 32DEgr1.2, 32DEgr1.5, 32DEgr1.10, 32DEgr1.14, 32DEgr1.16, 32DEgr1.19, and 32DEgr1.25 cells stimulated for 10 days with GM-CSF showed morphological characteristics of macrophage differentiation similar to those shown for 32DEgr1.6 cells, whereas none of the 32Dneo clones underwent macrophage differentiation.

CSF underwent both granulocytic and monocytic differentiation, with fourfold more mature granulocytes than monocytes, and the remainder of the population either remained blasts or differentiated to early intermediate stages of granulocytes or macrophages (Table 3). In contrast to the parental 32Dcl3 and control 32Dneo cells, 32DEgr1 cells primed with G-CSF and then treated with GM-CSF underwent terminal macrophage differentiation (Table 3), with most of the cells adhering to the culture dish and with a loss of proliferative capability associated with the terminally differentiated phenotype. These data further corroborate the previous observations that *Egr*-1 restricts differentiation to the monocytic lineage and support the notion that *Egr*-1 potentiates terminal monocytic differentiation. Furthermore, this line of experimentation reinforces the observation that the effect of ectopic expression of *Egr*-1 on the response of 32D cells to GM-CSF is not mediated by up-regulation of GM-CSFR.

DISCUSSION

In this work it was shown that IL-3-dependent hematopoietic precursor cells, which do not differentiate in response to GM-CSF, have acquired the ability to be induced by GM-CSF to undergo differentiation exclusively along the macrophage lineage when genetically manipulated to express an *Egr*-1 transgene. These findings provide the first experimental evidence to indicate that Egr-1, previously identified as a zinc finger nuclear regulator that may be essential for and restricts differentiation of myeloid precursor cells along the macrophage lineage (20), also potentiates terminal macrophage differentiation, suggestive of a role for *Egr*-1 in determining the development of hematopoietic precursor cells along the macrophage lineage.

By using antisense oligonucleotides in the culture medium it had been demonstrated that *Egr*-1 may be essential for monocytic differentiation of normal bone marrow, as well as for various cell lines (20). In addition, it was shown that ectopic expression of *Egr*-1 in HL60 cells, which can be induced to undergo either granulocytic or monocytic differentiation, restricts differentiation of the cells to the macrophage lineage. In this work, we showed that ectopic expression of *Egr*-1 in

TABLE 2. Differentiation characteristics following stimulation with GM-CSF in 32Dneo versus 32DEgr1 clones[a]

Cell line	Treatment	Cell type (%)[b]					% of cells NSE positive[c]
		Granulocyte			Macrophage		
		Blast	Intermediate	Mature	Intermediate	Mature	
32Dneo1	IL-3	89	11	0	0	0	≤1
	GM-CSF	81	18	≤1	0	0	4
32Dneo7	IL-3	85	15	0	0	0	≤1
	GM-CSF	82	17	≤1	0	0	5
32DEgr1.2	IL-3	87	13	0	0	0	22
	GM-CSF	2	0	0	7	91	97
32DEgr1.5	IL-3	89	11	0	0	0	31
	GM-CSF	3	0	0	5	92	99
32DEgr1.6	IL-3	90	10	0	0	0	37
	GM-CSF	≤1	0	0	6	93	99
32DEgr1.25	IL-3	89	11	0	0	0	14
	GM-CSF	2	0	0	12	86	92

[a] Results of all experiments represent the means of at least three independent determinations, with standard deviations up to 15% (e.g., 13% = 13% ± 1.95%).
[b] Cell morphology was determined following 4 days with IL-3 or 10 days with GM-CSF. Morphological differentiation was determined by counting at least 300 cells on May-Grunwald-Giemsa-stained cytospin smears and scoring the proportion of immature blast cells, cells at intermediate stages of differentiation, and mature myeloid cells.
[c] After stimulation for 4 days with IL-3 or 10 days with GM-CSF, NSE staining was determined by staining cells on plates; positive cells contain red (NSE) granules in the cytoplasm.

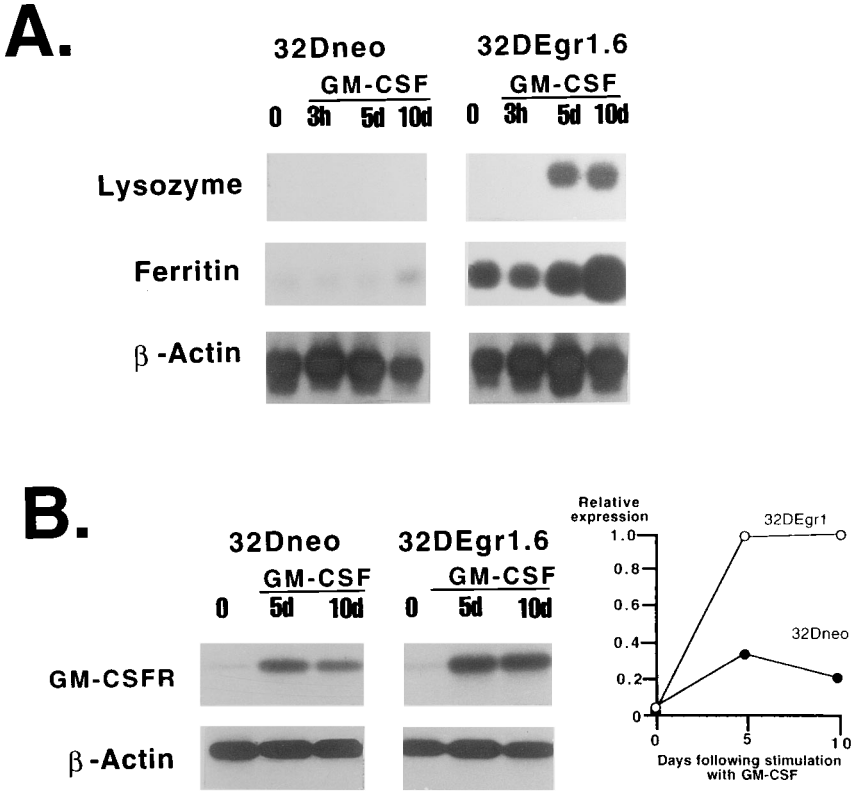

FIG. 5. Analysis of gene expression of 32Dneo and 32DEgr1 cells following stimulation with GM-CSF. (A) Analysis of the expression of lysozyme and ferritin light-chain mRNA in 32DEgr1.6 cells compared with that in 32Dneo7 cells, before and after stimulation with GM-CSF. Northern blots of total RNA (10 μg per lane) were prepared as described in Materials and Methods and hybridized with probes specific for lysozyme or ferritin light-chain mRNAs. (B) RT-PCR analysis of the level of transcripts encoding GM-CSF α subunit receptors in 32DEgr1.6 cells compared with that in 32Dneo7 cells, before and after stimulation with GM-CSF. RT-PCR was performed, and PCR products were visualized and quantitated as described in Materials and Methods. These are representative experiments, where similar results were obtained three times. 32DEgr1.2, 32DEgr1.5, and 32DEgr1.25 cells gave results similar to those of 32DEgr1.6; 32Dneo1 and parental cells gave results similar to those of 32Dneo7.

32Dcl3 cells blocked G-CSF-induced terminal granulocytic differentiation, corroborating previous observations with HL60 cells. It was also shown that the effect on granulocytic differentiation was not mediated via down-regulation of G-CSF receptors.

By identifying Egr-1 target genes, it should be possible to dissect the role of Egr-1 in both restricting and potentiating monocytic differentiation. To understand the molecular controls of blood cell development, it would also be useful to identify additional primary-response genes specific for either granulocytic or monocytic pathways, keeping in mind roles for these genes in restriction and determination.

TABLE 3. Effect of G-CSF priming on GM-CSF responsiveness of 32Dneo versus 32DEgr1 clones[a]

Cell line	Cell type (%)[b]					% of cells NSE positive[c]
	Granulocyte			Macrophage		
	Blast	Intermediate	Mature	Intermediate	Mature	
32Dneo1	5	30	41	15	9	11
32Dneo7	3	33	39	17	8	12
32DEgr1.2	4	0	0	7	89	99
32DEgr1.5	3	0	0	5	92	97
32DEgr1.6	5	0	0	4	91	98
32DEgr1.25	2	0	0	3	95	99

[a] Results of all experiments represent the means of at least three independent determinations, with standard deviations up to 15% (e.g., 17% = 17% ± 2.5%).
[b] All cells were treated with G-CSF for 3 days and then with GM-CSF. Cell morphology was determined following 10 days of treatment with GM-CSF. Morphological differentiation was determined as described in Table 2, footnote *b*.
[c] Staining for NSE was determined following 10 days of treatment with GM-CSF as described in Table 2, footnote *c*.

The similar levels of GM-CSFR transcripts in the uninduced 32Dneo and 32DEgr1 cells led to the conclusion that the acquired ability of the 32DEgr1 cells to undergo GM-CSF-induced macrophage differentiation is not due to *Egr*-1-mediated up-regulation of GM-CSFR, suggesting the involvement of other target genes. It was observed that expression of some of the markers specific for macrophage differentiation, including NSE and ferritin, already was elevated in unstimulated 32DEgr1 cells, raising the possibility that the genes that encode either NSE or ferritin, or regulate the expression of NSE and ferritin, are direct targets of Egr-1. Various strategies are currently being employed to identify Egr-1 target genes in hematopoietic cells and then to assess a role for these target genes in restricting and determining monocytic differentiation. On the basis of our previous observations that enforced expression of c-*fos*, and to a lesser extent *junB*, in the myeloid leukemic M1 cell line resulted in an increased propensity to be induced for terminal differentiation (15), the levels of c-*fos* and *junB* expression in parental and *Egr*-1-expressing 32Dcl3 cells were ascertained, and no significant differences were detected in the low levels of expression of either transcript (data not shown).

The work of Kreider et al. (7) has shown that priming 32Dcl3(G) cells with G-CSF, which induced GM-CSFR, and then exposing the primed cells to GM-CSF result in cells which undergo granulocytic differentiation, with some of the cells undergoing monocytic differentiation. We have gone on to show that in the presence of ectopic Egr-1 these cells exclusively underwent monocytic differentiation. These data further corroborate the previous observations that Egr-1 restricts differentiation to the monocytic lineage and support the notion that Egr-1 potentiates terminal monocytic differentiation. Most importantly, this line of experimentation is highly suggestive that the effects of *Egr*-1 are mediated upstream from the ligand receptor signal transduction cascade, since both parental and *Egr*-1-expressing 32Dcl3 cells respond to GM-CSF, with ectopic Egr-1 expression modulating the response.

It has been shown for two distinct cell lines, HL-60 and 32Dcl3, that ectopic expression of *Egr*-1 blocks terminal granulocytic differentiation. Interestingly, *Egr*-1 transcripts are induced at late times following granulocytic differentiation (this work and reference 20), suggesting that Egr-1 plays a role in mature myeloid cells, perhaps one related to the maintenance and/or regulation of a certain function(s) of differentiated cells. Egr-1 is a transcription factor which is expressed in a variety of tissues under many different physiological conditions. In addition to being a growth response gene in cultured fibroblasts (4, 8, 11, 28) and being induced in response to B-cell maturation, *Egr*-1 is induced during differentiation of nerve, bone, and myeloid cells (2, 18, 27, 29, 30). Thus, Egr-1 is an example of a regulator of gene expression which can play a role in a variety of developmental programs. If the target genes regulated by Egr-1 are the same in these different tissues has not yet been determined. If *Egr*-1 mediates its effect in monocytic versus granulocytic differentiation by the timing of its expression, the actual target genes activated by Egr-1, or both is an open question. Identifying Egr-1 target genes in the different contexts when it is expressed will increase our understanding of the role of this transcription factor as a regulator of development, as well as how different developmental pathways are controlled.

Localization of *Egr*-1 to the smallest region of chromosome 5 commonly deleted in malignant myeloid diseases (9) is consistent with our observations, demonstrating a role for *Egr*-1 as a central genetic switch regulating blood cell development. Homozygous mutant *NGF1*-A (*Egr*-1) mice appear to be phenotypically normal (10). Analysis of the repertoire of hematopoietic cells generated from bone marrow of these mice and their normal counterparts following treatment with different cytokines may delineate alterations in developmental pathways of blood cells in the absence of functional *Egr*-1. Additional studies, already under way, utilizing retrovirus-mediated gene transfer to genetically manipulate hematopoietic precursor-enriched bone marrow cells to express *Egr*-1, in order to analyze their differentiation profiles in vitro and in vivo, undoubtedly will be instrumental towards further delineating the role(s) that *Egr*-1 plays in normal hematopoietic cell development on the one hand and in leukemogenicity on the other.

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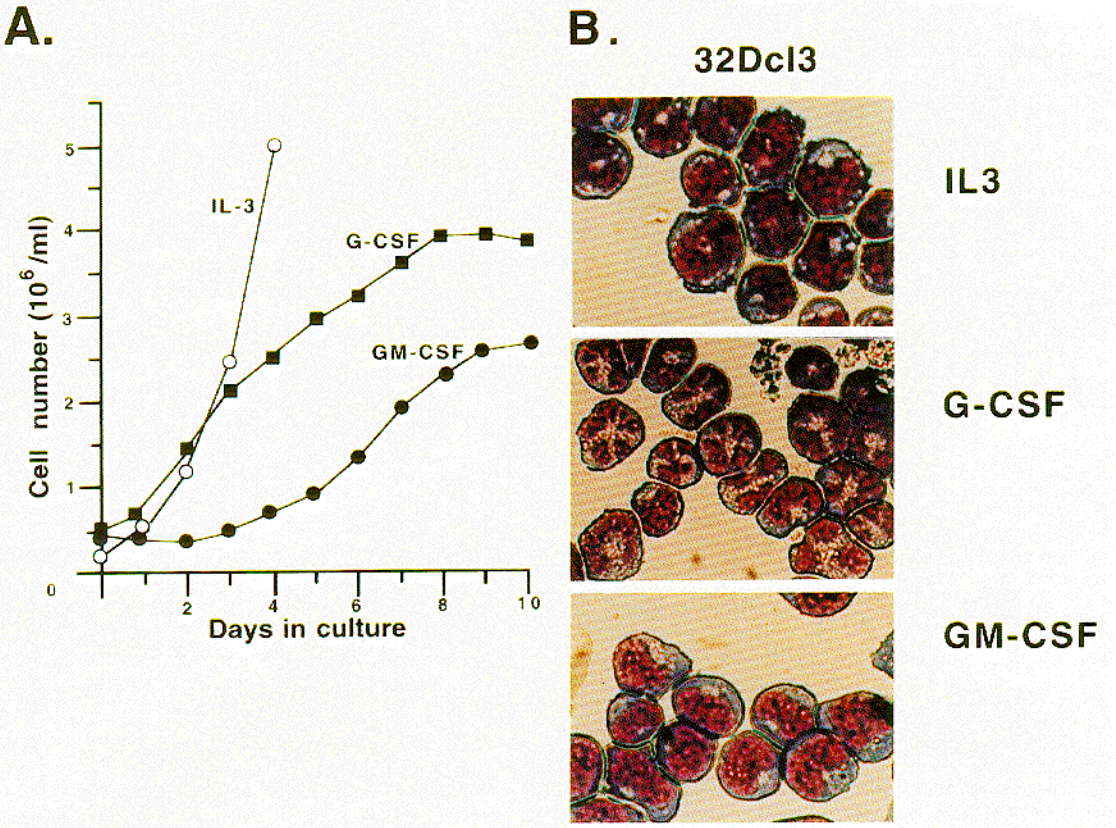
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IL-3
G-CSF
GM-CSF
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Days in culture
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2
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5
2
4
6
8
10
IL3
G-CSF
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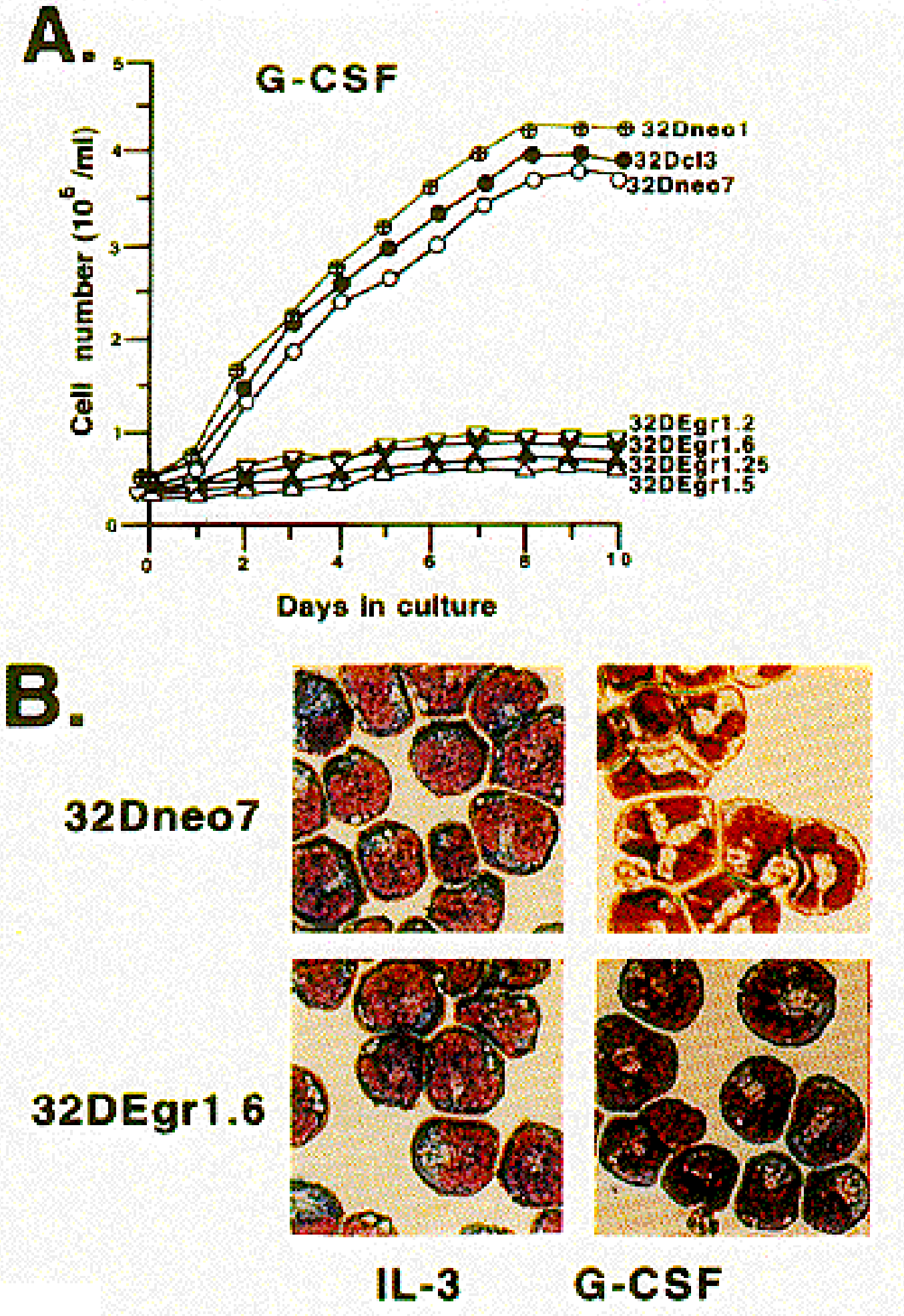
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G-CSF
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0
1
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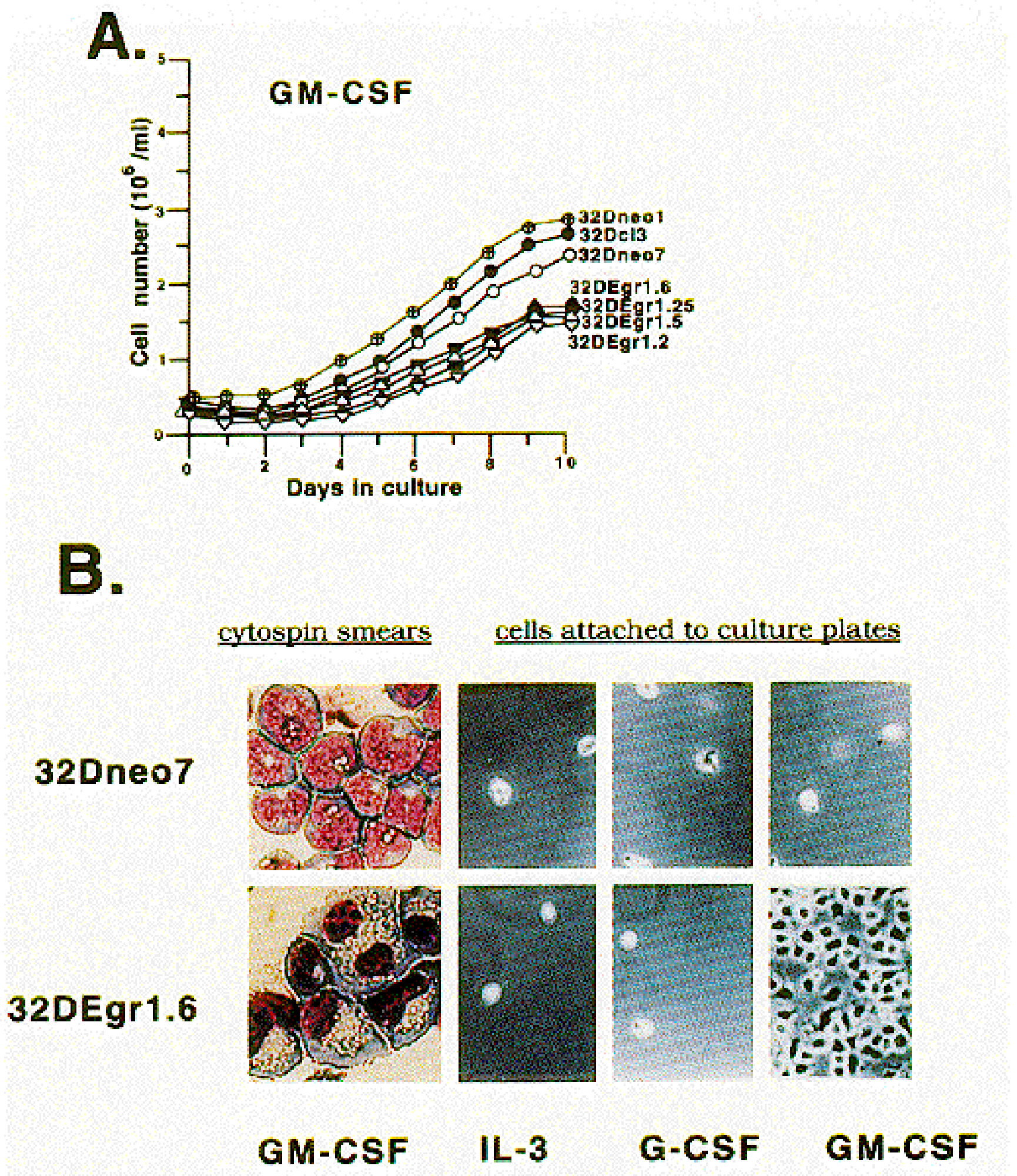
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GM-CSF
Cell number (10^5 /ml)
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32DEgr1.2
B.
cytospin smears
cells attached to culture plates
32Dneo7
32DEgr1.6
GM-CSF
IL-3
G-CSF
GM-CSF