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Granulocyte Colony-Stimulating Factor Induction of Normal Human Bone Marrow Progenitors Results in Neutrophil-Specific Gene Expression

By Nancy Berliner, Andrew Hsing, Timothy Graubert, Fridbjorn Sigurdsson, Mona Zain, Edward Bruno, and Ronald Hoffman

We have used a combination of hematopoietic growth factors to induce in vitro granulocytic maturation. A fraction of marrow cells enriched for hematopoietic progenitor cells (CD34+, HLA-DR+) was isolated from normal human bone marrow by monoclonal antibody staining and fluorescence-activated cell sorting. Cells were cultured in a suspension system for 3 days in the presence of stem cell factor and interleukin-3 (IL-3), after which granulocyte colony-stimulating factor (G-CSF) was added. Cells were harvested daily and analyzed for phenotypic maturation by morphologic criteria, and total RNA was obtained for analysis of myeloid gene expression. Maturation was observed to progress to the late metamyelocyte and band stage over a period of 10 to 12 days. Neutrophil-specific gene expression was assayed by reverse transcription-polymerase chain reaction (RT-PCR). Induction with G-CSF resulted in sequential expression of primary and secondary granule proteins, with asynchronous expression of primary granule proteins starting from days 1 to 5, and synchronous expression of lactoferrin and transcobalamin I (secondary granule proteins) from days 7 to 8. Interestingly, myeloperoxidase (MPO) mRNA expression was easily detected in both the freshly isolated CD34+, HLA-DR+ cells and cells at all subsequent stages of induction. This suggests that MPO mRNA is expressed very early during neutrophil development, perhaps before the development of significant numbers of phenotypically recognizable granules. This recapitulation of a program of sequential expression of primary and secondary granule protein genes suggests that in vitro marrow culture suspensions to which appropriate growth factors are added can mimic normal granulocytic maturation. This system should provide an important model for the study of neutrophil-specific gene expression.

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STUDY OF THE granulocyte differentiation pathway has depended largely on the investigation of patterns of maturation and gene expression in cell lines derived from patients with acute leukemia. Many of these cell lines can be induced to undergo partial maturation toward neutrophils with the use of exogenous chemical agents. For example, HL60 cells can be induced with retinoic acid or dimethyl sulfoxide (DMSO) to undergo phenotypic maturation toward metamyelocytes; the cells become phagocytic and reduce nitroblue tetrazolium.[1] Similarly, the promyelocytic leukemia cell line NB4 can be induced to mature with all-*trans* retinoic acid.[2] Patterns of gene expression in such cell lines, however, must be interpreted with caution because of the abnormality of maturation induced by the leukemic phenotype. Leukemic cell lines do not respond to the physiologic mediators of neutrophil maturation, and after induction with other agents, their phenotypic maturation is defective. Leukemic cell lines induced to differentiate consistently do not express late neutrophil proteins in an appropriate manner.[3-6] They also have variable expression of early-acting transcription factors, which may reflect the leukemic phenotype rather than represent correlates of normal gene expression.[7] Consequently, understanding of normal myeloid differentiation could be enhanced by investigation of a more physiologic model for the neutrophil maturation pathway.

Recent understanding of hematopoiesis has been advanced by the identification of antigens that characterize human primitive hematopoietic progenitor cells, as well as continued identification of hematopoietic growth factors that enhance their proliferation and maturation in vivo. Cell separation techniques based on antibody staining and flow cytometry for the isolation of CD34+ cells, with additional separation based on HLA-DR expression, rhodamine staining,[8-10] or markers of slightly more mature precursors,[11] has become the routine method for isolating hematopoietic progenitor cells. In vitro expansion of precursors has been successful using a variety of growth factor combinations, with the most consistently successful cytokines in inducing expansion of early progenitors including interleukin-3 (IL-3) and c-*kit* ligand.[12-14]

These advances in the understanding of primitive hematopoietic progenitors have had critical impact on the development of techniques for bone marrow transplantation and gene therapy by allowing better identification of the bone marrow repopulating cells and by offering the potential for ex vivo expansion of these primitive progenitors to enhance efficiency of engraftment. Implicit in these studies is the assumption that the induced proliferation and maturation of marrow progenitors in vitro will recapitulate the normal hematopoietic differentiation pathway. Therefore, primary bone marrow cultures offer potential insight into the normal patterns of gene expression in developing hematopoietic cells. In this study, we have evaluated the induction of normal marrow progenitors along the granulocytic pathway as a model for neutrophil differentiation. We have confirmed a pattern of neutrophil-specific gene expression in cells induced toward neutrophils, and suggest that this represents a powerful model system for the study of normal neutrophil maturation.

MATERIALS AND METHODS

Isolation and in vitro culture of bone marrow progenitors. Bone marrow was obtained with informed consent from normal volunteers.

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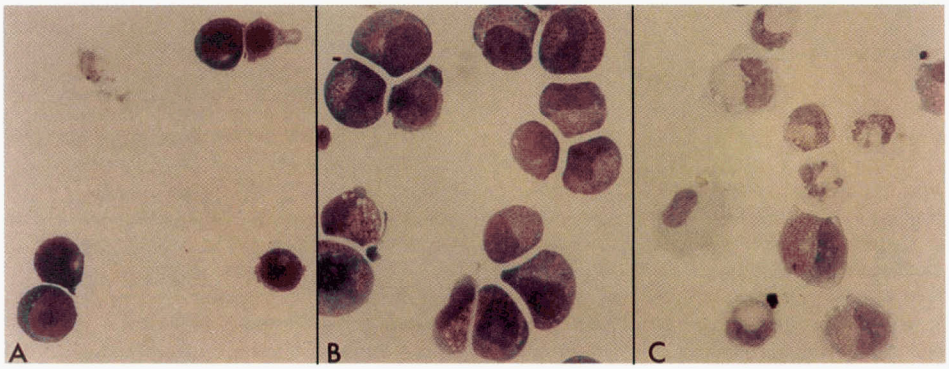

Fig 1. Growth factor-induced maturation of bone marrow progenitors. (A) Sorted CD34+, HLA-DR+ progenitors. (B) Cells on day 4 of culture, at time of addition of G-CSF to culture medium. (C) Cells on day 10 of culture, showing full maturation to bands and segmented forms.

Progenitor cells were isolated by monoclonal antibody staining and fluorescence-activated sorting to enrich for CD34+, HLA-DR+ cells as previously described.[4] Purified progenitor cells were grown at a concentration of 1×10^4 to 1×10^5 cells per milliliter in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% heat-inactivated fetal calf serum. Cells were grown for 3 days in medium supplemented with 100 ng/mL IL-3 and 100 ng/mL c-*kit* ligand. After 3 days, cells were placed in medium containing the same growth factors plus granulocyte colony-stimulating factor (G-CSF), or in G-CSF alone (10 ng/mL). Cells were fed with medium containing the appropriate cytokines every 48 hours. Cells were harvested daily and analyzed for phenotypic maturation by morphologic criteria, and total RNA was obtained for analysis of myeloid gene expression. Cell counts were obtained, and maturation was monitored by Wright-Giemsa staining of cytospin smears. Cells (1×10^5) were lysed for RNA preparation. Volume was replaced with medium of the same composition.

The induction protocol was repeated four times, with preparation of cytospins, RNA isolation, and reverse transcription-polymerase chain reaction (RT-PCR) analysis for each experiment.

RNA isolation and RT-PCR of bone marrow progenitors. Approximately 1×10^5 cells were solubilized in 300 μL guanidinium isothiocyanate and extracted with chloroform/isoamyl alcohol. RNA was pelleted with isopropanol using 10 μg glycogen as a carrier.

Approximately 100 ng of total RNA was mixed with 50 ng of oligo dT, denatured at 65°C for 10 minutes, and mixed with reverse transcription buffer, dNTPs, RNAsin, and dithiothreitol (DTT) in a final volume of 50 μL. Reactions were incubated at 37°C for 1 hour in the presence of reverse transcriptase. A 1-μL aliquot of the resultant cDNA was then subjected to 30 to 40 cycles of PCR under standard conditions, using 100 ng of the appropriate primers and 1 to 3 U of Taq polymerase. Approximate cDNA concentrations were standardized by comparison with PCR of β_2 microglobulin transcripts. As a control for the PCR of the less abundant secondary granule protein gene mRNAs, PCR of an aliquot of a chronic myeloid leukemia (CML) cDNA library known to contain the secondary granule cDNAs was used.[4]

Results are presented from a representative set of cDNA samples prepared from one of the four induction experiments.

RESULTS

Wright-Giemsa staining of newly sorted cells showed phenotypic characteristics of undifferentiated blast cells (Fig 1A). Cells cultured for 3 days in the presence of IL-3 and c-*kit* ligand were observed to undergo phenotypic maturation toward promyelocytes (Fig 1B). Further culture with the addition of G-CSF resulted in phenotypic maturation toward the late metamyelocyte to the band stage over the course of 10 to 12 days (Fig 1C).

Daily differential counts were performed on Wright-stained smears and showed increasing heterogeneity of the myeloid cell population over the course of 10 to 12 days (Table 1). Because in some IL-3–dependent cell lines successful induction of myeloid differentiation with G-CSF requires the removal of IL-3,[15] we also attempted induction with the addition of G-CSF at day 4 accompanied by removal of IL-3 and c-*kit* ligand. Maturation appeared similar under these circumstances but was not improved; there was, in fact, some delay in maturation to later forms under the latter conditions (data not shown).

Neutrophil-specific gene expression was assessed by RT-PCR of RNA isolated from cells induced with IL-3, c-*kit* ligand, and G-CSF as outlined in Table 1. Using PCR of mRNA encoding β_2 microglobulin as a housekeeping gene, cDNA was roughly quantified, and the concentrations of the different samples were equalized. Expression of the primary granule proteins myeloperoxidase (MPO), neutrophil elastase (NE), lysozyme, and defensin and the secondary granule proteins lactoferrin and transcobalamin I was then assayed. Surprisingly, MPO was expressed at relatively high levels in cells at day 0. Expression increased to maximum over the first 2 to 3 days in culture (Fig 2, Table 2). In contrast, the other primary granule protein genes showed later expression, which was asynchronous. Lysozyme expression was detect-

Table 1. Differential Granulocyte Counts

Day of Culture	Blasts	Pros	Myelos	Metas	Bands	Segs
0	100					
1	100					
2	100					
3	90	10				
4	65	35				
5	37	37	26			
6	22	22	35	21		
7	23	14	36	21	6	
8	14	6	40	32	7	
9	9	7	42	30	9	5
10	5	10	40	30	13	2
11	4	1	24	35	27	9

Numbers refer to percentages of cells of each stage in a differential count of 100 cells.

Abbreviations: Blasts, myeloblasts; Pros, promyelocytes; Myelos, myelocytes; Metas, metamyelocytes; Segs, segmented neutrophils.

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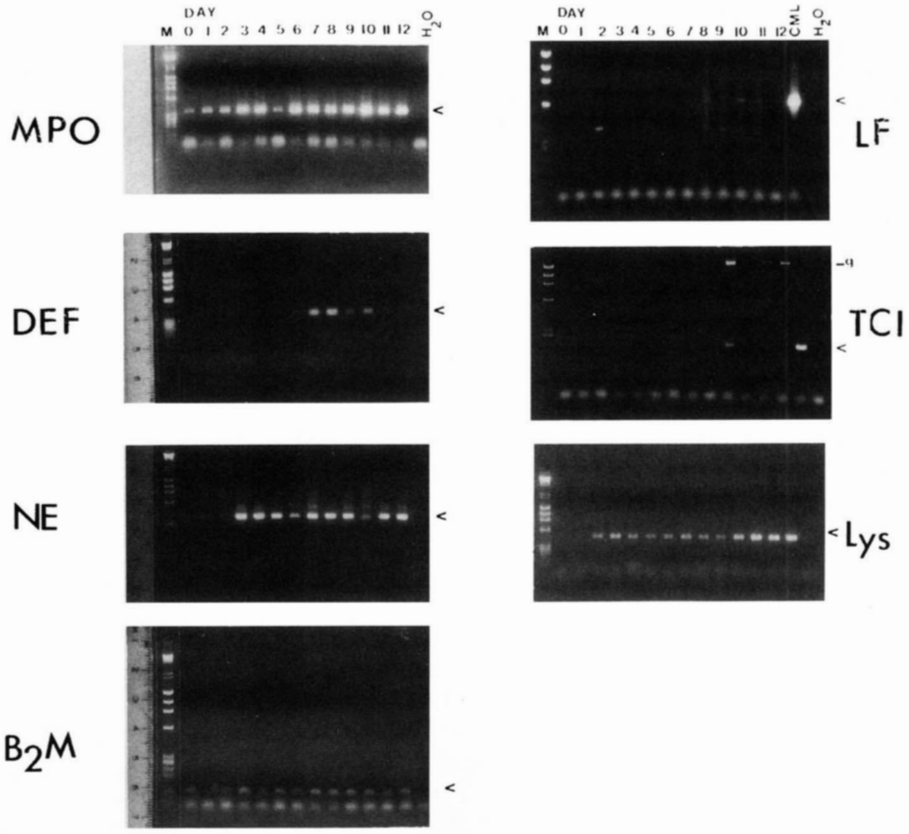

Fig 2. RT-PCR of mRNA from cultured progenitor cells. Numbers refer to day of culture. B_2M, β_2 microglobulin; LF, lactoferrin; TCI, transcobalamin I; Lys, lysozyme. Amplification of contaminating genomic DNA is marked "g" in TCI assay. CML denotes PCR of an aliquot of a CML cDNA library[4] as a positive control for PCR of secondary granule protein gene mRNA.

able at day 2 and beyond; NE was expressed at high levels from day 3, although trace amounts of NE mRNA were detectable from day 1. In contrast, defensin, another primary granule protein, was expressed at high levels from days 6 to 7, with only trace amounts detectable from day 5 (Fig 2, Table 2).

Expression of the secondary granule protein lactoferrin was undetectable until days 7 to 8 (Fig 2, Table 2). These results correlated closely with the results seen with transcobalamin I, another content protein of the secondary granule.

DISCUSSION

These experiments confirm that in vitro granulocytic differentiation of hematopoietic precursors to the late band and mature neutrophil stage can be achieved using an appropriate combination of hematopoietic growth factors. Interestingly, growth for 3 days in IL-3 and c-*kit* ligand alone resulted in phenotypic maturation to the promyelocyte stage, because 35% of the cells at day 4, the time G-CSF was added to the medium, were promyelocytes. However, further growth in IL-3 and c-*kit* ligand did not lead to significant further maturation (data not shown); progression to the later stages of myeloid differentiation appeared to depend on the presence of G-CSF. Despite previous reports in an inducible tissue culture line that IL-3 inhibits the maturation induced by G-CSF, we found here that, in this system, growth and maturation were enhanced by maintaining the cells in IL-3 and c-*kit* ligand during G-CSF induction.

The maturation seen in these experiments is not synchronous but mimics the pattern of differentiation seen in marrow colonies, in which cells at all stages of differentiation can be seen within a single colony. The phenotypic maturation seen appears to be normal, with the sequential development of primary and secondary granules at the appropriate developmental stages and normal changes in nuclear morphology. This maturation has been correlated with expression of neutrophil-specific genes by RT-PCR. One interesting finding is the observation that MPO is detectable from the earliest stages of differentiation. Although another primary granule constituent, defensin, was detectable in newly isolated CD34+ cells, this probably reflects more mature cells contaminating the initial sorted population; these cells appeared to die rapidly in culture, and defensin expression became undetectable until the promyelocyte stage. In contrast, the MPO expression persisted at a high level throughout the culture period. These results suggest that MPO expression may occur as a very early event in the myeloid differentiation pathway, before the stage at which one can detect primary granules by histologic examination. These data correlate with the previously reported observation that a subset of CD34+, HLA-DR+ cells have MPO that is immunocytochemically detectable by fluorescence-activated cell sorting.[16]

The expression of the other primary granule constituents, namely lysozyme, NE, and defensin are quite disparate. Lysozyme is detectable from day 2 of culture; trace amounts of NE are detectable early, but expression is clearly seen somewhat later, around day 3. In contrast, defensin is unde-

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Table 2. mRNA Expression by RT-PCR

	Day of Culture												
Gene	0	1	2	3	4	5	6	7	8	9	10	11	12
B_2 MCB	+	+	+	+	+	+	+	+	+	+	+	+	+
MPO	+	+	+	+	+	+	+	+	+	+	+	+	+
NE	+/−	+/−	+/−	+	+	+	+	+	+	+	+	+	+
Lysozyme	−	−	+	+	+	+	+	+	+	+	+	+	+
Defensin	+	−	−	−	−	+	+	+	+	+	+	+	+
LF	−	−	−	−	−	−	−	+	+	+	+	+	+
TCI	−	−	−	−	−	−	+/−	+	+	+	+	+	+

Abbreviations: B_2 MCB, β_2 microglobulin; LF, lactoferrin; TCI, transcobalamin I; +, expression detected; −, no expression detected.

tectable until days 5 to 6 of culture. The potential significance of this relatively late expression of defensin is discussed in greater detail below.

The expression of mRNA encoding the secondary granule protein genes lactoferrin and transcobalamin I was first detectable at days 6 to 7 of culture, when a significant number of the cell population had reached the myelocyte and metamyelocyte stage. This relatively coordinate expression of secondary granule protein genes is in agreement with the hypothesized late, coordinate expression of these genes in terminally differentiated neutrophil precursors.[17] In this context, the relatively late expression of the defensin gene is of interest, because its peak expression is more closely synchronized with the expression of the secondary granule proteins. This intriguing finding may have important implications for the control of defensin gene expression. Specific granule deficiency is a rare syndrome associated with absence of neutrophil secondary granules, which has been shown to be associated with a defect in mRNA expression of all of the secondary granule content protein genes. Interestingly, defensin is the only primary granule protein whose expression is also markedly reduced in this disease.[18] We have postulated that the finding of coordinate loss of mRNA expression in that disease may reflect dysfunction or deletion of a shared regulatory factor; the delayed expression of defensin expression seen here may support the contention that high-level expression of that gene is dependent on the same regulatory pathway.

Because the maturation pattern seen in this system is not uniform, it cannot be delineated with certainty which cells within the culture population contribute the mRNA detected by PCR. The stage-specific correlation of maturation with gene expression, as well as a further delineation of the source of early MPO expression in freshly sorted progenitor cells, could be best achieved by in situ hybridization studies. In previous studies, Fouret et al[19] analyzed the pattern of NE and MPO mRNA expression in unsorted human bone marrow cells by in situ hybridization and found the expression of these genes to be relatively synchronous.[19] Similar studies aimed at delineating granule protein gene expression in this system are in progress, as are attempts to better synchronize the maturation of the in vitro cultures.

In summary, cultured marrow CD34+, HLA DR+ progenitor cells can be induced toward a granulocytic phenotype with appropriate growth factors. G-CSF allows their in vitro differentiation into late metamyelocytes, bands, and mature neutrophils in a 10 to 12-day period, similar to that which occurs in vivo. Unlike the results seen in inducible leukemic cell lines, these cells show evidence for the sequential expression of primary and secondary granule protein genes in a manner that appears to recapitulate the normal neutrophil maturation pathway. This system offers a potentially important tool for the study of myeloid-specific gene expression.

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