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 in 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.
Progenitor cells were isolated by monoclonal antibody staining and fluorescence-activated sorting to enrich for CD34+, HLA-DR+ cells as previously described. Purified progenitor cells were grown at a ligand. After medium supplemented with heat-inactivated fetal calf serum. Cells were grown for 3 days in fluorescence-activated sorting to enrich for CD34+, HLA-DR+ cells modified Dulbecco’s medium (IMDM) supplemented with 10% CSF), containing the appropriate cytokines every harvest 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 cytopsins, 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 guanidium isothiocyanate and extracted with chloroform/isooamyl alcohol. RNA was pelleted with isopropanol using 10 μg glycerogen 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 β2 microglobulin transcripts. As a control for the PCR of the less abundant granulocyte gene mRNAs, PCR of an aliquot of a chronic myeloid leukemia (CML) cDNA library known to contain the secondary granule cDNAs was used.

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, 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).

**Table 1. Differential Granulocyte Counts**

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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.
G-CSF INDUCTION OF NORMAL MARROW PROGENITORS

Fig 2. RT-PCR of mRNA from cultured progenitor cells. Numbers refer to day of culture. B2M, \( \beta_2 \) 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 as a positive control for PCR of secondary granule protein gene mRNA.

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, 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+ cells, 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 is 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-
This intriguing finding may have important implications for regulatory factor; the delayed expression of defensin expres-
some interesting, because its peak expression is more closely synchro-
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 expres-
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 expres-
Table 2. mRNA Expression by RT-PCR

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Abbreviations: B2 MCB, β2 microglobulin; LF, lactoferrin; TCI, transcobalamin I; +, expression detected; -, no expression detected.

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