Expression of Protooncogene c-myb in Normal Human Hematopoietic Cells

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Expression of the protooncogene, c-myb, in various subpopulations of normal human hematopoietic cells was characterized. Cells expressing the immature cell surface marker, CD34 (My10), were isolated by immune adherence with the “panning” technique or immunomagnetic microspheres and were shown to be strongly positive for c-myb protein expression in an immunoperoxidase assay. The CD34+ progenitor cell population was further separated into myeloid plus erythroid progenitors (CD34+, CD10+) v B-lymphoid precursors (coexpressing CD34 and CD10) by two-color FACS. Both CD34+ progenitor cell subsets strongly expressed c-myb protein by the immunoperoxidase assay. A flow cytometric assay was then developed which permitted simultaneous detection of a cell-surface antigen (to characterize lineage and stage of maturation) and the nuclear oncoprotein. This assay confirmed that CD34+ cells were strongly positive for c-myb expression and also allowed quantitative comparisons of c-myb expression in selected populations of other normal hematopoietic cells. Most human bone marrow cells appear to express some level of c-myb protein, although the CD34+ progenitor cell population expresses the highest amount.

The protooncogene c-myb encodes a highly conserved, 75 to 83 Kd, nuclear, DNA binding protein which is the normal cellular homologue of the transforming gene (v-myb) of the avian myeloblastosis virus. The v-myb gene induces myeloblastosis in vivo, whereas in vitro the gene was initially shown to transform immature myelomonocytic cells. The potential role of the c-myb gene product in induction or maintenance of the transformed phenotype is not yet understood; neither has its function in normal cells been discovered.

Expression of the c-myb gene has been primarily characterized in malignant hematopoietic cell lines in culture. Chemically induced differentiation of the myeloid leukemia cell lines HL-60, ML-1 and WEHI-3B results in decreased levels of c-myb expression. Experimental models in which “natural” separations of normal cell lineages occur (such as chicken bursa, yolk sac, or thymus and mouse fetal liver) suggest that c-myb mRNA levels are higher in immature normal hematopoietic cells than in more differentiated cells. These findings led to suggestions that the myb protein has a significant function in hematopoietic cell growth and/or differentiation pathways. However, caution must be used in interpretation of these findings, since differential expression of cell-surface or intracellular proteins in hematopoietic cell lines or leukemias may not accurately reflect normal differentiation pathways.

Characterization of c-myb expression in vivo has been difficult in normal developing human hematopoietic cells as a function of lineage and/or stage of maturation because of the cellular heterogeneity of normal bone marrow and the rarity of many of the differentiating cell populations. These problems have made it difficult to apply the usual assays for mRNA by Northern blots and protein by Western blots or immunoprecipitations. Initial attempts to circumvent these problems have used crude separations of heterogeneous cell populations, followed by Northern blots, or in situ RNA hybridization, coupled with morphologic characterization of cell types, to characterize c-myb expression in individual cell types. These approaches, however, are only semiquantitative, and cell types cannot be defined precisely in these assays.

Normal human hematopoietic cell lineage and maturational stage have been correlated with cell-surface antigen expression by multiparameter flow cytometric analysis. The Myl0 (CD34) cell-surface antigen has been a particularly useful marker in hematopoiesis, since it is expressed only on the most immature myeloid, erythroid, and B-lymphoid precursor cells. In this study, we used two methods to evaluate expression of the c-myb protein in specific types of normal human hematopoietic cells characterized by their cell-surface antigen expression: (a) Cells were isolated based on cell-surface marker expression and then qualitatively analyzed for c-myb expression by an immunoperoxidase assay; and (b) two-color flow cytometry was used to quantitate relative levels of c-myb protein in discrete cell types defined simultaneously by their cell-surface antigen expression.

MATERIALS AND METHODS

Antibodies. Previously, polyclonal rabbit sera directed against recombinant c-myb protein were generated and characterized; mybl and myb1 represent sera from two different animals. Because of limiting amounts of preimmune sera from the same rabbits, normal rabbit serum was used as a control in most experiments; however, no significant differences were noted between preimmune serum from the immunized animal and normal rabbit serum in either assay (data not shown). Phycoerythrin (PE)-conjugated or unlabeled monoclonal antibodies (MoAbs) to CD34, CD10, CD20,
CD22, CD5, CD8, CD45, and Leu19 were obtained from Becton Dickinson Immunocytometry Systems (Mountain View, CA). When unlabeled antibodies were used to stain cell-surface antigens, PE-conjugated, affinity-purified, isotype-specific goat anti-mouse antibodies (Southern Biotechnology Associates, Birmingham, AL) were used as a secondary labeling reagent. Affinity-purified, FITC-conjugated, goat anti-rabbit IgG (Caltag, San Francisco) was used to label oncoproteins indirectly.

**Cell preparations and separations.** Bone marrow aspirates were obtained from consenting normal adult volunteers, as approved by the Institutional Review Board and Department of Health and Human Services. CD34+ cells were isolated from low-density mononuclear cells (1.077 g/mL, Ficoll-Hypaque, Pharmacia, Piscataway, NJ) by immune adherence by panning as previously described or by immunomagnetic microspheres. Enrichment for progenitor cells was assessed by morphology, colony-forming assays, and flow cytometry.

Low-density leukocytes were obtained, and cell-surface antigens were labeled, directly or indirectly, with PE-conjugated antibodies as previously described. In two-color cell-sorting experiments, cell-surfaces were labeled with FITC and PE-conjugated antibodies. Cells stained for two colors of immunofluorescence were sorted on an Epic 752 flow cytometer (Coulter Electronics, Hialeah, FL). After isolation, sorted cell populations were reanalyzed for purity on a FACSscan flow cytometer (Becton Dickinson) before immunohistochemical analysis.

**Immunoperoxidase assay.** Cytosin preparations were made (Shandon Cytospin II, Pittsburgh) of unseparated low-density leukocytes, of CD34+ cells enriched by immune adherence, and of cell populations isolated by cell sorting. The cells were then analyzed for c-myb expression, by the avidin-biotin-peroxidase complex method, according to manufacturer's instructions (Vector, Burlingame, CA). Before assay for c-myb, cells were fixed in 1% paraformaldehyde (Polysciences, Warrington, PA) in phosphate-buffered saline (PBS). PBS with 0.2% bovine serum albumin (BSA, Sigma, St Louis), and 0.05% Tween 20 (Sigma) was used as the sample and wash buffer. DAB (Sigma) was the peroxidase substrate, and the cells were counterstained with fast green. Control sera and anti-c-myb sera were used at dilutions of 1:200 (optimal ratio of signal to background based on titration; data not shown).

**Flow cytometric assay for c-myb.** Cell-surface antigens on KG1a cells or low-density leukocytes from normal human bone marrow were labeled with PE as described above (Fig 1). The subsequent fixation, permeabilization, and nuclear protein labeling technique were adapted from Clevinger et al. All procedures were done at 4°C. The cells (1 × 10⁶) were fixed in 2 mL 1% paraformaldehyde (Polysciences) in PBS for 15 minutes, centrifuged (200 g for ten minutes), and resuspended in 2 mL 0.1% Triton X-100 (Sigma) in 10 mmol/L Hepes-saline, pH 7.4, 4% newborn calf serum (IFA buffer) for three minutes.

The permeabilized cells were centrifuged (450 g for ten minutes), resuspended in 0.1 mL control rabbit serum or anti-c-myb rabbit serum diluted 1:400 (optimal dilution for this assay; data not shown) in IFA buffer, and incubated for one hour. The cells were washed with 2 mL Triton X-100/IFA buffer, resuspended in 0.1 mL FITC-conjugated goat anti-rabbit IgG diluted 1:300 (optimal titration; data not shown) in IFA, and incubated for 30 minutes. Two percent goat serum and human AB+ serum were added to block nonspecific binding of immune-species antibodies during both primary and secondary nuclear antibody incubations. The cells were washed twice with 2 mL Triton X-100/IFA buffer and resuspended in 1 mL IFA buffer for flow cytometric analysis. Two-color immunofluorescence was analyzed on a FACSscan flow cytometer after appropriate compensations of the FITC and PE channels using Calibrite beads (Becton Dickinson) according to manufacturer's instructions.

**RESULTS**

**Immunohistochemical staining for c-myb in progenitor cells.** Cells that express the cell-surface antigen CD34 constitute only 1.5% (on average) of all human bone marrow cells but selectively include the progenitor cells of at least the myeloid, erythroid, and B-lymphoid lineages. In these experiments, cells expressing CD34 were initially isolated by immune adherence by panning on Petri plates or immunomagnetic microspheres. These types of separation reproducibly resulted in recovery of most colony-forming cells, with purities of 70% to 99% morphologic blast cells (data not shown); refs. 20, 28; C.A. Cremo and C.I. Civin, unpublished observations, February 1988).

Cytosin preparations of these "progenitor-enriched" populations were prepared, and the cells were probed for c-myb protein expression by immunohistochemical staining with two different rabbit polyclonal antisera. These antisera were raised against the carboxyl terminal portion of the c-myb protein, which had been expressed from a bacterially promoted human c-myb recombinant gene in Escherichia coli. Both antisera were characterized previously and recognize c-myb-specific epitopes in an 83-Kd protein (in humans) localized to the cell nucleus. Immunoperoxidase staining of unseparated bone marrow low-density leukocytes with either of these c-myb antisera (myb1 or myb11) resulted in darkly stained ("positive") nuclei in ~30% to 40% of cells (Fig 2A and B). In contrast, 80% to 95% of CD34+ cells isolated by immune adherence had darkly stained nuclei (Fig 2D, isolated using immunomagnetic microspheres). Use of control rabbit serum rather than anti-myb serum on this progenitor cell population resulted in little to no nuclear staining (Fig 2C; CD34+ cells in this photograph were isolated by FACS, discussed below). Thus, CD34+ progenitor cells enriched from normal human bone marrow express relatively high levels of c-myb.

To characterize c-myb expression in progenitor cells further, the CD34+ cell population was subdivided by FACS
into B-lymphoid progenitors + myeloid plus erythroid progenitors. The most immature identifiable B-lymphoid lineage cell (stage I) coexpresses CD34 and CD10 common acute lymphocytic leukemia antigen (CALLA) on its surface. Normal human bone marrow cells were incubated with monoclonal antibodies (MoAbs) to CD34 and CD10, which were then differentially labeled with the fluorochromes FITC and PE. Cells were sorted into four populations (CD34+CD10–; CD34–CD10+; CD34–CD10–; and CD34–CD10–) on the basis of two-color immunofluorescence (by FACS as previously described), and the sorted populations were evaluated for c-myb expression by the immunoperoxidase assay.

Virtually all (>95%) of the isolated myeloid plus erythroid precursors (CD34+/CD10–) were darkly stained by both c-myb antibodies by the immunoperoxidase assay (Fig 2E and F). More than 90% of stage I B-lymphoid precursors (CD34+/CD10–) also strongly expressed c-myb (Fig 2G). In addition, stage II and III B-lymphoid cells (CD34+/CD10+) stained positively for c-myb (Fig 2H), although the staining appeared less intense as compared with either CD34+ population. c-myb staining of the CD34+/CD10– population was not significantly different from unseparated bone marrow (data not shown). This last result is not unexpected, since 90% to 95% of the starting bone marrow population is present in this sort window. These findings further support the concept that myeloid, erythroid, and B-lymphoid progenitor cells, which represent a small proportion of all bone marrow cells, express high levels of the c-myb protein relative to other cells in the bone marrow. In addition, a significant fraction of other bone marrow cells also appear to have detectable levels of c-myb protein.

Flow cytometric analysis of c-myb. The use of FACS to isolate discrete populations of cells for subsequent analysis of c-myb expression by an immunoperoxidase assay was not an ideal methodology since: (a) it involves a time-consuming isolation (especially for rare populations of cells, such as those isolated above), which can result in the death of a significant percentage of cells prepared for immunohistochemical analysis; (b) only a few hematopoietic cell types (identified by cell-surface markers) can conveniently be analyzed in any single experiment; and (c) quantification of relative levels of c-myb expression in various cell types with the subjective immunoperoxidase assay is difficult. For these reasons, we adapted a two-color flow cytometry technique (previously designed to correlate expression of nuclear proteins and stage of cell cycle) to detect a cell-surface marker of differentiation and the c-myb nuclear oncoprotein simultaneously. The conditions of this assay had been previously optimized for simultaneous nuclear protein detection and DNA staining. To use this technique to characterize c-myb expression in specific cell types, we had to show that fixation/permeabilization conditions could be found which left the cell membrane sufficiently intact to retain the cell surface marker, while effectively permeabilizing the cell and nuclear membranes to allow antibodies into the nucleus.

A primitive myeloid leukemia cell line, KG1a, which expresses high levels of CD34 on its cell membrane and nuclear c-myb, was used to evaluate and optimize this technique (Fig 1). Two-color flow cytometric analysis revealed that virtually 100% of the KG1a cells were specifically labeled with both CD34 and c-myb antibodies (Fig 3). The quadrants in Fig 3A were defined by the fluorescence observed with the control antibodies (MOPC 21 IgG, myeloma protein controlling for CD34 and control rabbit antiserum); thus, quadrants 1 and 2 represent CD34+ cells, and quadrants 2 and 4 represent c-myb+ cells. Separate histogram analysis of each fluorescent label also showed that >97% of KG1a cells express c-myb above control levels (Fig 3B) and more importantly technically, that detergent treatment of the cells does not significantly alter levels of cell-surface marker staining (Fig 3C). Fluorescence microscopy confirmed that the PE staining was retained on the cell surface after fixation and permeabilization and that the FITC staining was nuclear (data not shown). The optimal concentration of Triton X-100 was 0.1% (Fig 3D). At 0.02% Triton X-100, very little FITC staining of the cells was noted, and at 0.05% Triton X-100, intranuclear staining...
was variable (with even greater variation in bone marrow cells than in the KG1a cells).

Flow cytometric analysis of c-myb in normal bone marrow. Human bone marrow low-density leukocytes isolated by Ficoll-Hypaque density-gradient centrifugation were labeled on the surface with one of several lineage- and/or stage-specific antibodies (directly or indirectly labeled with PE), and then fixed, permeabilized, and assayed for c-myb protein as described above (Fig 1). For each cell-surface marker evaluated, nuclear binding of rabbit control serum and c-myb serum were assayed, resulting in plots as shown in Fig 4. Unpermeabilized controls were also run for every cell-surface marker used. The only cell-membrane marker affected by the detergent treatment was CD45, which showed a small quantitative decrease in fluorescence intensity as compared with unpermeabilized controls.

Fluorescence of human bone marrow low-density leukocytes labeled with CD34(PE) on the surface and control serum(FITC) in the nucleus is shown in Fig 4A. The quadrants were drawn to show all CD34+ cells above the horizontal quadrant axis and control nuclear FITC binding levels at left of the vertical quadrant axis. The change in FITC fluorescence when c-myb antiserum was used (Fig 4C) is apparent. Almost 100% of the CD34+ bone marrow cells appeared to be strongly positive for c-myb expression, in agreement with the immunoperoxidase results described above. In addition, ~30% to 40% of all bone marrow cells were shifted to the right of the vertical axis and therefore appeared to be positive for c-myb. This observation is also in agreement with the immunohistochemical analysis (described above). This labeling pattern of the bulk of bone marrow cells (cells below the horizontal quadrant axis = non-PE-labeled cells) was reproducibly observed in every experiment.

In contrast to CD34+ cells, cells labeled on the surface with CD20 antibodies (stages III and IV B-lymphoid cells) did not shift to the right of the vertical quadrant axis with c-myb serum labeling (Fig 4D), indicating lower levels of c-myb expression in the more mature B-lymphoid CD20+ cells. However, although the CD20+ cells labeled with c-myb serum did not shift past the right of this vertical axis based on control serum binding in whole bone marrow (Fig 4D), they did shift slightly relative to the CD20+ cells labeled with control rabbit serum (upper quadrants in Fig 4B as compared with D). Therefore, when control whole bone marrow cells were compared with CD20+ cells, the CD20+ cells did not appear to express c-myb above background control levels.

The two-color flow cytometric assay allows "analytic" separation of interesting cell populations as opposed to the "physical" separation methods used earlier. Nuclear fluorescence data can be collected on a population of cells expressing only a given cell-surface marker, rather than on the entire bone marrow population. As shown in Fig 4, this is achieved by "gating" on the PE (CD34+ or CD20+) cells above the horizontal axis and comparing c-myb nuclear fluorescence...
with control nuclear fluorescence in those PE\(^+\) cells (to determine if c-myb is expressed above background levels in a given cell type). In addition, comparison of c-myb fluorescence in PE\(^+\) cells labeled with different cell-surface antibodies permits determination of relative levels of c-myb expression among various cell types.

Examples of this type of analysis are shown in the histograms in Figs 5 and 6. Selective analysis of the rare CD34\(^+\) population of bone marrow cells for c-myb expression is shown in Fig 5A. Staining with either of the two different polyclonal myb sera gave identical levels of fluorescence, with >95% of the CD34\(^+\) cells above the fluorescence levels using control rabbit serum. The two polyclonal sera also appeared identical in their labeling of CD10\(^+\) cells (Fig 5B), although the level of c-myb signal relative to control was less than in the CD34\(^+\) population.

With this approach, levels of c-myb expression in various cell types can also be compared. CD34\(^+\) cells have significantly higher levels of c-myb protein than B-lymphoid lineage cells identified by either CD10 or CD22(stage IV B cells) (Figs 5C and 6). The average c-myb content of CD34\(^+\) cells is also much greater than that of unseparated bone marrow cells (on the average), whereas c-myb levels in CD10\(^+\) and CD22\(^+\) cells are not distinguishable from those of whole bone marrow (Fig 5C).

Direct comparisons of various other cell types revealed higher c-myb levels in progenitor cells (CD34\(^+\)) than in T lymphocytes (CD5\(^+\)), which in turn appeared to have slightly more c-myb than early or late B-lymphocytes (CD10\(^+\), CD20\(^+\) or CD22\(^+\); Fig 6A, C, and D). The two anti-c-myb sera gave identical results in this comparison of c-myb expression across cell types (Fig 6A and C). The suppressor T-cell subpopulation (CD8\(^+\)) was not detectably different from the total T-cell population (CD5\(^+\)) or from subpopulations of natural killer (NK) cells (Leu 19\(^+\)) (Fig 6B). Relatively mature nucleated erythroid precursors can be identified by lack of expression of the common human leukocyte antigen, HLe-1 (CD45).\(^{18}\) This population of cells was identified by labeling bone marrow cells with CD45 and selectively gating on cells negative for this marker. Most of these late nucleated erythroid cells appeared to contain little if any c-myb (Fig 6D), although a few of these cells contained significant levels of c-myb. Anucleate (mature) RBCs do not survive the detergent treatment and cannot be evaluated in this assay.

**DISCUSSION**

Previous work on malignant hematopoietic cell lines has led to the suggestion that the protooncogene c-myb is expressed at higher levels in immature cells than in mature cells.\(^4,12\) Addressing this question adequately in normal hematopoietic cells has been difficult because of the heterogeneity of normal bone marrow and the difficulties associated with isolating adequate numbers of pure cell populations for analysis of c-myb expression. We successfully used two methods to isolate or identify selected subpopulations of normal human hematopoietic cells and to detect c-myb protein expression immunologically in these subpopulations using previously characterized rabbit antisera to c-myb.\(^1\)
C-MYB IN NORMAL HUMAN HEMATOPOIETIC CELLS

The first method consisted of physically isolating hematopoietic progenitor cells (identified by cell surface markers CD34 and CD10) by immune adherence or FACS, followed by immunohistochemical staining for c-MYB. Our results using this method confirmed the prediction that normal hematopoietic progenitor cells (including B-lymphoid progenitors) express c-MYB (Fig 2). However, that a significant number (at least 30%) of other bone marrow cells had detectable levels of c-MYB protein was also obvious. In addition, determination of relative levels of expression of c-MYB in different cell types was difficult, since it was based on subjective visual assessment of degrees of immunohistochemical staining intensity.

To facilitate and improve quantitative assessment of relative oncoprotein levels across cell types, we adapted a flow cytometric assay to allow simultaneous identification of cell types (by cell-surface marker) and nuclear protein levels. This assay has the advantages of being relatively rapid and quantitative and allows assessment of numerous normal hematopoietic cell types in any given experiment. We confirmed the relatively high level of c-MYB expression in CD34+ progenitor cells using this technique (Figs 5 and 6). We also showed that selection of an identical cell population, rather than heterogeneous bone marrow cells, to define control levels of binding with preimmune serum influenced assessment of c-MYB expression within a particular cell population. This selection of control values also influences assessment of the number of total bone marrow cells expressing c-MYB. The histochemical stain and the quadrants used in Fig 3 suggest that 30% to 40% of bone marrow cells stain more intensely with c-MYB antiserum than with control serum. However, almost all cell types examined in these studies had some detectable binding above background in that cell type [except perhaps the maturing nucleated erythroid cells (Fig 6)], suggesting that most low-density bone marrow cells have detectable c-MYB protein.

The relative levels of c-MYB protein in the various normal hematopoietic cell types we report (approximately fourfold difference in mean fluorescence intensity between CD34+ cells and CD22+ cells) appear to vary less than the relative changes in c-MYB mRNA expression at various stages of chemically-induced maturation of some leukemic cell lines. One difference in experimental design is our measurement of c-MYB protein rather than mRNA. Regulation of c-MYB expression appears to be very complex, occurring at both transcriptional and posttranscriptional steps. This complexity could produce a nonlinear relationship between c-MYB mRNA levels and protein levels in various cell types. Protein levels, however, are probably a more significant variable in determining (patho)physiologic roles. Alternatively, the absence of c-MYB mRNA could be a characteristic of induced, differentiating cultured cell lines but not accurately reflect normal in vivo differentiation pathways. Stimulation of normal hematopoietic cells by growth factors in vitro also results in marked, though transient, alterations in protooncogene expression. Such transient changes in the levels of c-MYB protein may not be evident in the specific types of unstimulated, freshly obtained human bone marrow cells evaluated in the experiments we reported.

Several laboratories have correlated levels of c-MYB with proliferation status in certain cell types. However, there are exceptions to this correlation, such as immature thymocytes which express c-MYB in a cell cycle-independent manner. CD34+ normal bone marrow cells have a lower proliferation index than CD10+ cells, and CD10+ cells have a much higher proliferation index than CD20+ cells. Yet we showed that CD34+ cells express higher levels of c-MYB than of CD10+ cells, which in turn express similar levels of c-MYB to CD20+ cells. Thus, the relative levels of c-MYB in CD34+...
CD10⁺, and CD20⁺ cells appear to be more closely related to maturation stage than to the fraction of proliferating cells in these populations.

Future experiments using these techniques should improve our understanding of the importance of the c-myb protein in proliferation and differentiation by examination of c-myb expression in specific populations of normal hematopoietic cells as a function of cell cycle with and without growth factor simulation. In addition, this flow cytometric technique is proving useful in assessing the significance of oncprotein levels in malignant cells by allowing comparison of the levels in various leukemic subtypes to those of phenotypically similar normal hematopoietic cells.

ACKNOWLEDGMENT

We thank Dr Lewis Strauss, Steve Welsh, and Carol Cremo for help with some experiments; Drs Ken Bauer, Mary Jo Fackler, and Craig Hurwitz for helpful discussions; Dr Michael Loken for critical review of the manuscript; and Jude Brown-Bellamy for excellent secretarial assistance.

REFERENCES

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32. Watson RJ: A transcriptional arrest mechanism involved in controlling constitutive levels of mouse c-myb mRNA. Oncogene 2:267, 1988
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