Expression of Human Interleukin-3 (multi-CSF) Is Restricted to Human Lymphocytes and T-Cell Tumor Lines

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While the cellular sources for granulocyte-macrophage colony-stimulating factor (GM-CSF) are known to be widely distributed among several cell types, interleukin-3 (IL-3) gene expression has been demonstrated in only certain T-cell clones and in blood mononuclear cells stimulated with phytohemagglutinin (PHA) and phorbol-myristate-acetate (PMA). To determine which blood cells were responsible for this expression, we fractionated PHA/PMA-stimulated mononuclear cells and identified T lymphocytes as the source of IL-3 mRNA. Low-level IL-3 expression was detected as well in several stimulated human T-cell lines. Hematopoietic stromal cells such as fibroblasts and endothelial cells could not be induced to express IL-3 mRNA. The kinetics of IL-3 mRNA induction in mononuclear cells and lymphocytes stimulated with PHA/PMA or anti-CD3 monoclonal antibody (MoAb) and interleukin-1 (IL-1) were similar to those observed for GM-CSF expression.

P ROGENITOR derived hematopoiesis depends on distinct hematopoietic growth factors (HGFs).1 For example, proliferation and differentiation of a substantial fraction1 of erythroid burst-forming units (BFU-E)4 requires both erythropoietin and an additional factor functionally referred to as burst-promoting activity (BPA).6 T lymphocytes have been identified as a particularly potent cellular source of BPA,6-11 which acts directly on BFU-E rather than through accessory cells.12,13

Soon after the genes for human interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF) were cloned and their glycoprotein products purified,14-16 both factors were recognized to have burst promoting activity.17,18 IL-3 is particularly potent in this regard.17 Studies of the cellular origins of these two HGFs have documented that GM-CSF gene expression occurs in a variety of cell types.19-22 Endothelial cells and fibroblasts, constituents of the hematopoietic microenvironment, produce GM-CSF in response to interleukin-1 (IL-1)23-25 or tumor necrosis factor (TNF).19,21 In contrast, recent data suggest that IL-3 expression occurs largely in lectin stimulated blood mononuclear cells13 and some T-cell clones.26

Since IL-3 has broad hematopoietic effects in experimental models in vivo27-28 its cellular source in humans is of great interest. To determine the sources of IL-3 we measured the capacity of human blood cells and hematopoietic stromal cells to produce IL-3. We demonstrate here that adequately stimulated T lymphocytes are the only normal cells in which IL-3 expression was detectable. The stimuli included phytohemagglutinin (PHA) and phorbol-myristate-acetate (PMA) or the combination of the monokine IL-1 with anti-CD3 monoclonal antibody (MoAb) attached to solid support. The data also demonstrated that although GM-CSF and IL-3 lie in close proximity on chromosome 5p9 they are regulated very differently. GM-CSF, but not IL-3, is expressed in a wide variety of cells, whereas IL-3 expression was detected solely in T lymphocytes and always in concert with GM-CSF. The mechanism of IL-3 gene regulation could be uncovered more readily by studies of human cell lines that express the gene. To find suitable cells we examined a series of T-cell tumor lines. IL-3 mRNA could be detected in several of the lines, but levels of mRNA expression were considerably lower than those observed in the gibbon T-cell leukemia line MLA 144, which served as the cloning source for primate IL-3. Furthermore, IL-3 expression in MLA 144 cells depended on stimulation with PMA, while the human T-cell line Jurkat produced IL-3 in response to the lectin PHA.

METHODS

Culture of endothelial cells and fibroblasts. Total cell RNA from primary umbilical vein endothelial cells (HUVE), dermal fibroblasts (DF) and endothelial cells immortalized with the Kirsten sarcoma virus pseudotyped with an amphotropic helper virus (HUVE-KSV) were generously provided by D. Faller (Dana Farber Cancer Institute, Boston). Cells were cultured as previously described26 and stimulated for 48 hours with recombinant human IL-1 beta (Genzyme Corp, Boston) at 10 U/mL or recombinant human TNF alpha (Genentech Inc, San Francisco) at 1,000 U/mL.

Separation of mononuclear cells and lymphocytes from blood. Heparinized blood obtained from healthy adult volunteers was separated over Ficoll-Paque (1.077 g/mL) (Pharmacia Fine Chemicals, Piscataway, NJ) at 400 g for 40 minutes at 20°C. The mononuclear cells at the interface were collected, washed, and resuspended in Iscove’s modified Dulbecco’s medium (IMDM) (GIBCO, Chagrin Falls, OH) containing 10% fetal calf serum (FCS). For separation of lymphocytes, heparinized blood samples were mixed 5:1 with Lymphocyte Separator Reagent (Technicon Instruments Corp, Tarrytown, NY) and incubated on a rotating platform at 37°C for one hour. This step allowed monocytes and polymorphonuclear leukocytes to phagocytose iron particles, thereby improving their depletion in following density gradients. Blood was

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then diluted with IMDM and separated over Ficoll-Paque as described above. The interface lymphocyte fraction was collected, washed, and cultured overnight in IMDM/10% FCS at 37°C. Nonadherent cells were harvested, washed, and resuspended in IMDM/10% FCS at 2 x 10⁶ cells/mL; they contained <1% monocytes by morphology using Wright-Giemsa staining.

Separation of monocytes and lymphocytes from leukocyte concentrates. Leukopheresis bags discarded after plateletheresis of healthy donors were used for purification of monocytes and lymphocytes, applying some modifications to a previously described procedure. Briefly, mononuclear cells from a single donor were separated on Ficoll-Paque and twice washed in RPMI (GIBCO) containing 10% FCS (Hyclone, Logan, UT) at 20°C. Cells were resuspended in culture medium at 5 x 10⁶ cells/mL in 15 mL polypropylene tubes (Corning, Medfield, MA) and placed horizontally on a rocker platform at 4°C. Under these conditions monocytes aggregated transiently. After 30 minutes the tubes were placed vertically on ice and aggregated cells were allowed to settle. To separate monocyte aggregates from lymphocytes, cells were underlaid with 3 mL FCS. The sedimented aggregates of monocytes were harvested after 15 minutes, resuspended vigorously, and adjusted to 2 x 10⁶ cells/mL in IMDM/10% FCS. Purity of the monocyte fraction was >97% by Wright-Giemsa staining. The lymphocyte-enriched fraction was collected at the interface above the FCS. Monocytes contaminating the lymphocyte fraction were removed by treatment with Lymphocyte Separator Reagent, Ficoll-Paque separation, and adherence to plastic as described above. B cells in the lymphocyte fraction were removed by immune absorption to rabbit anti-mouse immunoglobulin-coated beads (Dynabeads; P and S Biochemical Inc, Gaithersburg, MD). Lymphocytes were labeled at 4°C for 30 minutes with anti-Leu 12 (Becton-Dickinson, Mountain View, CA). Cells were then washed twice in IMDM, once in phosphate-buffered saline (PBS)/5% FCS and added to the immunomagnetic beads at approximately five beads per mixture. The mixture was resuspended and incubated for 30 minutes at 4°C. A magnet was used to remove cells bound to the beads. Nonbinding lymphocytes were washed twice and resuspended at 2 x 10⁶ cells/mL in IMDM/10% FCS. By fluorescence-activated cell analysis lymphocytes contained <1% monocytes and B cells, 10% to 20% CD15 positive natural killer (NK) lymphocytes, and 70% to 85% CD3 positive T cells.

Culture of T-cell lines and clones. The T-cell lines Jurkat, Hut 78, HPB-ALL, HSB-2, CEM, Molt 3, Molt 4, Peer, and MLA 144 were maintained in RPMI/10% FCS. An antigen-specific CD4+ T-cell line clone (gift of S. Burakoff, Dana-Farber Cancer Institute) was maintained with periodic antigen stimulation in RPMI/10% FCS supplemented with 10% interleukin-2 (IL-2)–containing human mononuclear cell conditioned media.

Stimulation of cell cultures with PHA/PMA or lipopolysaccharides. Cells to be stimulated were resuspended in IMDM/10% FCS at 2 x 10⁶ cells/mL. PHA (Difco Laboratories, Inc, Detroit) at 0.1% vol/vol and PMA (Sigma Chemical Co, St Louis) at 10 ng/mL or lipopolysaccharides (LPS) (Difco) at 15 μg/mL were added. Cultures were then incubated in 75 cm² Falcon Flasks (Becton Dickinson, Lincoln Park, NJ) at 37°C for various times. After incubation, nonadherent cells from mononuclear cell or lymphocyte cultures were removed, centrifuged at 400 g for 15 minutes, and washed twice in cold PBS before RNA extraction. Conditioned media was stored at -20°C to be tested for HGF activity in a CML blast assay. For monocyte cultures conditioned media was removed from the flask and the adherent cell layer washed three times with cold PBS before RNA extraction.

Stimulation of lymphocytes with anti-CD3 MoAb and IL-1. One hundred fifty square centimeter culture flasks (Costar, Cambridge, MA) were coated overnight at 4°C with the anti-CD3 MoAb OKT3 (American Type Culture Collection, Rockville, MD) at 10 μg/mL dissolved in 20 mmol/L carbonate buffer (pH 9.6). This concentration of anti-CD3 MoAb induced maximal proliferation of purified T cells in the presence of IL-1 (data not shown). Flasks were then washed three times with cold PBS and one with RPMI/10% FCS. Lymphocytes resuspended in IMDM/10% FCS at 2 x 10⁶ cells/mL were added to the culture flasks and incubated at 37°C in the presence of recombinant IL-1 at 10 U/mL.

RNA isolation and Northern analysis. Cells were lysed in guanidinium thiocyanate solution, and total RNA was extracted as described. Fifteen μg of total RNA per lane was separated by electrophoresis in 1% agarose gels and transferred to nitrocellulose filters. mRNAs were detected by hybridization with 32P-radiolabeled full-length cDNA probes of IL-3 or GM-CSF or with their respective anti-sense RNA probes. For construction of the anti-sense RNA probes the full-length cDNA fragment of IL-3 cDNA was inserted into the pSP78 vector (Boehringer Mannheim, Indianapolis) and the full-length human GM-CSF cDNA into the pSP64 vector (Promega Biotec, Madison, WI). Prehybridization was for four hours and hybridization for 20 hours in 50% formamide, 2.5 mmol/L phosphate buffer (pH 6.5), 5 x SSC, 5 x Denhardt’s, 60 μg/mL salmon sperm DNA, and 0.1% SDS. Hybridization was allowed to proceed at 42°C (DNA probes) or 56°C (RNA probes). Filters were washed twice at 58°C and once at 68°C in 0.2 x SSC, 0.1% SDS. After hybridization with RNA probes some filters were subsequently RNase treated. Blots were rinsed three times in 2 x SSC for five minutes, incubated for 15 minutes in 50 ng/mL RNAse A (Sigma Chemical Co) in 2 x SSC, and washed for 30 minutes at 50°C in 0.1 x SSC, 0.1% SDS. All filters were then exposed to Kodak X-OMAT AR films at −70°C with a Dupont Cronex (Wilmington, DE) intensifier screen.

RESULTS

IL-3 mRNA is not detectable in endothelial cells, fibroblasts, or monocytes, but only in T lymphocytes. GM-CSF production is known to occur in primary HUVE and human DF after stimulation with IL-1 (10 U/mL) or TNF (1,000 U/mL). In addition, HUVE cells immortalized by Kirsten sarcoma virus (HUVE-KSV) constitutively express GM-CSF. However, we were unable to detect IL-3 mRNA in HUVE or DF cells, either unstimulated or stimulated with IL-1 or TNF. Similarly, there was no constitutive IL-3 expression in the immortalized HUVE-KSV cell line. IL-3 mRNA was, however, readily detectable in human blood mononuclear cells. While unstimulated mononuclear cells did not express detectable levels of IL-3 mRNA, gene expression was readily detected 12 hours after stimulation with PHA/PMA (Fig 1). Separation of the mononuclear cell culture into monocyte and lymphocyte fractions revealed that IL-3 mRNA was expressed in PHA/PMA-stimulated lymphocytes but not in monocytes stimulated with either PHA/PMA (Fig 1) or LPS (data not shown). Fractionation of the lymphocyte population by depletion of B cells showed that the remaining population of T and NK lymphocytes was responsible for the IL-3 expression. As we did not directly analyze B lymphocytes we cannot rule out the possibility that B cells may have made a small contribution to the IL-3 signal observed in the unfractionated lymphocytes. Further evidence that T cells are capable of expressing IL-3 mRNA is demonstrated by high message levels accumulated in an IL-2-dependent antigen reactive CD4 positive T-cell clone proliferating with periodic antigen stimulation.
IL-3 and GM-CSF mRNA are expressed with similar kinetics in stimulated mononuclear cell and lymphocyte cultures. IL-3 mRNA first became detectable in mononuclear cell cultures two hours after addition of PHA/PMA (Fig 2A). Expression gradually increased and peaked eight hours after the onset of stimulation. A subsequent slight decrease in message level was followed by a second rise at 18 hours. This coincided with the onset of cell proliferation in the cultures (data not shown). Maximal gene expression was seen at 24 hours and was maintained while the cultured cells continued to proliferate. The pattern of IL-3 gene expression characterized by an early peak and subsequent rise to higher plateau levels was a consistent finding in PHA/PMA stimulated mononuclear cell and lymphocyte cultures. There were, however, modest variations in the timing of the different phases of expression; eg, in PHA/PMA-stimulated lymphocyte cultures depleted of monocytes the peak of the early phase of IL-3 expression was seen 12 hours after the addition of PHA/PMA.

The kinetics of GM-CSF expression were similar to those of IL-3 (Fig 2B). In contrast to IL-3, low level GM-CSF mRNA expression could be detected in unstimulated mononuclear cells (Fig 2). This mRNA was absent in unstimulated monocyte-depleted lymphocyte cultures (data not shown). IL-3 and GM-CSF mRNA expression correlated with bioactivity analyzed in the CML blast cell assay geomet32'33 (data not shown).

IL-3 and GM-CSF mRNA are expressed in T lymphocytes after activation with IL-1 and anti-CD3 MoAb. Our experiments show that T lymphocytes express IL-3 mRNA on activation with PHA/PMA. To evaluate T-cell activation through the T-cell receptor-CD3 complex, resting lymphocytes were stimulated with solid phase anti-CD3 MoAb and the monokine IL-1 (Fig 3). Lymphocytes purified by vigor-
Inducible expression of IL-3 or GM-CSF mRNA.

Ous monocyte depletion were cultured in media containing IL-1 (10 U/mL) in the presence of anti-CD3 MoAb attached to solid support. As shown in Fig 3, IL-3 mRNA could be detected two hours after the addition of IL-1 and anti-CD3 MoAb. IL-3 expression peaked at 12 hours and subsequently decreased. The second peak of expression, consistently observed in PHA/PMA-stimulated cell cultures (Fig 2), was absent in the lymphocyte culture stimulated with anti-CD3 MoAb/IL-1, even after stimulation for 30 hours (Fig 3) and in the presence of cell proliferation (data not shown). Again, the expression of GM-CSF mRNA followed kinetics essentially identical to those of the IL-3 transcript (data not shown).

IL-3 and GM-CSF mRNA are expressed in human T-cell tumor lines. Studies of regulation of human IL-3 gene expression could be greatly facilitated if established human cell lines were to produce high levels of specific mRNA and protein. To that end we screened a series of human T-cell tumor lines (Table 1). IL-3 and GM-CSF mRNA were detected in the Jurkat, HPB-ALL, HSB-2, and Peer cell lines 12 hours after the addition of PHA/PMA. Levels of expression of GM-CSF were significantly higher than those observed for IL-3. Neither GM-CSF nor IL-3 was detectable in Molt 3, Molt 4, or CEM cells. Constitutive as well as inducible GM-CSF expression was noted in the cell line HUT 78, but IL-3 message could not be demonstrated in these cells even after treatment with PHA/PMA.

Regulation of IL-3 gene expression in Jurkat is distinct from MLA 144. The requirements for the induction of IL-3 mRNA in Jurkat cells were investigated. IL-3 gene expression was observed in Jurkat cells stimulated with PHA, but not after addition of PMA alone (Fig 4). The same mechanism of induction was demonstrated for GM-CSF and IL-2 (data not shown). These results differ from those observed in the gibbon T-cell line MLA 144. This cell line, which is infected with a gibbon ape leukemia virus, expressed high levels of IL-3 (Fig 4), GM-CSF, and IL-2 (data not shown) after PMA but not after PHA stimulation.

**DISCUSSION**

While hematopoietic stromal cells have been shown to synthesize several HGFs, including GM-CSF, in response to stimulation with endotoxin or the monokines IL-1 or TNF, IL-3 expression was not detectable. In contrast to mesenchymal cells, blood mononuclear cells stimulated with PHA/PMA did express the IL-3 gene. We showed that the T-cell population and possibly NK lymphocytes in the mononuclear cell cultures were the actual site of IL-3 production. Our finding of IL-3 expression in stimulated human T lymphocytes is consistent with observations in murine cells.

Antigenic or mitogenic stimulation of T lymphocytes is required for IL-3 expression. Transcription of IL-3 is detectable in stimulated mononuclear cells two hours after addition of PHA/PMA, and the first peak of expression occurs at about eight hours. A second more pronounced and higher increase in IL-3 mRNA was observed at 18 hours. This steady state level persisted as long as the cells continued to proliferate (data not shown). We do not know whether this expression is due to enhanced transcription in the lymphocyte population responsible for the early phase of expression or to the induction of IL-3 in a different subset. Identification of the cells expressing IL-3 at any given time during stimulation of the culture will require the use of in situ hybridization.

In PHA/PMA-stimulated mononuclear cells or lymphocytes GM-CSF gene expression parallels that described for IL-3; this was also true for IL-2 mRNA expression (data not shown). Although activation was necessary for GM-CSF mRNA induction, we did observe low level expression in the mononuclear cells before addition of PHA/PMA. This low level, which was absent in the monocyte depleted lymphocyte fraction, is probably due to expression in monocytes that were mildly activated following the purification procedure.

Stimulation of resting T cells through the CD3 T-cell receptor-complex in the presence of IL-1 promptly induced IL-3 and GM-CSF expression. The kinetics of the first wave of mRNA expression were similar to those shown for PHA/PMA-stimulated lymphocyte cultures. The second peak of expression was, however, not observed. Since PMA was omitted in these cultures, activation of contaminating monocytes by the phorbol ester would not have occurred. Thus, it is possible that the induction of the second peak is dependent.
on the presence of activated monocytes. Although monocytes do not synthesize IL-3 themselves, they may play an important role in regulating IL-3 production in T lymphocytes through cellular interactions or the secretion of monokines.

Elucidation of the mechanisms of human IL-3 gene regulation would be facilitated through the availability of a cell line that produces relatively high levels of specific mRNA and protein. To that end, we screened the mRNA of a series of human T-cell tumor lines. After PHA/PMA stimulation, low levels of IL-3 message were detectable in Jurkat, HPB-ALL, HSB-2, and Peer, all of which constitutively expressed GM-CSF. In fact, among the GM-CSF mRNA expressing T-cell lines only HUT 78 failed to display IL-3 expression, even though it exhibited constitutive and highly inducible GM-CSF mRNA levels. The lines found capable of IL-3 and GM-CSF expression were heterogeneous with respect to stage of maturation, expression of surface antigens, and the ability to produce IL-2. This is in agreement with previous work in which release of BPA activity by T-cell lines varied with no clear correlation between BPA production and phenotypic characteristics of the cells.44,45 Interestingly, the Peer cell line, which expresses both IL-3 and GM-CSF, encodes a T-cell receptor comprised of gamma and delta chains instead of the more common alpha and beta heterodimer.46,47 This suggests that the subset of immature thymocytes and adult blood T cells that express the gamma/delta T-cell receptor48,49 is capable of transcribing both CSFs on stimulation. If this is generally the case, we may begin to understand the capacity of lymphocytes of patients with severe combined immunodeficiency to express GM-CSF and IL-3. The residual lymphocytes in some of these patients express a similar gamma/delta T-cell receptor.49 This may explain the normal steady state and stress hematopoiesis observed in these immunodeficient patients.8

Although much can be learned from the study of IL-3 expression in human T-cell lines, it must be emphasized that the level of expression is considerably lower than that observed in freshly isolated lymphocytes stimulated with PHA/PMA. The reasons for this difference are unclear and elucidation of the molecular events leading to IL-3 gene activation may shed light in that respect. High levels of IL-3 mRNA were observed only in the gibbon T-cell leukemia line MLA 144. Interestingly, the mechanism of IL-3 gene regulation in MLA 144 cells was different from that observed in Jurkat cells. The latter expressed IL-3 after PHA stimulation, but not after PMA exposure. The same pattern of induction has been reported for IL-2 production in these cells.50 In MLA 144 cells the signal required for IL-3 expression was PMA and not PHA. The observation that MLA 144 may not express a functional T cell receptor (Niemeyer CM, unpublished observation) may explain their unresponsiveness to PHA. PMA induced protein kinase C activation is thought to bypass cell surface receptor expression. MLA 144 cells contain a variant of the San Francisco strain of gibbon ape leukemia virus integrated in their genome.51 In fact, constitutive expression of IL-2 by these cells was shown to result from integration of two proviruses in the 5' and 3' vicinity of the IL-2 gene.52 Whether other proviruses play a direct or indirect role in IL-3 and GM-CSF regulation is not known. Studies to answer these questions are in progress.

While in all our experiments the presence of IL-3 transcription was associated with GM-CSF expression and both genes are known to be tightly linked,32,53 our study did not address the question of differential control mechanism of these two lymphokines. Independent regulation for the two CSFs in T cells has recently been claimed.54,55 GM-CSF expression occurs without IL-3 production in induced monocytes, endothelial cells, and fibroblasts. Since synthesis of the multipotential hematopoietic growth factor IL-3 appears to be confined to T lymphocytes, these cells may play a key role in the rapid derivation of blood cells from progenitors during the host response to antigens.

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