Insulin-Like Growth Factor-1 Potentiates Expansion of Interleukin-7–Dependent Pro-B Cells

By Laura F. Gibson, Debra Piktel, and Kenneth S. Landreth

Commitment to B-lymphocyte differentiation is characterized by expression of the B220 form of the common leukocyte antigen (Ly-5) and D-J rearrangement of the Ig heavy chain gene complex. B-lineage progenitor cells, or pro-B cells, that have initiated Ig gene rearrangement, but do not express detectable Ig heavy or light chain protein, have recently been shown to retain substantial capacity for expansion in vitro in the presence of bone marrow (BM) stromal cells and interleukin-7 (IL-7). Although the potentiating effect of stromal cells on pro-B–cell proliferation can be partially attributed to the ligand for the proto-oncogene receptor c-kit (c-kit ligand [KL] or stem cell factor), several lines of evidence suggest that c-kit–mediated cell signaling is not required for pro-B–cell expansion. Previous studies from this laboratory demonstrated that insulin-like growth factor-1 (IGF-1) potentiated the proliferative effect of IL-7 on nonadherent cells from lymphoid long-term BM cultures in a manner similar to that shown for KL. To further delineate specific cell stages that respond to lymphopoietic cytokines, we derived continuously proliferating pro-B–cell lines from day-14 murine fetal liver in the presence of IL-7 and BM stromal cell clone S10. Initial expansion and continued proliferation of these pro-B–cell lines was absolutely dependent on the presence of both IL-7 and stromal cells. In the absence of KL, IL-7–stimulated proliferation of these cells in short-term cultures and addition of either recombinant IGF-1 or KL significantly potentiated this proliferative response. Although IGF-2 and insulin also potentiated the effect of IL-7, our data suggest that neither IGF-2 nor insulin represent normal regulators of intramyeloid lymphocyte development. IGF-1 and KL activate unique cascades of intracellular signaling events and inclusion of both cytokines in cultures of IL-7–stimulated pro-B cells resulted in additive potentiation of the proliferative response. Taken together, these results suggest that expansion of pro-B cells in vivo is maintained by at least three stromal cell-derived cytokines. IL-7 appears to be unique in delivering the primary proliferative signal for pro-B–cell expansion; however, both KL and IGF-1 potentiate the proliferative effect of IL-7 on these cells. The functional redundancy and additive effects of IGF-1 and KL as amplification signals for developing B-lineage cells underscore the essential nature of clonal expansion and diversification in development of immunocompetent lymphoid cells.

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KL are additive and reflect the fact that these cytokines activate separable sets of intracellular signalling events. These data show that, whereas the role of IL-7 in B lymphopoiesis appears to be unique in directly stimulating proliferation of pro-B cells, potentiation of this primary proliferative signal and significant expansion of pro-B cell clones in vivo is also regulated by both KL and IGF-1.

MATERIALS AND METHODS

Mice. BALB/c mice (3 to 6 weeks old) were obtained from Taconic Laboratories (Germantown, NY) and housed in the vivarium of the West Virginia University Health Sciences Center. Timed pregnancies were calculated from the appearance of vaginal plugs after overnight mating. The plug date was considered day 0.

Cytokines. Recombinant murine IL-7 (10^7 U/mg), recombinant human IGF-1, and recombinant human IGF-2 were obtained from Biosource International (Westlake Village, CA). Recombinant human IGF-I, and recombinant human IGF-2 were obtained from Genzyme, Inc (Boston, MA).

Stromal cell lines. Characterization and maintenance of BM stromal cell line S10 has been previously described in detail.

Briefly, stromal cells were grown to confluence in 25-cm² or 75-cm² flasks in α-Modified Eagle’s Medium (αMEM; GIBCO, Grand Island, NY) containing 5% fetal calf serum (Hyclone Lot No. 11111041; Hyclone, Logan, UT). Cells were passaged weekly by trypsinization.

Derivation of pro-B–cell lines. Derivation of pro-B–cell lines and clones has been described in detail. Briefly, day-14 fetal livers were removed and dispersed into single-cell suspensions by repeated aspiration through progressively smaller bore hypodermic needles (18 to 22 gauge). Pro-B–cell lines were derived by serially passing day-14 fetal liver nonadherent cells in the presence of BM stromal cell line S10 and 50 U/mL recombinant murine IL-7. Cells that proliferated for 4 weeks in vitro were expanded in bulk culture under the same conditions and carried as a fetal liver cell line (FL). FL cells were cloned at limiting dilution and expanded in the presence of S10 stromal cells and IL-7. A panel of cloned derivatives of FL were characterized for further study. Data from one of the resulting clones, C1.92, are presented.

Cell depletion. Nonadherent pro-B–cells were depleted of residual adherent stromal cells on G10 Sephadex exactly as previously described. Briefly, up to 10^8 cells in αMEM were loaded onto 8-mL columns of G10 Sephadex equilibrated in 37°C αMEM and held for 30 minutes. Nonadherent cells were then eluted with 30 mL of the same medium. In all experiments, G10 nonadherent cells were evaluated microscopically after the depletion procedure. In all of the experiments reported, nonadherent pro-B–cells were of uniform lymphoid morphology and did not contain detectable stromal cells.

Fluorescence microscopy. For cytoplasmic Ig staining, 10^5 cells were cytocentrifuged onto glass slides, fixed in ethanol containing 5% glacial acetic acid, rehydrated in phosphate-buffered saline (PBS), and incubated with fluorescein isothiocyanate (FITC)-goat antimouse κ and FITC-goat antimouse λ light chain antibodies (50 µg/mL; Southern Biotechnology Associates, Inc [SBA], Birmingham, AL) for 30 minutes at room temperature. After an overnight wash in PBS, slides were mounted in Fluoromount G (SBA) and examined under epi-illumination on a Zeiss Universal microscope (Carl Zeiss, Inc, Thornwood, NY). All cells with detectable Ig light chain were considered B cells. Cells with cytoplasmic κ heavy chain of Ig but no detectable light chain protein were considered pre-B cells. For B220 staining, cells were incubated for 30 minutes at 4°C with monoclonal antibody 14.8 and then for 30 minutes with FITC-goat antirat Ig (SBA). Cells were washed to remove excess antibody and examined by epi-fluorescent microscopy.

Characterization of Ig gene rearrangement status. For Ig gene rearrangement analysis, high molecular weight DNA was organically extracted from 2 × 10^6 cells and precipitated with 100% ETOH. DNA was air-dried, resuspended in 100 µL of dH2O, and restricted with 20 U EcoRI for 6 to 8 hours at 37°C. DNA was then reprecipitated with 2 vol of 100% ETOH in the presence of 20 µg Escherichia coli tRNA as carrier for use as polymerase chain reaction (PCR) template. Ig heavy chain gene rearrangement status was determined using primer sets that defined intervening sequence 5′ of DFL16.1 and 5′ of Jκ1 (Fig 1). The sequence of primer sets used was exactly as previously reported. Amplification was performed for 35 cycles with denaturation at 95°C for 1 minute, annealing at 58°C for 3 minutes, and extension for 4 minutes at 72°C, with an additional 15-minute extension added to the final cycle. Germline configuration was confirmed with identically prepared DNA samples from stromal cell line S10. Amplification of glucose-aldehyde-phosphate dehydrogenase (GAPDH) using primer sets previously described was used throughout as a control for template integrity and normalization of data to a constitutively expressed transcript. PCR products were separated on 2% ethidium bromide-stained agarose gels and photographed for densitometric analysis using BioScan Optimas (BioScan, Inc, Edwards, WA).

Proliferation assays. Nonadherent FL or C1.92 cells were cultured at a final concentration of 2.5 × 10^4 cells/mL (5 × 10^4 in 200 µL) in αMEM-containing 5% heat-inactivated fetal calf serum in 96-well tissue culture plates (Corning Glassworks, Corning, NY).
REGULATION OF PRO-B-CELL PROLIFERATION

Table 1. Phenotype of Culture-Expanded Fetal Liver Pro-B Cells

<table>
<thead>
<tr>
<th></th>
<th>B220</th>
<th>cx</th>
<th>cx + cl</th>
<th>sigM</th>
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<tr>
<td>FL</td>
<td>83 ± 8</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
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<tr>
<td>C1.92</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
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FL or C1.92 cells were depleted of residual stromal cells and stained with fluorescent-labeled antibodies as described in the Materials and Methods. Immunofluorescent staining was evaluated microscopically under epi-illumination. Data are representative of three similar experiments.

Where indicated, KL (100 ng/mL), IGF-1 (5 to 100 ng/mL), IGF-2 (5 to 100 ng/mL), insulin (5 to 100 ng/mL), or IL-7 (50 U/mL) were added to these cultures. Wells were pulsed with $1 \mu$Ci $^{3}H$-Tdr at 24 hours and harvested 18 hours later onto glass wool fiber strips with an automated cell harvester (Cambridge Instruments, Boston, MA). Incorporated radioactivity was determined by liquid scintillation counting (LKB/Wallac Model 1410; Gaithersburg, MD) in an aqueous fluor (Biosafe-II; Research Products International, Mount Prospect, IL).

RESULTS

Derivation of pro-B-cell lines. To directly investigate the effect of insulin-like growth factors on pro-B-cell proliferation, we initiated cultures of day-14 murine fetal liver cells on BM stromal cell line S10 in the presence of 50 U/mL of IL-7 as previously described. As noted in that study, addition of exogenous IL-7 was absolutely required to maintain lymphoid cell proliferation in these cultures. Cells passaged under these conditions for at least 4 weeks were used in all studies. Nonadherent cells from the uncloned pro-B-cell line (FL) and a panel of cloned derivatives of this cell line were morphologically identical and characterized as large to medium immature lymphoid cells with characteristic large nucleus to cytoplasmic ratio and leptochromatric nuclear morphology. This cell morphology is consistent with that previously described for fetal liver and BM B-cell precursors.

The cell surface molecule Ly-5, or the common leukocyte antigen (CD45), is expressed differentially on hemopoietic lineages. B-lineage cells in the marrow express the 220-Kd form of this molecule (B220) with initiation of expression apparently coincident with initial rearrangement of Ig heavy chain genes. We found that the majority of uncloned culture expanded pro-B cells expressed the B220 form of Ly-5, but did not have detectable cytoplasmic or cell surface expression of heavy or light chain Ig protein (Table 1). Cloned pro-B-cell line derivatives of FL, of which C1.92 is representative, did not express B220 or detectable Ig heavy or light chain protein (Table 1). However, subsequent to the completion of data for this report, this cell line initiated B220 expression and has remained B220+ in continuous culture (data not shown). This alteration in B220 expression at 12 to 15 weeks of continuous culture did not correlate with a change in cell morphology, Ig protein expression, or characteristic pattern of Ig gene rearrangement (see below). In addition, expression of B220 did not correlate with any change in cytokine or stromal dependence or proliferative response in the studies similar to those reported here (data not shown).

Ig gene rearrangements in pro-B-cell lines. To further confirm the developmental staging of pro-B-cell lines used in this study, Ig gene rearrangements were evaluated using PCR amplification of germline sequence 5' of JH1 and 5' of DFL16.1 (Fig 1). This strategy has been used previously to detect loss of germline sequence after D → JH and Vμ → DJμ rearrangements, respectively. As shown in Fig 1, both FL and its cloned derivative C1.92 had apparent complete rearrangement at the DJH locus, indicated by absence of amplifiable sequence 5' of JH1-specific amplicon (572-bp amplicon). As previously described for pro-B cells, both FL and clone C1.92 retained 5' DFL16.1 amplifiable sequence, showing at least some remaining germline configuration of the Vμ-D locus (Fig 1). Densitometry of these amplicons, and comparison to GAPDH and stromal cell controls, suggested that FL and C1.92 were unrearranged at the Vμ-D locus on both alleles (data not shown). However, verification of this phenotype will require PCR primer strategies that show amplicon appearance after Ig rearrangement. The Ig gene rearrangement status and lack of expression of Ig heavy or light chain gene products in these pro-B-cell lines is consistent with that previously reported for early IL-7 and stromal cell-dependent pro-B-cell lines.

Only IL-7 directly stimulated pro-B-cell proliferation. Both FL and C1.92 proliferated in short-term cultures containing only IL-7 (Figs 2 and 3). However, these
cells could not be maintained for extended periods (>2 days) in the absence of stromal cells (data not presented). Neither IGF-1, insulin, IGF-2, nor KL stimulated proliferation of pro-B cells in the absence of IL-7 at any concentration tested (Figs 2 and 3).

Pro-B–cell proliferation is potentiated by IGF-1, insulin, IGF-2, and KL. Addition of IGF-1 to cultures of pro-B cells that contained saturating levels of IL-7 resulted in marked amplification of the proliferative response for all of the cell lines tested. This effect is shown here using both the uncloned cell line FL (Fig 2) and a cloned derivative line, C1.92 (Fig 3). Similar results were observed with 5 additional independent pro-B–cell lines established from day-13 to day-16 fetal livers (data not presented). Optimal potentiation of IL-7–mediated proliferation was achieved with 20 to 100 ng/mL recombinant IGF-1 and this effect was essentially identical to that found with saturating concentrations of KL (100 ng/mL; Figs 2 and 3).

It is well documented in several experimental systems that IGF-1 and the structurally related cytokines IGF-2 and insulin often stimulate the same cell types. For that reason, we investigated the effect of IGF-2 and insulin on expanded cultures of pro-B cells. IGF-2 potentiated the effect of IL-7 on proliferation of the pro-B–cell line C1.92 (Fig 3), but had no effect when added to cultures in the absence of IL-7. However, IL-7 potentiation was achieved with a lower concentration of IGF-1 (20 ng/mL) than with IGF-2 (100 ng/mL). Although insulin appeared to minimally potentiate the effect of IL-7 at a low concentration (20 ng/mL), this result was both significantly lower than that achieved with either IGF-1 or IGF-2 and no elevation of nucleotide incorporation was achieved by increasing concentrations of insulin (Fig 3).

In some experiments, duplicate cultures were analyzed in the absence of serum. No significant differences in 3H-TdR incorporation were found between cultures that contained either no serum or 5% of the selected batch of serum used in this study (data not presented).

The effects of IGF-1 and KL are additive on pro-B–cell lines. Intracellular events that accompany IGF-1 receptor binding and KL binding are known to differ, suggesting that these two cytokines potentiate IL-7 stimulation by independent molecular mechanisms. As shown in Fig 4, when IGF-1 (20 ng/mL) and KL (100 ng/mL) were added to cultures of C1.92 cells that also contained saturating levels of IL-7, pro-B–cell proliferation was further enhanced as compared to cultures that contained either IGF-1 or KL (Fig 4). This enhanced proliferative effect in the presence of both IGF-1 and KL was additive and could not be reproduced by increasing the concentration of either single cytokine over a wide concentration range (2 to 500 ng/mL; data not presented).

DISCUSSION

The regulatory role of stromal cells in proliferation and differentiation of immature B-lineage cells has been well documented. Previous studies of BM stromal cell clones have shown that, whereas cell contact is clearly important, most of the regulatory effects described for these cells can be attributed to secreted cytokines. It is also clear that the full complement of stromal cell–derived signals necessary for pro-B–cell expansion and differentiation is not yet known. It has been generally accepted that proliferation of pro-B cells in hematopoietic tissues requires the presence of both IL-7 and additional stromal cell–derived signals. We and others have previously shown that stromal cell–derived KL potentiated the proliferative effect of IL-7 on heterogeneous long-term BM culture cells. We also reported that IGF-1 potentiated the proliferative effect of IL-7 on nonadherent lymphoid long-term BM culture cells in a manner analogous to that described for KL. In the present report, we show that cloned fetal liver pro-B–cell lines that have initiated Ig heavy chain rearrangement but do not yet express Ig protein proliferated in the presence of IL-7 alone.
This proliferative activity of IL-7 was significantly potentiated by either IGF-1 or KL and inclusion of both IGF-1 and KL resulted in additive potentiation of the proliferative effect of IL-7. This additive effect of IGF-1 and KL presumably reflects the activation of separable intracellular signaling mechanisms that result in maximal potentiation of IL-7 function.

IL-7 was originally described as a cytokine secreted by BM stromal cells that stimulated proliferation of pre-B cells. Subsequent studies have shown that IL-7 is functionally pleiotropic and stimulates proliferation of both immature and mature T lymphocytes. It has also been generally accepted that proliferation of early B-lineage cells, or pro-B cells, requires stromal cell-associated signalling in addition to IL-7 and that ability to respond to IL-7 alone is acquired relatively late in this developmental pathway. However, studies with isolated human B-cell precursors suggested that target cells for IL-7 were likely earlier developmentally than pre-B cells characterized by μ expression. We have directly addressed this question in the present study using a set of pro-B-cell lines derived from day-14 fetal liver. These pro-B-cell lines were characterized by partial rearrangement of the Ig heavy chain locus and lack of expression of Ig heavy chain protein, a phenotype consistent with previous reports. All of the pro-B-cell clones tested proliferated in response to IL-7 stimulation alone.

We previously demonstrated that proliferation of lymphoid cells from long-term BM cultures could be enhanced by coculture with BM stromal cells. The potentiating effect of stromal cells could be reproduced, in part, with recombinant KL, a cytokine produced by the BM stromal cell line used in that study. We also showed that IGF-1 stimulated differentiation of pro-B cells in the complex environment of the BM, and enhanced the proliferation of nonadherent cells from long-term BM cultures. The experiments presented herein show that both KL and IGF-1 potentiate the proliferative effect of IL-7 on isolated fetal liver pro-B-cell lines as well. These data firmly establish that the effect of IGF-1 is direct on B-lineage cells and that IGF-1-responsive cells include pro-B cells that have initiated D-J rearrangement, but lack μ-heavy chain expression.

Proliferation of IL-7-dependent pro-B-cell lines was also potentiated by both IGF-2 and insulin, albeit to a lesser degree than that found with either IGF-1 or KL. It is known that there is some cross-reactivity of insulin family cytokines with regard to binding to specific receptors for IGF-1, IGF-2, and insulin. Because of the possibility that the activity of IGF-2 or insulin was due to binding to the IGF-1 receptor, we addressed the question of whether pro-B cells express receptors for all three cytokines using previously described primer sets in reverse transcriptase PCR analyses (unpublished data). This analysis showed that pro-B cells have mRNA transcripts for both IGF-1 and insulin receptors, but no IGF-2 receptor transcripts were detected. Although these experiments did not quantitate the relative abundance of receptor mRNAs nor the actual expression of functional receptors on the cell surface, they are consistent with both previously published data and the biologic data presented here for pro-B cells. That is, the potentiating effect of IGF-1 is approximately fivefold to 10-fold higher than that found for IGF-2. This reduced effectiveness of IGF-2 correlates with the known lower affinity of IGF-1 receptor for IGF-2 compared with its cognate cytokine. The effect of insulin, on the other hand, is minimal and it seems highly unlikely that insulin is a biologically relevant ligand for this proliferative activity. However, these data do not rule out the possibility that insulin could significantly potentiate pro-B-cell proliferation at high concentrations. Interestingly, insulin has been reported to enhance growth potential of early B-lineage cells in limiting dilution assays.
and our data suggest that, in fact, IGF-1, rather than insulin, may be the biologically relevant and more active cytokine in this respect.

The role of IGF-1 in proliferation and differentiation of pro-B cells has only recently been appreciated. Like KL, IGF-1 is a pleiotropic cytokine, with endocrine effects on somatic cell growth, as well as paracrine effects on proliferation and differentiation of a number of developing cell lineages. We reported previously that stromal cell production of IGF-I was necessary for differentiation of BM pro-B cells in α-heavy chain expression in vitro. Clinically, the association of IGF-1 deficiency and B-cell immunodeficiency has also been well documented, and one recent report showed a similar immunodeficiency syndrome in mice rendered IGF-1 deficient by administration of antibodies to tumor necrosis factor α. It seems reasonable, then, to now include IGF-1 as an essential component of stromal cell-regulated B lymphopoiesis.

Our data suggest that IGF-1 and KL have both redundant and additive amplifying functions in the expansion of pro-B cells in the presence of IL-7. The functional redundancy of this amplification signal would largely explain the failure to find defects in pre-B-cell production in W-anemic mice that lack functional c-kit receptors, and in mice treated with blocking antibodies to c-kit. However, the observation that the effects of IGF-1 and KL are additive on pro-B cells in the presence of IL-7 and that IGF-1 is required for differentiation to c-kit expression suggests that, at least in embryonic development, both cytokines may be required for normal expansion and differentiation of progenitor cells for the developing B lineage. It will now be important to address the mechanisms by which stromal cell production of IGF-1 and KL are regulated and to investigate specific intracellular events that accompany ligand binding and result in differential regulation of pro-B-cell proliferation and differentiation. It will also be essential to determine whether similar proliferative activity can be shown with BM pro-B cells and whether fetal or postnatal cells differ in regulation of their developmental options.

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