Interleukin 7 independent development of human B cells
(hematopoietic stem cells)

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ABSTRACT Mammalian hematopoietic stem cell (HSC) commitment and differentiation into lymphoid lineage cells proceed through a series of developmentally restricted progenitor compartments. A complete understanding of this process, and how it differs from HSC commitment and differentiation into cells of the myeloid/erythroid lineages, requires the development of model systems that support HSC commitment to the lymphoid lineages. We now describe a human bone marrow stromal cell culture that preferentially supports commitment and differentiation of human HSC to CD19+ B-lineage cells. Fluorescence activated cell sorter-purified CD34++/lineage− cells were isolated from fetal bone marrow and cultured on human fetal bone marrow stromal cells in serum-free conditions containing no exogenous cytokines. Over a period of 3 weeks, CD34+/*lineage− cells underwent commitment, differentiation, and expansion into the B lineage. Progressive changes included: loss of CD34, acquisition of and graded increases in the level of cell surface CD19, and appearance of immature B cells expressing γ/α or μ/λ cell surface Ig receptors. The tempo and phenotype of B-cell development was not influenced by the addition of IL-7 (10 ng/ml), or by the addition of goat anti-IL-7 neutralizing antibody. These results indicate a profound difference between mouse and human in the requirement for IL-7 in normal B-cell development, and provide an experimental system to identify and characterize human bone marrow stromal cell-derived molecules crucial for human B lymphopoiesis.

Hematopoietic stem cells (HSC) are defined by their long-term self-renewing capacity and their potential to differentiate into all the major lineages of blood-forming cells. CD34 is expressed on all human HSC and restricted hematopoietic progenitors (1), and anti-CD34 monoclonal antibodies (mAbs) can be used to purify HSC for fundamental studies of blood cell development and clinical bone marrow/cord blood transplantation (1). One of the unresolved questions in blood cell development is the mechanism by which HSC commit to the lymphoid lineages. Most lymphohematopoietic schemes portray the existence of a lymphoid stem cell—i.e., a cell with the capacity to become a T, B, or natural killer cell—but no other hematopoietic cell. Formal proof supporting the existence of a lymphoid stem cell is lacking (2), but two recent reports provided insight into the nature of putative lymphoid stem cells. Georgopoulos et al. (3) reported that targeted disruption of the Ikaros gene results in a mouse lacking T, B, and natural killer cells, whereas the myeloid/erythroid compartments are essentially normal. Although this does not prove the existence of a common lymphoid stem cell, it does suggest that the Ikaros transcription factor may play a role in lymphoid cell fate. In a different approach using human bone marrow, Galy et al. (4) showed that a progenitor population with the phenotype CD34+/lineage−/CD45RA+/CD10+ could give rise to T, B, natural killer, and dendritic cells, but not myeloid/erythroid cells. This is the strongest evidence to date that normal human bone marrow contains a common lymphoid stem cell.

We know relatively little about the role of the bone marrow microenvironment in regulating human HSC commitment to the B lineage. Purified CD34+ human fetal bone marrow stem cells have been examined for their capacity to differentiate into the B lineage. Two studies found that plating CD34+ lineage human fetal bone marrow cells on a murine bone marrow stromal cell (BMSC) line yielded CD33+ and CD19+ B-lineage cells after 4–6 weeks of culture (5, 6). Similarly, cord-blood enriched CD34+ stem cells plated on the S17 murine BMSC line gave rise to CD19+/surface Ig− B-cell precursors with the capacity to proliferate for 4–12 weeks (7). Another study cultured CD34+/CD38+/HLA-DR− fetal bone marrow stem cells in medium containing interleukin (IL)-3, IL-6, stem cell factor (SCF), granulocyte/macrophage colony-stimulating factor, erythropoietin, basic fibroblast growth factor, and insulin-like growth factor 1 (8). In the absence of a BMSC feeder layer the stem cells developed into multiple lymphohematopoietic lineages, including CD10+/CD19+ B-lineage cells (8).

Our laboratory has developed a serum-free human BMSC culture that supports the IL-7-enhanced growth of CD19+ or CD34+ human pro-B cells (9, 10). In the current study we have adapted this culture system to evaluate the earliest stages of human HSC commitment to the B lineage. We demonstrate that ontogenically matched human BMSC maintained in serum-free culture conditions, with no exogenous cytokines, are sufficient to support HSC commitment and differentiation to immature B cells expressing surface Ig.

MATERIALS AND METHODS

Cell Origin and Isolation. A pool of CD19+/surface μ− B-cell precursors (≥95%) and CD19+/CD34++ HSC (≤5%) were isolated from human fetal bone marrow by mAb/magnetic bead depletion (11). In this study, anti-CD7 (T3-3A1; American Type Culture Collection) was added to the mAb cocktail (11) to enhance elimination of early T/natural killer cell precursors. HSC and pro-B (CD19+/CD34+−) cells were purified from the B-cell precursor/BMSC pool using mAb to CD19 and CD34, and sorting on a FACStarPlus (Becton Dickinson). The recovery of purified cells ranged from 0.33 × 10⁶ to 4.4 × 10⁶ for HSC and 1.74 × 10⁶ to 7.5 × 10⁶ for pro-B cells in 12 experiments. Postsort purity was assessed on a FACScan.

Human fetal BMSC cultures were initiated and maintained as described (9, 10) in X-VIVO 10 serum-free medium (Bio-
Whittaker). CD19+ B-lineage cells were not detected in control BMSC cultures not receiving purified HSC. The use of human tissue was approved by the Institutional Review Board: Human Subjects Committee of the University of Minnesota. Cell Culture. HSC or pro-B cells were cultured at $5 \times 10^3$ cells/well, or at successively lower concentrations to 3 cells/well for limiting dilution assays, in 96-well flat-bottom microtiter plates on BMSC plated at 200 μl X-VIVO 10. Cultures were maintained ± IL-7 (10 ng/ml; Peprotech, Rocky Hill, NJ) at 37°C in 5% CO2. Neutralizing goat antibody recognizing IL-6, IL-7, and SCF, all from R & D Systems, or control goat IgG were added (10 μg/ml) in selected experiments. The efficacy of each goat antibody in blocking the biological activity of IL-6, IL-7, or SCF was evaluated by using the human IL-6-dependent myeloma cell line ANBL-6 (kindly provided by Brian Van Ness, University of Minnesota), the murine IL-7-dependent pre-B-cell line 2E8 (TIB239; American Type Culture Collection), and SCF-responsive human HSC. Blocking effectiveness of the antibody was 88% for IL-6, >99% for IL-7, and 98% for SCF. Cultures were fed by demidepletion twice a week. Cells were harvested by using cell dissociation solution (Sigma) and subsequently stained with mAb for phenotypic analysis.

**Antibodies, Flow Cytometry, and Cell Quantitation.** Cells were stained with mAb to CD10 (J5; Coulter); CD19 (25C1); CD33 (LeuM9) and CD34 (HPCA-2) – both from Becton Dickinson; μ heavy (H) chain (HB57), and κ (HB61) and λ (HP6054) light (L) chains all from American Type Culture Collection; and IL7R/CDw127 (M27, kindly provided by Richard Armitage and Michael Widmer, Immunex). The HP6054 anti-λ mAb does not crossreact with surrogate light chains. mAbs were conjugated to fluorescein isothiocyanate, phycoerythrin, or biotin as described (12). Streptavidin-phycocerythrin was used to detect biotinylated mAb in one- or two-color analysis. Streptavidin-QuantumRed (Sigma) was used to detect biotinylated mAb in three-color analysis. Quantitation of CD19+ cells by the flow cytometry-based microsphere assay was conducted as described (13). The microsphere assay is sensitive to a detection level of 100 CD19+ cells/well.

**Cytoplasmic μ Staining.** Acetone-fixed cytosplins were stained with rhodamine-conjugated goat antibody to μ or γH chains (Southern Biotechnology Associates) as described (12).

**Statistics.** Analysis of variance was performed using the STATWORKS program (Data Metrics and Heyden and Son, Philadelphia). Poisson and binomial statistical models were used to evaluate limiting dilution assays. CTLF software (obtained from E. Katsanis, University of Minnesota) was used to calculate cloning efficiencies.

### RESULTS

**Isolation of HSC and Pro-B Cells.** We have described a BMSC culture which supports the survival, growth, and differentiation of pro-B cells (10). We sought to determine whether this BMSC-dependent/serum-free culture could support HSC commitment to the B lineage. We isolated CD34+ HSC depleted of lineage-committed progenitors, since CD34+ HSC contain the developmental capacity to differentiate into T, B, and myeloid cells (6). Fig. 1 *Upper* shows the sorting gates used to isolate CD19+/CD34+ HSC (lower right quadrant) and CD19+/CD34+ pro-B cells (upper right quadrant). The postsort purity of the HSC and pro-B cells was 99.3% (CD34 mean fluorescent intensity = 673), and 98.1% (CD34 mean fluorescent intensity = 555), respectively. Postsort purities of 95% to >99% were obtained by this method.

**BMSC Support the Commitment and Expansion of HSC to the B Lineage.** HSC or pro-B cells ($5 \times 10^3$) were cultured on BMSC in serum-free medium alone for up to 20 days. Day 0 HSC exhibited low forward/low 90° light scatter characteristics by flow cytometry, and these cells increased in size and granularity by day 7 (data not shown). CD33+ cells were present in the larger, more granular cell population. Microscopic analysis showed a mixture of large blast-like and small lymphoid-like cells during the first week of culture. By day 19 a uniform population of cells with small lymphoid cell light scatter characteristics was evident.

HSC cultured on BMSC were harvested and stained for CD19 expression and quantitation. The results of one such experiment are shown in Fig. 2. On day 0, <150 residual cell...
CD19+ cells (solid bars) were present in the HSC population. By 10 days of culture 34,056 ± 3978 CD19+ cells were present, and this number increased to 56,222 ± 2459 CD19+ cells by day 19. In contrast, cultures initiated with pro-B cells (hatched bars) only increased from 5 x 10^3 CD19+ cells on day 0 to 21,534 ± 3979 by day 10, and decreased to 5962 ± 2437 CD19+ cells by day 19. This minimal expansion of sorted cells during 3 weeks of culture argues against the possibility that CD19+ cells emerging in cultures initiated with HSC were derived from the expansion of residual CD19+ pro-B cells present at day 0.

Limiting dilution analysis was used to more rigorously test whether CD19+ B-lineage cells developed from HSC. In one experiment, HSC (containing ≈2% pro-B cells) were plated at 100, 33, 11, and 3 cells/well on BMSC. Wells were scored positive if ≧100 CD19+ cells/well were detected after 5 weeks culture. CD19+ cells were detected in 100% of wells initiated at 100, 33, and 11 HSC/well. In cultures initiated at 3 HSC/well 32 of 59 wells (54.2%) contained ≥100 CD19+ cells. This calculated to a responding cell frequency of one in three using a single-hit model to determine cloning efficiency. The mean ± SD (range) of CD19+ cells in the individual 32 positive wells was 367 ± 338 (118-1651). Two statistical models were used to reject the hypothesis that CD19+ cell outgrowth was derived from the 2% pro-B cells present at day 0. The binomial and Poisson models predict, with 95% confidence, that ≧13.5% or ≧12.6% pro-B cells would have to have been present in the initial HSC population to account for the presence of CD19+ cells in 32/59 wells. CD19+ B-lineage cells were detected in 10 of 29 wells initiated with a minimum of 11 HSC/well in a second experiment.

**BMSC Support the Differentiation of HSC to the B Lineage.** The expression of CD10, CD19, CD34, and μ H chain is shown in Fig. 3. Following 10-day culture of HSC >95% of the analyzed cells were CD19+, and virtually all CD19+ cells were CD10+. Twenty-two percent of pro-B cells were present by day 10; this number decreased to 4% by day 19. Conversely, CD19+/surface μ H chain+ immature B cells increased from 17% at day 10 to 27% at day 19. An increase in CD19 chain expression was observed between days 10 and 19 (Fig. 3), consistent with an ongoing maturation of the total B-lineage population.

A time course experiment was conducted to evaluate the kinetics of HSC commitment and differentiation into the B lineage (Fig. 4). Less than 0.25% of the HSC (or ≧10 cells/well) plated at day 0 were CD19+. By day 4, the number of CD19+ cells had increased to ~4400/well (Fig. 4A) and comprised ~65% of the analyzed cells (Fig. 4B). The CD19+ cell number expanded to ~23,000 by day 19 (Fig. 4A) and comprised 93% of the analyzed cells (Fig. 4B). Cells expressing μ H chain were detected on day 7 (0.5%), but L chain+ cells were not detected. By day 12, ~6% and ~3% of the CD19+ cells were H chain+ and L chain+, respectively (Fig. 4C). By day 19, ~35% and ~28% of the CD19+ cells were H chain+ and L chain+, respectively (Fig. 4C). In addition, 30–40% cytoplasmic μ+ cells were present in three separate experi-

**Fig. 4.** HSC were plated at 5 × 10^3 cells/well on BMSC in serum-free medium. Cells were analyzed on days 4, 7, 12, and 19. Each data point represents the mean of duplicate wells. The number and percentage of CD19+ cells present at day 0 in the HSC was 10 (0.25% of 5 × 10^3), based on postsort analysis of CD19+ cells. (A) Absolute number of CD19+ cells. (B) Percentage of CD19+ cells as a function of the total number of viable cells. (C) Percentage of CD19+ cells expressing H chains (solid bars) or L chains (hatched bars). nd, Not determined.
ments by day 19 (data not shown). Fig. 5 shows the two-color analysis of CD19 and Ig expression on day 19.

**IL-7 Is Not Required for Commitment and Expansion of HSC to the B Lineage.** We have earlier shown that IL-7R/CDw127 is expressed on most pro-B and pre-B cells, and IL-7 can enhance the proliferation of pro-B cells on BMSC (10). Three-color flow cytometry showed that IL-7R/CDw127 is expressed on ~30% of HSC (data not shown). HSC and pro-B cells were therefore cultured ± IL-7, and CD19+ cells were quantitated after 10 or 19 days.

In 12/12 experiments where HSC were cultured without IL-7, HSC committed to the B-lineage and CD19+ cells expanded for the duration of the culture (Table 1). From an initial plating population of 5 x 10^3 HSC, 1.7 x 10^6 (±1.0 x 10^5) CD19+ cells emerged by day 10, and 2.1 x 10^6 (±1.3 x 10^5) CD19+ cells emerged by day 19 (Table 1). The commitment and expansion of HSC to the B lineage was not consistently enhanced by the addition of exogenous IL-7. In five of nine experiments the number of CD19+ cells was greater with IL-7 than without IL-7 by day 19 (Table 1). However, analysis of variance showed that the contribution of IL-7 was insignificant when cells were analyzed on day 10 (P = 0.081, n = 8 experiments) and day 19 (P = 0.490, n = 8 experiments). In contrast, expansion of pro-B cells from an initial plating population of 5 x 10^3 was limited without IL-7. When analyzed at day 10, 1.6 x 10^4 (range, 0.3 x 10^4 to 3.9 x 10^4, n = 9 experiments) CD19+ cells were present, and in all experiments CD19+ cells declined by day 19, at which time only 0.4 x 10^4 (range, 0.1 x 10^4 to 1.1 x 10^4, n = 9 experiments) CD19+ cells were present. These collective results are consistent with the experiment shown in Fig. 2. As expected (9, 10), analysis of variance showed that the addition of exogenous IL-7 significantly enhanced the proliferation of pro-B cells (P = 0.015, n = 8 experiments on day 10, and P = 0.003, n = 7 experiments on day 19).

Because endogenous IL-7 production by BMSC could be partially responsible for the commitment and expansion of HSC to the B lineage, we quantitated IL-7 production by ELISA. Fetal BMSC produced ~1-2 pg/ml of IL-7, and this concentration was not altered by coinubcation of BMSC with HSC or pro-B cells for up to 20 days. This level of IL-7 does not support the growth of the IL-7-dependent murine pre-B-cell line 2E8 (data not shown). To evaluate the potential contribution of this low level of BMSC IL-7 production, we added a goat anti-human IL-7 neutralizing antibody to the HSC/fetal BMSC cultures. Anti-IL-7 had no effect on CD19+ cell numbers emerging after 19 days in culture, compared with medium containing normal goat IgG or medium alone (n = 4 experiments, data not shown). Antibody to IL-6 and SCF also had no effect on the number of CD19+ cells recovered from HSC/fetal BMSC cultures.

**DISCUSSION**

Human B-cell development occurs in multiple organ sites during embryonic life (i.e., bone marrow, liver, lung, kidney, and omentum), and only in the bone marrow from neonatal through adult life (14–16). However, the molecular basis that underlies HSC commitment to the myeloid/erythroid lineages, and how this differs from HSC commitment to the B lineage, has not been elucidated. Our results indicate that human HSC can commit to the B lineage and progressively differentiate through individual developmental compartments, culminating in surface Ig+ immature B cells. The CD34+ (CD34 high density) HSC isolated from fetal bone marrow (Fig. 1) are capable of differentiating into myeloid, monocyctoid, erythroid, and T and B lineages (6). In contrast, CD34+ (CD34 low density) cells do not contain multilineage potential (6).
preliminary studies we have shown that CD19+CD34+ (CD34 low density) fetal bone marrow cells fail to give rise to CD19+ B-lineage cells (unpublished observations), confirming the importance of initiating the cultures with CD34+ HSC to achieve B-cell development.

The two most salient attributes of this study are (i) the cellular components of the culture system are all of human fetal bone marrow origin, and (ii) the BMSC culture supports multiple stages of human B-cell development. We used human fetal bone marrow as a tissue source for BMSC and HSC, and the cells were supported in an exogenous serum/cytokine-free environment. This distinguishes our study from prior studies that used murine BMSC maintained in fetal bovine serum (5–7), or exogenous cytokine cocktails in a fetal bovine serum/horse serum-based culture (8), to derive CD19+ B-lineage cells from HSC. Results shown in Figs. 3–5 demonstrate that B-cell development in our BMSC culture is characterized by progressive changes consonant with successful rearrangement of H and L chain genes. This includes loss of CD34+ cells, acquisition of CD19 and an increase in the level of cell surface CD19, and the appearance of immature B cells expressing μ/κ or μ/λ Ig receptors. The gradual appearance of surface μ−immature B cells (Fig. 4) is a defining feature of our culture system. Prior studies (5–8) did not achieve this degree of B-cell differentiation, and in the study by Rawlings et al. (7) the predominant CD19+ population present after 3 months in culture had germ-line H chain genes.

Our results imply that the BMSC fibroblast-like cells provide the contact-dependent interactions and/or soluble mediators essential to support commitment and differentiation of HSC into the B lineage. The identity of the BMSC molecules critical for this process are unknown, but IL-6, IL-7, and SCF play little or no role. Neutralizing antibody against these three molecules had no effect on the tempo or phenotype of developing B cells. Formal exclusion of a role for these cytokines would require the use of human BMSC that fail to express of the respective genes or proteins.

The role of IL-7 in human B-cell development deserves special comment. IL-7 was not required for B-cell development in our BMSC culture, since in every experiment CD19+ cells emerged and expanded in the absence of exogenous IL-7 (Table 1). The results in Table 1 show enhancement of CD19+ B-lineage cell growth in cultures supplemented with IL-7 in some experiments, but this result was neither consistently obtained or statistically significant. Importantly, even though BMSC in our culture system secrete small amounts of IL-7, neutralizing anti-IL-7 had no effect on the development or expansion of CD19+ B-lineage cells. IL-7 nonresponsive human B-cell progenitors were also described in a prior report (7). We cannot dismiss the evidence supporting some role for IL-7 in human B-cell development. Pro-B cells respond to IL-7 signaling by (i) modestly enhanced growth on BMSC (10), (ii) specific enhancement of cell surface CD19 (17, 18), and (iii) downregulation of TdT, RAG-1, and RAG-2 (18). In situ IL-7 expression at the mRNA level has also been detected in human bone marrow (19).

Our results are consistent with the apparent IL-7-independent development of human B cells in vivo. Patients with X-linked severe combined immunodeficiency (XSCID) have mutations in the γc subunit (20, 21) that severely disrupt signaling through the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15. T-cell development is severely impaired in XSCID, but B-cell numbers are normal or elevated (22). Since γc is an essential signaling subunit of the IL-7R (23, 24), one concludes that B-cell precursors in XSCID patients fail to respond to IL-7 (although to our knowledge this has not been formally demonstrated using B-cell precursors from XSCID patients). Further evidence for a negligible role of IL-7R/γc signaling in human B-cell development is the recent description of an autosomal severe combined immunodeficiency with mutations in the Janus family tyrosine kinase, Jak-3 (25, 26). Patients with Jak-3 mutations have immunological characteristics similar to XSCID, including normal numbers of circulating B cells (25, 26). These results are consistent with the known physical association between Jak-3 and γc (27, 28). Therefore, our in vitro results and experiments of nature point to little or no role for IL-7 in at least the normal numerical development of human B cells. This is in striking contrast to the absolute dependency of murine B-cell development on IL-7/IL-7 receptor interactions (29–32), and subsequent downstream signaling involving γc (33, 34) and Jak-3 (35, 36). We have shown that human CD34+ HSC can give rise to surface Ig+ immature B cells over a 2- to 3-week period in a culture that may at least partially recapitulate the in vivo bone marrow microenvironment. This culture can now be used to evaluate the role of BMSC and their cytokine products in regulating the patterns of gene expression (10, 18, 37) that characterize developing human B cells. It may also be a starting point for identifying potentially unknown molecules critical for the normal development of human B cells.

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