Production of Interleukin 1β by Human Hematopoietic Progenitor Cells

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Abstract

The production of interleukin 1β (IL-1β) by human hematopoietic stem/progenitor cells was studied to explore the concept that these cells are not merely responders to stimuli from their microenvironment, but can themselves produce a powerful biomodulator. Cells with a $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ phenotype were purified from human umbilical cord blood and cultured one per well in serum-free medium with a mixture of cytokines. Cells that had divided over 2–5 d to form doublets were identified and the daughter cells were studied individually. 91% (460/506) of daughter cells had clonogenic potential. Analysis of these individual daughter cells by reverse transcription–polymerase chain reaction showed that 29% of them (14/48) were positive for IL-1β mRNA. One of the cells that was strongly positive for IL-1β mRNA had a sibling that generated 366,000 cells of multiple lineages after 14 d. IL-1β converting enzyme mRNA, which is necessary to produce IL-1β, was also detected by reverse transcription–polymerase chain reaction at the single-cell level. Moreover, enzyme immunoassay for mature secreted IL-1β in culture supernatants demonstrated the production of IL-1β protein by these cells. This was confirmed by fluorescent immunostaining of the cells for human IL-1β which showed a significant portion of positive cells. Taken together, the results demonstrate the capacity of early hematopoietic cells to synthesize IL-1β. The capacity of human hematopoietic stem/progenitor cells to produce IL-1β may be involved in regulation of their proliferation and differentiation under certain circumstances and dysregulation of this process may be modified in leukemogenesis. (*J. Clin. Invest.* 1996. 97:1666–1674.) Key words: reverse transcription–polymerase chain reaction • IL-1β converting enzyme • enzyme immunoassay • immunostaining • daughter cells

Introduction

Pluripotent hematopoietic stem cells are believed to self-renew, proliferate and differentiate into multiple lineages in response to external stimuli from their microenvironment, including a series of cytokines such as interleukin 3 (IL-3), IL-6, and Steel factor (SLF)[1] (1). Stem cells have thus been regarded as passive and regulated by surrounding cells. Recent advances in flow cytometry have enabled us to obtain highly enriched fractions of hematopoietic stem cells (2–5) and to ask whether these cells could themselves express cytokine genes. It is difficult to draw conclusions about the expression of genes in stem cells using standard techniques because they require relatively large numbers of cells, which makes it difficult to exclude contaminating cells as a source of cytokine mRNA. Therefore, we have used a sensitive reverse transcription (RT)-PCR technique that allows analysis of single cells. We have shown with this approach that IL-1β mRNA was expressed at single-cell level in $> 20\%$ of candidate human hematopoietic stem cells with the phenotype of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ (6). However, due to a relatively low cloning efficiency of these cells ($\sim 20\%$), we could not directly demonstrate that the IL-1β–positive cells were truly stem cells or progenitor cells. To overcome this problem we have therefore performed the experiments reported here using cells with much higher cloning efficiency. We took cells with the phenotype of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ from human umbilical cord blood and cultured them individually to obtain daughter cells, which we showed had a very high probability (> 0.9) of generating a colony or cluster of their progeny. We analyzed these cells for expression of IL-1β, CD34, and β-actin genes. To provide evidence that early hematopoietic cells produced IL-1β protein, we investigated the expression of a specific proteinase termed interleukin 1β converting enzyme (ICE) (7–11) that is required to cleave the precursor of IL-1β and produce mature IL-1β. Moreover, we studied production of a mature form of IL-1β by $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells using a sensitive enzyme immunoassay and immunostaining. The results demonstrated the capacity of hematopoietic stem/progenitor cells to produce IL-1β.

Methods

Candidate human hematopoietic stem cell separation. Umbilical cord blood cells, collected according to institutional guidelines, were obtained during normal full-term deliveries. Low-density cells (< 1.077 grams/ml) were isolated using Ficoll-Paque (Pharmacia, Uppsala, Sweden), washed twice in PBS, resuspended in Iscove's medium containing 2.5% human serum albumin and 7.5% DMSO or in Hanks' Hepes-buffered salt solution containing 30% FCS and 7.5% DMSO, aliquoted, and frozen until used. Vials of frozen cells were rapidly thawed and slowly diluted with Iscove's medium containing 30% FCS and 0.1 mg/ml DNase (type II-S, D4513; Sigma Chemical Co., St. Louis, MO). Cells were then washed twice and resuspended in Hanks' Hepes-buffered salt solution containing 2% FCS and 0.1% sodium azide (HFN) for subsequent staining. Cells were stained as

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1. *Abbreviations used in this paper:* ICE, interleukin 1β converting enzyme; RT, reverse transcription; SLF, Steel factor.

described previously (2–5). Briefly, cells (10^7/ml) were incubated simultaneously with mAbs specific for CD34 (8G12 labeled with cyanine 5-succinimidylester [Cy5]), CD71 (OKT9 labeled with FITC), and CD45RA (8d2 labeled with phycoerythrin [RPE]), at 20, 1, and 4 μg/ml, respectively, for 30 min at 4°C. Controls included cells stained with single-reagent and using three-color with anti–trinitrophenol-RPE instead of 8d2-RPE. Cells were then washed twice and resuspended in HFN containing 2 μg/ml propidium iodide before sorting. Cells were sorted on a FACStar Plus® (Becton Dickinson, San Jose, CA) equipped with a 5-W argon and a 30-mW helium neon laser. Specific fluorescence of FITC, RPE, propidium iodide, and Cy5 excited at 488 nm (0.4 W) and 633 nm (30 mW), as well as forward and orthogonal light scatter signals, was used to establish sort windows. Cells were separated into fractions expressing low or undetectable levels of any of the antigens (e.g., $CD71^{lo}$) and cells expressing intermediate or high levels of antigen (e.g., $CD71^{+}$).

Cell culture. Purified $CD34^{+}$ $CD45RA^{lo}$ $CD71^{lo}$ cells were cultured in serum-free medium consisting of Iscove's modified Dulbecco's medium supplemented with bovine serum albumin (2%), insulin (10 μg/ml), transferrin (200 μg/ml), 2-mercaptoethanol (10^{-5} mol/liter), low-density lipoprotein (40 μg/ml), and penicillin–streptomycin (100 U and 50 μg/ml, respectively). In most experiments, the medium was supplemented with combinations of the following recombinant hematopoietic cytokines: SLF (50 ng/ml), IL-3 (20 ng/ml), IL-6 (10 ng/ml), M-CSF (10 ng/ml), erythropoietin (3 U/ml), IL-3/GM-CSF fusion protein (20 ng/ml) (12), and/or granulocyte CSF (G-CSF; 20 ng/ml). Control cultures contained no recombinant cytokines. Cells were cultured in at 37°C, 5% CO_2, at a concentration of 1–1,000 cells/well. In experiments for ELISA of IL-1β, 10^4 cells with the $CD34^{+}$ $CD45RA^{lo}$ $CD71^{lo}$ phenotype were cultured in 200 μl of serum-free medium alone, or of the medium supplemented with SLF (50 ng/ml), or IL-1α (10 ng/ml) or TNF-α (40 ng/ml) or a mixture of SLF (50 ng/ml), IL-3 (20 ng/ml), IL-6 (10 ng/ml), M-CSF (10 ng/ml), erythropoietin (3 U/ml), and G-CSF (20 ng/ml). Control for ELISA was the serum-free medium containing all cytokines used for the experiments at the same concentration to rule out false positives. SLF, IL-3, IL-6, and IL-3/GM-CSF fusion protein were kindly provided by Dr. D.E. Williams (Immunex, Seattle, WA). Erythropoietin and G-CSF were provided by colleagues in The Terry Fox Laboratory. M-CSF was a gift from Genetics Institute (Cambridge, MA).

Manipulation of single daughter cells (Fig. 1). Individual $CD34^{+}$ $CD45RA^{lo}$ $CD71^{lo}$ cells were plated into wells of 96-well tissue culture plates (Nunc, Kamstrup, Denmark) by using an automated cell deposition unit (Becton Dickinson). Each well contained 100 μl of serum-free media with a mixture of cytokines described above. The cultures were inspected daily using an inverted microscope and after the first cell division (two-cell stage), which occurred on days 2–5 of culture, the cells were resuspended and the contents of each well were divided into four aliquots of 25 μl each that were transferred into four wells, each containing 80 μl of serum-free medium supplemented with the same mixture of cytokines as mentioned above. By chance, daughter cells tended to be distributed into different wells; the presence of a single daughter cell in a well was directly confirmed by microscopic inspection. To isolate mRNA from a single daughter cell, culture volume was reduced carefully to 10 μl. Lysis buffer (described below) was then added to the well and the mixture was transferred to a tube.

Isolation of mRNA from single or small numbers of cells. 1–1,000 cells were lysed in a 1.5-ml microfuge tube containing 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 0.5% SDS, 1 mg/ml proteinase K). After incubation for 1 h at 37°C, 20 μl of 2.5 M NaCl and 50 μl of oligo-dT cellulose suspension (~ 50 μg) were added, and the mixture was rotated overnight at room temperature. The oligo-dT cellulose was washed three times with binding buffer (10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM EDTA, 0.5% SDS), followed by one wash with washing buffer (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl). The mRNA was eluted by incubating the oligo-dT cellulose for 10 min at room temperature with 50 μl of elution buffer (10 mM Tris-HCl, pH 7.4). mRNA was ethanol precipitated in the presence of 5 μg tRNA, centrifuged, and then washed in 70% ethanol. The dried RNA pellet was redissolved directly in the RT mixture.

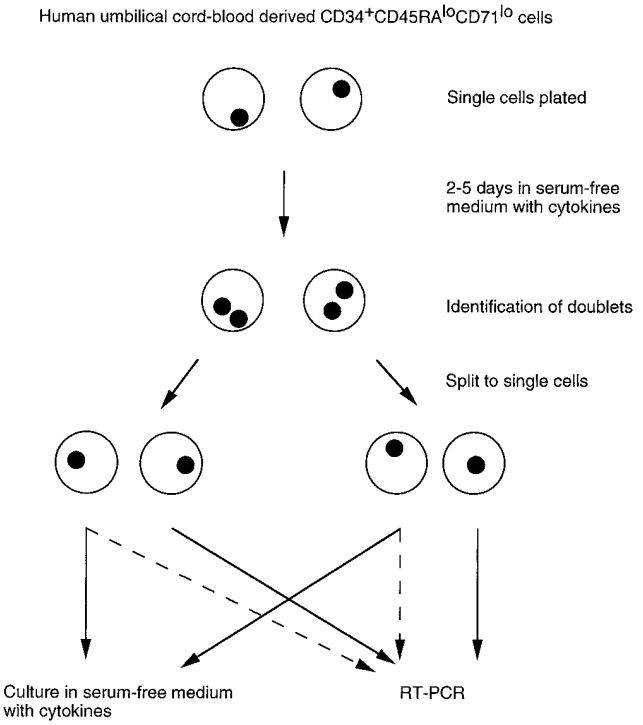


Figure 1. Strategy for analysis of single daughter cells for expression of IL-1β, CD34, β-actin, and ICE mRNA by using RT-PCR. Dashed lines indicate that in some experiments all daughter cells underwent RT-PCR analysis (see Figs. 3 and 4); solid lines indicate that in other experiments one daughter cell was analyzed for gene expression by RT-PCR, while the sibling was cultured in serum-free culture with a mixture of cytokines (see Fig. 5).

RT. RT was performed by incubation of mRNA at 37°C for 60 min in 20 μl of final volume with 400 U of Moloney murine leukemia virus–reverse transcriptase (GIBCO-BRL, Gaithersburg, MD), 10 mM of DTT, 1:5 (vol/vol) 5× RT buffer (GIBCO-BRL), 10 U of human placental RNase inhibitor (GIBCO-BRL), 5 μM of random hexamer [$pd(N)_6$; Pharmacia], and 0.5 mM of each dNTP (Pharmacia). RT products were incubated at 95°C for 5 min and then quickly chilled on ice.

The first round of PCR. The first PCR was performed by mixing one aliquot (5 ~ 20 μl) of RT product to a final volume of 100 μl with 2.5 U of *Taq* DNA polymerase (Promega, Madison, WI), 0.25 mM dNTP, 1 μM of 5′ sense and 3′ antisense primer of up to three genes, 2 mM of $MgCl_2$ (or 1 mM of $MgCl_2$ for ICE amplification), and 1:10 (vol/vol) 10× PCR buffer (Promega). The mixture was overlaid by 100 μl of mineral oil and then amplified for 40 cycles by the Thermocycler programmable heating block (Perkin-Elmer Cetus Corp., Norwalk, CT). The durations and temperatures used in the first cycle were 30 s at 94°C for denaturing, 1 min at 50°C for annealing, and 2 min at 72°C for polymerizing. The polymerizing phase was extended 5 s per cycle. In some experiments, we used half of the first PCR reaction to analyze expression of a gene (see Fig. 7 *A*).

Seminested PCR. Seminested PCR was performed by adding 1 μl of the first PCR product to a final volume of 50 μl which included the same reagents as for the first PCR, except that the sense primer and the nested antisense primer (at 1 μM) for amplification of a specific gene were substituted for the primers used in the first PCR. The mixture was overlaid by 50 μl of mineral oil and then amplified for up to 40 cycles as in the first PCR, with conditions the same, except for an annealing temperature of 60°C for β-actin.

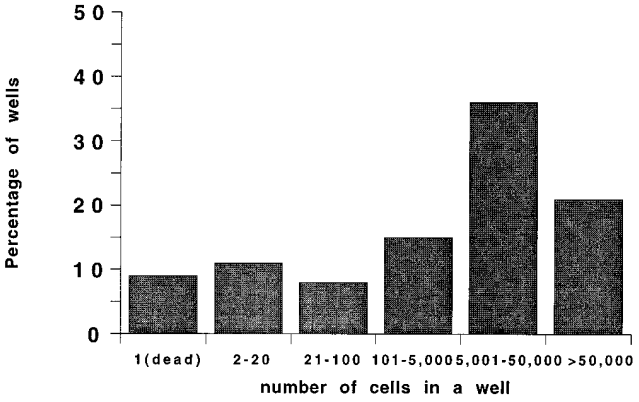


Figure 2. Colony/cluster forming capacity of daughter cells that had undergone a first cell division in the serum-free liquid culture with a mixture of cytokines. Individual daughter cells from pairs split after the first cell division were cultured in the same conditions. Numbers of progeny of the individual daughter cells were counted 14 d after culture.

Primers. The primers used for PCR amplification and the expected size of products were as follows, in order, (*i*) 5′ sense, (*ii*) 3′ antisense for the first PCR (with the expected size of the first PCR product), and (*iii*) 3′ antisense primer for seminested PCR (with the expected size of seminested PCR product). (XY) indicates 50% sharing by the base X and Y. IL-1β: (*i*) 5′-GGAATTCTGAGCACCTTCTTT(CT)CCTTC-3′, (*ii*) 5′-GGAATTCT(GT)(CT)TCTGCTTG(TA)GAGGTGCT-3′ (466 bp), (*iii*) 5′-GGAATTCGATGTACCAGTTGGGGAAGT-3′(446 bp); CD34: (*i*) 5′-GGAATTCGAGGCCACAACAAACATCAC-3′, (*ii*) 5′-GGAATTCGCAGATGCCCTGAGTCAATT-3′ (330 bp), (*iii*) 5′-GGAATTCCACTTCTCTGATGCCTGAAC-3′ (309 bp); β-actin: (*i*) 5′-(CT)GT(CT)ACCAACTGGGACGACATGGAGAA-3′, (*ii*) 5′-A(GA)GAAGGAAGGCTGGAA(GA)AG(TA)GCCTCA-3′ (576 bp), (*iii*) 5′-TTGCCAAT(GA)GTGATGAC(CT)TGGCCGTCA-3′ (531 bp); ICE: (*i*) 5′-GGAATTCCTGCCCAAGTTTGAAGGACA-3′, (*ii*) 5′-GGAATTCACGATCTCTTCACTTCCTGC-3′ (456 bp), (*iii*) 5′-GGAATTCATGTCCTGGGAAGAGGTAGA-3′ (406 bp).

Sample analysis. 20 μl of sample was loaded onto 1 ∼ 2% agarose gel and electrophoresed at 80 V for 90 min. The gel was denatured in 0.5 M NaOH, 1.5 M NaCl for 45 min, rinsed briefly in distilled water, neutralized in 0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl for 45 min, and blotted overnight to Hybond N (Amersham, Bucks, United Kingdom) in 20× SSC (1.5 M NaCl, 0.15 M sodium citrate [pH 7.0]). In some experiments, a Vacugene (Bio-Rad Laboratories, Richmond, CA) was used for blotting. DNA was fixed by ultraviolet cross-linking in UV Stratalinker (Stratagene, La Jolla, CA). The membrane carrying transferred DNA was prehybridized in 50 mM Tris-HCl (pH 8.0) containing 10 mM EDTA, 6× SSC, 1% SDS, 5× Denhardt's solution, and 50% formamide for > 4 h. Hybridization was performed for 16 h at 42°C with ^{32}P-labeled cDNA probes in a replaced solution of the same mixture as the prehybridization buffer. The DNA probes used were 1.5-kb human full-length IL-1β cDNA (kindly provided by Dr. Neil Reiner, Vancouver, BC), 1.0-kb (AvaII-AvaII site) human CD34 cDNA (Dr. Brian Seed, Boston, MA), and 1.5-kb bovine β-actin cDNA (Dr. Neil Reiner). 0.4-kb ICE cDNA was purified from the PCR amplified product of 30 $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells (see Fig. 6 *A*), which was confirmed to be ICE gene by DraI digestion (see Fig. 6 *B*). They were radiolabeled with [^{32}P]dCTP (Du Pont NEN Research Products, Wilmington, DE) using the multiprime, random DNA labeling method. Blots were washed finally to 65°C for 1 h and were exposed to x-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen for 30 min to 3 d at −70°C. Negative controls were always confirmed as negative by a long (up to 3 d) exposure.

ELISA. ELISA was performed using a kit for human IL-1β (Quantikine™ HS; R&D Systems, Minneapolis, MN) as manufacturers recommended. This kit does not cross-react with human SLF, IL-3, IL-6, M-CSF, G-CSF, and erythropoietin which were used in the present study for culture of candidate stem cells. Briefly, 200 μl of standard or sample per well, which was coated by mouse anti–human IL-1β mAb, was incubated with 50 μl of the assay diluent and incubated at room temperature for 16 h. After washing four times with 400 μl of the wash buffer, 200 μl of alkaline phosphatase–linked anti–human IL-1β polyclonal antibody was added, and incubated at room temperature for 2 h. The well was washed four times as before and in-

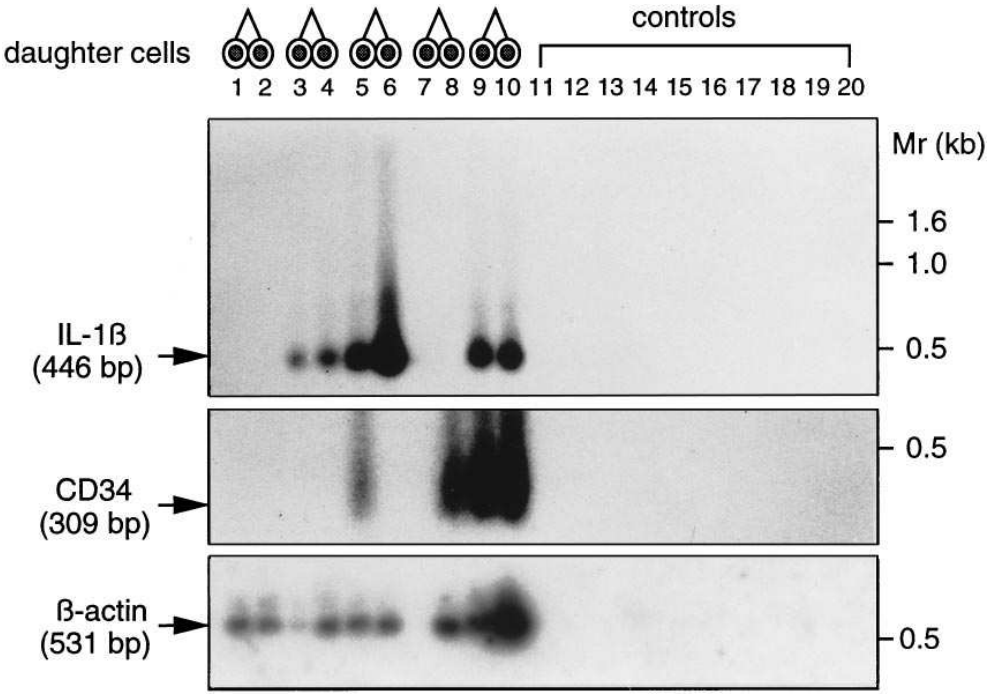


Figure 3. A representative result of seminested RT-PCR-Southern hybridization analysis of expression of IL-1β, CD34, and β-actin mRNA in single daughter cells. The top, middle, and bottom show the results for IL-1β, CD34, and β-actin mRNA, respectively. Positions of IL-1β, CD34, and β-actin PCR products are indicated. Negative controls containing medium alone (no cells) were prepared in the same number of tubes to exclude contamination. Mr indicates molecular size standards.

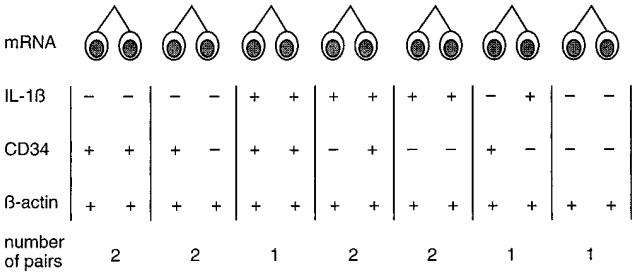


Figure 4. Expression of IL-1β and CD34 mRNA in paired daughter cells, both of which were positive for β-actin mRNA.

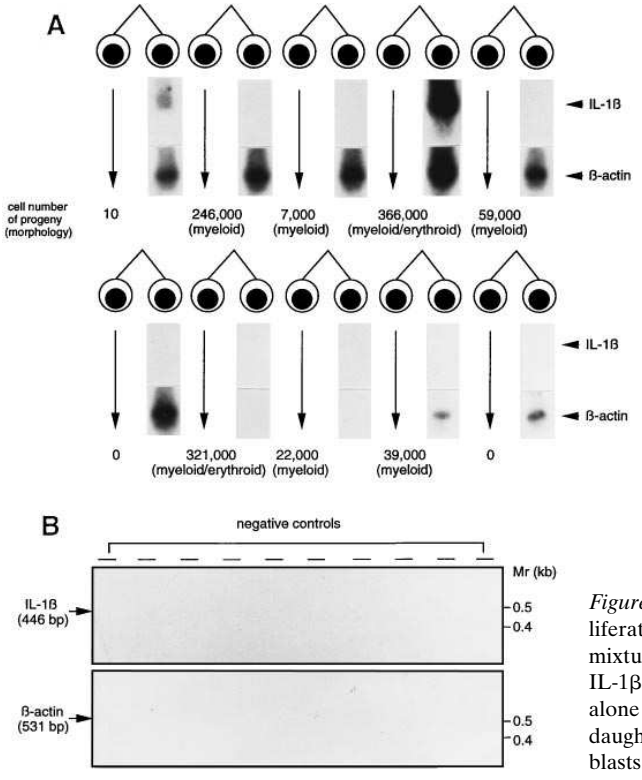
cubated with 50 μl of substrate (NADPH) for 45 min at room temperature. 50 μl of amplifier solution, containing alcohol dehydrogenase and diaphorase, was then added, and incubated for 45 min at room temperature. Finally, 50 μl of stop solution (2 N sulfuric acid) was added, and the optical density of the solution was measured at 490 nm with wavelength correction at 650 nm using a microplate reader. Human IL-1β values of the test sample were determined by superimposing them on the standard curve.

Indirect immunofluorescent staining. Wells of an 8-well slide (Fisher, Ottawa, Ontario, Canada) were coated with poly-L-lysine by drying up a drop (70 μl) of poly-L-lysine solution (10 μg/ml) on a well of the 8-well slide at 37°C for 30 min. A 70-μl cell suspension (1,000 cells/ml) was then mounted and dried up at 37°C for 30 min. Samples were incubated with 70 μl of PBS containing 50% of normal goat serum for 3 h. 70 μl of rabbit anti–human IL-1β serum (kindly provided by Dr. Hermann Ziltener (The Biomedical Research Centre, UBC, Vancouver) and used at 1:10,000 dilution in PBS containing 0.5% BSA) or normal rabbit serum (as control, used at 1:10,000 dilution in PBS containing 0.5% BSA) was mounted and incubated for 1 h. Washing was performed by mounting a drop of PBS containing 0.05% Tween 20 and incubated at room temperature for 15 min each time for three times. In some experiments, we washed a whole slide in a chamber containing 50 ml of PBS containing 0.05% Tween 20 for 15 min for three times. 70 μl of FITC-labeled goat anti–rabbit IgG (1:1,000 dilution in PBS containing 0.5% BSA) was used as a secondary antibody and incubated for 1 h at room temperature. After washing as before, we covered the wells with a PermaFluor aqueous mountent (Fisher) and a coverglass and observed the results by a fluorescent microscope. Evaluation of fluorescent intensity of cells was performed individually. We recognized brightest cells as positive cells, and cells with less or no intensity of fluorescence as negative cells.

Results

To derive a population of cells with a high cloning efficiency, $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells, derived from human umbilical cord blood, were plated one per well and cultured in serum-free liquid culture with SLF, IL-3, IL-6, M-CSF, erythropoietin, G-CSF, and IL-3/GM-CSF fusion protein (12) as reported previously (13). After 2–5 d, we identified cells that had given rise to doublets of daughter cells. We obtained 506 daughter cells that had undergone a first cell division in the culture. Of these, 91% (460/506) gave rise to colonies or clusters containing > 6 cells, and 57% (288/506) of those clones consisted of > 5,000 cells (Fig. 2).

To minimize risks of contamination, the optimal number of samples to be processed was 20. Therefore, we used 10 paired daughter cells in each experiment with the same number of negative controls (medium alone or no cells) and analyzed expression of IL-1β, CD34, and β-actin mRNA in each daughter cell. In a representative experiment (Fig. 3), 6 of 10 paired daughter cells were positive for IL-1β mRNA, 4 for CD34 mRNA, and 9 for β-actin mRNA. 10 controls containing only medium were processed identically in parallel for all the steps of mRNA extraction, RT, and PCR and all were negative. Re-



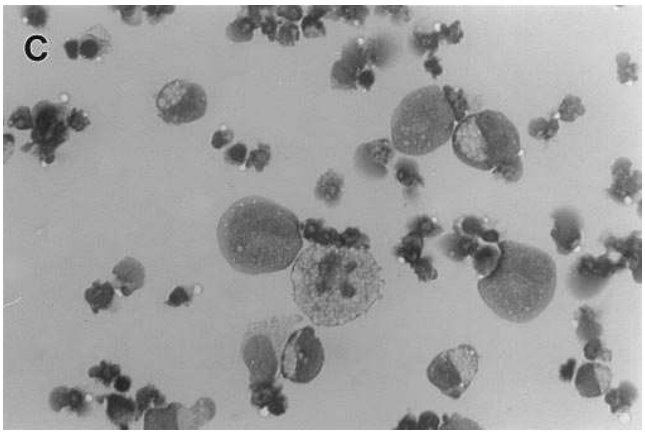


Figure 5. (*A*) Expression of IL-1β and β-actin mRNA in daughter cells and proliferative potential of their siblings after 14 d of serum-free liquid culture with a mixture of cytokines as described in Methods. (*B*) Results of parallel analysis for IL-1β and β-actin mRNA expression of negative control samples of medium alone (no cells). (*C*) Stained smear of progeny of a sibling of the IL-1β–positive daughter progenitor cell which gave rise to 366,000 cells. Myeloblasts, erythroblasts, and macrophages are observed in this field.

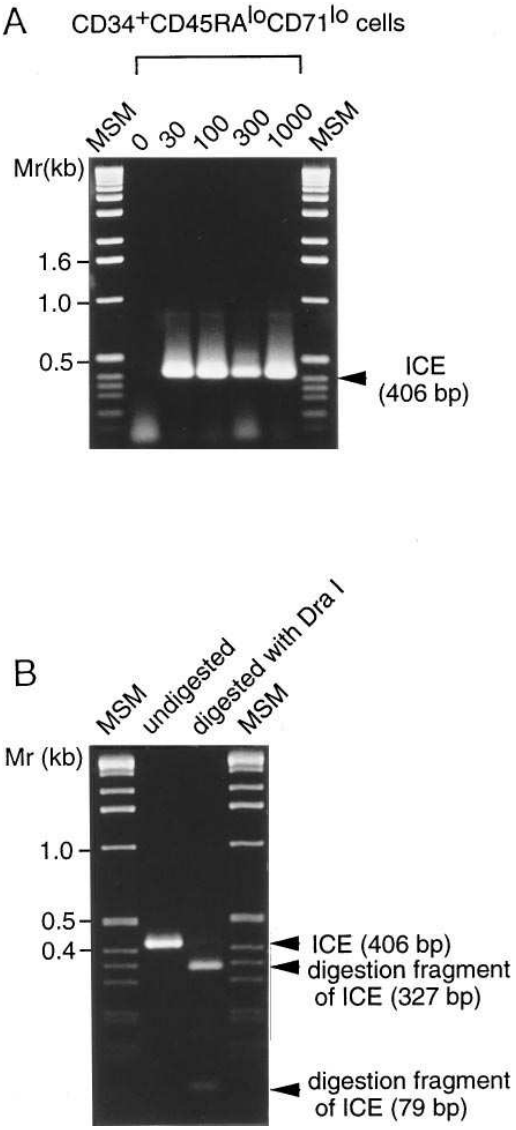


Figure 6. (*A*) Seminested RT-PCR analysis of human ICE mRNA in titrated candidate human hematopoietic stem cells derived from human umbilical cord and with the phenotype $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$. MSM indicates molecular size marker. (*B*) DraI digestion of PCR products of the 30 cells. Digestion of ICE amplicon is supposed to give rise to 327- and 79-bp products.

sults from five independent experiments on a total of 48 daughter cells showed that 14 cells of the 48 cells (29%) were positive for IL-1β mRNA, 21/48 (44%) for CD34 mRNA, and 37/48 (77%) for β-actin mRNA. In three experiments in which daughter cells were identified and analyzed by RT-PCR after 2 d of culture, the results showed that 39±20% (mean±SD) of paired daughter cells were positive for IL-1β mRNA, 51±11% for CD34 mRNA, and 83±21% for β-actin mRNA. These experiments also showed that up to 30% of daughter cells were positive for both CD34 and IL-1β mRNA.

In this study we used β-actin gene as a marker for successful recovery of mRNA from single cell. Fig. 4 shows that in 5 (10 cells) of 11 pairs (22 cells) in which β-actin mRNA was detected in both daughter cells, there were differences in the expression of IL-1β and/or CD34 mRNA.

In some experiments, we cultured the daughter cells for 2 wk in the liquid culture to observe their progeny, while their sibling daughter cells were analyzed for IL-1β and β-actin mRNA expression. Fig. 5 *A* shows analysis of such a pair in which one daughter cell was strongly positive for IL-1β mRNA while its sibling generated in 14 d 366,000 cells that were of multiple lineages, including myeloblasts, erythroblasts, and macrophages (Fig. 5 *C*). However, another daughter cell that expressed IL-1β mRNA had a sibling which gave rise to only 10 cells, and we could not analyze their lineages. No IL-1β and β-actin mRNA were detected in a cell whose sibling generated 321,000 cells with multiple lineages. Interestingly, daughter cells in which only β-actin was detected had siblings that had only myeloid progeny or failed to divide (Fig. 5 *A*).

As a further approach to assess the likelihood that the candidate human hematopoietic stem cell might produce IL-1β, we analyzed expression of the ICE gene. Titrated numbers (30–1,000) of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells were cultured overnight in serum-free medium in the absence of cytokine, after which cDNA was prepared and one half of each aliquot of cDNA was analyzed by seminested PCR. All fractions, including that containing only 30 cells, were clearly positive for ICE mRNA (Fig. 6 *A*). DraI digestion of the ICE amplicon from the half of the RT product from 30 cells gave rise to 327- and 79-bp bands which were expected from the sequence (Fig. 6 *B*). Moreover, seminested PCR-Southern blot of half of the first PCR reaction for genes in the daughter cells examined in Fig. 3 revealed that 1 of the 10 cells was clearly positive for ICE mRNA expression (Fig. 7 *A*). This showed that the cell was positive for IL-1β, ICE, and β-actin mRNA and negative for CD34 mRNA. Another experiment using a different set of 10 daughter cells and the entirety of the RT products (cDNA) gave a similar result, in that 1 of the 10 paired progenitor cells was positive for ICE mRNA (Fig. 7 *B*).

To obtain a statistical estimate of the frequency of candidate stem cells with the $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ phenotype that could express ICE mRNA, we analyzed wells containing three cells each. This allows analysis of more cells while reducing the risk of contamination, which increases with the number of tubes to be handled. These cells were cultured overnight with a combination of cytokines (SLF, IL-3, IL-6, M-CSF, erythropoietin, G-CSF, and IL-3/GM-CSF fusion protein). Of 10 wells, each of which had been confirmed to contain exactly 3 cells by direct observation, 1 was positive for ICE mRNA. In contrast, 10 control wells containing medium alone were all negative for ICE mRNA (Fig. 8). β-Actin mRNA was detected in all of the 10 wells containing 3 cells (data not shown), demonstrating successful recovery of mRNA from these samples. From this result, we estimated the fraction of cells expressing ICE mRNA among these cells to be $> 3.5\%$ with 95% confidence limit of 0.00083 to 0.18 (*t* test). RT-PCR amplifications of ICE using human B cell lines HS-Sultan and Daudi were both negative for the gene (data not shown).

To add more evidence to show IL-1β production by hematopoietic progenitor cells, we performed a sensitive ELISA for IL-1β and immunostaining by adapting anti–IL-1β serum. The ELISA for human mature-form IL-1β in the culture supernatants of 10^4 candidate hematopoietic stem cells with the $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ phenotype showed secretion of IL-1β by various cytokine stimulations. Fig. 9 showed a representative result of two independent experiments. Production of IL-1β was observed even by cells cultured in serum-free me-

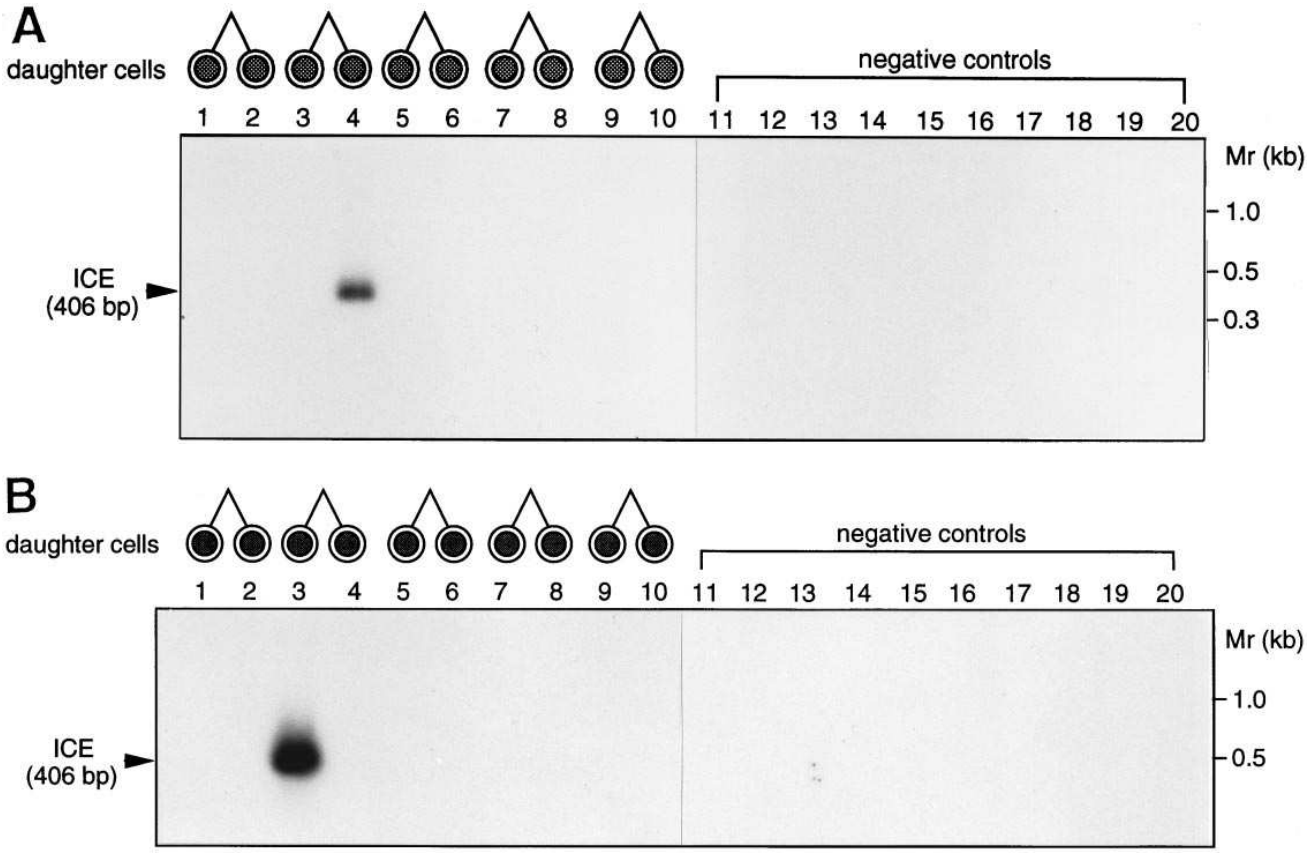


Figure 7. (*A*) Seminested RT-PCR Southern analysis of ICE mRNA in single daughter progenitor cells. These daughter cells were the same as those in Fig. 3, showing that the fourth daughter cell expressed both IL-1β and ICE mRNA. (*B*) Seminested RT-PCR Southern analysis of ICE mRNA in the different daughter cells from the cells in *A*. This experiment showed the same frequency of ICE mRNA positive cells as that in *A*.

dium alone but induced effectively by the stimulation of IL-1α. Control sample of serum-free medium containing cytokines used (SLF, IL-1α, TNF-α, IL-3, IL-6, M-CSF, erythropoietin, G-CSF) showed no cross-reactivity of this system between IL-1β and other cytokines.

Fluorescent immunostaining for IL-1β showed a significant increase of positive cells compared with a control sample. In two independent experiments, $CD34^{+}$ $CD45RA^{lo}$ $CD71^{lo}$ cells were cultured overnight in serum-free media with a mixture of cytokines. Both experiments gave similar results and Fig. 10 shows a representative result in which the number of IL-1β–positive cells (22 cells in 88 cells evaluated) was significantly higher than that of false positive cells (6 cells in 69 cells evaluated) ($P < 0.01$). Each of these positive cells was a round-shaped small lymphocytoid and not a macrophage-like or fibroblastic cell.

Discussion

It is believed that a very small fraction of cells in the bone marrow and peripheral blood cells is responsible for the continuous generation of blood cells. These hematopoietic stem/progenitor cells are characterized by extensive capacities for self-renewal and the generation of progenitors of multiple lin-

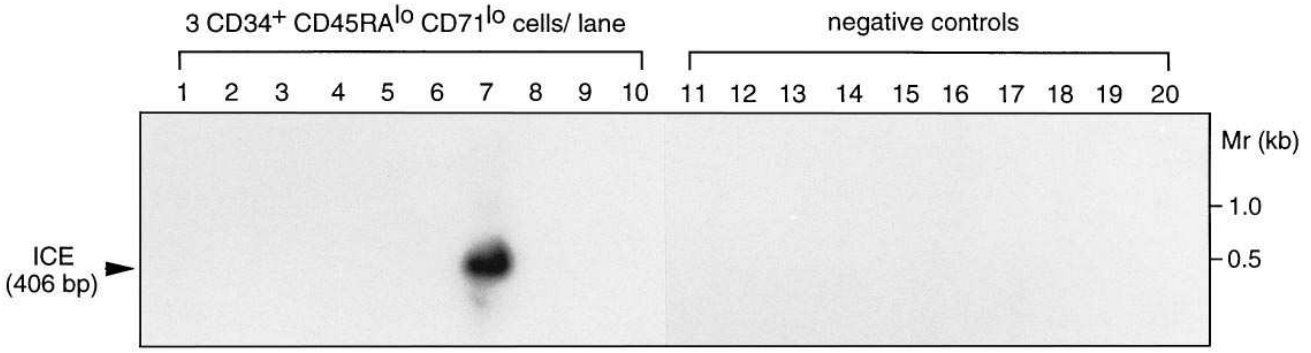


Figure 8. Seminested RT-PCR Southern analysis of ICE mRNA. *Lanes 1–10* correspond to 3 cells per lane. *Lanes 11–20* are negative controls containing medium alone (no cells).

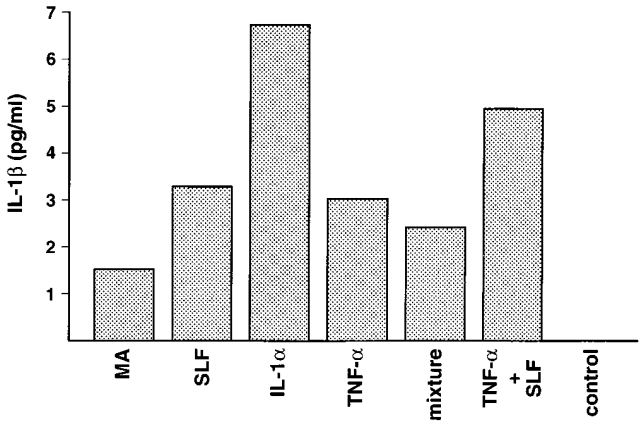


Figure 9. Enzyme immunoassay for a mature form of human IL-1β in culture supernatants of $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells. Production of IL-1β was induced by various cytokines. *MA*, medium alone.

eages. Much is now known of the stimuli that regulate the growth, differentiation, and survival of stem cells and progenitor cells, and > 20 cytokines or growth factors produced by stromal cells or other cells have been shown to influence stem cells and progenitor cells. In all of these studies, stem cells and progenitor cells have been considered as passive cells that respond to signals from the hematopoietic microenvironment rather than as cells that themselves might regulate their environment. However, based on the observation of expression of IL-1β gene in candidate stem and progenitor cells, we have proposed that human hematopoietic stem/progenitor cells can produce IL-1β, which is a powerful, pleiotropic biomodulator of other cytokines and adhesion molecules. However, our conclusions were limited by the fact that the cell fraction we studied, although highly purified and phenotypically homogeneous, had an in vitro cloning efficiency of only 13–26% (3).

Although RT-PCR provides a very sensitive technique for detection of mRNA in a single cell, the destructive nature of the procedure makes it impossible to simultaneously analyze gene expression and observe the capacity of the tested cell to generate progeny. To conclude that we have truly analyzed gene expression in hematopoietic stem/progenitor cells, we needed to adopt a statistical approach, and this required us to identify cells with a very high cloning efficiency. We observed that the daughter cells of individual human cord blood derived $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells, that had undergone a first cell division in the liquid culture, had a remarkably high colony/cluster forming efficiency (91%). This compared with that of the parent population of $\sim$ 32% (5). Therefore, we chose this population to investigate gene expression in individual hematopoietic stem/progenitor cells.

Our results showed that 30 $\sim$ 40% of the daughter cells were positive for IL-1β mRNA. Because of the high colony/cluster forming efficiency of these daughter cells (91%), it is evident that the 30–40% of cells expressing the IL-1β gene must have included hematopoietic stem/progenitor cells with a capacity to generate colonies or clusters.

When focusing on coexpression of CD34 and IL-1β mRNA, up to 30% of the single daughter cells were positive for both CD34 and IL-1β mRNA. We observed that some of

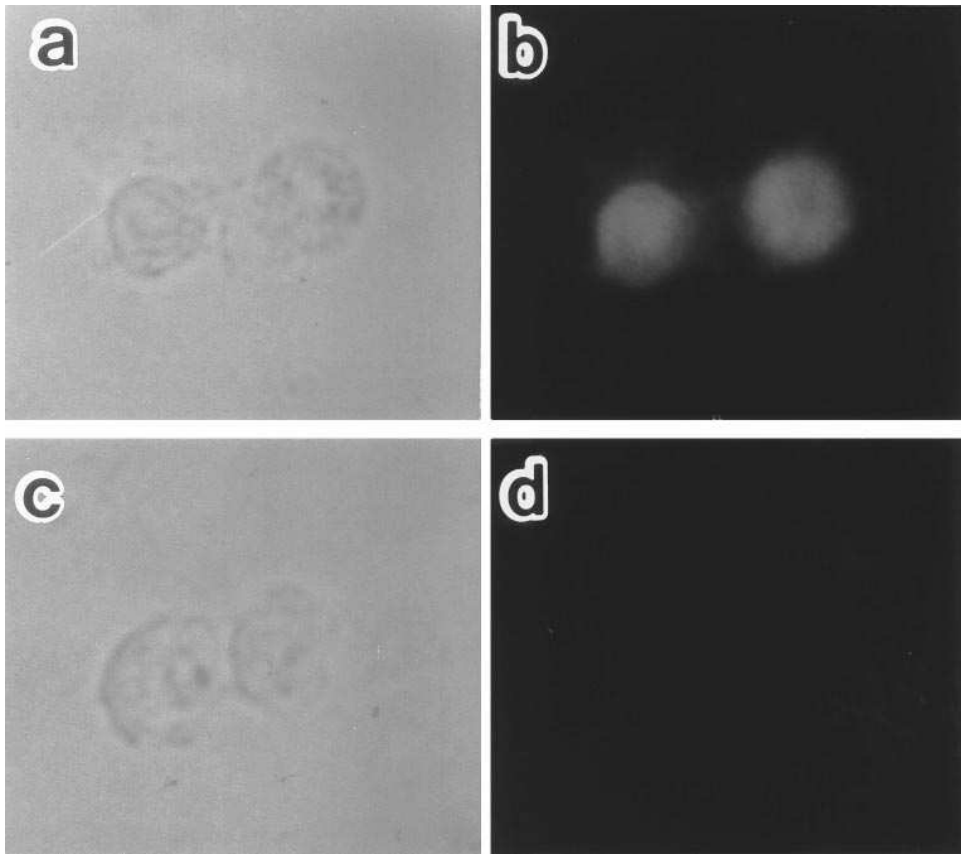


Figure 10. Indirect fluorescent immunostaining for IL-1β in $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells. Cells were cultured for 24 h and were prepared according to Methods. *a* and *b* show IL-1β–positive cells under a conventional and a fluorescent microscope, respectively. *c* and *d* show control cells.

the daughter cells that expressed IL-1β mRNA lacked CD34 mRNA. However, it has been reported previously that $CD34^+$ cells can be negative for CD34 mRNA, presumably indicating that the mRNA has a short half-life relative to that of the CD34 protein on the cell membrane (14, 15). This suggested that more of the daughter cells that had IL-1β mRNA could have been expressing CD34 protein.

There were differences in the patterns of genes expressed in pairs of daughter cells in which β-actin was successfully detected. For example, one daughter cell had detectable levels of CD34 mRNA, whereas the sibling did not (Fig. 4). However, we do not conclude that this necessarily reflects qualitative asymmetry in gene expression in the two hematopoietic progenitor cells, because the differences could be due to the amount of mRNA in one cell being below the threshold of detection of our RT-PCR method.

In other experiments, we analyzed IL-1β and β-actin mRNA expression in one of a pair of daughter cells, while allowing the sibling cell to proliferate in liquid culture (Fig. 5, *A–C*). There was variation in the generative capacity of siblings of IL-1β mRNA positive cells. Thus, we observed one instance in which an IL-1β–positive daughter cell had a sibling that generated > 300,000 progeny comprising erythroid and myeloid lineages and another in which the IL-1β–positive cell had a sibling that generated only 10 progeny. These findings demonstrated that IL-1β mRNA was present in cells that were early in the hierarchy of hematopoiesis and had a significant capacity to generate progeny.

The presence of IL-1β mRNA in these hematopoietic stem/progenitor cells does not prove that they produced the mature form of IL-1β (16) but does suggest that they might have the capacity to do so. Further evidence for their potential to produce IL-1β came from our observations on expression of ICE gene. The mature IL-1β is produced as a 17-kD protein as a result of cleavage of a 33-kD inactive precursor by a specific cysteine protease, ICE (7, 8). ICE mRNA was clearly detected in aliquots of 30 $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells, and in 1 out of 10 of the daughter cells, in which one of the daughter cells had both IL-1β and ICE mRNA. We prepared cDNA from human B cell lines HS-Sultan and Daudi as negative controls for expression of ICE. The average frequency of ICE mRNA positive cells in the daughter cell population was 10%. Experiments on groups of three cells of the $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ parental cells indicated a frequency of ICE mRNA positive cells of > 3%. Thus, primitive hematopoietic cells, including those with a higher cloning efficiency, exhibited expression of the ICE gene, at a significant frequency. Although we have not shown that they are producing ICE protein or the active enzyme, the fact that both IL-1β and ICE genes were expressed in these cells is strongly suggestive that they may be capable, under some circumstances, of producing functional IL-1β.

In this respect, ELISA data added more support to the notion that hematopoietic stem/progenitor cells were capable of producing IL-1β. This system measured only the biologically active secreted form of IL-1β and is specific and sensitive, detecting levels of IL-1β as low as 50 fg/ml. Cells with the $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ phenotype secreted a significant amount of IL-1β into culture supernatants even without stimulation. Interestingly, presence of various cytokines, especially IL-1α, induced production of IL-1β by candidate hematopoietic stem cells. A report of IL-1 receptor mRNA expression in hematopoietic primitive cells in G_0 phase is consistent with our observation (17). Since the ELISA data were derived from culture supernatants of relatively large numbers of highly purified $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells (purity > 98.8%), we could not completely exclude that the IL-1β arose from contaminating mature cells. However, the results of immunostaining for IL-1β indicated that a significant fraction of these cells contained IL-1β. Moreover, these positive cells were small round lymphocytoid cells and not macrophage- or fibroblast-like cells, which would be the most likely contaminating cellular sources of IL-1β.

The demonstration that IL-1β or ICE mRNA can be present in "true" human hematopoietic progenitor cells and that $CD34^+$ $CD45RA^{lo}$ $CD71^{lo}$ cells produced a mature form of IL-1β raises the question of the functional role of IL-1β and ICE in these early cells. Recently ICE knock-out as well as IL-1β knock-out mice have been generated (18, 19). These mice did not appear to have a major defect in steady state hematopoiesis, although as in the case of other gene knock-outs, it is conceivable that further work could reveal a defect that is compensated for by redundant mechanisms. It will also be important to determine in knock-out and normal mice whether upregulation of IL-1 production by hematopoietic stem/progenitor cells plays a role in inflammatory or other responses to stress.

In conclusion, whatever the mechanisms involved, our observations suggest that some human hematopoietic stem/progenitor cells may regulate their proliferation and differentiation through production of IL-1β and that possible contribution of IL-1β to leukemogenesis as described before (20) may reflect dysregulation of production of IL-1β by these cells.

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