RAPID COMMUNICATION

Cellular and Molecular Characterization of the Role of the FLK-2/FLT-3 Receptor Tyrosine Kinase in Hematopoietic Stem Cells

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The flk-2/ft-3 receptor tyrosine kinase was cloned from a hematopoietic stem cell population and is considered to play a potential role in the developmental fate of the stem cell. Using antibodies derived against the extracellular domain of the receptor, we show that stem cells from both murine fetal liver and bone marrow can express flk-2/ft-3. However, in both these tissues, there are stem cell populations that do not express the receptor. Cell cycle analysis shows that stem cells that do not express the receptor have a greater percentage of the population in G0 when compared with the flk-2/ft-3-positive population. Development of agonist antibodies to the receptor shows a proliferative role for the receptor in stem cell populations. Stimulation with an agonist antibody gives rise to an expansion of both myeloid and lymphoid cells and this effect is enhanced by the addition of kit ligand. These studies serve to further illustrate the importance of the flk-2/ft-3 receptor in the regulation of the hematopoietic stem cell.

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MATERIALS AND METHODS

Isolation of hematopoietic stem cell populations. Hematopoietic stem cell populations were isolated from AA4+ cells derived from midgestation fetal liver as previously described.10 The AA4+ cells were fractionated into Sca+ and Sca− subpopulations using the Ly6 A/E phycoerythrin conjugate (Pharmingen, San Diego, CA). The AA4+CD34+ or CD34− populations were derived using an affinity purified rabbit antimouse CD34.12 Antimurine flk-2/ft-3 antibodies were raised in rabbits or Syrian hamsters using a flk-2/ft-3-IgG chimeric protein as immunogen. Hamster antimurine flk-2 hybridomas IC2-514 and IC2-310 were derived from Syrian hamster splenocytes fused with murine myeloma P3X63 Ag8.1.13 For fluorescence-activated cell sorter (FACS) analysis, the IC2-514 monoclonal was purified by protein A affinity and conjugated to biotin, phycoerythrin, or fluorescein isothiocyanate (FITC). flk-2/ft-3 specificity of the antibodies was assessed by flow cytometry analysis of BAF-3 cells transfected with the full-length receptor. The nontransfected parental line was used as control (data not shown). The 579A rabbit polyclonal sera and antimurine flk-2/ft-3-IgG chimeric protein as immunogen. Hamster antimurine flk-2 hybridomas IC2-514 and IC2-310 were derived from Syrian hamster splenocytes fused with murine myeloma P3X63 Ag8.1.13 For fluorescence-activated cell sorter (FACS) analysis, the IC2-514 monoclonal was purified by protein A affinity and conjugated to biotin, phycoerythrin, or fluorescein isothiocyanate (FITC). flk-2/ft-3 specificity of the antibodies was assessed by flow cytometry analysis of BAF-3 cells transfected with the full-length receptor. The nontransfected parental line was used as control (data not shown). The 579A rabbit polyclonal sera and IC2-514 monoclonal antibody were also shown to immunoprecipitate the flk-2/ft-3 receptor from 25S-methionine labelled transfected cell lines (data not shown). The antimouse c-kit biotin conjugate was purchased from Pharmingen and all secondary and Lin cocktail antibodies were purchased from Caltag (South San Francisco, CA).

Bone marrow hematopoietic stem cells were obtained from 8- to 12-week-old C57Bl/6 Ly 5.1 (A20.1) or 5.2 (AL1-4A2) mice. The mononuclear cell fraction was isolated by density gradient centrifugation (Accudenz; Accurate Biochemicals, Westbury, NY) and stained with the Lin cocktail antibodies as previously described.11 Lin stained cells were removed via magnetic bead depletion (Dynal, Inc, Great Neck, NY).14 The Lin− population was then stained using the appropriate antibodies. Viable cells were selected by propidium iodide (1 μg/mL) exclusion and separated on an Elite flow cytometer (Coulter Electronics, Hialeah, FL).

Competitive repopulation. Stem cell populations were isolated from the bone marrow of lethally irradiated (950 rads) C57Bl/6 Ly 5.1 (A20.1) or 5.2 (AL1-4A2) mice. These are six- to eight-week-old mice, with body weights of 25-27 g. They are housed in a specific pathogen-free environment on autoclaved bedding and provided with autoclaved food and water. They are maintained in a 12-h light/12-h dark cycle. The mice are housed in microisolator cages (绿地 isolation, Inc., Houston, TX) that are pressure monitored and filtered. The mice are observed daily for mortality and morbidity.

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0006-4971/94/8408-0047$3.00/0

from young adult C57 Bl/6 Ly 5.1 mice. Young adult male C57Bl/6 Ly 5.2 mice were obtained from the National Cancer Institute (NCI; Bethesda, MD) and used as recipients. A minimum of five animals was used per experimental group. Whole body irradiation (1,100 cGy, 190 cGy/min) was administered as a single dose from a $^{137}$Cs source. One million or 5 x $10^6$ bone marrow cells from the 5.2 mice were used as competitor. Titration experiments were performed with an AA4$^+$Sca$^+$ stem cell population to ensure we were in the linear range of the competitive repopulation assay. In general, the repopulation capacity of $1 \times 10^3$ cells of the potential stem cell populations being compared were measured relative to the competitor population. Cell doses were equivalent to the representation of each population in the fetal liver or marrow. Cells were administered via tail vein injection and peripheral blood samples (50 to 100 $\mu$L) were obtained via the retro-orbital sinus 4 weeks, 12 weeks, and 6 months postreconstitution. The percentage of Ly 5.1 (A20.1) donor cells was determined by staining with biotin-conjugated Ly 5.2 (AL1-4A2) monoclonal (A20.1.7). Briefly, peripheral blood was collected in 10 U/mL heparin, 1 mmol/L EDTA in phosphate-buffered saline (PBS) and immediately placed on ice. Erythrocytes were removed by the addition of 5 vol 2% dextran T500 in PBS followed by incubation at room temperature for 20 minutes. The red blood cell-depleted supernatant was then centrifuged for 5 minutes at approximately 200g. The pellet was resuspended in 450 $\mu$L D H2O on ice followed immediately by the addition of 50 mL 1.5 mmol/L NaCl and 500 mL of PBS/10% fetal bovine serum (PBS/FBS) and centrifuged as before. The resuspended pellet was resuspended in PBS/FBS before staining. This procedure removed nearly 100% of the erythrocytes (the remaining red blood cells were excluded by size gating) while leaving the leukocytes 95% viable by propidium iodide exclusion. FACS staining of Ly 5.2 peripheral blood mononuclear cells prepared by density gradient centrifugation for Ly 5.2 antigen was nearly identical to cells prepared by lysing. Controls for the specificity of Ly 5.2 staining included naive Ly 5.1 cells (negative control), naive Ly 5.2 cells (positive control), and cells from animals repopulated with Ly 5.2 competitor cells only. To confirm repopulation by the donor Ly 5.1 cells in all lineages, peripheral blood cells and the bone marrow mononuclear fraction were stained in conjunction with Ly 5.2 for the following antigens: B220 (B-cell lineages), CD4/8 (T-cell lineages), and Gr-1/Mac-1 (myeloid lineages). The relative repopulating ability of each putative stem cell population was compared by calculating repopulation units (RU) as described by Harrison et al. Each RU represents the same repopulating ability as $1 \times 10^3$ fresh marrow cells. Harrison et al have confirmed that, for long-term repopulating stem cells, the stem cell content of fresh marrow is 1 stem cell per $10^3$ total marrow cells. Therefore, the number of RU from each donor population is defined by the following equation: $R_U = %$ donor repopulation / 100(RU/RU + C); where C is the number of fresh competitor marrow cells/10$^3$ and RU = % (C)/(100 - %). Cloning of murine flk-2/flt-3 receptor. The murine flk-2/flt-3 receptor was cloned by reverse transcription-PCR (RT-PCR) from RNA isolated from midgestation mouse fetal livers. Six sets of overlapping primers were designed to the murine flk-3 sequence. These primers were designed to amplify three segments of the gene, nucleotides 1-1307, 1308-1992, and 1993-3096. The PCR products were then subcloned into pRKS-A. The sequence of the full-length flk-2/flt-3 gene was identical to the published murine flt-3 gene. A flk-2/flt-3-IgG extracellular domain-IgG fusion gene was constructed and fusion protein produced as previously described. Assays for agonistic antibodies. The BAF-3 cell line was electroporated with the full-length murine flk-2/flt-3 receptor as previously described. Tyrosine phosphorylation experiments were performed as previously described. Briefly, BAF-3 cells were incubated with hybridoma supernatant or polyclonal antisera diluted 1:20 for 30 minutes, lysed in IP lysis buffer (1% NP-40, 1 mmol/L EDTA, 200 mmol/L NaCl, 50 mmol/L Tris-Cl, pH 8.0, 2.5 mmol/L phenylmethylsulfonyl fluoride [PMSE], 2.5 mmol/L Na3VO4), and immunoprecipitated with 579A anti-murine flk-2/flt-3 polyclonal sera. Immunoprecipitated lysates were separated on a 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gradient gel and analyzed by antiphosphotyrosine Western blot. For the thymidine incorporation assay, BAF-3 cells were starved of interleukin-3 (IL-3) for 24 hours and then stimulated with antibody overnight, followed by an 8-hour pulse of 1 $\mu$Ci [H] thymidine. Thymidine incorporation was then determined using a cell harvester. After the discovery of I2C-310 agonist activity, the hybridoma supernatant was purified by protein-A affinity chromatography. The purified antibody retained activity at concentrations between 10 and 40 $\mu$g/mL. We routinely used 40 $\mu$g/mL in all subsequent in vitro assays. Colony assays. Standard myeloid myeloblast cell colony assays were performed using complete methylcellulose medium (M3430; Stem Cell Technologies, Inc, Vancouver, British Columbia, Canada) with the addition of 50 ng/mL KL (R & D Systems, Minneapolis, MN). Colonies were counted after 10 days in culture; only colonies of greater than 50 cells were scored. Lymphoid colonies were produced using basic methylcellulose (Stem Cell Technologies) with 50 ng/mL KL and 50 ng/mL murine IL-7 (R&D Systems). Cytospin analyses of the resultant colonies were performed as previously described. Stromal cell production. Fetal liver stromal cell lines were isolated by infecting primary cultures of fetal stroma with the recombinant retrovirus $^{+}40$A5A8 as previously described. One of the resultant stromal cell lines, designated 7-4, was used in these experiments. Suspension culture assays. Hematopoietic stem cell populations were seeded at 10$^3$ cells/mL in Dulbecco’s modified Eagle’s medium (DMEM/F12 media supplemented with IGF-1 at 10 ng/mL (Genentech, Inc, South San Francisco, CA), KL at 50 ng/mL (R & D Systems), PDGF-BB at 2 ng/mL, and bFGF at 25 ng/mL (Boehringer Mannheim, Indianapolis, IN). Stromal cell/stem cell coculture assays. Hematopoietic stem cell populations were seeded at 10$^3$ cells/mL on the fetal liver stromal line 7-4 in DMEM/F12 media supplemented with 10% fetal calf serum (FCS). Cocultures were incubated at 37°C for 7 days and the contents of each well were then removed for analysis. The results obtained from each in vitro assay system were confirmed in a minimum of three independent experiments. Growth factors were used at the following concentrations: KL at 50 ng/mL; IL-3 at 1 ng/mL (Genentech, Inc, South San Francisco, CA); granulocyte-macrophage colony-stimulating factor (GM-CSF) at 0.2 ng/mL (R & D Systems); PDGF-BB at 2 ng/mL; and bFGF at 2.5 ng/mL (Boehringer Mannheim). Cultured hematopoietic cells were phenotyped by staining using direct antibody conjugates to the following antigens: CD4, CD8, Mac-1, GR-1, and B220 (Caltag Inc, South San Francisco, CA). All antibodies were titered against peripheral blood white blood cells and controls for specificity included irrelevant isotype-matched direct conjugates at similar dilutions. Staining profiles were also obtained in the presence of an FcR blocking antibody (Pharmingen). Cell cycle analysis. Two-step acridine orange staining was performed as detailed previously. Briefly, cells that had been sorted after dual-parameter immunofluorescence staining were centrifuged and resuspended in RPMI 1640 cell culture medium with 10% FBS at a final concentration of 10$^6$/mL. To 0.3 mL of this cell suspension, a solution consisting of 0.45 mL of 0.1% Triton X-100 in 0.15 N NaCl and 0.08 N HCl was added and the mixture was incubated for 45 seconds on ice. To this mixture, 1.8 mL of a solution consisting of 12 $\mu$m acridine orange (Polysciences, Inc, Warrington, PA) in...
0.2 mol/L Na₂HPO₄, 0.1 mol/L citric acid, 10⁻² mol/L Na-EDTA, and 0.15 mol/L NaCl was added and the sample was immediately analyzed by flow cytometry. Green fluorescence (DNA content) was collected through a 560-nm dichroic long-pass filter coupled with a 525 ± 15 nm bandpass while red fluorescence (RNA) was simultaneously collected through a 630-nm long-pass filter.

The G₀ subpopulation was defined on the basis of the red fluorescence (RNA content) of peripheral blood mononuclear cells (PBMC) stained in parallel to the previously sorted samples. A cursor was placed at the position corresponding to the red fluorescence intensity of 97% of PBMC, with cells having higher RNA contents above this position classified as cycling (ie, G₁, S, and G₂M) populations and those at or below the cursor classified as G₀. 22 Enumeration of the proportions of cycling cells was performed by conventional cell cycle analysis using the algorithm of Dean and Jett 21 available in the Multicycle software (Phoenix Flow Systems, San Diego, CA).

RESULTS

Characterization of flk-2/flt-3 expression in hematopoietic cell populations. Investigation of flk-2/flt-3 mRNA expression showed that flk-2/flt-3 was preferentially expressed in primitive hematopoietic cell populations. However, it was unknown whether the flk-2/flt-3 receptor was expressed on hematopoietic stem cells capable of long-term engraftment of lethally irradiated hosts. The production of hamster monoclonal antibodies raised against the murine flk-2/flt-3 receptor allowed this question to be addressed. Additionally, to characterize further murine hematopoietic stem cell populations, an affinity-purified rabbit antimurine CD34 polyclonal antibody was raised as previously described.

The stem cell expression of CD34 and flk-2 was characterized using previously defined stem cell populations. In the fetal liver, the AA4.1 antibody (AA4) delineates a population of approximately 1% of the cells in which all the totipotent stem cell activity resides. 23 The AA4⁺ population can be further enriched using antibodies to the Sca-1 antigen (Ly6A/E). The AA4⁺ Sca⁻ population contains all the totipotent stem cell activity. 4 Experiments using the CD34 polyclonal antibody demonstrated that 60% of the AA4⁺ cells are CD34⁺ and that all AA4⁺CD34⁺ cells are positive for c-kit expression. Furthermore, all AA4⁺Sca⁻ cells are also CD34⁺ (data not shown). Repopulation studies showed that all the long-term reconstituting stem cell activity resides in the AA4⁺CD34⁺ kit⁺ population and that the AA4⁺CD34⁺ kit⁺ population does not repopulate (Table 1). Similarly, all the colony-forming potential in methylcellulose assays was found in the AA4⁺CD34⁺ kit⁺ fraction (30 colonies per 1 × 10⁶ AA4⁺CD34⁺ kit⁺ cells plated v 0 colonies per 5 × 10³ AA4⁺CD34⁺ kit⁻ cells plated).

We used the AA4⁺ Sca⁻ and the AA4⁺CD34⁺ populations to investigate stem cell expression of the flk-2/flt-3 receptor (Fig 1). FACS analysis of these populations showed that all of the stem cell populations could be subdivided into flk-2⁺ or flk-2⁻ fractions (Fig 1). Repopulation studies demonstrated that the flk-2/flt-3 receptor can be expressed on stem cells but that both flk-2/flt-3⁺-positive and flk-2/flt-3⁻-negative cell fractions give rise to long-term reconstitution (Table 1). In experiments using bone marrow as the source for stem cells, the mononuclear fraction was segregated into a Lin⁻ population by immunomagnetic bead separation using the Lin cocktail of antibodies to mature hematopoietic cell types. 24 The resulting Lin⁺ population contained both Lin⁻ and Lin⁺ cells by FACS analysis; however, for simplicity, it will be referred to as Lin⁺. The Lin⁺ mononuclear cells were fractionated into a Lin⁻Sca⁺ stem cell fraction (Fig 1). In accordance with the fetal liver populations, this bone marrow stem cell population also gave rise to both flk-2/flt-3⁺-positive or flk-2/flt-3⁻-negative subpopulations. Both of these subpopulations gave rise to long-term repopulation (Table 1). The Lin⁻CD34⁺ bone marrow population also gave rise to long-term repopulation (data not shown).

To compare the relative repopulating abilities of each of the different donor populations from the same experiment, RU were calculated as described in Materials and Methods (Table 1). These experiments showed the high stem cell content of the AA4⁺CD34⁺ kit⁺ and AA4⁺Sca⁻ subpopulations. Interestingly, in both the AA4⁺CD34⁺ fraction from fetal liver and the Lin⁻Sca⁻ fraction from bone marrow, the flk-2⁻ cell populations had a statistically significant increase in RU when compared with the relevant flk-2⁺ population (AA4⁺CD34⁺ flk⁻2+ ≈ AA4⁺CD34⁺ flk⁻2⁻ P < .05; Lin⁻Sca⁻ flk⁻2⁺ ≈ Lin⁻Sca⁻ flk⁻2⁻ P < .001; Table 1).

Cell cycle analysis of flk-2/flt-3—expressing stem cell populations. Cell cycle analysis of hematopoietic stem cell populations has previously indicated that stem cell populations are heterogeneous in relation to cell cycle status. 25,26 Furthermore, enhanced repopulation has been attributed to those stem cells in the G₀/G₁ phase compared with the actively proliferating S/G₂M subset. 26 If flk-2/flt-3 expression on stem cells represents a potentially more committed stem cell population, exhibiting decreased repopulation capacity, this may be reflected in the cell cycle status. Additionally, it has been suggested that flk-2/flt-3 mRNA expression is correlated with cycling stem cells. 27,28 Therefore, we determined the cell cycle status of the fetal liver stem cell population AA4⁺ Sca⁻ with respect to flk-2/flt-3 expression (Fig 2).

We used a two-step acridine orange staining technique to differentiate G₀ from G₁ phase cells. 23 When coupled with conventional cell cycle analysis of the DNA content histogram, this method allows for the simultaneous analysis of the G₀, G₁, S, and G₂M cell cycle phase compartments of any cell population. Using this technique, we showed that a major difference between these two subpopulations was the greatly increased percentage of cells residing in G₀ in the AA4⁺Sca⁻ flk⁻2⁻ population (Fig 2). Additionally, the percentage of cells in S/G₂M phase of the cell cycle argues that these two fetal liver stem cell populations contain many actively cycling cells. Similarly, the bone marrow stem cell population, Lin⁻Sca⁻, was also found to contain a greater percentage of G₀ cells in the flk⁻2⁻ population (Lin⁻Sca⁻ flk⁻2⁻ G₀ [39%] v Lin⁻Sca⁻ flk⁻2⁻ G₀ [26%]).

Cell cycle analysis of the AA4⁺ kit⁺ stem cell populations demonstrated a much lower percentage of cells in G₀ when compared with the AA4⁺Sca⁻ flk⁻2⁻ population. However, little difference was found between the AA4⁺ kit⁺ flk⁻2⁻ (G₀ [12%], G₁ [39%], S [41%], G₂M/8%)) and the AA4⁺ kit⁻ flk⁻2⁻ (G₀ [7%], G₁ [47%], S [52%], G₂M/5%)) populations.

Development of agonist antibodies to the flk-2/flt-3 receptor. We approached functional studies of the biologic role...
Table 1. Competitive Repopulation Assays of Purified Cell Populations

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>% 5.1 4 wk</th>
<th>RU</th>
<th>% 5.1 12 wk</th>
<th>RU</th>
<th>% 5.1 24 wk</th>
<th>RU</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA4+ 'Sca-1' flk-2'</td>
<td>72 ± 3</td>
<td>13</td>
<td>83 ± 4</td>
<td>24</td>
<td>90 ± 2</td>
<td>45</td>
</tr>
<tr>
<td>AA4+ 'Sca-1' flk-2(-)</td>
<td>68 ± 3</td>
<td>11</td>
<td>89 ± 5</td>
<td>41</td>
<td>92 ± 2</td>
<td>57</td>
</tr>
<tr>
<td>AA4 'CD34+ flk-2'</td>
<td>41 ± 12</td>
<td>4</td>
<td>66 ± 10</td>
<td>10</td>
<td>68 ± 9</td>
<td>11</td>
</tr>
<tr>
<td>AA4 'CD34+ flk-2(-)'</td>
<td>58 ± 18</td>
<td>7</td>
<td>77 ± 8</td>
<td>17</td>
<td>84 ± 4</td>
<td>26</td>
</tr>
<tr>
<td>AA4 'CD34+ kit'</td>
<td>75 ± 4</td>
<td>30</td>
<td>84 ± 5</td>
<td>39</td>
<td>70 ± 3</td>
<td>23</td>
</tr>
<tr>
<td>AA4 'kit' CD34(-)</td>
<td>2 ± 2</td>
<td>0</td>
<td>23 ± 6</td>
<td>3</td>
<td>4 ± 3</td>
<td>0</td>
</tr>
<tr>
<td>Lin- 'Sca-1' flk-2'</td>
<td>59 ± 8</td>
<td>10</td>
<td>40 ± 6</td>
<td>7</td>
<td>24 ± 5</td>
<td>3</td>
</tr>
<tr>
<td>Lin- 'Sca-1' flk-2(-)</td>
<td>57 ± 12</td>
<td>13</td>
<td>53 ± 10</td>
<td>11</td>
<td>67 ± 20</td>
<td>20</td>
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Fetal liver and bone marrow stem cell populations were isolated as described. All AA4+ populations were from the fetal liver and all Lin- populations were from the bone marrow. Long-term repopulation by fetal liver- or bone marrow-derived populations was determined using competitive repopulation analysis of genetically marked fetal liver or bone marrow in C57BL/6 mice allelic at the Ly 5 locus, designated Ly 5.1 (A20.1) and Ly 5.2 (AL1-4A2). Five animals were used per stem cell population as described in Materials and Methods. Ly 5.1 expression in the Ly 5.2 mice was determined 4, 12, and 24 weeks postengraftment. Only the 24-week time point is shown. RU were calculated as described in Materials and Methods. Contribution of the stem cell populations to all lineages was determined by staining both peripheral blood leukocytes and bone marrow cells for the following antigens: B220, CD-4/8, and Gr-1/MAC-1. These experiments showed that all lineages were reconstituted by donor 5.1 cells (data not shown). Additionally, no preferential reconstitution of any lineage by the different donor populations was observed.

Fig 1. Fractionation of fetal liver and bone marrow stem cell populations. Fractionation of AA4 cells from day 14 gestation fetal liver, AA4+ cells were enriched by immune-panning. AA4+ cells were subsequently stained using antibodies against Sca-1, CD34, flk-2, and c-kit. AA4+ cells were stained for (A) flk-2 and Sca-1; (B) c-kit and CD34; (C) flk-2 and CD34; and (D) flk-2 and c-kit. Fractionation of Lin- bone marrow progenitor cells with antibodies against Sca, flk-2, c-kit, and CD34. Lin- bone marrow progenitor cells were isolated by indirect magnetic bead panning. The Lin cocktail comprised RA3-6B2, YTS 191.1, YTS 169.4, B6S, TER-119, M1/70.15, and CG16. The Lin- bone marrow cells were stained for (E) flk-2 and CD34 and (F) Sca-1 and flk-2 (shown as a dot-plot because of the very small population of Lin- 'Sca-1' flk-2' cells in the marrow). These experiments were repeated a minimum of four times and gave similar staining profiles on each occasion.
Fig 2. Cell cycle analysis of hematopoietic stem cell populations. Dual-parameter fluorescence histograms of AA4+Sca+flk-2+ enriched (upper left) and AA4+Sca+flk-2+ enriched (upper right) stem cell populations after cell sorting. The lower panels illustrate corresponding red fluorescence histograms obtained after acridine orange for AA4+Sca+flk-2+ (lower left) and for AA4+Sca+flk-2+ populations (lower right). The cursor illustrated the fluorescence intensity used to discriminate G0 (lower fluorescence intensity) from cycling cells (see Materials and Methods). The percentages of cells in each phase of the cell cycle is provided in the inset.

of the flk-2/flt-3 receptor using antibodies raised to the extracellular domain of the receptor. A polyclonal antibody (579A) was raised in rabbits by producing a flk-2/flt-3-IgG extracellular domain fusion protein and using this as antigen. The 579A polyclonal antisera was subsequently shown to be capable of activating tyrosine phosphorylation of the flk-2/flt-3 receptor (Fig 3). Therefore, the flk-2-IgG fusion protein was used to raise monoclonal antibodies in Syrian hamsters. The hybridomas resulting from the subsequent fusions were screened for the ability to activate the flk-2/flt-3 receptor.

Agonistic activity of these resultant hybridomas was determined using two assay systems. A phosphotyrosine assay using the full-length murine flk-2/flt-3 receptor expressed in the IL-3-dependent cell line BAF-314 and a thymidine incorporation assay using IL-3-dependent BAF-3 cells expressing the full-length receptor.

In the phosphotyrosine assay, the transfected/non-transfected BAF-3 lines were treated with various antibodies followed by immunoprecipitation of the flk-2/flt-3 receptor using the 579A polyclonal antibody. Immunoblotting of the immunoprecipitated material using antiphosphotyrosine antibodies demonstrated the phosphorylation of the flk-2/flt-3 receptor in response to both IC2-310 or 579A (Fig 3). The phosphorylated flk-2/flt-3 receptor migrated at an apparent molecular weight of approximately 160 kD. Similar molecular weight values for the full-length receptor have been obtained in other studies. In the thymidine incorporation assay, cells were starved of IL-3 for 24 hours and then incubated with antibody. Both 579A and IC2-310 gave a significant stimulation of thymidine uptake in the transfected BAF-3 cells (Fig 4). The specificity of these responses was demonstrated by the lack of response to irrelevant antibodies and to hamster IgG.

Hematopoietic assays of the flk-2r agonist monoclonal antibody IC2-310. To assist in the evaluation of the biologic function of the IC2-310 agonist antibody, we developed a Dexter culture assay system using immortalized stromal cell lines from the fetal liver (see Materials and Methods). Stem cell populations were plated onto the fetal liver stromal cell line 7-4. The stem cell content was determined by competitive repopulation analysis prior to and after 7 days of coculture. After 7 days of coculture the resulting cell populations could only sustain short-term repopulation of the irradiated host as evidenced by contribution of donor cocultivated cells at 4 weeks postengraftment (data not shown). However, after this early time point no further contribution from the cocultivated cells was observed.
Cocultivation upon the 7-4 stromal cell line gave rise to a dramatic expansion in cell number (Table 2). Lineage analysis of the resultant cell populations was performed using flow cytometric analysis and Wright-Giemsa staining of cytospin material. These analyses showed the presence of immature progenitor, myeloid, and lymphoid cells (Table 3).

Stem cells plated in the presence of the IC2-310 agonist antibody gave rise to a greater proliferative event than seen on 7-4 alone. However, IC2-310 did not induce proliferation of the non-flk-2/flt-3—expressing stem cell populations or the non-stem cell populations AA4+ Sca−. The lack of effect on the AA4+Sca− population is noteworthy because we can detect low levels of flk-2/flt-3-positive cells in this population (data not shown). Furthermore, IC2-310 had no effect on the non-flk-2/flt-3—expressing stem cell populations (Table 2). FACS analysis of these cells again demonstrated the presence of several potential lineages (Table 3). As with cells grown on 7-4 alone, the IC2-310 stimulated cells were only capable of repopulating in the short term (data not shown). The proliferative event enhanced by the IC2-310 antibody was greatly increased in combination with KL (Table 2). Cytospin analysis of the cocultivated cells showed a significant decrease in the percentage of blast cells with a concomitant increase in the percentage of cells from the myeloid lineages, including myeloblasts, myelocytes, promyelocytes, or metamyelocytes (Table 3). Collectively, these data show the overall proliferation resulting from stimulation of the flk-2/flt-3 receptor. Furthermore, they illustrate that, in the context of cocultivation on the 7-4 stromal cell line, this proliferative event was accompanied by differentiation to more mature hematopoietic phenotypes.

To develop stroma-free suspension cultures, we investigated the ability of various growth factor combinations to support the growth of hematopoietic cells in the absence of stroma. We found that a combination of IGF-I, bFGF, KL, and PDGF-BB was a very potent stimulator of multilineage expansion of stem cells cocultivated on 7-4, with expansion exceeding 200-fold over 7 days. Suspension cultures performed in the presence of these factors, without stroma, gave rise to a 41±10-fold expansion in cell number. The addition of IC2-310 to this growth factor cocktail increased expansion to 85±12-fold. These resultant cells once again represented multiple lineages as assessed by FACS analysis (data not shown). However, stem cells plated in suspension with IC2-310 alone did not give rise to viable cultures.
Effect of IC2-310 monoclonal antibody on methylcellulose colony formation. Hematopoietic colony assays were performed to determine the effects of the IC2-310 antibody on the colony-forming potential of primitive hematopoietic populations. The methylcellulose assays were performed in the presence of WEHI-conditioned media supplemented with KL to test the myeloid potential of the input cell or, alternatively, in the presence of IL-7 and KL to test the B-lymphoid potential.

Stem cell populations plated directly into methylcellulose with the addition of IC2-310 alone showed no induction of colony growth. However, IC2-310 did promote a small increase in methylcellulose colony formation when added in combination with WEHI-conditioned media compared with the use of WEHI-conditioned media alone (135 ± 3 v 104 ± 7 per 10³ cells plated). The most dramatic increases in colony formation using IC2-310 were observed when stem cells were first cocultivated on the stromal cell-line 7-4.

Hematopoietic stem cell populations plated onto 7-4 cells and then removed after 7 days were capable of yielding both myeloid colony-forming cells (CFC) and lymphoid CFC. When the cocultivation on 7-4 was performed in the presence of IC2-310 agonist antibody there was an approximate 10-fold increase in myeloid CFC and a ninefold increase in lymphoid CFC (Fig 5). Cytospin data showed the myeloid colonies to be of mixed lineage but principally they represented the granulocyte/macrophage subset (data not shown).

### Table 2. Cell Proliferation Assays on Fetal Liver Stroma

<table>
<thead>
<tr>
<th>Cell Population</th>
<th>Control</th>
<th>IC2-310</th>
<th>KL</th>
<th>KL + IC2-310</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA4'kit'flk-2'</td>
<td>33 ± 14</td>
<td>52 ± 2</td>
<td>210 ± 18</td>
<td>276 ± 12</td>
</tr>
<tr>
<td>AA4'kit'+flk-2'</td>
<td>32 ± 3</td>
<td>28 ± 1</td>
<td>71 ± 12</td>
<td>84 ± 5</td>
</tr>
<tr>
<td>AA4'Sca'</td>
<td>8 ± 0.71</td>
<td>31 ± 2.1</td>
<td>120 ± 14</td>
<td>180 ± 13</td>
</tr>
<tr>
<td>AA4'Sca'</td>
<td>6 ± 0.71</td>
<td>6 ± 0.64</td>
<td>13 ± 0.35</td>
<td>12 ± 0.35</td>
</tr>
<tr>
<td>AA4'CD34'kit'</td>
<td>12 ± 2.8</td>
<td>22 ± 2.3</td>
<td>96 ± 6.1</td>
<td>129 ± 7</td>
</tr>
<tr>
<td>AA4'CD34'flk-2'</td>
<td>25 ± 5</td>
<td>52 ± 11</td>
<td>132 ± 11</td>
<td></td>
</tr>
<tr>
<td>AA4'CD34'flk-2'</td>
<td>12 ± 3</td>
<td>14 ± 5</td>
<td>125 ± 5</td>
<td></td>
</tr>
<tr>
<td>Lin'CD34'flk-2'</td>
<td>52 ± 7</td>
<td>713 ± 36</td>
<td>713 ± 36</td>
<td></td>
</tr>
</tbody>
</table>

Stem cell populations were isolated as described. Ten thousand cells from the relevant stem cell population were plated onto 7-4 stromal line and incubated at 37°C. After 7 days, hematopoietic cells were removed from the stroma and cell counts were performed. Data are presented as fold expansion of the initial 10,000 cells. Assays were performed in duplicate wells and repeated in three independent experiments. Control wells were media containing hamster-lgG at 40 μg/mL.

### Table 3. Lineage Analysis From Cell Proliferation Assays

<table>
<thead>
<tr>
<th>Cytopsin Analysis</th>
<th>% Blasts</th>
<th>% Ig MNC</th>
<th>% Myeloid</th>
<th>% Lymphoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA4'Sca'</td>
<td>Media alone</td>
<td>28</td>
<td>47</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>IC2-310</td>
<td>10</td>
<td>52</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>IC2-310/KL</td>
<td>15</td>
<td>35</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>IC2-310/GM-CSF</td>
<td>12</td>
<td>31</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>IC2-310/KL/GM-CSF</td>
<td>3</td>
<td>23</td>
<td>70</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>FACS Analysis</th>
<th>% MAC-1</th>
<th>% Gr-1</th>
<th>% B220</th>
<th>% CD4/CD8</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA4'Sca'</td>
<td>Control</td>
<td>65</td>
<td>64</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>310</td>
<td>76</td>
<td>63</td>
<td>22</td>
</tr>
</tbody>
</table>

Cytospin differentials from the AA4'SCA' stem cell population after cocultivation with stromal cell line 7-4 for 7 days. Cells were cocultivated in the presence of the growth factors indicated. “Blasts” are cells of immature phenotype characterized by intermediate to high nuclear to cytoplasmic ratio with basophilic nuclei and pale cytoplasm. Large mononuclear cells (Lg MNC) are cells of intermediate size and low nuclear to cytoplasmic ratio. The majority of these cells show pale to light blue cytoplasm and coarsely reticulated chromatin. Some of these cells are smaller, more basophilic, and more clearly granulocytic. “Myeloid” are cells of mature myeloid lineages. “Lymphoid” are cells displaying lymphocyte or plasma cell characteristics. For FACS analysis, cells from all control wells or all IC2-310 treated wells were pooled and stained using antibodies against MAC-1 Gr-1, B220, and CD4/8. These data are presented from one representative experiment. The experiments were repeated a minimum of three times.
Analysis of the colonies produced in the presence of IL-7 and KL demonstrated a B220 + IgM - phenotype. Once again, the proliferative effect of IC2-310 was restricted to the stem cell population. No effect was seen on the AA4 + Sca - cell population that does not contain stem cells (Fig 5). These results support the observations that the IC2-310 antibody is only capable of stimulating proliferation of hematopoietic cell populations containing early progenitors.

DISCUSSION

Hematopoiesis is dependent on the capacity of the hematopoietic stem cell to self-renew and commit to a pathway of differentiation. In recent years, progress has been made in identifying some of the molecules that are involved in these events. The flk-2/flt-3 receptor was cloned from an enriched stem cell population and it was speculated that it may play a role in determining the early developmental fate of the stem cell. However, previous studies of this receptor have all determined expression at the level of the mRNA. The development of monoclonal antibodies that recognize the native receptor allowed us to examine the expression of this molecule on stem cell populations.

Reconstitution experiments of lethally irradiated mice show that the flk-2/flt-3 receptor tyrosine kinase is expressed on stem cell populations, but, quite clearly, not all stem cells express flk-2/flt-3. This finding was confirmed in all the different fetal liver and bone marrow stem cell populations isolated. Cell cycle analysis of the stem cell populations from fetal liver and bone marrow demonstrated that the flk-2 - stem cell fractions had significantly fewer cycling cells than the corresponding flk-2 + fraction. Furthermore, in both the AA4 + CD34 + and the Lin - Sca - populations, the flk-2 - subpopulations had significantly less repopulating capacity than their flk-2 + counterparts. These data suggest the hypothesis that the flk-2/flt-3 receptor is expressed by a subset of hematopoietic stem cells destined to differentiate to more committed progenitor cells. This hypothesis gains support from studies that have demonstrated decreased radioprotective capacity in cycling stem cells and from the expression of flk-2/flt-3 mRNA in stem cell fractions believed to be actively cycling.

The most widely used marker for the study of human hematopoietic cells is cell surface expression of CD34. Various functional assays have shown that the CD34 + subpopulation from human marrow contains virtually all primitive hematopoietic cells. The monospecific polyclonal antibody to murine CD34 clearly demonstrated that, in accordance with the human homologue, the stem cell activity in murine hematopoiesis is confined to the CD34 + fraction. Therefore, the phenotype of the murine hematopoietic stem cell from the fetal liver is AA4 + Lin - Sca - CD34 + kit - flk-2 -. From the bone marrow, the phenotype of the stem cell appears to be Lin - Sca - Kit + CD34 + flk-2 +.

From the current experiments, it is clear that activation of the flk-2/flt-3 receptor promotes the proliferation and differentiation of hematopoietic stem cells when they are cocultivated with stroma. This proliferation is most clearly evidenced by the increases in both cell number and CFC obtained on activation of stem cells with the IC2-310 agonist antibody. Conversely, the agonist antibody has little effect on non-stem cell populations. In these experiments, activation of flk-2/flt-3 expressing stem cells does not lead to an increase in the number of cells capable of long-term reconstitution. However, it is possible that the assays used on the stromal cell line 7-4 initiate a differentiation program that could prevent self-renewal of the most primitive stem cell. We are currently addressing the effects of the agonist antibody in vivo, where the ability of the stem cell to self renew should not be compromised.

Recently, the cognate ligand of the flk-2/flt-3 receptor has been cloned. Based on thymidine incorporation studies and methylcellulose assays, the flt-3 ligand induces proliferation of fetal liver and bone marrow primitive hematopoietic populations. As with IC2-310, the proliferative effects are greatly enhanced in cooperation with KL. It is interesting to note that we see greater proliferative effects of KL on the AA4 + Kit + flk-2 - stem cell population than on its flk-2 + counterpart. These results may indicate that the expression of flk-2/flt-3 and c-kit on stem cell populations represent potentially different stages of stem cell development. However, with the current availability of reagents to activate these receptors, the opportunity exists to dissect the early proliferative events of the stem cell. Such information should prove valuable for the biology of hematopoiesis as well as for clinical transplantation therapy.

ACKNOWLEDGMENT

We thank Dr David Goeddel and Dr Tim Austin for critical comments on the manuscript. We also gratefully acknowledge Christopher Donahue and James Chin for expert flow cytometry. The patient and expert assistance of Sunita Sohrabji in preparing the manuscript is greatly appreciated. We also thank Linda Werner and Gabrielle Hatami for assistance with the cytospin analyses.

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