c-kit Expression by CD34+ Bone Marrow Progenitors and Inhibition of Response to Recombinant Human Interleukin-3 Following Exposure to c-kit Antisense Oligonucleotides

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The c-kit proto-oncogene encodes a receptor having tyrosine-specific kinase activity and has been mapped to chromosome 4 in the human and chromosome 5 in the mouse, at the dominant white spotting locus (W). Mutations at the W locus affect various aspects of murine hematopoiesis. The c-kit proto-oncogene has been shown to be expressed by leukemic myeloblasts, but not by normal unseparated human bone marrow cells. The role of this oncogene in differentiation and proliferation of human hematopoietic progenitors is presently undefined. To determine c-kit expression by normal hematopoietic progenitors, CD34+ cells were isolated from disease-free human bone marrow, and RNA-based polymerase chain reaction (PCR) techniques were used to assess expression. By this method, we have demonstrated c-kit expression by CD34+ bone marrow progenitors. To address the functional requirement for c-kit expression in normal human hematopoiesis, CD34+ cells were incubated in the presence of sense, antisense, or missense oligonucleotides to c-kit, and subsequently cultured in the presence of either recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) or recombinant human interleukin-3 (rhlL-3). Exposure of CD34+ cells to c-kit antisense oligonucleotides significantly inhibited colony-forming ability of cells cultured in the presence of rhlL-3, but had no effect on colony formation of cells cultured in rhGM-CSF. Together, these data suggest a possible role for c-kit in hematopoietic proliferation and differentiation that may be linked to some, but not all, stimulatory factors.

Mutations at the W locus have pleiotropic effects on embryonic development and regulation of hematopoiesis in W/W" doubly heterozygous mice, which include absent coat pigmentation, sterility, and severe macrocytic anemia. Nocka et al have demonstrated that fetal tissue from homozygotes (W/W") completely lacks c-kit mRNA transcripts; these mice die in utero from severe anemia.

Deficiencies in hematopoiesis, identical to those of W/W" doubly heterozygous mice, have been previously noted in mice harboring mutations at the steel (Sl) locus on murine chromosome 10. While the defect in W/W" mice was considered to be a cellular one, the defect in mice with mutations at the Sl locus (Sl/Sl" or Sl/Sl") was considered to be a microenvironmental one. Similar to W/W" homozygotes, Sl/Sl" homozygotes die in utero from severe macrocytic anemia. As previously suspected, the S1 locus encodes the ligand for c-kit, stem cell factor (SCF), which is a recently described hematopoietic growth factor. The c-kit mRNA is expressed by fresh human leukemic myeloblasts from patients with acute myelogenous leukemia (AML; French-American-British classification [FAB] M1, M4, and M5) and by erythroleukemia cell lines (HEL and LAMA). A number of myeloid cell lines, including HL-60, KG1, and U937, lymphoid leukemia cell lines LEUCO2 (T cell), RAJI (B cell), and BJAB (B cell), normal human lymphocytes, and leukemic lymphoblasts do not express c-kit message. In addition, normal unseparated human bone marrow cells, which consist primarily of maturing committed cells, lack expression of c-kit mRNA as determined by Northern analysis. These data suggest that the c-kit product may be expressed normally in humans by early erythroid and myeloid lineage cell types. Because c-kit plays a major role in normal murine hematopoiesis, the following studies were conducted, which showed expression of c-kit by CD34+ human hematopoietic progenitors. In addition, a functional role for c-kit during normal hematopoiesis was investigated using antisense technology.

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MATERIALS AND METHODS

Bone marrow procurement. Bone marrow aspirates were obtained from young healthy male and female donors who had given informed consent for the procedure. Under sterile conditions and local anesthesia, approximately 5 to 10 cc of bone marrow was aspirated from the posterior iliac spine into a heparinized syringe using a sterile Rosenthal needle. None of the donors suffered complications as a result of this procedure.

Isolation of mononuclear bone marrow cells and CD34+ cells. Mononuclear cells were separated by centrifugation at 500g over a Ficoll-Hypaque (LSM, Organon Teknika, Durham, NC) gradient. The cells were adherence depleted for 90 minutes at 37°C in 1X α-MEM (additives: 1-glutamine, 40 mmol/L; penicillin and streptomycin, 0.5 mg/L; and neomycin, 1 mg/L) (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Gibco). For isolation of CD34+ cells, the ficolled and adherence-depleted cells were exposed to a murine anti-human monoclonal antibody to the CD34 antigen (human progenitor cell antigen-1 [HPCA-1], Becton-Dickinson, Mountain View, CA) at a concentration of 10 µg/10^7 cells for 1 hour at 4°C to prevent phagocytosis of the antibody/receptor complex. This was followed by incubation for 30 minutes at 4°C (rocking) with magnetic beads coated with goat anti-mouse immunoglobulin (Dynabeads M-450, Dynal, Oslo, Norway) at a target cell to bead ratio of 1:3. CD34+ cells were isolated using a magnetic particle concentrator (Dynal MPC-6). The percent yield of CD34+ cells varied from donor to donor, but was usually in the range of 0.5% to 1.0% of the initial cell count after density gradient separation. Absolute cell numbers ranged from 2 × 10^6 to 2 × 10^8 per sample. Differential counts performed on cytospin preparations stained with May-Grünwald Giemsa showed that 85% to 95% of these cells demonstrated a blast-like phenotype consisting of a high nuclear to cytoplasmic ratio and multiple nucleoli as previously described. To further assess the purity of this population, the cells were incubated for 14 to 16 hours at 37°C in 1X α-MEM/10% FCS to facilitate magnabead detachment from the cells as suggested by the manufacturer (Dynal). Following repeat centrifugation over a Ficoll-Hypaque gradient to remove loose beads, the cells were reincubated with Anti-HPCA-1 (Becton-Dickinson) at a concentration of 0.05 µg/mL for 1 hour at 4°C protected from light. After washing in phosphate-buffered saline (PBS; Gibco) containing 0.5% bovine serum albumin (BSA; Sigma, St Louis, MO) and 0.1% sodium azide (Sigma), the cells were incubated with a biotinylated goat antimouse Ig (Southern Biotechnology Associates, Birmingham, AL) at a concentration of 0.01 µg/mL in PBS/BSA/sodium azide solution for 30 minutes on ice. The cells were washed again and incubated with Strep-Avidin (0.002 mg/mL conjugated phycoerythrin (0.003 µL/mL; Tago, Burlingame, CA) in PBS/10% FCS for 10 minutes on ice, protected from light. After washing twice with PBS, cytospin preparations showed positive staining for CD34 antigen in 95% to 98% of the cells, thus determining purity of the study population.

Isolation of leukemic myeloblasts. Blast cells were obtained from the peripheral blood and bone marrow of patients diagnosed with AML who were hospitalized at the Medical College of Virginia Hospitals, Richmond, VA or the Westchester County Medical Center, Valhalla, NY (courtesy of Dr Zalmen Arlin). These cells were subjected to Ficoll gradient centrifugation and adherent cell depletion, as described above, as well as T-cell depletion following the method of Weiner et al.

Isolation of RNA. Total cellular RNA was isolated from CD34+ cells, normal unseparated bone marrow, and leukemic myeloblasts by the guanidine thiocyanate-cecum chloride method of Chirgwin et al.

Reverse transcription and polymerase chain reaction. Reverse transcription was performed on total RNA of CD34+ cells, normal unseparated bone marrow, and leukemic myeloblasts using a primer specific for the published c-kit message: 3' primer (4386) 5'-GGCTACACGTCTAAAGGGTTAAA-3'. The resulting cDNA was amplified using the polymerase chain reaction (PCR) and primers specific for a 500-base sequence of the c-kit message: 5' primer (3868) 5'-CAGCTTCAAGATGGCATTGTA-3', 3' primer (4386) 5'-GGCTACAGTCTAAAGGGTTAAA-3'. For reverse transcription and PCR, the following were added to a reaction tube: total RNA 0.16 µg to 0.2 µg (CD34+ cells) or 2 µg (AML blasts and unseparated bone marrow), 4 µL 5X reverse transcriptase buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 50 mmol/L dithiothreitol, 15 mmol/L MgCl2) (BRL, Gaithersburg, MD), 1 µg each of 5' and 3' primers, 10 µL dNTPs (250 µmol/L each dCTP, dATP, dTTP, dGTP) (Perkin Elmer Cetus, Norwalk, CT). This mixture was heated to 65°C for 3 minutes and 300 U of reverse transcriptase (200 U/µL, M-MLV RT, BRL) was then added. Reverse transcription occurred for 1 hour at 37°C. The reaction mixture was transferred to a DNA thermal cycler (Perkin Elmer Cetus), and 10 µL of Taq polymerase (0.2 U/µL, Perkin Elmer Cetus) was added and the mixture was overlayed with 50 µL of light mineral oil (Sigma). The reaction was cycled as follows: 94°C for 2 minutes, annealing at 37°C for 2 minutes, extension at 72°C for 4 minutes to 35 cycles. In addition, reverse transcription and PCR were performed on a titer of RNA from normal unseparated marrow cells that was equivalent to amounts of RNA obtained from CD34+ cells (0.1 µg to 1 µg).

Southern blotting. The PCR products were extracted using 50 µL chloroform (Fisher, Fairlawn, NJ) to reverse the phases. The resulting amplified cDNA was electrophoresed through a 0.8% agarose gel and transferred to Nitran filters (Schleicher & Schuell, Keene, NH).

5' End-labeling of the probe and Southern blot hybridization. The oligonucleotide probe (100 ng) (3876) 5'-TCACTAAAATTTTGAACATTCAGCTTCAAGATGGCATTGTAA-3' (5' DNA Terminator Labeling System, BRL), 1 µL 32P-γ-ATP (150 mCi/mL, NE Research Products, Dupont, Boston, MA), and 1 µL T4 kinase (5 U/µL, BRL) were added to a reaction tube. Following incubation at 37°C for 30 minutes, labeled probe was isolated using a nucleic acid purification cartridge, Nensorb 20 column (Dupont, Wilmington, DE) and specific activity averaged 4 × 106 cpm/µg. Southern blots (Schleicher & Schuell) were prehybridized for 1 hour using a 50:50 mix of deionized formamide (Fluka, Ronkonkoma, NY) and 2X prehybridization mixture (total yeast RNA [Boehringer, Germany] 0.5 mg/mL, sodium dodecyl sulfate [SDS] [Sigma], 0.2%, ficoll [Sigma] 0.02%, polynucleotidolone [Sigma] 0.02%, bovine serum albumin [fraction V, Sigma] 0.02%, 100 mmol/L NaPO4, pH 6.5 [Sigma], and 10X SSC [1X SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate]). The 5' end-labeled oligonucleotide probe was added to the hybridization mixture (50% 2X prehybridization mix, 50% deionized formamide) at a concentration of 106 cpm/µL. The blots were hybridized overnight and then washed as follows: 2X SSC, 0.1% SDS for 5 minutes, 3X SSC, 0.025% SDS at 65°C for 30 minutes. Autoradiography occurred at ~70°C using X-Omat AR Kodak diagnostic film (Rochester, NY) with a Dupont Quanta III intensifying screen.

Oligonucleotide synthesis and preparation. A 15-base antisense oligonucleotide complementary to the c-kit transcripts located 133 nucleotides 3' of the start codon was synthesized using a 380A DNA synthesizer (Applied Biosystems, Foster City, CA): antisense oligonucleotide: 5'-CCACCGGGACTATTTA-3'. In addition, the sense control oligonucleotide: 5'-TAATAGTCCGGTGG-3', and missense oligonucleotide of the same antisense oligonucleotide
RESULTS

Detection of c-kit expression by CD34+ bone marrow progenitors and leukemic myeloblasts. To evaluate the expression of c-kit by human hematopoietic progenitor cells, we isolated cells from normal human bone marrow bearing the CD34 antigen. This population of cells is enriched for a variety of primitive, multipotent, and committed progenitors including CFU-GM. CD34+ cells represent approximately 1% of normal marrow cells. In the presence of appropriate stimuli, these cells can differentiate into myeloid or erythroid colonies in vitro and are capable of reconstituting normal marrow function in lethally irradiated primates. Since these cells are markedly diluted in normal marrow, detection of RNAs expressed specifically by these cells using Northern analysis of total bone marrow is difficult. To overcome this problem, we used PCR to detect c-kit expression, first in normal bone marrow and leukemic myeloblasts, and subsequently in CD34+ bone marrow cells.

RNA was isolated from normal unseparated bone marrow and leukemic myeloblasts. Reverse transcription was performed on total cellular RNA from these cells using a primer specific for the published c-kit message. The resulting cDNA was amplified by PCR using primers specific for a 500-base sequence of the c-kit message located approximately 1,000 bases 3' of the tyrosine kinase domain. The amplified material was electrophoresed through a 0.8% agarose gel, transferred to a nylon filter, and probed with a 31-base 5' end-labeled oligonucleotide probe specific for the amplified sequence.

We detected c-kit expression in leukemic myeloblasts of several patients with various subcategories of AML as shown in the autoradiograph in Fig 1. FAB subclasses 2 (Fig 1, lanes 1 and 2) and 3 (Fig 1, lanes 3 and 4) demonstrate a strong signal at 0.5 kb, consistent with the amplified sequence. Lane 5 represents an unknown subtype of AML, and lane 6 is a negative control.

We also detected c-kit expression using PCR of 2 μg of RNA from normal bone marrow (data not shown). To determine whether a subpopulation of progenitor cells could be responsible for this expression, RNA was isolated from CD34+ cells and subjected to PCR. As shown in Fig 2, autoradiography of these filters demonstrates strong signals at 0.5 kb in those lanes containing amplified cDNA from CD34+ cells (Fig 2, lanes 2, 3, and 4) and leukemic myeloblasts (Fig 2, lane 1) consistent with the amplified sequence. For comparison, reverse transcription and PCR were performed on a titer of RNA from normal unseparated marrow in amounts equivalent to that obtained from CD34+ cells (0.1 μg to 1 μg). Expression of c-kit was not demonstrated by unseparated bone marrow cells using an equivalent amount of RNA to that from CD34+ cells and is represented in lane 5 of Fig 2.
Inhibition of proliferative response to IL-3 following exposure to c-kit antisense oligonucleotides. Inhibition of specific gene function by exposure of target cells to synthetic antisense oligonucleotides has been used to investigate the requirement of proto-oncogene function in normal hematopoiesis. To determine a functional requirement of c-kit during normal human hematopoiesis, we exposed CD34+ bone marrow cells to c-kit antisense oligonucleotides before culture in rhIL-3. These cells were incubated with sense, antisense, or missense oligonucleotides to c-kit for 3 hours at 37°C and subsequently cultured in soft agar with 50 ng/mL of rhIL-3. Colonies consisting of greater than 50 cells were enumerated following 14 days incubation.

Exposure of CD34+ cells to c-kit antisense oligonucleotides significantly inhibited the colony-forming ability of cells cultured in the presence of rhIL-3 compared with that of cells grown in rhIL-3 alone, or rhIL-3 and sense or missense oligonucleotides (Student’s t-test, P < .05) (Fig 3).

The c-kit antisense oligonucleotides have no effect on proliferative response to GM-CSF. GM-CSF is also a multilineage growth factor that stimulates proliferation of CD34+ cells. To determine an effect of antisense oligonucleotides to c-kit on the proliferative response to GM-CSF, CD34+ cells were cultured in the presence of 50 ng/mL of rhGM-CSF following exposure to antisense oligonucleotides. These cell cultures were performed in parallel with the IL-3 cultures, using cells from the same donor. In contrast to the response of cells cultured in rhIL-3, CD34+ cells cultured in the presence of rhGM-CSF following exposure to c-kit antisense oligonucleotides demonstrated no significant inhibition of colony formation compared with cells cultured in rhGM-CSF alone or following exposure to sense or missense oligonucleotide controls (Student’s t-test, P < .05) (Fig 4).

DISCUSSION

Previous efforts have failed to demonstrate c-kit expression by normal unseparated human bone marrow using Northern analysis. These attempts may have been unsuccessful due to the marked dilution of progenitor cells in normal marrow. The present studies demonstrate c-kit expression by a population of cells enriched for primitive, multipotent, and committed bone marrow progenitors, and represent the first demonstration of SCF-R expression by normal human hematopoietic progenitors. This finding, along with evidence for inhibition of colony formation by c-kit antisense oligonucleotides, suggests the c-kit product may have a specific function in the regulation of proliferation and differentiation of these cells.

We have also demonstrated expression of c-kit by normal, unseparated bone marrow cells using PCR. This finding would be expected, since CD34+ cells represent approximately 1% of this population. However, c-kit expression was not demonstrated by unseparated bone marrow cells using amounts of RNA equivalent to that obtained from CD34+ cells. This finding further supports the evidence that CD34+ cells are indeed responsible for the detected c-kit message and shows that strong expression of c-kit can be demonstrated when these cells are isolated from whole bone marrow. To further document the purity of this population so as to determine if contaminating cells could have accounted for our findings, we used the Strep-Avidin conjugated phycoerythrin stain technique to determine what percentage of these cells were indeed CD34+. Purity varied from sample to sample, but fell in the range of 95% to 98% of cells staining positively for the CD34 antigen, effectively ruling out expression by contaminating cells.
Furthermore, the experiments involving leukemic myeloblasts demonstrate c-kit expression by two additional FAB subtypes of AML: M2 and M3. The implication of c-kit expression by both normal and neoplastic hematopoietic cells is unclear. The present data do not directly support any mechanism or stage specific expression.

Only those cells cultured in rhIL-3, but not in rhGM-CSF, exhibited a decrease in colony formation following exposure to antisense oligonucleotides to c-kit. This observation is of particular interest especially in view of recent findings by Zsebo et al, demonstrating that W/W bone marrow cells, which lack a normal c-kit product, respond normally to GM-CSF, but not to SCF. Other recent data indicate that IL-3 and SCF have a synergistic proliferative effect on murine progenitor cells identified by their ability to bind rhodamine 123 ("rhodamine bright"). However, more primitive murine progenitors ("rhodamine dull") depend on the presence of both SCF and IL-3 for proliferation and differentiation. Taken together, these data suggest the presence of a transmembrane or intracellular communication between c-kit and the IL-3 receptor, which could account for these observations. Further evidence supporting this view was recently reported by Welham and Schrader, who showed that IL-3 down-regulates c-kit expression in IL-3 factor-dependent and -independent cell lines and in marrow mast cells. Indeed, this observation further strengthens our speculation that an association may exist between these two receptors.

IL-3 is a hematopoietic growth factor produced predominantly by activated T cells and supports the proliferation and differentiation of early stem cells, as well as cells committed to various lineages. Signal transduction through the IL-3 receptor results in phosphorylation of proteins containing tyrosine residues and subsequent transmission of the signal to the nucleus. However, the detailed mechanisms involved in this process are largely unknown. Cell surface receptors for IL-3 are currently being characterized, and early results have suggested that a 65-Kd protein is the predominant IL-3 binding component. The 65-Kd IL-3 receptor does not itself possess intrinsic tyrosine kinase activity, but requires another unknown protein, or proteins, to provide this activity, which is critical for signal transduction. In addition to IL-3, other growth factors such as IL-2, IL-4, and granulocyte-macrophage colony-stimulating factor (GM-CSF), do not contain tyrosine kinase domains, but are capable of activating tyrosine phosphorylation of proteins in their signal transduction pathways. These findings suggest that the receptors must be "coupled" or "complexed" to intracellular tyrosine kinases. Mammalian cells do possess nonreceptor tyrosine kinases, which like kit, have been discovered through their homologies with retroviral oncogenes such as src, fms, and abl. Regulatory mechanisms involving these oncogenes are not understood, but it is possible that these intracellular tyrosine kinases are coupled with different receptors such as those for IL-2, IL-3, and GM-CSF. These kinases may exist as a component of the receptor complex and be activated through direct protein-protein interaction or may function through a coupling protein such as the G proteins. Based on the present data, we propose that expression of c-kit is necessary for the function of IL-3 and is inhibited following exposure to antisense oligos before IL-3 stimulation. We interpret that signal transduction through IL-3 is interrupted and results in inhibition of proliferation in response to IL-3. This may explain the difference in cytokine response following exposure to antisense oligonucleotides. We have observed that this effect occurs very early in culture, ie, few colonies ever develop in response to IL-3 throughout the culture period.

In conclusion, the present studies demonstrate that c-kit is expressed by progenitor cell-enriched populations of normal human bone marrow cells, and that modulation of the expression of this proto-oncogene affects the response of the IL-3 receptor to its ligand. Collectively, these findings suggest the presence of a transmembrane or intracellular communication previously undescribed between c-kit and the IL-3 receptor.

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