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 (rhIL-3). Exposure of CD34+ cells to c-kit antisense oligonucleotides significantly inhibited colony-forming ability of cells cultured in the presence of rhIL-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.

R ECEPTOR TYROSINE kinases (RTK) are a group of closely related proteins that transduce growth regulatory signals across the plasma membrane. The c-kit proto-oncogene product, platelet-derived growth factor receptor (PDGF-R) and macrophage colony-stimulating factor receptor (CSF-1R), are RTKs that share strong structural homology, indicating a common evolutionary origin. The structural organization of these glycoproteins consists of an extracellular ligand-binding domain, a transmembrane segment, and an intracellular domain possessing tyrosine-specific kinase activity. To initiate a mitogenic signal, growth factors bind to these receptors and activate the kinase. This chain of events, the detailed steps of which are unknown, transmits the growth signal from the cell surface to the nucleus, resulting in proliferation and differentiation of the progenitor cell. Oncogenic versions of these glycoproteins are derived from mutations or deletions in the receptor gene. These aberrant receptors have constitutively activated kinases, which appear to function in the absence of a ligand. This deregulation of receptor kinase activity results in uncontrolled cell growth and transformation.

The c-kit proto-oncogene, located on chromosome 4 in the human, has been mapped to the dominant white spotting locus (W) on chromosome 5 in the mouse. 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 SI 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 SI locus (SI/SI) was considered a microenvironmental one. Similar to W/W homozygotes, SI/SI homozygotes die in utero from severe macrocytic anemia. As previously suspected, the SI 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: L-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 ficoll 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 × 10^7 per milliliter. 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 magnagbage 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 Biotech, Birmingham, AL) at a concentration of 0.02 μg/mL. The cells were washed 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 Aulin). These cells were subjected to Ficol gradient centrifugation and adherent cell depletion, as described above, as well as T-cell depletion following the method of Weiner et al.18

**Isolation of RNA.** Total cellular RNA was isolated from CD34+ cells, normal unseparated bone marrow, and leukemic myeloblasts by the guanidine thiocyanate-cesium chloride method of Chirgwin et al.19 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'-GGCTACAGTCTAAAGGGTATAAA-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'-CAGCTTCAGAATGGCTTATA-3', 3' primer (4386) 5'-GGCTACAGTCTAAAGGGTATAAA-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 μL/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 for 35 to 50 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 nitro filters (Schleicher & Schuell, Keene, NH).

**5' End-labeling of the probe and Southern blot hybridization.** The oligonucleotide probe (100 ng): 5'-TACGTAACCTTTGGTCAAGGAGCATCACAAGGCGGTTACCG-3' was fluorescently labeled at the 5' end using a 5' DNA Terminus Labeling System, BRL), 1 μL 33P-γ-ATP (150 mCi/mL, NEN 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, Nen sorb 20 column (Dupont, Wilmington, DE) and specific activity averaged 4 × 10^6 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%, polyvinylpyrrolidone [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 10^6 cpm/mL. The blots were hybridized overnight and then washed as follows: 2X SSC, 0.1% SDS for 5 minutes x 4 at 37°C, followed by 0.5X 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'-CCAAGGGGACTTTAGA-3'. In addition, the sense control oligonucleotide: 5'-TAATAGTCGCCGCGTG-3', and missense oligonucleotide of the same antisense oligonucleotide.
compositions (random order): 5'-TTAGGCACACTGCA-3' were also prepared. The oligonucleotides were deblocked for 18 hours at 55°C and dried for 6 hours in a speed vacuum centrifuge. The dried oligonucleotides were then washed in a 75% ethanol slurry and the resulting pellet dried in a vacuum centrifuge for 30 to 60 minutes. The resulting dried material was resuspended in sterile water and the OD_{260} value assisted in determining concentration.

**Soft agar culture of CD34+ cells.** CD34+ cells were incubated at a concentration of 5 x 10^4/mL in 1X aMEM (with additives, see above) supplemented with 20% FCS for 3 hours at 37°C in the presence of 30 μg/mL (6.67 μmol/L) of sense, antisense, or missense oligonucleotides. The CD34+ cells were then plated in 0.3% agar (Difco, Detroit, MI) to a final concentration of 5 x 10^4/mL, with addition of either rhIL-3 (Genzyme, Boston, MA) or rhGM-CSF (Shering-Plough, Kenilworth, NJ) (both having final concentrations of 50 ng/mL) and final oligonucleotide concentration of 6.67 μmol/L, over agar underlayers having final concentrations of 0.48% agar, 20% FBS, and 1X aMEM (with additives) for a final volume of 1 mL. Following 14 days at 37°C, 5% O₂, 5% CO₂, colony-forming unit-granulocyte-macrophage (CFU-GM) colonies of greater than 50 cells were enumerated.

**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.1.20 CD34+ cells represent approximately 1% of normal marrow cells. In the presence of appropriate stimuli, these cells can differentiate into myeloid17 or erythroid17,20 colonies in vitro and are capable of reconstituting normal marrow function in lethally irradiated primates.21 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 30-base 5' end-labeled oligonucleotide probe specific for the amplified sequence.

We detected c-kit expression in leukemic myeloblasts of several patients with various subclasses of AML as shown in the autoradiograph in Fig 1. FAB subtypes 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 titration 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.

**Fig 1.** Autoradiograph demonstrating expression of c-kit from five patients with AML. Strong signals are noted at 0.5 kb, indicating probe hybridization to the amplified sequence. Lanes 1 and 2 represent FAB M2, lanes 3 and 4 represent FAB M3, and lane 5 is of unknown FAB subtype. Lane 6 is a negative control (human plasmid pckit-171, ATCC, Rockville, MD) lacking the amplified sequence.

**Fig 2.** Autoradiograph demonstrating expression of c-kit by CD34+ cells. Signals are noted at 500 bases, indicating probe hybridization to the amplified sequence. AML control is present in lane 1. CD34+ cells from three donors are represented by lanes 2, 3, and 4. Expression of c-kit is not demonstrated in unseparated bone marrow cells using an amount of RNA equivalent to that obtained from CD34+ cells (0.16 μg) (lane 5). Human plasmid pckit-171 (ATCC), which lacks the amplified sequence, is shown in lane 6 (negative control).
**c-kit** EXPRESSION BY CD34+ BM PROGENITORS

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 multi-lineage 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.13 Other recent data indicate that IL-3 and SCF have a synergistic proliferative effect on murine progenitor cells that are able 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.24 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.25 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 cells5 and supports the proliferation and differentiation of early stem cells, as well as cells committed to various lineages.27,28 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.26 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.26 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.29 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.30 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.32 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.33 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 oligonucleotides 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, i.e., 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.

REFERENCES


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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