Expression and Physiologic Significance of Kit Ligand and Stem Cell Tyrosine Kinase-1 Receptor Ligand in Normal Human CD34+ c-Kit+ Marrow Cells

By Mariusz Z. Ratajczak, Wojciech I. Kuczynski, Deborah L. Sokol, Jonni S. Moore, Charles H. Pletcher Jr., and Alan M. Gewirtz

To determine the potential role of autocrine growth factor production in regulating primitive human hematopoietic cell development, we examined highly purified CD34+, c-Kit+ marrow mononuclear cells for expression of c-Kit ligand (KL) and stem cell tyrosine kinase 1 (sttk1) ligand (STK1-L). Normal marrow mononuclear cells coexpressing CD34 and c-Kit were isolated by a combination of immunomagnetic bead isolation and fluorescence-activated cell sorting. Purified cells were then screened for expression of KL and sttk1-L mRNA using a sensitive reverse transcription-polymerase chain reaction method. Using this approach, expression of both cytokine genes at the mRNA level was found in this highly enriched cell population. We then examined the functional significance of these mRNAs by inhibiting their expression with antisense (AS) oligodeoxynucleotides (ODN).

Hematopoietic stem and progenitor cells reside in and are sustained by the bone marrow microenvironment. Stem/progenitor cells and the microenvironment form a complex “ecosystem” whose intricacies are now slowly being resolved. An important component of this environment is the hematopoietic growth factors produced by cells of the marrow stroma. The various cytokines elaborated by these cells are thought to regulate hematopoietic cell development in an endocrine or paracrine manner. More recently, it has been postulated that hematopoietic stem/progenitor cell development may also be regulated by autocrine synthesis of growth factors. In support of this hypothesis it has been reported that murine embryonic stem cells, some leukemic cell lines, and primary leukemic blast cells may simultaneously express a hematopoietic ligand and its corresponding receptors. Recently, normal progenitor enriched marrow have also been found to express mRNA for growth factors known to regulate early hematopoietic cell proliferation. Examples of this phenomenon include interleukin-1β (IL-1β), transforming growth factor-β (TGF-β), IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In addition, antisense oligodeoxynucleotides (AS ODN) disrupting the erythropoietin (Epo)/Epo receptor (Epo-R) or GM-CSF/GM-CSF-R2 axes have also been found to inhibit in vitro growth of normal murine erythroid and granulocyte-macrophage progenitors, respectively. Accordingly, it is reasonable to hypothesize that autocrine stimulation of hematopoietic progenitor cells at some stage or stages of their life cycle is important for their proper development.

The studies described above do not unambiguously address the possibility that an autocrine growth loop(s) plays a role in lineage commitment or the very earliest stages of committed progenitor cell growth. Because this is now a timely and important issue, we sought to address this question in a highly purified, primitive population of human hematopoietic progenitor cells. We were particularly interested in examining a CD34+ marrow population that coexpressed the c-Kit and STK1 tyrosine kinase receptors because of the acknowledged importance of the interaction of the c-Kit and stkJ receptors with their ligands during the very earliest stages of hematopoietic cell development. We also sought to impart functional significance to these expression studies by determining if interruption of putative autocrine growth loops with AS ODN would lead to perturbation of hematopoietic cell growth.

MATERIALS AND METHODS

Cells. Light-density bone marrow mononuclear cells (MNC) were obtained from four normal consenting donors and depleted of adherent cells and T lymphocytes (A-T-MNC) as described. CD34+ were enriched from the A-T-MNC population by incubation with anti–HPCA-1 murine monoclonal antibodies (MoAbs; Becton Dickinson, San Jose, CA) and subsequent immunoselection of antibody-labeled cells with magnetic beads according to the manufacturer’s protocol (Dynal, Oslo, Norway), as described. The c-Kit+ subset of CD34+ cells was isolated using fluorescence-activated cell sorting (FACS). In brief, 2 × 10^6 human A-T-MNC were suspended in phosphate-buffered saline (PBS) supplemented with 5% bovine calf serum (BCS) and labeled for 30 minutes at 4°C with anti-c-Kit MoAbs (SR-1; 1:1,000; generously provided by V. Broudy; CA54384 and CA51083 (A.M.G.).

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University of Washington, Seattle, WA). Cells were washed three times in ice-cold PBS supplemented with 5% BCS and then incubated with anti-CD34 MoAb directly conjugated with fluorescein isothiocyanate (FITC; anti-CD34: Becton Dickinson; 20 μL/10⁶ cells) along with phycoerythrin-conjugated goat antimosue MoAb (Sigma, St Louis, MO; 1:80) for 30 minutes at 4°C. After incubation, the cells were again washed three times in ice-cold PBS supplemented with 5% BCS and then subjected to FACS using a FACS Star Plus equipped with an argon-ion laser tuned to 488 nm (Becton Dickinson).

Marrow stromal cells were obtained by culturing MNC in 75 mL plastic flasks (Corning, Corning, NY). A total of 5 × 10⁶ cells were initially plated in 20 mL of Dulbecco modified Eagle's medium (DMEM; Gibco, Grand Island, NY) supplemented with 12.5% BCS, 12.5% horse serum (Hyclone, Logan, UT), 1% L-glutamine (Gibco), penicillin (100 U/mL), and streptomycin (100 mg/mL: Gibco).

After incubating for 3 days, nonadherent cells were removed and 50% of the culture medium was exchanged. This procedure was repeated once per week. When the cells became confluent, they were trypsinized and then suspended again in propylene tubes in 0.2 mL of the same medium supplemented with 5% FCS, 12.5% horse serum (Hyclone, Logan, UT), 1% L-glutamine (Gibco), and 100 U/mL IL-3 (10 U/mL: kind gift of Genetics Institute, Cambridge, MA), and KL (100 ng/mL: kind gift of Amgen, Thousand Oaks, CA).

ODN targeted to various regions of the stkl ligand cDNA were added to cells in a manner identical to the protocol described above. ODN were targeted to codons 2 through 6 (STKI-L sense ODN, 5′ACA GTG CTT GGC CCC GCC-3′; STKI-L antisense ODN, 5′GGC TGG CGC CAG CAC TGT-3′; STKI-L scrambled ODN, 5′-CAG CTG GCC AGC TGC GTG-3′) and to a potential stem loop (nts −68 to −51 and −60 to −43) with reference to the start site adenine (nts 275-292) as predicted by the Wisconsin Sequence Analysis Package (Genetics Computer Group, Madison, WI). Sequences used were as follows: sense, 5′-GCC CCT GCC CAG TGC TCC ACA CCC AAC-3′; antisense, 5′-GGT GTG TGT GGA AGG GCC-3′; scrambled, 5′-TGG AGG GTC GCC AGG GTG-3′; antisense, 5′-CAC ACC CAA CCG GGG CAA-3′; antisense, 5′-TCT CCC CAG TCG TTG GTG-3′; scrambled, 5′-TGC TGT GTC TGC TGG AGG-3′; nts 275-292 (STKI-L DSRUN); sense, 5′-CCA AGA TGC AAG GCT GTG-3′; 5′-TAT ATG TGT GTC TCT CTC-3′; KL scrambled-2, ODN. 5′-TAT ATG TGT GTC TCT CTC-3′. KL scrambled-2 has the identical base content as KL AS ODN.

After an additional 8 hours of incubation, cells were plated, unwashed, in serum-free methylcellulose cultures performed as follows. A total of 10⁵ cells in 0.2 mL of Iscove’s DMEM were added to 1 mL of Iscove’s DMEM medium containing 0.8% methylcellulose (Methocel MC, Fluka, Switzerland); 1% delipidated, deionized, charcoal-treated bovine serum albumin (BSA; Sigma); 270 μg/mL iron-saturated transferrin (Sigma); 5.6 μg/mL cholesterol (Sigma), and 2 mmol/L L-glutamine. Cells were suspended in the mix by pipetting and then transferred to 3.5-cm plastic petri dishes for incubation (37°C, 95% humidity, 5% CO₂). Seven days after seeding, colony growth was stimulated by the addition of recombinant human colony stimulating factor 1 (rH) Epo (5 U/mL), IL-3 (10 U/mL; kind gift of Genetics Institute, Cambridge, MA), and KL (100 ng/mL; kind gift of Amgen, Thousand Oaks, CA).

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antisense, 5'-GCA AGC TCT GCA TCT TGG-3'; scrambled, 5'-GTC CGT ACT CGA GCT AGT-3'.

Growth of colony-forming units-Mix (CFU-Mix), burst-forming unit-erythroid (BFU-E), and CFU-granulocyte-macrophage (CFU-GM) colonies in untreated control cultures and in cultures containing the ODN was scored visually with an inverted microscope 15 days after the addition of the growth factors. Effects of the ODN on target gene mRNAs were assessed as previously reported.11

**RNA extraction.** RNA was extracted from cells isolated by magnetic beads or FACS using poly A-mRNA QuickPrep Micro mRNA purification kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. RNA was extracted from stromal cells using RNAzol (Tel-Test, Inc, Friendswood, TX) as instructed by the manufacturer.11 The isolated mRNA was dissolved in triple-distilled water and stored at -20°C until used.

**Reverse transcription-polymerase chain reaction (RT-PCR).** RT-PCR was performed as reported previously.11 Briefly, RNA from FACS-sorted CD34+ c-Kit+ MNC (from 1 to 1,000) or 0.5 μg of RNA isolated from cultured stromal cells was reverse transcribed with 500 U of Moloney murine leukemia virus reverse transcriptase (GIBCO-BRL, Gaithersburg, MD) and 50 pmol of a 3' primer complementary to one of the following sequences: (1) KL, nt 777-754 (ACT GCT GTC ATT CCT AAG GGA GCT); or (2) STK-1L, nt 337-317 (GGC ACA TTT GGT GAC AAA GTG). The resulting cDNA fragments were amplified (35 cycles) using 5 U of Taq polymerase (Promega, Madison, WI) and 5' primers specific for nt 418-441 (GAC AGC TGG ACT GAT CTT GAC) of the reported KL sequence15 or nt 30-50 (AAC AAC GTA TCT CCT GCT) of the reported STK-1L sequence.16 Detection of STK-IR mRNA16 and c-Kit R mRNA17 was performed in a similar manner with the following primers: (1) STK-1 R, 5'-CTC GGT GTT TCT TCT GCA ATG (nts 97-117); 3'-GAT GGA AGC AGA TAC ATC CAC (nts 324-304); and (2) c-Kit R primers, 5'-CGT TGA CTA TCA TGA GTT CAG CAG G (nts 842-864); 3'-CTA GGA ATG TGT AAG TGC CTC C (nts 1201-1179). The predicted sizes of the STK-1 R and c-Kit R products were 228 and 369 bp, respectively.

**Southern blot of RT-PCR products.** Ten microliters of amplified product was electrophoresed on a 4% agarose gel and transferred to a nylon filter. Filters were prehybridized and then probed with an oligonucleotide 3'-end labeled using T4 polynucleotide kinase (Promega, WI) and a nylon filter. Filters were prehybridized and then probed with an oligonucleotide 5'-end labeled using T4 polynucleotide kinase (Promega, WI) and hybridization was detected by autoradiography as described previously.11

**Statistical analysis.** P values were calculated with a statistical software package developed for Macintosh computers (Instat; GraphPad Software, San Diego, CA).

**RESULTS**

**Isolation of CD34+.** c-Kit+ marrow mononuclear cells. MNC depleted of adherent cells and T lymphocytes were immunocompetently labeled and subjected to flow cytometry as described in the Materials and Methods. Results of a typical multiparameter sort repeated three times are shown in Fig 1. The lymphocyte region (R2) was identified by low forward and orthogonal scatter analysis (Fig 1A). Within this region (~8% of the total cell population), cells were further analyzed for coexpression of c-Kit and CD34 (Fig 1B). Positivity was determined by comparison with isotype-labeled control cells. Cells coexpressing each marker in the brightest 5% of the dual-labeled population are indicated by R1 (Fig 1C). Logical gating was used to sort only those cells that satisfied R2 and R1 criteria for mRNA phenotyping.

The sorted cell population was greater than 98% pure (Fig 1D) and represented ~3.5% of the total lymphocyte population present in the original gate.

**mRNA phenotyping of CD34+.** c-Kit+ hematopoietic cells. RT-PCR was used to characterize cells in this population for expression of the mRNAs of interest. Using this strategy, we were able to amplify KL mRNA in this cell population (Fig 2A, lane 1) as well as in marrow stromal cells (Fig 2A, lane 2), which are known to express KL mRNA and to synthesize this protein. A water control RT-PCR, run simultaneously with all reaction constituents save cellular RNA, showed no bands, making carry-over artifact an unlikely explanation for the signals detected (Fig 2A, lane 3). To further exclude the possibility that a contaminating cell type was contributing to this result, serial dilutions of the CD34+, c-Kit+ MNC were performed to determine the smallest number of cells from which signal could be detected. As shown in Fig 2B, KL mRNA was easily detected in 50 cells, and faint signal could be discerned from as few as 10 cells.
We were also able to document that this same population coexpressed both stkl receptor (Fig 3A) and stkl-L (Fig 3B, lane 1). Marrow stromal cells were also analyzed for expression of the latter as a positive control yielding the expected 307-bp band (Fig 3B, lane 2). A water control RT-PCR reaction yielded no bands (Fig 3B, lane 3), and signal could be detected in as few as 10 cells (Fig 3C).

Functional analysis of the c-Kit/KL and stkl/stkl-L axis. Using an antisense inhibition strategy, we have previously shown that disruption of c-Kit and stkl receptor expression in CD34+ marrow cells impairs hematopoietic cell growth. In the case of stkl receptor, growth of all lineages is inhibited, with the most marked effect being observed in the most primitive cells assayed, the long-term culture-initiating cell. When c-Kit expression was perturbed, development of erythroid cells was the most severely affected in standard colony assays. We used a similar approach to determine the functional significance of downregulating KL and stkl-L expression in the CD34+, c-Kit+ cell population.

It was first necessary to show that expression of the targeted mRNAs could be inhibited. This was accomplished relatively easily in the case of KL. ODN sequences directed against codons 2 through 6 of the mature mRNA were synthesized. When added to the purified primitive progenitor cells, inhibition of KL mRNA was only observed with the AS ODN (Fig 4A). Various control ODN, including a scrambled sequence with the identical base content as the KL AS ODN, failed to inhibit KL mRNA expression (Fig 4A).
Simultaneous assay of β-actin mRNA expression showed no apparent change with any of the sequences used, suggesting that downregulation of KL mRNA was specific for the KL AS ODN.

Inhibition of stkl-L mRNA proved more difficult. Several AS ODN preparations targeted to codons 2 through 6 of the mature mRNA had no effect on stkl-L mRNA levels. Similarly, a downstream antisense PCR primer (21mer) was also ineffective. We therefore used a computer software program (Wisconsin Sequence Analysis Package, Madison, WI) to identify mRNA regions potentially accessible to ODN based on free energy calculations of predicted mRNA folding. Two promising regions were identified in this manner (Fig 5). One was a stem loop structure at nts -63 to -43 (relative to the start site), and the other was a downstream region encompassed by nts 275-292 that was predicted to have a run of 12 unpaired bases, followed by another run of 3 unpaired bases separated by flanking paired dinucleotides. The stem loop was targeted by two complete sets (sense, antisense, and scrambled) of overlapping ODN and the downstream unpaired run was targeted by another complete set. Only one of the two antisense sequences targeted to the stem loop, STEM ODN #1, inhibited stkl-L mRNA levels (Fig 4B). All the other sequences used (AS, sense, or scrambled) produced no inhibition of the target mRNA. These results suggest that targeting based on computer generated folding predictions may be no more reliable than educated guessing. It is important to note that the STEM ODN #1 scrambled control sequence also contained a “G quartet” in the identical location as that of the STEM ODN #1 AS.

Once it was established that the mRNA of either ligand could be inhibited, the biologic consequences of such inhibition were then determined. For these experiments, cells were cultured in serum-free, chemically defined medium to exclude the possibility that exogenously derived KL or stkl-L might substitute for KL or stkl-L elaborated by the marrow hematopoietic cells. KL targeting results from four separate experiments, each performed in quadruplicate, are shown in Fig 6. CFU-GM colony growth was not inhibited in these experiments (Fig 6A). In contrast, BFU-E (Fig 6B) and CFU-MIX (Fig 6C) colonies were inhibited ~70% and ~89%, respectively (P < .0001). It is important to note that, during the 7-day period that cultures were deprived of exogenous growth factors, daily examination under an inverted microscope failed to show evidence of proliferative activity. STKI-L targeting gave quite different results (Table 1). By itself, inhibition of STKI-L with STEM ODN #1 had no discernible effect on CFU-GM, BFU-E, or CFU-MIX colony formation. However, when STKI-L STEM ODN #1 and KL AS ODN were combined, significant synergistic inhibition...
of CFU-MIX and CFU-GM was observed. No additive inhibition of BFU-E over that observed with KL AS ODN alone was observed. None of the other STK1-L AS ODN gave inhibition when combined with the KL AS ODN (data not shown). These results further show that colony inhibition was likely related to downregulation of both messages, with consequent loss of the respective proteins.

**DISCUSSION**

The KL/c-Kit receptor and stk1-L/stk1 receptor axes are important for the development of early human hematopoietic progenitors. Both receptors belong to a family of type III tyrosine kinase receptors, are expressed on very early progenitors, and are most probably present on stem cells. The ligands for both receptors are produced by bone marrow stromal cells and exist in both membrane-bound and secreted forms. They act directly in a paracrine fashion on cells residing within the niches of the hematopoietic microenvironment.

As noted above, there is increasing evidence that hematopoietic progenitor cells synthesize ligands for receptors that they display on their surfaces in an apparently autocrine manner. There are also data suggesting that these autocrine growth loops are physiologically significant and exert some role in the development of hematopoietic cells. The results reported here extend these observations by demonstrating that the mRNAs for the ligands of receptors expressed on the earliest progenitor/stem cells (c-Kit and Stk1) are also present in these same cells. One must always be concerned that a contaminating cell type could be contributing to these results. Stromal cells in particular would be a significant concern, but it is unlikely that cells of this type were present in the FACS-purified population because, in our hands (unpublished observation) and the reports of others, stromal cells do not express c-Kit. Accordingly, they would not have been included in the sorted population. However, even if a small percentage of stromal cells contaminated the sort, they are statistically unlikely to be present after limiting dilutions down to the 10 cell level were performed. Accordingly, these studies provide highly suggestive data that the growth factor mRNAs detected are in fact expressed by the CD34+ hematopoietic cells and not by any of the cells potentially contaminating this population. In this same regard, these data further suggest that previously noted autocrine synthesis of growth factors by leukemic cell lines and primary blast cells may not be a marker of the neoplastic phenotype but is merely a manifestation of a normal growth- or apoptosis-suppressing mechanism.

Our studies also suggest, but do not prove, that, in addition to expressing the STK1-L and KL mRNAs, these growth factors are also synthesized by cells of the population studied. This statement is supported by the fact that AS ODN that inhibited expression of the mRNAs for these growth factors led to a significant inhibition of colony formation under serum-free conditions. It is important to note also that AS sequences that did not suppress the target mRNA were ineffective in this regard. Although we do not provide direct evidence of the role of these sequences in the regulation of hematopoietic colony formation, the results provide highly suggestive data that these sequences may be involved in the regulation of colony formation.

**Table 1. Hematopoietic Colony Formation by CD34+ c-Kit+ Marrow MNC: Effect of Inhibiting Stk1-L mRNA Expression**

<table>
<thead>
<tr>
<th>ODN Added</th>
<th>Colony Type</th>
<th>CFU-GM</th>
<th>BFU-E</th>
<th>CFU-MIX</th>
</tr>
</thead>
<tbody>
<tr>
<td>STK1-L ODN alone</td>
<td>None (untreated)</td>
<td>100 ± 30</td>
<td>80 ± 27</td>
<td>9 ± 4</td>
</tr>
<tr>
<td></td>
<td>Stk1-L sense</td>
<td>88 ± 30</td>
<td>71 ± 26</td>
<td>9 ± 3</td>
</tr>
<tr>
<td></td>
<td>STK1-L AS</td>
<td>81 ± 41</td>
<td>77 ± 31</td>
<td>9 ± 4</td>
</tr>
<tr>
<td></td>
<td>STK1-L scrambled</td>
<td>98 ± 34</td>
<td>73 ± 22</td>
<td>8 ± 4</td>
</tr>
<tr>
<td>STK1-L + KL ODN</td>
<td>None (untreated)</td>
<td>131 ± 59</td>
<td>82 ± 31</td>
<td>10 ± 5</td>
</tr>
<tr>
<td></td>
<td>KL AS</td>
<td>121 ± 69</td>
<td>72 ± 19</td>
<td>12 ± 6</td>
</tr>
<tr>
<td></td>
<td>STK1-L + KL AS</td>
<td>63 ± 26</td>
<td>22 ± 13</td>
<td>0.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>STK1-L + KL sense</td>
<td>125 ± 60</td>
<td>72 ± 26</td>
<td>12 ± 5</td>
</tr>
<tr>
<td></td>
<td>STK1-L + KL scrambled</td>
<td>115 ± 39</td>
<td>75 ± 21</td>
<td>10 ± 2</td>
</tr>
</tbody>
</table>

Hematopoietic cells were purified from normal consenting donors and exposed to ODN as detailed in the Materials and Methods. Colonies were counted using an inverted microscope according to standard criteria. Numbers of colonies are presented as mean ± SD of four separate experiments. Each data point is derived from duplicate or quadruplicate dishes. Stk1-L STEM no. 1 ODN data are shown.
proof that the mRNAs are translated into proteins in these cells, the biologic data presented are certainly consistent and supportive of this hypothesis. Accordingly, these data also suggest that a functional autocrine growth loop exists in this population. Whether this is of the internal or secreted type is unclear at this time.

The potential regulatory role of these autocrine growth loops is interesting to speculate on. It would appear that both growth factors, at the least, play a role in cell survival. When the mRNAs were inhibited with antisense ODN, cells did not persist in the cultures in the absence of exogenously added growth factors. In addition, during the 7-day observation period in which growth factors were withheld from these serum-free cultures, direct observation failed to show any proliferative activity. Accordingly, we believe that the putative autocrine loops do not function as mitogenic stimuli but rather support previous suggestions that these loops prevent primitive hematopoietic cells from entering an apoptotic pathway. Our data are also of interest because they appear to suggest that KL and STK1-L may exert these effects on progenitor cells with different lineage potentials. In this regard, the data support the preliminary observations of Olweus et al. who suggest that differential expression and modulation of c-Kit receptor on primitive hematopoietic cells may help explain the interaction of different growth factors on hematopoietic progenitor cell subtypes. Nevertheless, the in vivo physiologic significance of autocrine expression of low levels of hematopoietic growth factors/receptors in primitive cells remains an intriguing issue with potentially important ramifications. Addressing this question may prove to be of value in defining conditions for identifying, preserving, and expanding human hematopoietic stem cells.

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Expression and physiologic significance of Kit ligand and stem cell tyrosine kinase-1 receptor ligand in normal human CD34+, c-Kit+ marrow cells

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