c-kit Ligand Gene Expression in Normal and Sublethally Irradiated Mice

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The c-kit ligand (KL; Steel factor, mast cell growth factor, stem cell factor) is a hematopoietic factor that has been shown to act as a potent cofactor for hematopoietic growth and differentiation in vitro. The in vivo effects of KL, however, have been variable. To study the hematopoietic role of KL in vivo, we evaluated KL gene expression in both normal mice and mice recovering from myelosuppressive radiation exposure using the reverse transcriptase-polymerase chain reaction (RT-PCR) technique. In a single RNA sample, we found that the RT-PCR technique has high precision (coefficient of variation, 15.7%). Amplifications of serial 1:2 dilutions of template RNA precisely correlated with starting RNA concentrations at 20 cycles or at 25 cycles, depending on the level of expression. Amplification of individual normal bone marrow and spleen cell RNA showed basal expression in all normal bone marrows but irregular expression in normal spleens. On day 2 after a sublethal 7.75-Gy (0.4 Gy/min) 152Co irradiation, spleen KL gene expression increased approximately 2.5-fold (P = .011), and bone marrow expression increased 15-fold (P = .004). During a 28-day postirradiation recovery period, KL expression increased in bone marrow on days 2 through 7. Spleenic expression during the same period was more variable. In conclusion, the KL gene is invariably expressed in all murine bone marrows and variably expressed in normal murine spleens. Postirradiation, recovering bone marrow and spleen both express increased levels of KL mRNA at day 2 and continue to express increased levels for several days postexposure. These data support a role for KL in the endogenous recovery of hematopoiesis after hypoplastic injury.

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The c-kit ligand (KL) is a cytokine known to be involved in both hematopoietic and nonhematopoietic development. This is evidenced by the fact that mice with viable mutations at the Steel genetic locus are sterile, have deficient melanin production, and exhibit macrocytic anemia as well as decreased megakaryocyte and granulocyte numbers. When KL is administered to Steel mice, partial reversal of the hematopoietic defects is observed. Multiple cell types have been shown to produce KL, including hematopoietic stromal cells, fibroblasts, hepatic parenchyma, and gonadal tissues. The active factor exists in both membrane-bound and soluble forms, with the soluble form representing a cleavage product of a surface-bound protein. KL binds the c-kit protooncogene product, the c-kit receptor, which among other cells is expressed on primitive hematopoietic progenitor cells.

Using cloned stromal cells from Steel mice that had been transformed with either membrane-bound or soluble KL, Toksoz et al showed that stromal cells expressing membrane-bound KL were more capable of inducing erythroid burst-forming unit (BFU-E), granulocyte-macrophage colony-forming unit (CFU-GM), and mixed colony-forming unit (CFU-Mix) proliferation in liquid culture than stromal cells expressing only soluble KL.

Because of the apparent role of KL in hematopoietic regulation, KL represents a factor that might aid in recovery of hypoplastic bone marrow (BM) or support engraftment in BM transplantation. In vitro, KL alone has little hematopoietic effect but synergizes with multiple cytokines, including interleukin-1 (IL-1), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin (Epo). Such effects have been shown with unfraccionated as well as fractioned BM cells.

When administered to normal primates, KL enhances hematopoiesis, as evidenced by increased BM CFU-GM and BFU-E and peripheral neutrophil, lymphocyte, and red blood cell counts. Andrews et al showed that KL administration in normal animals stimulated the circulation of cells capable of engraftment lethally irradiated baboons. Radiation studies suggest involvement of KL in the regulation of hematopoietic regeneration after BM injury. In studying the role of KL in the radioprotectant effect of IL-1, Neta et al incidentally showed that an antibody to KL increased the radiation sensitivity of irradiated control mice, strongly suggesting that endogenous KL plays a role in recovery from radiations-induced hematopoietic hypoplasia. In a series of experiments using a murine radiation model, Patchen et al have shown that recovery of splenic colony-forming units (CFU-S) and CFU-GM, as well as BM CFU-GM, can be accelerated by postirradiation KL administration. Furthermore, they have shown that transplantation of lethally irradiated mice with BM from recovering irradiated mice treated with KL increased survival compared with cells from recovering saline-treated controls. The ability of KL to accelerate postirradiation hematopoietic recovery in canine radiation models has also been shown.

Consequently, there is significant data to support the conclusion that KL is a major stromal factor responsible for growth and differentiation in normal hematopoiesis and in recovery from hypoplastic injury. However, as KL is a stromal factor, whether radiation or cytotoxic regimens may cause stromal injury and alter expression of this important factor is not known. In our study we used a mouse model of sublethal radiation-induced hypoplasia to evaluate the effects of radiation on KL ligand expression and the potential involvement of KL in the recovery of radiation-induced hematopoietic injury. This study shows that, after sublethal radiation injury, increases in KL gene transcripts can be detected in murine spleen and BM.

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MATERIALS AND METHODS

Experimental design. BM and splenic KL expression was first studied in normal animals to determine if KL expression was constitutive in either organ. After establishing the pattern of BM and splenic expression in normal animals, BM and splenic KL expression in mice 48 hours after sublethal 7.75-Gy irradiation was evaluated to determine the acute effects of radiation injury. Four to six sets of pooled samples at each time point were analyzed for KL expression. Each set represented RNA from three normal animals or seven irradiated animals (because of the differences in cellularity after irradiation). Later experiments were designed to look at the expression of KL during prolonged recovery from sublethal irradiation. In each of two experiments, BM or splenic RNA from three to seven animals on days 1, 2, 3, 4, 7, 10, 14, 17, 21, 24, and 28 after irradiation was pooled and analyzed.

Mice. B6D2F1 female mice were purchased from Jackson Laboratories (Bar Harbor, ME) and used for experimentation at 8 to 10 weeks of age (weighing approximately 20 g). Mice were maintained in a facility accredited by the American Association for Accreditation of Laboratory Animal Care (AAALAC) in Micro-Isolator cages on hardwood chip contact bedding and were provided commercial rodent chow and acidified (pH 2.5) water ad libitum. Animals were maintained on a 12-h light/dark cycle at 70 ± 2°F and 50% ± 10% relative humidity, with at least 10 air changes per hour of conditioned fresh air. Upon arrival, all mice were tested for Pseudomonas infection and were quarantined until test results were obtained. Only healthy mice were used for experiments. All animal experiments were approved by the Institute’s Animal Care and Use Committee. Research was conducted according to the principles of the Institute for Animal Resources, National Research Council.31

Irradiation. Mice were irradiated (whole-body) in the bilateral gamma field of the Armed Forces Radiobiology Research Institute (AFRRI) 60Co facility. During irradiation, animals were confined within individual cubicles of a plexiglass restrainer to restrict movement. The midline tissue dose to animals was 7.75 Gy (delivered at a dose rate of 0.4 Gy/min). Before animal irradiations, dosimetry was performed in an acrylic mouse phantom with a 0.5-cm tissue equivalent ionization chamber (calibration traceable to the National Institute of Standards and Technology). The tissue-to-air ratio was 0.96, and the dose variation within the exposure field was less than 3%. Dosimetric measurements were made in accordance with the American Association of Physicians in Medicine (AAPM) protocol for the determination of absorbed dose from high-energy photon and electron beams.32

Cell suspensions. Femurs and tibia were obtained by cervical dislocation after mice had been killed. Cell suspensions for each of two experiments, BM or splenic RNA from three to seven irradiated animals (because of the differences in cellularity after irradiation). Later experiments were designed to look at the expression of KL during prolonged recovery from sublethal irradiation. In each of two experiments, BM or splenic RNA from three to seven animals on days 1, 2, 3, 4, 7, 10, 14, 17, 21, 24, and 28 after irradiation was pooled and analyzed.

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RNA preparation. Total cellular RNA was obtained from intact spleens and single-cell suspensions of BM, using the RNAzol (Tel-Test Inc, Friendswood, TX) modification of the Chomczynski method according to manufacturer’s directions. Each spleen was lysed in 2 mL RNAzol and homogenized with a tissue homogenizer. BM cells were lysed in 1 to 2 mL RNAzol. Lysates were extracted with chloroform, and the extract was centrifuged at 12,000 rpm to collect the aqueous supernatant. Supernatants were precipitated with isopropanol on ice and centrifuged to pellet the RNA. The pellets were washed with 75% ethanol and recentrifuged. The ethanol was decanted, and pellets were air-dried. Pellets were resuspended in Tris-EDTA buffer (20 mMol/L Tris, pH 8.0; 1 mMol/L EDTA), and samples were quantified in a Beckman DU-65 ultraviolet spectrophotometer (Beckman Industries, Fullerton, CA).

Reverse transcriptase-polymerase chain reaction (RT-PCR). At each time point, 0.5 µg of pooled splenic RNA or 0.1 µg of pooled BM RNA was reverse-transcribed to single-stranded cDNA in 50 µmol/L Tris-HEC (pH 8.3), 75 µmol/L KCl, 3 µmol/L MgCl2, 1 mmol deoxyxynucleoside triphosphate (dNTP) mix, and 50 U of Moloney murine leukemia virus reverse transcriptase (BRL, Gaithersburg, MD) at 37°C for 1 hour. The reaction was stopped by heating to 90°C for 10 minutes, and the mixture cooled on ice for 10 minutes. PCR master mix was added to the final concentration of 50 µmol/L KCl, 10 µmol/L Tris-HEC (pH 8.3), 1.5 µmol/L MgCl2, 1 µmol/L 1.5’ primer, 1 µmol/L 3’ primer, and 1.25 U Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). Samples were amplified for 20 to 25 cycles with 1-minute denaturation at 94°C, annealing at 60°C for 2 minutes, and extension at 72°C for 3 minutes. A final extension at 72°C for 7 minutes was performed after the last cycle. In each set of reactions, RNA samples were subjected to PCR without undergoing the RT procedure to exclude genomic DNA contamination.

PCR primers and probes. PCR primers for murine KL and glycerophosphate-dehydrogenase (GAPDH) were obtained commercially (Synthetic Genetics, San Diego, CA). The following were the sequences for each primer: KL sense, AAG-GAG-ATC-TGC-GGG-GAT-GCT-CTG-ATG-A; KL antisense, ACT-GCT-ATG-CCT-GAT-ATC-CCT-AAAG-G; GAPDH sense, CCA-TGG-AGA-AGG-GGG; and GAPDH antisense, CAA-AGT-GTG-CAT-GGA-TGA-CC. The GAPDH probe was obtained from American Type Culture Collection (ATCC; Rockville, MD). The KL probe was a gift from the Immunex Corporation representing the Sal I fragment of murine mast cell growth factor (MGF) cloned into the Sal I site of pBluescript (Stratagene, La Jolla, CA).

Southern blots and hybridization. Each PCR product (10 µL) was electrophoresed in a 1× Tris-borate-EDTA agarose gel (89 µmol/L Tris (pH 8.0), 89 µmol/L boric acid, 2 mMol/L EDTA) and transferred onto Nytran membrane (Schleicher and Schuell, Keene, NH). Blots were prehybridized overnight at 42°C in 6× saline sodium citrate (1× SSC: 150 mmol/L sodium chloride; 15 mmol/L sodium citrate, pH 7.0), 5× Denhardt’s solution (1× Denhardt’s solution: 0.2 mg/mL Ficoll, 0.2 mg/mL poly(vinylpyrrolidone), 0.2 mg/mL bovine serum albumin), 0.1 mg/mL sheared denatured salmon sperm DNA, 50% formamide, 1% sodium dodecyl sulfate (SDS), and 50 µmol/L Tris (pH 8.0). Blots were then hybridized overnight in the same solution after addition of nick-translated, radiolabeled plasmid probes to a final concentration of 1×106 to 2×107 cpm/mL of prehybridization fluid. Blots were then washed three times at 42°C in 2× SSC/0.5% SDS and finally washed at 62°C for 1 hour in 0.1× SSC/0.1% SDS. Excess fluid was removed, and the blots were exposed to Kodak XAR autoradiography film (Eastman Kodak, Rochester, NY).

Quantitation and analysis. Using a scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA), each sample’s densitometric volume was measured. PCR product quantification was based on relative expression differences between irradiated and control samples. GAPDH was used to normalize for tube-to-tube variations in RNA loading due to pipette error or reaction efficiency. To normalize, the densitometric volume of each set of GAPDH RT-PCR reactions was averaged, and each specific GAPDH densitometric volume was expressed as a percent of the average to obtain a correction factor (CFv). Each KL densitometric volume was then divided by the corresponding GAPDH correction factor to obtain a corrected KL densitometric volume (ie, [GAPDH/GAPDHcorr] = CFv; [KLcorr] = KLcorr/CFv). All statistical comparisons were made using the Mann-Whitney U test.

RESULTS

Cellular response to irradiation. The cellular response to radiation was a predictable decrease in cellularity on the
second day postirradiation. In BM, the cellularity decreased from \(9.93 \times 10^9 \pm 0.45 \times 10^9\) cells per bone in normals to \(0.60 \times 10^9 \pm 0.03 \times 10^9\) cells by the second day after irradiation. Splenic cellularity decreased from \(131.98 \times 10^9 \pm 11.98 \times 10^9\) cells per spleen in normals to \(19.99 \times 10^9 \pm 0.38 \times 10^9\) cells on the second day postirradiation.

**RNA yield postirradiation.** The total RNA yield per animal also decreased after irradiation. The BM RNA yield per animal decreased from \(11.48 \pm 0.89 \mu g\) in normals to \(2.90 \pm 0.24 \mu g\) on the second day after irradiation. In whole spleens, the RNA yield decreased from \(180.99 \pm 16.96 \mu g\) to \(59.95 \pm 4.48 \mu g\) per spleen. However, the degree of decrease in BM RNA did not correlate with the decrease in BM cellularity, as the RNA yield per cell rose from \(0.31 \pm 0.22 \mu g/10^9\) cells to \(1.21 \pm 0.06 \mu g/10^9\) cells. Comparable data are not available in spleens, as the RNA yields were obtained from whole-spleen lysates and not from single-cell spleen suspensions.

**Reaction efficiency.** To evaluate the efficiency of the combined RT and PCRs, splenic RNA from a single subject was aliquoted into 14 separate reaction tubes, reverse-transcribed, and then amplified using substrate, buffer, and enzymes prepared as RT and PCR master mixes. The resulting blot is shown in Fig 1. Absolute densitometric volume averaged \(201.7 \pm 8.5\). Although the absolute densitometric volume is a function of exposure time and film saturation kinetics, the sample showed remarkably little variation (coefficient of variation, 15.7%).

**Quantitation efficiency.** To assess the effects of amplification cycle number and exposure duration on quantitation efficiency, serial twofold dilutions of a single sample of splenic RNA were amplified simultaneously under identical conditions as described above at 20, 25, and 30 cycles. The resulting blots were first autoradiographed for 4 hours and then overnight to compare differences in observed results. The short-exposure (Fig 2) blot easily displays expression at 30 cycles but not in a serial twofold fashion, reflecting either saturation of the reaction or the film. While the 20-cycle samples were detected, the densitometer could not differentiate results at the lower dilutions in a twofold manner. However, the 25-cycle PCR products did show serial twofold differences at the lower concentrations of RNA but not at the higher concentrations. As shown in the accompanying data table to Fig 3A, the long-exposure blots show near-linear twofold decreases in densitometric volume at 25 cycles. Similar linear twofold decreasing results were observed at 25 cycles, but only at the lower concentrations. The results of 20- and 25-cycle long-exposure autoradiographs suggest that somewhat longer exposures can be used for the 20-cycle reactions without significant loss of quantification efficiency.

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**Fig 1.** Precision of the RT-PCR assay in the measurement of c-kit ligand expression. (A) Data represent the simultaneous amplification of 14 equal aliquots of a single sample of splenic RNA (mean, 201.7; median, 199.85; SD, 30.55; coefficient of variation, 15.71%). (B) Photograph represents the corresponding autoradiograph. Exposure time was 4 hours.
Normal KL expression. Bone marrow and splenic RNA from individual animals was amplified under identical conditions for each tissue to determine whether KL was constitutively expressed and if there were significant differences between individual animals. In BM, KL appeared to be regularly expressed at comparable levels in all animals (Fig 4A). However, in spleens the range of normal expression was quite broad, with levels in many individuals undetectable at amplification cycles and exposures designed to detect modest levels (Fig 4B).

Expression on the second day postirradiation. To evaluate the acute effects of irradiation on KL expression, BM and splenic RNA from three sets of normal mice or mice 2 days after radiation exposure (each set of RNA was pooled from three to seven mice) was amplified in duplicate under identical conditions. An equal aliquot of RNA was separately subjected to RT-PCR for GAPDH as a control. Figure 5A and B shows that, after GAPDH correction, BM and splenic KL transcript levels in irradiated mice increased 15-fold and threefold, respectively. When evaluated without correcting for GAPDH expression, the difference in BM KL expression after 7.75-Gy irradiation increased 25-fold (Fig 6A), as BM GAPDH expression alone increased threefold in irradiated mice (Fig 6B). Splenic GAPDH expression did not change after irradiation (data not shown).

KL expression during prolonged recovery from sublethal irradiation. To evaluate the time course of KL expression during prolonged recovery from 7.75-Gy irradiation, RNA from three to seven animals was harvested at various times and subjected to RT-PCR. As shown in Fig 7A, BM transcript levels showed a uniphasic change in expression, with an increase on day 2 postirradiation and a return to normal on day 10. Splenic KL transcript levels increased on day 1 and returned to normal on day 4 postirradiation, only to increase again on day 10 and return to baseline on day 17 (Fig 7B).

DISCUSSION
This study shows that KL transcript levels increase in hematopoietic tissues immediately and for several days after sublethal radiation injury. This response to injury may be an integral part of the host response to hypoplastic injury and appears to partially explain previously reported data on the role of endogenous KL in radioprotection and the effects of exogenous KL in therapy for radiation injury.
As shown, KL transcript levels were uniformly present in normal BM but, although common, were not uniformly present in normal splenic tissue. Several possible explanations exist for this difference. Before RNA preparation, BM cells were extracted from femurs and tibias, and some activation of gene transcription could have occurred due to manipulation of the cells during the harvest process; in contrast, splenic RNA was prepared within seconds of spleen removal. However, if gene activation did occur it was not maximal as further dramatic increases in transcript levels were seen in irradiated mice. An alternate explanation is that basal hematopoiesis is the primary function of BM and is supported to some unknown extent by this basal level of the KL transcript. The variable KL expression of the normal spleen may represent its secondary role in basal hematopoiesis.

The postirradiation increases in BM and splenic KL transcript levels seen in this study may have numerous explanations. The existing data on the role of KL as a growth factor on the basis of the interactions of KL with the c-kit product, as shown in the Steel and White mutant mouse model systems. However, the in vitro data suggest that this role may be a necessary but insufficient one to drive maximal hematopoiesis. This appears to be borne out by in vivo data in murine hypoplasia models where exogenously administered KL produced, at best, modestly accelerated hematopoietic regeneration compared with other growth factors evaluated in similar models. If the transcript levels reported here are matched by protein expression, then these results may serve to explain some of the conflicts on the basis of the interaction between exogenous KL and the c-kit receptor in competition with endogenous KL produced after a hypoplastic injury. Recent data reported by Avraham et al on the role of KL in the interaction between megakaryocytes and BM stroma, as well Caceres-Cortes and Hoang’s data on leukemic cell interaction, provide further evidence in support of this hypothesis. Neta et al’s data showing that anti-KL antibodies block the radioprotectant effect of IL-1 argue for an interme...
diary role of KL as an explanation for the radioprotectant effect of IL-1; however, an alternate explanation may be that the function of the radiation-induced expression of membrane-associated or -secreted KL was simply blocked by the anti-KL antibody. This is especially likely based on the increased radiation sensitivity observed in otherwise normal animals treated with the anti-KL antibody.21 Furthermore, while early data suggested that basal endothelial production of KL was upregulated by IL-1, more recent data have shown that IL-1 has no direct effect on KL production by either cultured endothelium or stromal cells.22,31 The overall apparent lack of specific effect on the BM CFU-S (but not CFU-GM) repopulation observed by Patchen et al22 in irradiated animals treated with KL may be explained by exogenously produced KL saturating the c-kit receptor on primitive progenitor cells, thus preventing interaction of the progenitors with marrow stromal cells. Alternatively, the expression of KL and other pivotal factors or the expression of their receptors may be regulated by the pharmacologic effects of KL so that, in vivo, the dynamic interactions yield vastly different results than those noted in vitro.

Other than increased transcription or stabilization of transcripts, an alternative explanation for the increased KL gene expression observed in our study includes relative homogenization of the cell populations in the BM and the spleen postirradiation. As stromal cells are relatively resistant to the radiation doses used in this study, this is a reasonable explanation. More likely, however, is a combined explanation including some degree of cellular homogenization along with true increases in transcript levels. In similar radiation experiments studying BM and splenic transforming growth factor β (TGF-β) gene expression, another stromal cell product, transcript levels did not change.30 The fact that no effect was seen on TGF-β suggests that the observed increases in KL expression cannot be explained simply on the basis of radiation-induced homogenization of radiation-resistant stromal and hematopoietic cell populations. In further support, Shirota and Tavassoli37 have described putative radiation-induced histologic changes in BM microvasculature at doses as low as 1 Gy and more prominent changes at higher radiation doses well below those used in our study. As human umbilical vein endothelial cells have been shown to normally express KL, it is reasonable to hypothesize that a radioresistant stromal cell population would be directly or indirectly affected by radiation at these doses.32
KIT LIGAND GENE EXPRESSION AFTER IRRADIATION

The use of the RT-PCR method to quantify levels of gene transcripts is a sensitive technique that must be carefully adapted to experimental conditions and the data requirements of the investigation. A number of methods to yield reliable results have aimed at controlling the supposed inherent variability of the RT-PCR process, including simultaneous amplifications of material, external standards for comparison, and various methods of internal standardization to control for variability. Additionally, the detection methods used to identify PCR products and the quantitation method to assign a value to the result must also be closely adapted to the data requirements to avoid saturation of the reaction, the film, or the scanning device. In this study, a semiquantitative method of transcript level comparison was obtained by simultaneous amplification of multiple sets of samples, each in duplicate. Attention was also directed to the length of exposure needed to achieve detection without causing film saturation to preserve the real differences between results. Consequently, the method chosen had a high degree of precision and was able to clearly differentiate relative differences in transcript levels.

In conclusion, this study shows that both BM and spleen express increased levels of KL transcripts after sublethal radiation injury and that the RT-PCR method, properly used, is a reliable method for semiquantitative analysis of changes in gene expression. The observed increases in KL expression may be an important response of hematopoietic stromal tissues in mediating hematopoietic recovery from hypoplastic injury.

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