Interaction of Human Bone Marrow Fibroblasts With Megakaryocytes: Role of the c-kit Ligand

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Human kit ligand (KL), also known as stem cell factor (SCF), steel factor, or mast cell growth factor, is a recently identified hematopoietic growth factor whose receptor is the product of the c-kit proto-oncogene. Alternative splicing of the pre-mRNA of KL/SCF results in secreted and membrane-bound forms of the protein. We and others have recently shown that the c-kit gene product is expressed on human megakaryocytes and that soluble KL/SCF in combination with granulocyte-macrophage colony-stimulating factor, interleukin-3 (IL-3), or IL-6 increased megakaryocyte progenitor colony formation (CFU-MEG) and stimulated mature megakaryocytes. Here we show that adhesion of human megakaryocytes to bone marrow stromal fibroblasts, which express the membrane-bound form of KL/SCF (mKL/SCF), is mediated in part by the interaction between mKL/SCF and the c-kit protein. This interaction also results in marrow fibroblast-stimulated proliferation but not an increase in ploidy of megakaryocytes; when the two cell types were separated by a trans soluble membrane, proliferation did not occur. Adhesion and proliferation of human megakaryocytes to an immortalized murine stromal cell line SI/SI lacking the KL/SCF gene was impaired, whereas transfection of SI/SI cells with human mKL/SCF significantly increased both adhesion and proliferation. Marrow stromal fibroblast mKL/SCF may serve both as an adhesion structure and as a growth-potentiating factor for megakaryocytes in the bone marrow.

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THE HUMAN kit ligand (KL), also known as stem cell factor (SCF), mast cell growth factor, or steel factor has recently been identified as the product of the SI locus. The cell surface receptor for KL/SCF is the product of the c-kit proto-oncogene. Mice with mutations in the SI locus have abnormalities in hematopoiesis, germ cells, and melanocytes. Two forms of KL/SCF have been described that arise through differential RNA processing: a membrane-bound species and a soluble secreted form. Soluble KL/SCF in vitro increased the growth response of progenitor cells to later-acting cytokines such as interleukin-3 (IL-3), erythropoietin, and granulocyte colony-stimulating factor (G-CSF). It has recently been observed that colony-forming unit-megakaryocyte progenitors (CFU-Meg) and mature human megakaryocytes proliferated in response to soluble recombinant KL/SCF.

Cos cells expressing surface murine KL/SCF adhered with murine mast cells, suggesting that the membrane-bound molecule may have a different function from the soluble species. A study of murine mast cell adhesion to mesenchymal cells derived from SI/SI mice found that the extracellular domain of mKL/SCF was required to mediate this adhesion. The interaction of human megakaryocytes with adhesion molecules present on bone marrow stromal cells has not been defined. To explore the potential role of mKL/SCF as an adhesion structure for megakaryocytes and other c-kit expressing hematopoietic cells, we studied adhesive interactions of human megakaryocytic cells with bone marrow stromal fibroblasts.

Our studies showed that adhesion of human megakaryocytes could be mediated in part via their c-kit receptor binding to membrane-associated KL/SCF expressed by bone marrow fibroblasts. Further, direct interaction between stromal fibroblasts and megakaryocytes induced DNA synthesis as measured by thymidine incorporation in the megakaryocytic cells. The dual function of mKL/SCF as both an adhesion structure and a regulator of proliferation is novel among hematopoietic growth factors, and provides a model whereby hematopoietic cells, such as megakaryocytes, mast cells, and early hematopoietic progenitors may be anchored to stromal elements within the bone marrow microenvironment and positioned to respond to cytokines.

MATERIALS AND METHODS

Cells. Human bone marrow was obtained by aspiration from the iliac crest of normal donors who gave informed consent in a protocol approved by the New England Deaconess Hospital Institutional Review Board. The marrow was aspirated into preservative-free heparin (Sigma Chemical Co, St Louis, MO) and separated by centrifugation through Ficoll-Hypaque (Pharmacia, Piscataway, NJ) at 1,200g at room temperature for 30 minutes. After two washes with sterile 1x phosphate-buffered saline (PBS), the cells were resuspended in Iscove's modified Dulbecco's medium (IMDM) with 20% fetal calf serum (FCS), penicillin/streptomycin (P/S), and L-glutamine; seeded onto T-75 tissue culture flasks (Corning, Corning, NY); and incubated at 37°C in 5% CO₂. After 48 hours, the nonadherent cells were gently removed, and the adherent cells were refed with fresh media. The cells were refed with fresh medium every 3 days and trypsinized and split after 1 week or when confluent. Cells underwent three cycles of trypsinization and splitting before characterization or use in experimental protocols. Cultures of marrow stromal fibroblasts prepared by this method were uniformly positive for vimentin, negative for cytokeratin, negative for von Willebrand's factor, and negative for nonspecific esterase by immunochemical or histochem-
rical staining. Human marrow megakaryocytes were isolated by a method employing immunomagnetic beads using antihuman glycoproteins GP Ib and GP Ia, monoclonal antibody (MoAb) as described previously. All of the isolated cells were recognizable as megakaryocytes by morphology and/or specific immunofluorescence using antiplatelet antibodies, GP Ib and GP Ia, synthesis of platelet factor 4, platelet derived growth factor, von Willebrand factor, and becomes polyploid on induction with phorbol esters.

The CMK cell line, provided by Dr T. Sato and derived from a child with megakaryoblastic leukemia, has properties of cells of megakaryocytic lineage, including surface expression of glycoproteins Ib and IIa, synthesis of platelet factor 4, platelet derived growth factor, von Willebrand factor, and becomes polyploid on induction with phorbol esters.

An immortalized stromal fibroblast cell line derived from the fetal hematopoietic microenvironment of a murine homozygous (SI/SI) embryo that lacks the entire coding sequence of the Steel gene has been derived. This stromal cell line (SI/SI) has been used for the transfection of a cDNA encoding a 220 amino acid SCF protein (hSCF) lacking the proteolytic cleavage site because of the exclusion of exon 6 by Toksoz et al. Therefore, the 220-amino acid polypeptide remains membrane-bound in the transfected SI/SI cells. Neither the original cell line (SI/SI) nor transfectants containing the 220 cDNA (SI/SI-SCF) secreted detectable amounts of KL/SCF. The SI/SI-hSCF transfectant showed a high level of membrane-associated bKL/SCF protein as measured by FITC goat antimouse staining of cells labeled with a primary MoAb to human KL/SCF.

Cell-cell adhesion assay. To measure cell-cell adhesion, 10⁵ CMK cells or bone marrow megakaryocytes in 2 mL of Dulbecco’s modified Eagle’s medium (DMEM) with 10% FCS were added to stromal fibroblast monolayers. The cells were allowed to settle for 1 hour at 37°C. Nonadherent cells were removed and the remaining adherent cells were collected, trypsinized, and counted as described above.

**DNA and RNA analysis.** RNA was isolated by the guanidinium isothiocyanate method. First-strand cDNA was synthesized at 37°C for 1 hour in a final volume of 10 μL with oligo-dT as primers; 4.5 μL RNA in DEPC-dH₂O, 2.0 μL 5 x buffer (250 mmol/L Tris-HCl, pH 8.3, 375 mmol/L KCl, 50 mmol/L dithiothreitol, 15 mmol/L MgCl₂, and 250 μg/mL actinomycin D), 0.5 μL RNAase (40 U/μL, Promega, Madison, WI), 1.0 μL dNTP (dATP, dCTP, dGTP, dTTP mix, 10 mmol/L each (Pharmacia), 1.0 μL oligo-dT, 1.0 μL Maloney murine leukemia virus (MMLV) reverse transcriptase (2,000 U/mL, Boehringer-Mannheim, Chicago, IL).

**Polymerase chain reaction (PCR).** Eighty microliters of PCR mix was added to 10 μL of first-strand cDNA. PCR mix contains: 53.5 μL sterile water, 10 μL 10 x buffer, 16 μL of dNTP mix (each at 1.25 mmol/L) and 0.5 μL (2.5 U) of the *Thermus aquaticus* thermostable DNA polymerase (Cetus-Perkin Elmer, Emeryville, CA).

**Fig 1.** PCR analysis of c-kit and KL/SCF cDNAs from megakaryocytes, CMK cells, H9 T-cells, and bone marrow fibroblasts. PCR was performed on cDNA prepared from RNA isolated from the indicated source of cells as described. The PCR products were separated on 2% or 1% agarose gels (c-kit and KL/SCF, respectively) run in Tris-borate EDTA buffer (TBE) containing 0.25% μg/mL ethidium bromide. Southern blot hybridization of the PCR products was performed using specific probes for c-kit and KL/SCF. The probe of KL/SCF was generated by linearizing pGEM3-hKL/SCF no. 9 with EcoRI-Hindlll using the 218-bp insert as a probe. The probe of c-kit was generated by linearizing pGEM3-hKL/SCF and using the 1.25-kb insert as a probe.
Adhesion of megakaryocytes to bone marrow fibroblasts. CMK cells and isolated human megakaryocytes expressed the c-kit proto-oncogene (Fig 1A and B). Bone marrow stromal fibroblasts expressed mRNAs related to both the c-kit proto-oncogene (Fig 1A and B). Bone marrow fibroblasts are visible as having fibroblast-like morphology and megakaryocytes as small round refractile cells.

Fig 3. (A) Effects of specific antibodies against c-kit, GP Ib/IIa, LFA-1, and Leu 8 on adhesion of megakaryocytic cells to bone marrow fibroblasts. CMK cells or isolated marrow megakaryocytes were incubated without antibodies, or with MoAbs for GP Ib, GP Ib/IIa, LFA-1, Leu 8, or c-kit for 30 minutes at 37°C. The cells were then washed and added to bone marrow fibroblasts for 1 hour at 37°C. Values are mean ± SEM, n = 6. Adhesion was significantly inhibited, P < .05, compared with control. (B) Immunofluorescence analysis using flow cytometry of CMK cells or isolated marrow megakaryocytes stained with specific antibodies for c-kit. CMK cells or megakaryocytes were incubated with purified control IgG ascites (100 ng/mL) or purified SR-1 antibody (100 ng/mL), which recognizes human c-kit. Cells, 10⁴, were analyzed in each instance and fluorescence intensity was displayed in relative intensity on a logarithmic scale. Percentage of positive cells, calculated between channel numbers 50 and 200, are indicated. An unrelated FITC-labeled conjugate (Swine antirabbit Ig) stained about 2% of each suspension (curves not shown).
soluble and membrane forms of KL/SCF (Fig 1C and D). Low but detectable amounts of secreted soluble KL/SCF (~0.3 ng/mL) were measured using a radioreceptor binding assay in the conditioned media of stromal fibroblasts. 

Incubation of CMK megakaryocytic cells with primary bone marrow stromal fibroblasts resulted in adhesion of the normally nonadherent CMK cells to the fibroblast monolayer. This interaction was rapid and was observed after 15 minutes (Fig 2A). Addition of soluble recombinant human KL/SCF at concentrations of 50 ng/mL inhibited subsequent adhesion of CMK cells to the fibroblasts (Fig 2A and B).

Adhesion also occurred between isolated human marrow megakaryocytes and bone marrow stromal fibroblasts. These studies showed adhesion of marrow megakaryocytes within 30 minutes that persisted for more than 24 hours (Fig 2C and E). Treatment with soluble recombinant KL/SCF (at 50 ng/mL) for 24 hours inhibited adhesion of marrow megakaryocytes to stromal fibroblasts (Fig 2D and F).

Inhibition of adhesion was also observed on pretreatment of CMK cells or marrow megakaryocytes with the SR-1 neutralizing MoAb to c-kit, but not in control cultures treated with an irrelevant MoAb of the same isotype, the control IgG ascites, or a control MoAb to HLA surface antigen (Fig 3A). Flow cytometry of CMK cells or marrow megakaryocytes treated with the anti–c-kit SR-1 antibody showed that 99% and 83%, respectively, stained positively (Fig 3B). Adhesion was also inhibited by pretreatment of stromal fibroblasts with polyclonal anti-KL/SCF antibodies, but not with control rabbit serum (data not shown).

To ascertain which other cell surface adhesion molecules could mediate cell-cell interaction between CMK cells and the marrow fibroblasts, cross-blocking cell adhesion experiments were performed using inhibitory MoAbs specific for the LFA-1 complex, GPIIb/IIIa, GPIb, and Leu 8 structures. MoAbs to LFA-1, GPIb, GPIIb/IIIa, and Leu 8 blocked adhesion of CMK cells. The combination of these MoAbs with the SR-1 antibody to c-kit antibodies resulted in a small increase in inhibition of adhesion of CMK cells (Fig 3A).

To further confirm that the mKL/SCF and c-kit interaction mediated adhesion and that the anti–c-kit antibody was not simply sterically blocking interactions involving other adhesion molecules on the megakaryocyte cell surface, two approaches were used. First, Fab-specific antibodies against c-kit, GPIIb/IIIa, GPIb, and Leu 8 structure blocked adhesion of CMK cells. The combination of these MoAbs with the SR-1 antibody to c-kit antibodies resulted in a small increase in inhibition of adhesion of CMK cells (Fig 3A).

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sion and additional adhesive molecules, such as GPIIb/IIIa, LFA-1, and Leu 8, also can participate in the adhesion process between megakaryocytes and marrow fibroblasts.

**DNA synthesis in megakaryocytes.** Additional experiments were performed to determine whether adhesion of bone marrow fibroblasts and CMK cells or isolated marrow megakaryocytes triggered DNA synthesis in these cells (Cig 4). In a coculture assay, bone marrow fibroblasts were incubated directly with CMK cells or isolated megakaryocytes. Stimulation of marrow megakaryocyte [3H] thymidine uptake was observed. Similar stimulation, though of a much lesser magnitude, was seen with the permanent CMK cell line, which grows autonomously. CMK cells or human megakaryocytes alone, bone marrow fibroblasts alone, bone marrow fibroblasts incubated in CMK or megakaryocyte conditioned media, or isolated megakaryocytes or CMK cells cultured in media conditioned by marrow fibroblasts did not achieve similar levels of [3H] thymidine incorporation. In addition, CMK cells or marrow megakaryocytes cultured in the presence of marrow fibroblasts but separated by a transuble membrane filter did not show augmented [3H] thymidine incorporation, indicating that direct contact was required for DNA synthesis and proliferation. Quantitation of viable cell number by Trypan blue exclusion confirmed proliferation results with [3H] thymidine incorporation (data not shown). Attachment of CMK cells or megakaryocytes to marrow fibroblasts resulted in cell division not nuclear endoreduplication. In addition, these experiments were repeated with irradiated bone marrow fibroblasts. The results were similar to that of nonirradiated bone marrow fibroblasts (data not shown).

**Adhesion of megakaryocytes to SI/SI-hSCF[220].** Another approach to further show that the membrane form of fibroblast KL/SCF mediated megakaryocyte adhesion was pursued. In a coculture assay, CMK or marrow megakaryocytes were incubated directly with murine SI/SI cells or SI/SI-hSCF[220], then floating megakaryocytes were washed away and the numbers of adherent cells determined. Megakaryocytes showed marked adhesion and spreading within 30 minutes and a maximal degree of adhesion was reached after 3 hours (54% ± 9%; mean ± SEM of five experiments). Only a small number of megakaryocytes adhered to the nontransfected murine SI/SI cells (21% ± 7%; mean ± SEM of five experiments), even after longer incubation times. The difference in adhesion of megakaryocytes to SI/SI-hSCF[220] compared with parent murine SI/SI cells was statistically significant (P < .05). These results indicated that murine SI/SI-hSCF[220] cells, which expressed the membrane form of human KL/SCF, promoted adhesion of human megakaryocytes, whereas parental murine SI/SI cells did not. Increased [3H] thymidine incorporation was observed in cocultures of adherent CMK cells or marrow megakaryocytes and irradiated SI/SI-hSCF[220] cells, but not with irradiated parental SI/SI cells (Fig 5), showing that direct interaction of c-kit and mKL/SCF resulted in DNA synthesis in the megakaryocytic cells.

**DISCUSSION**

Our results show that human megakaryocytes can adhere to bone marrow stromal fibroblasts, which results in induction of DNA synthesis in the megakaryocytes. This adhesion may be mediated in part by the specific interaction of the c-kit product on the megakaryocyte cell surface with the membrane-associated form of KL/SCF on the marrow fibroblast. This conclusion is based on the observations that treatment of megakaryocytes with soluble recombinant KL/SCF inhibited this adhesion, and that inhibition of adhesion was also observed with the SR-1 antibody to c-kit or with polyclonal antibodies to KL/SCF. Direct cell-cell interaction of megakaryocytes with stromal fibroblasts was required for DNA synthesis in the megakaryocytes, as shown by the coculture studies. This was most clearly seen using primary bone marrow megakaryocytes, which are likely to be more physiologically relevant compared with the immortalized CMK cell line.

During the course of our experiments, two other research groups presented data indicating that the product of the Steel gene may mediate mast cell adhesion. Planagan et al[10] transfected cos cells with murine KL/SCF and observed aggregation of murine mast cell lines. Kaneko et al[11] also reported murine mast cell adhesion with the extracellular domain of KL/SCF. Our work addressed the functions of membrane-associated human KL/SCF in the context of megakaryocytopenes. The bone marrow stromal microen-
vironment may serve a number of functions in megakaryocyte growth and maturation, acting as a support framework for developing progenitor cells as well as a rich source of growth factors.\textsuperscript{14,15} Expression of alternative forms of KL/SCF by stromal fibroblasts may be a point of regulation of megakaryocytes and other c-kit expressing cells, because such hematopoietic cells may use this surface structure as an adhesion molecule. Further more, direct interaction of mKL/SCF with the c-kit receptor may result in potentiation of cell growth, particularly in conjunction with other cytokines such as IL-3, IL-6, and granulocyte-macrophage colony-stimulating factor.\textsuperscript{4,5,16} The dual function of mKL/SCF—mediating megakaryocyte adhesion and proliferation—is novel among the hematopoietic growth factors reported to modulate cells of this lineage.\textsuperscript{15} Whether adhesion and proliferation are regulated by independent functional domains within mKL/SCF or its receptor, c-kit, is subject to further study.

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