RAPID COMMUNICATION

Cloning of the Human Homologue of the Murine flt3 Ligand: A Growth Factor for Early Hematopoietic Progenitor Cells

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Using a fragment of the murine flt3 ligand as a probe, we have succeeded in cloning a human flt3 ligand from a human T-cell λgt10 cDNA library. The human and murine ligands are 72% identical at the amino acid level. Analysis of multiple cDNA clones shows that alternative splicing of the human flt3 mRNA can occur at a number of positions. A recombinant soluble form of the human flt3 ligand stimulates the proliferation and colony formation of a subpopulation of human bone marrow cells that are CD34+ and are enriched for primitive hematopoietic cells. In addition, the human flt3 ligand also stimulates the proliferation of cells expressing murine flt3 receptors. Northern blot analysis shows widespread expression of flt3 ligand mRNA transcripts in human tissues.

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A NUMBER OF LIGANDS for tyrosine kinase receptors play a role in regulating the proliferation and differentiation of cells in the hematopoietic system. These include colony-stimulating factor 1 (CSF-1), which regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells,1 and Steel factor (SLF) (also known as mast cell growth factor, stem cell factor, or kit ligand), which affects the proliferation of both myeloid and lymphoid cells.2 Several years ago a new tyrosine kinase receptor was cloned, alternately named as ft3 or flk-2, that was structurally related to the CSF-1 and SLF receptors.3,4 This receptor was described by one group as being selectively expressed on hematopoietic stem and progenitor cell-enriched fetal liver populations.5 We have recently cloned a novel hematopoietic growth factor from a murine T-cell line that is a ligand for the ft3/flk-2 tyrosine kinase receptor.6 This growth factor, which we refer to as a flt3 ligand, stimulates the proliferation of hematopoietic progenitor cells isolated from mouse fetal liver or adult mouse bone marrow (BM).6 In addition, the murine factor was functionally active on human CD34+ BM cells that are enriched for hematopoietic stem cells.5

The flt3 ligand is similar in size and structure to SLF and CSF-1 in that all three proteins are type I transmembrane proteins with short cytoplasmic domains; four cysteine residues appear to be conserved in the extracellular domains of all three of these growth factors. A fragment of the murine flt3 ligand cDNA was used as a probe to screen a human T-cell cDNA library for a human flt3 ligand cDNA. We now report the cloning of a human flt3 ligand, which is similar in structure to the murine protein and shares 72% amino acid identity. A soluble form of the human flt3 ligand stimulates the proliferation and colony formation of a subpopulation of human BM cells that are CD34+ and are enriched for hematopoietic progenitor cells.

MATERIALS AND METHODS

Cloning of the human flt3 ligand. The human flt3 ligand was cloned from a random-primed cDNA library in λgt10 constructed from human clone 22 T-cell mRNA4 by screening the library with a 413-bp PstI fragment from the extracellular domain of the murine flt3 ligand (bp 103 through 516 of the murine flt3 ligand clone no. 6C cDNA).5 The fragment was random primed with [32P]dCTP, hybridized overnight to the library filters at 55°C in oligo prehybridization buffer (60 mM sodium phosphate, pH 8.0, 2 mM EDTA, 5× Denhardt's solution, 6× SSC, 0.1% N-lauryl sarcosine, 0.5% NP40, 200 μg/mL salmon sperm DNA), and then washed at 55°C in 2× SSC/0.1% sodium dodecyl sulfate (SDS) for 1 hour, then 1× SSC/0.1% SDS for 1 hour, and finally 0.5× SSC/0.1% SDS for 1 hour. DNA from positive plaques was extracted, inserted were amplified by PCR using oligonucleotides specific for the phage arms, and the DNA was sequenced. A total of six cDNAs were isolated from this library (after screening 480,000 phage clones) that corresponded to the human flt3 ligand sequence. Two clones appeared to be identical by restriction digestion, so only five clones were sequenced. We were also able to isolate human flt3 ligand cDNA clones using a probe derived from the murine flt3 ligand 5′H cDNA clone (data not shown).

Construction of a splicing variant of the human flt3 ligand. A 179 bp deletion seen in the clone 14 flt3 ligand cDNA (Fig 1) was transferred into the wild-type clone 9 flt3 ligand cDNA as follows. The wild-type clone 9 cDNA (in the pBluescript [Stratagene, La Jolla, CA] vector) was cut with EcoNI, and a 319-bp fragment containing the 179-bp segment was removed (bp 478 to 796 in Fig 2). Similarly, the clone 14 flt3 ligand cDNA (in the pBluescript vector) was also cut with EcoNI and a 140-bp fragment that spans the desired 179-bp deletion was isolated. The 140-bp EcoNI fragment isolated from clone 14 was then ligated into the clone 9 cDNA, and a recombinant clone was identified by restriction digestion. The recombinant clone was sequenced to confirm the endpoints of the deletion as well as the orientation of the insert. Both the clone 9/Δ179 flt3 ligand as well as the wild-type clone 9 flt3 ligand cDNAs were then transferred into the pDC302 expression vector6 for expression of the protein in CV-1/EBNA cells.

DNA and protein sequence analysis. DNA and protein sequence analysis was performed using the GCG software package (University of Wisconsin, Madison). The signal peptide was identified using the algorithm of von Heijne,7 and the transmembrane domain was identified using the algorithm of Eisenberg et al.8 DNA sequencing was performed by primer walking using the ABI Taq DyeDeoxy Terminator Cycle Sequencing kit on an automated DNA sequencer (model 373A; Applied Biosystems, Foster City, CA). Both strands of each of the cDNA clones were sequenced in their entirety.

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Northern blot analysis of flt3 ligand expression. Human tissue Northern blots were purchased from Clontech (Palo Alto, CA); each lane contains approximately 2 μg of poly A+ mRNA. The blots were hybridized overnight at 55°C with a polymerase chain reaction (PCR) generated fragment of the human flt3 ligand containing nucleotides 161 through 574 (Fig 2) that had been random-primed. The blot was then washed to 2X SSC/0.1% SDS for 1 hour, then 1X SSC/0.1% SDS for 1 hour, and finally 0.5X SSC/0.1% SDS for 1 hour, then put to film. The blots were later probed without being stripped with an α actin probe as a loading control for the various mRNAs according to the manufacturer's directions.

Activity of human flt3 ligand on human CD34+ BM cells and cells expressing the murine flt3 receptor. Recombinant soluble human flt3 ligand was produced in yeast essentially as described before for the murine flt3 receptor as described previously. Therefore, we decided to screen a random-primed cDNA library in λgt10 constructed from human clone 22 T-cell mRNA to isolate human flt3 ligand clones. Five human flt3 ligand cDNA clones were isolated (Fig 1) by screening this library with a 413-bp Ple I fragment from the extracellular domain of the murine flt3 ligand. Of the five cDNA clones isolated, two clones (nos. 9 and 8) encoded full-length proteins corresponding to the murine flt3 ligand sequence, and one clone (no. 22) contained a partial flt3 ligand sequence. Two other clones, nos. 14 and 24, contained flt3 ligand clones that have undergone alternate splicing that disrupts the reading frame as described below. The sequence shown in Fig 2 is a composite that represents clone no. 9, with additional 5' and 3' noncoding regions from clone no. 8.

Hematopoietic colony assay. Human BM or cord blood-derived CD34+ cells were isolated as described previously. Approximately 90% of the enriched cell population was CD34+ (data not shown). Cells were plated in 0.5 mL of methylcellulose media/well (methocult H4230; Terry Fox Laboratory, Vancouver, Canada) in 24-well plates (600 cells/well for BM and 800 cells/well for cord blood). Cytokines were added at the following concentrations: 2 U/mL erythropoietin (EPO) (Terry Fox Laboratory), 10 ng/mL IL-3, 20 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 250 ng/mL SLF, and 20 ng/mL Pixy 321, which is a GM-CSF–IL-3 fusion protein. Flt3 ligand was added at 100 ng/mL alone and in combination with other factors. The plates were incubated in a 37°C, 7% O2, 6.5% CO2 incubator and total colony numbers were counted on day 14.

Binding assays. CV-1/EBNA cells were transfected with various flt3 ligand cDNA expression constructs and assayed 2 days later for their capacity to bind a flt3-Fc fusion protein (a soluble version of the flt3 receptor) using methods described previously.

RESULTS

Cloning of the human flt3 ligand. The murine flt3 ligand was cloned from a murine T-cell line and is expressed on a number of murine T-cell lines (S.D.L., unpublished data). Therefore, we decided to screen a random-primed cDNA library in λgt10 constructed from human clone 22 T-cell mRNA to isolate human flt3 ligand clones. Five human flt3 ligand cDNA clones were isolated (Fig 1) by screening this library with a 413-bp Ple I fragment from the extracellular domain of the murine flt3 ligand. Of the five cDNA clones isolated, two clones (nos. 9 and 8) encoded full-length proteins corresponding to the murine flt3 ligand sequence, and one clone (no. 22) contained a partial flt3 ligand sequence. Two other clones, nos. 14 and 24, contained flt3 ligand clones that have undergone alternate splicing that disrupts the reading frame as described below. The sequence shown in Fig 2 is a composite that represents clone no. 9, with additional 5' and 3' noncoding regions from clone no. 8.

Analysis of the sequence of a composite human flt3 ligand cDNA (Fig 2) showed an open reading frame of 705 bp surrounded by 83 bp of 5' noncoding sequence and 321 bp of 3' noncoding sequence (Fig 2). There were no in-frame stop codons upstream of what we are designating the initiator methionine. The open reading frame encodes a type I transmembrane protein of 235 amino acids. Analysis of the amino acid sequence indicates that the protein has an N-terminal signal peptide of 26 amino acids, followed by a 156-amino acid extracellular domain, a 23-amino acid transmembrane domain, and a 30-amino acid cytoplasmic domain. There are two potential sites for N-linked glycosylation in the extracellular domain of the protein that are at the same positions as seen in the murine protein. The mouse and human flt3 ligand sequences are 72% identical at the amino acid level (Fig 3) and 76% identical at the nucleotide level in the coding region (data not shown). This level of homology is consistent with
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Fig 2. Nucleotide and amino acid sequence of the human flt3 ligand. The sequence shown is a composite constructed from the sequence of clone no. 9, to which 5' and 3' untranslated regions from clone no. 8 have been added (the ends of no. 9 are denoted by that number in the figure). The signal peptide is designated with the bold underline and the predicted transmembrane region is designated by the boxed amino acids. Two potential sites of N-linked glycosylation are underlined with a thin line; the putative poly A addition signal (TATAAA) is underlined twice. Nucleotides missing from the coding region of clones no. 14 (A30 bp, nucleotides 426 through 455; A179 bp, nucleotides 565 through 743) and no. 24 (A139 bp, nucleotides 426 through 564) are indicated by brackets, as are the 51 bp missing from the 3' noncoding region of clones no. 8, 14, and 24 (A51 bp 812 through 862). The position of a 17-bp insert in the leader peptide in clone 14 (CACCCGCTCCCCTG-CAG) is indicated by an inverted triangle. The four cysteines that are conserved between flt3 ligand, SLF, and CSF-l are circled. An asterisk denotes the point at which murine clones no. 6C and no. 5H diverge. The ATTTA mRNA instability motif in the 3' noncoding region is overlined. The filled circle indicates the end of clone no. 22-1. These sequence data are available from Genbank (Bethesda, MD) under the accession number U03858.

Fig 3. Human (bottom line) and murine (top line) flt3 ligand amino acid sequences are 72% identical. Amino acid identities are indicated by vertical lines between the corresponding amino acids; two dots indicate a high likelihood of amino acid replacement during evolution, and one dot indicates a somewhat lower likelihood of amino acid replacement during evolution.
overnight on day 3 and then harvesting the cells on day 4. The data control (no ligand).

Biologic activity of the human flt3 ligand. Recombinant soluble flt3 ligand was produced in yeast (see Materials and Methods) and tested for its capacity to stimulate the proliferation of a subpopulation of human BM cells that are low density, soybean agglutinin-negative, and CD34⁺. This subpopulation of BM cells is considered to be enriched for hematopoietic stem cell and progenitor cells and proliferated in response to the human flt3 ligand (Fig 4). The maximum response observed with these human cells (3.8-fold stimulation index) was similar to that seen with the murine flt3 ligand (data not shown). The CD34⁺ fraction of the low-density, soybean agglutinin-negative cells did not proliferate in response to flt3 ligand (data not shown).

The capacity of human flt3 ligand to stimulate clonal colony formation from CD34⁺ progenitor cells derived from BM and cord blood was also examined (Table 1). flt3 ligand alone induced significant colony formation from the BM progenitors but not the cord blood progenitors. The colonies observed were all of the granulocyte/monocyte (GM) type. The possibility that flt3 ligand could synergize with a number of cytokines known to induce colony formation was also tested. Synergy with flt3 ligand was observed in all three experiments with Pcs 321 and in two of the three experiments with IL-3 and GM-CSF. Colony size was also in-

Results obtained by Southern blot analysis in which hybridization of the murine ligand to human genomic DNA could not be detected after washing at moderate stringency (0.5× SSC at 63°C). The 3’ noncoding region of clone no. 8 ended in a poly A tail; 15 bp upstream of the tail was a sequence similar to a consensus poly A addition signal (TATAAA as compared with the consensus AATAAA) that likely directs polyadenylation of the message. The TATAAA sequence was found in both clones no. 8 and 24 (data not shown). Polyadenylation signals that are not AATAAA occur relatively infrequently, and this uncommon hexanucleotide sequence is used in hepadnaviruses to signal polyadenylation. Also seen in the 3’ noncoding region is a sequence motif (bp 1015 through 1019; ATTTA) that may be responsible for rapid turnover of the mRNA.

The coding regions of clones 8, 9, and 22-1 are identical except that clone 22-1 is truncated and ends approximately 13 amino acids before the termination codon. Clone 14 is unlikely to encode a biologically active protein as a result of the frame shift caused by the insertion of a 17-bp DNA segment in the middle of the signal peptide sequence (Fig 2). The 30-bp deletion seen in the middle of the clone no. 14 sequence begins at the same nucleotide as the 139-bp deletion seen in clone no. 24. The 30 nucleotides deleted in clone no. 14 by this splicing would remove 10 amino acids in the middle of the extracellular domain, but would preserve the reading frame. None of the four cysteines conserved between flt3 ligand, CSF-1, and SLF are in this segment, which is located between the third and fourth helices in our four helix bundle model of the flt3 ligand. The point at which the 139-bp deletion in clone no. 24 ends is at exactly the same point at which the 179-bp deletion in clone no. 14 begins; moreover, this point is also the exact point of divergence between two murine flt3 ligand cDNAs isolated previously. Thus, this splice junction has been retained in both species. Three of the clones (nos. 8, 14, and 22-1) have had 51 bp spliced out of their 3’ noncoding region; all of these splices begin and end at the same nucleotides.

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>flt3 Ligand</th>
<th>BM 1</th>
<th>BM 2</th>
<th>CB 1</th>
</tr>
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<tr>
<td>Media</td>
<td>—</td>
<td>3.0 ± 1.8</td>
<td>0.3 ± 0.5</td>
<td>2.5 ± 0.6</td>
</tr>
<tr>
<td>IL-3</td>
<td>+</td>
<td>18.5 ± 2.1</td>
<td>6.5 ± 3.3</td>
<td>9.0 ± 2.3</td>
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<tr>
<td>GM-CSF</td>
<td>—</td>
<td>ND</td>
<td>12.8 ± 2.2</td>
<td>28.5 ± 4.7</td>
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<tr>
<td>Pixy 321</td>
<td>—</td>
<td>37.8 ± 11.3</td>
<td>27.8 ± 6.04</td>
<td>47.8 ± 5.44</td>
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<tr>
<td>EPO</td>
<td>+</td>
<td>ND</td>
<td>36.5 ± 14.7</td>
<td>59.0 ± 8.04</td>
</tr>
<tr>
<td>SLF</td>
<td>—</td>
<td>7.5 ± 1.3</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SLF + IL-3</td>
<td>+</td>
<td>22.8 ± 4.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

CD34⁺ cells from two BM samples (BM 1, BM 2) and one cord blood sample (CB 1) were plated in clonal colony assays, and cytokines were added as described in Materials and Methods. Total colony numbers were scored on day 14 (ND, not done). The numbers represent the mean of quadruplicate wells ± SD. The degree of significance was tested using t-tests for contrasts from an analysis of variance, using all the data.

*P < .05, 1P > .05 but <.1 for flt3 ligand compared with media alone. All other figures derived from single factors were significant (P < .05) when compared to media, with the exception of SLF in BM 1 and IL-3 in BM 2.

† Colony numbers for flt3 ligand + growth factor were greater than additive, P < .05.
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Fig 5. Soluble human flt3 ligand stimulates the proliferation of BAF/BO3 cells transfected with the murine flt3 receptor. flt3-expressing BAF/BO3 cells (10,000/well) were plated in medium containing flt3 ligand and assayed for proliferation after 2 days of growth by pulsing the cells with [3H]-thymidine for 6 hours and then harvesting the cells. Each point represents the mean number of cpm from duplicate wells; the hatched bar represents the mean cpm ± SEM of duplicate samples for the medium alone control (no ligand). Stimulation of flt3-transfected BAF/BO3 cells with 29 ng/mL of IL-3 gave a mean cpm maximum of 22,424. Non-flt3 receptor-transfected BAF/BO3 cells showed no proliferative response to flt3 ligand (data not shown).

Fig 6. Northern blot analysis of flt3 ligand expression. Expression of the flt3 ligand in various organs was examined by probing human tissue Northern blots with a probe generated by PCR from the middle of the coding region of the human ligand (see Materials and Methods). mRNA for the ligand was detected in every tissue examined except brain (Fig 6). The size of the predominant flt3 ligand mRNA transcript was about 1.2 kb, which is consistent with the length of the composite cDNA clone shown in Fig 2. Weaker signals for larger mRNA transcripts were also detected in several tissues, but the significance of these bands is unclear.

Generation of a soluble form of ligand by mRNA splicing. The most interesting of the alternative splices seen in the flt3 ligand cDNAs was the Δ179-bp deletion in clone 14 (Fig 1). That deletion splices out part of the extracellular domain, the transmembrane region, and about half of the cytoplasmic tail of the protein; the entire C-terminus of the protein is replaced by 26 amino acids encoded in a different reading frame. An flt3 ligand cDNA containing this deletion would be predicted to produce only a soluble form of the ligand. To test this hypothesis, the Δ179-bp deletion was introduced into the full-length clone 9 cDNA, which was then transfected into CV-1/EBNA cells. The transfected cells were examined 2 days later for cell-surface expression of the ligand as well as for the presence of ligand activity in medium conditioned by the cells. In contrast to the wild-type clone, no expression of the flt3 ligand Δ179 was seen on the cell surface (Fig 7A), consistent with the removal of the transmembrane region. Bioassay of the medium conditioned by the transfected cells showed high levels of flt3 ligand activity in both wild-type (clone 9) and Δ179-transfected cells (Fig 7B). Generation of a soluble form of the flt3 ligand after transfection of the full-length cDNA has now been shown for both human (this report) and murine flt3 ligand proteins and presumably results from proteolytic cleavage of the membrane-bound form. Some cell types may lack the putative protease responsible for ligand cleavage; thus, alternative splicing provides a second method for generating a soluble form of the ligand.

DISCUSSION

We have succeeded in isolating a human homologue of the murine flt3 ligand gene and shown that this protein is biologically active on primitive human hematopoietic cells. The human ligand is 72% identical to the murine protein at the amino acid level and conserves many of the features of the murine protein, including glycosylation sites, key cysteine residues, and splice junctions. The human ligand can bind to a soluble version of the murine flt3 receptor (data not shown) and stimulates the proliferation of BAF/BO3 cells transfected with the murine receptor (Fig 5).
The murine and human flt3 ligands have different amino acids at their C-termini (Fig 3) and neither of these residues is valine. A carboxy terminal valine residue appears to be a critical determinant for extracellular cleavage of membrane-bound transforming growth factor α (TGFα) into a soluble growth factor. A C-terminal valine residue is also seen in SLF and CSF-1 (murine or human), which are also membrane-bound proteins that generate soluble, biologically active growth factors. Although the flt3 ligand appears to have a similar overall structure to SLF and CSF-1, and also generates a soluble form, it seems that either amino acids other than valine are capable of substituting for that residue to allow extracellular domain cleavage, or there is a different mechanism that leads to the generation of a soluble form of flt3 ligand. It would be of interest to determine if a C-terminal proline residue (as seen in mouse flt3 ligand) or histidine residue (human flt3 ligand) can substitute for a C-terminal valine residue to facilitate the extracellular cleavage of TGFα, SLF, or CSF-1.

The murine and human flt3 ligands are each capable of stimulating the proliferation of both murine and human cells, whereas the human factor acts on human cells but is 100 to 1,000 times less active on murine cells. In contrast to SLF, where the murine factor stimulates the proliferation of both murine and human cells, whereas the human factor acts on human cells but is 100 to 1,000 times less active on murine cells. The human flt3 ligand and, if so, if there is an alteration in the biologic activity, expression, or intracellular localization of the ligand associated with an expansion in this region may not be translated efficiently. However, the expression pattern of the ligand suggests that restrictive expression of the receptor may be the dominant factor in regulating ligand-receptor interactions. The apparent lack of flt3 ligand expression in human brain is interesting because the flt3 receptor is expressed in both fetal and adult mouse brain. Expression of the ligand may be localized to a small region of brain that either cannot be detected at the level of Northern blot analysis or was not part of the tissue from which the RNA was isolated. In situ hybridization analysis should help to resolve this issue.

The widespread expression of flt3 ligand mRNA was surprising, given that only a few of a large number of cell lines screened were capable of binding a soluble version of the flt3 receptor. It is not clear if expression of flt3 ligand protein mirrors expression of mRNA transcripts (the mRNA may not be translated efficiently). However, the expression pattern of the ligand suggests that restrictive expression of the receptor may be the dominant factor in regulating ligand-receptor interactions. The apparent lack of flt3 ligand expression in human brain is interesting because the flt3 receptor is expressed in both fetal and adult mouse brain. Expression of the ligand may be localized to a small region of brain that either cannot be detected at the level of Northern blot analysis or was not part of the tissue from which the RNA was isolated. In situ hybridization analysis should help to resolve this issue.
Now that the flt3 ligand has been cloned, we will be able
to determine which lineages of hematopoietic cells proliferate
in response to this factor, and at what stages within these
lineages the cells are responsive. Data obtained thus far
suggests that flt3 ligand alone induces modest prolifera-
tion and colony formation of CD34+ BM-derived progenitor
cells. However, in conjunction with the hematopoietic fac-
tors IL-3, GM-CSF, or Piyx321, synergistic effects were
observed. However, unlike SLF, no effect was observed on
erythroid colony formation when flt3 ligand was added with
EPO, nor did SLF appear to synergize with flt3 ligand (Table 1).
Experiments are now underway to determine which sub-
fractions of CD34+ cells are responsive to flt3 ligand.

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