Plasma/Serum Levels of flt3 Ligand Are Low in Normal Individuals and Highly Elevated in Patients With Fanconi Anemia and Acquired Aplastic Anemia

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The flt3 ligand is a growth factor that stimulates the proliferation of hematopoietic progenitor and stem cells. We established a sensitive enzyme-linked immunosorbent assay (ELISA) to measure the concentration of flt3 ligand in plasma or serum from normal individuals, as well as in patients with hematopoietic disorders. Concentrations of flt3 ligand in plasma or serum from normal individuals were quite low: only 12% (7 of 60) of normal individuals had flt3 ligand levels above 100 pg/mL (the limit of detection). In contrast, 86% (19 of 22) of samples from patients with Fanconi anemia and 100% (eight of eight) of samples from patients with acquired aplastic anemia had plasma or serum levels above 100 pg/mL. Mean plasma or serum concentrations (calculated by assigning a value of 0 pg/mL to any sample reading below the level of detection) were as follows: normal volunteers, 14 pg/mL; patients with Fanconi anemia, 1,331 pg/mL; and patients with acquired aplastic anemia, 460 pg/mL. Concentrations of flt3 ligand in blood are, therefore, specifically elevated to a level that may be physiologically relevant in hematopoietic disorders with a suspected stem cell component. The elevated flt3 ligand concentrations in these individuals may be part of a compensatory hematopoietic response to boost the level of progenitor cells.

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MATERIALS AND METHODS

Generation of antibodies to human flt3 ligand. Antibodies to human flt3 ligand were made against recombinant FLAG yeast-derived flt3 ligand, as outlined below. The term "FLAG" designates the presence of an eight-amino acid sequence at the N-terminus of the protein that facilitates its purification via anti-FLAG antibodies.13

Human flt3 ligand polyclonal antibody production. A New Zealand white rabbit was immunized subcutaneously with 25 μg FLAG yeast-derived human flt3 ligand extracellular domain (FLAG-human flt3 ligand) to generate polyclonal antisera. This antiserum was designated P1 (polyclonal 1).

Production of anti-human flt3 ligand monoclonal antibody. Lewis rats were injected subcutaneously with 10 μg FLAG yeast-derived human flt3 ligand extracellular domain in Freund's complete adjuvant. After two immunizations, one rat had immunoglobulin titers greater than 1:6,400, with no reactivity to irrelevant FLAG protein. That rat was given an intrapleural immunization of 2 μg FLAG yeast-derived human flt3 ligand in saline, and 3 days later, the spleen cells were fused with the NS1 myeloma cell line using polyethylene glycol. Fusion plates and cloning plates were screened by an antibody capture plate assay using biotinylated FLAG yeast-derived human flt3 ligand and by fluorescence-activated cell sorting (FACS) analysis using CV1 cells transfected with human flt3 ligand cDNA. Antibodies positive by FACS were also tested by ELISA against an irrelevant FLAG protein to be sure that they were not FLAG-reactive. Monoclonal antibody M5 (IgG2a isotype) tested positive in these assays and in an immunoprecipitation assay using flt3 ligand cDNA-transfected CV1 cells. Preliminary data indicate that the M5 antibody captures the FLAG-tagged mouse and human flt3 ligand extracellular domains and does not detect an irrelevant FLAG protein.

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antibody recognizes an epitope within the last 22 amino acids of the extracellular domain of flt3 ligand (data not shown).

**Human flt3 ligand ELISA.** Wells of polystyrene microtiter plates (Maxisorp; NUNC, Roskilde, Denmark) were coated with 100 μL of a 0.1 μg/mL solution of ascites-produced human flt3 ligand-specific monoclonal antibody M5 in 0.01 mol/L phosphate-buffered saline (PBS), pH 7.2, and incubated at 2°C to 8°C overnight. A Chinese hamster ovary (CHO)-derived human flt3 ligand standard (Immunex Corp, Seattle, WA) and human serum were diluted in a sample buffer comprised of PBS with 0.05% Tween-20 (PBST) with an additional 0.5 mol/L NaCl and 5% normal rat serum. The human flt3 ligand standard curve ranged from 1,600 to 25 pg/mL in twofold increments. Sera were tested at 1:4, 1:8, 1:16, and 1:32 dilutions in duplicate. All reagents were dispensed in 100 μL per well volumes, and the wells were washed with PBST before the addition and incubation of each successive reactant. Standards and sera were incubated for 1 hour at room temperature (RT), followed by a 1-hour RT incubation of a solution of rabbit anti-human flt3 ligand polyclonal P1, followed by a 1-hour RT incubation of a solution of peroxidase-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Color was developed with 3,3′,5,5′-tetramethylbenzidine (TMB) peroxidase substrate/chromogen solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 10 minutes at RT. The reaction was stopped with 1 mol/L H2PO4, and optical densities were determined at a wavelength of 450 nm. DeltaSoft microplate analysis software (BioMetallics, Princeton, NJ) was used to fit the standard curve by a four-parameter logistic model and to estimate sample concentrations by interpolation from the fitted curve. Standards used for flt3 ligand (CHO-derived or yeast-derived) were amino acid-analyzed to determine protein concentration. The ELISA protocols were performed blinded, without knowledge of the hematopoietic status of the sample donors.

Inhibition of detection of human flt3 ligand by soluble human flt3 receptor (huFlt3-Fc). A soluble version of the human flt3 receptor was constructed using the same method that was used to engineer a soluble version of the murine flt3 receptor. The extracellular domain of the receptor is fused to the Fc region of human IgG, and the protein is referred to as huFlt3-Fc. Samples containing Flt3 ligand and huFlt3-Fc in molar ratios ranging from 1:1 to 10:8 were prepared and preincubated at RT for 2 hours before analysis in the ELISA. The Flt3 ligand concentration was held constant at 800 pg/mL, while the concentration of huFlt3-Fc was varied. A sample of Flt3 ligand without huFlt3-Fc served as a reference control for calculations of percent Flt3 ligand detection. Flt3 ligand bioassay. Bioactivity of Flt3 ligand in human plasma samples was determined by measuring their capacity to stimulate the proliferation of the murine WWF7 cell line in a [3H]-thymidine incorporation assay, as previously described. Recombinant, soluble human Flt3 ligand produced in CHO cells was used as a positive control in these bioassays and was spiked into pooled normal human male serum (Sigma, St Louis, MO). A soluble form of the human Flt3 receptor (huFlt3-Fc fusion protein, see above) was used to inhibit Flt3 ligand bioactivity and thereby demonstrate the specificity of the proliferative response. The final concentration of the Flt3-Fc fusion protein in those assays where it was included was 2 μg/mL.

**Collection of human blood samples.** All blood samples were collected in compliance with each institution’s guidelines for human subjects. Blood samples were collected from normal volunteers after obtaining informed consent. Samples from patients with hematologic disorders were collected in the course of clinical evaluation. Plasma samples were prepared by collecting blood in tubes containing heparin and then removing the cells by centrifugation. Serum samples were prepared by allowing the blood to clot and then removing the clot by centrifugation. Total numbers of samples evaluated for each group are reported below, with the number of plasma/number of serum samples in parentheses: normal controls, 60 (49/11); Fanconi anemia, 22 (22/0); aplastic anemia, eight (seven/one); pure red cell aplasia, 13 (one/12); polycythemias, eight (none/eight); Diamond-Blackfan anemia, five (two/three); α thalassemia (two-gene deletion), three (three/none); anemia of undetermined origin with intact myelopoiesis, seven (seven/none); idiopathic thrombocytopenia purpura, one (one/none). Plasma and serum samples were stored frozen at −20°C or at −70°C before use.

A diagnosis of Fanconi anemia was established by the presence of various congenital malformations and/or hematologic abnormalities and by the demonstration of an increased frequency of chromosomal breakage in the presence of diepoxybutane. Patients with acquired aplastic anemia fulfilled the following criteria: hemoglobin level ≥10 g/dL or hematocrit ≥30%; platelet count ≥20 × 10⁹/μL, and granulocyte count ≥0.5 × 10⁹/μL. The bone marrow cellularity (ascertained by bone marrow biopsy) was less than 25% in all patients and was devoid of any neoplastic infiltration or significant fibrosis. None of the patients with acquired aplastic anemia exhibited congenital anomalies, growth retardation, or increased chromosomal breakage in the presence of diepoxybutane.

Patients with pure red cell aplasia had severe anemia (hematocrit less than 23%) and reticulocytopenia, while granulocyte and platelet counts were normal. Hemoglobinized cells comprised less than 2% of nucleated cells in the marrow aspirate. Patients with Diamond-Blackfan anemia had marrow erythropoietin hypoplasia and either a high mean corpuscular volume or fetal hemoglobin level. All patients met standard diagnostic criteria.

Eight patients in our study had polycythemias, three of whom had polycythemia rubra vera. These three subjects had documented elevation of hemoglobin concentration with confirmed elevation of red cell mass, as measured by [51Cr]-labeling of red cells using standard methods. All subjects had elevated platelet counts. The diagnosis of polycythaemia vera was confirmed by demonstration of a normal or low serum erythropoietin level, normal hemoglobin oxygen dissociation-P50 determination, and erythropoietin-independent colonies in clonogenic assays of erythropoietin progenitors.

Three additional subjects had secondary congenital polycythemia, normal P50, normal arterial blood gases, and stable, significantly elevated erythropoietin levels. Polycythemia was confirmed by red cell blood volume measured by [51Cr] assay. Two other subjects had primary congenital polycythemia with a low erythropoietin level, increased sensitivity of erythroid progenitors to erythropoietin, and normal white blood cells and platelets. These two unrelated patients had different mutations of the erythropoietin receptor in its cytoplasmic domain, which was shown to result in the hypersensitivity of erythroid progenitors to erythropoietin.

**Statistical analysis.** Wilcoxon two-sample tests were performed pairwise to compare the various patient and control groups statistically.

**RESULTS**

**Establishment of the ELISA.** Monoclonal and polyclonal antibodies raised against human yeast-derived Flt3 ligand were used to establish a sandwich ELISA. Although the assay was developed using antibodies directed against Flt3 ligand made in yeast, no difference was seen in standard curves calibrated using either yeast-derived Flt3 ligand or Flt3 ligand produced by CHO cells (Fig 1). We were unable to use the native Flt3 ligand protein as an ELISA standard because it has not been purified from human blood, and we have not determined the exact C-terminus of the native protein. However, there appeared to be no difficulties in measuring this
SERUM LEVELS OF flt3L ARE ELEVATED IN FA/AA

Fig 1. Standard curve of yeast-derived and CHO-derived flt3 ligand in the ELISA. The graph shows the standard curve used to determine plasma or serum levels of flt3 ligand. Recombinant soluble human flt3 ligand produced in either yeast (■) or CHO cells (○) read out identically in this assay.

Fig 2. Effect of soluble flt3 receptor on detection of human flt3 ligand by ELISA. Human flt3 ligand (800 pg/mL) and varying amounts of soluble human flt3 receptor (a human Flt3-Fc fusion protein) were mixed in molar ratios ranging from 1:0.8 to 1:1,000 and incubated at room temperature for 2 hours before analysis in the ELISA. A sample of human flt3 ligand without Flt3-Fc served as a reference control for calculations of percent human flt3 ligand detection.

There have been no reports in the literature of soluble flt3 receptors in human blood. However, given the high serum levels (mean, 324 ng/mL) reported for soluble c-kit receptor, we tested whether soluble human flt3 receptor could inhibit the capacity of the ELISA to measure flt3 ligand. This was accomplished using a fusion protein that links the extracellular domain of the human flt3 receptor to the Ig portion of human IgG. No inhibition of ligand binding was seen until a sixfold molar ratio of receptor to ligand was reached (Fig 2). Maximal inhibition of about 70% occurred at approximately a 100-fold molar excess; increasing the amount of soluble receptor to a 1,000-fold molar excess produced no additional inhibition.

Plasma/serum levels of flt3 ligand. In most normal individuals, the flt3 ligand plasma/serum concentration was less than 100 pg/mL, which is the limit of detection of our ELISA (Fig 3). The highest level measured in a normal person was only 152 pg/mL. Normal plasma/serum levels of flt3 ligand are, therefore, significantly lower than the normal levels of two related growth factors, CSF-1 and Steel factor, both of which are normally found in the 1- to 8-ng/mL range. Plasma/serum flt3 ligand levels measured in patients with pure red cell aplasia (n = 12), α thalassemia (n = 3), Diamond-Blackfan anemia (n = 5), anemia of undetermined origin (n = 7), or idiopathic thrombocytopenia purpura (n = 1) were also quite low, with most of the samples being below our level of detection. We also measured flt3 ligand levels in serum from patients with various polycythemias (n = 8) and found these to be in the normal range (Fig 3).

In contrast with these results, flt3 ligand plasma levels in patients with either Fanconi or aplastic anemias were greatly elevated (Fig 3). Whereas only 12% (7 of 60) of normal individuals had flt3 ligand levels above 100 pg/mL, 86% (19 of 22) of patients with Fanconi anemia and 100% (eight of eight) of patients with acquired aplastic anemia had plasma levels above this amount. We assigned a value of 0 pg/mL to any sample that was below our ELISA level of detection of 100 pg/mL to calculate mean plasma/serum levels. This enabled us to calculate the following mean ± SD plasma/serum flt3 ligand concentrations: normal individuals, 14 ± 39 pg/mL (median, 0 pg/mL; range, 0 to 152 pg/mL); Fanconi anemia, 1,331 ± 2,350 pg/mL (median, 453 pg/mL; range, 0 to 10,815 pg/mL); and aplastic anemia, 460 ± 187 pg/mL (median, 426 pg/mL; range, 223 to 887 pg/mL). Plasma/serum concentrations of flt3 ligand in the Fanconi or aplastic anemia groups were significantly greater than both the normal donor group (P = .0001) and the pure red cell aplasia (mean, 27 ± 64 pg/mL; median, 0 pg/mL; range, 0 to 191 pg/mL) or polycytemia (mean, 15 ± 41 pg/mL; median, 0 pg/mL; range, 0 to 123 pg/mL) groups (P = .0001). Thus, flt3 ligand levels are substantially elevated in patients with these two stem cell-based anemias compared with normal individuals or those with several other hematopoietic disorders. Analysis of the levels of flt3 ligand in patients with Fanconi anemia as a function of age, transfusion status, hemoglobin level, or disease severity showed that there was neither a positive nor negative correlation between these parameters (data not shown). The variations in flt3 ligand levels are much greater in the samples from patients with Fanconi anemia than in those from patients with aplastic anemia. We believe that most of the increased
variability is likely due to the sample size, which is almost three times larger for the Fanconi group (22 patients) relative to the aplastic anemia group (8 patients).

Plasma samples from the three Fanconi anemia patients with flt3 ligand concentrations above 3,500 pg/mL (Fig 3) were tested in a proliferation assay to determine if the flt3 ligand measured in the ELISA was biologically active. All three of these Fanconi anemia plasma samples stimulated the proliferation of WWF7 cells, and this activity was specifically inhibited by the addition of soluble flt3 receptor (Fig 4). In contrast, plasma samples from the three patients with Fanconi anemia that had no detectable flt3 ligand by ELISA did not stimulate the proliferation of the WWF7 cells (data not shown). The specific activity of endogenous flt3 ligand in the Fanconi plasma samples is very similar to the specific activity of the recombinant flt3 ligand produced in CHO cells (data not shown). Whether the elevated levels of flt3 ligand in patients with Fanconi anemia and acquired aplastic anemia are physiologically effective in stimulating hematopoiesis cannot be experimentally resolved.

One of the patients with Fanconi anemia (patient E.M.) received an HLA-matched cord blood transplant from a sib-

![Fig 3](image1)

**Fig 3.** Levels of flt3 ligand in plasma or serum from normal individuals and those with various hematopoietic disorders. The numbers shown below the 100-pg/ml line are the numbers of individuals whose serum levels measured below the limit of detection in the ELISA. Mean flt3 ligand concentrations in plasma and serum are shown beneath each group. To determine this number, a value of 0 pg/mL was given to all samples that read out below the level of detection in the ELISA. "Anemia-Other" refers to anemias of undetermined origin with intact myelopoiesis.

![Fig 4](image2)

**Fig 4.** Bioactivity of flt3 ligand in plasma from patients with Fanconi anemia. (A) Proliferative response of the WWF7 cell line to recombinant, CHO-derived flt3 ligand that was spiked into normal human serum (Sigma). The highest concentration of flt3 ligand in the assay was 25 ng/mL; serial dilutions are twofold. This activity was completely inhibited by the addition of 2 μg/mL soluble flt3 receptor. (B) Proliferative response of WWF7 cells to plasma from three patients with Fanconi anemia in the absence or presence of 2 μg/mL Flt3-Fc. Plasma levels of flt3 ligand (determined by ELISA) for these three patients were as follows: S.P., 10,815 pg/mL; L.K., 3,560 pg/mL; D.W., 4,755 pg/mL. The Flt3-Fc completely inhibited the flt3 ligand activity in plasma from all three patients.
Serum levels of FLT3 are elevated in FAAN

**DISCUSSION**

The data presented here show that FLT3 ligand levels in plasma/serum from humans are normally quite low, but are elevated to a high degree in acquired and constitutional aplastic anemias. Our hypothesis is that the elevated FLT3 ligand levels in Fanconi anemia and acquired aplastic anemia patients result from a physiologic attempt to compensate for an intrinsic deficiency in their stem cell compartment. In this model, the hematopoietic system attempts to compensate for a stem cell deficiency by inducing production of a factor, FLT3 ligand, that stimulates the proliferation of these cells. In contrast, FLT3 ligand plasma/serum levels are not elevated in pure red cell aplasia, Diamond-Blackfan anemia, anemias of undetermined origin, and α thalassemia, because these hematopoietic disorders are not at the level of the stem cell and instead are restricted primarily to the erythroid lineage.

Polycythemia vera is a myeloproliferative disorder associated with the overproduction of cells from multiple hematopoietic lineages, primary the erythroid lineage. According to our hypothesis outlined above, patients with polycythemia vera would not be expected to have elevated FLT3 ligand levels because they have no stem cell deficiency and, therefore, no need to produce a factor that functions to produce more of these cells. Levels of FLT3 ligand were not elevated in any of the polycythemia samples, including the three patients with polycythemia vera (Fig 3), supporting our hypothesis that FLT3 ligand blood levels are only elevated in disorders where there is a lack of stem or progenitor cells.

We have shown in this report that the elevated levels of soluble FLT3 ligand observed in some of the Fanconi anemia plasma samples are high enough to stimulate the proliferation of a FLT3 ligand-responsive cell line. However, these levels of FLT3 ligand would not be predicted to be high enough to saturate FLT3 receptors and, therefore, would be unlikely to produce a maximal physiologic response. The level of FLT3 ligand measured in plasma/serum from normal individuals (generally less than 100 pg/mL) is significantly below the amount required to see a proliferative response in vitro, suggesting that normal circulating levels of FLT3 ligand are not high enough to be physiologically active. However, our measurements of plasma/serum levels of FLT3 ligand do not address the role cell surface FLT3 ligand plays in hematopoiesis. Soluble Steel factor has been shown to have different biologic effects in vivo compared with cell surface-expressed Steel factor; the same may be true for FLT3 ligand as well. Several of the patients with Fanconi anemia that had elevated plasma FLT3 ligand concentrations also provided bone marrow aspirates. Levels of FLT3 ligand in plasma and bone marrow were very similar (data not shown).

One complicating factor in attempting to determine circulating levels of FLT3 ligand is the fact that there may be soluble FLT3 receptors present in serum that interfere with our ELISA. No reports of soluble FLT3 receptor have been published to date, but levels of a soluble form of the c-kit receptor, which is structurally related to FLT3, have been reported to be extremely high in human serum. The high levels of c-kit did not, however, prevent measurement of soluble Steel factor in serum. We acknowledge that it is possible that our ELISA is reflecting differences in the serum levels of FLT3 receptor, which may result in differences in free (ie, unbound) ligand that can be measured in our assay.

One issue that we have not addressed is the mechanism responsible for generating both the normal and elevated levels of circulating, soluble FLT3 ligand in human serum. Soluble FLT3 ligand can be generated by either proteolytic cleavage of a transmembrane protein or alternative splicing of mRNAs. The exact tissue source(s) that produces the soluble FLT3 ligand is the fact that there may be soluble FLT3 receptors present in serum that interfere with our ELISA

A comparison of the levels of FLT3 ligand and Steel factor in normal individuals and those with hematopoietic disorders reveals some striking differences. Levels of Steel factor (as well as CSF-1) in normal human serum average several nanograms per milliliter, much higher than the levels of FLT3 ligand reported here. Mean Steel factor serum levels were somewhat lower in patients with aplastic anemia compared with normal individuals, and the same is true for patients with Fanconi anemia (N.T.S. and J.C.S., unpublished observation, February 1995). Similarly, mean serum levels of Steel factor were about 25% lower than normal in patients with a variety of preleukemic disorders compared

![Graph showing plasma levels of FLT3 ligand in patient E.M. with Fanconi anemia before and after undergoing an HLA-matched cord blood transplant.](image-url)
with normal individuals. Serum levels of Steel factor appeared to be somewhat elevated in only 2 of 15 patients with Diamond-Blackfan anemia (J.L.A., unpublished data, May 1995). Our central conclusion from these published studies as well as our data presented here is that circulating levels of flt3 ligand are significantly elevated in patients with Fanconi anemia or acquired aplastic anemia, whereas serum levels of Steel factor appear to be essentially normal or somewhat decreased. These results underscore the fact that while both flt3 ligand and Steel factor stimulate the proliferation of hematopoietic progenitor cells, there appear to be major differences in their biologic regulation.

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