Flt3 Ligand Level Reflects Hematopoietic Progenitor Cell Function in Aplastic Anemia and Chemotherapy-Induced Bone Marrow Aplasia

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Flt3 ligand (flt3L) is a member of a small family of cytokines acting as tyrosine kinase receptor ligands that stimulate the proliferation of primitive hematopoietic progenitors in vitro. To gain insight into the physiological role of flt3L in early hematopoiesis, levels of flt3L were determined in serum of patients with multilineage bone marrow failure and related to the severity of stem cell depletion. In patients with aplastic anemia (AA) and in cancer patients with chemotherapy-induced transient suppression of hematopoiesis, flt3L fluctuated in an inverse relationship to the degree of bone marrow failure. In severe AA at diagnosis, levels of circulating soluble flt3L were highly elevated (2,653 ± 353 pg/mL) as compared with normal blood serum values of 14 ± 39 pg/mL. Flt3L returned to near normal levels within the first 3 months following successful bone marrow transplantation and in autologous remission induced by immunosuppressive therapy with antithymocyte globulin (ALG; 100 ± 31 and 183 ± 14 pg/mL, respectively). In contrast, rejection of the graft or relapse of the disease after ALG was accompanied by an increase to high pretreatment concentrations of the circulating cytokine (3,170 ± 2,485 and 1,788 ± 233 pg/mL, respectively). Flt3L in serum inversely correlated with the colony-forming ability of AA bone marrow precursors in vitro (R = -0.86), indicating that the concentration of the ligand reflects hematopoiesis at the progenitor cell level. Flt3L increased to 2,500 pg/mL in the serum of leukemia patients during chemoradiotherapy-induced bone marrow suppression and returned to normal values along with hematopoietic recovery. Expression of the membrane-bound form of flt3L was significantly elevated in mononuclear bone marrow and peripheral blood cells from patients with severe pancytopenia, suggesting de novo synthesis of the factor in response to bone marrow failure. The data provide a strong argument for the involvement of flt3L in the regulation of early hematopoiesis in vivo.

Hematopoietic growth factors play an important role in the control of self-renewal and differentiation of hematopoietic stem cells within the bone marrow microenvironment. A large number of these cytokines are known to act on purified progenitor cell populations in vitro (reviewed in Ogawa). However, less is known about the mechanisms that regulate the proliferation and differentiation of stem cells in vivo. In the mouse, spontaneous or targeted disruption of genes encoding hematopoietic cytokines or their receptors helps in the understanding of early hematopoietic regulation. In humans, naturally occurring or treatment-induced bone marrow failure provides a model for the study of cytokine regulation at the stem cell level.

Aplastic anemia (AA) is an acquired disorder characterized by unexplained failure of the bone marrow to produce blood cells. Phenotypic and functional analysis of the stem cell pool has demonstrated a strong reduction of CD34 anti- gen-bearing progenitor cells with in vitro clonogenic potential. Immune processes have been implicated as the major pathophysiological mechanism responsible for destruction of the bone marrow in AA. The severe form of AA (SAA) is fatal if untreated. Relapse is frequent despite improvement of therapeutic strategies that involve either bone marrow transplantation (BMT) or immunosuppression with antithymocyte globulin (ALG) and cyclosporin A.

Flt3 ligand (flt3L) is a novel hematopoietic growth factor, which acts as a ligand for the tyrosine kinase receptor flt3. While the ligand is expressed ubiquitously by a number of hematopoietic and nonhematopoietic tissues, expression of its receptor is restricted to early hematopoietic progenitors. In human bone marrow, flt3 is detectable on the surface of CD34+ cells, but not CD34- cells. In vitro, flt3L was shown to support maintenance and to stimulate proliferation of stem and progenitor cells, suggesting that this growth factor plays a role in regulation of early hematopoiesis. Biologically active flt3L exists in a soluble and a membrane-bound form. The properties and structure of flt3L and flt3 receptor resemble those of stem cell factor (SCF) and its receptor, c-kit, the tyrosine kinase ligand-receptor pair known to influence the function of very immature hematopoietic cells.

It has recently been reported that levels of circulating soluble flt3L are elevated in serum of patients with AA and its congenital form, Fanconi anemia. To find out whether the level of circulating flt3L reflects hematopoietic activity in vivo, we monitored its concentration in patients with AA at presentation, in remission, and in relapse, as well as in leukemia patients with transient bone marrow aplasia induced by chemoradiotherapy. Results demonstrated a significant inverse correlation between flt3L levels and the degree of stem cell depletion, suggesting that flt3L reflects the function of hematopoietic progenitor cells in spontaneous and treatment-induced failure of hematopoiesis.

MATERIALS AND METHODS

Serum from AA patients. A bank of AA sera had been stored in the context of a long-term study on the treatment of SAA at our...
Clusters of dead cells from analysis, propidium iodide at formation assay was performed in 1% methylcellulose cultures containing flt3L for the detection of apoptotic cells. Analysis of membrane-bound flt3L was performed on a FACScan (Becton Dickinson, San Jose, CA).

Sera from leukemia patients. The serum of three patients who underwent myeloablative therapy for acute leukemia was examined daily. Patient N.C. was treated for acute lymphocytic leukemia (B-cell ALL) and died of infection while pancycopenic. Patient J.K. was treated with high-dose chemotherapy, without total body irradiation, as preparation for a peripheral blood stem cell transplant (PBSCT) for relapsing ALL. Patient D.S. was prepared for an autologous PBSCT with chemotherapy and total body irradiation for acute nonlymphocytic leukemia (ANLL).

Controls. Peripheral blood from 60 donors was used with informed consent.

Preparation of peripheral blood serum. Serum was collected from native peripheral blood after centrifugation for 10 minutes at 25,000 rpm. Samples were aliquoted and stored at -70°C until used.

Mononuclear cells. Mononuclear cells were isolated from heparinized peripheral blood or bone marrow by Ficoll-Hypaque density gradient centrifugation (d = 1.077). Cells were immediately used for colony assay or for fluorescence-activated cell sorter (FACS) analysis, without prior freezing.

Determination of flt3L. Concentrations of soluble flt3L in control and patient serum were measured by an enzyme-linked immunosorbent assay (ELISA) assay and calculated as described. To detect cell surface flt3L, 5 x 10^5 mononuclear peripheral blood or bone marrow cells were washed in phosphate-buffered saline and incubated for 20 minutes with 10 mg/mL of monoclonal antibodies against human flt3 ligand (rat antihuman IgG2a) followed by fluorescein isothiocyanate (FITC)-conjugated secondary sheep antirat IgG antibody (Silenes, Hawthorn, Victoria, Australia). To exclude dead cells from analysis, propidium iodide at 10 mg/mL was included in the final wash. Gates were set according to forward and sideward light scatter to exclude residual granulocytes, erythroblasts, and apoptotic cells. Analysis of membrane-bound flt3L was performed on a FACSscan (Becton Dickinson, San Jose, CA).

Hematopoietic colony formation assay. Hematopoietic colony formation assay was performed in 1% methylcellulose cultures containing 2 x 10^5 bone marrow mononuclear bone marrow cells/mL, as described. Hematopoietic colonies formed by myeloid and erythroid precursors were counted after 14 days in culture.

Statistical analysis. Spearman rank correlation test was used to analyze the relationship between flt3L serum levels with the peripheral blood neutrophil counts and with the number of hematopoietic colonies formed by bone marrow precursors.

RESULTS

Serum levels of flt3 ligand in aplastic anemia in relation to patients' clinical status. Using a recently established ELISA assay, we determined the concentration of circulating flt3L in serum of patients with AA at different time points in relation to therapy. The analyzed collection of 122 sera from 34 patients included 18 pretreatment sera, 16 sera from patients after BMT and 88 sera collected following treatment with ALG (Table 1). At diagnosis, flt3L levels were highly elevated (2,653 ± 353 pg/mL) compared with normal (14 ± 39 pg/mL). The lowest amount of 489 pg/mL was measured in a patient with moderate AA, while in 17 patients with severe AA, the values ranged from 1,000 to 5,900 pg/mL. Flt3L concentrations returned to near normal mean values (100 ± 31 pg/mL) in patients who were transplanted successfully, and in those who achieved stable autologous hematopoietic function after ALG (183 ± 14 pg/mL). In contrast, rejection of the graft and relapse of the disease after ALG were associated with high flt3L (3,770 ± 2,485 and 1,788 ± 233 pg/mL, respectively), resembling pretreatment values. For all measured samples, there was a significant negative correlation between serum flt3L and the peripheral blood neutrophil count, a transfusion independent parameter of hematopoietic function (R = -0.73, P < 0.001).

When examined in individual patients at multiple time points, the serum levels of flt3L fluctuated in relation to the clinical status (Fig 1). High pretreatment values normalized within 3 months in transplanted patients (Fig 1A and B). Flt3L concentrations remained low in those with a stable graft (patients 1 through 4), but increased again when clinical parameters indicated rejection (patients 5 and 6). A similar observation was made in patients treated with ALG (Fig 1C and D). Initially high flt3L levels decreased and remained low in patients who had long-lasting remission (patients 7 through 10). In those who never achieved stable hematopoietic improvement, flt3L remained elevated throughout the observation period (patients 11 through 16).

Serum levels of flt3 ligand and colony-forming ability of bone marrow cells in aplastic anemia. Because clonogenicity of precursor cells is a measure of multilineage marrow function, we investigated the relationship between serum flt3L and the ability of patients' bone marrow to form hematopoietic colonies in vitro. Bone marrow, collected at the time of serum sampling, was examined for colony formation by myeloid and erythroid precursors (Fig 2). Results of the analysis demonstrated an inverse relationship between total colony numbers and serum flt3L (Fig 2A). When flt3L levels were elevated to over 1,000 pg/mL, the corresponding marrow colonies formed on average only 0.9 ± 0.3 colonies/2 x 10^5 cells, indicating that high flt3L reflects severe loss or damage to clonogenic precursor cells. The correlation between flt3L and a number of colonies was not linear, but hyperbolic. The Spearman rank correlation test showed a highly significant negative correlation of the two parameters (R = -0.86; P <
FLT3L IS AN INDICATOR OF HEMATOPOIETIC FUNCTION

**A. BMT → remission**

![Graph](image1)

**B. BMT → graft rejection**

![Graph](image2)

**C. ALG → remission**

![Graph](image3)

**D. ALG → relapse of AA**

![Graph](image4)

**Fig 1.** Changes in FLT3L serum levels in patients at diagnosis of severe AA (dark bars) and at different time points (months, m; years, y) after BMT (A and B) or immunosuppression with ALG (C and D). Some patients underwent additional BMT or more than one course of ALG treatment, as indicated in the figure. Remission was defined as: Hb ≥ 12 g/dL; neutrophils ≥ 1.5 × 10^9/L; platelets ≥ 100 × 10^9/L. Relapse was defined as transfusion dependence after ≥3 months of independence or transition from complete to partial remission.

.0001). The inverse relationship between the level of growth factor in serum and hematopoietic bone marrow activity in vitro is further illustrated by examples of individual patients analyzed at multiple time points at diagnosis and following therapy (Fig 2B). It is apparent that changes in FLT3L in serum are reciprocal to the colony forming ability in vitro and the neutrophil counts in vivo.

**Serum levels of FLT3 ligand in leukemia patients undergoing chemoradiotherapy.** To find out whether an increase of FLT3L in the serum is specific for primary bone marrow failure syndromes or also occurs in transient pancytopenia induced by chemotherapy, we tested serum from leukemia patients before, during, and after chemotherapy or pretransplant conditioning. Figure 3 shows results of daily FLT3L measurements and peripheral blood leukocyte counts in three individual patients. Changes in FLT3L levels and cell counts were reciprocal. Myeloablative therapy was associated with an abrupt increase of FLT3L to over 2,000 pg/mL. The FLT3L level remained high in patient N.C. who developed severe infection and died while severely pancytopenic. Patient J.K. underwent peripheral blood stem cell transplantation (PBSCT) after high-dose chemotherapy; the FLT3L level returned to normal when increasing leukocyte counts indicated engraftment. The FLT3L serum levels mirrored the leukocyte counts in patient D.S. who was prepared for PBSCT with high-dose chemotherapy and total body irradiation. In all three patients, FLT3L correlated inversely with peripheral leukocyte counts (R = -0.69; P < .0001). FLT3L was also measured in serum of two patients undergoing chemotherapy for solid tumors, mammary, and bladder carcinoma and found to be elevated to 893 and 192 pg/mL when peripheral blood leukocyte counts were 0.49 and 0.95 × 10^9/L, respectively.

**Expression of membrane-bound FLT3 on mononuclear cells from peripheral blood and bone marrow.** To test whether the increase of soluble FLT3L in serum of patients with severe pancytopenia is associated with an increase of its membrane-bound isoform, we examined expression of FLT3L on the surface of peripheral blood and bone marrow cells. Cell surface bound ligand was detected by flow cytometry using specific monoclonal antibodies against human FLT3L (Fig 4). Expression of FLT3L was weak on the surface of control mononuclear cells, but was significantly higher on cells derived from bone marrow of a patient with SAA and from peripheral blood of a leukemia patient undergoing chemotherapy. In chemotherapy-induced aplasia, membrane-bound FLT3L was found on the surface of T lymphocytes (not shown), although contribution from other mononuclear cell populations cannot, at present, be excluded.

**DISCUSSION**

In vitro studies suggest that FLT3L plays a role in regulating the proliferation and differentiation steps of early hematopoietic progenitor cells. To assess the role of FLT3L in human hematopoiesis in vivo, we monitored serum levels of the
Several lines of evidence suggest that changes in FLT3 reflect regulatory events at the early progenitor cell level. It has previously been shown that increase of FLT3 is specific to anemias resulting from multilineage bone marrow failure and is not found in cytopenias involving single cell lineages. In bone marrow of AA patients with highly elevated FLT3, in vitro clonogenic precursor cells are virtually absent, indicating that high FLT3 levels reflect severe damage to the progenitor cell compartment. When platelets or red blood cells are replaced in severely pancytopenic patients or when their neutrophil count is raised by granulocyte colony-stimulating factor (G-CSF), FLT3 does not return to normal levels (unpublished observations, August 1995), suggesting that single lineage correction is not sufficient for normalization of the ligand. Interestingly, in AA patients who were in clinical remission after ALG treatment, FLT3 decreased significantly, but did not normalize completely, confirming the residual stem cell defect after immunosuppression, as documented also by persisting growth defect in vitro. It appears unlikely that elevated FLT3 itself causes or contributes to bone marrow suppression because daily measurements in patients undergoing chemotherapy have shown that increases in FLT3 levels did not precede, but precisely coincided with the drop of peripheral blood counts. The time relationship between endogenous circulating ligand and treatment-induced secondary bone marrow aplasia further supports the assumption that an increase of FLT3 reflects a physiological response to depletion of the stem cell compartment.

Disease-dependent fluctuations of circulating ligand distinguish it from a related cytokine, SCF. Although FLT3 and SCF both act at a primitive level of hematopoiesis and activate structurally similar tyrosine kinase receptors, their serum levels in steady-state condition and in human bone marrow failure syndromes differ profoundly. SCF is present in normal serum in a relatively high concentration (3,300 ± 1,000 pg/mL). In AA, the level of SCF is at the low range of normal with wide interindividual variations at diagnosis and without obvious changes in relation to therapy. Serum SCF also remains unchanged during conditioning chemotherapy for PBSCRT. Therefore, in contrast to FLT3, the level of circulating SCF does not reflect hematopoietic bone marrow function in vivo, implicating major differences in the biological role of FLT3 and SCF in early human hematopoiesis. Additive function of the two cytokines in hematopoietic development is implicated by the results of the recently reported targeted disruption of their receptors in double mutant mice, displaying severe abnormalities in B lymphoid and stem cell compartments.

The absence of correlation between SCF serum levels and hematopoietic function in patients with bone marrow failure syndromes, has been taken as an argument against the clinical value of SCF serum measurements. In contrast, circulating erythropoietin (Epo) and thrombopoietin (TPO), which are elevated in response to peripheral requirements, are acknowledged as indicators of hematopoietic activity in the erythroid and the thrombopoietic line, respectively. In analogy, serum FLT3 may be a clinically useful indicator of hematopoietic activity at the stem cell level.
The source of elevated Flt3L in serum during pancytopenia remains unknown. The widespread expression of Flt3L mRNA in human adult tissues including peripheral blood cells, kidney, and spleen suggests that several organs are potentially capable of producing the ligand in response to bone marrow failure. We found elevated expression of the membrane-bound Flt3L form on the surface of mononuclear cells, indicating that peripheral blood and bone marrow cells can serve as a source of the soluble isoform that is known to arise from proteolytic cleavage of the membrane-associated ligand or to be generated independently by an alternatively spliced mRNA. Regardless of which cells or organs provide
the ligand, two mechanisms regulating levels of flt3L under condition of bone marrow failure can be envisaged. The amount of circulating flt3L may reflect the rate of its uptake and destruction by cells expressing flt3 receptors, as has been described for TPO, which is regulated by the platelet level. However, in contrast to platelets, hematopoietic precursors carrying flt3 receptors circulate at very low numbers and are, therefore, unlikely to metabolize flt3L if constitutively produced in the nanograms per milliliter range. Alternatively, high serum flt3L may be due to its increased synthesis, in analogy to the transcriptional activation of the Epo gene in response to anemia or hypoxia. The increase of membrane-bound flt3L in mononuclear cells in severe pancytopenia suggests that at least part of the ligand becomes newly produced in response to stem cell depletion. In conclusion, we propose that flt3L might be a regulator of the stem cell pool. In analogy to the feed-back mechanism in which an "oxygen-sensor" regulates expression of Epo in the kidney, a putative "stem cell sensor" responding to bone marrow hypoplasia may be controlling the level of flt3L.

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