Flt3-mediated signaling in human acute myelogenous leukemia (AML) blasts: a functional characterization of the effects of Flt3-ligand in AML cell populations with and without genetic Flt3 abnormalities

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Background and Objectives. Intracellular signaling initiated via Flt3 seems important in both leukemogenesis and chemosensitivity in acute myelogenous leukemia (AML). Flt3 is activated by binding of its natural Flt3-ligand (Flt3-L), but Flt3 genes with internal tandem duplications (Flt3-ITD) or Asp(D)-835 point mutations encode molecules with constitutive activation. The aim of this study was to compare functional effects of exogenous Flt3-L on AML blast populations with and without genetic Flt3 abnormalities.

Design and Methods. Native AML blasts were derived from 64 consecutive patients with high blast counts in peripheral blood, and *in vitro* models were used to characterize the Flt3-L effects.

Results. The Flt3 protein levels showed a similar wide variation between AML blast populations with and without genetic Flt3 abnormalities. Flt3-L was an autocrine growth factor only for 2 patients. Flt3-ITD$^+$ AML cells had lower responsiveness to exogenous cytokines than cell populations without Flt3 abnormalities, but exogenous Flt3-L increased blast proliferation both for patients without Flt3 abnormalities and patients with Flt3-ITD as well as D835 mutations. This enhancement was observed even in the presence of other exogenous cytokines and included clonogenic AML progenitors. Flt3-L inhibited proliferation only for 1 patient, but had divergent effects on AML blast cytokine release. Flt3-L affected AML blast differentiation (inhibition of erythroid colonies, increased neutrophil granulation) only in a minority of patients, whereas it had an anti-apoptotic effect for a larger subset of patients.

Interpretation and Conclusions. Intracellular signaling initiated by Flt3 ligation modulates the functional phenotype for native human AML blasts both with and without genetic Flt3 abnormalities.

Key words: acute myelogenous leukemia, Flt3 internal tandem duplications, Flt3-D835 mutations, Flt3 ligand, cytokines, *in vitro* effects.

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Acute myelogenous leukemia (AML) is characterized by clonal proliferation of immature myeloid cells and has an overall disease-free survival after intensive chemotherapy of less than 50%.[1-4] The most important cause of death is AML relapse,[1-4] and the two most important predictors of relapse have been cytogenetic abnormalities and response to induction therapy.[2] However, recent studies have demonstrated that abnormalities of the Flt3 gene (a membrane-anchored receptor tyrosine kinase) are also associated with an increased relapse risk.[3-12] At diagnosis nearly 30% of AML patients have genetic Flt3 abnormalities,[10-12] and new abnormalities can develop later in leukemia relapse.[13,14] The adverse prognostic impact of Flt3 internal tandem duplications (Flt3-ITD) is now well established,[3,4] and recent evidence suggests that Asp(D)-835 point mutations have a similar prognostic effect.[12]

The Flt3 gene encodes for a tyrosine kinase with an extracellular ligand-binding part and an intracellular catalytic unit.[6-10] Ligation of the Flt3 molecule induces activation of the tyrosine kinase through ligand-induced receptor oligomerization and autophosphorylation with subsequent phosphorylation of cytoplasmic substrates.[5-9] However, genetic Flt3 abnormalities result in the expression of a tyrosine kinase with constitutive activity in the AML blasts.[8,9,15,16] Previous experimental evidence suggests that the constitutive activation is implicated in leukemogenesis, and recent clinical studies have demonstrated that constitutive kinase activation is also important for chemosensitivity in AML.[3,4,12]

The clinical and experimental studies discussed above suggest that intracellular signaling events initiated by Flt3 activation are important for the regulation of functional characteristics of AML blasts.[3,4,8-10] However, it is not known whether the functional effects of Flt3-ligation by the natural Flt3-L differ between AML blasts without genetic Flt3 abnormalities and leukemia cells that express abnormal Flt3 molecules with constitutive activity. The aim of the present study was, therefore, to characterize the functional effects of natural Flt3 ligation in detail in a large group of consecutive patients, and to compare these effects in native AML blasts with and without genetic Flt3 abnormalities (Flt3-ITD or D835 mutations).

Design and Methods

Patients

The study was approved by the local Ethics Committee and samples were collected after informed consent had

Table 1. Clinical and biological characteristics of AML patients.

					Membrane molecule expression[1]							
Pat.	*Sex*	*Age*	*Previous malignant or pre malignant disease*	*FAB classification*	*CD13*	*CD14*	*CD15*	*CD33*	*CD34*	*Cytogenetic analysis*	*FLT3-abnormality*[2]	*WBC counts*[3]
1.	M	44		AML-M5	+	–	+	+	+	inv(16)	PM	351
2.	F	36	Neurofibromatosis, malignant Schwannoma	AML-M5	+	–	+	+	–	t(9;11)	–	37.6
3.	M	49		AML-M4	–	–	+	+	–	nt3	ITD	78
4.	M	69		AML-M2	+	–	nt	+	–	inv(16)	–	89
5.	F	87		AML-M1	–	–	nt	+	–	nt	–	51.2
6.	M	83		AML-M1	–	–	–	+	+	nt	–	80
7.	M	72		AML-M4	+	+	+	+	–	+11	ITD	290
8.	M	49		AML-M5	+	+	nt	+	–	Normal	ITD	63.5
9.	F	58		AML-M2	+	–	+	+	–	Normal	ITD, wt–	40.7
10.	F	56		AML-M2	+	–	–	+	–	+21	–	69.2
11.	F	38		AML-M5	+	+	+	+	+	Normal	nt	182
12.	F	55		AML-M0	+	–	–	–	+	Normal	ITD	43.6
13.	M	51		AML-M4	+	+	+	+	–	Normal	–	31.4
14.	F	49		AML-M1	–	–	–	+	+	+21	–	121
15.	M	65		AML-M1	+	–	–	+	–	Normal	ITD, wt–	166
16.	M	64		AML-M2	+	–	+	+	+	nt	–	23.5
17.	F	63		AML-M5	–	+	+	+	–	t(2;3), (q37;q21), (q13;q21;q21) der (11q), 19q+	–	57.8
18.	F	36		AML-M5	+	+	+	+	–	Normal	nt	88.6
19.	F	82		AML-M2	+	–	–	+	+	nt	ITD	49
20.	F	63		AML-M4	–	–	–	+	–	nt	nt	126

Patients were regarded as positive when more than 20% of blast cells stained positive judged by flow cytometric analysis. All AML populations were negative for T-lymphocyte (CD2, CD3) and B-lymphocyte (CD19, CD20) markers; Flt3- abnormalities were internal tandem duplications (ITD), Asp-D835 point mutations (PM) and loss of wild type (wt–), nt, not tested; white blood cell (WBC) counts in peripheral blood are expressed as $\times 10^9$/L (normal range 3.5-10.5$\times 10^9$/L).The WBC included at least 80% leukemia blasts.

been provided. During the period 1991-2001 AML blasts were taken from 64 consecutive patients with high peripheral blood blast counts. The patients were classified as having AML-M0/M1 (21 patients), AML-M2 (20 patients) and AML-M4/M5 (23 patients). Forty-six patients had newly diagnosed *de novo* AML, 6 patients had AML relapse, and 12 patients had AML secondary to chemotherapy (4 patients), chronic myeloproliferative disorders (2 patients) or primary myelodysplastic syndromes (6 patients). Leukemic cells from the last 20 patients were used in most experiments, and the characteristics of these patients are presented in Table 1.

Cytogenetic analyses were performed for the last 48 patients included in our study; of these, 28

patients had a normal karyotype, 3 patients had a favorable karyotype (all inv(16)) and 5 had an unfavorable karyotype according to the definitions used by Wheatley *et al.*[2] and Kottaridis *et al.*[3] A total of 98 patients with AML were admitted to our institution during the same period. The distribution of karyotypes in the patients selected for our present study did not differ significantly from that in the whole series of patients seen during the same period. The relatively low frequency of patients (3/48) with favorable karyotypes is different from that reported in other studies[2,3] and was also observed for the whole series of patients from the same period (6/98).

Preparation of AML blasts

Native AML blasts. Leukemic peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) from the peripheral blood of patients with a high percentage of AML blasts among blood leukocytes (Table 1). Cells were stored frozen in liquid nitrogen.[23] The percentage of blasts among leukemic PBMC exceeded 95% for all patients,[24-26] the contaminating cells being small lymphocytes.

Enriched AML blasts. Immunomagnetic beads coated with anti-CD2 and anti-CD19 specific monoclonal antibodies (Dynabeads; Dynal, Oslo, Norway) were used for depletion of $CD2^+$ and $CD19^+$ cells, respectively.[24] Depletion was performed in two separate steps before adherent cells were removed, and the enriched populations contained <1% of $CD2^+$ T-cells and $CD19^+$ B-cells.[24]

Analysis of Flt3 abnormalities in AML blasts

Our method for analysis of Flt3-ITD has recently been described in detail.[14] Analysis of D835 point mutations (PM) was performed using the restriction fragment gene length polymorphism at codon 835/836 as described previously.[27] Briefly, after amplification of a 111 bp fragment from exon 20 using genomic DNA with primer 20F-5'-CCGCCAGGAACGTGCTTG-3' and 20R-5'-GCCTCACATTGCCCCTGA-3', polymerase chain reaction (PCR) products were digested by EcoRV and the fluorescence (6-FAM, 6-carboxylfluorescein)-labeled product analyzed.[14] Undigested products served as template for a new polymerase chain reaction with the same primer combination, and the products were thereafter cleaned using ExoSAP-IT before being directly sequenced using an ABI BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Analysis of Flt3 expression

Cell suspensions were lysed at 4°C in 60-100 μL of lysis buffer (10 mM trishydroxymethyl-aminomethane (Tris) with pH 7.5, 400 mM NaCl, 10% glycerol, 0.5% detergent Nonidet P-40 (Amersham Biosciences, Uppsala, Sweden), 5 mM NaF, 0.5 mM Na-orthovanadate, 1 mM dithiotreitol (DTT), and protease inhibitor cocktail Complete (Roche, Basel, Switzerland). Samples were kept at 4°C, homogenized and centrifuged (14,000g, 15 minutes). 3X sodium dodecyl sulphate (SDS) loading buffer (0.5 M Tris pH 6.8, 2 M β-mercaptoethanol, 12% SDS, 30% glycerol, bromphenol blue) was added to supernatant aliquots containing 40 μg of protein, and thereafter boiled for 7 minutes before separation in SDS-polyacrylamide gel electrophoresis (PAGE) minigels with 7.5% polyacrylamide. After electroblotting to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) and blocking for 1 h in phosphate-buffered saline with 0.5% Tween (PBS-T), the filters were incubated with primary anti-Flt3 antibody (the rabbit polyclonal S18 and C20 antibodies diluted 1:250 in PBS-T; Santa Cruz, CA, USA) for 1 h (room temperature) or overnight (4°C) before washing for 1 hour in PBS-T. Both antibodies recognized the p160 and p130 isoforms of Flt3. The C-20 antibody reacts with a C-terminal epitope whereas S-18 reacts with the kinase insert region. The PVDF membranes were thereafter washed (1 hour in PBS-T), incubated for 1 hour with a secondary anti-rabbit antibody conjugated to alkaline phosphatase (the antibody dissolved in PBS-T), washed (1 hour in PBS-T), and finally incubated with CDP-Star Chemiluminisence Substrate (Applied Biosystems, Foster City, CA, USA). The membranes were then exposed to Kodak X-ray films which were scanned for densiometric analysis of the 130 plus 160 kDa bands (Microtek Scanmaker 5700, NIH Image ver. 1.60 for Apple Macintosh). The intensity for each AML sample was normalized to the lower 70 kDa anti-Flt3-reactive band in Jurkat control extracts which were included in each gel (equal intensity defined as 1.0). Equal protein loading was confirmed by staining the minigels with Coomassie blue. Actin could not be used as the loading control because of differences in the molecular weight of the immunoreactive bands between patients.

Reagents for tissue culture

Cytokines. Recombinant human Flt3-L (Peprotech; Rocky Hill, NJ, USA) was used at a concentration of 20 (only ^{3}H-thymidine incorporation) or 50 ng/mL; this was based on previous studies of *in vitro* cultured AML blasts showing that Flt3-L effects reach a plateau at concentrations ≥10 ng/mL.[8,18,21] Other recombinant human cytokines were used at the following concentrations: interleukin 1β (IL1β, Peprotech) 50 ng/mL; IL3 (Peprotech) 20 ng/mL, stem cell factor (SCF; Peprotech) 20 ng/mL, thrombopoietin (TPO; Peprotech) 50 ng/mL, vascular endothelial growth factor (VEGF;

Peprotech) 50 ng/mL, macrophage colony-stimulating factor (M-CSF, Peprotech) 50 ng/mL, granulocyte-macrophage colony-stimulating factor (GM-CSF; Sandoz, Basel, Switzerland) 100 ng/mL, G-CSF (Roche) 100 ng/mL.

Culture media. Unless otherwise stated, the culture medium was RPMI 1640 with HEPES and glutamine (BioWhitacker; Walkersville, MA, USA) and supplemented with 10% inactivated fetal calf serum (FCS; BioWhitacker).[26] The serum-free media X-vivo 10®, X-vivo 15® (BioWhitacker) and StemSpan SFEM™ (referred to as StemSpan; Stem Cell Technologies, Vancouver, BC, Canada) were used in certain experiments.[26] All the media contained 100 μg/mL of gentamicin.

Antibodies. The monoclonal Flt3-L specific neutralizing antibody (clone 40416.111; R&D Systems, Abingdon, UK) was always tested in parallel with an isotypic control antibody; 0.02-0.06 μg/mL of this anti-Flt3-L antibody will neutralize 50% of the bioactivity of 5 ng/mL of recombinant human Flt3-L (manufacturer's information).

Assays for AML blast proliferation

Suspension cultures. As described previously,[24-26] 5×10^4 cells/well were cultured in 150 μL medium in flat-bottomed microtiter plates (Costar 3796; Cambridge, MA, USA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After six days ^{3}H-thymidine (37 kBq/well; TRA 310, Amersham International, Amersham, UK) was added in 20 μL of 0.9% NaCl solution and nuclear radioactivity assayed 18 hours later by liquid scintillation counting.

Colony formation assays. AML blasts were cultured in different methylcellulose-based media: (i) medium alone (MethoCult H4230; Stem Cell Technologies, referred to as spontaneous colony formation) or the same medium supplemented with GM-CSF (GM-CSF-dependent colony formation); (ii) medium with erythropoietin plus phytohemagglutinin-leukocyte conditioned medium (MethoCult H4433; Stem Cells Technologies). Cells were cultured in 24 well tissue culture plates (Costar 3524) with 10^5 cells in 0.5 mL medium per well. Cultures were incubated for 14 days before the number of colonies containing at least 20 cells was determined by light microscopy (duplicate analysis). The colonies were classified as erythroid (red color in the whole or a part of the colony) and non-erythroid.

Cytokine analysis

Analysis of AML cell cytokine secretion. As described previously,[25] 1×10^6 AML blasts/mL were cultured in 24-well tissue culture plates (Costar 3524; 2 mL medium/well) for 48 hours before supernatants were harvested. ELISA analyses were used to determine levels of IL1β, IL6, tumor necrosis factor (TNF) α (Pelikine compact ELISA kits; Central Laboratory of the Netherlands' Red Cross Blood Transfusion Services, Amsterdam, The Netherlands), Flt3-L, G-CSF and GM-CSF (Quantikine ELISA kits; R&D Systems) in the supernatants. The minimal detectable levels were IL1β 0.8 pg/mL, IL6 0.8 pg/mL, TNFα 1.0 pg/mL, Flt3-L 7 pg/mL, GM-CSF 3 pg/mL and G-CSF 8 pg/mL.

Cytokine-specific RNA levels. AML blasts (2×10^6 cells in 2 mL FCS-containing medium per well; Costar 3524 culture plates) were cultured for 48 hours before cells were harvested and washed in phosphate-buffered saline. The cell pellets were stored frozen at -70°C until total RNA was isolated.[29] For quantification of IL1β- and IL6-specific RNA the samples and calibrators were hybridized in microwells with gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes, and cytokine-specific RNA levels then determined in a calorimetric microplate assay (Quantikine RNA assay, R&D Systems). The results are expressed as concentrations of IL1β- and IL6-specific RNA when testing total RNA at the concentration of 2.5 μg/mL.

Studies of apoptotic cell death

Estimation of the number of apoptotic cells. AML blasts were incubated for 24 and 48 hours before cell death was analyzed as described in detail previously.[24,25,30-32] Firstly, AML blasts were stained with DNA-specific bisbenzimide H33258 (Hoechst; Basel, Switzerland) or daunorubicin (Pharmacia), and the percentage of cells showing chromatin distribution consistent with apoptosis was determined by fluorescence microscopy.[25,30] Secondly, detection of phosphoserine exposure on the cell surface was used as a marker for apoptosis; flow cytometric analysis was then performed on FITC-annexin V stained cells as described previously.[31] Thirdly, JC-1 staining (Molecular Probes) was used to determine the mitochondrial status, and the number of cells with depolarized mitochondria consistent with apoptosis was determined by flow cytometric analysis.[31,32]

Caspase-3 activity in AML cells. Caspase-3 activity was measured in cell extracts using a specific caspase-3 cellular activity assay kit (Calbiochem; La Jolla, CA, USA). Briefly, 1×10^6/mL AML blasts (2 mL of medium per well, 24-well Costar 3524 culture plates) were cultured for 48 hours before cells were harvested, washed twice, and cell concentration adjusted to 1×10^6 cells/mL in lysis buffer. These cell extracts were stored at -70°C until enzymatic activity was assayed according to the manufacturer's instructions. The results are presented as pmol/min for 80 μL of sample volume.

Patient	Jurkat	1	8	30	4	31	32	33	37	38	7	39	40	41
Flt3-ITD		-	+	-	-	-	-	-	-	-	+	+	-	+
D835		+	-	-	-	-	-	-	+	+	-	-	-	-
Expr.	1.0	1.1	0.1	nd	1.5	0.4	nd	3.0	nd	2.9	2.3	1.2	0.1	0.2

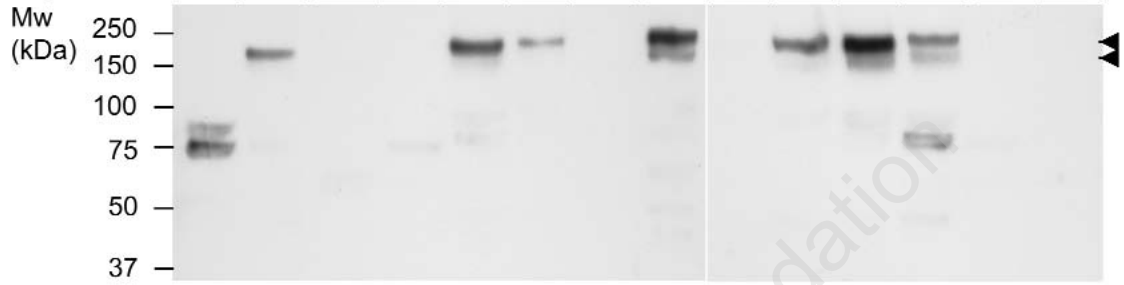

Figure 1. Protein expression of Flt3 by Jurkat cells and native AML blasts derived from 12 patients. An antibody reactive with the C-terminal domain (C-20 antibody) was used as described in Design and methods. The molecular weight (Mw) in kDa is indicated on the left side, and the arrows on the right side indicate that the antibody bound two bands corresponding to the 130 and 160 kDa isoforms. The patients' number is given at the top of the figure. For each individual patient it is also indicated whether Flt3-ITD was detected, and detection of D835 mutations is indicated by +. The Flt3 protein level is presented as the relative expression (Expr) compared with the 70 kDa band of the Jurkat cell (nd, not detectable). The figure illustrates the wide variation in the expression of both the 130 and 160 kDa bands.

Presentation of the data

^{3}H-thymidine incorporation was assayed in triplicate and the mean counts per minute (cpm) used for all calculations. The *incremental response* was defined as the cpm for cultures with AML blasts minus cpm for negative controls, and significant blast proliferation was defined as an incremental response exceeding 1,000 cpm. A *significant alteration* of proliferation was defined as a difference in incremental responses (i) exceeding 2000 cpm, and (ii) the difference in cpm being >20% of the control response. For cytokine combinations an *additive enhancing* or *inhibitory* effect was defined as a proliferative response exceeding the highest/lowest of the two single responses by at least 2000 cpm and 20%; smaller differences are referred to as intermediate. A significant *alteration of AML blast colony formation* was defined as a difference corresponding to >20% of the control response and with an absolute value >10 per 10^5 seeded cells. Cytokine concentrations were transformed to logarithmic values that were used for statistical comparisons. The Sign test, χ^2 test and Wilcoxon's test for paired samples were used for statistical analysis, and differences were regarded as significant when $p<0.05$.

Results

Flt3 protein expression in native AML blasts

The protein level of Flt3 in native AML blasts was examined for 42 patients. Two molecules corresponding to the 130 and 160 kDa isoforms of Flt3 were detected by both antibodies. The protein level of Flt3 showed a wide variation between patients, and the relative expression ranged from not detectable to 3.0. The relative levels of Flt3 did not differ significantly between Flt3-ITD$^+$ (median 0.31, range 0.01-2.40) and ITD$^-$ (median 0.38, range <0.01-3.00) AML cell populations. A wide variation was also observed for the 3 ITD$^-$PM$^+$ patients (range <0.01-1.5). This is illustrated by the results presented in Figure 1.

The proliferative capacity of AML blast populations with and without Flt3 abnormalities

AML blasts derived from 55 patients were available for analysis of Flt3 abnormalities. Flt3-ITD was detected for 21 patients (38%). A D835 point mutation was detected for 7 patients (13%), and 3 of these patients had both ITD and point mutations.

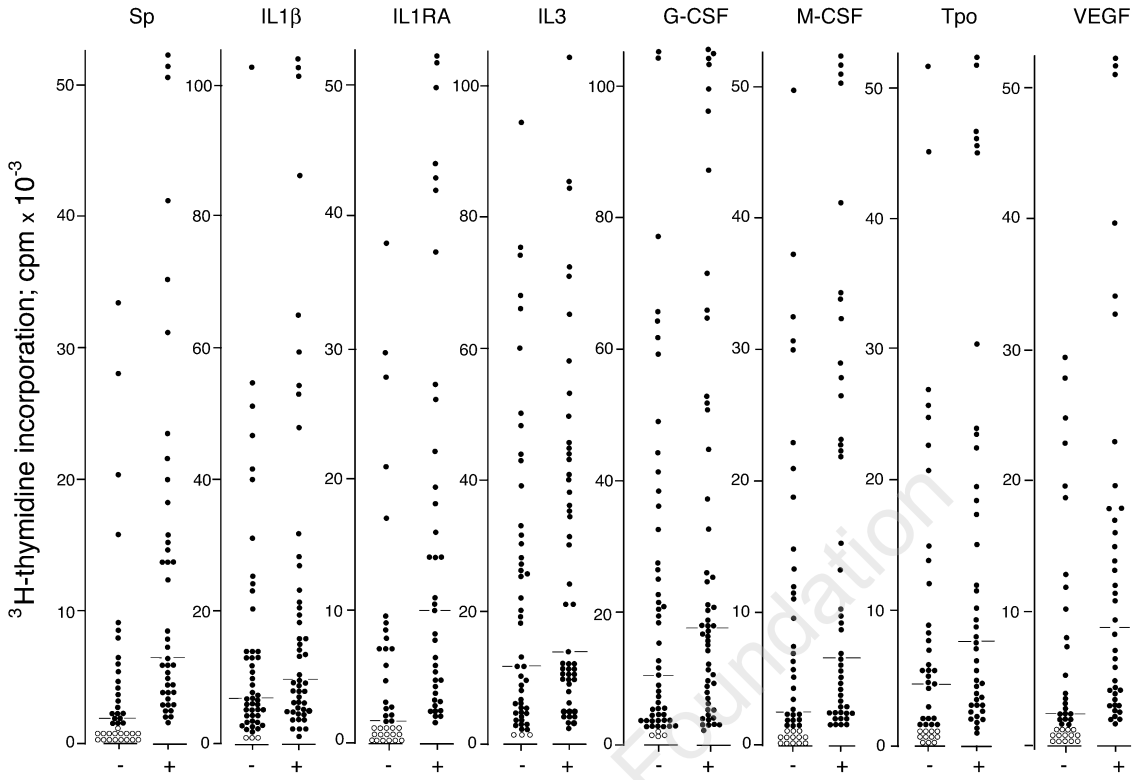

Figure 2. The effect of Flt3-L on AML blast proliferation. Leukemia blasts were derived from 64 consecutive patients, but the figure presents the results only for those patients showing detectable ^{3}H-thymidine incorporation for cultures without (⁻) or with (⁺) Flt3-L 20 ng/mL. AML blasts were cultured either in medium alone (Sp, 40 out of 64 patients showing detectable proliferation) or in the presence of IL1β (49/64), IL1RA (37/64), IL3 (49/64), G-CSF (52/64), M-CSF (44/64), TPO (41/64) or VEGF (38/64). All these exogenous cytokines were tested at 50 ng/mL except G-CSF that was tested at 100 ng/mL. The results for each patient are presented as the mean cpm of triplicate determinations, and undetectable proliferation is indicated in the figure (o). The median ^{3}H-thymidine incorporation for all these patients is also indicated in the figure (—).

The presence of Flt3-ITD showed no association with FAB classification, the expression of the stem cell marker CD34 or the ability of autocrine proliferation (*data not shown*). We also investigated the proliferative capacity in the presence of exogenous cytokines (IL1β, IL3, G-CSF, M-CSF, GM-CSF, SCF, Flt3-L, TPO, VEGF). This wide range of cytokines was used because the growth factor responsiveness of AML populations is heterogeneous. A total of 495 patient/cytokine combinations were thus examined. The frequency of combinations with undetectable ^{3}H-thymidine incorporation (<1000 cpm) was significantly higher for Flt3-ITD⁺ patients (n=21, 90 out of 189 combinations) than for Flt3⁻ITD⁻ patients (n=34, 89 out of 306 combinations, χ^2 test, $p<0.001$). This difference was also statistically significant when Flt3-PM⁺ patients were excluded from the control group. Undetectable ^{3}H-thymidine incorporation in the presence of all nine cytokines was most common among patients with Flt3 abnormalities (Flt3-ITD⁺ or PM⁺ 6/21, no Flt3 abnormalities 4/34), but this difference did not reach statistical significance.

Effects of exogenous Flt3-L on proliferation of AML blasts in suspension cultures

The proliferation of native AML blasts derived from 64 consecutive patients was assayed when cells were cultured with and without exogenous Flt3-L 20 ng/mL in FCS-containing medium alone and medium with various exogenous cytokines. AML blasts from 10 patients did not proliferate *in vitro* either in medium alone or in the presence of any exogenous cytokine. The overall results for the other 54 patients with detectable ^{3}H-thymidine incorporation (corresponding to >1000 cpm) are presented in Figure 2 and summarized in the upper part of Table 2. When the statistical analysis only included those patients with detectable proliferation (>1000 cpm), (i) Flt3-L increased AML blast proliferation significantly both for cells cultured in medium alone and cells cultured with exogenous IL1β, IL1RA, IL3, G-CSF, M-CSF, TPO, and VEGF (Sign test, $p<0.002$ for each), and (ii) the enhancement reached a significant level (for definitions see Design and methods, presentation of the data) for

Table 2. Effects of Flt3-L on *in vitro* proliferation of native AML blasts; studies of ^{3}H-thymidine incorporation of AML blasts derived from 64 patients.

Comparison (patient number)	*Effect*	*Exogenous cytokine added alone and together with Flt3-L[1] (number of patients)*							
		None	*IL1*	*IL1RA*	*IL3*	*G-CSF*	*M-CSF*	*TPO*	*VEGF*
Cultures with exogenous Flt3-L compared with corresponding controls (n=64)	Increase[2]	34	26	29	29	31	23	26	28
	Intermediate[2]	5	21	7	17	15	20	14	8
	Decrease[2]	1	1	1	3	6	1	1	2
	No detectable proliferation[2]	24	16	27	15	12	20	23	26
Cultures with exogenous Flt3-L compared with corresponding controls (n=64)	Additive growth-enhancing effects[3]	–	15	0	27	28	16	14	2
Flt3-ITD$^+$ AML blasts (n=21)[4]	Flt3-L induced increase of proliferation[2]	9	8	7	9	13	7	7	6
Flt3-PM$^+$ AML blasts (n=4)[4]	Flt3-L induced increase of proliferation	2	2	3	2	1	1	1	2
Flt3-ITD$^-$ AML blasts (n=30)[4]	Flt3-L induced increase of proliferation	19	13	16	12	15	12	14	15

The results for 64 patients are presented.[1] For each cytokine the effects were classified (see below) and the number of patients showing enhanced/indifferent/decreased/no significant proliferation in the presence of exogenous Flt3-L is given for each cytokine. The effect of Flt3-L was investigated for AML blasts cultured in medium alone or medium with exogenous cytokines (IL1β, IL1RA, IL3, G-CSF, TPO, M-CSF, VEGF). The upper part of the table describes the effect of exogenous Flt3-L in the presence of other mediators, the middle part states the number of patients with an additive effect of Flt3-L and other exogenous cytokines (for detailed definitions see Design and Methods, presentation of the data), and the lower part compares the numbers of patients with Flt3-L-induced growth enhancement for Flt3-ITD$^+$ and -ITD- AML blast populations.[2] A significant alteration was defined as a difference being (i) >20% of the corresponding Flt3-L-free control and (ii) with an absolute value >2000 cpm. Smaller differences were classified as intermediate. No detectable proliferation was defined as ^{3}H-thymidine incorporation <1000 cpm both for cultures with and without Flt3-L.[3] An additive effect of Flt3-L and another cytokine was defined as a proliferative response exceeding the highest of the two responses by at least 20% and this difference being >2000 cpm.[4] Nine patients were not available for Flt3-ITD testing. The group of Flt3-ITD$^+$ patients includes 3 patients with additional Flt3-PM, whereas patients with Flt3-PM alone are presented as a separate group.

a large group of patients both when cells were cultured in medium alone and when they were cultured with medium and exogenous cytokines (Table 2, upper part). These differences were also statistically significant when all patients were included in the analysis (*data not shown*). Flt3-L and the other cytokines had additive effects only for a subset of the patients (Table 2, middle part). When comparing the overall results for the 54 patients with detectable proliferation, Flt3-L had significant effects on blast proliferation in medium alone or in the presence of at least one exogenous cytokine for 50 of these patients. However, the Flt3-L effect showed no correlation with the Flt3 protein levels (*data not shown*), and the growth-enhancing effect was detected for blast populations both with and without Flt3 abnormalities (Table 2, lower part).

AML blast expression of wild-type Flt3 could not be detected for 3 ITD$^+$ patients. However, exogenous Flt3$^-$L could modulate AML blast proliferation even for these patients (Table 3).

The effects on spontaneous and cytokine-dependent blast proliferation were also compared for Flt3-L, GM-CSF and SCF (Figure 3). All three cytokines caused strong enhancement of the proliferation for a majority of patients both when cells were cultured in medium alone and when the cells were cultured with medium in the presence of exogenous cytokines (Figure 3). Flt3-L (Table 2), GM-CSF and SCF (*data not shown*) had additive growth-enhancing effects with other cytokines only for a subset of patients.

Enriched AML blasts were prepared for 5 patients (Table 1, patients #7, 8, 11, 14 and 20), and Flt3-L increased the proliferation of enriched cells for all these patients (*data not shown*).

Flt3-L as an autocrine growth factor for native AML blasts

AML blasts derived from 64 patients were cultured for 48 hours before concentrations of Flt3-L were determined in the supernatants. Flt3-L did not reach detectable levels (<7 pg/mL) for any patient. Leukemia cells derived from the 9 patients with the highest spontaneous *in vitro* proliferation were also cultured with Flt3-L specific monoclonal antibody and isotypic control antibodies. Anti-Flt3-L caused a dose-dependent inhibition of spontaneous blast proliferation only for patient 8 (Flt3-ITD$^+$PM$^-$) and 10 (Flt3-ITD$^-$PM$^-$), but anti-Flt3-L did not have an antiproliferative effect for any patient in the presence of exogenous GM-CSF (Figure 4).

Table 3. Effects of exogenous Flt3-L on *in vitro* proliferation of native AML blasts: studies of Flt3-ITD+ leukemia cells with undetectable expression of wildtype Flt3.

Patient and culture characteristics	*Flt3-L 20 ng/mL*	*Patient 9*	*Patient 15*	*Patient 24*
Flt3-ITD (base pairs)		24	63/33	48
Flt3-PM (D835)		–	–	–
Exogenous mediator				
None	–	1084±172	147±29	299±27
	+	**5891±998**	696±98	497±98
IL1	–	3456±627	568±108	14.836±1982
	+	**7090±882**	939±111	17.005±2035
IL1RA	–	478±87	183±23	485±82
	+	**2769±524**	724±74	612±54
IL3	–	68.911+3760	542±225	8653±1230
	+	80.511+4072	939±339	**11.783±1074**
G-CSF	–	2156±524	524±92	21.284±2310
	+	**10.330±1099**	1706±233	21.058±1962
M-CSF	–	564±38	510±44	539±27
	+	**3166±422**	833±92	591±83
TPO	–	378±88	228±76	4836±623
	+	**2962±499**	876±73	4471±826
VEGF	–	259±78	127±29	312±27
	+	**2654±881**	594±127	483±99

AML blasts were cultured in suspension cultures with and without Flt3-L 20 ng/mL. The Flt3-L effects were assayed for cells cultured in medium alone and medium supplemented with various exogenous cytokines. ^{3}H-thymidine incorporation was assayed after 7 days of culture, and the results are presented as the mean+standard deviation of triplicate determinations. Results in bold represent significant alterations induced by Flt3-L (the difference corresponding to >20% of the controls and an absolute value >2,000 cpm).

Table 4. The effect of Flt3-L on clonogenic AML cells; effects of pre-incubation with Flt3-L before analysis of colony formation.

			Colony-formation with erythropoietin plus conditioned medium (number of colonies per 10^5 seeded cells)	
Patient	*FLT3-abnormality*	*Flt3-L 50 ng/mL*	*Non Erythroid*	*Erythroid*
1.	PM	–	16.5±0.7	0
		+	17.5±0.7	0
2.	–	+	117.0±26.9	150.0±6.3
			115.0±9.9	**170.0±14.0**
3.	ITD	–	85.0±15.4	0
		+	**125.0±4.2**	2.0±1.4
4.	–	–	170.0±11.3	96.0±25.4
		+	**144.0±11.3**	**23.0±7.0**
5.	–	–	194.0±31.1	30.0±12.5
		+	**265.0±29.6**	**1.0±1.4**
6.	–	-	15.0+712.5	0
		+	**123.0+12.6**	0
7.	ITD	–	41.5±3.5	0
		+	**91.0±14.7**	0
8.	ITD	–	118.0±14.1	71.5±18.3
		+	**168.0±24**	**0.5±0.7**
9.	ITD, wt–	–	72.0±25.5	23.0±9.1
		+	**126.0±1.4**	**1.5±0.7**
10.	–	–	329.0±43.8	0
		+	**371.0±49.5**	0
12.	ITD	–	39.0±5.6	4.0±2.8
		+	**64.0±21.0**	1±1.4
13.	–	–	24.5±2.1	23.5±10.5
		+	28.0±0	23.0±13.3
14.	–	–	57.7±6.3	0
		+	**45.0±2.8**	0
15.	ITD, wt-	–	82.0±24.8	3.0±2.8
		+	**154.0±4.2**	0
16.	–	–	176.0±34.3	0
		+	**127.5±33.6**	0
17.	–	–	4.5±3.5	4.0±5.6
		+	4.0±5.6	**44.5±9.1**
18.	nt	–	50.0±4.2	0
		+	**90.0±7.0**	0

Cells were pre-cultured in suspension cultures with and without Flt3-L 50 ng/mL for 7 days before the number of colonies was determined in the erythropoietin-conditioned medium assay. Values for patients with significant effects of Flt3-L are marked in bold: this was defined as differences >10 and exceeding the Flt3-L-negative control by 10%. The results are presented as the mean+SD of duplicate determinations.

Effects of exogenous Flt3-L on AML blast colony-formation

AML blasts derived from 17 patients (Table 1, patients #1-10 and 12-18) were pre-incubated in serum-free StemSpan™ medium without and with Flt3-L 50 ng/mL for 7 days before the frequency of colony-forming cells was determined under Flt3-L-free conditions by using the erythropoietin+conditioned medium assay. Patients with Flt3 abnormalities showed lower frequencies of clonogenic cells than did the other patients, but this difference did not reach statistical significance (Table 4). For most patients Flt3-L either increased (10 of 17 patients) or did not alter (4/17) the frequencies of non-erythroid colonies, but a reduction was observed for 3 patients. Erythroid colonies were detected for 10 patients; pre-incubation with Flt3-L reduced this frequency for 4 patients and increased it for 2 patients. The Flt3-L effects did not differ between

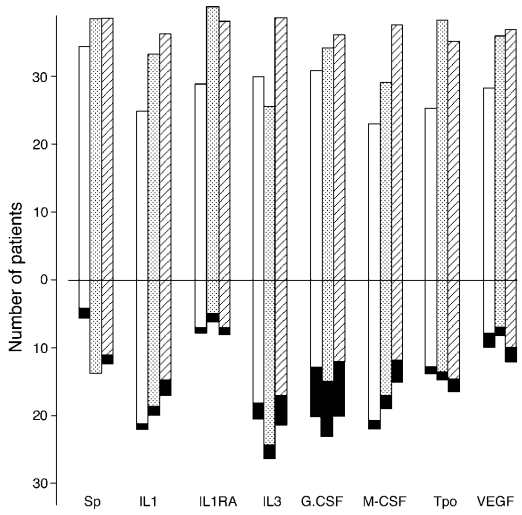

Figure 3. Effects of Flt3-L, GM-CSF and SCF on AML blast proliferation. The proliferation of native AML blasts cultured in medium alone or with Flt3-L 20 ng/mL (open columns), GM-CSF 100 ng/mL (stippled) or SCF 20 ng/mL (stripes) was first compared (Sp, i.e. spontaneous proliferation in medium alone). The effects of these three cytokines were also examined when the culture medium was supplemented with other exogenous cytokines (IL1, ILβ1RA, IL3, G-CSF, M-CSF, TPO, VEGF; see the bottom of the figure), and proliferation in Flt3-L/GM-CSF/SCF containing cultures was then compared with that in the corresponding cytokine-containing controls. An increase or decrease in blast proliferation was defined as an alteration corresponding to (i) >20% of the corresponding control and (ii) exceeding 2000 cpm; smaller differences are referred to as intermediate responses. A total of 64 patients were examined, and the figure presents the results for those patients who showed detectable proliferation (corresponding to >1000 cpm) for each of the cytokines. The figure shows the number of patients with increased (the part of the columns above the X-axis) and intermediate/decreased (the part of the column below the X-axis) proliferation in the presence of Flt3-L/GM-CSF/SCF. The number of patients with decreased responses is indicated in black at the lowest part of each column.

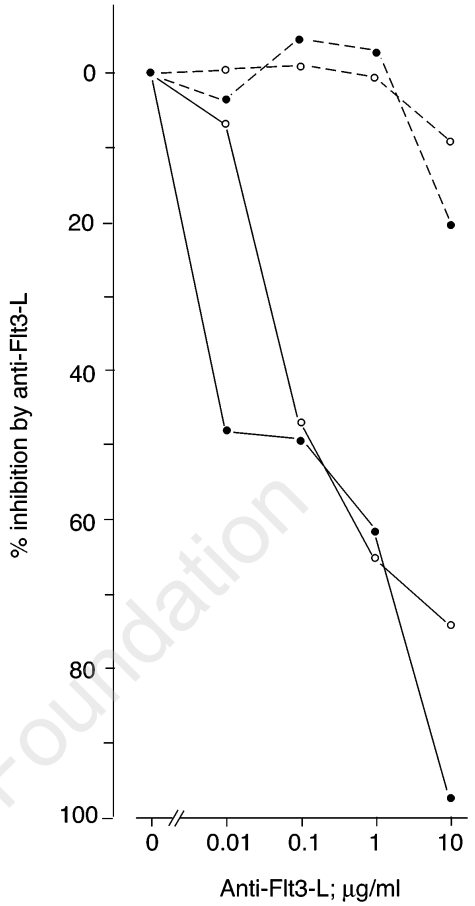

Figure 4. The effect of Flt3-L specific neutralizing antibodies on spontaneous and GM-CSF-dependent AML blast proliferation. Native AML blasts derived from two patients (Table 1; patients 8 •, 10 o) were cultured either in medium alone (____) or in the presence of GM-CSF 100 ng/mL (—). Proliferative responses were then compared for cultures with Flt3-L-specific neutralizing monoclonal antibodies and cultures containing isotypic control antibodies at the same concentration (see x-axis). The results are presented as the percent inhibition by neutralizing antibodies, i.e. the incremental cpm for cultures with Flt3-L specific antibodies, relative to the incremental cpm for corresponding isotypic control cultures. The responses in isotypic control cultures were: patient #8, spontaneous proliferation 8302+1876 cpm, GM-CSF dependent proliferation 36.813+852 cpm; patient # 10, spontaneous proliferation 17.206+1428 cpm, GM-CSF dependent proliferation 94.865+3474 cpm.

patients with and without Flt3 abnormalities and were reproduced for 5 patients (Table 1, patients 1#, 2, 6, 8, 9) when using various media for the pre-incubation (RPMI + 10% FCS, X-vivo 10®, X-vivo 15®, StemSpan™, *data not shown*). Spontaneous and GM-CSF-dependent colony formation were examined for 8 patients (Table 1, patients #7-14); the assays were then prepared with and without Flt3-L 50 ng/mL. Only non-erythroid colonies were detected. Flt3-L could increase both spontaneous (1 out of 8 patients) and GM-CSF-dependent (3/8) colony formation, and decreased colony-formation was not observed for any patients.

Effects of exogenous Flt3-L on constitutive cytokine secretion by native AML blasts

Flt3-L 50 ng/mL had divergent effects on the release of IL1β, IL6, TNFα, G-CSF and GM-CSF by native human AML blasts (Figure 5). This divergence was observed for native blasts and was

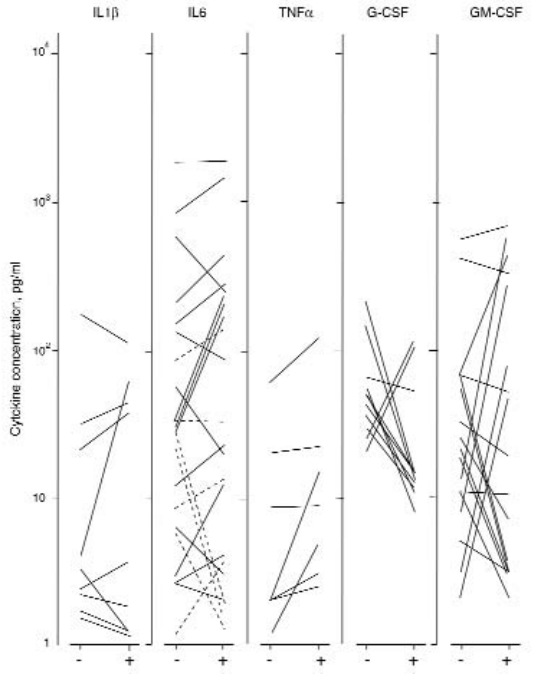

Figure 5. The effect of Flt3-L on AML blast cytokine secretion. The AML cells were cultured without and with Flt3-L 50 ng/mL, and cultures were prepared with either native (____) or enriched (----) AML blasts. The cells were cultured at 1×10^6 cells/mL for 48 hours before supernatants were harvested and cytokine levels (IL1β, IL6, TNFβ, G-CSF, GM-CSF; see top of the figure) determined by ELISA analyses. The figure compares the cytokine levels (pg/mL) for cultures without (⁻) and with (⁺) Flt3-L, and only the results for patients showing detectable cytokine levels for at least one of these cultures are presented: - IL1β: 25 patients tested, 9 patients showed detectable levels; - IL6: 15 out of 25 patients showed detectable levels; in addition the figure presents the results for enriched AML blasts derived from 7 of these patients (----); - TNFα: detectable levels were observed for 7 out of 25 patients; - G-CSF, 11 out of 19 patients showed detectable levels; - GM-CSF, 17 out of 19 patients showed detectable levels.

reproduced for enriched leukemia cells (7 patients examined), and neither the cytokine levels nor the divergent effects of exogenous Flt3-L differed between patients with and without Flt3 abnormalities. Exogenous SCF also had divergent effects on the secretion of IL1β, IL6 and TNFα (*data not shown*). Furthermore, AML blasts derived from 5 patients (Table 1, patients 1-4 and 7) were cultured with and without Flt3-L 50 ng/mL for 48 hours before IL1β- and IL6-specific RNA was quantified. IL1β- (range 15.0-39.0 amol/μg total RNA) and IL6-specific RNA (range 32.0-86 amol/μg RNA) were detected for all patients, and Flt3-L had divergent effects on these levels (*data not shown*).

Effects of exogenous Flt3-L on AML blast morphology

Native AML blasts derived from 10 consecutive patients (Table 1, patients 1-10) were cultured with and without Flt3-L 50 ng/mL for 2 and 7 days before examination of cell morphology (May-Grünwald-Giemsa staining). After 2 days neutrophil differentiation was observed in ≤ 10% of the cells for all patients, and these percentages were not significantly altered (i.e. ≤ 5%) by Flt3-L. In contrast, after 7 days of culture with Flt3-L an increased percentage of cells with neutrophil granulation was observed for patients 1 (ITD^-PM^+, 17 versus 5%), 2 (ITD^-PM^-, 28 versus 1%), 4 (ITD^-PM^-, 12 versus 1%) and 7 (ITD^+PM^-, 8 versus 2%). However, neutrophil differentiation further than the promyelocyte stage and erythroid or monocytoid differentiation was not observed for any patient.

Effects of Flt3-L on regulation of apoptosis in native AML cells

The frequency of apoptotic AML blasts (H33258 staining) after 24 and 48 hours of culture was first examined for 11 patients (Table 1, patients 1 and 10-19), and this frequency exceeded 30% only for patient 17 (ITD^- PM^-, 54% after 48 hours). Flt3-L reduced apoptosis significantly (i.e. ≥5% difference) for patients 1 (ITD^- PM^+), 10 (ITD^- PM^-), 13 (ITD^- PM^-), 17 (ITD^- PM^-) and 18 (ITD not tested, PM^-). Furthermore, the effects of Flt3-L, GM-CSF and SCF on apoptosis were compared for an additional group of 12 consecutive patients, 4 of whom were $Flt3^-ITD^+$ and 1 of whom was $Flt3^-PM^+$ (chromatin staining, JC-1 staining, annexin-V labeling). For $Flt3^-$L the morphologic examination demonstrated reduced apoptosis (≥5% alteration) for 6 of the 12 patients, whereas increased apoptosis was observed for 2 patients. A similar divergence was observed both after 24 and 48 hours of culture and with all three methods (*data not shown*). The anti-apoptotic effect was observed for AML blast populations both with and without Flt3 abnormalities (including the $Flt3^-PM^+$ patients). SCF and GM-CSF also had divergent effects on apoptosis (*data not shown*).

Caspase-3 activity in AML blasts was examined after 48 hours of *in vitro* culture with and without $Flt3^-$ L 50 ng/mL for patients 1 (ITD^- PM^+), 10 (ITD^- PM^-) and 19 (ITD^+ PM^-). These patients were selected because Flt3-L increased the blast proliferation both in cultures with medium alone and in cultures in the presence of all the exogenous cytokines. In contrast, Flt3-L had divergent effects on caspase-3 activity and caused either minimally altered (patient 10: without Flt3-L 34.8 pmol/min, with Flt3-L 42.9 pmol/min), decreased (patient 1: 107 versus <13.4 pmol/min) or increased activity (patient 19: 6.7 versus 68 pmol/min). Caspase-inhibitory activity was not detected for any patient.

Discussion

In our present study we included consecutive patients with high peripheral blood blast counts, and thus highly enriched populations of native AML blasts could be prepared by density gradient separation from peripheral blood samples. This simple technique has a minimal risk of inducing functional alterations in the blasts,[35,36] but a high degree of *leukemization* may reflect biologic differences[2,37] and thus our results are only representative of this particular subset of patients. The cytogenetic studies demonstrated a normal karyotype in nearly 60% of our patients, whereas favorable and unfavorable karyotypes were detected only for small subsets of patients (6% and 11% respectively). This distribution is not much different from that found in other studies, except for the low frequency of favorable karyotypes, which was also observed when we analyzed all patients admitted to our institution during the same study period.[2,3] However, the relatively high frequencies of Flt3-ITD (38 versus 27%)[3] and D-835 mutations (13 versus 7%),[28] in our present study possibly reflect a selected patient population. The relatively low number of patients examined may then explain why the frequencies of abnormal karyotypes did not differ from that found in our overall population and in other studies.[2,3]

Previous studies have reported a wide variation in the percentage of patients with Flt3-L-responsive AML blasts (range 62-88%).[22] In contrast to these studies we examined the effects of Flt3-L in a large group of consecutive patients. We detected Flt3 expression by AML blasts in more than 80% of patients, and exogenous Flt3-L altered blast proliferation in at least one *in vitro* model for a large majority of patients. The expression of Flt3 isoforms showed a wide quantitative variation between patients without there being any correlation with the effects of Flt3-L on blast proliferation, suggesting that downstream mechanisms are more important than receptor density for these functional Flt3-L effects.

Ligation of wild-type Flt3 leads to activation of the intracellular tyrosine kinase,[5] whereas abnormal Flt3 genes encode molecules with constitutive enzyme activity.[8,9] Our present results demonstrated that Flt3-L was a growth factor even for AML blasts with genetic Flt3 abnormalities, an observation suggesting that receptor ligation can induce additional growth-stimulatory signaling even in cells with constitutive kinase activity. Although it seems likely that this effect is caused by ligation of wild-type Flt3, the observation of increased proliferation in the presence of exogenous Flt3–L for Flt3–ITD$^+$ blasts without detectable wild-type suggests that ligation of mutated Flt3 may also contribute. However, this last observation should be interpreted with great care because relatively few patients were examined and the presence of small, undetectable amounts of wild-type Flt3 may influence these results. Furthermore, Flt3 ligation could also modulate colony formation and spontaneous apoptosis of *in vitro* cultured Flt3-ITD$^+$ and -ITD$^-$ blast populations. Our results thereby support the hypothesis that exogenous Flt3–L is an important growth factor for AML blasts in a major part of patients. In contrast, Flt3–L is an autocrine growth factor only for a small subset, and the importance of the autocrine secretion is also reduced by the presence of other exogenous cytokines (e.g. GM-CSF).

ITD$^+$ AML blasts have decreased proliferative capacity in stroma-supported cultures.[33,34] Our present study demonstrated an association between Flt3-ITD and decreased AML blast proliferation in suspension cultures. This observation suggests that the decreased proliferative capacity is caused by a direct effect of the Flt3-ITD on intracellular signaling and not by indirect effects with modulation of paracrine growth-regulatory mechanisms.

GM-CSF and SCF can increase AML blast proliferation for most patients,[37,38] but their effects on the proliferative capacity of native human AML blasts have not been compared with those of Flt3-L. All three growth factors had similar effects with regard to growth enhancement and anti-apoptosis and divergent effects on cytokine release (Figure 5),[37-40] including the autocrine growth factors IL1β, G-CSF and GM-CSF. Thus, the overall results demonstrate that these three cytokines have growth-enhancing effects that are only minimally influenced by local cytokine networks and are independent of autocrine growth factor release and regulation of apoptosis.

The growth-enhancing effect of Flt3-L was characterized in detail. Firstly, Flt3-L could not be detected in culture supernatants for any patient, but Flt3-L specific neutralizing antibodies inhibited spontaneous AML blast proliferation for two patients. This observation suggests that Flt3-L can function as an autocrine growth factor in a small minority of patients. Secondly, the growth-enhancing effects of Flt3-L were not due to increased IL1β release because (i) the Flt3-L effects on proliferation and IL1β levels showed no correlation; (ii) Flt3-L had divergent effects on cytokine-specific RNA levels both for IL1β and IL6 independently of its growth-enhancing effect; and (iii) Flt3-L enhanced proliferation even in the presence of the antagonistic IL1RA. Thirdly, the growth-enhancing effect of Flt3-L involved the clonogenic AML cell subset, and this effect could be detected when using various experimental approaches. Lastly, control experiments with serum-free conditions and enriched AML blast populations showed that the Flt3-L effects were not dependent on unidentified serum components or the small lymphocyte contamination.

The possible interaction between differentiation status and Flt3-L effects was investigated by various experimental approaches. Flt3-L had only minimal effects on AML blast differentiation in suspension cultures. Differentiation was then analyzed by light microscopy because we regard morphologic alterations as a more robust sign of differentiation than flow-cytometric detection of altered expression of a single or a few membrane molecules.[35,36,41-43] Flt3-L did not induce erythroid differentiation in the colony formation assays either. Thus, the effects of Flt3-L seem to be only minimally affected by and have only a minimal influence on the differentiation status of native human AML blasts.

To conclude, Flt3-L was able to modulate the functional phenotype of native human AML blasts, and the most important effect seemed to be enhanced proliferation. This growth-enhancing effect was observed both for AML cells with and those without genetic Flt3 abnormalities, and our results thereby suggest that intracellular signaling initiated by Flt3-ligation in the AML blasts is involved in leukemogenesis and possibly also chemosensitivity for a majority of patients.

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Pre-Publication Report & Outcomes of Peer Review

Contributions

All authors took part in the design of the study, interpretation of data and preparation of the manuscript. All authors approved the final version of the manuscript. The authors especially contributed to the following parts: ØB: collection of cells, investigation of AML blast proliferation, cytokine secretion and differentiation. RH: analysis of Flt3-internal tandem duplication. LW and BTG: analysis of apoptosis in AML blasts. TsH: analysis of cytokine RNA levels.

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This manuscript was peer-reviewed by two external referees and by Professor Francesco Lo Coco, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Lo Coco and the Editors. Manuscript September 16, 2002; accepted February 25, 2003.

In the following paragraphs, the Deputy Editor summarizes the peer-review process and its outcomes.

What is already known on this topic

FLT3 signaling is increasingly relevant in AML, particularly after the discovery that constitutive activation of the receptor by gene mutation is common in this disease and may be targeted by recently developed inhibitors.

What this study adds

The study shows that FLT3 ligation initiates signaling and modulates AML phenotype irrespective of the FLT3 status (i.e. mutated or not).

Caveats

The study includes a selection of hyperleukocytic cases. It would be interesting to figure out whether similar observations are obtained in patients with low initial WBC counts.