From www.bloodjournal.org by guest on May 6, 2015. For personal use only.
Blood First Edition Paper, prepublished online October 25, 2005; DOI 10.1182/blood-2005-06-2530

VEGFR-1 (FLT-1) activation modulates acute lymphoblastic leukemia localization and survival within the bone marrow, determining the onset of extramedullary disease

Rita Fragoso¶†, Teresa Pereira°, Yan Wu^, Zhenping Zhu^, José Cabeçadas°, Sérgio Dias¶†‡*

¶ Angiogenesis Lab., Centro Investigação Patobiologia Molecular, Instituto Português de Oncologia Francisco Gentil (IPOFG), Rua Professor Lima Basto, 1099-023 Lisboa, Portugal

° Serviço de Anatomia Patológica, IPOFG
‡ Instituto de Medicina Molecular (IMM), Faculdade de Medicina, Lisboa
† Instituto Gulbenkian de Ciência, Oeiras

^ ImClone Systems, New York, USA

* corresponding author
sergidias@ipolisboa.min-saude.pt

Fax: 351 21 722 98 95

Copyright © 2005 American Society of Hematology

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Abstract

The presence of persistent circulating leukemia cells, or engrafted into extramedullary tissues, is a bad prognostic factor for acute leukemia patients. However, little is known about the mechanisms that regulate the exit of leukemia cells from the bone marrow (BM) microenvironment. We reveal that Vascular Endothelial Growth Factor Receptor 1 (FLT-1) modulates acute leukemia distribution within the BM, along VEGF and PlGF gradients, regulating leukemia survival and exit into the peripheral circulation. FLT-1 activation on acute lymphoblastic leukemia (ALL) cells results in cell migration and proliferation *in vitro*, while *in vivo* FLT-1 overexpressing cells accumulate in the BM epiphysis of NOD-SCID recipients, and are detected in circulation 2 weeks after inoculation. In turn, FLT-1 neutralization affects leukemia localization (now in the BM diaphysis), increases leukemia apoptosis and impedes the exit of ALL cells, prolonging the survival of inoculated mice. We demonstrate further that FLT-1-induced cell migration involves actin polimerization and lipid raft formation. Taken together, we show that FLT-1 regulates the BM localization of ALL cells, determining their survival and exit into the circulation, and ultimately the survival of inoculated recipients. FLT-1 targeting on subsets of acute leukemias may delay the onset of extramedullary disease, which may be advantageous in combinatorial therapeutic settings.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Introduction

The early detection of acute leukemia cells outside the BM microenvironment is considered an unfavourable prognostic factor, both in lymphoblastic as well as in myeloid leukemias (1-5). Moreover, leukemia infiltration into extra-medullary sites may also reduce leukemia responsiveness to induction chemotherapy (6, 7), while persisting blasts correlate with decreased overall survival and confer poor prognosis in ALL patients (8, 9). Thus, there is great interest in determining the molecular mechanisms that regulate acute leukemia exit from the BM microenvironment into the peripheral circulation and into extramedullary organs such as the spleen, among others.
The importance of angiogenesis and angiogenesis-related signalling pathways for the growth and expansion of acute leukemias has been amply demonstrated (references 10 and 11 provide updated references on the subject). For instance, we and others have shown that subsets of acute leukemia cells express Vascular Endothelial growth factor and its receptors (namely VEGFR-2/KDR and VEGFR-3/FLT-4), resulting in autocrine loops that modulate leukemia survival, proliferation and migration (12, 13). Thus, acute leukemia growth involves autocrine and paracrine VEGF/VEGF receptor loops that regulate expansion of the leukemia clones within the BM microenvironment. This has generated great interest in designing strategies to block such VEGF/VEGF receptor loops for the treatment of subsets of acute leukemias (see 14 as an example).

Regarding the actions of VEGF, several studies suggested it modulates cell proliferation and survival, via KDR, while FLT-1 activation has been linked mostly with cell migration (15, 16). In the case of BM diseases, such as acute leukemias, there is little information concerning a putative function for FLT-1, although a previous report suggested it may induce proliferation of myeloid leukemia cell lines (17) and may also regulate the localization of immature malignant precursors within the BM, in myelodisplastic syndromes (18). More recently, work done in multiple myeloma reinforced the idea that FLT-1 may regulate the migration and possibly the proliferation of the malignant plasma cells (19). In acute leukemias, it remains to be demonstrated whether FLT-1 conveys similar signals, and its importance during disease onset and progression.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

In the present report we used acute lymphoblastic leukemia cells as a model to study the role of FLT-1 in leukemia biology. We demonstrate *in vitro* and *in vivo* that FLT-1 activation results mainly in leukemia cell migration, while having a modest effect in cell proliferation. Strikingly, FLT-1 activation on leukemia cells *in vivo* results in distinct cell localizations within the BM; FLT-1 overexpressing cells localize predominantly close to articulations (epiphysis) of long bones, while FLT-1 non-expressing cells (or those where the activation of the receptor was blocked) localize in the central portions (diaphysis) of the bone marrows. This selective localization of leukemia cells conditions their survival, the exit of blasts into the peripheral circulation and ultimately the survival of inoculated recipients (*in vivo*).

Taken together, we demonstrate that the intra-BM localization of acute leukemia cells is modulated by FLT-1 activation, and determines the rate of leukemia exit from the BM microenvironment, and ultimately the survival of inoculated recipients.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Material and Methods

Primary Leukemia samples

All the primary samples analyzed consisted of BM biopsies and were collected at diagnosis with the informed consent of the patients, according to Institutional guidelines.

Leukemia cells were enriched by density gradient centrifugation prior to further analysis. All the patient samples studied were obtained from BM biopsies with a minimum of 75% leukemia blasts (as determined by the Pathology Department at IPOFG, following Institutional Protocols).

Cell culture

The leukemia cell lines studied included Acute myeloid leukemias (HL-60, HEL, kasumi, MV4;11), CML (K562, KCL-22) and ALL (TOM-1, RS4;11, 697, REH, BV-173). The ALL cell lines studied in detail were RS4;11, BV-173, REH and 697. These were cultured in complete RPMI medium (10% FBS, L-Glutamine 1X, antimicotic-antibiotic 1X) (Gibco – Invitrogen Corporation, Grand Island, NY, USA).

RNA extraction, cDNA synthesis and RT-PCR

Leukemia cells were first analysed for VEGF receptors, VEGF and PlGF expression by RT-PCR (reverse transcriptase-polimerase chain reaction). Total cellular RNA was extracted and cDNA synthesized following conventional protocols. PCR was performed using a PCR thermal cycler (Uno II, Biometra, Goettingen, Germany). The PCR program used to amplify VEGF, VEGFR-1 (FLT-1) and VEGFR-3 (FLT-4) and PLGF consisted of a denaturation step of 4 minutes at 94ºC, followed by 35 cycles of 1 minute at 94ºC, 45 seconds at 60ºC and 1 minute at 72ºC and concluded with a final extension of 7 minutes at 72ºC. For VEGFR-2 (KDR) amplification the program was the same, preceded by a pre-cycle of 4 minutes at 94ºC, 45 seconds at 55ºC and 1 minute at 72ºC. The primer sequences were as follows: FLT-1 forward primer: CAC CAA GAG CGA CGT GTG; FLT-1 reverse primer: TTT TGG GTC TCT GTG CCA G (FLT-1 PCR product: 196 bp); FLT-4 forward primer: CTG CTG GAG GAA AAG TCT GG; FLT-4 reverse primer: CTT GCA GAA CTC CAC GAT CA (FLT-4 PCR product: 550 bp); KDR forward primer: CTG GCA TGG TCT TCT GTG AAG CA; KDR reverse primer: AAT ACC AGT GGA TGT GAT GCG G (KDR PCR product: 660 bp); VEGF forward primer: AGC TTC CTA CAG CAC AAC AAA TGT; VEGF

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

reverse primer: CGC CTC GGC TTG TCA CA (VEGF PCR product: 212 bp); PlGF forward primer: CTC CTA AAG ATC CGT TCT GG; PlGF reverse primer: GGT AAT AAA TAC ACG AGC CG (PlGF PCR product:). The PCR program used to amplify Human CD19 expression from circulating mononuclear cells from inoculated NOD-SCID mice was as follows: denaturing step of 95ºC for 4 minutes, followed by 30 cycles of 1 minute at 95ºC, 2 minutes at 65ºC, 2 minutes at 72ºC, and finally by an elongation step of 10 minutes at 72ºC. The Human CD19 primer sequence was as follows: CD19 forward primer: TCACCGTGGCAACCTGACCATG; CD19 reverse primer: GAGACAGCACGTTCCCGTACTG. CD19 PCR product size: 267 bp.

FLT-1 Quantification by real Time PCR (RQ-PCR)

FLT-1 mRNA quantification was performed using the ABI Prism 7700 Sequence Detection System and the SYBR Green Master Mix kit (both from Applied Biosystems, Foster City, CA). BCR gene was used as standard reference (normalizer). The relative expression of FLT-1 was calculated by using the comparative threshold cycle (CT) method. The primer sequences were as followed: FLT-1 sense: 5' CACCAAGAGCGACGTGTG 3'; antisense: 5' TTTTGGGTCTCTGTGCCAG 3'; BCR sense 5' GAGCGTGCAGAGTGGAGGGAGAACA 3'; antisense: 5' CACAGTATCCTCAGGGTCTGGGA 3'.

VEGF ELISA

Human VEGF production by the leukemia cell lines used in the present study was determined by ELISA. Briefly, 1 x 10^6 leukemia cells were placed in 1mL serum-free RPMI for 24 hrs. After this period, the supernatant was collected and the VEGF production was quantified by ELISA (Calbiochem, Dalmstadt, Germany) following the manufacturer protocol.

FLT-1 Protein expression

Total protein extracts from ALL cell lines were obtained by lysing the cells in cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-110 and 0.1% SDS), in the presence of protease and phosphatase inhibitors. After 30 minutes on ice, lysates were centrifuged for 15 minutes at 4° C and 13000 rpm. Equal protein amounts were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (8% gels) under reducing conditions (in the presence of ß-

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

mercaptoethanol) and transferred to nitrocellulose membranes. Blots were blocked in PBS/1% BSA/0,1% Tween-20 for 1 hr at room temperature (RT) followed by incubation with primary (o/n at 4ºC) and secondary antibodies (1hr at RT). Rabbit polyclonal anti-FLT-1 (Santa Cruz Biotechnology, Santa cruz, USA) antibody was used at a concentration of 0,5 µg/mL and secondary anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, USA) was used at 1:6000. The ECL chemiluminescence detection system (Amersham Biociences, Buckinghamshire, UK) was used to visualize the presence of specific proteins on the nitrocellulose blots.

For immunofluorescent detection of FLT-1, the different leukemia cell lines were spun (2 minutes at 400 rpm) onto glass microscope slides. The cells were fixed in 4% (vol/vol) formaldehyde/phosphate-buffered saline (PBS) for 10 minutes and washed in PBS. After blocking with 5% goat serum in PBS, the cells were incubated with a mouse anti-human FLT-1 antibody o/n at 4° C (FB6, a gift from ImClone Systems, NY, USA). Then, the cells were washed and incubated with a goat anti-mouse Alexa Fluor 488 antibody (Molecular Probes, Eugene, OR, USA) for 1 hour at RT. The samples were mounted in Vectashield and analyzed by fluorescence microscopy (Axioplan Microscope, Zeiss, Oberkochen, Germany).

Transfection

RS4;11 cells were transfected with human full length FLT-1, inserted in a pcDNA3.1 vector (Invitrogen, Barcelona, Spain) using the reagent Lipofectamine 2000 (Invitrogen, Barcelona, Spain). The protocol followed was performed according to the conditions of the transfection reagent used. Transfection efficiency was checked by RQ-PCR and functional assays (described below).

Proliferation assay

For proliferation experiments, leukemia cells were cultured in serum-free RPMI at a cell density of 5 x 10^5 cells/ml. Cells were treated (10-30 ng/mL VEGF or 10 ng/ml PLGF, Sigma Aldrich, Madrid, Spain) or untreated (RPMI alone) and cultured in the presence or absence of 1 µg/mL of 6.12 monoclonal Ab (a mouse monoclonal specific anti-Human FLT-1 neutralizing Ab, from ImClone Systems, NY, USA). To each condition 5U/mL of heparin (Sigma Aldrich, Madrid, Spain) was added. After 24 and 48 hours, viable cells (as determined by trypan blue exclusion) were counted using a

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

hemocytometer. Both the FLT-1 neutralizing Ab, VEGF and PlGF were re-added every 24h. Each experimental condition was done in triplicate.

Migration assay

Cells ($1x10^6$/mL) were placed in serum free medium for 1hour in the presence/absence of the FLT-1 neutralizing Ab (6.12 mAb, 1µg/mL). Cell aliquots (100µl) were subsequently added to 5-µm-pore transwell (Corning-Costar Corp., Cambridge, Massachusetts, USA) inserts and plated into the wells of a 24 plate. The lower compartment contained 600 µl of serum free medium with or without VEGF (20ng/mL) or PlGF (10ng/mL) and heparin (5 U/mL). Migration was determined after 4 hours by counting the number of migrated cells in 6 high power fields (400x magnification) in an optical microscope.

Leukemia cell survival assay

$5x10^5$/ml cells were cultured in serum free for 60h in the presence of an anti-VEGF Ab (1µg/mL) (clone 4.6.1, a gift from Genentech, South San Francisco, USA) or the FLT-1 neutralizing mAb (clone 6.12, 1µg/mL). All antibodies were re-added every 24h. Cell viability was determined at 24h and 48h by trypan blue exclusion and cell counts (with the aid of a hemocytometer). Each experiment was done in triplicate.

***In vivo* experiments**

RS4;11, RS4;11 FLT-1 transfected and 697 cells were xenotransplanted into sub-lethally irradiated (250rads) NOD/SCID mice. $1x10^7$ leukemia cells /mouse were injected intravenously 24h after irradiation. For each experiment, 3 groups were established: the control group (vehicle alone), the FLT-1 neutralizing Ab treated group (200-500 ng/injection of 6.12 mAb) and the KDR neutralizing Ab treated group (1 µg/injection of IMC1-C11 Ab, mouse anti-Human monoclonal neutralizing antibody) (from ImClone Systems, NY, USA). The neutralizing Abs were injected 3 days after leukemia cell inoculation, and were administered every other day for the remaining of the experiment. When presenting signs of disease (such as decreased locomotion, loss of weight, etc), the mice were sacrificed, tissues and blood collected for further analysis.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Apoptosis analysis

To assess leukemia cells apoptosis *in vivo*, we performed analysis of DNA fragmentation by TUNEL in paraffin embedded BM sections. We used the *In Situ* Cell Death Detection Kit, POD and DAB Substrate Kits (Roche, Brandeburg, New Jersey, USA) and followed the manufacturer's instructions. The percentage of apoptotic cells was determined by counting a total of 200 cells/area (epiphysis or diaphysis, 400x magnification) and calculating the proportion of stained (TUNEL positive) nuclei. These quantifications were done in triplicate (3 independent counts/bone marrow section).

***In vivo* evidence for cell migration**

To determine if FLT-1 neutralization *in vivo* resulted in the impairment of leukemia cells migration within the BM microenvironment, BM paraffin sections were stained with Phalloidin (Sigma Aldrich, Madrid, Spain) to detect polymerized actin. The slides were previously deparaffinized and dehydrated using conventional methods. Next, slides were blocked for 1h with PBS1X/0,1% BSA/5% goat serum/0,1% TX-100 and incubated for 30 minutes with Phalloidin (0,5µg/mL in PBS 1X). Slides were washed with PBS, mounted in Vectashield and analyzed by fluorescence microscopy (Axioplan Microscope, Zeiss, Germany).

Immunohistochemistry

To assess the distribution of the leukemia cells in the bone marrow we studied the long bones of the sacrificed animals. The bone was fixed in 10% buffered formalin for a minimum of 24h and decalcified in a rapid bone decalcifier (Perudo00-008, Eurobio, France) for 3 hours and paraffin embedded. For the immunostaining 2µm sections were deparafinized in xylene and rehydrated in decreasing concentrations of ethanol.

Slides for VEGF and PLGF staining were blocked with the biotin blocking solution SP2001 from Vector Laboratories inc., Burlingame, California, USA for 30 minutes. Slides for TdT and Ki67 were blocked with a hydrogen peroxide solution (Dako Cytomation, Denmark).

Antigen retrieval was performed as follows: 20 min in 0.01M pH6.0 citrate buffer in a Phillips Whirlpool Jet1000 microwave oven set at 750W for VEGF and PlGF; 2 min in EDTA for TdT and 6 min in citrate buffer for Ki67 in a pressure cooker.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

VEGF (Santa Cruz Biotechnology inc., California, USA) was used diluted at 1:100 and PlGF (Santa Cruz Biotechnology inc., California, USA) at 1:50. Both were incubated overnight at 4ºCelsius and detected with a peroxidase method. TdT and Ki67 Abs were obtained from Dako Cytomation. Staining was performed in a Dako Thechmate500 Plus from Dako Cytomation at room temperature. TdT was used at 1:30 and Ki67 at 1:200.

Statistical analysis

Results are expressed as mean ± standard deviation. Data were analyzed using the unpaired two-tailed student's t test or the one-way ANOVA Tukey test . P values of <0.05 were considered significant.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Results

FLT-1 is the most common VEGF receptor expressed by leukemia cells

We started our study by analysing the global VEGF/PLGF and VEGF receptor expression pattern in primary (patient) acute and chronic leukemia samples and also in different cell lines. In total, we evaluated VEGF receptor expression, by RT-PCR, in 11 cell lines (myeloid and lymphoid leukemias) and on 83 patient BM samples. As shown in Table 1, FLT-1 is expressed by 80% of the total samples analyzed (namely all the cell lines) whereas only 18% and 24% of the samples express KDR and FLT-4, respectively. Regardless of the leukemia subtype, FLT-1 was most abundantly expressed, and particularly frequent in CML and AML (Table 1). In representative samples, cell sorting was performed to enrich for leukemia blasts; further analysis confirmed the expression of VEGF receptors could be attributed to the leukemia population (data not shown).
With regards to the ligands, VEGF was expressed by all samples studied, at different levels, while PlGF was expressed by only 1/3 of all samples studied.

FLT-1 modulates leukemia proliferation and migration

Given the high percentage of FLT-1 expressing leukemias, we reasoned FLT-1 might have a significant role in regulating leukemia biology, such as proliferation, survival and/or migration. We focused our analysis on acute lymphoblastic leukemias, since a putative role for FLT-1 in these leukemias has not been described.
We performed proliferation and migration assays with 4 ALL cell lines corresponding to different B cell stages, and expressing VEGF and VEGF receptors (Table 2). In these cell lines, we quantified FLT-1 expression by Western blotting, immunofluorescent staining and RQ-PCR (Fig 1A: WB and Fig1B: Real Time PCR). Of the 4 ALL cell lines used in this study, 697 and REH appeared to express more FLT-1, while RS4;11 expressed the least. With regards to the ligands, VEGF and PlGF, all 4 cell lines produce VEGF, in varying (but modest, see Table 2) amounts, while PlGF is not expressed by these cell lines.

For the proliferation assay, the cells were placed in serum free medium in the presence or absence of PlGF (FLT-1-specific ligand). As shown in Fig 2A, after 48h several cell lines proliferated more in response to PlGF, but significance was obtained only with the 697 cell line ($p<0,05$).

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

With regards to cell migration, PlGF-induced cell migration was analyzed in transwell migration assays. As shown in Fig 2B, the 697 cell line shows the most robust chemotactic response to PlGF (with a 2.5-fold ratio of PlGF-migrated cells/control, $P<0.01$), with the remaining 3 cell lines showing comparable migration capacity. Taken together, these data show PlGF induces proliferation and migration of subsets of ALL cells *in vitro*.

FLT-1 overexpression confers a more invasive phenotype to leukemia cells *in vivo*

In order to test the putative function of FLT-1 in modulating leukemia cell migration and BM localization *in vivo*, we used 2 distinct approaches: 1) gain of function experiment, where an ALL cell line that expresses modest FLT-1 levels (RS 4;11) was transfected with full-length FLT-1, and changes in its *in vitro* and *in vivo* phenotype were determined; 2) *in vivo* inoculation of the ALL cell line that expresses more FLT-1 and shows the strongest responses to VEGF/PlGF *in vitro* (697 cells), subsequent treatment of inoculated mice with FLT-1 monoclonal neutralizing antibodies, and observation of its *in vivo* phenotype. The results obtained were as follows:

1) Starting with the gain of function experiments, we demonstrated by RQ-PCR that transfected cells express significantly more FLT-1 than their untransfected counterparts (Fig 2C). In these transfectants, we observed a significant increase in VEGF-induced cell migration (compared to untransfected cells: $p<0.05$) but not proliferation (not shown) *in vitro*; importantly, this effect was blocked by the FLT-1 monoclonal neutralizing Ab ($p<0.05$ compared to cells exposed to VEGF alone), proving the transfected receptor is functional, and proving FLT-1 is the receptor that mediates VEGF-induced cell migration. Similar results were obtained using PlGF as the chemoattractant (not shown).

Inoculation of the transfected or untransfected cells *in vivo* also resulted in distinct phenotypes. FLT-1 overexpressing RS 4;11 cells localized mostly close to the articulations (epiphysis) of the BM in long bones (Fig 3A), and could also be found in extramedullary organs such as the spleen (Fig 3B), while untransfected cells were detected predominantly in the central part of the BM (diaphysis), and – at the same time-points- no extramedullary engraftment was observed. Interestingly, inoculation of this cell line was always associated with significant oedema in the BM cavity (Fig 3, white arrows), which may result from VEGF production by the leukemia cells.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

These data suggested the *in vitro* phenotype conferred by FLT-1 overexpression (enhanced migration in response to the ligands) is also observed *in vivo*, and may regulate also the exit of leukemia blasts into the peripheral circulation and into extramedullary sites such as the spleen.

2) As shown above, the 697 cell line is the one that expresses more FLT-1, which is reflected in the ability of these cells to migrate and proliferate in response to VEGF or PlGF. Inoculation of 697 cells *in vivo* largely reproduced the phenotype observed with the RS4;11 FLT-1 overexpressing cell line (mentioned above). Shortly after inoculation, 697 cells accumulate mostly in the BM epiphysis of inoculated recipients (Fig 4A). As disease develops (after 15-20 days), 697 cells are detected in the circulation of inoculated mice (as determined by RT-PCR, Table 3), and we could also detect leukemia blasts engrafted into extramedullary sites such as the spleen and lungs (not shown). This phenotype is reversed upon treatment of inoculated mice with the neutralizing monoclonal Ab to FLT-1 (6.12 Ab), but not with an Ab against KDR (as a control). In 6.12 treated mice, 697 cells are detected in the BM diaphysis of inoculated mice (Fig 4A), and the presence of leukemia cells in the peripheral circulation is impeded up to day 20 following leukemia inoculation (Table 3). As a consequence of FLT-1 neutralization, the group of mice that received the 6.12 Ab therapy survived significantly longer than control or KDR Ab treated mice (Fig 4B), and at day 20 post-inoculation, have reduced circulating human VEGF levels, which again suggests reduced leukemia cell exit from the BM into the peripheral circulation (Fig 5A). Notably, at day 40 post-inoculation, 6.12 Ab-treated mice still had reduced (below 20 pg/mL) Human VEGF levels (not shown), demonstrating the efficacy of the treatment in delaying the onset of extramedullary disease.

These data suggest one function of FLT-1 may be to determine leukemia cell localization within the BM microenvironment. The selective localization of leukemia cells within the BM seems to regulate their exit into the peripheral circulation and eventual engraftment into extramedullary tissues such as the spleen.

FLT-1 neutralization results in increased leukemia cell apoptosis in the BM

Next, we sought to determine the mechanisms whereby FLT-1 neutralization and re-distribution of leukemia cells within the BM resulted in increased survival of inoculated mice, and a decrease in circulating blasts. For this, we stained BM sections at day 18

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

after inoculation (we chose areas packed with blasts: over 80% of the total cells) with Ki67 (proliferation marker) and TUNEL (to detect apoptosis). FLT-1 neutralization resulted in a significant increase in the proportion of apoptotic leukemia cells in the BM diaphysis and epiphysis of inoculated mice (Table 4), while there was no difference in the proportion of proliferating (Ki67 positive) cells and these were evenly distributed throughout the BM of control and 6.12 Ab treated mice (not shown). In parallel, we treated 697 cells with the Ab 6.12 *in vitr*o, but surprisingly there was very little effect in cell apoptosis (data not shown). Thus, the intra-medullary localization of leukemia cells, which is modulated by FLT-1 activation, regulates leukemia survival *in vivo*. As a consequence of FLT-1 neutralization, the presence of leukemia cells in the peripheral blood of inoculated mice was significantly delayed (Table 3)

Mechanisms

PlGF and VEGF gradients explain the BM distribution of leukemia cells

To define the mechanisms whereby FLT-1 modulated the localization of leukemia cells within the BM, we started by determining the normal distribution of its ligands, PlGF and VEGF. As shown in Figure 5B, PlGF and VEGF BM staining patterns suggest the existence of a defined gradient, from weak or diffused staining in the diaphysis to a clear accumulation in the epiphysis (Fig 5B shows sections from a littermate that was not inoculated with leukemia cells).

Next we sought to visualize leukemia cell movement within the BM, by looking for evidence of "polarized" leukemia cells. As shown in Figure 6A, phaloidin staining (to detect polimerized actin) demonstrated the existence of polarized cells in the epiphysis of control mice, while those treated with the neutralizing Ab 6.12 had little evidence of actin polarization (an almost complete absence of phalloiding staining, Fig 6A).

These results fit with the notion proposed above, and with the *in vitro* results: inoculated leukemia cells respond to paracrine VEGF/PlGF gradients within the BM microenvironment, migrate and localize to those areas where VEGF and PLGF production is most abundant. FLT-1 neutralization, in turn, impedes cell movement within the BM microenvironment, thereby conditioning cell survival and resulting in leukemia apoptosis.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

PlGF-mediated leukemia migration involves lipid raft formation

Next, we investigated some of the molecular mechanisms whereby PlGF induced leukemia cell migration. Besides demonstrating VEGF increases matrix metalloproteinase secretion by leukemia cells (not shown), we also sought to define the biochemical mechanisms whereby FLT-1 activation resulted in distinct cell distribution patterns *in vivo*. As shown in Figure 6B, PlGF-induced leukemia migration was blocked by pre-treating the leukaemia cell line 697 with nystatin, a cholesterol sequestering agent that blocks lipid raft formation. These results suggest PlGF-induced leukemia cell migration involves polarized cell movement requiring lipid raft formation and actin polimerization.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Discussion

Similarly to solid tumour growth, leukemia expansion within the BM microenvironment has been correlated with an increase in vascular content (angiogenesis). This has been held true in acute as well as chronic leukemias, as shown in both laboratory and clinical studies (20-24). Several facts stand out from most studies published to date: leukemia cells release significant amounts of VEGF, and a proportion of leukemia blasts co-express its receptors VEGFR-1 (FLT-1), VEGFR-2 (KDR) and VEGFR-3 (FLT-4). In the case of KDR, we and others have demonstrated the existence of functional autocrine loops that support acute myeloid leukemia migration and survival both *in vitro* and *in vivo* (12, 13, 26). Interestingly, in the case of FLT-4, its functions appear to be exerted in a paracrine manner, mediating the proliferation and resistance to chemotherapy in subsets of acute myeloid leukemias (13). However, and also reflecting the lack of knowledge in other cell types, much less is known concerning a role for FLT-1 in leukemia biology. In the present report, we sought to define a role for FLT-1 in acute lymphoblastic leukemia, independent of the expression or activation of the other VEGF receptors.

We demonstrate that FLT-1 activation on ALL cells has little effect in proliferation but mediates cell migration *in vitro*, in agreement with previous studies in other cell types (Malignant T cells: reference 25; smooth muscle cells: 27; monocytes: 28). We add on to these findings, and demonstrate *in vivo* that FLT-1 activation, or its neutralization, changes the behaviour of ALL cells. Using a NOD-SCID mouse model, we observed that FLT-1 overexpressing ALL cells engraft and localize close to articulations (epiphysis) of long bones of inoculated recipients, whereas those expressing less FLT-1 (or where FLT-1 activation was blocked by specific neutralizing monoclonal Abs) localize predominantly to the central portions (diaphysis) of the BM of long bones. This phenotype correlates with a delay in the presence of ALL cells in extramedullary organs or the peripheral circulation, and a consequent increase in the survival of inoculated recipients. As a consequence of FLT-1 neutralization, treated mice survive significantly longer than their untreated counterparts, and there is significant reduction in circulating ALL cells.

In human leukemia, the presence of extramedullary disease is considered an unfavourable prognostic factor (1-5), while the persistence of circulating blasts after induction regimens indicates patients at higher risk of relapse (6-8). This is particularly

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

relevant in pediatric ALL patients who may develop extramedullary disease, especially in the CNS (9). Thus, there is great interest in determining the mechanisms that may explain the exit of acute leukemia cells from the BM microenvironment. In the present report we reveal a crucial role for FLT-1 in regulating the distribution of leukemia cells within the BM microenvironment, their survival, and consequently their exit into the peripheral circulation.

The data obtained from our *in vivo* studies should be interpreted in the context of the vascularization of long bones. Previous studies have revealed that the BM of long bones such as femurs, is vascularized by the afferent (arterial) and the efferent (venous) vascular systems. The principal components of the afferent vascular system of a long bone have been suggested to "enter" the BM in the diaphysis, supplying nutrients and oxygen to the hematopoietic elements, whereas those of the efferent system leave the BM draining the hematopoietic elements predominantly in the epiphysis (29).
In our mouse models, we observed that ALL cells expressing FLT-1 localize predominantly in the epiphysis, supporting the concept that such a subset of acute leukemia cells leave the BM via the efferent circulatory system, en route to establish extramedullary growth. Thus, ALL cells are attracted to these areas of the BM via FLT-1 activation. In agreement, we demonstrate that normal (non-leukemic) BM sections exhibit a clear gradient of murine VEGF and PlGF expression, increasing from the dyaphisys to the epiphysis. Conversely, the 697 cell line used in our *in vivo* models produces very modest VEGF levels (at the assay detection limit, Table 2), and does not express PlGF, suggesting the phenotype seen *in vivo* results from paracrine stimulation by endogenous murine VEGF/PlGF. The cells that express these ligands in abundance within the BM, and the regulation of such expression, are still undisclosed, although previous reports have shown VEGF is abundantly expressed by osteoclasts and osteoblasts in the epiphysial areas of long bones, where it was shown to be essential for fracture healing (30, 31).

The effects of FLT-1 neutralization extend beyond the blockade of ALL cell movement, and appear also to result in leukemia apoptosis. Our results in this regard support the notion that the attraction/tropism of subsets of leukemia cells for particular areas (niches) within the BM microenvironment may in fact condition their survival and expansion. FLT-1 activation on ALL cells could be exerted in a paracrine (external) or

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

autocrine (internal) VEGF/PLGF production. In the case of the ALL cell lines we studied, none expressed PlGF, although VEGF was produced in modest amounts by all of them. However, *in vitro* neutralization of VEGF or FLT-1 resulted only in a minor delay in leukemia growth and a modest increase in cell apoptosis (results were not significant, data not shown), supporting the notion that autocrine VEGF may not promote relevant FLT-1 activation, at least on the ALL cells studied.
In vivo, the neutralizing Ab used in our studies should block both paracrine and autocrine FLT-1 activation on the leukemia blasts, thus producing a more evident phenotype. There are also examples of heterotypic engagement of adhesion molecules by malignant cells resulting in increased expression and activation of angiogenic molecules such as VEGF (see 11 as an extensive review of such mechanisms in multiple myeloma). If we extrapolate the studies in multiple myeloma to our model, the interaction of ALL cells with the stromal elements of the BM might result in a modulation of VEGF and FLT-1 and consequently result in greater dependence of the leukemia cells on FLT-1 signalling *in vivo*.

Taken together, we observed that *in vivo* FLT-1 neutralization in subsets of leukemias such as ALL, directly impedes cell migration into more "suitable" niches in the BM, induces leukemia apoptosis, and as a consequence delays leukemia exit into the peripheral circulation, reduces circulating Human VEGF levels and delays extramedullary growth (see model proposed in Figure 7).

To our knowledge, it is the first time that FLT-1 activation is clearly implicated in regulating leukemia cell migration inside the BM, and that neutralization of FLT-1 results in cell apoptosis, thus impeding cell exit into the peripheral circulation. Recent studies have suggested FLT-1 activation might be involved in situations of abnormal localization of immature precursors (ALIP), particularly frequent in Myelodisplastic Sindromes (MDS) (17). We add on to these findings, and demonstrate that the paracrine activation of FLT-1 may result in migration and survival also of acute leukemia cells, such as subsets of acute lymphoblastic leukemias, within the BM microenvironment.

With regards to the mechanisms whereby FLT-1 may mediate ALL cell migration, we demonstrate for the first time that this involves actin polimerization and lipid raft formation. These results are not surprising, given previously published data

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

demonstrating the importance of actin polimerization (and focal adhesion kinase) for cell migration in other cell types (32) and also a putative role for lipid rafts in cell chemotaxis and migration (33, 34). Concerning the effects of nystatin, namely its capacity to block lipid raft formation, and the mechanisms underlying its migration-inhibitory effect, it remains to be established whether this is required for FLT-1 re-distribution along the cell membrane, modulating polarized cell movement, or whether this is connected with receptor turnover and internalization (our ongoing studies). These results not withstanding, we demonstrate active and directed cell movement *in vivo* and *in vitro*, and the involvement of particular biochemical mechanisms in this process, which adds to our understanding of the biology of ALL growth within the BM microenvironment.

Our data suggest the use of FLT-1 blockers for treating subsets of acute leukemia, namely ALL, may be of therapeutic benefit. This may prove beneficial also in combination with chemotherapeutic agents used to treat ALL patients, such as vincristine or daunorubicin (among others); since FLT-1 neutralization impedes cell movement within the BM, localizing the leukemia blasts mostly in areas of afferent (arterial) vascularization, these should be more exposed to inoculated (circulating) chemotherapy.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

References

1. Gaynon PS, Desai AA, Bostrom BC, et al. Early response to therapy and outcome in childhood acute lymphoblastic leukemia: a review. Cancer. 1997; 80(9): 1717-26.
2. Rautonen J, Hovi L, Siimes MA. Slow disappearance of peripheral blast cells: an independent risk factor indicating poor prognosis in children with acute lymphoblastic leukemia. Blood. 1988; 71(4): 989-91.
3. Rautonen J, Siimes MA. Can late relapse be predicted at initial diagnosis in childhood acute lymphoblastic leukemia? Eur J Haematol. 1989; 43(3): 215-9.
4. Griffin TC, Shuster JJ, Buchanan GR, Murphy SB, Camitta BM, Amylon MD. Slow disappearance of peripheral blood blasts is an adverse prognostic factor in childhood T cell acute lymphoblastic leukemia: a Pediatric Oncology Group study. Leukemia. 2000; 14(5): 792-5.
5. Dusenbery KE, Howells WB, Arthur DC, Alonzo T, Lee JW, Kobrinsky N, et al. Extramedullary leukemia in children with newly diagnosed acute myeloid leukemia: a report from the Children's Cancer Group. J Pediatr Hematol Oncol. 2003; 25(10): 760-8.
6. Rivera GK, Zhou Y, Hancock ML, Gajjar A, Rubnitz J, Ribeiro RC, et al. Bone marrow recurrence after initial intensive treatment for childhood acute lymphoblastic leukemia. Cancer. 2005; 103(2): 368-76.
7. Gajjar A, Harrison PL, Sandlund JT, Rivera GK, Ribeiro RC, Rubnitz JE, et al. Traumatic lumbar puncture at diagnosis adversely affects outcome in childhood acute lymphoblastic leukemia. Blood. 2000; 96(10): 3381-4.
8. Sandlund JT, Harrison PL, Rivera G, Behm FG, Head D, Boyett J, et al. Persistence of lymphoblasts in bone marrow on day 15 and days 22 to 25 of remission induction predicts a dismal treatment outcome in children with acute lymphoblastic leukemia. Blood. 2002; 100(1): 43-7.
9. Gajjar A, Ribeiro R, Hancock ML, Rivera GK, Mahmoud H, Sandlund JT, et al. Persistence of circulating blasts after 1 week of multiagent chemotherapy confers a poor prognosis in childhood acute lymphoblastic leukemia. Blood. 1995; 86(4): 1292-5.
10. Aguayo A, Giles F, Albitar M. Vascularity, angiogenesis and angiogenic factors in leukemias and myelodysplastic syndromes. Leuk Lymphoma. 2003; 44(2): 213-22.
11. Podar K, Anderson KC. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. Blood. 2005; 105(4): 1383-95.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

12. Dias S, Shmelkov SV, Lam G, Rafii S. VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. Blood. 2002; 99(7): 2532-40.
13. Dias S, Choy M, Alitalo K, Rafii S. Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood. 2002; 99(6): 2179-84.
14. Giles FJ, Cooper MA, Silverman L, Karp JE, Lancet JE, Zangari M, et al. Phase II study of SU5416--a small-molecule, vascular endothelial growth factor tyrosine-kinase receptor inhibitor--in patients with refractory myeloproliferative diseases. Cancer. 2003; 97(8): 1920-8.
15. Hicklin DJ, Ellis LM. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol. 2005; 23(5): 1011-27.
16. Ferrara N. Vascular endothelial growth factor: basic science and clinical progres. Endocr Rev. 2004; 25(4): 581-611.
17. List AF, Glinsmann-Gibson B, Stadheim C, Meuillet EJ, Bellamy W, Powis G. Vascular endothelial growth factor receptor-1 and receptor-2 initiate a phosphatidylinositide 3-kinase-dependent clonogenic response in acute myeloid leukemia cells. Exp Hematol. 2004; 32(6):526-535.
18. Bellamy WT, Richter L, Sirjani D, Roxas C, Glinsmann-Gibson B, Frutiger Y, et al. Vascular endothelial cell growth factor is an autocrine promoter of abnormal localized immature myeloid precursors and leukemia progenitor formation in myelodysplastic syndromes. Blood. 2001; 97(5): 1427-34.
19. Vincent L, Jin DK, Karajannis MA, Shido K, Hooper AT, Rashbaum WK, et al. Fetal stromal-dependent paracrine and intracrine vascular endothelial growth factor-a/vascular endothelial growth factor receptor-1 signaling promotes proliferation and motility of human primary myeloma cells. Cancer Res. 2005; 65(8): 3185-92.
20. Aguayo A, Kantarjian H, Manshouri T, Gidel C, Estey E, Thomas D, et al. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood. 2000; 96(6): 2240-5.
21. Padro T, Ruiz S, Bieker R, Burger H, Steins M, Kienast J, et al. Increased angiogenesis in the bone marrow of patients with acute myeloid leukemia. Blood. 2000; 95(8): 2637-44.
22. Kini AR, Kay NE, Peterson LC. Increased bone marrow angiogenesis in B cell chronic lymphocytic leukemia. Leukemia. 2000; 14(8); 1414-8.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

23. Kini AR, Peterson LA, Tallman MS, Lingen MW. Angiogenesis in acute promyelocytic leukemia: induction by vascular endothelial growth factor and inhibition by all-trans retinoic acid. Blood. 2001; 97(12): 3919-24.
24. Molica S, Vacca A, Levato D, Merchionne F, Ribatti D. Angiogenesis in acute and chronic lymphocytic leukemia. Leuk Res. 2004; 28(4): 321-4.
25. Hayashibara T, Yamada Y, Miyanishi T, Mori H, Joh T, Maeda T, et al. Vascular endothelial growth factor and cellular chemotaxis: a possible autocrine pathway in adult T-cell leukemia cell invasion. Clin Cancer Res. 2001; 7(9): 2719-26.
26. Dias S, Hattori K, Zhu Z, Heissig B, Choy M, Lane W, et al. Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. J Clin Invest. 2000; 106(4): 511-21.
27. Wang H, Keiser JÁ. Vascular endothelial growth factor upregulates the expression of matrix metalloproteinases in vascular smooth muscle cells: role of flt-1. Circ Res. 1998; 83(8): 832-40.
28. Sawano A, Iwai S, Sakurai Y, Ito M, Shitara K, Nakahata T, et al. Flt-1, vascular endothelial growth factor receptor 1, is a novel cell surface marker for the lineage of monocyte-macrophages in humans. Blood. 2001; 97(3): 785-91.
29. Rhinelander FW, Stewart CL, Wilson JW. Bone Vascular Supply. In Skeletal Res. 1979; pp 3. New York Academic Press.
30. Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z, Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med. 1999; 5(6): 623-8.
31. Street J, Bao M, deGuzman L, Bunting S, Peale FV, Jr, Ferrara N, et al. Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover. Proc Natl Acad Sci U S A. 2002; 99(15): 9656-61.
32. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. Nat Rev Mol Cell Biol. 2005; 6(1): 56-68.
33. Wysoczynski M, Reca R, Ratajczak J, Kucia M, Shirvaikar N, Honczarenko M, et al. Incorporation of CXCR4 into membrane lipid rafts primes homing-related responses of hematopoietic stem/progenitor cells to an SDF-1 gradient. Blood. 2005; 105(1): 40-8.
34. Gomez-Mouton C, Lacalle RA, Mira E, Jimenez-Baranda S, Barber DF, Carrera AC, et al. Dynamic redistribution of raft domains as an organizing platform for signaling during cell chemotaxis. J Cell Biol. 2004; 164(5): 759-68.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Acknowledgments

This work was supported by a grant from Fundação para a Ciencia e Tecnologia (POCTI/CBO/38391/2001). Rita Fragoso is supported by a Fellowship from FCT (PRAXIS/SFRH/BD/14162/2003). The authors would like to acknowledge other members of the Angiogenesis Laboratory and the Hematology group (CIPM), for critically reading this manuscript, Dolores Bonaparte and Cristina Rodrigues (Instituto Gulbenkian Ciencia) for their help with the *in vivo* work.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Figure Legends

Figure 1.

VEGFR-1 (FLT-1) expression level of the different ALL cell lines used in this study, as determined by western blotting (A) and Real Time quantitative PCR (B). The 697 cell line expresses the highest levels of FLT-1. All procedures are described in Materials and Methods.

Figure 2.

A. Proliferation assays to test the mitogenic effects of PlGF stimulation of the 4 ALL cell lines used in this study. Results show cells that were stimulated for 24 hrs with PlGF (10ng/mL) in the presence of heparin, and counted in triplicate experiments (using the trypan blue exclusion test). *: PlGF induces a significant increase in cell proliferation in the 697 cell line ($p<0.05$).

B. Transwell migration assay to test the chemotactic effects of PlGF on the 4 ALL cell lines used in this study. Results show the ratio of PlGF-induced cell migration/control cells (calculated from the mean cell number in 6 high power fields, HPF: 400x magnification) after 4 hours of PlGF (10ng/mL) stimulation. *: 697 migrated significantly more in the presence of PlGF (3 independent experiments, $p<0.01$ for 697 cells).

2 C..RS4;11 cells were transfected with full length FLT-1, to determine the result of FLT-1 overexpression *in vitro* and *in vivo*. FLT-1 mRNA expression, as determined by Real Time PCR. Note the increased expression of FLT-1 mRNA in FLT-1 transfected cells (RS4;11 FLT-1T). **D.** Transwell migration assay was performed with non-transfected RS4;11 and those transfected with full length FLT-1, in the presence of VEGF (30 ng/mL) for 4 hrs. Results are shown as the mean cell number in 6 HPF (400x magnification), and demonstrate that RS4;11 FLT-1T cells migrate significantly more in the presence of VEGF than their untransfected counterparts (*: 3 independent experiments, $p<0.05$). **E.** Migration assay to demonstrate the transfected full length FLT-1 modulates cell migration. Results show RS4;11 FLT-1 transfected cells exposed to VEGF (30 ng/mL) for 4 hrs, alone or in the presence of the FLT-1 neutralizing Ab, 6.12 (1 μg/mL). *: VEGF-induced RS4;11 FLT-1T migration is significantly reduced in the presence of the 6.12 Ab (3 independent experiments, $p<0.05$), demonstrating the transfected receptor is functional and modulates cell migration.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Figure 3.

FLT-1 transfected ALL cells show different *in vivo* phenotype. Native RS4;11 or those transfected with full length FLT-1 were inoculated into sub-lethally irradiated NOD-SCID mice, as described in Methods. **A.** Results show the transfected cells (lower panels) formed masses of leukemia cells in the epiphysis (here shown at 400x magnification) of the long bones, 3-5 days after inoculation, whereas the untransfected cell line localized predominantly in the diaphysis (upper panels, 200x magnification). Black arrows: leukemia cells in the different areas of the BM of long bones. White arrows: evidence for blood leakage in the bones of inoculated recipients. Results shown are representative of 3 mice/condition.

B. Evidence for the earlier appearance of extramedulary disease in mice inoculated with FLT-1 transfected RS4;11 cells. Lower panel shows a spleen section (200x magnification) of a NOD-SCID recipient, 10 days after RS4;11 FLT-1T cells inoculation, clearly evidencing the engraftment of leukemia cells. The results are representative of 3 independent animals.

Figure 4.

A. Evidence for distinct BM localization of 697 cells, in untreated recipients or those treated with the neutralizing monoclonal Ab to FLT-1, 6.12 (FLT-1 Ab, administered every 2 days at 500ng/injection). Lower panels show evidence for the preferential localization of inoculated 697 cells in the epiphysis of the long bones of NOD-SCID mice (right panel (150x magnification and inset, 200x magnification), visualized by human TdT staining as described in Materials and Methods. Note the almost complete absence of TdT staining in the diaphysis of Control or KDR Ab treated mice (lower left panel and inset). Upper panels show BM of long bones of NOD-SCID recipients treated every 2 days with the FLT-1 neutralizing Ab 6.12. Results show a strong accumulation of TdT positive leukemia cells in the diaphysis of the bone (upper left panel and inset), whereas the epiphysis shows no evidence for the presence of 697 cells (upper right panel and inset). The results shown are representative of 12 recipients for each condition, as determined in 3 independent experiments.

Figure 4B.

FLT-1 neutralization prolongs the survival of NOD-SCID mice inoculated with ALL cells. The results show a significant increase ($p<0.05$, Kaplan meyer curve) in the

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

survival of NOD-SCID mice inoculated with 697 cells and treated every 2 days with the FLT-1 neutralizing Ab 6.12 (FLT-1 Ab, 500ng/injection). The results shown were obtained from 3 independent experiments (4 mice per condition in all experiments).

Figure 5.

A. FLT-1 neutralization reduces the levels of circulating Human VEGF plasma levels in NOD-SCID mice inoculated with 697 ALL cells. Results show a significant decrease ($p<0.05$) in Human VEGF levels (ELISA) in the plasma of NOD-SCID mice inoculated the 697 cell line (after 10 days) and treated every other day with the FLT-1 neutralizing Ab 6.12 (FLT-1 Ab, 500ng/injection). These results were obtained in 3 independent experiments.

Figure 5B.

VEGF and PlGF accumulate in the epiphysis of long bones of normal (non-leukemic) NOD-SCID mice, littermate controls of mice used in all the other experiments described in this paper. Images (at 200x magnification) show BM sections, localized to the diaphysis or epiphysis, stained with mVEGF and mPlGF specific Abs. Note the clear accumulation of VEGF and PlGF (brown staining) close to the epiphyseal regions of the bone, whereas the diaphysis shows little evidence for VEGF or PlGF staining. Results shown were obtained from 2 independent determinations, done in animals of different ages.

Figure 6.

A. FLT-1 neutralization reduces actin polimerization in leukemia cells within the BM of inoculated NOD-SCID mice. Upper panels (630x magnification) show BM sections of control (untreated) NOD-SCID mice, 10 days after leukemia cells inoculation, stained and visualized in a fluorescence microscope as described in Materials and Methods. Green staining shows leukemia cells with evidence for polarization/movement (Phalloidin, stains polimerized actin). Note the almost complete absence of Phalloidin staining in the epiphyseal regions of BM of mice treated with the 6.12 FLT-1 neutralizing Ab (FLT-1Ab, lower panels, 630x magnification). These results were obtained from 3 independent experiments.

Figure 6B.

Nystatin, a cholesterol sequestering agent that impedes lipid raft formation, blocks PLGF-induced leukemia cell migration. Results show transwell migration assay data,

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

demonstrating that 697 cell migration (mean cell number in 6 HPF, 400x) in response to PLGF (10ng/mL) is significantly ($p<0.05$) impeded by co-treatment with nystatin, used as described in Materials and Methods. Results shown are representative of 2 independent experiments.

Figure 7.

Proposed model for the role of FLT-1 in regulating leukemia growth and localization within the BM, and in modulating the exit of ALL cells into the peripheral circulation.

Table 1.

VEGF receptors are expressed at different frequencies in acute and chronic leukemia samples (primary leukemias and cell lines). Leukemia blasts were obtained from BM samples of patients with different types of acute and chronic leukemias, and FLT-1, KDR and FLT-4 positivity was determined by RT-PCR as described in Materials and Methods.

Table 2.

The different ALL cell lines used in this study correspond to distinct stages of B cell differentiation. Of note, the cell lines that correspond to more "differentiated" B cells express higher levels of FLT-1. Also shown are the VEGF receptor expression profile of the 4 cell lines studied, and their Human VEGF production *in vitro*. Note that the 4 cell lines express FLT-1 and none express KDR.

Table 3.

FLT-1 neutralization impedes the exit of ALL cells from the BM to the peripheral circulation. As described in Materials and Methods, total mononuclear cells were isolated from the PB of inoculated NOD-SCID mice, and RNA was extracted to allow screening for human cells (as determined by Human CD19 expression) in the circulation, by RT-PCR. Results show that at day 19 after leukemia cell inoculation, it was possible to detect circulating blasts in untreated mice and those treated with the KDR Ab. Conversely, mice treated with the neutralizing FLT-1Ab had no evidence for circulating leukemia cells, at this same time-point. These data are representative of 3 independent experiments, with the same results.

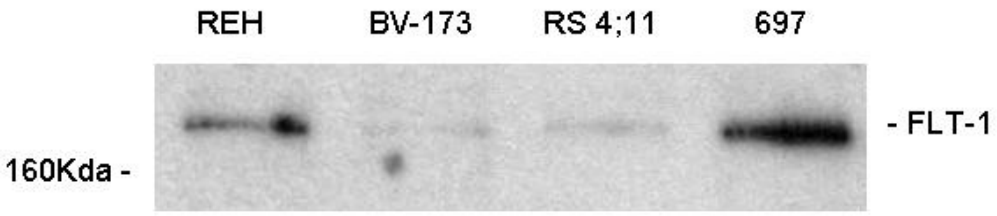
From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Table 4.

FLT-1 neutralization increases leukemia apoptosis within the BM. Results show the percentage of TUNEL-positive leukemia cells (out of a total of 200 cells counted/BM area). Note the increase in TUNEL-positive cells in the BM of mice treated with the neutralizing Ab to FLT-1 (in the diaphysis and the epiphysis). Results shown are representative of 3 independent determinations/experiments.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

A.



B.

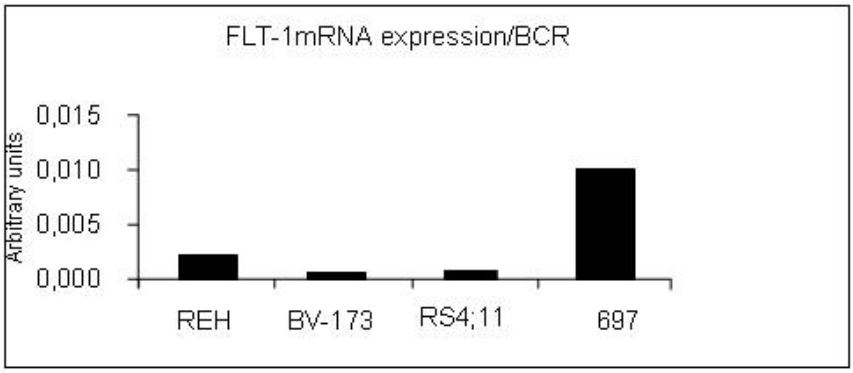


Fig 1

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

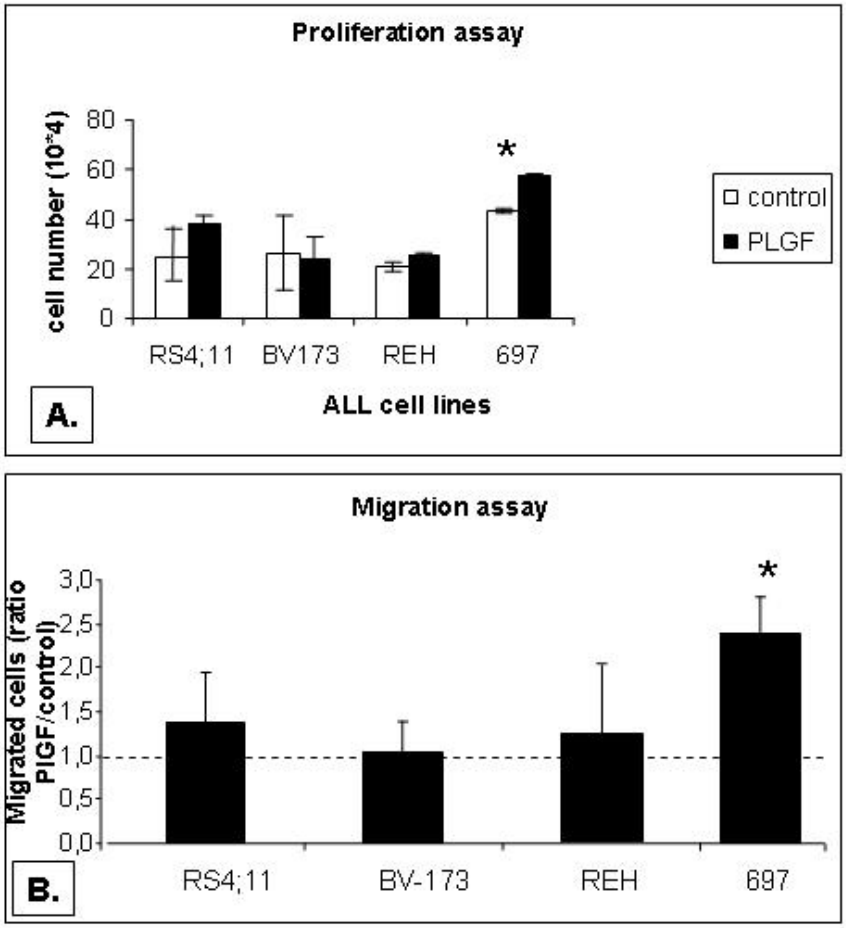
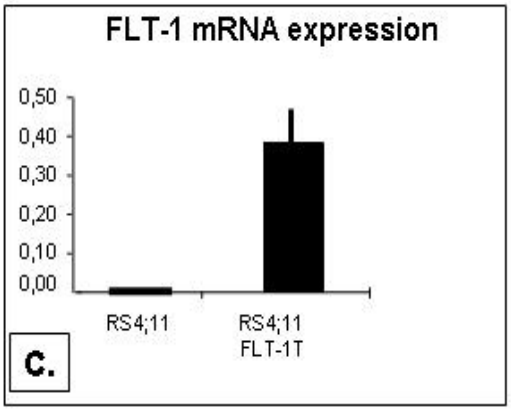
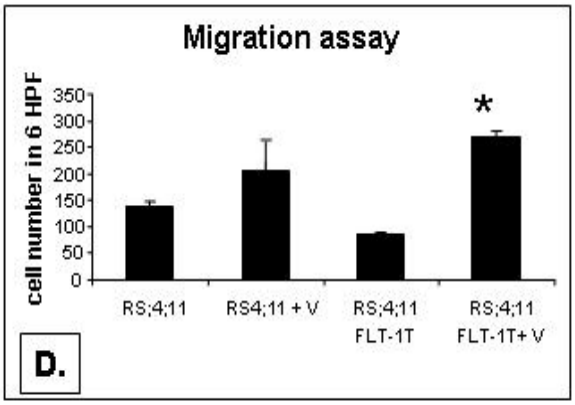


Fig 2

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.





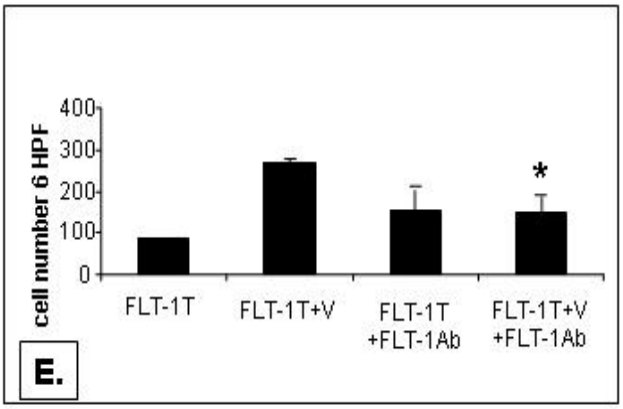


Fig 2

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

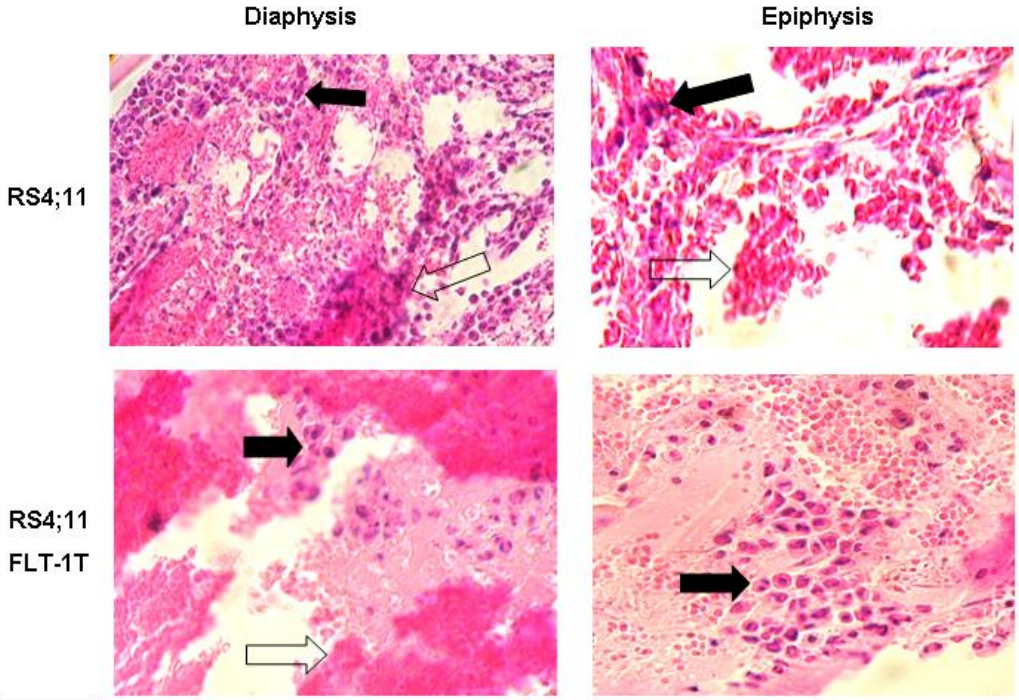
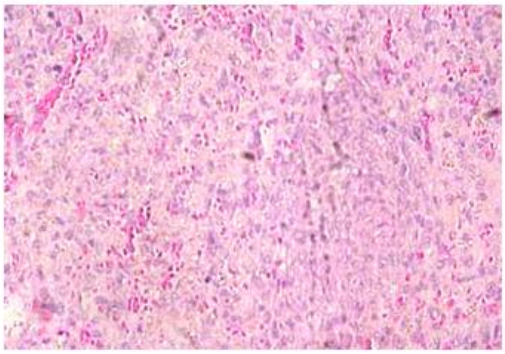


Fig 3A

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

RS4;11



RS4;11
FLT-1T

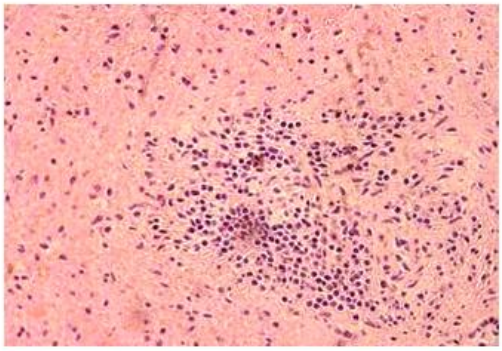


Fig. 3B

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

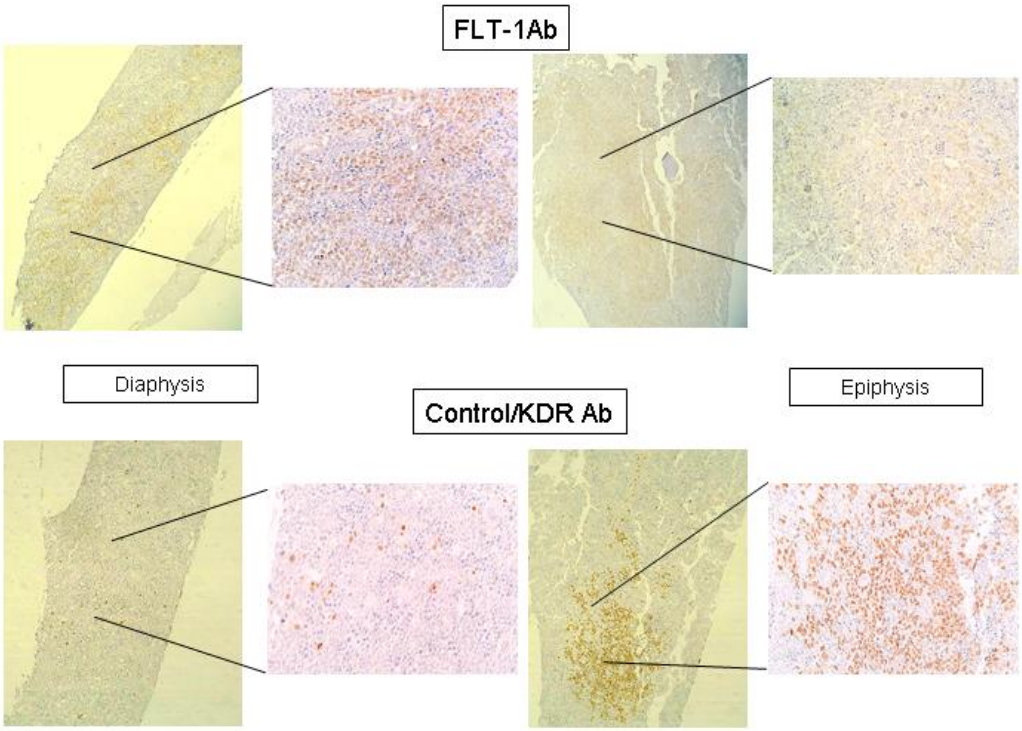


Fig 4A

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

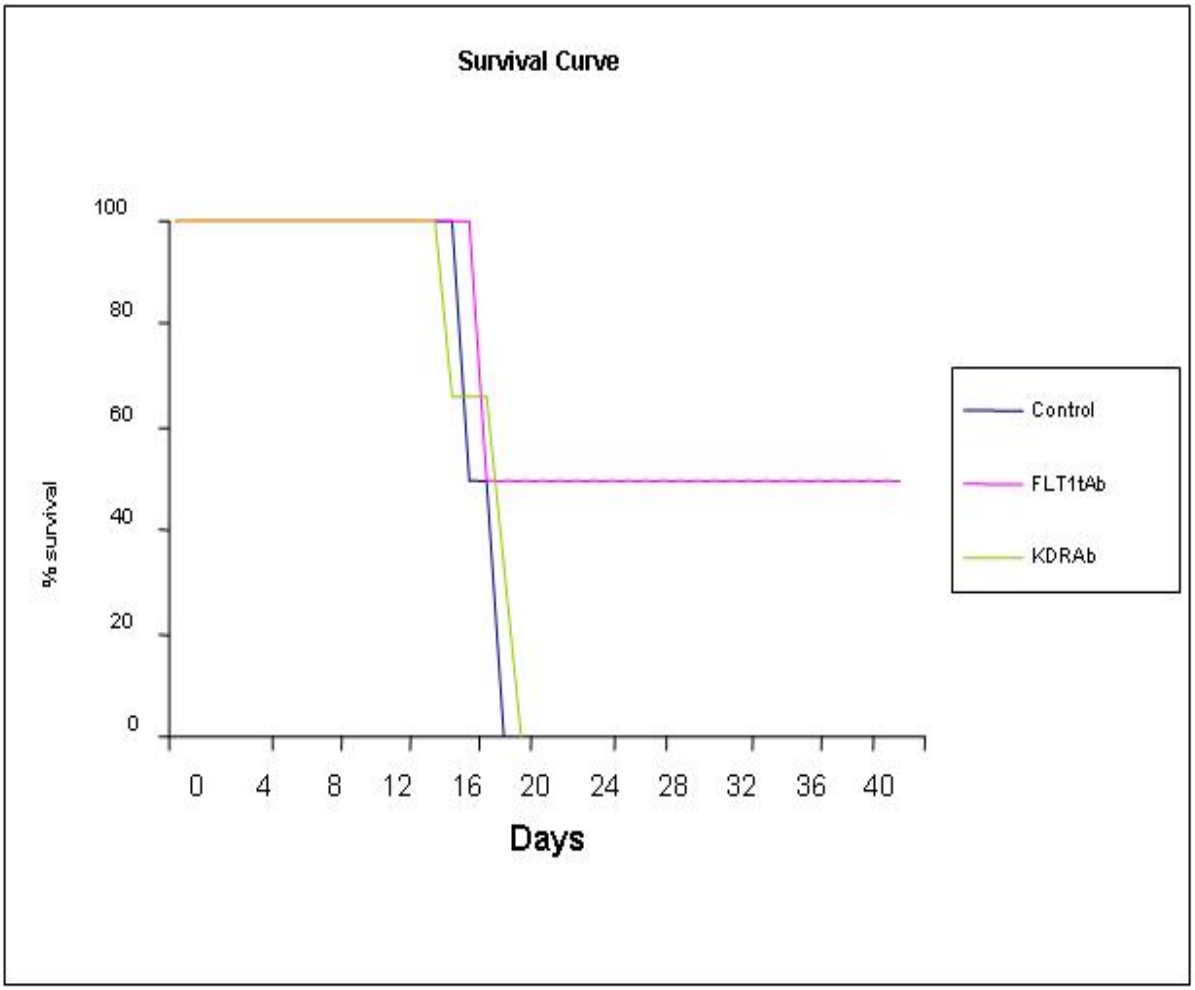


Fig 4B

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

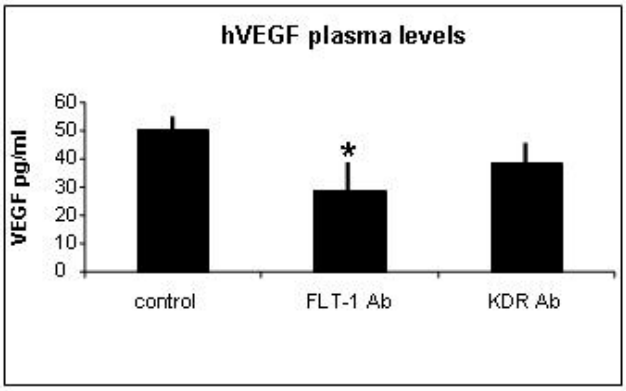


Fig 5A

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

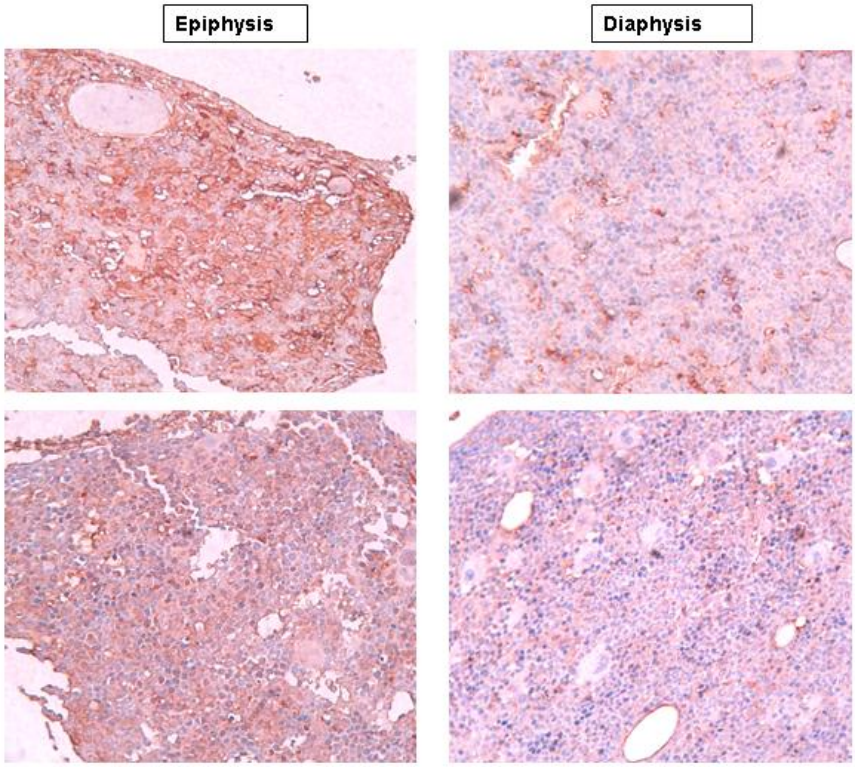


Fig 5B

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

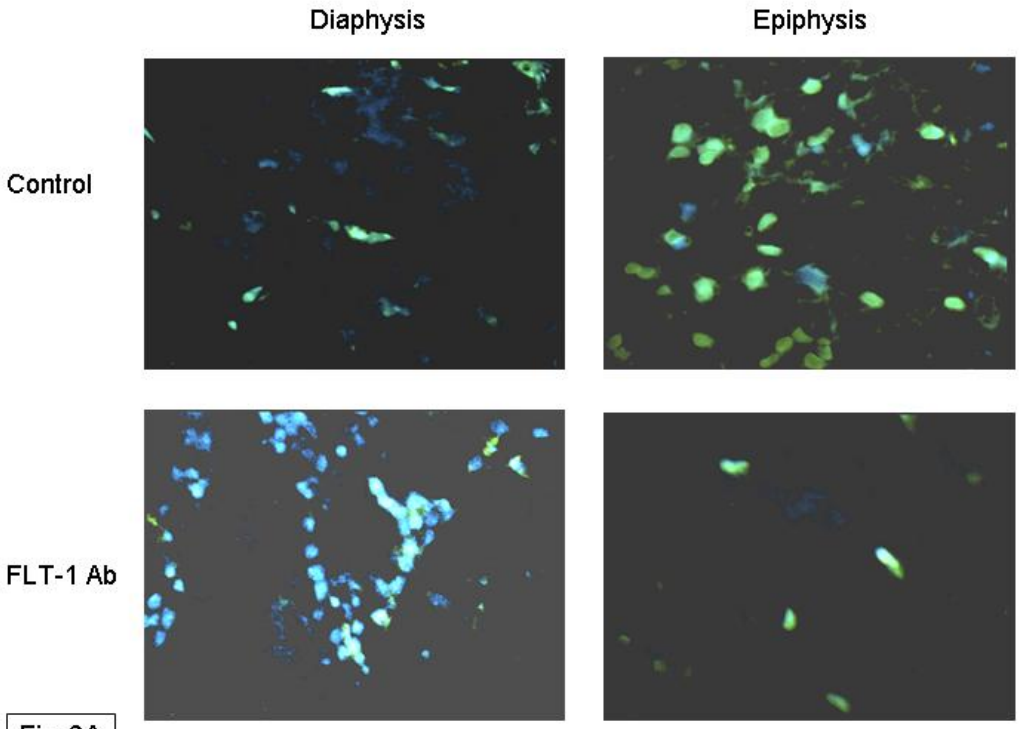


Fig 6A

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

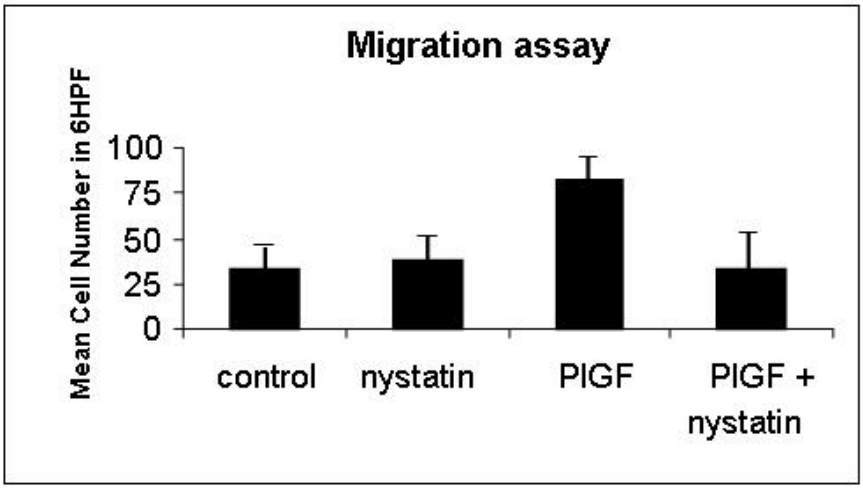
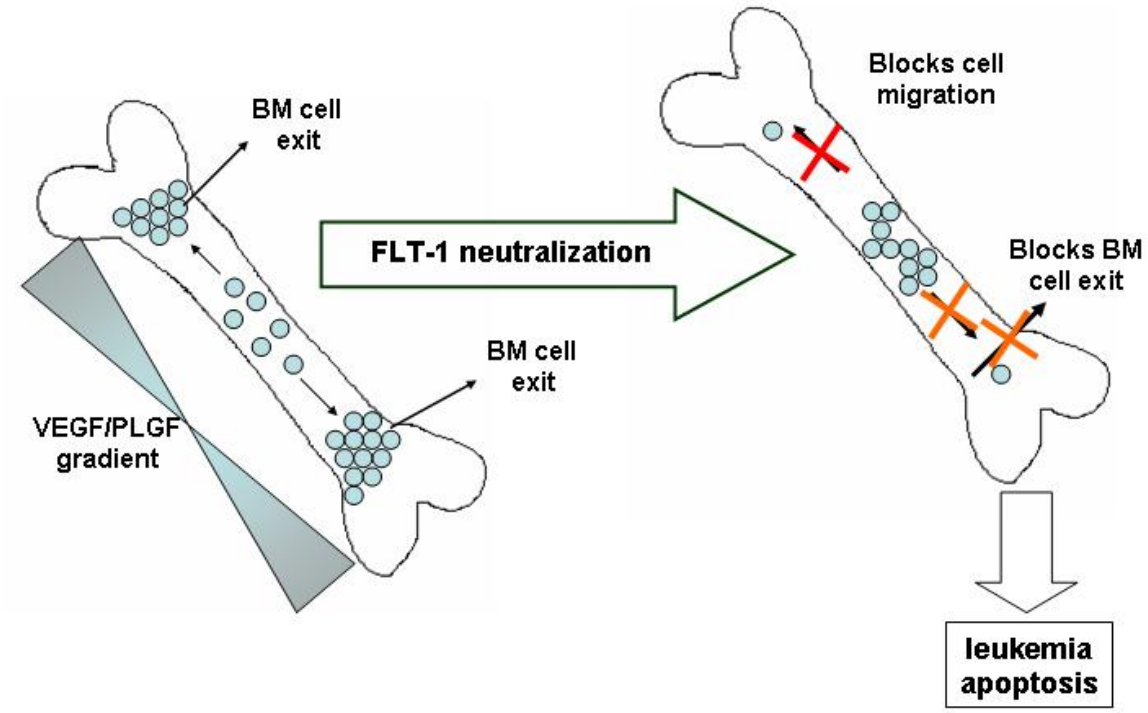


Fig 6B

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.



Mechanistic view of the role of FLT-1 in regulating leukemia growth in the BM

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Type of Leukemia	%FLT-1 positive	%KDR positive	%FLT-4 positive
CML (n=30)	91%	17,6%	29%
ALL (n=23)	71%	9%	39%
AML (n=30)	78%	28%	2,8%

Table 1.

Cell line	B cell stage	FLT-1	KDR	FLT-4	VEGF (pg/mL)
RS4;11	B cell precursor leukemia CD10- CD19+ CD20-	+	-	+	10,3
BV173	B cell precursor leukemia CD10+ CD19+ CD20+	+	-	+	2,4
REH	pre-B cell leukemia CD10+ CD19+ CD20+	+	-	-	20
697	pre B cell leukemia CD10+ CD19+ CD20+	+	-	-	1,5

Table 2.

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

Table 3.

	Days	
	10	19
Control/KDR Ab	-	+
FLT-1 Ab	-	-

Table 4.

	Diaphysis	Epiphysis
Control	25%	35%
FLT-1Ab	45%	54%

From www.bloodjournal.org by guest on May 6, 2015. For personal use only.

blood

Prepublished online October 25, 2005;
doi:10.1182/blood-2005-06-2530

VEGFR-1 (FLT-1) activation modulates acute lymphoblastic leukemia localization and survival within the bone marrow, determining the onset of extramedullary disease

Rita Fragoso, Teresa Pereira, Yan Wu, Zhenping Zhu, Jose Cabecadas and Sergio Dias

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml

Advance online articles have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Advance online articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Advance online articles must include digital object identifier (DOIs) and date of initial publication.

Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.
Copyright 2011 by The American Society of Hematology; all rights reserved.