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ORIGINAL ARTICLE

Regulation of FLT3 and its ligand in normal hematopoietic progenitor cells

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Abstract FLT3 and its ligand (FL) are one of the regulators of normal hematopoiesis. Ligand-independent activation of FLT3 occurs in about 30% of acute myeloid leukemia cases and is one goal for selectively targeted therapies. However, the function of FLT3/FL in the regulation of non-malignant immature hematopoietic cells is poorly characterized. In order to elucidate the role of FLT3 in normal hematopoiesis, human adult $CD34^+$ hematopoietic progenitor cells were cultured in cytokine-supplemented liquid culture in the presence or absence of FLT3 inhibition by CEP-701 (lestaurtinib). Total cell number, lineage-committed, and primitive progenitors and apoptosis were assayed. FLT3 expression and FL secretion in various conditions were analyzed by fluorescent activated cell sorter and enzyme-linked immunosorbent assay. Effects of nonspecific targeting of FLT3 were evaluated with addition of imatinib (Gleevec®) to cell cultures. It is demonstrated that FLT3 inhibition impaired cell and progenitor cell growth and increased the rate in apoptosis. Effects were observed independent of addition of FL. The dose-dependent growth inhibition was partially equalized by inhibiting FL with a neutralizing antibody. FLT3 inhibition resulted in markedly increased production of FL by cultured $CD34^+$ cells as well as upregulation of FLT3 expression. Imatinib mimicked effects of selective FLT3 inhibition. In conclusion, FLT3 and its ligand regulate proliferation of hematopoietic progenitor cells in an autocrine/paracrine manner Nonspecific inhibition of FLT3 may contribute to hematotoxicity caused by imatinib treatment.

Keywords FLT3 · FL · Hematopoiesis · Expansion

Introduction

Hematopoiesis is an unidirectional multilinear process where hematopoietic stem cells (HSC) differentiate into mature hematopoietic cells by progressive loss of developmental options and restriction to one lineage. Expansion and regeneration of specific lineages are largely regulated by different hematopoietic cytokines [1]. Ligand-mediated activation of the FMS-like tyrosine kinase-3 (FLT3) receptor is one of the regulators of HSC self-renewal and differentiation (reviewed in [2, 3]). FLT3 is member of the type III RTK subfamily that also includes c-kit, c-FMS, and PDGFα/β [4–7]. The role of FLT3 differs between the mouse and the human system. In the mouse system, FLT3 expression is correlated with short-term reconstituting HSC (Lin-Sca-1+c-kit+FLT3+), whereas there is no evidence so far that it is also expressed in long-term repopulating (HSC) [8, 9]. In human adult bone marrow (BM), FLT3 expression is restricted to $CD34^+$ cells and a subset of dendritic precursors [10]. Recently, it was shown that human cord blood $CD34^+$ cells capable of multilineage engrafting express FLT3 [11, 12]. However, cobblestone-area forming cells (CAFCs) [13] are equally distributed between $CD34^+FLT3^+$ and $CD34^+FLT3^-$ cells [14]. In general, expression levels of FLT3 decrease with increasing differentiation of multipotent progenitor cells. So far, the detailed role of FLT3 in human HSC regulation still has to be defined.

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FLT3 ligand (FL) is a type 1 membrane protein existing in a membrane-bound and soluble isoforms. Soluble forms activate the corresponding FLT3 receptor [3] Although its messenger RNA is detected in most hematopoietic and non-hematopoietic tissues, protein expression is restricted to T lymphocytes and BM stromal cells composing the hematopoietic microenvironment [15–17]. Generally, FL acts in combination with other cytokines, resulting in strongly synergistic effects; isolated addition of FL to stem/progenitor cell cultures has no significant effect on proliferation of myeloid and lymphoid progenitors [18–21].

Ligand-independent activation of FLT3 has recently been identified as an important oncogenic event in pathogenesis of acute myeloid leukemia (AML) [22, 23]; activating mutations in the kinase domain correlate with a dismal prognosis and a high risk for relapse [24].

The emerging role of FLT3 in leukemia pathogenesis exposed it as a therapeutic target for specific therapy. Several tyrosine kinase inhibitors have recently been described. CEP-701 (lestaurtinib; Cephalon West Chester, PA, USA) is an indolocarbazole alkaloid which inhibits FLT3 (wild type, ITD, and PM) in an IC_{50} of 3 nM [25]. The most closely related kinases [platelet-derived growth factor (PDGF), FMS, and kit] are inhibited at concentrations of 500–1000 nM or greater. CEP-701 is cytotoxic to primary AML and ALL blast samples and showed clinical activity in a phase I/II trial in patients with relapsed or refractory AML [26–28]. In addition, FLT3 is a nonspecific target of various other tyrosine kinase inhibitors. A research group from our institution had previously shown that imatinib (Gleevec™), which is widely used in the therapy of chronic myeloid leukemia, inhibits stem and progenitor cell activity of HSC. Potential signaling pathways responsible for this inhibitory effect were investigated, but at this point, it was excluded that this mechanism is c-kit- or PDGF-dependent [29]. The group discussed FLT3 as a possibly targeted pathway.

In this study, we investigated the effect of FLT3 inhibition on normal $CD34^+$ HSC using CEP-701. This method allowed us to uncover the interaction of FLT3 and its ligand in HSC. In addition, we went back to the results of Bartolovic et al. [29] and compared the effects of FLT3 inhibition with those of imatinib in $CD34^+$ cell culture. From our results obtained, we conclude that FLT3 inhibition most likely contributes to the hematotoxic side effects of imatinib.

Materials and methods

Reagents

CEP-701 (lestaurtinib) was generously provided by Cephalon. For in vitro experiments, stock solutions of lestaurtinib were prepared at 80 and 20 μg/ml by dissolving the compound in dimethyl sulfoxide (DMSO)/X-VIVO 20 medium (BioWhittaker, Walkersville, MD, USA; 1:4) and stored at −20°C.

Cell samples

Peripheral blood mononuclear cells (MNC) were obtained from healthy donors and patients with hematopoietic and non-hematopoietic disorders undergoing stem cell mobilization with G-CSF and were collected after individuals gave their informed consent. MNC were purified using Ficoll–Hypaque density gradient centrifugation (Biochrom, Berlin, Germany). The study was approved by the ethics review board of the medical faculty of the University of Tuebingen (project number 268/2003V, University of Tuebingen).

Purification of stem and progenitor cells

$CD34^+$ cells from fresh leukapheresis samples were selected using a Midi-MACS CD34 isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. The purity of $CD34^+$ cells ranged between 92% and 99% in all samples as determined by flow cytometry.

Cytokine-supplemented liquid culture

Isolated hematopoietic progenitor cells (HPC, 1×10^5) were cultured for 1, 2, 4, and 7 days in 5 ml serum-free medium (X-VIVO 20, BioWhittaker, Walkersville, MD, USA) supplemented with a combination of cytokines: SCF 50 ng/ml, IL-6 10 ng/ml and, in selected cultures, FL (50 ng/ml) (all from Tebu, Frankfurt, Germany). Lestaurtinib was added at increasing doses from 0 to 50 nM. Cells were incubated at 37°C in humidified atmosphere with 5% CO_2 in air. In liquid cultures, all cells could be recovered without trypsination.

Flow cytometry

Cells ($1–2\times10^5$) were incubated for 20 min at 4°C with the fluorescein isothiocyanate or PE-conjugated monoclonal antibody (mAb) CD34, CD45, and CD38 (Becton-Dickinson, Heidelberg, Germany) or FLT3 (R&D Systems, Minneapolis, MN, USA). An isotype-identical mAb served as a control. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson). To calculate the percentage of positive cells, a proportion of ≤1% false positive events was accepted in the negative control sample. Cell acquisition and analysis was performed using the CellQuest software program (Becton Dickinson).

Assay for clonogenic progenitors

One thousand five hundred cells were plated in triplicate in 35-mm tissue culture dishes (Greiner, Frickenhausen, Germany) containing 0.9% methycellulose, 30% fetal bovine serum, 1% bovine serum albumin, 70% Iscove's DMEM (Stem Cell Technologies, Vancouver, Canada), and a combination of five cytokines: human SCF (20 ng/mL, Pepro Tech Hamburg, Gemany), human interleukin-3 (50 ng/ml, Tebu Offenbach, Germany), interleukin-6 (20 ng/ml, Tebu Offenbach), human granulocyte colony-stimulating factor (rhumet-G-SCF, 100 ng/ml, Amgen, Thousand Oaks, CA, USA), and human erythropoietin (6 U/ml, Jannsen-Cilag, Neuss, Germany). In selected cultures, increasing concentrations of CEP-701 (lestaurtinib, 0–20 nM) were added. The plates were cultured for 14 days at 37°C, 100% humidity, and 5% CO_2. Colonies (>40 cells) were scored using an inverted microscope. By this technique, erythroid (burst-forming and colony-forming units erythroid: BFU-E, CFU-E), myeloid colonies (colony-forming units granulocyte, macrophage, and granulocyte–macrophage: CFU-G, CFU-M, CFU-GM), and mixed colonies were detected.

MTT assay

Cell proliferation and survival were measured by cellular uptake of 3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich). Fresh $CD34^+$ cells were resuspended in growth-factor-supplemented serum-free medium at a concentration of 3×10^4 cells/150 μl. Cells were pre-incubated for 24 h in 96-well flat-bottomed microtiter plates (Becton Dickinson) before CEP-701 (lestaurtinib) was added at increasing concentrations (0–50 nM). All analyses were performed in triplicate. After 48 h, 10 μl MTT solution (10 mg/ml) was added to each well. Plates were incubated for another 2 h before 90 μl MTT lysis solution (15% sodium dodecyl sulfate, 50% dimethylformamide) was added to each well, and the absorbance was measured after 12 h on an enzyme-linked immunosorbent assay (ELISA) plate reader (Dynatech MR7000; Dynatech, Denkendorf, Germany). The IC_{50} is defined as the concentration of drug that produces 50% cell growth inhibition and corresponds to the affected fraction (F_a value) of 0.5.

Apoptosis (annexin V/propidium iodide)

$CD34^+$ cells (1×10^5 cells/well) were cultured in six-well tissue plates under the liquid culture conditions described. Without pre-incubation, lestaurtinib was added at increasing doses from 0 to 20 nM. Cells were incubated either for 24, 48, or 96 h. Propidium iodide (PI) staining solution was used to assess plasma membrane integrity in annexin V apoptosis assays. PI is used in two-color annexin V flow cytometric assays to distinguish cells that are in the early stages of apoptosis (annexin V pos./PI neg) from those that are in the later stages of apoptosis or already dead (annexin V pos./PI pos.). Cells were analyzed after 24, 48, and 96 h.

Cobblestone area formation

For determination of CAFC formation, the contact-inhibited MS-5 murine stromal cell line was used. When stromal layers cultured in T12.5 flasks (Falcon) reached confluency, $CD34^+$ or cultured cells (1×10^5) were added. Stromal cultures were incubated at 37°C and 10% CO_2 for 5 weeks in human long-term culture medium consisting of α-MEM (Gibco) supplemented with 12.5% fetal bovine serum (HyClone), 12.5% horse serum (Sigma), 1% L-Glutamin (Gibco), 50 μM hydrocortison (Sigma), 50 mM mercaptoethanol (Gibco), and 10,000 U/ml penicillin/streptomycin (Gibco) with weekly half-medium changes (demidepopulation). At the end of the 5-week period, phase-dark cobblestone areas of a least five cells were counted.

Inhibition of FLT-3 signaling

To investigate whether the growth inhibitory effect of lestaurtinib on normal HPC can be abrogated by addition of FL antibody, 1×10^5 $CD34^+$ were expanded in liquid culture conditions. Expansion was performed with or without the addition of the FL inhibitory antibody according to the manufacturer's instructions (0.1 mg/ml; Peprotech, USA).

Statistical analysis

Data from independent experiments were expressed as mean±SEM (standard error of the mean) of at least three experiments. Differences between groups were compared by the parameter-free Mann–Whitney rank sum test for statistical significance.

Results

Short-term expansion of $CD34^+$ cells in serum-free liquid culture

Mobilized $CD34^+$ cells were expanded for 7 days in serum-free liquid culture starting with 1×10^5 cells/per well supplemented either with SCF and IL-6 or with SCF, IL-6, and FL. CEP-701 was added at day 0 in increasing doses (0–50 nM). Total cell counts after 1 week of culture are shown in Fig. 1a. Cell counts decreased continuously with increasing concentrations of CEP-701 due to induction of apoptosis (see below). The effect observed was independent from addition of FL to the cell culture. Looking on the rates

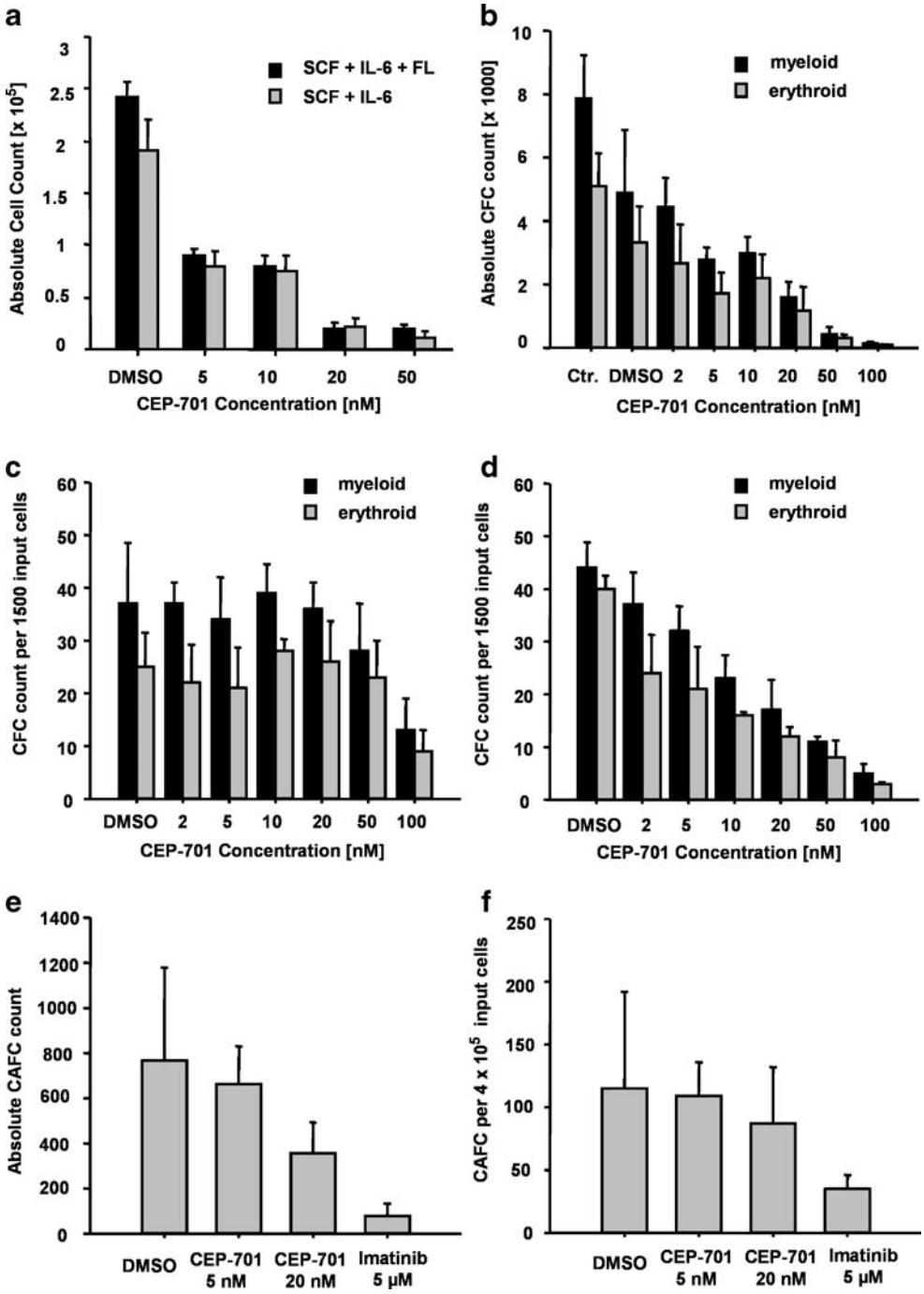

Fig. 1 **a** Absolute cell counts after a 7-day culture period in cytokine-supplemented serum-free liquid culture. Input $CD34^+$ cells were exposed to increasing concentrations of CEP-701 (5–100 nM). Addition of DMSO as the solvent for CEP-701 served as control. Cell counts were obtained using trypan blue dye exclusion. **b**, **c** Myeloid and erythroid colony formation of day 7 cells cultured in cytokine-supplemented (SCF, IL-6) liquid cultures under increasing concentrations of CEP-701 (5–100 nM). Absolute and relative CFC numbers per 1,500 input cells are shown. **d** Colony formation of day 0 input $CD34^+$ cells when CEP-701 was added directly into semisolid methylcellulose cultures with increasing concentrations (5–100 nM). **e**, **f** Cobblestone area formation (*CAFC*) of expanded $CD34^+$ cells, absolute and relative counts. After 96 h in serum-free, cytokine-supplemented liquid culture (IL6, SCF) under addition of CEP-701 (5 and 20 nM) or imatinib, expanded cells were assayed for CAFC formation without any further addition of CEP-701 or imatinib

of $CD34^+$ cells after 7 days in culture, $CD34^+$ rates also continuously decreased with increasing concentration of CEP-701 (Table 1).

Effect of FLT3 inhibition on lineage-committed and primitive progenitors

In order to evaluate whether the effect of FLT3 inhibition extends to both, lineage-committed and more primitive HPC, colony and cobblestone area formation was assayed. Again, $CD34^+$ cells were cultured in cytokine-supplemented (IL-6, SCF) liquid culture conditions with increasing concentrations of CEP-701 as described above. For determination of colony formation, cells were transferred to semisolid cytokine-supplemented methylcellulose media without further addition of CEP-701 after 7 days of liquid culture. Colony formation was assayed after 14 days. As shown in Fig. 1b, absolute counts of colony-forming units decrease continuously when cells were exposed to CEP-701 for 7 days in increasing concentrations. The decrease occurs in parallel to

Table 1 Percentage of $CD34^+$ cells after 7 days of liquid culture under supplementation with SCF, IL-6 or SCF, IL-6 and FL

	% $CD34^+$ cells (SCF, IL-6)	% $CD34^+$ cells (SCF, IL-6, FL)
DMSO	64.2±8.7	57.2±5.5
CEP-701 5 nM	49.3±8.2	47.7±3.9
CEP-701 10 nM	41.0±6.6	40.3±2.7
CEP-701 20 nM	27.8±4.6	18.3±4.1
CEP-701 50 nM	13.7±3.7	11.2±4.2

the absolute cell counts measured under different concentrations of CEP-701. However, relative counts of colony-forming units were not significantly different, indicating that non-apoptotic, surviving progenitor cells are not impaired in function (Fig. 1c). However, if CEP-701 is directly added into day 0 methylcellulose cultures, a dose-dependent decrease in colony formation was detected, as it was shown for cell counts in liquid culture system (Fig. 1d).

In addition, we investigated cobblestone area formation of the cultured $CD34^+$ cells to obtain more information about the earlier progenitor cell population [30]. Similar to the findings in lineage-committed colony-forming units, absolute CAFC counts, assayed after 5 weeks of co-culture of 96 h liquid culture cells with MS5 stroma, decreased with exposure to increasing concentrations of CEP-701 during the liquid culture period (Fig. 1e). Again, relative CAFC counts did not decrease after culturing the cells with 5 nM CEP-701 (Fig. 1f), although this concentration resulted in a significant growth inhibition (Fig. 1a). Using higher concentrations of CEP-701 (20 nM) relative CAFC counts were also affected. With concentrations higher than 20 nM, CAFC assays were not realizable due to increasing amount of apoptotic cells in culture. These results indicate that exposure of HPC to CEP-701 for 7 days reduces the number of CAFC rather than affecting the cloning efficiency of the cells when transmitted to the CAFC assay (Fig. 1e, f). Addition of imatinib (5 μM) leads to a similar effect in absolute but a more pronounced decrease in relative CAFC counts.

Cytotoxicity and apoptosis

A tetrazolium-based MTT assay was used to quantify 50% growth inhibition (IC_{50}) of cytokine-stimulated $CD34^+$ cells after 48 h of exposure to CEP-701. The mean IC_{50} (±SD) for CEP-701 on normal $CD34^+$ cells was 56±19 nM.

To determine whether CEP-701 cytotoxicity was due to induction of apoptosis, we performed annexin-V binding flow cytometric assays. With increasing concentrations of CEP-701, an increasing rate of apoptosis in cultures was detected (analysis after 48 h of culture). These data indicate that apoptosis is the predominant mechanism of cytotoxicity of CEP-701 (Fig. 2).

Effects of FLT3 inhibition can be partially equalized by addition of FL Ab

The above findings showed cytotoxic effects of FLT3 inhibition in the early stem and progenitor cell population. To further demonstrate that these effects are specific due to FLT3 inhibition and not due to nonspecific inhibitory events, activation of the FLT3 tyrosine kinase was down-regulated by inhibition of its ligand FL with a neutralizing antibody (FL-Ab). When FL-Ab was added into the cytokine-supplemented liquid culture system, inhibitory effects of CEP-701 were partially equalized (Fig. 3). These findings were even present when FL was not added into the culture system, indicating that the FLT3 receptor in this condition is activated by presence of FL in culture obviously produced by the culture cells itself.

FLT3 inhibition results in marked upregulation of FL production in cell culture

Observing the effects of FLT3 inhibition independent of FL substitution, we had to conclude that FL is autonomously produced by input $CD34^+$ cells and their progeny in liquid culture conditions. Measuring FL with ELISA in the supernatant showed a baseline production of 1.3, 2.1, and 0.8 ng/ml FL after 24, 72, and 96 h, respectively. In the case of FLT3 inhibition by CEP-701 (20 nM), FL production by the cultured $CD34^+$ cells markedly increased up to 841, 709, and 511 ng/ml FL after 24, 72, and 96 h. Addition of imatinib, targeting the abl kinase but also having nonspecific inhibitory effects on FLT3, lead to a similar effect (Fig. 4a). After 24 h, virtually all cultured cells were still $CD34^+$ (Fig. 4b), indicating that also $CD34^+$ progenitors and not only their progeny are able to produce FL.

FLT3 inhibition results in upregulation of FLT3 expression

In a next step, we investigated the effect of FLT3 inhibition with CEP-701 of FLT3 expression in liquid-culture $CD34^+$ cells. After 24 h in liquid culture with cytokine supplementation, FLT3 is partially expressed in $CD34^+$ cells assayed by flow cytometry. However, adding CEP-701 into the culture system, FLT3 expression increased (Fig. 4b). Again, a similar effect was observed by adding imatinib to the cell cultures.

Discussion

Hematopoiesis is a complex process in which survival, proliferation, differentiation, and other functions are at least

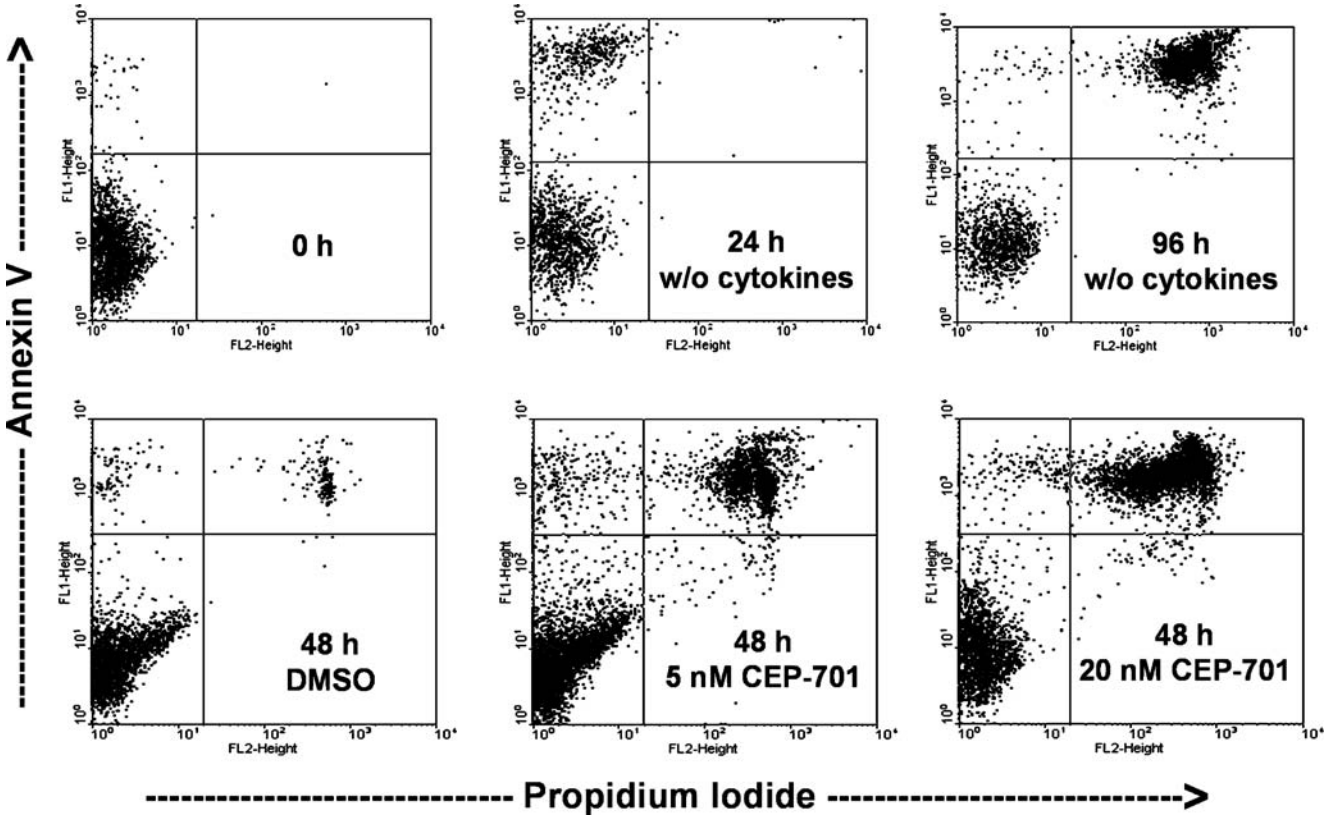

Fig. 2 Immunophenotypic analysis of apoptosis. $CD34^+$ cells were cultured for 96 h in cytokine-supplemented liquid culture conditions with increasing concentrations of CEP-701 (5 and 20 nM). Cells were tested for the externalization of phosphatidylserine by annexin V binding using a flow cytometer

partially regulated through a network of growth factors [1, 31–33]. FLT3 and its ligand FL are known regulators in this process, mainly enhancing effects of other cytokines more than having specific effects by itself. Due to the role of FLT3 and its activating mutations in AML, it became also a therapeutic target.

In this study, we investigated the role of FLT3 and its ligand in normal early hematopoiesis. We used a cytokine-supplemented, serum-free liquid culture system that had been shown to be effective for ex vivo expansion of adult $CD34^+$ cells [20]. FLT3 inhibition was performed with CEP-701 (lestaurtinib) which is known to act as a potent inhibitor in nanomolar concentrations.

It was shown that cell counts in the liquid culture system decreased in a significant and dose-dependent fashion in cells treated with CEP-701 even in low nanomolar concentrations (5–10 nM). Dose-dependent inhibition of $CD34^+$ cell growth and expansion was also reflected in investigation of progenitor cell functions (CFC and CAFC formation). Decrease of these colony- or cobblestone-forming units was in parallel with the absolute cell counts in culture. Looking on the relative counts, surviving non-apoptotic progenitor cells showed nearly the same capacity of colony and cobblestone formation.

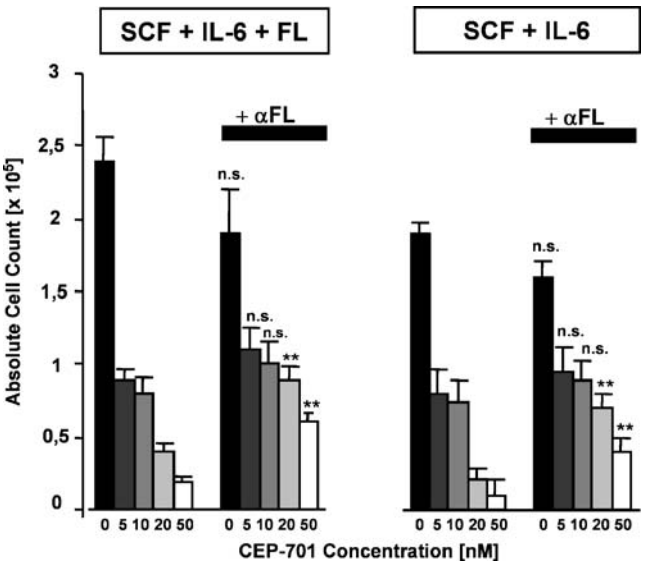

Fig. 3 Inhibition of FL by neutralizing antibody. $CD34^+$ cells were cultured for 96 h in serum-free, cytokine supplemented (Il-6, SCF or IL-6, SCF, and FL) liquid culture in the presence of various concentrations of CEP-701 (5–50 nM) with or without a neutralizing antibody against FL (α-FL, $n=6$). $^{**}p<0.01$, *ns* not significant

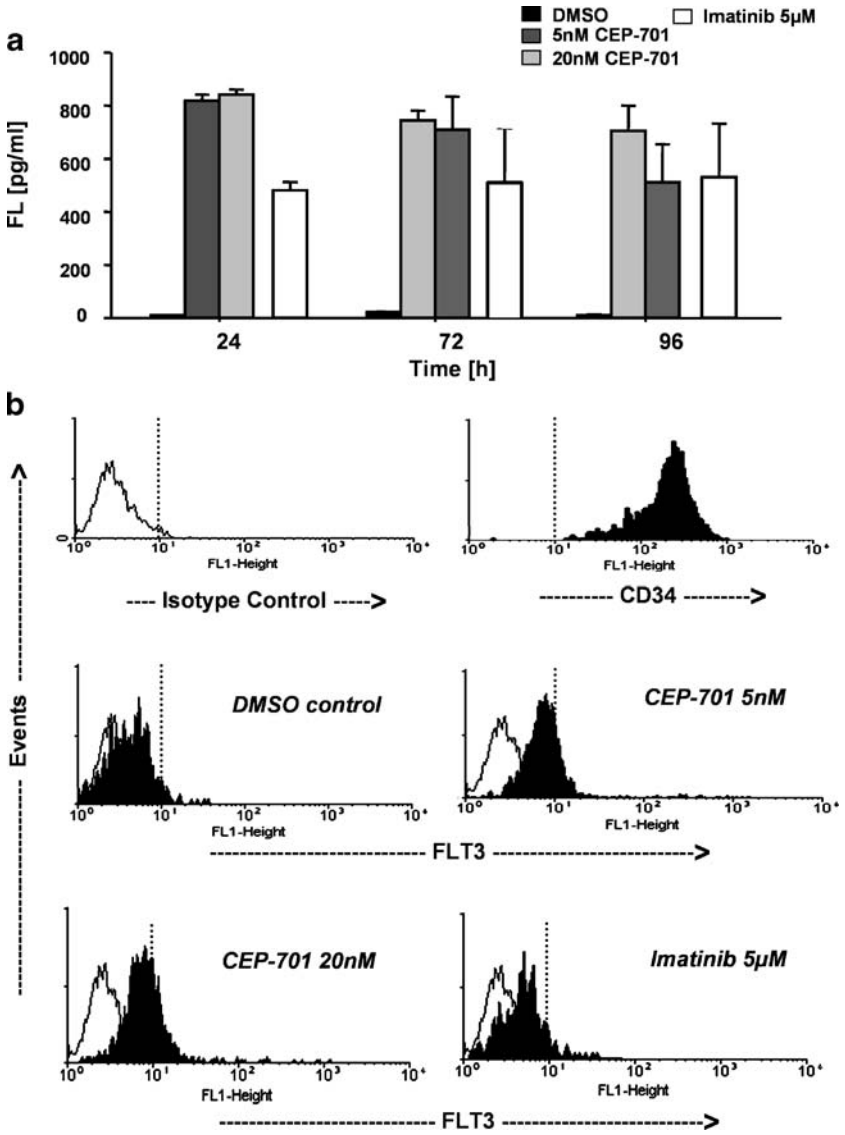

Fig. 4 **a** Production of FL by $CD34^+$ cells after 24, 72, and 96 h in serum-free, cytokine-supplemented (IL-6, SCF) liquid culture in presence of various concentrations of CEP-701 (5 and 20 nM) or imatinib (5 μM). Concentrations of FL in the supernatant were determined by ELISA. **b** Determination of CD34 and FLT3 expression in progenitor cells cultured for 24 h in serum-free, cytokine-supplemented liquid culture conditions under exposure ton CEP-701 (5 and 20 nM) or imatinib

The mechanism CEP-701 acts on $CD34^+$ progenitor cells was mainly apoptotic, as shown using the annexin V staining. Interestingly, the growth inhibitory effects of FLT3 inhibition were independently of FL supplementation in the liquid-culture system. This was the first hint that the cultured cells by themselves produce significant amounts of FL, leading to a continuous tyrosine kinase activation.

To confirm that the observed effects of FLT3 inhibition were specific, we used a neutralizing antibody against FL to block ligand activation of FLT3. With addition of the antibody, inhibitory effects of CEP-701 were partially equalized. This effect was again observed in the absence of supplementation with FL.

Focusing more in detail on the suspicion that FL and FLT3 are acting in an autocrine- or paracrine-independent manner in cell culture, FL production in serum-free liquid culture conditions was analyzed. Using ELISA technique, we did not only show for the first time that FL is produced in these culture conditions by the progenitor cells, we also could show that this production is markedly upregulated by FLT3 inhibition. The same conditions had impact on FLT3 expression itself. Inhibition of FLT3 by CEP-701 results in an upregulation of FLT3 in the $CD34^+$ progenitors. In conclusion, the simultaneous upregulation of FLT3 and FL expression under inhibiting conditions showed a compensatory mechanism in a self-regulated feedback system.

Looking on the inhibitory effect of imatinib on $CD34^+$ hematopoietic progenitor cells, it is comparable to the effect we found with FLT3 inhibition [29]. Here, we demonstrated in a direct comparison that imatinib used in concentrations based on the data of Bartolovic et al. and those usually achieved in patients showed the same effect on FL and FLT3 expression as was observed with CEP-701. Inhibition of progenitor cell function was documented by reduction of

CAFC formation. More importantly, imatinib lead to an enhanced production of FL in culture and to an increased expression of FLT3 in $CD34^+$ cells cultured for 24 h, comparable to the data generated with direct FLT3 inhibition. Imatinib is known to have inhibitory effects on the FLT3 tyrosine kinase. It is therefore concluded that the hematotoxicity known as one of the most relevant side effects of imatinib therapy is at least partially mediated by nonspecific FLT3 inhibition.

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